

The Pennsylvania State University  
The Graduate School  
Department Civil and Environmental Engineering

**THE DEVELOPMENT OF AN ADVANCED TREATMENT PROCESS TO REMOVE  
ENDOCRINE-DISRUPTING COMPOUNDS FROM WASTEWATER USING  
*PHANEROCHAETE CHRYSOSPORIUM***

A Thesis in  
Environmental Engineering

by  
Erin Henry

© 2011 Erin Henry

Submitted in Partial Fulfillment  
of the Requirements  
for the Degree of

Master of Science

May 2011

The thesis of Erin Henry was reviewed and approved\* by the following:

Rachel A. Brennan  
Assistant Professor of Environmental Engineering  
Thesis Advisor

John M. Regan  
Associate Professor of Environmental Engineering

Stephanie B. Velegol  
Instructor of Environmental Engineering

Peggy A. Johnson  
Professor of Civil Engineering  
Head of the Department of Civil and Environmental Engineering

\*Signatures are on file in the Graduate School

## ABSTRACT

Endocrine-disrupting compounds (EDCs) have entered our wastewater in many forms including pesticides and prescription medications. Because typical wastewater treatment processes are not designed to remove EDCs, some compounds remain in the effluent. Upon discharge, EDCs are released into the environment, where they can harm aquatic species or reenter potable water supplies.

One potential solution to this problem involves the use of the white-rot fungus (WRF), *Phanerochaete chrysosporium*. *P. chrysosporium* produces lignin-degrading extracellular enzymes, which have the capacity to biodegrade a variety of organic pollutants. Although there have been numerous experiments exploring the degradation capacity of WRF, there have not been adequate studies involving wastewater applications. This bench-scale study investigated conditions for fungal growth and extracellular enzyme activity in wastewater for eventual large-scale applications. Degradation of an EDC, atrazine, by *P. chrysosporium* was also evaluated.

Three batch experiments were carried out to evaluate the growth and extracellular enzyme activity of immobilized *P. chrysosporium*. The effects of sterile and non-sterile conditions, an organic carbon amendment, pH, wastewater effluent treatment, and three packing (immobilization) materials on *P. chrysosporium* were evaluated. Additionally, wastewater was spiked with atrazine, and degradation was monitored. In anticipation of scale-up, continuous-flow column reactors were designed and built; however, experimental data was not obtained from these columns.

The batch experiments indicated that 1) enzyme activity can be produced within a pH range of 3.4 – 5.1; 2) supplemental organic carbon positively affected growth, but did not affect enzyme activity; 3) fluctuations in pH negatively affected enzyme activity; 4) enzyme activity was produced in non-sterile wastewater; and 5) removal of atrazine by fungal treatments was not

significantly different from controls; however, one fungal treatment may have started to degrade atrazine via enzymatic biocatalysis by the end of the experiment.

## TABLE OF CONTENTS

LIST OF FIGURES .....	viii
LIST OF TABLES .....	xii
ACKNOWLEDGEMENTS .....	xiii
1. Introduction .....	1
1.1. The Endocrine System, Endocrine-Disrupting Compounds and Health Risks .....	1
1.1.1. The Endocrine System .....	1
1.1.2. Endocrine-Disrupting Compounds and Health Risks .....	2
1.2. EDC Occurrence in Wastewater .....	3
1.3. Removal of EDCs from Wastewater: Physical, Chemical, and Biological Techniques .....	5
1.3.1. Physical and Chemical Removal .....	6
1.3.2. Biological Removal: Microbial and Fungal .....	8
1.4. White-Rot Fungi and Lignin Degradation .....	9
1.5. <i>Phanerochaete chrysosporium</i> and Degradation of EDCs .....	13
1.6. Atrazine .....	15
1.6.1. Use, Occurrence in the Environment, and Health Risks .....	15
1.6.2. Atrazine Degradation .....	17
1.7. Scope of Study .....	18
2. Materials and Methods .....	20
2.1. Water Source and Atrazine .....	20
2.2. Packing Materials .....	20
2.3. Organism, Maintenance, and Cultivation Conditions .....	21
2.4. Inoculum Preparation for Batch Experiments .....	22
2.5. Batch Reactor Setup .....	23
2.6. Batch Experiments .....	24
2.6.1. Batch Experiment 1 .....	24
2.6.2. Batch Experiment 2 .....	26
2.6.3. Batch Experiment 3 .....	27
2.7. Analytical Methods .....	28
2.8. Statistical Analysis .....	29
3. Results .....	30
3.1. Wastewater Characteristics .....	30
3.2. Batch Experiment 1 .....	31
3.2.1. pH .....	31
3.2.2. Attached Biomass and Enzyme Activity .....	33
3.3. Batch Experiment 2 .....	36
3.4. Batch Experiment 3 .....	39
3.4.1. pH and Enzyme Activity .....	39

3.4.2. Atrazine Degradation .....	43
4. Discussion .....	46
4.1. Batch Experiment 1 .....	46
4.1.1. Growth and Enzyme Activity at the pH of Wastewater .....	46
4.1.2. Growth and Enzyme Activity in Non-Sterile Wastewater .....	47
4.1.3. Effect of Supplemental Organic Carbon on Growth and Enzyme Activity ..	49
4.1.4. Relationship Between Biomass and Enzyme Activity .....	50
4.2. Batch Experiment 2 .....	50
4.3. Batch Experiment 3 .....	52
4.3.1. Effect of Packing Material on Enzyme Activity .....	52
4.3.2. Transformation of Atrazine and Degradation Kinetics .....	53
4.4. Overall Effects of the Experimental Parameters on <i>P. chrysosporium</i> .....	55
4.4.1. pH .....	55
4.4.2. Organic Carbon Amendment .....	55
4.4.3. Sterile vs Non-Sterile .....	56
4.4.4. Natural vs Synthetic Packing Media .....	56
5. Conclusions, Engineering Significance, and Future Work .....	57
5.1. Conclusions .....	57
5.2. Engineering Significance .....	59
5.3. Future Work .....	60
REFERENCES .....	63
Appendix A Wastewater Collection Site .....	72
Appendix B Photographs of Materials Used in Experimental Setups .....	74
Appendix C Media Recipes and Inoculum Preparation Methods .....	77
C.1. YMPG Media Recipe .....	77
C.2. Low Nitrogen Media Recipe .....	78
C.3. Basal III Media Recipe .....	79
C.4. Trace Elements Solution Recipe .....	80
C.5. Preparation of Mycelia Inoculum for Batch Reactors .....	81
C.5.1. Spore Solution .....	81
C.5.2. Mycelia Solution .....	83
C.5.3. Homogenization/Inoculum Prep .....	83
Appendix D Fungal Batch Flask Sacrificing Procedures .....	84
D.1. Flask Selection .....	84
D.2. Sample Preservation for Atrazine Analysis (Batch Experiment 3 only) .....	84
D.3. pH, Filtration and Drying (Batch Experiments 1 and 2 only) .....	85
Appendix E Enzyme Activity Assays .....	88

E.1. Lignin Peroxidase Assay.....	88
E.2. Manganese Peroxidase Assay .....	89
E.3. Solution Preparation Methods.....	90
E.4. Enzyme Activity Calculations.....	92
Appendix F Photographs of Packing Materials from Sacrificed Reactors.....	94
Appendix G LC/MS/MS Analytical Method .....	98
Appendix H Preliminary Aeration Experiment .....	109
Appendix I Batch Experiment 2: Initial Attempt.....	111
Appendix J Experimental Evaluation of the Effects of Glucose, Woodchips, Buffers, and Autoclaving on Solution pH.....	114
Appendix K Oxygen Mass-Transfer into Solution from Bubbles vs Oxygen Utilization by Aerobic Microbes (Calculations).....	118
Appendix L Adsorption of Atrazine onto Packing Media (Calculations).....	123

## LIST OF FIGURES

Figure 1-1. Several common EDCs that share structural similarities to lignin.....	14
Figure 1-2. Atrazine metabolites.....	17
Figure 2-1. Aerated batch reactor setup, illustrating the aeration system and individual reactors.....	24
Figure 3-1. The effect of indigenous wastewater microorganisms and supplemental glucose on pH in aerated batch reactors containing <i>P. chrysosporium</i> immobilized on Tri-pack in secondary clarifier effluent (Batch Experiment 1). Data points represent mean values of triplicate flask reactors; error bars represent $\pm 1$ standard deviation. Sterile treatments are represented by solid lines; non-sterile treatments are represented by dotted lines. pH-adjusted treatments are represented by circles; un-adjusted treatments are represented by triangles. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols. ....	32
Figure 3-2. Attached growth on Tri-pack and enzyme activities observed for <i>P. chrysosporium</i> in aerated batch reactors containing secondary clarifier effluent (Batch Experiment 1). Treatment codes are provided in Table 2-2. a.) S-4.3-10; b.) N-4.3-10; c.) S-4.3-0; d.) N-4.3-0; e.) S-WW-10; f.) N-WW-10; g.) S-WW-0; f.) N-WW-0. Data points represent mean values from triplicate flask reactors; error bars represent $\pm 1$ standard deviation. ....	35
Figure 3-3. Changes in pH in non-sterile, aerated batch reactors containing wastewater from different locations in the treatment train inoculated with <i>P. chrysosporium</i> (Batch Experiment 2). Circles indicate points of pH adjustments. Data points represent mean values of triplicate flask reactors (except for points of pH adjustment, which are mean values of all remaining reactors within the treatment); error bars represent $\pm 1$ standard deviation.....	37
Figure 3-4. The effect of wastewater treatment level on biomass (fungi + wastewater microbes) attachment to Tri-pack in aerated batch reactors inoculated with <i>P. chrysosporium</i> (Batch Experiment 2). Data points represent mean values of triplicate flask reactors; error bars represent $\pm 1$ standard deviation.....	38
Figure 3-5. The effect of packing material (wood chips or foam) and supplemental glucose on pH in aerated batch reactors containing sterile secondary clarifier effluent inoculated with <i>P. chrysosporium</i> (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (uninoculated control); error bars represent $\pm 1$ standard deviation. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols.....	40



Figure 3-6. LiP and MnP enzyme activities observed for <i>P. chrysosporium</i> attached to wood chips or foam in aerated batch flasks containing wastewater (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (control); error bars represent $\pm 1$ standard deviation. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols. ....	42
Figure 3-7. Atrazine concentrations for <i>P. chrysosporium</i> and control treatments in aerated batch reactors containing wastewater (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (control); error bars represent $\pm 1$ standard deviation, with the exception of the final F-0-C (0 g/L glucose, foam, control) point which is from a single flask. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols. ....	43
Figure 3-8. Percent atrazine removal that was not attributed to the initial, non-linear sorption in a) wood chip and b) foam treatments. Remaining time points were normalized by dividing the atrazine concentration at each time point by the concentration at $t = 2.4d$ (first sacrificial point in the experiment). Final F-0-C (0 g/L glucose, foam, control) point is from a single flask. ....	45
Figure A-1. Penn State wastewater treatment plant schematic. ....	72
Figure A-2. Penn State wastewater treatment plant process flow-diagram. Stars indicate the seven effluent locations that were considered for Batch Experiment 2. Encircled stars indicate the four selected effluent locations. ....	73
Figure B-1. Jaeger Tri-Pack® (1"). ....	74
Figure B-2. Birch woodchips (0.131" – 0.25"). ....	74
Figure B-3. Foamex SIF II® Foam (ZS15D) cubes (0.5"). ....	74
Figure B- 4. Aeration apparatus used to seal each flask. ....	75
Figure B- 5. Humidification apparatus. Photograph is courtesy of Michael Shreve. ....	75
Figure B- 6. Experimental setup. This photograph illustrates Batch Experiment 1 reactors connected to aeration manifolds. ....	76
Figure C-1. Glass funnel containing glass wool for filtering spore solution. ....	82

Figure F-1. Representative photograph of <i>P. chrysosporium</i> attachment to Tri-pack®. The Tri-pack® pictured were from an aerated flask in a preliminary aeration experiment at t = 1 day. ....	94
Figure F-2. Representative photograph of woodchips from a control treatment (no fungus). These woodchips were taken from a W0-C treatment flask at t = 12.7 days. ....	95
Figure F-3. Representative photograph of woodchips with fungal attachment. These woodchips were taken from a W0-P treatment flask at t = 12.7 days. Arrows point to visible mycelia. ....	95
Figure F-4. Representative photograph of foam without fungal attachment. This foam was taken from a F0-C treatment flask at t = 12.7 days. ....	96
Figure F-5. Representative photograph of foam with fungal attachment from a glucose treatment. This foam was taken from a F10-P treatment flask at t = 12.7 days. Arrows point to visible mycelia. ....	96
Figure F-6. Representative photograph of foam with fungal attachment from a treatment without glucose amendments. The foam was taken from a F0-P treatment flask at t = 12.7 days. Arrows point to visible mycelia. ....	97
Figure H-1. pH data for Preliminary Aeration Experiment. Points represent mean values of triplicate reactors. Standard deviation for each point is less than 0.0 mg/m <sup>2</sup> . ....	110
Figure H-2. Attached fungal biomass for Preliminary Aeration Experiment. Points represent mean values of triplicate flask reactors. Error bars represent ± 1 standard deviation. ....	110
Figure H-3. Preliminary Aeration Experiment reactors at t = 7 days. The arrows point to mycelia mats that have formed on the solution surface of treatments that had not been aerated by day 1. ....	110
Figure I-1. pH data from initial attempt of Batch Experiment 2. ....	112
Figure I-2. Attached biomass from initial attempt of Batch Experiment 2. ....	113
Figure J-1. Sterile (a.) and non-sterile (b.) treatments with no initial pH adjustments (Treatments # 1-8). Treatments without woodchips have empty squares; treatments with woodchips have filled squares. Treatments with glucose were represented with green lines; treatments without glucose were represented with purple lines. ....	116
Figure J-2. Sterile + glucose (a.), non-sterile + glucose (b.), sterile + no glucose (c.), and non-sterile + no glucose (d.) treatments with initial pH adjustment to 4.3. ....	

Treatments without woodchips have empty squares; treatments with woodchips have filled squares. Treatments with glucose were represented with green lines; treatments without glucose were represented with purple lines. Buffered treatments have dotted lines; un-buffered treatments have solid lines..... 117

Figure K-1. Predictive model (using Excel) to determine the time at which oxygen utilization by aerobic bacteria,  $r$ -utilization, overcomes the estimated mass-transfer of oxygen into solutions when the initial substrate concentration is ~10.67 g BOD/L (i.e. in non-sterile glucose treatments). ..... 121

Figure L-1. Selected atoms (horizontally-striped red and white circles) within the atrazine molecule to determine the maximum molecular diameter. Adapted from Dyer, 2010..... 124

## LIST OF TABLES

Table 1-1. Frequently detected EDCs in surface and groundwater samples studied by Focazio et al. (2008). Adapted from Table 3 in: <i>A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States – II) Untreated drinking water sources</i> (Focazio et al., 2008).....	4
Table 1-2. Compounds detected in the waters of the Living Filter in State College, PA. Adapted from Nemitz and Parizek (2005). .....	5
Table 1-3. Chemical oxidizers used for the removal of EDCs. Adapted from Lui et al. (2009). .....	7
Table 1-4. Some white-rot fungal species and selected EDCs that they are known to degrade. Adapted from Cabana et al. (2007).....	9
Table 1-5. Reactions involved in contaminant oxidation by LiP and MnP. ....	12
Table 1-6. Experimentally determined redox potentials of Lac, LiP, and MnP. ....	13
Table 1-7. Characteristic properties of atrazine (US EPA, 2003).....	16
Table 2-1. Packing materials used in batch reactors. ....	21
Table 2-2. Treatment matrix for Batch Experiment 1.....	25
Table 2-3. Treatment matrix for Batch Experiment 3.....	27
Table 3-1. Wastewater effluent characteristics from seven locations within the Penn State WWTP.....	30
Table 3-2. Maximum average (mean) attached biomass on Tri-pack and the time at which it was observed in Batch Experiment 1. Biomass in sterile treatments is assumed to be fungal; biomass in non-sterile treatments is assumed to be fungal + microbial. Treatment codes are provided in Table 2-2. ....	33
Table 3-3. Maximum average (mean) attached biomass on Tri-pack and the time at which it was observed in Batch Experiment 2. ....	39
Table 3-4. Percent atrazine removal and calculated removal rate based on linear degradation following the initial atrazine removal via non-linear sorption. ....	44
Table J-1. Experimental design to compare the effects of sterility, glucose, woodchips, and buffer on pH. ....	115
Table L-1. Average bond lengths used in the estimation of the projected area of an atrazine molecule. Adapted from Schwarzenbach et al. (2003). ....	123

## **ACKNOWLEDGEMENTS**

Many thanks to my advisor, Dr. Rachel Brennan, for her guidance, support, and well-timed words of encouragement throughout my research experience. Thanks to Dr. John Regan and Dr. Stephanie Velegol for serving on my committee and contributing to the review of my thesis. Thanks also to Dr. Robert Shannon, Associate Professor of Agricultural Engineering, for his support over my many years at Penn State.

I would also like to thank the environmental engineering graduate students, especially those in the basement of Sackett, for their technical support, advice, and mood-lightening conversations. Thanks to the many faculty and staff members who have graciously given their time and imparted their knowledge along the way. Thanks also to Dr. Ming Tien, Professor of Biochemistry, Dr. Frank Dorman, Associate Professor of Biochemistry and Molecular Biology, and Dr. John Janowiak, Professor of Wood Products Engineering.

Finally, I would like to thank my family and friends for believing in me. Without them, my journey through graduate school would have been a much bumpier ride.

# **1. Introduction**

## **1.1. The Endocrine System, Endocrine-Disrupting Compounds and Health Risks**

### **1.1.1. The Endocrine System**

The endocrine system, or the hormone system, is “the internal system of chemical communication involving hormones, the ductless glands that secrete hormones, and the molecular receptors on or in target cells that respond to hormones” (Campbell et al., 1999). In vertebrates, the endocrine system regulates biological processes including sexual development, growth, and metabolism (Campbell et al., 1999). To regulate these processes, glands release hormones (US EPA, 2010b). The hormones travel through the body to locate and bind to their matching receptors. After binding, the receptor interprets the hormone’s chemical signal by altering proteins or turning on genes to synthesize new proteins (US EPA, 2010b).

The major glands of the vertebrate endocrine system are the hypothalamus, pituitary glands, thyroid gland, pancreas, adrenal glands, gonads, pineal gland, and thymus (Campbell et al., 1999; US EPA, 2010b). The hypothalamus links the endocrine system and the nervous system (Campbell et al., 1999; US EPA, 2010b). The pituitary gland is controlled by the hypothalamus and 1) secretes hormones made by the hypothalamus and 2) produces hormones to regulate other endocrine glands (US EPA, 2010b). The thyroid gland is involved in development, bioenergetics, and homeostasis, whereas the pancreas regulates blood glucose concentration (Campbell et al., 1999). The adrenal glands produce hormones to respond to stress and regulate blood pressure, glucose metabolism, and the body’s salt and water balance (Campbell et al., 1999;

US EPA, 2010b). The gonads, testes in males and ovaries in females, regulate growth, development, reproductive cycles, and sexual behavior (Campbell et al., 1999).

### **1.1.2. Endocrine-Disrupting Compounds and Health Risks**

An endocrine disruptor, as defined by Kavlock et al. (1996) and adopted by the United States Environmental Protection Agency (US EPA), is an “exogenous agent that interferes with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes”. Numerous compounds, man-made and natural, are considered to be endocrine-disrupting compounds (EDCs). EDCs can be found in many pharmaceutical drugs, personal care products, household cleaning agents, and pesticides. Some of the well-known EDCs include atrazine (a pesticide), 17 $\beta$ -estradiol (a hormone), and phthalates (a plasticizer).

Natural hormone concentrations in animals are very low (for example, 17 $\beta$ -estradiol is typically < 50 pmol/L in women (Ziomkiewicz et al., 2008)); therefore, it is possible that even low concentrations of EDCs could interrupt the normal functions of the endocrine system (Diamanti-Kandarakis et al., 2009). The potential health risks are those that would be associated with endocrine disruption in general. Risks include different types of cancers, attention-deficit disorder, birth defects, infertility, sexual under-development, changes in sexual behavior, and changes in the function of the thyroid and adrenal glands (Vogel, 2004). Currently, there is much debate over the issue of EDC-related health risks. Although some scientists believe that research supports a causative relationship between certain EDCs and negative health effects (Ostby, 1999; Thayer et al., 2001), others believe that many studies lack necessary considerations and parameters to make these relationships (Kavlock et al., 1996; Safe, 2000; International Programme on Chemical Safety, 2002; Diamanti-Kandarakis et al., 2009). Currently, through the

Endocrine Disruptor Screening Program (EDSP), the EPA is determining: 1) which EDCs should be regulated; 2) screening methods for monitoring EDCs in drinking water and food; and 3) policies and procedures for screening contaminants (US EPA, 2010a).

## **1.2. EDC Occurrence in Wastewater**

Over the years, EDCs have entered our wastewater in many forms including: 1) pesticides in agricultural runoff; 2) herbicides in private-lawn runoff; 3) prescription medications and metabolites in human excretion; and 4) personal care products in water that had been washed down a household drain. EDCs have been detected in wastewater and effluent-receiving waters across the United States (Kolpin et al., 2002; Glassmeyer et al., 2005; Bartelt-Hunt et al., 2009) and in foreign countries such as England (Jiang et al., 2005), Germany (Petrovic et al., 2004), Sweden (Petrovic et al., 2004), China (Zhang et al., 2008), and Spain (Kuster et al., 2008). Typically, compound concentrations are on the scale of nanograms per liter (ppt) or micrograms per liter (ppb) (Pennsylvania Department of Environmental Protection, 2011).

In 2008, the United States Geological Survey (USGS) reported that the most commonly detected EDCs in surface waters were cholesterol (natural sterol), metolachlor (herbicide), cotinine (nicotine metabolite),  $\beta$ -sitosterol (natural plant sterol), and 1,7-dimethylxantine (caffeine metabolite) (Focazio et al., 2008). In groundwater, the most common compounds were tetrachlorethylene (solvent), carbamazepine (pharmaceutical), bisphenol-A (plasticizer), 1,7-dimethylxantine (caffeine metabolite), and tri (2-chloroethyl) phosphate (fire retardant) (Focazio et al., 2008). Table 1-1 lists the overall most frequently detected compounds in surface and groundwater as reported by Focazio et al. (2008).



**Table 1-1.** Frequently detected EDCs in surface and groundwater samples studied by Focazio et al. (2008). Adapted from Table 3 in: *A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States – II) Untreated drinking water sources* (Focazio et al., 2008).

Compound	Associated Use/Occurrence	Detection Limit (µg/L)	Detection Frequency (%)	Maximum Concentration (µg/L)
β-sitosterol	natural plant sterol	2	24	NQ; >DL
cholesterol	natural sterol	2	42	NQ; >DL
metolachlor	herbicide	0.5	40	0.67
prometon	herbicide	0.5	26	NQ
cotinine	nicotine metabolite	0.023	35	0.10
1,7-dimethylxanthine	caffeine metabolite	0.018	23	0.30
cabamazepine	anticonvulsant	0.011	22	0.19
tri (2-chloroethyl) phosphate	manufacturing additive, fire retardant	0.5	20	NQ

Detection frequency is the percentage of surface and groundwater samples (combined) in which the compound was detected; NQ= Not quantified; >DL = Concentration is suspected to be greater than the detection limit.

Locally, EDCs have been detected in the Penn State Wastewater Treatment Plant (WWTP) effluent and in the groundwater and soils at the Penn State Living Filter (Table 1-2; Nemitz and Parizek, 2005). Nemitz and Parizek (2005) screened for 16 EDCs in the feed-water, groundwater, and soil of the Living Filter, an area of vegetated land on which Penn State WWTP effluent is spray-irrigated year-round. Of the 16 EDCs screened, 14 were detected in various water samples (Table 1-2; Nemitz and Parizek, 2005).

**Table 1-2.** Compounds detected in the waters of the Living Filter in State College, PA. Adapted from Nemitz and Parizek (2005).

Compound	Associated Use/Occurrence	Occurrence Frequency in WWTP Effluent Samples (%)	Occurrence Frequency in All Samples (%)	Maximum Concentration (µg/L)
triclosan	anti-microbial, disinfectant	8	3	0.02
cholesterol	natural sterol	25	5	0.02
caffeine	stimulant	42	11	0.13
bis (2-ethylhexyl) phthalate	plasticizer	17	35	0.29
trimethoprim	antibiotic	25	5	0.13
DEET	insect repellent	25	20	0.46
atrazine	fungicide	17	5	0.1
2,6 di-tert-butylphenol	UV-stabilizer, anti-oxidant	17	15	<0.1
acetaminophen	fever-reducer	25	4	<0.1
5-methyl-1H-benzotriazole	manufacturing additive, anti-corrosive	33	5	<0.1
ibuprofen	anti-inflammatory	17	1	<0.1
sulfamethoxazole	antibiotic	0	1	<0.1
estradiol	natural estrogenic hormone	0	1	<0.1
17α-ethinylestradiol	estrogenic, contraceptive	0	1	<0.1

Occurrence frequency in WWTP effluent samples is the percentage of the 12 samples in which the compound was detected. Occurrence frequency in all samples is the percentage of all samples (12 effluent, 14 overland flow, 13 wetland, 16 soil water, and 19 groundwater samples) in which the compound was detected. Maximum concentration is the maximum for all samples.

A survey of five streams in south-central Pennsylvania (PA) showed that discharged wastewater effluent contributed detectable levels of multiple EDCs to each stream (Loper et al., 2007). A sample from one stream, Killinger Creek, contained 20 of the 46 EDCs included in the sample screening. Overall, 13 pharmaceuticals and 11 antibiotics were detected in samples at least once throughout the six-month study (Loper et al., 2007).

### 1.3. Removal of EDCs from Wastewater: Physical, Chemical, and Biological Techniques

Typical wastewater treatment processes are designed for the removal of suspended solids, phosphorus and nitrogen; however, they do not provide complete removal of EDCs (Auriol et al., 2006). Because of the potential ecological and human health risks of EDCs, public concern has

grown (Koplin et al., 2002; Westerhoff et al., 2005), and there is a clear need to develop cost-effective techniques for greater removal of EDCs from wastewater.

### **1.3.1. Physical and Chemical Removal**

Currently available technologies for physical and chemical removal include activated carbon (AC), membrane processes, and chemical advanced oxidation (CAO). Although these processes have been proven to be effective at removing EDCs (Lui et al., 2009; Auriol et al., 2006), they are quite expensive to operate and maintain.

Powdered activated carbon (PAC) has been shown to effectively remove many types of EDCs from synthetic and natural wastewater (reviewed in Lui et al., 2009). The physicochemical properties of the EDCs and the type of material used to create the PAC influence the removal of EDCs by adsorption. Although PAC has been shown to remove EDCs, removal capacity is greatly decreased as the complexity of the aqueous solution increases (Fukuhara et al., 2006; Snyder et al., 2007); therefore, it may be more suitable to include a pre-treatment step (Lui et al., 2009), such as membrane filtration to remove excess organic material that could block PAC pores or compete with EDCs for sorption sites (Snyder et al., 2007).

Membrane processes, such as filtration (ultra-, micro-, and nano-) and reverse osmosis, have also been shown to remove EDCs from wastewater (reviewed in Auriol et al., 2006, and Lui et al., 2009). Similar to removal by PAC, the physicochemical properties of the EDCs are important factors for removal efficiency. Some of the properties to consider include molecular weight, octanol-water partition coefficient, solubility, and molecular charge (Lui et al., 2009). High molecular weight, high octanol-water partition coefficient, low solubility, and high molecular charge are all favorable properties for EDC removal via membrane processes. In addition, the properties of the membrane will affect removal efficiency. In most cases, removal

(rejection) by membranes occurs via size exclusion, charge repulsion, and adsorption (Lui et al., 2009). Although membrane processes can be effective tools for removing EDCs from wastewater, they are costly and require frequent maintenance to avoid fouling issues (Auriol et al., 2006).

Chemical removal of EDCs may be achieved using CAO. Table 1-3 lists common oxidizers used. Certain oxidizers, like ozone or hydrogen peroxide, may be combined with UV to increase removal efficiency by the production of hydroxyl radicals. Unfortunately, combining these techniques increases the cost of CAO.

**Table 1-3.** Chemical oxidizers used for the removal of EDCs. Adapted from Lui et al. (2009).

Chemical Oxidizer	Half Reaction	Redox Potential, $E^0$ (V)
Ferrate	$\frac{1}{3} \text{FeO}_4^{2-} + \frac{8}{3} \text{H}^+ + \text{e}^- \rightarrow \frac{1}{3} \text{Fe}^{3+} + \frac{4}{3} \text{H}_2\text{O}$	0.73
Ozone	$\frac{1}{2} \text{O}_3 + \text{H}^+ + \text{e}^- \rightarrow \frac{1}{2} \text{O}_2 + \frac{1}{2} \text{H}_2\text{O}$	1.04
Dithionite	$\frac{1}{2} \text{S}_2\text{O}_4^{2-} + \text{e}^- \rightarrow \frac{1}{2} \text{SO}_4^{2-}$	1.01
Hydrogen Peroxide	$\frac{1}{2} \text{H}_2\text{O}_2 + \text{H}^+ + \text{e}^- \rightarrow \text{H}_2\text{O}$	0.89
Chlorine	$\frac{1}{2} \text{Cl}_2 + \text{e}^- \rightarrow \text{Cl}^-$	0.68
Chlorine Dioxide	$\text{ClO}_2 + \text{e}^- \rightarrow \text{ClO}_2^-$	0.95
Hypochlorite	$\frac{1}{2} \text{ClO}^- + \frac{1}{2} \text{H}_2\text{O} + \text{e}^- \rightarrow \frac{1}{2} \text{Cl}^- + \text{OH}^-$	0.42

Effective treatment of many EDCs can be achieved with CAO, however, complete mineralization of all compounds in a wastewater stream may not be achieved. For instance, the use of chlorination as a chemical oxidant is not effective at removing estrogenic activity (Hu et al., 2002; Hu et al., 2003; Moriyama et al., 2004). In addition, mutagenic and carcinogenic

chlorinated by-products may be formed from the chlorination of certain EDCs (Moriyama et al., 2004).

### **1.3.2. Biological Removal: Microbial and Fungal**

Aerobic microbial degradation of certain EDCs is possible with the use of activated sludge or trickling filter treatments (Auriol et al., 2006). Anaerobic degradation may be achieved using anaerobic digesters. Of these treatments, the activated sludge process is the only one that provides at least partial removal of estrogenic activity; increasing the solid-retention time may increase the effectiveness of the activated sludge process (Auriol et al., 2006; Lui et al., 2009).

Aerobic fungal degradation of EDCs via enzymatic biocatalysis is currently a growing field of research. Many species of fungi produce enzymes that have been shown to degrade multiple EDCs (Table 1-4; Reddy, 1995; Bumpus and Aust, 2005; Cabana et al., 2007; Hwang et al., 2008). The degradation of EDCs by fungal mycelia, also known as mycoremediation, has been shown to occur in batch systems supported by nutrient media (Soares et al., 2005; Blázquez and Guieysse, 2008; Hwang et al., 2008). In addition, bench-scale continuous-flow systems in nutrient media have successfully produced enzyme activity (Shim and Kawamoto, 2002; Pocić et al., 2009) and/or shown degradation of organic contaminants such as azo dye (Orange II; Zhang et al., 1999), 2-chlorophenol (Lewandowski et al., 1990), 4-chlorophenol (Yum and Pierce, 1998), 2,4-dichlorophenol (Yum and Pierce, 1998), and 2,4,6-trichlorophenol (Ehlers and Rose, 2005).

**Table 1-4.** Some white-rot fungal species and selected EDCs that they are known to degrade. Adapted from Cabana et al. (2007).

<b>Fungus</b>	<b>EDC Degraded</b>	<b>Spiked EDC Concentration (mg/L)</b>	<b>Percent Removal (%)</b>	<b>Reference</b>
<i>Bjerkandera sp.</i>	nonylphenol	45	95 (after 5 days)	Soares et al., 2006
<i>Daldinia concentrica</i>	dibutylphthalate	28	94 (after 1 day); 100 (after 6 days)	Lee et al., 2004
<i>Heterobasidium insulare</i>	bisphenol A	200	77 (after 3 days); 100 (after 14 days)	Dorado et al., 2000
<i>Phanerochaete chrysosporium</i>	dibutylphthalate	28	85 (after 6 and 20 days)	Lee et al., 2004
<i>Phanerochaete sordida</i>	estrone	2.7	70 (after 3 days); 100 (after 6 days)	Tamagawa et al., 2006
<i>Trametes versicolor</i>	nonylphenol	45	90 (after 15 days)	Soares et al., 2006
	triclosan	72.4	60 (after 1 week); 90 (after 4 weeks)	Hundt et al., 2000
	dibutylphthalate	28	83 (after 1 day); 100 (after 6 days)	Lee et al., 2004

Although there have been many experiments exploring the degradation capacity of these fungi, there still have not been adequate studies involving large-scale application of these fungi in wastewater treatment, specifically for the removal of EDCs.

#### 1.4. White-Rot Fungi and Lignin Degradation

In nature, the enzymes involved in EDC biocatalysis are employed by white-rot fungi (WRF) for the purpose of degrading lignin, a recalcitrant and complex aromatic biopolymer that is biosynthesized from three precursor alcohols – coumaryl (*p*-hydroxyphenyl lignin units), coniferyl (guaiacyl lignin units), and sinapyl (syringyl lignin units) (Kirk and Farrell, 1987). Wood generally contains 20-30% lignin, and most of the lignin is intertwined with hemicellulose (Kirk and Farrell, 1987). This combination of lignin and hemicellulose surround the cellulose

(Kirk and Farrell, 1987), forming a matrix known as lignocellulose (Howard et al., 2003; Dashtban et al., 2010). This matrix gives protection to the hemicellulose and cellulose against microbial degradation (Dashtban et al., 2010). Degradation of hemicellulose and cellulose occurs via primary metabolism by fungi and other microbes (Pointing, 2001). Due to the complexity of lignin, the initial biodegradation must be non-specific and extracellular (Kirk and Farrell, 1987). This means that the enzymes involved in the initial degradation must have the ability to break down a variety of organic compounds. Although most microbes cannot degrade lignin, several fungi, including WRF, have the capacity to degrade this complex polymer (Dashtban et al., 2010).

Lignin degradation, a secondary metabolism process, occurs only if the fungi are nutrient-limited (Kirk and Farrell, 1987). Depending on the fungal species, secondary metabolism can be induced by limiting nitrogen, carbon, or sulfur; however, nitrogen-limited environments are typical for WRF because wood naturally has a low nitrogen content (Cowling and Merrill, 1966; Dill and Kraepelin, 1986). Additionally, WRF cannot gain energy from the breakdown of lignin (Pointing, 2001); therefore, an additional carbon energy source must be present (Kirk et al., 1976; Leatham, 1986).

In addition to nutrient limitations, the WRF must also have suitable pH, sufficient oxygen, and protection from mechanical stress. Most WRF prefer a pH range between 4 and 6, with an optimal pH of 4 – 4.5 (Kirk et al, 1978; Singh and Chen, 2008). In cultures, higher oxygen concentrations have resulted in greater ligninolytic activities (Kirk and Farrell, 1987). Some researchers have maintained liquid-culture dissolved oxygen (DO) between 5 and 7 mg/L (Lewandowski et al., 1990; Yum and Peirce, 1998; Zhang et al., 1999); however most investigators do not report DO, especially for stationary cultures. In stationary culture flasks, pure oxygen is usually added to the head space (Tien and Kirk, 1988). If agitation or continuous aeration is used to introduce oxygen into solution, a surfactant like Tween 80™ is typically added

to the solution to prevent suppression of enzyme activity via mechanical stresses (Asther et al., 1987; Tien and Kirk, 1988). Additionally, the fungi perform best in aerated or agitated cultures when they are immobilized. Although some studies have shown enzyme activity or compound degradation in agitated cultures without fungal immobilization (Zhang et al., 1998; Ergül et al., 2009), most research supports using immobilization techniques, like encapsulation in alginate spheres (Pallerla and Chambers, 1997; Enayatzamir et al., 2010) or attachment to a support material (Lewandowski et al., 1990; Venkatadri and Irvine, 1993; Yum and Peirce, 1998; Siddiqui et al., 2009), to avoid enzymatic suppression. To stimulate the ligninolytic system, specifically lignin peroxidase catalyzed reactions in cultures, it has been suggested to add a substrate, like veratryl alcohol (VA) (Leisola et al., 1984; Faison and Kirk, 1985). VA is a product of secondary metabolism, and it is used in a radical form (in conjunction with lignin peroxidase) to oxidize components of lignin (Singh and Chen, 2008).

To carry out the degradation of lignocellulose material, WRF employ extracellular ligninolytic and accessory enzymes, as well as mediating substrates (Dashtban et al., 2010). Ligninolytic enzymes include the phenol oxidase enzyme, laccase (Lac), and heme peroxidase enzymes, such as manganese peroxidase (MnP), lignin peroxidase (LiP), and versatile peroxidase (VP) (Martinez et al., 2005). Lac enzymes oxidize aromatic and non-aromatic compounds using oxygen as the electron acceptor (Mai et al., 2004). The heme peroxidases, LiP, MnP, and VP, are stable in a reduced form. The enzymes are activated (oxidized; two-electron loss) by hydrogen peroxide (Table 1-5) (Singh and Chen, 2008). After this step, LiP and MnP behave differently; however it is important to note here that VP can perform the tasks of LiP and MnP (Wessenberg et al., 2003; Asgher et al., 2008). Following oxidation, LiP can then 1) directly oxidize non-phenolic and phenolic compounds or 2) oxidize a mediator substrate, usually veratryl alcohol, which forms a radical. The substrate radical can then oxidize phenolic and non-phenolic compounds.



**Table 1-5.** Reactions involved in contaminant oxidation by LiP and MnP.

Enzyme	Reactions	Reference
LiP	$\text{LiP} + \text{H}_2\text{O}_2 \rightarrow \text{LiPI} + \text{H}_2\text{O}$ $\text{LiPI} + \text{Substrate} \rightarrow \text{LiPII} + \text{Substrate}\cdot$ $\text{LiPII} + \text{Substrate} \rightarrow \text{LiP} + \text{Substrate}\cdot$	Singh and Chen, 2008
MnP	$\text{MnP} + \text{H}_2\text{O}_2 \rightarrow \text{MnPI} + \text{H}_2\text{O}$ $\text{Mn(II)} + \text{MnPI} \rightarrow \text{Mn(III)} + \text{MnPII}$ $\text{Mn(III)} + \text{Chelator}_2 \rightarrow \text{Mn(Chelator)}_2^{3+}$ $\text{Mn(II)} + \text{MnPII} + \text{Chelator}_2 \rightarrow \text{Mn(Chelator)}_2^{3+} + \text{MnP}$ $\text{Mn(Chelator)}_2^{3+} + \text{Substrate} \rightarrow \text{Mn(II)} + \text{Chelator}_2 + \text{Substrate}\cdot$	Wariishi et al., 1989; Singh and Chen, 2008

LiPI = intermediate 2 electron-oxidized enzyme; Substrate = organic substrate; LiPII = intermediate 1 electron-oxidized enzyme; Substrate $\cdot$  = oxidized substrate

MnPI = intermediate 2 electron-oxidized enzyme; MnPII = intermediate 1 electron-oxidized enzyme; Chelator = organic acid (ex. oxalate and malonate)

Unlike LiP, MnP cannot directly oxidize the lignin components; instead, MnP oxidizes Mn(II) to Mn(III), which reacts with a chelator, typically an organic acid produced by the fungus (Table 1-5) (Martinez et al., 2005). This chelated complex is then released from the enzyme surface to oxidize phenolic compounds (Wessenberg et al., 2003). In addition to the lignin-degrading enzymes, important accessory enzymes are produced. Accessory enzymes include 1) oxidases that generate the  $\text{H}_2\text{O}_2$  required by peroxidases and 2) dehydrogenases that oxidize products originating from lignin (Martinez et al., 2005). Experimentally determined redox potentials of the ligninolytic enzymes (except VP) from multiple sources are listed in Table 1-6. In comparison to the chemical oxidants previously listed (Table 1-3), Lac appears to be comparable to hypochlorite, chlorine, and ferrate; however, the redox potentials for MnP and LiP appear to be much lower than the inorganic oxidants.

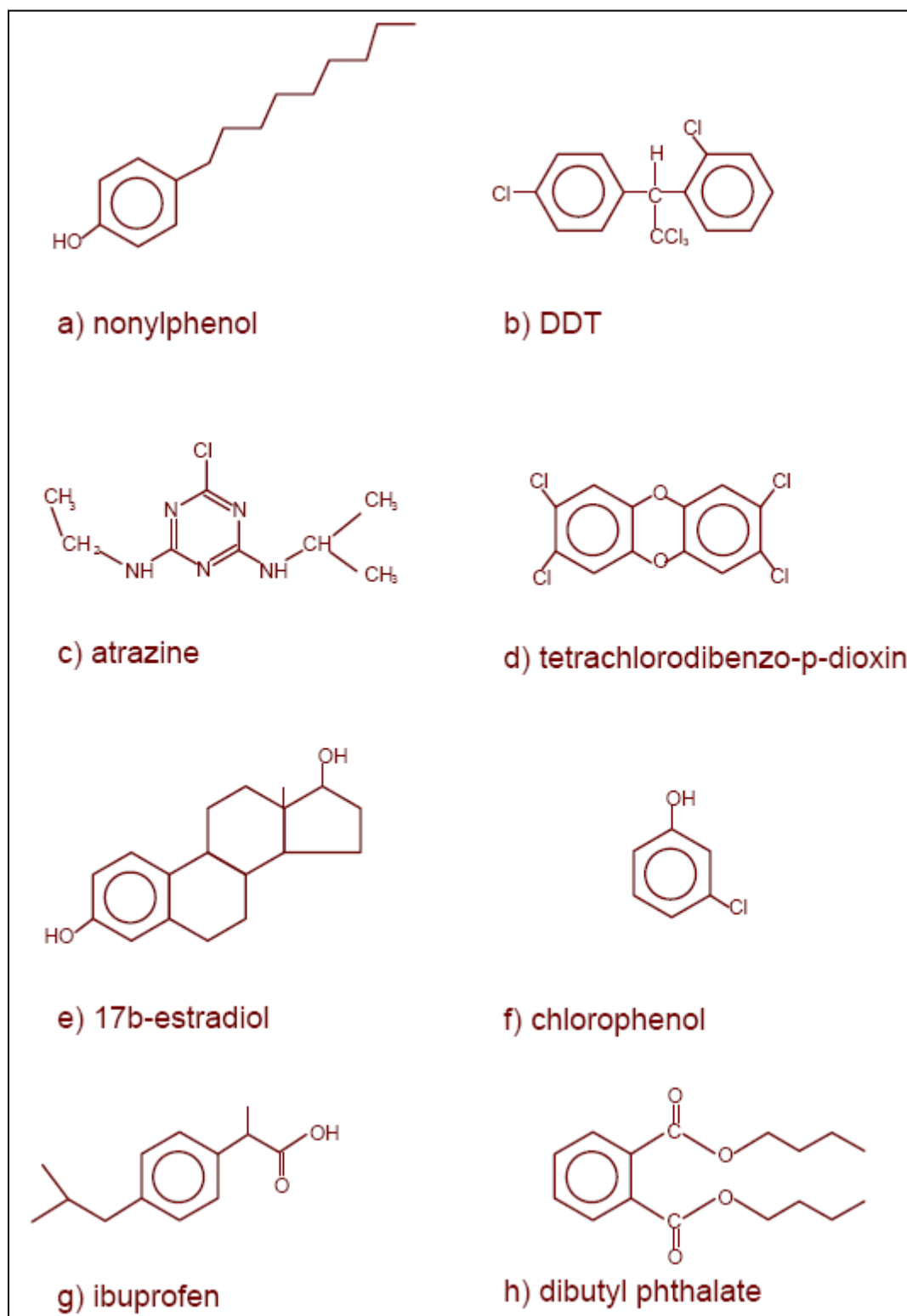
**Table 1-6.** Experimentally determined redox potentials of Lac, LiP, and MnP.

Isoenzyme	Fungus	Redox Potential,		Reference
		E (V)	Conditions	
Lac1 (T1* site)	Basidiomycete C30	0.73	T = 55°C; pH 5.7	Klonowska et al., 2002
Lac2 (T1 site)	Basidiomycete C30	0.56	T = 55°C; pH 5.7	Klonowska et al., 2002
Lac** (T1 site)	<i>Polyporus pinsitus</i>	0.79	T = 20°C; pH 5.3	Xu et al., 1996
Lac** (T1 site)	<i>Myceliophthora thermophila</i>	0.48	T = 20°C; pH 5.3	Xu et al., 1996
LiP (H1)	<i>Phanerochaete chrysosporium</i>	0.064	T = 25°C; pH 3.5	Millis et al., 1989
LiP (H8)	<i>Phanerochaete chrysosporium</i>	0.069	T = 25°C; pH 3.5	Millis et al., 1989
MnP (H4)	<i>Phanerochaete chrysosporium</i>	0.079	T = 25°C; pH 3.5	Millis et al., 1989

\*T1 is 1 of 4 copper sites; \*\*Isoenzyme was not specified.

### 1.5. *Phanerochaete chrysosporium* and Degradation of EDCs

*Phanerochaete chrysosporium*, a well-known WRF, has been studied extensively under ideal conditions. It is considered to be a model WRF for studying the ligninolytic system because it produces a more complete enzyme complex than most (Singh and Chen, 2008). *P. chrysosporium* produces several LiP and MnP isoenzymes with reported activities on the order of  $10^3$  -  $10^4$  U/L for LiP (Singh and Chen, 2008) and  $10^1$  –  $10^2$  U/L for MnP (Moreira et al., 1998; Pickard et al., 1999). *P. chrysosporium* does not produce Lac (Kirk and Farrell, 1987) or VP (Dashtban et al., 2009); however, it is one of the most efficient lignin-degrading microorganisms that have been studied (Dashtban et al., 2009). Because of this, it can also degrade recalcitrant contaminants, like many EDCs, that are structurally analogous to lignin (Figure 1-1).



**Figure 1-1.** Several common EDCs that share structural similarities to lignin.

A few of the EDCs that have been shown to be degraded by *P. chrysosporium* include nonylphenol (Soares et al., 2005), chlorinated phenols (Aust, 1990), dibutyl phthalate (Lee et al., 2004), 17 $\beta$ -estradiol (Mao et al., 2010), tetrachlorodibenzo-*p*-dioxin, and 1,1-*bis*(4-chlorophenyl)-2,2,2-trichloroethane (DDT) (Bumpus et al., 1985). In this study, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine), a commonly used herbicide, was selected for use.

## **1.6. Atrazine**

### **1.6.1. Use, Occurrence in the Environment, and Health Risks**

Atrazine (Figure 1-3) is the most heavily used agricultural herbicide in the United States (US EPA, 2011). An estimated 76.4 million pounds of atrazine are applied each year; 86% of this is applied to corn, 10% to sorghum, and 4% to other crops. In the US, agricultural use of atrazine is highest in Iowa, Illinois, Indiana, Ohio, Nebraska, and Delaware. Use of atrazine on lawns is highest in Florida and other southeastern states (US EPA, 2011). Several properties of atrazine are listed in Table 1-7. Based on these properties, atrazine is likely to sorb to organic matter. Although atrazine has a low vapor pressure, it is possible for it to co-evaporate with water (Scribner et al., 2005).

**Table 1-7.** Characteristic properties of atrazine (US EPA, 2003).

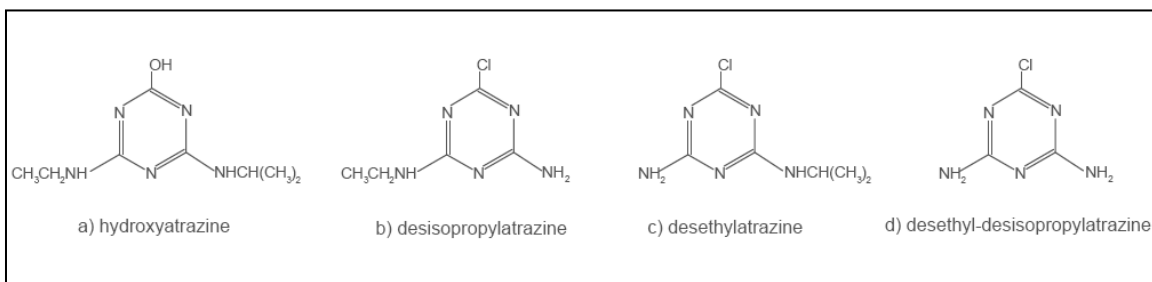
<b>Property</b>	<b>Value</b>
Octanol-Water Partition Coefficient (K <sub>ow</sub> )	10 <sup>2.75</sup>
Soil Organic Carbon Partition Coefficient (K <sub>oc</sub> )	10 <sup>2.09</sup>
Henry's Law Coefficient (K <sub>H</sub> )	10 <sup>-6.92</sup>
Vapor Pressure	3 x 10 <sup>-7</sup> mm Hg
Density	1.19 g/mL
Aqueous Solubility	0.03 g/L

Values were measured at T = 20°C.

The maximum contaminant level (MCL) established by the Safe Drinking Water Act for atrazine is 3.0 ppb (US EPA, 2011). Although concentrations are rarely above the MCL, atrazine is one of the most commonly detected surface and groundwater contaminants in agricultural areas of the country (Wu et al., 2010). An extensive USGS study of surface water, groundwater, and precipitation in the Midwest found that, of the waters sampled, approximately 76% of stream samples (at time of harvest), 82% of reservoir samples (from April, 1992 to September, 1993), 24% of groundwater samples (in 1993), and 30% of precipitation samples (from March 1990 to September, 1991) had detectable (> 0.05 ppb) concentrations of atrazine (Scribner et al., 2005). In addition, atrazine is one of the 14 EDCs that have been detected in the effluent of the Penn State WWTP at a maximum concentration of 0.1 ppb (Nemitz and Parizek, 2005).

The major metabolites of atrazine are hydroxyatrazine, desethylated atrazine (DEA), desisopropyl atrazine (DIA), and diaminochlorotriazine (DACT; Figure 1-2). These metabolites are also metabolites of other triazine pesticides. The metabolites are of concern because their persistence and toxicity are comparable to those of the parent compounds (Gammon et al., 2001). Additionally, aqueous concentrations of triazine metabolites may be equal to or greater than parent compound concentrations (Scribner et al., 2000). Metabolite concentrations in groundwater are typically higher than parent compound concentrations (Scribner et al., 2000) because the metabolites are more polar and, therefore, more susceptible to leaching from soil into

groundwater (Gammon et al., 2001). In surface water, the parent compound concentrations are generally higher than metabolite concentrations during times of heavy herbicide application; however, as the growing season continues, the parent compound concentrations decrease and metabolite concentrations increase (Scribner et al., 2000).



**Figure 1-2.** Atrazine metabolites.

Atrazine has low acute toxicity and is likely not a human carcinogen (US EPA, 2011); however, chronic exposure may cause adverse health effects to humans and wildlife. A recent study suggests that chronic low-dose exposure to atrazine can contribute to insulin resistance and obesity in rats (Lim et al., 2009). Another study found that exposure to atrazine at concentrations below the MCL impaired reproduction of the Fathead minnow (Tillet et al., 2010). Other adverse effects of atrazine exposure include altered gene expression in tadpoles (Langerveld et al., 2009), altered morphology of Rainbow trout (Fischer-Scherl et al., 1991), and increased incidence of benign and malignant mammary tumors in rats (Gammon et al., 2001).

### 1.6.2. Atrazine Degradation

Abiotic hydrolysis of atrazine does not appear to occur at environmental pH and temperature conditions; however, at a pH below 5, it may be possible to cleave the triazine ring (Gammon et al., 2001). Atrazine breakdown via photolysis does not appear to be a significant degradation pathway (Gammon et al., 2001).

Microbial degradation of atrazine occurs naturally in soils by at least four species of bacteria and 21 species of fungi (Gammon et al., 2001). Soil bacteria that participate in atrazine degradation include *Pseudomonas* and *Cytophaga* species (Alvey and Crowley, 1996). The major forms of microbial degradation are dealkylation, hydrolysis at the chlorine atom to form hydroxyatrazine, and cleavage of the triazine ring (Gammon et al., 2001).

Because atrazine is an extremely common EDC, much research employing WRF for degradation has already been done, providing a valuable knowledge base upon which to test innovative technologies. Although the complete mineralization of atrazine by WRF has not been observed, the production of the hydroxyatrazine and dealkylated metabolites in liquid culture has been documented, and correlations between growth and enzyme production have been well described (Mougin et al., 1994; Masaphy et al., 1996). The majority of previous studies on WRF degradation of atrazine were carried out in soil (Hickey et al., 1994; Bastos and Magan, 2009) or in nutrient broth (Mougin et al., 1994; Masaphy et al., 1996; Mougin et al., 1997; Bending et al., 2002); no studies of atrazine degradation by WRF in wastewater were encountered during the literature review. In addition, of all the mycodegradable EDCs documented in the effluent of the PSU WWTP, atrazine gives the best response on liquid chromatography-mass spectrometry, allowing for the lowest detection limit. For these reasons, atrazine was selected as a prototypical compound to aid in technology and method development for the fungal biocatalysis of EDCs in wastewater.

## **1.7. Scope of Study**

This study was designed to investigate how the growth and enzyme production of the commonly-studied WRF, *Phanerochaete chrysosporium*, could be optimized for the treatment of EDCs in wastewater. Multiple nutrient conditions, packing materials (for immobilization), and

locations within a typical WWTP train were evaluated through a series of three batch experiments. The degradation capacity of *P. chrysosporium* for the prevalent EDC, atrazine, was also evaluated. **The goal of these batch experiments was to answer the following questions:**

#### **Batch Experiment 1**

- Will there be growth and enzyme activity at the natural pH (~7) of wastewater?
- Will there be growth and enzyme activity in non-sterile wastewater conditions?
- Does *P. chrysosporium* require supplemental organic carbon for growth and enzyme activity in wastewater?
- Is there a direct relationship between biomass and enzyme activity?

#### **Batch Experiment 2**

- Where in the treatment train would *P. chrysosporium* perform the best based on growth and enzyme activity?
- Will enzyme activity be inhibited by available nitrogen in the wastewater?
- Will the natural organic carbon in the wastewater serve as an acceptable carbon source?

#### **Batch Experiment 3**

- How are growth and enzyme activity affected by the packing (immobilization) material used (natural vs synthetic; slow-release carbon source vs non-degraded synthetic material)?
- Will *P. chrysosporium* degrade atrazine under experimental conditions? If so, what are the associated degradation kinetics?



## **2. Materials and Methods**

### **2.1. Water Source and Atrazine**

Wastewater used in this study was obtained from the Pennsylvania State University Wastewater Treatment Plant (WWTP) (State College, PA). Effluent from the secondary activated-sludge clarifier was used in Batch Experiment 1, 2, and 3 (Appendix A). In Batch Experiment 2, effluent from the rag removal, primary settling tank, and trickling filter were also evaluated (Appendix A). Wastewater was collected in 1 L and 2 L glass media bottles for immediate use.

Powdered atrazine (Chem Service, West Chester, PA) was dissolved in acetonitrile (EMD, USA) to a final concentration of 1000 mg/L. This stock solution was used to spike treatment solutions in Batch Experiment 3. Similarly, powdered atrazine-d5 (Sigma-Aldrich, St. Louis, MO) was dissolved in acetonitrile to a final concentration of 1000 mg/L. This atrazine-d5 (d5) was used as an internal standard in samples analyzed using liquid chromatography with tandem mass spectrometry (LC/MS/MS).

### **2.2. Packing Materials**

Polypropylene Tri-pack® (1"; Jaeger Products Inc., Houston, TX), birch wood chips (0.131" - 0.25"; genus *Betula*, Kish Lumber Co., Belleville, PA), and polyether polyurethane SIF II® Foam (ZS15D) cubes (0.5"; FXI Foamex Innovations, Media, PA) were used as packing materials (Table 2-1; Appendix B). Tri-pack® and ZS15D foam were not altered prior to use; however, the wood chips were further reduced in size by milling. A Wiley mill (Model No.

3; Arthur H. Thomas Co.,) was used to grind the chips (1 – 2” length), and chips were sieved to a particle size of 0.131” – 0.25” using U.S. Standard #1/4 and #6 sieves (Fisher-Scientific, USA). At the time of use, the wood chips contained approximately 7% moisture.

**Table 2-1.** Packing materials used in batch reactors.

Name	Material	Shape	Size (in)	Geometric Surface Area (ft <sup>2</sup> /ft <sup>3</sup> )
Tri-Pack®	Polypropylene	Sphere	1	85
SIF II® Foam (ZS15D)	Polyether Polyurethane Foam	Cube	0.5	240
Wood chip, <i>Betula</i>	Untreated Wood	Irregular/Cuboid	0.131 - 0.25	240 - 1100*

\*Wood chip geometric surface area range was estimated based on observed particle sizes.

Tri-pack® was used in Batch Experiment 1 and 2 solely as an attachment surface to immobilize the fungal mycelia. In Batch Experiment 3, the wood chips and foam were used as attachment surfaces and potential slow-release carbon sources.

### 2.3. Organism, Maintenance, and Cultivation Conditions

*P. chrysosporium* (ATCC 24725) was obtained from Dr. Ming Tien, Professor of Biochemistry in the Department of Biochemistry and Molecular Biology at The Pennsylvania State University. *P. chrysosporium* was maintained on YMPG media as described by Tien and Kirk (1988) and stored at 4 °C. See Appendix C for the complete YMPG medium recipe. In brief, the YMPG medium consisted of the following: 1 L of deionized water, 10 g of glucose (EMD, Gibbstown, NJ), 10 g of malt extract (BD, Sparks, MD), 2 g of peptone (BD, Sparks, MD), 2 g of yeast extract (BD, Sparks, MD), 1 g of L-asparagine (USB Corp., Cleveland, OH), 2 g of KH<sub>2</sub>PO<sub>4</sub> (Fisher Scientific, Fair Lawn, NJ), 1 g of MgSO<sub>4</sub> · 7H<sub>2</sub>O (Alfa Aesar, Ward Hill,

MA), 1 mg of thiamine (Sigma-Aldrich, St. Louis, MO), and 20 g of agar (BD, Sparks, MD). To maintain the fungal strain, fresh YMPG plates and slants were poured and inoculated every 3 - 5 months. The plates and slants were inoculated, aseptically, using small pieces of fungus-covered agar from an older plate and cultivated in a warm room at 30 °C until fungal mycelia covered the agar (approximately 3 to 5 days).

## **2.4. Inoculum Preparation for Batch Experiments**

Inoculum for the batch reactors was prepared, aseptically, from a 10% spore suspension in Low Nitrogen (LN) medium as adapted from Tien and Kirk (1988). See Appendix C for the complete LN medium recipe and inoculum preparation method. In brief, 1 L of medium contained: 10 g of glucose, 0.2 g of ammonium tartrate (Alfa Aesar, Ward Hill, MA), 1 mg thiamine, 100 mL of 0.1 M *trans*-aconitic acid (pH 4.3; TCI, Tokyo, Japan), 100 mL of Basal III medium, 60 mL of trace elements solution, and 740 mL of deionized water. The medium recipe varies slightly from Tien and Kirk's (1988) recipe in that *trans*-aconitic acid was used as the buffer instead of 2,2-dimethylsuccinate.

To make the spore suspension, sterile distilled deionized water was pipetted into fungal slants, and the fungus was gently scraped from the agar into solution. Solutions were then filtered through sterile glass wool to remove the mycelia from the spores. The absorbance of the resulting spore solution was measured at a wavelength of 650 nm using a UV-Spectrophotometer (UV-1601; Shimadzu Scientific Equipment, Columbia, MD). The spore suspension was diluted with sterile deionized water to an absorbance of 0.5 cm<sup>-1</sup>, which corresponded to a concentration of ~2.5 x 10<sup>6</sup> spores/mL (Kirk et al. 1978).

To a 2 L Erlenmeyer flask, 10 mL of spore suspension and 90 mL of LN medium were added and incubated at 30 °C for approximately five days. During the stationary incubation

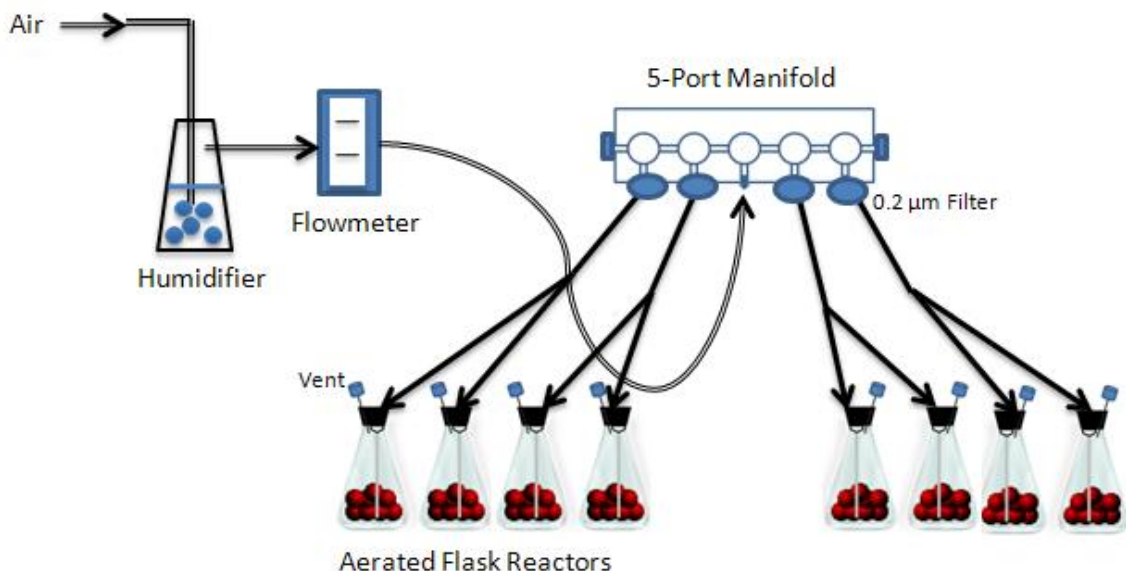
period, a mycelia mat formed on the solution surface. The mat and solution were homogenized using a hand-held blender (Model No. 2605; OSTER, USA). The homogenized mycelia solution was used as a 10% (v/v) inoculum in batch reactors, with the exception of Batch Experiment 1. In Batch Experiment 1, the inoculum was 5% (v/v). During the 5 day stationary growing period, the Batch Experiment 1 was augmented to include more variables, and the number of reactors was doubled.

## **2.5. Batch Reactor Setup**

Each sacrificial reactor consisted of a 150 mL glass Erlenmeyer flask containing 135 mL of solution and enough packing material to displace the solution and fill the flask to the 150 mL mark (see Chapter 2.6.1 through 2.6.3 for experiment-specific solution makeup and packing materials). Each reactor was sealed with an aeration apparatus consisting of a one-hole rubber stopper (No. 6.5) with a 20-gauge stainless steel venting needle and pass-through 1/16" inside diameter (ID) Tygon® tubing (Saint-Gobain, USA) connected to a 20-gauge stainless steel needle (Appendix B). For sterile reactors, aeration apparatus (except needles), flasks, packing materials, and prepared solutions (except mycelia solution) were autoclaved. At the time of reactor assembly, sterile 20-gauge needles were added to the aeration apparatus, and venting needles were filled with glass wool to prevent contamination.

Humidified lab air was used in the aeration system to minimize solution volume losses by evaporation (Appendix B). The air was introduced into a central port in the aeration manifold consisting of 5-port (female luer) polycarbonate manifolds connected by polypropylene (male luer) adapters (Figure 2-1). Cellulose acetate syringe filters (0.2 µm) were attached to remaining ports, and 1/8" ID Tygon® tubing (Saint-Gobain, USA) was attached to each filter. Each aeration line was split with polypropylene wyes and reduced to 1/16" ID tubing with

polypropylene reducing couplers. The reduction in tube diameter was necessary in order to pass the tubing through the stopper hole. Each reactor was aerated at a rate of  $\sim 0.3$  SCFH ( $2 \text{ cm}^3/\text{s}$ ).



**Figure 2-1.** Aerated batch reactor setup, illustrating the aeration system and individual reactors.

## 2.6. Batch Experiments

### 2.6.1. Batch Experiment 1

In the first batch experiment, the effects of sterile conditions (sterile vs. non-sterile), pH (optimal pH of 4.3 vs. wastewater pH of  $\sim 7.5$ ), and an organic carbon amendment (0 g/L glucose vs. 10 g/L glucose) on fungal growth and extracellular MnP and LiP activity were evaluated (Table 2-2). Note that a treatment of 0 g/L glucose implies that no additional glucose was added; however, there may be residual glucose or available organic carbon metabolites present within the fungal inoculum that was added to each treatment. Any residual glucose present within the inoculum was not accounted for during treatment preparations.

**Table 2-2.** Treatment matrix for Batch Experiment 1.

<b>Treatment #</b>	<b>Treatment Name</b>	<b>Sterile (+/-)</b>	<b>pH 4.3 (+/-)</b>	<b>Glucose (+/-)</b>
1	S-4.3-10	+	+	+
2	S-4.3-0	+	+	-
3	S-WW-10	+	-	+
4	S-WW-0	+	-	-
5	N-4.3-10	-	+	+
6	N-4.3-0	-	+	-
7	N-WW-10	-	-	+
8	N-WW-0	-	-	-

S = Sterile; N = Non-sterile; 4.3 = Initial pH of wastewater was adjusted to 4.3; WW = Initial pH of wastewater was unaltered; 10 = 10 g/L glucose; 0 = 0 g/L glucose; + = Yes; - = No.

All sacrificial reactors (120 flasks) contained: 9 Tri-pack®, 7 mL of mycelia solution, 6.75 mL of 1% Tween 80™ (Sigma-Aldrich, St. Louis, Missouri), and 121.25 mL of wastewater. Tween 80™, a surfactant, was added to protect the extracellular enzymes from mechanical degradation. On day 4 of the experiment, the reactors were augmented with 1 mL of 54 mM veratryl alcohol (0.4 mM in reactor solution; TCI, Tokyo, Japan) to stimulate enzyme activity.

Wastewater pH was either left unaltered or adjusted to 4.3, the optimal pH for enzyme production (Tien and Kirk, 1988). pH adjustments were made to the wastewater with 6.2 N hydrochloric acid (HCl; EMD, Gibbstown, NJ) prior to the addition of any other amendment. For glucose-amended treatments, powdered glucose was added to the wastewater to achieve a total solution concentration of 10 g/L, since this is the concentration used in a typical enzyme production medium (Tien and Kirk, 1988). Sterile treatments were prepared as described in Chapter 2.5 prior to the introduction of mycelia.

Treatment flasks were sacrificed in triplicate over a period of ~2 weeks using the methods in Appendix D. At each time point, solution pH, attached growth, suspended growth, and enzyme activities were measured. Attached and suspended growth were measured by drying the respective fungal, or fungal + microbial, biomasses on pre-weighed filter paper at 40 °C until a constant mass was reached. Attached growth was considered to be the growth that was physically attached to the packing material; suspended growth was considered to be the unattached biomass in solution. Approximately 10 mL of solution from each reactor was stored at -20 °C for analysis.

### **2.6.2. Batch Experiment 2**

In the second batch experiment, total biomass (fungus + naturally-occurring wastewater microbes) and extracellular MnP and LiP activity were evaluated in non-sterile, pH-adjusted wastewater effluent from four stages of treatment – rag removal, primary settling, trickling filtration, and secondary clarification (Appendix A).

All sacrificial reactors (60) contained: 9 Tri-pack®, 13.3 mL of mycelia solution, 6.75 mL of 1% Tween 80<sup>TM</sup>, and 114.95 mL of wastewater. On day 4 of the experiment, the reactors were augmented with 1 mL of 54 mM veratryl alcohol to yield a final concentration of 4 mM in reactor solution.

Initial pH adjustments to 4.3 were made to the wastewater effluents. Unlike Batch Experiment 1, pH changes throughout the experiment were occasionally corrected to 4.3. In Batch Experiment 2, all pH adjustments were made using 1 N HCl and 1 N sodium hydroxide (NaOH; J.T. Baker, Phillipsburg, NJ).

Treatment flasks were sacrificed and sampled as described in Chapter 2.6.1.

### 2.6.3. Batch Experiment 3

In the final batch experiment, the effects of a slow-release organic carbon-containing packing material (12 g wood chips vs. 25 ZS15D foam cubes) and an organic carbon amendment (0 g/L glucose vs. 10 g/L glucose) on LiP and MnP activities, pH, and fungus-catalyzed degradation of atrazine were evaluated under sterile conditions (Table 2-3). As in Batch Experiment 1, 0 g/L glucose does not imply the complete absence of glucose; residual glucose may be present within the fungal inoculum.

**Table 2-3.** Treatment matrix for Batch Experiment 3.

Treatment #	Treatment Name	Wood Chips (+/-)	Foam (+/-)	Glucose (+/-)	<i>P. chrysosporium</i> (+/-)
1	W-10-P	+	-	+	+
2	W-10-C	+	-	+	-
3	W-0-P	+	-	-	+
4	W-0-C	+	-	-	-
5	F-10-P	-	+	+	+
6	F-10-C	-	+	+	-
7	F-0-P	-	+	-	+
8	F-0-C	-	+	-	-

W = 12 g wood chips in each reactor; F = 25 foam cubes in each reactor; 10 = 10 g/L glucose; 0 = 0 g/L glucose; P = inoculated with *P. chrysosporium* ; C = Control with no fungus

All sacrificial reactors (100 flasks) contained: 12 g of birch wood chips or 25 ZS15D foam cubes (1.9 g), 13.5 mL of mycelia solution (active treatments) or LN medium (control treatments), 6.75 mL of 1% Tween 80<sup>TM</sup>, 114.75 mL of pH-adjusted wastewater with 0 g/L glucose or 10 g/L glucose amendments, and 0.68 mL of atrazine stock solution (1000 mg/L) to a final concentration of 5 mg/L atrazine. On day 4 of the experiment, the reactors were augmented with 1 mL of 54 mM veratryl alcohol.



Wastewater pH was adjusted to 4.3 with 1 N HCl prior to the addition of any other amendment. Glucose-amended treatments were prepared as in Chapter 2.6.1. Prior to the introduction of mycelia (active treatments) or LN medium (control treatments), wastewater treatments were sterilized as described in Chapter 2.5. To make the controls as similar as possible to the actives, sterile LN medium was used as the mycelia substitute since the mycelia were grown and homogenized in LN medium.

Active flasks were sacrificed in triplicate and control flasks were sacrificed in duplicate as described in Chapter 2.6.1. At each time point, solution pH, atrazine concentration, packing material mass with fungus (actives) and without fungus (controls), and enzyme activities were measured. Sacrificed packing materials were dried on pre-weighed pieces of aluminum foil at 40 °C until a constant weight was reached. Approximately 10 mL of solution from each reactor was stored at -20 °C for later analysis.

## **2.7. Analytical Methods**

Ammonium was measured using an electrode (Thermo Scientific Orion) connected to a pH/mV meter (Accumet® Basic AB15; Fisher Scientific, USA). Total organic carbon was measured using a total organic carbon analyzer (TOC-V CSN; Shimadzu Scientific Equipment, Columbia, MD). pH was measured using an electrode (Thermo Scientific Orion) connected to a pH meter (SympHony® SP70P; VWR, USA). The activities of lignin peroxidase and manganese peroxidase were measured spectrophotometrically by methods adapted from Tien and Kirk (1984) and Paszczyński et al. (1986), respectively (Appendix E). Atrazine removal was analyzed using an LC/MS/MS (3200 Q-TRAP LC/MS/MS System; AB Sciex, Foster City, CA) using a method created for the Brennan group by Dr. Rebecca Wittrig, AB Sciex Market Development Specialist (Appendix G).

## **2.8. Statistical Analysis**

Analysis of variance (ANOVA) was used to determine if there was a significant difference between atrazine removal in fungal and control treatments of Batch Experiment 3. Pair-wise comparison was completed using a Tukey's test. A 95% confidence interval ( $p = 0.05$ ) was used in the analyses. The analyses were completed using Minitab® 16 statistical software.

### 3. Results

#### 3.1. Wastewater Characteristics

To help determine the best location for a potential fungal bioreactor, wastewater was collected from seven different locations within the Penn State Wastewater Treatment Plant (Appendix A) and analyzed for ammonium and total organic carbon content (Table 3-1). As would be expected, ammonium and TOC generally decrease as wastewater flows through the plant. Effluent from the secondary activated-sludge clarifier contained the smallest amount of organic carbon and available nitrogen; therefore, it was used in all tests to represent the minimum nutrient levels that could be available to future fungal growth (Batch Experiments 1 – 3). To evaluate the feasibility of other potential fungal locations, effluent from the rag removal step, primary settling tank, and trickling filter was also evaluated in Batch Experiment 2.

**Table 3-1.** Wastewater effluent characteristics from seven locations within the Penn State WWTP.

Effluent Location	pH	Ammonium (mg/L $\text{NH}_4^+$ - N)	Total Organic Carbon (mg/L C)
Rag Removal	7.8	27	159
Primary Aeration	7.5	35	155
Primary Settling	7.5	35	149
Trickling Filtration	7.3	24	94.9
Anoxic/Trickling Filtration Combined	7.1	9.1	119
Secondary Aeration	7.0	0.7	63.4
Secondary Clarification	7.1	<0.5*	38.7

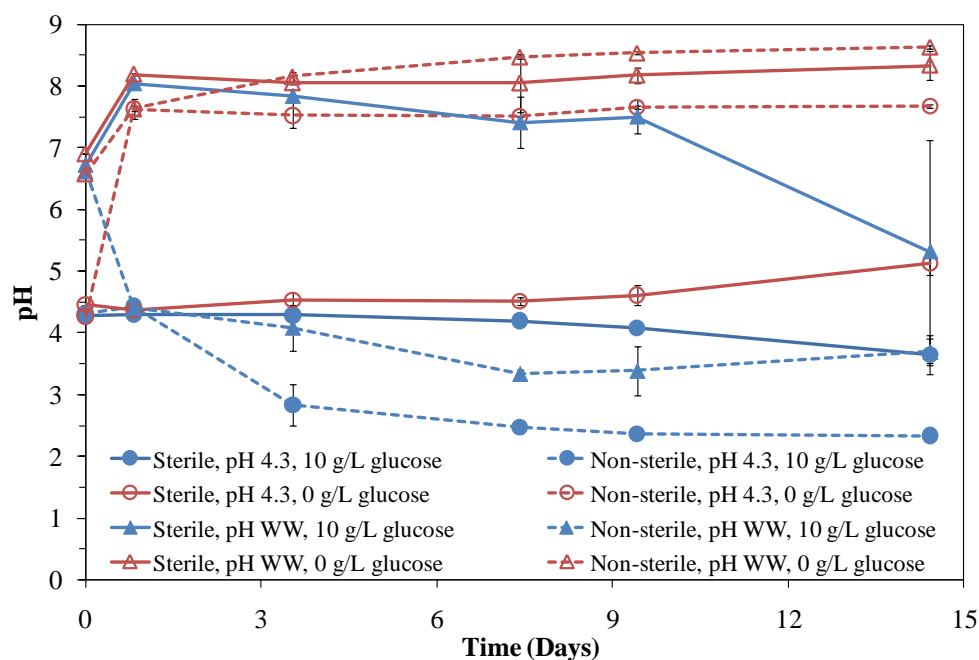
Shaded effluent locations were used in Batch Experiment 2. \*Detection limit = 0.5 mg/L  $\text{NH}_4^+$  - N.

### **3.2. Batch Experiment 1**

In this experiment, the effect of pH, sterile and non-sterile environments, and an organic carbon amendment on growth and enzyme activity in secondary clarifier effluent were evaluated in aerated batch flasks over a two-week period.

#### **3.2.1. pH**

By the first sampling time point ( $t = 0.8$  days), the pH of the non-sterile treatments without glucose amendments (N-4.3-0 and N-WW-0) increased to values above the initial pH of unamended secondary clarifier effluent ( $\sim 7$ ; Figure 3-1). After  $t = 3.5$  days, these treatments remained fairly stable at pH 7.6 (N-4.3-0) and 8.5 (N-WW-0). The pH of non-sterile treatments with glucose amendments (N-4.3-10 and N-WW-10) dropped below the optimal pH range (4.0 - 4.5) for fungal enzyme production; the pH of N-4.3-10 stabilized at pH 2.3 and N-WW-10 stabilized near pH 3.4 (Figure 3-1). The immediate drop in pH of the N-WW-10 (non-sterile,  $\text{pH}_0 \sim 7$ , 10 g/L glucose) treatment likely indicates the rapid aerobic and anaerobic microbial metabolism of the added glucose and production of acidic metabolites, whereas the rise in pH of the N-4.3-0 (non-sterile,  $\text{pH}_0$  4.3, 0 g/L glucose) treatment suggests rapid aerobic microbial metabolism of existing organic acids and  $\text{CO}_2$  stripping. Additionally, the rapidly increasing pH (from acid removal) could have shocked the fungus population, causing some die-off. If the microbes then degraded the fungal cells and liberated nitrogen in the form of ammonia, a weak base, the solution pH could be driven up.



**Figure 3-1.** The effect of indigenous wastewater microorganisms and supplemental glucose on pH in aerated batch reactors containing *P. chrysosporium* immobilized on Tri-pack in secondary clarifier effluent (Batch Experiment 1). Data points represent mean values of triplicate flask reactors; error bars represent  $\pm 1$  standard deviation. Sterile treatments are represented by solid lines; non-sterile treatments are represented by dotted lines. pH-adjusted treatments are represented by circles; un-adjusted treatments are represented by triangles. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by unfilled symbols.

The pH of the sterile treatments remained fairly stable, with the exception of S-WW-10 (sterile, pH<sub>0</sub> 4.3, 10 g/L glucose). By the last sampling point, two of the three remaining sterile, glucose-amended flasks (S-WW-10) were observed to be contaminated with microbial growth. The only flask assumed to be contaminant-free exhibited a pH of 7.38. Additionally, the sterile treatments which had been adjusted to pH 4.3 (S-4.3-0 and S-4.3-10) were the only treatments to remain close to the optimal pH of 4.3.

### 3.2.2. Attached Biomass and Enzyme Activity

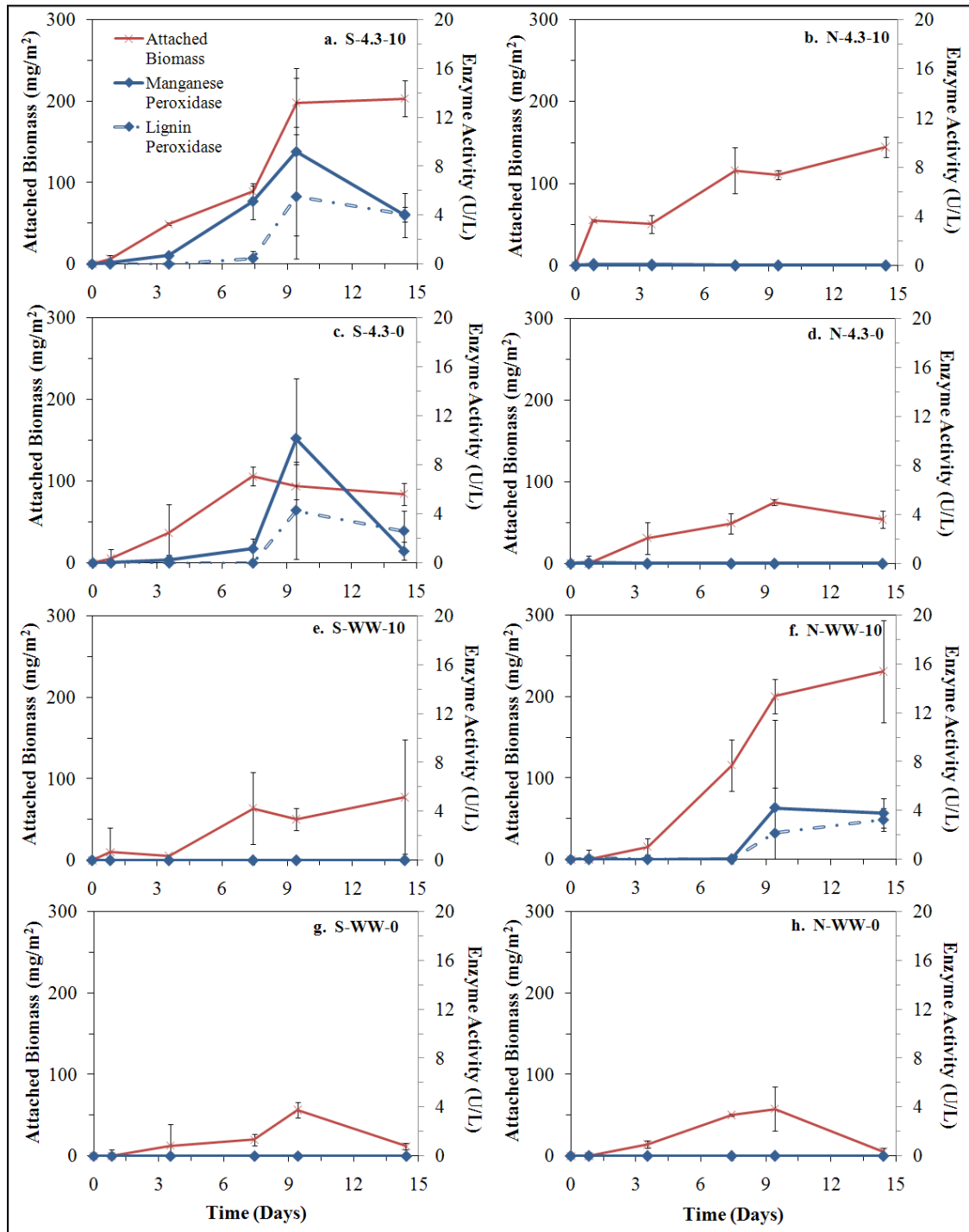
In all cases, treatments with glucose amendments resulted in greater attached biomass than their counterparts without glucose amendments. Treatments N-WW-10 (non-sterile, pH<sub>o</sub> ~7, 10 g/L glucose) and S-4.3-10 (sterile, pH<sub>o</sub> 4.3, 10 g/L glucose) resulted in the highest attached biomass with a maximum of  $230 \pm 62$  and  $203 \pm 22$  mg biomass/m<sup>2</sup> packing material, respectively, at t = 14.4 days (Table 3-2). Additionally, all treatments without glucose amendments exhibited losses in attached biomass by the end of the experiment (Figure 3-2).

**Table 3-2.** Maximum average (mean) attached biomass on Tri-pack and the time at which it was observed in Batch Experiment 1. Biomass in sterile treatments is assumed to be fungal; biomass in non-sterile treatments is assumed to be fungal + microbial. Treatment codes are provided in Table 2-2.

Treatment #	Treatment Name	Max. Avg.	Time (d)
		Attached Biomass (mg/m <sup>2</sup> )	
1	S-4.3-10	$203 \pm 22$	14.4
2	S-4.3-0	$106 \pm 11$	7.4
3	S-WW-10	$78 \pm 70$	14.4
4	S-WW-0	$56 \pm 10$	9.4
5	N-4.3-10	$144 \pm 12$	14.4
6	N-4.3-0	$75 \pm 4$	9.4
7	N-WW-10	$230 \pm 62$	14.4
8	N-WW-0	$58 \pm 27$	9.4

Of the eight treatments, only three treatments (S-4.3-10, S-4.3-0, and N-WW-10) exhibited MnP and LiP activities (Figure 3-2). In all cases, maximum observed enzyme activities occurred at t = 9.4 days, and MnP activity was greater than LiP activity. Maximum MnP activity was observed in treatment S-4.3-0 ( $10 \pm 5$  U/L), while maximum LiP activity was observed in

treatment S-4.3-10 ( $5.5 \pm 5$  U/L). The standard deviations for the enzyme activity measurements are quite high because of the high variability in the activity produced within the three representative treatment flasks sacrificed at each time point. For example, the maximum recorded LiP activity at  $t = 9.4$  days for treatment S-4.3-10 was 10 U/L and the minimum was 0 U/L. The MnP activity for this treatment at the same time point was a maximum of 14 U/L and a minimum of 3 U/L. For treatment S-4.3-0, the maximum recorded LiP activity at  $t = 9.4$  days was 8 U/L and the minimum was 0 U/L. For the same treatment and time, the maximum MnP activity was 16 U/L and the minimum was 6 U/L.



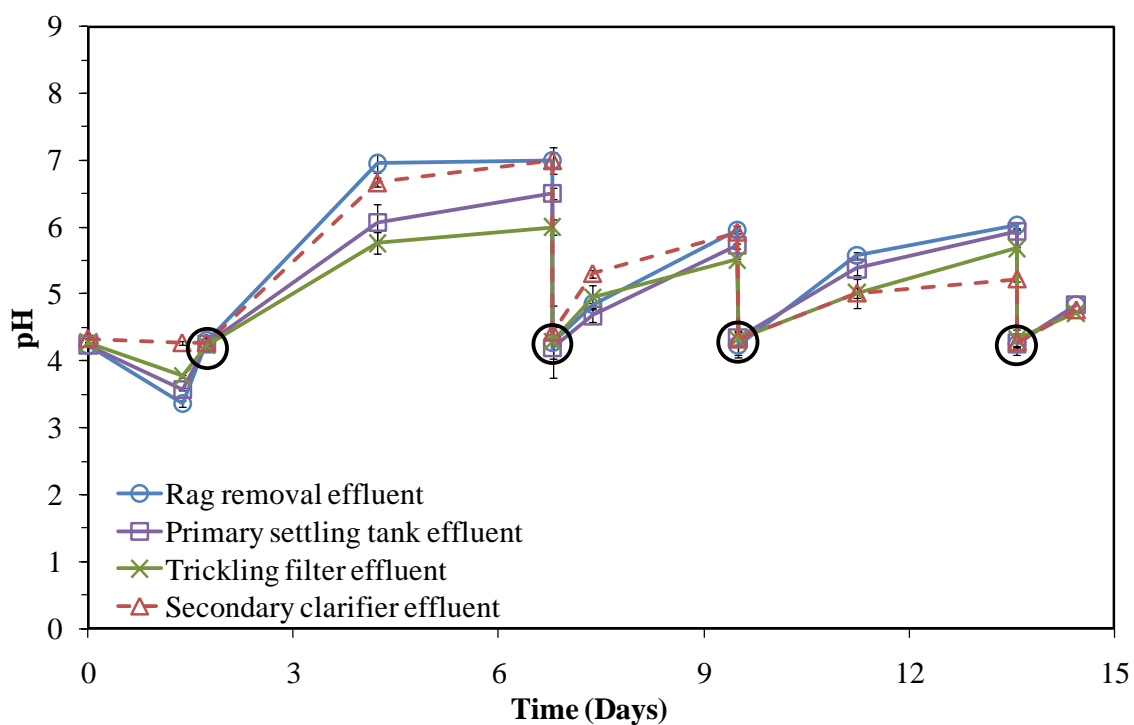
**Figure 3-2.** Attached growth on Tri-pack and enzyme activities observed for *P. chrysosporium* in aerated batch reactors containing secondary clarifier effluent (Batch Experiment 1). Treatment codes are provided in Table 2-2. a.) S-4.3-10; b.) N-4.3-10; c.) S-4.3-0; d.) N-4.3-0; e.) S-WW-10; f.) N-WW-10; g.) S-WW-0; h.) N-WW-0. Data points



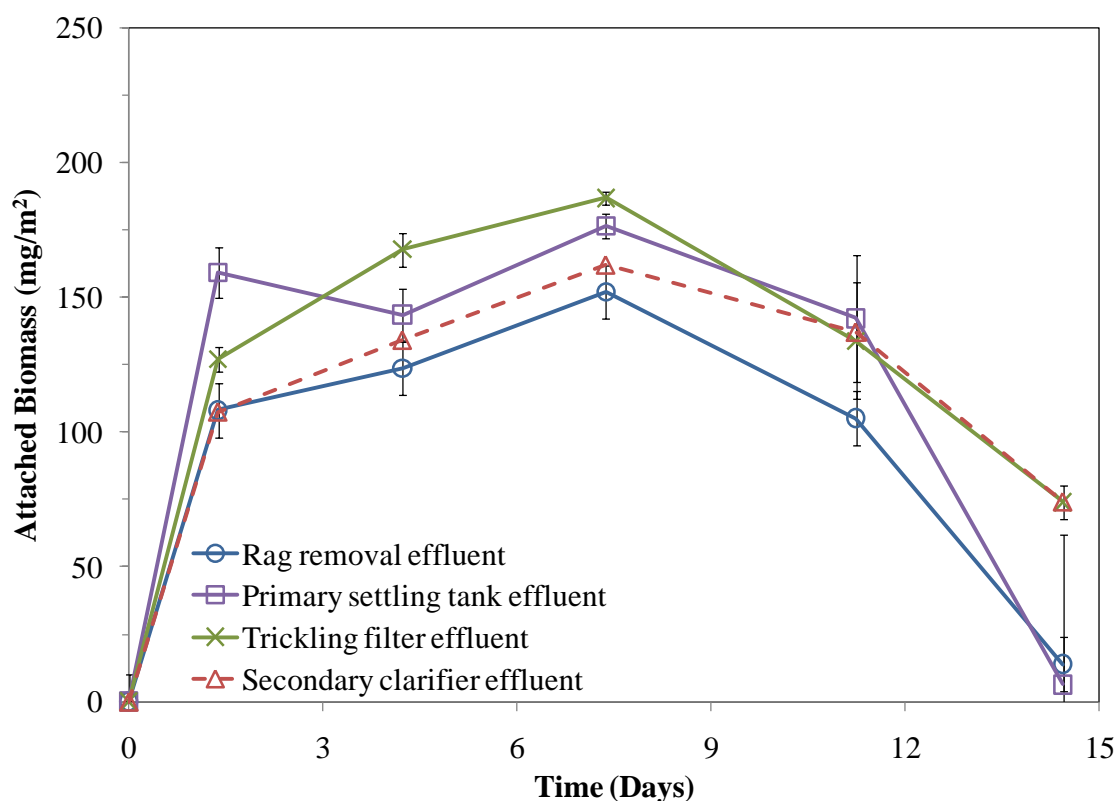
represent mean values from triplicate flask reactors; error bars represent  $\pm 1$  standard deviation.

### **3.3. Batch Experiment 2**

In Batch Experiment 2, the growth and enzyme activity of *P. chrysosporium* was evaluated in wastewater from four parts of the Penn State WWTP treatment train (i.e. effluent from the rag removal, primary settling tank, trickling filter, and secondary clarifier). All treatments were pH-adjusted, non-sterile, and not amended with organic carbon (i.e., glucose). Throughout the experiment, pH was not stable (Figure 3-3) and there was no enzyme activity. It is unclear if the addition of acid (for altering pH) throughout the study negatively affected the attached biomass (Figure 3-4). Note that the results of the first attempt at Batch Experiment 2 can be found in Appendix I.



**Figure 3-3.** Changes in pH in non-sterile, aerated batch reactors containing wastewater from different locations in the treatment train inoculated with *P. chrysosporium* (Batch Experiment 2). Circles indicate points of pH adjustments. Data points represent mean values of triplicate flask reactors (except for points of pH adjustment, which are mean values of all remaining reactors within the treatment); error bars represent  $\pm 1$  standard deviation.



**Figure 3-4.** The effect of wastewater treatment level on biomass (fungi + wastewater microbes) attachment to Tri-pack in aerated batch reactors inoculated with *P. chrysosporium* (Batch Experiment 2). Data points represent mean values of triplicate flask reactors; error bars represent  $\pm 1$  standard deviation

Fungal attachment was observed in all treatments; however, fungal mass could not be distinguished from total (microbial + fungal) mass, so total attached biomass is reported here. The maximum biomass was observed at  $t = 7.4$  days for all treatments (Table 3-3). With the exception of the rag removal effluent treatment, the initial mass data seems to correlate to the varying amounts of nitrogen and organic carbon within the different effluents (i.e. the primary settling tank effluent treatment had the highest attached biomass, which correlates to it having the highest measured ammonium and second-highest organic carbon content).

**Table 3-3.** Maximum average (mean) attached biomass on Tri-pack and the time at which it was observed in Batch Experiment 2.

<b>Treatment #</b>	<b>Treatment Name</b>	<b>Max. Avg. Attached Biomass (mg/m<sup>2</sup>)</b>	<b>Time (d)</b>
1	Rag Removal Effluent	162 ± 8	7.4
2	Primary Settling Tank Effluent	187 ± 5	7.4
3	Trickling Filter Effluent	177 ± 2	7.4
4	Secondary Clarifier Effluent	152 ± 9	7.4

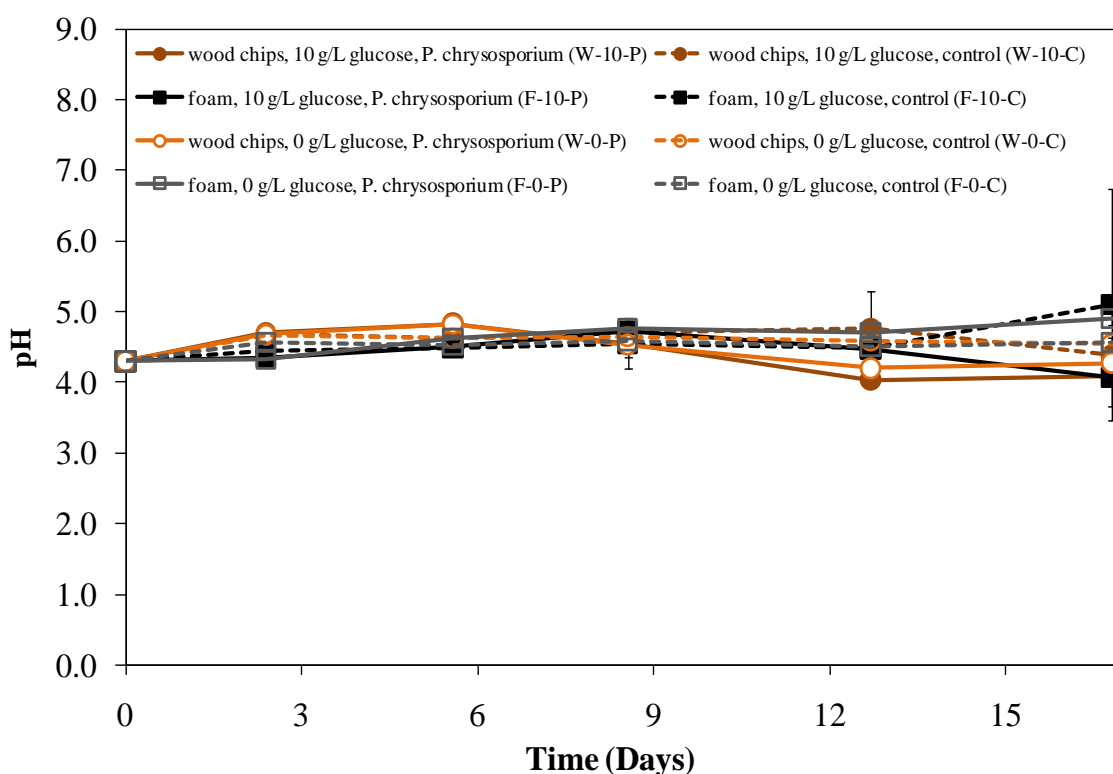
### 3.4. Batch Experiment 3

In this experiment, *P. chrysosporium* was grown in triplicate aerated batch reactors on either birch wood chips or polyether polyurethane foam cubes. Negative controls were run in duplicate. The flasks were filled with sterile secondary clarifier effluent which was either amended with 10 g/L glucose or left unamended. All treatments were spiked with atrazine to an initial concentration of approximately 5 ppm. pH, enzyme activity, and atrazine concentrations were monitored throughout the experiment.

#### 3.4.1. pH and Enzyme Activity

In general, the pH of all treatments remained fairly stable throughout the 17-day experiment. Figure 3-5 illustrates this stability within a pH range of approximately 4 – 5. By the last time point, microbial contamination is the likely the cause of the decreased pH of the F-10-P (foam, 10 g/L glucose, *P. chrysosporium*) treatment and the high standard deviation of the F-0-P (foam, 10 g/L glucose, *P. chrysosporium*) treatment. In addition, the duplicate F-10-C (controls) treatment flasks were contaminated by the last time point.

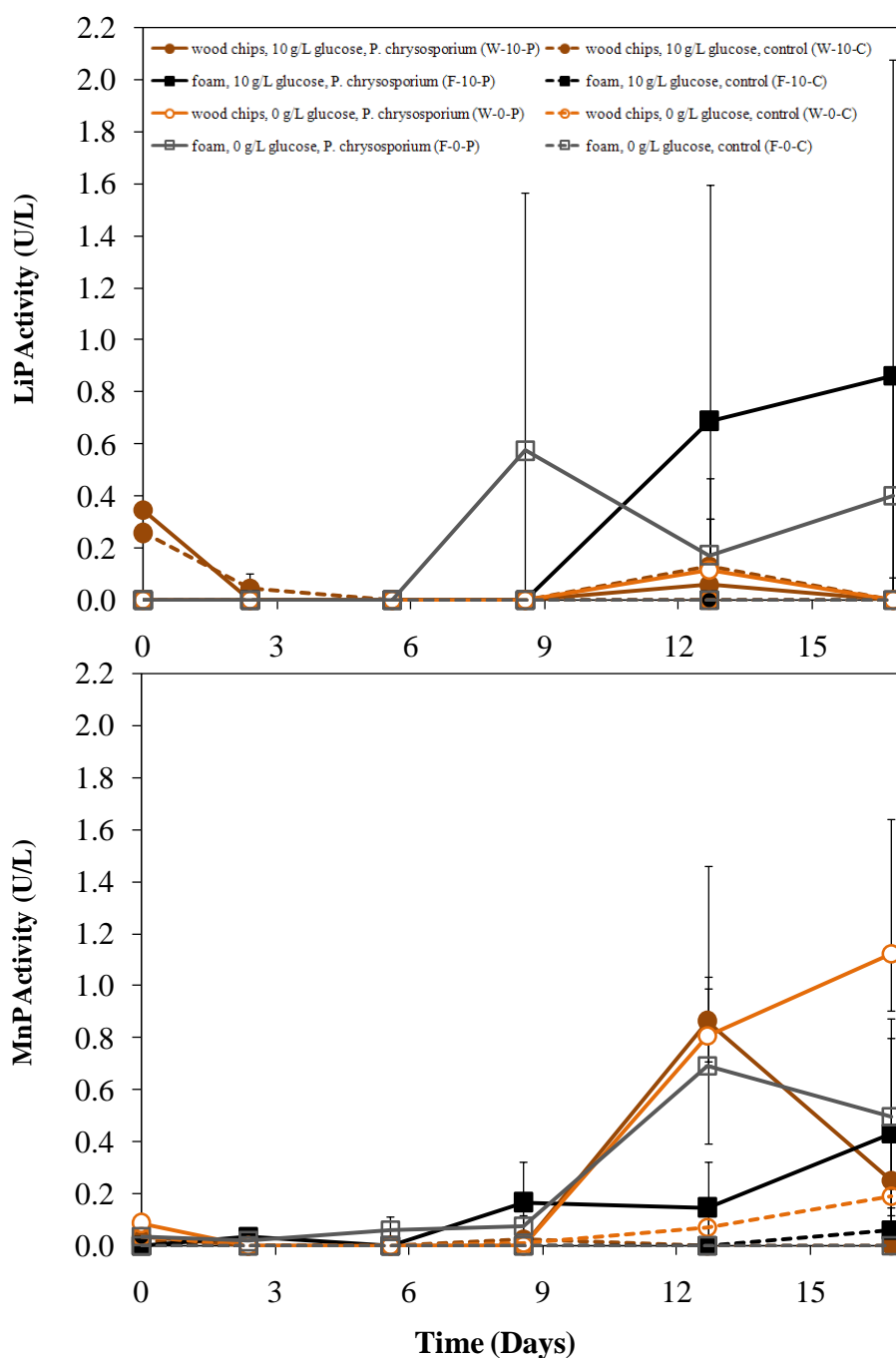
The slight decrease in pH of the inoculated wood chip treatments (W-0-P and W-10-P) is likely due to fungal degradation of the lignocellulose and release of organic wood acids (aromatic and alkyl carboxylic acids, Kirk and Farrell, 1987). This small change in pH was accompanied by a change in solution color. By the 10<sup>th</sup> day of the experiment, all of the inoculated wood reactor solutions (W-0-P and W-10-P) turned a dark amber color that persisted throughout the remainder of the experiment. The controls for these two treatments, W-0-C and W-10-C, did not exhibit this color change; therefore, the color change is likely not abiotic.



**Figure 3-5.** The effect of packing material (wood chips or foam) and supplemental glucose on pH in aerated batch reactors containing sterile secondary clarifier effluent inoculated with *P. chrysosporium* (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (uninoculated control); error bars represent  $\pm 1$  standard deviation. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols.

LiP and MnP activities were observed; however, they were quite low (Figure 3-6).

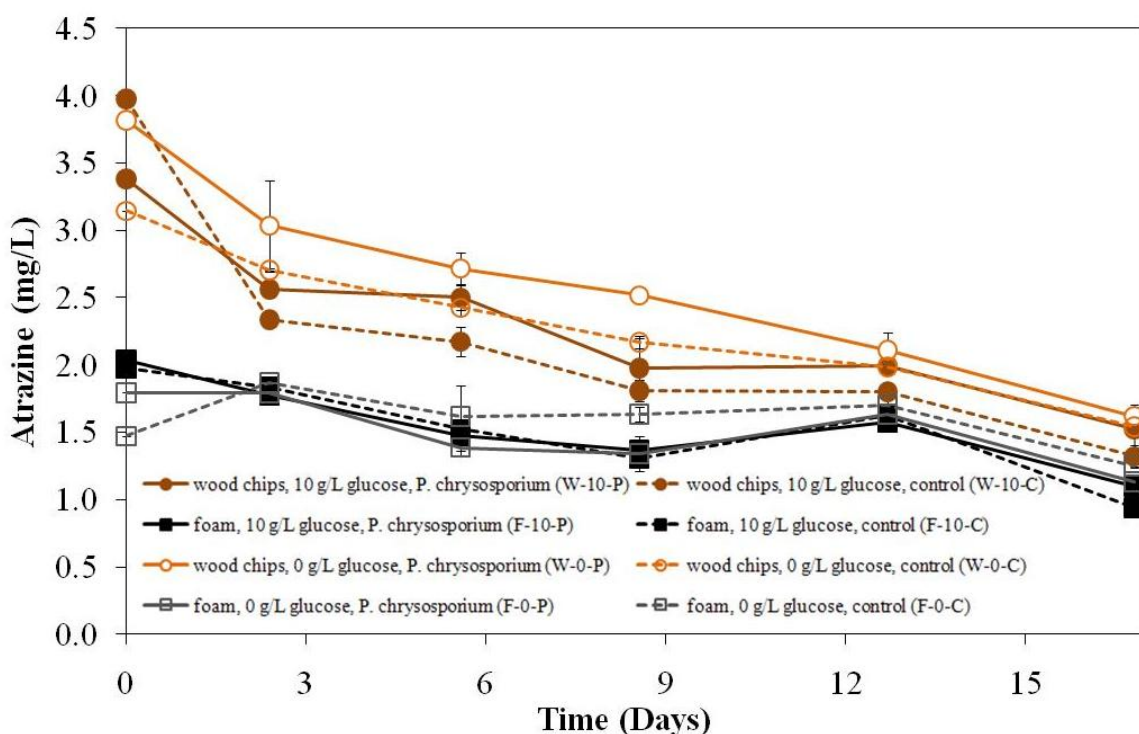
Treatments with foam (F-0-P and F-10-P) exhibited the highest LiP activities:  $0.6 \pm 0.9$  U/L at  $t = 8.6$  days and  $0.9 \pm 1.2$  U/L at  $t = 16.8$  days, respectively. These treatments also exhibited some MnP activity:  $0.7 \pm 0.3$  U/L at  $t = 12.7$  days for F-0-P and  $0.4 \pm 0.4$  U/L at  $t = 16.8$  days for F-10-P. Treatments with woodchips (W-0-P and W-10-P) exhibited the highest MnP activities:  $1.1 \pm 0.5$  U/L at  $t = 16.8$  days and  $0.9 \pm 0.2$  U/L at  $t = 12.7$  days, respectively. Although enzyme activity was low for all treatments, LiP and MnP activities in F-10-P appear to still be increasing by the end of the experiment; MnP activity in W-0-P also appears to be increasing.



**Figure 3-6.** LiP and MnP enzyme activities observed for *P. chrysosporium* attached to wood chips or foam in aerated batch flasks containing wastewater (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (control); error bars represent  $\pm 1$  standard deviation. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols.

### 3.4.2. Atrazine Degradation

Figure 3-7 illustrates the change in atrazine concentration of each treatment during the experiment. Although each reactor was spiked to an initial atrazine concentration of 5 to 5.5 mg/L, there was apparently an immediate loss of atrazine from solution before the  $t = 0$  flasks could be sacrificed. The rapid initial loss of atrazine was likely due to sorption to the packing material (wood chips or foam).



**Figure 3-7.** Atrazine concentrations for *P. chrysosporium* and control treatments in aerated batch reactors containing wastewater (Batch Experiment 3). Data points represent mean values of triplicate flask reactors (inoculated) or duplicate flask reactors (control); error bars represent  $\pm 1$  standard deviation, with the exception of the final F-0-C (0 g/L glucose, foam, control) point which is from a single flask. Inoculated fungal treatments are represented by solid lines; control treatments are represented by dotted lines. Wood chip treatments are represented by circles; foam treatments are represented by squares. Glucose-amended treatments are represented by filled symbols; un-amended treatments are represented by un-filled symbols.



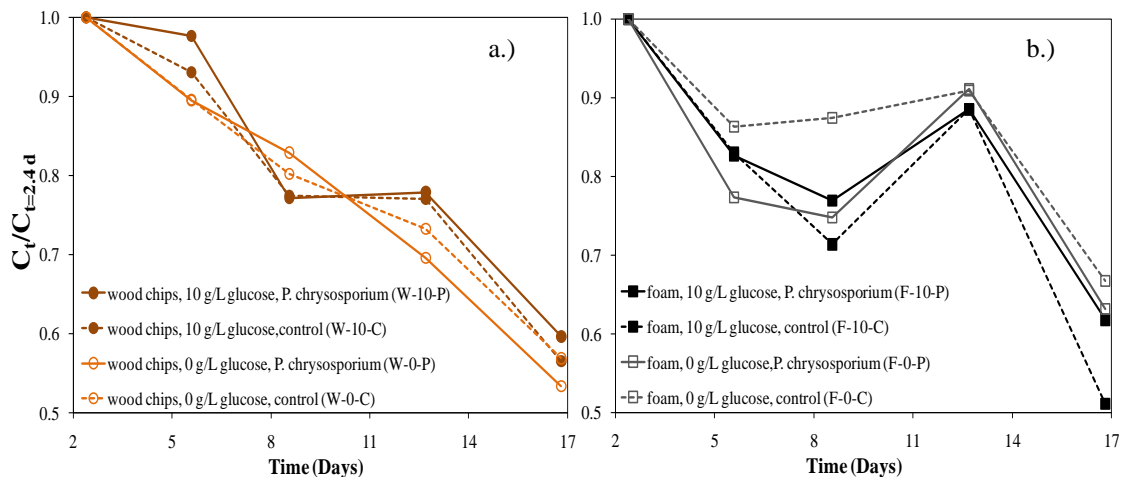
Statistically, there is no significant difference between the percent atrazine removals in each inoculated treatment and its equivalent uninoculated control (95% confidence interval; Tukey's pairwise comparison intervals contain zero; Table 3-4). In general, foam treatments removed significantly more atrazine than wood treatments (ANOVA p-value = 0.000); however, this is likely due to the initial sorption. In addition, the removal rate (dC/dt) was estimated for the fairly linear removal that was observed following the initial, non-linear sorption (from point t = 2.4 d to t = 16.8 d). In general, wood treatment removal rates were significantly greater than foam treatments (2-sample t-test p-value = 0.005).

**Table 3-4.** Percent atrazine removal and calculated removal rate based on linear degradation following the initial atrazine removal via non-linear sorption.

Treatment	Description	Atrazine <sup>a</sup> at t = 0 (mg/L)	Final Concentration (mg/L)	Total Removal <sup>b</sup> (%)	Calculated dC/dt * (mg/L-d)	R <sup>2</sup> ** (-)
W-10-P	wood chips, 10 g/L glucose, <i>P. chrysosporium</i>	3.38	1.53 ± 0.15	69 - 72	-0.071	0.9021
W-10-C	wood chips, 10 g/L glucose, control	3.98	1.32 ± 0.080	74	-0.067	0.9332
W-0-P	wood chips, 0 g/L glucose, <i>P. chrysosporium</i>	3.82	1.62 ± 0.084	68 - 71	-0.096	0.9925
W-0-C	wood chips, 0 g/L glucose, control	3.14	1.54 ± 0.013	69	-0.077	0.9849
F-10-P	foam, 10 g/L glucose, <i>P. chrysosporium</i>	2.03	1.10 ± 0.085	78 - 80	-0.035	0.6131
F-10-C	foam, 10 g/L glucose, control	1.97	0.94 ± 0.10	81	-0.047	0.6107
F-0-P	foam, 0 g/L glucose, <i>P. chrysosporium</i>	1.79	1.13 ± 0.018	77 - 79	-0.029	0.4152
F-0-C	foam, 0 g/L glucose, control	1.47	1.25 ± 0 <sup>y</sup>	75	-0.033	0.6672

<sup>a</sup>Initial concentrations were measured in singlet. <sup>b</sup>Total removal is expressed as a range (min - max) because the initial concentration was unknown but between 5.0 - 5.5 ppm; \*Calculated rate of removal was the slope of the line of best-fit from t = 2.7 d to t = 16.8 d (linear data after non-linear sorption); \*\*Coefficient of correlation for the line of best-fit; <sup>y</sup>This was a singlet control.

Since accurate initial atrazine concentrations could not be measured and concentrations could not be assumed to be equal for controls and inoculated treatments, the data could not be normalized over the entire experiment. This means that the fraction of remaining atrazine,  $C_t/C_0$ , could not be estimated over time. Although the total percent removal was very similar for all treatments, it is unclear if the fungus had any effect, or was beginning to have an effect, on atrazine removal because the data could not be normalized for all time points; however, an attempt at normalization for at least a portion of the experiment was made. Figure 3-8 illustrates the normalization of the data from  $t = 2.4$  days (first sacrificial time point) to  $t = 16.8$  days (final sacrificial time point). Although there is no clear trend in the foam treatments, the wood treatments appear to be fairly linear. Additionally, the fraction of the  $C_{t=2.4d}$  remaining in the W-0-P (wood chips, 0 g/L glucose, *P. chrysosporium*) treatment appears to be slightly lower than the equivalent control (W-0-C).



**Figure 3-8.** Percent atrazine removal that was not attributed to the initial, non-linear sorption in a) wood chip and b) foam treatments. Remaining time points were normalized by dividing the atrazine concentration at each time point by the concentration at  $t = 2.4d$  (first sacrificial point in the experiment). Final F-0-C (0 g/L glucose, foam, control) point is from a single flask.

## **4. Discussion**

Over the duration of the study, the effects of parameters such as pH, organic carbon, and packing media on fungal growth and enzyme activity were evaluated in three batch experiments. Atrazine removal and removal rates were also studied in the final batch experiment. In addition to these batch studies, the following was also completed:

- A preliminary aeration experiment to determine when to begin aerating the batch flasks (Appendix H)
- A buffer test to determine if the pH of non-sterile wastewater treatments could be stabilized for use in batch experiments (Chapter 4.3; Appendix J)

### **4.1. Batch Experiment 1**

In Batch Experiment 1, the effects of pH, non-sterile and sterile solutions, and the presence or absence of a 10 g/L glucose amendment on fungal biomass and enzyme activity were monitored in secondary clarifier effluent. The goals of this experiment were to determine if:

- 1) *P. chrysosporium* would grow and produce enzyme activity at the natural pH of wastewater;
- 2) *P. chrysosporium* would grow and produce enzyme activity in non-sterile conditions;
- 3) *P. chrysosporium* required supplemental organic carbon for growth and enzyme activity; and
- 4) a direct relationship between fungal biomass and enzyme activity existed.

#### **4.1.1. Growth and Enzyme Activity at the pH of Wastewater**

The pH of the wastewater had a significant effect on the growth and enzyme activity of *P. chrysosporium*. Treatments in which the pH stabilized at or above the natural pH of wastewater (pH ~7) (Figure 3.2 d, e, g, and h) exhibited lower growth and enzyme activity. Although the fungus could grow and attach to the packing material at near neutral pH, total biomass production was fairly low ( $< 100 \text{ mg/m}^2$ ) in comparison to other treatments, and LiP and MnP were not detected. This is likely because LiP and MnP enzymes are most stable at pH 3 and 4, respectively (Odier and Artaud, 1992; Singh and Chen, 2008). From these results, the importance of maintaining a low pH for this application has been confirmed in wastewater.

#### **4.1.2. Growth and Enzyme Activity in Non-Sterile Wastewater**

In non-sterile wastewater, total biomass (fungus + wastewater microbes) was comparable to sterile wastewater, and enzyme activity was only present in one treatment (Figure 3.2b, d, f, and h). The fungus was able to produce enzyme activity in the N-WW-10 (non-sterile, pH<sub>0</sub> ~7, 10 g/L glucose) treatment. This treatment produced LiP and MnP activities, and it had the highest amount of total attached biomass ( $230 \pm 62 \text{ mg/m}^2$ ) in the experiment.

The N-WW-10 treatment had an initial pH of ~7; however, the pH quickly dropped to 4.4 less than one day into the experiment. The pH continued to drop and stabilized near pH 3.4. Although this pH is not ideal for *P. chrysosporium* growth, it is ideal for enzyme stability. The cause of the quick drop in pH is likely due to the activities of the native wastewater microbes since this pH-drop was not observed in the equivalent treatment in sterile wastewater (S-WW-10). The most likely explanation is: 1) the excess of available substrate (10 g/L glucose) caused the aerobic microbial populations to grow exponentially and rapidly exhaust the dissolved oxygen, at least in certain areas within the flask reactors; and 2) anaerobic conditions formed, and facultative anaerobic populations grew and produced acidic fermentation products. Typical acidic products

include volatile fatty acids such as acetic, propionic, and butyric acid and other carboxylic acids such as formic and lactic acid.

To determine, theoretically, if the decreased pH was caused by products of anaerobic metabolism, 1) oxygen mass-transfer into solution and 2) oxygen utilization rate by aerobic microbes were estimated (Appendix K). The rate of oxygen being transferred into solution was calculated to be 1 mg O<sub>2</sub>/L-s; however, only a fraction of this will be transferred into the biomass. Using a small time step, the change in oxygen utilization over time was predicted, incrementally, using the conditions of my experiment. It was estimated that it would take approximately 3.55 days for the oxygen utilization rate to equal the oxygen mass-transfer rate into solution; however, anaerobic pockets (in solution) could begin to form before that time. At  $t = 3.55$  days under completely aerobic conditions, this simple model predicts that 30% of the glucose would remain. These calculations support the theory that the flasks experienced at least temporary anaerobic conditions, which would facilitate the production of VFAs and other carboxylic acids.

Although there is evidence of the formation of anaerobic pockets within the N-WW-10 (non-sterile, pH<sub>0</sub> ~7, 10 g/L glucose) treatment flasks, there is also evidence of aerobic conditions. *P. chrysosporium* is an aerobic fungus and will not produce ligninolytic enzymes in an oxygen-limited environment (Kirk et al., 1978; Singh and Chen, 2008). Although the LiP and MnP activities were not as high in this non-sterile treatment as they were in the sterile treatments (S-4.3-10 and S-4.3-0), they were still produced. This means that at least a portion of the fungus was not oxygen-limited. However, it appears as if there might have been greater lag in enzyme production for the N-WW-10 treatment in comparison to the sterile treatments. This lag could have been caused by an initially low dissolved oxygen concentration during the period of high glucose utilization and microbial population growth. In future studies, DO concentrations should be monitored to ensure that it is not a limiting factor for fungal productivity.

#### 4.1.3. Effect of Supplemental Organic Carbon on Growth and Enzyme Activity

In all cases, treatments with glucose resulted in greater attached biomass than their counterparts without glucose amendments. Treatments N-WW-10 (non-sterile, pH<sub>o</sub> ~7, 10 g/L glucose) and S-4.3-10 (sterile, pH<sub>o</sub> 4.3, 10 g/L glucose) resulted in the highest attached biomass with a maximum of 230±62 and 203±22 mg biomass/m<sup>2</sup>, respectively, at t = 14.4 days (Table 3-2). Additionally, all treatments without glucose exhibited losses in attached biomass by the end of the experiment (Figure 3-2).

Although glucose amendments positively affected growth, they did not appear to positively affect LiP and MnP enzyme production and activity. For instance, treatment S-4.3-0 (sterile, pH<sub>o</sub> 4.3, 0 g/L glucose), resulted in comparable maximum LiP and MnP enzyme activities (Figure 3-2 a.) and c.)). The lack of activity of the oxygen-dependent LiP and MnP enzymes may be linked to the penetration limits of dissolved oxygen into the fungal mycelia. In the case of greater biomass, if the biomass surface area (contacting the solution) is not increased and the biofilm is simply thicker, then the oxygen-dependent production of enzymes would be limited based on oxygen penetration into the biofilm. In addition, *P. chrysosporium* has been shown to produce a build-up of extracellular polysaccharides when glucose is the organic carbon substrate (Ntwampe et al., 2010). These extracellular polysaccharides are thought to exacerbate the existing oxygen mass-transfer limitations into fungal mycelia (Ntwampe et al., 2010).

Although large concentrations of glucose may not be beneficial for enzyme activity, some additional glucose might help to reduce the lag between experimental start-up and observed enzyme activity. MnP activity was detected earlier in the sterile treatment amended with glucose, and the only non-sterile treatment to produce enzyme activity (N-WW-10) contained glucose; however, it is unclear if it was 1) the organic carbon supplied by the glucose and the decreased

pH (3.4) from microbial transformation of the glucose or 2) purely the drop in pH provided by microbial actions that actually facilitated the enzyme production.

#### **4.1.4. Relationship Between Biomass and Enzyme Activity**

From the previously discussed results, there does not appear to be a direct relationship between fungal biomass and production of enzyme activity. Although treatment S-4.3-0 (sterile, pH<sub>0</sub> 4.3, 0 g/L glucose) exhibited only half of the fungal growth observed in treatment S-4.3-10 (sterile, pH<sub>0</sub> 4.3, 10 g/L glucose), it (S-4.3-10) still resulted in approximately the same levels of enzyme activity over the 2-week experiment.

#### **4.2. Batch Experiment 2**

In Batch Experiment 2, fungal growth and enzyme activity were evaluated under non-sterile conditions in four different stages of unamended wastewater effluent (rag removal, primary settling tank, trickling filter, and secondary clarifier effluent). The goals of this experiment were to: 1) determine the best location for a fungal reactor within a WWTP treatment train; 2) determine if enzyme activity would be inhibited by the available nitrogen in the different effluents; and 3) determine if the different organic carbon compound concentrations and make-up would affect growth and enzyme activity. The experimental conditions (non-sterile, unamended wastewater with an initial pH adjustment to 4.3) for Batch Experiment 2 were determined based on the results of Batch Experiment 1; however, the data for two of the Batch Experiment 1 treatments, N-WW-10 (non-sterile, pH<sub>0</sub> ~7, 10 g/L glucose) and N-4.3-0 (non-sterile, pH<sub>0</sub> 4.3, 0 g/L glucose), were erroneously switched. A total organic carbon analysis was used to determine the actual identity of the treatments (correct results were presented in Chapter 3); however this

determination was not made until after Batch Experiment 2 was completed. This mistake led to the initial conclusions that 1) the pH of unamended, non-sterile wastewater would remain somewhat close to the optimum range (4.0-4.5) and 2) enzyme activity could be produced under these experimental conditions.

During the experiment, pH was not stable (Figure 3-3) and there was no enzyme activity. It is not clear whether the acid additions throughout the study negatively affected the attached biomass; however, the instability of the pH is likely the reason that there was no LiP or MnP activity.

At the first sampling point ( $t = 1.4$  days), the treatments in the effluents of the rag removal, primary settling tank, and trickling filter all experienced slight decreases in pH. Of these three treatments, the rag removal effluent treatment had the largest drop in pH while the trickling filter effluent treatment had the smallest pH drop. This pH trend correlates to the initial total organic carbon (TOC) content of each of these effluents. The rag removal effluent has the highest TOC content ( $\sim 160$  mg C/L), the primary settling tank effluent had the second highest TOC content ( $\sim 150$  mg C/L), and the trickling filter effluent has the lowest TOC content ( $\sim 95$  mg C/L). The treatment in secondary clarifier effluent held stable at pH 4.3 and had a TOC content of  $\sim 40$  mg C/L.

After sampling, pH adjustments were made to the flasks of each treatment (with 1N NaOH) except for the treatment in secondary clarifier effluent. After that point ( $t = \sim 2$  days), all pH adjustments made were to decrease pH, not increase. Since there was no buffer in these treatments, the pH of the treatments continued to rise after adjustments were made. The rising pH was likely caused by organic matter degradation and ammonia production.

Although fungal attachment was observed in all treatments, fungal mass could not be distinguished from total (microbial + fungal) mass. Because of this and the lack of enzyme activity, no conclusions could be drawn about: 1) where, in the wastewater treatment train, a



fungal reactor would be well-suited; 2) how the available nitrogen concentration affects enzyme activity; and 3) how the different TOC content of the effluents would affect growth and enzyme activity. Even though no conclusions could be drawn for the original objectives, the results of Batch Experiment 2 led me to the errors of my conclusions for Batch Experiment 1. Additionally, this study led to the experimental evaluation of multiple buffers in various secondary clarifier effluent treatments (40 treatments in total) to determine if there was a suitable buffer to stabilize non-sterile wastewater pH within the optimum range (4.0 – 4.5).

### **4.3. Batch Experiment 3**

In this 17-day experiment, all treatments were sterile and spiked with atrazine to an initial concentration of approximately 5 ppm. The eight treatments were combinations of the following factors: 1) 10% fungal inoculate or 0% inoculate (control); 2) 12 g wood chips or 25 foam cubes; and 3) 10 g/L glucose or 0 g/L glucose. The goals of this experiment were to: 1) determine how enzyme activity is affected by the packing (immobilization) material used; 2) determine if *P. chrysosporium* will transform atrazine under the experiment conditions; and 3) determine degradation kinetics if *P. chrysosporium* does transform atrazine.

#### **4.3.1. Effect of Packing Material on Enzyme Activity**

Overall, wood treatments had higher MnP activities while foam treatments had higher LiP activities; however, all activity measurements were low and highly variable. In addition, there did not appear to be LiP activity in the wood treatments. A possible explanation for this is inhibition by: a) phenolic products released from the wood (Ferapontova et al., 1996) ; and/or b) excessive Mn(II) in solution (Rothschild et al., 1999). Phenols can have an inhibitory affect on

LiP, specifically in the first reaction involving the reduction of hydrogen peroxide and the production of the oxidized LiP-I complex (Table 1-6) (Ferapontova et al., 1996). Ferapontova et al. found that adding veratryl alcohol corrected this issue; however, there was no specification as to how much veratryl alcohol must be added to reverse the effects of inhibition (1996). In addition to phenol, high concentration of Mn(II) in solution can also inhibit LiP activity. In a study done by Rothschild et al., LiP was shown to be completely inactive at Mn(II) concentrations of 13 mg/L; however, no minimum concentration for the onset of inhibition was given (1999). Additionally, the mechanism of LiP activity suppression was unknown; however, the authors speculated that the way in which Mn and veratryl alcohol play regulatory roles in enzyme activities was involved. Because an Mn-deficiency in another study enhanced production of veratryl alcohol and LiP activity (Mester et al., 1995), Rothschild et al. concluded that an excess of Mn could suppress veratryl alcohol production, which would suppress LiP activity (1999).

#### **4.3.2. Transformation of Atrazine and Degradation Kinetics**

As stated in the results (Chapter 3), there was a rapid loss of atrazine prior to the  $t = 0$  measurement for the experiment. This rapid loss, which was significantly greater in the foam treatments ( $-3.43 \pm 0.29$  mg/L) than the wood treatments ( $-1.67 \pm 0.47$  mg/L), was most likely caused by adsorption to the material surface and absorption into the material matrix. For both wood and foam, adsorption of atrazine onto the packing material, assuming 50% total packing material coverage by atrazine, would likely be on the order of  $10^{-2} - 10^{-1}$  mg/L (Appendix L). Losses from absorption into the packing material are unknown, but this was likely another mechanism of removal. Additionally, atrazine may have been lost via abiotic hydrolysis (i.e. cleavage of the chlorine atom and replacement with a hydroxyl group) (Armstrong et al., 1967).

Armstrong et al. reported greater rates of abiotic atrazine hydrolysis under acidic conditions, especially in the presence of organic matter on which the atrazine could adsorb (1967). In Batch Experiment 3, pH was maintained below 5 and organic matter (i.e., packing media, wastewater suspended solids, and/or fungus) was present, which would have facilitated greater transformation of atrazine via hydrolysis.

Overall, the initial sorption of atrazine by the foam likely caused the foam treatments to have a higher total atrazine removal from solution than the wood chip treatments by the end of the 17-day experiment. In addition, there was no significant difference between the percent removals in each inoculated treatment and its equivalent control. Based on this, there is no support that atrazine was transformed by *P. chrysosporium*; however, the study may not have been run long enough for enhanced atrazine removal by the fungus. Support for this theory can be seen if atrazine removal rate ( $dC/dt$ ) is considered. Additionally, enzyme production occurred late in the study, so the fungus may have just been starting to degrade atrazine when the study was ending.

In general, the wood chip treatment removal rates were significantly greater than the foam treatments. Treatment W-0-P (wood chips, 0 g/L glucose, *P. chrysosporium*) resulted in the highest atrazine removal rate ( $-0.096 \text{ mg/L-d}$ ;  $R^2 = 0.99$ ). Because treatment W-0-P had a higher removal rate in comparison to its control (W-0-C), it is possible that the fungus was slowly starting to contribute to atrazine removal; however, the experiment was not carried out for a sufficient amount of time to make this conclusion with certainty. Some support for fungal contributions to degradation include: 1) the solution color change associated with fungal breakdown of lignocellulose (starting at day ~8); and 2) enzyme activity production at approximately the same time (day ~10) that the fraction of atrazine remaining ( $C_t/C_{t=2.4d}$ ) in the W-0-P treatment dropped below the fraction remaining in the W-0-C treatment (Figure 3-8).

#### **4.4. Overall Effects of the Experimental Parameters on *P. chrysosporium***

##### **4.4.1. pH**

In all three batch experiments, maintaining a stable pH near the optimum range (4.0 – 4.5) proved to be only somewhat important for growth (at least in a short time period) and very important for enzyme activity; however, enzyme activity can still be produced at pH values that are slightly outside of the optimum range (Batch Experiment 1; Batch Experiment 3). No enzyme activity was recorded when the flask solution pH was below 3.4 or above 5.1.

##### **4.4.2. Organic Carbon Amendment**

Organic carbon amendments, in the form of glucose, were used in half of the treatments in Batch Experiment 1 and 3. In Batch Experiment 1, it was found that glucose amendments resulted in more biomass, but enzyme activity was not enhanced. It might be possible to shorten the lag period between the start of the experiment and the observation of enzyme activity by adding glucose; however, there is not enough evidence to prove this. In Batch Experiment 3, glucose additions had no observable effect on atrazine degradation and enzyme activities. From the results of Batch Experiment 1 and 3, it does not appear that organic carbon additions (i.e. in addition to any organic carbon that may be present in the fungal inoculum) are necessary in batch flask reactors, unless the purpose of the addition is to drive down the pH of a non-sterile treatment (Batch Experiment 1).

#### **4.4.3. Sterile vs Non-Sterile**

In the batch flask reactors employed in Batch Experiment 1, 2, and 3, maintaining sterile conditions is recommended based on the previously discussed results. Although enzyme activity was observed in a non-sterile treatment (Batch Experiment 1), the pH cannot be controlled and maintained within the optimum range. If the reactors are successfully scaled up to continuous-flow columns, it could be possible, especially with the aid of an auto-titrator (for pH control), to evaluate enzyme activity or compound degradation under non-sterile conditions.

#### **4.4.4. Natural vs Synthetic Packing Media**

Synthetic packing materials were used in all of the batch experiments. Batch Experiment 3 was the only one in which a natural packing material was also used. Good biomass attachment and enzyme activity were observed in several treatments with each type of packing material (Tri-pack®, birch wood chips, and polyether polyurethane foam); therefore, on a small time-scale, packing material does not seem to affect these measurements. On a longer time-scale, the natural packing material could double as a slow-release carbon source to avoid organic carbon starvation.

In Batch Experiment 3, the importance of considering the contaminant sorption rate and capacity of the packing media was illustrated. In this experiment, atrazine sorbed immediately to the foam, and the foam did not appear to continue sorbing much, if any, atrazine throughout the remainder of the experiment. In contrast, the wood chips sorbed much less atrazine before  $t = 0$  than the foam, but then appeared to exhibit linear sorption of the chemical over time.

## 5. Conclusions, Engineering Significance, and Future Work

### 5.1. Conclusions

Based on the results of this batch study evaluating growth, enzyme activity, and atrazine degradation capacity of immobilized *P. chrysosporium* in multiple wastewater effluent conditions, the following conclusions can be made:

- ❖ **Both LiP and MnP enzymes can be produced in non-sterile secondary clarifier effluent; however, under the conditions tested in these experiments, it was quite low in comparison to reported values in the literature.** Within this study, maximum LiP and MnP enzyme activities were on the scale of  $10^0 - 10^1$  U/L. Typical LiP activity reported in the literature is from  $10^2 - 10^3$  U/L; MnP activity typically ranges from  $10^1 - 10^2$  U/L.
- ❖ **pH stability near 4.3 is necessary for enzyme activity, but not for growth; large fluctuations in pH result in no enzyme activity.** Most of the research in the literature suggests maintaining the solution pH within an optimum range of 4.0 – 4.5; however enzyme activity can be obtained at *stable* pH values that are outside of this range but still relatively close. In this study, enzyme activity was achieved within a range of 3.4 – 5.1; however, the highest activity was observed when the pH was within the optimum range.
- ❖ **Glucose amendments, above the residual carbon provided in the fungal inoculum, are not necessary for growth in wastewater; however, better growth was observed in glucose-amended treatments.** In Batch Experiment 1, attached biomass in all of the

glucose-amended treatments was greater than in the equivalent unamended treatments; however the fungus still grew when no additional glucose was provided. In the sterile, pH-adjusted wastewater treatments, the maximum attached fungal biomass in flasks without glucose was only 50% of the maximum attached fungal biomass in glucose-amended flasks.

- ❖ **Glucose amendments do not appear to enhance the activity of LiP and MnP in sterile wastewater.** In Batch Experiment 1, the maximum observed MnP and LiP activities were 16 U/L and 8 U/L, respectively, under sterile conditions without glucose amendments. Under sterile conditions with glucose amendments, the maximum MnP and LiP activities observed were 14 U/L and 10 U/L, respectively. Additionally, glucose amendments in Batch Experiment 3 did not appear to enhance enzyme activity; maximum observed activities were comparable in glucose-amended and unamended treatments containing the same packing media.
- ❖ **Atrazine removal, most likely due to sorption, was significantly higher in flasks containing foam packing media than in flasks containing wood chips.** The foam treatments (fungal and control) of Batch Experiment 3 resulted in approximately 80% atrazine removal by day 17. The wood chip treatments (fungal and control) resulted in approximately 70% atrazine removal within the same amount of time.
- ❖ **After initial sorption, the rate of linear atrazine removal was highest in treatments containing *P. chrysosporium*, wood chips, and no glucose amendments.** The removal rate for this treatment was -0.096 mg/L-d ( $R^2 = 0.9925$ ). All other wood chip treatments fell within a range of -0.067 to -0.077 mg/L-d; foam treatments fell within a range of -0.029 to -0.047 mg/L-d.

- ❖ **During the short duration of the tests (~2 weeks), overall atrazine removal was not significantly different between the fungal treatments and equivalent controls; however, as enzyme production was only observed toward the end of this period, atrazine removal may be observed with longer testing.**

## **5.2. Engineering Significance**

- ❖ **Additional bench-scale experiments must be done before scale-up is logistically and economically feasible.** Although enzyme activities have been observed, they are quite low in comparison to reported activities in the literature. Enzyme activity must be increased and sustained for scale-up. In addition, white-rot fungi have very specific pH requirements that are not within the typical range of wastewater pH. Because of this, associated maintenance and operation (O&M) costs may be high depending on the method of decreasing the pH for a fungal reactor and increasing the pH prior to effluent discharge. If conditions for scale-up are achieved, the cost of pH adjustments and other potential O&M costs should be compared to the cost of available physical and chemical treatment technologies to determine the overall economic feasibility of system.
- ❖ **Fungal performance can be achieved without significant organic carbon amendments.** This is especially important if the biological treatment method for emerging contaminant removal were to be installed near the end of a wastewater treatment train (i.e. just before a clarification step or just before chlorination and discharge). If additional carbon is added near the end of the treatment train, the BOD in the effluent could exceed the typical 30 mg/L limit, which would negatively impact the natural ecosystem of the receiving water. Additionally, if the effluent is disinfected via



chlorination prior to release, hazardous by-products, such as haloacetic acids, haloacetonitriles, and haloketones, will likely form from reactions between the chlorine and the excess organic material. The use of fungi, however, does not require a continuously high supply of organic carbon to induce enzyme production, thereby limiting concerns related to BOD addition during the final stages of treatment. It is important to note though, that fungi would periodically require organic carbon and nutrient supplements to maintain performance if immobilized on an inorganic support structure. More work must be done to determine the actual requirements of a fungal reactor for proper design and protection against high levels of BOD in discharge.

- ❖ **Fungus can grow outside of the optimum pH range.** Although there is no enzyme production when the pH is far outside of the optimum range, the fungus can still grow and remain attached. This characteristic of the fungus is desirable for scale-up because, in the event that the pH of the fungal reactor was temporarily un-controlled, the fungus would not likely be completely killed or washed out.

### 5.3. Future Work

- ❖ **Perform a carbon-balance for experimental treatments.** A carbon analysis should be carried out in future experiments to determine 1) total organic carbon in solution and rate of degradation and 2) total inorganic carbon in solution and rate of carbon dioxide production. Additionally, if anaerobic conditions could potentially arise, then the future experiments should also determine if methane and VFAs are being produced.
- ❖ **Evaluate the use of fine-bubble diffusers and the affect of aeration rate on fungal productivity under non-sterile conditions.** In these experiments, aeration was initially

assumed to not be limiting; however, in non-sterile conditions, the presence of native wastewater bacteria must be considered. In general, these bacteria grow faster and can out-compete the fungus for oxygen.

- ❖ **Determine if a period of fungal establishment on the packing media would reduce problems associated with non-sterile conditions (i.e. increased pH and decreased biomass).** A period of fungal attachment and growth on the packing material in batch flasks in a low-nutrient media may enhance the fungal biomass and give it a competitive advantage over native wastewater microbes. This period would follow the homogenation and introduction of mycelia inoculum to batch flasks. Within this period (1 – 3 days), the fungus would be grown in a low nutrient solution in batch flasks. After this establishment period, the nutrient solution would be replaced with a wastewater treatment solution.
- ❖ **Further explore the use of wood and lignocellulose-containing materials as packing media.** Lignocellulose-containing materials, such as wood, are what white-rot fungi degrade in nature. These types of materials may help to provide conditions that are more ideal for fungal growth and contaminant degradation in an unfamiliar environment – wastewater.
- ❖ **Evaluate degradation capacities of other strains of white-rot fungi in wastewater.** Other strains or a combination of strains should be considered for future studies. Co-culturing may enhance contaminant removal, especially if the fungi produce different isoenzymes, allowing for more flexibility and adaptability to environmental conditions.
- ❖ **Test the removal of other emerging contaminants, in addition to atrazine.** Other types of contaminants that are commonly found in wastewater, including estrogenic

hormones and prescription medication, should be selected for study based on: 1) prevalence, persistence, and typical concentrations found in wastewaters, environmental waters, and drinking waters; 2) representative structures found in lignocellulose; 3) structural differences between classes of EDCs (i.e. select a variety of structurally different compounds); and 4) severity of known health effects of the compounds. These compounds should be evaluated individually and in combination to determine the differences in degradation kinetics and potential toxicity to the fungus.

❖ **Evaluate potential seasonal effects of wastewater on the performance of fungi.**

Seasonal increases and decreases of influent and influent characteristics should be considered, and increases in influent by storm-water contributions should be considered in conjunction with agricultural activity. In a wastewater treatment plant, EDC concentrations will vary; therefore multiple contaminant concentrations should be evaluated to determine degradation kinetics and potential inhibition effects of higher contaminant concentrations.

## REFERENCES

- Alvey, S. and Crowley, D.E. (1996). "Survival and activity of an atrazine-mineralizing bacterial consortium in rhizosphere soil". *Environmental Science and Technology*, 30, 1596-1603.
- Armstrong, D.E., Chesters, G., and Harris, R.F. (1967). "Atrazine hydrolysis in soils". *Soil Science Society of America Proceedings*, 31, 61-66.
- Asgher, M., Bhatti, H.N., Ashraf, M., and Legge, R.L. (2008). "Recent developments in biodegradation of industrial pollutants by white rot fungi and their enzyme system". *Biodegradation*, 19, 771-783.
- Asther, M., Corrieu, G., Drapron, R., and Odier, E. (1987). "Effect of Tween 80 and oleic acid on ligninase production by *Phanerochaete chrysosporium* INA-12". *Enzyme and Microbial Technology*, 9, 245-249.
- Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., Adams, C.D., and Surampalli, R.Y. (2006). "Endocrine disrupting compounds removal from wastewater, a new challenge". *Process Biochemistry*, 41, 525-539.
- Aust, S.D. (1990). "Degradation of environmental pollutants by *Phanerochaete chrysosporium*". *Microbial Ecology*, 20, 197-209.
- Bailey, J.E. and Ollis, D.F. (1986). *Biochemical Engineering Fundamentals*, 2<sup>nd</sup> ed., New York, NY, McGraw-Hill Book Co.
- Bastos, A.C. and Magan, N. (2009). "*Trametes versicolor*: Potential for atrazine bioremediation in calcareous clay soil, under low water availability conditions". *International Biodeterioration and Biodegradation*, 63, 389-394.
- Bending, G.D., Friloux, M., and Walker, A. (2002). "Degradation of contrasting pesticides by white rot fungi and its relationship with ligninolytic potential". *FEMS Microbiology Letters*, 212, 59-63.
- Blázquez, P. and Guieysse, B. (2008). "Continuous biodegradation of 17 $\beta$ -estradiol and 17 $\alpha$ -ethynylestradiol by *Trametes versicolor*". *Journal of Hazardous Materials*, 150(2), 459-462.
- Bumpus, J.A. and Aust, S.D. (2005). "Biodegradation of environmental pollutants by the white rot fungus *Phanerochaete chrysosporium*: Involvement of the lignin degrading system". *BioEssays*, 6(4), 166-170.
- Bumpus, J.A., Tien, M., Wright, D., and Aust, S.D. (1985). "Oxidation of persistent environmental pollutants by a white rot fungus". *Science*, 228(4706), 1434-1436.
- Cabana, H., Jones, J.P., and Agathos, S.N. (2007). "Elimination of endocrine disrupting chemicals using white rot fungi and their lignin modifying enzymes: A review". *Engineering in Life Sciences*, 7(5), 429-456.

- Campbell, N.A., Reece, J.B., and Mitchell, L.G. (1999). *Biology*, 5<sup>th</sup> ed., New York, NY, Addison Wesley Longman, Inc.
- Cowling, E.B. and Merrill, W. (1966). "Nitrogen in wood and its role in wood deterioration". *Canadian Journal of Botany*, 44, 1539-1554.
- Dashtban, M., Schraft, H., Syed, T.A., and Qin, W. (2010). "Fungal biodegradation and enzymatic modification of lignin". *International Journal of Biochemistry and Molecular Biology*, 1(1), 36-50.
- Diamanti-Kandarakis, E., Bourguignon, J., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., and Gore, A.C. (2009). "Endocrine-disrupting chemicals: An Endocrine Society scientific statement". *Endocrine Reviews*, 30(4), 293-342.
- Dill, I. and Kraepelin, G. (1986). "Palo Podrido: Model for extensive delignification of wood by *Ganoderma applanatum*". *Applied Environmental Microbiology*, 52(6), 1305-1312.
- Dorado, J., Claassen, F.W., van Beek, T.A., Lenon, G., Wijnberg, J.B., and Sierra-Alvarez, R. (2000). "Elimination and detoxification of softwood extractives by white-rot fungi". *Journal of Biotechnology*, 80, 231-240.
- Dyer, Kerul (2010). "Chemical warfare on native species". *Environmental Protection Information Center*. Updated March 8, 2010: <http://www.wildcalifornia.org/blog/chemical-warfare-on-native-species/>.
- Ehlers, G.A. and Rose, P.D. (2005). "Immobilized white-rot fungal biodegradation of phenol and chlorinated phenol in trickling packed-bed reactors by employing sequencing batch operation". *Bioresource Technology*, 96, 1264-1275.
- Enayatzamir, K., Alikhani, H.A., Yahchali, B., Tabandeh, F., and Rodríguez-Couto, S. (2010). "Decolouration of azo dyes by *Phanerochaete chrysosporium* immobilized into alginate beads". *Environmental Science and Pollution Research*, 17, 145-153.
- Ergül, F.E., Sargin, S., Öngen, G., and Sukan, F.V. (2009). "Dephenolization of olive mill wastewater using adapted *Trametes versicolor*". *International Biodeterioration and Biodegradation*, 63, 1-6.
- Faison, B.D. and Kirk, T.K. (1985). "Factors involved in the regulation of a ligninase activity in *Phanerochaete chrysosporium*". *Applied Environmental Microbiology*, 49, 299-304.
- Ferapontova, E.E., Castillo, J. and Gorton, L. (2006). "Bioelectrocatalytic properties of lignin peroxidase from *Phanerochaete chrysosporium* in reactions with phenols, catechols, and lignin-model compounds". *Biochimica et Biophysica Acta*, 1760(9), 1343-1354.
- Fischer-Scherl, T., Veesser, A., Hoffmann, R.W., Kühnhauser, C., Negele, R.D., and Ewringmann, T. (1991). "Morphological effects of acute and chronic atrazine exposure in rainbow trout (*Oncorhynchus mykiss*)". *Archives of Environmental Contaminant Toxicology*, 20(4), 454-461.

- Focazio, M.J., Koplin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., and Thurman, M.E. (2008). "A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States—II) untreated drinking water sources". *Science of the Total Environment*, 402(2-3), 201-216.
- Fukuhara, T., Iwasaki, S., Kawashima, M., Shinohara, O., and Abe, I. (2006). "Adsorbability of estrone and 17- $\beta$ -estradiol in water onto activated carbon". *Water Research*, 40, 241-248.
- Gammon, D.W., Silva, M., Aldous, C., Sanborn, J.R., Carr, W.C., Pfeifer, K., and Schreider, J.P. (2001). "Atrazine: Risk Characterization Document". California Environmental Protection Agency. Report: Atrazine 8-15-01.
- Glassmeyer, S.T., Furlong, E.T., Koplin, D.W., Cahill, J.D., Zaugg, S.D., Werner, S.L., Meyer, M.T., and Kryak, D.D. (2005). "Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination". *Environmental Science and Technology*, 39(14), 5157-5169.
- Hickey, W.J., Fuster, D.J., and Lamar, R.T. (1994). "Transformation of atrazine in soil by *Phanerochaete chrysosporium*". *Soil Biology and Biochemistry*, 26(12), 1665-1671.
- Howard, R.L., Abotsi, E., Jansen van Rensburg, E.L., and Howard, S. (2003). "Lignocellulose biotechnology: Issues of bioconversion and enzyme production". *African Journal of Biotechnology*, 2(12), 602-619.
- Hu, J.Y., Aizawa, T., and Kubo, S. (2002). "Products of aqueous chlorination of bisphenol A and their estrogenic activity". *Environmental Science and Technology*, 36, 1980-1987.
- Hu, J.Y., Cheng, S.J., Aizawa, T., Terao, Y., and Kunikane, S. (2003). "Products of aqueous chlorination of 17 $\beta$ -estradiol and their estrogenic activities". *Environmental Science and Technology*, 37, 5665-5670.
- Hundt, K., Martin, D., Hammer, E., Jonas, U., Kindermann, M.K., and Schauer, F. (2000). "Transformation of triclosan by *Trametes versicolor* and *Pycnoporus cinnabarinus*". *Applied Environmental Microbiology*, 66, 4157-4160.
- Hwang, S.S., Choi, H.T., and Song, H.G. (2008). "Biodegradation of endocrine-disrupting phthalates by *Pleurotus ostreatus*". *Journal of Microbiology and Biotechnology*, 18(4), 767-772.
- Ingham, E.R. (2007). "Chapter 4: Soil Fungi". *The Soil Biology Primer*. National Resources Conservation Service (NRCS).
- International Programme on Chemical Safety (IPCS). (2002). "Global assessment of the state-of-the-science of endocrine disruptors". Eds. Damstra, T., Barlow, S., Bergman, A., Kavlock, R., and Van der Kraak, G. Prepared for: the World Health Organization, the International Labour Organisation, and the United Nations Environment Programme.

- Jiang, J.Q., Yin, Q., Zhou, J.L., and Pearce, P. (2005). "Occurrence and treatment trials of endocrine disrupting chemicals (EDCs) in wastewaters". *Chemosphere*, 61(4), 544-550.
- Kavlock, R. J., Daston, G.P., DeRosa, C., Fenner-Crisp, P., Gray, L. E., Kaattari, S., Lucier, G., Luster, M., Mac, M. J., Maczka, C., Miller, R., Moore, J., Rosalind, R., Scott, G., Sheehan, D. M., Sinks, T., and Tilson, H. A. (1996). "Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U.S. EPA-sponsored Workshop". *Environmental Health Perspectives*, 104(4), 715-740.
- Klonowska, A., Gaudin, C., Fournel, A., Asso, M., Le Petit, J., Giorgi, M. and Tron, T. (2002). "Characterization of a low redox potential laccase from the basidiomycete C30". *European Journal of Biochemistry*, 269, 6119-6125.
- Kuster, M., López de Alda, M.J., Hernando, M.D., Petrovic, M., Martín-Alonso, J., and Barceló, D. (2008). "Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain)". *Journal of Hydrology*, 358(1-2), 112-123.
- Kirk, T.K. and Farrell, R.L. (1987). "Enzymatic "combustion": The microbial degradation of lignin". *Annual Review of Microbiology*, 41, 465-505.
- Kirk, T.K., Connors, J., and Zeikus, G. (1976). "Requirement for a growth substrate during lignin decomposition by two wood-rotting fungi". *Applied and Environmental Microbiology*, 32(1), 192-194.
- Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., and Zeikus, J.G. (1978). "Influence of culture parameters on lignin metabolism by *Phanerochaete chrysosporium*". *Archives of Microbiology*, 117, 277-285.
- Koplin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., and Buxton, H.T. (2002). "Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance". *Environmental Science and Technology*, 36(6), 1202-1211.
- Langerveld, A.J., Celestine, R., Zaya, R., Mihalko, D., and Ide, C.F. (2009). "Chronic exposure to high levels of atrazine alters expression of genes that regulate immune and growth-related functions in developing *Xenopus laevis* tadpoles". *Environmental Research*, 109, 379-389.
- Leatham, G.F. (1986). "The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium*". *Applied Microbiology and Biotechnology*, 24, 51-58.
- Lee, S.M., Koo, B.W., Lee, S.S., Kim, M.K., Choi, D.H., Hong, E.J., Jeung, E.B., and Choi, I.G. (2004). "Biodegradation of dibutylphthalate by white rot fungi and evaluation on its estrogenic activity". *Enzyme and Microbial Technology*, 35(5), 417-423.
- Leisola, M.S.A., Ulmer, D.C., Waldner, R., and Fiechter, A. (1984). "Role of veratryl alcohol in lignin degradation by *Phanerochaete chrysosporium*". *Journal of Biotechnology*, 1, 331-339.

- Lewandowski, G.A., Armenante, P.M., and Pak, D. (1990). "Reactor design for hazardous waste treatment using a white rot fungus". *Water Research*, 24(1), 75-82.
- Lim, S., Ahn, S.Y., Song, I.C., Chung, M.H., Jang, H.C., Park, K.S., Lee, K.U., Pak, Y.K., and Lee, H.K. (2009). "Chronic exposure to the herbicide, atrazine, causes mitochondrial dysfunction and insulin resistance". *Plos One*, 4(4), e5186, 1-11.
- Logan, B.E. (1999). *Environmental Transport Processes*, New York, NY, John Wiley & Sons, Inc.
- Loper, C.A., Crawford, J.K., Otto, K.L., Manning, R.L., Meyer, M.T., and Furlong, E.T. (2007) "Concentrations of selected pharmaceuticals and antibiotics in south-central Pennsylvania waters, March through September 2006". *U.S. Geological Survey Data Series 300*, 101p.
- Lui, Z., Kanjo, Y., and Mizutani, S. (2009). "Removal mechanisms for endocrine disrupting compounds (EDCs) in wastewater treatment – physical means, biodegradation, and chemical advanced oxidation: A review". *Science of the Total Environment*, 407, 731-748.
- Mai, C., Kues, U., and Militz, H. (2004). "Biotechnology in the wood industry". *Applied Microbiology and Biotechnology*, 63, 477-494.
- Mao, L., Lu, J., Gao, S., and Huang, Q. (2010). "Transformation of 17beta-estradiol mediated by lignin peroxidase: The role of veratryl alcohol". *Archives of Environmental Contamination and Toxicology*, 59(1), 13-9.
- Martinez, A.T., Speranza, M., Ruiz-Duenas, F.J., Ferreira, P., Camarero, S., Guillen, F., Martinez, M.J., Gutierrez, A., and del Rio, J.C. (2005). "Biodegradation of lignocellulosics: Microbial, chemical, and enzymatic aspects of the fungal attack of lignin". *International Microbiology*, 8, 195-204.
- Masaphy, S., Henis, Y., and Levanon, D. (1996). "Manganese-enhanced biotransformation of atrazine by the white rot fungus *Pleurotus pulmonarius* and its correlation with oxidation activity". *Applied and Environmental Microbiology*, 62(10), 3587-3593.
- Millis, C.D., Cai, D., Stankovich, M.T., and Tien, M. (1989). "Oxidation-reduction potentials and ionization states of extracellular peroxidases from the lignin-degrading fungus *Phanerochaete chrysosporium*". *Biochemistry*, 28, 8484-8489.
- Moreira, M.T., Palma, C., Feijoo, G., and Lema, J.M. (1998). "Strategies for the continuous production of ligninolytic enzymes in fixed and fluidized bed bioreactors". *Journal of Biotechnology*, 66, 27-39.
- Moriyama, K., Matsufuji, H., Chino, M., and Takeda, M. (2004). "Identification and behavior of reaction products formed by chlorination of ethynylestradiol". *Chemosphere*, 55, 839-847.



- Mougin, C., Laugero, C., Asther, M., and Chaplain, V. (1997). "Biotransformation of *s*-triazine herbicides and related degradation products in liquid cultures by the white rot fungus *Phanerochaete chrysosporium*". *Pesticide Science*, 49, 169-177.
- Mougin, C., Laugero, C., Asther, M., Dubroca, J., Frasse, P., and Asther, M. (1994). "Biotransformation of the herbicide atrazine by the white rot fungus *Phanerochaete chrysosporium*". *Applied Environmental Microbiology*, 60, 705-708.
- Nemitz, J.L and Parizek, R.R. (2005). "Occurrence, fate and transport of pharmaceuticals and personal care products in at Penn State's Living Filter Project". *2004 Forecast of Loading Report, Office of the Physical Plant*. The Pennsylvania State University.
- Ntwampe, S., Chowdhury, F., Sheldon, M., and Volschenk, H. (2010). "Overview of parameters influencing biomass and bioreactor performance used for extracellular ligninase production from *Phanerochaete chrysosporium*". *Brazilian Archives of Biology and Technology*, 53(5), 1057-1066.
- Odier, E. and Artaud, I. (1992). "Degradation of lignin". In: Winkelmann, G. (Ed.), *Microbial degradation of natural products*, VCH, Weinheim, Germany.
- Ostby, J., Monosson, E., Kelce, W.R., and Gray, L.E., (1999). "Environmental antiandrogens: Low doses of the fungicide vinclozolin alter sexual differentiation of the male rat". *Toxicology and Industrial Health*, 15, 48-64.
- Pallerla, S. and Chambers, R.P. (1997). "Characterization of a Ca-alginate-immobilized *Trametes versicolor* bioreactor for decolorization and AOX reduction of papermill effluents". *Bioresource Technology*, 60(1), 1-8.
- Paszczyński, A., Huynh, V., and Crawford, R. (1986). "Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium*". *Archives of Biochemistry and Biophysics*, 244 (2), 750-765.
- Petrovic, M., Eljarrat, E., Lopez de Alda, M.J., and Barceló, D. (2004). "Endocrine disrupting compounds and other emerging contaminants in the environment: A survey on new monitoring strategies and occurrence data". *Analytical and Bioanalytical Chemistry*, 378, 549-562.
- Pennsylvania Department of Environmental Protection (PA DEP). 2011. "Emerging Contaminants". *Public Drinking Water*. Updated 2011:  
[http://www.portal.state.pa.us/portal/server.pt/community/public\\_drinking\\_water/10549/merging\\_contaminants/553903#ques6](http://www.portal.state.pa.us/portal/server.pt/community/public_drinking_water/10549/merging_contaminants/553903#ques6).
- Pickard, M.A., Vandertol, H., Roman, R., and Vazquez-Duhalt, R. (1999). "High production of ligninolytic enzymes from white rot fungi in cereal bran liquid medium". *Canadian Journal of Microbiology*, 45, 627-631.
- Pocedič, J., Hasal, P., and Novotný, Č. (2009). "Decolorization of organic dyes by *Irpex lacteus* in a laboratory trickle-bed biofilter using various mycelium supports". *Journal of Chemical Technology and Biotechnology*, 84, 1031-1042.

- Pointing, S.B. (2001). "Feasibility of bioremediation by white-rot fungi". *Applied Microbiology and Biotechnology*, 57, 20-33.
- Research Collaboratory for Structural Bioinformatics (RCSB). (2011). *Protein Data Base*. Updated March 8, 2011: <http://www.rcsb.org/pdb/home/home.do>.
- Reddy, C.A. (1995). "The potential for white-rot fungi in the treatment of pollutants". *Current Opinion in Biotechnology*, 6, 320-328.
- Rittmann, B.E. and McCarty, P.L. (2001). *Environmental Biotechnology: Principles and Applications*, New York, NY, McGraw-Hill.
- Rothschild, N., Levkowitz, A., Hadar, Y., and Dosoretz, C.G. (1999). "Manganese deficiency can replace high oxygen levels needed for lignin peroxidase formation by *Phanerochaete chrysosporium*". *Applied and Environmental Microbiology*, 65(2), 222-238.
- Safe, S.H. (2000). "Endocrine disruptors and human health-Is there a problem? An update". *Environmental Health Perspectives*, 108 (6), 487-493.
- Sanderson, J.T., Letcher, R.J., Heneweer, M., Giesy, J.P., and van den Berg, M. (2001). "Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes". *Environmental Health Perspectives*, 109(10), 1027-1031.
- Scribner, E.A., Thurman, E.M., Goolsby, D.A., Meyer, M.T., Battaglin, W.A., and Koplin, D.W. (2005). "Summary of significant results from studies of triazine herbicides and their degradation products in surface water, ground water, and precipitation in the Midwestern United States during the 1990s". USGS Scientific Investigations, Report 2005-5094.
- Scribner, E.A., Thurman, E.M. and Zimmerman, L.R. (2000). "Analysis of selected herbicide metabolites in surface and groundwater of the United States". *Science of the Total Environment*, 248, 157-168.
- Shim, S.S. and Kawamoto, K. (2002). "Enzyme production activity of *Phanerochaete chrysosporium* and degradation of pentachlorophenol in a bioreactor". *Water Research*, 36, 4445-4454.
- Siddiqui, M.F., Andleeb, S., Ali, N., Ghumro, P.B., and Ahmed, S. (2009). "Up-flow immobilized fungal column reactor for the treatment of anthraquinone dye diramarene blue K2RL". *African Journal of Biotechnology*, 8(20), 5570-5577.
- Singh, D. and Chen, S. (2008). "The white-rot fungus *Phanerochaete chrysosporium*: Conditions for the production of lignin-degrading enzymes". *Applied Microbiology and Biotechnology*, 81, 399-417.
- Snyder, S.A., Adham, S., Redding, A.M., Cannon, F.S., Decarolis, J., Oppenheimer, J., Wert, E.C., and Yoon, Y. (2007). "Role of membranes and activated carbon in the removal of endocrine disruptors and pharmaceuticals". *Desalination*, 202, 156-81.

- Soares, A., Jonasson, K., Terrazas, E., Guieysse, B., and Mattiasson, B. (2005). "The ability of white-rot fungi to degrade the endocrine-disrupting compound nonylphenol". *Applied Microbiology and Biotechnology*, 66, 719-725.
- Soares, A., Guieysse, B., and Mattiasson, B. (2006). "Influence of agitation on the removal of nonylphenol by the white-rot fungi *Trametes versicolor* and *Bjerkandera sp.* BOL 13". *Biotechnology Letters*, 28, 139-143.
- Tamagawa, Y., Yamaki, R., Hirai, H., Kawai, S., Nishida, T. (2006). "Removal of estrogenic activity of natural steroidal hormone estrone by ligninolytic enzymes from white rot fungi". *Chemosphere*, 65, 97-101.
- Thayer, K.A., Ruhlen, R.L., Howdeshell, K.L., Buchanan, D.L., Cooke, P.S., Preziosi, P.S., Welshons, W.V., Haseman, J., and vom Saal, F.S. (2001). "Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17 $\alpha$ -ethinyl estradiol". *Human Reproduction*, 16(5), 988-996.
- Tien, M. and Kirk, T. K. (1984). "Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization, and catalytic properties of a unique H<sub>2</sub>O<sub>2</sub>-requiring oxygenase". *Biochemistry*, 81, 2280-2284.
- Tien, M. and Kirk, T. K. (1988). "Lignin peroxidase of *Phanerochaete chrysosporium*". *Methods in Enzymology*, 161, 238-249.
- Tillet, D.E., Papoulias, D.M., Whyte, J.J., and Richter, C.A. (2010). "Atrazine reduces reproduction in fathead minnow (*Pimephales promelas*)". *Aquatic Toxicology*, 99(2), 149-159.
- United States Environmental Protection Agency (US EPA) (2003). "Technical fact sheet on: Atrazine". <http://www.epa.gov/safewater/pdfs/factsheets/soc/tech/altrazine.pdf>.
- US EPA (2010a). "EDSP background". *Endocrine Disruptor Screening Program (EDSP)*. Updated April 22, 2010: <http://www.epa.gov/endo/pubs/edspoverview/background.htm>.
- US EPA (2010b). "Endocrine Primer". *Endocrine Disruptor Screening Program (EDSP)*. Updated April 22, 2010: <http://www.epa.gov/endo/pubs/edspoverview/primer.htm#1>.
- US EPA (2011). "Atrazine background". *Pesticides: Topical and Chemical Fact Sheets*. Updated February 16, 2011: [http://www.epa.gov/pesticides/factsheets/atrazine\\_background.htm](http://www.epa.gov/pesticides/factsheets/atrazine_background.htm).
- Vogel, J.M. (2004). "Tunnel vision: The regulation of endocrine disruptors". *Policy Science*, 37, 277-303.
- Venkatadri, R. and Irvine, R.L. (1990). "Effect of agitation on ligninase activity and ligninase production by *Phanerochaete chrysosporium*". *Applied Environmental Microbiology*, 56, 2684-2691.

- Venkatadri, R., and Irvine, R.L. (1993). "Cultivation of *Phanerochaete chrysosporium* and production of lignin peroxidase in novel biofilm reactor systems: Hollow fiber reactor and silicone membrane reactor". *Water Research*, 27(4), 591-596.
- Wariishi, H., Dunford, H.B., MacDonald, I.D., and Gold, M.H. (1989). "Manganese peroxidase from the lignin-degrading basidiomycete *Phanerochaete chrysosporium*: Transient state kinetics and reaction mechanism". *Journal of Biological Chemistry*, 264(6), 3335-3340.
- Wessenberg, D., Kyriakides, I., and Agathos, S.N. (2003). "White-rot fungi and their enzymes for the treatment of industrial dye effluents". *Biotechnology Advances*, 22, 161-187.
- Westerhoff, P., Yoon, Y., Snyder, S., and Wert, E. (2005). "Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes". *Environmental Science and Technology*, 39(17), 6649-6663.
- Wu, M., Quirindongo, M., Sass, J., and Wetzler, A. (2010). "Still poisoning the well: Atrazine continues to contaminate surface and drinking water in the United States". National Resource Defense Council. Report.
- Yum, K.J., and Peirce, J.J. (1998). "Biodegradation kinetics of chlorophenols in immobilized-cell reactors using a white-rot fungus on wood chips". *Water Environment Research*, 70(2), 205-213.
- Xu, F., Shin, W., Brown, S.H., Wahleithner, J.A., Sundaram, U.M., and Solomon, E.I. (1996). "A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability". *Biochimica et Biophysica Acta*, 1292, 303-311.
- Zhang, F.M., Knapp, J.S., and Tapley, K.N. (1999). "Development of bioreactor systems for decolorization of Orange II using white rot fungus". *Enzyme and Microbial Technology*, 24, 48-53.
- Zhang, X., Yao, Y., Zeng, X., Qian, G., Guo, Y., Wu, M., Sheng, G., and Fu, J. (2008). "Synthetic musks in the aquatic environment and personal care products in Shanghai, China". *Chemosphere*, 72, 1553-1558.
- Ziomkiewicz, A., Ellison, P.T., Lipson, S.F., Thune, I., and Jasienska, G. (2008). "Body fat, energy balance, and estradiol levels: A study based on hormonal profiles from complete menstrual cycles". *Human Reproduction*, 23(11), 2555-2563.

## Appendix A

### Wastewater Collection Site

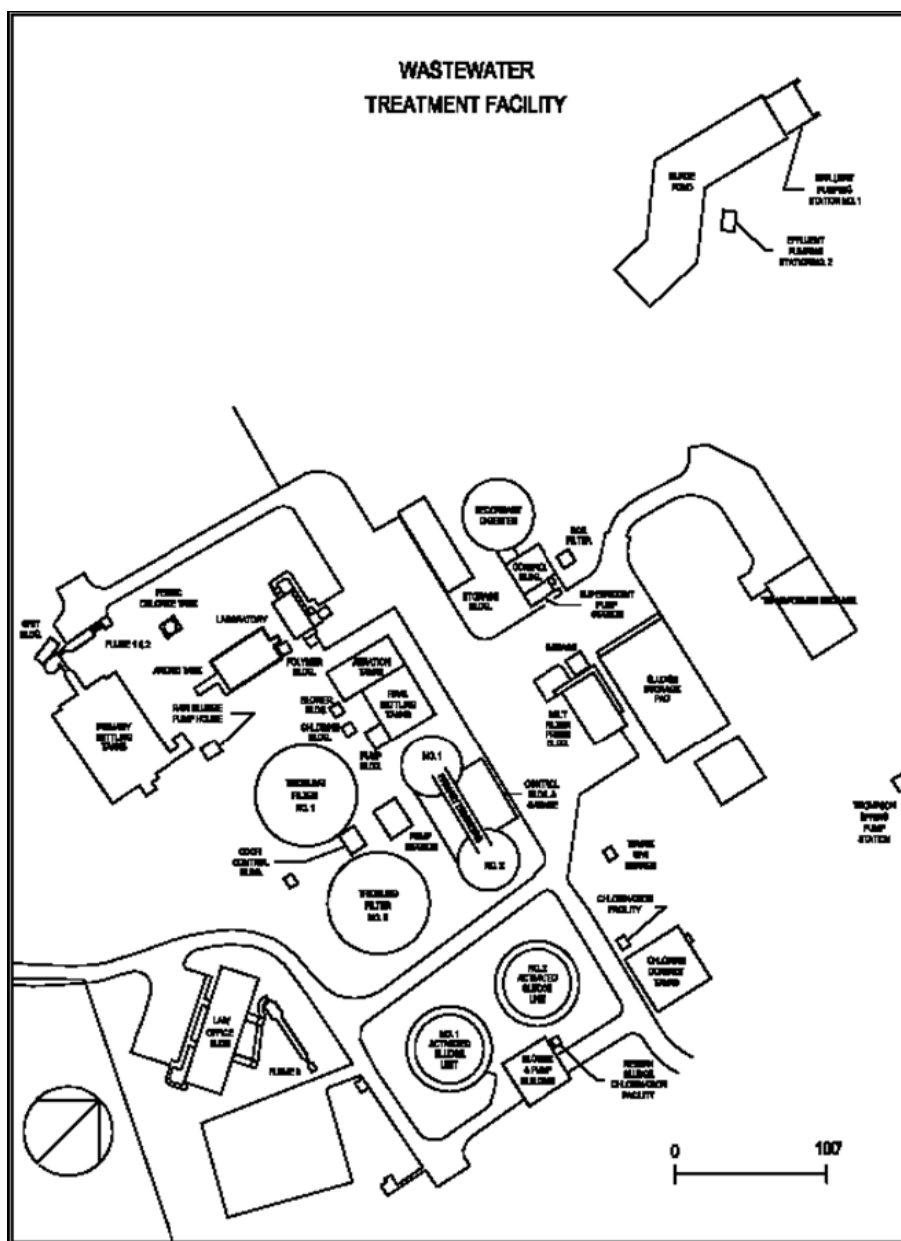
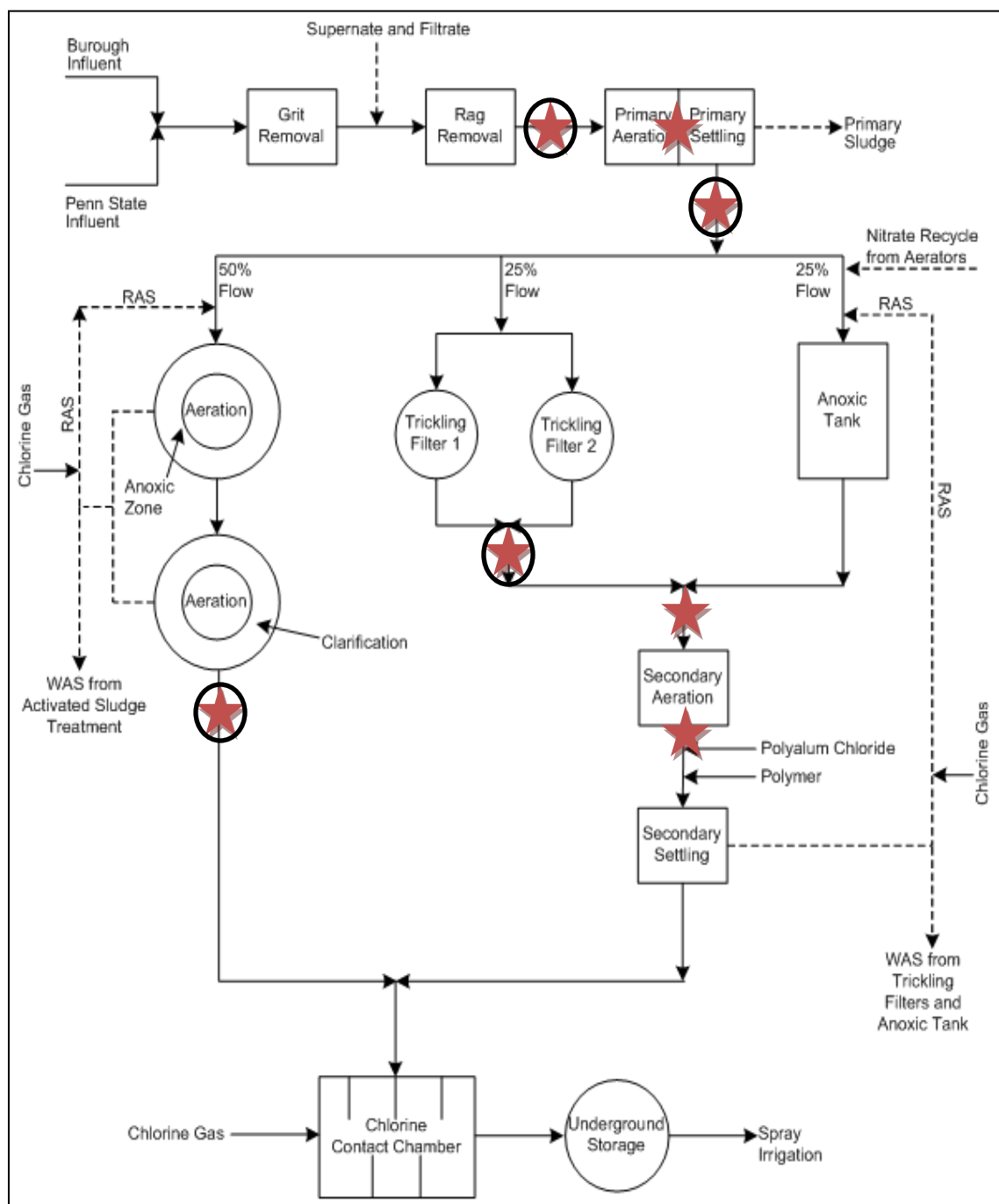


Figure A-1. Penn State wastewater treatment plant schematic.



**Figure A-2.** Penn State wastewater treatment plant process flow-diagram. Stars indicate the seven effluent locations that were considered for Batch Experiment 2. Encircled stars indicate the four selected effluent locations.

## Appendix B

### Photographs of Materials Used in Experimental Setups



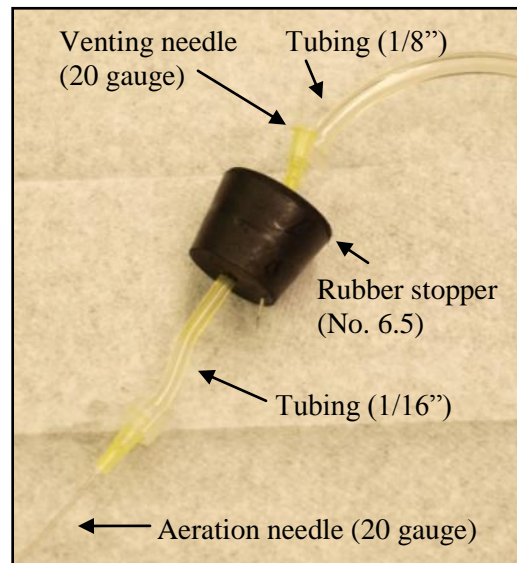
**Figure B-1.** Jaeger Tri-Pack® (1").



**Figure B-2.** Birch woodchips (0.131" – 0.25").



**Figure B-3.** Foamex SIF II® Foam (ZS15D) cubes (0.5").



**Figure B- 4.** Aeration apparatus used to seal each flask.



**Figure B- 5.** Humidification apparatus. Photograph is courtesy of Michael Shreve.





**Figure B- 6.** Experimental setup. This photograph illustrates Batch Experiment 1 reactors connected to aeration manifolds.

## Appendix C

### Media Recipes and Inoculum Preparation Methods

Note: The following recipes are for 1 L of each media solution; however solutions can be made in any volume by adjusting the mass of ingredients accordingly.

#### C.1. YMPG Media Recipe

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

<b>D – Glucose, Anhydrous</b>	<b>10g</b>
<b>Malt extract</b>	<b>10g</b>
<b>Peptone</b>	<b>2g</b>
<b>Yeast extract</b>	<b>2g</b>
<b>L – Asparagine, Anhydrous</b>	<b>1g</b>
<b>KH<sub>2</sub>PO<sub>4</sub></b>	<b>2g</b>
<b>MgSO<sub>4</sub> •7H<sub>2</sub>O</b>	<b>1g</b>

3. **If making slants, add 20g of agar.** (Omit agar if making liquid media.)
4. Remove the stir bar, and take the final volume to 1 L with DDI water.
5. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
6. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.

7. Autoclave for 30 minutes.
8. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.
9. Add 1ml of sterile **1mg/ml thiamine** (vitamin B1) solution to the cooled media to yield a final concentration of 1mg/L, and swirl by hand to mix.
  - a. To make a 1mg/ml thiamine solution:
    - i. Mix 50mg of thiamine with 50mL of DDI H<sub>2</sub>O in a volumetric flask (yields a 1mg/mL solution).
    - ii. Invert the flask until the solution is completely mixed.
    - iii. Filter sterilize the thiamine solution into a sterile serum bottle (see sterilizing serum bottles and venting procedure).
10. In laminar flow hood with Bunsen burner lit, aliquot out 3mL of media into sterile 2-position snap tubes (17 x 100 mm polypropylene, Dot Scientific Inc, Product # 592-S) using a pipettor bulb and sterile volumetric pipette. Cap to first (loose) position to vent.
11. Place capped tubes at a 20° angle or less to allow for maximum surface area once cooled.
12. Once cooled, push the cap to the bottom position to seal.
13. Place all tubes into a Ziploc bag, and store in refrigerator until ready to streak with fungi. If storing individually, wrap the cap with plastic wrap or parafilm.

## C.2. Low Nitrogen Media Recipe

1. To a 2-L autoclavable glass flask, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

<b>Basal III media</b>	<b>100ml</b>
<b>D – Glucose, Anhydrous</b>	<b>10g</b>

<b>0.1M transaconitic acid,pH 4.3</b>	<b>100mL</b>
<b>Ammonium tartrate</b>	<b>0.2g</b>
<b>Trace elements</b>	<b>60mL</b>

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Wrap aluminum foil over the opening of the flask and add a piece of autoclave tape.
5. Place flask in an autoclavable bin and fill with several inches of tap water to aid in equal heating.
6. Autoclave for 30 minutes.
7. Remove from autoclave and allow media to cool until you can place your hands on the sides of the flask without being burnt.
8. Add 1mL of sterile **1mg/ml thiamine** solution to the cooled media to yield a final concentration of 1mg/L, and swirl by hand to mix.
9. For stationary cultures, add 100mL of sterile **4mM veratryl alcohol** on day 3. For agitated cultures, add the veratryl alcohol and 50mL of sterile **1% Tween 80 solution**.

### **C.3. Basal III Media Recipe**

1. To a 1-L glass container, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add the following:

<b>KH<sub>2</sub>PO<sub>4</sub></b> (Fisher Scientific, Fair Lawn, NJ)	<b>20g</b>
<b>MgSO<sub>4</sub>•7H<sub>2</sub>O</b>	<b>5g</b>
<b>CaCl<sub>2</sub></b> (Fisher Scientific)	<b>1g</b>
<b>Trace elements solution</b>	<b>100mL</b>

3. Remove the stir bar, and take the final volume to 1 L with DDI water.

4. Store in refrigerator.

#### C.4. Trace Elements Solution Recipe

##### Procedure

1. To a 1-L glass container, add a stir bar and ~500ml DDI water. Place on a stir plate.
2. While stirring, add:

**Nitrilotriacetic acid (NTA; TCI, Tokyo, Japan) 1.5g**

*Adjust pH to 6.5 with concentrated NaOH or HCl (~1-2mL)*

*(some minerals will not go into solution if pH drifts from 6.5 significantly)*

Continue adding in order, allowing each to dissolve in turn:

<b>MgSO<sub>4</sub>•7H<sub>2</sub>O</b>	<b>3g</b>
<b>MnSO<sub>4</sub>•H<sub>2</sub>O</b> (J.T. Baker, Phillipsburg, NJ)	<b>0.5g</b>
<b>NaCl</b> (J.T. Baker)	<b>1g</b>
<b>FeSO<sub>4</sub>•7H<sub>2</sub>O</b> (J.T. Baker)	<b>0.1g</b>
<b>CoCl<sub>2</sub>•6H<sub>2</sub>O</b> (J.T. Baker)	<b>0.1g</b>
<b>ZnSO<sub>4</sub>•7H<sub>2</sub>O</b> (Sigma-Aldrich)	<b>0.1g</b>
<b>CuSO<sub>4</sub>•5H<sub>2</sub>O</b> (J.T. Baker)	<b>0.1g</b>
<b>Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>•18H<sub>2</sub>O</b> (Sigma-Aldrich)	<b>10mg</b>
<b>H<sub>3</sub>BO<sub>3</sub></b> (VWR International, France)	<b>10mg</b>
<b>Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O</b> (Sigma-Aldrich)	<b>10mg</b>

3. Remove the stir bar, and take the final volume to 1 L with DDI water.
4. Store in refrigerator.

## C.5. Preparation of Mycelia Inoculum for Batch Reactors

### C.5.1. Spore Solution

1. Calculate the total volume of mycelia inoculum needed for your experiment.
  - a. Inoculum = 10% of total solution volume in your flask reactor; consists of 10 mL spore solution + 90 mL Low Nitrogen Media
  - b. For one reactor flask (135 mL solution), 13.5 mL of this should be mycelia inoculum. For **X** number of reactor flasks, **total volume** of inoculum needed = **X \* 13.5 mL**.
2. Calculate the total number of 2 L Erlenmeyer flasks needed to grow up mycelia from spore solution.
  - a. Use 1 flask per 100 mL of inoculum (i.e. **flask #** = **[X \* 13.5 mL]/100 mL**). If you do not have an even # of flasks needed (i.e. you find you need 8.1 flasks) round up to the next flask and **DO NOT make partial batches** (i.e. all flasks will have 100 mL of inoculum)
3. Gather approximately 10 – 12 slants per each 100 mL of *spore solution* needed.
4. In the laminar flow hood using aseptic technique:
  - a. Open one 50 mL sterile serological pipet tip, and insert it into the pipet bulb.
  - b. While holding the pipet, open the bottle containing sterile water, set the bottle cap on the counter (threaded side up), flame the lip of the bottle, and insert the pipet tip. Draw up ~50 mL of water, remove the pipet tip from the bottle, flame the bottle lip, and recap the water.
  - c. While holding the pipet, open one slant tube and pass the open end of the tube through the flame once. Pipette water into the tube (fill tube ~3/4 to top). Re-

flame and re-cap the tube. Repeat for remaining tubes. Repeat step b. as needed. Remember to change out pipet tips (or flame if the tip is glass) if the tip touches any surface.

- d. Re-open the first tube, flame, and gently scrape the growth into solution using a sterile 10 mL serological pipet tip. Pour the solution into the filtration apparatus (Figure C-1). Re-flame and re-cap the tube. Repeat for remaining tubes making sure to flame the glass pipet tip after each use.



**Figure C-1.** Glass funnel containing glass wool for filtering spore solution.

- e. Open and flame the first tube again. Aseptically pipette water into the first tube and re-scrape. **Instead of filtering**, pour this solution into the next tube and re-scrape. Continue pouring from one tube to the next. After scraping the last tube, pour the solution into the filtering apparatus.
- f. Aseptically pipette approximately 1 mL of the filtered spore solution into a cuvette, and read the absorbance at a wavelength of 650 nm. Final absorbance should be ~0.5. Dilute the spore solution with water as needed and re-measure

absorbance until it is ~0.5. Be sure not to use the same pipet tip for water and spore solution.

### **C.5.2. Mycelia Solution**

1. In the laminar flow hood using aseptic technique:
  - a. Pipette 90 mL of Low Nitrogen media to each 2L flask.
  - b. Pipette 10 mL of spore solution to each 2L flask (total of 100 mL in each flask)
  - c. Ensure that the foil completely covers the opening of each flask.
2. Incubate the 2L flasks in the 30°C room for 5 days. **Do not move or disturb the flasks for at least 2 days.**

### **C.5.3. Homogenization/Inoculum Prep**

1. After 5 days, remove the 2 L flasks from the 30°C room.
2. In the laminar flow hood using aseptic technique:
  - a. Combine the contents of all 2L flasks in a 2L (or 3-4L) beaker.
  - b. Homogenize for ~1 min (or until there are no chunks) using the blender (sterilized with isopropyl alcohol and flamed).



## **Appendix D**

### **Fungal Batch Flask Sacrificing Procedures**

#### **D.1. Flask Selection**

1. Close the aeration ports to 3 (or 2 if sampling in duplicate) flasks of each treatment and remove them from the aeration manifold.
2. Calculate the reduction in aeration that is needed to maintain the 0.3 SCFH airflow to the remaining flasks. Disconnect the airline from the manifold, connect it to the flow meter, and adjust the airflow to meet the new total airflow needed. Reconnect the airline to the manifold.

#### **D.2. Sample Preservation for Atrazine Analysis (Batch Experiment 3 only)**

1. Collect and label 1 of the 2 mL amber glass autosampler vials and 1 of the 1.5 mL eppendorf tubes for each flask. Collect 1 of the 4 mL amber vials for each flask.
2. Pipette 10  $\mu\text{L}$  of 2N  $\text{H}_2\text{SO}_4$  (EMD, Gibbstown, NJ) into each 2 mL vial to acidify to a  $\text{pH} < 2$ .
3. Pipette 0.25  $\mu\text{L}$  of atrazine-D5 (d5) stock solution (1000 mg/L) into a 15 mL vial. This will serve as a 5 mg/L internal standard for the LC/MS/MS analysis.
4. Pipette 5 mL of reactor solution into the 15 mL vial containing d5. Mix the solution by pulling solution into the pipette tip and expelling multiple times.
5. Pipette 1 mL of the solution into an eppendorf tube.
6. Repeat steps 3 – 5 for the remaining reactor flasks.
7. Centrifuge the eppendorf tubes for 10 minutes at 3000 rpm.

8. For each sample, pipette 990  $\mu\text{L}$  of the centrifuged solution into a 2 mL vial. The vial should now contain 1 mL of solution (10  $\mu\text{L}$  sulfuric acid + 990  $\mu\text{L}$  sample = 1000  $\mu\text{L}$ ). Cap securely so that the septa is slightly concaved, but do not over-tighten or the septa may leak around the edges. Repeat this step for all samples.
9. Store acidified samples in a small box (ex. old tip box) in the refrigerator.

### **D.3. pH, Filtration and Drying (Batch Experiments 1 and 2 only)**

1. Measure and record pH for each flask.
2. Set up the filtering apparatus. Attach the filtering flasks to laboratory vacuum lines and turn on the vacuum lines. In general, filtration is slow, so setting up multiple filtering apparatus is recommended. If you are measuring suspended and attached growth, it may be convenient to use two filtering apparatus for each reactor (i.e. one filtering apparatus for suspended growth/solution filtration and one for drying the attached growth).
3. Place the **pre-weighed** and labeled filter paper in the funnels with the labeled side down.
4. For **attached** growth:
  - a. Wet the filter paper with a small amount of DDI water.
  - b. Using forceps, remove the support media (ex. Tri-Pack) and attached biomass from the reactor flask and place into the funnel.
  - c. Using forceps again, remove the attached growth from the support media and place it onto the filter paper.
  - d. After all biomass is collected and no solution pools are present on the filter paper, remove the funnel from the top of the flask (or turn off the vacuum) and carefully fold the paper in half using forceps. Fold the paper in half again (filter should be folded into a quarter of a circle).

- e. Place the folded filter paper onto the aluminum foil.
5. For **suspended** growth:
- a. Do NOT wet the filter paper with DDI water.
  - b. Slowly pour remaining flask contents into the funnel. Do NOT over-fill the funnel – solids will pass under the filter paper!
  - c. After all suspended biomass is collected and no solution pools are present on the filter paper, remove the funnel from the top of the flask (or turn off the vacuum) and carefully fold the paper in half using forceps. Fold the paper in half again (filter should be folded into a quarter of a circle).
  - d. Place the folded filter paper onto the aluminum foil.
  - e. Pour ~10mL of filtered solution into a 15mL centrifuge tube.
  - f. Using a 10mL syringe and tubing, pull up ~5mL of filtered solution. Rinse the syringe and discard the solution.
  - g. Pull up ~10mL of filtered solution and store in the freezer.
  - h. Discard the remaining solution in the filtering flask. Rinse the filtering flask twice with DDI water.
6. After filtration for all reactor solutions is complete, fold the aluminum foil over or add an additional piece to cover the filter paper. Place into the oven at 40°C for 3 days.
7. Remove the foil (with filter paper) from the oven and allow to cool in the dessicator.
8. Using the analytical balance, weigh the filter papers.

### **D.3. pH, Solution Volume Estimating and Drying (Batch Experiments 3 only)**

1. Complete the following steps after atrazine-sampling preservation.
2. Measure the pH of the solution in each reactor flask.

3. Pipette 10 mL of reactor solution into a labeled 15 mL centrifuge tube. Repeat for all flasks.  
Store these samples in the freezer.
4. Decant remaining flask solution into 100 mL and 50 mL cylinders to measure the remaining volume. Record the volume and repeat for all flasks.
5. Remove the packing media (wood or foam) and place it on a pre-weighed piece of foil. Mass the foil + packing media to obtain the wet mass. Repeat for all flasks.
6. Place the sacrificed packing media into the oven at 40°C for 4 - 5 days.
7. Remove the samples from the oven and allow to cool in the dessicator.
8. Using the analytical balance, weigh the samples

## **Appendix E**

### **Enzyme Activity Assays**

#### **E.1. Lignin Peroxidase Assay**

5. Set the UV spectrophotometer to 310 nm wavelength.
6. To a 1.5 mL cuvette, add:
  - a. 50  $\mu$ L of 20 mM VA
  - b. 100  $\mu$ L of 0.2 M Sodium tartrate buffer (pH 3.0)
  - c. X  $\mu$ L of sample solution
  - d. 750 – X  $\mu$ L of DDI water
7. Mix the cuvette solution well by drawing up and expelling the solution a few times with the 100 – 1000  $\mu$ L pipettor.
8. Put the cuvette into the UV spec. Make sure that the arrow on the cuvette is facing forward (toward you).
9. Pipette 100  $\mu$ L of 2 mM hydrogen peroxide into the cuvette. Mix. Close the top of the UV spec and hit “auto-zero” immediately.
10. When the display reads zero (the machine will beep), immediately start the timer. Record the absorbance (ABS) readings at time = 10, 20, 60, 90, and 120 seconds. More or less time points can be used depending on the observed rate of change.
11. In Excel, plot absorbance vs time. Determine the slope (dABS/dt) of the linear portion of the line. To determine the slope, use the equation of a best-fit line that runs through at least 3 points.

12. Multiply the slope by 60 seconds to get the change in absorbance after 1 minute. Use this

$\Delta$ ABS to calculate enzyme activity using the following equation:

$$\text{Lignin Peroxidase Activity} \left( \frac{U}{mL} \right) = \frac{\Delta ABS}{9.3} \times \frac{1000}{X}$$

Where X ( $\mu$ L) = sample volume added to the cuvette.

## **E.2. Manganese Peroxidase Assay**

1. Set the UV spectrophotometer to 470 nm wavelength.
2. To a 1.5 mL cuvette, add:
  - a. 50  $\mu$ L of 20 mM 2,6-Dimethoxyphenol
  - b. 100  $\mu$ L of 0.5 M Sodium tartrate buffer (pH 4.5)
  - c. 50  $\mu$ L of 20 mM Manganese sulfate
  - d. X  $\mu$ L of sample solution
  - e. 700 – X  $\mu$ L of DDI water
3. Mix the cuvette solution well by drawing up and expelling the solution a few times with the 100 – 1000  $\mu$ L pipettor.
4. Put the cuvette into the UV spec. Make sure that the arrow on the cuvette is facing forward.
5. Pipette 100  $\mu$ L of 2 mM hydrogen peroxide into the cuvette. Mix. Close the top of the UV spec and hit “auto-zero” immediately.
6. When the display reads zero (the machine will beep), immediately start the timer. Record the absorbance (ABS) readings at time = 10, 20, 60, 90, and 120 seconds. More or less time points can be used depending on the observed rate of change.

7. In Excel, plot absorbance vs time. Determine the slope (dABS/dt) of the linear portion of the line. To determine the slope, use the equation of a best-fit line that runs through at least 3 points.
8. Multiply the slope by 60 seconds to get the change in absorbance after 1 minute. Use this  $\Delta$ ABS to calculate enzyme activity using the following equation:

$$\text{Manganese Peroxidase Activity} \left( \frac{U}{mL} \right) = \frac{\Delta ABS}{49.6} \times \frac{1000}{X}$$

Where X ( $\mu$ L) = sample volume added to the cuvette.

### E.3. Solution Preparation Methods

The following solutions are made to a volume of **15 or 30 mL** and stored in 50 mL centrifuge tubes in the refrigerator. If solutions have been stored for several months prior to use, make sure that they are still good by using them to test for enzyme activity in samples with confirmed activity. The **hydrogen peroxide solution must be made fresh** right before assaying.

#### ■ 20 mM Veratryl alcohol (VA)

$$\frac{20mmol}{L} \times \frac{1L}{1000mL} \times 15mL = 0.3mmol VA$$

$$0.3mmol \times \frac{1mol}{1000mmol} \times \frac{168.19g}{1mol} \times \frac{1mL}{1.157g} \times \frac{1000\mu L}{1mL}$$

$$= 43.6\mu L \text{ of VA added to 15 mL DDI water}$$

#### ■ 0.2 M (pH 3.0) Sodium tartrate buffer

$$\frac{0.2\text{mol}}{1000\text{mL}} \times 15\text{mL} = 0.003\text{mol sodium tartrate}$$

$$0.003\text{mol} \times \frac{194.051\text{g}}{\text{mol}} = 0.582\text{g sodium tartrate}$$

Add 0.582g sodium tartrate to 10mL DDI water. Shake tube well. Adjust the pH of the buffer to 3.0 using 1N HCl (it should take approximately 1 mL), ensuring complete mixing after each addition of HCl by swirling the pH probe and measuring pH twice. Bring the final volume to 15 mL.

■ 2 mM Hydrogen peroxide (must be made fresh daily)

$$\frac{2\text{mmol}}{1000\text{mL}} \times \frac{1\text{mol}}{1000\text{mmol}} \times 30\text{mL} = 6 \times 10^{-5}\text{mol hydrogen peroxide}$$

$$\text{Using 30\% Hydrogen Peroxide: } \frac{3\text{mL}}{10\text{mL}} \times \frac{1.463\text{g}}{1\text{mL}} \times \frac{1\text{mol}}{34\text{g}} \times \frac{1000\text{mL}}{1\text{L}} = 12.9\text{M}$$

$$6 \times 10^{-5}\text{mol} \times \frac{1\text{L}}{12.9\text{mol}} \times \frac{10^6\mu\text{L}}{1\text{L}} = 4.65\mu\text{L 30\% hydrogen peroxide in 30mL DDI water}$$

■ 20 mM 2,6-Dimethoxyphenol

$$\frac{20\text{mmol}}{1000\text{mL}} \times 30\text{mL} = 0.6\text{mmol}$$

$$0.6\text{mmol} \times \frac{1\text{mol}}{1000\text{mmol}} \times \frac{154.17\text{g}}{1\text{mol}} = 0.0925\text{g 2,6 - dimethoxyphenol in 30mL DDI water}$$

■ 0.5 M (pH 4.5) Sodium tartrate buffer



$$\frac{0.5\text{mol}}{1000\text{mL}} \times 15\text{mL} = 0.0075\text{mol sodium tartrate}$$

$$0.0075\text{mol} \times \frac{194.051\text{g}}{\text{mol}} = 1.46\text{g sodium tartrate}$$

Add 1.46g sodium tartrate to 10mL DDI water. Shake tube well. Adjust the pH of the buffer to 4.5 using 1N HCl (it should take approximately 1mL), ensuring complete mixing after each addition of HCl. Bring the final volume to 15 mL.

■ 20 mM Manganese sulfate

$$\frac{20\text{mmol}}{1000\text{mL}} \times 15\text{mL} = 0.3\text{mmol sodium tartrate}$$

$$0.3\text{mmol} \times \frac{1\text{mol}}{1000\text{mmol}} \times \frac{169\text{g}}{\text{mol}} = 0.0507\text{g manganese sulfate in 15mL DDI water}$$

#### E.4. Enzyme Activity Calculations

$$\text{Enzyme Activity} \left( \frac{U}{\text{mL}} \right) = \frac{\Delta\text{ABS} \times 1/\epsilon}{X}$$

Where:

U = amount of enzyme that will catalyze the reaction of 1 μmol of substrate per minute

ΔABS = calculated change in absorbance over 1 minute; ε (1/cm) x substrate concentration  
(mol/L) x 1cm

ε = molar extinction coefficient of product formed (veratryl aldehyde or 2,6-dimethoxyquinone);  
measurement of how strongly the product absorbs light at the set wavelength

X = volume of sample added to the cuvette

Unit Conversions:

$$\text{Enzyme Activity} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\Delta \text{ABS} \left[ \left( \frac{1}{\text{cm}} \right) \times \left( \frac{\text{mol}}{\text{L}} \right) \times 1 \text{ cm} \right] \times 1/\varepsilon [\text{cm}]}{X \mu\text{L}}$$

$$\text{Enzyme Activity} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\Delta \text{ABS} \left[ \left( \frac{\text{mol}}{\text{L}} \right) \times \left( \frac{1\text{L}}{1000\text{mL}} \right) \right] \times 1/\varepsilon \left[ \text{cm} \times \frac{1\text{mL}}{1\text{cm}} \right]}{X \mu\text{L} \times \frac{1\text{mL}}{1000\mu\text{L}}}$$

$$\text{Enzyme Activity} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\Delta \text{ABS} \left[ \left( \frac{1\text{mol}}{1000\text{mL}} \right) \times \frac{10^6 \mu\text{mol}}{1\text{mol}} \right] \times 1/\varepsilon [1\text{mL}]}{X \frac{1\text{mL}}{1000}}$$

$$\text{Enzyme Activity} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\frac{\Delta \text{ABS}}{\varepsilon} \left[ \frac{10^6 \mu\text{mol}}{1000} \right]}{X \frac{1\text{mL}}{1000}} = \frac{\Delta \text{ABS}}{X} \times 10^6 \frac{\mu\text{mol}}{\text{mL}}$$

Lignin Peroxidase Activity Example:  $\Delta \text{ABS} = 0.950$ ;  $\varepsilon \text{ (VA)} = 9.3 \times 10^3/\text{cm}$ ;  $X = 750\mu\text{L}$

$$\text{LiP} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\frac{0.950}{9.3 \times 10^3/\text{cm}} \times 10^6}{750\mu\text{L}}$$

$$\text{LiP} \left( \frac{\text{U}}{\text{mL}} \right) = \frac{\frac{0.950}{9.3 \times 10^3/\text{cm}} \times 10^6}{750\mu\text{L}} = 0.136 \frac{\text{U}}{\text{mL}} = \mathbf{136 \frac{U}{L}}$$

## Appendix F

### Photographs of Packing Materials from Sacrificed Reactors



**Figure F-1.** Representative photograph of *P. chrysosporium* attachment to Tri-pack®. The Tri-pack® pictured were from an aerated flask in a preliminary aeration experiment at  $t = 1$  day.



**Figure F- 2.** Representative photograph of woodchips from a control treatment (no fungus). These woodchips were taken from a W0-C treatment flask at  $t = 12.7$  days.



**Figure F- 3.** Representative photograph of woodchips with fungal attachment. These woodchips were taken from a W0-P treatment flask at  $t = 12.7$  days. Arrows point to visible mycelia.



**Figure F-4.** Representative photograph of foam without fungal attachment. This foam was taken from a F0-C treatment flask at  $t = 12.7$  days.



**Figure F-5.** Representative photograph of foam with fungal attachment from a glucose treatment. This foam was taken from a F10-P treatment flask at  $t = 12.7$  days. Arrows point to visible mycelia.



**Figure F-6.** Representative photograph of foam with fungal attachment from a treatment without glucose amendments. The foam was taken from a F0-P treatment flask at  $t = 12.7$  days. Arrows point to visible mycelia.

## **Appendix G**

### **LC/MS/MS Analytical Method**

The following EDC detection method was created by Dr. Rebecca Wittrig of AB Sciex for use on an API 3200™ LC/MS/MS System. The method was initially created on an API 4000™ LC/MS/MS System.



File Name: EDCs Brennan 6-15-10.wiff  
 File Path: D:\Analyst Data\Projects\PPCP\_RW\2010\_06\_14\Data\  
 Original Name: EDCs Brennan 6-15-10.wiff  
 Software Version: Analyst 1.5.1

**Log Information from Devices at Start of acquisition:**

Integrated System	Shimadzu Controller	CBM20A
Serial#	L20234752182	
ROM Version	1.21	
Pressure Units	psi	
Time from start =0.0000 min		Pump
Shimadzu LC20AD		
Serial#	L20434650001	
ROM Version	1.21	
Time from start =0.0000 min		Pump
Shimadzu LC20AD		
Serial#	L20434650003	
ROM Version	1.21	
Time from start =0.0000 min		AutoSampler
Shimadzu SIL20AC		
Serial#	L20454750020	
ROM Version	1.22	
Time from start =0.0000 min		Column Oven
Shimadzu CTO20AC		
Serial#	L20214750544	
ROM Version	1.07	
Time from start =0.0000 min		
Time from start =0.0000 min	Injection Volume used	50.00
µl		
Time from start =0.0000 min	Mass Spectrometer	4000
Q TRAP	0	
Config Table Version	10	
Firmware Version	M401402 B4T0301 M3L1417 B3T0300	
Component Name	Linear Ion Trap Quadrupole LC/MS/MS Mass Spectrometer	
Component ID	Q Trap4000	
Manufacturer	AB Sciex Instruments	
Model	1004229-A	
Serial Number	U0020208PT	
Time from start =0.0000 min	Mass Spectrometer	4000
Q TRAP	0	
Start of Run - Detailed Status		
Vacuum Status	At Pressure	
Vacuum Gauge (10e-5 Torr)	3.2	
Backing Pump	Ok	
Interface Turbo Pump	Normal	
Analyzer Turbo Pump	Normal	



Sample Introduction Status	Ready	
Source/Ion Path Electronics	On	
Source Type	Turbo Spray	
Source Temperature (at setpoint)	600.0 C	
Source Exhaust Pump	Ok	
Interface Heater	Ready	
Time from start =0.0167 min	Mass Spectrometer	4000
Q TRAP 0		
End of Run - Detailed Status		
Vacuum Status	At Pressure	
Vacuum Gauge (10e-5 Torr)	3.2	
Backing Pump	Ok	
Interface Turbo Pump	Normal	
Analyzer Turbo Pump	Normal	
Sample Introduction Status	Ready	
Source/Ion Path Electronics	On	
Source Type	Turbo Spray	
Source Temperature (at setpoint)	600.0 C	
Source Exhaust Pump	Ok	
Interface Heater	Ready	

Time from start =10.3167 min

#### Acquisition Info

Acquisition Method:	\EDCs Brennan 6-15-10 POS.dam
Acquisition Path:	D:\Analyst Data\Projects\PPCP_RW\2010_06_14\Acquisition
Methods\	
First Sample Started:	Tuesday, June 15, 2010 11:42:33 AM
Last Sample Finished:	Tuesday, June 15, 2010 2:05:22 PM
Sample Acq Time:	Tuesday, June 15, 2010 1:54:59 PM
Sample Acq Duration:	9min59sec
Number of Scans:	0
Periods in File:	1
Batch Name:	\EDCs Brennan 6-15-10.dab
Batch Path:	D:\Analyst Data\Projects\PPCP_RW\2010_06_14\Batch\
Submitted by:	AMCONW-3H4B2D1\Administrator()
Logged-on User:	AMCONW-3H4B2D1\Administrator
Synchronization Mode:	LC Sync
Auto-Equilibration:	Off
Comment:	Ultra II Biphenyl 3um 50x2.1mm
Software Version:	Analyst 1.5.1
Set Name:	SET1
Sample Name	EDC Brennan 1ppb Std
Sample ID	
Sample Comments:	
Autosampler Vial:	13
Rack Code:	1.5mL Standard
Rack Position:	1
Plate Code:	1.5mL Standard

Plate Position

1

### Shimadzu LC Method Properties

Shimadzu LC system Equilibration time = 0.00 min

Shimadzu LC system Injection Volume = 50.00 ul

Shimadzu LC Method Parameters

Pumps

=====

Pump A Model: LC-20AD

Pump B Model: LC-20AD

Pumping Mode: Binary Flow

Total Flow: 0.6000 mL/min.

Pump B Conc: 10.0 %

B Curve: 0

Pressure Range (Pump A/B): 0 - 5000 psi

Autosampler

=====

Model: SIL-20AC

Rinsing Volume: 200 uL

Needle Stroke: 52 mm.

Rinsing Speed: 35 uL/sec.

Sampling Speed: 15.0 uL/sec.

Purge Time: 25.0 min.

Rinse Dip Time: 0 sec.

Rinse Mode: No rinsing

Cooler Enabled: Yes

Cooler Temperature: 15 deg. C

Control Vial Needle Stroke: 52 mm

Oven

=====

Model: CTO-20AC

Temperature Control: Enabled

Temperature: 35 deg. C

Max. Temperature: 90 deg. C

System Controller

=====

Model: CBM-20A

Power: On

Event 1: Off

Event 2: Off

Event 3: Off

Event 4: Off

Time Program

=====

Time	Module	Events	Parameter
1.00	Pumps	Pump B Conc.	10
6.00	Pumps	Pump B Conc.	90

7.50	Pumps	Pump B Conc.	90
7.60	Pumps	Pump B Conc.	10
10.00	System Controller	Stop	

#### Quantitation Information:

Sample Type: Standard  
Dilution Factor: 1.000000

Custom Data:

Quantitation Table:

#### Period 1:

-----  
Scans in Period: 555  
Relative Start Time: 0.00 msec  
Experiments in Period: 1

#### Period 1 Experiment 1:

-----  
Scan Type: MRM (MRM)  
Scheduled MRM: No  
Polarity: Positive  
Scan Mode: N/A  
Ion Source: Turbo Spray  
Resolution Q1: Unit  
Resolution Q3: Unit  
Intensity Thres.: 0.00 cps  
Settling Time: 0.0000 msec  
MR Pause: 5.0070 msec  
MCA: No  
Step Size: 0.00 Da

Q1 Mass (Da) 152.400	Q3 Mass (Da) 110.000 Acetaminophen 1	Dwell(msec) 50.00	Param DP  CE CXP	Start 56.00  24.00 6.00	Stop 56.00  24.00 6.00	ID
Q1 Mass (Da) 152.400	Q3 Mass (Da) 65.100 Acetaminophen 2	Dwell(msec) 50.00	Param DP  CE CXP	Start 56.00  42.00 6.00	Stop 56.00  42.00 6.00	ID
Q1 Mass (Da) 192.100	Q3 Mass (Da) 118.900	Dwell(msec) 50.00	Param DP CE CXP	Start 86.00 26.00 8.00	Stop 86.00 26.00 8.00	ID DEET 1
Q1 Mass (Da) 195.500	Q3 Mass (Da) 138.100	Dwell(msec) 50.00	Param DP CE CXP	Start 71.00 28.00 8.00	Stop 71.00 28.00 8.00	ID Caffeine 1
Q1 Mass (Da)	Q3 Mass (Da)	Dwell(msec)	Param	Start	Stop	ID

195.500	110.100	50.00	DP CE CXP	71.00 32.00 8.00	71.00 32.00 8.00	Caffeine 2
Q1 Mass (Da) 216.600	Q3 Mass (Da) 174.100	Dwell(msec) 50.00	Param DP CE CXP	Start 71.00 24.00 12.00	Stop 71.00 24.00 12.00	ID Atrazine 1
Q1 Mass (Da) 216.600	Q3 Mass (Da) 68.100	Dwell(msec) 50.00	Param DP CE CXP	Start 71.00 52.00 6.00	Stop 71.00 52.00 6.00	ID Atrazine 2
Q1 Mass (Da) 229.400	Q3 Mass (Da) 151.100 Oxybenzone 1	Dwell(msec) 50.00	Param DP  CE CXP	Start 61.00  28.00 10.00	Stop 61.00  28.00 10.00	ID
Q1 Mass (Da) 229.400	Q3 Mass (Da) 105.100 Oxybenzone 2	Dwell(msec) 50.00	Param DP  CE CXP	Start 61.00  28.00 8.00	Stop 61.00  28.00 8.00	ID

Q1 Mass (Da) 310.000 1	Q3 Mass (Da) 147.600	Dwell(msec) 50.00	Param DP	Start 21.00	Stop 21.00	ID Fluoxetine
			CE	12.00	12.00	
			CXP	12.00	12.00	
Q1 Mass (Da) 221.600 d5 IS 1	Q3 Mass (Da) 179.300	Dwell(msec) 50.00	Param DP	Start 71.00	Stop 71.00	ID Atrazine
			CE	24.00	24.00	
			CXP	12.00	12.00	
Q1 Mass (Da) 221.600 d5 IS 2	Q3 Mass (Da) 101.400	Dwell(msec) 50.00	Param DP	Start 71.00	Stop 71.00	ID Atrazine
			CE	52.00	52.00	
			CXP	6.00	6.00	
Q1 Mass (Da) 231.100 1	Q3 Mass (Da) 185.000	Dwell(msec) 100.00	Param DP	Start 56.00	Stop 56.00	ID Naproxen
			CE	19.00	19.00	
			CXP	12.00	12.00	

Q1 Mass (Da) 231.100 2	Q3 Mass (Da) 170.100	Dwell(msec) 100.00	Param DP	Start 56.00	Stop 56.00	ID Naproxen
			CE CXP	37.00 30.00	37.00 30.00	
Q1 Mass (Da) 273.200 Estradiol 1	Q3 Mass (Da) 107.200	Dwell(msec) 100.00	Param DP	Start 51.00	Stop 51.00	ID 17beta-
			CE CXP	47.00 16.00	47.00 16.00	
Q1 Mass (Da) 273.200 Estradiol 2	Q3 Mass (Da) 76.800	Dwell(msec) 100.00	Param DP	Start 51.00	Stop 51.00	ID 17beta-
			CE CXP	79.00 4.00	79.00 4.00	

### Parameter Table(Period 1 Experiment 1)

CUR:	20.00
IS:	5000.00
TEM:	600.00
GS1:	75.00
GS2:	65.00
ihe:	ON
CAD:	Medium
EP	10.00
IQ2	-18.00

### Resolution tables

Quad 1	Positive	Unit
--------	----------	------

Last Modification Date Time: June 14, 2010 12:03:57

IE1	0.900
Mass (Da)	Offset Value
59.050	0.053
175.133	0.107
616.464	0.299
906.673	0.420
1254.925	0.569
1545.134	0.690
2010.469	0.890

Quad 3	Positive	Unit
--------	----------	------

Last Modification Date Time: June 14, 2010 14:10:33

IE3	0.000
Mass (Da)	Offset Value
59.050	0.052
175.133	0.122
616.464	0.359
906.673	0.515
1254.925	0.690
1545.134	0.843
2010.469	1.094
2242.637	1.215

### Calibration tables

Quad 1	Positive	Unit Resolution
--------	----------	-----------------

Last Modification Date Time: June 14, 2010 12:18:41

Mass (Da)	Dac Value
59.050	1100



175.133	3312
616.464	11729
906.673	17263
1254.925	23906
1545.134	29441
2010.469	38318
2242.637	42746

Quad 3                      Positive                      Unit Resolution  
 Last Modification Date Time: June 14, 2010 13:17:16

Mass (Da)	Dac Value
59.050	1101
175.133	3310
616.464	11718
906.673	17247
1254.925	23882
1545.134	29412
2010.469	38278
2242.637	42703

#### **Instrument Parameters:**

Detector Parameters (Positive):  
 CEM                      2000.0

#### **Keyed Text:**

File was created with the software version: Analyst 1.5.1

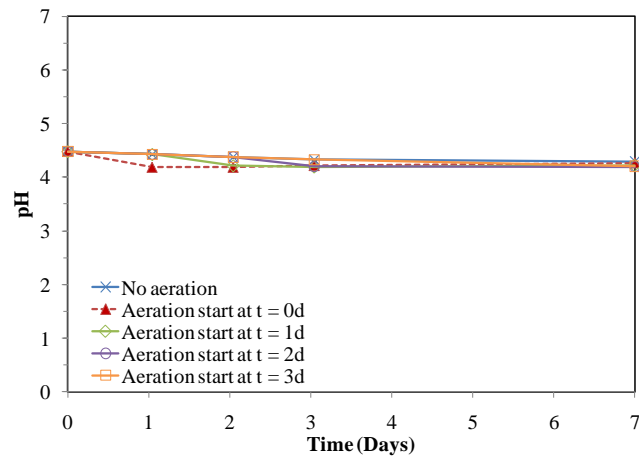
## **Appendix H**

### **Preliminary Aeration Experiment**

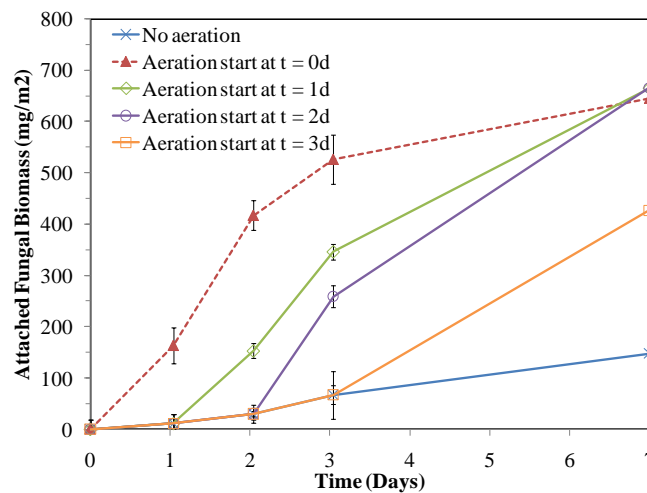
The Preliminary Aeration Experiment was carried out to determine if the fungus needed an acclimation period or additional time for mycelia reformation and attachment prior to aeration. The batch flasks were assigned to one of five treatments: no aeration, immediate aeration (aeration start at  $t = 0d$ ), aeration after 1 day (aeration start at  $t = 1d$ ), aeration after 2 days (aeration start at  $t = 2d$ ), and aeration after 3 days (aeration start at  $t = 3d$ ) of stationary conditions in the reactors.

All sacrificial reactors contained 9 Tri-pack®, 10% (v/v) mycelia solution, 0.01% Tween 80™ in LN media. On day 4 of the experiment, the reactors were augmented with 1 mL of 54 mM veratryl alcohol. All treatments were sterile.

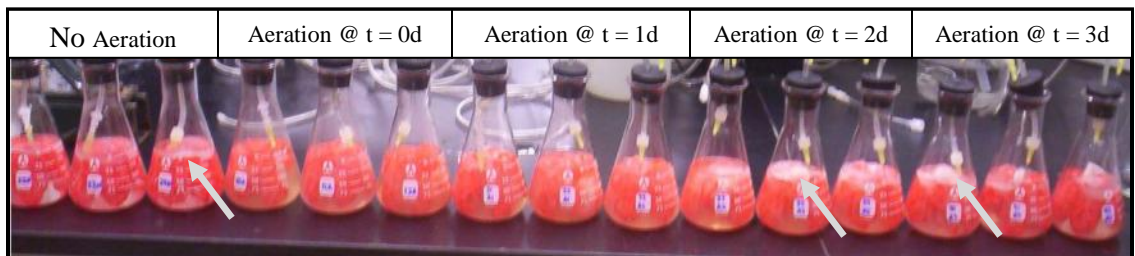
Treatment flasks were sacrificed in triplicate over a period of 1 week using the methods in Appendix D. At each time point, solution pH, attached growth and suspended growth were measured. Over the course of the experiment, pH remained stable (Figure H-1). From the attached biomass data (Figure H-2), it can be seen that a stationary period is unnecessary. Additionally, a short stationary (un-aerated) period does not appear to affect the ability of the fungus to attach and grow once aeration and agitation (via aeration) has commenced. However, it can be seen in Figure H-3 that attachment is not uniform and mycelia mats will form if reactors are not aerated by day 1. The mats likely form because dissolved oxygen is too low to sustain the aerobic fungus in solution.



**Figure H-1.** pH data for Preliminary Aeration Experiment. Points represent mean values of triplicate reactors. Standard deviation for each point is less than 0.0 mg/m<sup>2</sup>.



**Figure H-2.** Attached fungal biomass for Preliminary Aeration Experiment. Points represent mean values of triplicate flask reactors. Error bars represent  $\pm 1$  standard deviation.



**Figure H-3.** Preliminary Aeration Experiment reactors at t = 7 days. The arrows point to mycelia mats that have formed on the solution surface of treatments that had not been aerated by day 1.

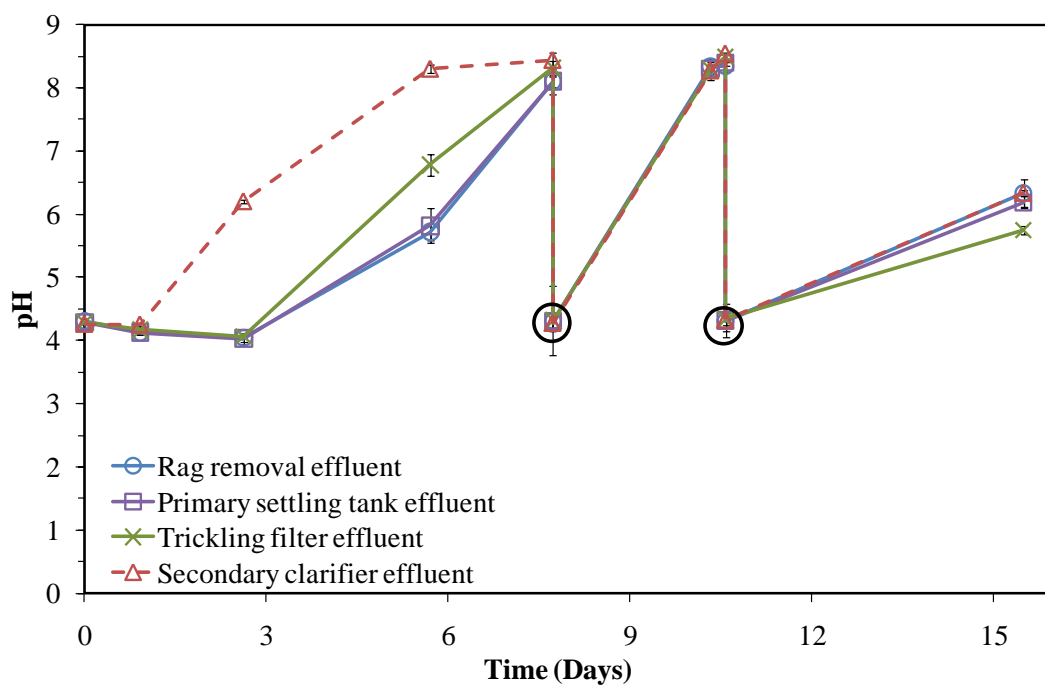
## Appendix I

### Batch Experiment 2: Initial Attempt

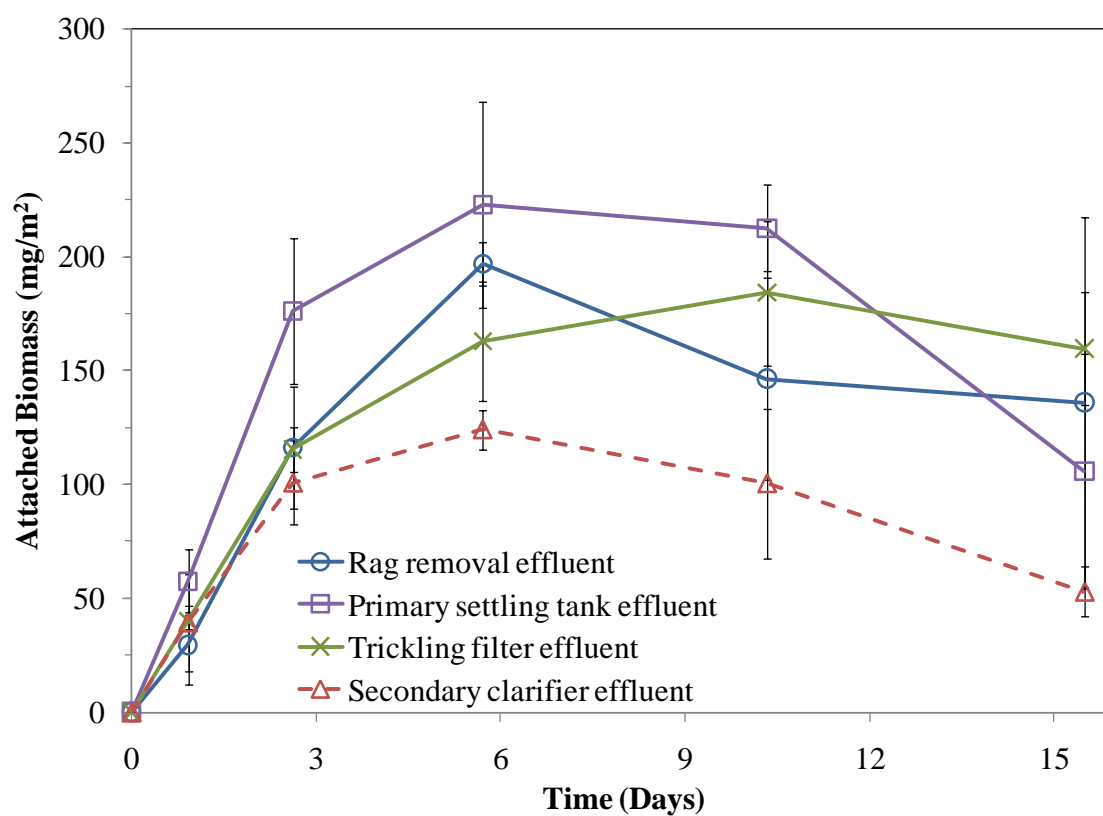
In the first attempt of Batch Experiment 2, the initial pH adjustments and the first (of two) corrective pH adjustments were made with 0.5 M *trans*-aconitic acid, an organic acid commonly used in LN media as a buffer. The second corrective pH adjustment was made using 1 N HCl (EMD, USA).

pH appeared to be fairly stable within the first 3 days (Figure I-1), with the exception of the secondary clarifier effluent treatment. The pH of the treatments appear to increase in order of least amount of organic carbon and nitrogen to most (amount of organic carbon and nitrogen), and pH increases likely correlate to the breakdown of organic carbon-containing materials and the stripping of CO<sub>2</sub>. Additionally, rate of growth and attachment began to decrease near day 3 (Figure I-2), and there may have been some death and decay of cells. This decay, in addition to the higher levels of available nitrogen within the treatment effluents, could release ammonia into solution, causing the pH to increase.

Overall, there was no enzyme activity recorded, and the effects of natural nitrogen and carbon content of the wastewater on enzyme activity and growth was not evaluated.



**Figure I-1.** pH data from initial attempt of Batch Experiment 2. Arrows indicate sampling points. Circles indicate points of pH adjustments. Each sampling point represents the mean value of triplicate reactors. Other points represent mean values of remaining flasks. Error bars represent  $\pm 1$  standard deviation.



**Figure I-2.** Attached biomass from initial attempt of Batch Experiment 2. Arrows indicate times when pH adjustments were made. Points represent the mean values of triplicate flasks. Error bars represent  $\pm 1$  standard deviation.

## **Appendix J**

### **Experimental Evaluation of the Effects of Glucose, Woodchips, Buffers, and Autoclaving on Solution pH**

This experiment was run to determine: 1) the feasibility of using a buffer to maintain the optimum pH (4.3) in secondary clarifier effluent; 2) the effects of glucose on pH; 3) the effects of woodchips on pH; and 4) the effects of autoclaving (especially with wood) on solution pH. No fungi were added to any treatments. The main purpose of this experiment was to determine which, if any, buffer could successfully maintain the pH within the optimum range under non-sterile, unamended conditions.

The treatment matrix is on the next page (Table J-1). The initial pH was either unadjusted (pH of the wastewater) or adjusted to pH 4.3. The adjusted wastewater was then either given one of three buffers (10mM) or no buffer. In addition, treatments were either given 1) 10 g/L glucose or no glucose and 2) 40 g wood chips or no wood chips. Finally, treatments were either sterile (autoclaved) or non-sterile. Each treatment was carried out in duplicate non-sacrificial aerated flask reactors. pH measurements were recorded until the sterile treatments became contaminated.

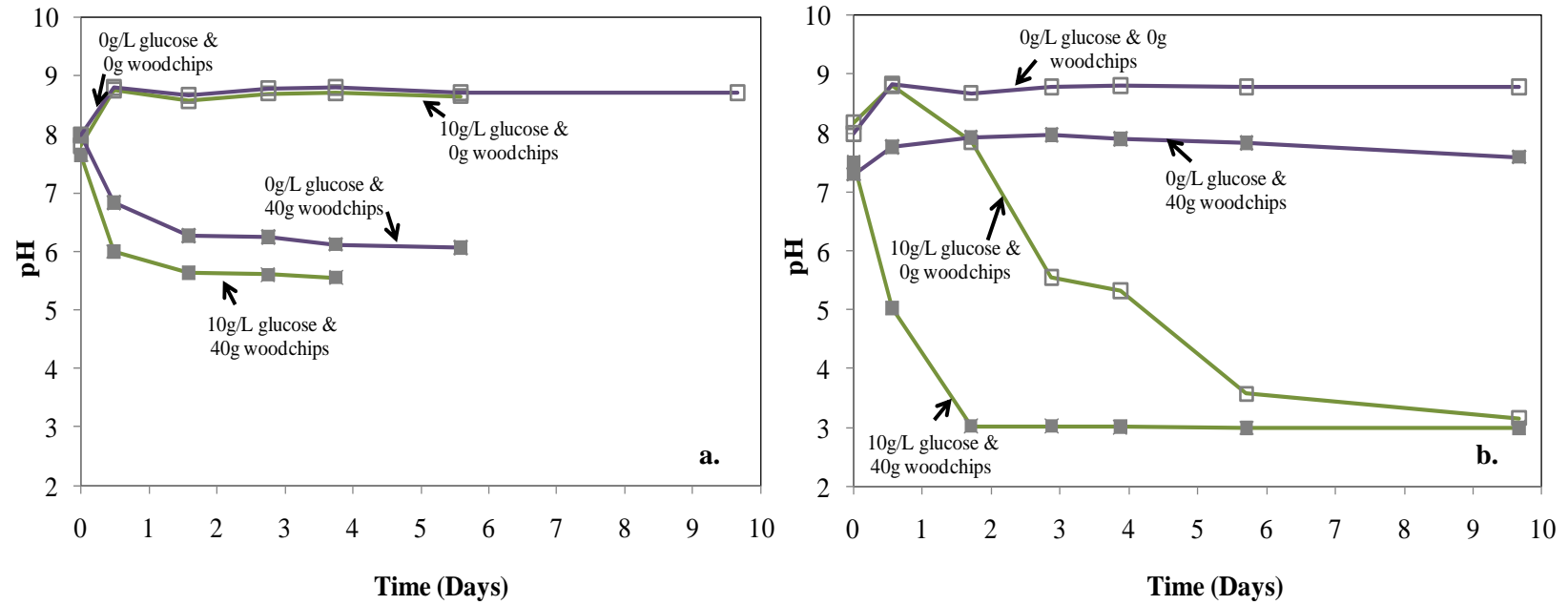
The results of the experiment can be found in Figure J-1 and Figure J-2. It appears as if autoclaving the wood causes the release of acids, and glucose has a synergistic effect on this (Figure J-1). Under non-sterile, glucose-amended conditions, the aconitate buffer performed the best; however, the pH of this treatment continued to drop. The final pH ( $t = \sim 10$  days) of this treatment was 3.1. Under non-sterile, unamended conditions, all three buffers lasted until  $t = \sim 2$  days; after that time point, the pH of these treatment solutions rose above 7. Overall, the pH of each non-sterile treatment could not be maintained within the optimum range.

**Table J-1.** Experimental design to compare the effects of sterility, glucose, woodchips, and buffer on pH.

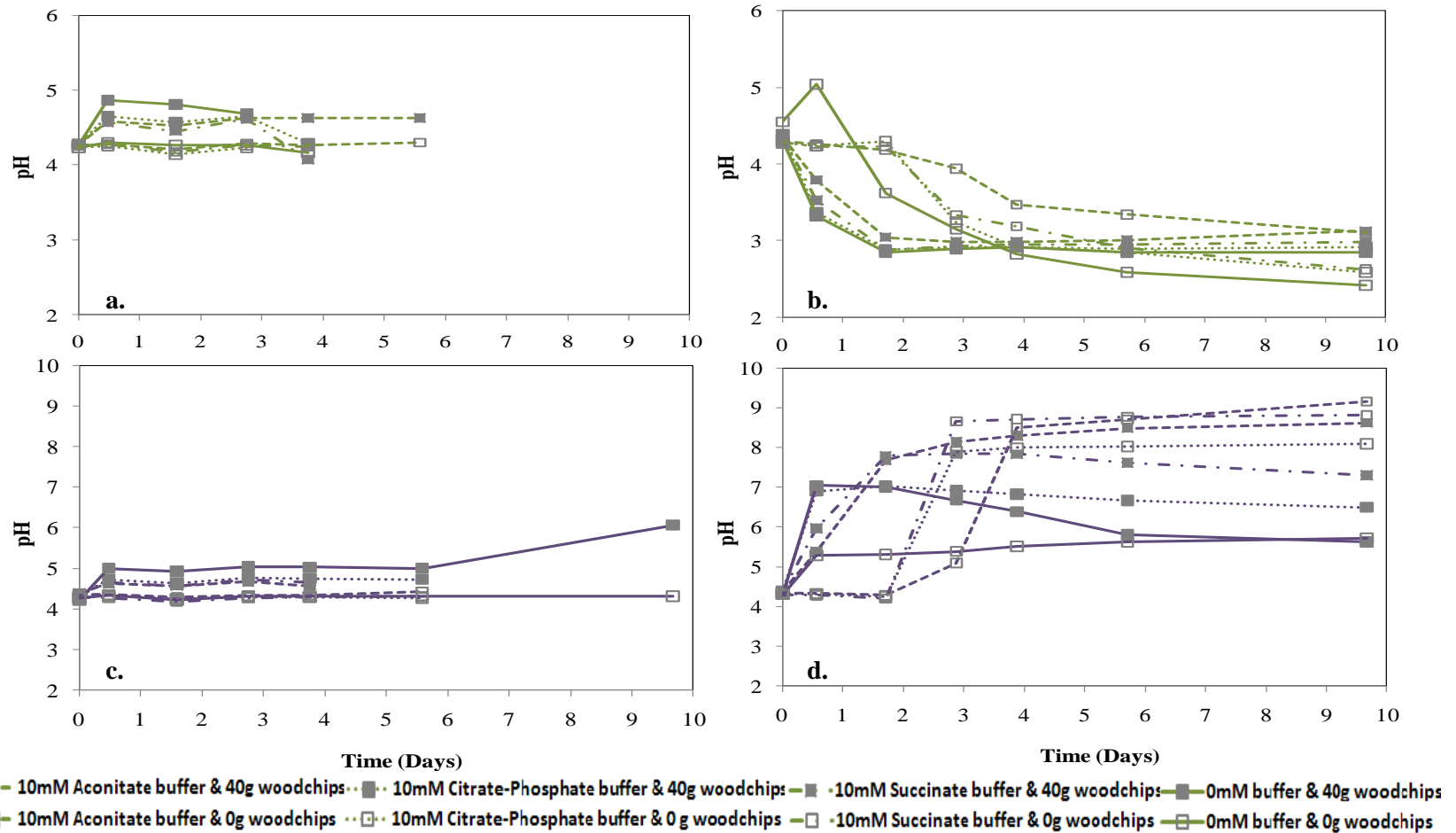
Treatment #	+/- Initial pH Adjustment to 4.3	+/- Sterile	+/- 10g/L Glucose	+/- Woodchips	+/- TAA Buffer	+/- Citrate- Phosphate Buffer	+/- Succinate Buffer	+/- No Buffer
1	-	+	+	+	-	-	-	+
2	-	+	+	-	-	-	-	+
3	-	+	-	+	-	-	-	+
4	-	+	-	-	-	-	-	+
5	-	-	+	+	-	-	-	+
6	-	-	+	-	-	-	-	+
7	-	-	-	+	-	-	-	+
8	-	-	-	-	-	-	-	+
9	+	+	+	+	+	-	-	-
10	+	+	+	+	-	+	-	-
11	+	+	+	+	-	-	+	-
12	+	+	+	+	-	-	-	+
13	+	+	+	-	+	-	-	-
14	+	+	+	-	-	+	-	-
15	+	+	+	-	-	-	+	-
16	+	+	+	-	-	-	-	+
17	+	+	-	+	+	-	-	-
18	+	+	-	+	-	+	-	-
19	+	+	-	+	-	-	+	-
20	+	+	-	+	-	-	-	+
21	+	+	-	-	+	-	-	-
22	+	+	-	-	-	+	-	-
23	+	+	-	-	-	-	+	-
24	+	+	-	-	-	-	-	+
25	+	-	+	+	+	-	-	-
26	+	-	+	+	-	+	-	-
27	+	-	+	+	-	-	+	-
28	+	-	+	+	-	-	-	+
29	+	-	+	-	+	-	-	-
30	+	-	+	-	-	+	-	-
31	+	-	+	-	-	-	+	-
32	+	-	+	-	-	-	-	+
33	+	-	-	+	+	-	-	-
34	+	-	-	+	-	+	-	-
35	+	-	-	+	-	-	+	-
36	+	-	-	+	-	-	-	+
37	+	-	-	-	+	-	-	-
38	+	-	-	-	-	+	-	-
39	+	-	-	-	-	-	+	-
40	+	-	-	-	-	-	-	+

Treatments that did not receive initial pH adjustments to 4.3 were not buffered. All buffers were 10 mM in solution.





**Figure J-1.** Sterile (a.) and non-sterile (b.) treatments with no initial pH adjustments (Treatments # 1-8). Treatments without woodchips have empty squares; treatments with woodchips have filled squares. Treatments with glucose were represented with green lines; treatments without glucose were represented with purple lines.



**Figure J-2.** Sterile + glucose (a.), non-sterile + glucose (b.), sterile + no glucose (c.), and non-sterile + no glucose (d.) treatments with initial pH adjustment to 4.3. Treatments without woodchips have empty squares; treatments with woodchips have filled squares. Treatments with glucose were represented with green lines; treatments without glucose were represented with purple lines. Buffered treatments have dotted lines; un-buffered treatments have solid lines.

## Appendix K

### Oxygen Mass-Transfer into Solution from Bubbles vs Oxygen Utilization by Aerobic Microbes (Calculations)

#### 1. Oxygen mass-transfer into solution from a swarm of bubbles

##### Assumptions:

- Bubbles are in a swarm and sufficiently small ( $R_b < 1.25\text{mm}$ )
- Bubble radius of a single bubble under similar conditions approximates the average radius of a bubble in the swarm
- Density of water  $\gg$  Density of air
- Reactor-specific parameters:  $F_a$  (air flow) =  $2\text{ cm}^3/\text{s}$ ; Needle (orifice)  $D = 0.584\text{ mm}$ ;  $V = 0.135\text{L}$ ;  $h = 2\text{ in.} = 5.08\text{cm}$
- Constants:  $\nu_w = 0.01\text{ cm}^2/\text{s}$ ;  $\mu_w = 10\text{ g/cm-s}$ ;  $D_{ow} = 2 \times 10^{-5}\text{ cm}^2/\text{s}$ ;  $\rho_w = 1\text{ g/cm}^3$ ;  $\rho_a = 1.275 \times 10^{-3}\text{ g/cm}^3$ ;  $g = 9.8\text{ m/s}^2$

##### Equations and Solutions:

- $D_b = D_o * 4.27$  (Eqn. 8.42, Bailey and Ollis, 1986), where  $D_b$  = bubble diameter and  $D_o$  = orifice diameter;  $D_b = D_o * 4.27 = 0.0584\text{cm} * 4.27 = \mathbf{0.25\text{cm}}$
- $t_b = h / (1.41(g * R_b^{0.5}))$ , (Eqn. 7-92, Logan, 1999), where  $t_b$  = time that the bubble is in the reactor,  $\nu_w$  = kinematic viscosity of water, and  $h$  = solution height;  $t_b = (5.08\text{cm}) / (1.41 * (980\text{ cm/s}^2 * 0.125\text{cm})^{0.5}) = \mathbf{0.326\text{ s}}$
- $a_v = (3F_a * t_b) / (V * R_b)$ , (Eqn. 7-90, Logan, 1999), where  $a_v$  = total interfacial area,  $F_a$  = air flow rate, and  $V$  = reactor volume;  $a_v = (3 * 2\text{ cm}^3/\text{s} * 0.326\text{ s}) / (135\text{ cm}^3 * 0.125\text{ cm}) = \mathbf{116 /cm}$

- $Sh = 0.31Ra^{1/3}$ , (Eqn. 7-86, Logan, 1999), where  $Sh$  is the Sherwood number and  $Ra$  is the Raleigh number;  $Sh = 0.31*(9570)^{1/3} = 6.58$ 
  - $Ra = R_b^3 * g * (\rho_w - \rho_a) / (D_{ow} * \mu_w)$ , (Eqn. 7-78, Logan, 1999), where  $\rho_w$  = water density,  $g$  = gravitational acceleration, and  $\rho_a$  = air density, and  $\mu_w$  = dynamic viscosity of water;  $Ra = (0.125\text{cm})^3 * 980\text{cm/s}^2 * (1\text{g/cm}^3 - \sim 0) / (2 \times 10^{-5} \text{ cm}^2/\text{s} * 10 \text{ g/cm-s}) = 9570$
- $K_{wa} = Sh * D_{ow} / R_b$ , (Eqn. 6-3, Logan, 1999), where  $K_{wa}$  = oxygen mass transport coefficient,  $R_b$  = bubble radius, and  $D_{ow}$  = oxygen diffusion coefficient in water;  $K_{wa} = 6.58 * 2 \times 10^{-5} \text{ cm}^2/\text{s} / 0.125\text{cm} = 1.05 \times 10^{-3} \text{ cm/s}$
- $W_{ow} = K_{wa} * a_v * V * (C_{eq} - C_{\infty})$ , (Eqn. 7-75, Logan, 1999), where  $W_{ow}$  = rate of oxygen transfer into water,  $C_{eq}$  = equilibrium concentration of oxygen in solution, and  $C_{\infty}$  = bulk concentration of oxygen in solution;  $W_{ow} = 1.05 \times 10^{-3} \text{ cm/s} * 116 \text{ /cm} * 0.135 \text{ L} * (8.7\text{mg/L} - 0) = 0.14 \text{ mg O}_2/\text{s}$ 
  - $C_{eq} = C_{l^*-ww} = \beta(C_{l^*-\text{clean water}})$ , (Eqn. 6.23, Rittmann and McCarty, 2001), where  $C_{l^*-ww}$  = liquid-phase oxygen concentration in equilibrium with the bulk phase in wastewater,  $\beta = 0.95$  for wastewater (fraction applied to convert  $C_{l^*-\text{clean water}}$  to  $C_{l^*-ww}$ ), and  $C_{l^*-\text{clean water}} = 9.2 \text{ mg/L}$  (liquid-phase oxygen concentration in equilibrium with the bulk phase in water);  $C_{eq} = C_{l^*-ww} = 0.95(9.2 \text{ mg/L}) = 8.7 \text{ mg/L}$

- $\text{Flux} = W_{ow} / V = 0.14 \text{ mg O}_2/\text{s} / 0.135 \text{ L} = 1 \text{ mg O}_2/\text{L-s}$

## 2. Oxygen Utilization by Aerobic Wastewater Microbes (MAX rate of utilization)

### Assumptions:

- Aerobic microbial metabolic activity >> fungal metabolic activity
- Carbonaceous oxygen demand >> nitrogenous oxygen demand; COD ~ BOD
- Assume no solution flow in or out of the system

- Assume the following constants for aerobic metabolism of carbohydrate substrate (oxygen as e- acceptor) from Rittmann and McCarty (2001):
  - $K = 0.02 \text{ g/L}$  (concentration giving  $\frac{1}{2}$  the maximum rate)
  - $Y = 0.49 \text{ g VSS/ gBOD}_L$  (true yield for cell synthesis)
  - $q\text{-hat} = 27 \text{ g BOD}_L / \text{g VSS-d}$  (maximum specific rate of substrate utilization)
  - $b = 0.15/\text{d}$  (endogenous decay coefficient)
- Reactor-specific parameters:  $F_a$  (air flow) =  $2 \text{ cm}^3/\text{s}$ ; Needle (orifice)  $D = 0.584 \text{ mm}$ ;  $V = 0.135\text{L}$ ;  $h = 2 \text{ in.} = 5.08\text{cm}$
- $X_a$  (initial) =  $0.009 \text{ g VSS/L}$  (Assuming  $10 \text{ mg VSS/L}$  in secondary clarifier effluent from the Penn State WWTP; Treatments contained 90% wastewater)
- $\Delta t = 0.05 \text{ days}$  for the predictive model

#### Equations:

- $dX_a/dt \sim \Delta X_a/\Delta t = Y \cdot X_a \cdot V \cdot (q\text{-hat} \cdot S)/(K+S) - bX_aV$ ;  $\Delta X_a = (Y \cdot X_a \cdot V \cdot (q\text{-hat} \cdot S)/(K+S) - bX_aV)\Delta t$ , (adaptation of Eqn. 3.16, Rittmann and McCarty, 2001)
- $dS/dt \sim \Delta S/\Delta t = -X_a \cdot V \cdot (q\text{-hat} \cdot S)/(K+S)$ ;  $\Delta S = (-X_a \cdot V \cdot (q\text{-hat} \cdot S)/(K+S))\Delta t$ , (adaptation of Eqn. 3.17, Rittmann and McCarty, 2001)
- **r-ut = rate of oxygen utilization =  $-(q\text{-hat} \cdot S \cdot X_a)/(K+S)$** , (Eqn. 3.6, Rittmann and McCarty, 2001)

#### Predictive Models:

- $X_{a(t+1)} = X_{a(t)} + \Delta X_a$
- $S_{(t+1)} = S_{(t)} + \Delta S$

delta t (d)	Initial Xa (g VSS/L)	K (g/L)	V (L)	Initial Substrate (~gBOD/L)	q-hat (g BOD/g VSS-d)	Y (g VSS/gBOD)	b (d-1)
0.05	0.009	0.02	0.135	10.67	27	0.49	0.15
time (d)	S	Xa	dS/ dt	r-utilization (g BOD/L-d)	r-ut (mg BOD/L-s)		
0	10.67	0.009					
0.05	10.66836282	0.00979	-0.0327	-0.24254537	-0.002807238		
0.1	10.66658136	0.01066	-0.0356	-0.263919101	-0.003054619		
0.15	10.66464292	0.0116	-0.0388	-0.287176327	-0.0033238		
0.2	10.66253366	0.01262	-0.0422	-0.312483023	-0.003616702		
0.25	10.66023853	0.01373	-0.0459	-0.340019788	-0.003935414		
0.3	10.65774114	0.01494	-0.0499	-0.369983137	-0.004282212		
0.35	10.65502368	0.01626	-0.0543	-0.402586899	-0.004659571		
0.4	10.65206675	0.01769	-0.0591	-0.438063747	-0.005070182		
0.45	10.64884925	0.01925	-0.0644	-0.476666855	-0.005516977		
0.5	10.64534822	0.02094	-0.07	-0.518671704	-0.006003145		
0.55	10.64153867	0.02279	-0.0762	-0.564378051	-0.006532153		
0.6	10.63739341	0.0248	-0.0829	-0.614112064	-0.007107779		
0.65	10.63288287	0.02698	-0.0902	-0.668228652	-0.007734128		
0.7	10.62797485	0.02936	-0.0982	-0.727113994	-0.008415671		
0.75	10.62263433	0.03195	-0.1068	-0.791188296	-0.009157272		
0.8	10.61682319	0.03476	-0.1162	-0.860908786	-0.009964222		
0.85	10.61049997	0.03782	-0.1265	-0.936772982	-0.01084228		
0.9	10.60361955	0.04116	-0.1376	-1.019322231	-0.011797711		
0.95	10.59613282	0.04478	-0.1497	-1.109145578	-0.012837333		
1	10.58798635	0.04873	-0.1629	-1.206883966	-0.013968564		
1.05	10.57912201	0.05302	-0.1773	-1.313234802	-0.015199477		
1.1	10.56947655	0.0577	-0.1929	-1.428956938	-0.016538853		
1.15	10.55898114	0.06278	-0.2099	-1.554876076	-0.017996251		
1.2	10.54756088	0.06831	-0.2284	-1.691890659	-0.019582068		
1.25	10.53513428	0.07433	-0.2485	-1.840978275	-0.021307619		
1.3	10.52161266	0.08088	-0.2704	-2.003202627	-0.023185216		
1.35	10.50689954	0.08801	-0.2943	-2.179721114	-0.025228254		
1.4	10.49088994	0.09577	-0.3202	-2.371793083	-0.027451309		
1.45	10.47346961	0.10421	-0.3484	-2.580788801	-0.029870241		
1.5	10.45451427	0.11339	-0.3791	-2.808199219	-0.032502306		
1.55	10.43388865	0.12338	-0.4125	-3.055646596	-0.03536628		
1.6	10.41144561	0.13425	-0.4489	-3.324896054	-0.038482593		
1.65	10.387025	0.14608	-0.4884	-3.617868147	-0.041873474		
3.15	6.891910367	1.83923	-6.1444	-45.5143383	-0.526786323		
3.2	6.557681257	2.00114	-6.6846	-49.51542373	-0.573095182		
3.25	6.194083046	2.17727	-7.272	-53.86640154	-0.623453722		
3.3	5.798552075	2.36888	-7.9106	-58.59718096	-0.678208113		
3.35	5.368307813	2.5773	-8.6049	-63.73989062	-0.737730216		
3.4	4.90033827	2.804	-9.3594	-69.32882129	-0.802416913		
3.45	4.391387215	3.05054	-10.179	-75.4001562	-0.872686993		
3.5	3.837946342	3.31864	-11.069	-81.9912405	-0.948972691		
3.55	3.236259614	3.61011	-12.034	-89.13877457	-1.03169878 *		
3.6	2.582358767	3.92686	-13.078	-96.8741995	-1.121229161		
3.65	1.872188186	4.27087	-14.203	-105.2104565	-1.217713617		
3.7	1.102049197	4.64391	-15.403	-114.094665	-1.320540105		
3.75	0.270781664	5.04653	-16.625	-123.1507455	-1.425355851 **		
3.8	-0.585689859	5.46109	-17.129	-126.8846702	-1.468572571		
*Maximum amount of time before O2 runs out!							
**Assuming aeration isn't limited; Complete substrate utilization after this time point							

**Figure K-1.** Predictive model (using Excel) to determine the time at which oxygen utilization by aerobic bacteria, r-utilization, overcomes the estimated mass-transfer of oxygen into solutions when the initial substrate concentration is ~10.67 g BOD/L (i.e. in non-sterile glucose treatments).

From the previous model, if the flasks were to remain completely aerobic until the point in which oxygen mass-transfer into solution was less than oxygen utilization rate (assuming 100% mass-transfer to the microbes, i.e. over-estimating the influent oxygen), the dissolved oxygen would be completely consumed by  $t = 3.55$  days. At that time point, 30% of the initial glucose would remain. Since the study lasted for about 2 weeks, it is likely that the glucose-treated flasks did not remain completely aerobic.

## Appendix L

### Adsorption of Atrazine onto Packing Media (Calculations)

#### Assumptions

1. The following assumptions were obtained from Dr. Stephanie Velegol:
  - a. Maximum surface area for atrazine adsorption = 50% of total packing media surface area
  - b. Adsorbed atrazine molecules will form a monolayer
2. The atrazine molecular shape can be approximated as a sphere

#### Data

1. Conversions:  $1 \text{ \AA} = 10^{-8} \text{ cm}$ ;  $1 \text{ ft} = 30.48 \text{ cm}$ ;  $1 \text{ mole} = 6.022 \times 10^{23} \text{ molecules}$
2. Atrazine molecular weight = 217.9 g/mole
3. Foam geometric surface area =  $240 \text{ ft}^2/\text{ft}^3$ ; Wood chip geometric surface area  $\sim (1100 + 240)/2 = 670 \text{ ft}^2/\text{ft}^3$
4. Internal angles of the ring structure are each  $120^\circ$ ; All other bond angles are assumed to be  $\sim 110^\circ$
5. Bond lengths are given in Table L-1:

**Table L-1.** Average bond lengths used in the estimation of the projected area of an atrazine molecule. Adapted from Schwarzenbach et al. (2003).

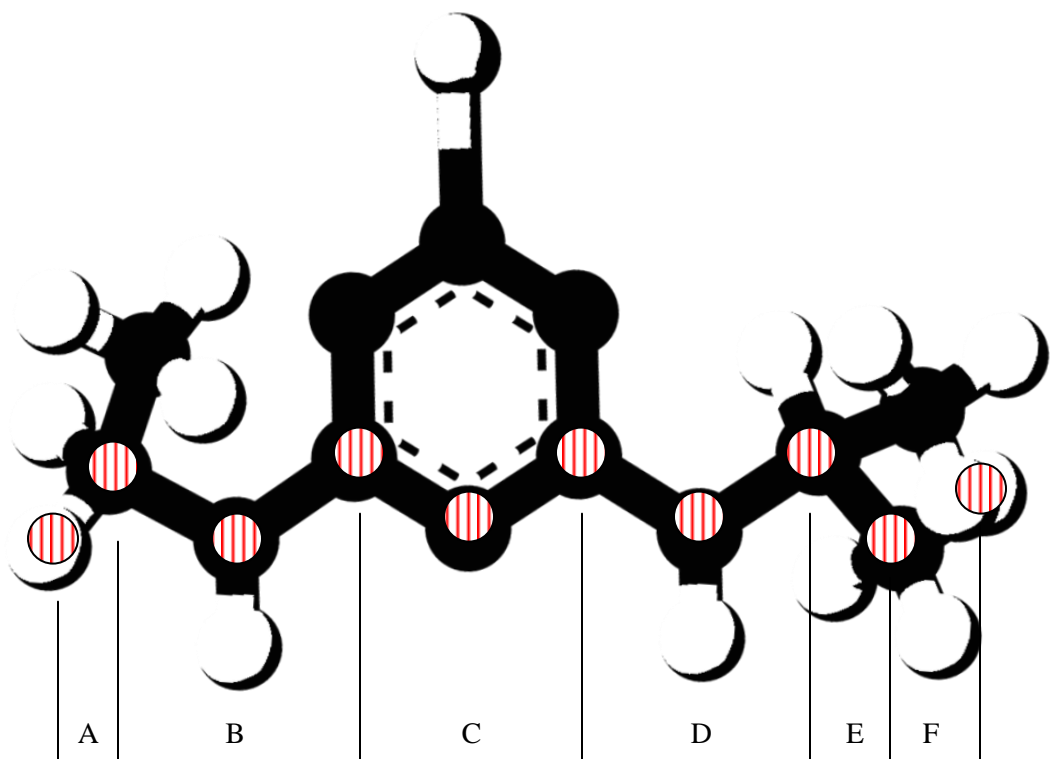
Bond	Length ( $\text{\AA}$ )
C-H	1.11
C=N	1.28
C-N	1.47
C-Cl	1.54
C-Cl	1.78



### Calculations

Minimum atrazine adsorption was based on maximum projected surface area of atrazine:

Figure L-2 illustrates the selected atoms to determine the maximum estimated diameter of a spherical molecule of atrazine. The diameter was calculated to be  $\sim 9 \text{ \AA}$  (lengths A, B, C, D, E, and F).



**Figure L-1.** Selected atoms (horizontally-striped red and white circles) within the atrazine molecule to determine the maximum molecular diameter. Adapted from Dyer, 2010.

**Length A = Length F = 0.91 Å**;  $\sin(\text{angle}) = (A)/(\text{C-H bond length})$ ;  $\sin(110/2) = A/1.11$

**Length B = Length D = 2.08 Å**;  $a^2 + b^2 = c^2$ ;  $(\text{C-N bond length})^2 + (\text{C-N bond length})^2 = c^2$ ;  $1.47^2 + 1.47^2 = 2.08^2$

**Length C = 1.98 Å**;  $a^2 + b^2 = c^2$ ;  $[(\text{C-N bond length} + \text{C=N bond length})/2]^2 + [(\text{C-N bond length} + \text{C=N bond length})/2]^2 = c^2$ ;  $[(1.47 + 1.28)/2]^2 + [(1.47 + 1.28)/2]^2 = 1.98^2$

**Length E = 1.26 Å**;  $\sin(\text{angle}) = E/(\text{C-C bond length})$ ;  $\sin(110/2) = E/1.54$

**Total diameter = 9.22 Å**;  $(0.91 \times 2 + 2.08 \times 2 + 1.98 + 1.26)$

**Projected Attachment Area/molecule =  $6.36 \times 10^{-15} \text{ cm}^2/\text{molecule}$** ;  $\pi * r^2 = \pi * [(9 \text{ Å}/2)(10^{-8} \text{ cm}/\text{Å})]^2$

**a. Sorption to wood chips**

15 mL (displaced solution) = **15 cm<sup>3</sup>** wood chips

**Maximum Available Surface Area = 165 cm<sup>2</sup>**;  $670 \text{ ft}^2/\text{ft}^3 \times 1 \text{ ft}^3/30.48^3 \text{ cm}^3 \times 30.48 \text{ cm}^2/\text{ft}^2 = 22 \text{ cm}^2/\text{cm}^3$ ;  $15 \text{ cm}^3 \times 22 \text{ cm}^2/\text{cm}^3 = 330 \text{ cm}^2$ ;  $0.5 \times 330 \text{ cm}^2 = 165 \text{ cm}^2$

**Maximum # of Molecules Adsorbed =  $2.6 \times 10^{16}$  molecules**; available surface area/surface area of a molecule of atrazine =  $165 \text{ cm}^2 / 6.36 \times 10^{-15} \text{ cm}^2/\text{molecule}$

**Concentration Removed from Solution via Adsorption =  $0.069 \text{ mg/L} \sim 10^{-2} - 10^{-1}$**

**mg/L**;  $2.6 \times 10^{16} \text{ molecules} \times (1 \text{ mole} / 6.022 \times 10^{23} \text{ molecules}) \times (215.7 \text{ g/mole}) \times (1000 \text{ mg/g}) \times (1/0.135 \text{ L reactor solution})$

**b. Sorption to foam**

$25 \text{ cubes} \times (.5'' \times 0.5'' \times 0.5'')/\text{cube} = 3.125 \text{ in}^3 \times 2.543 \text{ cm}^3/\text{in}^3 = \mathbf{51.2 \text{ cm}^3}$  foam cubes (solid + voids)

**Maximum Available Surface Area = 202 cm<sup>2</sup>**;  $240 \text{ ft}^2/\text{ft}^3 \times 1 \text{ ft}^3/30.48^3 \text{ cm}^3 \times 30.48 \text{ cm}^2/\text{ft}^2 = 7.87 \text{ cm}^2/\text{cm}^3$ ;  $51.2 \text{ cm}^3 \times 7.87 \text{ cm}^2/\text{cm}^3 = 403 \text{ cm}^2$ ;  $0.5 \times 403 \text{ cm}^2 = 202 \text{ cm}^2$

**Maximum # of Molecules Adsorbed =  $3.18 \times 10^{16}$  molecules;** available surface

area/surface area of a molecule of atrazine =  $202 \text{ cm}^2 / 6.36 \times 10^{-15} \text{ cm}^2/\text{molecule}$

**Concentration Removed from Solution via Adsorption =  $0.084 \text{ mg/L} \sim 10^{-2} - 10^{-1}$**

**mg/L;**  $3.18 \times 10^{16} \text{ molecules} \times (1 \text{ mole} / 6.022 \times 10^{23} \text{ molecules}) \times (215.7 \text{ g/mole}) \times (1000 \text{ mg/g}) \times (1/0.135 \text{ L reactor solution})$

Based on these rough estimations, adsorption to either packing material would result in removal atrazine (from solution) on the order of  $10^{-2} - 10^{-1} \text{ mg/L}$ .