

# **THE COMPARATIVE TOXICITIES OF FILTRATES FROM CONVENTIONAL AND ALTERNATIVE BLEACHING AGENTS**

A Dissertation Submitted by

Teri A. Ard

B.S., 1991, University of Washington

M.S., 1993, Institute of Paper Science and Technology

in partial fulfillment of the requirements  
from the Institute of Paper Science and Technology  
for the degree of Doctor of Philosophy

Atlanta, Georgia

Publication Rights Reserved by the  
Institute of Paper Science and Technology

October 14, 1996



## EXECUTIVE SUMMARY

In response to environmental concerns, the pulp and paper industry is investigating alternatives to conventional bleaching sequences, which have generally utilized chlorine in the first bleaching stage. Many alternative bleaching agents are being considered for chlorine replacement. However, little research has gone into quantifying the toxicity reduction generated by modifying bleaching sequences.

At the onset of this thesis, it was important to know under what storage conditions the generated filtrates should be stored to maintain initial toxicity. It was also important to identify how storage effects would affect filtrates from bleaching agents of interest: chlorine, chlorine dioxide, and ozone. Another factor of interest was the effect of chemical charge in the first stage, not only upon final toxicity, but how the storage effects could impact filtrates created under the various charges. In this study, each bleaching agent was evaluated with two chemical charges. Both chlorine and chlorine dioxide were utilized at kappa factors of 0.15 and 0.25. Ozone was applied at 0.4% and 1.2% (as O.D. pulp).

A laboratory study was conducted to comparatively evaluate the toxicities of chlorine and chlorine dioxide bleaching filtrates with the three toxicity assay systems: Microtox™ (an ocean-dwelling photobacterium), XTT (utilizing human liver cells), and a stress gene assay. These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen predelignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and

washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. The combined filtrates were then subjected to an ether extraction in order to fractionate the whole filtrate into ether-extractable fractions and non-ether extractable fractions. The ether-extractable fraction is considered to be of the most environmental relevance.

Chlorine bleaching results in the most toxic filtrates for the Microtox assay, but the lowest toxicity for the XTT assay. Background filtrates were not significantly different from chlorine dioxide filtrates in the Microtox assay, but were significantly more toxic than the chlorine or chlorine dioxide filtrates in the XTT assay. Neither background, chlorine dioxide, or chlorine filtrates showed significant inductions in the stress gene assay.

## TABLE OF CONTENTS

Executive Summary	i
Chapter 1	1
Terminology	1
Introduction	2
Thesis Objectives	4
Summary of Papers Included in Thesis	5
Literature Review	12
Paper #1:     “Toxicity Assays in the Pulp and Paper Industry - A Review and Analysis”	13
Chapter 2	28
Terminology	28
Experimental Methods and Materials	29
Filtrate Generation	29
Ether Extraction and Fractionation	32
Biotreatment	32
Total Organic Carbon (TOC)	33
Chemical Oxygen Demand (COD)	34
Microtox Assay	35
XTT Assay	36
Stress Gene Assay	37
Statistical Methods	39

Chapter 3	42
Terminology	42
The Effect of Storage Conditions on the Toxicity of Filtrates from Chlorine, Chlorine Dioxide, and Ozone Bleaching	43
Paper #2:     “Comparative Toxicities of Oxygen, Ozone, Chlorine Dioxide, and Chlorine Pulp Bleaching Filtrates – Microtox Toxicities of Untreated Filtrates”	45
Chapter 4	61
Terminology	61
Comparative Toxicities of Filtrates from Chlorine and Chlorine Dioxide Bleaching	64
Paper #3:     “Microtox Toxicities of Pulp Bleaching Filtrates - Evaluating Both Biotreatment and Bioaccumulable Fractions”	66
Paper #4:     “Use of a Soluble Tetrazolium/Formazan Assay (XTT) with HepG2 Cells for Toxicity Evaluation of Pulp Bleaching Filtrates”	90
Paper #5:     “The Use of Stress Genes to Evaluate Pulp Bleaching Filtrates”	114
Paper #6:     “Innovative Methods for Determining Pulp Bleaching Filtrate Toxicity”	141
Chapter 5	175
Conclusions and Final Results	175
Conclusions	175
Suggestions for Future Research	176
Acknowledgments	177

Appendices	179
Appendix A: Bleaching Conditions	179
Appendix B: Total Organic Chlorine Data (TOC)	196
Appendix C: Chemical Oxygen Demand (COD)	198
Appendix D: Environmental Protocol for Pro-Tox (C) Xenometrix Assay Kit	200
Appendix E: Pro-Tox Stress Gene Names and Promoter Fusion Descriptions	205
Appendix F: Factorial Experimental Design and Summary Results for Storage Effects Experiments	213
Appendix G: Microtox Data Compilation for Bleaching Filtrates and Fractions	215
Appendix H: Graphical Representations of Microtox Data	217
Appendix I: XTT Data Compilation for Bleaching Filtrates and	222
Appendix J: XTT Curves for Determining EC50	232





## CHAPTER 1 TERMINOLOGY

<b>AOX</b>	absorbable organic halides; in pulp and paper industry generally refers to chlorinated organics
<b>Bioaccumulate</b>	accumulation of xenobiotics within an organism
<b>Biotreatment</b>	commonly referred to as secondary treatment; treatment with microbes to reduce environmental and other variables prior to effluent release
<b>DNA Damage</b>	break or rearrangement in DNA strand
<b>EC50</b>	concentration that results in a 50% reduction in response being measured
<b>EE</b>	ether-extractable fraction
<b>HepG2</b>	human liver cell line used in this research obtained from American Type Culture Collection (Rockville, Maryland)
<b>K<sub>ow</sub></b>	octanol/water partition coefficient; ratio of amount dissolved in octanol vs. water
<b>Microtox</b>	assay utilizing the light output of <i>Vibrio fischeri lux</i> , a marine organism
<b>NEE</b>	non-ether extractable fraction
<b>NVW</b>	non-volatile whole
<b>Osmotic Pressure</b>	the external pressure sufficient to oppose osmosis and stop it
<b>Oxidative Damage</b>	damage caused by oxidative-reduction reactions
<b>Oxidative Extraction</b>	extraction of pulp with NaOH and oxygen
<b>Stress Genes</b>	genes that become active under biological or environmental stress; in this thesis, it specifically applies to genes that have been modified with stress promoters, which increase the production of RNA when a particular stress is experienced
<b>TU</b>	toxicity units = 100/EC50
<b>XTT</b>	sodium 3,3'-[1[(phenylamino) carbonyl] 3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate)

## Chapter 1 INTRODUCTION

In response to environmental concerns, the pulp and paper industry is investigating alternatives to conventional bleaching sequences, which have generally utilized chlorine in the first bleaching stage. Many alternative bleaching agents are being considered for chlorine replacement. However, little research has gone into quantifying the toxicity reduction generated by modifying bleaching sequences.

Initially, alternative bleaching technologies were investigated to reduce the amount of dioxin formed during the bleaching process. Although there is still doubt to the actual health implications of dioxin, a large amount of time, effort, and money was invested by the pulp industry to reduce dioxin emissions in wastewater [Culliton (1991); Hanson (1991); Schmidt (1992); Yanchinski (1989)]. Pulp mills were able to reduce the amount of dioxin released to non-detectable limits without eliminating chlorine from the bleaching sequence. However, newer regulations regarding effluent AOX restrictions are having a greater impact on the use of chlorine and chlorine-containing compounds in the pulp industry [PIMA (1990)]. The use of chlorine dioxide results in a significant decrease in AOX produced [Cox (1989); Liebergott *et al.*(1990)]. Ironically, no relationship has been found between current mill levels of AOX and toxicity [Borton (1990); Owens (1990)]. Neither has any correlation been found between toxicity and mill levels of BOD or TOC [Walden (1976); Walden and Howard (1977)]. A relationship has been found between toxicity and the concentration of resin acids present [Priha and Talka (1986)].

Despite the fact that chlorine-containing organic molecules are only found in low concentrations in bleach effluents, emphasis has been placed on these compounds due to their non-polar nature and lipid-soluble character, which may lead to bioaccumulation in marine organisms. The octanol/water partition coefficient ( $K_{ow}$ ) is often used to predict the ability of a compound to bioaccumulate. However, when it is desired to evaluate solutions of unknown organic content, such as bleach plant filtrates, for bioaccumulation potential, it is more practical to use ether extractions to separate compounds by polarity.

Bioaccumulation complicates toxicity concerns. Organochlorine chemicals tend to be persistent in the environment due to their inherent resistance to biological degradation [Voss *et al.* (1980)]. Many of the chlorinated compounds possess the potential to migrate widely throughout the ecosystem, ultimately accumulating in fatty tissues. Cumulative effects may be substantial [Suntio *et al.* (1988)]. Chlorinated organics have been found in the tissue of fish, which have been exposed to diluted spent bleaching filtrates [Lindstrom and Nordin (1979)]. It has been shown, however, that once exposure is discontinued, clearance of the chlorinated compounds from the fish tissues is relatively rapid and compounds may even be metabolized before excretion [Landner *et al.* (1977)].

Another area for concern is synergism among chemical compounds. Although all of the components of a mill effluent are not known, concern arises when streams are mixed. There can be additive effects ( $2 \text{ toxicity units} + 2 = 4$ ), antagonistic effects (2

toxicity units + 5 = 1), synergistic effects (2 toxicity units + 3 = 10), or inhibitory effects (10 toxicity units + 2 = 0).

## **THESIS OBJECTIVES**

The objective of this research was to determine the relative toxicity of various bleaching filtrates. Filtrates from chlorine and chlorine dioxide bleaching stages, as well as the subsequent oxidative extraction stage, were evaluated for toxicity. Toxicity was quantified by evaluating toxic responses by human liver cells and utilizing the Microtox and stress gene assays. The toxicity of fractions of the whole filtrate was evaluated in order to determine the relative toxicities the individual fractions contributed to the toxicity of the whole filtrate. Means to achieve these objectives are outlined below.

- Prepared filtrates under controlled bleaching conditions so that equitable toxicity comparisons were made between filtrates. The filtrates were prepared by bleaching an oxygen-prebleached softwood pulp. The effect of the chemical charge in the first stage on toxicity of the filtrates was also evaluated.
- Used lab-scale biological treatment to evaluate the effect of biotreatment on filtrate toxicity. The goal was not to reproduce all of the effects of biotreatment achieved in the mill setting, but to achieve a more realistic evaluation of the effluent released to the environment.
- Separated the whole filtrates into ether-soluble and non-ether soluble fractions.
- Identified the fractions most responsible for human liver cell toxicity and determined the dose/response relationships for the adverse effects.
- Evaluated whole filtrates and fractions with the Microtox analyzer, a standard industry assay.
- Evaluated whole filtrates with stress gene assays to determine mechanism of toxicity.

## SUMMARY OF PAPERS INCLUDED IN THESIS

The thesis is written by joining six papers with clarifying information and additional data. The six papers are summarized below. Additional information can be found in each chapter before and after the presented paper. Appendices were used abundantly for data and supplementary descriptions.

### **Paper Number One, Chapter One**

**“Toxicity Assays in the Pulp and Paper Industry — A Review and Analysis”** by T.A. Ard and T.J. McDonough, presented at the 1996 TAPPI International Environmental Conference in Orlando, Florida, May 1996.

This paper provides a summary and clarification of knowledge regarding toxicity assays utilized in the pulp and paper industry. A wide variety of toxicity assays are described and evaluated, including those based on *Ceriodaphnia*, fathead minnow, salmonids and trout, Microtox, *Nitzschia closterium*, Ames *Salmonella* Assay, and enzyme induction including EROD. Standard toxicological terminology and definitions are discussed. Also included is a discussion of relationships between toxicity and effluent parameters, including chlorophenols, chlorinated dioxins and furans, and extractives.

## Paper Number Two, Chapter Three

**“Comparative Toxicities of Oxygen, Ozone, Chlorine Dioxide, and Chlorine Pulp Bleaching Filtrates — Microtox Toxicities of Untreated Filtrates”** by T.A. Ard and T.J. McDonough, prepared for submission to Environmental Science and Technology.

A laboratory study was conducted to comparatively evaluate the toxicities of filtrates from conventional and alternative wood pulp bleaching processes. Filtrates from ozone, chlorine dioxide, and chlorine bleaching were evaluated in comparison to control filtrates representing background toxicity. In each case, a sample of oxygen predelignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately. The persistence of their toxicities was also assessed by examining the effects of the duration, pH, and temperature of storage before testing.

For the first bleaching stage, averaging over all levels of these factors, the toxicities associated with the bleaching chemicals and charges could be ranked as follows:  $0.15 \text{ KF ClO}_2 > 1.2\% \text{ O}_3 > 0.25 \text{ KF Cl}_2 \geq \text{Background} \geq 0.25 \text{ KF ClO}_2 \geq 0.15 \text{ KF Cl}_2 \geq 0.4\% \text{ O}_3$ . The effects of all other factors were also found to be statistically significant.

For the second bleaching stage, storage factors were not found to significantly affect toxicity. However, the bleaching chemical and charge utilized in the first bleaching stage significantly affected the toxicity of the second bleaching stage filtrate. Averaging

over all levels of storage factors, the toxicities associated with the bleaching chemicals and charges could be ranked as follows: Background > 1.2% O<sub>3</sub> ≥ 0.15 KF ClO<sub>2</sub> ≥ 0.15 KF Cl<sub>2</sub> ≥ 0.25 KF Cl<sub>2</sub> > 0.25 KF Cl<sub>2</sub> > 0.4% ClO<sub>2</sub>.

### **Paper Number Three, Chapter Four**

**"Microtox Toxicities of Pulp Bleaching Filtrates—Evaluating Both Biotreatment and Bioaccumulable Fractions"** by T.A. Ard and T.J. McDonough, prepared for submission to Water Research.

A laboratory study was conducted to comparatively evaluate the toxicities of filtrates from chlorine and chlorine dioxide bleaching using the Microtox™ assay. These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen predelignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. The combined filtrates were then subjected to an ether extraction in order to fractionate the whole filtrate into ether-extractable fractions and non-ether extractable fractions. Generally, the ether-extractable fraction is considered to be of the most environmental importance. The toxicity of the whole filtrates and fractions was determined by the use of the Microtox™ toxicity assay.

In general, both charges of chlorine resulted in statistically significantly higher toxicity filtrates than both chlorine dioxide filtrates and the background filtrates. The

background filtrates were not significantly different from either chlorine dioxide charge filtrates. Future research should examine the source of the toxicity of the background filtrate, which may also explain chlorine dioxide filtrate toxicity and remove concerns regarding the use of this chemical for pulp bleaching purposes.

The first bleaching stage produced filtrates which are statistically significantly higher in toxicity than the filtrates from the second bleaching stage. The whole, combined filtrates were approximately equal to the average of the first and second stages indicating no synergistic effects. If the toxicities of the ether-extractable (EE) fractions and the non-ether extractable (NEE) fractions of the whole filtrates are compared to the non-volatile whole (NVW) toxicity, it appears that there are additive effects for the background filtrates and low charge chlorine filtrates. Both chlorine dioxide charges show antagonistic effects. The high chlorine charge filtrates show a synergistic effect when combined.

The use of laboratory-simulated biotreatment in this study was not effective in reducing Microtox toxicity. However, this is not necessarily indicative of the treatability of the filtrates for Microtox toxicity because another type of treatment system or bacteria used could potentially be successful in reducing Microtox toxicity.

#### **Paper Number Four, Chapter Four**

**"Use of a Soluble Tetrazolium/Formazan Assay (XTT) with HepG2 Cells for Toxicity Evaluation of Pulp Bleaching Filtrates"** by T.A. Ard, T.J. McDonough, and M.J. DeLong, prepared for submission to Ecotoxicology and Environmental Safety.



A laboratory study was conducted to comparatively evaluate the toxicities of filtrates from chlorine and chlorine dioxide bleaching with a XTT toxicity assay utilizing human liver cells (HepG2). These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen predelignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. The combined filtrates were then subjected to an ether extraction in order to fractionate the whole filtrate into ether-extractable fractions and non-ether extractable fractions. Generally, the ether extractable fraction is considered to be of the most environmental importance. The toxicity of the whole filtrates and fractions was determined by the use of the XTT assay utilizing human liver cells.

The XTT assay utilizing human liver cells (HepG2) was chosen to evaluate potential human liver toxicity due to concerns from environmental groups that humans could be exposed to compounds found in bleaching filtrates through consumption of aquatic organisms, specifically fish. The XTT assay measures the ability of the electron transport system of the cells to function. If cells are functioning properly, the electron transport system will convert the XTT tetrazolium salt into a formazan, which is soluble and colorimetric, spectrophotometrically active at 600 nm.

Analysis of variance indicated that bleaching agent and bleaching charge used in the first stage were significant at the 95% confidence level. The background filtrates

resulted in the highest toxicity. The resulting toxicities (in decreasing order) are B > D (low charge) > D (high charge) > C (low charge) > C (high charge).

The first bleaching stage produced filtrates which are higher in toxicity than the filtrates from the second bleaching stage. The background filtrates showed a slight antagonistic effect when combined, while the low charge chlorine dioxide filtrates also showed an antagonistic effect. The high charge chlorine dioxide and both chemical charges of chlorine showed synergistic effects when the first and second stages were combined. With regard to the filtrate fractions, all of the bleaching agents/charges show extremely low or unmeasurable EE fraction toxicity. This is significant due to the fact that the EE fraction is considered to be the fraction of most relevance and concern to the environment. In all bleaching agent/charges, the NEE fraction and the NVW fraction are not significantly different from each other, leading to the conclusion that the majority, if not all, of the filtrate toxicity assayed by XTT is induced by the NEE fraction, which is not considered to be of environmental importance relating to bioaccumulation or biomagnification of toxicants through the food chain.

Labscale biotreatment was highly successful in reducing the toxicity of the filtrates' XTT toxicity. The background filtrates resulted in the highest reduction of toxicity from biotreatment.

**Paper Number Five, Chapter Four**

**“Use of Prokaryotic Stress Promoters as Indicators of Toxicity Mechanisms for Pulp Bleaching Filtrates”** by T.A. Ard, T.J. McDonough, J. Cairney, M. DeLong, and J. Williams, prepared for submission to Tappi Journal.

Bacterial stress promoters were used to evaluate untreated filtrates from chlorine and chlorine dioxide bleaching of an oxygen predelignified softwood kraft pulp. We utilized an E.coli stress gene assay kit containing sixteen bacterial strains, each comprised of a different stress gene promoter fused to a lacZ structural gene. The modified test organisms are designed to reveal the occurrence of specific toxicity mechanisms, including DNA damage of various types, oxidative damage, adverse osmotic conditions, oxidative stress, protein damage, and heavy metal effects. None of the stress genes was induced in media containing filtrates from either chlorine or chlorine dioxide bleaching at kappa factors of 0.15 and 0.25, at concentrations as high as 80%. The bacteria grew normally in the presence of all filtrates. These observations indicate that the bleaching filtrates have none of the indicated toxic effects on the test organism.

## **Paper Number Six, Chapter Five**

**“Innovative Methods for Determining Pulp Bleaching Filtrate Toxicity”** by T.A. Ard and T.J. McDonough, prepared for submission to Tappi Journal.

This paper provides a review and comparison of the results described in the above-mentioned (papers numbered three through five) papers. Differences such as the high toxicity of chlorine in the Microtox assay, but low toxicity in the XTT assay. Similarities between assays are also discussed. For example, the filtrates which were generated with a higher charge of chlorine dioxide had a lower toxicity than the filtrates generated with the lower charge of chlorine dioxide. The advantages and disadvantages of the Microtox, XTT, and Xenometrix assay are considered and placed in perspective.

## **LITERATURE REVIEW**

In order to provide the reader with a background of the literature on the topic of bioassays used in the pulp and paper industry, “Toxicity Assays in the Pulp and Paper Industry — A Review and Analysis” by T.A. Ard and T.J. McDonough follows. Further information on the assays utilized in this thesis is described in more detail throughout the additional papers.

## TOXICITY ASSAYS IN THE PULP AND PAPER INDUSTRY - A REVIEW AND ANALYSIS

T.A. Ard  
Graduate Student  
IPST  
Atlanta, GA

T.J. McDonough  
Professor  
IPST  
Atlanta, GA

### ABSTRACT

---

This paper endeavors to summarize and clarify knowledge regarding toxicity assays utilized in the pulp and paper industry. A wide variety of toxicity assays are described and evaluated including those based on: *Ceriodaphnia*, the fathead minnow, salmonids and trout, Microtox, *Nitzschia closterium*, Ames *Salmonella* Assay, and enzyme induction including MFO and EROD.

Standard toxicological terminology and definitions are discussed. Also included is a discussion of relationships between toxicity and effluent parameters, including chlorophenols, chlorinated dioxins and furans, and extractives.

---

### INTRODUCTION

The field of toxicity testing is complicated by lack of agreement between experts in the field and regulatory agencies. The result is that there are many complex protocols with few objective criteria to facilitate a preference for the use of one protocol over another(1). Another difficulty is that terminology is extensive, highly specialized, and varies from one research group to another(1). This paper endeavors to summarize and clarify knowledge of toxicity assays currently utilized in the pulp and paper industry.

### DEFINITION OF TERMS

The terms "acute" and "chronic" are often utilized ambiguously to describe toxicity. Both can refer to either the length of exposure or length of response. In general, acute toxicity

implies effects having a sudden onset and lasting a short time. When referring to a stimulus, acute means the stimulus is severe enough to induce a response rapidly. The duration of an acute aquatic toxicity test is generally 4 days or less and mortality is the response measured(2).

A chronic stimulus lingers or continues for a long time. The term often signifies periods from several weeks to years, depending on the reproductive life cycle of the aquatic species in question. Chronic exposure typically induces response with slow progress and long continuance(2). Often the terms chronic and sublethal are used interchangeably. It is important to understand that lethal or nonlethal responses can occur either acutely (quickly) or chronically (longer response time).

A life cycle study is one in which all the significant life stages of an organism are exposed to the test material(2). Usually, this involves an entire reproductive cycle of the test organism.

The definitions of typical toxicity assay endpoints also need clarification. The median lethal concentration, LC50, is the concentration that results in death for 50% of the test organisms. Likewise, the median inhibition concentration, IC50, is the concentration that reduces the fecundity of the test organism by 50%. The median effective concentration, EC50, is the concentration that induces a sublethal response of 50% in the test organism. If an EC50 is cited, a statement of the sublethal response needs to be clearly defined. LC50, IC50, and EC50 are time-dependent values. Therefore, the test duration (e.g., 24-hr or 96-hr) that yielded the observed values must be stated.

The lowest observed effect concentration (LOEC) is the lowest concentration of the test material used in the toxicity assay that has a statistically significant adverse effect on the test organism compared with the control. Likewise, the no observed effect concentration (NOEC) is the highest concentration of a test material, used in the assay, which has no statistically significant adverse effect on the exposed test organism compared with the control.

If an assay is referred to as *in vivo*, it is an assay occurring within an intact animal or organism. This is common for aquatic assays. On the other hand, if the assay is *in vitro*, it is outside the intact organism. This term is generally applied to experiments involving biochemical reactions in tissue fragments or fractions.

## MEASUREMENTS OF TOXICITY

Because there are many possible toxic responses, it is difficult to detect and measure all of them. One test method, or even a battery of test methods, can not evaluate all possible responses. Often a particular bioassay will be weak at determining toxicants that are in a particular class of compounds. By using a battery of assays, there is a higher probability of detecting a potential toxin than by using only one test. However, when the number of bioassays is increased, there is also an increase in capital equipment, consumable supplies, and required personnel. In the long run, a balance must be found between the cost of a number of tests and the potential increase in information gathered(3).

### *Ceriodaphnia*

Use of *Ceriodaphnia dubia* as a test species is the most common method for evaluating toxicity in the pulp and paper industry. *Ceriodaphnia*, a water flea, is an invertebrate.

The 7-day test method consists of exposing early life stage organisms to various concentrations of mill effluents. The mill effluent is diluted with either preconditioned or natural water which may need to be enriched. Dietary needs are met with trout chow, yeast, and Cerophyll (a cereal leaf product), which is administered regularly to the test species(4). *Ceriodaphnia* survival and reproduction rates are calculated and compared at five effluent concentrations(5). The neonate (baby) should be able to develop to sexual maturity and produce three broods of offspring during the 7-day test period.

It is generally accepted that reproduction is a more sensitive endpoint for toxicity evaluation than mortality(6). Reproduction is more sensitive than mortality by at least a factor of five. Reductions in fecundity are indicative of a chronic response, whereas mortality can be either an acute or chronic response.

Advantages of the *Ceriodaphnia* test method include completion of an entire life cycle evaluation during a relatively short (7-day) test period. Although the paper industry utilizes a 7-day test, the *Ceriodaphnia's* average life span is 21 days. When compared to other organisms, such as the fathead minnow, *Ceriodaphnia* has been determined to be the most sensitive species(5). Another advantage is that a specific protocol has been developed by the EPA. Other environmental regulatory agencies and technical associations have also drafted procedures for *Ceriodaphnia* toxicity determinations(7).

One major difficulty in utilizing the *Ceriodaphnia* method is the time and cost commitment. The extensive time commitment and cost constraints limit the sample size as well as the number of concentrations that can be tested(8). Labor requirements are high; approximately 90 hours of labor are needed over a 7-day test period. Culture maintenance and expenses are additional(9).

Other problems involve the lack of understanding of *Ceriodaphnia* health and survival requirements. The nutritional needs are not well understood, and therefore, could be leading to death or disease(4). Water quality parameters, such as pH, hardness, alkalinity, and conductivity are not understood with regard to the health of *Ceriodaphnia*. There have been cases where the ambient water toxicity was reduced by the addition of effluents(9). One cause of "toxic" water may simply be lack of nutrients.

Other concerns include maintenance of *Ceriodaphnia* populations in the laboratory. Although *Ceriodaphnia* are relatively easy to culture, population fluctuations are frequent, and occasional crashes (death of all control organisms) occur(4, 9).

In using the 7-day test, neonates must be between 2 and 24 hours old and all test specimens must be within four hours age of each other. One major difficulty arises in gathering sufficient numbers of neonates for testing. For this reason, as well as the fragility of the organisms, suggestions have been made to increase the age of the neonates to 24-48 hours old(9).

Little work has been done in the area of reproducibility and applicability of the *Ceriodaphnia* method, and demand is growing for further research in this area. In May 1990, The National Council of the Paper Industry for Air and Stream Improvement (NCASI) published a report on the intra- and inter-laboratory reproducibility of the *Ceriodaphnia* method(10).

Results showed that “about half (56 percent) of the laboratories participating in the study were able to routinely complete the test successfully”. The laboratories that participated were from industry, contractors, EPA, and universities that routinely conducted these tests.

Several test specimens were provided to the labs and the five concentrations to be tested were specified. Even with identical test specimens and concentrations, the interlaboratory coefficients of variation are extremely high as can be seen in Table 1.

Although the NCASI report investigated inter- and intralaboratory reproducibility, only interlaboratory results are cited here since many labs investigated were unable to successfully repeat the experiments.

Test Material	CV (%)* Lethality (LC50)	CV (%)* Reproduction (IC50)
NaCl #1	6.7	46.4
NaCl #2	61.9	65.0
potassium dichromate #1	70.8	30.8
potassium dichromate #2	38.2	48.9
utility effluent #1	56.4	57.7
utility effluent #2	0.0	27.3
pulp and paper effluent #1	0.0	28.3
pulp and paper effluent #2	12.0	7.5
overall mean	30.8	39.0

\* all < or > values considered as actual values

**Table 1.** Interlaboratory coefficients of variation for seven-day lethality (LC50) and reproduction (IC50) with *Ceriodaphnia* toxicity assay(10).

For comparison purposes, inter- and intra-laboratory reproducibility have also been compiled for common analytical test methods. These are shown in Table 2.

EPA method	Intralaboratory CV range (%)	Interlaboratory CV range (%)
601 purgeable halocarbons by purge and trap	5-28	13-58
602 purgeable aromatics	7-18	15-71
604 phenols	15-37	20-45
605 benzidines	24-40	38-69
606 phthalate esters	1-80	12-73
607 nitroamines	13-36	21-46
608 organochlorine pesticides and PCBs	10-33	12-45
609 Nitroaromatics and Isophorone	13-45	26-60
610 PNAs	11-50	16-91
611 Haloethers	15-31	32-53
612 Chlorinated Hydrocarbons	16-24	26-41

**Table 2.** Inter- and Intralaboratory coefficients of variation for various EPA methods(10).

### *Pimephales promelas* (Fathead Minnow)

This 7-day method relies on measurements of growth and survival of newly hatched larval minnows. The method can be used to study several concentrations and effluents, but unlike the *Ceriodaphnia* method, the fathead minnow's weight is also monitored as a measure of growth.

The fathead minnow has the same advantages as the short 7-day *Ceriodaphnia* test method and a protocol has been established by the EPA. Unfortunately, using the fathead minnow assay also has several disadvantages.

According to the EPA protocol, the fathead minnow is fed brine shrimp. This poses two problems. First, newly hatched fry may be unable to eat large shrimp for several days. Secondly, since fathead minnows are sight-feeders, color effects of effluents may reduce

their ability to locate food. Ambient laboratory lighting may also limit sight-feeding(4, 9). Concern also surrounds the young age of the organism. Due to the fragility of the newly hatched fry during the first days of life, being placed in a foreign solution may cause shock, damage, or death from even the safest of effluent samples. The delicacy of these small, fragile organisms can lead to damage during handling prior to and during the study.

Like *Ceriodaphnia*, the fathead minnow's dietary, water quality, and biological requirements are uncertain. These areas of uncertainty must be explored further in order to ensure accurate results in testing for chronic toxicity.

### Salmonids and Trout

Salmonids, trout, and other species indigenous to pulp and paper mill outflow areas have been used for chronic toxicity testing. Trout and salmonids are monitored for growth changes (both length and weight) as well as survival and reproduction.

Advantages include using an organism higher in the food chain. These organisms are also more likely to be found directly adjacent to effluent release sites, thus giving a more accurate representation of chronic dangers to the aquatic environment.

The lengthy testing period (8 months to 2 years) is most often the factor discouraging use of these methods. The testing period is based on the life cycle of the organism; a salmon goes through a life cycle of approximately one year(11).

Storage and living areas required for the fish are extensive. As an example, Seim *et al.* used six wooden troughs 3.3 meters long, 66 cm wide, and 25 cm deep. Equipment needed to support the study were one 800 gallon tank, two refrigerated 350 gallon and several 500 gallon tanks(12). When using salmonids and trout for toxicity evaluations, capital investment is relatively large in comparison with other methods.

When dealing with more complex organisms, variability of results becomes higher. Younger fish are fairly uniform in size and weight,

resulting in lower levels of variability. But as organisms age, variability, unrelated to the effluent being tested, increases(5).

As in the *Ceriodaphnia* and fathead minnow methods, there are several analytical procedural problems with utilizing Salmonids and trout. For the first three weeks of life, trout retain a yolk sac. For thorough chronic toxicity testing, the parent would need to be exposed to the effluent before and during spawning in order for offspring to be exposed for a full lifetime. Salmonids are also sight feeders. Growth may be minimized by the effluent's inhibition of the salmonid's ability to seek nourishment.

James River Corporation has published extensively on chronic toxicity testing methods and results. They have determined water fleas and fathead minnows more "appropriate, acceptable, and logically defensible" than salmonids for chronic toxicity testing(5).

### Respiration Rate

Oxygen consumption is a useful measure of sublethal toxicity effects since energy processes are indicators of overall physiological health(2). Toxic agents damage respiratory membranes of aquatic animals. Asphyxiations can be brought on by toxicants reacting with some constituent of the mucus secreted by fish gills. Exposure to a toxicant may also result in gill tissue deterioration including swelling of epithelial cells, adhesion of secondary lamellae, detachment of epithelium from the pillar cell system, discharge of mucous cells, and hematomas. Monitoring respiration may be justifiable for pollutants that greatly affect respiratory membranes or respiratory processes, but organochlorines do not appear to be in this category. However, trace metals do result in respiratory damage(2).

### Microtox

The Microtox™ assay, marketed by Microbics Incorporated, indirectly monitors the metabolic response of an ocean-dwelling bacterium to determine toxicity. *Vibrio fischeri lux* (previously referred to in the literature as *Photobacterium phosphoreum*) naturally emits visible light as a result of metabolic reactions which liberate



energy(13). When exposed to toxic compounds, the enzymes which promote this reaction are inhibited; the resulting decrease in light output is directly proportional to the toxicity of the sample. Light reduction is the direct measurement; EC50 is the concentration of the test sample which results in a 50% reduction in light output.

Many researchers have evaluated the comparative sensitivity of Microtox™ to that of other common aquatic assays, with conflicting conclusions. Qureshi *et al.* demonstrated that for organic compounds, pulp and paper effluents, and oil refinery effluents, Microtox™ sensitivity was comparable to *Salmo gairdneri* (rainbow trout), *Spirillum volutans* (a bacterium), and *Daphnia magna* (a crustacean) bioassays(14). Microtox™, in general, was found to be the most sensitive to industrial effluents; in some cases, Microtox™ was substantially more sensitive.

Firth and Backman found Microtox™ to have good correlations with acute rainbow trout LC50 values and *Ceriodaphnia* chronic toxicity values for both treated and untreated pulp and paper mill wastewaters(8, 15). Microtox™ values were more sensitive than rainbow trout mortality but less sensitive than *Ceriodaphnia* reproduction. However, in another study, Microtox™ was found to be more sensitive than the *daphnid* chronic assay(16). Fraser completed a correlation study comparing Microtox™ with Rainbow trout and *Daphnia magna*. Results indicate that Microtox™ results correlate well with rainbow trout, but not with *Daphnia magna*(17). Stauber *et al.* compared the Microtox™ toxicity of bleached eucalypt kraft mill effluents to a phytoplankton (*Nitzschia closterium*) growth inhibition test. The effluents exhibited greater toxicity to *Nitzschia* (1-20x more toxic) than Microtox™(18, 19).

When Renberg evaluated data from both mill and laboratory experiments, no correlation was found between Microtox™ toxicity and *Ceriodaphnia* or algal toxicity. On the other hand, Renberg did find that when the use of Microtox™ was limited to samples from similar sources, such as pulp mill effluents, the response agreed with that of *Ceriodaphnia*(20). Springer and Bazarow evaluated compounds typically found in mill effluents, but found no significant correlation between Microtox™ and rainbow

trout, fathead minnow, *daphnia magna*, or *selenastrum capricornutum*(21).

Microtox™ has been shown to exhibit satisfactory sensitivity to pulp and paper process effluents and is a relatively quick, easy, and economical method for evaluating toxicity. No culture maintenance is necessary since the bacteria arrive freeze-dried and remain frozen until immediately prior to use. Another benefit of the Microtox™ assay is the small sample volume required for testing.

The EC50 is determined by the decrease in luminescence of bacteria after exposure to the effluent in question. This test method gives no information or indication as to what, if any, specific effects the effluent may impose on marine life.

### *Nitzschia closterium*

*Nitzschia closterium* is a unicellular marine diatom (colonial algae) which is widely distributed in Australian waters. Algal species have been widely used for toxicity assays; however, this species is given attention in this paper because there is currently a strong movement in Australia to begin toxicity testing with native organisms to evaluate kraft effluents. The *Nitzschia* cells divide 1.4 times per day so that a number of generations of cells are exposed to the effluent over the 3-day bioassay(18). This allows for a short-term, sublethal, chronic bioassay.

When *Nitzschia* EC50 (72 hours; reduction of cell division rate) was compared to Microtox 15-min EC50, the effluent was found to be 1-20x more toxic to *Nitzschia* than to Microtox(18). The *Nitzschia* assay has also been compared to other Australian native organisms, such as *Hormosira banksii* (brown macroalgal fertilization test) and Tasmanian blenny (survival of larval stages of a tidepool fish), and the *Nitzschia* was the most sensitive and the most reproducible(19). This assay needs further development, but appears to have much promise in providing Australian researchers with a native toxicity assay.

### Ames *Salmonella* Test

The Ames test, developed by Ames in 1971, is the best known and accepted method for determining mutagenic potential and is accepted around the world as a standard method(22). It is based on specially prepared mutants of the bacterium *Salmonella typhimurium*. As a result of treatment, the bacteria have lost the ability to synthesize histidine, an amino acid. The bacteria can not grow unless histidine is added to the culture medium. When exposed to a mutagenic compound, the introduced damage to the histidine mechanism can be repaired by mutation and the bacteria will regain the ability to grow on a histidine-free medium(23).

Numerous strains of *Salmonella typhimurium* have been isolated in order to detect a multitude of mutation mechanisms. Many of the strains have been used by different researchers investigating kraft bleaching effluents. For pulp bleaching effluents, the strain that has been found to be the most sensitive for detecting mutagens is TA 100(24).

In order to simulate the conditions in a mammalian body, rat liver microsomes (S9 fraction), which contain the enzymes responsible for the most important metabolism of chemicals in the mammalian body, can be added to the bacterial cultures. Most of the known substances which give rise to cancer, such as vinyl chloride, aflatoxin, benzopyrene, and benzidine, require a metabolic change in order to become active carcinogens(25). However, chlorination stage effluents have been found to have a reduction in mutagenic potential when liver microsomes are present, indicating a metabolic detoxification of the mutagens(25).

It has been determined that spent chlorination liquors of most pulp types exhibit mutagenic activity when tested according to the Ames test(24, 26). The effect is apparently caused by various chlorinated compounds with relatively low molecular masses(27). The bacterial mutagenicity of chlorinated effluents was initially reported in Sweden in 1977, and has since been confirmed by workers at the Pulp and Paper Research Institute of Canada (PPRIC) and elsewhere(28). A majority of the total mutagenic activity, estimated to be 75%, has been found to originate from the lipid-soluble

fraction(24, 27, 29). In spent chlorination liquors, approximately 85% of the mutagenicity has been found in the fraction containing compounds with low molecular weights(23, 30). Chlorination liquors tested for mutagenicity with the Ames test have shown the number of mutant revertants to be linearly related to the chlorine ratio(31). Hypochlorite, extraction, and chlorine dioxide bleaching effluents have been found to have little or no mutagenic activity(25). It has been estimated that oxygen prebleaching may reduce the total mutagenicity of a subsequent CD stage effluent by 50%(23). A linear relationship exists between the number of mutants and the chlorine dioxide/chlorine ratio, the number of mutants decreasing with increasing chlorine dioxide/chlorine ratio. With pure chlorine dioxide, the number of mutants is close to the number found in the control sample(23).

### Enzyme Induction

Enzymes are biological catalysts, substances that increase biological reactions without being changed themselves in the overall process. When a toxic compound is present, three types of observations are possible in enzyme systems. First, enzymatic production may not be affected. Secondly, enzymatic production may be increased to aid detoxification reactions. Thirdly, enzymatic production may be inhibited by the toxicant and results in a reduction of enzyme available for necessary biological reactions.

Many toxicity assays have used enzyme monitoring to evaluate the toxicity of a compound or solution. The following invertebrates have been monitored for an induction of mixed-function oxygenase (MFO) enzymes: *Nereis virens* (sandworm), *Uca pugilator* (fiddler crab), *Mytilus galloprovincialis* (mussel), and *Collinectes sapidus* (blue crab). Vertebrates have also been used for this purpose, such as: *Fundulus heteroclitus* (mummichog), *Cittarichthys sordidus* (pacific sanddab), *Leuciscus cephalus* (chub), *Salmo gairdneri* (rainbow trout), *Barbus barbus* (barbel), *Chondrostoma nasus* (nase), and *Perca fluviatilis* (perch)(32).

When an organism such as a salmonid, trout, or even a human is exposed to a xenobiotic (toxicant), enzymes, primarily in the liver, react

in order to remove the toxicant from the body(33). There are many enzyme systems available; however, the major system is the cytochrome P-450, otherwise known as the mixed-function oxygenase (MFO) system. The MFO enzymes are found in the phospholipid matrix of the endoplasmic reticulum(34).

In brief, the enzymes attempt to detoxify the xenobiotic by adding a hydroxyl moiety which will result in increasing the water solubility of the toxicant allowing it to be excreted from the organism(35). When exposed to a xenobiotic, the enzyme system has the ability to increase its capacity to metabolize the toxicants(36). To determine toxicity, enzyme induction, or the increase in enzymes present in an organism exposed to a test material, can be compared to a control organism.

There are two substrates used for measuring monooxygenase activity: benzo(a)pyrene (i.e., arylhydrocarbon hydroxylase activity, AHH) and 7-ethoxyresorufin deoxyethylase (i.e., EROD activity). Since there is a strong linear correlation between AHH and EROD activity, only one assay, preferably EROD, is necessary(37).

Preliminary evidence indicates that the kraft process may be a source of MFO-inducing substances(38). However, MFO response patterns have been found not to reflect the distribution of AOX in the water(39).

MFO induction responses did parallel the gradients of phenolics in water and the levels of TCDD equivalents found in fish tissues(39). On other occasions, EROD induction was found not to correlate with either dioxins or furan body burdens(37). It should be emphasized that EROD induction is a response to a broad spectrum of hydrophobic compounds, so induction is not evidence of the presence of any particular class of compounds whether it be furans, dioxins, or other organochlorines. In fact, Birkholz and Swanson suggest that the compound(s) causing MFO induction are non-chlorinated since induction is seen in fish exposed to both bleached and unbleached effluents(36).

Researchers found that MFO increased for both bleached and unbleached kraft effluents over controls(38). The presence of oxygen delignification, high substitution, and secondary treatment did not eliminate the MFO response(38). Data show that the replacement of chlorine in the bleaching of kraft pulp by chlorine dioxide or nonchlorine-containing compounds such as peroxide does not significantly alter an effluent's ability to induce MFO activity(38). The use of chlorine for kraft pulp bleaching did not appear to be an important factor in determining the ability of final mill effluents to cause MFO enzyme induction in fish(40). New bleaching technologies with secondary treatment also did not eliminate the MFO response(38).

#### **EFFLUENT PARAMETERS AND THEIR RELATIONSHIP TO TOXICITY**

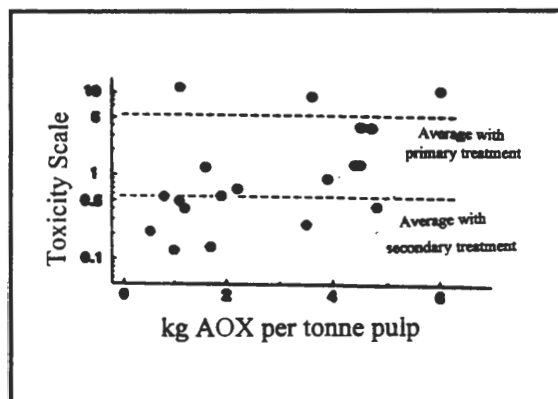
The impact of pulp mill discharges on receiving waters is dependent upon the oxygen demand, suspended solids, pH, color, and toxicity of the effluent. It is important to note that frequently it is impossible to segregate effects due to toxicity from those due to other effluent characteristics(41). Variables which affect effluent composition include: raw materials, woodyard operation, pulping processes, degree of closure and spill control, handling and use of condensates, process chemicals, and control of operations outside of the pulp stream(42). In natural waters, the impact of the effluent upon the receiving water is dependent upon not only effluent characteristics, but also the composition of the receiving water, relative flows, and conditions affecting dilution and dispersal of the wastes(43).

Toxicity relationships to groups of compounds are discussed below. However, it is important to preface this discussion by noting that additive and synergistic effects complicate the toxicity analysis of bleaching liquors. Assessing the risks posed by chemicals in industry is especially difficult, not only because of the large number of chemicals involved, but also because these chemicals often occur as complex mixtures of poorly known composition. The effluents from pulp and paper mills include dissolved lignin and cellulose degradation products, other wood extracts, and chlorinated organics. It is more practical and relevant in experimental efforts to

study the whole mixture rather than specific compounds since it is the entire mixture that is released into the environment(3).

## AOX

AOX is a chemical analysis procedure which measures the total organically bound chlorine, without reference to species(44). Researchers agree that AOX is not related to toxicity and that appropriate internal measures taken at kraft pulp mills combined with biological treatment of the effluents eliminate acute lethal toxicity, and the remaining sub-acute toxicity that can be characterized as “weak toxic effects” are not caused by organochlorine substances, but rather by neutral, non-chlorinated compounds(45). O’Conner *et al.* conducted tests on a variety of bleached and unbleached kraft mill effluents, sampled before and after biological treatment systems, and showed that there was no correlation between AOX and acute or sub-lethal toxicity to *Ceriodaphnia* or fathead minnows(45). Figure 1 illustrates the lack of correlation between AOX and toxicity.



**Figure 1.** AOX vs. Chronic *Ceriodaphnia* Toxicity (after biological treatment) of Effluents from Bleached and Unbleached Pulping Facilities(47)

Lehtinen found when chlorine use was reduced or when effluents were more thoroughly treated, a different spectrum of chlorinated compounds was detected in the effluent and toxicity (mortality, growth, enzyme induction) decreased(64,65). One mill showed significant correlations between low (<1000) MW TOX concentrations and acute toxicity in rainbow trout and *Ceriodaphnia* chronic toxicity(48).

When a mill implements process changes to reduce AOX discharges, many other characteristics of the effluents also change, systematically reducing discharges of many pollutants(45). The reduction in total organics discharged, which accompanies implementation of AOX control measures, is probably the reason for the toxicity reduction observed when effluents from mills with differing AOX discharge rates are compared(45).

There is abundant evidence that AOX measurements are ineffective in characterizing the toxicity of pulp mill effluents or for use as a parameter indicative of environmental impact(45, 49). An independent scientific advisory group, convened by Procter and Gamble with the assistance of NCASI, determined that “available data do not support a conclusion that chlorinated organic compounds discharged from bleached pulp mills are largely responsible for the damage to the aquatic environment observed in Sweden.”(50) Rather than AOX, other properties of effluents, such as chronic toxicity measured with appropriate aquatic test organisms, or composition with respect to specific, persistent, bioaccumulable, and potentially toxic substances are more relevant(49).

## Chlorophenols

Salkinoja-Salonen *et al.* were unable to demonstrate a positive correlation between sample toxicity and concentration of specific chlorophenolic compounds(50). Information on structure-activity relationships demonstrates that the degree of chlorination is the major factor determining the toxicity of phenolic compounds rather than specific parent ring structure(44).

## Chlorinated Dioxins and Furans

Lab exposures of fish to chlorinated dioxins and furans result in epithelial cell damage, embryol- larval mortality, a possibility of impaired regulation of reproductive hormones, and a strong induction of hepatic MFO enzymes(52-56). Since most mills in the United States have reached non-detectable levels of both chlorinated dioxins and furans, this is becoming less of a toxicity issue. Attention must be paid to the bioaccumulation potential which could lead to contamination of the higher food chain.

## Extractives

Since alkaline pulping conditions ensure maximum solubilization of extractives into the pulping liquor(57), wood resin accounts for approximately 10% of the COD in pulping liquors(58, 59). Certain resin acids are known to bioaccumulate and to be toxic to aquatic organisms(60-62). A positive relationship has been found between toxicity and the concentration of resin acids present(63). Lab exposure of fish to resin acids causes lysis of red blood cells, increased breakdown of hemoglobin to bilirubin, and overloading of the conjugation pathway(64).

Scandinavian studies also suggest that resin and fatty acids and perhaps plant sterols originating from wood extractives are important toxicologically(65-68). In fact, much of softwood pulp effluent toxicity has been attributed to the resin acids, particularly dehydroabietic acid(69).

## Summary

Chemical and biological data specific to a mill are usually necessary to evaluate and to define the relationship between the operation of the mill and the health of its receiving ecosystem(42). However, there are numerous toxicity assays that can be used to evaluate the potential impact upon various aquatic populations and to determine potential sources of toxicity.

No single test system is adequate to ensure the detection of all toxic effects of a particular compound or of a complex effluent. There is also no single "most sensitive species." For this reason, batteries of tests are now frequently used. However, increasing the number of bioassays results in additional expense for capital equipment, consumable supplies, personnel, and testing. In the final decision, a balance must be gained between the cost of a battery of tests and the potential increase in information.

No correlation has been found between toxicity and BOD, AOX, or TOC(41, 43, 70, 71). The relative contribution of chlorinated organic waste to acute toxicity has not been isolated from the nonchlorinated waste contribution(72). It is dangerous to isolate compounds or compound classes and assign toxicity blame since

synergistic effects most likely occur between compounds found in mill effluents.

## ACKNOWLEDGMENTS

The authors thank the Institute of Paper Science and Technology and its member companies for financial support. Portions of this work will be used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

## LITERATURE CITED

1. Hodgson, E. and Levi, P.E., *A Textbook of Modern Toxicology*, Elsevier, New York, N.Y., 1987.
2. Rand, G.M. and Petrocelli, S.R., *Fundamentals of Aquatic Toxicology - Methods and Applications*, Hemisphere Publishing Corporation, New York, N.Y., 1985.
3. Zimmermann, F.K. and Taylor-Mayer, R.E., *Mutagenicity Testing in Environmental Pollution Control*, Ellis Horwood Limited, New York, N.Y.
4. Hall, T.J. and Borton, D.L., *Tappi Journal*, "Chronic effluent bioassays and their limitations to characterizing effluents," 70:57-59(1987).
5. Kraus, G.L., *Tappi Journal*, "Comparative sensitivity of warm and cold water species of fish and *Ceriodaphnia* to treated bleached kraft mill effluents," 73(11):163-166(1990).
6. Williams, P.L., Evaluation of *Caenorhabditis elegans* as an Acute Lethality and Neurotoxicity Screening Model, Ph.D. Thesis, Georgia Institute of Technology, Atlanta, GA, 1988.
7. Gersich, Hopkins, Applegath, Mendoza and Milazzo, "The sensitivity of chronic endpoints used in *Daphnia Magna* Straus life cycle tests," *Aquatic Toxicology and Hazard Assessment: Eighth Symposium (ASTM STP 891)*, 1985.

8. Firth, B.K. and Backman, C.J., *Tappi Journal*, "Comparison of Microtox testing with rainbow trout (acute) and *Ceriodaphnia* (chronic) bioassays in mill wastewaters," 73(12):169-174(1990).
9. Kraus, G. and Kornder, S., *Tappi Journal*, "Experience with EPA chronic bioassay methods," 70:62-66(1987).
10. DeGraeve, M., Cooney, J. and Borton, D., "Precision of the EPA seven-day *Ceriodaphnia* dubia survival and reproduction test intra- and interlaboratory study," Technical Bulletin NCASI, 588, 1990.
11. *Bioassay Techniques and Environmental Chemistry*, Ann Arbor Science, 1973.
12. Seim, W.K., Lichatowich, J.A., Ellis, R.H. and Davis, G.E., *Water Research*, "Effects of kraft mill effluent on juvenile salmon production in laboratory streams," 11:189-196.
13. Ribo, J.M. and Kaiser, K.L.E., *Toxicity Assessment: An International Quarterly*, 2:305(1987).
14. Qureshi, A.A., Flood, K.W., Thompson, S.R., Janhurst, S.M., Inniss, C.S. and Rokosh, D.A., "Comparison of a Luminescent Bacterial Test with Other Bioassays for Determining Toxicity of Pure Compounds and Complex Effluents," Aquatic Toxicology and Hazard Assessment: Fifth Conference ASTM STP 766, 179-195, 1982.
15. Firth, B.K. and Backman, C.J., "A Comparison of Microtox Testing with Rainbow Trout (Acute) and *Ceriodaphnia* (Chronic) Bioassays Using Pulp- and Paper-Mill Wastewaters," 1990 Environmental Conference Proceedings, 621-626, 1990.
16. Suckling, I.D., Pasco, M.F. and Gifford, J.S., "Origin of Effluent Toxicity from a Simulated BCTMP Process," 1993 Environmental Conference, Boston, 449-456, 1993.
17. Jamieson, S., *Pulp & Paper Canada*, "In Environmental Monitoring, What You Don't Know Can Hurt You," 93(2):9-10(1992).
18. Stauber, J., "Development and Application of Algal Bioassays for Determining the Toxicity of Bleached Eucalypt Kraft Mill Effluents," Appita Annual General Conference, 789-796, 1993.
19. Stauber, J., Gunthorpe, L., Deavin, J., Munday, B. and Ahsanullah, M., "Application of New Marine Bioassays for Assessing the Toxicity of Bleached Eucalypt Kraft Mill Effluents," 48th Appita General Conference, 511-518, 1994.
20. Renberg, L., "Use of Cost-Effective Chemical and Biological Tests for the Estimation of the Environmental Impact of Bleaching-Plant Effluents," 1992 Environmental Conference, Richmond, VA, 317-329, 1992.
21. Springer, A.M. and Bazarow, J., "An Assessment of the Microtox Toxicity Analyser as a Screening Test for an Integrated Bleached Kraft Pulp and Paper Mill Effluent," 1993 Environmental Conference, Boston, MA, 427-448, 1993.
22. Li, Y., Dunipace, A.J. and Stookey, G.K., *Mutation Research*, "Absence of mutagenic and antimutagenic activities of fluoride in Ames Salmonella assays," 190:229-236(1987).
23. Stockman, L., Stromberg, L. and de Sousa, F., *Cellulose Chemistry and Technology*, "Mutagenic properties of bleach plant effluents: Present state of knowledge," 14:517-526(1980).
24. Kringstad, K.P., Lindquist, P.O. and Stromberg, L.M., *Environmental Science and Technology*, "Evaluation of lipophilic properties of mutagens present in the spent chlorination liquor from pulp bleaching," 18(3):200-203(1984).
25. Ander, P., Eriksson, K.E., Kolar, M.C. and Kringstad, K., *Svensk Papperstidning*, "Studies on the mutagenic properties of bleaching effluents," 80(14):454-459(1977).
26. Kringstad, K.P., Ljungquist, P.O., de Sousa, F. and Stromberg, L.M., *Environmental Science and Technology*, "Stability of 2-chloropropenol and some other mutagens formed in the chlorination of softwood kraft pulp," 17(8):468-471(1983).

27. Kringstad, K.P., Ljungquist, P.O., de Sousa, F. and Stromberg, L.M., *Environmental Science and Technology*, "Identification and mutagenic properties of some chlorinated aliphatic compounds in the spent liquor from kraft pulp chlorination," **15**(5):562-566(1981).
28. Holmbom, B.R., Voss, R.H., Mortimer, R.D. and Wong, A., *Tappi Journal*, "Isolation and identification of Ames-mutagenic compounds present in kraft chlorination effluents," **64**(3):172-174(1981).
29. Rao, S.S., Burnison, B.K., Rokosh, D.A. and Taylor, C.M., *Chemosphere*, "Mutagenicity and toxicity assessment of pulp mill effluent," **28**(10):1859-1870(1994).
30. Rannug, U., Jenssen, D., Ramel, C., Eriksson, K.E. and Kringstad, K., *Journal of Toxicology and Environmental Health*, "Mutagenic effects of effluents from chlorine bleaching of pulp," **7**:33-47(1981).
31. Annergren, G., Rees-Andersson, A.M., Lindblad, P.O., McKague, B., Stromberg, L.M. and Kringstad, K.P., "Minimizing the formation of Chlorinated Organic Material Through Controlled Chlorination in the Production of High Quality Softwood Kraft Pulp," Environmental Conference, 313-318, 1987.
32. Payne, J.F., Fancey, L.L., Rahimtula, A.D. and Porter, E.L., *Comp. Biochem. Physiol.*, "Review and perspective on the use of mixed-function oxygenase enzymes in biological monitoring," **86C**(2):233-245(1987).
33. Ahokas, J.T., Holdway, D.A. and Brennan, S.E., "The Use of Bioindicators in the Monitoring of Effluents from Pulp and Paper Industry," Appita Annual General Conference, 4B41:797-802, 1993.
34. *Casarett and Doull's Toxicology: The Basic Science of Poisons*, 4th ed, Pergamon Press, Inc., New York, N.Y., 1991.
35. Nebert, D.W. and Gonzalez, F.J., *Ann. Rev. Biochem.*, "P-450 Genes: Structure, evolution and regulation," **56**:945-993(1987).
36. Birkholz, D.A. and Swanson, S.M., "The Relationship Between Mixed Function Oxygenase Induction and other Health Parameters for Fish Exposed to Bleached Kraft Mill Effluent," Environmental Conference, 85-88, 1993.
37. Martel, P.H., Kovacs, T.G., O'Connor, B.I. and Voss, R.H., "A Survey of Pulp and Paper Mill Effluents for Their Potential to Induce Mixed Function Oxidase Enzyme Activity In Fish," 79th Annual Meeting, Technical Section, CPPA, A165-A178.
38. Hodson, P.V., McWhirter, M., Ralph, K., Gray, B., Thivierge, D., Carey, J.H., Van Der Kraak, G., Whittle, D.M. and Levesque, M.-C., *Environmental Toxicology and Chemistry*, "Effects of bleached kraft mill effluent on fish in the St. Maurice River, Quebec," **11**:1635 - 1651(1992).
39. O'Connor, B.I., Kovacs, T.G., Voss, R.H., Martel, P.H. and Van Lierop, B., *Pulp & Paper Canada*, "A laboratory assessment of the environmental quality of alternative pulp bleaching effluents," **95**(3):47 - 55(1994).
40. Walden, C.C., *Water Research*, "Review paper - the toxicity of pulp and paper mill effluents and corresponding measurement procedures," **10**:639-664(1976).
41. Owens, J.W., "Assessing the Environmental Impact of Pulping and Bleaching Operations," 1991 Bleach Plant Operations Short Course, 269-280, 1991.
42. Walden, C.C. and Howard, T.E., *Tappi Journal*, "Toxicity of pulp and paper mill effluents - a review of regulations and research," **60**(1):122-125
43. Holloran, M.F., Willes, R.F., Palmiere, M.J. and Wilson, D.G., "Estimating Effluent Toxicity from Molecular-Chlorine-Gas-Free Bleaching in Terms of Toxicity Equivalence," Pacific Paper Expo, 7-1-0 - 7-1-12, 1990.
44. McCubbin, N. and Folke, J., "Significance of AOX vs Unchlorinated Organics," 1993 Environmental Conference, 21-29, 1993.



45. O'Connor, B.I., Kovacs, T.G., Voss, R.H. and Martel, P.H., "A Study of the Relationship between Laboratory Bioassay Response and the AOX Content for Pulp Mill Effluents," Annual Meeting CPPA Technical Section, Montreal, 223, 1993.
46. Wrist, P.E., "Bleached Pulp in a Global Marketplace," PIMA Annual International Management Conference Proceedings: Going Global, 197 - 210, 1991.
47. Bryant, C.W. and Amy, G.L., "Organic halide in draft mill wastewaters: factors affecting in-mill formation and removal by biological treatment," 1988 Environmental Conference, Charleston, SC, 438, 1988.
48. Berry, R.M., Luthe, C.E., Voss, R.H., Wrist, P.E., Axegard, P., Gellerstedt, G., Lindblad, P.-O. and Popke, I., *Pulp and Paper Canada*, "The effect of recent changes in bleached softwood kraft mill technology on organochlorine emissions: an international perspective," 92(6):T155-165(1991).
49. Smith, J.W. and Sprague, J.B., "Compatibility of Pulping Effluents with the Aquatic Environment," CPPA Annual Meeting, B301 - B305, 1990.
50. Salkinoja-Salonen, M., Saxelin, M.L. and Pere, J., *Industrial Wastewater Analysis*, "Analysis of toxicity and biodegradability of organochlorine compounds released in the environment in bleaching effluents of kraft pulping," 2:266(1981).
51. Muir, D.C.G., Yarechewski, A.L., Metner, D.A., Lockhart, W.L., Barrie Webster, G.R. and Friesen, K.J., *Environ. Toxicol. Chem.*, "Dietary accumulation and sustained hepatic mixed function oxidase enzyme induction by 2,3,4,7,8-pentachlorodibenzofuran in rainbow trout," 9:1463-1472(1990).
52. Lindstrom-Seppa, P. and Oikari, A., *Mar. Environ. Res.*, "Bleached kraft pulp mill effluents and TCDD: Effects on biotransformation and testosterone status of mature male rainbow trout (*Salmo gairdneri*)," 28:99-103(1989).
53. Helder, T., *Toxicology*, "Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on early life stages of rainbow trout (*Salmo gairdneri*)," 19:101-112(1981).
54. Spitsbergen, J.M., Kleeman, J.M. and Peterson, R.E., *J. Toxicol. Environ. Health*, "2,3,7,8-Tetrachlorodibenzo-*p*-dioxin toxicity in yellow perch (*Perca flavescens*)," 23:359-383(1988).
55. Spitsbergen, J.M., Kleeman, J.M. and Peterson, R.E., *J. Toxicol. Environ. Health*, "Morphologic lesions and acute toxicity in rainbow trout (*Salmo gairdneri*) treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin," 23:333-358(1988).
56. Sierra-Alvarez, R., Field, J.A., Kortekaas, S. and Lettinga, G., *Wat. Sci. Tech.*, "Overview of the anaerobic toxicity caused by organic forest industry wastewater pollutants," 29(5-6):353-363(1994).
57. Welander, T. and Andersson, P.E., *Wat. Sci. Tech.*, "Anaerobic treatment of wastewater from the production of chemi-thermomechanical pulp," 17(1):103-112(1985).
58. Field, J.A., Vicent, T., Sierra, R. and Paris, J., "The Anaerobic Treatment and Detoxification of Black Liquors Derived from Non-Woody Feedstocks," Internal Report Autonomous University of Barcelona, Department of Chemical Engineering, 1991.
59. Leach, J.M. and Thakore, A.N., *Prog. Water Tech.*, "Compounds toxic to fish in pulp mill waste streams," 9(4):787(1978).
60. Leach, J.M. and Thakore, A.N., *Tappi Journal*, "Toxic constituents in mechanical pulping effluents," 59(2):12(1976).
61. Leach, J.M. and Thakore, A.N., *J. Fish. Res. Board Can.*, "Identification of the constituents of kraft pulping effluent that are toxic to juvenile coho salmon (*Oncorhynchus kisutch*)," 30(4):479(1973).
62. Priha, M.H. and Talka, E.T., *Pulp and Paper Canada*, "Biological activity of bleached kraft mill effluent (BKME) fractions and process streams," 87(12):143-14



63. Mattsoff, L. and Oikari, A., *Comp. Biochem. Physiol.*, "Acute hyperbilirubinaemia in rainbow trout *Salmo gairdneri* caused by resin acids," **88C**:263-268(1987).
64. Lehtinen, K.-J., "Inhibitory and Stimulatory Responses in Fish Exposed to BKME," International Conference on Bleaching Kraft Pulp Mills - Technical and Environmental Issues, 219, 1991.
65. Lehtinen, K.-J., "Effects of BKME on Marine Communities Studied in Mesocosms," International Conference on Bleaching Kraft Pulp Mills - Technical and Environmental Issues, 255, 1991.
66. Hillis, W.E., "Unique Qualities of Eucalypt Wood," International Conference on Bleached Kraft Pulp Mills - Technical and Environmental Issues, Melbourne, 39, 1991.
67. Borton, D., "Examination of the Relationship Between the Adsorbable Organic Halide (AOX) Content of Paper-Industry Waste Waters and Potential Aquatic Biological Effects: First Progress Report," NCASI Special Report, 90-07, 1990.
68. Owens, W., "Review of Recent Scandinavian Research on the Aquatic Effects of Bleached Pulp Mill Effluents," NCASI Special Report, 90-11, 1990.
69. Paasivirta, J. and Koistinen, J., "Environmental Significance of Organochlorines in Water Ecosystems. Relative Risk of Damages from Pulp Chlorobleaching Wastes," Pacific Paper Expo, (7-2-0)-(7-2-16), 1990.

## LITERATURE CITED - CHAPTER 1

Borton, D. (1990) Examination of the Relationship Between the Adsorbable Organic Halide (AOX) Content of Paper-Industry Waste Waters and Potential Aquatic Biological Effects: First Progress Report. **NCASI Special Report**, No. 90-07.

Cox, J. (1989) BC Mills to Spend C\$800 Million to Meet Toxicity Regulations. *American Papermaker*, **52**(7), 27.

Culliton, B.J. (1991) US Government Orders New Look at Dioxin. *Nature*, Aug. 29, 753.

Hanson, D.J. (1991) Dioxin Toxicity: New Studies Prompt Debate, Regulatory Action. *Chemical Engineering News*. Aug. 12, 7-14.

Landner, L., Lindstrom, K., Karlsson, M., Nordin, J., Sorensen, L. (1977) Bioaccumulation in Fish, of Chlorinated Phenols from Kraft Pulp Mill Bleachery Effluents. *Bulletin of Environmental Contamination & Toxicology*, **18**(6), 663-673.

Liebergott, N., van Lierop, B., Kovacs, T., Nolin, A. (1990) Comparison of the Order of Addition of Chlorine and Chlorine Dioxide in the Chlorination Stage. *Tappi Journal*, **73**(10), 207-213.

Lindstrom, K. and Nordin, J. (1979) Identification of Some Neutral Chlorinated Organic Compounds in Spent Bleach Liquors. *Svensk Papperstidning*, **55**.

Owens, W. (1990) Review of Recent Scandinavian Research on the Aquatic Effects of Bleached Pulp Mill Effluents. **NCASI Special Report**, No. 90-11.

PIMA Roundtable (1990) Pulping Trends. *PIMA*, **73**(10), 29-35.

Priha, M.H. and Talka, E.T. (1986) Biological Activity of Bleached Kraft Mill Effluent (BKME) Fractions and Process Streams. *Pulp and Paper Canada*, **87**(12), 143-144.

Schmidt, K.F. (1992) Dioxin's Other Face: Portrait of an Environmental Hormone, *Science News*, Jan. 11, 24-27.

Suntio, L.R., Shiu, W.Y., and Mackay, D. (1988) A Review of the Nature and Properties of Chemicals Present in Pulp Mill Effluents. *Chemosphere*, **17**(7), 1249-1290.

Voss, R.H., Wearing, J.T., Mortimer, R.D., Kovacs, T., Wong, A. (1980) Chlorinated Organics in Kraft Bleachery Effluents. *Paperi ja Puu - Papper och Tra*, **12**, 809-814.

Walden, C.C. (1976) Review Paper-The Toxicity of Pulp and Paper Mill Effluents and Corresponding Measurement Procedures. *Water Research*, **10**, 639-664.

Walden, C.C. and Howard, T.E. (1977) Toxicity of Pulp and Paper Mill Effluents-A Review of Regulations and Research. *Tappi Journal*, **60**(1), 122-125.

Yanchinski, S. (1989) New Analysis Links Dioxin to Cancer. *New Scientist*, Oct. 28, 24.

**CHAPTER 2 TERMINOLOGY**

<b>β-galactosidase</b>	lactase; an enzyme that hydrolyzes lactose to glucose and galactose <sup>i</sup>
<b>BOD</b>	biological oxygen demand
<b>COD</b>	chemical oxygen demand
<b>CV</b>	coefficient of variation = standard deviation/mean
<b>EC50</b>	the concentration that results in a 50% reduction of the response being measured
<b>EO</b>	second bleaching stage utilizing NaOH and oxygen
<b>KF</b>	kappa factor = total active chlorine, % / kappa #
<b>O.D.</b>	oven dry weight
<b>ONPG</b>	<i>o</i> -nitrophenyl-β-D-galactoside; for this research used in a buffer
<b>TOC</b>	total organic carbon
<b>TU</b>	toxicity units = 100/EC50

<sup>i</sup> Walker, J.M. and Cox, M., The Language of Biotechnology. A Dictionary of Terms, Second Edition, American Chemical Society, Washington, D.C., 1995.

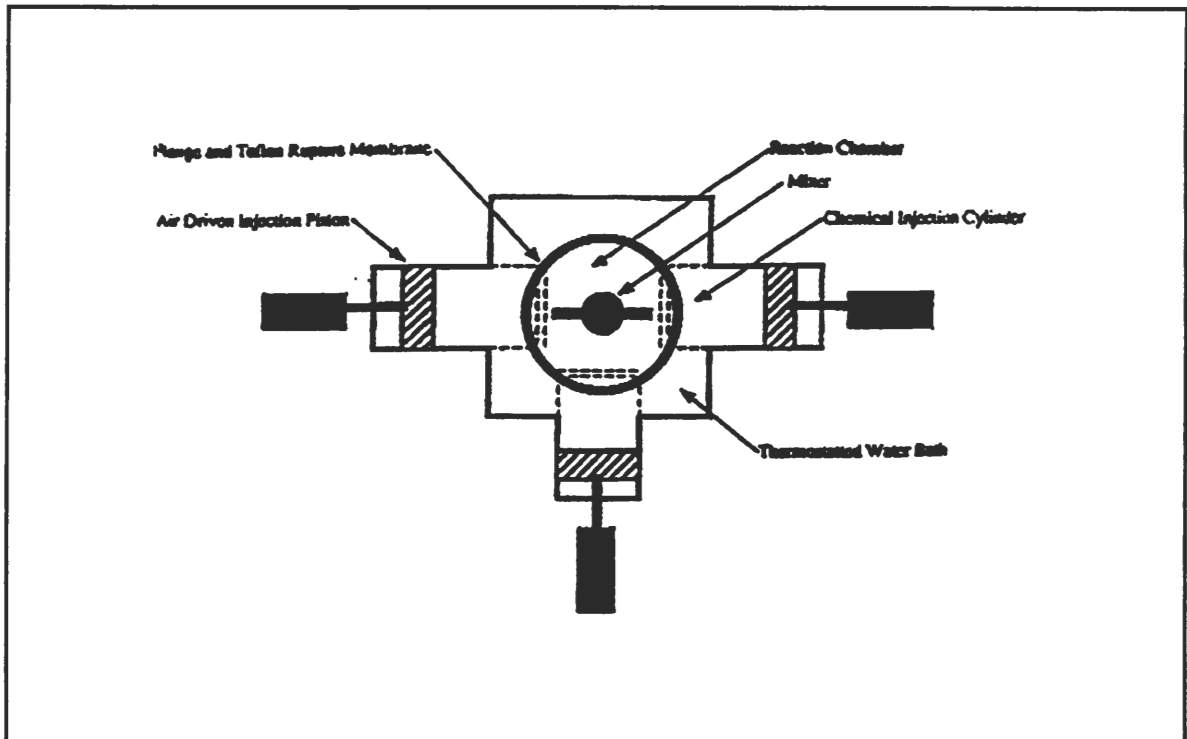
## **Chapter 2 EXPERIMENTAL METHODS AND MATERIALS**

### **Filtrate Generation**

Oxygen delignified pulp was obtained from a commercial facility immediately following the washing stage. Oxygen stage carryover was obtained immediately prior to the wash stage. In the laboratory, the pulp was washed thoroughly and centrifuged in cycles until no color remained in the wash water; a minimum of three cycles was used. The pulp was then centrifuged to approximately 35% solids, fluffed, and stored at 4°C. Prior to bleaching, the moisture content was determined by drying three samples and averaging the results. Three hundred grams, expressed as O.D. pulp, was weighed for bleaching.

The bleaching reactor used for the first bleaching stage was designed by T.A. Schwantes and is shown in Figure 2-1 (Schwantes, 1992). The reactor was designed with several important considerations in mind. First, the reactor attained complete mixing rapidly. Schwantes evaluated the quality of mixing obtained in the reactor and found that complete mixing occurred within 0.5 seconds. Secondly, the reactor was designed to maintain the mixing chamber at the desired bleaching temperature. An outer heated water shell surrounds a portion of the reactor to warm the mixture. Thirdly, both the chlorine and chlorine dioxide solutions need to be kept cool in order to keep the chemical charge in solution constant and to avoid flashing. The bleaching chemical chamber is surrounded by another water shell designed to hold water and ice mixture to keep the chemical chamber cold. The bleaching chemical is separated from the pulp solution by a

membrane. When it is desired to mix the bleaching chemical into the pulp, piston pressure is applied to one side of the bleaching chemical chamber, forcing the membrane to break and mixing to occur.



**Figure 2-1.** High Shear Mixer Designed by Schwantes.

In preparation for bleaching, pulp, enough acid to reduce the pulp solution to less than pH 2, oxygen carryover, and enough dilution water to make the solution 3% consistency were placed in the bleaching chamber. The amount of oxygen carryover was determined by assuming a well-washed system, achieving only 10 kg COD carryover per ton of pulp. Sulfuric acid was used to reduce the pH. Distilled water, preheated to 70°C, was used for dilution. Chlorine and chlorine dioxide solution concentrations were determined immediately prior to bleaching using idometric titration. For the

“background” bleaches, distilled water was substituted for bleaching chemical.

Bleaching conditions for each bleach are shown in Appendix A.

Immediately following the first bleaching stage (30 minutes in duration), the pulp and filtrate were immediately filtered and the pulp washed thoroughly with fresh distilled water. Pulp washing and filtering were repeated at least three times.

For the second bleaching stage, an oxidative extraction (EO), the appropriate amount of dilution water at room temperature was added to the pulp so that the consistency was 10% minus the required sodium hydroxide solution. The pulp slurry was allowed to heat to 70°C prior to the sodium hydroxide solution and the oxygen charge addition. Sodium hydroxide was added as a 40 g/l solution at the appropriate charge. Oxygen was injected into the reactor under pressure. For the initial experiments to evaluate storage effects (described in Chapter 3), a Quantum reactor was used for the (EO) stage. For the remainder of the experiments, a peg mixer was used to complete the (EO) stage.

When the 70 minute bleaching time was complete, the pulp and filtrate were filtered, and again, the pulp was washed well. Individual stage filtrates were stored immediately at negative 70°C. Individual stage (1<sup>st</sup> and 2<sup>nd</sup> stages) filtrates were also combined immediately; one sample was stored immediately at -70°C; one sample was vacuum evaporated for 24 hours and then stored at -70°C; and 4 liters of the combined filtrate were immediately placed in the ether extractor for fractionation.

## **Ether Extraction and Fractionation**

Ether extractions were performed in 4 L glass continuous extractors. Four liters of combined (first and second stage) filtrates were added to the extractor and connected to a 1 L round bottom flask containing 500 mls ether. The ether was refluxed slowly for four days, and the refluxing condensate continuously passed through the aqueous phase before returning to the boiling flask, extracting what has been considered the bioaccumulable fraction from the whole filtrate. A rotary vacuum evaporator was used for 24 hours to remove the ether from both the ether-extractable fraction and the non-ether extractable fraction. Residual ether, determined by TOC, was found to be minor.

## **Biotreatment**

It was desired to emulate the effects of pulp and paper secondary biotreatment facilities in order to have a more realistic view of environmental impact of effluents in the aquatic environment. Most U.S. mills are equipped with a minimum of secondary treatment. Untreated filtrates are not discharged directly into the aquatic environment. Therefore, laboratory-scale biotreatment was completed on all whole, combined filtrates. Filtrates were diluted to 25% v/v with double deionized, double filtered water, and then the pH was adjusted to  $7.0 \pm 1.0$ . Four hundred mls of each diluted filtrate were added to a flask, and then one Hach BOD capsule (Hach, Loveland, Colorado) was added to each flask. The flasks were placed on a shaker at 200 rpm at room temperature. After five days of treatment, the contents of each flask were placed in a 1000 ml round bottom flask. The flask was then placed on a rotary evaporator in a water bath at 40°C until



approximately 50 mls of filtrate remained. The filtrate was then washed from the flask with double deionized, double filtered water and diluted back to its original concentration (100 mls). The filtrate pH was adjusted to  $7.4 \pm 0.05$ . The filtrate was then filtered with 0.22  $\mu\text{m}$  filter and stored at  $-70^{\circ}\text{C}$  until tested for toxicity.

### **Total Organic Carbon (TOC)**

In order to determine total organic carbon (TOC) directly, an aliquot of each filtrate and fraction was adjusted with HCl to a pH of 2 or less. At a pH of 3 or less, carbon dioxide is separated from the carbonate so that TOC measurements can be made directly rather than subtracting inorganic carbon from total carbon, each determined separately. To protect against contamination, each aliquot was discarded after TOC determinations.

TOC was determined utilizing the Shimadzu Model TOC-5050 Total Organic Carbon Analyzer. TOC values for filtrates and fractions are given in Appendix B.

Three calibration curves were constructed each day that TOCs were determined. Each calibration point solution was made independently of the other calibration solutions. Reagent grade potassium hydrogen phthalate was used as the calibration chemical compound.

Prior to making solutions, the potassium hydrogen phthalate  $[\text{C}_6\text{H}_4(\text{COOK})(\text{COOH})]$  was dried in an oven at  $105-110^{\circ}\text{C}$  for two hours and then cooled in a desiccator. A sample was then weighed and transferred to a volumetric flask and

diluted with double deionized water at pH 1.5 +/- 0.1. Solutions were mixed by inverting the flask and then stored in a refrigerator. New solutions were made as needed, with a maximum storage time of two weeks.

### **Chemical Oxygen Demand (COD)**

Hach COD test method was used to measure the chemical oxygen demand of each filtrate and fraction. COD is an important measure of the amount of oxygen available for marine organisms. It is not as accurate, for determining oxygen available for marine organisms, as BOD but is less expensive, quicker, and requires less sample. This micro-sample digestion method is approved by the U.S. Environmental Protection Agency for NPDES reporting. Following the Hach COD System for Wastewater Testing, the method consists of pipetting two mls of sample into a Hach high COD vial, which contains potassium dichromate, mercuric sulfate, and silver sulfate. Mercuric sulfate eliminates chloride interferences. Oxidizable organic compounds react with the dichromate ion ( $\text{Cr}_2\text{O}_7^{2-}$ ) to form green chromic ion ( $\text{Cr}^{3+}$ ). Silver sulfate acts as a catalyst to promote oxidation of resistant organic compounds. The sample and vial contents are mixed by inverting the vial repeatedly. The vial is then placed in a COD reactor at 150°C for 120 minutes. The reactor is allowed to cool until the temperature reaches 120°C. At that time, the vial is removed from the reactor, mixed by inversion, and allowed to cool in a test tube rack until the vial and its contents reached room temperature. A control (0 mg/L COD) and a potassium hydrogen phthalate standard (300 mg/L COD) were also tested. COD is determined by comparing the light absorbance of the sample to the control. A

standard is performed with each reactor run to confirm the equipment is working properly. COD results are given in Appendix C.

### **Microtox™ Assay**

Microtox™ is a trademark of Microbics Corporation. The assay uses photoluminescent bacteria (*Vibrio fischeri lux*, previously referred to as *Photobacterium phosphoreum*) to evaluate the toxicity of samples. The bacterium strain NRRL B-1117 is deposited with the Northern Regional Laboratory in Peoria, Illinois, U.S.A. (Environmental Protection Series, 1992). Blue-green photoluminescence is produced by enzymatic reactions and is directly proportional to the health of the organism (Ribo and Kaiser, 1987). EC50 is the sample concentration that reduces the reagent light output by 50%. For this research, 15-minute EC50 values were used. The EC50 term can be confusing since toxicity increases as EC50 decreases and vice versa. The use of toxicity units, TU, eliminates this problem. The toxicity in TU is defined as 100 divided by the EC50, expressed as a percent.

In the 100% test, the primary method used in this research, four concentrations of each filtrate were evaluated (90, 45, 22.5, and 11.25%). If the average EC50 was less than 11.25% for a sample, the sample was retested using the Basic Test Method. The Basic Test evaluates 45, 22.5, 11.25, and 5.625% concentrations. Approximately  $10^6$  microorganisms are used to evaluate each test concentration. Prior to testing, an aliquot of each filtrate and fraction is removed from storage at  $-70^{\circ}\text{C}$  where the filtrate/fraction had been pH adjusted to  $7.4 \pm 0.05$  and sterilized with a  $0.22 \mu\text{m}$  filter. Each test sample

was evaluated in triplicate following the protocol described in the Microbics' manual (Microbics, 1992).

### **XTT Assay**

The procedure used for the XTT assay was acquired from Scudiero *et al.* (Scudiero *et al.*, 1988). Scudiero *et al.* developed the XTT assay to replace the MTT assay, which requires the use of DMSO solubilization of MTT-formazan which is not only laborious, but also poses risk exposure to laboratory personnel because there is potential for hazardous solutions to be dissolved in the DMSO. On the other hand, the metabolic reduction of XTT results in a soluble colored formazan, simplifying the procedure and reducing risk to laboratory personnel. The procedure is described thoroughly in Scudiero *et al.*

For the experiments in our laboratory, HepG2 (human liver) cells were grown in 75 cm<sup>2</sup> flasks in an incubator at 37°C. Prior to XTT testing, the cells were trypsinized and transferred to 96-well plates. After the cells had grown to confluence (full coverage of cells on plate), five concentrations (25, 10, 5, 1, ½ %) of each filtrate were tested in quadruplicate along with a control. Four filtrates could be tested per 96-well plate. The cells were exposed for 24 hours to the test solutions. The 0.025 mM PMS-XTT solution was prepared by mixing 25 µl of the 5mM PMS solution to each 5 ml of XTT (1 mg/ml). Fifty µl of PMS-XTT solution were then added to each well. The 96-well plate was then placed back in the incubator for another four hours. At this time, the 96-well plate was read in a microplate reader at 450 nm. Each test concentration was compared to the

control in the same row. A dose-response curve was then created by averaging the results of each quadruplet test result and the corresponding other test concentrations and performing a linear regression. The EC<sub>50</sub> was then calculated from the linear regression equation.

### **Stress Gene Assay**

The Pro-Tox (C) kit for running stress gene assays was obtained from Xenometrix. It was determined that the Environmental Protocol, which allows for the testing of higher concentrations of filtrate, was most appropriate for the bleaching filtrates and fractions. Since the Environmental Protocol is not, at this time, permanently printed in any citable material and the protocol was altered slightly to account for the small amount of filtrate available, the protocol provided by Xenometrix and used in this study is described in detail in Appendix D. All materials used, except for the 96-well plates, pipettes, and pipette tips, were provided by Xenometrix. Again, the protocol was developed by Xenometrix and only altered to test two plates per filtrate rather than three.

All protocol steps referred to are from the Pro-Tox (C) version 1.5 manual (Xenometrix, 1996). First, the overnight bacterial cultures (all 16 strains) are prepared and placed in an incubator at 37°C with agitation at approximately 300 rpm. Pro-Tox (C) strain names and promoter fusion descriptions are given in Appendix E. After approximately 15 hours, the overnight cultures are subcultured in order to obtain log phase cultures. The overnight cultures were diluted 1:50 with 1X Pro-Tox Medium. The diluted cultures are incubated at 37°C with agitation at 300 rpm until an OD<sub>600</sub>

(absorbance at 600 nm in a spectrophotometer) reading between 0.3 to 0.4 is reached.

This takes approximately 3 hours. When the cultures begin to have OD<sub>600</sub> readings between 0.3 to 0.4, read the OD<sub>600</sub> of all the cultures. The cell densities are then adjusted so that all the strains are equal.

Stock solutions are prepared at 111% of the desired exposure concentration to allow the addition of bacteria. The stock solutions of the aqueous environmental samples are prepared in 10X Pro-Tox Medium.

Stock Conc.	0%	22%	33%	44%	56%	67%	78%	89%
Test Concentration	0%	20%	30%	40%	50%	60%	70%	80%
Amt. Test Sample (ml)	0.0	2.2	3.3	4.4	5.6	6.7	7.8	8.9
Amt. 10X Medium (ml)	3.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Amt. dH <sub>2</sub> O (ml)	27.0	6.8	5.7	4.6	3.4	2.3	1.2	0.1

**Table 2-1.** Stock and Test Concentrations of Filtrate Solutions.

The bacterial growth plates are then prepared as described by the Environmental Protocol (Appendix D). An OD<sub>600</sub> reading of both the slow and the fast plates is taken. Twenty-five µl of bacteria is transferred to each test well. Another OD<sub>600</sub> reading is taken of each plate. The plates are then incubated for 2 hours at 37°C with agitation at 300 rpm. The final OD<sub>600</sub> reading is taken at the end of the incubation period. The OD<sub>600</sub> readings provide the survival and growth information of the bacterial strains.

At this point, the procedure follows the standard procedure described in the Xenometrix manual Version 1.5(C). In a fume hood, chloroform is added to each well in columns 2 through 11 of a polypropylene microtiter plate. One chloroform plate is prepared for each bacterial plate. Solution from each well of the bacterial plates is

transferred to a chloroform plate. When the cells are added to the chloroform, they are lysed.

The  $\beta$ -galactosidase plates are prepared by adding Pro-Tox Buffer and ONPG to polystyrene 96-well plates. ONPG contains 4 mg/ml O-nitrophenyl- $\beta$ -D-galactoside in a buffer containing 16.1 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 5.5 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.75 g KCl, 0.246 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  in 1 L of sterile ddH<sub>2</sub>O, adjusted to pH 7. To begin the  $\beta$ -galactosidase assay, a portion of the upper aqueous phase of the chloroform plate is added to the  $\beta$ -galactosidase assay plate. Each plate is read at OD<sub>420</sub>. The slow plates are read immediately and then allowed to set for 30 minutes before being read at OD<sub>420</sub> again. The fast plates are allowed to set for only 10 minutes before repeating the OD<sub>420</sub> reading. The computer calculations use the final three 600 readings and both 420 readings to determine  $\beta$ -galactosidase activity. Equations used are as follows:

$$\text{Activity} = c [(\Delta\text{OD}_{420}) / (\text{lag time})(\Delta\text{OD}_{600})] \quad (2-1)$$

$c$  = constant for Miller units conversion

$$\Delta\text{OD}_{420} = [(\text{OD}_{420} \text{ Reading \#2}) - (\text{OD}_{420} \text{ Reading \#1})] \quad (2-2)$$

$$\Delta\text{OD}_{600} = [\text{Post-Exposure} - (\text{Post-Dose} - \text{Pre-Dose})] \quad (2-3)$$

## Statistical Methods

For the experiments evaluating storage effects, a factorial experiment was used to separately evaluate the first and second stage filtrates. Significance was determined at the

95% level. For the Microtox and XTT experiments evaluating the toxicity of chlorine, chlorine dioxide, and background filtrates and fractions, one sample (C<sub>H</sub>-2) was lost to a laboratory accident. This left the experimental block unbalanced. For the purpose of statistical analysis, the toxicity of the destroyed sample was estimated by the method of fitting constants. Significant factors were determined by use of a split-plot design analysis. Significance within factors was determined by using the q-test. Significance in all tests was determined at the 95% confidence level. Standard error was used to determine error bars for all figures.



## LITERATURE CITED - CHAPTER 2

Environmental Protection Series, Environment Canada, "Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*)," Report EPS 1/RM/24, November 1992.

Hach DR/3000 Spectrophotometer Manual, 1993.

"Hach COD System for Wastewater Testing", A Hach Company technical bulletin, 1993.

Microtox Manual, A Toxicity Testing Handbook, Microbics Corporation, 1992.

Ribo, J.M. and Kaiser, K.L.E., "Photobacterium Phosphoreum Toxicity Bioassay. I. Test Procedures and Applications," Toxicity Assessment: An International Quarterly, Vol. 2, 305-323 (1987).

Schwantes, T.A., "A Fundamental Characterization of Pulp Bleaching Effluents Produced Under Various Low-AOX Process Conditions," Ph.D. Thesis, Institute of Paper Science and Technology, 1994.

Schwantes, T.A., "A Fundamental Characterization of Pulp Bleaching Effluents Produced Under Various Low-AOX Conditions," Ph.D. Thesis Progress Report Number 5, Institute of Paper Science and Technology, 1992.

Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., and Boyd, M.R., "Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines," Cancer Research, **48**, 4827-4833 (1988).

Xenometrix; 2860 Wilderness Place; Boulder, Colorado 80301; 1-800-436-2869.

**CHAPTER 3 TERMINOLOGY**

<b>AOX</b>	absorbable organic halides; in pulp and paper industry generally refers to chlorinated organics
<b>B</b>	background bleaching (no bleaching chemical used in first stage)
<b>Cl<sub>2</sub></b>	chlorine
<b>ClO<sub>2</sub></b>	chlorine dioxide
<b>C<sub>L</sub></b>	chlorine bleaching, low charge (0.15 KF)
<b>C<sub>H</sub></b>	chlorine bleaching, high charge (0.25 KF)
<b>CV</b>	coefficient of variation = standard deviation/mean
<b>Debarking</b>	removal of bark from tree prior to chipping
<b>D<sub>L</sub></b>	chlorine dioxide bleaching, low charge (0.15 KF)
<b>D<sub>H</sub></b>	chlorine dioxide bleaching, high charge (0.25 KF)
<b>EC50</b>	the concentration that results in a 50% reduction of the response being measured
<b>EO</b>	second bleaching stage utilizing NaOH and oxygen
<b>Factorial Design</b>	statistical design which allows for evaluation of variables at different levels to determine significant variables
<b>KF</b>	kappa factor = total active chlorine, % / kappa #
<b>O<sub>3</sub></b>	ozone
<b>O.D.</b>	oven-dry weight
<b>TU</b>	toxicity units = 100/EC50

### **Chapter 3 THE EFFECT OF STORAGE CONDITIONS ON THE TOXICITY OF FILTRATES FROM CHLORINE, CHLORINE DIOXIDE, AND OZONE BLEACHING**

At the onset of this thesis, it was important to know under what storage conditions the generated filtrates should be stored to maintain initial toxicity. It was also important to identify how storage effects would affect filtrates from bleaching agents of interest: chlorine, chlorine dioxide, and ozone. Another factor of interest was the effect of chemical charge in the first stage, not only upon final toxicity, but how the storage effects could impact filtrates created under the various charges. When toxicity research is done in the pulp and paper industry, often there is little continuity in storage factors across researchers, which leads to concern about comparing results and the validity of the results.

In this study, each bleaching agent was evaluated with two chemical charges. Both chlorine and chlorine dioxide were utilized at kappa factors of 0.15 and 0.25. Ozone was applied at 0.4% and 1.2% (as O.D. pulp). Details of bleaching conditions for each bleach are given in Appendix A.

The following paper, titled “Comparative Toxicities of Oxygen, Ozone, Chlorine Dioxide, and Chlorine Pulp Bleaching Filtrates—Microtox Toxicities of Untreated Filtrates,” describes the background of Microtox usage, the methods and materials utilized, results, discussion of results, and conclusions. The factorial design and summary of results are given in Appendix F.

The implications of the results of this paper are widespread. Besides providing general information regarding the relative toxicities of filtrates from chlorine, chlorine dioxide, and ozone, this research also shows the significant difference or lack of differences caused by

varying the chemical charge in the first bleaching stage. This research also indicates that there is a significant contribution to toxicity from the process stages prior to the bleach plant or from the raw material. Finally, the paper emphasizes the importance of proper storage of filtrates prior to toxicity testing.

Paper #2 - Prepared for Submission to Environmental Science and Technology

**COMPARATIVE TOXICITIES OF OXYGEN, OZONE, CHLORINE DIOXIDE,  
AND CHLORINE PULP BLEACHING FILTRATES --MICROTOX TOXICITIES  
OF UNTREATED FILTRATES**

T.A. Ard and T.J. McDonough  
Institute of Paper Science and Technology  
Atlanta, GA

**ABSTRACT**

A laboratory study was conducted to comparatively evaluate the toxicities of filtrates from conventional and alternative wood pulp bleaching processes. Filtrates from ozone, chlorine dioxide, and chlorine bleaching were evaluated in comparison to control filtrates representing background toxicity. In each case, a sample of oxygen pre-delignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately. The persistence of their toxicities was also assessed by examining the effects of the duration, pH, and temperature of storage before testing.

For the first bleaching stage, averaging over all levels of these factors, the toxicities associated with the bleaching chemicals and charges could be ranked as follows:  $0.15 \text{ KF ClO}_2 > 1.2\% \text{ O}_3 > 0.25 \text{ KF Cl}_2 \geq \text{Background} \geq 0.25 \text{ KF ClO}_2 \geq 0.15 \text{ KF Cl}_2 \geq 0.4\% \text{ O}_3$ . The effects of all other factors were also found to be statistically significant.

For the second bleaching stage, storage factors were not found to significantly affect toxicity. However, the bleaching chemical and charge utilized in the first bleaching stage significantly affected the toxicity of the second bleaching stage filtrate. Averaging over all levels of storage factors, the toxicities associated with the bleaching chemicals and charges could be ranked as follows:  $\text{Background} > 1.2\% \text{ O}_3 \geq 0.15 \text{ KF ClO}_2 \geq 0.15 \text{ KF Cl}_2 \geq 0.25 \text{ KF Cl}_2 > 0.25 \text{ KF ClO}_2 > 0.4\% \text{ O}_3$ .

## INTRODUCTION

We have conducted a laboratory study of the toxicity of filtrates from chlorine-based and non-chlorine bleaching processes. In the course of this study, we have also examined two related issues.

The first is whether any toxicants generated during bleaching are rendered harmless by neutralization or storage. This is relevant because bleach plant filtrates are neutralized by mixing with filtrates of dissimilar pH before being discharged to the environment. What occurs during storage is also of interest because filtrates are not discharged as soon as they are generated, but are retained in the mill for periods ranging from a few hours to several days. A portion of this storage takes place within a secondary biological treatment system, the effect of which is not directly addressed in this paper. Nevertheless, any toxicity reduction that occurs simply as a result of storage would also occur in the treatment system.

The second issue is whether any toxicity observed in mill filtrates actually originates in the bleaching process, as opposed to being due to raw material components or compounds formed during the pulping step that precedes bleaching. This was investigated by conducting control experiments in which no bleaching agents were used but for which conditions and measurements were otherwise identical to those used in the bleaching experiments. Since all bleaching experiments employed oxygen stage filtrate carryover to simulate upstream washer inefficiency, these control experiments effectively

measured the toxicity contribution from oxygen bleaching and upstream sources, including the wood raw material.

The toxicity assay utilized for this study was the Microtox™ assay by Microbics Incorporated. The Microtox™ assay indirectly monitors the metabolic response of an ocean-dwelling bacterium to determine toxicity. *Vibrio fischeri lux* (previously referred to in the literature as *Photobacterium phosphoreum*) naturally emits visible light as a result of metabolic reactions which liberate energy.<sup>1</sup> When exposed to toxic compounds, the enzymes which promote this reaction are inhibited; the resulting decrease in light output is directly proportional to the toxicity of the sample. Light reduction is the direct measurement; EC50 is the concentration of the test sample which results in a 50% reduction in light output.

Many researchers have evaluated the comparative sensitivity of Microtox™ to that of other common aquatic assays, with conflicting conclusions. Qureshi *et al.* demonstrated that, for organic compounds, pulp and paper effluents, and oil refinery effluents, Microtox™ sensitivity was comparable to *Salmo gairdneri* (rainbow trout), *Spirillum volutans* (a bacterium), and *Daphnia magna* (a crustacean) bioassays.<sup>2</sup> Microtox™, in general, was found to be the most sensitive to industrial effluents; in some cases, Microtox™ was substantially more sensitive.

Firth and Backman found Microtox™ results to have good correlations with acute rainbow trout LC50 values and *Ceriodaphnia* chronic toxicity values for both treated and untreated pulp and paper mill wastewaters.<sup>3,4</sup> Microtox™ values were more sensitive than

rainbow trout mortality but less sensitive than *Ceriodaphnia* reproduction. However, Microtox™ has been found to be more sensitive than the *daphnid* chronic assay.<sup>5</sup> Jamieson reported a correlation study completed by Fraser comparing Microtox™ with Rainbow trout and *Daphnia magna* and reported that Microtox™ results correlated well with rainbow trout, but not with *Daphnia magna*.<sup>6</sup> Stauber *et al.* compared the Microtox™ toxicity of bleached eucalypt kraft mill effluents to a phytoplankton (*Nitzschia closterium*) growth inhibition test. The effluents exhibited greater toxicity to *Nitzschia* (1-20x more toxic) than Microtox™.<sup>7,8</sup>

Conversely, when Renberg evaluated data from both pulp mill and laboratory experiments, no correlation was found between Microtox™ toxicity and *Ceriodaphnia* or algal toxicity. On the other hand, Renberg did find that when the use of Microtox™ was limited to samples from similar sources, such as pulp mill effluents, the response agreed with that of *Ceriodaphnia*.<sup>9</sup>

Although no one assay can adequately evaluate the environmental impact of a test sample, Microtox™ has been shown to exhibit satisfactory sensitivity to pulp and paper process effluents and is a relatively quick, easy, and economical method for evaluating toxicity. No culture maintenance is necessary since the bacteria arrive freeze-dried and remain frozen until immediately prior to use. Another benefit of the Microtox™ assay is the small sample volume required for testing.



## EXPERIMENTAL METHODS

### Pulp Bleaching

Oxygen-delignified softwood kraft pulp and its associated filtrate were obtained from an operating pulp mill and further delignified in the laboratory with ozone (Z), chlorine dioxide (D), or chlorine (C) prior to a standard oxygen-reinforced caustic extraction (EO) stage. The initial kappa number (kappa no.  $\approx 6.7 \cdot \text{lignin, \%}$ ) of the softwood kraft pulp was 15.2. C and D stages were conducted at kappa factors (KF) 0, 0.15, and 0.25. The kappa factor is defined as follows:

$$\text{kappa factor} = \frac{\text{Equivalent Cl}_2 \text{ charge, \% dry pulp weight}}{\text{kappa number}} \quad (1)$$

The chlorine equivalency of  $\text{ClO}_2$  is given by the corresponding equivalent weight ratio (equivalent  $\text{Cl}_2 = \text{ClO}_2 \times 2.63$ ). Z stages were conducted in a CRS high-shear mixer at medium consistency with ozone charges of 0.44% and 1.2% (dry pulp basis).

All Z, D, C, and EO filtrates were collected at 3% consistency and were subjected to triplicate determinations of Microtox toxicity after being stored for either 24 hours or two weeks, with or without neutralization, at either 4°C or 20°C, providing a complete factorial experimental design. The resulting data were expressed as toxicity units ( $\text{TU} = 100/\text{EC}_{50}$ ), where  $\text{EC}_{50}$  is the concentration that results in a 50% reduction in light output by the test organisms. The data were subjected to analysis of variance.

After the first bleaching stage, the filtrate was collected and the pulp washed thoroughly. The pulp was then subjected to a standard oxygen-enhanced sodium hydroxide (EO) extraction. Again, the filtrates were collected. In addition to evaluating the above-mentioned bleaching agents, a control experiment was used to evaluate whether any toxicity observed in mill filtrates actually originates in the bleaching process, as opposed to being due to raw material components or compounds formed during the pulping step that precedes bleaching. The “background” (control) experiment was identical to the chlorine and chlorine dioxide experiments except distilled water was used in the first stage.

### **Storage Conditions**

Filtrates were stored for either 24 hours or two weeks prior to toxicity testing. All filtrates were stored in the dark. Storage temperature was either 4°C or 20°C. The pH of the filtrates was either allowed to remain at the pH resulting from the bleaching stage or adjusted to nearly neutral pH. Acidic filtrates were adjusted with sodium hydroxide, and alkaline filtrates were adjusted with sulfuric acid to a pH of 7.0 +/- 1.0 pH unit. Regardless of storage pH, all filtrates were pH adjusted prior to toxicity evaluation.

### **Microtox™ Evaluation**

Samples were maintained at their designated storage conditions until immediately prior to toxicity testing. Microtox™ testing was in all cases conducted at nearly neutral pH. The pH of samples, which had not previously been neutralized, was adjusted to a

value of 7.0 +/- 1.0 with either sodium hydroxide or sulfuric acid. The Microtox™ 100% test protocol was used to evaluate the toxicity of the samples. If the initial testing showed an EC50 level greater than 9.1 TU, then the Basic test protocol was utilized. The Basic and 100% test protocols differ in both accuracy and test concentrations. While the Basic test is more accurate, it only allows for testing filtrate concentrations from 5.5 to 45%. On the other hand, the 100% test allows for evaluating filtrate concentrations from 11 to 90%. With proper operator pipetting techniques, the accuracy of the 100% test approaches that of the Basic test.

Others have used the Basic protocol to evaluate the entire range of toxicities when testing bleaching filtrates.<sup>10</sup> This forces an extensive and inappropriate extrapolation for filtrates with EC50s close to 100%.

## **Data Analysis**

Samples were evaluated by Microtox™ in triplicate. Although both 5-minute and 15-minute EC50s were recorded, 15-minute EC50s were used for final statistical analysis in order to facilitate comparisons with data obtained by other researchers.

The detection limit of the test was determined to be  $EC_{50} = 90\%$  or  $TU = 1.111$  since this was the highest concentration tested. Toxicity unit (TU) measurements lower than this were replaced by  $TU = 0.556$  (one-half the detection limit). The data were analyzed in logarithmic form to satisfy the requirement for constant variance.

The analysis model used was a  $7 \times 2^4$  factorial design. The seven “levels” of the bleaching chemical factor were background, 0.15 KF chlorine, 0.25 KF chlorine, 0.15 KF chlorine dioxide, 0.25 KF chlorine dioxide, 0.4% ozone, and 1.2% ozone. The three storage factors evaluated were time (length of storage), pH during storage, and temperature during storage. The confidence level used for determination of significance was 95%. The q-test, at 95% significance level, was used to determine differences within significant factors. Standard error was used to determine the values of all error bars.

## **RESULTS AND DISCUSSION**

### **Comparisons of Bleaching Filtrates**

The bleaching filtrates were stored under various storage conditions as shown in Tables 1a-1c. To arrive at overall toxicity rankings for the filtrates, the toxicity data were averaged over all levels of the other variables. For filtrates from the first bleaching stage, this gave the following ranking (in order of decreasing toxicity):  $0.15 \text{ KF ClO}_2 > 1.2\% \text{ O}_3 > 0.25 \text{ KF Cl}_2 \geq \text{Background} \geq 0.25 \text{ KF ClO}_2 \geq 0.15 \text{ KF Cl}_2 \geq 0.4\% \text{ O}_3$ . For filtrates from the second bleaching stage, the toxicity was determined solely by the bleaching agent and charge used in the first stage; storage factors had no significant effect on second stage toxicity. The toxicity rankings for the (EO) stage are as follows (in order of decreasing toxicity):  $\text{Background} > 1.2\% \text{ O}_3 \geq 0.15 \text{ KF ClO}_2 \geq 0.15 \text{ KF Cl}_2 \geq 0.25 \text{ KF Cl}_2 > 0.25 \text{ KF ClO}_2 > 0.4\% \text{ O}_3$ . These results are illustrated in Figures 1 and 2.

Several pertinent observations may be made. The use of chlorine in bleaching does not result in the most toxic filtrates. A possible explanation is that the chlorinated

compounds formed during bleaching are not primarily responsible for toxicity. Likewise, the background filtrates are not the least toxic filtrates. In fact, background second stage filtrates have the highest toxicity. This observation indicates that the toxicity may be primarily due to wood raw material or the pulping process prior to bleaching. It is possible that bleaching agents reduce toxicity by “neutralizing” toxic compounds by either destroying the compound or by substituting onto the structure. Either of these reasons help to explain the next finding.

Although it has been well-proven that AOX is not related to aquatic toxicity, it might be hypothesized that since AOX increases with increasing chlorine charge that toxicity would also increase with increasing chlorine charge. However, the data presented here show that there is clearly no significant difference in filtrate toxicity between filtrates prepared under 0.15 KF or 0.25 KF chlorine bleaching conditions.

Since the use of chlorine dioxide resulted in decreased toxicity with increasing applied chemical charge, it could be postulated that this bleaching agent reduces toxicity by “neutralizing” toxic compounds, either by destroying the compound or by substituting onto the main structure.

According to these results, since background toxicity is significant, it could be suggested that removing chlorine and chlorine dioxide from the bleach plant and replacing with a non-chlorine containing bleaching agent may not be warranted. An examination of the toxicity of compounds naturally found in the pulp mill wood source as well as the formation of toxic compounds in pulping processes is warranted. If necessary,

release of compounds from the wood source or the pulping process can be prevented through dry debarking and spill control technologies.

### **Evaluation of Additional Factors**

Length of storage, pH of filtrate, and temperature during storage had statistically significant effects on first stage filtrate toxicity. No interactions between storage factors were found to be significant. Results are illustrated in Figure 3. ANOVA results for first stage filtrates is given in Table 2. Storage factors did not significantly affect second stage filtrate toxicity as shown in Table 3.

The average toxicity for filtrates stored for two weeks is 34% of the toxicity of filtrates tested 24 hours after bleaching. This gives an indication of the ephemeral nature of the toxic constituents in the filtrates. It may be assumed that the toxic constituents are denatured during the storage period and, given time, decrease in toxicity.

The pH of the filtrate during storage either remained at the pH of the bleaching stage (approximately pH 2 for the first stage) or the filtrate was neutralized prior to storage. Figure 3 illustrates the 59% decrease in toxicity achieved by neutralizing the filtrates prior to storage. It should be noted that all filtrates were neutralized immediately prior to toxicity testing; the decrease in toxicity achieved by neutralization is not an artifact of the toxicity assay's requirement for a neutral pH.

Temperature during storage was either 20°C or 4°C. A decrease of 65% was observed when the filtrates were stored at room temperature (20°C) rather than in a cold

room (4°C). A possible explanation is that either bacterial degradation or chemical degradation would be increased at higher temperatures which favor such reactions.

The first and second bleaching stages were evaluated separately for toxicity. First stage filtrates, on average, are 47% the toxicity of filtrates from the second bleaching stage. However, it is unlikely that the toxic compounds are formed in the extraction stage. More likely, compounds present in the first bleaching stage (whether naturally present or generated during the bleaching stage) may have difficulty escaping the fiber matrix. Perhaps only when the lignin is solubilized from the fiber matrix during the second stage can these compounds dissolve into the filtrate. Another explanation for the higher toxicity in the (EO) stage would be that the lignin liberated during the second stage is a major source of toxicity.

## CONCLUSIONS

Several conclusions may be drawn from the data presented above, which are summarized as follows:

1. There is a background level of toxicity which originates in the oxygen stage, process steps prior to bleaching, or in the wood raw material. It is decreased by neutralization and storage, but residual toxicity is still detected after two weeks. More complete removal may be achievable in biological treatment systems, which were not investigated in this study.

2. The toxicity of filtrates from the chlorine sequence is insensitive to  $\text{Cl}_2$  charge; the use of chlorine does not result in the most toxic filtrates.
3. When evaluating the average toxicity of filtrates from chlorine dioxide bleaching sequences, the higher the chemical charge in the first stage, the lower the toxicity.
4. Temperature during storage, pH of filtrate, length of storage, and bleaching stage are all statistically significant factors affecting first stage filtrate toxicity. No significant interactions between factors were observed.
5. Storage factors did not significantly reduce the toxicity of second stage filtrates. The compounds released in the second stage are not of an ephemeral nature.
6. This study evaluated the toxicity of conventional and alternative bleaching filtrates and the effect of storage conditions, but did not evaluate the impact of biotreatment on toxicity or the biotreatability of these filtrates. Further research needs to be conducted to evaluate the effect of biological treatment on toxicity of conventional and alternative bleaching filtrates.

#### **ACKNOWLEDGMENTS**

The authors thank Dr. Lucy Sonnenberg and Dr. Mary DeLong for technical advice and guidance, and the Institute of Paper Science and Technology and its Member Companies for the financial support provided for student research. Portions of this work were used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.



factor s	pH during storage	length of storage	temp. during storage	back- ground	0.15 KF chlorine	0.25 KF chlorine	0.15 KF chlorine dioxide	0.25 KF chlorine dioxide	0.4% ozone	1.2% ozone
(1)	-	-	-	0.33	-0.01	0.29	0.42	0.17	0.09	0.93
a	+	-	-	0.26	0.21	0.26	0.82	0.16	0.48	0.41
b	-	+	-	-0.15	-0.20	0.24	0.33	0.19	-0.25	0.36
ab	+	+	-	0.63	0.15	0.17	0.98	0.01	-0.25	0.51
c	-	-	+	0.23	0.15	0.29	0.24	0.07	-0.25	0.38
ac	+	-	+	0.20	-0.15	0.17	0.17	0.29	0.34	0.63
bc	-	+	+	-0.25	0.11	-0.03	-0.05	-0.15	-0.25	-0.25
abc	+	+	+	-0.09	0.15	0.11	0.70	-0.14	0.14	-0.15

**Table 1a.** Factorial Design for the Evaluation of Storage Effects on First Stage Filtrates from Chlorine, Chlorine Dioxide, and Ozone Bleaching. Data reported as log TU.

factor s	pH during storage	length of storage	temp. during storage	back- ground	0.15 KF chlorine	0.25 KF chlorine	0.15 KF chlorine dioxide	0.25 KF chlorine dioxide	0.4% ozone	1.2% ozone
(1)	-	-	-	0.68	0.32	0.32	0.53	0.35	0.32	0.79
a	+	-	-	0.86	0.45	0.56	0.66	0.27	0.36	0.21
b	-	+	-	0.97	0.45	0.34	0.26	0.25	-0.02	0.49
ab	+	+	-	1.08	0.42	0.36	0.85	0.01	0.10	0.71
c	-	-	+	0.79	0.31	0.48	0.34	0.24	0.04	0.51
ac	+	-	+	0.83	0.48	0.25	0.49	0.18	0.21	1.01
bc	-	+	+	-0.25	0.54	0.57	0.05	-0.15	-0.25	0.11
abc	+	+	+	0.80	0.36	0.17	0.55	0	-0.15	0

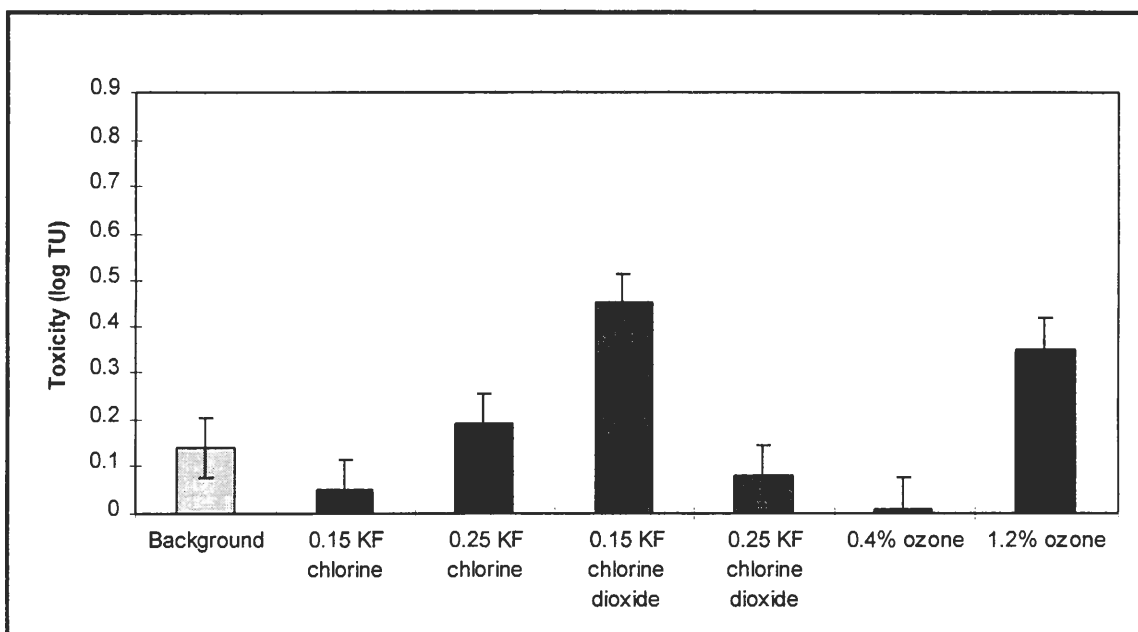
**Table 1b.** Factorial Design for the Evaluation of Storage Effects on Second Stage (EO) Filtrates from Chlorine, Chlorine Dioxide, and Ozone Bleaching. Data reported as log TU.

variable	-	+
pH during storage	neutralized	bleaching stage end pH
length of storage (days)	1	14
temperature during storage (°C)	4	20

**Table 1c.** Levels of Factors Used in Factorial Design

<u>Source</u>	<u>DF</u>	<u>Sum-Squares</u>	<u>Mean Square</u>	<u>F-Ratio</u>	<u>Prob&gt;F</u>
pH during storage	1	0.314	0.314	9.47	0.022 *
length of storage	1	0.449	0.449	13.53	0.010 *
temp. during storage	1	0.433	0.433	13.05	0.011 *
bleaching chemical/charge	6	1.297	.216	6.51	0.019 *

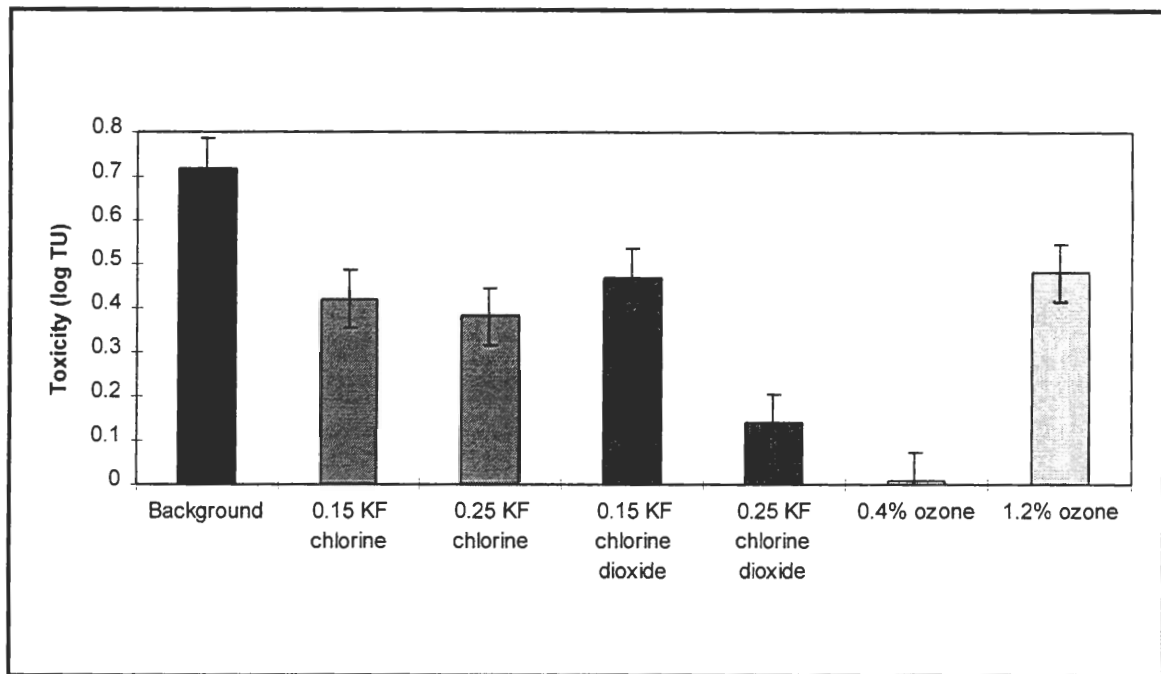
**Table 2.** ANOVA Table of Storage and Chemical/Charge Variables for First Stage Filtrates. \* signifies significance at the 95% confidence level



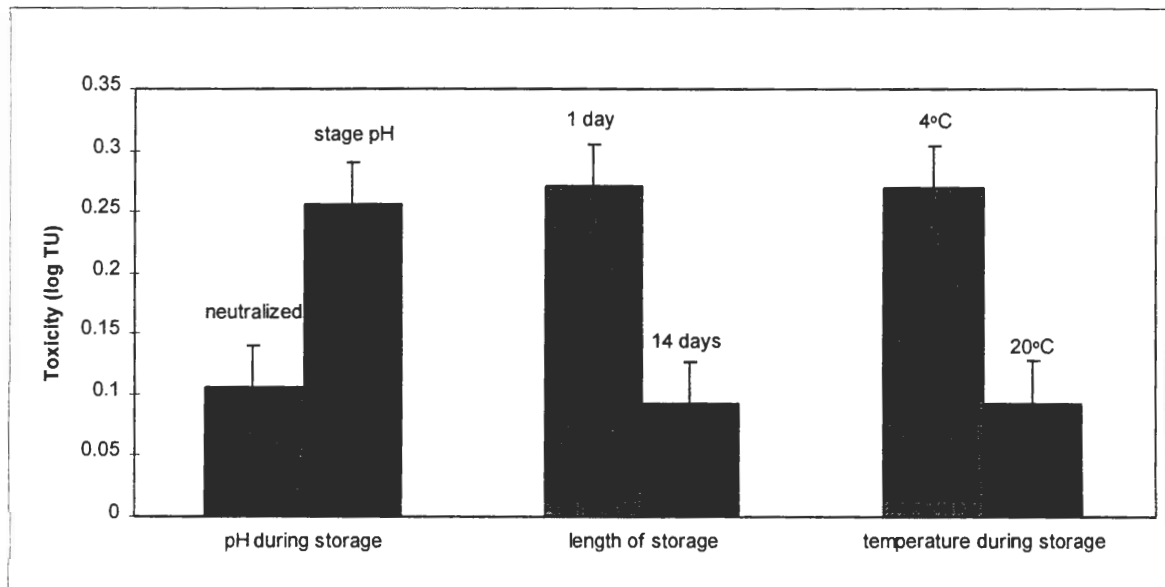
**Figure 1.** Average Toxicity of First Stage Bleaching Filtrates. (Toxicity Expressed as log TU. TU=100/EC50(%).)

<u>Source</u>	<u>DF</u>	<u>Sum-Squares</u>	<u>Mean Square</u>	<u>F-Ratio</u>	<u>Prob&gt;F</u>
pH during storage	1	0.131	0.131	1.87	0.220
length of storage	1	0.319	0.319	4.57	0.076
temp. during storage	1	0.351	0.351	5.04	0.066
bleaching chemical/charge	6	2.257	0.376	5.39	0.030 *

**Table 3.** ANOVA Table of Storage and Chemical/Charge Variables for Second Stage Filtrates. \* signifies significance at the 95% confidence level



**Figure 2.** Average Toxicity of Second Stage Bleaching Filtrates.



**Figure 3.** Average Toxicity Effect of Storage Factors on First Stage Bleaching Filtrates.

---

## LITERATURE CITED

1. Ribo, J.M.; Kaiser, K.L.E. *Toxicity Assessment: An International Quarterly* **1987**, 2, 305.
2. Qureshi, A.A.; Flood, K.W.; Thompson, S.R.; Janhurst, S.M.; Inniss, C.S.; Rokosh, D.A. *Aquatic Toxicology and Hazard Assessment: Fifth Conference, ASTM STP 766*; Pearson, J.G., Foster, R.B., and Bishop, W.E., eds., American Society for Testing and Materials, 1982; pp. 179-195.
3. Firth, B.K.; Backman, C.J. *Tappi Journal*, **1990**, 73(12), 169.
4. Firth, B.K.; Backman, C.J. *1990 Environmental Conference Proceedings*; TAPPI Press: Atlanta, GA, 1990; pp. 621-626.
5. Suckling, I.D.; Pasco, M.F.; Gifford, J.S. *1993 Environmental Conference Proceedings*; TAPPI Press: Atlanta, GA, 1993; pp. 449-456.
6. Jamieson, S. *Pulp & Paper Canada* **1992**, 93(2), 9.
7. Stauber, J. *Appita Annual General Conference Proceedings*, Vol. 2; Appita: Australia, 1993; pp. 789-796.
8. Stauber, J.; Gunthorpe, L.; Deavin, J.; Munday, B.; Ahsanullah, M. *48th Appita General Conference*; Appita: Australia, 1994; pp. 511-518.
9. Renberg, L. *1992 Environmental Conference Proceedings*; TAPPI Press: Atlanta, GA, 1992; pp. 317-329.
10. Graves, J.W.; Joyce, T.W., Jameel, H. *Tappi Journal*, **1993**, 76(7), 153.

**CHAPTER 4 TERMINOLOGY**

<b>AOX</b>	absorbable organic halides; in pulp and paper industry, generally refers to chlorinated organics
<b>B</b>	background bleaching (no bleaching chemical used in first stage)
<b>Biotreatment</b>	commonly referred to as secondary treatment; treatment with microbes to reduce environmental and other variables prior to effluent release
<b>Cl<sub>2</sub></b>	chlorine
<b>ClO<sub>2</sub></b>	chlorine dioxide
<b>C<sub>L</sub></b>	chlorine bleaching, low charge (0.15 KF)
<b>C<sub>H</sub></b>	chlorine bleaching, high charge (0.25 KF)
<b>CV</b>	coefficient of variation = standard deviation/mean
<b>Debarking</b>	removal of bark from tree prior to chipping
<b>D<sub>L</sub></b>	chlorine dioxide bleaching, low charge (0.15 KF)
<b>D<sub>H</sub></b>	chlorine dioxide bleaching, high charge (0.25 KF)
<b>DNA</b>	deoxyribonucleic acid; double stranded combination of four bases, which carries genetic information
<b>DNA Damage</b>	break or rearrangement in DNA strand
<b>EC50</b>	the concentration that results in a 50% reduction of the response being measured
<b>EE</b>	ether-extractable fraction
<b>Elongation</b>	second step of RNA conversion to protein; stepwise formation of peptide bonds
<b>EO</b>	second bleaching stage utilizing NaOH and oxygen
<b>Factorial Design</b>	statistical design which allows for evaluation of variables at different levels to determine significant variables

<b>HepG2</b>	human liver cell line used in this research obtained from the American Type Culture Collection (Rockville, Maryland)
<b>Initiation</b>	the first step in RNA conversion to protein
<b>KF</b>	kappa factor = total active chlorine, %/ kappa #
<b>Microtox</b>	assay utilizing the light output of <i>Vibrio fischeri lux</i> , a marine organism
<b>mRNA</b>	messenger RNA; involved in second step of protein translation; an RNA molecule synthesized from a DNA template by the enzyme RNA polymerase; the mRNA sequence is complementary to the DNA sequence <sup>i</sup>
<b>NEE</b>	non-ether extractable fraction
<b>NVW</b>	non-volatile whole
<b>O<sub>3</sub></b>	ozone
<b>O.D.</b>	oven-dry weight
<b>Operon</b>	a group of functionally related genes regulated and transcribed as a unit <sup>i</sup>
<b>Osmotic Pressure</b>	the external pressure sufficient to oppose osmosis and stop it
<b>Oxidative Damage</b>	damage caused by oxidative-reduction reactions
<b>Oxidative Extraction</b>	extraction of pulp with NaOH and oxygen (EO)
<b>Oxygen Delignified Pulp</b>	pulp exposed to oxygen after the pulping stage
<b>Oxygen Stage Carryover</b>	liquid phase accompanying the oxygen delignified pulp
<b>Promoter</b>	a nucleotide sequence found upstream of a gene that acts as a signal for the binding of RNA polymerase <sup>i</sup>
<b>Ribosome</b>	a particle comprising RNA and protein found in all cells; each cell contains many thousand ribosomes; the site of protein synthesis in which the code in a molecule of messenger of mRNA is translated into a protein sequence <sup>i</sup>

<b>RNA</b>	ribonucleic acid; involved in replication of DNA; encodes information for synthesis of a protein and replication
<b>Secondary Treatment</b>	biological treatment to reduce environmental and other effluent parameters prior to release
<b>Stress Genes</b>	genes that become active under biological or environmental stress; in this thesis, it specifically applies to genes that have been modified with stress promoters, which increase the production of RNA when a particular stress is experienced
<b>Structural Genes</b>	nucleotide sequences coding for any RNA or protein product other than a regulator <sup>ii</sup>
<b>Termination</b>	the final step of RNA conversion to protein; the completed protein is released from the ribosome
<b>Transcription</b>	the process whereby a molecule of RNA is synthesized by the enzyme RNA polymerase using DNA as a template; the process involves complementary base-pairing; the genetic information encoded in the DNA is transferred to an RNA molecule, which can diffuse into the cytoplasm; the encoded message in the RNA can then be used to direct the synthesis of the protein by the process of translation, which occurs at the ribosome <sup>i</sup>
<b>XTT</b>	sodium 3,3'-[1[(phenylamino) carbonyl] 3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate)
<b>W</b>	whole filtrates (combined 1 <sup>st</sup> and 2 <sup>nd</sup> stages, volatiles not removed)

<sup>i</sup> Walker, J.M. and Cox, M., The Language of Biotechnology. A Dictionary of Terms, Second Edition, American Chemical Society, Washington, D.C., 1995.

<sup>ii</sup> Lewin, B., Genes IV, Oxford University Press, Cambridge, 1990.

## **Chapter 4 COMPARATIVE TOXICITIES OF FILTRATES FROM CHLORINE AND CHLORINE DIOXIDE BLEACHING**

All of the research discussed in this chapter utilized the same filtrates evaluated with various toxicity assays. Once the filtrates were produced, individual stage filtrates were pH adjusted to 7.4 +/- 0.05, filtered with a 0.22  $\mu\text{m}$  filter for sterilization, and then frozen at  $-70^{\circ}\text{C}$ . For the combined filtrates, equal amounts of first and second stage filtrates were added together and mixed well. Four liters of combined filtrate were added to the ether extractor and the extractor started. Another sample of the combined filtrate was pH adjusted, filtered, and frozen at  $-70^{\circ}\text{C}$ . A final sample of combined filtrate was vacuum evaporated for 24 hours prior to pH adjustment, filtering, and freezing. After four days, the ether extraction was terminated. The two fractions, ether extractable (EE) and non-ether extractable (NEE) were each vacuum evaporated for 24 hours prior to pH adjustment, filtering, and freezing. This was repeated for all ten bleaches - a background bleach, 0.15 KF chlorine dioxide, 0.25 KF chlorine dioxide, 0.15 KF chlorine, and 0.25 KF chlorine, and a second replication of all of the bleaches listed.

Four papers are presented in this chapter. The first discusses the results obtained from testing these filtrates with the Microtox assay. The second paper discusses the results obtained from testing the filtrates with a tetrazolium assay on HepG2 (human liver cells). The third paper discusses a stress gene assay system that was used to evaluate the combined whole filtrates, and the final paper summarizes the previous papers and discusses and analyzes the differences in assay results and their potential meanings.



The first paper, entitled, “Microtox Toxicities of Pulp Bleaching Filtrates—Evaluating Both Biotreatment and Bioaccumulable Fractions,” discusses the results obtained by testing the filtrates with the Microtox™ assay. Raw data compilation is given in Appendix G, while graphical representation of results can be found in Appendix H. The Microtox™ assay is a pulp and paper industry-accepted assay that is widely used for preliminary analysis of effluents prior to more extensive (and expensive) testing using such organisms as *Ceriodaphnia* or fathead minnows.

While evaluating the data presented in the following paper, it may be helpful to note the Microtox™ toxicities of common solutions presented in Table 4-1.

COMMON SOLUTION	MICROTOX TOXICITY (TU)
Diet Cola	1.3
Beer	6.2
Wine	3.2
Atlanta Tap Water	4.1

**Table 4-1.** Microtox™ Toxicities of Common Solutions.

Paper #3 - Prepared for Submission to Water Research

**MICROTOX TOXICITIES OF PULP BLEACHING FILTRATES -  
EVALUATING BOTH BIOTREATMENT AND BIOACCUMULABLE FRACTIONS**

T.A. Ard and T.J. McDonough  
Institute of Paper Science and Technology  
Atlanta, GA

**ABSTRACT**

A laboratory study was conducted to comparatively evaluate the toxicities of chlorine and chlorine dioxide bleaching filtrates with the Microtox™ assay. These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen pre-delignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. The combined filtrates were then subjected to an ether extraction in order to fractionate the whole filtrate into ether-extractable fractions and non-ether extractable fractions. The ether-extractable fraction is considered to be of the most environmental relevance. The toxicities of the whole filtrates and fractions were determined by using the Microtox™ toxicity assay.

Both charges of chlorine resulted in significantly more highly toxic filtrates than either chlorine dioxide or background filtrates. The background filtrates were not significantly different from either chlorine dioxide filtrate. Future research should examine the source of the toxicity of the background filtrate, which may also explain

chlorine dioxide filtrate toxicity and remove concerns regarding the use of this chemical for pulp bleaching purposes.

Filtrates from the first bleaching stage were significantly higher in toxicity than filtrates from the second bleaching stage and the whole combined filtrate. The whole, combined filtrates were approximately equal in toxicity to the average of the first and second stages, indicating no synergistic effects for filtrates for all bleaching agents except high chemical charge of chlorine. If the toxicities of the ether-extractable (EE) fractions and the non-ether extractable (NEE) fractions of the whole filtrates are compared to the non-volatile whole (NVW) toxicity, it appears that there are additive effects for the background filtrates. Both chlorine dioxide charges show synergistic effects. The low chlorine charge filtrates may show a slight synergistic effect. The high chlorine charge shows a definite antagonistic effect.

The use of biotreatment in this study was not effective in reducing Microtox™ toxicity. However, this is not necessarily indicative of the treatability of the filtrates for Microtox™ toxicity since another type of treatment system or bacteria used could potentially be successful in reducing Microtox™ toxicity.

## **KEY WORDS**

bleaching, filtrates, biotreatment, dioxide, chlorine, bioaccumulation, toxicity, Microtox

## INTRODUCTION

Environmental concerns are causing the pulp and paper industry to investigate alternatives to conventional bleaching sequences, which have generally utilized chlorine in the first bleaching stage. Little research has gone into quantifying the toxicity reduction achieved when alternative bleaching agents are substituted for chlorine.

Regulatory restrictions on effluent AOX are having a substantial impact on the use of chlorine and chlorine-containing compounds in the pulp and paper industry. Replacement of chlorine with chlorine dioxide results in a significant decrease in the amount of AOX produced [Cox (1989); Liebergott *et al.* (1990)]. However, there appears to be little relationship between AOX and toxicity [Borton (1990); Owens (1990)]. Neither has any correlation been found between toxicity and BOD or TOC [Walden (1976); Walden and Howard (1977)]. A relationship has been found between toxicity and the concentration of resin acids present [Priha and Talka (1986)].

Despite the fact that chlorine-containing organic molecules are only found in low concentrations in bleach effluents, emphasis has been placed on these compounds due to their non-polar nature and lipid-soluble character, which may lead to bioaccumulation in marine organisms. The octanol/water partition coefficient ( $K_{ow}$ ) is often used to predict the ability of a compound to bioaccumulate. However, when it is desired to evaluate the bioaccumulation potential of solutions of unknown organic content, such as bleach plant filtrates, it is more practical to use ether extractions to separate compounds by polarity.

For this study, the Microtox™ bioassay was chosen to evaluate the toxicity of the samples. The Microtox™ assay, marketed by Microbics Incorporated, indirectly monitors

the metabolic response of an ocean-dwelling bacterium to determine toxicity. *Vibrio fischeri lux* (previously referred to in the literature as *Photobacterium phosphoreum*) naturally emits visible light as a result of metabolic reactions which liberate energy [Ribo and Kaiser (1987)]. When exposed to toxic compounds, the enzymes which promote this reaction are inhibited; the resulting decrease in light output is directly proportional to the toxicity of the sample. Light reduction is the direct measurement; EC50 is the concentration of the test sample which results in a 50% reduction in light output.

Many researchers have evaluated the comparative sensitivity of Microtox™ to that of other common aquatic assays, with conflicting conclusions. Qureshi *et al.* demonstrated that for organic compounds, pulp and paper effluents, and oil refinery effluents, Microtox™ sensitivity was comparable to *Salmo gairdneri* (rainbow trout), *Spirillum volutans* (a bacterium), and *Daphnia magna* (a crustacean) bioassays [Qureshi *et al.* (1982)]. Microtox™, in general, was found to be the most sensitive to industrial effluents; in some cases, Microtox™ was substantially more sensitive.

Firth and Backman found Microtox™ to have good correlations with acute rainbow trout LC50 values and *Ceriodaphnia* chronic toxicity values for both treated and untreated pulp and paper mill wastewaters [Firth and Backman (1990a); Firth and Backman (1990b)]. Microtox™ values were more sensitive than rainbow trout mortality but less sensitive than *Ceriodaphnia* reproduction. However, in another study, Microtox™ was found to be more sensitive than the *daphnid* chronic assay [Suckling *et al.* (1993)]. Fraser completed a correlation study comparing Microtox™ with Rainbow

trout and *Daphnia magna*. Results indicate that Microtox™ correlates well with rainbow trout, but not with *Daphnia magna* [Jamieson (1992)]. Stauber *et al.* compared the Microtox™ toxicity of bleached eucalypt kraft mill effluents to a phytoplankton (*Nitzschia closterium*) growth inhibition test. The effluents exhibited greater toxicity to *Nitzschia* (1-20x more toxic) than Microtox™ [Stauber (1993); Stauber *et al.* (1994)].

When Renberg evaluated data from both mill and laboratory experiments, no correlation was found between Microtox™ toxicity and *Ceriodaphnia* or algal toxicity. On the other hand, Renberg did find that when the use of Microtox™ was limited to samples from similar sources, such as pulp mill effluents, the response agreed with that of *Ceriodaphnia* [Renberg (1992)].

Microtox™ has been shown to exhibit satisfactory sensitivity to pulp and paper process effluents and is a relatively quick, easy, and economical method for evaluating toxicity. No culture maintenance is necessary since the bacteria arrive freeze-dried and remain frozen until immediately prior to use. Another benefit of the Microtox™ assay is the small sample volume required for testing.

## **MATERIALS AND METHODS**

### **Bleaching and Filtrate Collection**

In bleaching kraft pulp, 45-90 kg of organic material is dissolved per metric ton of pulp. Of the dissolved organic material, 75-90% is usually produced during the first two

bleaching stages [Annergren *et al.* (1986)]. For this reason, only the filtrates from the first oxidative bleaching stage and the first extraction stage were tested. All filtrates tested were generated in the Institute of Paper Science and Technology bleaching facilities.

Oxygen delignified pulp was obtained from a commercial facility immediately following the washing stage. A sample of the liquid phase accompanying the oxygen delignified pulp (“oxygen stage carryover”) was obtained from the oxygen washer simultaneously. In the laboratory, the pulp was repeatedly washed and centrifuged until no color remained in the wash water. The pulp was then centrifuged to approximately 35% consistency (consistency = weight of pulp/weight of pulp and liquid, expressed as a percent), fluffed, and stored at 4°C. In preparation for bleaching, 300 g pulp, enough sulfuric acid to reduce the pulp solution to pH 1.8-2.0, oxygen carryover, and dilution water to make the solution 3% consistency were mixed in the reactor. The amount of oxygen carryover required was determined by assuming a well-washed system, achieving only 10 kg COD carryover per ton of pulp. Distilled water, preheated to 70°C, was used as dilution water. Chlorine and chlorine dioxide solution concentrations were determined immediately prior to bleaching using iodometric titration. For the “background” bleaches, distilled water was substituted for bleaching chemical.

The initial kappa number (kappa no.  $\approx 6.7 \times \text{lignin, \%}$ ) of the softwood kraft pulp was 17.2. Chlorine (C) and chlorine dioxide (D) stages were conducted at kappa factors of 0.15 and 0.25. The kappa factor is defined as follows:

$$\text{kappa factor} = \frac{\text{Equivalent Cl}_2 \text{ charge, \% dry pulp weight}}{\text{kappa number}}$$

The chlorine equivalency of  $\text{ClO}_2$  is given by the corresponding equivalent weight ratio (equivalent  $\text{Cl}_2 = \text{ClO}_2 \times 2.63$ ).

Immediately following the first bleaching stage, 30 minutes in duration, the pulp and filtrate were separated by filtration. The filtrate was collected for later testing. The pulp was then washed well with distilled water.

The second bleaching stage, an oxidative extraction (EO), was completed at 10% consistency. The pulp slurry was heated to  $70^\circ\text{C}$  prior to adding sodium hydroxide and oxygen. When the 70 minute bleaching time was complete, the pulp suspension was diluted with cool water to 3% consistency and then mixed. The pulp and filtrate were separated by filtration and the filtrate collected for testing, while the pulp was again washed well with distilled water. A kappa number test was used to determine the extent of lignin removal.

Once the filtrates were produced, individual stage filtrates were pH adjusted to  $7.4 \pm 0.05$ , filtered with a  $0.22 \mu\text{m}$  filter for sterilization, and then frozen at  $-70^\circ\text{C}$ . For the combined filtrates, equal amounts of first and second stage filtrates were added together, mixed well, and acidified to pH 2. Four liters of combined filtrate were added to the ether extractor and the extractor started. Another sample of the combined filtrate was pH adjusted, filtered, and frozen at  $-70^\circ\text{C}$ . A final sample of combined filtrate was vacuum evaporated for 24 hours prior to pH adjustment, filtering, and freezing.



## Ether Extractions

Continuous ether extractions were performed in 4 L glass extractors. Four liters of combined (first and second stage) filtrates were added to the extractor and connected to a 1 L round bottom flask containing 500 mls ether. Extraction with refluxing ether was continued for four days. A rotary evaporator, under vacuum, was used for 24 hours to remove the ether from both the ether-extract and the extracted filtrate.

All of the ether-extract (EE) was evaporated to dryness and then reconstituted with filtered double deionized water to 250 mls. There were four liters of extracted filtrate following the ether fractionation procedure; however, it required another two liters of double deionized water,  $\text{H}_2\text{SO}_4$ , and NaOH to completely remove the extracted filtrate from the extractor. An aliquot of 500 mls of extracted filtrate, now referred to as non-ether extractable (NEE) fraction was evaporated for 24 hours and then reconstituted to 500 mls. The remainder (5500 mls) of the extracted filtrate and wash solution was disposed of properly.

In order to accurately compare the toxicities of the whole filtrates to the fractions, the measured toxicities had to be recalculated to account for the various dilutions and concentrations. The 250 mls of the EE fraction actually represent the compounds from 4L of filtrate; likewise, the 500 mls of NEE fraction were taken from a diluted source (6L) of 4L of filtrate. Therefore, the toxicity of the EE fraction was multiplied by 16 to put the toxicity on an equivalent basis with the whole filtrate. The toxicity of the NEE fraction was multiplied by (4/6) in order to put the NEE toxicity on an equivalent basis

with the whole filtrate. All toxicity numbers presented in the paper have already been placed on an equivalent basis.

### **Microtox™**

Microtox™ is a trademark of Microbics Corporation. The assay uses photoluminescent bacteria (*Vibrio fischeri lux*) as the test organism. The bacterium strain NRRL B-1117 is deposited with the Northern Regional Laboratory in Peoria, Illinois, USA [Environment Canada (1992)]. This strain of bacteria diverts approximately ten percent of its metabolic energy into a metabolic pathway that converts chemical energy into visible light. Light emission results from the interaction of an enzyme, luciferase, and a long chain aldehyde in the presence of oxygen. This interaction constitutes a fraction of the cell electron transport system. The emission of light is dependent on this electron flow. Therefore, the light output reflects changes in the metabolic activity of the organism [Ribo and Kaiser (1987)]. The metabolic pathway is directly related to the respiration of the organism. A change in cellular metabolism or a disruption of the cellular structure results in a change of respiration and a proportional change in bioluminescence [Ross].

EC50 is the sample concentration that reduces the light output by 50%. For this research, 15-minute EC50 values were used. A 15-minute EC50 is the concentration that results in a 50% reduction in light output after 15 minutes of organism exposure to the test compound. For this reason, we used the 100% assay, which evaluates four concentrations of each filtrate (90, 45, 22.5, and 11.25%) and compares to a control. Approximately  $10^6$  microorganisms are used to evaluate each test concentration [Ross].

The EC50 term can be confusing since toxicity increases as EC50 decreases and vice versa. The use of toxicity unit, TU, eliminates this problem. TU is defined as 100 divided by the EC50 (expressed as a percent).

Immediately after bleaching or ether fractionation, the filtrate is adjusted to a pH of 7.4  $\pm$  0.05, filtered with a 0.22 $\mu$ m filter, and then stored at -70°C until tested. Each test sample was evaluated in triplicate following the protocol described by the Microtox manual [Microbics (1994)].

### **Biotreatment**

All U.S. mills are equipped with a minimum of secondary, or biological, treatment. Untreated filtrates are not discharged directly into the aquatic environment. It was desired to emulate the effects of pulp and paper secondary biotreatment facilities in order to have a more realistic view of environmental impact of effluents in the aquatic environment. Therefore, laboratory-scale biotreatment was completed on all whole, combined filtrates.

Filtrates were diluted to 25% v/v with double deionized, double filtered water, and then the pH was adjusted to 7.0  $\pm$  1.0. Four hundred mls of each diluted filtrate were added to an Erlenmeyer flask, and then one Hach BOD capsule (Hach, Loveland, Colorado) was added to each flask. The flasks were placed on a shaker in an unstopped flask at 200 rpm at room temperature. After five days of treatment, the contents of each flask were placed in a 1000 ml round bottom flask. The round bottom flask was then placed on a rotary evaporator in a water bath at 40°C until approximately 50 mls of filtrate remained. The filtrate was then washed from the round bottom flask with double

deionized, double filtered water and diluted back to its original concentration (100 mls). The filtrate pH was adjusted to 7.4 +/- 0.05. The filtrate was then filtered with a 0.22 $\mu$ m filter and stored at -70°C until tested for toxicity.

### **Statistical Methods**

One sample (high charge chlorine, first stage, second replicate) was lost to a laboratory accident. This left the experimental design unbalanced. For the purpose of statistical analysis, the toxicity of the destroyed sample was estimated by the method of fitting constants [Hicks (1964)]. Significant factors were identified by use of a split-plot design analysis of variance. Significant differences between means were identified by using Tukey's Least Significant Difference Test (q-test) [Walpole and Myers (1989)]. Significance in all tests was determined at the 95% confidence level. Standard error was used to determine the values of all error bars.

### **RESULTS AND DISCUSSION**

All data points are averages of triplicate determinations on the same sample. The data are presented in Table 1. A split-plot analysis of variance was performed with the results presented in Table 2. A two-way table of means is provided in Table 3.

		B	D <sub>L</sub>	D <sub>H</sub>	C <sub>L</sub>	C <sub>H</sub>
REP 1	1 <sup>st</sup> stage	2.59	0.92	1.71	4.55	8.45
	2 <sup>nd</sup> stage	0.79	0.56	0.56	0.56	0.56
	combined	1.51	0.78	0.99	3.25	3.20
	NVW	1.65	0.56	0.56	1.28	3.06
	EE	0.56	3.72	1.03	0.56	0.56
	NEE	0.56	0.56	0.56	1.02	0.77
REP 2	1 <sup>st</sup> stage	0.56	1.54	1.70	5.39	8.31
	2 <sup>nd</sup> stage	1.31	0.56	0.56	1.05	1.16
	combined	0.56	1.18	0.56	2.49	3.80
	NVW	0.56	0.56	0.56	1.79	3.65
	EE	0.60	1.93	0.79	1.37	1.87
	NEE	0.56	0.56	0.56	0.56	0.56

**Table 1.** Microtox Results, Expressed in Toxicity Units, Obtained in Triplicate.

The presence of a high number of 0.56 TUs reported should be noted. This is accountable due to the detection limit of the test being determined to be  $EC_{50} = 90\%$  or  $TU = 1.111$  since this was the highest concentration tested. Toxicity unit (TU) measurements lower than this were replaced by  $TU = 0.556$ , one-half the detection limit.

whole plot	factor	df	SS	MS	F-ratio	F-critical	significant?
	R	1	0.0104	0.0104	0.0218	4.034	no
	B	4	39.3797	9.8449	20.6522	2.557	yes
whole plot error		4	1.9068	0.4767			
split plot	F	5	57.0702	11.4140	44.3952	2.534	yes
	BF	20	66.8401	3.3420	12.9988	1.932	yes
split plot error		24	6.17	0.2571			
total		58	171.35				

**Table 2.** Split-Plot Analysis of Variance of Microtox™ Toxicity Data. (R=repetition; B=bleaching agent/charge; F=filtrate stage/fraction)

	Background	chlorine dioxide (low)	chlorine dioxide (high)	chlorine (low)	chlorine (high)	mean
1 <sup>st</sup> stage	1.58	1.23	1.70	4.97	8.38	3.57
2 <sup>nd</sup> stage	1.05	0.56	0.56	0.81	0.86	0.78
whole	1.04	0.98	0.78	2.87	3.50	1.83
non-volatile whole	1.11	0.56	0.56	1.54	3.36	1.42
ether extractable	0.58	2.82	0.91	0.96	1.22	1.30
non-ether extractable	0.56	0.56	0.56	0.79	0.66	0.63
mean	0.98	1.12	0.84	1.99	3.00	

**Table 3.** Two-Way Table of Means for Bleaching/Fraction Interaction.

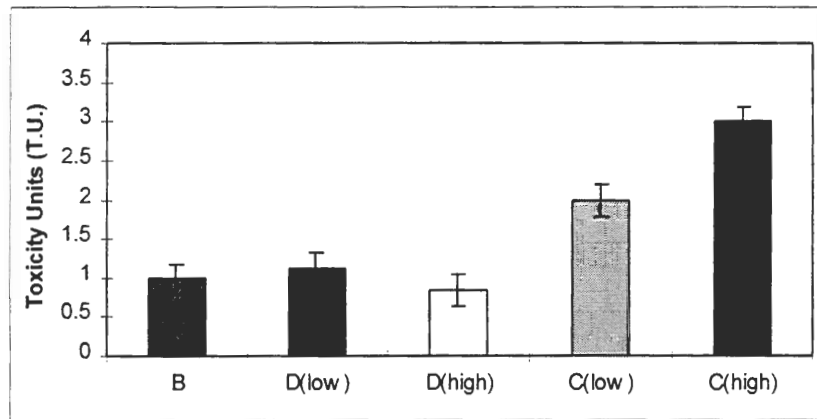
### Bleaching Filtrate Toxicity

The two bleaching agents (chlorine and chlorine dioxide) were both evaluated at two bleaching charges - 0.15 KF and 0.25 KF. The 0.15 KF bleaching, whether chlorine or chlorine dioxide, is referred to as the “low” charge; the 0.25 KF bleaching is referred to as the “high” charge. Chlorine, chlorine dioxide, and background bleaching filtrate toxicities are discussed in this section.

The bleaching agent and charge used in the first bleaching stage were significant at the 95% confidence level. The q-test was used to determine the differences between toxicities for bleaching agents and charges. The least significant difference used to evaluate these differences was found to be 0.338 toxicity units.

The high chlorine charge bleaching resulted in higher toxicity filtrates than the low chlorine charge bleaching. Both low and high chlorine charge filtrates were

significantly higher than both chlorine dioxide charges and the background filtrates as shown in Figure 1.



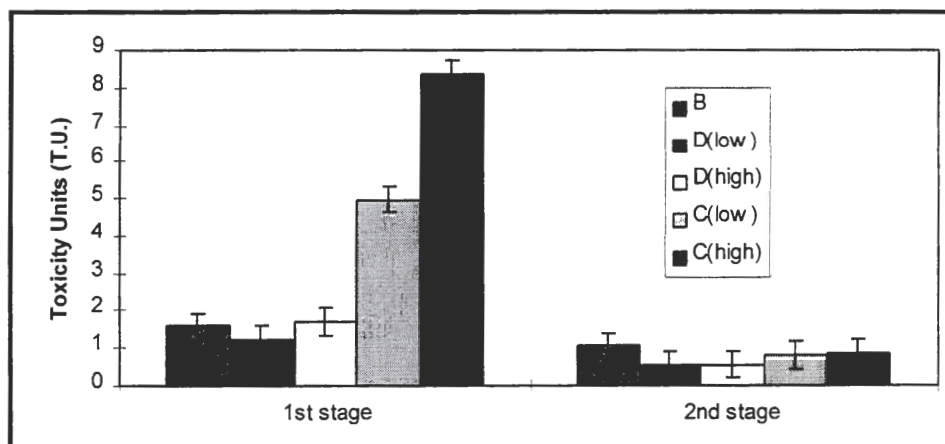
**Figure 1.** Average Toxicity of All Bleaching Filtrates and Fractions.

Both charges of chlorine dioxide were found to be lower in toxicity than the chlorine filtrates, but were not significantly different than the toxicity of the background filtrates. The background toxicity was also found to be significantly lower than both of the chlorine charge filtrates, but not significantly different from the chlorine dioxide filtrates.

### **Bleaching Stage Toxicity**

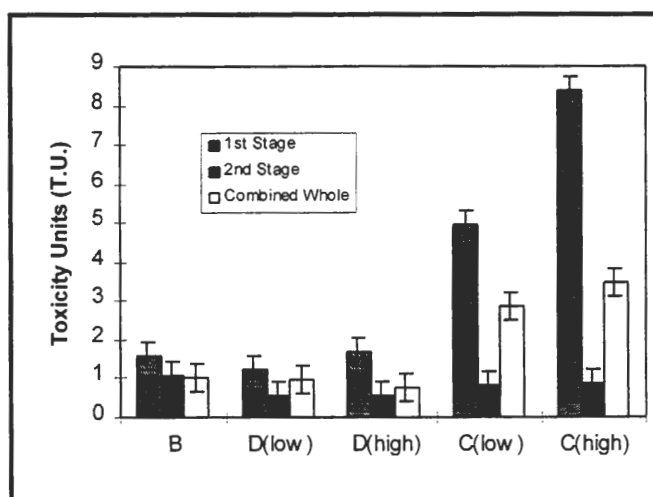
As shown in Table 2, the filtrate bleaching stages are significant factors. Again, utilizing the q-test, with the least significant difference of 0.324 toxicity units, differences between bleaching stages were determined. The first bleaching stage filtrates are significantly more toxic than the second stage bleaching filtrates. The mean toxicities of

the bleaching filtrates from the first and second bleaching stages for the various bleaching agents and charges are shown in Figure 2.



**Figure 2.** Toxicity of First and Second Stage Bleaching Filtrates.

Because there is a significant bleaching agent and bleaching stage interaction, it is interesting to observe the variations in relationships of the first and second stages in conjunction with the combined whole filtrates in reference to the various bleaching agents. Figure 3 illustrates the bleaching agent and bleaching stage interactions.

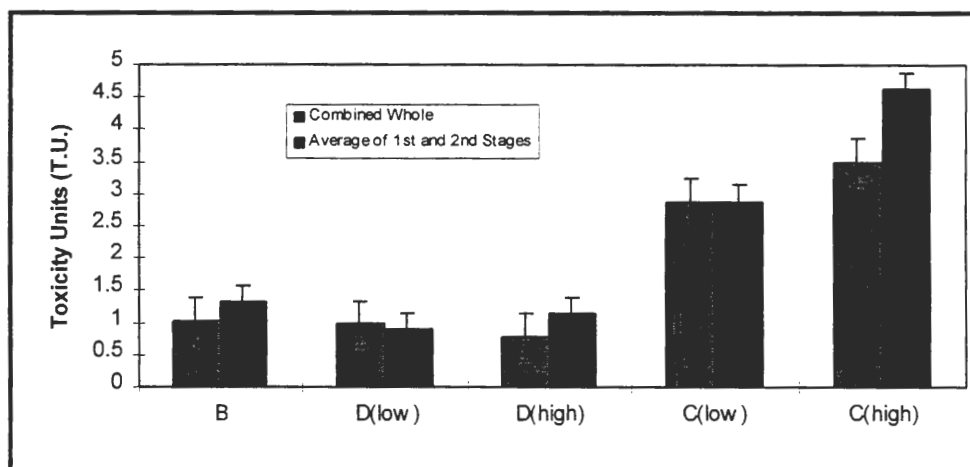


**Figure 3.** Toxicity Comparison of 1<sup>st</sup>, 2<sup>nd</sup>, and Combined Whole Bleaching Filtrates.



When combining chemical compounds, it is of interest whether the toxicity of the combined solution shows increased or decreased toxicity of the individual compounds. When two compounds are combined and the toxicity of the first and the second add to the sum of the combined solution, the addition is considered additive. If the first and second compounds combine to produce a solution higher than the sum of their toxicities, the addition is considered synergistic. If the first and second compounds combine to produce a solution lower in toxicity than the sum of their toxicities, the addition is considered antagonistic.

Figure 4 illustrates the comparison of the whole (combined) filtrates with the average of the first and second stage filtrates. The average of the 1<sup>st</sup> and 2<sup>nd</sup> stages is approximately the same as the combined whole, indicating an additive effect of the two bleaching stages when mixed, except for the filtrates prepared using high charge chlorine. The high chemical charge of chlorine resulted in filtrates, which act antagonistically when combined.

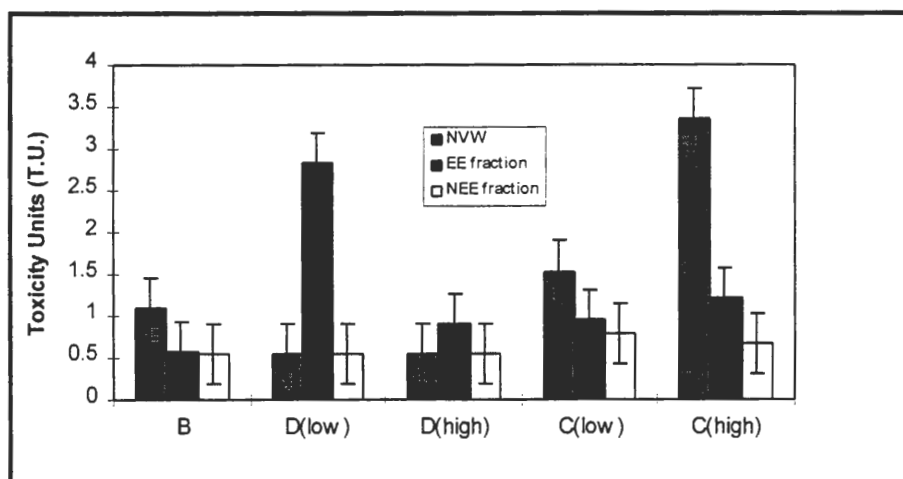


**Figure 4.** Comparison of Theoretical Combined Whole and Actual Combined Whole.

## Filtrate Fractions Toxicity

As shown in Table 2, the filtrate fractions are also significant factors. Again, utilizing the q-test, with a least significant difference of 1.18 toxicity units, differences between filtrate fractions were determined.

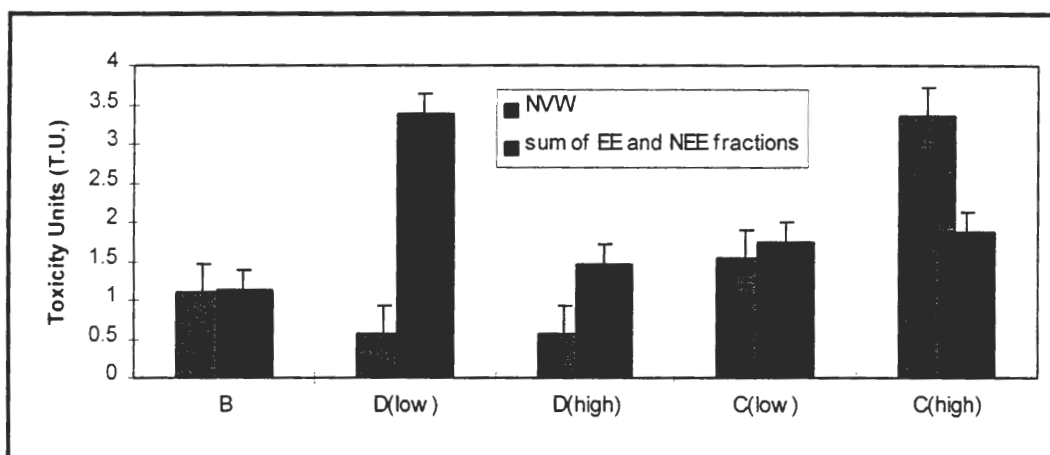
Figure 5 illustrates the bleaching agent and ether fraction interaction. The most notable difference between bleaching agents is that the D(low) has a significantly higher toxicity in the ether-extractable fraction than the other bleaching agent filtrates.



**Figure 5.** Comparative Toxicity of Filtrates from Non-Volatile Whole (NVW), Ether-Extractable Fraction (EE) and Non-Ether Extractable (NEE) Fraction (data from Table 3, rows 4, 5, and 6)

Figure 6 illustrates the comparison of the non-volatile whole (NVW) filtrate with the average of the ether-extractable (EE) and non-ether extractable (NEE) fractions in order to identify synergistic, additive, or antagonistic effects. The D(high) filtrates are obviously additive. The background filtrates are close to being considered additive, but would more accurately be described as synergistic when the two fractions are not

separated. Likewise, both chlorine filtrates show synergistic effects with the C(high) showing strong synergistic effects when the fractions are not separated. The D(low) shows strong antagonistic effects when the filtrates are not fractionated.



**Figure 6.** Comparison of NVW Filtrate Toxicity and Sum of EE and NEE Fraction Toxicity.

### Effect of Biotreatment on Toxicity

Toxicity reductions as a result of benchtop biotreatment is shown in Table 4. Caution must be exerted when examining these results due to several reasons. The first is that the biotreatment utilized in this research varies greatly from the biotreatment used in an industrial setting. Secondly, it is typical in industry to optimize the microbes used for biotreatment in order to utilize the most effective biotreatment system. This could not be done in our system without biasing the results. Finally, in a industrial biotreatment system, there are many other pulp and paper streams added to the bleaching stream which may aid biotreatment and detoxification by breaking down molecules for ease of consumption by the microbes.

Sample	Untreated	Biotreated	Reduction
B whole rep 1	1.5 +/- 1.2	0.6 +/- 1.2	0.9 +/- 1.2
B whole rep 2	0.6 +/- 1.2	0.6 +/- 1.2	0 +/- 1.2
D(low) whole rep 1	0.8 +/- 1.2	2.6 +/- 1.2	-1.8 +/- 1.2
D(low) whole rep 2	1.2 +/- 1.2	2.7 +/- 1.2	-1.5 +/- 1.2
D(high) whole rep 1	1.0 +/- 1.2	0.6 +/- 1.2	0.4 +/- 1.2
D(high) whole rep 2	0.6 +/- 1.2	3.9 +/- 1.2	-3.3 +/- 1.2
C(low) whole rep 1	3.2 +/- 1.2	2.7 +/- 1.2	0.5 +/- 1.2
C(low) whole rep 2	2.5 +/- 1.2	0.6 +/- 1.2	1.9 +/- 1.2
C(high) whole rep 1	3.2 +/- 1.2	3.5 +/- 1.2	-0.3 +/- 1.2
C(high) whole rep 2	3.8 +/- 1.2	3.4 +/- 1.2	0.4 +/- 1.2

**Table 4.** Microtox Toxicity. (as Toxicity Units)

## CONCLUSIONS

1. There is no significant difference between low and high chlorine dioxide charge and background filtrate toxicity. There is also no significant difference between low and high chlorine charge filtrates. Both chlorine charge filtrates resulted in significantly higher toxicity filtrates than the background or chlorine dioxide bleaching filtrates.
2. In all cases examined, the first bleaching stage (including the background) resulted in higher toxicity than the second bleaching stage (oxidative extraction).
3. The whole, combined filtrates were approximately equal to the average of the first and second stages, indicating no synergistic effects.
4. Since the background toxicity is not significantly different from the chlorine dioxide bleaching filtrates, future research should examine the source of the toxicity of the

background filtrate, which may also explain chlorine dioxide filtrate toxicity and remove concerns regarding the use of this chemical for pulp bleaching purposes.

5. The use of biotreatment in this study was not effective in reducing Microtox toxicity. However, this is not necessarily indicative of the treatability of the filtrates for Microtox toxicity since another type of treatment system or bacteria used could potentially be successful in reducing Microtox toxicity.

## ACKNOWLEDGMENTS

The authors thank Dr. Lucy Sonnenberg, Dr. Mary DeLong, and Dr. John Cairney for technical advice and guidance, and the Institute of Paper Science and Technology and its Member Companies for the financial support provided for student research. Portions of this work were used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

## LITERATURE CITED

- Annergren, G., Lindblad, P., Carlsson, G., and Norrby, M. (1986) Spent Liquors from Bleaching of Softwood Kraft Pulps. Effect of Chlorination Variables on the Content of Lipophilic Chlorinated Organic Matter with Low Molecular Mass. **Proceedings of the EuCePa Symposium "Environmental Protection in the '90's,"** Helsinki, May 19-22.
- Borton, D. (1990) Examination of the Relationship Between the Adsorbable Organic Halide (AOX) Content of Paper-Industry Waste Waters and Potential Aquatic Biological Effects: First Progress Report. **NCASI Special Report**, No. 90-07.
- Cox, J. (1989) BC Mills to Spend C\$800 Million to Meet Toxicity Regulations. *American Papermaker*, **52**(7), 27.
- Environment Canada (1992) Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*). **Environmental Protection Series**, Report EPS 1/RM/24.

Firth, B.K. and Backman, C.J. (1990a) A Comparison of Microtox Testing with Rainbow Trout (Acute) and *Ceriodaphnia* (Chronic) Bioassays Using Pulp- and Paper-Mill Wastewaters. 1990 TAPPI Environmental Conference Proceedings, 621-626.

Firth, B.K. and Backman, C.J. (1990b) Comparison of Microtox Testing with Rainbow Trout (Acute) and *Ceriodaphnia* (Chronic) Bioassays in Mill Wastewaters. *Tappi Journal*, **73**(12), 169-174.

Hicks, C.R. (1964) **Fundamental Concepts in the Design of Experiments**, Chapter 9. Holt, Rinehart, and Winston Publishers, New York, N.Y., 130-147.

Jamieson, S. (1992) In Environmental Monitoring, What You Don't Know Can Hurt You. *Pulp & Paper Canada*, **93**(2), 9-10.

Liebergott, N., van Lierop, B., Kovacs, T., and Nolin, A. (1990) Comparison of the Order of Addition of Chlorine and Chlorine Dioxide in the Chlorination Stage. *Tappi Journal*, **73**(10), 207-213.

Microbics Corporation (1994), **Microtox Operator Manual**.

Owens, W. (1990) Review of Recent Scandinavian Research on the Aquatic Effects of Bleached Pulp Mill Effluents. **NCASI Special Report**, No. 90-11.

Pima Roundtable (1990) Pulping Trends. *PIMA*, **73**(10), 29-35.

Priha, M.H. and Talka, E.T. (1986) Biological Activity of Bleached Kraft Mill Effluent (BKME) Fractions and Process Streams. *Pulp and Paper Canada*, **87**(12), 143-144.

Qureshi, A.A., Flood, K.W., Thompson, S.R., Janhurst, S.M., Inniss, C.S., and Rokosh, D.A. (1982) Comparison of a Luminescent Bacterial Test with Other Bioassays for Determining Toxicity of Pure Compounds and Complex Effluents. *Aquatic Toxicology and Hazard Assessment: Fifth Conference ASTM STP 766*, 179-195.

Renberg, L. (1992) Use of Cost-Effective Chemical and Biological Tests for the Estimation of the Environmental Impact of Bleaching-Plant Effluents. 1992 TAPPI Environmental Conference Proceedings, 317-329.

Ribo, J.M. and Kaiser, K.L.E. (1987) *Photobacterium Phosporeum* Toxicity Bioassay. I. Test Procedures and Applications. *Toxicity Assessment: An International Quarterly*, **2**, 305-323.

Ross, P., "The Role of the Microtox System in Aquatic Toxicity Testing." **Illinois Natural History Survey**, 607 E. Peabody Drive, Champaign, IL 61820-6970.

Stauber, J. (1993) Development and Application of Algal Bioassays for Determining the Toxicity of Bleached Eucalypt Kraft Mill Effluents. Appita Annual General Conference Proceedings, 789-796.

Stauber, J., Gunthorpe, L., Deavin, J., Munday, B., and Ahsanullah, M. (1994) Application of New Marine Bioassays for Assessing the Toxicity of Bleached Eucalypt Kraft Mill Effluents. 48<sup>th</sup> Appita Annual General Conference, 511-518.

Suckling, I.D., Pasco, M.F., and Gifford, J.S. (1993) Origin of Effluent Toxicity from a Simulated BCTMP Process. 1993 TAPPI Environmental Conference Proceedings, 449-456.

Walden, C.C. (1976) Review Paper-The Toxicity of Pulp and Paper Mill Effluents and Corresponding Measurement Procedures. *Water Research*, **10**, 639-664.

Walden, C.C. and Howard, T.E. (1977) Toxicity of Pulp and Paper Mill Effluents-A Review of Regulations and Research. *Tappi Journal*, **60**(1), 122-125.

Walpole, R.E. and Myers, R.H. (1989) **Probability and Statistics for Engineers and Scientists, Fourth Edition**, Chapter 11, MacMillan Publishing Company, New York, N.Y., 486.

The second paper in this chapter discusses the results of a tetrazolium (XTT) assay completed on HepG2 (human liver cells) exposed to the filtrates and fractions. The XTT (sodium 3,3'-[1[(phenylamino) carbonyl] 3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) assay is essentially a MTT assay modified by Scuderio. The water-soluble XTT-formazan product simplifies measurements by eliminating the need for solvent-extraction steps, which are necessary in the MTT assay (Meshulam *et al*). The XTT assay measures the reduction in mitochondrial dehydrogenase activity (Gressner *et al*). Bioreduction of XTT requires the addition of an intermediate electron acceptor, phenazine methosulphate (PMS) (Goodwin *et al*).

The HepG2 cells were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland). The cell line was derived from a 15-year-old Caucasian. The cells were grown in monolayer in 75 cm<sup>2</sup> flasks. The HepG2 cells were cultured and maintained in an incubator at 37°C. The HepG2 line was maintained as stock in EMEM (Atlanta Biologicals, Atlanta, Georgia) supplemented with 10% fetal bovine serum and 5% donor horse serum, sodium pyruvate, non-essential amino acids, gentamycin, and Hanks medium. The horse serum was only used during cell maintenance and was deleted from the medium preparation during testing. Approximately once per week, the cells were trypsinized and transferred to new flasks or plates depending upon the need of cells for research at that particular time.

The raw data compilation is presented in Appendix I. The curves used to determine EC50s from the XTT data points are shown in Appendix J.



While evaluating the data presented in the following paper, it may be helpful to note the XTT toxicities of common solutions, presented in Table 4-2.

COMMON SOLUTION	XTT TOXICITY (IN TOXICITY UNITS)
Diet Cola	<1.0
Beer	<1.0
Wine	<1.0
Atlanta Tap Water	<1.0

**Table 4-2.** XTT Toxicities of Common Solutions.

Paper #4 - Prepared for Submission to Ecotoxicology and Environmental Safety

**USE OF A SOLUBLE TETRAZOLIUM/FORMAZAN ASSAY (XTT) WITH HEPG2 CELLS FOR  
TOXICITY EVALUATION OF PULP BLEACHING FILTRATES**

T.A. Ard and T.J. McDonough  
Institute of Paper Science and Technology  
Atlanta, GA

and  
M.J. DeLong  
School of Public Health  
Emory University  
Atlanta, GA

**ABSTRACT**

A laboratory study was conducted to comparatively evaluate the toxicities of chlorine and chlorine dioxide bleaching filtrates applying an XTT assay utilizing human liver cells (HepG2). These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen pre-delignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. Each of the combined filtrates was then subjected to an ether extraction to separate it into ether-extractable and non-ether extractable fractions. The ether-extractable fraction is considered to be of the most environmental importance. The toxicity of each of the whole filtrates and fractions was determined by the use of the XTT assay utilizing human liver cells.

The bleaching agent used in the first bleaching stage resulted in significantly different filtrate toxicities. The bleaching agents resulted in filtrates with the following ranking of toxicities (in decreasing order): background > 0.15 KF chlorine dioxide > 0.25 KF chlorine dioxide > 0.25 KF chlorine > 0.15 KF chlorine. There was a marginally significant interaction between the bleaching agent used in the first bleaching stage and the bleaching stage filtrates and bleaching fractions tested.

There were significant differences found between bleaching stages and fractions. The first bleaching stage filtrates were significantly more toxic than the filtrates from the second bleaching stage. Also, the ether-extractable (EE) fraction was extremely low; however, the non-ether extractable (NEE) fraction was slightly more toxic than the non-volatile whole (NVW) filtrates but not significantly different from the whole filtrate. This indicates that the toxicity of the filtrates comes from the least environmentally troublesome fraction.

## **KEY WORDS**

bleaching, filtrates, biotreatment, dioxide, chlorine, bioaccumulation, toxicity, XTT, HepG2

## **INTRODUCTION**

Environmental concerns are causing the pulp and paper industry to investigate alternatives to conventional bleaching sequences, which have generally utilized chlorine in the first bleaching stage. Little research has gone into quantifying the toxicity reduction achieved when alternative bleaching agents are substituted for chlorine.

Regulatory restrictions on effluent AOX are having a substantial impact on the use of chlorine and chlorine-containing compounds in the pulp and paper industry. Replacement of chlorine with chlorine dioxide results in a significant decrease in the amount of AOX produced.<sup>1,2</sup> However, there appears to be little relationship between AOX and toxicity.<sup>3,4</sup> Neither has any correlation been found between toxicity and BOD or TOC.<sup>5,6</sup> A relationship has been found between toxicity and the concentration of resin acids present.<sup>7</sup>

Despite the fact that chlorine-containing organic molecules are only found in low concentrations in bleach effluents, emphasis has been placed on these compounds due to their non-polar nature and lipid-soluble character, which may lead to bioaccumulation in marine organisms. The octanol/water partition coefficient ( $K_{ow}$ ) is often used to predict the ability of a compound to bioaccumulate. However, when it is desired to evaluate the bioaccumulation potential of solutions of unknown organic content, such as bleach plant filtrates, it is more practical to use ether extractions to separate compounds by polarity.

The XTT assay utilizing human liver cells (HepG2) was chosen to evaluate potential human liver toxicity due to concerns from environmental groups that humans could be exposed to compounds found in bleaching filtrates through consumption of aquatic organisms, specifically fish. The XTT assay measures the ability of the electron transport system of the cells to function. If cells are functioning properly, the electron transport system will convert the XTT tetrazolium salt into a formazan, which is soluble and colorimetric, spectrophotometrically active at 600 nm. The EC50 is determined by a reduction of 50% in the absorbance of the test solution at 600 nm.

## **MATERIALS AND METHODS**

### **Bleaching and Filtrate Collection**

In bleaching kraft pulp, 45-90 kg of organic material are dissolved per metric ton of pulp. Of the dissolved organic material, 75-90% is usually produced during the first two bleaching stages.<sup>8</sup> For this reason, only the filtrates from the first oxidative bleaching stage and the first extraction stage were tested. All filtrates tested were generated in the Institute of Paper Science and Technology bleaching facilities.

Oxygen delignified pulp was obtained from a commercial facility immediately following the washing stage. A sample of the liquid phase accompanying the oxygen delignified pulp ("oxygen stage carryover") was separately obtained. In the laboratory, the pulp was repeatedly washed and centrifuged until no color remained in the wash water. The pulp was then centrifuged to approximately 35% consistency (consistency = weight of pulp/weight of pulp and liquid, expressed as a percent), fluffed, and stored at 4°C. In preparation for bleaching, 300 g pulp, enough sulfuric acid to reduce the pulp solution to pH 1.8-2.0, oxygen carryover, and dilution water to make the solution 3% consistency were mixed in the reactor. The amount of oxygen carryover required was determined by assuming a well-washed system, achieving only 10 kg COD carryover per ton of pulp. Distilled water, preheated to 70°C, was used as dilution water. Chlorine and chlorine dioxide solution concentrations were determined immediately prior to bleaching

combined filtrates, equal amounts of first and second stage filtrates were added together, mixed well, and pH adjusted to less than 2. Four liters of combined filtrate were added to the ether extractor and the extractor started. Another sample of the combined filtrate was pH adjusted, filtered, and frozen at  $-70^{\circ}\text{C}$ . A final sample of combined filtrate was vacuum evaporated for 24 hours prior to pH adjustment, filtering, and freezing.

### **Ether Extractions**

Continuous ether extractions were performed in 4 L glass extractors. Four liters of combined (first and second stage) filtrates were added to the extractor and connected to a 1 L round bottom flask containing 500 mls ether. Extraction with refluxing ether was continued for four days. A rotary evaporator, under vacuum, was used for 24 hours to remove the ether from both the ether-extract and the extracted filtrate.

All of the ether-extract (EE) was evaporated to dryness and then reconstituted with filtered double deionized water to 250 mls. There were four liters of extracted filtrate following the ether fractionation procedure; however, it required another two liters of double deionized water,  $\text{H}_2\text{SO}_4$ , and  $\text{NaOH}$  to completely remove the extracted filtrate from the extractor. An aliquot of 500 mls of extracted filtrate, now referred to as non-ether extract (NEE) was evaporated for 24 hours and then reconstituted to 500 mls. The remainder (5500 mls) of the extracted filtrate and wash solution was disposed.

In order to accurately compare the toxicities of the whole filtrates to the fractions, the measured toxicities had to be recalculated to account for the various dilutions and concentrations. The 250 mls of the EE fraction actually represent the compounds from

4L of filtrate; likewise, the 500 mls of NEE fraction were taken from a diluted source (6L) of 4L of filtrate. Therefore, the toxicity of the EE fraction was multiplied by 16 to put the toxicity on an equivalent basis with the whole filtrate. The toxicity of the NEE fraction was multiplied by (4/6) in order to put the NEE toxicity on an equivalent basis with the whole filtrate. All toxicity numbers presented in the paper have already been placed on an equivalent basis.

### **XTT Assay**

HepG2 (human liver) cells, obtained from the American Type Culture Collection (ATCC; Rockville, Maryland), were used in the XTT assay analysis. The HepG2 cells were cultured and maintained in an incubator at 37°C. The HepG2 line was maintained as stock in EMEM (Atlanta Biologicals, Atlanta, Georgia) supplemented with 10% fetal bovine serum and 5% donor horse serum, sodium pyruvate, non-essential amino acids, gentamycin, and Hanks medium. The horse serum was only used during cell maintenance and was deleted from the medium preparation during testing. The cell cultures were passaged once or twice weekly using trypsin-EDTA for detachment from the culture flask.

Prior to testing, cells were trypsinized from their 75 cm<sup>2</sup> flasks and placed into 96-well plates at approximately 10,000 cells/well. Twenty-four hours later, the appropriate concentrations of filtrates were added to the wells. For each column of twelve wells, there were two control wells and five concentrations (25, 10, 5, 1, and ½%) of each of two filtrates. Four columns were used as replicates for each filtrate. After 24 hours of cell exposure to filtrates, a 0.025 mM PMS-XTT solution was prepared and administered

to the 96-well plates as described by Scudiero et al.<sup>9</sup> The plates were placed back in the incubator for exactly four hours. They were then removed, placed in a 96-well plate reader, and evaluated for absorbance at 600 nm.

### **Biotreatment**

All U.S. mills are equipped with a minimum of secondary, or biological, treatment. Untreated filtrates are not discharged directly into the aquatic environment. It was desired to emulate the effects of pulp and paper secondary biotreatment facilities in order to have a more realistic view of environmental impact of effluents in the aquatic environment. Therefore, laboratory-scale biotreatment was completed on all whole, combined filtrates.

Filtrates were diluted to 25% v/v with double deionized, double filtered water, and then the pH was adjusted to 7.0 +/- 1.0. Four hundred mls of each diluted filtrate were added to an Erlenmeyer flask, and then one Hach BOD capsule (Hach, Loveland, Colorado) was added to each flask. The flasks were placed on a shaker at 200 rpm at room temperature. After five days of treatment, the contents of each flask were placed in a 1000 ml round bottom flask. The round bottom flask was then placed on a rotary evaporator in a water bath at 40°C until approximately 50 mls of filtrate remained. The filtrate was then washed from the round bottom flask with double deionized, double filtered water and diluted back to its original concentration (100 mls). The filtrate pH was adjusted to 7.4 +/- 0.05. The filtrate was then filtered with a 0.22µm filter and stored at -70°C until tested for toxicity.



## **Statistical Methods**

One sample (high charge chlorine, first stage, second replicate) was lost to a laboratory accident. This left the experimental design unbalanced. For the purpose of statistical analysis, the toxicity of the destroyed sample was estimated by the method of fitting constants.<sup>10</sup> Significant factors were identified by using a split-plot design analysis of variance. Significant differences between means were identified by using Tukey's Least Significant Difference Test (q-test).<sup>11</sup> Significance in all tests was determined at the 95% confidence level. Standard error was used to determine the values of all error bars.

## **RESULTS AND DISCUSSION**

All data points are averages of quadruplet determinations on the same sample. The data are presented in Table I. A split-plot analysis of variance was performed with the results presented in Tables II and III.

		Background	Chlorine Dioxide (low)	Chlorine Dioxide (high)	Chlorine (low)	Chlorine (high)
rep 1	1 <sup>st</sup> bleaching stage	34	28	28	7	7
	2 <sup>nd</sup> bleaching stage	1	1	2	0	0
	whole	15	14	22	5	7
	non-volatile whole	11	20	18	6	8
	ether extractable fraction	0	0	0	0	0
	non-ether extractable fraction	15	16	17	3	9
rep 2	1 <sup>st</sup> bleaching stage	43	16	0	6	0
	2 <sup>nd</sup> bleaching stage	1	19	2	0	10
	whole	20	10	8	7	4
	non-volatile whole	17	8	9	2	2
	ether extractable fraction	0	0	2	0	0
	non-ether extractable fraction	12	14	12	9	4

**Table I.** XTT Results Obtained in Quadruplet. (expressed as TU=100/EC50(%))

<b>whole plot</b>	factor	df	SS	MS	F-ratio	F-critical	significant?
	R	1	0.542	0.542	0.9186	4.03	no
	B	4	10.426	2.607	4.4186	2.56	yes
	whole plot error	4	2.358	0.590			
<b>split plot</b>	F	5	17.934	3.587	10.969	2.53	yes
	BF	20	12.652	0.633	1.936	1.93	yes
	split plot error	24	7.845	0.327			
	<b>total</b>	<b>58</b>	<b>51.757</b>				

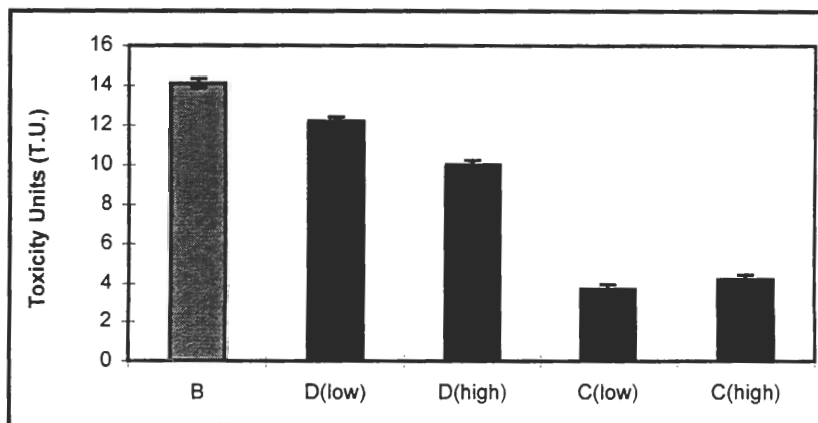
**Table II.** Split-Plot Design Analysis of XTT Toxicity Data. (R=repetitions 1 and 2; B=bleaching agent/charge; F=filtrate stage/fraction)

	background	chlorine dioxide (low)	chlorine dioxide (high)	chlorine (low)	chlorine (high)	average
1 <sup>st</sup> stage	38.5	22.0	14.0	6.5	3.5	16.9
2 <sup>nd</sup> stage	1.0	10.0	2.0	0.0	5.0	3.6
whole, combined	17.5	12.0	15.0	6.0	5.5	11.2
non-volatile whole	14.0	14.0	13.5	4.0	5.0	10.1
ether extractable fraction	0.0	0.0	1.0	0.0	0.0	0.2
non-ether extractable fraction	13.5	15.0	14.5	6.0	6.5	11.1
average	14.0	12.1	10.0	3.5	4.25	

**Table III.** Two-Way Table of Means Expressed in TU.

### **Bleaching Agent Toxicity**

As shown in Table II, and also illustrated in Figure I, the bleaching agent used in the first stage and the chemical charge used had a significant effect on the resulting filtrate. The background filtrates, or filtrates produced by using no chemical bleaching agent in the first bleaching stage, were significantly more toxic than those produced by using chlorine or chlorine dioxide as bleaching agents.

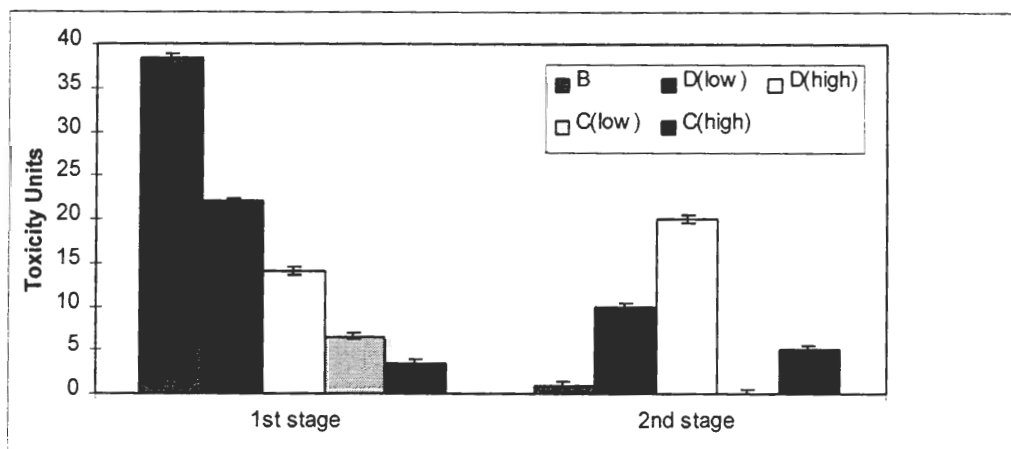


**Figure I.** Average Toxicity of All Bleaching Filtrates and Fractions.

Both chemical charges of chlorine dioxide evaluated resulted in more toxic filtrates than either chemical charge of chlorine. A lower charge of chlorine dioxide resulted in filtrates that were significantly more toxic than the higher charge of chlorine dioxide. Chlorine bleaching filtrates were the lowest in toxicity of those filtrates tested. The lower the chemical charge used in chlorine bleaching, the lower the toxicity of the bleaching filtrates.

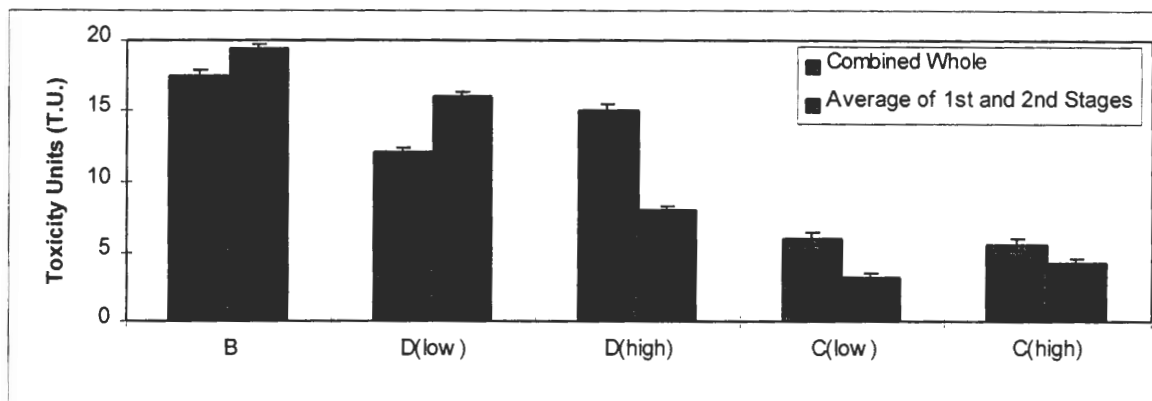
### **Bleaching Stage Filtrate Toxicity**

There were statistically significant differences in the toxicity of filtrates from different bleaching stages. The first bleaching stage, as shown in Figure II, is higher in toxicity than the filtrates from the second bleaching stage (oxidative extraction).



**Figure II.** Toxicity of First and Second Stage Bleaching Filtrates.

The combined filtrates (1<sup>st</sup> and 2<sup>nd</sup> stages), which theoretically, if no synergistic activity between stages was occurring, would equal the mean of the first and second stages. As Figure III illustrates, the theoretical combined toxicity (average of 1<sup>st</sup> and 2<sup>nd</sup> stages) does not accurately predict the toxicity of the combined whole filtrate. For the background filtrate, the combined whole is slightly less than the average of the first two stages, while the D(low) combined whole is significantly lower than the average of the first two stages. This indicates antagonistic effects. On the other hand, the D(high), C(low), and C(high) all show synergistic effects when the first two stages are mixed.



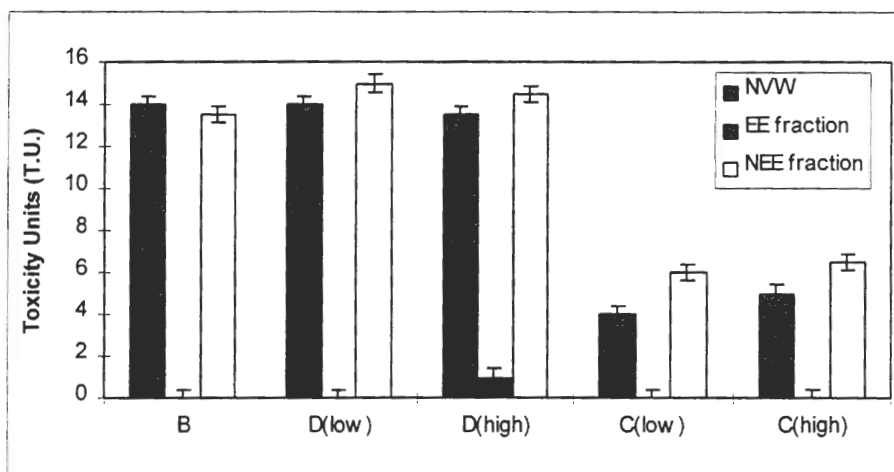
**Figure III.** Comparison of Theoretical Combined Whole and Actual Combined Whole.

### Filtrate Fraction Toxicity

After the ether extraction and vacuum evaporation of the ether, there were two fractions to test: the non-ether extractable (NEE) fraction and the ether-extractable (EE) fraction. Another filtrate, the non-volatile whole (NVW), was created by taking the whole filtrate and subjecting it to the same vacuum evaporation technique as the NEE and EE fractions. This allows for accurate comparison between the whole (without volatiles) and the fractions created by ether extraction.

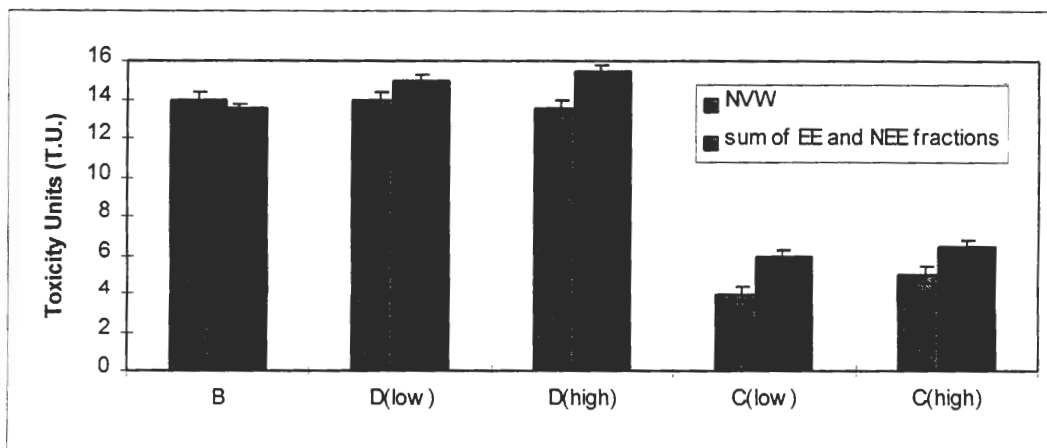
Figure IV shows the relative toxicities of the NVW, NEE, and EE filtrates. As can be observed from the figure, all of the bleaching agents/charges show extremely low or unmeasurable EE fraction toxicity. This is significant due to the fact that the EE fraction is considered to be the fraction of most relevance and concern to the environment due to bioaccumulation. It is interesting to note that in all bleaching agent/charges the NEE fraction is slightly higher in toxicity than the NVW fraction. This information combined with the extremely low EE fraction toxicity could lead to the conclusion that

the majority, if not all, of the filtrate toxicity assayed by XTT is induced by the NEE fraction, which is not considered to be of environmental relevance in regards to bioaccumulation or biomagnification of toxicants through the food chain.



**Figure IV.** Comparative Toxicity of Filtrates from Non-Volatile Whole (NVW), Ether-Extractable Fraction (EE) and Non-Ether Extractable (NEE) Fraction.

As discussed earlier, the NVW filtrates, which theoretically, if no synergistic activity between stages was occurring, would equal the average of the EE and NEE fractions. As Figure V illustrates, the background EE and NEE filtrates add to approximately the toxicity of the NVW fraction, once again indicating an additive effect.



**Figure V.** Comparison of NVW Filtrate Toxicity and Average of EE and NEE Fraction Toxicity.

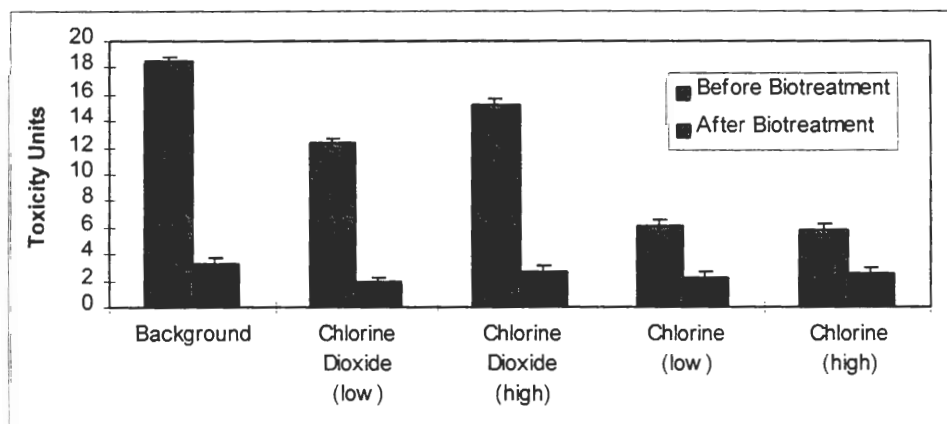
### Effect of Biotreatment on Toxicity

As shown in Table IV, and illustrated in Figure VI, lab-scale biotreatment was successful in reducing the filtrate toxicity to human liver cells as measured by the XTT assay. The background filtrates were reduced an average of 82.8%, while the low charge and high charge chlorine dioxide were reduced an average of 83.1% and 77.5%, respectively. The reduction in XTT toxicity for chlorine filtrates was not as high as the reduction in the background and chlorine dioxide filtrates. The low charge chlorine filtrates were reduced by an average of 63.4%, and the high charge filtrates were reduced by an average of 54.0%. It is encouraging that the highest toxicity filtrates resulted in the highest reduction in toxicity due to biotreatment, indicating that the toxicity is easily reduced or removed.



Sample	Untreated	Biotreated	% reduction
B+E-1	15.2	2.0	86.8
B+E-2	21.7	4.6	78.8
D (low charge) +E rep 1	14.5	1.2	91.7
D (low charge) +E rep 2	10.2	2.6	74.5
D (high charge) +E rep 1	22.2	2.8	87.4
D (high charge) +E rep 2	8.3	2.7	67.5
C (low charge) +E rep 1	5.1	1.8	64.7
C (low charge) +E rep 2	7.1	2.7	62.0
C (high charge) +E rep 1	7.2	2.7	62.5
C (high charge) +E rep 2	4.4	2.4	45.5

**Table IV.** XTT Toxicity (as Toxicity Units) of Whole Filtrates Before and After Biotreatment.



**Figure VI.** Toxicity Reduction After Labscale Biotreatment.

## ACKNOWLEDGMENTS

The authors thank Dr. Lucy Sonnenberg and Dr. John Cairney for technical advice and guidance, and the Institute of Paper Science and Technology and its Member Companies for the financial support provided for student research. Portions of this work were used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

## LITERATURE CITED

1. Cox, J. (1989) BC Mills to Spend C\$800 Million to Meet Toxicity Regulations. *American Papermaker*, **52**(7), 27.
2. Liebergott, N., van Lierop, B., Kovacs, T., Nolin, A. (1990) Comparison of the Order of Addition of Chlorine and Chlorine Dioxide in the Chlorination Stage. *Tappi Journal*, **73**(10), 207-213.
3. Borton, D. (1990) Examination of the Relationship Between the Adsorbable Organic Halide (AOX) Content of Paper-Industry Waste Waters and Potential Aquatic Biological Effects: First Progress Report. **NCASI Special Report**, No. 90-07.
4. Owens, W. (1990) Review of Recent Scandinavian Research on the Aquatic Effects of Bleached Pulp Mill Effluents. **NCASI Special Report**, No. 90-11.
5. Walden, C.C. (1976) Review Paper - The Toxicity of Pulp and Paper Mill Effluents and Corresponding Measurement Procedures. *Water Research*, **10**, 639-664.
6. Walden, C.C. and Howard, T.E. (1977) Toxicity of Pulp and Paper Mill Effluents - A Review of Regulations and Research. *Tappi Journal*, **60**(1), 122-125.
7. Priha, M.H. and Talka, E.T. (1986) Biological Activity of Bleached Kraft Mill Effluent (BKME) Fractions and Process Streams. *Pulp and Paper Canada*, **87**(12), 143-144.
8. Annergren, G. Lindblad, P., Carlsson, G., and Norrby, M. (1986) Spent Liquors

- 
- from Bleaching of Softwood Kraft Pulps. Effect of Chlorination Variables on the Content of Lipophilic Chlorinated Organic Matter with Low Molecular Mass. **Proceedings of the EuCePa Symposium "Environmental Protection in the 90's,"** Helsinki, May 19-22.
9. Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., and Boyd, M.R. (1988). Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. *Cancer Research* **48**, 4827-4833.
  10. Hicks, C.R. (1964) **Fundamental Concepts in the Design of Experiments**, Chapter 9. Holt, Rinehart, and Winston Publishers, New York, N.Y., 130-147.
  11. Walpole, R.E. and Myers, R.H. (1989) **Probability and Statistics for Engineers and Scientists, Fourth Edition**, Chapter 11, MacMillan Publishing Company, New York, N.Y., 486.

The next paper, titled, "The Use of Stress Genes to Evaluate Pulp Bleaching Filtrates" by T.A. Ard, T.J. McDonough, J. Cairney, M. DeLong, and J. Williams, covers the results obtained by using stress promoter genes to evaluate filtrates from chlorine and chlorine dioxide bleaching. Stress promoter genes are fused to a structural reporter gene whose product is easily measured. In this research, the stress gene promoters are fused to the *lacZ* ( $\beta$ -galactosidase) gene in *E. coli*. The stress gene activity is measured by the degradation of o-nitrophenyl- $\beta$ -galactopyranoside (ONPG) to the products galactose and the intensely yellow ortho-nitrophenol. This change can be measured as a colorimetric change at 420 nm. The colorimetric change is directly related to the degree of stress promoter induction.

A discussion of the function of stress genes follows along with the paper titled, "The Use of Stress Genes to Evaluate Pulp Bleaching Filtrates".

## **FUNCTION OF STRESS GENE PROMOTERS**

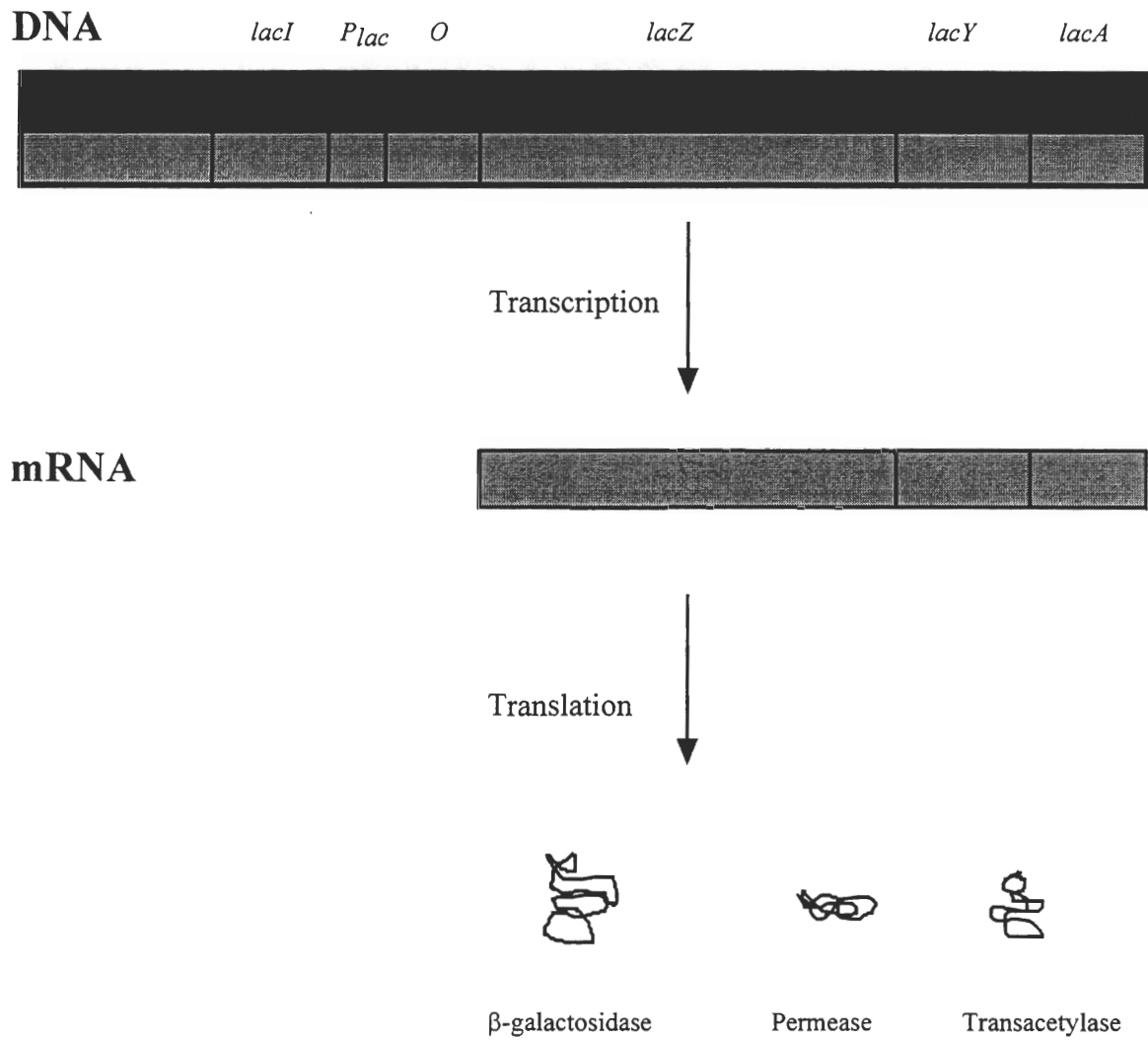
In order to understand the theory of the stress gene assays, it is important first to understand the structures and terminology involved. Often, the enzymes needed for a specific bacterial process are encoded by a number of genes located in series on the same region of DNA. Each structural gene encodes a specific enzyme required for one step in the synthesis of a cellular biochemical product. The cell ensures that these enzymes are produced in the correct amount and at the correct time by coordinately regulating their synthesis. This is efficiently managed by co-transcribing these genes in an operon. As

shown in Figure 4-1, an operon contains both protein coding information and gene regulatory sequences.

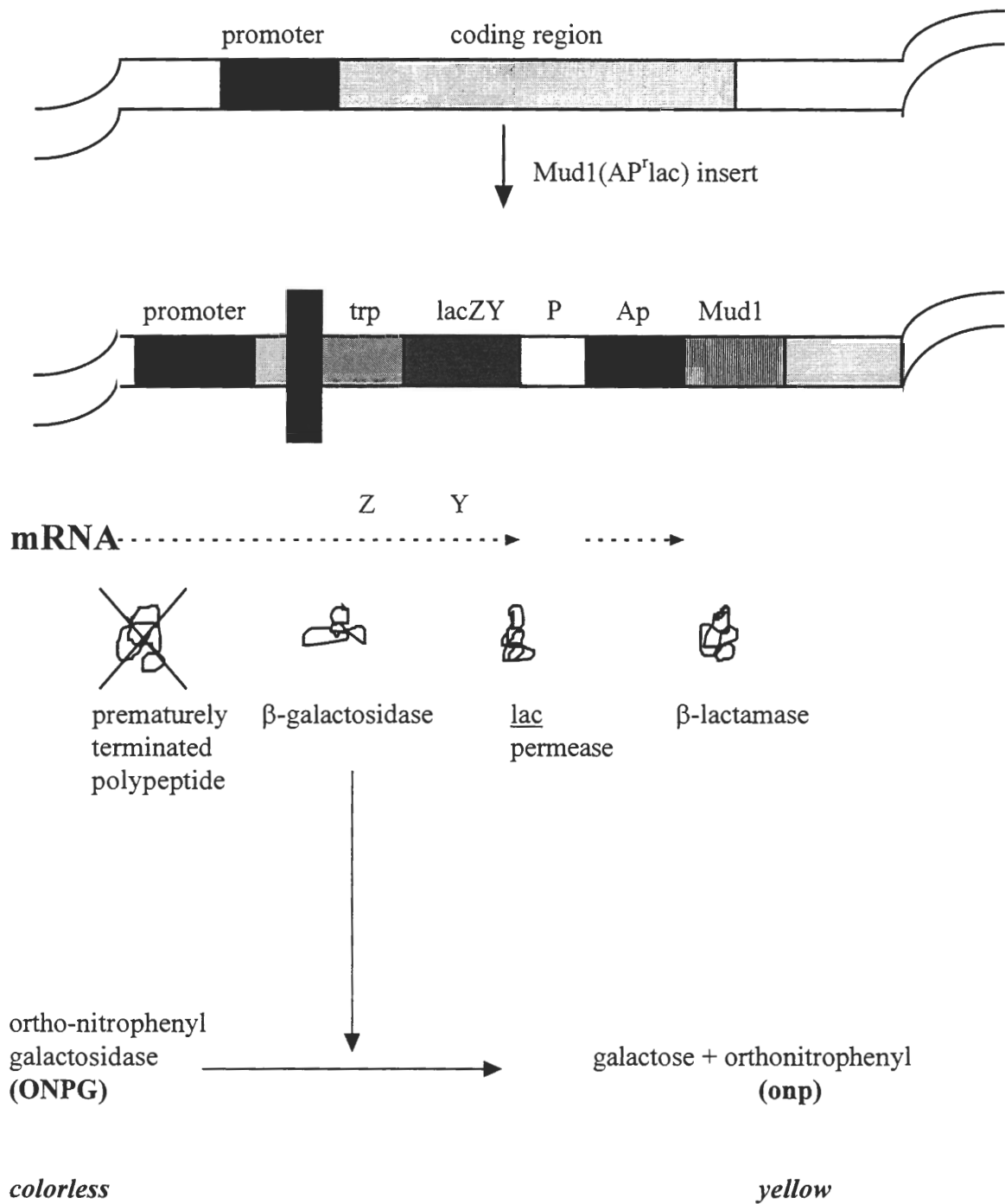
In the Xenometrix assay, a number of bacterial strains, each containing a *lacZ* structural gene fused to a different stress promoter, are used to assay toxic substances in the media. Figure 4-2 illustrates the manner in which these gene fusions are constructed. A promoterless *lacZY* gene, inserted within a Mud1 transposon, is used to mutagenize a Lac-minus *E. coli*. Insertions within a gene of interest (stress inducible gene) are selected.

The stress gene activity is measured by the degradation of o-nitrophenyl- $\beta$ -galactopyranoside (ONPG) by  $\beta$ -galactosidase to the products galactose and the intensely yellow ortho-nitrophenol. This change can be measured as a colorimetric change at 420 nm. The colorimetric change is directly related to the degree of stress promoter induction.

When inducer is present, RNA promoter is activated and RNA synthesis occurs. The quantity of RNA is reflected by the activity of its encoded product (e.g.  $\beta$ -galactosidase). Thus, by measuring  $\beta$ -galactosidase activity, we gain a sense of the degree to which a gene is induced.



**Figure 4-1.** The Lac Operon



**Figure 4-2.** Stress Gene Formation and Production of  $\beta$ -galactosidase and Other Products

Paper #5 - Prepared for Submission to TAPPI Journal

## **THE USE OF STRESS GENES TO EVALUATE PULP BLEACHING FILTRATES**

**T.A. Ard, T.J. McDonough, and J. Cairney**  
**Institute of Paper Science and Technology**  
**Atlanta, GA**

**M. DeLong**  
**Emory University School of Public Health**  
**Atlanta, GA**

**J. Williams**  
**Xenometrix, Inc.**  
**Boulder, Colorado**

### **ABSTRACT**

Bacterial stress promoters were used to evaluate untreated filtrates from chlorine and chlorine dioxide bleaching of an oxygen predelignified softwood kraft pulp. In each case, a sample of oxygen pre-delignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected, combined and then assayed. In order to evaluate the compounds that enter the bleach plant from the pulping processes, "background" filtrates were prepared by performing control experiments in which the oxidant solution was replaced with distilled water.

We utilized an *E. coli* stress gene assay kit containing sixteen bacterial strains, each Strain containing a different stress gene promoter fused to a lacZ structural gene<sup>1</sup>. The modified test organisms are designed to reveal the occurrence of specific toxic



conditions including those which cause DNA damage of various types, oxidative stress, fluctuations in osmotic conditions, oxidative stress, protein damage, and heavy metal effects. None of the stress genes was induced in media containing filtrates from either chlorine or chlorine dioxide bleaching at kappa factors of 0.15 and 0.25, at concentrations as high as 80%. The bacteria grew normally in the presence of all filtrates. These observations indicate that the bleaching effluents have none of the indicated toxic effects on the test organism within the limits of this assay.

## **KEY WORDS**

bleaching, filtrates, stress, genes, promoters, toxicity, mechanisms

## **INTRODUCTION**

Effluents from bleached kraft pulp mills have come under increasing scrutiny since the discovery that chlorine bleaching under inappropriate conditions can produce effluents containing chlorinated dioxins. In order to prevent a recurrence of such a surprising discovery, it is important to gain as complete an understanding as possible of the potential for bleach plant effluents to impart environmentally undesirable characteristics to the treated whole mill effluent that is discharged to the receiving water. It is equally important to anticipate the application of the most recently available techniques for assessing this potential for environmental effects. In accordance with these principles, we undertook an investigation of bleaching filtrate toxicity as measured by the application of stress gene assays, and report the results here. We chose to examine the

“worst case scenario” of untreated filtrates, since knowledge of their potential for toxicity is needed to enable the selection of the best methods for ensuring that the effluent ultimately discharged from the mill’s secondary biological treatment system. In interpreting the results of such a study, it is important to remember that the observed effects represent the *potential* for effect on the final mill effluent, which undergoes extensive secondary biological treatment before being discharged.

Until now, the Ames assay has generally been used to evaluate the mutagenicity of pulp bleaching filtrates. Most spent pulp chlorination liquors exhibit mutagenic activity when tested according to the Ames test<sup>2,3</sup>. The effect is apparently caused by various chlorinated compounds with relatively low molecular masses<sup>4</sup>. The bacterial mutagenicity of chlorinated effluents was initially reported in Sweden in 1977, and has since been confirmed by workers at the Pulp and Paper Research Institute of Canada and elsewhere<sup>5</sup>.

A major portion of the total mutagenic activity, estimated to be 75%, has been found to originate in the lipid-soluble fraction<sup>2,4,6</sup>. In spent chlorination liquors, approximately 85% of the mutagenicity has been found in the fraction containing compounds with low molecular weights (<1000)<sup>7,8</sup>. Ames testing of chlorination liquors tested for mutagenicity have shown that the number of mutant revertants is linearly related to the amount of molecular chlorine applied.<sup>9</sup> Hypochlorite, caustic extraction, and chlorine dioxide bleaching effluents have been found to have little or no mutagenic activity<sup>10</sup>. It has been estimated that oxygen prebleaching may reduce the total

mutagenicity of the subsequent chlorine dioxide/chlorine stage effluent by 50%<sup>7</sup>. A linear relationship has been reported between the number of mutants and the chlorine dioxide/chlorine ratio in this stage, the number of mutants decreasing with increasing chlorine dioxide/chlorine ratio. With pure chlorine dioxide, the number of mutants is close to the number found in the control sample<sup>7</sup>.

An alternative to the Ames test has been developed recently (Pro-Tox Assay, Xenometrix). In addition to evaluating mutagenicity, this assay provides information on the mechanism of toxicity. It employs stress gene promoters fused to a reporter gene. These promoters are the control regions which dictate the activity of defense and repair genes in bacteria. In most cases, the promoters act autonomously, such that when placed adjacent to a reporter gene by DNA technology, the adjacent gene becomes regulated by the promoter. In the Xenometrix Pro-Tox assay, the stress gene promoters are fused to the *lacZ* ( $\beta$ -galactosidase) structural gene in *E. coli*. The stress gene activity is measured by the hydrolysis of *o*-nitrophenyl- $\beta$ -galactopyranoside (ONPG) to the products galactose and the intensely yellow *ortho*-nitrophenol<sup>11</sup>. This change can be measured colorimetrically at 420 nm. The colorimetric change is directly related to the degree of stress promoter activity. Exposure to a toxicant that acts by a mechanism specific to the particular stress gene promoter triggers the production, by the reporter gene, of  $\beta$ -galactosidase whose activity is then measured colorimetrically. A battery of different stress promoter-*lacZ* bacterial strains are provided, each of which is sensitive to different

stress conditions. A flowsheet describing the steps involved in the Environmental Protocol for the Xenometrix assay is provided in Figure 1.

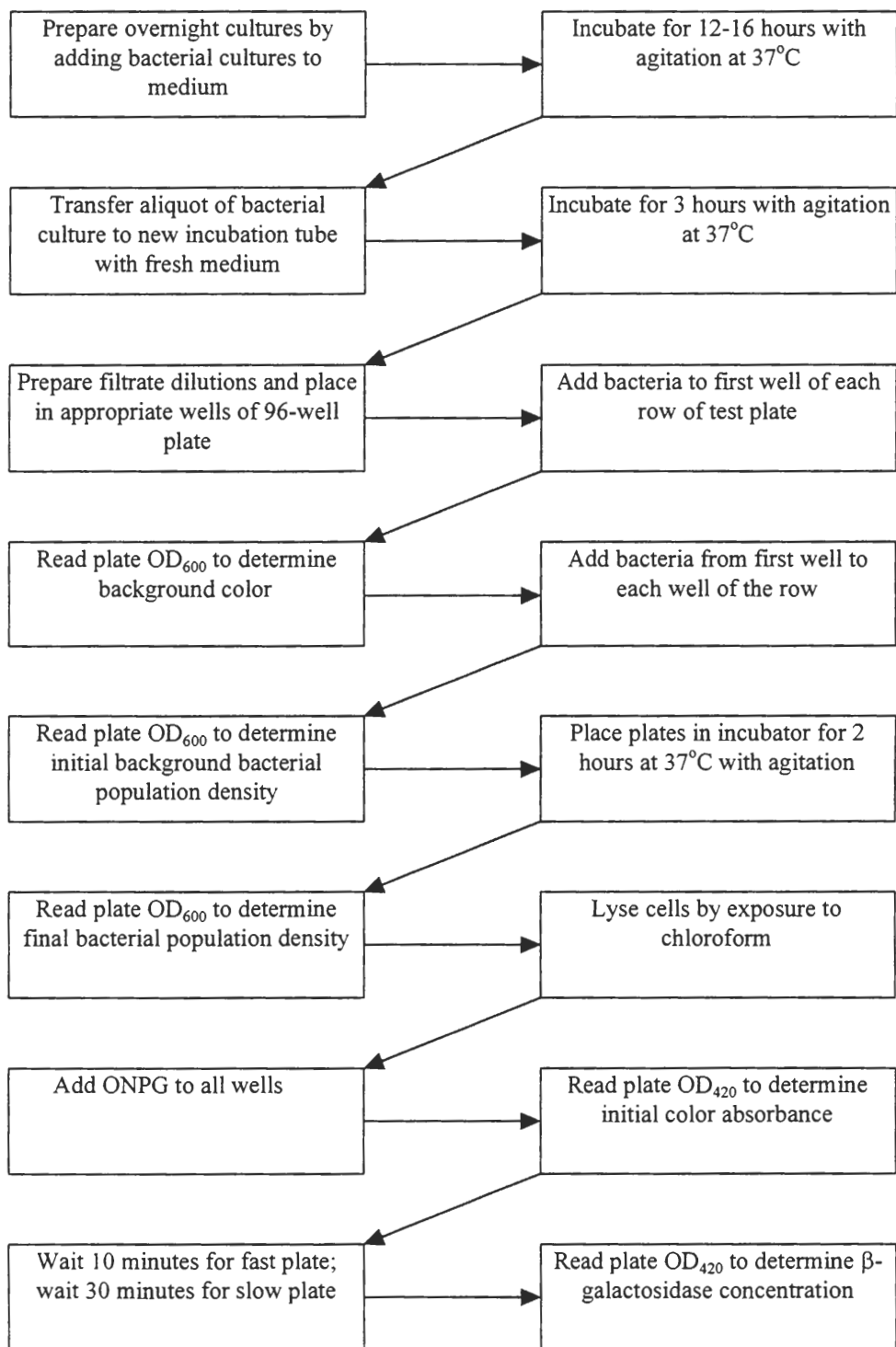
As shown in Figure 2, these assays can be performed in a 96-well plate format, which requires only small amounts of test compound and allows rapid testing of many mechanisms which may potentially explain the toxicity of a particular compound or solution. Seven concentrations of a compound can be tested for sixteen strains of *E. coli* bacteria altered with stress genes in a six hour period.

## **MATERIALS AND METHODS**

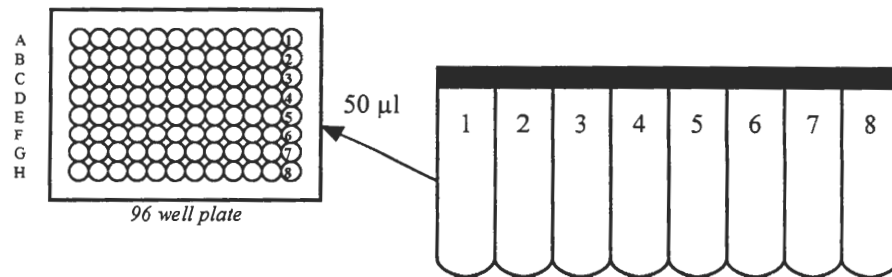
### **Bleaching and Filtrate Collection**

In bleaching kraft pulp, 45-90 kg of organic material is dissolved per metric ton of pulp. Of the dissolved organic material, 75-90% is usually produced during the first two bleaching stages<sup>9</sup>. For this reason, only the filtrates from the first oxidative bleaching stage and the first extraction stage were tested. All filtrates tested were generated in the laboratory.

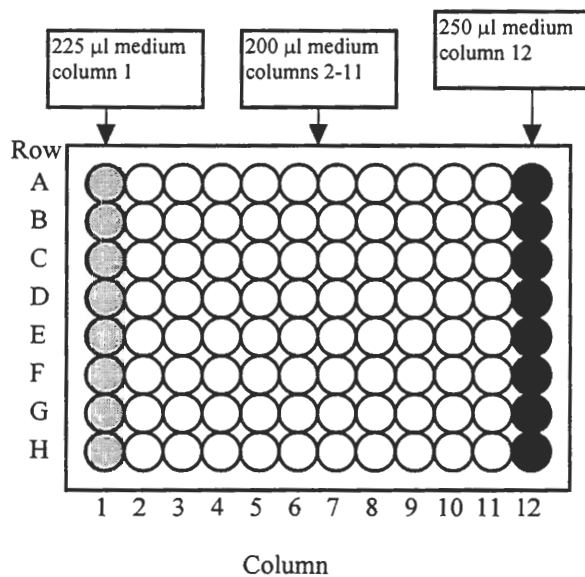
Oxygen delignified pulp was obtained from an operating pulp mill commercial facility immediately following the washing stage. A sample of the liquid phase accompanying the oxygen delignified pulp ("oxygen stage carryover") was separately obtained. In the laboratory, the pulp was repeatedly washed and centrifuged until no color remained in the wash water. The pulp was then centrifuged to approximately 35% consistency (consistency = weight of pulp/weight of pulp and liquid, expressed as a percent), fluffed, and stored at 4°C. In preparation for bleaching, 300 g. pulp, enough



**Figure 1.** Flowsheet of Environmental Protocol to Determine  $\beta$ -galactosidase Induced Activity. ( $OD_{600}$  = optical density at 600nm;  $OD_{420}$  = optical density at 420 nm.)



**Figure 2.** 96-Well Plate Format Used in Stress Gene Assay (Culture tubes are shown on right; 50µl of each strain added to one well shown on left. One 96-well plate was used for slow promoters and one plate used for fast promoters.)



**Figure 3.** Diagram of Bacterial Growth Plates

sulfuric acid to reduce the pulp solution to pH 1.8-2.0, oxygen stage carryover, and dilution water to make the solution 3% consistency were mixed in the reactor. The amount of oxygen carryover used corresponded to 10 kg chemical oxygen demand (COD) carryover per ton of pulp. Deionized water, preheated to 70°C, was used as dilution water. Chlorine and chlorine dioxide solution concentrations were determined immediately prior to bleaching using iodometric titration. For the “background” bleaches, deionized water was substituted for bleaching chemical solution.

The initial kappa number (kappa no.  $\approx 6.7 \times$  lignin, %) of the softwood kraft pulp was 17.2. Chlorine (C) and chlorine dioxide (D) stages were conducted at kappa factors of 0.15 and 0.25. The kappa factor is defined as follows:

$$\text{kappa factor} = \frac{\text{Equivalent Cl}_2 \text{ charge, \% dry pulp weight}}{\text{kappa number}}$$

The chlorine equivalency of  $\text{ClO}_2$  is given by the corresponding equivalent weight ratio (equivalent  $\text{Cl}_2 = \text{ClO}_2 \times 2.63$ ).

Immediately following the first bleaching stage, 30 minutes in duration, the pulp and filtrate were separated by filtration. The filtrate was collected for later testing. The pulp was then well washed with distilled water.

The second bleaching stage, an oxidative extraction (EO), was completed at 10% consistency. The pulp slurry was heated to 70°C prior to adding sodium hydroxide and oxygen. When the 70 minute bleaching time was complete, the pulp and suspension was diluted with cool water to 3% consistency and then mixed. The pulp and filtrate were

separated by filtration and the filtrate collected for testing, while the pulp was again well washed with distilled water. A kappa number test was used to determine the extent of lignin removal.

The first and second stage bleaching filtrates were combined for testing. No biological (secondary) treatment was used to reduce toxicity.

### **Stress Gene Assay**

The stress gene assay utilized a specialized kit (PRO-TOX C) prepared by Xenometrix, Inc (Boulder, CO)<sup>1</sup>. Sixteen *E. coli* strains were provided in the kit. Each strain had a different stress promoter fused to a *lacZ* structural gene. The stress promoters are listed in Tables 1 and 2. Fast promoters are described as those with a basal, or control,  $\beta$ -galactosidase activity greater than 100 units, while slow promoters are described as those with basal  $\beta$ -galactosidase activity lower than 100 units<sup>12</sup>. The strains were constructed by fusing the *lacZ* structural gene to the different stress promoters as described previously<sup>12</sup>.

All materials used, except for the 96-well plates, pipettes, and pipette tips, were provided by Xenometrix. Test solutions that were needed for the assay, such as sterile distilled water, growth medium and buffer were also provided. The buffer contained 16.1 g  $\text{Na}_2\text{HPO}_4 \bullet 7\text{H}_2\text{O}$ , 5.5 g  $\text{NaH}_2\text{PO}_4 \bullet \text{H}_2\text{O}$ , 0.75 g KCl, and 0.246 g  $\text{MgSO}_4 \bullet 7 \text{H}_2\text{O}$  in 1 L of sterile double deionized  $\text{H}_2\text{O}$ , adjusted to pH 7<sup>12</sup>. A solution containing 4 mg/ml *o*-nitrophenyl- $\beta$ -D-galactoside (ONPG) in the same buffer was also provided<sup>12</sup>.



For testing relatively non-toxic bleaching filtrates, it was necessary to use the “Environmental Protocol” since it allows for the testing of higher concentrations of the filtrates. Due to limited filtrate availability, each filtrate was run in duplicate, rather than the recommended triplicate.

The *E. coli* arrived in a freeze-dried form and were resuspended in growth medium and placed on a stir plate at 300 rpm in a 37°C incubator approximately 12-16 hours prior to testing. After approximately 15 hours, the overnight cultures were subcultured in order to obtain log phase cultures. The overnight cultures were diluted 1:50 with 1X growth medium. The diluted cultures were incubated at 37°C with agitation at 300 rpm until an optical density at 600 nm (OD<sub>600</sub>) reading between 0.3 and 0.4 was obtained; this takes approximately 3 hours.

Stock solutions were prepared at 111% of the desired exposure concentrations, to allow for the addition of bacteria, and added to the appropriate wells of the 96-well plate. The stock solutions of the aqueous environmental samples were prepared in 10X growth medium.

The bacterial growth plates were then prepared as shown in Figure 3.<sup>13</sup> An OD<sub>600</sub> reading was taken of all 96 well plates with control and filtrate solutions present to determine bacteria population density. Then, 25µl of bacteria were transferred to each test well. Another OD<sub>600</sub> reading of each plate was then taken. The plates were incubated for 2 hours at 37°C with agitation at 300 rpm to allow growth of bacteria in the presence of bleach effluents. Another OD<sub>600</sub> reading was taken at the end of the

incubation period to assess bacterial growth during that period. This provides information on the survival and growth of the bacterial strains in the presence of bleaching filtrates.

The bacterial cells were then lysed as follows to allow assay of synthesized  $\beta$ -galactosidase. In a hood, 200 $\mu$ l of solution from each well of the bacterial plates was transferred to a chloroform plate containing 50 $\mu$ l of chloroform per well. The plates were allowed to stand for 5-10 minutes in the fume hood to fully lyse the cells. The  $\beta$ -galactosidase plates were prepared by adding 170 $\mu$ l of buffer and 40 $\mu$ l ONPG to all test wells. To start the  $\beta$ -galactosidase assay plate, 40 $\mu$ l of the upper aqueous phase of the chloroform plate was transferred to the assay plate.

Each plate was read with a Bio-Tech Instruments biokinetic reader (EL312e) to obtain OD<sub>420</sub> (reading #1) to determine initial absorbance. The slow plates were then allowed to stand for 30 minutes before being read again (OD<sub>420</sub> reading #2). The fast plates were allowed to stand for only 10 minutes before the repeat OD<sub>420</sub> reading (OD<sub>420</sub> reading #2). The final three OD<sub>600</sub> readings and both OD<sub>420</sub> readings were used to determine  $\beta$ -galactosidase activity. The equation used to determine activity was:<sup>13</sup>

$$\text{Activity} = c [(\Delta\text{OD}_{420})/(\text{lag time} \times \Delta\text{OD}_{600})] \quad (1)$$

where: c=constant for Miller units conversion

lag time=length of time for  $\beta$ -galactose assay

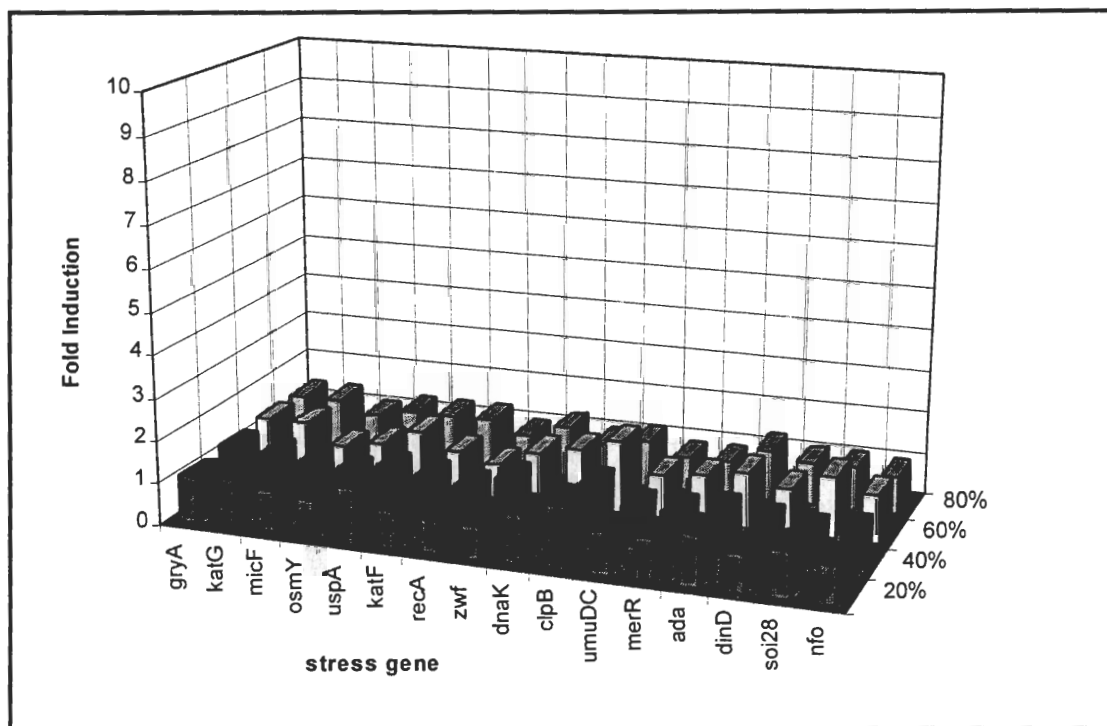
(10 minutes for fast promoters; 30 minutes for slow promoters)

$$\Delta\text{OD}_{420} = (\text{OD}_{420} \text{ reading \#2}) - (\text{OD}_{420} \text{ reading \#1}) \quad (2)$$

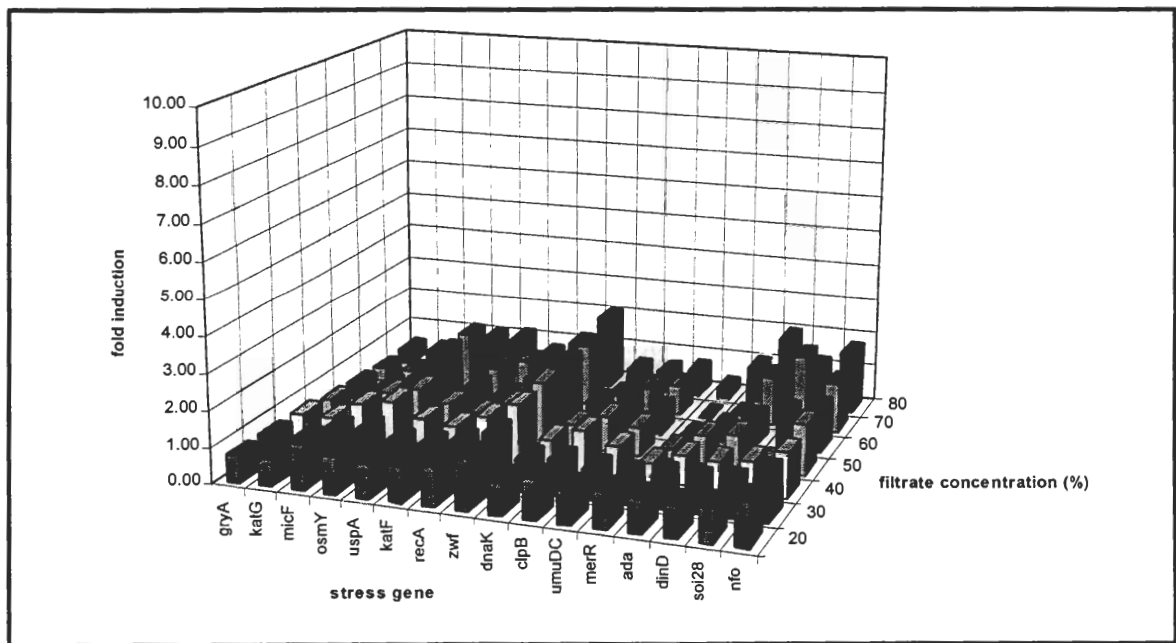
$$\Delta\text{OD}_{600} = [\text{OD}_{600} \text{ Post-Exposure} - (\text{OD}_{600} \text{ Post-Dose} - \text{OD}_{600} \text{ Pre-Dose})] \quad (3)$$

## RESULTS AND DISCUSSION

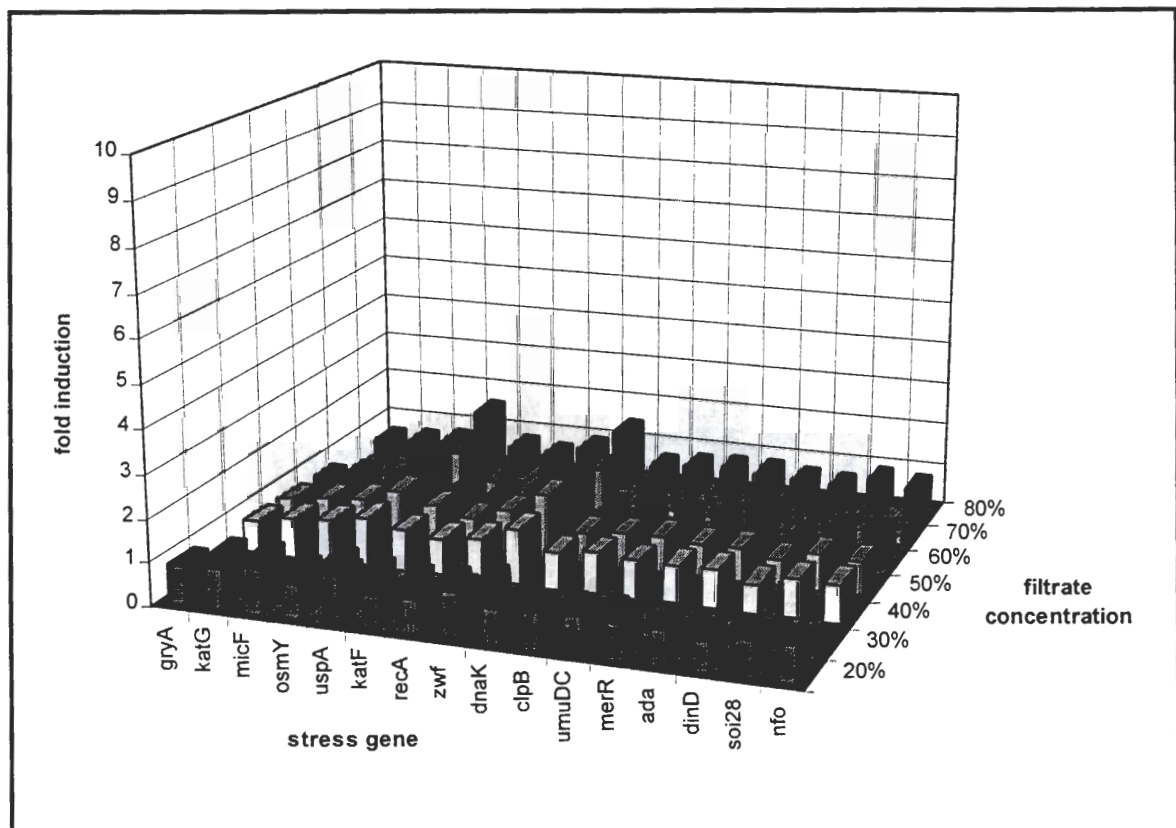
The extent of induction for each of the stress gene promoter types is shown graphically in Figures 4-8. Each vertical bar in the figures represents the average of duplicate measurements on each of two replicate bleaches. As can be observed in Figures 4-8, no filtrates showed any significant (greater than 2 fold) induction of any stress promoter.



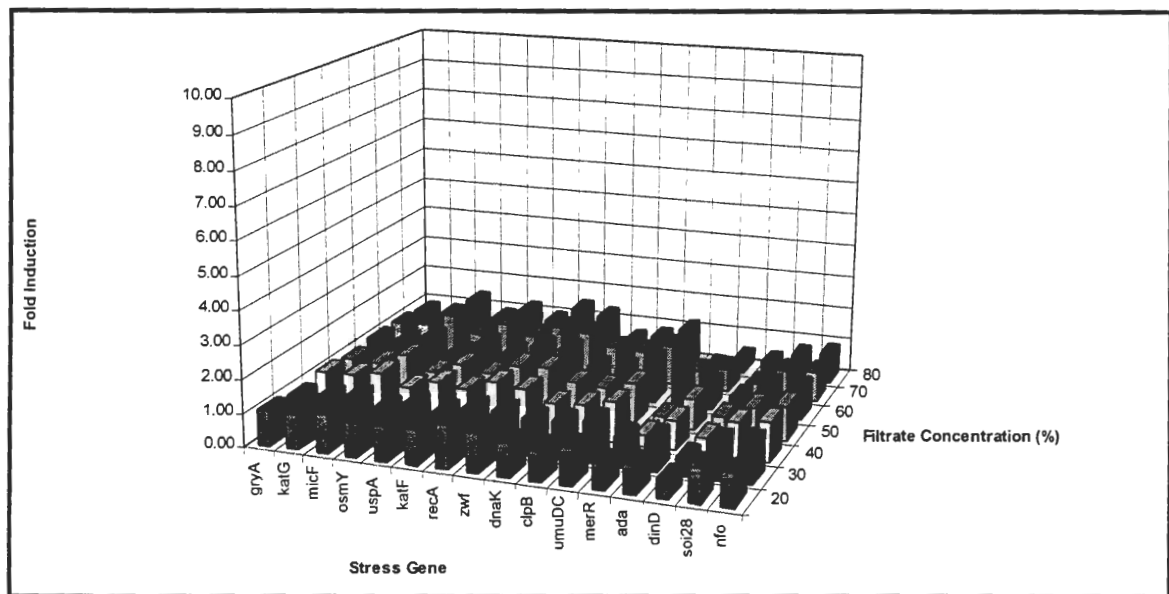
**Figure 4.** Induction of Stress Gene Activity When Exposed to Background Filtrates.



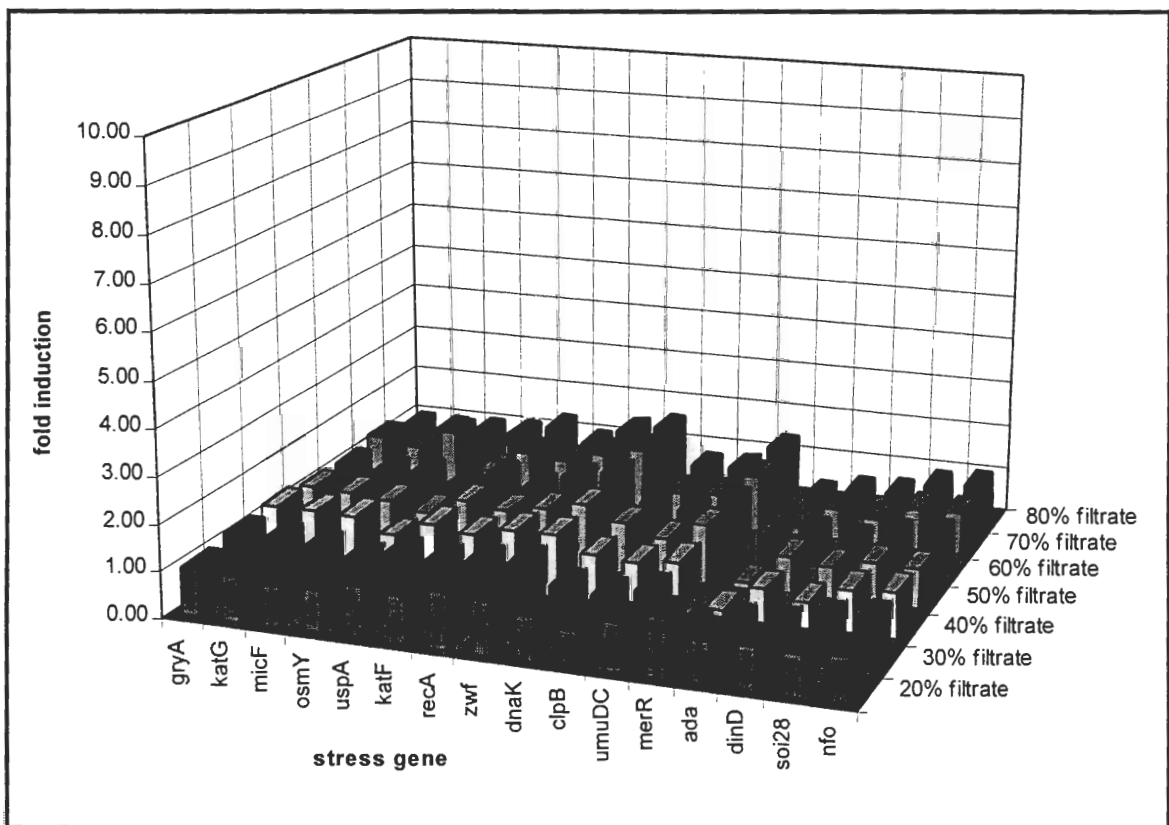
**Figure 5.** Induction of Stress Gene Activity When Exposed to 0.15 KF Chlorine Dioxide Bleaching Filtrates



**Figure 6.** Induction of Stress Gene Activity When Exposed to 0.25 KF Chlorine Dioxide Bleaching Filtrates



**Figure 7.** Induction of Stress Gene Activity When Exposed to 0.15 KF Chlorine Bleaching Filtrates



**Figure 8.** Induction of Stress Gene Activity When Exposed to 0.25 KF Chlorine Bleaching Filtrates

The observation that neither chlorine nor chlorine dioxide bleaching filtrates caused significant induction of any of the stress promoters suggests that neither bleaching agent generates toxicants that act by any of the mechanisms indicated in Table 2. Before this conclusion can be reached, however, other possible interpretations must be examined. Two such alternative interpretations, as suggested by Orser *et al.*,<sup>12</sup> are that toxic compounds which act by these mechanisms are present but are of a molecular size that is too large to permit entry into the cell, or that they require metabolic activation for toxicity. The first of these cannot be eliminated, but the second is unlikely since chlorination stage effluents have been found to undergo a reduction in mutagenic potential when liver microsomes are present, indicating a metabolic detoxification of the mutagens.<sup>10</sup>

Two other possibilities were eliminated on the basis of experimental evidence. These were (1) that the cells were dying at a rate that would not allow significant induction and (2) that the cells were stressed by the filtrate to a point that there was interference with their ability to participate in transcription or translation.

PROMOTER FUSION	INDUCING AGENTS
<i>gyrA</i>	responds to DNA damaging agents such as mitomycin C and 4-nitroquinoline 1-oxide
<i>katG</i>	responds to oxidative damage as caused by compounds like hydrogen peroxide
<i>micF</i>	responds to fluctuations in osmotic conditions as well as chemicals that effect the outer membrane such as ethanol
<i>osmY</i>	responds to changes in growth conditions and osmotic conditions and chemicals like sodium nitrite and ethanol
<i>uspA</i>	responds to DNA damaging agents such as 4-nitroquinoline 1-oxide and nalidixic acid
<i>katF</i>	responds to DNA damage, oxidative stress, and chemicals like mitomycin C and potassium cyanide
<i>recA</i>	responds to DNA damage and chemicals like methyl methanesulfonate and mitomycin C
<i>zwf</i>	Responds to oxidative stress as mediated by chemicals with paraquat and 4-nitroquinoline 1-oxide

**Table 2a.** Description of PRO-TOX C Fast Plate Promoters

PROMOTER FUSION	INDUCING AGENTS
<i>dnaK</i>	responds to growth temperature fluctuations and changes in growth conditions and compounds like 1-propanol and ethanol
<i>clpB</i>	responds to agents affecting proteins, such as acetone chloroform, and p-chloroaniline
<i>umuDC</i>	induced by DNA damaging agents like benzo[a]pyrene, mitomycin C, and 4-nitroquinoline 1-oxide
<i>merR</i>	Responds to the presence of heavy metals, eg. mercuric chloride, cadmium sulfate
<i>ada</i>	responds to DNA damaging agents like MMS, MNNG, and p-chloroaniline
<i>dinD</i>	responds to DNA damaging agents such as mitomycin C, MMS, MNNG, nalidixic acid, and 4-nitroquinoline 1-oxide
<i>soi28</i>	responds to superoxide radical anion generating compounds like paraquat and 4-nitroquinoline 1-oxide
<i>nfo</i>	responds to the presence of apurinic and apyrimidinic sites as well as ssDNA and dsDNA breaks caused by bleomycin

**Table 2b.** Description of PRO-TOX C Slow Plate Promoters.













The first possibility, that the cells exposed to filtrates are not alive, is addressed in Tables 3-8 and Figures 9-13, which show growth rates at all testing conditions, as inferred from the change in OD<sub>600</sub> readings over the course of the experiment as described earlier in this paper,. As shown in Figures 9-13, growth rates were greater than 80% of the control growth rates under all conditions, except at the highest levels of chlorine dioxide filtrate exposure. At these high levels, the growth rates were only moderately depressed, from which we conclude that it is possible, but unlikely, that the observed lack of induction at these levels was due to failure of the bacteria to reproduce at a sufficiently high rate.

The possibility that the cells were stressed to a point that there was interference with their ability to induce  $\beta$ -galactosidase activity, even when presented with an ideal inducer, is addressed in Figures 14 and 15. Figure 14 illustrates the evaluation of *E.coli* growth when exposed to 0.15 KF chlorine dioxide bleaching filtrates at a concentration of 50% with and without the addition of stress inducers known to induce specific stress gene activity. When exposed to the bleaching filtrate alone, all cultures grew at the same rate as the control cultures as shown in Figure 14. In parallel experiments, cultures of several *E. coli* strains were grown with bleaching filtrate plus a specific inducer, present at concentrations known to activate the appropriate promoter (Table 8).<sup>12</sup> Growth of the cultures was inhibited when both the bleaching filtrate and a known inducing agent were present in the cases of *katG*, *katF*, *merR*, and *osmY* strains, i.e. when menadione, methanol or sodium nitrite were present, together with the bleaching filtrate as shown in Figure 14. This indicates the existence of a synergistic growth inhibition between these

compounds (when present at the concentrations indicated in Table 8) and the bleaching filtrate. It may be speculated that sodium chlorite, which is frequently present in chlorine dioxide bleaching filtrates, is responsible for this synergistic effect. Sodium chlorite can be oxidized to chlorine dioxide, a known bactericide, and can itself oxidize appropriate substrates, with the possible production of species responsible for the observed effect. It is unlikely that sodium chlorite would survive the secondary biological treatment system used by virtually all U.S. bleached pulp mills.

The observation of synergistic negative effects of certain inducers and bleaching filtrate on growth rates in these experiments is not relevant to the interpretation of the lack of induction in the experiments described earlier, where no inducers was added.

Figure 15 examines the stress induction, expressed as fold induction (or ratio of induction of control to induction over treatment), over a two hour time period. Measurements of both OD<sub>600</sub> and OD<sub>420</sub> were taken every 15 minutes for 120 minutes. The bleaching filtrates did not impair the response of the cultures to known inducers, except in the cases of *katF* and *clpB*. The lack of response of *katF* may be attributed to its failure to grow in the presence of both the inducer and the bleaching filtrate. In the case of *clpB* either the bleaching filtrate did compromise the organism's ability to express the enzyme or the organism as supplied lacked that ability.

<b>Stress Gene Evaluated</b>	<b>Inducer</b>	<b>Inducer Concentration in Filtrate</b>
clp B	propanol	2.5% by volume
mer R	methanol	20% by volume
soi 28	paraquat	500 µg/ml
nfo	paraquat	500 µg/ml
kat G	menadione	30 µg/ml
kat F	menadione	30 µg/ml
osmY	sodium nitrite	5000 µg/ml
zwf	paraquat	125 µg/ml

**Table 8.** Stress Gene Promoters Used with in Conjunction with Bleaching Filtrate to Determine Induction

## CONCLUSIONS

Sixteen bacterial strains of *E.coli*, modified by fusing different stress promoters to a lacZ structural gene, have been used to estimate and compare the toxicities of untreated filtrates from chlorine and chlorine dioxide laboratory bleaching of oxygen delignified kraft pulp. The modified test organisms are designed to reveal the occurrence of specific toxicity mechanisms, including DNA damage of various types, oxidative damage, fluctuations in osmotic conditions, oxidative stress, protein damage, and heavy metal effects. None of the stress genes was induced in media containing filtrates from either chlorine or chlorine dioxide bleaching at kappa factors of 0.15 and 0.25, at concentrations as high as 80%. The bacteria grew normally in the presence of all filtrates. These

observations indicate that the bleaching effluents have none of the indicated toxic effects on the test organism.

## ACKNOWLEDGMENTS

The authors thank Dr. Earl Malcolm and Dr. Lucy Sonnenberg for technical advice and guidance, and the Institute of Paper Science and Technology and its member companies for the financial support provided for student research. Portions of this work were used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

## LITERATURE CITED

- 
1. Xenometrix, Inc., 2860 Wilderness Place; Boulder, Colorado 80301; 1-800-436-2869.
  2. Kringstad, K.P., Lindquist, P.O. and Stromberg, L.M., "Evaluation of Lipophilic Properties of Mutagens Present in the Spent Chlorination Liquor from Pulp Bleaching", *Environmental Science and Technology*, 18(3):200-203 (1984).
  3. Kringstad, K.P., Ljungquist, P.O., de Sousa, F. and Stromberg, L.M., "Stability of 2-chloropropenol and some other mutagens formed in the chlorination of softwood kraft pulp", *Environmental Science and Technology*, 17(8):468-471 (1983).
  4. Kringstad, K.P., Ljungquist, P.O., deSousa, F., and Stromberg, L.M., "Identification and mutagenic properties of some chlorinated aliphatic compounds in the spent liquor from kraft pulp chlorination", *Environmental Science and Technology*, 15(5):562-566 (1981).
  5. Holmbom, B.R., Voss, R.H., Mortimer, R.D., and Wong, A., "Isolation and Identification of Ames-Mutagenic Compounds Present in Kraft Chlorination Effluents", *TAPPI Journal*, 64(3):172-174 (1981).
  6. Rao, S.S., Burnison, B.K., and Rokosh, D.A., "Mutagenicity and Toxicity Assessment of Pulp Mill Effluent", *Chemosphere*, 28(10):1859-1870 (1994).



- 
7. Stockman, L., Stromberg, L., and de Sousa, F., "Mutagenic Properties of Bleach Plant Effluents: Present State of Knowledge", *Cellulose Chemistry and Technology*, 14:517-526.
  8. Rannug, U., Jenssen, D., Ramel, C., Erksso, K.E., and Kringstad, K., "Mutagenic Effects of Effluents from Chlorine Bleaching of Pulp", 7:33-47 (1981).
  9. Annergren, G., Rees-Andersson, A.M., Lindblad, P.O., McKague, B., Stromberg, L.M. and Kringstad, K.P., "Minimizing the Formation of Chlorinated Organic Material Through Controlled Chlorination in the Production of High Quality Softwood Kraft Pulp", *TAPPI Environmental Conference Proceedings*, p. 313-318 (1987).
  10. Ander, P., Eriksson, K.E., Kolar, M.C. and Kringstad, K., "Studies on the Mutagenic Properties of Bleaching Effluents", *Svensk Papperstidning*, 80(14):454-459 (1977).
  11. MacGregor, J.T., Faar, S., Tucker, J.D., Heddle, J.A., Tice, R.R., and Turteltaub, K.W., "New Molecular Endpoints and Methods for Routine Toxicity Testing", *Fundam. Appl. Toxicol.*, 26:153-173 (1995).
  12. Orser, C.S., Foong, F.C.F., Capaldi, S.R., Nalezny, J., MacKay, W., Benjamin, M., and Farr, S.B., "Use of Prokaryotic Stress Promoters as Indicators of the Mechanisms of Chemical Toxicity", *In Vitro Toxicology*, 8(1):71-85 (1995).
  13. Xenometrix Operator Manual, 1996, Boulder, Colorado.

The final paper of this chapter, and thesis, is titled, “Innovative Methods for Determining Pulp Bleaching Filtrate Toxicity”, by T.A. Ard and T.J. McDonough summarizes the previous three papers with additional discussion on similarities and differences between assays as well as the advantages and disadvantages of utilizing the assays for bleach plant filtrates.

**INNOVATIVE METHODS FOR DETERMINING PULP BLEACHING FILTRATE TOXICITY****T.A. Ard**Weyerhaeuser New Bern Pulp Mill  
New Bern, NC

and

**T.J. McDonough**Institute of Paper Science and Technology  
Atlanta, GA**ABSTRACT**

A laboratory study was conducted to comparatively evaluate the toxicities of chlorine and chlorine dioxide bleaching filtrates. These filtrates were compared to a control filtrate representing background toxicity. In each case, a sample of oxygen pre-delignified kraft pulp was treated with one of two commercially feasible oxidant charge levels, then filtered and washed before being subjected to a standard oxygen-reinforced alkaline extraction. The filtrates from both stages were collected and assayed separately and then in combination. The combined filtrates were then subjected to an ether extraction in order to fractionate the whole filtrate into ether-extractable fractions and non-ether extractable fractions. The ether-extractable fraction is considered to be of the most environmental importance. The toxicities of the whole filtrates and fractions were determined by using the Microtox™, XTT assay utilizing human liver cells (HepG2), and stress gene promoters utilizing 16 bacterial strains.

**KEY WORDS**

bleaching, filtrates, toxicity, stress, XTT, liver, Microtox, gene, promoters, stress

## INTRODUCTION

Environmental concerns are causing the pulp and paper industry to investigate alternatives to conventional bleaching sequences, which have generally utilized chlorine in the first bleaching stage. Little research has gone into quantifying the toxicity reduction achieved when alternative bleaching agents are substituted for chlorine.

Regulatory restrictions on effluent AOX are having a substantial impact on the use of chlorine and chlorine-containing compounds in the pulp and paper industry. Replacement of chlorine with chlorine dioxide results in a significant decrease in the amount of AOX produced.<sup>1,2</sup> However, there appears to be little relationship between AOX and toxicity.<sup>3,4</sup> Neither has any correlation been found between toxicity and BOD or TOC.<sup>5,6</sup> A relationship has been found between toxicity and the concentration of resin acids present.<sup>7</sup>

Despite the fact that chlorine-containing organic molecules are only found in low concentrations in bleach effluents, emphasis has been placed on these compounds due to their non-polar nature and lipid soluble character, which may lead to bioaccumulation in marine organisms. The octanol/water partition coefficient ( $K_{ow}$ ) is often used to predict the ability of a compound to bioaccumulate. However, when it is desired to evaluate the bioaccumulation potential of solutions of unknown organic content, such as bleach plant filtrates, it is more practical to use ether extractions to separate compounds by polarity.

For this study, the Microtox™ bioassay was chosen to evaluate the toxicity of the samples due to minimal time requirements, low cost, and protocol simplicity. The

Microtox assay allows for multiple evaluation of the toxicity endpoint for more statistically reliable determinations than standard aquatic toxicity assays.

The XTT assay utilizing human liver cells (HepG2) was chosen to evaluate potential human liver toxicity due to concerns from environmental groups that humans could be exposed to compounds found in bleaching filtrates through consumption of aquatic organisms, specifically fish. The XTT assay measures the ability of the electron transport system of the cells to function. If cells are functioning properly, the electron transport system will convert the XTT tetrazolium salt into a formazan, which is soluble and colorimetric, spectrophotometrically active at 600 nm.

The stress gene assay is used to determine toxicity mechanisms. Stress gene promoters are fused to a structural reporter gene whose product is easily measured. In the Pro-Tox assay, the stress gene promoters are fused to the *lacZ* ( $\beta$ -galactosidase) structural gene in *Escherichia coli*. The stress gene activity is measured by the degradation of *o*-nitrophenyl- $\beta$ -galactopyranoside (ONPG) to the products galactose and the intensely yellow *ortho*-nitrophenol.<sup>8</sup> This change can be measured as a colorimetric change at 420 nm. The colorimetric change is directly related to the degree of stress promoter induction.

## **MATERIALS AND METHODS**

### **Bleaching and Filtrate Collection**

In bleaching kraft pulp, 45-90 kg of organic material are dissolved per metric ton of pulp. Of the dissolved organic material, 75-90% is usually produced during the first two bleaching stages.<sup>9</sup> For this reason, only the filtrates from the first oxidative bleaching stage and the first extraction stage were tested. All filtrates tested were generated in laboratory bleaching facilities.

Oxygen delignified pulp was obtained from a commercial pulping facility immediately following the washing stage. A sample of the liquid phase accompanying the oxygen delignified pulp ("oxygen stage carryover") was separately obtained. In the laboratory, the pulp was repeatedly washed and centrifuged until no color remained in the wash water. The pulp was then centrifuged to approximately 35% consistency (consistency = weight of pulp/weight of pulp and liquid, expressed as a percent), fluffed, and stored at 4°C. In preparation for bleaching, 300 g pulp, enough sulfuric acid to reduce the pulp solution to pH 1.8-2.0, oxygen carryover, and dilution water to make the solution 3% consistency were mixed in the reactor. The amount of oxygen carryover required was determined by assuming a well-washed system, achieving only 10 kg COD carryover per ton of pulp. Distilled water, preheated to 70°C, was used as dilution water. Chlorine and chlorine dioxide solution concentrations were determined immediately prior to bleaching using iodometric titration. For the "background" bleaches, distilled water was substituted for bleaching chemical.

The initial kappa number (kappa no.  $\approx 6.7 \times$  lignin, %) of the softwood kraft pulp was 17.2. Chlorine (C) and chlorine dioxide (D) stages were conducted at kappa factors of 0.15 and 0.25. The kappa factor is defined as follows:

$$\text{kappa factor} = \frac{\text{Equivalent Cl}_2 \text{ charge, \% dry pulp weight}}{\text{kappa number}}$$

The chlorine equivalency of  $\text{ClO}_2$  is given by the corresponding equivalent weight ratio (equivalent  $\text{Cl}_2 = \text{ClO}_2 \times 2.63$ ).

Immediately following the first bleaching stage, 30 minutes in duration, the pulp and filtrate were separated by filtration. The filtrate was collected for later testing. The pulp was then washed well with distilled water.

The second bleaching stage, an oxidative extraction (EO), was completed at 10% consistency. The pulp slurry was heated to  $70^\circ\text{C}$  prior to adding sodium hydroxide and oxygen. When the 70-minute bleaching time was complete, the pulp and suspension were diluted with cool water to 3% consistency and then mixed. The pulp and filtrate were separated by filtration and the filtrate collected for testing, while the pulp was again washed well with distilled water. A kappa number test was used to determine the extent of lignin removal. The first and second stages were added together in equal volumes, mixed well, and pH adjusted to less than 2.

### **Ether Extractions**

Continuous ether extractions were performed in 4 L glass extractors. First and second bleaching stage filtrates were combined in equal volumes and acidified to pH 2.

Four liters of combined (first and second stage) filtrates were added to the extractor and connected to a 1 L round bottom flask containing 500 mls ether. Extraction with refluxing ether was continued for four days. A rotary evaporator, under vacuum, was used for 24 hours to remove the ether from both the ether-extract and the extracted filtrate.

All of the ether-extract (EE) was evaporated to dryness and then reconstituted with filtered double deionized water to 250 mls. There were four liters of extracted filtrate following the ether fractionation procedure; however, it required another two liters of double deionized water,  $\text{H}_2\text{SO}_4$ , and NaOH to completely remove the extracted filtrate from the extractor. An aliquot of 500 mls of extracted filtrate, now referred to as non-ether extract (NEE) was evaporated for 24 hours and then reconstituted to 500 mls. The remainder (5500 mls) of the extracted filtrate and wash solution was disposed.

In order to accurately compare the toxicities of the whole filtrates to the fractions, the measured toxicities had to be recalculated to account for the various dilutions and concentrations. The 250 mls of the EE fraction actually represent the compounds from 4L of filtrate; likewise, the 500 mls of NEE fraction were taken from a diluted source (6L) of 4L of filtrate. Therefore, the toxicity of the EE fraction was multiplied by 16 to put the toxicity on an equivalent basis with the whole filtrate. The toxicity of the NEE fraction was multiplied by (4/6) in order to put the NEE toxicity on an equivalent basis with the whole filtrate. All toxicity numbers presented in the paper have already been placed on an equivalent basis.



**Microtox™**

Microtox™ is a trademark of Microbics Corporation. The assay uses photoluminescent bacteria (*Vibrio fischeri lux*) as the test organism. The bacterium strain NRRL B-1117 is deposited with the Northern Regional Laboratory in Peoria, Illinois, USA.<sup>10</sup> This strain of bacteria diverts approximately 10% of its metabolic energy into a metabolic pathway that converts chemical energy into visible light. Light emission results from the interaction of an enzyme, luciferase, and a long chain aldehyde in the presence of oxygen. This interaction constitutes a fraction of the cell electron transport system. The emission of light is dependent on this electron flow. Therefore, the light output reflects changes in the metabolic activity of the organism.<sup>11</sup> The metabolic pathway is directly related to the respiration of the organism. A change in cellular metabolism or a disruption of the cellular structure results in a change of respiration and a proportional change in bioluminescence<sup>12</sup>.

EC50 is the sample concentration that reduces the light output by 50%. For this research, 15 minute EC50 values were used. A 15 minute EC50 is the concentration that results in a 50% reduction in light output after 15 minutes of organism exposure to the test compound. For this reason, we used the 100% assay which evaluates four concentrations of each filtrate (90, 45, 22.5, and 11.25%) and compares to a control. Approximately  $10^6$  microorganisms are used to evaluate each test concentration.<sup>12</sup> The EC50 term can be confusing since toxicity increases as EC50 decreases and vice versa. The use of toxicity unit, TU, eliminates this problem. TU is defined as 100 divided by the EC50 (expressed as a percent).

Immediately after bleaching or ether fractionation, the filtrate is adjusted to a pH of 7.4  $\pm$  0.05, filtered with a 0.22 $\mu$ m filter, and then stored at -70°C until tested. Each test sample was evaluated in triplicate following the protocol described by the Microtox manual.<sup>13</sup>

### **XTT Assay**

HepG2 (human liver) cells were used in the XTT assay analysis. The HepG2 cells were cultured and maintained in an incubator at 37°C. The HepG2 line was maintained as stock in EMEM (Atlanta Biologicals, Atlanta, Georgia) supplemented with 10% fetal bovine serum and 5% donor horse serum, sodium pyruvate, non-essential amino acids, gentamycin, and Hanks medium. The horse serum was only used during cell maintenance and was deleted from the medium preparation during testing. The cell cultures were passaged once or twice weekly using trypsin-EDTA for detachment from the culture flask.

Prior to testing, cells were trypsinized from their 75 cm<sup>2</sup> flasks and placed into 96-well plates at approximately 10,000 cells/well. Twenty-four hours later, the appropriate concentrations of filtrates were added to the wells. For each column of twelve wells, there were two control wells and five concentrations (25, 10, 5, 1, and ½%) of each of two filtrates. Four columns were used as replicates for each filtrate. After 24 hours of cell exposure to filtrates, a 0.025 mM PMS-XTT solution was prepared and administered to the 96-well plates as described by Scudiero et al.<sup>14</sup> The plates were placed back in the incubator for exactly four hours. They were then removed, placed in a 96-well plate reader and evaluated for absorbance at 600 nm.

## Stress Gene Assay

The stress gene assay utilized a specialized kit (PRO-TOX C) prepared by Xenometrix, Inc.<sup>15</sup> Sixteen *E. coli* strains were provided in the kit. Each strain had a different stress promoter fused to a *lacZ* structural gene. The stress promoters are listed in Tables 1 and 2. Fast promoters are described as those with a basal  $\beta$ -galactosidase activity greater than 100 units, while slow promoters are described as those with basal  $\beta$ -galactosidase activity lower than 100 units.<sup>8</sup> The strains were constructed by fusing the *lacZ* structural gene to the different stress promoters as described previously.<sup>8</sup>

All materials used, except for the 96-well plates, pipettes, and pipette tips, were provided by Xenometrix. Test solutions that were needed for the assay, such as sterile distilled water, Pro-Tox Medium, ONPG, and Pro-Tox Buffer were also provided. Pro-Tox Medium contains nutrients for the growth of the bacterial strains. Pro-Tox Buffer contains 16.1 g  $\text{Na}_2\text{HPO}_4 \cdot 7 \text{H}_2\text{O}$ , 5.5 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 0.75 g KCl, 0.246 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  in 1 L of sterile ddH<sub>2</sub>O, adjusted to pH 7.<sup>15</sup> ONPG solution contains 4 mg/ml *o*-nitrophenyl- $\beta$ -D-galactoside in Pro-Tox Buffer.<sup>15</sup>

For testing relatively non-toxic bleaching filtrates, it was necessary to use the “Environmental Protocol” since it allows for the testing of higher concentrations of the filtrates. Due to the limited amount of filtrate available, each filtrate was run in duplicate, rather than the recommended triplicate.

PROMOTER FUSION	INDUCING AGENTS
<i>gyrA</i>	responds to DNA damaging agents such as mitomycin C and 4-nitroquinoline 1-oxide
<i>katG</i>	responds to oxidative damage as caused by compounds like hydrogen peroxide
<i>micF</i>	responds to fluctuations in osmotic conditions as well as chemicals that effect the outer membrane such as ethanol
<i>osmY</i>	responds to changes in growth conditions and osmotic conditions and chemicals like sodium nitrite and ethanol
<i>uspA</i>	responds to DNA damaging agents such as 4-nitroquinoline 1-oxide and nalidixic acid
<i>katF</i>	responds to DNA damage, oxidative stress, and chemicals like mitomycin C and potassium cyanide
<i>recA</i>	responds to DNA damage and chemicals like methyl methanesulfonate and mitomycin C
<i>zwf</i>	Responds to oxidative stress as mediated by chemicals with paraquat and 4-nitroquinoline 1-oxide

**Table 1.** Description of PRO-TOX C Fast Plate Promoters

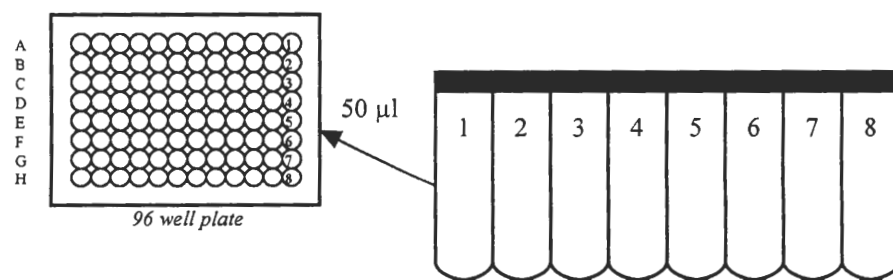
PROMOTER FUSION	INDUCING AGENTS
<i>dnaK</i>	responds to growth temperature fluctuations and changes in growth conditions and compounds like 1-propanol and ethanol
<i>clpB</i>	responds to agents affecting proteins, such as acetone chloroform, and p-chloroaniline
<i>umuDC</i>	induced by DNA damaging agents like benzo[a]pyrene, mitomycin C, and 4-nitroquinoline 1-oxide
<i>merR</i>	Responds to the presence of heavy metals, eg. mercuric chloride, cadmium sulfate
<i>ada</i>	responds to DNA damaging agents like MMS, MNNG, and p-chloroaniline
<i>dinD</i>	responds to DNA damaging agents such as mitomycin C, MMS, MNNG, nalidixic acid, and 4-nitroquinoline 1-oxide
<i>soi28</i>	responds to superoxide radical anion generating compounds like paraquat and 4-nitroquinoline 1-oxide
<i>nfo</i>	responds to the presence of apurinic and apyrimidinic sites as well as ssDNA and dsDNA breaks caused by bleomycin

**Table 2.** Description of PRO-TOX C Slow Plate Promoters.

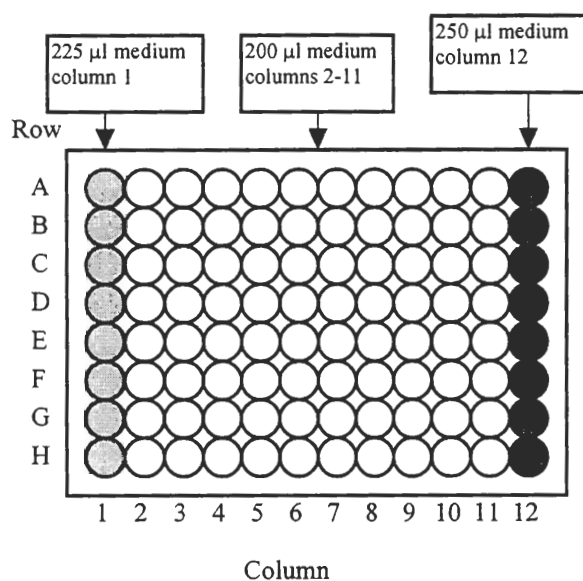
The *E. coli* arrive in a freeze-dried form and are resuspended in medium and placed on a stir plate at 300 rpm in a 37°C incubator approximately 12-16 hours prior to testing. After approximately 15 hours, the overnight cultures were subcultured in order to obtain log phase cultures. The overnight cultures were diluted 1:50 with 1X Pro-Tox Medium. The diluted cultures were incubated at 37°C with agitation at 300 rpm until an OD<sub>600</sub> reading between 0.3 and 0.4 was obtained; this takes approximately 3 hours.

Stock solutions are prepared at 111% of the desired exposure concentration to allow the addition of bacteria. The stock solutions of the aqueous environmental samples are prepared in 10X Pro-Tox Medium.

The bacterial growth plates are then prepared as shown in Figure 1.<sup>16</sup> An OD<sub>600</sub> reading was taken of all plates. Then, 25 µl of bacteria were transferred to each test well. Another OD<sub>600</sub> reading of each plate was then taken. The plates were incubated for 2 hours at 37°C with agitation at 300 rpm. Another OD<sub>600</sub> reading is taken at the end of the incubation period. This information provides the survival and growth of the bacterial strains.



**Figure 1a.** 96-Well Plate Format Used in Stress Gene Assay (Culture tubes are shown on right; 50 $\mu$ l of each strain added to one well shown on left. One 96-well plate was used for slow promoters and one plate used for fast promoters.)



**Figure 1b.** Diagram of Bacterial Growth Plates

The bacterial cells were then lysed as follows to allow assay of synthesized  $\beta$ -galactosidase. In a hood, 200  $\mu$ l of solution of each well of the bacterial plates are transferred to a chloroform plate containing 50  $\mu$ l of chloroform per well. The plates are allowed to stand for 5-10 minutes in the fume hood to fully lyse the cells. The  $\beta$ -galactosidase plates are prepared by adding 170  $\mu$ l of Pro-Tox Buffer and 40  $\mu$ l ONPG to all test wells. To start the  $\beta$ -galactosidase assay plate, 40  $\mu$ l from only the upper aqueous phase of the chloroform plate is transferred to the assay plate.

Each plate was read with a Bio-Tech Instruments biokinetic reader (EL312e) to obtain OD<sub>420</sub> (reading #1) to determine initial absorbance. The slow plates were then allowed to stand for 30 minutes before being read again (OD<sub>420</sub> reading #2). The fast plates are allowed to set for only 10 minutes before the repeat reading (OD<sub>420</sub> reading #2). The final three 600 readings and both 420 readings were used to determine  $\beta$ -galactosidase activity. The equation used to determine activity was:<sup>17</sup>

$$\text{Activity} = c [(\Delta\text{OD}_{420})/(\text{lag time})(\Delta\text{OD}_{600})] \quad (1)$$

where:  $c$  = constant for Miller units conversion  
lag time = length of time for  $\beta$ -galactose assay  
(10 min. for fast promoters; 30 min. for slow promoters)

$$\Delta\text{OD}_{420} = [(\text{OD}_{420} \text{ Reading \#2}) - (\text{OD}_{420} \text{ Reading \#1})] \quad (2)$$

$$\Delta\text{OD}_{600} = [\text{OD}_{600} \text{Post-Exposure} - (\text{OD}_{600} \text{Post-Dose} - \text{OD}_{600} \text{Pre-Dose})] \quad (3)$$

## **Biotreatment**

All U.S. mills are equipped with a minimum of secondary, or biological, treatment. Untreated filtrates are not discharged directly into the aquatic environment. It was desired to emulate the effects of pulp and paper secondary biotreatment facilities in order to have a more realistic view of environmental impact of effluents in the aquatic environment. Therefore, laboratory scale biotreatment was completed on all whole, combined filtrates.

Filtrates were diluted to 25% v/v with double deionized, double filtered water, and then the pH was adjusted to 7.0 +/- 1.0. Four hundred (400) mls of each diluted filtrate were added to an Erlenmeyer flask, and then one Hach BOD capsule (Hach, Loveland, Colorado) was added to each flask. The flasks were placed on a shaker at 200 rpm at room temperature. After five days of treatment, the contents of each flask were placed in a 1000 ml round bottom flask. The round bottom flask was then placed on a rotary evaporator in a water bath at 40°C until approximately 50 mls of filtrate remained. The filtrate was then washed from the round bottom flask with double deionized, double filtered water and diluted back to its original concentration (100 mls). The filtrate pH was adjusted to 7.4 +/- 0.05. The filtrate was then filtered with a 0.22µm filter and stored at -70°C until tested for toxicity.

## **Statistical Methods**

One sample (high charge chlorine, first stage, second replicate) was lost to a laboratory accident. This left the experimental design unbalanced. For the purpose of statistical analysis, the toxicity of the destroyed sample was estimated by the method of



fitting constants.<sup>18</sup> Significant factors were identified by use of a split-plot design analysis of variance. Significant differences between means were identified by using Tukey's Least Significant Difference Test (q-test).<sup>19</sup> Significance in all tests was determined at the 95% confidence level. Standard error was used to determine the values of all error bars in graphs.

## **RESULTS AND DISCUSSION**

Complete discussions of results are published elsewhere, but a summary of all results is included below.

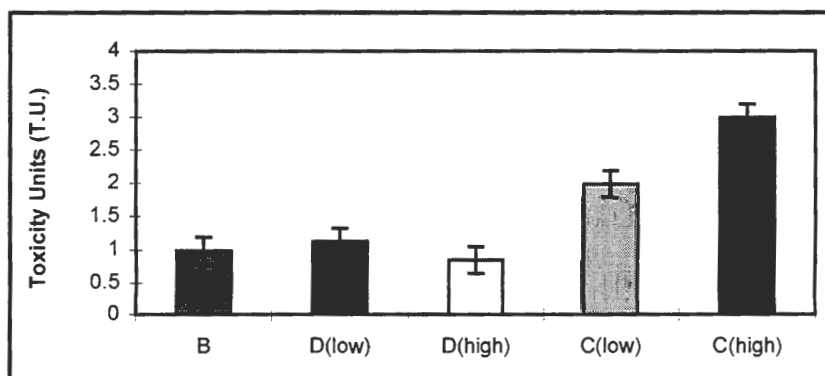
### **Microtox**

#### **Bleaching Filtrate Toxicity**

The two bleaching agents (chlorine and chlorine dioxide) were both evaluated at two bleaching charges - 0.15 KF and 0.25 KF. The 0.15 KF bleaching, whether chlorine or chlorine dioxide, is referred to as the "low" charge; the 0.25 KF bleaching is referred to as the "high" charge. Chlorine, chlorine dioxide, and background bleaching filtrate toxicities are discussed in this section.

The bleaching agent and charge used in the first bleaching stage were significant at the 95% confidence level. The q-test was used to determine the differences between bleaching agents and charges. The least significant difference used to evaluate these differences was found to be 0.63 toxicity units.

Both, low and high, chlorine charge filtrates were significantly higher than both chlorine dioxide charges and the background filtrates as shown in Figure 2.

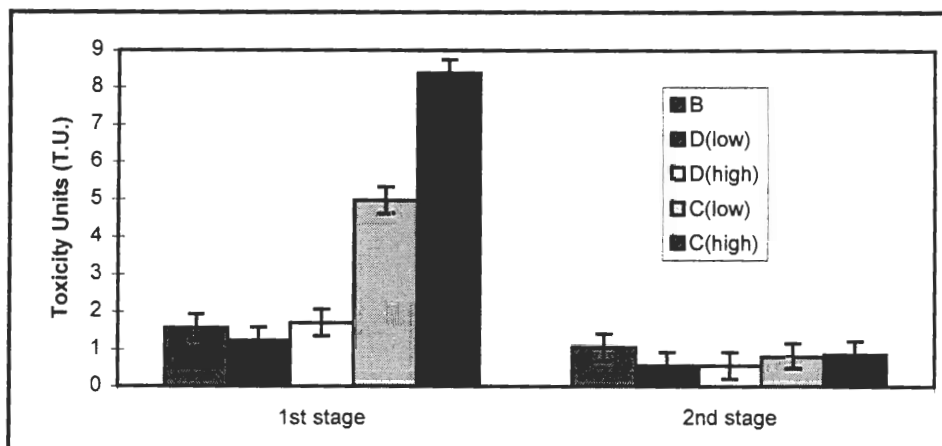


**Figure 2.** Average Toxicity of All Bleaching Filtrates and Fractions.

Both charges of chlorine dioxide were found to be lower in toxicity than the chlorine filtrates, but were not significantly different than the toxicity of the background filtrates. The background toxicity was also found to be significantly lower than both of the chlorine charge filtrates, but not significantly different from the chlorine dioxide filtrates.

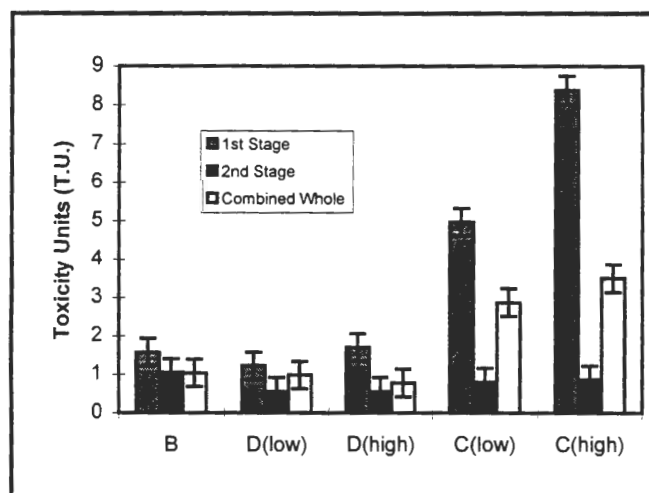
#### Bleaching Agent and Bleaching Stage Interaction

As shown in Table 3, the filtrate bleaching stages and fractions are significant factors. Again, utilizing the q-test with a least significance difference of 1.18 toxicity units, differences between bleaching stages and ether extraction fractions were determined. The first bleaching stage filtrates are significantly more toxic than the second stage bleaching filtrates. The toxicities of the bleaching filtrates from the first and second bleaching stages for the various bleaching agents and charges are shown in Figure 3.



**Figure 3.** Toxicity of First and Second Stage Bleaching Filtrates

Because there is also a significant bleaching agent and bleaching stage interaction, it is interesting to observe the variations in relationships of the first and second stages in conjunction with the combined whole filtrates in reference to the various bleaching agents. Figure 4 illustrates the bleaching agent and bleaching stage interactions.

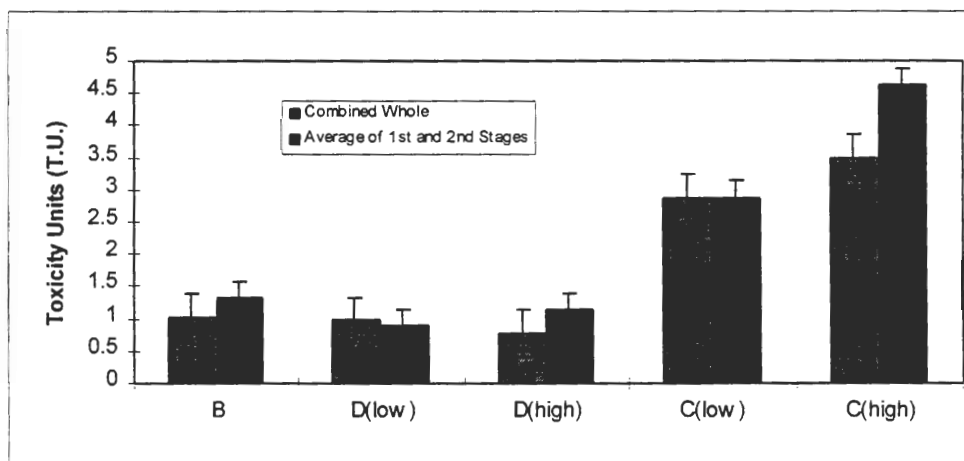


**Figure 4.** Toxicity Comparison of 1<sup>st</sup>, 2<sup>nd</sup>, and Combined Whole Bleaching Filtrates.

### Additive Effects of First and Second Bleaching Filtrate Toxicities

When combining chemical compounds, it is of interest whether the toxicity of the combined solution shows increased or decreased toxicity of the individual compounds. When two compounds are combined and the toxicity of the first and the second add to the sum of the combined solution, the addition is considered additive. If the first and second compounds combined to produce a solution higher than the sum of their toxicities, the addition is considered synergistic. If the first and second compounds combine to produce a solution lower in toxicity than the sum of their toxicities, the addition is considered antagonistic.

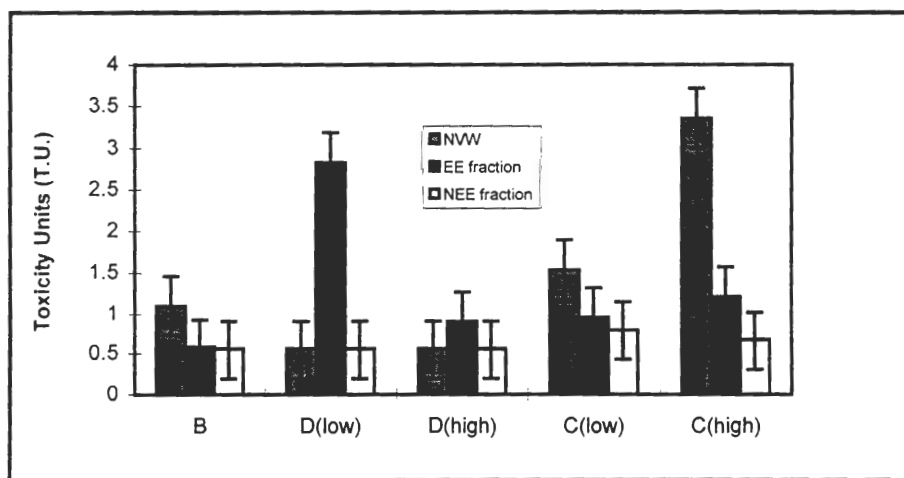
Figure 5 illustrates the comparison of the whole (combined) filtrates with the average of the first and second stage filtrates. The average of the 1<sup>st</sup> and 2<sup>nd</sup> stages is approximately the same as the combined whole, indicating an additive effect of the two bleaching stages when mixed.



**Figure 5.** Comparison of Theoretical Combined Whole and Actual Combined Whole.

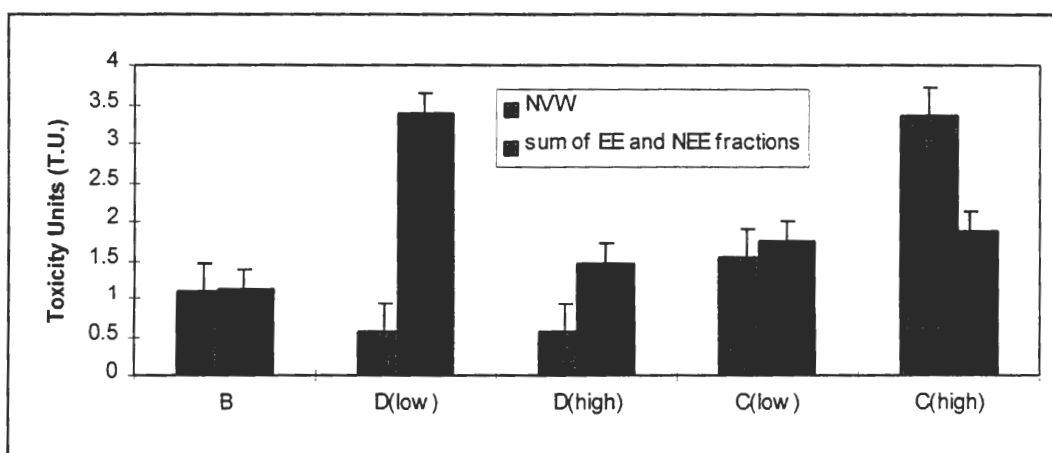
## Ether Filtrate Fractions and Bleaching Agent Interactions

Figure 6 illustrates the bleaching agent and ether fraction interaction. The most notable difference between bleaching agents is that the D(low) has a significantly higher ether extractable fraction than the other bleaching agent filtrates.



**Figure 6.** Comparative Toxicity of Filtrates from Non-Volatile Whole (NVW), Ether Extractable Fraction (EE) and Non-Ether Extractable (NEE) Fraction.

Figure 7 illustrates the comparison of the non-volatile whole (NVW) filtrate with the average of the ether extractable (EE) and non-ether extractable (NEE) fractions in order to identify synergistic, additive, or antagonistic effects. Both the background filtrates and the low charge chlorine filtrates showed additive effects when combined. Both chemical charges of chlorine dioxide filtrates showed antagonistic effects when combined. On the other hand, the high charge chlorine filtrates showed synergistic effects when combined.



**Figure 7.** Comparison of NVW Filtrate Toxicity and Sum of EE and NEE Fraction Toxicity.

### Effect of Biotreatment on Toxicity

Toxicity reductions as a result of benchtop biotreatment are shown in Table 4.

Caution must be exerted when examining these results due to several reasons. The first is that the biotreatment utilized in this research varies greatly from the biotreatment used in an industrial setting. Secondly, it is typical in industry to optimize the microbes used for biotreatment in order to utilize the most effective biotreatment system. This could not be done in our system without biasing the results. Finally, in an industrial biotreatment system, there are many other pulp and paper streams added to the bleaching stream which may aid biotreatment and detoxification by breaking down molecules for easy consumption by the microbes.

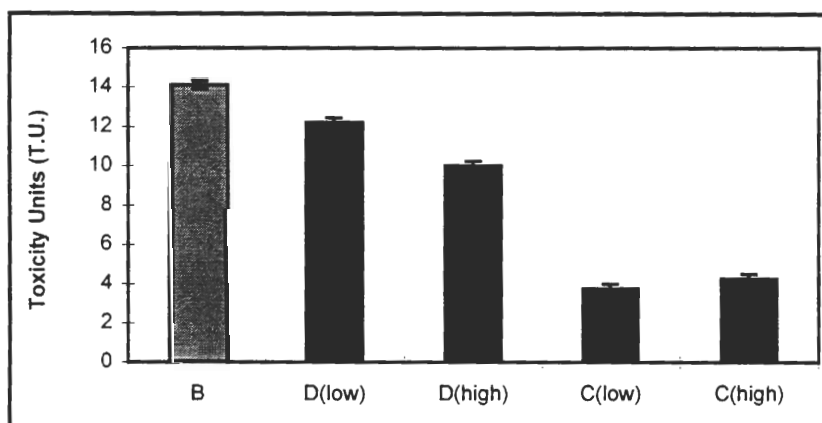
Sample	Untreated	Biotreated	Reduction
B whole rep 1	1.5 +/- 1.2	0.6 +/- 1.2	0.9 +/- 1.2
B whole rep 2	0.6 +/- 1.2	0.6 +/- 1.2	0 +/- 1.2
D(low) whole rep 1	0.8 +/- 1.2	2.6 +/- 1.2	-1.8 +/- 1.2
D(low) whole rep 2	1.2 +/- 1.2	2.7 +/- 1.2	-1.5 +/- 1.2
D(high) whole rep 1	1.0 +/- 1.2	0.6 +/- 1.2	0.4 +/- 1.2
D(high) whole rep 2	0.6 +/- 1.2	3.9 +/- 1.2	-3.3 +/- 1.2
C(low) whole rep 1	3.2 +/- 1.2	2.7 +/- 1.2	0.5 +/- 1.2
C(low) whole rep 2	2.5 +/- 1.2	0.6 +/- 1.2	1.9 +/- 1.2
C(high) whole rep 1	3.2 +/- 1.2	3.5 +/- 1.2	-0.3 +/- 1.2
C(high) whole rep 2	3.8 +/- 1.2	3.4 +/- 1.2	0.4 +/- 1.2

**Table 4.** Microtox Toxicity. (as Toxicity Units)

### XTT (HepG2) Assay

#### Bleaching Agent Effect on Toxicity

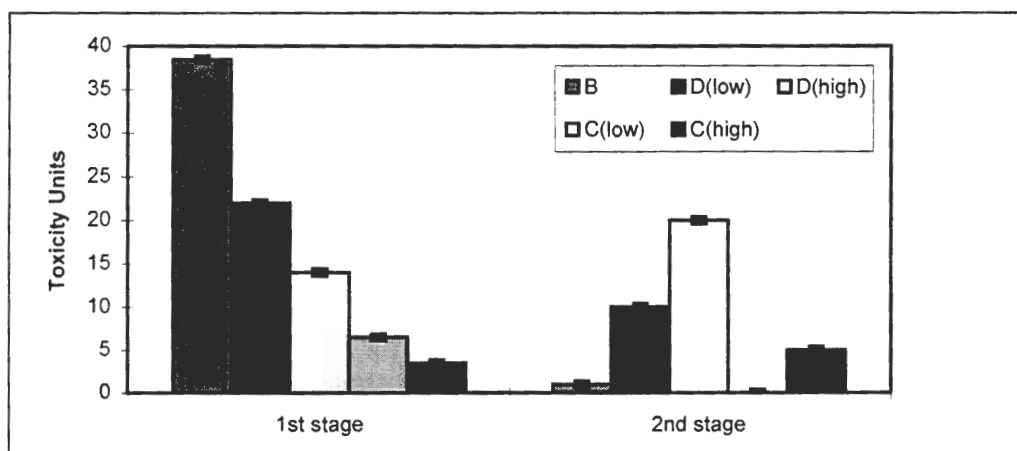
As determined by analysis of variance and illustrated in Figure 8, the bleaching agent used in the first stage had a significant effect on the resulting filtrate. The background filtrates, or filtrates produced by using no chemical bleaching agent in the first bleaching stage, were the most toxic. Filtrate toxicity was found to be as follows (in decreasing order): Background > D (low charge) > D (high charge) > C (high charge) > C (low charge).



**Figure 8.** Average Toxicity of All Bleaching Filtrates and Fractions.

## Bleaching Filtrate Stage

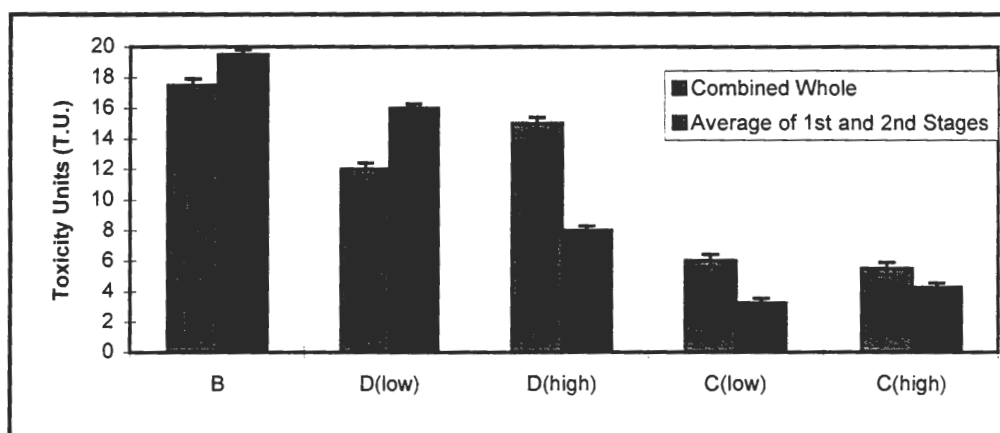
There were also significant differences between bleaching stages. The first bleaching stage, as shown in Figure 9, is higher in toxicity than the filtrates from the second bleaching stage (oxidative extraction).



**Figure 9.** Toxicity of First and Second Stage Bleaching Filtrates.

The combined filtrates (1<sup>st</sup> and 2<sup>nd</sup> stages), which theoretically, if no synergistic activity between stages was occurring, would equal the mean of the first and second stages. As Figure 10 illustrates, low charge chlorine dioxide results in antagonistic effects when the stages are mixed. Likewise, the background filtrates show slight antagonistic effects. Both the high charge chlorine dioxide and low charge chlorine result in filtrates which show synergistic effects when added, while the high charge chlorine filtrates result in slight synergistic effects when added.





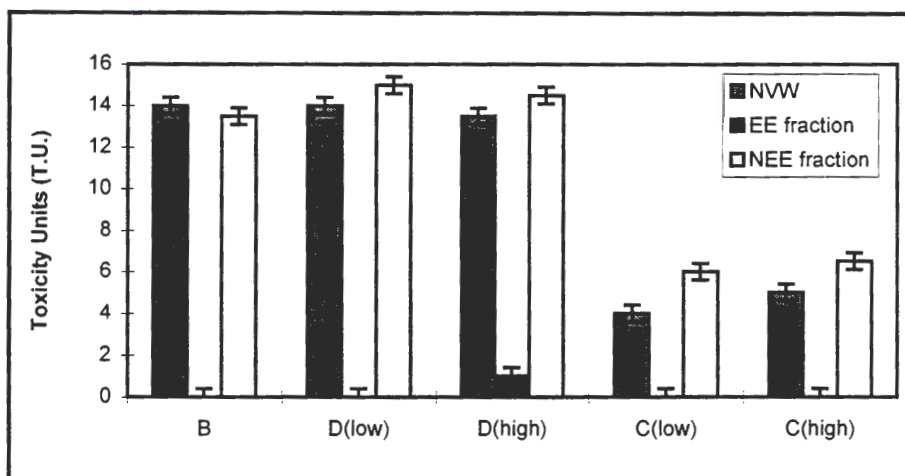
**Figure 10.** Comparison of Theoretical Combined Whole and Actual Combined Whole.

### Filtrate Fraction Toxicity

After the ether extraction and vacuum evaporation of the ether, there were two fractions to test: the non-ether extractable (NEE) fraction and the ether extractable (EE) fraction. Another filtrate, the non-volatile whole (NVW), was created by taking the whole filtrate and subjecting it to the same vacuum evaporation technique as the NEE and EE fractions. This allows for accurate comparison between the whole (without volatiles) and the fractions created by ether extraction.

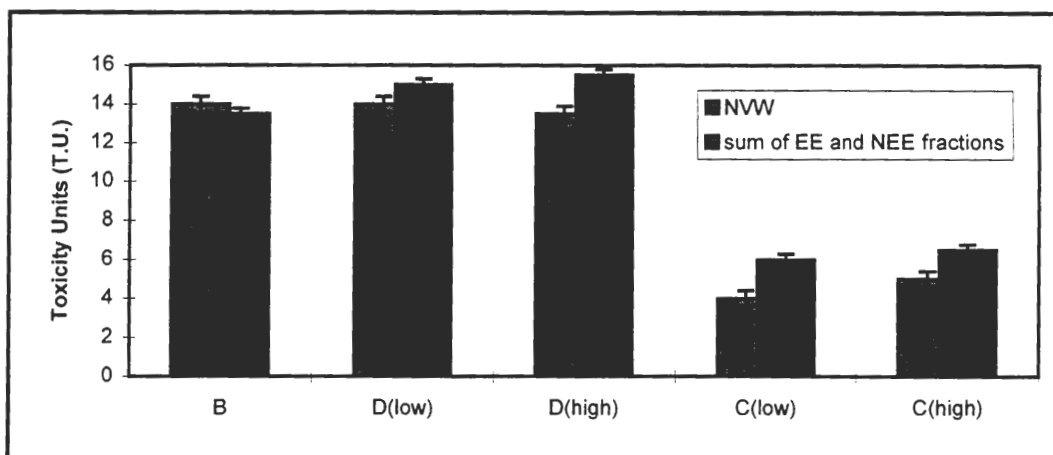
Figure 11 shows the relative toxicities of the NVW, NEE, and EE filtrates. As can be observed from the figure, all of the bleaching agents/charges show extremely low or unmeasurable EE fraction toxicity. This is significant due to the fact that the EE fraction is considered to be the fraction of most relevance and concern to the environment. It is interesting to note that in all bleaching agent/charges the NEE fraction and the NVW fraction are not significantly different from each other. This information combined with the extremely low EE fraction toxicity could lead to the conclusion that

the majority, if not all, of the filtrate toxicity assayed by XTT is induced by the NEE fraction, which is not considered to be of environmental importance relating to bioaccumulation or biomagnification of toxicants through the food chain.



**Figure 11.** Comparative Toxicity of Filtrates from Non-Volatile Whole (NVW), Ether Extractable Fraction (EE) and Non-Ether Extractable (NEE) Fraction.

As discussed earlier, the NVW filtrates, which theoretically, if no synergistic activity between stages was occurring, would equal the average of the EE and NEE fractions. As Figure 12 illustrates, the background EE and NEE filtrates add to approximately the toxicity of the NVW fraction, once again indicating an additive effect. However, both chlorine dioxide chemical charges and both chlorine chemical charges show slight antagonistic effects.



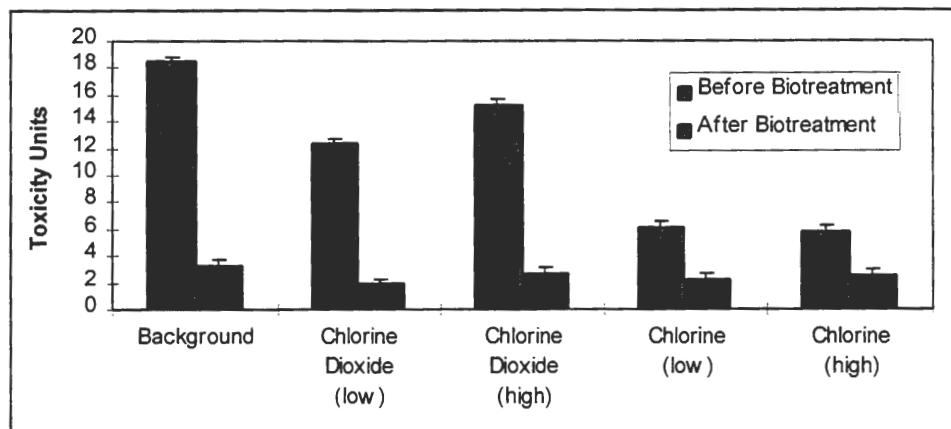
**Figure 12.** Comparison of NVW Filtrate Toxicity and Average of EE and NEE Fraction Toxicity.

#### Effect of Biotreatment on Toxicity

As shown in Table 5, and illustrated in Figure 13, lab-scale biotreatment was successful in reducing the filtrate toxicity to human liver cells as measured by the XTT assay. The background filtrates were reduced an average of 82.8%, while the low charge and high charge chlorine dioxide were reduced an average of 83.1% and 77.5%, respectively. The reduction in XTT toxicity for chlorine filtrates was not as high as the reduction in the background and chlorine dioxide filtrates. The low charge chlorine filtrates were reduced by an average of 63.4%, and the high charge filtrates were reduced by an average of 54.0%. It is encouraging that the highest toxicity filtrates resulted in the highest reduction in toxicity due to biotreatment indicating that the toxicity is easily reduced or removed.

Sample	Untreated	Biotreated	% Reduction
B+E-1	15.2	2.0	86.8
B+E-2	21.7	4.6	78.8
D <sub>L</sub> +E-1	14.5	1.2	91.7
D <sub>L</sub> +E-2	10.2	2.6	74.5
D <sub>H</sub> +E-1	22.2	2.8	87.4
D <sub>H</sub> +E-2	8.3	2.7	67.5
C <sub>L</sub> +E-1	5.1	1.8	64.7
C <sub>L</sub> +E-2	7.1	2.7	62.0
C <sub>H</sub> +E-1	7.2	2.7	62.5
C <sub>H</sub> +E-2	4.4	2.4	45.5

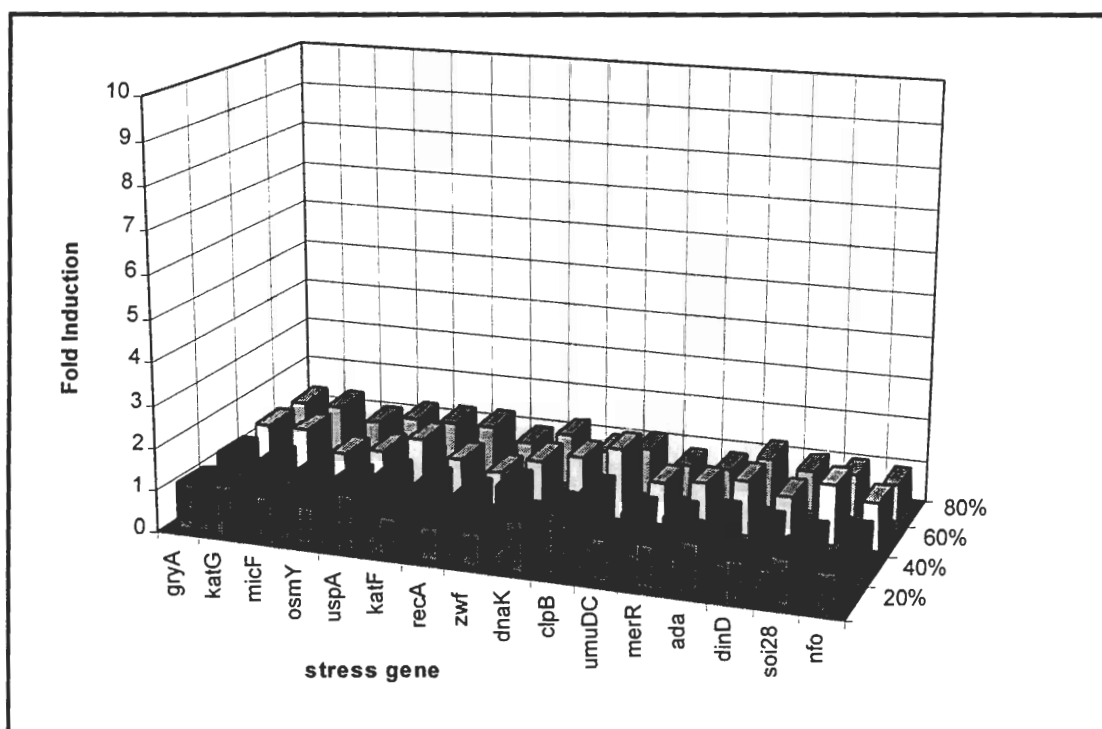
**Table 5.** XTT Toxicity (as Toxicity Units) of Whole Filtrates Before and After Biotreatment.



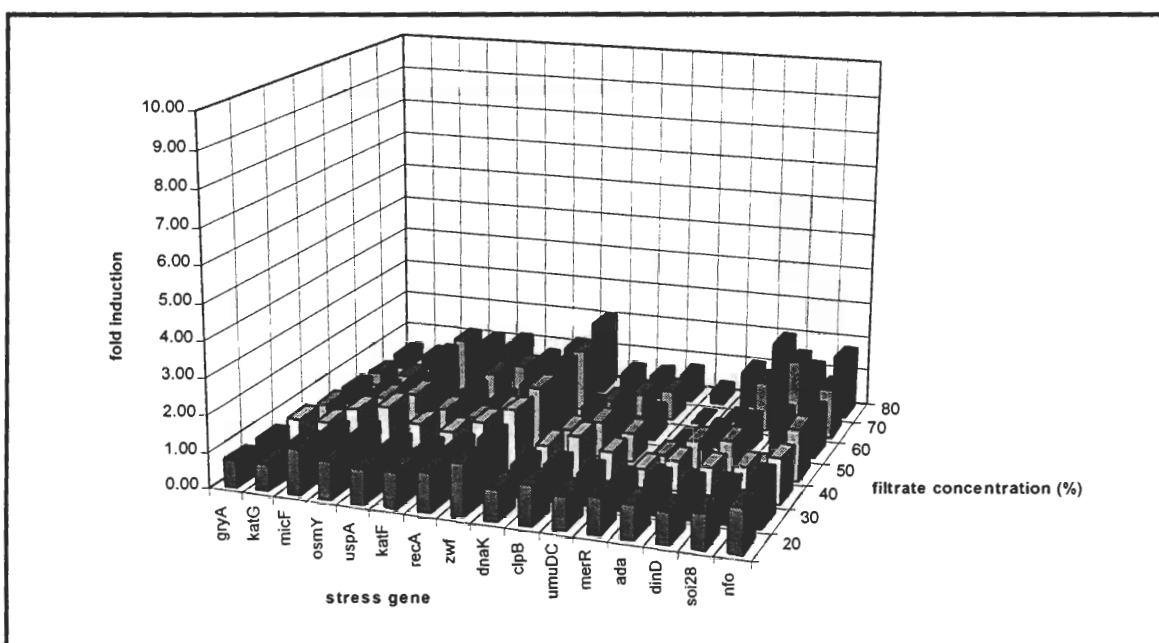
**Figure 13.** Toxicity Reduction After Labscale Biotreatment.

### Stress Gene Promoter Assay

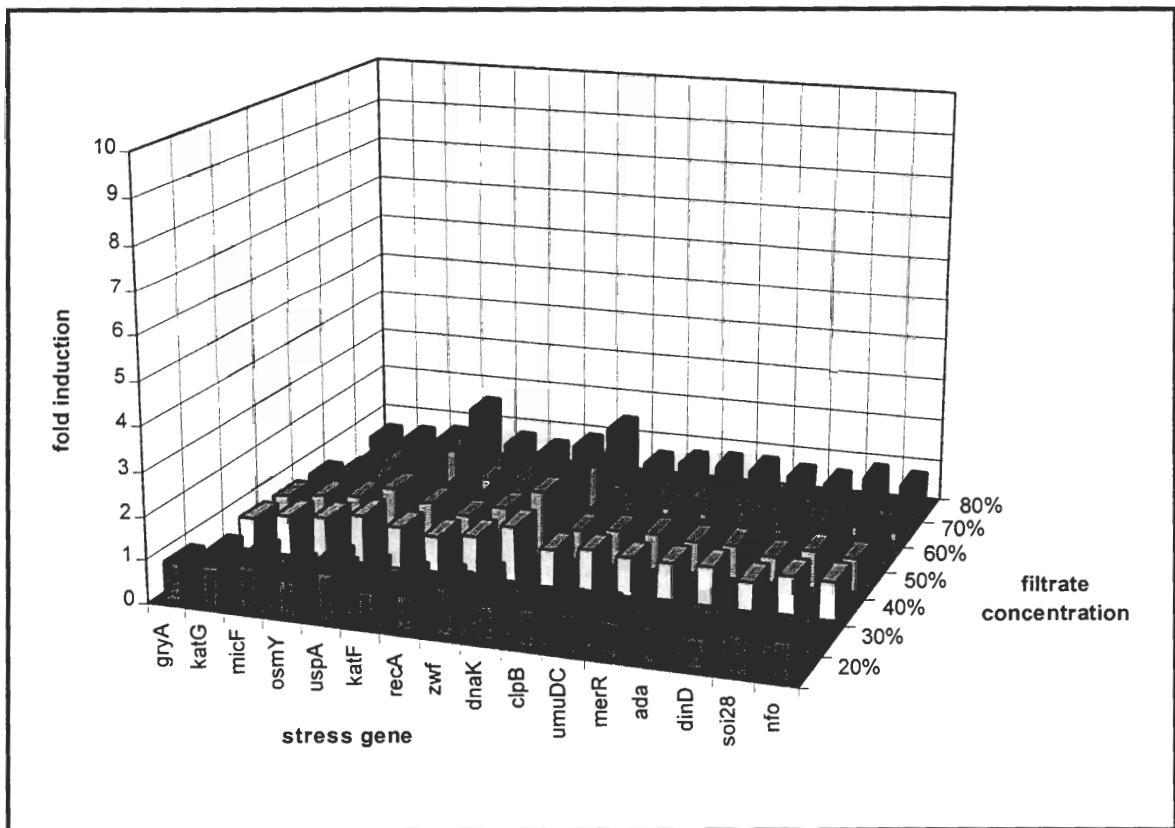
The extent of induction for each of the stress gene promoter types is shown graphically in Figures 14-18. Each vertical bar in the figures represents the average of duplicate measurements on each of two replicate bleaches. As can be observed in Figure 14-18, no filtrates showed any significant (greater than 2 fold) induction of any stress promoter.



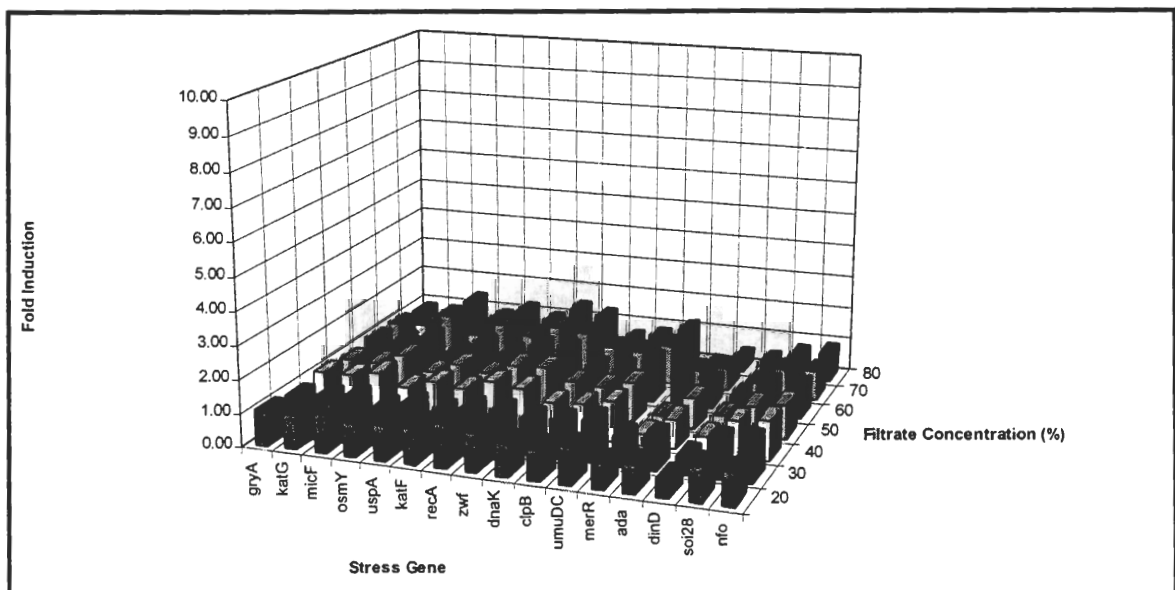
**Figure 14.** Induction of Stress Gene Activity When Exposed to Background Filtrates.



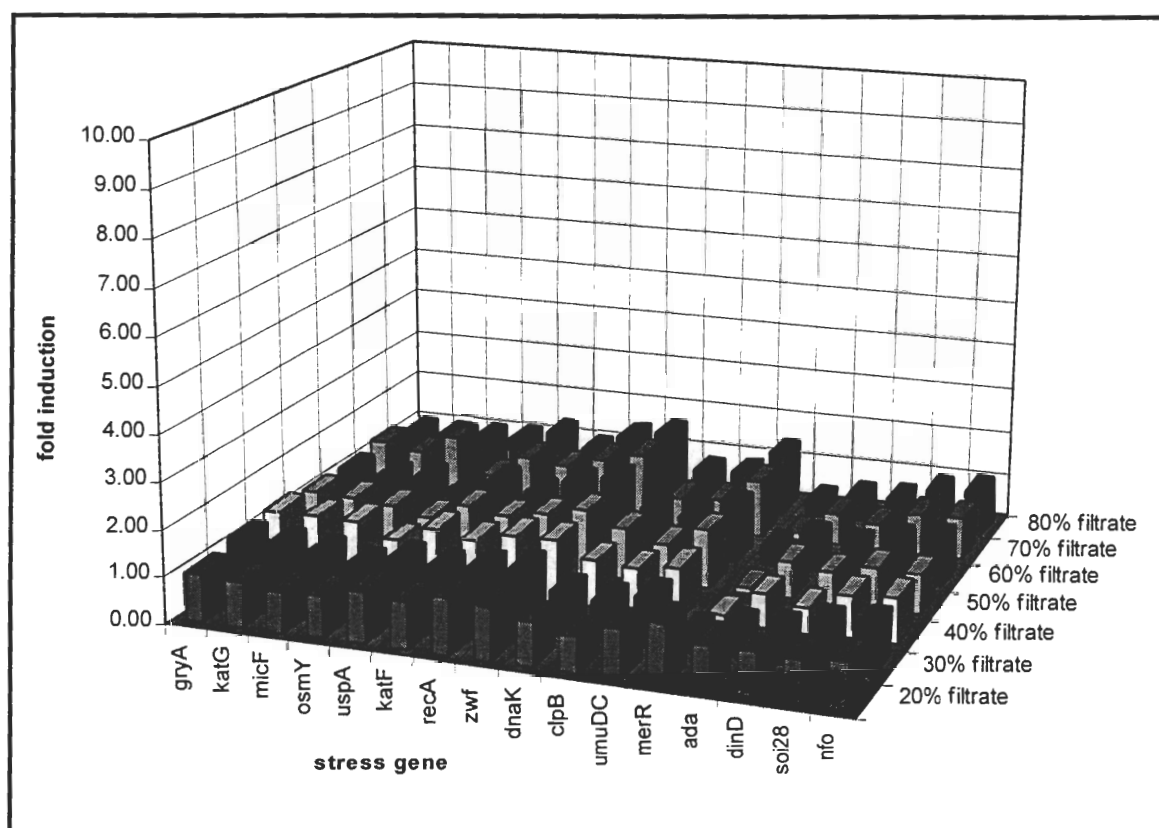
**Figure 15.** Induction of Stress Gene Activity When Exposed to 0.15 KF Chlorine Dioxide Bleaching Filtrates



**Figure 16.** Induction of Stress Gene Activity When Exposed to 0.25 KF Chlorine Dioxide Bleaching Filtrates



**Figure 17.** Induction of Stress Gene Activity When Exposed to 0.15 KF Chlorine Bleaching Filtrates



**Figure 18.** Induction of Stress Gene Activity When Exposed to 0.25 KF Chlorine Bleaching Filtrates

The observation that neither chlorine nor chlorine dioxide bleaching filtrates caused significant induction of any of the stress promoters suggests that neither bleaching agent generates toxicants that act by any of the mechanisms indicated in Tables 1 and 2. Before this conclusion can be reached, however, other possible interpretations must be examined. Two such alternative interpretations, as suggested by Orser et al.,<sup>8</sup> are that toxic compounds which act by these mechanisms are present but are of a molecular size that is too large to permit entry into the cell, or that they require metabolic activation for toxicity. The first of these cannot be eliminated, but the second is unlikely since

chlorination stage effluents have been found to undergo a reduction in mutagenic potential when liver microsomes are present, indicating a metabolic detoxification of the mutagens.<sup>20</sup>

## COMPARISON OF TOXICITY RESULTS

As could be expected, there are both similarities and differences between Microtox and the XTT assay utilizing human liver cells. While Microtox showed higher toxicities of filtrates from chlorine bleaching than background or chlorine dioxide bleaching, the XTT assay showed no significant differences between filtrates. Observing the ranking of toxicities of filtrates to the XTT assay, chlorine would be the least toxic.

It is important to remember that Microtox, a bacterium system, has a different response to xenobiotics than a human liver system. Bacteria have a regulated response to DNA damage and other cellular traumas referred to as the SOS response. The key components of the regulatory system are the *lexA* repressor, which regulates the expression of the genes that are induced in SOS, and the RecA protein. Induction of the SOS system requires the inactivation of the *lexA* repressor. The cleavage is catalyzed by *lexA* itself. The signal for induction of the SOS system is the appearance of single-stranded DNA gaps as a result of DNA damage. The Microtox system utilized measures an acute bacterial response, the Xenometrix assay measures a longer term acute bacterial response, while the human liver cells response is a mammalian response of a more chronic nature.



On the other hand, human liver cells respond to xenobiotics by utilizing detoxifying enzymes to add -OH subunits to the xenobiotics, which results in creating more water-soluble compounds, which allows them to be removed from the system. It should also be noted, however, that detoxifying enzymes also have the potential to increase the toxicity of compounds by structural modification.

## CONCLUSIONS

The Microtox assay is a relatively simple and inexpensive aquatic assay utilizing a marine bacterium. The assay indicated a higher toxicity to chlorine filtrates than background or chlorine dioxide in contrast to the XTT assay, in which there was no significant statistical difference in toxicities of filtrates from different bleaching agents. The XTT assay utilized human liver cells and the health of their electron transport system. Both assays showed significant background toxicity. In the future, emphasis should not be placed only on the bleach plant, but consideration must also be given to pulping and other process steps prior to the bleach plant which may contribute to toxicity of waste streams.

The most important statement to come from this research is that no one bioassay can be used to adequately assess the toxicity of an environmental sample. Different organisms respond in diverse methods to exposure to xenobiotics. In addressing environmental impact, a wide diversity of organisms must be considered; and with diversity of organisms, comes a wide diversity of responses which must be adequately

considered. Unfortunately, increasing the number of bioassays also increases the cost of toxicity evaluations. A balance must be found between cost and information gained.

#### **ACKNOWLEDGMENTS**

The authors thank Dr. Earl Malcolm, Dr. Lucy Sonnenberg, Dr. John Cairney, and Dr. Mary DeLong for technical advice and guidance, and the Institute of Paper Science and Technology and its Member Companies for the financial support provided for student research. Portions of this work were used by T.A.A. as partial fulfillment of the requirements for the Ph.D. degree at the Institute of Paper Science and Technology.

## LITERATURE CITED

1. Cox, J. (1989) BC Mills to Spend C\$800 Million to Meet Toxicity Regulations. *American Papermaker*, **52**(7), 27.
2. Liebergott, N., van Lierop, B., Kovacs, T., and Nolin, A. (1990) Comparison of the Order of Addition of Chlorine and Chlorine Dioxide in the Chlorination Stage. *Tappi Journal*, **73**(10), 207-213.
3. Borton, D. (1990) Examination of the Relationship Between the Adsorbable Organic Halide (AOX) Content of Paper-Industry Waste Waters and Potential Aquatic Biological Effects: First Progress Report. **NCASI Special Report**, No. 90-07.
4. Owens, W. (1990) Review of Recent Scandinavian Research on the Aquatic Effects of Bleached Pulp Mill Effluents. **NCASI Special Report**, No. 90-11.
5. Walden, C.C. (1976) Review Paper - The Toxicity of Pulp and Paper Mill Effluents and Corresponding Measurement Procedures. *Water Research*, **10**, 639-664.
6. Walden, C.C. and Howard, T.E. (1977) Toxicity of Pulp and Paper Mill Effluents - A Review of Regulations and Research. *Tappi Journal*, **60**(1), 122-125.
7. Priha, M.H. and Talka, E.T. (1986) Biological Activity of Bleached Kraft Mill Effluent (BKME) Fractions and Process Streams. *Pulp and Paper Canada*, **87**(12), 143-144.
8. Orser, C.S., Foong, F.C.F., Capaldi, S.R., Nalezny, J., Mackay, W., Benjamin, M., and Farr, S.B., "Use of Prokaryotic Stress Promoters as Indicators of the Mechanisms of Chemical Toxicity," *In Vitro Toxicology*, **8**(1):71-85 (1995).
9. Annergren, G. Lindblad, P., Carlsson, G., and Norrby, M. (1986) Spent Liquors from Bleaching of Softwood Kraft Pulps. Effect of Chlorination Variables on the Content of Lipophilic Chlorinated Organic Matter with Low Molecular Mass. **Proceedings of the EuCePa Symposium "Environmental Protection in the 90's,"** Helsinki, May 19-22.
10. Environment Canada, Biological Test Method: Toxicity Test Using Luminescent Bacteria (*Photobacterium phosphoreum*), Environmental Protection Series, Report EPS 1/RM/24, (1992)

- 
- I. 11. Ribo, J.M. and Kaiser, K.L.E., "*Photobacterium Phosphoreum* Toxicity Bioassay. Test Procedures and Applications," Toxicity Assessment: An International Quarterly, **2**:305-323.
  12. Ross, P., The Role of the Microtox System in Aquatic Toxicity Testing, Illinois Natural History Survey, 607 E. Peabody Drive, Champaign, IL 61820-6970.
  13. Microbics Corporation, Microtox Operator Manual (1994).
  14. Scudiero, D.A., Shoemaker, R.H., Paull, K.D., Monks, A., Tierney, S., Nofziger, T.H., Currens, M.J., Seniff, D., and Boyd, M.R. (1988). Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines. *Cancer Research* **48**, 4827-4833.
  15. Xenometrix, Inc., 2860 Wilderness Place; Boulder, Colorado 80301; 1-800-436-2869.
  16. Xenometrix Instruction Manual (1996).
  17. Miller, J.H., Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, N.Y., (1972).
  18. Hicks, C.R. (1964) **Fundamental Concepts in the Design of Experiments**, Chapter 9. Holt, Rinehart, and Winston Publishers, N.Y., N.Y., 130-147.
  19. Walpole, R.E. and Myers, R.H. (1989) **Probability and Statistics for Engineers and Scientists, Fourth Edition**, Chapter 11, MacMillan Publishing Company, New York, N.Y., 486.
  20. Ander, P., Ericksson, K.E., Kolar, M.C., and Kringstad, K., "Studies on the Mutagenic Properties of Bleaching Effluents," *Svensk Papperstidning*, 80(14):454-459 (1977).

## **Chapter 5 CONCLUSIONS AND FINAL RESULTS**

### **CONCLUSIONS**

The most important assertion to come from this research is that no one bioassay can be used to adequately assess the toxicity of an environmental sample. Different organisms respond in diverse methods to exposure to xenobiotics. In considering a environmental impact, a wide diversity of organisms must be evaluated. And with diversity of organisms, comes a wide diversity of responses which must be adequately considered. Unfortunately, increasing the number of bioassays also increases the cost of toxicity evaluations. A balance must be found between cost and information gained.

The Microtox assay is a relatively simple and inexpensive aquatic assay utilizing a marine bacterium. The assay indicated a higher toxicity in chlorine filtrates than background or chlorine dioxide in contrast to the XTT assay, in which the chlorine filtrates showed the lowest toxicity. The XTT assay utilized human liver cells and the health of their electron transport stem. In the XTT assay, chlorine bleaching filtrates showed the lowest toxicity levels. Both assays showed significant background toxicity. In the future, emphasis should not be placed only on the bleach plant, but consideration must be given to pulping and other process steps prior to the bleach plant which may contribute to toxicity of waste streams.

The Xenometrix Pro-Tox (C) assay kit provides simplified access to stress promoters as indicators of toxicity mechanisms. Sixteen bacterial strains of E.coli which

have been modified by fusing different stress promoters to a lacZ structural genes were tested. Laboratory prepared, untreated filtrates from chlorine and chlorine dioxide bleaching had no significant induction to any of the stress gene promoters. A background filtrate was prepared to evaluate the induction by compounds coming into the bleach plant, and again, no significant induction was observed. Several potential reasons for the lack of response were discussed or evaluated; however, no definitive reason can be attributed to the lack of response except lack of induction by bleaching filtrates.

#### **SUGGESTIONS FOR FUTURE RESEARCH**

1. Use in vitro toxicology to evaluate “at risk” human and aquatic organisms.
2. Evaluate the toxicity of raw material components as well as pulping streams which may add to bleaching waste stream toxicity.
3. Use in vitro toxicology to evaluate emerging new pulping and bleaching technologies as well as modifications to current technology in order to assess environmental impact improvements.
4. Evaluate additional stress gene promoters for induction by bleaching filtrates.
5. Evaluate bleaching filtrates from industrial facilities for in vitro toxicity as well as stress gene assay induction.
6. Evaluate influents and effluents from industrial biotreatment facilities to evaluate in vitro toxicity and stress gene induction.

## ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Tom McDonough, for all his advice, guidance, and encouragement during these difficult years of my life. Special thanks also go to my committee members Drs. John Cairney, Lucy Sonnenberg, and especially Mary DeLong who had the perserverance to teach an engineer how to successfully work with cell cultures.

I can only begin to thank the people who have helped me achieve my goal of obtaining my Ph.D. My Weyerhauser mentors, Bill Fuller and Jim Ferris, have been there throughout my education to give advice and encouragement whenever needed. Thanks to Comer and Pearson for truly being there through thick and thin, and there was a lot of thin. And without my fellow student, Denise Martin, I may have never made it; I am not sure if I would have starved to death or lost my mind first without Denise to provide her great cooking and, more importantly, words of wisdom. Eric, Cammie, and Catherine Stephens have helped me keep my sanity by giving advice about life and relaxation. And Eric provided more than one solution to major computer problems. But only Catherine, now 13 months old, was capable of teaching what is really important in life.

To my other friends, the staff, faculty, and students at the Institute, my sincere thanks for all the help you have provided. No student can make it through a thesis by themselves, only with the assistance of those around them can a quality thesis be

completed. Special thanks go to Dr. Malcolm, Dr. Rudie, Dr. Ragauskas, Chuck Courchene, Mark Turner, Charlie Burney, Dani Denton, Todd Schwantes, and Pete Froass. The biology technicians, including Sonya, Shannon, Cammie, Yolanda, Karen, and Bobbie, have been instrumental in showing me the ropes of biology techniques with patience, even when I was messing up their lab. To all the other staff that have assisted my learning, my most sincere thanks.



## Appendix A

### BLEACHING CONDITIONS

#### Oxidative Stage

Identification: **Storage Effects; Background Bleach**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): 0

ClO<sub>2</sub> Concentration (g/l): 0

Total Oxidant Volume (L): 0

TAC(g): 0

TAC (%on pulp): 0

Kappa Factor (TAC, %/Kappa): 0

Pulp (g): 300

Water (g): 8,730

Carryover Volume (mls): 970

Consistency (%): 3

Slurry pH, initial: 2.0

Slurry pH, final: 2.0

Oxidant Temp. (°C): n/a

Initial Slurry Temp. (°C): 58

Reaction Time (min): 30

#### Extraction Stage

Identification: **Storage Effects; Background Bleach**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 150

NaOH Charge (% on pulp): 2%

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Water (mls): 1,580

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> Volume (L): 1.1

O<sub>2</sub> Pressure (atm): 1

**APPENDIX A, CONT'D**  
**BLEACHING CONDITIONS**

**Oxidative Stage**

Identification: **Storage Effects; 0.15 KF Chlorine**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/1): 6.5

ClO<sub>2</sub> Concentration (g/1): 0.0

Total Oxidant Volume (L): 1.1

TAC(g): 6.84

TAC (%on pulp): 2.28

Kappa Factor (TAC, %/Kappa): 0.15

Pulp (g): 300

Water (g): 7,630

Carryover Volume (mls): 970

Consistency (%): 3

Slurry pH, initial: 1.9

Slurry pH, final: 1.9

Oxidant Temp. (°C): 0

Initial Slurry Temp. (°C): 56

Reaction Time (min): 30

**Extraction Stage**

Identification: **Storage Effects; 0.15 KF Chlorine**

NaOH Concentration (g/1): 40

NaOH Volume (mls): 94

NaOH Charge (% on pulp): 1.25

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Water (mls): 1,636

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> Volume (L): 1.1

O<sub>2</sub> Pressure (atm): 1

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Storage Effects; 0.25 KF Chlorine**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): 7.8ClO<sub>2</sub> Concentration (g/l): 0.0

Total Oxidant Volume (L): 1.5

TAC(g): 11.4

TAC (%on pulp): 3.8

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Water (g): 7,230

Carryover Volume (mls): 970

Consistency (%): 3

Slurry pH, initial: 2.0

Slurry pH, final: 2.0

Oxidant Temp. (°C): 0

Initial Slurry Temp. (°C): 57

Reaction Time (min): 30

**Extraction Stage**Identification: **Storage Effects; 0.25 KF Chlorine**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 157

NaOH Charge (% on pulp): 2.09

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Water (mls): 1,573

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> Volume (L): 1.1O<sub>2</sub> Pressure (atm): 1

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Storage Effects; 0.15 KF Chlorine Dioxide**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): 0.0ClO<sub>2</sub> Concentration (g/l): 5.3

Total Oxidant Volume (L): 1.3

TAC(g): 6.9

TAC (%on pulp): 2.3

Kappa Factor (TAC, %/Kappa): 0.15

Pulp (g): 300

Water (g): 7,430

Carryover Volume (mls): 970

Consistency (%): 3

Slurry pH, initial: 1.8

Slurry pH, final: 1.9

Oxidant Temp. (°C): 0

Initial Slurry Temp. (°C): 59

Reaction Time (min): 30

**Extraction Stage**Identification: **Storage Effects; 0.15 KF Chlorine Dioxide**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 94

NaOH Charge (% on pulp): 1.25

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Water (mls): 1636

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> Volume (L): 1.1O<sub>2</sub> Pressure (atm): 1

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Storage Effects; 0.25 KF Chlorine Dioxide**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): 0.0ClO<sub>2</sub> Concentration (g/l): 5.7

Total Oxidant Volume (L): 2.0

TAC(g): 11.4

TAC (%on pulp): 3.8

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Water (g): 6,730

Carryover Volume (mls): 970

Consistency (%): **3**

Slurry pH, initial: 1.8

Slurry pH, final: 1.9

Oxidant Temp. (°C): 0

Initial Slurry Temp. (°C): 62

Reaction Time (min): 30

**Extraction Stage**Identification: **Storage Effects; 0.25 KF Chlorine Dioxide**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 157

NaOH Charge (% on pulp): 2.09

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Water (mls): 1,573

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> Volume (L): 1.1O<sub>2</sub> Pressure (atm): 1

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Storage Effects; 0.4% Ozone**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): n/aClO<sub>2</sub> Concentration (g/l): n/a

Total Oxidant Volume (L): n/a

TAC(g): n/a

Ozone applied (% on pulp): 0.4

Kappa Factor (TAC, %/Kappa): n/a

Pulp (g): 300

Water (g): 865

Carryover Volume (mls): 970

Consistency (%): 10

Slurry pH, initial: 2.0

Slurry pH, final: 2.0

Oxidant Temp. (°C): n/a

Initial Slurry Temp. (°C): 20

**Extraction Stage**Identification: **Storage Effects; 0.4% Ozone**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 38

NaOH Charge (% on pulp): 1.0

Oxidative Stage Carryover (mls): 135

Total Stage Volume (L): 1.5

Carryover (% of Oxidative Stage): 10

Water (mls): 1,177

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub>Applied (g): 0.64

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Storage Effects; 1.2% Ozone**

Initial Kappa: 15.2

Cl<sub>2</sub> Concentration (g/l): n/aClO<sub>2</sub> Concentration (g/l): n/a

Total Oxidant Volume (L): n/a

TAC(g): n/a

Ozone applied (% on pulp): 1.2%

Kappa Factor (TAC, %/Kappa): n/a

Pulp (g): 300

Water (g): 865

Carryover Volume (mls): 485

Consistency (%): 10

Slurry pH, initial: 2.0

Slurry pH, final: 2.0

Oxidant Temp. (°C): n/a

Initial Slurry Temp. (°C): 20

**Extraction Stage**Identification: **Storage Effects; 1.2% Ozone**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 56

NaOH Charge (% on pulp): 1.5

Oxidative Stage Carryover (mls): 135

Total Stage Volume (L): 1.5

Carryover (% of Oxidative Stage): 10

Water (mls): 1159

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

O<sub>2</sub> applied (g): 0.75

## APPENDIX A, CONT'D

## BLEACHING CONDITIONS

**Oxidative Stage**Identification: **Comparative Study; Background, rep 1**

Initial Kappa: 17.6

Cl<sub>2</sub> Concentration (g/l): n/aClO<sub>2</sub> Concentration (g/l): n/a

Total Oxidant Volume (L): n/a

TAC(g): n/a

TAC (%on pulp): n/a

Kappa Factor (TAC, %/Kappa): 0

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

**Extraction Stage**Identification: **Comparative Study; Background, rep 1**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 146

NaOH Charge (% on pulp): 1.9

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 10

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 11.0



**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**Identification: **Comparative Study; Background, rep 2**

Initial Kappa: 17.6

Cl<sub>2</sub> Concentration (g/l): n/aClO<sub>2</sub> Concentration (g/l): n/a

Total Oxidant Volume (L): n/a

TAC(g): n/a

TAC (%on pulp): n/a

Kappa Factor (TAC, %/Kappa): 0

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

**Extraction Stage**Identification: **Comparative Study; Background, rep 2**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 146

NaOH Charge (% on pulp): 1.9

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 14.3

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.15 KF Chlorine, rep 1**

Initial Kappa: 17.6	TAC(g): 7.92
TAC (%on pulp): 2.6	Kappa Factor (TAC, %/Kappa): 0.15
Pulp (g): 300	Carryover Volume (kg COD/ton): 10
Consistency (%): 3	Slurry pH, initial: 2.0
Oxidant Temp. (°C): 0	Reaction Time (min): 30

**Extraction Stage**

Identification: **Comparative Study; 0.15 KF Chlorine, rep 1**

NAOH Concentration (g/l): 40	NAOH Volume (mls): 109
NAOH Charge (% on pulp): 1.45	Oxidative Stage Carryover (mls): 970
Total Stage Volume (L): 3	Carryover (% of Oxidative Stage): 10
Consistency (%): 10	Temperature (°C): 70
Reaction Time (min): 70	
Extracted Kappa: 3.9	

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.15 KF Chlorine, rep 2**

Initial Kappa: 17.6

TAC(g): 7.92

TAC (%on pulp): 2.6

Kappa Factor (TAC, %/Kappa): 0.15

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

**Extraction Stage**

Identification: **Comparative Study; 0.15 KF Chlorine, rep 2**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 109

NaOH Charge (% on pulp): 1.45

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 3.5

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.25 KF Chlorine, rep 1**

Initial Kappa: 17.6

TAC(g): 13.20

TAC (%on pulp): 4.4

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

**Extraction Stage**

Identification: **Comparative Study; 0.25 KF Chlorine, rep 1**

NAOH Concentration (g/l): 40

NAOH Volume (mls): 182

NAOH Charge (% on pulp): 2.42

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 1.3

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.25 KF Chlorine, rep 2**

Initial Kappa: 17.6

TAC(g): 7.92

TAC (%on pulp): 2.6

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

**Extraction Stage**

Identification: **Comparative Study; 0.25 KF Chlorine, rep 2**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 182

NaOH Charge (% on pulp): 2.42

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 1.2

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.15 KF Chlorine Dioxide, rep 1**

Initial Kappa: 17.6

TAC(g): 7.92

TAC (%on pulp): 2.6

Kappa Factor (TAC, %/Kappa): 0.15

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

ClO<sub>2</sub> (g): 3.01

**Extraction Stage**

Identification: **Comparative Study; 0.15 KF Chlorine Dioxide, rep 1**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 109

NaOH Charge (% on pulp): 1.45

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Kappa Factor (TAC, %/Kappa): 0.15

Extracted Kappa: 5.8

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.15 KF Chlorine Dioxide, rep 2**

Initial Kappa: 17.6

TAC(g): 7.92

TAC (%on pulp): 2.6

Kappa Factor (TAC, %/Kappa): 0.15

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

ClO<sub>2</sub> (g): 3.01

**Extraction Stage**

Identification: **Comparative Study; 0.15 KF Chlorine Dioxide, rep 2**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 109

NaOH Charge (% on pulp): 1.45

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 5.8

**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.25 KF Chlorine Dioxide, rep 1**

Initial Kappa: 17.6

TAC(g): 13.2

TAC (%on pulp): 4.4

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

ClO<sub>2</sub> (g): 5.02

**Extraction Stage**

Identification: **Comparative Study; 0.25 KF Chlorine Dioxide, rep 1**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 182

NaOH Charge (% on pulp): 2.42

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 3.6



**APPENDIX A, CONT'D****BLEACHING CONDITIONS****Oxidative Stage**

Identification: **Comparative Study; 0.25 KF Chlorine Dioxide, rep 2**

Initial Kappa: 17.6

TAC(g): 13.2

TAC (%on pulp): 4.4

Kappa Factor (TAC, %/Kappa): 0.25

Pulp (g): 300

Carryover Volume (kg COD/ton): 10

Consistency (%): 3

Slurry pH, initial: 2.0

Oxidant Temp. (°C): 0

Reaction Time (min): 30

ClO<sub>2</sub> (g): 5.02

**Extraction Stage**

Identification: **Comparative Study; 0.25 KF Chlorine Dioxide, rep 2**

NaOH Concentration (g/l): 40

NaOH Volume (mls): 182

NaOH Charge (% on pulp): 2.42

Oxidative Stage Carryover (mls): 970

Total Stage Volume (L): 3

Carryover (% of Oxidative Stage): 10

Consistency (%): 10

Temperature (°C): 70

Reaction Time (min): 70

Extracted Kappa: 3.6

## Appendix B

## TOC DATA FOR COMPARATIVE TOXICITY STUDY (CHAPTER 4)

FILTRATE FRACTION	TOC (PPM) REP A	TOC (PPM) REP B	TOC (PPM) REP C	AVERAGE	CV (%)
B-1 stage	69.87	68.52	69.99	69.46	1.2%
B-2 stage	64.25	64.55	64.23	64.34	0.3%
E(B)-1 stage	89.70	90.65	90.38	90.24	0.6%
E(B)-2 stage	89.21	90.63	89.29	89.71	0.9%
B+E-1 whole	77.40	76.05	77.06	76.84	0.7%
B+E-2 whole	73.59	74.53	73.94	74.02	0.5%
B+E-1 NVW	71.26	72.88	72.27	72.14	0.8%
B+E-2 NVW	71.91	71.05	71.67	71.54	0.6%
B+E-1 EE	2967.6	2980.0	2923.6	2958.0	1.0%
B+E-2 EE	3222.8	3233.6	3252.8	3236.4	0.5%
B+E-1 NEE	190.4	190.2	190.4	190.3	0.1%
B+E-2 NEE	226.9	226.8	228.8	227.5	0.5%
D <sub>L</sub> -1 stage	131.0	131.3	130.9	131.1	0.2%
D <sub>L</sub> -2 stage	69.9	68.5	70.0	69.5	1.2%
E(D <sub>L</sub> )-1 stage	133.8	132.7	131.4	132.6	0.9%
E(D <sub>L</sub> )-2 stage	89.7	90.7	90.4	90.2	0.6%
D <sub>L</sub> +E-1 whole	98.1	103.0	107.6	102.9	4.7%
D <sub>L</sub> +E-2 whole	77.4	76.1	77.1	76.8	0.9%
D <sub>L</sub> +E-1 NVW	88.1	87.4	96.0	90.5	5.4%
D <sub>L</sub> +E-2 NVW	71.3	72.9	72.3	72.1	1.2%
D <sub>L</sub> +E-1 EE	634.4	626.8	626.8	629.2	0.7%
D <sub>L</sub> +E-2 EE	79.4	79.5	79.9	79.6	0.4%
D <sub>L</sub> +E-1 NEE	195.9	194.7	194.2	194.9	0.4%
D <sub>L</sub> +E-2 NEE	190.4	190.2	190.4	190.3	0.1%
D <sub>H</sub> -1 stage	219.6	210.2	215.3	215.0	2.4%
D <sub>H</sub> -2 stage	64.3	64.6	64.2	64.3	0.3%
E(D <sub>H</sub> )-1 stage	200.3	205.8	204.4	203.5	1.4%
E(D <sub>H</sub> )-2 stage	89.2	90.6	89.3	89.7	0.9%
D <sub>H</sub> +E-1 whole	184.9	185.0	184.3	184.8	0.2%
D <sub>H</sub> +E-2 whole	73.6	74.5	73.9	74.0	0.6%
D <sub>H</sub> +E-1 NVW	163.8	159.8	160.7	161.5	1.3%
D <sub>H</sub> +E-2 NVW	71.9	71.1	71.7	71.5	0.6%
D <sub>H</sub> +E-1 EE	107.0	106.2	105.8	106.3	0.7%
D <sub>H</sub> +E-2 EE	174.3	174.8	174.9	174.7	0.2%

D <sub>H</sub> +E-1 NEE	193.6	215.0	187.7	198.8	7.3%
D <sub>H</sub> +E-2 NEE	226.9	226.8	228.6	227.5	0.5%
C <sub>L</sub> -1 stage	131.0	131.3	130.9	131.1	0.2%
C <sub>L</sub> -2 stage	197.8	197.6	197.0	197.5	0.2%
E(C <sub>L</sub> )-1 stage	133.8	132.7	131.4	132.6	0.9%
E(C <sub>L</sub> )-2 stage	253.0	253.5	251.6	252.7	1.0%
C <sub>L</sub> +E-1 whole	98.1	103.0	107.6	102.9	4.7%
C <sub>L</sub> +E-2 whole	209.6	216.4	215.1	213.7	1.6%
C <sub>L</sub> +E-1 NVW	88.1	87.4	96.0	90.5	5.4%
C <sub>L</sub> +E-2 NVW	55.4	53.7	53.6	54.2	2.9%
C <sub>L</sub> +E-1 EE	62.2	62.7	62.7	62.5	0.7%
C <sub>L</sub> +E-2 EE	31.8	32.3	31.8	32.0	0.8%
C <sub>L</sub> +E-1 NEE	195.9	194.7	194.2	194.9	0.8%
C <sub>L</sub> +E-2 NEE	86.5	86.5	86.7	86.6	0.2%
C <sub>H</sub> -1 stage	105.5	104.3	104.5	104.8	0.8%
C <sub>H</sub> -2 stage					
E(C <sub>H</sub> )-1 stage	78.1	77.8	79.6	78.5	1.7%
E(C <sub>H</sub> )-2 stage	126.4	126.2	125.1	125.9	0.7%
C <sub>H</sub> +E-1 whole	89.8	89.9	89.6	89.8	0.1%
C <sub>H</sub> +E-2 whole	156.2	154.1	154.8	155.0	0.7%
C <sub>H</sub> +E-1 NVW	49.8	51.1	50.1	50.4	1.3%
C <sub>H</sub> +E-2 NVW	120.5	120.1	122.8	121.1	1.4%
C <sub>H</sub> +E-1 EE	237.5	240.8	240.2	239.5	0.7%
C <sub>H</sub> +E-2 EE	197.8	197.6	197.0	197.5	0.2%
C <sub>H</sub> +E-1 NEE	124.3	127.5	126.8	126.2	1.3%
C <sub>H</sub> +E-2 NEE	60.9	60.9	62.6	61.5	2.4%

## Appendix C

## COD DATA FOR COMPARATIVE TOXICITY STUDY (CHAPTER 3)

<b>FILTRATE FRACTION</b>	<b>COD (MG/L) REP A</b>	<b>COD (MG/L) REP B</b>	<b>AVERAGE</b>
B-1 stage	241	234	238
B-2 stage	242	242	242
E(B)-1 stage	333	326	330
E(B)-2 stage	320	345	333
B+E-1 whole	293	272	283
B+E-2 whole	263	288	276
B+E-1 NVW	294	270	282
B+E-2 NVW	253	256	255
B+E-1 EE	15,440	15,110	15,275
B+E-2 EE	18,660	17,340	18,000
B+E-1 NEE	884	875	880
B+E-2 NEE	1079	1043	1061
D <sub>L</sub> -1 stage	393	394	394
D <sub>L</sub> -2 stage	454	445	450
E(D <sub>L</sub> )-1 stage	477	447	462
E(D <sub>L</sub> )-2 stage	565	569	567
D <sub>L</sub> +E-1 whole	427	417	422
D <sub>L</sub> +E-2 whole	503	502	503
D <sub>L</sub> +E-1 NVW	352	342	347
D <sub>L</sub> +E-2 NVW	471	469	470
D <sub>L</sub> +E-1 EE	3,710	3,520	3,620
D <sub>L</sub> +E-2 EE	10,320	10,820	10,570
D <sub>L</sub> +E-1 NEE	825	822	824
D <sub>L</sub> +E-2 NEE	1218	1224	1221
D <sub>H</sub> -1 stage	441	540	491
D <sub>H</sub> -2 stage	441	437	439
E(D <sub>H</sub> )-1 stage	716	710	713
E(D <sub>H</sub> )-2 stage	761	758	760
D <sub>H</sub> +E-1 whole	582	574	578
D <sub>H</sub> +E-2 whole	583	576	580
D <sub>H</sub> +E-1 NVW	494	495	495
D <sub>H</sub> +E-2 NVW	514	521	518
D <sub>H</sub> +E-1 EE	1,720	1,810	1,770
D <sub>H</sub> +E-2 EE	10,180	10,700	10,440

D <sub>H</sub> +E-1 NEE	735	745	740
D <sub>H</sub> +E-2 NEE	1493	1482	1488
C <sub>L</sub> -1 stage	603	596	600
C <sub>L</sub> -2 stage	657	657	657
E(C <sub>L</sub> )-1 stage	681	687	684
E(C <sub>L</sub> )-2 stage	719	723	721
C <sub>L</sub> +E-1 whole	637	636	637
C <sub>L</sub> +E-2 whole	686	689	688
C <sub>L</sub> +E-1 NVW	584	594	589
C <sub>L</sub> +E-2 NVW	635	644	640
C <sub>L</sub> +E-1 EE	10,520	11,000	10,760
C <sub>L</sub> +E-2 EE	11,680	11,380	11,530
C <sub>L</sub> +E-1 NEE	1241	1230	1236
C <sub>L</sub> +E-2 NEE	2720	2660	2690
C <sub>H</sub> -1 stage	769	784	777
C <sub>H</sub> -2 stage			
E(C <sub>H</sub> )-1 stage	820	812	816
E(C <sub>H</sub> )-2 stage	768	785	777
C <sub>H</sub> +E-1 whole	796	796	796
C <sub>H</sub> +E-2 whole	653	610	632
C <sub>H</sub> +E-1 NVW	707	710	709
C <sub>H</sub> +E-2 NVW	534	545	540
C <sub>H</sub> +E-1 EE	8960	9540	9250
C <sub>H</sub> +E-2 EE	10,180	10,700	10,490
C <sub>H</sub> +E-1 NEE	2480	2660	2570
C <sub>H</sub> +E-2 NEE	3260	3280	3270

## **Appendix D**

### **ENVIRONMENTAL PROTOCOL FOR PRO-TOX (C) XENOMETRIX ASSAY KIT**

The Pro-Tox (C) kit for running stress gene assays was obtained from Xenometrix. It was determined that the Environmental Protocol, which allows for the testing of higher concentrations of filtrate, was most appropriate for the bleaching filtrates and fractions. Since the Environmental Protocol is not, at this time, permanently printed in any citable material and the protocol was altered slightly to account for the small amount of filtrate available, the protocol provided by Xenometrix and used in this study is described in detail below. All materials used, except for the 96-well plates, pipettes, and pipette tips, were provided by Xenometrix. Again, the protocol was developed by Xenometrix and only altered to test two plates per filtrate rather than three.

All protocol steps referred to are from the Pro-Tox (C) version 1.5 manual (Xenometrix, 1996). First, the overnight bacterial cultures (all 16 strains) are prepared and placed in incubator at 37°C with agitation at approximately 300 rpm. After approximately 15 hours, the overnight cultures are subcultured in order to obtain log phase cultures. The overnight cultures were diluted 1:50 with 1X Pro-Tox Medium. Into sterile culture tubes, 120 µl overnight culture was added with 6 mls 1X Pro-Tox Medium. The diluted cultures are incubated at 37°C with agitation at 300 rpm until an OD<sub>600</sub> (absorbance at 600 nm in a spectrophotometer) reading between 0.3 to 0.4 is reached. This takes approximately 3 hours.

When the cultures begin to have OD<sub>600</sub> readings between 0.3 to 0.4, read the OD<sub>600</sub> of all the cultures. The cell densities are now adjusted so that all the strains are the same. In order to calculate the amount of 1X Pre-Tox Medium that must be added to each culture tube by using the following calculation for each strain:

$$\text{volume to add} = [(\text{OD}_{600} \text{ of strain}) / \text{lowest OD}_{600} \text{ value of all strains}] \times (5.8 \text{ mls}) - 5.8 \text{ ml}$$

The equation allows adjustment for all strains to have the same number of organisms per volume. The 5.8 mls number comes from 6 mls of total bacterial solution prepared minus 0.2 mls of solution used to test for the OD<sub>600</sub>.

In order to determine the concentrations of stock solutions that must be used to achieve the final desired exposure concentration, the chart below is used as a guide. Stock solutions are prepared at 111% of the desired exposure concentration to allow the addition of bacteria.

The stock solutions of the aqueous environmental samples are prepared in 10X Pro-Tox Medium. In order to prepare sufficient stock for testing in duplicate, follow the following chart.

Stock Conc.	0%	22%	33%	44%	56%	67%	78%	89%
Amt. Test Sample (ml)	0.0	2.2	3.3	4.4	5.6	6.7	7.8	8.9
Amt. 10X Medium (ml)	3.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Amt. dH <sub>2</sub> O (ml)	27.0	6.8	5.7	4.6	3.4	2.3	1.2	0.1

In order to prepare sufficient stock for testing in triplicate, follow the following chart.

Stock Conc.	0%	22%	33%	44%	56%	67%	78%	89%
Amt. Test Sample (ml)	0	3.3	4.9	6.6	8.4	10.1	11.7	13.4
Amt. 10X Medium (ml)	4.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Amt. dH <sub>2</sub> O (ml)	40.5	10.2	8.6	6.9	5.1	3.4	1.8	0.1

The bacterial growth plates are then prepared by performing the following steps:

- a. Add 225 µl of the 0% stock solution to all the wells of columns 1 through 4 of a sterile polystyrene microtiter plate.
- b. Add 225 µl of the lowest stock concentration (stock #1) to all the wells of column 5.
- c. Add 225 µl of stock solution #2 to all the wells of column 6.
- d. Add 225 µl of stock solution #3 to all the wells of column 7.
- e. Add 225 µl of stock solution #4 to all the wells of column 8.
- f. Add 225 µl of stock solution #5 to all the wells of column 9.
- g. Add 225 µl of stock solution #6 to all the wells of column 10.
- h. Add 225 µl of stock solution #7 to all the wells of column 11.
- i. Repeat steps a-h for three more sterile polystyrene microtiter plates if testing in duplicate and five more sterile polystyrene microtiter plates if testing in triplicate.
- j. Transfer 300 µl of the fast promoter cultures to column 12, rows A-H respectively, in each of the two (or three) fast plates.



- k. Transfer 300  $\mu$ l of the slow promoter cultures to column 12, rows A-H respectively, in each of the two (or three) slow plates.

Open a new file in the Pro-Tox program. Enter the pertinent information being sure to choose “Pro-Tox (C) Environmental” choice under assay type in the software interface. Take an OD<sub>600</sub> reading of both the slow and the fast plates. Within each plate, transfer 25  $\mu$ l of bacteria from column 12 to columns 2 through 11. Before each transfer, pipet up and down in the wells of column 12 to keep the cells well distributed. Take another OD<sub>600</sub> reading of each plate. Incubate the plates for 2 hours at 37°C with agitation at 300 rpm. Take another OD<sub>600</sub> reading at the end of the incubation period. This information provides the survival and growth of the bacterial strains.

At this point, the procedure follows the standard procedure described in the Xenometrix manual Version 1.5(C), beginning on page 19. In a fume hood, 50  $\mu$ l of chloroform is added to each well in columns 2 through 11 of a polypropylene microtiter plate. Prepare one chloroform plate for each bacterial plate. 200  $\mu$ l of solution of each well of the bacterial plates is transferred to a chloroform plate. The chloroform plates should be covered to prevent evaporative loss. Allow the plates to stand for 5-10 minutes in the fume hood to fully lyse the cells.

While the cells are being exposed to chloroform, prepare  $\beta$ -galactosidase assay plates. Prepare one plate for each chloroform plate. The  $\beta$ -galactosidase plates are prepared by adding 170  $\mu$ l of Pro-Tox Buffer to all wells of columns 1 through 11. Add 40  $\mu$ l of sterile water to all wells of column 1. Add 40  $\mu$ l of ONPG to all wells of

columns 1 through 11. To start the  $\beta$ -galactosidase assay plate transfer 40  $\mu$ l from only the upper aqueous phase of the chloroform plate to the assay plate. Columns 2-11 are to be transferred and must be done in a row by row manner using a 12 channel pipettor. Mix thoroughly by pipetting up and down 2-3 times after each row is transferred to the assay plate. It is possible to reuse the same pipet tips for all the transfers within a plate if the tips are rinsed between rows using a trough of sterile deionized water. Each plate is read at OD<sub>420</sub>. The slow plates are read immediately and then allowed to set for 30 minutes before being read at OD<sub>420</sub> again. The fast plates are allowed to set for only 10 minutes before having the repeat OD<sub>420</sub> reading.

The computer calculations uses the final three 600 readings and both 420 reading to determine  $\beta$ -galactosidase activity. Equations used are as follows:

$$\text{Activity} = c [(\Delta \text{OD}_{420}) / (\text{lag time})(\Delta \text{OD}_{600})]$$

c = constant for Miller units conversion

$$\Delta \text{OD}_{420} = [(\text{OD}_{420} \text{ Reading \#2}) - (\text{OD}_{420} \text{ Reading \#1})]$$

$$\Delta \text{OD}_{600} = [\text{Post-Exposure} - (\text{Post-Dose} - \text{Pre-Dose})]$$

## Appendix E

### PRO-TOX (C) STRESS NAMES AND PROMOTER FUSION DESCRIPTIONS

PROMOTER FUSION	INDUCING AGENTS
<i>gryA</i>	responds to DNA damaging agents such as mitomycin C and 4-nitroquinoline 1-oxide
<i>katG</i>	responds to oxidative damage as caused by compounds like hydrogen peroxide
<i>micF</i>	responds to fluctuations in osmotic conditions as well as chemicals that effect the outer membrane such as ethanol
<i>osmY</i>	responds to changes in growth conditions and osmotic conditions and chemicals like sodium nitrite and ethanol
<i>uspA</i>	responds to DNA damaging agents such as 4-nitroquinoline 1-oxide and nalidixic acid
<i>katF</i>	responds to DNA damage, oxidative stress, and chemicals like mitomycin C and potassium cyanide
<i>recA</i>	responds to DNA damage and chemicals like methyl methanesulfonate and mitomycin C
<i>zwf</i>	Responds to oxidative stress as mediated by chemicals with paraquat and 4-nitroquinoline 1-oxide

**Table 1.** Description of PRO-TOX C Fast Plate Promoters

PROMOTER FUSION	INDUCING AGENTS
<i>dnaK</i>	responds to growth temperature fluctuations and changes in growth conditions and compounds like 1-propanol and ethanol
<i>clpB</i>	responds to agents affecting proteins, such as acetone chloroform, and p-chloroaniline
<i>umuDC</i>	induced by DNA damaging agents like benzo[a]pyrene, mitomycin C, and 4-nitroquinoline 1-oxide
<i>merR</i>	Responds to the presence of heavy metals, eg. mercuric chloride, cadmium sulfate
<i>ada</i>	responds to DNA damaging agents like MMS, MNNG, and p-chloroaniline
<i>dinD</i>	responds to DNA damaging agents such as mitomycin C, MMS, MNNG, nalidixic acid, and 4-nitroquinoline 1-oxide
<i>soi28</i>	responds to superoxide radical anion generating compounds like paraquat and 4-nitroquinoline 1-oxide
<i>nfo</i>	responds to the presence of apurinic and apyrimidinic sites as well as ssDNA and dsDNA breaks caused by bleomycin

**Table 2.** Description of PRO-TOX C Slow Plate Promoters.

***gyrA*** - DNA gyrase is responsible for the introduction of negative supercoils into bacterial and plasmid genomes in *E.coli*. DNA gyrases ability to introduce and remove negative supercoils in its DNA implicates its involvement in both the replication of the chromosome and also in transcriptional activation of many promoters. The *gyrA-lacZ* fusion is induced by DNA damaging agents such as mitomycin C and 4-nitroquinoline 1-oxide.

***katG*** - The level of hydrogen peroxide in the bacterial cell is controlled through the catalases and peroxidases, collectively called hydroperoxidases. In *E. coli* there are three hydroperoxidases, HPI, HPII, and HPIII. These enzymes provide an important function as hydrogen peroxide causes direct DNA damage through the occurrence of single-strand breaks. Hydrogen peroxide can accumulate as a photoproduct of near-ultraviolet radiation as well as being a product of superoxide-dismutase-mediated catalysis of the superoxide anion ( $O_2^-$ ). KatG encodes hydrogen peroxidase I, a tetrameric bifunctional catalase and *o*-dianiside peroxidase. The *katG-lacZ* fusion responds to oxidative damage and is induced by hydrogen peroxide.

***micF*** - The major outer membrane proteins of *E.coli* are OmpF and OmpC whose abundance are reflective of the osmotic environment of the cell. As the osmolarity of the culture medium increases, the OmpF production decreases while OmpC increases, maintaining the same amount of Omp proteins in the outer membrane. The regulation of the translation of OmpF is in part mediated by a short antisense RNA which is the complement to the 5' end of the OmpF mRNA. This antisense molecule is *micF*, which

is short for mRNA-interfering complementary RNA. Simplistically, the production of mic RNA is in proportion to the level of *ompC* mRNA, creating an efficient way to maintain a constant level of Omp proteins in the outer membrane. The *ompF* and *ompC* genes are also positively regulated by OmpR. The *micF-lacZ* fusion responds to fluctuations in temperature and osmotic conditions as well as a wide range of chemicals that interfere with the integrity of the outer membrane, including *p*-chloroaniline, DMSO, ethanol, nalidixic acid, and phenol.

***osmY*** - There are many genes and more than 20 proteins which are induced following exposure to high osmolarity. This is not surprising given the natural habitat of *E. coli* is the gut and that it can survive seawater. The function of the protein encoded by *osmY* has not been resolved. The *osmY-lacZ* fusion responds to compounds such as sodium nitrite, ethanol, and nalidixic acid.

***uspA*** - *E. coli*, like most bacteria, is able to withstand long periods of growth arrest without the production of dormant structures like spores. The protection of inherent cellular macromolecules is a complex stress response. Many proteins have been identified by 2-dimensional gel electrophoresis to accumulate under various stress conditions. The universal stress protein was found to increase in abundance in the cytoplasm of *E. coli* under conditions of growth inhibition. The growth inhibiting conditions which induced the presence of this protein included nutrient exhaustion of carbon, nitrogen, phosphate, or sulphate, as well as the presence of toxic agents like heavy metals, oxidants, acids, and antibiotics. The actual function of this small molecular

weight protein is unknown at this time. The *uspA-lacZ* fusion responds to DNA damaging compounds such as 4-nitroquinoline 1-oxide and nalidixic acid.

***katF*** - One component of the cellular response of *E. coli* to oxidative stress is controlled by the product of the *katF* gene. KatF encodes a trans-acting positive regulator or sigma factor for exonuclease III, encoded by *xthA* and hydrogen peroxidase II (HP-II), encoded by *katE*. Both exonuclease III and HP-II are involved in the cellular response to oxidative damage. HP-II helps in the elimination of potentially damaging hydrogen peroxide and exonuclease III help to repair DNA damage caused in part by reactive oxygen species. The *katF-lacZ* fusion responds to DNA damage and chemicals such as mitomycin C, KCN, menadione, and chloroform.

***recA*** - In enteric bacteria, agents which cause DNA damage or block DNA replication induce an extensive regulon called the SOS response. The genes that make up this regulon are all negatively regulated by the LexA repressor. The *recA* gene encodes a protein which is a key member of the SOS response regulon. RecA is involved in DNA repair and recombination. The inducibility of *recA* under the SOS response is mediated thorough the proteolytic cleavage of the LexA repressor by an activated-RecA protein. Therefore, a fairly high basal level of RecA exists in the cell for processes not immediately involved in the SOS response but required for activation by RecA. The *recA-lacZ* fusion responds to DNA damage as induced by chemicals like methyl methanesulfonate and mitomycin C.

***zwf*** - One of the two dehydrogenases of the pentose phosphate pathway is glucose-6-phosphate dehydrogenase (G6PD) encoded by *zwf*. The activity of this enzyme increases with increasing growth rate, but by a manner dissimilar to that of the translational machinery. With ribosomal components the increase is immediate and with G6PD the increase is the same as that of total protein. This lag time is indicative of a transcriptionally regulated growth dependent response of *zwf*. In addition, the level of G6PD also increases following exposure to redox-cycling agents through transcriptional activation. This fusion is induced by oxidative stress as mediated by drugs such as paraquat and 4-nitroquinoline 1-oxide.

***dnaK*** - Following exposure to elevated temperature, organisms including *E. coli* transiently produce several proteins, collectively referred to as heat shock proteins. The heat shock proteins are highly conserved among organisms. The *dnaK-lacZ* fusion responds to changes in growth conditions and compounds like 1-propanol and ethanol.

***clpB*** - Induction of the heat shock response occurs coordinately at the level of transcription in part due to the presence of a RNA polymerase heat shock sigma factor 32 in *E. coli*. The *clpB-lacZ* fusion responds to agents which affect proteins, such as acetone, chloroform, methapyrilene, and *p*-chloroaniline.

***umuDC*** - In enteric bacteria, agents which cause DNA damage or block DNA replication induce an extensive regulon called the SOS response. Agents which cause DNA damage

derepress the SOS regulon. The umuDC transcript falls within this LexA repressed regulon. The gene products of umuDC play a role in radiation and chemically inducible mutagenesis. The umuDC-lacZ fusion is induced by benzo[a]pyrene, mitomycin C, and 4-nitroquinoline 1-oxide.

***merR*** - Mercury resistance is widespread in bacteria. The typical mercury operon is located in a transposon and functions to detoxify mercury by first transporting the mercury into the cell and then reducing it to the less toxic, volatile metallic  $Hg^0$ . The regulation of the mer operon is mediated by the repressor protein, merR. MerR induces transcription of the structural mer genes in the presence of nanomolar  $Hg(II)$ . Transcription of the mer genes is also affected by the level of DNA supercoiling. MerR is autoregulated. The merR-lacZ fusion is very sensitive to the presence of heavy metals, particularly mercury.

***ada*** - Alkylating agents are toxic and mutagenic in bacterial systems and carcinogenic to mammalian cells. The ada gene is the regulatory gene and also serves as one of the structural genes of the adaptive response to alkylation damage in *E.coli*. Following DNA alkylation, the ada protein transfers the methyl group from O-alkylated lesions in DNA to one of two cysteine residues in itself. This self methylation causes the ada protein to become a strong activator of its own transcription and also of alkA. The ada-lacZ fusion responds to the presence of methyl adducts on DNA and therefore is induced by DNA damaging agents like MMS, MNNG, and *p*-chloroaniline.



***dinD*** - Following the occurrence of extensive DNA damage to the *E. coli* genome, the SOS response becomes activated. The goal of this SOS response is to minimize the impact of the incurred DNA lesions. The genes involved in this DNA-damage inducible response encode enzymes required for the processing of damaged DNA, including DNA repair enzymes and enzymes involved in DNA recombination. The function of *dinD* is unknown. The *dinD-lacZ* fusion responds to many different types of DNA damaging chemicals like mitomycin C, MMS, MNNG, nalidixic acid,  $\beta$ -naphthoflavone, and 4-nitroquinoline 1-oxide.

***soi28*** - Aerobic organisms, including *E. coli*, are under continual insult from reactive oxygen species. These oxygen species can arise from numerous sources including incomplete reduction of molecular oxygen during respiration. Reactive oxygen species include the superoxide radical anion ( $O_2^-$ ), hydrogen peroxide, and the hydroxy radical. The *soi* (for superoxide radical inducible) genes were isolated based on their induction by superoxide radical anion generators such as paraquat and lumbagin. However, the *soi28* gene is not induced by hydrogen peroxide. The specific function of the protein encoded by *soi28* is under investigation. The *soi28-lacZ* fusion is induced specifically by  $O_2$  generating agents such as paraquat and 4-nitroquinoline 1-oxide.

***nfo*** - There are two enzymes present in *E. coli* which respond to the presence of apurinic and apyrimidinic sites. These so-called AP endonucleases which cleave 5' to the AP site are exonuclease III and endonuclease IV. The product of the *nfo* gene is endonuclease IV, which is predominately involved in the DNA repair of cells treated with bleomycin and t-butyl hydroperoxide. The *nfo-lacZ* fusion responds to the presence of apurinic and

apyrimidinic sites as well as DNA breaks as caused by bleomycin. This fusion also responds to oxidative damage of DNA such as caused by paraquat

**Appendix F**  
**FACTORIAL EXPERIMENTAL DESIGN AND SUMMARY RESULTS**  
**FOR STORAGE EFFECTS EXPERIMENTS**

factor s	pH during storage	length of storage	temp. during storage	back- ground	0.15 KF chlorine	0.25 KF chlorine	0.15 KF chlorine dioxide	0.25 KF chlorine dioxide	0.4% ozone	1.2% ozone
(1)	-	-	-	0.33	-0.01	0.29	0.42	0.17	0.09	0.93
a	+	-	-	0.26	0.21	0.26	0.82	0.16	0.48	0.41
b	-	+	-	-0.15	-0.20	0.24	0.33	0.19	-0.25	0.36
ab	+	+	-	0.63	0.15	0.17	0.98	0.01	-0.25	0.51
c	-	-	+	0.23	0.15	0.29	0.24	0.07	-0.25	0.38
ac	+	-	+	0.20	-0.15	0.17	0.17	0.29	0.34	0.63
bc	-	+	+	-0.25	0.11	-0.03	-0.05	-0.15	-0.25	-0.25
abc	+	+	+	-0.09	0.15	0.11	0.70	-0.14	0.14	-0.15

Table 1a. Factorial Design for the Evaluation of Storage Effects on First Stage Filtrates from Chlorine, Chlorine Dioxide, and Ozone Bleaching. Results expressed as log TU (toxicity units) and are the result of quadruplet testing

factor s	pH during storage	length of storage	temp. during storage	back- ground	0.15 KF chlorine	0.25 KF chlorine	0.15 KF chlorine dioxide	0.25 KF chlorine dioxide	0.4% ozone	1.2% ozone
(1)	-	-	-	0.68	0.32	0.32	0.53	0.35	0.32	0.79
a	+	-	-	0.86	0.45	0.56	0.66	0.27	0.36	0.21
b	-	+	-	0.97	0.45	0.34	0.26	0.25	-0.02	0.49
ab	+	+	-	1.08	0.42	0.36	0.85	0.01	0.10	0.71
c	-	-	+	0.79	0.31	0.48	0.34	0.24	0.04	0.51
ac	+	-	+	0.83	0.48	0.25	0.49	0.18	0.21	1.01
bc	-	+	+	-0.25	0.54	0.57	0.05	-0.15	-0.25	0.11
abc	+	+	+	0.80	0.36	0.17	0.55	0	-0.15	0

Table 1b. Factorial Design for the Evaluation of Storage Effects on Second Stage (EO) Filtrates from Chlorine, Chlorine Dioxide, and Ozone Bleaching. Results expressed as log TU (toxicity units) and are the result of quadruplet testing

variable	-	+
pH during storage	neutralized	bleaching stage end pH
length of storage (days)	1	14
temperature during storage (°C)	4	20

Table 1c. Levels of Factors Used in Factorial Design

## APPENDIX F, CONT'D

**FACTORIAL EXPERIMENTAL DESIGN AND SUMMARY RESULTS  
FOR STORAGE EFFECTS EXPERIMENTS**

**Table 2.** ANOVA Table of Storage and Chemical/Charge Variables for First Stage Filtrates

<u>Source</u>	<u>DF</u>	<u>Sum-Squares</u>	<u>Mean Square</u>	<u>F-Ratio</u>	<u>Prob&gt;F</u>
pH during storage	1	0.314	0.314	9.47	0.022 *
length of storage	1	0.449	0.449	13.53	0.010 *
temp. during storage	1	0.433	0.433	13.05	0.011 *
bleaching chemical/charge	6	1.297	.216	6.51	0.019 *

\* signifies significance at the 95% confidence level

**Table 3.** ANOVA Table of Storage and Chemical/Charge Variables for Second Stage Filtrates

<u>Source</u>	<u>DF</u>	<u>Sum-Squares</u>	<u>Mean Square</u>	<u>F-Ratio</u>	<u>Prob&gt;F</u>
pH during storage	1	0.131	0.131	1.87	0.220
length of storage	1	0.319	0.319	4.57	0.076
temp. during storage	1	0.351	0.351	5.04	0.066
bleaching chemical/charge	6	2.257	0.376	5.39	0.030*

\* signifies significance at the 95% confidence level

## Appendix G

### MICROTOX DATA COMPILATION FOR BLEACHING FILTRATES AND FRACTIONS

(RESULTS EXPRESSED AS TOXICITY UNITS (TU))

				REP 1						REP 2				
	filtrate	fraction	a	b	c	average	std dev.	CV (%)	a	b	c	average	std dev.	CV (%)
B		whole	2.9	2.1	2.8	2.6	0.43	16.5%	0.6	0.6	0.6	0.6	0.00	0.0%
E(B)		whole	1.2	0.6	0.6	0.8	0.40	50.7%	1.4	1.3	1.3	1.3	0.08	5.8%
B+E		whole	1.2	1.6	1.7	1.5	0.29	19.0%	0.6	0.6	0.6	0.6	0.00	0.0%
B+E		NVW	0.6	1.7	2.7	1.6	1.06	64.6%	0.6	0.6	0.6	0.6	0.00	0.0%
B+E		EE	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
B+E		NEE	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>L</sub>		whole	0.6	0.6	1.6	0.9	0.62	68.1%	1.5	2.0	1.1	1.5	0.49	31.9%
E(D <sub>L</sub> )		whole	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>L</sub> +E		whole	0.6	0.6	1.2	0.8	0.38	49.2%	0.6	2.4	0.6	1.2	1.08	91.7%
D <sub>L</sub> +E		NVW	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>L</sub> +E		EE	3.9	4.4	2.9	3.7	0.76	20.4%	2.2	1.9	1.7	1.9	0.25	13.0%
D <sub>L</sub> +E		NEE	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>H</sub>		whole	1.9	1.2	2.0	1.7	0.41	23.8%	1.4	1.7	1.9	1.7	0.25	14.9%
E(D <sub>H</sub> )		whole	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>H</sub> +E		whole	0.6	1.2	1.3	1.0	0.38	38.6%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>H</sub> +E		NVW	0.6	0.6	0.6	0.6	0.00	0.0%	0.6	0.6	0.6	0.6	0.00	0.0%
D <sub>H</sub> +E		EE	0.6	1.9	0.6	1.0	0.75	72.6%	0.6	0.6	1.3	0.8	0.41	51.5%

[illegible]

## Appendix H

## GRAPHICAL REPRESENTATIONS OF MICROTOX DATA

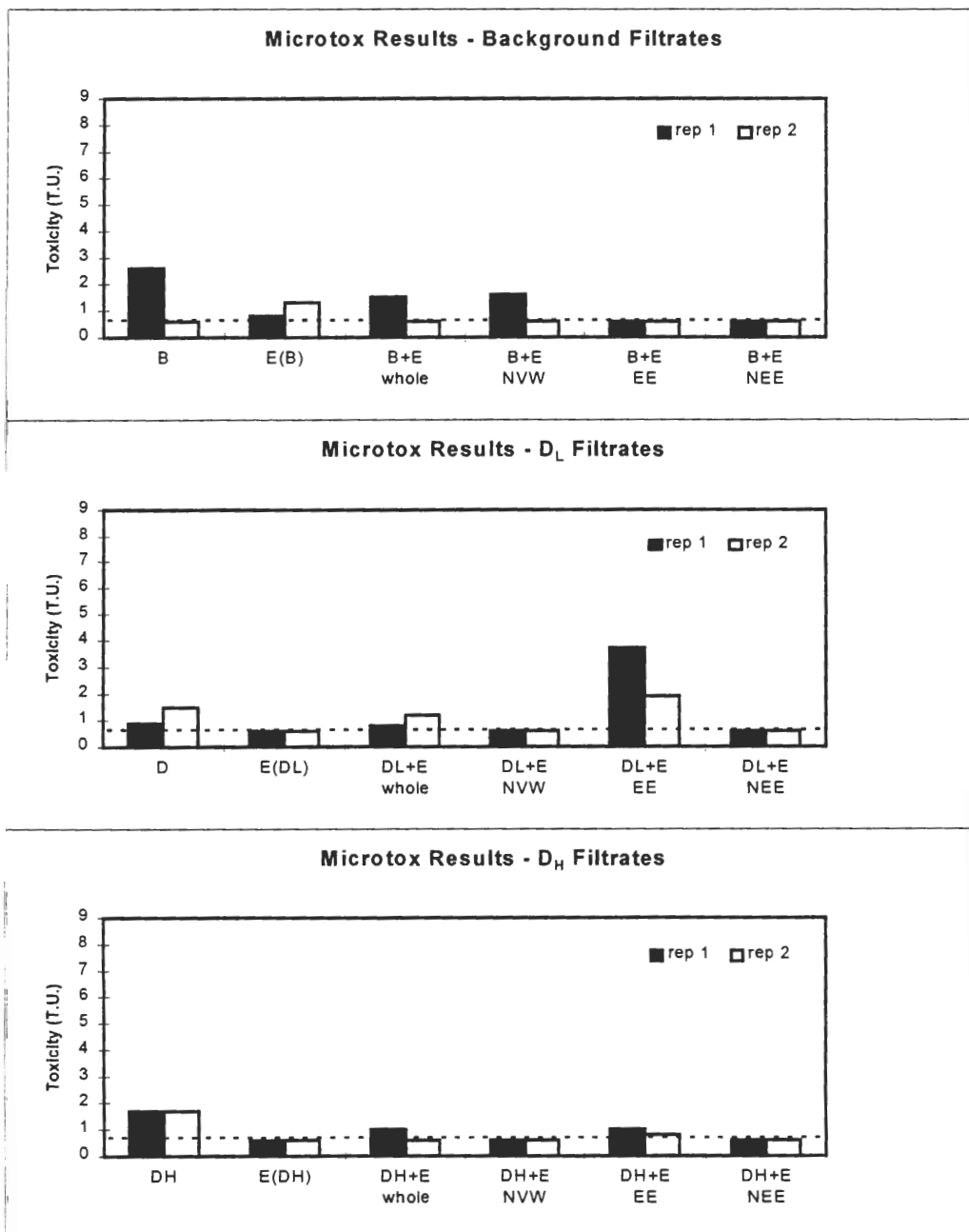


Figure 1. Microtox Toxicities Evaluated By Bleaching Agent/Charge

## APPENDIX H, CONT'D

## GRAPHICAL REPRESENTATIONS OF MICROTOX DATA

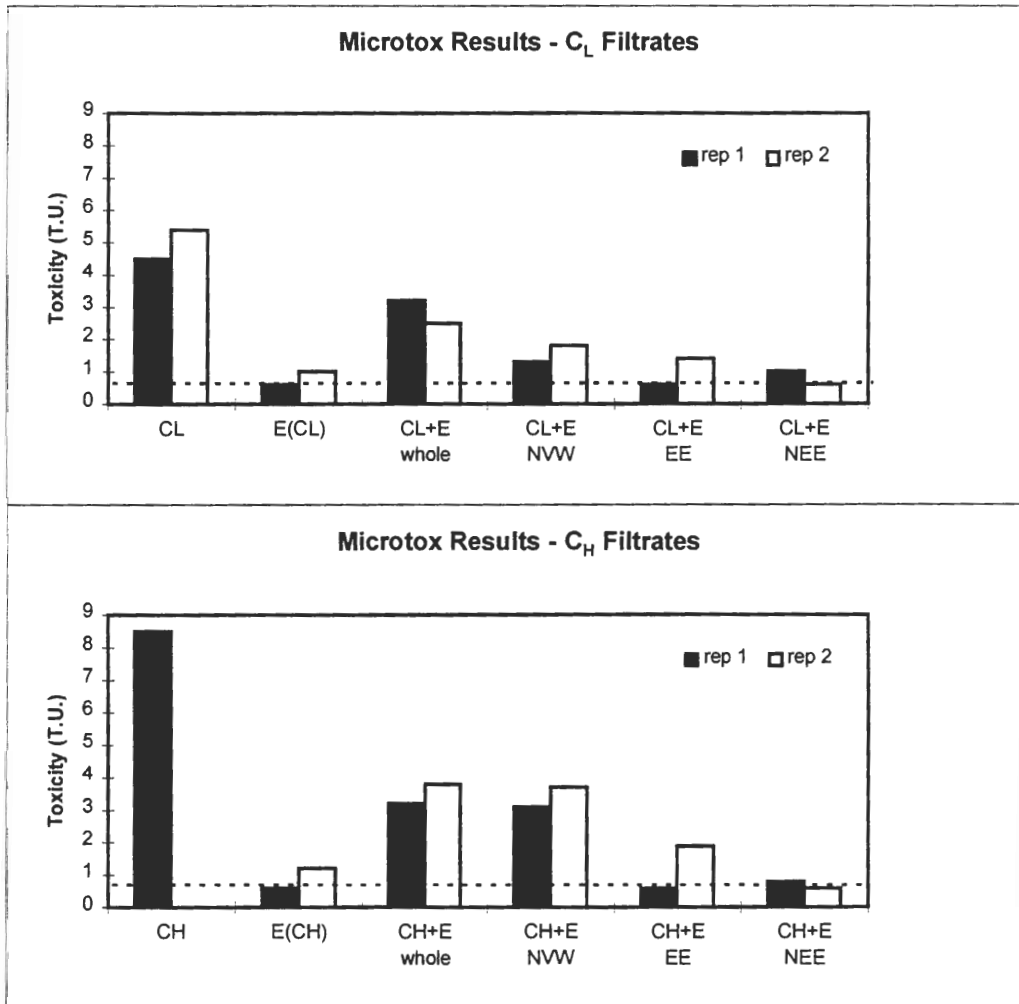
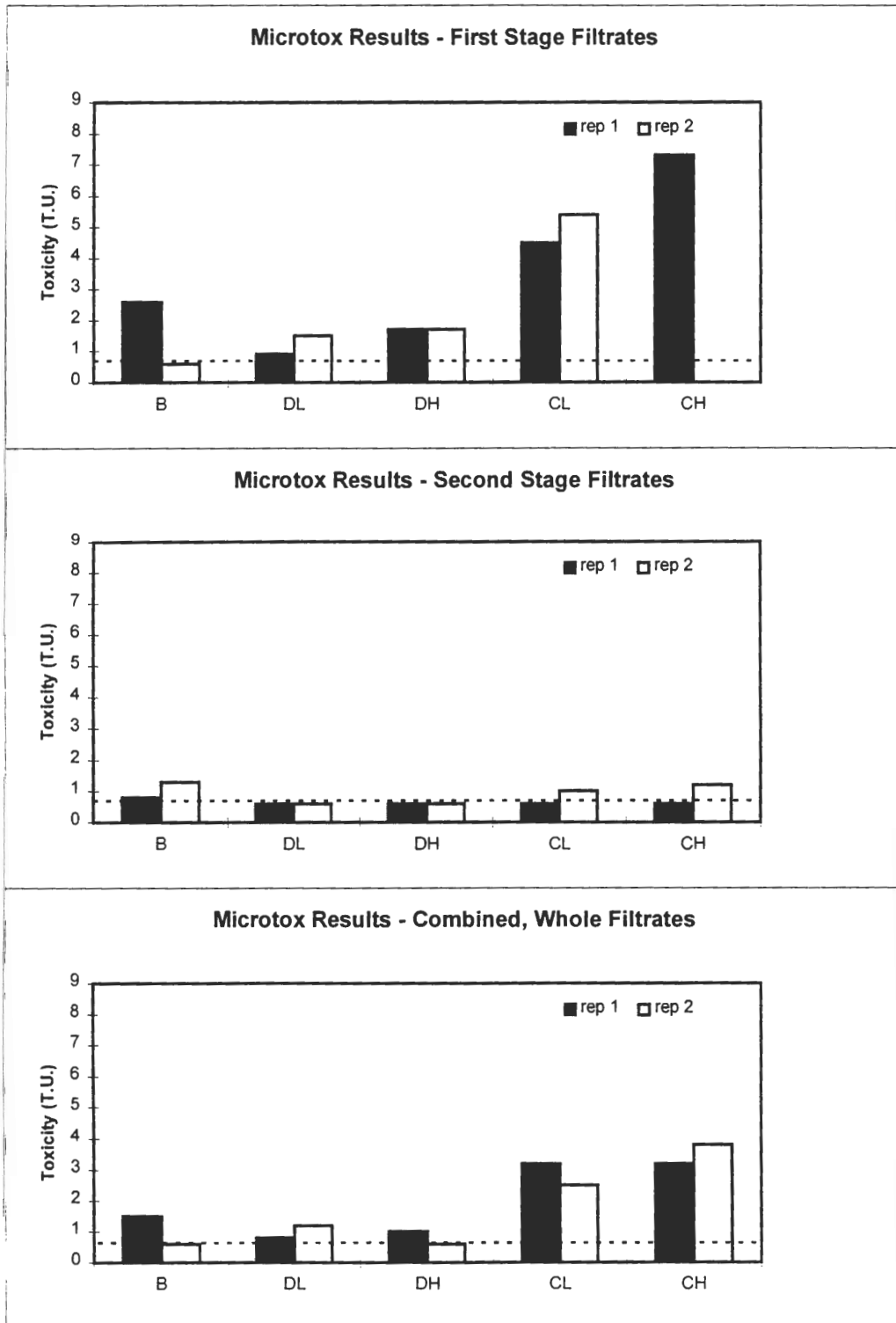


Figure 1. Microtox Toxicities Evaluated By Bleaching Agent/Charge, cont'd



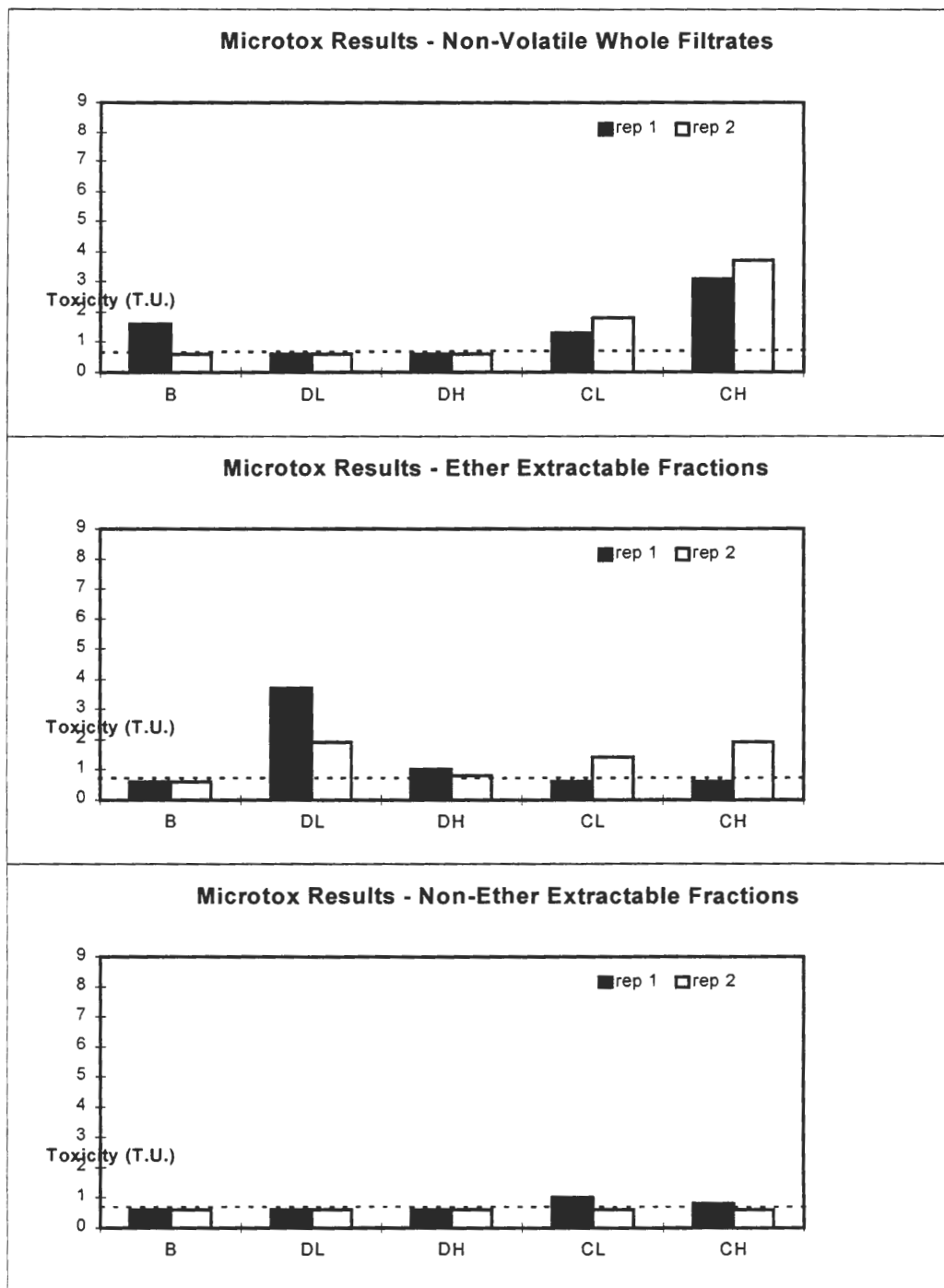
## APPENDIX H, CONT'D

## GRAPHICAL REPRESENTATIONS OF MICROTOX DATA,

**Figure 2.** Microtox Toxicities Evaluated By Bleaching Agent

## APPENDIX H, CONT'D

## GRAPHICAL REPRESENTATIONS OF MICROTOX DATA

**Figure 2.** Microtox Toxicities Evaluated By Bleaching Agent, cont'd

## Appendix I

### XTT DATA COMPILATION FOR BLEACHING FILTRATES AND FRACTIONS

#### (RESULTS EXPRESSED AS TOXICITY UNITS (TU))

Column 1 filtrate description B=background stage C=chlorine stage D=chlorine dioxide stage E=extraction stage -1= rep 1 -2= rep 2 L=low charge H=high charge	Column 6 average of columns 4 and 5	Column 12 column 7 divided by column 6 = response as a % of control for 0.5% exposure concentration	Column 18 average of four repeats (a,b,c, and d)
Column 2 fraction description W=whole NVW=non-volatile whole EE=ether extractable fraction NEE=non-ether extractable fraction	Column 7 absorbance reading for 0.5% exposure concentration	Column 13 column 8 divided by column 6 =response as a % of control for 1% exposure conc.	Column 19 std.dev. of four repeats (a,b,c, and d)
Column 3 repeat description (a,b,c, or d)	Column 8 absorbance reading for 1.0% exposure concentration	Column 14 column 9 divided by column 6 =response as a % of control for 5% exposure conc.	
column 4 absorbance reading for 1 <sup>st</sup> control	Column 9 absorbance reading for 5% exposure concentration	Column 15 column 10 divided by column 6 =response as a % of control for 10% exposure conc.	
Column 5 absorbance reading for 2 <sup>nd</sup> control	Column 10 absorbance reading for 10% exposure concentration	Column 16 column 11 divided by column 6 =response as a % of control for 25% exp. conc.	
	Column 11 absorbance reading for 25% exposure concentration	Column 17 conc. tested	

filtrate	fraction	repeat	control a	control b	avg. control	0.50 %	1%	5%	10%	25%	0.5%/control	1%/control	5%/control	10%/control	25%/control	averages	std. dev
----------	----------	--------	--------------	--------------	-----------------	-----------	----	----	-----	-----	--------------	------------	------------	-------------	-------------	----------	-------------

# XTT DATA COMPILATION FOR BLEACHING FILTRATES AND FRACTIONS

(RESULTS EXPRESSED AS TOXICITY UNITS (TU))

filtrate	fraction	repeat	control	control	avg.	0.50	1%	5%	10%	25%	0.5%/control	1%/control	5%/control	10%/control	25%/control	average	std.
			a	b	control	%										s	dev
DL-1		a	2.232	2.242	2.237	1.286	1.614	1.047	0.678	0.493	57.5%	72.2%	46.8%	30.3%	22.0%	0.50%	81.3%
		b	2.022	2.075	2.049	1.717	1.239	0.811	0.807	0.496	83.8%	60.5%	39.6%	39.4%	24.2%	1%	68.3%
		c	2.025	1.712	1.869	2.051	1.420	0.791	0.702	0.516	109.8%	76.0%	42.3%	37.6%	27.6%	5%	40.9%
		d	2.372	2.291	2.332	1.726	1.504	0.810	0.723	0.523	74.0%	64.5%	34.7%	31.0%	22.4%	10%	34.6%
																25%	24.1%
DL+E-1	Whole	a	2.490	1.847	2.169	1.808	1.380	1.029	0.984	0.720	83.4%	63.6%	47.5%	45.4%	33.2%	0.50%	90.2%
		b	1.881	2.038	1.960	1.473	1.438	1.110	0.849	0.542	75.2%	73.4%	56.6%	43.3%	27.7%	1%	77.5%
		c	2.365	2.096	2.231	2.163	1.962	1.115	1.012	0.711	97.0%	88.0%	50.0%	45.4%	31.9%	5%	53.0%
		d	1.823	2.203	2.013	2.122	1.714	1.162	1.016	0.621	105.4%	85.1%	57.7%	50.5%	30.8%	10%	46.1%
																25%	30.9%
EDL-1		a	2.232	2.242	2.237	1.705	2.007	1.409	1.524	1.109	76.2%	89.7%	63.0%	68.1%	49.6%	0.50%	81.6%
		b	2.022	2.075	2.049	1.661	1.702	1.369	1.459	1.125	81.1%	83.1%	66.8%	71.2%	54.9%	1%	91.4%
		c	2.025	1.712	1.869	1.839	1.895	1.492	1.499	1.240	98.4%	101.4%	79.9%	80.2%	66.4%	5%	66.4%
		d	2.372	2.291	2.332	1.646	2.130	1.302	1.462	1.213	70.6%	91.4%	55.8%	62.7%	52.0%	10%	70.6%
																25%	55.7%
DL+E-1	NW	a	2.490	1.847	2.169	1.954	1.303	0.982	0.809	0.550	90.1%	60.1%	45.3%	37.3%	25.4%	0.50%	83.5%
		b	1.881	2.038	1.960	2.016	1.305	1.053	0.901	0.495	102.9%	66.6%	53.7%	46.0%	25.3%	1%	71.1%
		c	2.365	2.096	2.231	1.475	1.712	0.867	1.047	0.485	66.1%	76.8%	38.9%	46.9%	21.7%	5%	46.0%
		d	1.823	2.203	2.013	1.506	1.630	0.926	0.985	0.623	74.8%	81.0%	46.0%	48.9%	30.9%	10%	44.8%
																25%	25.8%

filtrate	fraction	repeat	control	control	avg.	0.50	1%	5%	10%	25%	0.5%/control	1%/control	5%/control	10%/control	25%/control	average	std.
			a	b	control	%										s	dev
DH-1		a	2.096	1.970	2.033	1.742	1.584	0.860	0.714	0.560	85.7%	77.9%	42.3%	35.1%	27.5%	0.50%	78.0%
		b	1.995	2.203	2.099	1.645	1.339	0.925	0.739	0.506	78.4%	63.8%	44.1%	35.2%	24.1%	1%	65.4%
		c	2.092	2.016	2.054	1.465	1.398	0.949	0.808	0.497	71.3%	68.1%	46.2%	39.3%	24.2%	5%	44.0%
		d	2.227	1.909	2.068	1.586	1.070	0.900	0.857	0.489	76.7%	51.7%	43.5%	41.4%	23.6%	10%	37.8%
																25%	24.9%
DH+E-1	W	a	1.727	1.606	1.667	1.368	1.385	0.794	0.693	0.558	82.1%	83.1%	47.6%	41.6%	33.5%	0.50%	78.5%
		b	2.107	2.320	2.214	1.675	1.533	0.818	0.890	0.569	75.7%	69.3%	37.0%	40.2%	25.7%	1%	73.1%
		c	2.100	2.038	2.069	1.493	1.579	1.037	0.671	0.490	72.2%	76.3%	50.1%	32.4%	23.7%	5%	45.2%
		d	1.781	2.258	2.020	1.694	1.290	0.929	0.947	0.517	83.9%	63.9%	46.0%	46.9%	25.6%	10%	40.3%
																25%	27.1%
EDH-1		a	1.970	2.096	2.033	2.146	2.013	1.540	1.597	1.122	105.6%	99.0%	75.8%	78.6%	55.2%	0.50%	92.9%
		b	2.203	1.995	2.099	2.194	1.924	1.507	1.401	1.178	104.5%	91.7%	71.8%	66.7%	56.1%	1%	95.2%
		c	2.016	2.092	2.054	1.520	1.995	1.316	1.563	1.104	74.0%	97.1%	64.1%	76.1%	53.7%	5%	68.6%
		d	1.909	2.227	2.068	1.808	1.926	1.295	1.561	1.114	87.4%	93.1%	62.6%	75.5%	53.9%	10%	74.2%

[illegible]

2.2.4

B+E-1		NVW	a	1.731	1.808	1.770	1.577	1.399	1.020	0.871	0.551	89.1%	79.1%	57.6%	49.2%	31.1%	0.50%	92.2%	4.2%
			b	2.038	2.142	2.090	1.938	1.839	1.493	1.071	0.612	92.7%	88.0%	71.4%	51.2%	29.3%	1%	81.3%	6.0%
			c	1.689	2.167	1.928	1.714	1.430	0.973	1.193	0.567	88.9%	74.2%	50.5%	61.9%	29.4%	5%	56.3%	11.2%
			d	2.082	2.320	2.201	2.154	1.845	1.006	1.492	0.603	97.9%	83.8%	45.7%	67.8%	27.4%	10%	57.5%	8.8%
																25%	29.3%	1.5%	
B-2			a	2.096	2.134	2.115	1.310	1.042	0.800	0.713	0.455	61.9%	49.3%	37.8%	33.7%	21.5%	0.50%	68.5%	10.9%
			b	2.103	2.198	2.151	1.376	1.111	0.958	0.713	0.468	64.0%	51.7%	44.5%	33.2%	21.8%	1%	54.5%	6.0%
			c	1.970	2.092	2.031	1.723	1.281	0.985	0.754	0.491	84.8%	63.1%	48.5%	37.1%	24.2%	5%	45.2%	5.4%
			d	2.029	2.439	2.234	1.412	1.204	1.112	0.766	0.478	63.2%	53.9%	49.8%	34.3%	21.4%	10%	34.6%	1.8%
																25%	22.2%	1.3%	
B+E-2		W	a	2.372	2.180	2.276	1.575	1.631	1.223	0.870	0.530	69.2%	71.7%	53.7%	38.2%	23.3%	0.50%	76.8%	7.2%
			b	1.955	2.142	2.054	1.513	1.428	1.168	0.822	0.505	73.7%	69.5%	56.9%	40.0%	24.6%	1%	74.3%	6.3%
			c	2.122	1.919	2.021	1.577	1.466	0.998	1.149	0.508	78.0%	72.6%	49.4%	56.9%	25.1%	5%	52.8%	3.3%
			d	2.163	2.423	2.293	1.976	1.916	1.171	0.966	0.521	86.2%	83.6%	51.1%	42.1%	22.7%	10%	44.3%	8.5%
																25%	23.9%	1.1%	
EB-2			a	2.134	2.096	2.115	1.817	1.886	1.696	1.687	1.154	85.9%	89.2%	80.2%	79.8%	54.6%	0.50%	97.1%	14.2%
			b	2.198	2.103	2.151	2.019	1.936	1.645	1.907	1.191	93.9%	90.0%	76.5%	88.7%	55.4%	1%	95.8%	9.3%
			c	2.092	1.970	2.031	2.393	2.217	1.755	1.839	1.211	117.8%	109.2%	86.4%	90.5%	59.6%	5%	77.1%	8.8%
			d	2.439	2.029	2.234	2.025	2.115	1.463	1.559	1.172	90.6%	94.7%	65.5%	69.8%	52.5%	10%	82.2%	9.5%
																25%	55.5%	3.0%	
B+E-2		NVW	a	2.180	2.372	2.276	1.502	1.740	0.889	1.070	0.556	66.0%	76.4%	39.1%	47.0%	24.4%	0.50%	77.7%	8.2%
			b	2.142	1.965	2.054	1.700	1.563	1.150	0.960	0.545	82.8%	76.1%	56.0%	46.7%	26.5%	1%	88.8%	15.9%
			c	1.919	2.122	2.021	1.577	2.212	0.985	0.893	0.547	78.0%	109.5%	48.8%	44.2%	27.1%	5%	49.0%	7.3%
			d	2.423	2.163	2.293	1.928	2.138	1.199	1.051	0.575	84.1%	93.2%	52.3%	45.8%	25.1%	10%	45.9%	1.3%
																25%	25.8%	1.2%	
B+E-1		EE	a	2.849	2.333	2.591	1.788	1.711	1.553	1.656	1.388	69.0%	66.0%	59.9%	63.9%	53.6%	0.50%	71.4%	2.7%
			b	2.379	2.439	2.409	1.812	1.560	1.392	1.426	1.314	75.2%	64.8%	57.8%	59.2%	54.5%	1%	67.9%	4.7%
			c	2.038	2.559	2.299	1.622	1.518	1.265	1.387	1.320	70.6%	66.0%	55.0%	60.3%	57.4%	5%	61.8%	8.6%
			d	2.339	2.455	2.397	1.696	1.795	1.782	1.552	1.320	70.8%	74.9%	74.3%	64.7%	55.1%	10%	62.0%	2.7%
																25%	55.2%	1.6%	
B+E-2		EE	a	2.320	2.408	2.364	1.455	1.401	1.359	1.540	1.340	61.5%	59.3%	57.5%	65.1%	56.7%	0.50%	69.2%	10.3%
			b	2.345	2.252	2.299	1.941	1.879	1.990	1.640	1.150	84.4%	81.7%	86.6%	71.4%	50.0%	1%	77.1%	12.4%
			c	1.981	1.843	1.912	1.259	1.680	1.699	1.426	1.222	65.8%	87.9%	88.9%	74.6%	63.9%	5%	76.2%	14.6%
			d	1.926	2.285	2.106	1.368	1.673	1.517	1.582	1.284	65.0%	79.5%	72.0%	75.1%	61.0%	10%	71.6%	4.6%
																25%	57.9%	6.0%	
B+E-1		NEE	a	2.333	2.849	2.591	2.333	1.856	1.793	1.223	0.730	90.0%	71.6%	69.2%	47.2%	28.2%	0.50%	93.8%	4.8%
			b	2.439	2.379	2.409	2.416	1.909	1.802	1.162	0.637	100.3%	79.2%	74.8%	48.2%	26.4%	1%	81.6%	7.7%
			c	2.559	2.038	2.299	2.171	1.995	1.734	1.314	0.637	94.5%	86.8%	75.4%	57.2%	27.7%	5%	74.4%	3.7%
			d	2.455	2.339	2.397	2.163	2.122	1.870	1.245	0.683	90.2%	88.5%	78.0%	51.9%	28.5%	10%	51.1%	4.5%
																25%	27.7%	0.9%	

B+E-2	NEE	a	2.408	2.320	2.364	2.089	1.729	1.712	1.396	0.657	88.4%	73.1%	72.4%	59.1%	27.8%	0.50%	82.4%	11.4%
		b	2.252	2.345	2.299	2.194	2.089	1.630	1.130	0.647	95.5%	90.9%	70.9%	49.2%	28.1%	1%	87.6%	10.3%
		c	1.843	1.981	1.912	1.379	1.866	1.557	1.029	0.643	72.1%	97.6%	81.4%	53.8%	33.6%	5%	73.9%	5.0%
		d	2.285	1.926	2.106	1.549	1.868	1.494	1.207	0.699	73.6%	88.7%	71.0%	57.3%	33.2%	10%	54.8%	4.4%
DL-2																25%	30.7%	3.2%
		a	2.339	2.528	2.434	1.819	2.227	1.765	1.262	0.507	74.7%	91.5%	72.5%	51.9%	20.8%	0.50%	82.0%	5.8%
		b	2.653	2.592	2.623	2.103	2.212	1.449	1.078	0.787	80.2%	84.3%	55.3%	41.1%	30.0%	1%	86.8%	5.4%
		c	2.627	2.455	2.541	2.159	2.308	1.419	1.063	0.560	85.0%	90.8%	55.8%	41.8%	22.0%	5%	57.5%	10.9%
		d	2.592	2.592	2.592	2.285	2.082	1.207	1.217	0.547	88.2%	80.3%	46.6%	47.0%	21.1%	10%	45.4%	5.0%
DH-2																25%	23.5%	4.4%
		a	2.333	2.592	2.463	2.227	2.580	2.189	1.926	1.525	90.4%	104.8%	88.9%	78.2%	61.9%	0.50%	99.6%	6.3%
		b	2.263	2.569	2.416	2.538	2.538	2.247	1.990	1.897	105.0%	105.0%	93.0%	82.4%	78.5%	1%	101.1%	6.0%
		c	2.285	2.548	2.417	2.439	2.473	2.068	2.263	1.793	100.9%	102.3%	85.6%	93.6%	74.2%	5%	88.5%	3.3%
		d	2.666	2.379	2.523	2.569	2.326	2.185	1.897	0.920	101.8%	92.2%	86.6%	75.2%	36.5%	10%	82.4%	8.1%
EDL-2																25%	62.8%	18.9%
		a	2.528	2.339	2.434	1.933	1.831	1.151	1.342	0.614	79.4%	75.2%	47.3%	55.1%	25.2%	0.50%	81.8%	4.8%
		b	2.592	2.653	2.623	2.001	1.697	1.309	0.996	0.510	76.3%	64.7%	49.9%	38.0%	19.4%	1%	76.6%	8.8%
		c	2.455	2.627	2.541	2.154	2.100	1.250	1.039	0.493	84.8%	82.6%	49.2%	40.9%	19.4%	5%	53.9%	10.3%
		d	2.592	2.592	2.592	2.252	2.176	1.795	1.168	0.615	86.9%	84.0%	69.3%	45.1%	23.7%	10%	44.8%	7.5%
EDH-2																25%	22.0%	3.0%
		a	2.592	2.333	2.463	2.709	2.490	2.092	1.845	1.231	110.0%	101.1%	85.0%	74.9%	50.0%	0.50%	98.7%	9.2%
		b	2.569	2.263	2.416	2.126	2.464	1.936	1.868	1.402	88.0%	102.0%	80.1%	77.3%	58.0%	1%	98.2%	6.7%
		c	2.548	2.285	2.417	2.431	2.130	2.217	1.897	1.306	100.6%	88.1%	91.7%	78.5%	54.0%	5%	84.3%	5.4%
		d	2.379	2.666	2.523	2.423	2.559	2.032	1.897	0.858	96.1%	101.4%	80.6%	75.2%	34.0%	10%	76.5%	1.7%
																25%	49.0%	10.5%

DL+E-2	W	a	2.615	2.580	2.598	1.886	2.416	1.763	1.509	0.586	72.6%	93.0%	67.9%	58.1%	22.6%	0.50%	83.2%	8.7%
		b	2.252	2.580	2.416	1.954	2.146	1.675	1.185	0.540	80.9%	88.8%	69.3%	49.0%	22.4%	1%	91.0%	4.2%
		c	2.490	2.431	2.461	2.291	2.359	1.856	1.311	0.776	93.1%	95.9%	75.4%	53.3%	31.5%	5%	71.4%	3.5%
		d	2.490	2.528	2.509	2.163	2.167	1.833	1.139	0.763	86.2%	86.4%	73.1%	45.4%	30.4%	10%	51.5%	5.5%
																25%	26.7%	4.9%
DH+E-2	W	a	2.653	2.189	2.421	2.431	2.154	1.812	1.521	0.681	100.4%	89.0%	74.8%	62.8%	28.1%	0.50%	98.5%	2.0%
		b	2.548	2.509	2.529	2.447	2.263	1.658	1.418	0.621	96.8%	89.5%	65.6%	56.1%	24.6%	1%	89.1%	3.2%
		c	2.592	2.528	2.560	2.559	2.176	1.907	1.419	0.838	100.0%	85.0%	74.5%	55.4%	32.7%	5%	73.7%	6.0%
		d	2.499	2.490	2.495	2.416	2.314	1.995	1.660	0.607	96.9%	92.8%	80.0%	66.5%	24.3%	10%	60.2%	5.4%
																25%	27.4%	3.9%
DL+E-2	NEE	a	2.580	2.615	2.598	2.326	2.089	1.616	1.387	0.808	89.5%	80.4%	62.2%	53.4%	31.1%	0.50%	87.7%	11.0%
		b	2.580	2.252	2.416	2.401	1.781	1.858	1.235	0.863	99.4%	73.7%	76.9%	51.1%	35.7%	1%	84.9%	10.8%
		c	2.431	2.490	2.461	2.185	2.122	1.895	1.514	0.702	88.8%	86.2%	77.0%	61.5%	28.5%	5%	70.5%	7.6%
		d	2.528	2.490	2.509	1.829	2.490	1.650	1.148	0.670	72.9%	99.2%	65.8%	45.8%	26.7%	10%	53.0%	6.6%
																25%	30.5%	3.9%



DL+E-2	NEE	a	2.189	2.653	2.421	1.829	2.022	1.916	1.489	0.533	75.5%	83.5%	79.1%	61.5%	22.0%	0.50%	84.5%	6.7%
		b	2.509	2.548	2.529	2.134	2.222	1.791	1.250	0.551	84.4%	87.9%	70.8%	49.4%	21.8%	1%	87.1%	2.4%
		c	2.528	2.592	2.560	2.339	2.268	1.799	1.729	0.767	91.4%	88.6%	70.3%	67.5%	30.0%	5%	75.1%	5.3%
		d	2.490	2.499	2.495	2.167	2.203	1.998	1.589	0.767	86.9%	88.3%	80.1%	63.7%	30.7%	10%	60.5%	7.8%
DL+E-2	EE	a	2.118	1.788	1.953	1.946	1.438	1.233	1.263	1.058	99.6%	73.6%	63.1%	64.7%	54.2%	0.50%	96.2%	7.5%
		b	1.919	1.775	1.847	1.574	1.347	1.265	1.283	1.107	85.2%	72.9%	68.5%	69.5%	59.9%	1%	75.3%	5.7%
		c	2.171	1.781	1.976	2.019	1.401	1.222	1.258	1.082	102.2%	70.9%	61.8%	63.7%	54.8%	5%	65.1%	3.1%
		d	1.860	1.777	1.819	1.775	1.522	1.214	1.271	1.064	97.6%	83.7%	66.8%	69.9%	58.5%	10%	66.9%	3.2%
DL+E-2	EE	a	1.788	2.118	1.953	1.504	1.463	0.822	0.678	0.660	77.0%	74.9%	42.1%	34.7%	33.8%	0.50%	82.0%	4.8%
		b	1.775	1.919	1.847	1.508	1.372	0.839	0.829	0.621	81.6%	74.3%	45.4%	44.9%	33.6%	1%	75.1%	0.9%
		c	1.781	2.171	1.976	1.597	1.477	0.782	0.766	0.647	80.8%	74.7%	39.6%	38.8%	32.7%	5%	43.8%	3.8%
		d	1.777	1.860	1.819	1.610	1.388	0.876	0.833	0.704	88.5%	76.3%	48.2%	45.8%	38.7%	10%	41.0%	5.2%
DL+E-2	NVEW	a	1.712	1.624	1.668	1.495	1.550	1.096	1.094	0.583	89.6%	92.9%	65.7%	65.6%	35.0%	0.50%	95.7%	9.7%
		b	1.729	1.856	1.793	1.533	1.819	1.095	0.802	0.566	85.5%	101.5%	61.1%	44.7%	31.6%	1%	94.1%	5.4%
		c	1.752	1.574	1.663	1.686	1.556	1.111	0.945	0.693	101.4%	93.6%	66.8%	56.8%	41.7%	5%	63.5%	3.3%
		d	2.007	1.615	1.811	1.921	1.603	1.091	0.984	0.699	106.1%	88.5%	60.2%	54.3%	38.6%	10%	55.4%	8.6%
DL+E-2	NVEW	a	1.624	1.712	1.668	1.296	1.339	1.004	0.943	0.645	77.7%	80.3%	60.2%	56.5%	38.7%	0.50%	75.6%	1.9%
		b	1.856	1.729	1.793	1.362	1.374	1.056	0.838	0.572	76.0%	76.7%	58.9%	46.8%	31.9%	1%	82.5%	6.1%
		c	1.574	1.752	1.663	1.215	1.365	1.177	1.025	0.646	73.1%	82.1%	70.8%	61.6%	38.8%	5%	61.2%	6.8%
		d	1.615	2.007	1.811	1.367	1.649	0.993	0.941	0.585	75.5%	91.1%	54.8%	52.0%	32.3%	10%	54.2%	6.4%
CL-1		a	1.699	1.686	1.693	1.424	1.428	1.057	0.835	0.686	84.1%	84.4%	62.5%	49.3%	40.5%	0.50%	89.2%	8.2%
		b	1.693	1.650	1.672	1.405	1.360	1.041	0.926	0.724	84.1%	81.4%	62.3%	55.4%	43.3%	1%	84.3%	2.1%
		c	1.672	1.621	1.647	1.438	1.419	1.091	0.890	0.670	87.3%	86.2%	66.3%	54.1%	40.7%	5%	63.9%	1.9%
		d	1.705	1.602	1.654	1.676	1.412	1.065	0.949	0.737	101.4%	85.4%	64.4%	57.4%	44.6%	10%	54.0%	3.4%
CL+E-1	W	a	1.684	1.627	1.656	1.446	1.494	1.301	1.081	0.850	87.3%	90.2%	78.6%	65.3%	51.9%	0.50%	89.2%	2.2%
		b	1.673	1.635	1.654	1.521	1.506	1.267	0.919	0.764	92.0%	91.1%	76.6%	55.6%	46.2%	1%	88.7%	3.3%
		c	1.949	1.658	1.804	1.626	1.511	1.176	1.090	0.656	90.2%	83.8%	65.2%	60.4%	36.4%	5%	71.5%	7.1%
		d	1.812	1.690	1.751	1.532	1.571	1.147	0.986	0.621	87.5%	89.7%	65.5%	56.3%	35.5%	10%	59.4%	4.5%
ECL-1		a	1.686	1.699	1.693	1.661	1.620	1.560	1.455	1.285	98.1%	95.7%	92.2%	86.0%	75.9%	0.50%	97.5%	0.7%
		b	1.650	1.693	1.672	1.624	1.629	1.546	1.551	1.158	97.2%	97.5%	92.5%	92.8%	69.3%	1%	97.2%	1.2%
		c	1.621	1.672	1.647	1.616	1.622	1.529	1.497	1.285	98.1%	98.5%	92.9%	90.9%	78.0%	5%	92.4%	0.4%
		d	1.602	1.705	1.654	1.599	1.603	1.521	1.446	1.248	96.7%	96.9%	92.0%	87.5%	75.5%	10%	89.3%	3.1%
																25%	74.7%	3.8%



CL+E-1	NVEW	a	1.627	1.684	1.656	1.549	1.553	1.289	0.930	0.673	93.6%	93.8%	77.9%	56.2%	40.7%	0.50%	91.7%	2.8%
		b	1.635	1.673	1.654	1.556	1.508	1.295	0.935	0.679	94.1%	91.2%	78.3%	56.5%	41.1%	1%	89.9%	3.6%
		c	1.658	1.949	1.804	1.587	1.539	1.340	1.093	0.684	88.0%	85.3%	74.3%	60.6%	37.9%	5%	72.5%	8.8%
		d	1.690	1.812	1.751	1.596	1.565	1.042	0.988	0.770	91.1%	89.4%	59.5%	56.4%	44.0%	10%	57.4%	2.1%
CL+E-1	EE	a	1.995	2.064	2.030	1.619	1.877	1.502	1.512	0.686	79.8%	92.5%	74.0%	74.5%	33.8%	0.50%	93.7%	9.9%
		b	1.879	2.171	2.025	1.987	2.100	1.483	1.203	0.732	98.1%	103.7%	73.2%	59.4%	36.1%	1%	99.8%	13.1%
		c	1.735	1.668	1.702	1.745	1.981	1.477	1.309	0.732	102.6%	116.4%	86.8%	76.9%	43.0%	5%	77.4%	6.3%
		d	1.694	1.897	1.796	1.693	1.557	1.359	1.265	0.693	94.3%	86.7%	75.7%	70.5%	38.6%	10%	70.3%	7.8%
CH-1		a	1.691	1.648	1.670	1.585	1.460	1.120	1.189	0.587	94.9%	87.5%	67.1%	71.2%	35.2%	0.50%	90.5%	9.2%
		b	2.115	1.839	1.977	1.578	1.670	1.500	0.928	0.735	79.8%	84.5%	75.9%	46.9%	37.2%	1%	90.2%	5.2%
		c	1.795	1.624	1.710	1.721	1.639	1.376	0.998	0.625	100.7%	95.9%	80.5%	58.4%	36.6%	5%	71.3%	8.5%
		d	1.690	1.884	1.787	1.549	1.664	1.101	0.888	0.693	86.7%	93.1%	61.6%	49.7%	38.8%	10%	56.6%	10.9%
CL+E-1	NEE	a	2.064	1.995	2.030	2.089	1.914	1.797	1.363	0.991	102.9%	94.3%	88.5%	67.2%	48.8%	0.50%	101.6%	1.7%
		b	2.171	1.879	2.025	2.010	1.715	1.795	1.614	1.098	99.3%	84.7%	88.6%	79.7%	54.2%	1%	90.0%	5.3%
		c	1.668	1.735	1.702	1.727	1.613	1.546	1.097	0.735	101.5%	94.8%	90.9%	64.5%	43.2%	5%	90.0%	1.7%
		d	1.897	1.694	1.796	1.847	1.550	1.652	1.223	0.868	102.9%	86.3%	92.0%	68.1%	48.3%	10%	69.9%	6.7%
ECH-1		a	1.648	1.691	1.670	1.837	1.687	1.687	1.635	1.227	110.0%	101.0%	101.0%	97.9%	73.5%	0.50%	102.6%	9.8%
		b	1.839	2.115	1.977	1.742	1.779	1.524	1.606	1.269	88.1%	90.0%	77.1%	81.2%	64.2%	1%	97.6%	8.6%
		c	1.624	1.795	1.710	1.812	1.847	1.641	1.368	1.046	106.0%	108.0%	96.0%	80.0%	61.2%	5%	91.0%	10.3%
		d	1.884	1.690	1.787	1.900	1.629	1.606	1.616	1.065	106.3%	91.2%	89.9%	90.4%	59.6%	10%	87.4%	8.4%
CH+E-1		a	1.727	1.732	1.730	1.566	1.529	1.193	0.959	0.733	90.5%	88.4%	69.0%	55.4%	42.4%	0.50%	90.6%	1.4%
		b	1.691	1.693	1.692	1.533	1.488	1.127	0.917	0.667	90.6%	87.9%	66.6%	54.2%	39.4%	1%	87.0%	2.9%
		c	1.706	1.727	1.717	1.525	1.527	1.129	0.921	0.768	88.8%	89.0%	65.8%	53.7%	44.7%	5%	65.8%	2.9%
		d	1.657	1.723	1.690	1.559	1.398	1.048	0.849	0.625	92.2%	82.7%	62.0%	50.2%	37.0%	10%	53.4%	2.2%
CH+E-1	EE	a	1.693	1.717	1.705	1.596	1.579	1.241	1.178	0.847	93.6%	92.6%	72.8%	69.1%	49.7%	0.50%	91.8%	2.2%
		b	1.696	1.740	1.718	1.563	1.504	1.147	0.948	0.644	91.0%	87.5%	66.8%	55.2%	37.5%	1%	90.5%	3.2%
		c	1.666	1.699	1.683	1.572	1.579	1.131	0.978	0.641	93.4%	93.8%	67.2%	58.1%	38.1%	5%	68.8%	2.7%
		d	1.696	1.768	1.732	1.543	1.527	1.186	1.080	0.721	89.1%	88.2%	68.5%	62.4%	41.6%	10%	61.2%	6.0%
CH+E-1	NVEW	a	1.732	1.727	1.730	1.639	1.160	1.194	0.991	0.777	94.8%	67.1%	69.0%	57.3%	44.9%	0.50%	92.7%	2.1%
		b	1.693	1.691	1.692	1.539	1.143	1.197	0.853	0.745	91.0%	67.6%	70.7%	50.4%	44.0%	1%	69.8%	3.3%
		c	1.727	1.706	1.717	1.558	1.277	1.073	0.875	0.648	90.8%	74.4%	62.5%	51.0%	37.8%	5%	66.6%	3.9%
		d	1.723	1.657	1.690	1.592	1.183	1.082	0.810	0.605	94.2%	70.0%	64.0%	47.9%	35.8%	10%	51.7%	4.0%
																25%	40.6%	4.5%

CH+E-1	NEE	a	1.717	1.693	1.705	1.641	1.270	1.267	1.053	0.773	96.2%	74.5%	74.3%	61.8%	45.3%		0.50%	94.2%	1.8%
		b	1.740	1.696	1.718	1.595	1.360	1.168	0.978	0.707	92.8%	79.2%	68.0%	56.9%	41.2%		1%	79.7%	5.2%
		c	1.699	1.666	1.683	1.558	1.318	1.165	0.906	0.593	92.6%	78.3%	69.2%	53.8%	35.2%		5%	70.4%	2.7%
		d	1.768	1.696	1.732	1.646	1.505	1.215	1.064	0.685	95.0%	86.9%	70.2%	61.4%	39.5%		10%	58.5%	3.8%
																	25%	40.3%	4.2%
tap H2O		a	1.656	1.492	1.574	1.752	1.676	1.429	1.361	1.140	111.3%	106.5%	90.8%	86.5%	72.4%		0.50%	97.3%	11.1%
		b	1.965	1.442	1.704	1.470	1.431	1.373	1.298	1.102	86.3%	84.0%	80.6%	76.2%	64.7%		1%	90.0%	11.9%
		c	2.085	1.442	1.764	1.600	1.396	1.547	1.282	1.080	90.7%	79.2%	87.7%	72.7%	61.2%		5%	84.7%	5.4%
		d	1.727	1.664	1.696	1.709	1.532	1.354	1.261	1.164	100.8%	90.4%	79.9%	74.4%	68.7%		10%	77.4%	6.2%
																	25%	66.8%	4.8%
CH+E-2	EE	a	1.815	1.793	1.804	1.584	1.486	1.951	1.323	1.770	87.8%	82.4%	108.1%	73.3%	98.1%		0.50%	94.4%	10.7%
		b	1.649	1.782	1.716	1.881	1.829	1.879	1.384	1.196	109.6%	106.6%	109.5%	80.7%	69.7%		1%	91.9%	10.4%
		c	1.512	1.831	1.672	1.571	1.488	1.299	1.232	1.201	94.0%	89.0%	77.7%	73.7%	71.9%		5%	93.4%	17.9%
		d	1.708	1.732	1.720	1.480	1.539	1.342	1.298	1.314	86.0%	89.5%	78.0%	75.5%	76.4%		10%	75.8%	3.4%
																	25%	79.0%	13.0%
CL+E-2	NVEW	a	1.492	1.656	1.574	1.700	1.535	1.282	1.077	0.895	108.0%	97.5%	81.4%	68.4%	56.9%		0.50%	94.4%	13.3%
		b	1.442	1.965	1.704	1.505	1.340	1.383	1.108	0.965	88.3%	78.7%	81.2%	65.0%	56.6%		1%	87.8%	9.8%
		c	1.442	2.085	1.764	1.387	1.410	1.240	1.123	0.973	78.7%	80.0%	70.3%	63.7%	55.2%		5%	77.2%	5.3%
		d	1.664	1.727	1.696	1.737	1.609	1.284	1.125	0.952	102.4%	94.9%	75.7%	66.4%	56.1%		10%	65.9%	2.0%
																	25%	56.2%	0.8%
wine		a	2.013	1.748	1.881	2.016	1.837	1.817	1.993	1.879	107.2%	97.7%	96.6%	106.0%	99.9%		0.50%	107.3%	9.2%
		b	1.693	2.029	1.861	1.835	1.804	1.849	2.038	2.007	98.6%	96.9%	99.4%	109.5%	107.8%		1%	110.6%	15.5%
		c	1.784	1.765	1.775	2.130	2.237	2.032	1.897	2.022	120.0%	126.1%	114.5%	106.9%	113.9%		5%	108.6%	12.9%
		d	1.711	1.715	1.713	1.772	2.085	2.122	1.954	2.252	103.4%	121.7%	123.9%	114.1%	131.5%		10%	109.1%	3.6%
																	25%	113.3%	13.4%
CH+E-2	NVEW	a	1.819	1.970	1.895	1.782	2.089	1.533	1.277	1.236	94.1%	110.3%	80.9%	67.4%	65.2%		0.50%	97.5%	3.8%
		b	1.696	2.189	1.943	1.995	1.946	1.711	1.391	1.066	102.7%	100.2%	88.1%	71.6%	54.9%		1%	100.8%	7.6%
		c	1.926	2.082	2.004	1.914	1.837	1.571	1.412	1.127	95.5%	91.7%	78.4%	70.5%	56.2%		5%	78.1%	9.6%
		d	1.888	1.833	1.861	1.817	1.879	1.212	1.164	0.682	97.7%	101.0%	65.1%	62.6%	36.7%		10%	68.0%	4.0%
																	25%	53.3%	12.0%
CL+E-2	EE	a	1.748	2.013	1.881	1.845	1.944	1.423	1.238	1.127	98.1%	103.4%	75.7%	65.8%	59.9%		0.50%	96.8%	11.4%
		b	2.029	1.693	1.861	1.726	1.416	1.412	1.138	1.021	92.7%	76.1%	75.9%	61.1%	54.9%		1%	93.6%	16.9%
		c	1.765	1.784	1.775	1.499	1.470	1.755	1.352	1.054	84.5%	82.8%	98.9%	76.2%	59.4%		5%	88.6%	14.9%
		d	1.715	1.711	1.713	1.914	1.919	1.779	1.664	1.473	111.7%	112.0%	103.9%	97.1%	86.0%		10%	75.1%	16.0%
																	25%	65.0%	14.1%
beer		a	1.970	1.819	1.895	2.089	2.359	2.379	2.285	2.724	110.3%	124.5%	125.6%	120.6%	143.8%		0.50%	110.7%	6.5%
		b	2.189	1.696	1.943	2.111	2.680	2.291	2.393	2.810	108.7%	138.0%	117.9%	123.2%	144.7%		1%	123.7%	10.6%
		c	2.082	1.926	2.004	2.089	2.274	2.439	2.263	2.810	104.2%	113.5%	121.7%	112.9%	140.2%		5%	129.6%	16.0%
		d	1.833	1.888	1.861	2.227	2.208	2.849	2.603	2.916	119.7%	118.7%	153.1%	139.9%	156.7%		10%	124.2%	11.4%
																	25%	146.3%	7.2%

[illegible]



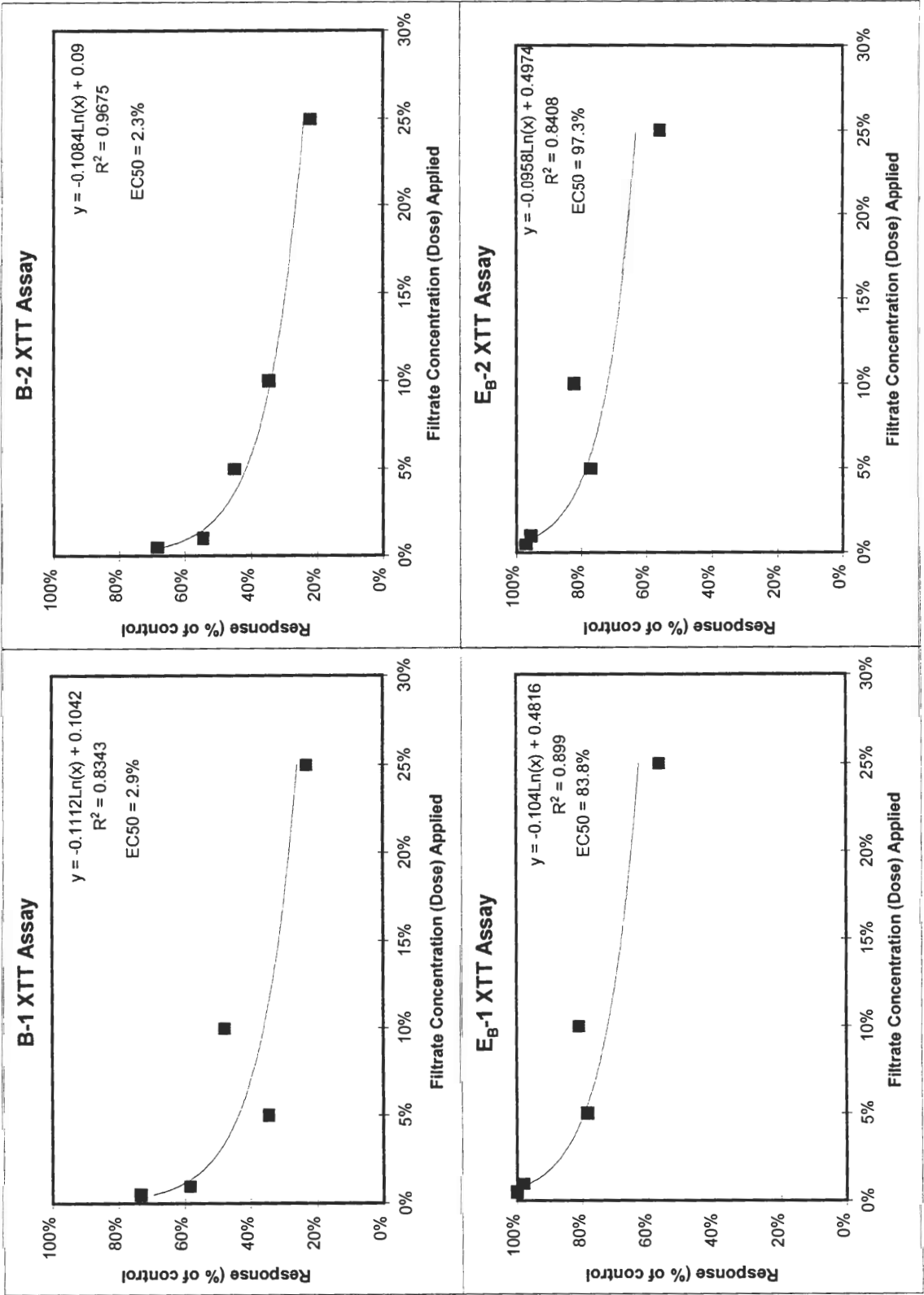
Bio	contro I 1	contro I 2	avg. contro I	25%	12.50%	6.25%	1.04%	0.52%		25% (% of control)	12.5% (% of control)	6.25% (% of control)	1.04 (% of control)	0.52% (% of control)		averages	std. dev
B+E-1																	
a	0.80	0.83	0.81	0.50	0.71	0.80	1.06	1.04		61.1%	87.3%	98.9%	130.2%	127.8%	0.52%	124.8%	0.126
b	0.83	0.81	0.82	0.41	0.68	0.69	0.93	0.90		50.3%	83.4%	83.9%	113.6%	110.5%	1.04%	123.6%	0.085
c	0.77	0.74	0.76	0.42	0.62	0.66	0.90	0.91		55.0%	81.9%	87.7%	119.3%	120.5%	6.25%	92.2%	0.076
d	0.78	0.65	0.71	0.40	0.55	0.70	0.94	1.00		55.6%	77.0%	98.5%	131.2%	140.3%	12.50 %	77.0%	0.043
															25.00 %	55.5%	0.044
Bio																	
CL+E-1																	
a	0.79	0.75	0.77	0.39	0.60	0.69	0.90	0.94		50.5%	77.2%	89.8%	116.5%	121.2%	0.52%	118.4%	0.020
b	0.73	0.72	0.72	0.37	0.57	0.70	0.91	0.86		51.6%	79.0%	96.6%	125.5%	118.5%	1.04%	119.7%	0.051
c	0.78	0.69	0.73	0.39	0.59	0.68	0.90	0.86		52.9%	80.7%	92.8%	122.2%	117.6%	6.25%	93.0%	0.028
d	0.79	0.76	0.78	0.41	0.63	0.72	0.89	0.90		52.4%	81.5%	92.8%	114.5%	116.3%	12.50 %	81.5%	0.019
															25.00 %	51.8%	0.010

[illegible]

[illegible]

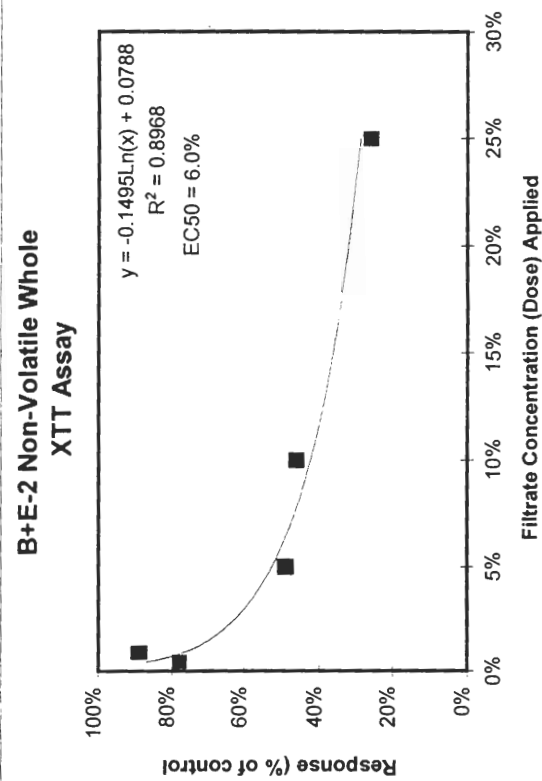
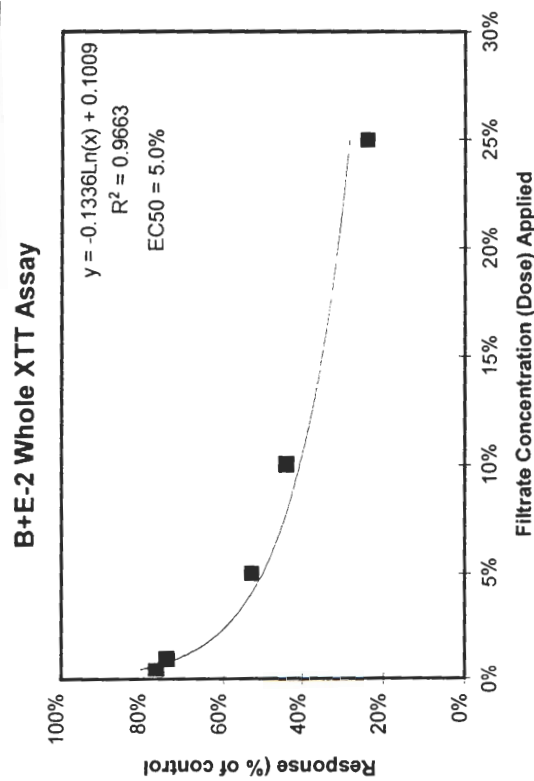
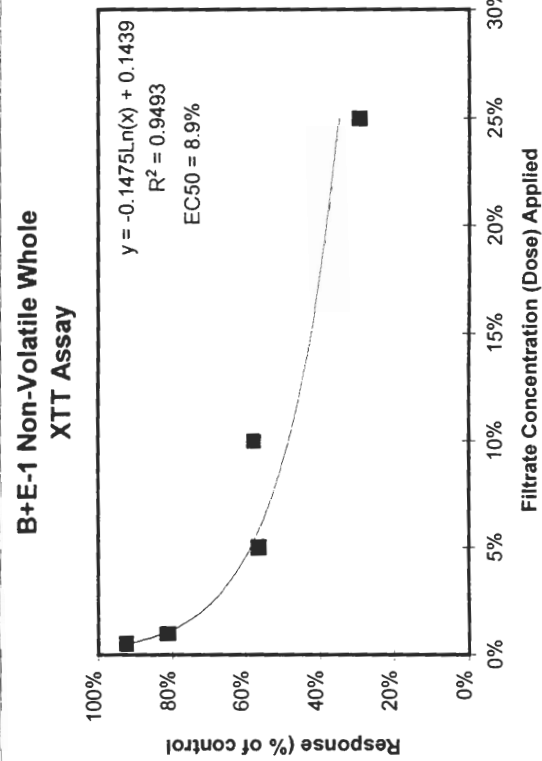
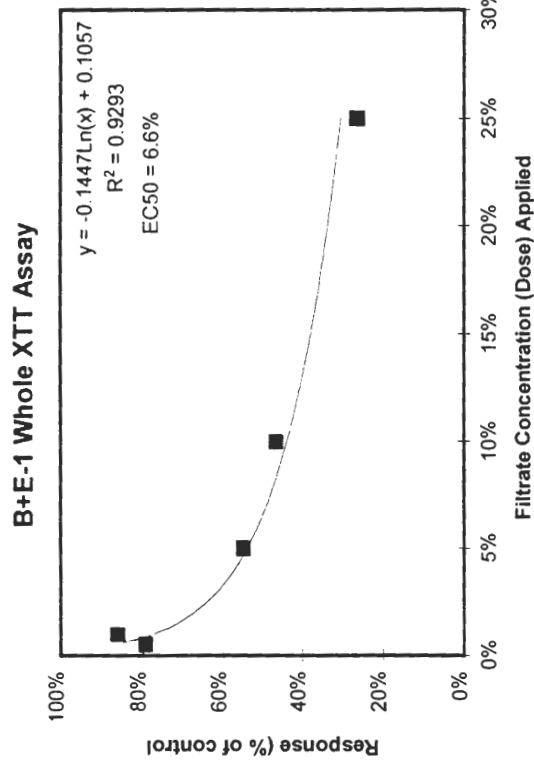
Appendix J

XTT CURVES FOR DETERMINING EC50

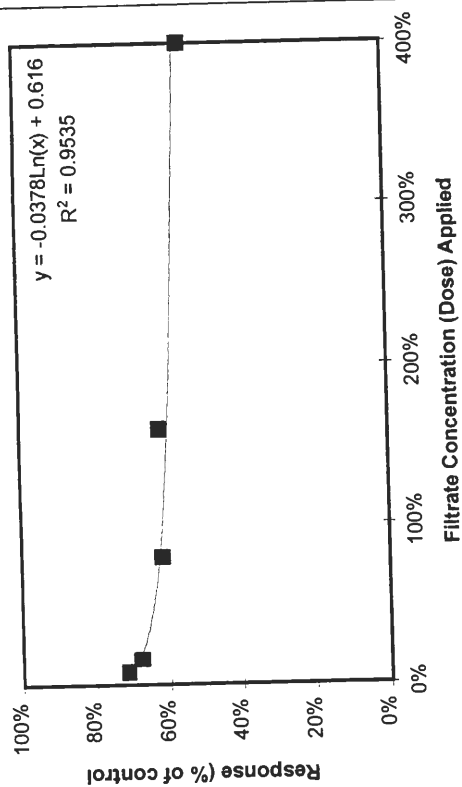
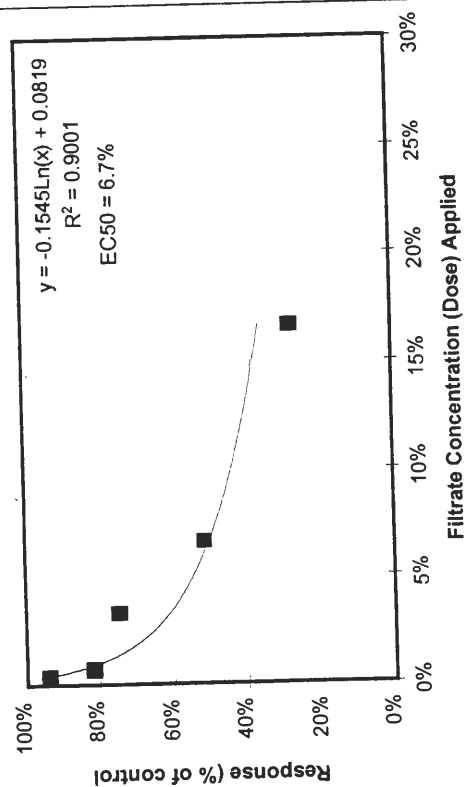
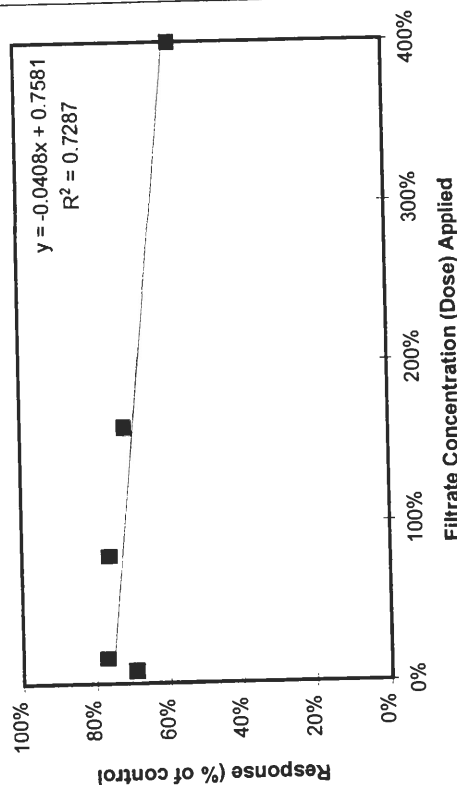
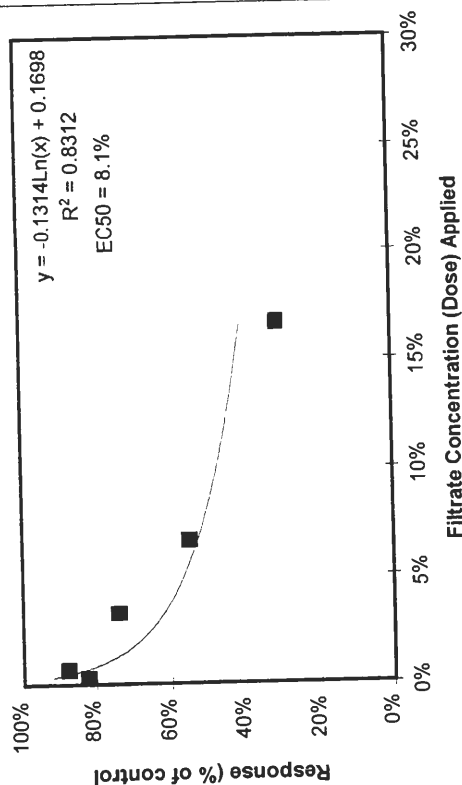


## APPENDIX J, CONT'D

## XTT CURVES FOR DETERMINING EC50

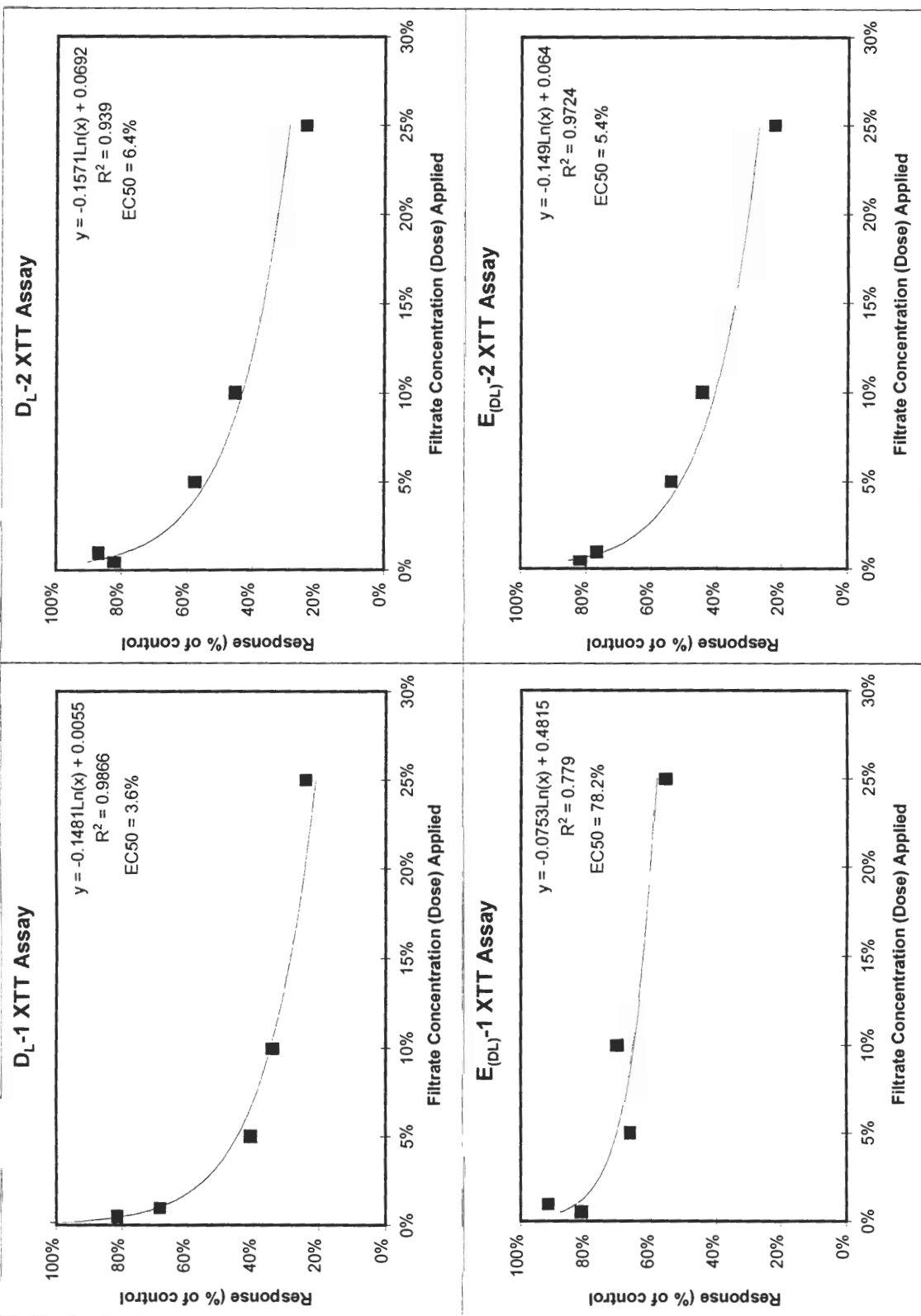


## XTT CURVES FOR DETERMINING EC50

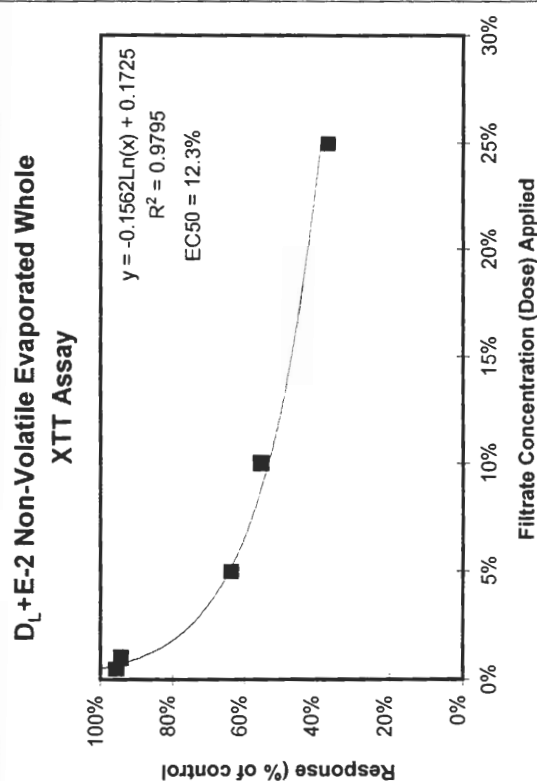
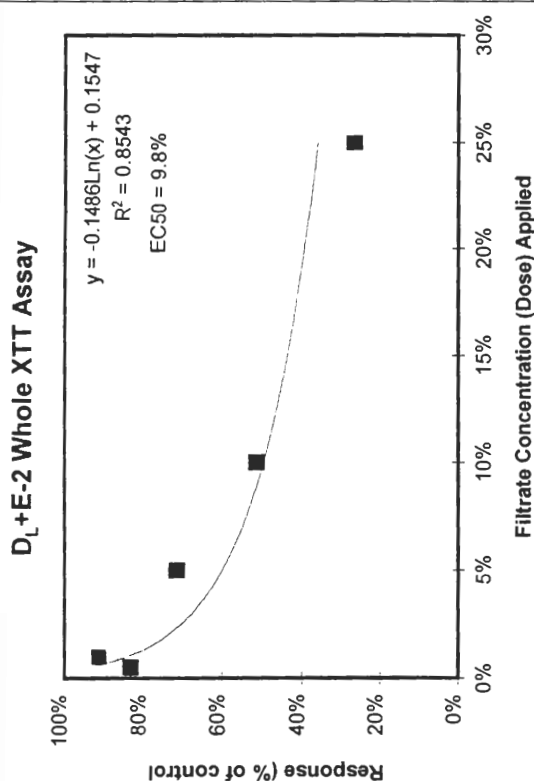
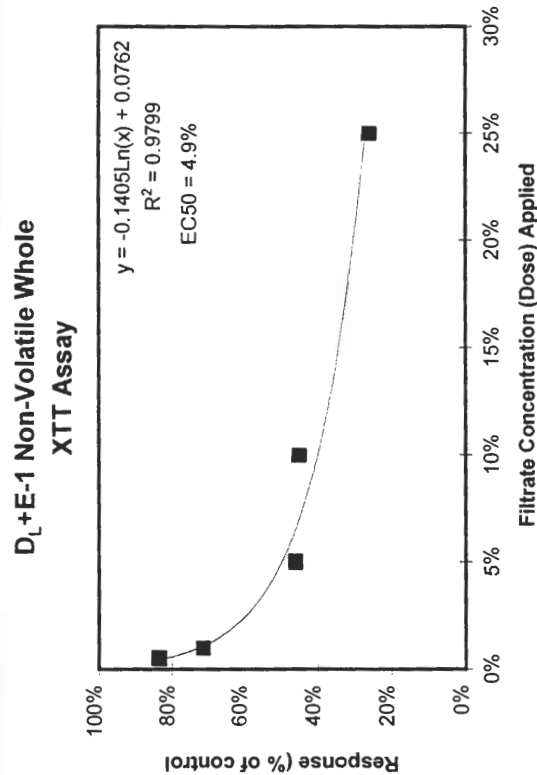
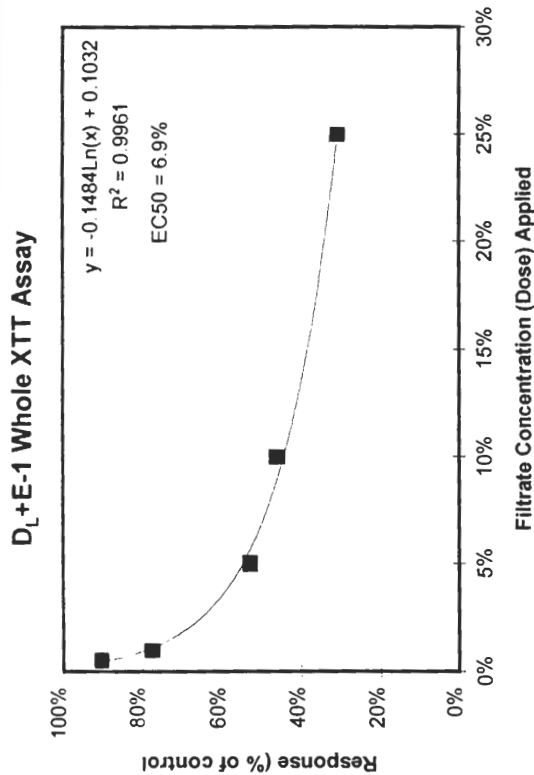
**B+E-1 Ether Extractable Fraction  
XTT Assay****B+E-1 Non-Ether Extractable  
XTT Assay****B+E-2 Ether Extractable Fraction  
XTT Assay****B+E-2 Non-Ether Extractable Fraction  
XTT Assay**



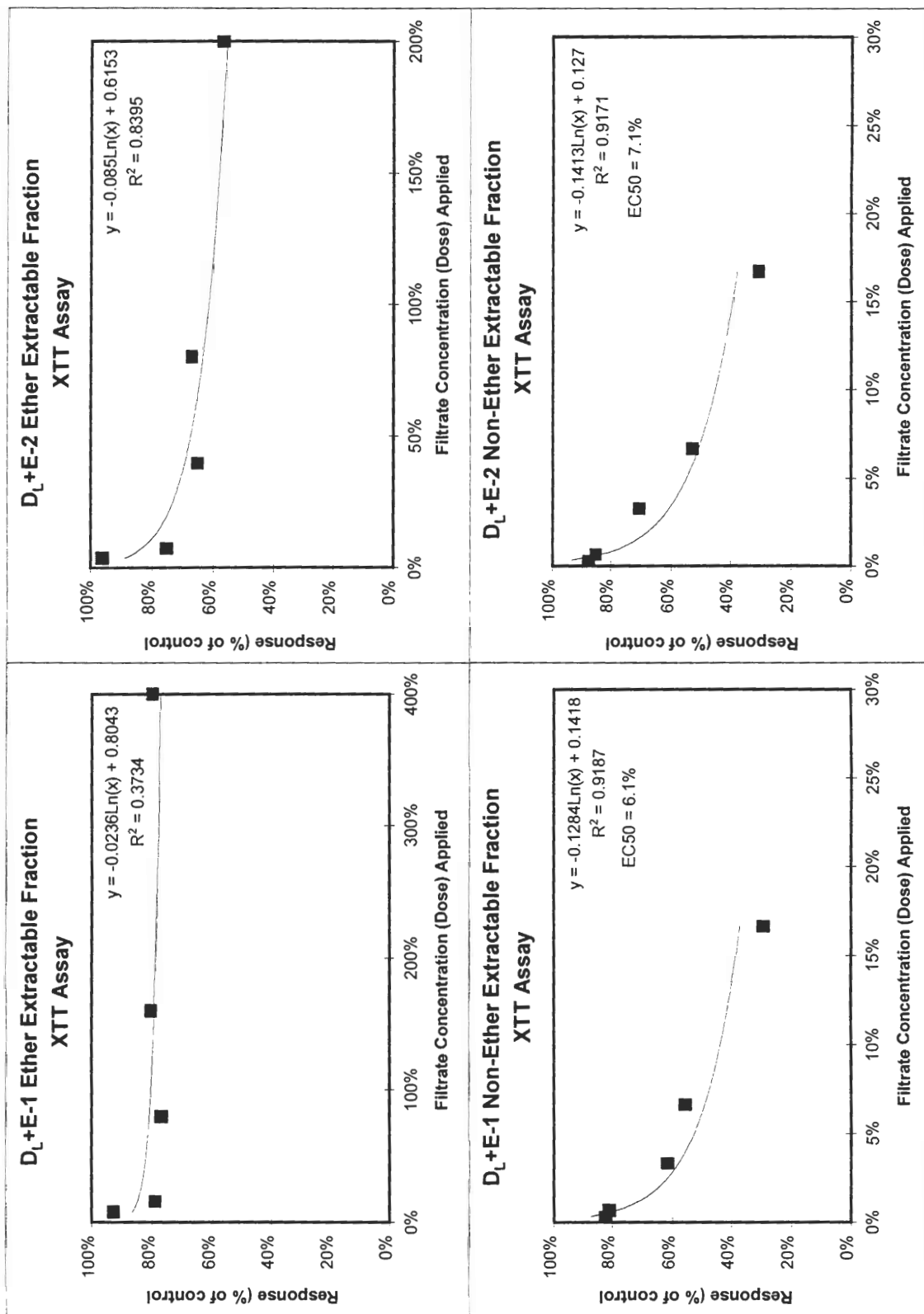
## XTT CURVES FOR DETERMINING EC50



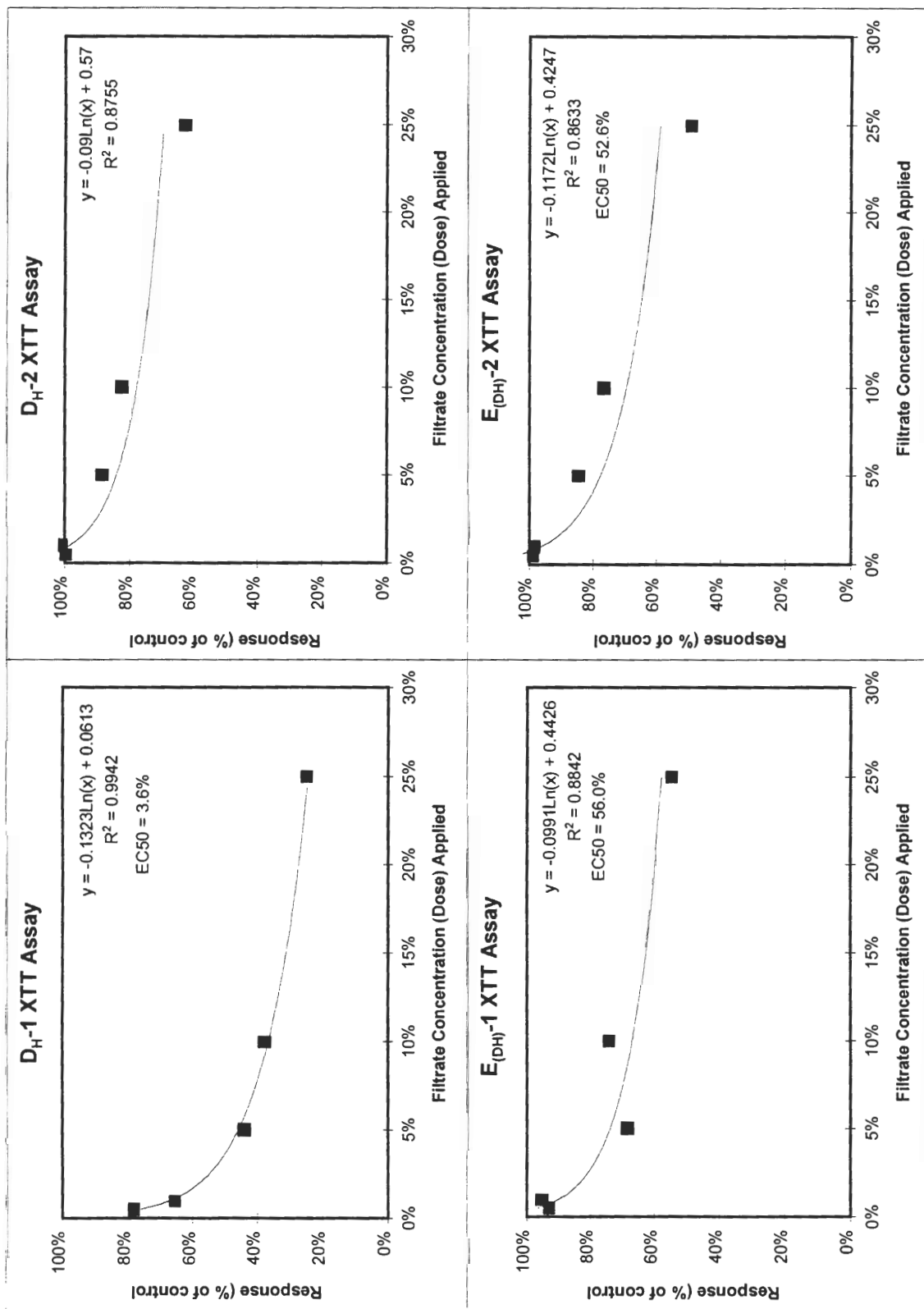
## XTT CURVES FOR DETERMINING EC50



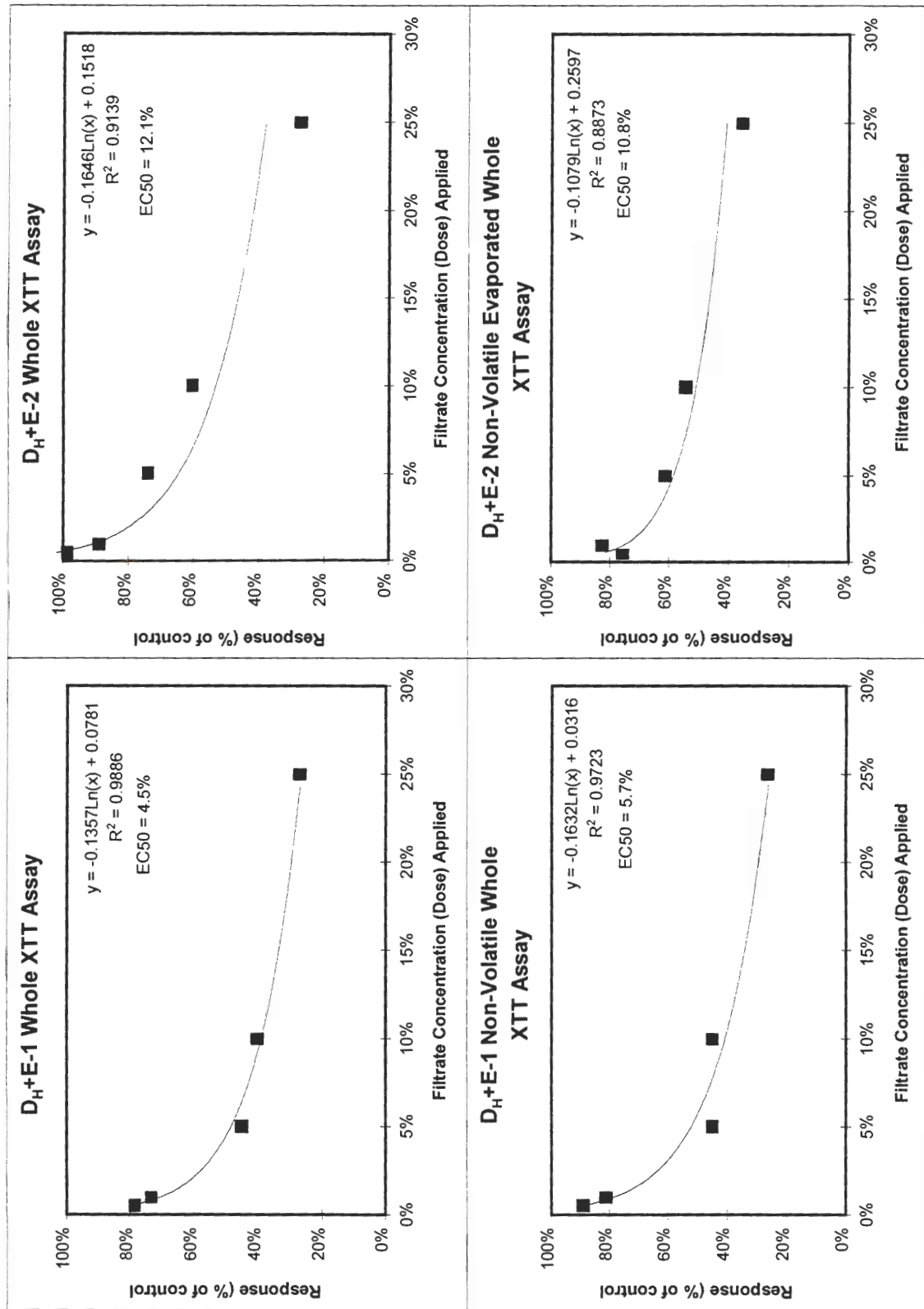
# XTT CURVES FOR DETERMINING EC50



## XTT CURVES FOR DETERMINING EC50



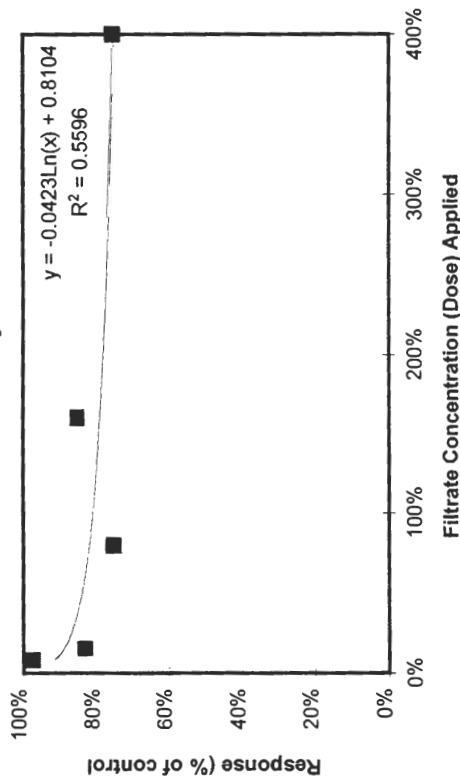
## XTT CURVES FOR DETERMINING EC50



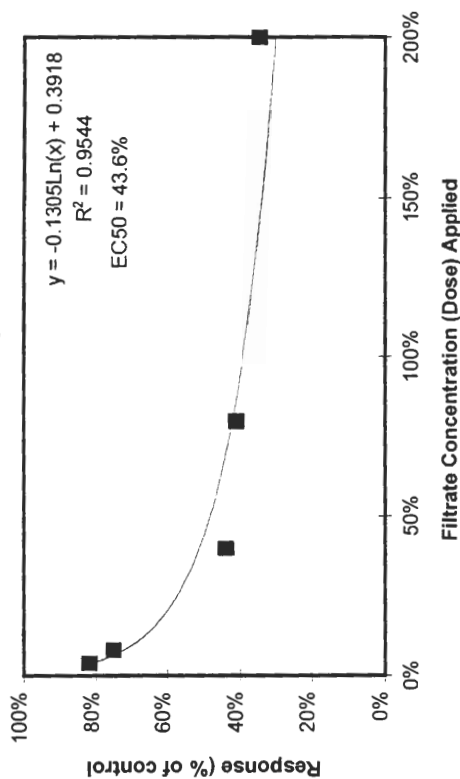
## APPENDIX J

## XTT CURVES FOR DETERMINING EC50, CONT'D

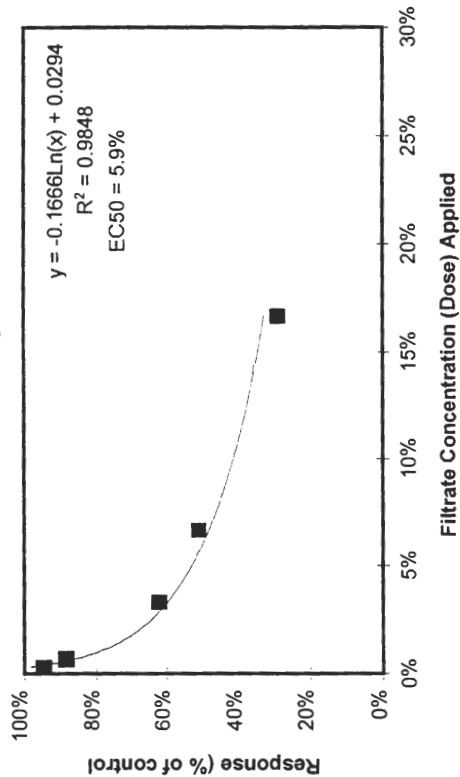
**D<sub>H</sub>+E-1 Ether Extractable Fraction  
XTT Assay**



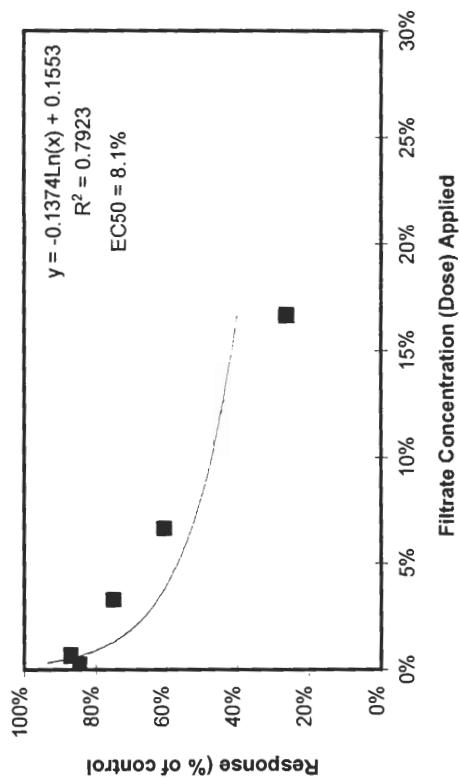
**D<sub>H</sub>+E-2 Ether Extractable Fraction  
XTT Assay**



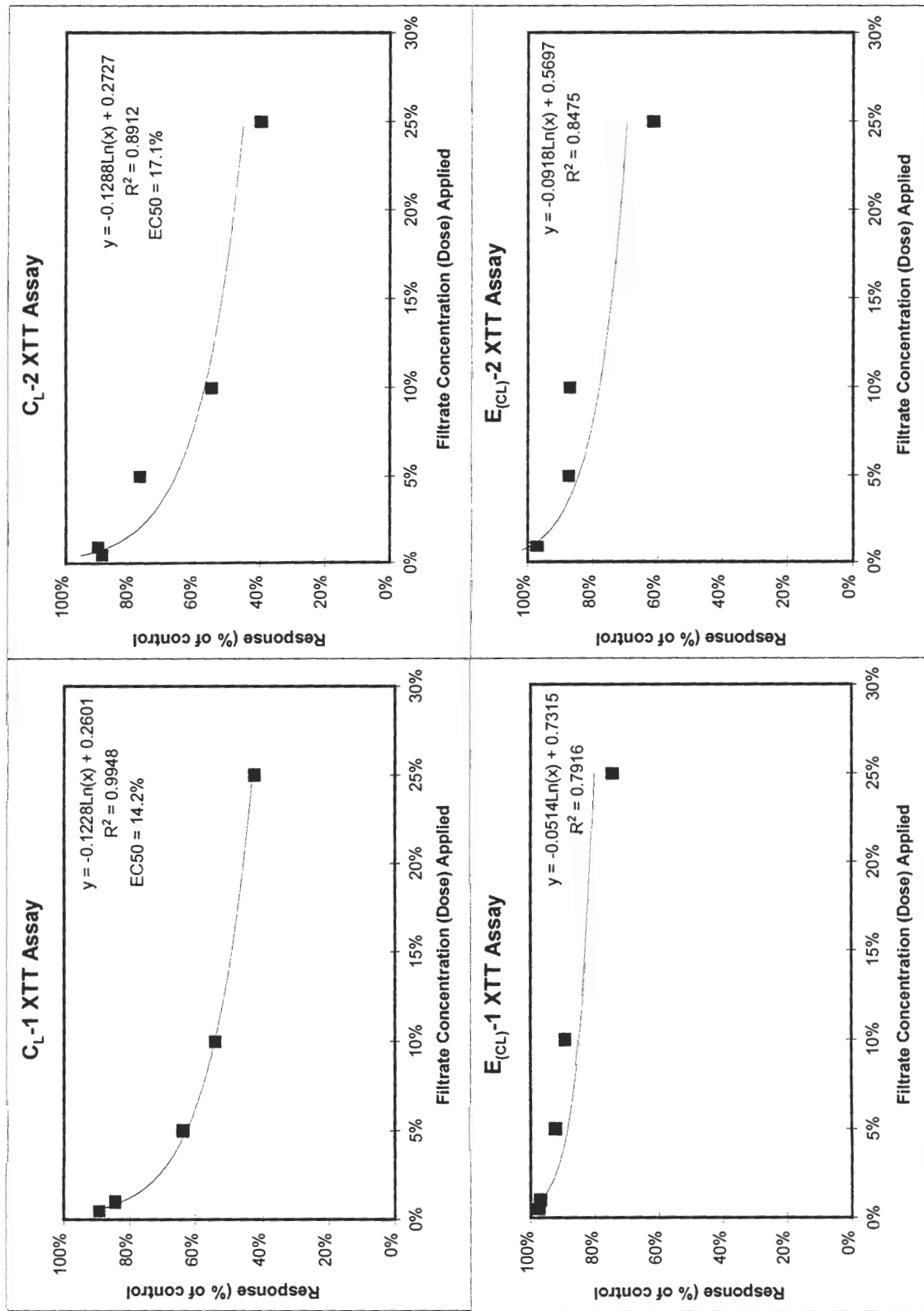
**D<sub>H</sub>+E-1 Non-Ether Extractable Fraction  
XTT Assay**



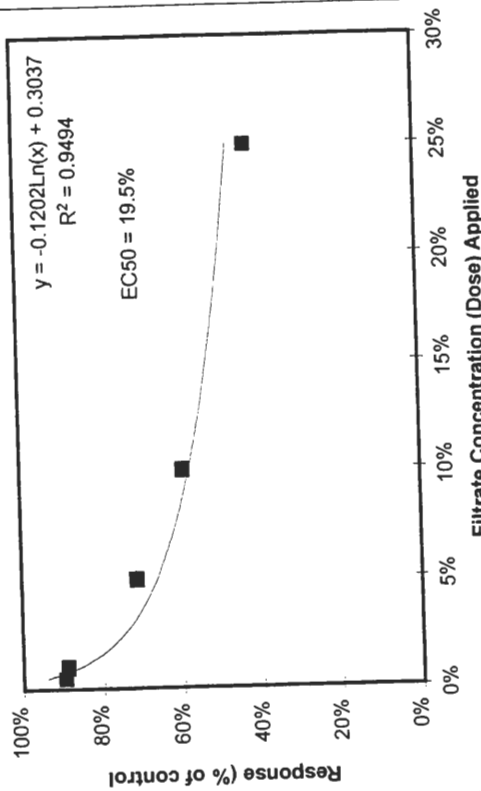
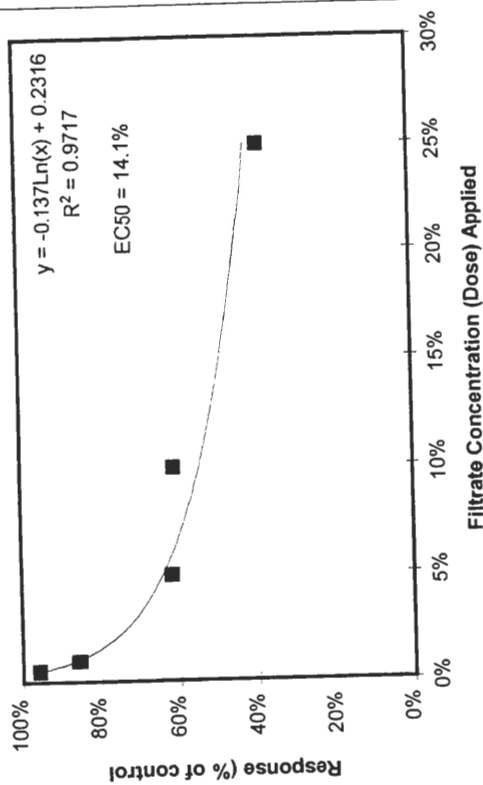
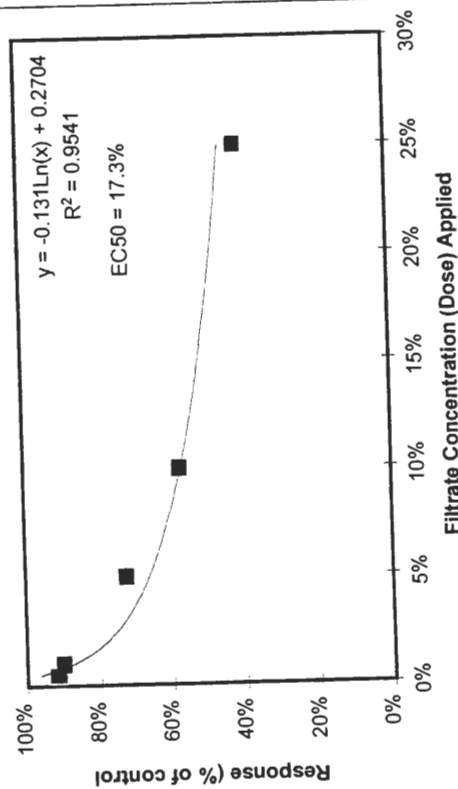
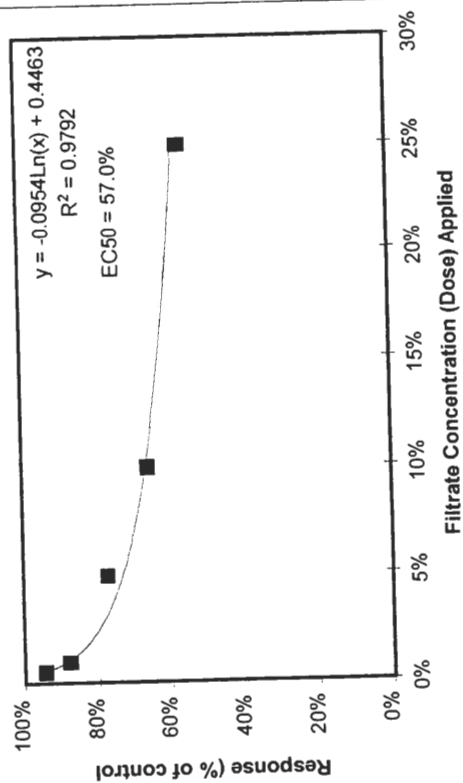
**D<sub>H</sub>+E-2 Non-Ether Extractable Fraction  
XTT Assay**



## XTT CURVES FOR DETERMINING EC50



## XTT CURVES FOR DETERMINING EC50

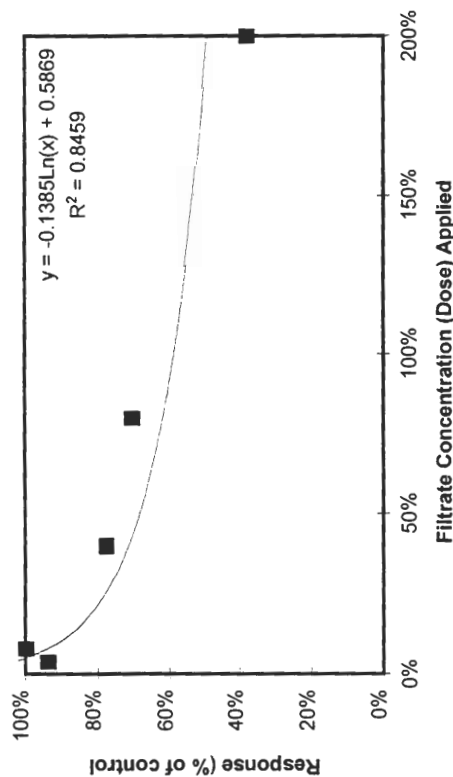
**C<sub>L</sub>+E-1 Whole XTT Assay****C<sub>L</sub>+E-2 Whole XTT Assay****C<sub>L</sub>+E-1 Non-Volatile Evaporated Whole XTT Assay****C<sub>L</sub>+E-2 Non-Volatile Evaporated Whole XTT Assay**



# XTT CURVES FOR DETERMINING EC50

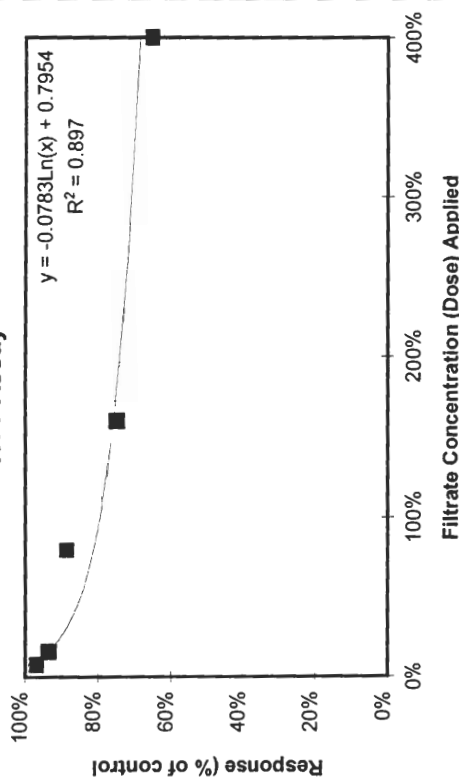
## C<sub>L</sub>+E-1 Ether Extractable Fraction

### XTT Assay



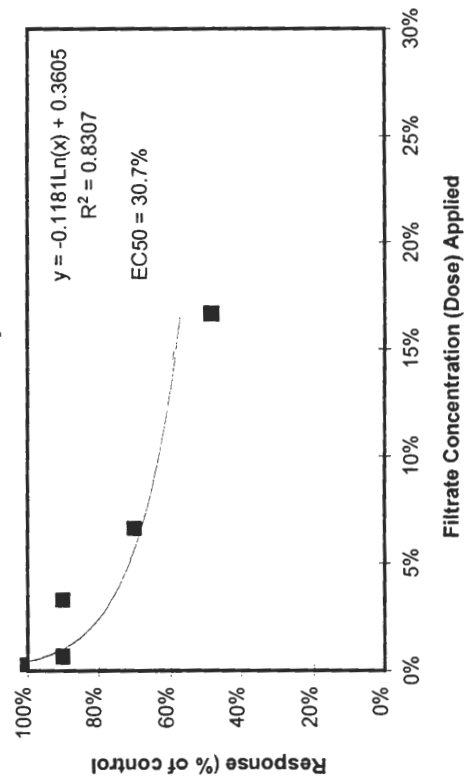
## C<sub>L</sub>+E-2 Ether Extractable Fraction

### XTT Assay



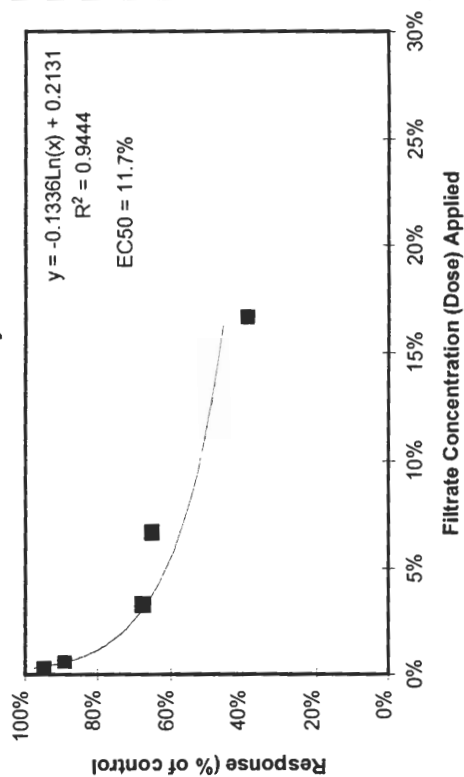
## C<sub>L</sub>+E-1 Non-Ether Extractable Fraction

### XTT Assay



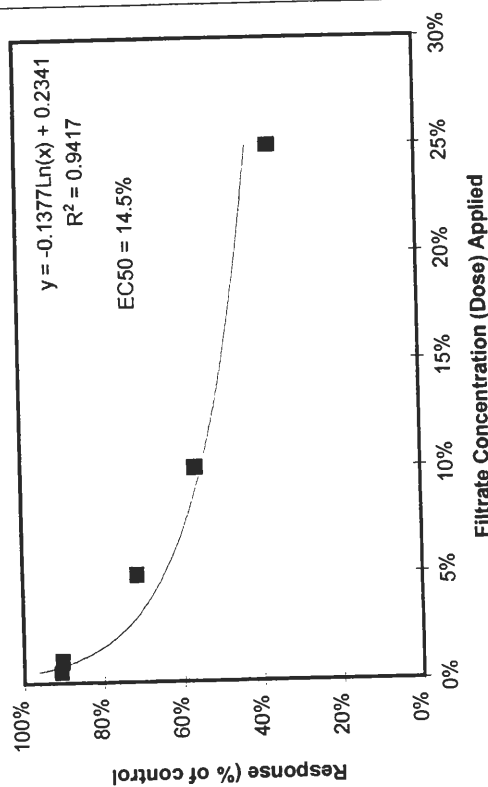
## C<sub>L</sub>+E-2 Non-Ether Extractable Fraction

### XTT Assay



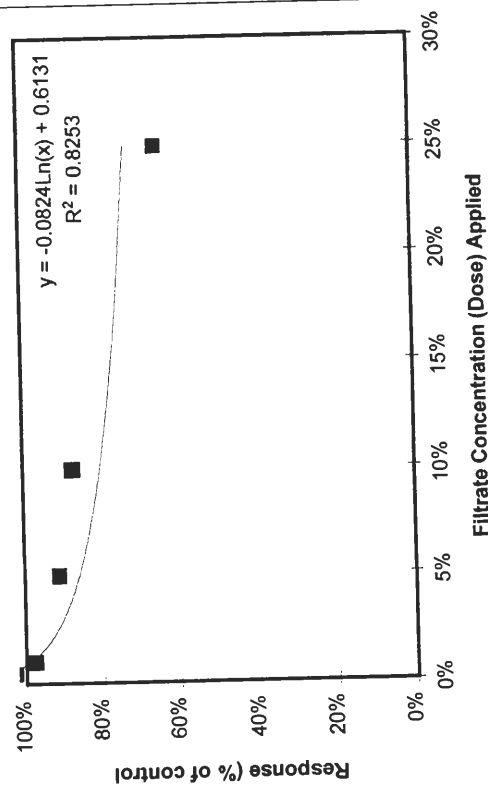
# XTT CURVES FOR DETERMINING EC50

**C<sub>H</sub>-1 XTT Assay**

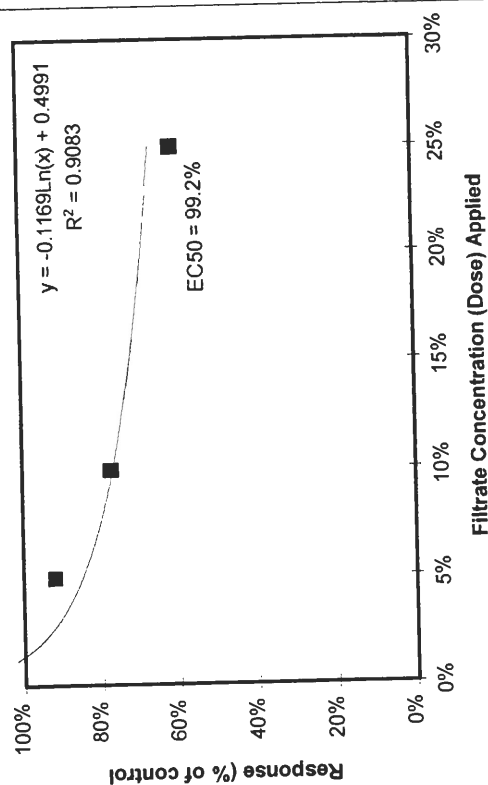


Sample C<sub>H</sub>-2  
destroyed in laboratory accident

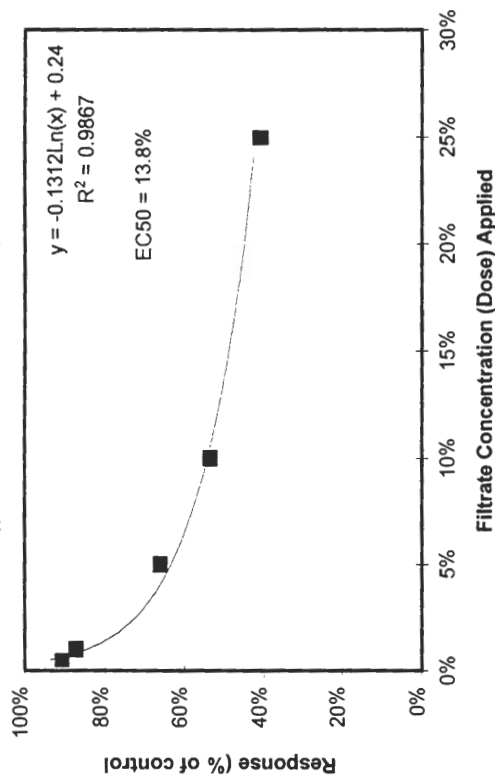
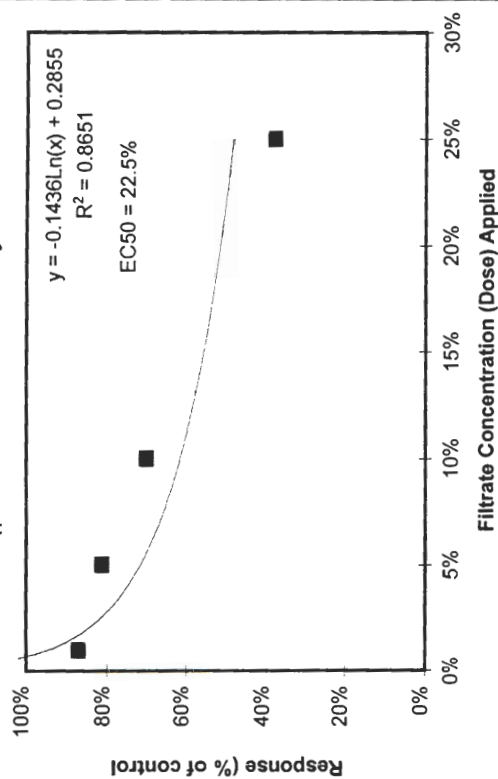
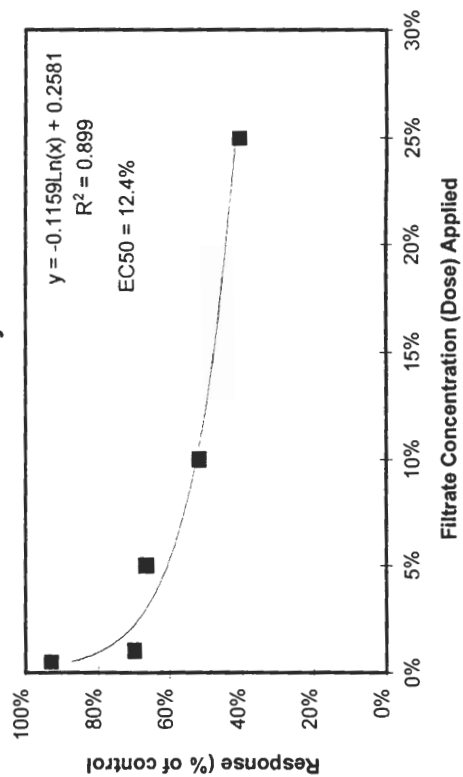
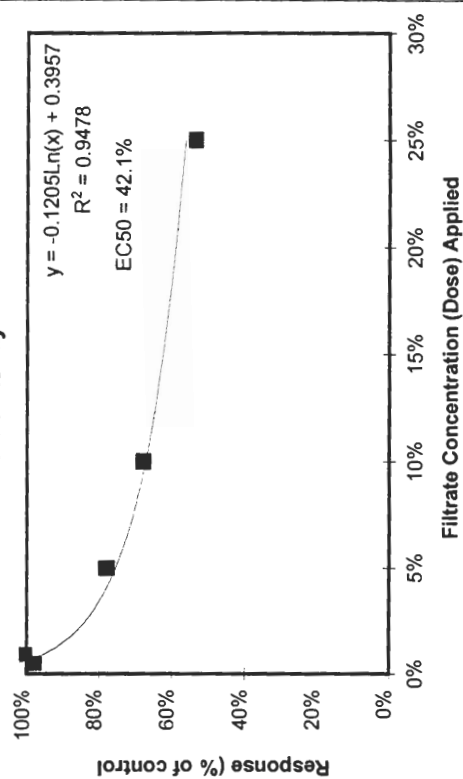
**E<sub>(CH)</sub>-1 XTT Assay**



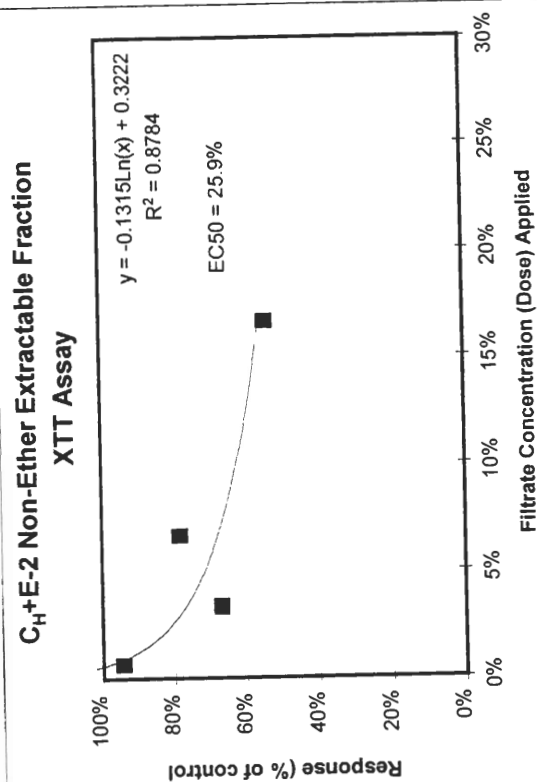
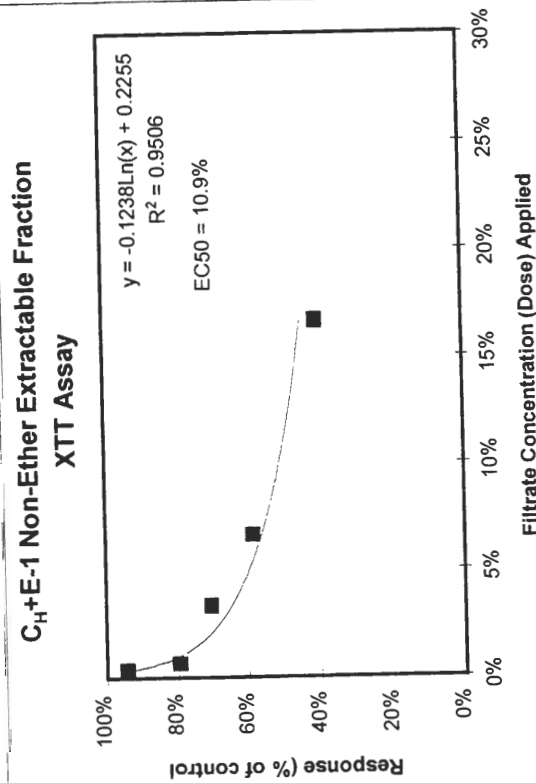
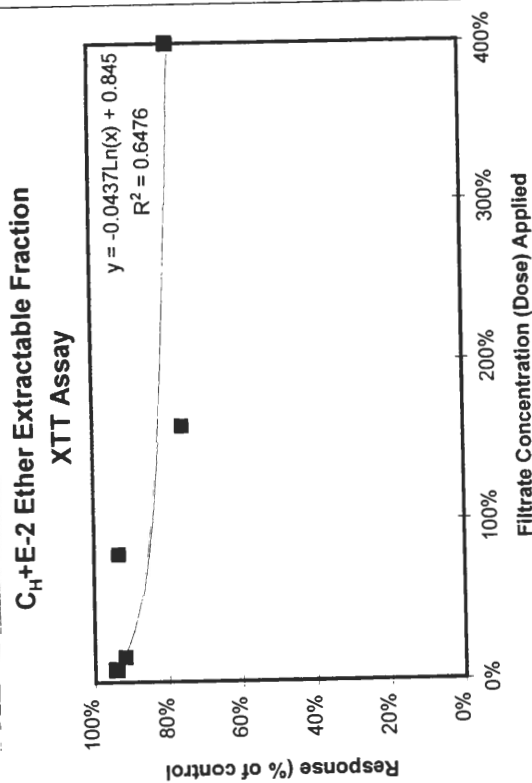
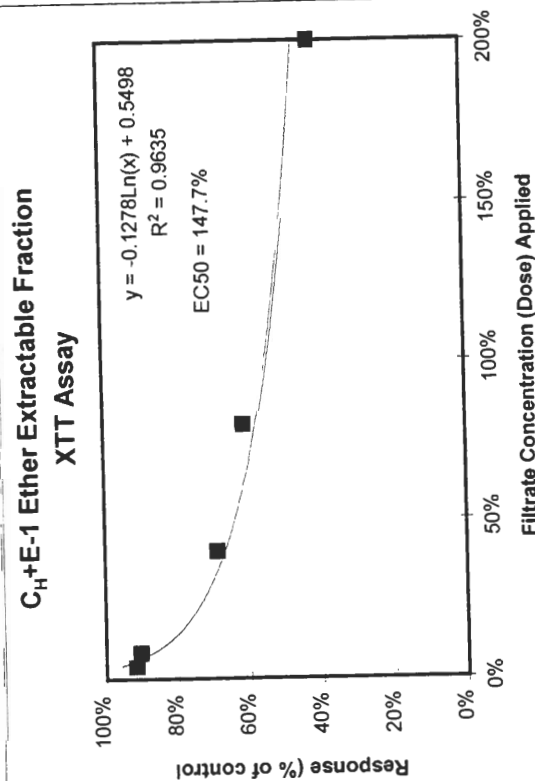
**E<sub>(CH)</sub>-2 XTT Assay**



## XTT CURVES FOR DETERMINING EC50

**C<sub>H</sub>+E-1 Whole XTT Assay****C<sub>H</sub>+E-2 Whole XTT Assay****C<sub>H</sub>+E-1 Non-Volatile Evaporated Whole XTT Assay****C<sub>H</sub>+E-2 Non-Volatile Evaporated Whole XTT Assay**

# XTT CURVES FOR DETERMINING EC50



## XTT CURVES FOR DETERMINING EC50

