A STUDY OF XENOESTROGENS IN THE GREATER PITTSBURGH AREA

by

Lara Renz

B. A. in Psychology, Duquesne University, 2002

M. P. H., University of Pittsburgh, 2008

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This dissertation was presented

by

Lara Renz

It was defended on

April 13, 2011

and approved by

Patricia K. Eagon, Ph.D, Associate Professor, Division of Gastroenterology, Hepatology, and Nutrition, School of Medicine, University of Pittsburgh

James Peterson, Ph.D, Associate Professor, Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Ravi K. Sharma, Ph.D, Assistant Professor, Behavioral and Community Health Sciences, Graduate School of Public Health, University of Pittsburgh

Dissertation Advisor: Conrad D. Volz, DrPH, Assistant Professor, Environmental and Occupational Health, Graduate School of Public Health, University of Pittsburgh

Conrad D. Volz, DrPH, M.P.H

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Lara Renz, DrPH

University of Pittsburgh, 2011

The Greater Pittsburgh Area is famous for its three rivers: the Allegheny, Monongahela, and Ohio Rivers. These rivers have a history of being polluted by decades of mine runoff and chemicals released by industrial sites. New problems, such as pollution from endocrine disrupting compounds and xenoestrogens, have recently been discovered in this well known aquatic environment and are suspected to be caused by the failing sewer system. Personal care products, pharmaceuticals and plasticizers all have the potential to enter the water supply though both treated and untreated sewage. Many of these compounds are known or suspected endocrine disruptors.

Estrogenic potential of fish extracts from flesh/fat tissue captured from Freeport and Ford City was studied via the E-Screen Assay on MCF-7, T47D and BT-20 human breast cancer cell lines. Results showed weak estrogenic responses in both MCF-7 and T47D cell lines, with no significant differences for fish gender, weight, or sample location.

Estrogenic potential of extracts from fish brain tissue was tested via Bromodeoxyuridine MCF-7 Analysis and paired with High Performance Liquid Chromatography-Mass Spectrometry to investigate the presence of specific xenoestrogens in the fish extracts. Fifty eight fish were sampled from rivers in the Greater Pittsburgh Area. All samples were non-detectable for methyl, ethyl, propyl and butyl parabens. Bisphenol A (BPA) was detected in 44 of the 58 samples, with

a range from non-detectable to 120 pg/gram. The Harmarville sample location had higher results for all analyses when compared to all other sample sites.

In summary, this dissertation supported all previous available literature leading to the conclusion that parabens are safe to remain on the market and are not a significant environmental concern. In particular, there does not seem to be any need for concern over paraben levels detected in the Greater Pittsburgh Area river system and water supply. The BPA portion of this research was in agreement with previous literature as to its bioconcentration tendencies; however, new implications regarding the public health significance of the effects from BPA in brain tissue may require some re-evaluation of concerns about BPA transport and fate in the environment around Pittsburgh and elsewhere.

TABLE OF CONTENTS

PREFACE ..... **XVI**

1.0 INTRODUCTION **1**

1.1 PUBLIC HEALTH SIGNIFICANCE **1**

1.2 INTRODUCTION TO ENDOCRINE DISRUPTING COMPOUNDS **2**

1.3 WASTE WATER TREATMENT PLANTS **7**

1.4 RESEARCH REVIEW FOR BISPHENOL A (BPA) **12**

1.4.1 Chemical Properties **12**

1.4.2 Transportation and Fate of BPA in the Environment **13**

1.4.3 Exposure to Bisphenol A **15**

1.4.4 Analytical Methods **17**

1.4.5 *In Vitro* Studies **19**

1.4.6 *In Vivo* Studies **21**

1.4.7 Human Studies **23**

1.4.8 Regulatory Actions **24**

1.4.9 Problems with Research on BPA **25**

1.5 RESEARCH REVIEW FOR PARABENS **26**

1.5.1 Chemical Properties **26**

1.5.2 Transportation and Fate of Parabens in the Environment **30**

1.5.3 Exposure to Parabens 30

1.5.4 Analytical Methods 35

1.5.5 *In Vitro* Studies 35

1.5.6 *In Vivo* Studies 39

1.5.7 Human Studies 40

1.5.8 Other Studies 42

1.5.9 Regulatory Actions 43

1.5.10 Problems with Research on Parabens 44

1.6 SUMMARY 45

2.0 ESTROGENICITY OF EXTRACTS FROM ALEWIFE (*ALOSA PSEUDOHARENGUS*) AND SHAD (*ALOSA SAPIDISSIMA*) CAPTURED IN THE GREATER PITTSBURGH AREA 46

2.1 OBJECTIVE 47

2.2 MATERIAL AND METHODS 48

2.2.1 Sampling Locations 48

2.2.2 Sampling Methods 50

2.2.3 Composite Preparation 50

2.2.4 Extraction of Fish Flesh 51

2.2.5 Cell Culture 51

2.2.6 E-Screen Assay 52

2.2.7 Proliferation Index (PI) and Estrogen Response Profile (ERP) 53

2.2.8 Estrogenicity Index (EI) 55

2.2.9 Statistical Analyses 56

2.3 RESULTS AND DISCUSSION 57

2.3.1 Proliferation Index 57

2.3.2 Descriptive Results from ERP 57

2.3.3 Estrogenicity Index 60

2.3.4 Statistical Results 62

2.4 PUBLIC HEALTH SIGNIFICANCE 70

3.0 ANALYSIS OF SURFACE WATER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY 72

3.1 BACKGROUND 73

3.2 OBJECTIVES 74

3.3 MATERIAL AND METHODS 75

3.3.1 Sample Locations 75

3.3.2 Surface Water Sampling Methods 77

3.3.3 Solid Phase Extraction 79

3.3.4 Analytical Method for Surface Water Analysis 80

3.4 RESULTS AND DISCUSSION 81

3.5 PUBLIC HEALTH SIGNIFICANCE 84

4.0 ANALYSES OF EXTRACTS FROM FISH BRAIN TISSUE FOR XENOESTROGENS AND ESTROGENIC POTENTIAL 85

4.1 OBJECTIVES 86

4.2 MATERIAL AND METHODS 87

4.2.1 Sampling Locations 87

4.2.2 Sampling Methods 89

4.2.3 Sample Extraction and Preparation for HPLC-MS Analysis..... 89
4.2.4 Derivatization for HPLC-MS Analysis..... 90
4.2.5 Analytical Method for HPLC-MS Analysis..... 90
4.2.6 Analytical Method for Bromodeoxyuridine (BrdU) Analysis..... 91
4.2.7 MCF-7 DNA Isolation..... 92
4.2.8 BPA and BrdU Data Comparisons and Corrections..... 93
4.2.9 Statistical Methods for BPA Analysis..... 93
4.2.10 Statistical Methods for BrdU Analysis..... 95
4.3 RESULTS..... 96
4.3.1 Analytical Results..... 96
4.3.2 Bioconcentration Factors (BCFs)..... 96
4.3.3 Statistical Results for BPA Analysis..... 99
4.3.4 Descriptive Results of Raw BrdU Data..... 103
4.3.5 Statistical Results for BrdU Analysis..... 104
4.3.6 Correlation between BPA and BrdU Results..... 106
4.4 CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE..... 106
5.0 OPINION OF POLICY AND REGULATIONS REGARDING PARABENS AND BISPHENOL A..... 113
5.1 POLICY AND REGULATION REGARDING PARABENS..... 113
5.1.1 Summary of Regulatory Actions for Parabens..... 113
5.1.2 Pros and Cons of Using Parabens..... 114
5.1.3 Alternatives to the Use of Parabens..... 115
5.1.4 Recommendations for Parabens..... 115

5.2 POLICY AND REGULATIONS REGARDING BISPHENOL A 116

5.2.1 Summary of Regulatory Actions for BPA 116

5.2.2 Pros and Cons of Using BPA..... 117

5.2.3 Alternatives to the Use of BPA 118

5.2.4 Recommendations for BPA..... 119

5.3 CONCLUSIONS..... 121

APPENDIX A : ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR BT-20 122

APPENDIX B : ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR MCF-7 134

APPENDIX C : ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR T47D 146

BIBLIOGRAPHY..... 158

LIST OF TABLES

Table 1. Chemical Properties of Bisphenol A 13

Table 2. Analytical Methods for Bisphenol A 18

Table 3. Chemical Properties of Various Parabens 29

Table 4. Summary of Eriksson's Results 32

Table 5. Summary of Analytical Methods for Parabens 36

Table 6. MCF-7 Proliferation Index Data Summary (Mean with SD and Range) 58

Table 7. T47D Proliferation Index Data Summary (Mean with SD and Range) 59

Table 8. Summary of the Estrogen Response Profile (ERP) for Cell Proliferation Assays by Composite 60

Table 9. MCF-7 Estrogenicity Index Data Summary (Mean with SD and Range) 61

Table 10. T47D Estrogenicity Index Data Summary (Mean with SD and Range) 61

Table 11. Surface Water Sampling Information 78

Table 12. Solid Phase Extraction Volumes 79

Table 13. Results of Surface Water Samples (ng/L or ppt) 83

Table 14. Individual Fish Data and Analytical Results for BPA 97

Table 15. Bioconcentration Factors (BCF) for BPA Using Individual Surface Water Results 99

Table 16. Bioconcentration Factors (BCF) for BPA Using Average Surface Water Results From All Locations 99

Table 17. Relationships Between BPA and Fish Characteristics from Preliminary Univariate Analysis..... 100

Table 18. The BPA Location Model..... 101

Table 19. The BPA Gender Model..... 101

Table 20. BrdU Analyses Results..... 102

Table 21. Relationships Between Average BrdU Analysis Results and Fish Characteristics from Preliminary Univariate Analysis..... 105

Table 22. The BrdU Model..... 105

LIST OF FIGURES

Figure 1. Chemical Structure of Bisphenol A 12

Figure 2. Chemical Structure of 5-(Dimethylamino)naphthalene-1-Sulphonyl Chloride (Dansyl Chloride) 18

Figure 3. Chemical Structure of Methyl 4-Hydroxybenzoate (Methyl Paraben) 27

Figure 4. Chemical Structure of Ethyl 4-Hydroxybenzoate (Ethyl Paraben) 27

Figure 5. Chemical Structure of Propyl 4-Hydroxybenzoate (Propyl Paraben) 28

Figure 6. Chemical Structure of Butyl 4-Hydroxybenzoate (Butyl Paraben) 28

Figure 7. Chemical Structure of 4-Hydroxybenzoic Acid 28

Figure 8. Map of Freeport and Ford City Fish Sample Locations with WWTP and CSO Outfalls 49

Figure 9. All MCF-7 Composites by Location 63

Figure 10. All T47D Composites by Location 63

Figure 11. MCF-7 Composites by Locations; Males Only 63

Figure 12. T47D Composites by Location; Males Only 64

Figure 13. MCF-7 Composites by Location; Females Only 64

Figure 14. T47D Composites by Location; Females Only 64

Figure 15. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 1 65

Figure 16. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 2 65
Figure 17. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 3 65
Figure 18. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 4 66
Figure 19. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 5 66
Figure 20. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 6 66
Figure 21. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 7 67
Figure 22. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 8 67
Figure 23. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 9 67
Figure 24. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 10 68
Figure 25. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 11 68
Figure 26. Correlation Plot of Estrogenicity Index for MCF-7 Versus T47D Analyses for All Composites 68
Figure 27. Map of Surface Water Sample Locations 77

Figure 28. Map of Seven Fish Sample Locations with Nearby Sewage Outfalls..... 88

Figure 29. Graph of Average Raw BrdU Data Compared to Estradiol Control..... 104

Figure 30. Graph of Average Corrected BrdU Data Compared to BPA Concentration..... 107

PREFACE

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1.0 INTRODUCTION

1.1 PUBLIC HEALTH SIGNIFICANCE

Endocrine Disrupting Compounds (EDCs) and xenoestrogens, such as Bisphenol A (BPA) and parabens, are a public health concern. Many prevalent diseases, potentially caused by a disruption of the endocrine system, are elevated in different societies throughout the world. Diabetes, a disorder of the endocrine system causing a build-up of glucose in the system due to a lack of or misuse of insulin, has had an increase in incidence rates, or new cases, for the last 20 years [1]. Additionally, reproductive cancers and sexual malformation disorders are prevalent across the globe. It is time to look at our efforts in determining the causes of such disorders. Environmental pollution from EDCs may be a contributing factor or a direct cause of these public health issues. Research on EDCs did not begin until the mid 1900s when it was discovered that diethylstilbestrol (DES), a pharmaceutical administered to prevent miscarriages, caused harsh health effects in the offspring of expectant mothers [2]. However, research interest was engendered in the 1990s when environmental studies discovered reptiles, specifically alligators in Florida and fish in the United Kingdom, with sexual malformations [3-5]. This discovery led researchers to begin the hunt for the cause of this environmental debacle. Since that time, academic and industrial researchers have been attempting to narrow down the growing problem of endocrine disruption. However, controversial studies, inconsistent results and the

ever expanding chemical market have bewildered the research community causing policy and legislation in the area of endocrine disruption to become quite contentious.

1.2 INTRODUCTION TO ENDOCRINE DISRUPTING COMPOUNDS

There has been a growing concern about the effects of man-made chemicals on the endocrine system in both humans and animals. The study of EDCs started decades ago, but slow progress has been made for many reasons: (1) the vast number of chemicals available on the market, (2) the difficulty in isolating risks from individual EDCs or those in combinations, and (3) the impact of confounding factors from natural hormones and phytoestrogens on any particular biological system. Although it is a widely accepted hypothesis that EDCs can cause detrimental changes to human endocrine systems, toxicological and risk assessment data have been unable to prove or disprove the potential for human health effects from a myriad of chemicals [6]. A great cause for environmental concern is the rapidly increasing amount of pharmaceutical and personal care products entering the consumer market. Most of these chemicals have not been thoroughly evaluated for endocrine activity. Although it is generally accepted that market products are safe, and some have research to support their claims, there are reasons to believe that products may have environmental endocrine effects. Some products have the sole purpose of altering the hormonal state of the user (e.g. contraceptives) and therefore may have involuntary effects to other humans and/or possible detrimental effects to organisms at lower doses [7]. Substances may also react with other environmental pollutants to form new endocrine active substances, and drugs may be metabolized to different unstudied forms and then excreted to the environment [7].

There may also be additive, synergistic or antagonistic effects of EDCs when present together in the environment [8].

The general public is exposed to these chemicals through various sources. It begins with the direct consumer, assume a female, applying hygiene products directly onto the skin and ingesting pharmaceutical contraceptives. Waste is disposed of down the drain and excreted into the sewer system. Sewer systems may treat water in a Wastewater Treatment Plant (WWTP) which may possibly remove some of the chemicals, while discharging the rest into the nearest body of water. Or, during high rain events, sewer systems may bypass treatment and dump untreated wastewater directly into the nearest body of water via Sanitary Sewer Overflows (SSOs) and Combined Sewer Overflows (CSOs). Surface water is generally used for the public drinking water supply and now contains low levels of hygiene products containing suspected EDCs and low levels of pharmaceutical contraceptives. These doses may or may not be harmful to the aquatic environment, and these chemicals may possibly be increasingly harmful in combination with other EDCs. Two new exposure pathways (via ingestion of the water and bathing in the water) are created for unsuspecting individuals, including men and children, two groups that should not be exposed to pharmaceutical contraceptives.

In endocrine disruption research, most attention has been directed towards aquatic species, primarily because the aquatic environment receives most of the pollutants released to the environment, either by direct point source discharges from industry and wastewater treatment facilities or indirect sources such as spills, pesticide application over large areas, runoff and deposition [5]. Generally, potential EDCs can get into the environment through various ways: disposal in landfills or into household drains, urination/sewage pathways, washing/bathing with hygiene products, or simply by direct use of the individual. Landfill disposal causes leachate

concerns which may transport potential EDCs to the local ground and surface waters. Sewer disposal allows for partial cleanup in WWTPs, if the sewage system is operating at its optimum potential. However, many sewer systems do not possess the ability to process all of the community sewage, and will dump untreated waste directly into receiving bodies of water during times of high flow and/or rain events. This is especially the case in Pittsburgh, Pennsylvania, and its surrounding areas which are known for the unusually high volumes of SSOs and CSOs [9].

The National Institute of Environmental Health Sciences (NIEHS) is a United States government entity that is appointed with the task of reducing the burden of human illness caused by environmental influences [10]. NIEHS defines endocrine disruptors as "naturally occurring compounds or man-made chemicals that may interfere with the production or activity of hormones of the endocrine system leading to adverse health effects" [11]. These chemicals can be found in many common household products that are used regularly, such as plastic bottles, detergents, food packaging, hygiene products and others [11, 12]. Research has proven that exogenous chemicals can alter the endocrine system in multiple ways: (1) chemicals can adhere to sites where natural hormones are expected to react, thus mimicking their effects and causing over stimulation of the hormone receptors with false messages; (2) chemicals compete with the endogenous hormones by blocking receptor sites and therefore prevent true signals from occurring; or (3) chemicals may breakdown natural hormones or prevent hormone synthesis [11, 13]. Sometimes the effects of a chemical are understood, but the methods of disruption are not [11, 13].

Several types of endocrine disruption can theoretically occur. Estrogenic chemicals, (termed 'xenoestrogens') cause the feminization of species, which has been demonstrated in

alligators from Florida lakes and fish from English rivers [5]. Xenoestrogens are also suspected to cause the proliferation of breast cancer cells [14]. The most strongly suspected estrogenic chemicals are the natural steroids 17B-estradiol (E2) and estrone (E1), and the synthetic active ingredient for contraceptive pharmaceuticals, ethinyl estradiol (EE2) [5]. Androgenic chemicals, in turn would masculinize species, which has been seen in fish downstream of paper and pulp mill effluents. The causative chemical has yet to be determined [5]. A known androgenic chemical that is suspected of having environmental consequences is 17B-trenbolone (TB), which is a metabolite of a growth steroid given to cattle [5]. It is theorized that chemicals may contain anti-estrogenic and/or anti-androgenic effects, and it is likely that there are chemicals which may have adverse effects on progesterone and/or the various thyroid hormones as well [5]. A specific concern is that possible EDCs may have additive effects when present together. Eight weak estrogenic chemicals were tested for additive effects at levels below all individual No Observed Effect Concentrations (NOECs) and it was concluded that there is a definitive additive estrogenic effect [15]. This poses a problem for studying individual EDCs as they are rarely present in the environment in such discrete manners.

The International Union of Pure and Applied Chemistry (IUPAC) is an international scientific committee dedicated to the study of global issues involving chemical sciences [16]. In 2003, they published a Special Topic Issue in their journal on the implications of endocrine active substances in humans and wildlife [17]. This special topic was a compilation of current research performed on endocrine active substances. The IUPAC developed some guidelines, such as the use of fish in endocrine disruptor research. They suggest using a full life-cycle test of fish for locations with a constant discharge of estrogenic chemicals, a fish partial life-cycle development/reproductive test when studying non-bioaccumulative pesticides, and short-term

assays for rapid screening of potential endocrine activity [18]. When screening for endocrine activity, three vital biomarker endpoints should be used: vitellogenin, gonado-somatic index (GSI) and gonad histopathology. Because it is well known that early life-stages in fish are sensitive to endocrine active substances, developmental tests, such as the Medaka developmental test, are useful. The Medaka developmental test is utilized by exposing fish, in prelarva to hatchling stages, to a suspected endocrine active substance, and monitoring the sex characteristics that develop. In this sex-reversal test, the natural sex-linked colors will not change, but the gonad histology and sexual characteristics can change when endocrine disruption is present [18]. Full life-cycle tests involve exposing newly fertilized embryos to a chemical until they reach adulthood, and breeding randomly selected pairs. The outcomes of spawning frequency, number of eggs produced, current generation fertility, viability of embryos, hatching success and growth/development of the second generation are all monitored [18].

Endocrine disruption research has some very critical problems. There are three main concerns about endocrine disruptors in the environment: very low doses can have profound effects on exposed species; mixtures of chemicals can have additive, synergistic or antagonistic effects; and health effects can be dependent upon the timing of exposure relative to the life cycle of a particular species (i.e. exposure at certain phases of growth and development may be more important than the amount of exposure) [13]. EDCs do not follow conventional scientific paradigms and do not follow conventional dose-response curves [13]. Dose-response curves for EDCs tend to yield results at extremely low doses and exhibit different behaviors over dose ranges, sometimes causing U-shaped or upside-down U-shaped dose response curves. Therefore, traditional linear dose response curves are not satisfactory prediction models. The mechanisms causing this are still unknown, but it is theorized that they are influenced by contrasting forces

acting concurrently within the biological system. Therefore, high dose studies cannot be fair predictors of the occurrence of health effects from low dose exposures and vice versa. Non-monotonic dose response curves are a fairly recent concept and many research study designs do not plan for such occurrences [13]. Embryos and fetuses tend to be extremely sensitive to EDCs, while adults are typically not. This challenges the concept of high dose animal studies being relevant to human exposure predictions. Human studies are more difficult to use for determining the effects of potential endocrine disruptors for a few reasons. There cannot be a true control group because the general population is already exposed to the chemical and conducting controlled endocrine disruption experiments on humans is unethical. Finally, it is also difficult to determine a causal relationship between a chemical and its potential effect because of the inevitability of confounding factors. Many chemicals are suspected of being EDCs. Few have been proven as such, and many have never been tested for their potential to disrupt the endocrine system.

Although advances have been made in the areas of endocrine disruption and its potential human health consequences, more research is needed to understand the intricacies of this field. Complicated research due to the ever increasing market for new chemical products, sophistication of the endocrine system itself and the likelihood for confounding factors, e.g. natural hormones and additive/synergistic effects, make advancement in this field challenging.

1.3 WASTE WATER TREATMENT PLANTS

Wastewater Treatment Plants (WWTPs) are a centralized way to remove household sewage and liquid wastes from communities. The use of a sewage collection system brings wastes to the

influent of the WWTP, where waste is then processed with intentions to separate harmful contaminants from the water in order to recycle the water back to receiving surface waters. Initial sewage collection systems were intended to remove rainwater from communities to prevent flooding. Later, the idea of household waste removal was developed and homes were tied in to this existing system. The mixture of water and sewage is called a combined sewer. The Greater Pittsburgh Area has problems with combined sewer overflow because these systems were historically combined [9]. As the population increased, most combined sewer systems were overburdened, causing CSOs to dump excess water directly to the receiving surface waters in times of high flow (i.e. during rainfall), resulting in large quantities of chemical pollutants and biological pathogens to enter our environment without the benefit of treatment. In newer communities, sanitary sewer systems were developed to prevent the mixing of wastes with rainwater. These areas have less potential to overflow pollutants into the environment, but still have the design capacity for SSOs to dump into surface waters during high flow events in order to prevent sewage line backups.

When wastewater actually reaches the WWTP, the pollutant potencies are drastically reduced. This is done though use of preliminary, primary, secondary and tertiary treatment processes [19]. Preliminary treatment employs the use of screens to remove coarse solids and floating objects from the influent. Sometimes a comminutor, a machine that grinds solids, is used to shred heavy material that may make it into the WWTP influent. Primary treatment involves the influent entering a grit chamber where sand and small stones may be allowed to settle to the bottom for removal to a landfill to prevent damage to the WWTP equipment. Wastewater then flows to a sedimentation tank which slows the flow rate to allow for organic and inorganic suspended solids to settle out and form primary sludge. Primary sludge will be

removed from the system. Secondary treatment employs the use of biological treatment processes to remove up to 90% of organic matter, using either attached growth processes (microbiological growth occurs on a surface which the wastewater runs across) or suspended growth processes (microbiological growth is suspended in an aerated tank) in WWTPs, usually followed by a secondary clarifier settling tank. Another type of secondary treatment utilized might be a lagoon, which works similarly to the microbiological growth processes but in a more natural manner. Finally, the tertiary treatment process step disinfects the water to make it safe for reuse by using chlorine, ozone, and/or ultraviolet radiation. Land Treatment (irrigating of crops with wastewater and allowing the soil to filter the sludge) and Constructed Wetlands are alternative methods to mechanical WWTPs [19].

Problems with current wastewater processing are numerous. These include, but are not limited to, the age of WWTP systems in use, the cost of replacing municipal WWTPs with newer models, the vast amounts of chemicals in household wastes and their individual properties for removal concerns, the increasing amount of waste caused by population growth, farm runoff and urban wastes that are not all collected by WWTPs, and the use of non-centralized septic systems [19].

While WWTP processes have developed over the last few decades, from primarily removing Biological Oxygen Demand (BOD) to current models for enhanced biological phosphorous removal, there is still much lacking in the ability to remove chemicals [20]. Any decomposition usually occurs as a first-order reaction [20]. However, the fate and transport of metabolites is of major relevance in the study of EDCs. Just because a chemical is broken down by bacteria does not guarantee that the metabolite becomes less potent; it may continue to have endocrine disrupting effects.

Some better methods of decomposition are available for use, but require a cost-benefit analysis. Although it is known that longer retention times help bacteria to develop methods to metabolize new chemicals, neither the United States nor the European Union operate with retention times long enough to satisfy this efficient decomposition. The solution would be to upgrade plants to allow for an increased retention time and therefore a longer sludge age of at least 12-15 days [20]. When WWTP effluents with little or no surface water dilution are to be used for irrigation purposes, ozonation should be considered. When only 5-10 g/m^3 of ozone are used, pharmaceutical concentrations can be reduced to below detectable limits, and the cost is minimal [20]. However, the required kilo-watt hours required for the WWTP process are significantly increased, leading to diminished cost-effectiveness, and the byproducts of ozonation have not been thoroughly investigated [20]. Two other expensive options for cleanup of effluents are nanofiltration and activated carbon adsorption.

Some methods of prevention may be useful to stop chemicals from entering the WWTP processes. They are, however, difficult to implement and will required much political support, encouragement and funding. The use of separate WWTPs for high pharmaceutical loads, such as hospitals and/or residential treatment facilities, would reduce public loading and could be specialized to meet the specific chemical demand rather than using expensive treatment processes on all water [20]. Discussion of environmental risks and labeling personal care products and pharmaceuticals packaging would help doctors and consumers to pick a more environmentally friendly product [20]. Special methods of disposal for products with environmental risks could be developed to keep dangers out of our water supply. Finally, a separate waste system for urine would contain the chemical load [20].

There are many things known about the transport and fate of generic environmental agents as they undergo the WWTP process. Personal care products tend to adsorb onto sludge and sediments because of their elevated lipophilicity [20]. As sludge age increases, biological decomposition becomes more effective most likely because slow growing bacteria have time to develop adequate numbers and/or diversified metabolism is developed to make use of new energy sources [20].

WWTPs are able to reduce the estrogenicity and androgenicity of human sewage. Estrogenicity of WWTP effluents has been proven by multiple methods, both *in vivo* and *in vitro*, using endpoints of induced vitellogenin (an egg yolk precursor protein found in female fish) production and shifted sex ratios of offspring [21]. In general, as wastewater moves through the sewage treatment process, estrogenic and androgenic activity decreases [22]. Plants with secondary and tertiary treatments tend to lower androgenic chemicals better than estrogenic compounds [23]. A test for androgenic potential was performed on the inlet water and outlet effluents of a WWTP in India. By performing the Hershberger assay, Kumar et. al. showed that outlet WWTP effluents, as well as the untreated influents, contained androgenic properties [24]. By using High Performance Liquid Chromatography (HPLC), twenty chemicals were found in the influent waters, and only five of the chemicals were found intact in the outlet samples [24]. Through Gas Chromatography-Mass Spectrometry methods (GC-MS), the effluent was found to contain four known androgenic compounds that were not removed by normal wastewater processing: nonylphenol, hexachlorobenzene, and two testosterone equivalents, *iso*androsterone and dehydroepiandrosterone [24]. In a study of five WWTP designs in the United Kingdom, overall estrogenic activity was removed between 70 - 100% and androgenic activity was removed between 93 - 100% in 4 out of the 5 designs [22]. Most of the estrogenic and

androgenic activity was removed during biological/secondary treatment processes [22]. Of the secondary treatment types available, aerated sludge secondary treatment was more effective at removing estrogenic activity than slag or plastic filtration [22].

1.4 RESEARCH REVIEW FOR BISPHENOL A (BPA)

1.4.1 Chemical Properties

Bisphenol A (BPA) or 4',4'-dihydroxy-2,2-diphenylpropane (CAS no. 80-05-7) is an organic compound containing two phenol functional groups. BPA has been classified as a suspected endocrine disruptor [12, 25]. Figure 1, Chemical Structure of Bisphenol A, shows its chemical structure. Table 1, Chemical Properties of Bisphenol A, lists known chemical properties of BPA.

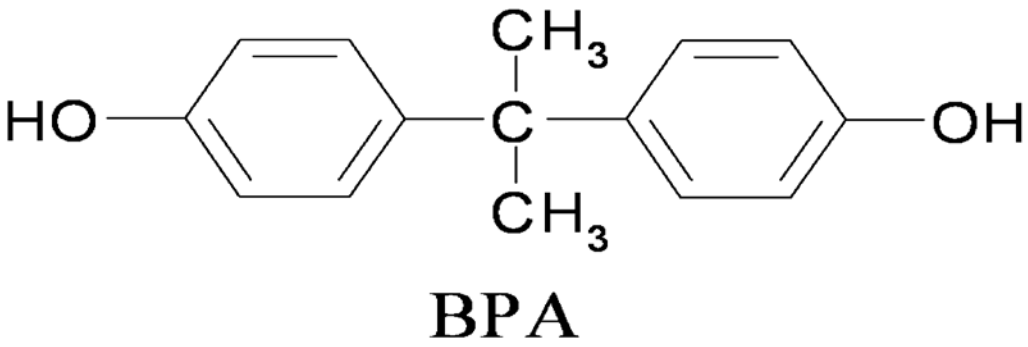


Figure 1. Chemical Structure of Bisphenol A

Table 1. Chemical Properties of Bisphenol A

Chemical Information	Value
CAS no. [26, 27]	80-05-7
Molecular Formula [26, 27]	$C_{15}H_{16}O_2$
Molecular Weight [26, 27]	228.29
Melting Point in deg C [26]	158 – 159
Solubility @ 20-25 deg C [27]	1000 mg/L
Vapor Pressure @ 20-25 deg C [27]	7.25e-7 mmHg
Henry's Law Constant @ 25 deg C [27]	2.18e-10
Sorption Coefficient K_{oc} [27]	2.74
Octanol-Water Partition Coefficient [26, 27]	3.32
Diffusion Coefficient in air [27]	0.05 cm^2/s
Diffusion Coefficient in water [27]	5.89 cm^2/s
Oral Reference Dose [27]	0.05 mg/kg/day
Inhalation Reference Concentration [27]	0.08 mg/m^3
Dermal Adsorption Fraction [27]	0.1
Gastrointestinal Adsorption Fraction [27]	0.5

1.4.2 Transportation and Fate of BPA in the Environment

BPA is used in polycarbonate plastics and epoxy resins for food packaging, plastic baby bottles, dental fillings, and in many other household products. BPA is known to enter the human body through oral exposure. It is partially excreted through human urine as free BPA and a conjugate form from liver enzymes [28]. BPA enters the environment from human urine and improperly disposed garbage when it leaches from plastics as they degrade. BPA may be processed by

WWTPs with a documented 73-93% removal efficiency [29]. It may also be directly deposited into the surface water system through untreated CSOs and SSOs or into groundwater from septic tank leachate.

The transport and fate of BPA in the environment has been studied to a great extent. The matrix in which BPA is studied can have a profound influence on the research results. BPA acts differently in deionized water compared to tertiary treated wastewater when exposed to hydroxyl radicals [30]. BPA exhibits a half-life of 3-5 days in river water and up to 30 days in seawater [31, 32]. BPA has a sediment adsorption coefficient (K_{oc}) of 1524, indicating that concentrations of BPA will be adsorbed onto sediments in concentrations higher than that of surface waters [33]. This adsorption is affected by many variables: adsorption to sediment is increased in higher salinity waters from decreasing solubility of BPA, competition with Dissolved Organic Matter results in a decrease to the amount of BPA adsorbed, and sediment conditions like temperature and pH can affect adsorption [33]. Li et. al. came to three conclusions: (1) manganese oxides can inhibit the adsorption of BPA onto sediment media in surface waters, (2) iron oxides and organic materials can encourage binding of BPA to sediments and (3) natural surface coatings samples contribute more to the pollution levels of surface waters than surficial sediments [34]. BPA has an Octanol-Water Partition Coefficient (K_{ow}) of 3.32, indicating that its lipophilicity will allow it to bioconcentrate in fatty tissues [33]. Fish bioconcentrate BPA via oral and gill exposure routes and have the potential to cause the bioaccumulation of BPA throughout the food chain.

Methods to remove BPA from the environment are being researched. BPA is broken down by photolysis according to the first-order reaction model and the use of diatomite-TiO_2 composites rather than pure TiO_2 powders seems to encourage this process [35]. Pan et. al.

discovered that carbon nanomaterials adsorb large amounts of BPA quickly and retain BPA due to a strong affinity. B PA pollution could be greatly altered in the presence of carbon nanomaterials and therefore this may be used as an environmental or WWTP clean-up method in the future [36].

1.4.3 Exposure to Bisphenol A

BPA is a large environmental contributor because of its high volume of production (over 6 billion pounds produced yearly and over 100 tons released into the atmosphere by yearly production) [13]. It is used extensively in the production of polycarbonate plastics, for canning and bottling industries. This allows for oral exposure through food and drink consumption. Plastic has become one of the fastest growing environmental waste concerns in the United States. BPA is a chemical used in the production of polycarbonate plastic and will leach out of the plastic after continued use, repeated washings, exposure to high temperatures and contact with acidic/basic substances [25].

BPA has been thoroughly studied in surface water systems across the world. BPA has been detected in Taiwan surface waters at ranges between 0.037 μg/L (limit of detection) to 4.23 μg/L in 59% of samples tested [37]. Surface waters analyzed in Spain and other Mediterranean areas discovered BPA in the μg/L range for all samples collected. United States waters ranged between <1.0 to 8.0 μg/L for BPA concentrations [38, 39]. BPA in Germany waters ranged from 0.0005 to 0.776 μg/L [39-44]. In Japan, levels varied from <0.005 to 1.9 μg/L [39, 45-50]. In China, BPA levels ranged from 0.03 to 0.083 μg/L and in the Netherlands, concentrations were found to be between <0.012 and 1 μg/L [39, 51-53].

Waterworks treatment methods for preparing public drinking water, using sand, ozone, and carbon filtrations, did reduce concentrations of BPA dramatically, but not completely [54]. BPA was also found in groundwater samples at low μg/L ranges, suggesting that degradation is slow in groundwater media [54]. BPA was detected in 51% of surface waters sampled in Portugal with analytical methods using solid phase extraction and Gas-Chromatography-Mass Spectrometry (GC-MS) in the range of 0.07- 4.0 μg/L [55]. BPA was detected in sewage treatment plant influents and effluents in the United Kingdom and Spain, with ranges of 884.7-1105.2 ng/L and 13.3-19.2 ng/L, respectively [56]. In Germany, WWTP effluents have BPA concentrations in the 18-33 ng/L ranges, and a surface water sample from the Czech Republic was 28 ng/L [57]. There has recently been new evidence published that previous aquatic hazard assessments of BPA may not be sufficiently conservative for all species [39]. A new weight of evidence approach was used to calculate a Predicted No Effect Concentration (PNEC) by nonparametric methods, and it determined that aquatic effects on mortality, growth, development and reproduction can occur between the concentrations of 0.0483 μg/L and 2280 μg/L with a PNEC of 0.06 μg/L [39]. Much of the published literature on BPA surface water concentrations exceeds this PNEC [37, 39, 55].

BPA has been detected in indoor and outdoor air samples, dusts and soils [13]. Many EDCs have been detected in indoor air and house dust. A study performed in the United States found 89 identified EDCs in 120 sampled homes, with chemicals from various classes of plasticizers, emulsifiers, disinfectants, adhesives, pesticides, personal care products and flame retardants [58]. From the samples, BPA was found above detection limits in 86% of the dust samples, ranging from below detectable limits to a maximum of 17.6 μg/g [58].

BPA has also been previously quantified in food products [13]. Milk samples purchased from markets in China had BPA concentrations in the range of 0.45-0.94 μg/L or approximately 0.41-0.85 $x10^{-3}$ mg/kg relative to milk density, which is well below the European Union's legal limit of 0.6 mg/kg [59]. Market seafood from Singapore was tested for concentrations of BPA. Basheer et. al. found BPA in prawn, crab, blood cockle, white clam, squid and fish in the range of 13.3 – 213.1 ng/g w/w, with the highest concentration found in crab and the lowest in the white clam [60]. The daily human oral intake of BPA has been estimated at less than 1 μg/kg bw/day [31]. Estimated oral exposure of children is in the range of 52-74 ng/kg bw/day and inhalation exposure at 0.24-0.41 ng/kg bw/day [61].

1.4.4 Analytical Methods

Analytical methods to determine the concentrations of BPA are available for many different matrixes. Table 2, Analytical Methods for Bisphenol A, provides a list of these matrixes and also describes the associated detection limits. HPLC with dansyl chloride derivatization has been successfully used to analyze for 4-nonylphenol and BPA in a sewage sludge matrix. Figure 2, Chemical Structure of 5-(Dimethylamino)naphthalene-1-Sulphonyl Chloride (Dansyl Chloride), shows the chemical structure of dansyl chloride. Dansyl chloride derivatization enhances the specificity of the determination of xenoestrogens because dansyl chloride easily reacts with phenolic hydroxyl and amino groups, which tend to be present in most suspected endocrine disrupting chemicals. Interference from other compounds decreases when detection is specified for the dansyl derivatives [62]. Derivatization with dansyl chloride has been used in the past with liquid chromatography methods to allow for greater sensitivity and selectivity of

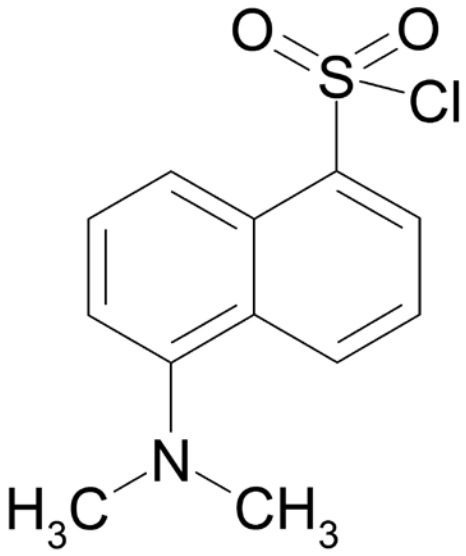


Figure 2. Chemical Structure of 5-(Dimethylamino)naphthalene-1-Sulphonyl Chloride (Dansyl Chloride)

Table 2. Analytical Methods for Bisphenol A

Matrix	Method	Detection Limits	Reference
Water	C18 solid phase extraction and LC-MS	0.1 ng/L	Pedersen, et. el. 1999 [63]
Water	Solid phase extraction with bamboo charcoal followed by HPLC	0.17-0.37 μg/L	Zhao, et. al. 2009 [64]
Surface water	Solid phase microextraction using OASIS cartridges followed by GC-MS	0.002 μg/L	Azevedo, et. al. 2001 [55]
Surface Water	Solid and/or liquid phase extraction and derivatization with N-methyl-N-(*tert.*-butyldimethyltrifluoroacetamide followed by GC-MS	4-6 ng/L	Mol, et. al. 2000 [65]
River water/Wine	Solid phase extraction with cryogel followed by HPLC	10 ng/L	Baggiani, et. al. 2010 [66]
Water/Milk	Solid phase extraction using magnetic molecularly imprinted polymer followed by HPLC and UV detection	Water = 14 ng/L Milk = 0.16 μg/L	Ji, et. al. 2009 [59]
Urine (humans)	Online solid phase extraction for HPLC-MS/MS	0.4 ng/mL	Ye, et. al. 2007[28]
Sewage sludge	Derivatization with dansyl chloride followed by HPLC	0.1 ng	Naassner, et. al 2002 [62]
Fish bile	Enzymatic hydrolysis and solid phase extraction using OASIS HLB cartridges followed by GC-MS/MS	0.1-0.7 ng/mL	Fenlon, et. al. 2010 [67]
Fish tissue (liver and muscle)	Microwave-assisted solvent extraction and solid phase extraction LC-MS	50 ng/g	Pedersen, et. el. 1999 [63]
Dust	Extraction and GC-MS	0.2 μg/g	Rudel, et. al. 2003 [58]

alkylphenols [68]. Other methods of derivatization are also available with gas or liquid chromatography [56, 65].

New methods to increase the efficiency and decrease the expense for performing solid phase extraction on environmental water samples are being developed, specifically to study the use of bamboo charcoal or molecularly imprinted polymer/cryogel composites for the extractant, both of which tend to yield promising results [64, 66]. The main drawbacks to currently available solid phase extraction cartridges is the low selectivity of the retention mechanism and the high likelihood for clogging when extracting from large volumes and/or dirty samples [66].

Analytical methods for testing fish tissue have been available since the 1990s, however, they have mostly focused on the edible tissues of fish, leaning towards a method to quantify exposure [63]. A method to test for bioconcentrated chemicals in fish bile was developed using solid phase extraction with gas-liquid chromatography-tandem mass spectrometry, however, the reference fish indicated laboratory contamination of BPA [67]. There is a need to further study the bioconcentration properties of BPA in fish and to develop methods for analyzing fish tissue.

1.4.5 *In Vitro* Studies

The effects of BPA have been studied *in vitro*. BPA is known to bind to the Estrogen Receptor (ER) and has an affinity towards ERβ that is 10 times greater than its affinity for ERα [13]. It is also known to bind to the thyroid hormone receptor and the aryl hydrocarbon receptor (AhR), and exhibits antiandrogenic properties [13]. Although BPA is approximately 10,000 times weaker than estradiol at exhibiting estrogenic effects, there is a great cause for concern to determine its other endocrine disrupting abilities [13]. BPA is known to mimic estrogen at low doses [25]. At higher doses, BPA impedes the binding of testosterone and thyroid hormone to

their associated receptors. Thus, BPA is classified as an endocrine disruptor [25]. BPA is also known to bind to persistent organic pollutants, such as dioxin and polychlorinated biphenyls (PCBs) causing longer exposures to BPA and increasing the environmental risk [25]. BPA tends to exhibit non-monotonic model dose response curves when studied over a wide range of doses [13]. Therefore, high dose studies cannot predict the occurrence of health effects at low doses and vice versa. Non-monotonic dose response curves are a fairly recent concept and many research study designs do not plan for such occurrences [13]. Low nanomolar exposure (equivalent to environmental levels) to BPA can antagonize the cytotoxic effects of anticancer chemotherapy drugs in both ERα-positive and –negative breast cancer cells, therefore, implicating that BPA interacts through an unknown method unrelated to the estrogen receptors ERα and ERβ [69]. Brominated BPA analogues are agonists of both ERα and ERβ estrogen receptors and stimulate ER-mediated luciferase induction *in vitro* [70]. BPA exposure at doses as low as 10 μg/L after a four week exposure period caused gene expression of cloned amino acid sequences and significantly increased mRNA levels in the testes of Nile tilapias [71]. Because of the massive influx of new chemicals available to the market every year, it is necessary to develop computer models to quickly screen chemicals for their endocrine disrupting potential. A Quantitative Structure – Activity Relationship (QSAR) model for androgen receptor antagonism has been developed with a sensitivity of 64%, a specificity of 84%, and a concordance of 76% [72]. The model was tested satisfactorily with 176103 chemicals; 47% were within the domain of the model, and of them, 8% were predicted to be AR antagonists, while BPA tested negative [72].

1.4.6 *In Vivo* Studies

Mammalian studies have shown that BPA has potential to cause many health effects. Exposure in female mice during pregnancy by oral ingestion of spiked drinking water at concentrations of 10 μg/mL caused allergic sensitization and bronchial inflammation in offspring [73]. Oral exposure of 2 and 20 μg/kg/day administered to pregnant mice in another study caused increases in prostate weight in male offspring [74]. BPA induced a significant uterotrophic response in prepubertal rats after oral exposure to doses of 200 mg/kg within three days. However, this response required doses 13,000 times higher than needed for ethinyl estradiol to induce the same response [75]. There are reports that BPA can advance the onset of puberty in female mice at doses as low as 2.4 μg/kg/day, although it is believed that differences in endogenous hormone levels from intrauterine positions, which may influence sensitivity to exogenous hormones, may have also affected the results [13, 76]. Subcutaneous injections of 5-10 mg/kg/day of BPA in the days following birth altered plasma levels of prolactin, a hormone involved in the regulation of lactation, and developmental patterns of prolactin in both male and female rats [77]. Meiotic aneuploidy, a condition that occurs during cell division in the egg causing daughter cells to receive the wrong number of chromosomes, has been proven to occur in female mice after exposure to BPA in environmental doses as low as 0.02-0.04 mg/kg/day [78]. Recent research on rats has proven that maternal exposure to BPA during lactation can cause decreased time to tumor latency and an increased amount of dimethylbenzanthracene-induced mammary tumors in female offspring [79]. BPA is known to cause molecular and morphological alterations in the uterus and vagina of rats in the microgram levels of exposure [80]. Low level exposure to BPA can bind to and activate the androgen receptor causing increased proliferation and tumor growth

in the presence of anti-cancer drug therapies when studying prostate cancer *in vivo* [81]. This indicates a very likely concern that certain groups, such as prostate cancer patients, might be significantly more susceptible to health effects from exposure to BPA, and impacts to sensitive groups need to be studied thoroughly.

Non-mammalian species are also susceptible to affects from BPA exposure. Insects are just one of these groups. R ankin and Grosjean found that when the ring-legged earwig is exposed by injection to 0.12 μg, the following significant effects occurred: males experience reduced weight gain, increased testis and increased seminal vesicle size; and females experience enhanced clutch size. However, higher doses did not always produce a dose-response effect, implying that there is a biological method to degrade BPA when detected by the body [82]. BPA displays genotoxic effects by causing DNA strand breaks after exposure to 0.3-30 μg/L for freshwater crustaceans and 5-500 μg/L for aquatic midge [83]. Male isopods exposed to soil concentrations of BPA of 10-1000 mg/kg soil for 10 weeks had a earlier molting period. Exposed juveniles experienced an altered sex ratio of one male per two females, while an equal gender distribution was observed in the controls [84]. BPA in the nanogram per liter ranges can cause superfeminization of freshwater snails, a condition characterized by the formation of excess female organs leading to increased female mortality. This in known to occur through the estrogen receptor, due to observed antagonism in experiments involving tamoxifen [85]. *In vivo* studies on t urbot blood showed a 6.7-fold increase of micronuclei after exposure to 50 ppb of BPA, indicating the potential for genotoxic effects [86].

1.4.7 Human Studies

Korean studies on breast cancer have not shown a significant risk from BPA exposure measured through blood serum levels. BPA, however, was detected in the blood serum levels in 50.8% of the subjects, at concentrations ranging from 0-13.87 μg/L [87]. H uman exposure has been quantified through blood serum levels of up to 20 ng/mL of serum [13]. BPA has been found to cross the maternal-fetal placental barrier [13]. Human exposure has also been quantified by detection in urine [13]. Ye et. al. tested urinary concentrations of 15 male and female volunteers with no doc umented occupational exposure to BPA and found positive results for BPA in all samples [28]. Concentrations were detected with a mean of 2.4 ng/mL, with a detection limit of 0.4 ng/mL. Acute oral exposure studies performed on human adults have shown that BPA levels in urine exhibit a half-life of approximately five hours in the body [88-90]. With this evidence, and the generally accepted assumption that most exposure to BPA is through the oral route primarily from food intake, it would be expected that BPA levels in a population would be inversely related to fasting times. Analysis of urinary data collected from the National Health and Nutrition Examination Survey (NHANES) showed that (1) the relationship between fasting time and BPA urine concentrations was weak, suggesting that the biological half-life determined from the above mentioned acute exposure studies is wrong; (2) accumulation of BPA in bodily tissues releases BPA slowly; (3) there is substantial chronic exposure to BPA that is not currently being addressed by the research; or (4) some combination of the three [91]. Another study using NHANES data documented positive associations between urinary concentrations of BPA and the prevalence of diabetes, heart disease and liver toxicity. However, it cannot be determined that the relationship is causal [13, 92]. Other studies have also associated BPA blood levels with obesity, endometrial hyperplasia, recurrent miscarriages, sterility and polycystic ovarian syndrome [13].

An occupational cohort study performed in China among workers in factories which manufacture BPA and epoxy resins observed a higher risk of self-reported sexual dysfunction than in unexposed workers. Exposed works noted significant changes in reduced sexual desire, erectile difficulty, ejaculation difficulty and reduced satisfaction with sex life after only one year of occupational exposure [93].

Premature infants in neonatal intensive care units are exposed to BPA. A study by Calafat et. al. shows levels of conjugated BPA in the infants' urine ranging from 1.6 – 946 μg/L, proving that infants have at least some capacity to metabolize the chemical and thereby refuting any claims to contaminated samples or analyses. BPA exposure was associated with the specific hospital location, but not the length of stay, method of feeding (i.e. breast-feed versus formula) or gender. Urinary concentrations were higher for lower gestational ages than for older infants [94].

1.4.8 Regulatory Actions

Although BPA is a hot topic for debate among regulatory agencies, little action has yet to be taken to remove BPA from the market. The maximum tolerated dose of BPA was determined to be 1000 mg/kg bw/day and the EPA calculated reference dose is 50 μg/kg/day. A No-Observed-Adverse-Effect-Level (NOAEL) has yet to be found because adverse responses have always been detected in even the lowest administered doses [13]. In 2008, the National Toxicology Program reported a subpanel critique of the data available on BPA exposure and potential low-dose health effects. They concluded that there is credible evidence available to show that low doses of BPA can cause specific effects, but that these effects have not been established as reproducible findings [95]. In the United States, though, most product withdraws have been

market driven, meaning that companies have voluntarily phased out BPA containing products in order to promote positive customer relations [96]. Some local and state governments have started to propose regulations and enforce them, but as of yet, nothing has been legislated on a federal government level. Canadian government officials have chosen to withdraw products containing BPA from their market. Prior to this decision, the Canadian governments respected limits for BPA of 0.1 mg/L for drinking water resulting from contact with BPA containing packaging [97]. A Provisional Tolerable Daily Uptake (PTDU) for BPA from food was established to be 25 μg/kg bw/day [97]. The European Scientific Committee on Food set its tolerable daily intake to 50 μg/kg bw/day in 2006 and exposure to BPA from migration out of food packaging was set at 0.6 mg/kg [97].

1.4.9 Problems with Research on BPA

Problems researching BPA vary greatly. The mechanism(s) for low dose effects from BPA is unknown. BPA has been referred to as a weak environmental estrogen, but it may still be a strong endocrine disruptor through another endocrine mechanism (i.e. thyroid hormone receptors) [13]. *In vitro* research methods do not account for the way a chemical reacts in a true biological system or at altered developmental stages. Furthermore, the same chemical can have different effects in different cell types or different organ tissues. *In vivo* research methods are complicated and it is unknown if animal mechanisms will be adequate to predict human health effects. With endocrine disruptor research, there will always be confounding factors, such as exposure to other environmental chemicals which may or may not be endocrine disruptors themselves or exposure to natural and endogenous hormones. Problems arise when dealing with

concerns for sensitive populations, such as prostate cancer patients, because BPA has been suggested through *in vivo* studies to alter the effects of anti-cancer drugs [81].

BPA is still considered safe for use at the current human exposure levels despite the substantial amount of research literature which concludes otherwise. The United States regulatory agencies involved in the decision to keep BPA in market products made the judgment based on a few industry-funded research studies employing Good Laboratory Practices (GLP). GLP is the government mandated method of research to ensure commercial industry does not falsify data for monetary benefit [98]. Non-industry funded researchers, some from National Institutes of Health (NIH) funded studies, rallied together to document their opposition to the lack of government regulation of BPA, stating that studies employing GLP are not necessarily superior to other research and GLP does not guarantee the use of appropriate protocols or the use of the most current and sensitive assays [98].

1.5 RESEARCH REVIEW FOR PARABENS

1.5.1 Chemical Properties

After initial introduction of their IUPAC names in the following figures, all parabens will be referred to in this document by their common names. Figure 3, Chemical Structure of Methyl 4-Hydroxybenzoate (Methyl Paraben), shows the chemical structure of methyl paraben. Figure 4, Chemical Structure of Ethyl 4-Hydroxybenzoate (Ethyl Paraben), Figure 5, Chemical Structure of Propyl 4-Hydroxybenzoate (Propyl Paraben), and Figure 6, Chemical Structure of Butyl 4-Hydroxybenzoate (Butyl Paraben), show the chemical structures of ethyl, propyl and butyl

paraben, respectively. Figure 7, Chemical Structure of 4-Hydroxybenzoic Acid, shows the chemical structure of 4-hydroxybenzoic acid. Table 3, Chemical Properties of Various Parabens, shows chemical properties of methyl, ethyl, propyl and butyl paraben, as well as 4-hydroxybenzoic acid.

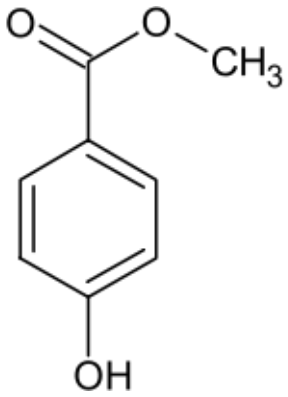


Figure 3. Chemical Structure of Methyl 4-Hydroxybenzoate (Methyl Paraben)

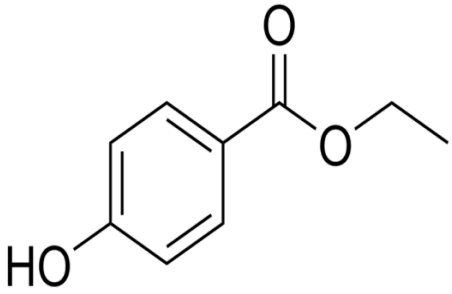


Figure 4. Chemical Structure of Ethyl 4-Hydroxybenzoate (Ethyl Paraben)

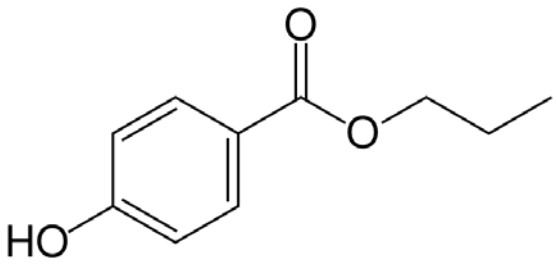


Figure 5. Chemical Structure of Propyl 4-Hydroxybenzoate (Propyl Paraben)

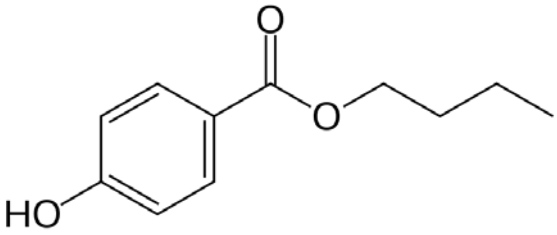


Figure 6. Chemical Structure of Butyl 4-Hydroxybenzoate (Butyl Paraben)

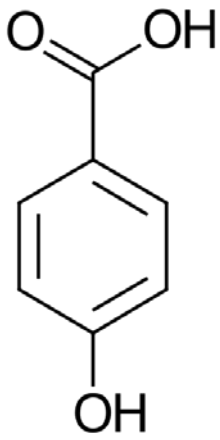


Figure 7. Chemical Structure of 4-Hydroxybenzoic Acid

Table 3. Chemical Properties of Various Parabens

Chemical Information	Methyl Paraben	Ethyl Paraben	Propyl Paraben	Butyl Paraben	4-Hydroxybenzoic Acid
CAS no. [99-103]	99-76-3	120-47-8	94-13-3	94-26-8	99-96-7
Molecular Formula [99-103]	$C_8H_8O_3$	$C_9H_{10}O_3$	$C_{10}H_{12}O_3$	$C_{11}H_{14}O_3$	$C_7H_6O_3$
Molecular Weight [104]	152.15	166.17	180.20	194.23	138.12
pKa [105]	8.87	8.90	8.87	8.79	
Solubility @ 25 deg C (g/100mL) [104]	0.25	0.17	0.05	0.02	0.6
Melting Point deg C [99-103]	125 - 128	114 – 117	95 – 98	67 - 70	213 - 217
Vapor Pressure Pa @ 100 deg C [106]					$3.9x10^{-3}$
Octanol-Water Partition Coefficient [104, 105, 107, 108]	1.87 - 1.96	2.34 - 2.51	2.90 - 3.04	3.46 - 3.57	1.56
Gibbs free energy ΔG_{sub} (kJ/mol) [109]	42.2	43.4	46.7	45.0	
Sublimation Enthalpy ΔH_{sub}(kJ/mol) [109]	98.8 $\pm$ 0.8	100.9 $\pm$ 0.7	123.7 $\pm$ 0.6	108.4 $\pm$ 0.8	
Entropy ΔS_{sub} (J/mol-K) [109]	190 $\pm$ 2	192 $\pm$ 2	258 $\pm$ 2	212 $\pm$ 3	
Entropy ΔS_{fus}(J/mol-K) [109]	63 $\pm$ 2	68 $\pm$ 2	74 $\pm$ 2	78 $\pm$ 2	
Heat of Fusion ΔH_{fus}(kJ/mol) [109]	25.3 $\pm$ 0.7	26.4 $\pm$ 0.8	27.2 $\pm$ 0.8	26.6 $\pm$ 0.8	
Heat of Vaporization ΔH_{vap}(kJ/mol) [109]	73.5	74.5	96.5	81.8	
Oral LD50- mouse (mg/kg) [99-102, 106]	>8000	3000	6332	13,200	2200
NOAEL-rat (mg/kg/day) [106]					1000

1.5.2 Transportation and Fate of Parabens in the Environment

Less is known about the transport and fate of parabens in the environment than BPA. Although it is generally believed that parabens do not persist for long periods of time, this may not be true. Any potential endocrine disrupting effects will most likely come from direct exposure from market products containing parabens. Wastewater treatment processes have been shown to adequately reduce paraben concentrations. Canosa et. al. showed influent concentrations of methyl paraben in two sets of samples were reduced from 2.92 ng/mL to less than detectable in the effluent [108]. Ethyl, propyl and butyl parabens were reduced to non-detectable as well, with benzyl paraben not being present in the influent [108]. With these removal efficiencies, it is not expected that parabens would be present in surface waters unless the body of water receives direct discharges of non-treated sewage water, which is a known problem in the Greater Pittsburgh Area [9, 108].

1.5.3 Exposure to Parabens

There is widespread exposure to parabens around the world. Parabens are used as food preservatives because of their antifungal properties and are therefore present in the edible coatings used on produce to extend the shelf life of these agricultural products. Parabens, however, only reduce the incidence and severity of citrus postharvest diseases caused by blue and green molds by less than 20%, with no synergistic effects noted for combinations of parabens [110, 111]. The poor inhibitory activity is suspected to be caused by the chemical characteristics of the coating and/or the fruit itself, suggesting that not all preservatives will be effective or should be used universally [110]. This method of exposure may be unnecessary if

the intended benefit is not satisfactory. While developing analytical methods, Zhang et. al. tested six food products: orange juice, soy sauce, pickle, strawberry jam, vinegar and soda. Methyl and ethyl parabens were found only in the soy sauce, at concentrations of 2.7 μg/g and 2.5 μg/g, respectively [112].

In Sweden, investigation of eleven paraben-containing compounds found the total paraben concentrations to be within the range of 0.43%-0.79% w/w for skin care products, 0.07%-0.44% for hair products, and 0.30%-0.52% for soaps, with all products containing methyl paraben and about 90% containing propyl paraben [107]. Two hundred and fifteen different cosmetic products found on the Danish market in the 1990s were analyzed by HPLC for paraben content. Of these, 93% were found to contain paraben(s), with a range of 0.01-0.59% w/w. Positive detection was found in 77% of rinse-off products, with a range of 0.01-0.50% w/w, and 99% in leave-on products, with a range of 0.01-0.59% w/w. Only one sunscreen had a concentration of 0.87% w/w which is higher than the limit of 0.80% w/w for mixtures set by the European Economic Community (EEC) [113].

Eriksson et. al. performed a substance flow analysis of parabens for Denmark based on data from 2004 [114]. Table 4, Summary of Eriksson's Results, describes a summary of the product analysis performed in Denmark. Total inflow was approximately 154 tonnes of pure chemicals and 7.2-73 tonnes via various commodities which correspond to an average wastewater concentration of 640-900 μg/L when excluding biodegradation, metabolism and sinks. Of personal care products on the Danish market, 272 out of 751 contained parabens (36%), with methyl and propyl parabens the most commonly used. Using the Households Products Database in the USA (over 6000 consumer brands) and the SkinDeep Personal Care

Table 4. Summary of Eriksson's Results

Commodity (Note: Reference [114] for the entire table)	Concentrations found in Products	Estimated Amounts on the Market	Estimated Usage or Theoretical Consumption	Annual Contribution in tonnes (unless otherwise noted)
Personal Care Products	0.024-0.8%	36%	Hair Conditioner = 12-39 Deodorant = 11-26 Hand Soaps = 10-16 Oral hygiene products = 5-14 Shampoo = 0-10 Skin care products = 1-6 (g/person/week)	4.0-132 (import) 2.0-68 (export) 0.16-5.2 (national consumption)
Pet Care Products	0.1-0.2%	15%		
Pharmaceuticals	0.3-3.16 mg/g	3.2%		0.26-0.91 (2004)
Herbal Medicines		4.9%		
Vitamins and Mineral Supplements	n/a	0%		
Veterinary Medicine	0.1 – 2.6 mg/g	7.9%		0.109 (MP and PP for 2005)
Filled Chocolate Candies	7.9-180 mg/kg	8.9%		Filled chocolates: <0.1 – 0.19 imported, <0.1-0.13 exported, <0.1-0.18 for domestic industry usage
Snacks, Nuts, and Candy	300 mg/kg (max. allowable)	Estimate 2%	4 g of snacks/day (adult) 9 g of snacks/day (child)	0.2 (imported 2004) 0.3 (exported 2004)
Meat Products	1000 mg/kg (max. allowable)	Estimate 2%		0.095-0.74 kg imported 0.35-0.96 kg exported
Soy Sauces	33.4-250 mg/L	26%		0.010-0.077 (imported 2004)
Food Supplements	2000 mg/kg (max. allowable)	Estimate 2%		0.3 tonnes imported 0.1 tonnes exported 0.2 national consumption
Household Cleaners	0.2% singles; 0.7% mixture	Estimate 11%		9-33 (imported 2004); 26-92 (exported 2004); 9-32 (natl' production 2004)
Spray Paints	<0.1%	0.007%	150-170 tonnes/year	<1.2 kg
Water-based Paints	0.27-0.6%	Estimate 2%		1 (import 2004) 1.3 (export 2004) 3.1 (internal consumption 2004)
Children's toys (i.e. finger paint, modeling clay, and sticky toys)	0.27%			0.3
Artificial Blood	0.2% MP	500 units (2005)		<1kg
Kitchen Rolls (made of virgin fibre)	2.9-3.1 mg/kg PP	18%	13 kg/year per person	37-40 kg
Industry				0.09-3.3

Product Safety Guide (over 14835 products), it was found that methyl paraben was present in 40% of personal care products, ethyl in 9%, propyl in 33%, butyl in 10%, and isobutyl in 10%. Personal care products containing parabens are considered to be stable for a period of 12 months after opened. For pharmaceuticals, liquid solutions generally have a shorter shelf-life than tablets. Digested pharmaceuticals are known to be excreted through human urine as either the paraben itself or the metabolite 4-hydroxybenzoic acid [114]. Eriksson et. al. estimated that 105-865 kg (digested waste from human urine), 44-104 kg (digested wastes from dog urine), and 3.4 tonnes (from garbage containing residual paraben products) will end up in solid waste. Using the assumptions that the daily consumption of water is 127 L/person with the estimated usage of 74% for grey wastewaters and 37% for personal hygiene, they also estimated 0.8-2.6 tonnes from bathroom grey wastewater-wastewater without toilets and 3.7-5.4 tonnes from point source emissions from industry to enter the wastewater stream [114].

Other studies have analyzed cosmetic products in the ranges of 35-977 mg/kg for methyl paraben, 14-735 mg/kg for ethyl paraben, 41-209 mg/kg for propyl paraben, and 300-466 mg/kg for butyl paraben [115]. Claver et. al. tested cleaning mousse and cleaning towels when developing new analytical methods and found concentrations of parabens in the range of 0.14-0.73 mg/g [105]. Wash-off cosmetic products were also tested and the results discovered Avon products with methyl paraben concentrations of approximately 1040 μg/g, Adidas products with propyl paraben at concentrations of 334 μg/g, and Clean & Clear products with methyl, ethyl, propyl and butyl parabens in the range of 114-317 μg/g [112].

EDCs have been detected in indoor air and house dust. A study performed in the United States found 89 identified EDCs in 120 sampled homes, with chemicals from classes of plasticizers, emulsifiers, disinfectants, adhesives, pesticides, personal care products and flame

retardants [58]. From the samples, methyl, ethyl and butyl parabens were present, with methyl paraben being found above detection limits in 67% of the air samples and 90% of the dust samples [58].

Cosmetic products get washed off and end up in WWTPs. In a Spanish test of three sewage sludge samples, methyl, propyl and benzyl parabens were found at concentrations between 5 and 202 μg/kg [116]. Due to the universal use of parabens in personal care products, especially those used for bathing, exposure is complicated by the development of by-products. Under experimental conditions, parabens will decay by pseudo-first-order-kinetics to one of two by-products (relative to each parent paraben compound) involving chlorination of the aromatic ring associated with a carbon in the *ortho*-positions relative to the hydroxyl group [117]. These Spanish researchers detected the formation of three new halogenated transformation products (for each parent paraben) when parabens were exposed to chlorinated tap water, making a total of five by-products for each parent paraben. They were identified as bromo- and bromochloro-parabens, because of the traces of bromine also found in tap water [117]. According to the study, this formation takes place within minutes, allowing dermal and possibly ingestion routes of exposure to be realistic while bathing. Formation of brominated by-products occurred when bromine was present in the tap water in only minimal amounts. This research group also found the presence of di-chlorinated forms of methyl and propyl parabens in raw sewage for the first time [117]. These by-products could be instrumental in the process of determining any dangers of using parabens, possibly more so that the endocrine disrupting potential of the parent parabens themselves.

After being processed in WWTPs, parabens are not expected to be persistent in the environment, but are being found in detectable levels in surface water samples. In a 2009

Spanish study, methyl paraben was detected in tap and surface water at 0.040 ng/mL and 0.037 ng/mL, respectively. Ethyl, propyl, butyl and benzyl parabens were all below the limits of quantification or non-detectable [118]. In a 2010 study of the same area (Northwest Spain), all parabens were present in levels ranging from 0.8 – 105 ng/L, with methyl paraben at 54 ng/L, ethyl paraben at 29 ng/L, and derivations of propyl paraben as the high and low values [119]. These two studies imply that there may be some seasonal effects on the transport and fate of this family of chemicals. Methyl paraben is also being detected in Spanish tap water in concentrations of 17 ng/L [120].

1.5.4 Analytical Methods

Some methods have been established to determine the concentrations of parabens in environmental matrices [121]. Surface water, WWTP influent and effluent, indoor air, and house dusts are the established analytical matrices for determination of methyl, ethyl, propyl, butyl and benzyl parabens [121]. Detection limits reported for GC/MS and LC/MS/MS are below 10 ng/L (ppt) [121]. Table 5, Summary of Analytical Methods for Parabens, provides a summary of analytical methods and the associated detection limits.

1.5.5 *In Vitro* Studies

Parabens have been studied extensively *in vitro* to determine their endocrine disrupting potential. It has been proposed that the mechanism for the estrogenicity of parabens is inhibition of sulfotransferase (SULT) activity [122]. Sulfotransferases are responsible for the metabolism of estrogen in the liver, skin and other organs. By inhibiting the SULT function, estrogens are not

metabolized to their inactive forms, leading to prolonged exposure from chronic topical application of parabens [122]. Prusakiewicz et. al. discovered SULT activity of skin cytosol to be inhibited by parabens, with potency increasing by ester chain length. The metabolite,

Table 5. Summary of Analytical Methods for Parabens

Matrix	Analytes	Method	Detection Limits	Reference
Surface water; sewage influent and effluent	MP, EP, PP, BuP, BzP	Solid-phase microextraction fibre for GC-MS/MS	0.001 – 0.025 ng/mL	Canosa, et. al. 2006 [108]
Surface water; sewage influent and effluent	MP, EP, PP, BzP	Solid-phase extraction and ultra-high performance liquid chromatography-electrospray ionisation-tandem mass spectrometry	1-5 ng/L	Pedrouzo, et. al. 2009 [123]
Urine (humans)	MP, EP, PP, n-BuP, iso-BuP, BzP	Online Solid-phase extraction for HPLC-isotope dilution tandem mass spectrometry	0.2 ng/mL	Ye, et. al. 2006 [124]
Sewage sludge	MP, EP, PP, BzP	Pressurized liquid extraction and ultra HPLC-tandem mass spectrometry	8 μg/kg	Nieto, et. al. 2009 [116]
Wash-off cosmetic products and food	MP, EP, PP, BuP	HPLC with chemiluminescence	MP = 1.9e-9 g/mL EP = 2.7e-9 g/mL PP = 3.9e-9 g/mL BuP = 5.3e-9 g/mL	Zhang, et. al. 2005 [112]
Cosmetic products	MP, EP, PP, BuP	Supercritical fluid extraction with LC-MS	MP = 4.7 ng/g EP = 13.5 ng/g PP = 13.4 ng/g BuP = 19.3 ng/g	Lee, et. al. 2006 [115]
Cosmetic products	MP, EP, PP, BuP	LPLC with monolithic column and chemiluminescence	MP = 1.9e-8 M EP = 2.8e-8 M PP = 2.3e-8 M BuP =4.2e-8 M	Claver, et. al. 2009 [105]
Indoor Air	MP, EP, BuP	Extraction and GC/MS	MP = 1 ng/m^3 EP = 1 ng/m^3 BuP =4 ng/m^3	Rudel, et. al. 2003 [58]
Dust	MP, EP, PP, BuP	Extraction and GC/MS	MP = 0.3μg/g EP = 0.2μg/g BuP = 0.2μg/g	Rudel, et. al. 2003 [58]

4-hydroxybenzoic acid, did not inhibit SULT activity [122]. Butyl paraben exhibited the most potent inhibitory effects, achieving complete inhibition at 1 mM, while propyl paraben could only achieve 50% inhibition, and methyl and ethyl only inhibited to a minor extent [122]. When tested in liver cytosol, the results were the same for butyl paraben, but methyl, ethyl and propyl parabens all achieved 50% inhibition [122]. When tested in normal human epidermal keratinocytes, butyl paraben potency for SULT inhibition increased three-fold, indicating that this phenomenon may be more potent in a cellular system than shown *in vitro* [122]. The MMT cell proliferation assay utilized human embryonic kidney cells to determine the antiandrogenic properties of parabens. Me thyl, propyl and butyl parabens significantly inhibited the transcriptional activity of testosterone at a concentration of 10 μM, while 4-hydroxybenzoic acid did not [125].

Van Meeuwen et. al. investigated the effects of parabens and their metabolites on human breast cancer cells using a MCF-7 cell proliferation assay. Each test compound was analyzed multiple times in the concentration range of 1nM - 10 mM, and three mixed compounds (containing combinations of parabens) were tested for an additive effect as well [126]. Parabens did not show cytotoxic effects up to a level of 1 mM [126]. Full concentration-response curves (relative to 17β-estradiol) from the MCF-7 proliferation assay were produced for all parabens tested except methyl paraben and 4-methoxybenzoic acid (this included ethyl, propyl, butyl, benzyl, isopropyl and isobutyl parabens). EC_{50} values ranged from 0.5 – 10 μM [126]. Estrogen equivalency factors (EEF) were derived from the EC_{50}s and determined to be around 10^{-5} for butyl, benzyl, isopropyl and isobutyl parabens and 10^{-6} for ethyl and propyl parabens [126]. EEFs for methyl paraben and 4-methoxybenzoic acid were derived from their EC_5 and EC_{10} values to be more accurate, as they did not produce a full curve equivalent to 17β-estradiol. The

values produced from EC_5 for methyl paraben and 4-methoxybenzoic acid were $7x10^{-7}$ and $1x10^{-7}$, respectively [126]. Results of the paraben mixtures demonstrate there is no interaction between ethyl and propyl parabens, but that there is an additive effect between 17β-estradiol and different parabens [126]. The second part of this study investigated the effects of parabens on aromatase using human placental microsomes (from one placenta sample to maintain background control) with a tritium water release assay. Compounds were tested in triplicate at a range of 100 nM – 100 μM, based on literature findings. This study confirmed with statistically significant results that aromatase activity was inhibited up to 55% by parabens at levels as low as 10 μM [126]. Metabolites of parabens did not inhibit aromatase activity [126]. Inhibition of aromatase is considered an anti-estrogenic effect because it is responsible for the conversion of androgens into estrogens. Inhibition occurs within one magnitude of the determined magnified estrogenic effects, so it is likely to see that these mechanisms are counteracting each other [126]. Van Meeuwen et. al. concluded that because effective concentrations determined in this study are orders of magnitude lower than actual concentrations found in human samples, it is unlikely that parabens would contribute greatly to the overall estrogenic burden, and that more research should focus on the additive effects of potential endocrine disrupting compounds [126].

Using the yeast screen assay, methyl, ethyl, propyl and butyl paraben were found to be estrogenic, but the main metabolite, 4-hydroxybenzoic acid, was not estrogenic [127]. The magnitude of response increased with alkyl group size, showing the following results compared to 17β-estradiol: methyl paraben was approximately 2,500,000-fold less; ethyl paraben was approximately 150,000-fold less; propyl paraben was approximately 30,000-fold less; and butyl paraben was approximately 10,000-fold less [127]. This means propyl paraben is equivalent in potency to 4-nonylphenol and butyl paraben was three-fold more potent [127]. To prove that this

mechanism was through the estrogen receptor, antiestrogenic 4-hydroxytamoxifen was used as an inhibitor, which effectively inhibited all parabens that had responded in a dose dependant manner [127].

1.5.6 *In Vivo* Studies

Parabens have been studied in mammalian species. Methyl paraben is known to activate TRPA1 channels in mice and cause pain sensations at an EC_{50} value of 4.4 mM. This concentration is well within the allowable range of 0.1 - 0.8% w/w which corresponds to 6.6 – 52.6 mM [128]. TRPA1 channels are members of the transient receptor potential (TRP) super family, and this particular channel has attracted attention for its role in nociception, or the neural processes of detecting stimuli which may cause damage to tissue [128]. Jewell et. al. compared the hydrolysis of parabens in human skin to the skin of minipigs. Parabens are hydrolyzed in tissue by carboxylesterases to the metabolite 4-hydroxybenzoic acid, however studies had not previously been evaluated using the skin [104]. The results showed that parabens are similarly absorbed through human and minipig skin, with minipig skin having a higher ability to hydrolyze more lipophilic esters than human skin [104]. It was also suggested that parabens with small alcohol leaving groups and low lipophilicity (such as methyl paraben) would be better used for pharmaceuticals needing quick systemic circulation, while esters with larger leaving groups and high lipophilicity (such as benzyl paraben) would be more efficient for pharmaceuticals which target the skin, due to the ability to be retained on the skin [104]. Butyl paraben competes with ^{3}H-estradiol for binding to the estrogen receptor in the uteri of immature rats five orders of magnitude lower than diethylstilbestrol and approximately two orders of magnitude less than 4-nonylphenol when tested via a competitive binding assay [127]. Methyl and butyl paraben (the

least and most potent from previous *in vitro* studies) were given to rats by oral and subcutaneous routes. Oral exposure to parabens did not produce an increase in uterus weights of immature rats [127]. The subcutaneous route did not show any significant results for methyl paraben, but butyl paraben significantly increased uterus weights at doses between 400 and 800 mg/kg/day. A butyl paraben dose of 1200 mg/kg/day increased the uterus wet weights by 170% above controls [127]. The lowest butyl paraben dose producing significant results was 200 mg/kg/day [127].

1.5.7 Human Studies

Systemic uptake of parabens has been studied in humans. In 2007, Janjua et. al. demonstrated that butyl paraben could be systemically absorbed through human skin after whole-body topical application of 2 mg/cm^2 basic cream formulation (mean cream amount of 40 grams) made up of 2% w/w compound. This provides an average exposure of 10 mg/kg bw/day. Blood sample serum levels of butyl paraben were found to increase rapidly, peak at a mean (SEM) level 135 μg/L three hours after the application, drop to 18 μg/L after 24 hours, and not return to baseline during the one week long testing period. Although butyl paraben was present, it did have a short-term effect on reproductive and thyroid hormone levels. When testing levels of testosterone, follicle stimulating hormone, sex hormone-binding globulin and T3 no significant changes were found. Some significant variations were reported for inhibin B, TSH, FT4 and estradiol levels. However, the authors concluded that the results of this study were caused by chance [129]. Janjua et. al. performed the same study methodology one year later but tested urinary concentrations, rather than blood. The results determined that the majority of butyl paraben was excreted 8-12 hours after whole-body topical application with the mean urine concentration found to be 2596 $\pm$ 136 μg [130]. The mean recovery rate for unmetabolized butyl

paraben was 0.32% [130]. This is expected to represent a huge underestimation of the amount actually absorbed as the metabolic change to 4-hydroxybenzoic acid was not analyzed because it is not specific to butyl paraben [130]. In a study of 22 demographically diverse adult volunteers with no known occupational exposure, Ye et. al. found parabens in all urinary samples [124]. Concentrations were detected as high as 41.4 ng/mL for methyl paraben and 10.2 ng/mL for propyl paraben, with a detection limit of 2 ng/mL [124].

Premature infants in neonatal intensive care units are exposed to parabens. A study by Calafat et. al. found that paraben concentrations were higher in the infants than adult concentrations from previously reported studies, and appeared to have correlated concentrations suggesting that methyl and propyl paraben exposure for premature infants was through the same route [94].

Cosmetics have had a suspected association with cancerous tumors for a long time. In, 2003, Darbre published a plea for more research involving the use of underarm cosmetics and breast cancer. Darbre suspected an association between the two because of supporting evidence that breast cancer occurs disproportionately in the upper outer quadrant (both in male and female cases) where deodorant/antiperspirant is applied, and because of the vast amounts of chemicals used in deodorant/antiperspirants, which include metal salts, antimicrobial agents and preservatives, among others [131]. Darbre, et. al. reported parabens accumulated in human breast tumours [132]. Eighteen out of twenty tumour extracts showed positive results (mean value of 20.6 ng/g) with methyl paraben present at the highest levels [132]. Benzyl paraben was not detected. This study is highly controversial and was greatly criticized. Peer-reviewers have concerns with the method of sample collection: (1) there was no data on patient use of paraben containing personal care products or paraben containing cancer treatment products; (2) no

control tissues were utilized; and (3) parabens were found in the analytical blanks leading to suspicion of contaminated equipment, although, according to Darbre, this was corrected for during the analysis [132-134]. Regardless of the controversy, Darbre's study showed that parabens can at least be accumulated in breast cancer tumors. Darbre suggests that the *in vitro* estrogenic activity of parabens may be stimulating growth of estrogen-dependent human breast cancer cells, but does not theorize a specific mechanism [131].

1.5.8 Other Studies

Because of the massive influx of new chemicals available to the market every year, it is necessary to develop computer models to quickly screen chemicals for their endocrine disrupting potential. A Quantitative Structure – Activity Relationship (QSAR) model for androgen receptor antagonism has been developed with a sensitivity of 64%, a specificity of 84%, and a concordance of 76% [72]. The model was tested satisfactorily with 176103 chemicals; 47% were within the domain of the model, and of them, 8% were predicted to be AR antagonists, while methyl, ethyl, propyl and butyl parabens and 4-hydroxybenzoic acid tested negative [72]. Guadarrama et. al. developed a simplified theoretical model using computers and mathematics to simulate the estrogenic activities of parabens. Th eir results showed that methyl paraben is theorized to be the most active (of all parabens tested, namely *n*-butyl, benzyl and isobutyl paraben) with fragments of the estrogen receptor [135]. The claim is that methyl paraben is the most estrogenic and the best antibacterial agent among other parabens [135].

1.5.9 Regulatory Actions

The European Commission (EEC) is a college of commissioners comprised of a member from each European Country. Its primary purpose is to simplify and improve the regulatory environment by proposing legislation to the European Parliament and the Council of Ministers to adopt [136]. In 1999, the EEC published Directive 76/768 on cosmetic legislation. In Annex VI, the EEC placed restrictions on parabens for the use of preservatives: products are not to exceed 0.4% w/w for one ester and 0.8% for mixtures of esters. These concentrations are not applicable if the use of parabens in the product is for something other than as a preservative [137]. In 2005 and 2006, the EEC published three documents written by scientific committees that explain their position on the potential dangers of parabens. One of the 2005 documents specifically addressed whether or not there was sufficient information to support a link between parabens and breast cancer, and determined that there is a lack of evidence [138]. The other document published in 2005 is a safety evaluation of parabens, and it summarized that the current literature supports the following:

- Acute toxicity is only seen at high doses.
- There is no evidence of mutagenicity, carcinogenicity, genotoxicity, teratogenicity.
- Research on developmental and reproductive effects is inadequate.
- There is efficient hydrolysis of parabens to the 4-hydroxybenzoic acid metabolite.
- There is no accumulation of either the parent or metabolites in tissues.
- Parabens are able to bind to the estrogen receptor but potential estrogenic potency is 1000 to 1,000,000 times lower than the potency of 17β-estradiol or testosterone.
- There are no interactions, additive, or synergistic effects of parabens [139].

Dietary administration studies *in vivo* calculated a NOAEL for methyl and ethyl paraben at 1000 mg/kg bw/day and a Lowest-Observed-Adverse-Effect-Level (LOAEL) of 10 mg/kg bw/day for propyl paraben [139]. Toxicological data has led the EU Scientific Committee on Food to establish an Acceptable Daily Intake (ADI) for total parabens at 10 mg/kg bw/day [139]. The opinion that methyl and ethyl parabens can be safely used up to maximum concentrations of 0.4% w/w remains unchanged, but more data was requested for propyl, butyl and isobutyl parabens [139]. The 2006 document was an extensive to-date literature review supporting the opinion of their earlier documents [140]. In the United States, the FDA has limited the use of heptyl paraben to a maximum level of 12 ppm in fermented malt beverages and 20 ppm in noncarbonated soft drinks and fruit-based beverages [141]. No other paraben derivatives have restricted uses in the United States.

1.5.10 Problems with Research on Parabens

Research on the endocrine disruptive effects of parabens has been controversial. Darbre's significant finding of paraben accumulation in breast cancer tissue has ignited the need for research on parabens, but due to its controversial nature, it has been highly criticized [132-134]. Issues with method of sample collections, confounding effects of other natural and xenoestrogens with proven higher potencies, paraben contamination issues and lack of adequate controls have been reoccurring problems in the study of potential health effects caused by paraben exposure.

1.6 SUMMARY

It is known that BPA can cause endocrine disruption in mammals and aquatic animals, and its endocrine disrupting effects have been proven *in vitro*. It is not unreasonable to suspect that BPA is causing health problems in humans as well. With the large exposure to BPA, it is necessary to thoroughly understand the effects of this chemical on the human endocrine system, which is determined by understanding its chemical and physical properties through *in vivo* and *in vitro* studies.

Parabens are known to cause endocrine disruption *in vitro* through their estrogenic properties, however they are not suspected to be a significant problem in biological systems. Parabens may still be a public health concern because of the wide exposure to high concentrations and volumes of parabens, caused by applying paraben containing products directly onto skin and on food products as preservatives. Little is known at this time about the transport and fate of parabens in the environment and they have not been thoroughly studied in the United States.

2.0 ESTROGENICITY OF EXTRACTS FROM ALEWIFE (*ALOSA PSEUDOHARENGUS*) AND SHAD (*ALOSA SAPIDISSIMA*) CAPTURED IN THE GREATER PITTSBURGH AREA

The Greater Pittsburgh Area is famous for its three rivers: the Allegheny, Monongahela, and Ohio Rivers. These rivers have a long history of being polluted by decades of mine runoff and chemicals released by industrial sites such as steel mills. New problems, such as pollution from Endocrine Disrupting Compounds (EDCs) and xenoestrogens, have recently been discovered in this well known aquatic environment and are suspected to be caused by the failing sewer system. The Greater Pittsburgh Area has a very old sewer system with approximately 317 documented Combined Sewer Overflows (CSOs) and Sanitary Sewer Overflows (SSOs) [142]. During wet weather conditions, CSOs and SSOs are known to discharge over 16 billion gallons per year of untreated wastewater into the Pittsburgh area rivers [9]. Untreated wastewater pollutes local surface waters with infectious organisms and EDCs. Newer research interests have questioned the use of personal care products (i.e. hygiene products and pharmaceuticals) and their effects on the environment. Both sewage treatment plant effluents and untreated sewage exhibit strong estrogenic activity in both *in vitro* and *in vivo* studies [22, 143-146]. Research in the United Kingdom has shown correlations between estrogenic substances found in sewage effluents and feminization of wild fish [147]. While this new research initiative was being pursued, efforts

were employed to standardized methods to utilize MCF-7 breast cancer cell lines for the screening of estrogenic compounds [14].

In 2007, T he University of Pittsburgh's Center for Healthy Environments and Communities (CHEC), the School of Medicine and the Veterans Research Foundation of the Pittsburgh Veterans Healthcare System participated in a study to detect the estrogenic potential of extracts derived from flesh, fat and skin sampled from channel catfish using the E-Screen Assay described by Soto et. al [14]. In this study, extracts derived from fish captured in the Pittsburgh lock and dam pool were compared to fish caught from up-river areas on the Allegheny River that were less impacted by CSO outfalls. The study found that extracts derived from fish caught in areas with higher CSO densities had significantly higher estrogenic potential than those less impacted by sewage effluent [148, 149]. This finding led CHEC, in conjunction with the Allegheny River Stewardship Project (ARSP), to continue research away from the city, into a small town model.

2.1 OBJECTIVE

The objective of this study was to investigate the presence of estrogenic compounds in extracts derived from the flesh, fat and skin taken from alewife (*Alosa pseudoharengus*) and shad (*Alosa sapidissima*) captured in the Greater Pittsburgh Area. Fish were caught in the Allegheny River from locations near both Freeport and Ford City, Pennsylvania. Estrogenic potential of extracts was tested utilizing the E-Screen Assay with human breast cancer cell lines MCF-7, T47D and BT-20.

2.2 MATERIAL AND METHODS

2.2.1 Sampling Locations

Ford City, Pennsylvania is located in Armstrong County on the Allegheny River approximately 40 miles northeast of the city of Pittsburgh and approximately four miles south of Kittanning. Ford City has one permitted Wastewater Treatment Plant (WWTP) and three permitted CSOs, as determined by Pennsylvania Department of Environmental Protection (PA DEP) data file searches. Fish samples were taken near Ford City, downstream of the area's WWTP and CSO outfalls. The nearest CSO discharge is approximately 1.8 miles and the furthest is approximately 2.9 miles, both upstream from the sample point. The sample point is the confluence of the Crooked Creek with the Allegheny River.

Freeport, Pennsylvania is located in Armstrong County on the Allegheny River approximately 28 miles northeast of Pittsburgh. Freeport has one permitted WWTP and a total of five permitted CSOs, as determined by PA DEP data file searches. Two of the CSOs and the WWTP discharge into Buffalo Creek, less than 0.5 miles from where the creek flows into the Allegheny River. The other three CSOs discharge directly into the Allegheny River, with the nearest discharge approximately 0.6 miles up-river of the Freeport sample point. All of the Freeport discharges are within one mile of the sample point.

The Freeport sample location is in the vicinity of two more CSOs than the Ford City location and is in closer proximity to the outfalls as well. It was suspected that extracts from the fish captured in the Freeport area would have higher estrogenicity levels than the ones from Ford City because of the higher number of CSOs in the Freeport area and because the sampling

location at Freeport is in closer vicinity to CSOs and WWTP effluent discharges than the Ford City sample location.

Figure 8, Map of Freeport and Ford City Fish Sample Locations with WWTP and CSO Outfalls, shows the two locations of fish sampling, the relative locations of WWTPs and CSO overflows and barriers of fish movement offered by lock and dam systems. Additionally, although fish are free to generally move within a specific lock and dam system, the fish sampled from these two locations are independent of one another. T wo lock and dam facilities exist along the Allegheny River between the sample locations making it very unlikely that the fish would travel across both dams to reach the other location [150].

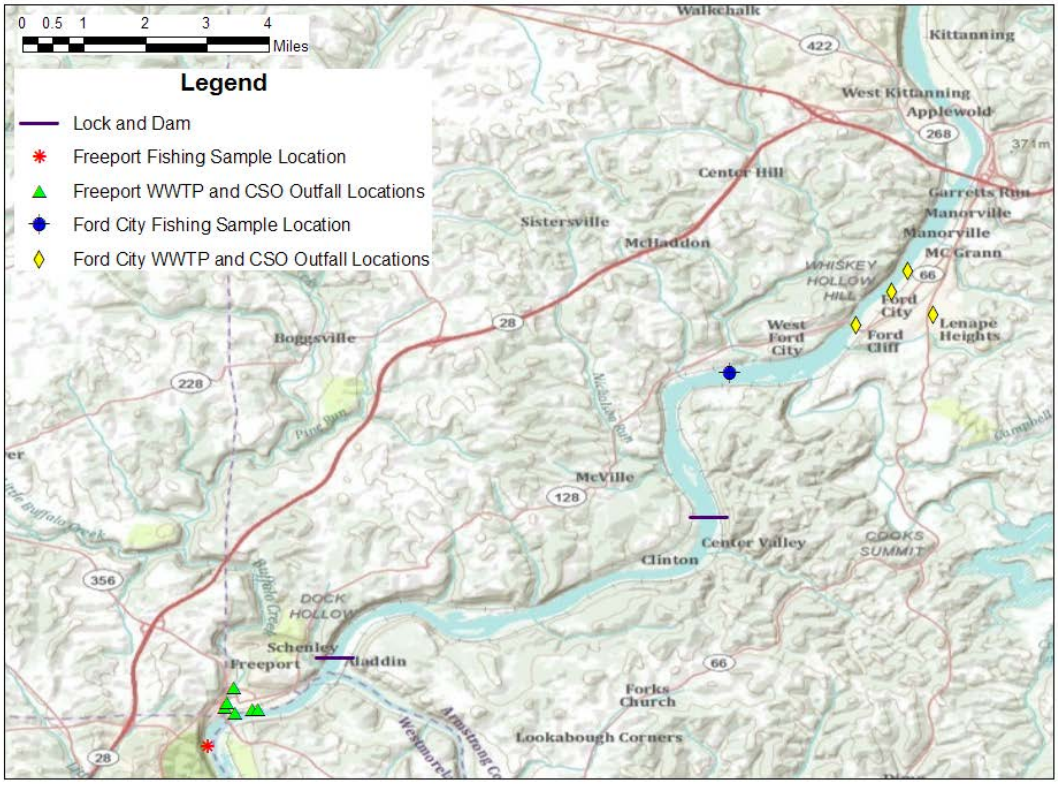


Figure 8. Map of Freeport and Ford City Fish Sample Locations with WWTP and CSO Outfalls

2.2.2 Sampling Methods

Fish were caught using rod and reel method by anglers on the shore of the river as well as boats provided by the research team of the ARSP and volunteers. All regulations of the Pennsylvania Fish and Boat Commission were followed, including ensuring that all anglers connected with this project held valid fishing licenses and that the ARSP obtained necessary scientific collection permits. Sample collection information consisting of river location, date and time of catch, GPS coordinates of catch, sampler's name and initial categorization of the fish species were recorded for each fish. The fish were then euthanized by pithing (protocol #0711563); the methods of capture and euthanasia have been approved by the Institutional Animal Care And Use Committee (IACUC) of the University of Pittsburgh. Each fish was then immediately packed on ice and transported to the laboratory for dissection.

Prior to dissection, each fish was assigned an identification number based on the date of catch, species and sample location. The gender and weight of the fish were recorded, and digital pictures were taken prior to dissection. The flesh/fat sample from each fish was standardized at approximately 200 grams. The multiple organs were removed and archived for future research. The organs and fillets were placed on dry ice until delivered to the laboratory where they were stored at $-80^{O}C$.

2.2.3 Composite Preparation

This study utilized a screening method of composites rather than individual fish samples due to cost-benefit evaluation between statistical power and analytical costs. Fish were composited according to sample location, gender and weight range. Each composite was comprised of two or

three fish. The fillet pieces used to construct each composite were allowed to thaw on ice and when pliable, each fillet was roughly minced using scissors. These explants were frozen in liquid nitrogen and pulverized into a fine powder using a cyropulvizer (Biospec Products Inc). The powder was collected into glass vials (Fisher Scientific) and stored under nitrogen at -80^{O}C.

2.2.4 Extraction of Fish Flesh

A one gram (±5%) sample of the composite powder was homogenized in normal saline using a Polytron homogenizer (Brinkmann Instruments) and extracted using a chloroform/methanol (9:1) extraction process. The resulting organic phase was evaporated using nitrogen. The remaining residue was stored under nitrogen at -20^{O}C in 15 x 45 mm threaded glass vials with rubber lined caps (Gerresheimer).

2.2.5 Cell Culture

The human breast cancer cell lines MCF-7 (ERα-positive), T47D (ERβ-positive) and BT-20 (ER-negative) were obtained from the American Type Cell Collection. These cell lines were maintained in a humidified 37^{O}C, 5% CO_2 incubator. The cells were grown in T-75 vented flasks (Greiner Bio-One). The growth medium consisted of RPMI 1640 with L-glutamine (Mediatech), supplemented with 10% Fetal Bovine Serum (FBS; Atlanta Biologicals), penicillin (50 units/mL; Invitrogen) and streptomycin (50 μg/mL; Invitrogen). Cell lines were passed once a week, and the growth medium was changed twice a week.

2.2.6 E-Screen Assay

The E-Screen Assay was performed as previously described in the literature [14, 151]. Seventy-two hours prior to performing the assay, a T-75 flask of each cell line had its growth medium removed, was rinsed with phenol-red free Hank's Balanced Salt Solution (HBSS; Mediatech), and the growth medium replaced with steroid-free medium consisting of RPMI 1640 with L-glutamine and without phenol red (Invitrogen), supplemented with 5% charcoal-dextran stripped FBS (Gemini Bio-Products), penicillin (50 units/mL) and streptomycin (50 μg/mL). After incubating for 72 hours, the steroid-free medium was removed and the monolayer rinsed with HBSS. Two mL of trypsin (0.25% trypsin, 2.21 nM EDTA in HBSS without sodium bicarbonate, calcium, and magnesium; Invitrogen) was added to the flask to detach the cells. The cells were resuspended in steroid-free medium and removed from the flask. The cell number was determined using a hemocytometer and the concentration of the cell suspension was adjusted to 50,000 cells per mL.

The E-Screen Assay was performed using 96-well plates (Greiner Bio-One). Each plate consisted of treated (steroid-free medium, cells, and fish extract), background control (steroid-free medium, fish extract and no cells), negative control (steroid-free medium, cells, and no fish extract) and positive control (steroid-free medium, cells, no fish extract, and 1 nM 17β-estradiol [Steraloids]) wells. One hundred μL of cell suspension was added to each treatment, negative control, and positive control well. The plates were returned to the incubator, and the cells allowed to adhere to the plate overnight.

The next morning, the composite fish extract residues to be tested were resuspended in one mL of ethanol:glycerol (70:30), kept at room temperature and shielded from light. The

resuspended residue was vortexed for one minute to ensure uniform distribution of fish extract. This suspension was used to make the stock solution of each fish extract. The stock solution was filtered using a 0.2 micron filter (Costar) to remove bacterial contamination. This stock solution was used to make eight dilutions of fish extract, with final concentrations of 1/4000, 1/3000, 1/2000, 1/1500, 1/1000, 1/500, 1/200, and 1/100 of composite extract diluted in steroid-free medium.

The plates were treated with 100 μL of eight dilutions of fish extract in the absence and presence of 1 nM estradiol (E2) and 10 nM hydroxytamoxifen. Each analysis was performed on five individual wells. The plates were returned to the incubator and allowed to incubate for 72 hours.

At the end of this period, the cell density per well was estimated following the procedure described by Soto et. al. [14, 151]. The medium was removed and the cells were fixed for one hour using a 10% trichloroacetic acid solution. The plate was then rinsed five times using tap water. The cells were stained using 0.4% w/v Sulforhodamine (SRB) in 1% acetic acid. After 30 minutes, the plates were rinsed with 1% acetic acid to remove unbound SRB and allowed to air dry. The protein-bound dye was solubilized with 10 mM Tris base per well. The plate was placed on a gyratory shaker for 10 minutes to homogenize the dye solution. The absorbance of each well was determined at a wave length of 564 nm using a Synergy HT plate reader (BioTek) with KC4 software (version 3.0) installed.

2.2.7 Proliferation Index (PI) and Estrogen Response Profile (ERP)

Raw absorbance values were collected for each well. These values converted to z-scores used to identify outliers. A z-score greater than 1.96 was considered to be an outlier and the well was

excluded from further analysis. The mean absorbance of the background control wells was calculated for each column and subtracted from the absorbance value of each well in that column. These background corrected absorbance values were used to calculate the Proliferation Index (PI) for each well. The PI is calculated according to the following equation:

PI = (background-corrected absorbance value of each well) / (mean absorbance of the negative control wells)

Where:

- background-corrected absorbance value of each well = absorbance value of the background control (steroid-free medium, fish extract and no cells) subtracted from the raw absorbance value of the treated well
- mean absorbance of the negative control wells = average of the raw absorbance values for the negative controls (steroid-free medium, cells, and no fish extract)

The PI is used to evaluate the data of each composite compared to its estradiol control. The PI for the negative control wells is not an assigned value, but was calculated in the same manner as the other conditions of the experiment. Once the PIs were calculated for each well, the mean and 95% Confidence Interval (CI) was calculated for each fish extract dilution, negative control and positive control. These data were plotted to compare their dose response curves against known responses of estradiol (positive estrogen responsive) and hydroxytamoxifen (anti-estrogen responsive) added extracts. Each analysis was graphed for the composite, the composite in combination with estradiol and the composite in combination with hydroxytamoxifen over the range of the eight dilutions for the MCF-7, T47D and BT-20 cell lines. In order to determine the receptor response, estradiol and hydroxytamoxifen were added to the extracts and thus compared

to expected estrogen and anti-estrogen receptor responses. BT-20 cells are ER-negative lines, therefore positive results would indicate that proliferation is occurring via an unknown mechanism that is not utilizing the estrogen receptor. The MCF-7 cell line is primarily ERα-positive and T47D are primarily ERβ-positive. Studying how the same sample reacts under all three conditions may give an indication to the type of mechanism causing any proliferation that may be observed in these experiments.

The Estrogen Response Profile (ERP) plots the mean and 95% CIs of the negative and positive controls, as well as the eight dilutions of fish extract along the X-axis. The Y-axis is a numeric scale of PI values. The 95% CIs of the negative and positive controls are used to define five response regions of the ERP. The 95% CI of the negative control establishes the non-estrogenic response range of the ERP. The 95% CI of the positive control defines the region of estrogenic response. The weak and moderate estrogenic responses are respectively defined as the midrange between the upper limit of the 95% CI of the negative control and the lower limit of the positive (E2) control range. A response above the upper limit of the 95% CI of the positive control represents a strong estrogenic response.

2.2.8 Estrogenicity Index (EI)

The data was analyzed by determining the Estrogenicity Index of each composite. The Estrogenicity Index (EI) is defined as the calculated value based on the proliferation of exposed cells after normalizing to the cell plate positive and negative control wells. The Proliferation Index is converted into the Estrogenicity Index according to following equation:

EI = (PI - mean of negative control wells) / (mean of positive control wells – the mean of negative control wells)

Where:

- PI (Proliferation Index) = (background-corrected absorbance value of each well) / (mean absorbance of the negative control wells)
- negative control wells = a blank matrix comprised of steroid-free medium, cells and no fish extract or any hormones
- positive control wells = a quality control comprised of steroid-free medium, cells, no fish extract, and 1nM 17β-estradiol

2.2.9 Statistical Analyses

This study design allowed for the creation of 440 analytical data points (5 runs * 8 dilutions * 11 composites = 440) which required a repeated measures statistical model. A Subject Specific Random Effects Model, a mixed model with fixed and random effects, was utilized to analyze the data. T he fish are the random effect because they were randomly sampled, and the composites are fixed effects because they were assigned based on the fixed effects gender, weight range and location. T his model does not assume normality of the sample population. The models were created in Stata using the xtreg command. The Estrogenicity Index (for both MCF-7 and T47D) was modeled as a function of location, gender or weight class, using the Subject Specific Random Effects Model. Spearman Rank Correlation testing was performed to determine the relationship between the MCF-7 and T47D analyses.

2.3 RESULTS AND DISCUSSION

2.3.1 Proliferation Index

Table 6, MCF-7 Proliferation Index Data Summary (Mean with SD and Range), displays the results of the Mean Proliferation Index with standard deviations and ranges for all eleven composites in each dilution range for the MCF-7 analysis, including extracts with estradiol or hydroxytamoxifen present. Table 7, T47D Proliferation Index Data Summary (Mean with SD and Range) displays the same results for the T47D analysis. BT-20 data is not shown because there was no effect.

2.3.2 Descriptive Results from ERP

Descriptive results for the three cell lines are described in Table 8, Summary of the Estrogen Response Profile (ERP) for Cell Proliferation Assays by Composite. Because BT-20 cell lines do not have estrogen receptors, the negative results seen in these cells give strength to the concept that all responses seen in the MCF-7 and T47D cell lines are due to the presence of estrogen receptors. Seven out of eleven of the MCF-7 analyses showed a weak estrogenic response. The other four showed no estrogenic response. There do not appear to be any obvious associations for fish weight, gender or location in these responses. Seven out of eleven of the T47D analyses showed a weak estrogenic response. Two out of eleven showed an estrogenic response and two out of eleven showed no estrogenic response. The two composites which showed no estrogenic response in the T47D analysis were both from Freeport (Composites 3 and 11). The two composites which showed estrogenic response in the T47D analysis were both

Table 6. MCF-7 Proliferation Index Data Summary (Mean with SD and Range)

	Neg Ctl	Pos Ctr	Tam	Tam + E2	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
	1.00 ± 0.08	2.03 ± 0.22	-	-	1.16 ± 0.05	1.14 ± 0.04	1.06 ± 0.06	1.02 ± 0.08	1.09 ± 0.10	1.15 ± 0.05	1.11 ± 0.09	1.24 ± 0.11
Composite 1	0.90 - 1.16	1.85 - 2.58	-	-	1.12 - 1.25	1.10 - 1.19	0.94 - 1.10	0.92 - 1.14	1.00 - 1.25	1.08 - 1.22	1.05 - 1.26	1.10 - 1.39
	1.00 ± 0.05	2.21 ± 0.17	-	-	2.14 ± 0.13	2.05 ± 0.18	2.23 ± 0.19	2.46 ± 0.36	2.26 ± 0.11	2.27 ± 0.09	2.01 ± 0.11	1.82 ± 0.17
Composite 1 with Estradiol (E2)	0.94 - 1.08	1.86 - 2.47	-	-	1.96 - 2.26	1.82 - 2.31	2.02 - 2.48	2.05 - 2.88	2.17 - 2.44	2.13 - 2.37	1.92 - 2.17	1.65 - 2.08
	1.00 ± 0.06	2.17 ± 0.14	0.97 ± 0.03	1.93 ± 0.05	-	-	1.05 ± 0.07	1.11 ± 0.06	1.11 ± 0.08	1.13 ± 0.02	1.10 ± 0.11	1.04 ± 0.05
Composite 1 with Hydroxytamoxifen (Tam)	0.89 - 1.11	2.00 - 2.38	0.93 - 1.00	1.86 - 1.99	-	-	0.97 - 1.13	1.06 - 1.21	1.01 - 1.23	1.11 - 1.16	1.00 - 1.29	0.97 - 1.10
	1.00 ± 0.04	2.84 ± 0.28	-	-	1.21 ± 0.14	1.05 ± 0.06	1.15 ± 0.08	1.22 ± 0.08	1.19 ± 0.10	1.20 ± 0.09	1.33 ± 0.09	1.42 ± 0.07
Composite 2	0.94 - 1.06	2.43 - 3.39	-	-	1.12 - 1.45	0.95 - 1.09	1.06 - 1.28	1.12 - 1.31	1.07 - 1.29	1.08 - 1.30	1.26 - 1.48	1.32 - 1.47
	1.00 ± 0.13	2.52 ± 0.27	-	-	2.72 ± 0.14	2.83 ± 0.14	2.72 ± 0.12	2.77 ± 0.09	2.74 ± 0.10	2.52 ± 0.11	2.48 ± 0.09	2.32 ± 0.11
Composite 2 with Estradiol	0.71 - 1.19	2.22 - 3.07	-	-	2.50 - 2.91	2.70 - 2.98	2.62 - 2.91	2.66 - 2.89	2.62 - 2.84	2.38 - 2.64	2.36 - 2.60	2.16 - 2.42
	1.00 ± 0.06	2.39 ± 0.10	1.13 ± 0.08	2.17 ± 0.09	-	-	1.20 ± 0.08	1.23 ± 0.06	1.12 ± 0.04	1.17 ± 0.05	1.17 ± 0.08	1.13 ± 0.06
Composite 2 with Hydroxytamoxifen	0.91 - 1.10	2.19 - 2.52	1.07 - 1.27	2.09 - 2.32	-	-	1.09 - 1.30	1.16 - 1.31	1.08 - 1.18	1.10 - 1.24	1.03 - 1.22	1.05 -1.20
	1.00 ± 0.04	2.12 ± 0.10	-	-	1.11 ± 0.09	1.00 ± 0.09	0.99 ± 0.06	1.00 ± 0.04	0.99 ± 0.05	1.04 ± 0.05	1.06 ± 0.06	1.08 ± 0.05
Composite 3	0.95 - 1.07	1.98 - 2.32	-	-	0.99 - 1.24	0.93 - 1.14	0.94 - 1.09	0.95 - 1.05	0.92 -1.04	0.97 - 1.08	1.01 - 1.14	1.00 - 1.14
	1.00 ± 0.06	1.92 ± 0.08	-	-	1.91 ± 0.14	1.92 ± 0.09	1.89 ± 0.08	1.81 ± 0.06	1.85 ± 0.07	1.84 ± 0.09	1.74 ± 0.07	1.66 ± 0.05
Composite 3 with Estradiol	0.90 - 1.11	1.82 - 2.06	-	-	1.76 - 2.05	1.77 - 2.00	1.79 - 1.99	1.74 - 1.91	1.77 - 1.96	1.77 - 2.00	1.67 - 1.81	1.59 - 1.73
	1.00 ± 0.10	2.07 ± 0.09	1.04 ± 0.04	2.18 ± 0.07	-	-	1.04 ± 0.04	1.02 ± 0.06	1.05 ± 0.04	1.04 ± 0.07	0.95 ± 0.06	0.94 ± 0.04
Composite 3 with Hydroxytamoxifen	0.85 - 1.19	1.87 - 2.17	1.01 - 1.09	2.07 - 2.24	-	-	1.00 - 1.09	0.93 - 1.08	1.00 - 1.11	0.97 - 1.15	0.86 - 1.01	0.89 - 0.98
	1.00 ± 0.09	1.87 ± 0.09	-	-	1.15 ± 0.06	1.20 ± 0.07	1.20 ± 0.04	1.19 ± 0.08	1.34 ± 0.11	1.41 ± 0.09	1.41 ± 0.09	1.35 ± 0.06
Composite 4	0.91 - 1.19	1.75 - 2.03	-	-	1.06 - 1.21	1.13 - 1.27	1.13 - 1.24	1.10 - 1.29	1.19 - 1.43	1.30 - 1.52	1.30 - 1.55	1.26 - 1.43
	1.00 ± 0.05	2.07 ± 0.13	-	-	2.12 ± 0.13	2.11 ± 0.09	2.12 ± 0.13	2.08 ± 0.11	2.12 ± 0.14	2.06 ± 0.05	1.98 ± 0.07	1.74 ± 0.10
Composite 4 with Estradiol	0.93 - 1.09	1.85 - 2.28	-	-	1.97 - 2.33	1.99 - 2.21	2.02 - 2.34	1.98 - 2.21	1.96 - 2.29	2.01 - 2.13	1.87 - 2.05	1.67 - 1.91
	1.00 ± 0.08	2.10 ± 0.18	0.91 ± 0.03	1.79 ± 0.09	-	-	0.93 ± 0.04	1.09 ± 0.00	1.05 ± 0.05	1.05 ± 0.11	1.07 ± 0.08	1.02 ± 0.04
Composite 4 with Hydroxytamoxifen	0.92 - 1.17	1.70 - 2.28	0.88 - 0.96	1.72 - 1.95	-	-	0.86 - 0.98	1.09 - 1.10	0.99 - 1.12	0.90 - 1.19	0.96 - 1.18	0.97 - 1.08
	1.00 ± 0.07	2.11 ± 0.10	-	-	1.00 ± 0.08	1.04 ± 0.07	1.04 ± 0.07	0.98 ± 0.05	0.98 ± 0.02	1.01 ± 0.04	1.13 ± 0.03	1.15 ± 0.06
Composite 5	0.87 - 1.10	1.97 - 2.29	-	-	0.90 - 1.11	0.96 - 1.14	0.96 - 1.12	0.92 - 1.04	0.95 - 1.01	0.97 - 1.08	1.10 - 1.18	1.08 - 1.23
	1.00 ± 0.06	2.25 ± 0.18	-	-	2.09 ± 0.06	2.09 ± 0.19	2.03 ± 0.05	2.11 ± 0.19	2.09 ± 0.13	2.02 ± 0.12	1.98 ± 0.14	2.07 ± 0.19
Composite 5 with Estradiol	0.86 - 1.11	2.02 - 2.60	-	-	2.01 - 2.17	1.78 - 2.30	1.97 - 2.09	1.86 - 2.31	1.88 - 2.20	1.81 - 2.10	1.83 - 2.17	1.75 - 2.24
	1.00 ± 0.05	2.27 ± 0.23	1.00 ± 0.03	1.80 ± 0.06	-	-	0.99 ± 0.06	1.07 ± 0.08	1.05 ± 0.06	1.04 ± 0.07	0.96 ± 0.07	1.02 ± 0.05
Composite 5 with Hydroxytamoxifen	0.91 - 1.06	1.94 - 2.65	0.97 - 1.05	1.74 - 1.91	-	-	0.94 - 1.07	0.98 - 1.17	1.00 - 1.14	0.94 - 1.12	0.88 - 1.04	0.95 - 1.07
	1.00 ± 0.04	2.04 ± 0.10	-	-	1.00 ± 0.02	0.99 ± 0.05	1.13 ± 0.03	1.11 ± 0.03	1.21 ± 0.06	1.33 ± 0.07	1.47 ± 0.04	1.40 ± 0.04
Composite 6	0.95 - 1.05	1.81 - 2.16	-	-	0.97 - 1.03	0.91 - 1.04	1.10 - 1.17	1.06 - 1.15	1.13 - 1.28	1.24 - 1.41	1.41 - 1.51	1.34 - 1.44
	1.00 ± 0.06	1.89 ± 0.11	-	-	1.90 ± 0.08	2.00 ± 0.08	1.83 ± 0.15	1.75 ± 0.06	1.97 ± 0.12	1.87 ± 0.06	1.73 ± 0.08	1.64 ± 0.15
Composite 6 with Estradiol	0.92 - 1.07	1.73 - 2.08	-	-	1.80 - 1.99	1.91 - 2.09	1.60 - 2.00	1.71 - 1.86	1.84 - 2.16	1.79 - 1.92	1.64 - 1.85	1.44 - 1.84
	1.00 ± 0.06	1.98 ± 0.16	1.08 ± 0.07	1.82 ± 0.15	-	-	1.07 ± 0.06	1.04 ± 0.07	1.06 ± 0.06	1.10 ± 0.07	1.03 ± 0.03	0.96 ± 0.10
Composite 6 with Hydroxytamoxifen	0.93 - 1.11	1.78 - 2.17	1.01 - 1.16	1.67 - 2.07	-	-	1.01 - 1.17	0.97 - 1.15	0.96 - 1.12	0.99 - 1.16	1.00 - 1.07	0.82 - 1.09
	1.00 ± 0.14	2.75 ± 0.17	-	-	1.36 ± 0.12	1.12 ± 0.07	1.21 ± 0.09	1.20 ± 0.04	1.36 ± 0.12	1.43 ± 0.02	1.62 ± 0.03	1.61 ± 0.07
Composite 7	0.80 - 1.18	2.51 - 2.98	-	-	1.18 - 1.47	1.04 - 1.22	1.12 - 1.32	1.13 - 1.24	1.22 - 1.50	1.40 - 1.46	1.59 - 1.65	1.55 - 1.72
	1.00 ± 0.07	2.73 ± 0.17	-	-	2.87 ± 0.14	2.90 ± 0.19	2.80 ± 0.27	2.94 ± 0.11	2.91 ± 0.13	2.87 ± 0.04	2.80 ± 0.13	2.48 ± 0.20
Composite 7 with Estradiol	0.90 - 1.12	2.43 - 2.98	-	-	2.70 - 3.02	2.75 - 3.22	2.61 - 3.26	2.82 - 3.12	2.73 - 3.02	2.84 - 2.92	2.71 - 3.01	2.21 - 2.69
	1.00 ± 0.08	2.89 ± 0.18	1.11 ± 0.13	2.62 ± 0.27	-	-	1.13 ± 0.07	1.22 ± 0.05	1.27 ± 0.03	1.32 ± 0.10	1.31 ± 0.04	1.18 ± 0.09
Composite 7 with Hydroxytamoxifen	0.83 - 1.13	2.66 - 3.30	0.96 - 1.26	2.37 - 3.03	-	-	1.06 - 1.25	1.17 - 1.30	1.24 - 1.31	1.20 - 1.44	1.25 - 1.36	1.06 - 1.28
	1.00 ± 0.07	1.79 ± 0.13	-	-	1.10 ± 0.06	1.12 ± 0.04	1.11 ± 0.08	1.03 ± 0.06	1.03 ± 0.03	1.09 ± 0.03	1.13 ± 0.06	1.14 ± 0.08
Composite 8	0.91 - 1.15	1.54 - 1.99	-	-	1.02 - 1.16	1.07 - 1.18	1.03 - 1.23	0.96 - 1.10	0.99 - 1.06	1.05 - 1.13	1.05 - 1.20	1.07 - 1.25
	1.00 ± 0.05	2.15 ± 0.06	-	-	2.03 ± 0.13	2.01 ± 0.10	2.11 ± 0.12	2.11 ± 0.08	2.23 ± 0.05	2.13 ± 0.13	2.14 ± 0.15	2.00 ± 0.03
Composite 8 with Estradiol	0.90 - 1.10	2.03 - 2.23	-	-	1.87 - 2.22	1.91 - 2.18	2.01 - 2.24	1.99 - 2.19	2.16 - 2.28	1.98 - 2.27	1.96 - 2.34	0.96 - 2.02
	1.00 ± 0.12	1.91 ± 0.07	0.84 ± 0.09	1.48 ± 0.08	-	-	0.90 ± 0.03	0.93 ± 0.08	0.89 ± 0.03	1.02 ± 0.10	0.94 ± 0.06	0.98 ± 0.05
Composite 8 with Hydroxytamoxifen	0.88 - 1.28	1.77 - 1.98	0.72 - 0.96	1.35 - 1.56	-	-	0.86 - 0.93	0.84 - 1.02	0.87 - 0.94	0.90 - 1.15	0.83 - 0.98	0.93 - 1.04
	1.00 ± 0.04	1.90 ± 0.14	-	-	1.16 ± 0.07	1.09 ± 0.07	0.99 ± 0.05	1.06 ± 0.03	1.03 ± 0.06	1.07 ± 0.05	1.18 ± 0.04	1.18 ± 0.05
Composite 9	0.94 - 1.07	1.65 - 2.06	-	-	1.09 - 1.24	1.02 - 1.19	0.93 - 1.04	1.01 - 1.09	0.95 - 1.09	1.00 - 1.14	1.13 - 1.24	1.12 - 1.25
	1.00 ± 0.05	2.16 ± 0.19	-	-	2.04 ± 0.14	2.01 ± 0.08	2.12 ± 0.11	2.08 ± 0.08	2.10 ± 0.14	2.05 ± 0.16	1.87 ± 0.07	1.89 ± 0.10
Composite 9 with Estradiol	0.91 - 1.06	1.91 - 2.45	-	-	1.91 - 2.22	1.92 - 2.11	2.00 - 2.25	1.99 - 2.17	1.96 - 2.29	1.92 - 2.24	1.81 - 1.98	1.81 - 2.03
	1.00 ± 0.05	2.10 ± 0.10	1.05 ± 0.04	1.88 ± 0.09	-	-	1.11 ± 0.09	1.07 ± 0.04	1.04 ± 0.02	1.04 ± 0.07	0.99 ± 0.05	0.95 ± 0.03
Composite 9 with Hydroxytamoxifen	0.92 - 1.07	1.96 - 2.26	1.00 - 1.12	1.76 - 2.01	-	-	0.99 - 1.22	1.02 - 1.11	1.03 - 1.06	0.97 - 1.10	0.93 - 1.05	0.93 - 1.00
	1.00 ± 0.06	2.30 ± 0.23	-	-	1.29 ± 0.06	1.16 ± 0.02	1.03 ± 0.07	1.03 ± 0.08	1.07 ± 0.04	1.01 ± 0.06	1.16 ± 0.07	1.15 ± 0.05
Composite 10	0.89 - 1.07	2.04 - 2.67	-	-	1.20 - 1.35	1.12 - 1.18	0.95 - 1.12	0.91 - 1.12	1.02 - 1.12	0.95 - 1.08	1.06 - 1.26	1.09 - 1.21
	1.00 ± 0.03	2.19 ± 0.28	-	-	2.17 ± 0.10	2.01 ± 0.13	2.26 ± 0.11	2.22 ± 0.11	2.17 ± 0.09	2.13 ± 0.09	2.05 ± 0.12	1.93 ± 0.17
Composite 10 with Estradiol	0.94 - 1.04	1.87 - 2.74	-	-	2.02 - 2.25	1.85 - 2.19	2.16 - 2.45	2.11 - 2.40	2.09 - 2.32	2.02 - 2.27	1.92 - 2.20	1.70 - 2.11
	1.00 ± 0.07	2.09 ± 0.13	0.96 ± 0.04	1.98 ± 0.09	-	-	1.03 ± 0.05	1.05 ± 0.05	1.08 ± 0.11	1.13 ± 0.05	1.02 ± 0.04	1.01 ± 0.03
Composite 10 with Hydroxytamoxifen	0.90 - 1.12	1.92 - 2.26	0.91 - 1.01	1.88 - 2.07	-	-	0.97 - 1.08	0.98 - 1.09	0.99 - 1.25	1.06 - 1.20	0.95 - 1.05	0.96 - 1.04
	1.00 ± 0.06	1.96 ± 0.10	-	-	1.10 ± 0.07	1.05 ± 0.06	1.02 ± 0.06	1.10 ± 0.05	1.02 ± 0.07	1.02 ± 0.04	1.01 ± 0.05	1.00 ± 0.06
Composite 11	0.94 - 1.09	1.86 - 2.17	-	-	1.03 - 1.19	1.00 - 1.13	0.93 - 1.10	0.96 - 1.07	0.94 - 1.11	0.98 - 1.06	0.97 - 1.08	0.95 - 1.09
	1.00 ± 0.08	2.14 ± 0.12	-	-	2.23 ± 0.10	2.20 ± 0.05	2.12 ± 0.04	2.10 ± 0.08	2.06 ± 0.12	2.09 ± 0.08	1.99 ± 0.09	1.78 ± 0.09
Composite 11 with Estradiol	0.90 - 1.16	2.05 - 2.45	-	-	2.07 - 2.33	2.14 - 2.28	2.09 - 2.19	2.00 - 2.16	1.91 - 2.18	1.99 - 2.17	1.88 - 2.10	1.68 - 1.89
	1.00 ± 0.03	1.92 ± 0.12	0.91 ± 0.08	1.87 ± 0.14	-	-	0.90 ± 0.03	0.90 ± 0.05	0.87 ± 0.04	0.89 ± 0.02	0.89 ± 0.05	0.85 ± 0.03
Composite 11 with Hydroxytamoxifen	0.94 - 1.04	1.74 - 2.09	0.84 - 1.01	1.71 - 2.03	-	-	0.86 - 0.94	0.83 - 0.96	0.82 - 0.93	0.87 - 0.92	0.83 - 0.96	0.82 - 0.89

Table 7. T47D Proliferation Index Data Summary (Mean with SD and Range)

	Neg Ctl	Pos Ctr	Tam	Tam + E2	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
	1.00 ± 0.07	1.77 ± 0.09	-	-	1.09 ± 0.08	1.07 ± 0.07	1.05 ± 0.04	1.08 ± 0.03	1.15 ± 0.06	1.17 ± 0.04	1.26 ± 0.08	1.39 ± 0.06
Composite 1	0.91 - 1.11	1.61 - 1.87	-	-	0.99 - 1.18	0.98 - 1.14	1.01 - 1.11	1.05 - 1.13	1.08 - 1.23	1.13 - 1.24	1.18 - 1.36	1.34 - 1.48
	1.00 ± 0.17	1.66 ± 0.31	-	-	2.13 ± 0.15	2.18 ± 0.08	2.03 ± 0.12	2.09 ± 0.06	2.04 ± 0.10	2.01 ± 0.13	1.59 ± 0.13	1.50 ± 0.08
Composite 1 with Estradiol	0.81 - 1.18	1.21 - 2.02	-	-	2.03 - 2.39	2.07 - 2.27	1.86 - 2.16	2.03 - 2.17	1.93 - 2.19	1.81 - 2.12	1.43 - 1.80	1.42 - 1.60
	1.00 ± 0.17	1.87 ± 0.23	1.43 ± 0.08	1.57 ± 0.10	-	-	1.16 ± 0.17	1.10 ± 0.08	1.01 ± 0.09	1.02 ± 0.07	1.10 ± 0.04	1.19 ± 0.11
Composite 1 with Hydroxytamoxifen	0.83 - 1.27	1.55 - 2.20	1.28 - 1.48	1.46 - 1.67	-	-	0.93 - 1.36	1.04 - 1.24	0.95 - 1.15	0.92 - 1.08	1.05 - 1.15	1.00 - 1.30
	1.00 ± 0.12	1.69 ± 0.09	-	-	1.10 ± 0.06	0.96 ± 0.12	1.02 ± 0.07	1.07 ± 0.12	1.24 ± 0.09	1.16 ± 0.03	1.24 ± 0.05	1.32 ± 0.07
Composite 2	0.78 - 1.14	1.48 - 1.77	-	-	1.03 - 1.16	0.84 - 1.15	0.92 - 1.09	0.93 - 1.19	1.15 - 1.37	1.12 - 1.19	1.20 - 1.31	1.23 - 1.41
	1.00 ± 0.05	1.62 ± 0.08	-	-	1.70 ± 0.11	1.75 ± 0.08	1.63 ± 0.09	1.68 ± 0.11	1.71 ± 0.02	1.70 ± 0.11	1.85 ± 0.11	1.80 ± 0.06
Composite 2 with Estradiol	0.90 - 1.06	1.50 - 1.77	-	-	1.60 - 1.87	1.67 - 1.85	1.56 - 1.77	1.60 - 1.87	1.69 - 1.75	1.52 - 1.80	1.76 - 2.03	1.73 - 1.89
	1.00 ± 0.06	1.70 ± 0.17	1.15 ± 0.05	1.43 ± 0.05	-	-	1.13 ± 0.10	1.14 ± 0.05	1.20 ± 0.06	1.21 ± 0.05	1.20 ± 0.03	1.23 ± 0.04
Composite 2 with Hydroxytamoxifen	0.92 - 1.10	1.47 - 2.15	1.08 - 1.20	1.37 - 1.48	-	-	1.01 - 1.25	1.08 - 1.22	1.12 - 1.27	1.13 - 1.25	1.16 - 1.22	1.19 - 1.28
	1.00 ± 0.08	1.51 ± 0.09	-	-	1.16 ± 0.06	1.11 ± 0.04	1.09 ± 0.06	1.03 ± 0.05	1.03 ± 0.08	1.07 ± 0.04	1.13 ± 0.03	1.19 ± 0.09
Composite 3	0.90 - 1.10	1.37 - 1.64	-	-	1.06 - 1.22	1.07 - 1.18	1.02 - 1.17	0.97 - 1.09	0.98 - 1.17	1.02 - 1.12	1.09 - 1.18	1.08 - 1.32
	1.00 ± 0.11	1.46 ± 0.13	-	-	1.58 ± 0.08	1.61 ± 0.12	1.55 ± 0.17	1.51 ± 0.14	1.53 ± 0.08	1.60 ± 0.09	1.61 ± 0.09	1.61 ± 0.11
Composite 3 with Estradiol	0.86 - 1.23	1.27 - 1.70	-	-	1.47 - 1.66	1.46 - 1.77	1.36 - 1.81	1.36 - 1.72	1.43 - 1.65	1.52 - 1.74	1.54 - 1.76	1.52 - 1.78
	1.00 ± 0.11	1.56 ± 0.15	1.16 ± 0.05	1.52 ± 0.04	-	-	1.03 ± 0.05	1.09 ± 0.12	1.08 ± 0.14	1.10 ± 0.12	1.11 ± 0.06	1.19 ± 0.11
Composite 3 with Hydroxytamoxifen	0.82 - 1.20	1.39 - 1.92	1.09 - 1.20	1.48 - 1.57	-	-	0.97 - 1.11	1.00 - 1.30	0.96 - 1.30	0.98 - 1.27	1.03 - 1.20	1.09 - 1.33
	1.00 ± 0.06	1.61 ± 0.06	-	-	1.20 ± 0.11	1.23 ± 0.06	1.16 ± 0.05	1.15 ± 0.10	1.29 ± 0.11	1.41 ± 0.09	1.58 ± 0.08	1.61 ± 0.07
Composite 4	0.89 - 1.10	1.52 - 1.68	-	-	1.08 - 1.36	1.15 - 1.29	1.08 - 1.21	1.05 - 1.30	1.13 - 1.39	1.27 - 1.50	1.49 - 1.70	1.51 - 1.70
	1.00 ± 0.06	1.76 ± 0.14	-	-	1.67 ± 0.05	1.67 ± 0.02	1.90 ± 0.09	1.84 ± 0.15	2.00 ± 0.06	1.93 ± 0.09	2.08 ± 0.08	1.99 ± 0.09
Composite 4 with Estradiol	0.92 - 1.10	1.56 - 1.95	-	-	1.63 - 1.75	1.64 - 1.69	1.78 - 2.00	1.71 - 2.08	1.94 - 2.09	1.81 - 2.02	1.94 - 2.14	1.91 - 2.10
	1.00 ± 0.06	1.80 ± 0.10	1.17 ± 0.07	1.45 ± 0.05	-	-	1.16 ± 0.04	1.17 ± 0.05	1.28 ± 0.03	1.27 ± 0.06	1.29 ± 0.09	1.23 ± 0.04
Composite 4 with Hydroxytamoxifen	0.95 - 1.14	1.62 - 1.96	1.08 - 1.27	1.38 - 1.49	-	-	1.12 - 1.23	1.10 - 1.23	1.23 - 1.32	1.19 - 1.33	1.22 - 1.44	1.19 - 1.29
	1.00 ± 0.05	1.56 ± 0.07	-	-	1.19 ± 0.04	1.10 ± 0.08	1.06 ± 0.09	1.05 ± 0.05	1.14 ± 0.04	1.11 ± 0.09	1.18 ± 0.11	1.22 ± 0.11
Composite 5	0.91 - 1.08	1.46 - 1.72	-	-	1.16 - 1.25	1.00 - 1.19	1.00 - 1.21	0.97 - 1.08	1.08 - 1.18	0.99 - 1.19	1.09 - 1.36	1.15 - 1.42
	1.00 ± 0.09	1.54 ± 0.13	-	-	1.55 ± 0.04	1.57 ± 0.07	1.51 ± 0.09	1.57 ± 0.10	1.58 ± 0.15	1.64 ± 0.08	1.55 ± 0.10	1.49 ± 0.03
Composite 5 with Estradiol	0.85 - 1.14	1.33 - 1.72	-	-	1.50 - 1.62	1.48 - 1.66	1.42 - 1.66	1.41 - 1.64	1.44 - 1.81	1.53 - 1.76	1.43 - 1.67	1.45 - 1.54
	1.00 ± 0.10	1.72 ± 0.17	1.11 ± 0.10	1.50 ± 0.16	-	-	1.08 ± 0.10	1.07 ± 0.05	1.07 ± 0.09	1.12 ± 0.05	1.14 ± 0.05	1.14 ± 0.12
Composite 5 with Hydroxytamoxifen	0.90 - 1.24	1.50 - 2.05	1.02 - 1.28	1.32 - 1.70	-	-	0.94 - 1.20	0.99 - 1.11	0.98 - 1.21	1.07 - 1.19	1.05 - 1.21	0.99 - 1.28
	1.00 ± 0.04	1.48 ± 0.07	-	-	1.00 ± 0.06	0.99 ± 0.05	1.02 ± 0.05	1.02 ± 0.05	1.05 ± 0.02	1.23 ± 0.02	1.32 ± 0.07	1.37 ± 0.07
Composite 6	0.94 - 1.07	1.33 - 1.58	-	-	0.91 - 1.07	0.91 - 1.03	0.94 - 1.08	0.96 - 1.08	1.02 - 1.06	1.21 - 1.25	1.23 - 1.39	1.29 - 1.48
	1.00 ± 0.06	1.54 ± 0.09	-	-	1.59 ± 0.13	1.54 ± 0.05	1.57 ± 0.07	1.52 ± 0.09	1.54 ± 0.13	1.62 ± 0.11	1.53 ± 0.07	1.56 ± 0.09
Composite 6 with Estradiol	0.90 - 1.09	1.39 - 1.69	-	-	1.42 - 1.73	1.46 - 1.61	1.50 - 1.66	1.42 - 1.63	1.40 - 1.75	1.50 - 1.74	1.43 - 1.60	1.47 - 1.71
	1.00 ± 0.05	1.58 ± 0.12	0.97 ± 0.02	1.27 ± 0.08	-	-	1.05 ± 0.03	1.08 ± 0.04	1.09 ± 0.02	1.10 ± 0.04	1.15 ± 0.04	1.13 ± 0.04
Composite 6 with Hydroxytamoxifen	0.93 - 1.11	1.40 - 1.76	0.94 - 1.00	1.18 - 1.40	-	-	1.02 - 1.09	1.02 - 1.11	1.07 - 1.13	1.06 - 1.15	1.09 - 1.20	1.09 - 1.19
	1.00 ± 0.08	1.61 ± 0.12	-	-	1.12 ± 0.07	1.05 ± 0.11	1.05 ± 0.01	1.04 ± 0.06	1.13 ± 0.06	1.17 ± 0.08	1.34 ± 0.09	1.38 ± 0.06
Composite 7	0.89 - 1.12	1.46 - 1.76	-	-	1.06 - 1.21	0.96 - 1.19	1.03 - 1.06	0.96 - 1.11	1.09 - 1.23	1.06 - 1.25	1.23 - 1.45	1.30 - 1.45
	1.00 ± 0.06	1.77 ± 0.11	-	-	1.93 ± 0.10	1.69 ± 0.11	1.78 ± 0.07	1.91 ± 0.07	1.96 ± 0.09	1.96 ± 0.09	2.00 ± 0.08	1.92 ± 0.13
Composite 7 with Estradiol	0.92 - 1.08	1.59 - 1.90	-	-	1.85 - 2.08	1.60 - 1.87	1.71 - 1.90	1.81 - 1.98	1.87 - 2.11	1.85 - 2.06	1.86 - 2.06	1.75 - 2.09
	1.00 ± 0.05	1.76 ± 0.10	1.19 ± 0.20	1.73 ± 0.43	-	-	1.19 ± 0.05	1.28 ± 0.07	1.29 ± 0.08	1.29 ± 0.04	1.36 ± 0.10	1.25 ± 0.07
Composite 7 with Hydroxytamoxifen	0.92 - 1.08	1.64 - 1.97	0.97 - 1.50	1.45 - 2.46	-	-	1.10 - 1.23	1.21 - 1.40	1.17 - 1.36	1.24 - 1.32	1.22 - 1.48	1.18 - 1.35
	1.00 ± 0.03	1.83 ± 0.14	-	-	1.00 ± 0.03	1.05 ± 0.11	1.11 ± 0.04	1.08 ± 0.10	1.13 ± 0.07	1.11 ± 0.06	1.25 ± 0.06	1.35 ± 0.06
Composite 8	0.94 - 1.04	1.51 - 2.02	-	-	0.96 - 1.03	0.96 - 1.22	1.04 - 1.15	0.97 - 1.22	1.06 - 1.22	1.01 - 1.16	1.18 - 1.34	1.30 - 1.44
	1.00 ± 0.08	1.83 ± 0.11	-	-	1.73 ± 0.09	1.81 ± 0.08	1.86 ± 0.07	1.93 ± 0.05	1.97 ± 0.09	1.92 ± 0.06	1.96 ± 0.09	1.99 ± 0.07
Composite 8 with Estradiol	0.90 - 1.15	1.63 - 1.98	-	-	1.63 - 1.84	1.70 - 1.93	1.75 - 1.93	1.89 - 2.02	1.83 - 2.06	1.83 - 1.98	1.80 - 2.04	1.90 - 2.10
	1.00 ± 0.07	1.93 ± 0.21	1.01 ± 0.07	1.29 ± 0.05	-	-	1.07 ± 0.03	1.15 ± 0.04	1.12 ± 0.03	1.17 ± 0.04	1.24 ± 0.09	1.31 ± 0.17
Composite 8 with Hydroxytamoxifen	0.87 - 1.12	1.72 - 2.39	0.93 - 1.12	1.25 - 1.36	-	-	1.03 - 1.12	1.11 - 1.20	1.07 - 1.16	1.12 - 1.22	1.14 - 1.36	1.12 - 1.53
	1.00 ± 0.07	1.56 ± 0.13	-	-	1.03 ± 0.03	1.04 ± 0.04	1.10 ± 0.10	1.08 ± 0.03	1.07 ± 0.03	1.13 ± 0.02	1.19 ± 0.03	1.24 ± 0.07
Composite 9	0.92 - 1.12	1.41 - 1.89	-	-	1.00 - 1.08	0.99 - 1.08	0.96 - 1.21	1.05 - 1.12	1.05 - 1.13	1.11 - 1.15	1.14 - 1.23	1.15 - 1.33
	1.00 ± 0.08	1.64 ± 0.14	-	-	1.59 ± 0.05	1.61 ± 0.10	1.69 ± 0.05	1.70 ± 0.12	1.73 ± 0.06	1.74 ± 0.09	1.70 ± 0.13	1.68 ± 0.09
Composite 9 with Estradiol	0.91 - 1.13	1.52 - 2.00	-	-	1.52 - 1.66	1.47 - 1.75	1.65 - 1.78	1.49 - 1.78	1.65 - 1.78	1.59 - 1.82	1.54 - 1.90	1.57 - 1.80
	1.00 ± 0.05	1.63 ± 0.08	1.13 ± 0.05	1.50 ± 0.12	-	-	1.12 ± 0.03	1.12 ± 0.05	1.13 ± 0.03	1.17 ± 0.10	1.08 ± 0.03	1.07 ± 0.01
Composite 9 with Hydroxytamoxifen	0.94 - 1.13	1.52 - 1.77	1.06 - 1.18	1.35 - 1.63	-	-	1.09 - 1.17	1.05 - 1.16	1.08 - 1.16	1.03 - 1.30	1.04 - 1.12	1.06 - 1.09
	1.00 ± 0.09	1.57 ± 0.12	-	-	1.00 ± 0.06	0.95 ± 0.06	1.01 ± 0.09	0.99 ± 0.08	1.04 ± 0.08	1.07 ± 0.07	1.18 ± 0.20	1.22 ± 0.12
Composite 10	0.89 - 1.16	1.39 - 1.78	-	-	0.93 - 1.08	0.85 - 0.99	0.88 - 1.11	0.87 - 1.08	0.94 - 1.16	0.97 - 1.15	0.92 - 1.41	1.10 - 1.41
	1.00 ± 0.07	1.62 ± 0.13	-	-	1.58 ± 0.09	1.56 ± 0.10	1.51 ± 0.04	1.64 ± 0.07	1.59 ± 0.06	1.69 ± 0.10	1.74 ± 0.08	1.69 ± 0.08
Composite 10 with Estradiol	0.90 - 1.10	1.44 - 1.80	-	-	1.47 - 1.67	1.45 - 1.68	1.44 - 1.54	1.53 - 1.72	1.54 - 1.69	1.56 - 1.80	1.66 - 1.88	1.60 - 1.79
	1.00 ± 0.16	1.56 ± 0.16	1.12 ± 0.14	1.49 ± 0.20	-	-	0.99 ± 0.06	1.10 ± 0.05	1.00 ± 0.07	1.14 ± 0.11	1.16 ± 0.09	1.16 ± 0.10
Composite 10 with Hydroxytamoxifen	0.83 - 1.35	1.24 - 1.79	1.02 - 1.35	1.17 - 1.71	-	-	0.91 - 1.07	1.02 - 1.14	0.89 - 1.07	1.02 - 1.33	1.04 - 1.25	1.08 - 1.28
	1.00 ± 0.07	1.44 ± 0.09	-	-	1.12 ± 0.16	1.04 ± 0.06	1.01 ± 0.09	1.10 ± 0.09	1.06 ± 0.08	1.08 ± 0.07	1.03 ± 0.03	1.05 ± 0.06
Composite 11	0.91 - 1.12	1.32 - 1.56	-	-	0.96 - 1.37	0.97 - 1.13	0.91 - 1.11	1.03 - 1.25	1.02 - 1.20	1.01 - 1.20	1.00 - 1.07	1.00 - 1.15
	1.00 ± 0.09	1.50 ± 0.14	-	-	1.59 ± 0.06	1.51 ± 0.10	1.45 ± 0.11	1.59 ± 0.20	1.49 ± 0.08	1.56 ± 0.09	1.34 ± 0.37	1.44 ± 0.06
Composite 11 with Estradiol	0.86 - 1.13	1.31 - 1.71	-	-	1.50 - 1.65	1.42 - 1.68	1.32 - 1.60	1.43 - 1.89	1.42 - 1.62	1.44 - 1.70	0.70 - 1.60	1.37 - 1.52
	1.00 ± 0.08	1.61 ± 0.10	1.04 ± 0.07	1.50 ± 0.11	-	-	1.10 ± 0.09	1.11 ± 0.05	1.17 ± 0.07	1.16 ± 0.04	1.09 ± 0.02	1.08 ± 0.04
Composite 11 with Hydroxytamoxifen	0.89 - 1.13	1.50 - 1.85	0.93 - 1.12	1.41 - 1.69	-	-	0.98 - 1.19	1.04 - 1.16	1.07 - 1.24	1.12 - 1.21	1.07 - 1.11	1.02 - 1.12

from Ford City (Composites 4 and 6). Gender does not appear to be a factor in either case. The graphs for BT-20, MCF-7 and T47D analyses are shown in Appendices A, B and C, respectively.

Table 8. Summary of the Estrogen Response Profile (ERP) for Cell Proliferation Assays by Composite

	1	2	3	4	5	6	7	8	9	10	11
BT20	-	-	-	-	-	-	-	-	-	-	-
MCF7	-	-/+	-	-/+	-/+	-/+	-/+	-	-/+	-/+	-
T47D	-/+	-/+	-	+	-/+	+	-/+	-/+	-/+	-/+	-

Key:
- **No Response**
-/+ **Weak (sub-estrogenic) Response**
+ **Estrogenic Response**
++ **Strong Response**

2.3.3 Estrogenicity Index

Table 9, MCF-7 Estrogenicity Index Data Summary (Mean with SD and Range), displays the results of the mean EI with standard deviations and ranges for all eleven composites in each dilution range for the MCF-7 analysis. Table 10, T47D Estrogenicity Index Data Summary (Mean with SD and Range), displays the same results for the T47D analysis.

Results for average EI of MCF-7 and T47D cell lines were plotted for location and gender by analyses and then for analyses by composite. Refer to Figures 9-25 for various plots of average EI versus dilution. Figures 9-14 show the cell lines plotted for location and gender by analyses. These figures clearly show that extracts from fish caught in Ford City exhibit higher estrogenic responses in both genders and in both MCF-7 and T47D analyses. In Figures 15-25, cell lines were plotted by composite. The T47D estrogenic responses were higher in all dilutions for Composites 3, 4, 5 and 11. The T47D cells appear to

Table 9. MCF-7 Estrogenicity Index Data Summary (Mean with SD and Range)

	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
Composite 1	0.16 ± 0.05 0.12 - 0.24	0.14 ± 0.04 0.10 - 0.18	0.05 ± 0.06 -0.06 - 0.10	0.02 ± 0.08 -0.08 - 0.14	0.09 ± 0.09 0.00 - 0.24	0.15 ± 0.05 0.08 - 0.21	0.11 ± 0.08 0.05 - 0.25	0.23 ± 0.11 0.10 - 0.38
Composite 2	0.11 ± 0.07 0.07 - 0.24	0.03 ± 0.03 -0.03 - 0.05	0.08 ± 0.05 0.03 - 0.15	0.12 ± 0.05 0.07 - 0.17	0.10 ± 0.05 0.04 - 0.16	0.11 ± 0.05 0.04 - 0.16	0.18 ± 0.05 0.14 - 0.26	0.23 ± 0.04 0.17 - 0.26
Composite 3	0.10 ± 0.08 -0.01 - 0.21	0.00 ± 0.08 -0.06 - 0.13	-0.01 ± 0.05 -0.05 - 0.08	0.00 ± 0.04 -0.04 - 0.04	0.00 ± 0.04 -0.07 - 0.04	0.03 ± 0.04 -0.03 - 0.07	0.06 ± 0.05 0.01 - 0.13	0.07 ± 0.05 0.00 - 0.13
Composite 4	0.18 ± 0.07 0.07 - 0.24	0.22 ± 0.08 0.15 - 0.31	0.23 ± 0.05 0.15 - 0.28	0.22 ± 0.10 0.11 - 0.33	0.39 ± 0.12 0.22 - 0.49	0.47 ± 0.11 0.34 - 0.60	0.48 ± 0.11 0.34 - 0.63	0.41 ± 0.07 0.30 - 0.49
Composite 5	0.00 ± 0.07 -0.09 - 0.10	0.03 ± 0.07 -0.04 - 0.13	0.03 ± 0.06 -0.04 - 0.11	-0.02 ± 0.04 -0.07 - 0.04	-0.02 ± 0.02 -0.05 - 0.01	0.01 ± 0.04 -0.03 - 0.07	0.12 ± 0.03 0.09 - 0.16	0.14 ± 0.05 0.07 - 0.21
Composite 6	0.00 ± 0.02 -0.03 - 0.03	-0.02 ± 0.05 -0.09 - 0.04	0.12 ± 0.02 0.10 - 0.16	0.10 ± 0.03 0.06 - 0.14	0.20 ± 0.06 0.13 - 0.27	0.31 ± 0.07 0.23 - 0.39	0.45 ± 0.04 0.39 - 0.49	0.39 ± 0.04 0.33 - 0.42
Composite 7	0.20 ± 0.07 0.10 - 0.27	0.07 ± 0.05 0.02 - 0.13	0.12 ± 0.05 0.07 - 0.18	0.12 ± 0.03 0.07 - 0.14	0.21 ± 0.07 0.13 - 0.29	0.24 ± 0.01 0.23 - 0.26	0.35 ± 0.02 0.34 - 0.37	0.35 ± 0.04 0.31 - 0.41
Composite 8	0.12 ± 0.08 0.03 - 0.20	0.15 ± 0.06 0.09 - 0.23	0.14 ± 0.09 0.04 - 0.29	0.03 ± 0.08 -0.05 - 0.13	0.04 ± 0.04 -0.01 - 0.08	0.11 ± 0.04 0.06 - 0.16	0.17 ± 0.08 0.06 - 0.25	0.18 ± 0.10 0.09 - 0.32
Composite 9	0.18 ± 0.07 0.10 - 0.27	0.10 ± 0.09 0.02 - 0.21	-0.02 ± 0.05 -0.08 - 0.04	0.07 ± 0.04 0.01 - 0.10	0.03 ± 0.07 -0.06 - 0.10	0.08 ± 0.06 0.00 - 0.16	0.20 ± 0.05 0.14 - 0.27	0.20 ± 0.05 0.13 - 0.28
Composite 10	0.22 ± 0.05 0.15 - 0.27	0.12 ± 0.02 0.09 - 0.14	0.02 ± 0.05 -0.04 - 0.09	0.02 ± 0.06 -0.07 - 0.09	0.05 ± 0.03 0.02 - 0.09	0.01 ± 0.05 -0.04 - 0.06	0.12 ± 0.06 0.05 - 0.20	0.12 ± 0.04 0.07 - 0.16
Composite 11	0.10 ± 0.07 0.03 - 0.20	0.06 ± 0.06 0.00 - 0.14	0.02 ± 0.07 -0.07 - 0.10	0.01 ± 0.05 -0.04 - 0.07	0.02 ± 0.07 -0.06 - 0.11	0.02 ± 0.04 -0.02 - 0.06	0.01 ± 0.04 -0.03 - 0.08	0.00 ± 0.05 -0.05 - 0.09

Table 10. T47D Estrogenicity Index Data Summary (Mean with SD and Range)

	1/4000	1/3000	1/2000	1/1500	1/1000	1/500	1/200	1/100
Composite 1	0.13 ± 0.11 -0.02 - 0.25	0.10 ± 0.10 -0.02 - 0.20	0.08 ± 0.06 0.02 - 0.16	0.12 ± 0.04 0.06 - 0.18	0.22 ± 0.08 0.11 - 0.32	0.24 ± 0.06 0.18 - 0.34	0.36 ± 0.11 0.25 - 0.51	0.54 ± 0.08 0.48 - 0.67
Composite 2	0.14 ± 0.08 0.04 - 0.24	-0.06 ± 0.17 -0.24 - 0.21	0.03 ± 0.10 -0.12 - 0.13	0.10 ± 0.17 -0.11 - 0.27	0.35 ± 0.14 0.22 - 0.53	0.23 ± 0.04 0.18 - 0.27	0.35 ± 0.08 0.28 - 0.45	0.46 ± 0.10 0.34 - 0.60
Composite 3	0.31 ± 0.12 0.12 - 0.42	0.22 ± 0.08 0.15 - 0.35	0.18 ± 0.12 0.03 - 0.33	0.06 ± 0.10 -0.06 - 0.18	0.07 ± 0.16 -0.03 - 0.34	0.14 ± 0.09 0.04 - 0.23	0.25 ± 0.07 0.17 - 0.35	0.38 ± 0.18 0.16 - 0.62
Composite 4	0.33 ± 0.18 0.13 - 0.59	0.37 ± 0.11 0.25 - 0.48	0.26 ± 0.08 0.13 - 0.35	0.24 ± 0.17 0.08 - 0.49	0.47 ± 0.18 0.21 - 0.63	0.68 ± 0.14 0.45 - 0.82	0.96 ± 0.13 0.81 - 1.15	1.00 ± 0.11 0.83 - 1.15
Composite 5	0.35 ± 0.07 0.28 - 0.44	0.18 ± 0.14 0.01 - 0.33	0.11 ± 0.16 0.00 - 0.37	0.09 ± 0.09 -0.05 - 0.15	0.24 ± 0.07 0.15 - 0.33	0.20 ± 0.15 -0.03 - 0.33	0.32 ± 0.19 0.17 - 0.64	0.40 ± 0.20 0.26 - 0.75
Composite 6	0.00 ± 0.13 -0.19 - 0.14	-0.03 ± 0.10 -0.18 - 0.06	0.03 ± 0.11 -0.11 - 0.16	0.03 ± 0.10 -0.08 - 0.17	0.10 ± 0.04 0.04 - 0.13	0.48 ± 0.04 0.43 - 0.52	0.66 ± 0.14 0.47 - 0.81	0.78 ± 0.15 0.60 - 0.99
Composite 7	0.20 ± 0.11 0.09 - 0.35	0.09 ± 0.17 -0.06 - 0.32	0.08 ± 0.02 0.06 - 0.10	0.06 ± 0.09 -0.07 - 0.18	0.22 ± 0.09 0.15 - 0.37	0.28 ± 0.14 0.09 - 0.41	0.55 ± 0.14 0.37 - 0.73	0.63 ± 0.10 0.50 - 0.73
Composite 8	-0.01 ± 0.03 -0.04 - 0.04	0.06 ± 0.13 -0.05 - 0.26	0.13 ± 0.05 0.05 - 0.19	0.10 ± 0.12 -0.04 - 0.27	0.15 ± 0.08 0.08 - 0.27	0.13 ± 0.07 0.01 - 0.19	0.30 ± 0.07 0.22 - 0.40	0.42 ± 0.07 0.37 - 0.53
Composite 9	0.06 ± 0.05 0.00 - 0.13	0.07 ± 0.07 -0.01 - 0.14	0.18 ± 0.17 -0.07 - 0.38	0.14 ± 0.05 0.09 - 0.22	0.13 ± 0.06 0.09 - 0.23	0.23 ± 0.03 0.20 - 0.27	0.34 ± 0.06 0.25 - 0.40	0.43 ± 0.13 0.27 - 0.60
Composite 10	0.01 ± 0.11 -0.12 - 0.14	-0.09 ± 0.10 -0.26 - -0.02	0.02 ± 0.16 -0.21 - 0.19	-0.02 ± 0.13 -0.23 - 0.14	0.06 ± 0.15 -0.10 - 0.27	0.12 ± 0.13 -0.05 - 0.26	0.31 ± 0.34 -0.14 - 0.72	0.39 ± 0.20 0.18 - 0.72
Composite 11	0.27 ± 0.37 -0.08 - 0.83	0.08 ± 0.14 -0.07 - 0.29	0.03 ± 0.19 -0.21 - 0.24	0.23 ± 0.21 0.06 - 0.58	0.14 ± 0.18 0.04 - 0.47	0.18 ± 0.17 0.02 - 0.46	0.06 ± 0.06 0.00 - 0.15	0.12 ± 0.13 0.01 - 0.34

show a stronger response in general than the MCF-7 cell line, which seems to imply that more of the estrogenic potential from these samples may be due to an ERβ response. However, because T47D have some content of alpha receptors, this could be a flawed assumption. T47D cell lines are primarily ERβ, but also exhibit some ERα response. This makes it difficult to determine the specific class of chemical that may be causing the estrogenic response in this case. If the T47D would have had a much greater response than the MCF-7, then the EI could have been contributed to chemicals only exhibiting ERβ receptor mechanisms. If the T47D response would have been much lower than the MCF-7 results, the EI could then have been contributed to chemicals only exhibiting ERα receptor mechanism. However, in this experiment the graphs trend quite well with each other, and this relationship was statistically verified by the Spearman's Rank Correlation for the overall analyses. Therefore, it cannot be determined that one class of chemicals is playing a more prominent role in the overall analysis.

2.3.4 Statistical Results

Statistical analysis of the calculated EI yielded a significant difference ($p < 0.05$) in MCF-7 estrogenicity when tested by location only, with samples from fish captured at Ford City having the higher mean EI. There were no significant differences when testing for differences in gender or weight class. A model including all three covariates did not produce significant results. Statistical analysis for estrogenicity in T47D cells did not show any significant results.

It was suspected that extracts from the fish captured in the Freeport area would have higher estrogenicity levels than the ones from Ford City because of the higher number of CSOs in the Freeport area and because the sampling location at Freeport is in closer vicinity to CSOs

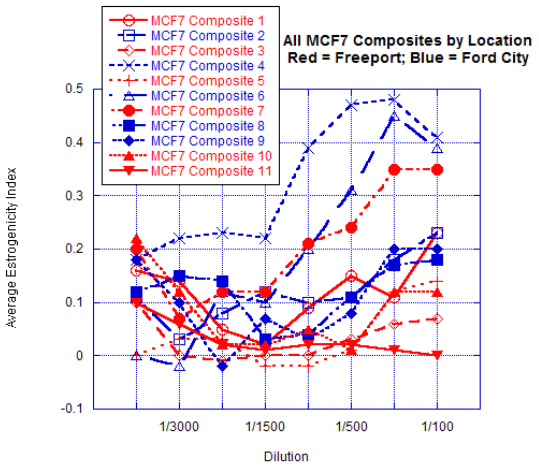


Figure 9. All MCF-7 Composites by Location

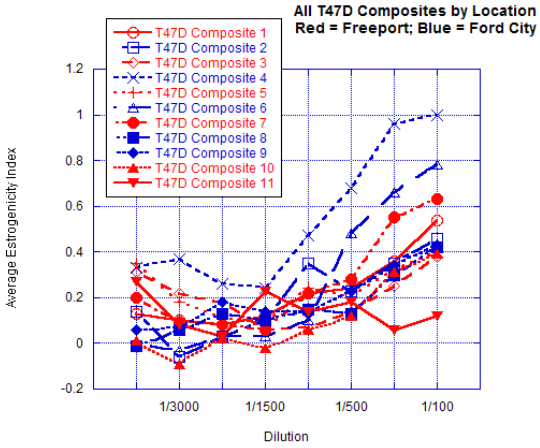


Figure 10. All T47D Composites by Location

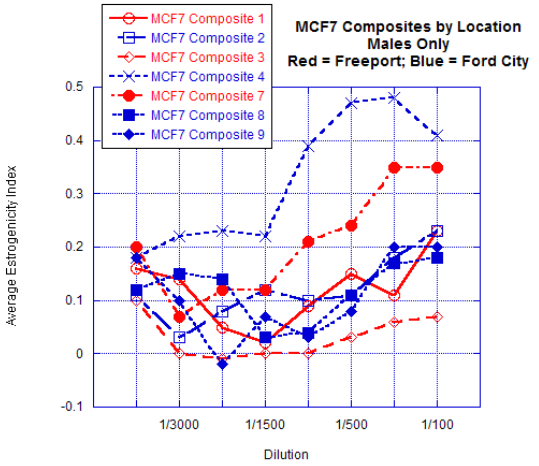


Figure 11. MCF-7 Composites by Locations; Males Only

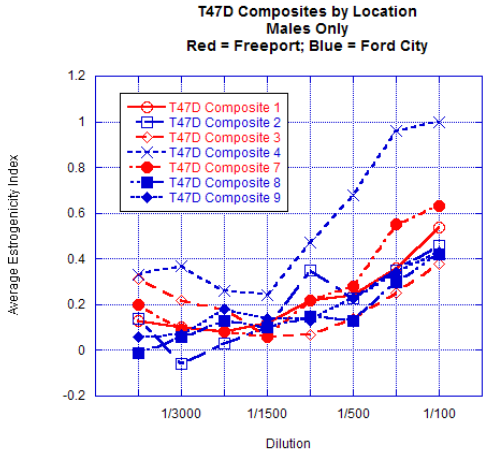


Figure 12. T47D Composites by Location; Males Only

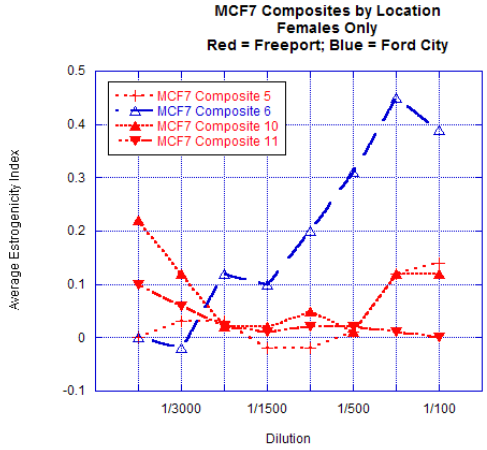


Figure 13. MCF-7 Composites by Location; Females Only

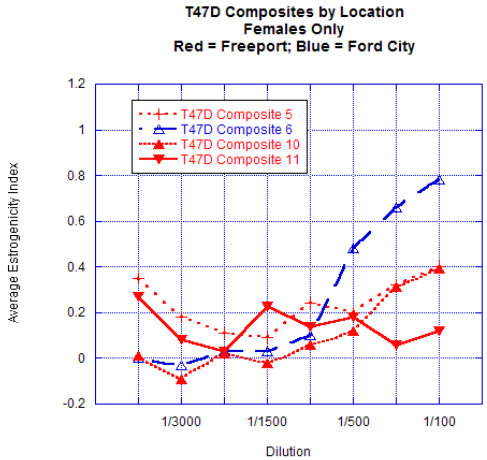


Figure 14. T47D Composites by Location; Females Only

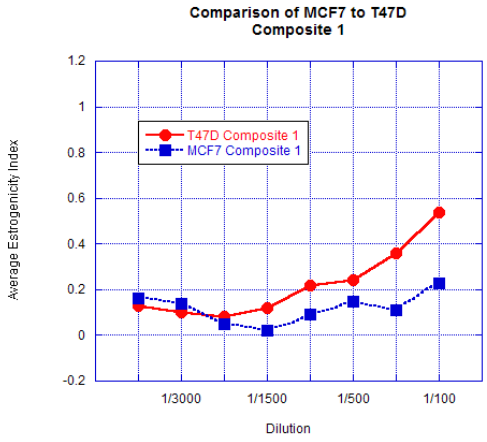


Figure 15. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 1

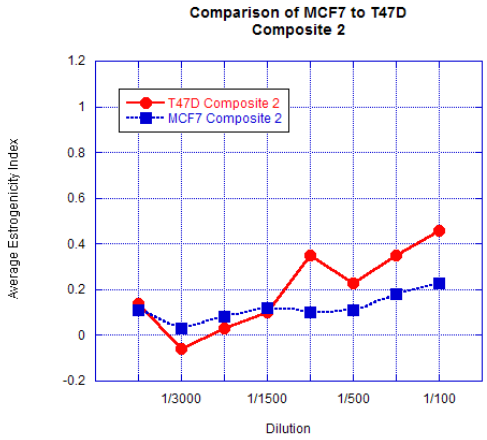


Figure 16. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 2

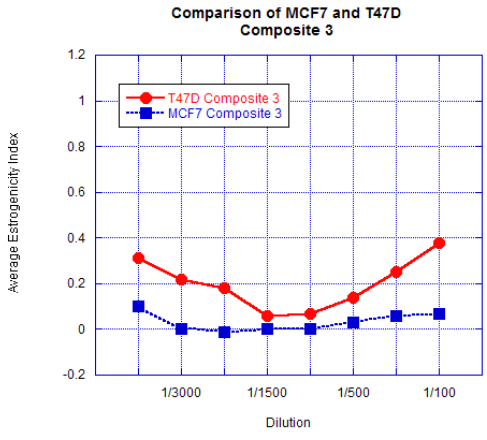


Figure 17. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 3

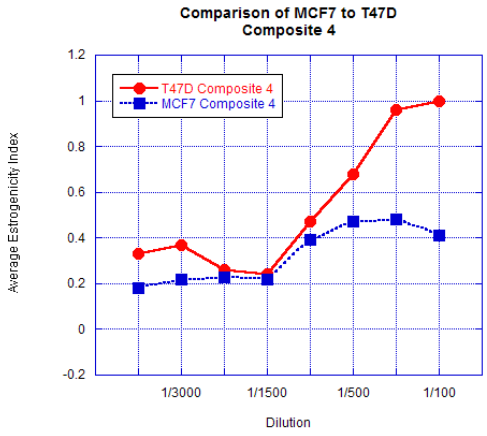


Figure 18. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 4

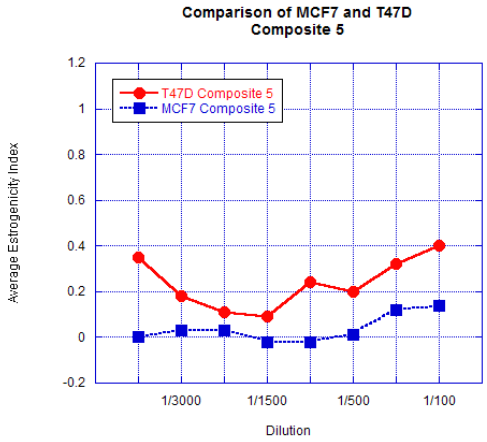


Figure 19. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 5

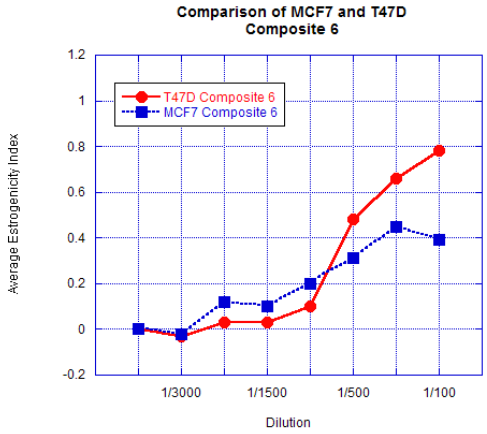


Figure 20. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 6

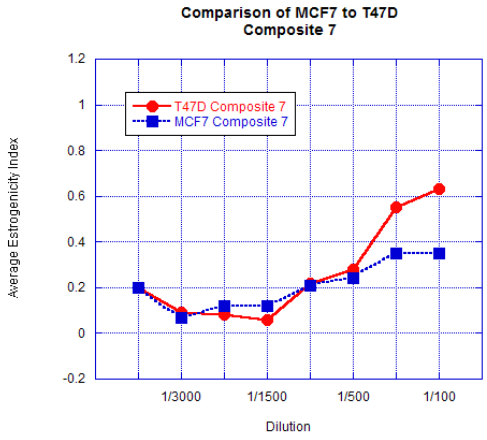


Figure 21. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 7

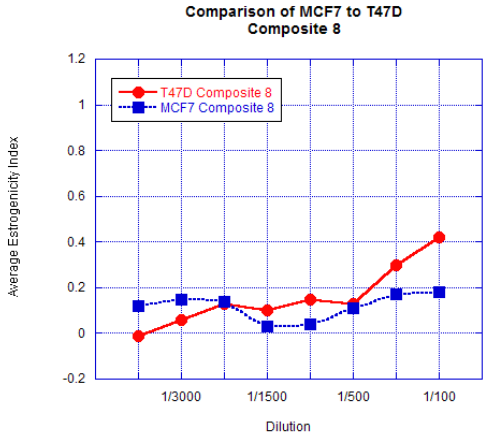


Figure 22. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 8

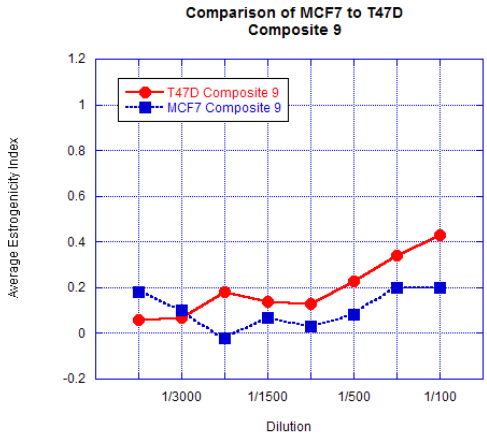


Figure 23. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 9

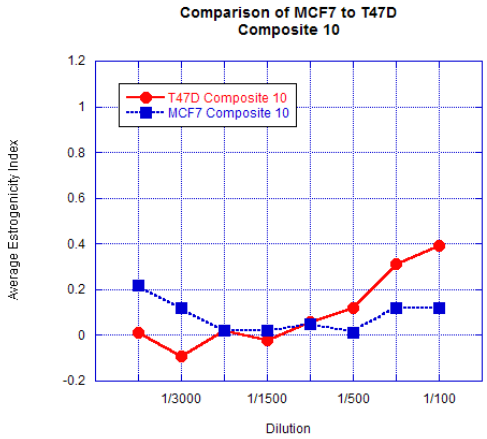


Figure 24. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 10

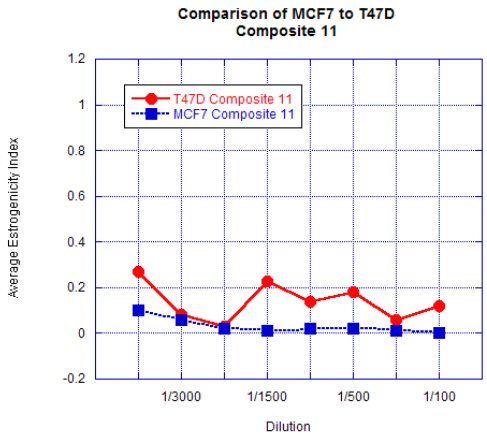


Figure 25. Comparison of Average Estrogenicity Index for MCF-7 and T47D Analyses for Composite 11

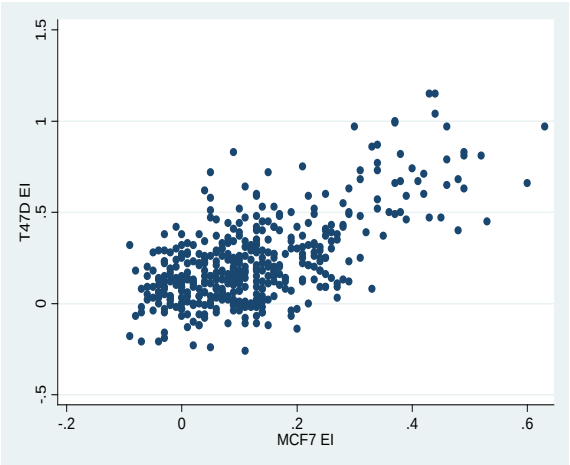


Figure 26. Correlation Plot of Estrogenicity Index for MCF-7 Versus T47D Analyses for All Composites

and WWTP effluent discharges than the Ford City sample location. However, this was not found to be the case. Although not significantly different from the Freeport location after adjusting for the covariates of gender and weight, samples from fish captured at Ford City had the higher mean Estrogenicity Index (e.g. 0.259 at dilution 1/100 vs. 0.171 for Freeport). This suggests that estrogenicity studies cannot be simply designed. There are multiple factors that have an effect on the outcome of the study, not merely CSO density. It would have been helpful to collect data on the amount of flow contributing from each WWTP and CSO outfall, rather than just the proximity. This is difficult because not even the PA DEP collects flow data for CSOs at this time. Previous studies have collected data on the amounts of total organic nitrogen in these areas. Higher nitrogen in the water is a sign that the water has not been processed by a WWTP because most WWTP processes will convert organic nitrogen and ammonia into nitrate before it leaves as WWTP effluent [19]. In 2003, the United States Geological Survey (USGS) sampled Allegheny River surface water in Kittanning (approximately four miles north of Ford City) and determined the total nitrogen content to be 0.67 mg/L [152]. Also in 2003, Three Rivers Second Nature Project, a group directed by artists and researchers in Allegheny County, PA in association with Carnegie Mellon University, sampled Buffalo Creek near the area where the creek enters into the Allegheny River and determined the total nitrogen content to be 0.30 mg/L [153]. Even though these studies were performed a few years before the current sampling in this study, and the sample locations are not exactly the same, the assessments of the areas are useful to show that although both areas have low total nitrogen values, the general area near Ford City had a total nitrogen value twice as high as that of Freeport. This data helps to explain why our original theory was incorrect. It is general knowledge that rural areas tend to have higher volumes of agricultural runoff and higher usage of septic tanks/outhouses. These two

confounding factors may explain why the initial assumption in this study was incorrect and could be contributing to xenoestrogenic pollution in the area, rather than CSO density alone.

Spearman Rank Correlation testing was performed to determine the relationship between MCF-7 and T47D analyses. Figure 26, Correlation Plot of Estrogenicity Index for MCF-7 Versus T47D Analyses for All Composites, is a plot of the correlation. There is a strong correlation between MCF-7 and T47D results, $\rho = 0.5056$, $p < 0.0001$. With a correlation this strong, it is safe to say that both the MCF-7 and T47D cell line analyses are equally valid in assessing for the estrogenicity of extracts from fish caught in surface waters.

2.4 PUBLIC HEALTH SIGNIFICANCE

Estrogenicity in fish extracts can imply environmental pollution by xenoestrogens. S ome examples of such xenoestrogens are pharmaceuticals (e.g. contraceptives or hormone replacement therapies), phytoestrogens (dietary xenoestrogens found in some plant products such as soy beans and flax seeds), plasticizers such as Bisphenol A, and hygiene product preservatives (e.g. parabens). As determined from this study, the correlation between the MCF-7 and T47D analyses results implies that there is probably an equal or mixed amount of ERα and ERβ receptor positive chemicals acting to cause the increase in estrogenic potential. Pharmaceutical products for hormone therapy are more likely to be the cause of ERα pollutants than phytoestrogens. Pharmaceutical products have a high potential to reach the water supply when excreted into human urine and feces, or are disposed of directly down household drains [19]. WWTPs are only partially efficient at removing pharmaceuticals and there is a lot of household water that is disposed of into community surface waters without the benefit of prior treatment

[20]. Although this research is not strong evidence that pharmaceutical products are causing estrogenic problems in the Ford City and Freeport area surface waters, it leads to stimulating results which support future hypothesis generating research.

3.0 ANALYSIS OF SURFACE WATER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Fish flesh and fat samples in the Greater Pittsburgh Area have been shown to exhibit estrogenic potential, as determined from research in Chapter 2 of this dissertation and previous Center for Healthy Environments and Communities (CHEC) studies [148, 149]. It can be inferred that Pittsburgh area surface waters would be the source of such estrogenicity in fish. A problem with screening fish extracts for estrogenic potential via the E-Screen Assay (or any similar cell proliferation assay) is that the analysis alone does nothing to discover which chemical, or combination of chemicals, is present in a sample to cause the estrogenic effect. Therefore, research efforts are needed to develop better techniques (e.g. more cost and time efficient analytical methods, as well as lower detection limits) to be used in conjunction with cell proliferation assays [154]. A method for analyzing several xenoestrogens in a surface water matrix was developed utilizing available literature and an attempt was made to quantify levels of Bisphenol A (BPA) and parabens in six locations along the Allegheny and Monongahela Rivers.

3.1 BACKGROUND

BPA has been thoroughly studied in surface water systems across the world. BPA has been detected in Taiwan surface waters at ranges between 0.037 μg/L (limit of detection) to 4.23 μg/L in 59% of samples tested [37]. Surface waters analyzed in Spain and other Mediterranean areas discovered BPA in the μg/L range for all samples collected. United States waters ranged between <1.0 to 8.0 μg/L for BPA concentrations [38, 39]. BPA in German waters ranged from 0.0005 to 0.776 μg/L, and a surface water sample from the Czech Republic was 28 ng/L [39-43, 57, 155]. Japanese levels varied from <0.005 to 1.9 μg/L, while in China, BPA levels ranged from 0.03 to 0.083 μg/L [39, 45-51]. In the Netherlands, concentrations were found to be between <0.012 and 1 μg/L [39, 52, 53]. BPA was detected in 51% of surface waters sampled in Portugal in the range of 0.07- 4.0 μg/L [55]. BPA was detected in sewage treatment plant influents and effluents in the United Kingdom and Spain, with ranges of 884.7-1105.2 ng/L and 13.3-19.2 ng/L, respectively [56]. In Germany, Wastewater Treatment Plant (WWTP) effluents have BPA concentrations in the 18-33 ng/L ranges [57].

Less is known about the transport and fate of parabens in the environment, but it is generally believed that they do not persist for long periods of time. Any potential endocrine disrupting effects will most likely come from direct exposure from market products containing parabens. Wastewater treatment processes have been shown to adequately reduce paraben concentrations. Canosa et. al. showed influent concentrations of methyl paraben in two sets of samples were reduced from 2.92 ng/mL to less than detectable in the effluent [108]. Ethyl, propyl and butyl parabens were reduced to non-detectable as well, with benzyl paraben not being present in the influent [108]. With these removal efficiencies, it is not expected that parabens

would be present in surface waters [108]. However, this was not found to be the case in Spanish research studies. In a 2009 Spanish study, methyl paraben was detected in tap and surface water at 0.040 ng/mL and 0.037 ng/mL, respectively, however ethyl, propyl, butyl and benzyl parabens were all below the limits of quantification or non-detectable [118]. In a 2010 study of the same area (Northwest Spain), all parabens were present in levels ranging from 0.8 – 105 ng/L, with methyl paraben at 54 ng/L, ethyl paraben at 29 ng/L, and derivations of propyl paraben as the high and low values [119]. These two studies imply that there may be some seasonal effects on the transport and fate of this family of chemicals. Methyl paraben is also being detected in Spanish tap water in concentrations of 17 ng/L [120].

3.2 OBJECTIVES

The objectives of this study were as follows:

- To develop a method for analyzing xenoestrogens, particularly BPA and parabens, utilizing available literature.
- To determine if concentrations of xenoestrogens are detectable in Greater Pittsburgh Area surface waters.

3.3 MATERIAL AND METHODS

3.3.1 Sample Locations

Six sample locations were chosen for this study to match the locations where fish were sampled, as described in Chapter 4 of this dissertation. Table 11, Surface Water Sampling Information, lists the coordinates of the sample points, as well as general information about sampling. Figure 27, Map of Surface Water Sample Locations, is a map of the sample locations with reference to the lock and dam system. All surface water locations from this chapter and fish sampling locations from Chapters 2 and 4 are in the same vicinity of one another. The Kittanning location was the only fish sampling point that was not sampled for surface water, due to logistical concerns.

Ford City, Pennsylvania is located in Armstrong County on the Allegheny River approximately 40 miles northeast of the city of Pittsburgh and approximately four miles south of Kittanning. Ford City has one permitted WWTP and three permitted Combined Sewer Overflows (CSOs), as determined by the Pennsylvania Department of Environmental Protection (PA DEP) data file searches. The sample point is the confluence of the Crooked Creek with the Allegheny River. Water samples were taken near Ford City downstream of the area's WWTP and CSO outfalls, between Allegheny Lock and Dam No 6 and 7, in the Lock and Dam 6 Pool [150].

Freeport, Pennsylvania is located in Armstrong County on the Allegheny River approximately 28 miles northeast of Pittsburgh. Freeport has one permitted WWTP and a total of five permitted CSOs, as determined by PA DEP data file searches. Two of the CSOs and the WWTP discharge into Buffalo Creek, less than 0.5 miles from where the creek flows into the

Allegheny River. The other three CSOs discharge directly into the Allegheny River, with the nearest discharge approximately 0.6 miles up-river of the Freeport sample point. All of the Freeport discharges are within one mile of the sample point, and the sample point is between Allegheny River Lock and Dam No 4 and 5, in the Lock and Dam 4 Pool [150].

Springdale and Harmarville sampling locations are both upstream of all Allegheny County Sanitary Authority (ALCOSAN) CSOs and Sanitary Sewer Overflows (SSOs). Springdale is located in Allegheny County on the Allegheny River approximately 18 miles northeast of Pittsburgh. The sample point is located between Allegheny River Lock and Dam No 4 and the C.W. Bill Young Lock and Dam, in the C.W. Bill Young Lock and Dam Pool [150]. Harmarville is located in Allegheny County on the Allegheny River northeast of Pittsburgh. The sample point is located between the Allegheny River Lock and Dam No 2 and the C.W. Bill Young Lock and Dam, in the Lock and Dam 2 Pool [150].

Braddock is located in Allegheny County on the Monongahela River southeast of Pittsburgh. The Braddock sample location has eleven CSOs within one mile of it; five are upstream and six are downstream. This sample site is located between the Monongahela River Lock and Dam No 3 and the Braddock Lock and Dam, within the Braddock Lock and Dam Pool [150].

Monessen is located in Westmoreland County on the Monongahela River south of Pittsburgh. There are a total of seventeen CSOs in the area, as determined by PA DEP data file searches. Six of the CSOs are upstream of the sample site and eleven downstream, all within approximately 2.5 miles on either side of the sample location. The Monessen sample location is upstream of the Monongahela River Lock and Dam No 3, in the Lock and Dam 3 Pool [150].

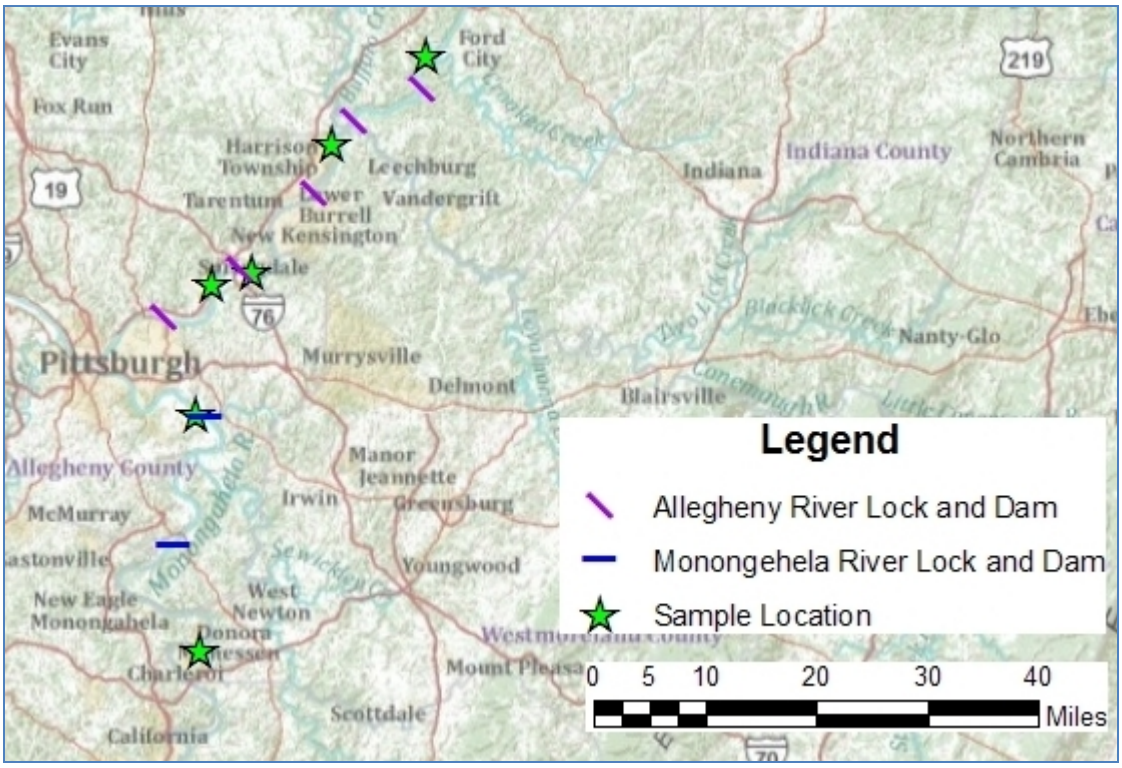


Figure 27. Map of Surface Water Sample Locations

3.3.2 Surface Water Sampling Methods

Sampling techniques were available for a wide variety of analytes and exposure pathways, but few exist specifically for suspected xenoestrogens. A written sampling plan was utilized, with specific sample locations, frequency, sample size and sample quantities predetermined so as to minimize bias [156, 157]. Sample contamination was avoided at all costs by employing good sampling techniques and minimizing the use of hygiene products during sample collection and preparation. This is especially important when analyzing for parabens because they are common hygiene product ingredients and could easily damage the integrity of the analysis.

Surface water samples were taken during the summer months. Amber glass sample jars were conditioned with environmental grade water, kept on ice, and in darkness to prevent

degradation. Grab samples were obtained using a 1.7L water sampler. Bottom, middle and top aliquots were composited into one liter samples. Top aliquots were all taken at one meter below the surface; middle and bottom sample depths are listed in Table 11, Surface Water Sampling Information. River conditions, such as temperature, depth, and Total Dissolved Solids (TDS), and GPS coordinates were noted for each sample. Samples were stored for no more than six days, in darkness at 4^{O}C, until solid phase extraction could be performed. Solid phase extraction was performed as described in the next section.

Table 11. Surface Water Sampling Information

Sample Location	Sample ID	Sample Date	Sample Time	Depth of river (m)	Depth of bottom aliquot (m)
Ford City	FC01	8/12/2009	1125	6.5	5.5
Freeport	FP01	8/12/2009	1306	13.5	12.2
Springdale	SP01	8/12/2009	1450	13.2	12.2
Harmarville	HV02	8/12/2009	1625	7.2	6.2
Braddock	BD01	8/13/2009	1102	4.8	3.6
Monessen	MN02	8/13/2009	1405	3.7	2.7

Sample Location	Depth of middle aliquot (m)	Latitude	Longitude	Temp. (C)
Ford City	4.5	N: 40.74939	W: 79.57368	23.1
Freeport	6.5	N: 40.66216	W: 79.69544	22.2
Springdale	4.1	N: 40.53582	W: 79.79764	23.2
Harmarville	3	N: 40.52304	W: 79.85004	23.1
Braddock	2.6	N: 40.39621	W: 79.87112	24.9
Monessen	1.75	N: 40.16156	W: 79.86620	25.3

3.3.3 Solid Phase Extraction

C18 Oasis HLB 5cc 200 mg LP glass cartridges were purchased from Waters Corporation. The columns were washed with 100% HPLC grade acetonitrile (Fisher Scientific), then conditioned with 1% acetonitrile. Acetonitrile was also added to all samples, reagents and blanks to make them 1% v/v. Only water that was both deionized and distilled was used for rinsing and standard preparation. Use of plastics were minimized in the laboratory environment, but could not be completely avoided. All glassware was triple rinsed with deionized and distilled water, then with acetonitrile. The volume extracted varied per sample due to solids in the river; exact extraction volumes are listed in Table 12, Solid Phase Extraction Volumes. Some samples needed to be filtered with Whatman filters (934-AH purchased from Fisher Scientific; cat. no. 1827 047). One blank, one quality control check, one duplicate sample and one sample with methyl paraben added were used to determine the accuracy and precision of the method. One liter of extracted sample was re-extracted in order to test if there was breakthrough of the columns. All samples were eluted with 5 mL of 100% acetonitrile, and the flask was then rinsed with 5 mL of acetonitrile to make a 10 mL sample stored in amber glass vials. The vials were then stored at $4^{O}C$ until delivered to the laboratory for analysis.

Table 12. Solid Phase Extraction Volumes

Sample Location	Sample ID	Filtered (Y/N)	Extraction Volume (mL)
Ford City	FC01A	No	130
Ford City Duplicate	FC01B	Yes	500
Freeport	FP01	No	1000
Springdale	SP01	Yes	400
Harmarville	HV02	No	900
Braddock	BD01	No	750
Monessen	MN02	No	1000

3.3.4 Analytical Method for Surface Water Analysis

Samples were analyzed using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) on triple quadrupole mass spectrometer (TSQ Discovery Max). The HPLC biphasic gradient (over 15 minutes) conditions were as follows: C-8 Hypersil gold column, 5 μ, 100 x 4.6 mm (Thermo Fisher) and H_2O : acetonitrile as mobile phase. Five microliters (5 μL) of sample were injected using the HPLC autosampler (Surveyor Plus), and the needle was washed in between each sample with 100 mL methanol to prevent cross contamination. All solutions were HPLC grade and the water was pre-treated trough C-18 columns (Thermo Fisher) to remove contaminating estrogenic chemicals prior to using it for the mobile phase gradient. The vial trays were kept chilled throughout the runs. The ions were assessed using multiple reaction monitoring of the product ions following argon gas collision fragmentation. The transitions monitored were: 151.04/92.1, 165.06/92.07, 170.07/92.13, 193.1/92.09 and 227.13/211.9 for methyl paraben, ethyl paraben, propyl paraben, butyl paraben and BPA; tube lens 66, 70, 50, 80 and 80; using collision energy at 23, 23, 24, 24 and 25 respectively and collision gas (argon) pressure at 1.5 psi. Electrospray ionization was chosen in the –ve ion mode with activated divert valve to monitor only the chromatogram within the expected retention time frame. Using neat standards (Sigma-Aldrich), the parent/product masses and the retention time for the parabens and BPA were assessed and the limitation of the detection using this method was 0.1-0.3 part per billion (μg/L) for the analytical vial. Solid phase extraction was performed in the laboratory and then concentration of the sample onto the ion chromatograph column allowed for sample concentrations as low as 0.2 ppt to be detectable.

The data was analyzed using Xcalibr software package (Thermo) and the results were expressed and calculated as total ion current of the area under the curve and were plotted against a standard curve with external neat standards.

3.4 RESULTS AND DISCUSSION

All samples were analyzed for the following chemicals: methyl paraben, ethyl paraben, propyl paraben, butyl paraben, BPA, simazine, atrazine, propazine, and cyanazine. The result of the quality control check completed for methyl paraben to test accuracy was satisfactory, being within 20% of the expected concentration. Methyl paraben was added to a second sample collected from Springdale, and it was determined that there was less than a 50% recovery from the solid phase extraction. This was confirmed by a second extraction of water performed on the Springdale sample, which continued to show carryover of methyl paraben, in a concentration nearly equal to the original sample, implying that not all methyl paraben is extracted by one pass through the C18 column. It is determined that the solid phase extraction process used in this method has a less than 50% recovery rate for methyl paraben in the river water matrix possibly because there was not enough C18 media for the unknown concentrations of chemicals or possibly due to clogging of the C18 media from solids in the sample. These results are different from the quality control check because it was a deionized water matrix, which would not cause clogging of the C18 media like the surface water matrix could. A duplicate sample analyses was attempted on samples collected from Ford City, however it was not a true duplicate sample because the analyses were not performed exactly the same way. Solid phase extraction volumes were different due to the turbidity and suspended solids in the sample. Before performing the

duplicate extraction, it was decided that filtration would be necessary to extract an adequate volume for analysis, thus slightly altering the true nature of a duplicate sample. The results of this duplicate sample were variable. Methyl and propyl parabens were reproducible within 25% of each other. Propazine and cyanazine were not detected in either sample. Atrazine was precise with a 4% difference between samples. However, ethyl paraben, butyl paraben and simazine were found to have low level positive results in one sample, but were not detected in the other. The BPA results had the most variation, with the first sample having a significant positive result of 15.4 ppt and the duplicate sample only having a small positive result (1.2 ppt). These discrepancies are likely attributed to the difference in volumes filtered for the sample, allowing for error in calculation caused by the extraction process. It may be possible that the discrepancies may be due to laboratory contamination, although this is less likely for these analytes than for the analytes that did not have discrepancies. It is also possible that some of the BPA was removed during the filtration process, because it is suspected that BPA will adsorb onto solids because of its high sediment adsorption coefficient [33].

Results were compared to a methanol blank and any values below this blank are determined to be non-detectable as well. Methyl paraben was detected in all water samples at concentrations above the methanol blank value ranging from 2.2 -17.3 ppt. Ethyl paraben was detected in only one sample, and the results were less than the methanol blank values, therefore, we can conclude that ethyl paraben was not detectable in the rivers of Pittsburgh. Propyl paraben was detected in one sample above the methanol blank values at the Ford City Location (confirmed by the duplicate sample analysis) at concentrations of 9.2 and 12.0 ppt. Butyl paraben was only detected in the duplicate analysis of Ford City, at the very low concentration of 0.2 ppt. BPA was detected in five out of six sample locations with concentrations ranging from

0.6-15.4 ppt. Simazine was detected in all six sample locations in the range of 0.4 -2.7 ppt. Atrazine was detected in all samples with concentrations ranging from 1.0-5.3 ppt. Propazine and cyanazine were not detected in any sample. Table 13, Results of Surface Water Samples (ng/L or ppt), lists the exact concentrations for all of these analytes.

Table 13. Results of Surface Water Samples (ng/L or ppt)

Sample Location	**Methyl Paraben**	**Ethyl Paraben**	**Propyl Paraben**	**Butyl Paraben**	**Bisphenol A**
Ford City	12.3	nd	9.2	nd	15.4
Ford City Duplicate	16.0	nd	12.0	0.2	1.2
Freeport	6.0	nd	nd	nd	0.6
Springdale	10.0	nd	nd	nd	2.0
Harmarville	2.2	nd	nd	nd	nd
Braddock	17.3	nd	nd	nd	0.8
Monessen	13.0	nd	nd	nd	0.6
Sample Location	**Simazine**	**Atrazine**	**Propazine**	**Cyanazine**	
Ford City	nd	3.8	nd	nd	
Ford City Duplicate	0.4	4.0	nd	nd	
Freeport	1.0	4.0	nd	nd	
Springdale	2.5	5.0	nd	nd	
Harmarville	1.1	4.4	nd	nd	
Braddock	2.7	5.3	nd	nd	
Monessen	2.0	1.0	nd	nd	
***'nd' = not detectable**					

3.5 PUBLIC HEALTH SIGNIFICANCE

The concentrations of BPA found in Pittsburgh rivers were fairly low compared to the range of 0.0005 – 8 μg/L reported in published literature from around the world [37-43, 45-53, 55, 57, 155]. The results were very similar to value of 28 ng/L reported for the Czech Republic [57]. The concentrations of parabens were also similar to published literature ranging from 0.8 – 105 ng/L [119]. While this research was obviously a pilot experiment with only six sample locations studied, it can be inferred that the rivers in the Greater Pittsburgh Area do show positive levels of suspected xenoestrogens in the surface water media.

4.0 ANALYSES OF EXTRACTS FROM FISH BRAIN TISSUE FOR XENOESTROGENS AND ESTROGENIC POTENTIAL

In order to further investigate the presence of xenoestrogens in the Greater Pittsburgh Area, fish brain tissue was selected for analysis because it has a high lipid content and would therefore be likely to have a higher potential to bioconcentrate chemicals in that area.

The method utilized in Chapter 3 to study xenoestrogens in a surface water matrix was further developed to allow for the analysis of parabens and Bisphenol A (BPA) in a tissue matrix. Because methods of solid phase extraction are not available for tissue samples, derivatization by dansyl chloride was necessary for the analysis via High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS). Dansyl chloride derivatization enhances the specificity of the determination of xenoestrogens. Dansyl chloride easily reacts with phenolic hydroxyl and amino groups, which tend to be present in most suspected Endocrine Disrupting Chemicals (EDCs). Interference from other compounds decreases when detection is specified for the dansyl derivatives [62]. Derivatization with dansyl chloride has been used previously with liquid chromatography methods to allow for greater sensitivity and selectivity of alkylphenols [68]. Fish were sampled from similar locations to the surface water study described in Chapter 3 so that observed Bioconcentration Factors (BCFs) could be calculated.

MCF-7 cell proliferation assays are becoming a common practice for determining the estrogenicity of a sample. Previous research has studied the total estrogenicity of fish

compounds by performing MCF-7 cell line analysis to determine cell proliferation rates after exposure to the fish serum [148, 149]. However, these methods do nothing to help determine which chemicals are causing the increases in estrogenicity of the fish. Little, if any, research has been published linking MCF-7 proliferation results to specific environmental xenoestrogen exposures. In this study, extracts from fish brain tissues were analyzed for estrogenicity in an attempt to correlate the results of the HPLC-MS analysis to the estrogenicity of the samples.

4.1 OBJECTIVES

The objectives of this study were as follows:

- To develop a method for analyzing xenoestrogens, particularly BPA and parabens, in a tissue matrix.
- To determine if concentrations of xenoestrogens were detectable in extracts from fish brain tissue sampled in the Greater Pittsburgh Area.
- To determine the estrogenicity of the extracts from fish brain tissues via Bromodeoxyuridine (BrdU) Analysis using MCF-7 human breast cancer cells.
- To determine if there are relationships between fish characteristics and the results of either of these analyses (HPLC-MS and/or BrdU).
- To determine if there is a correlation between the detected xenoestrogens and estrogenicity, if applicable.

4.2 MATERIAL AND METHODS

4.2.1 Sampling Locations

A total of seven sampling sites were selected for this study. The Center for Healthy Environments and Communities (CHEC) and Allegheny River Stewardship Project (ARSP) researchers collected samples from Freeport, Fort City, and Springdale locations based on investigation from previous studies from those same locations described in Chapter 2 of this dissertation [148, 149]. CHEC then paired with researchers from the United States Geological Survey (USGS) who were taking fish samples in the area to extend this research to the other four locations, (Kittanning, Harmarville, Braddock, and Monessen) which also overlapped with previous CHEC research locations.

Six of the seven sampling locations are the same as listed in Chapter 3 for surface water analysis via HPLC-MS. Fish from Kittanning, Pennsylvania were available from USGS and were determined to be valuable to this study.

Kittanning, Pennsylvania is located on the Allegheny River approximately 44 miles northeast of Pittsburgh. The sampling site is located downstream from one Combined Sewer Overflow (CSO), as determined by the Pennsylvania Department of Environmental Protection (PA DEP) data file searches, and is upstream from the Allegheny River Lock and Dam No 7, in the Lock and Dam 7 Pool [150].

Refer to Chapter 3 for descriptions of CSO outfalls and lock and dam pool information for all other sample locations. Figure 28, Map of Seven Fish Sample Locations with Nearby Sewage Outfalls, shows the seven locations of fish sampling, the relative locations of WWTPs and CSO overflows and barriers of fish movement offered by lock and dam systems. Although

fish are generally free to move within a specific lock and dam system, the fish sampled from these seven locations are independent of one another. Lock and dam facilities exist along the Allegheny and Monongahela Rivers between each sample location, making it unlikely that the fish would travel across dams to reach the other locations [150].

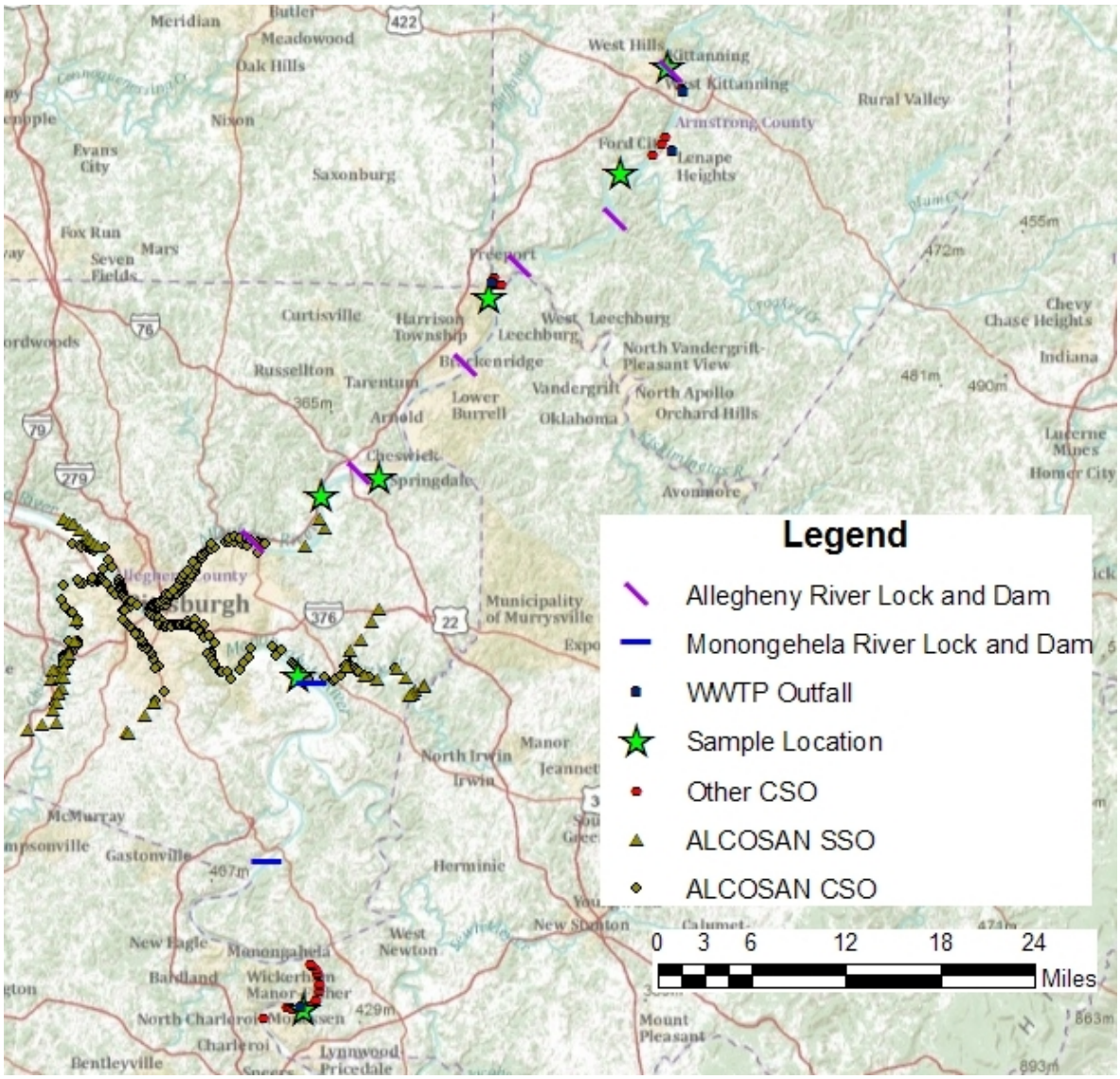


Figure 28. Map of Seven Fish Sample Locations with Nearby Sewage Outfalls

4.2.2 Sampling Methods

The methods for sampling and dissecting fish were described in Chapter 2 of this dissertation. All of the same regulations and protocols were followed for this study as well.

4.2.3 Sample Extraction and Preparation for HPLC-MS Analysis

Samples were placed in a polypropylene round bottom tube (Falcon-352063) and 2 mL of Environmental Grade Water Optima LC/MS (Fisher-W6-4) were added. Using Tissue Tearor (Biospec Products-985370), the tissues were homogenized in the polypropylene tubes. Each homogenized tissue was then divided into two 1 mL aliquots, stored in disposable culture tubes (Fisher-14-958-G). Next, two mL of ethyl acetate was added to each tube, and tubes were mixed at high speed for 20 seconds. Samples were then placed in the centrifuge at 3000 RPM for three minutes. The solvent phase of the mixture was transferred to a new culture tube and vacuum dried using Speed Vac Plus (Savant-SC110A) and Refrigerated Vapor Trap (Savant-RVT100) in the Gel Dryer Vacuum System. Two mL of hexane was added to the aqueous phase of the centrifuged sample, mixed for 20 seconds at maximum speed and centrifuged again at 3000 RPM for three minutes. The solvent phase was then transferred to a new tube and vacuum dried as described above. After drying, all samples were stored at -20^{o}C and protected from light. Each sample was then extracted into four tubes, two from the ethyl acetate solvent phase and two from the hexane solvent phase. Three hundred μL of methanol was then added to each tube and all were vortexed. One hundred twenty μL was extracted for straight analysis, 120 μL for was extracted for dBPA present analyses, and the remaining 60 μL was derivatized as described in the next section.

4.2.4 Derivatization for HPLC-MS Analysis

The background concentrations of BPA and parabens in the mobile phase generated measurable signals because of the concentration effect of the C-8 column. In an effort to increase the sensitivity of the assay, a method that relies on chemical derivatization of OH-containing compounds was adopted. The samples were extracted as described above from fish brains. Environmental-grade water used for the extraction contained 100 ppt of 16d (deuterium) BPA as an internal quality control. Following vacuum desiccation of the organic phase, the samples were resuspended in 100 μL sodium bicarbonate (0.1 M in water). Dansyl chloride (Sigma-Aldrich) was added (100 mL, 1 mg/mL) in acetone, briefly mixed with vortexing and incubated in a water bath for 10 minutes at 60^{o}C. The samples were then cooled and extracted with 1.5 μL ethyl acetate. The organic phase was separated by centrifugation and dried under vacuum. The derivatized samples were resuspended in 100 mL methanol and moved to HPLC screw top vials.

4.2.5 Analytical Method for HPLC-MS Analysis

The newly derivatized ions were assayed via HPLC-MS on a triple quadrupole mass spectrometer (TSQ Discovery Max) using electrospray ionization in the positive ion mode. The new m/z for the dansylated (DS) compounds were: 386.14, 399.93, 4 14.08, 428.08, 440.25, 440.25, 454.26, 522.3, 530.3, 695.29, 709.31 f or DS-MP, DS-EP, DS-PP, DS-BP, DS-octyl phenol, DS-tert-octyl phenol, DS-nonyl phenol, DS-estriol, DS-ethynyl estradiol, DS-BPA and DS-16d-BPA respectively. The prominent product ions after argon gas collision were 171.1.1, 171.06, 171.06, 170.97, 170.97, 170.97, 171.06, 170.95, 170.1 and 170 and the collision energy used was 27, 25, 28, 27, 29, 29, 34, 34, 37, 36 respectively. The samples were resolved on C-8

reverse phase column (C-8 Hypersil gold) using mobile phase gradient for 30 minutes with 0.1% formic acid (A) and 100% acetonitrile (D). The gradient for this HPLC method was: 0 min 99% A 1% D, 3 min 60% A 40% D, 22 min 100% D, hold 100% D for 3 minutes, 29 min 99% A 1% D and 2 minutes hold for the last condition. The injection volume was 5 μL and the needle was washed between the samples in 100% methanol. This method was unsuccessful for DS-octyl phenol, DS-tert-octyl phenol, and DS-nonyl phenol due to interferences at the peak retention time and/or background contamination. These problems did not occur for BPA or parabens.

The data was analyzed using Xcalibr software package (Thermo) and the results were expressed and calculated as total ion current of the area under the curve and were plotted against a standard curve with external neat standards. With the derivatized compounds, DS-16d-BPA was used as internal calibrator or quality control for the extraction and loading of the samples. The limitation of the detection using this method was 0.1-0.3 part per billion (μg/L) in the sample vial.

4.2.6 Analytical Method for Bromodeoxyuridine (BrdU) Analysis

Fish brain samples were weighed and their masses were recorded before liquid phase extraction with environmental grade water and ethyl acetate occurred, as described above. The solvent phase was then extracted and dried. One hundred μL of methanol was added. Five μL of the brain extract in methanol was added to MCF-7 breast cancer cells and left to sit for one day. Bromodeoxyuridine (BrdU) was added to the cell cultures. BrdU works by substituting the 'T' in DNA synthesis with a 'U' attached to bromine. U-Br on the newly synthesized DNA can then be detected with an antibody. Media was removed from the cell plate after an incubation period. This leaves the cells attached to the plate. D NA was then extracted out of the cells and

dissolved. The protein was removed and the DNA precipitated. DNA was reconstituted in TE Buffer.

4.2.7 MCF-7 DNA Isolation

Cells were collected with 100 μL lysis solution. One μL RNase A was added to the lysate, then inverted 25 times and incubated for five minutes at 37°C. Samples were cooled to room temperature in order to precipitate the protein by using ice for one minute. Next, 33 μL of protein precipitate was added to the solution, vortexed vigorously at high speed for 20 seconds, and then centrifuged at 13,000-16,000 xg for 1 minute. This was repeated until a tight pellet was formed. The supernate was poured into clean 1.5 mL eppendorf tubes containing 150 μL of 100% isopropanol (2-propanol) used to precipitate the DNA. It was then mixed by inverting 50 times and centrifuged at 13,000-16,000 xg for one minute or until the DNA was visible. Again, the supernate was poured off and drained. One hundred microliters (100 μL) of 70% ethanol was added, inverted several times, then centrifuged for one minute. The ethanol was poured off and dried. Fifty μL TE Buffer was added.

SSC Buffer was added to the sample during membrane preparation. DNA adheres to membrane and is dot-blotted on a Nitro-cellulous membrane using a western blot. The membrane was soaked in water and treated with UV light, then fixed with an anti-BrdU antibody. A second anti-body was added to detect the first, and light is emitted based on the intensity of the signal.

4.2.8 BPA and BrdU Data Comparisons and Corrections

Raw BrdU data was analyzed alongside of a known estradiol standard (3 ppb), as well as a known ethinyl estradiol standard (3 ppb), in order to compare results from the samples to determine their relative estrogenicity.

There is, however, the potential for error in pipetting to the initial cell plate during BrdU analysis, extraction of the DNA, and blot loading. An internal control check was performed to verify that equal amounts of DNA were loaded on to the blot. MCF-7 proliferation data were divided by the internal control check to correct for errors within DNA synthesis.

The fish extracts prepared for either analysis (HPLC-MS and MCF-7 cell proliferation assay) were not originally corrected for sample size. The whole brain sample was used during extraction, and therefore concentrations were different for each sample. To correct for this, the BrdU results corrected for DNA synthesis were also corrected for the weight of the fish brain sample. BPA vial concentrations in ng/L were back calculated based upon the weight of the fish brain sample for reporting results in pg/gram of fish brain.

4.2.9 Statistical Methods for BPA Analysis

Preliminary data analyses utilized BPA concentrations rather than BCF calculations because BPA concentrations were available for all fish (non-detectable results were considered to be zero for statistical purposes) and BCF concentrations were not available for all fish. BCFs could not be calculated in locations where surface water concentrations were non-detectable (Harmarville) nor could they be calculated for the Kittanning location because surface water samples were not collected. BCF is calculated from the BPA concentrations, so statistical results should be

similar. The preliminary statistics will provide information on which effects may be significant when creating the Subject Specific Random Effects Model to be used with the BPA data. To examine relationships between BPA concentration and certain fish characteristics, three nonparametric tests were utilized. Spearman's Rank Correlation was utilized for the continuous variables of weight and length. The Kruskal-Wallis test (the nonparametric alternative to one-way ANOVA) was utilized for the categorical variables species and location, and Wilcoxon Mann-Whitney was used for gender.

Univariate analysis of the BPA statistics showed that location and species may significantly influence BPA concentration in fish brain. A Subject Specific Random Effects Model was utilized to determine if these relationships would hold true after adjusting for additional covariates. BPA concentrations were utilized rather than the BCF because they were available for all 58 fish, as BCF was only able to be calculated in five of the seven locations.

The covariates considered for entry into the model were species, gender, weight, length and location. All fish had complete information on species and location. One Freeport sample was missing information on gender. Gender, weight and length data were not available from the Braddock, Harmarville, Kittanning and Monessen locations. A version of step-wise model selection was utilized to determine the optimal model for the data [158]. Because of collinearity errors, two models were selected. The incomplete information on gender, weight and length, as well as small sample sizes caused collinearity issues and was prohibitive of the full investigation of effects from all factors.

4.2.10 Statistical Methods for BrdU Analysis

Preliminary data analyses utilized average BrdU corrected analysis results rather than individual repeated results because univariate analysis is not adequate for repeated measures statistics. The preliminary statistics will provide information on which effects may be significant when creating the Subject Specific Random Effects Model to be used with the BrdU data. To examine relationships between BrdU analysis results and certain fish characteristics, three nonparametric tests were utilized. Spearman's Rank Correlation was utilized for the continuous variables of weight and length. The Kruskal-Wallis test (the nonparametric alternative to one-way ANOVA) was utilized for the categorical variables species and location, and Wilcoxon Mann-Whitney was used for gender.

Univariate analysis of the Average BrdU statistics showed that length and species may significantly influence BrdU analysis results. A Subject Specific Random Effects Model was utilized to determine if these relationships would hold true after adjusting for additional covariates. Average BrdU statistics were utilized rather than the individual analysis for each fish because univariate analysis does not account for repeated measured analytical results.

The covariates considered for entry into the model were species, gender, weight, length and location. All fish had complete information on species and location. One Freeport sample was missing information on gender. Gender, weight and length data were not available from the Braddock, Harmarville, Kittanning and Monessen locations. Collinearity errors were a concern for this model, as it was for the BPA models. However, adjustments for these errors did not reveal any new findings and were therefore excluded from this summary. A version of step-wise model selection was utilized to determine the optimal model for the data [158].

Spearman's Rank Correlation testing will also be utilized to determine if there is a relationship between the concentration of BPA and the average BrdU analysis results.

4.3 RESULTS

4.3.1 Analytical Results

Fifty eight fish were sampled from the rivers in the Greater Pittsburgh Area. All fish were tested for methyl, ethyl, propyl and butyl paraben, as well as BPA. All samples were non-detectable for methyl, ethyl, propyl and butyl parabens. BPA was detected in 44 of the 58 samples, with a range from non-detectable up to 120 pg/gram of fish brain (or ppt by weight) and an average of 16.4 pg/gram fish brain. dBPA was found positive in all samples at the correct retention time to validate the accuracy of BPA detection. Table 14, Individual Fish Data and Analytical Results for BPA, contains all individual fish concentrations of Bisphenol A, as well as fish characteristics for all samples.

4.3.2 Bioconcentration Factors (BCFs)

Bioconcentrations factors (BCFs) were calculated for fish using the analytical results from the surface water study described in Chapter 3 of this dissertation. BCFs were calculated as a ratio of the final BPA concentration in pg/gram of fish brain (or ppt by weight) to the surface water concentration in ng/L (or ppt). BCFs could not be calculated for the Harmarville location

Table 14. Individual Fish Data and Analytical Results for BPA

Fish	Species	Gender	Location	Weight (g)	Length (cm)	BPA (pg/g)
1	Shad	Male	Freeport	200	30.3	2.0
2	Alewife	Male	Ford City	150	25.5	3.0
3	Shad	Male	Freeport	160	26.8	15.7
4	Alewife	Male	Ford City	250	30.0	13.0
5	Shad	Male	Freeport	175	25.1	11.5
6	Alewife	Female	Ford City	350	31.9	4.4
7	Shad	Female	Ford City	203	28.4	4.1
8	Small Mouth Bass		Freeport	530	34.5	7.1
9	Shad	Male	Freeport	150	27.5	4.0
10	Shad	Male	Freeport	550	37.4	3.8
11	Alewife	Male	Ford City	175	26.5	7.5
12	Small Mouth Bass	Male	Springdale	107	21.6	2.5
13	Small Mouth Bass	Male	Freeport	125	22.4	3.3
14	Small Mouth Bass	Male	Freeport	400	35.1	nd
15	Alewife	Male	Ford City	165	26.5	3.7
16	Small Mouth Bass	Male	Freeport	200	26.5	15.0
17	Alewife	Male	Ford City	275	31.4	nd
18	Small Mouth Bass	Male	Springdale	205	25.9	8.2
19	Shad	Female	Freeport	250	30.7	nd
20	Shad	Male	Freeport	200	29.3	3.1
21	Shad	Female	Freeport	225	29.8	66.7
22	Alewife	Male	Ford City	175	26.3	nd
23	Alewife	Male	Ford City	230	28.4	2.9
24	Small Mouth Bass	Male	Ford City	100	22.5	3.7
25	Shad	Male	Freeport	650	37.9	nd
26	Shad	Male	Ford City	167	26.6	nd
27	Small Mouth Bass	Male	Ford City	150	26.5	22.1
28	Alewife	Male	Ford City	200	27.0	6.9
29	Small Mouth Bass	Male	Springdale	395	31.9	1.3
30	Small Mouth Bass	Male	Springdale	150	23.5	7.5
31	Small Mouth Bass	Male	Freeport	135	24.0	5.9
32	Small Mouth Bass	Male	Ford City	190	25.2	27.3
33	Small Mouth Bass	Male	Freeport	190	19.9	nd
34	Small Mouth Bass	Male	Springdale	435	33.4	nd
35	Small Mouth Bass	Male	Springdale	400	32.0	nd
36	Alewife	Male	Ford City	197	27.5	nd
37	Small Mouth Bass	Female	Ford City	317	29.5	23.5
38	Small Mouth Bass	Male	Springdale	222	26.7	20.0
39	Small Mouth Bass	Male	Ford City	375	31.2	25.0
40	Small Mouth Bass		Monessen			8.9
41	Small Mouth Bass		Monessen			22.5
42	Small Mouth Bass		Monessen			85.7

Table 14 Continued

Fish	Species	Gender	Location	Weight (g)	Length (cm)	BPA (pg/g)
43	Small Mouth Bass		Monessen			32.0
44	Small Mouth Bass		Braddock			11.5
45	Alewife	Male	FordCity	300	31.7	7.5
46	Shad	Male	Freeport	180	27.3	nd
47	Alewife	Male	Freeport	200	28.4	nd
48	Alewife	Male	Freeport	160	26.4	nd
49	Alewife	Female	Freeport	320	31.9	40.0
50	Shad	Male	Freeport	140	24.4	nd
51	Shad	Female	Freeport	600	42.9	3.0
52	Shad	Female	Freeport	450	35.7	0.1
53	Small Mouth Bass		Kittanning			6.9
54	Small Mouth Bass		Kittanning			9.0
55	Small Mouth Bass		Kittanning			0.3
56	Small Mouth Bass		Harmarville			120.0
57	Small Mouth Bass		Harmarville			60.0
58	Small Mouth Bass		Harmarville			3.6
"nd" = not detectable						

because surface water results were non-detectable. BCFs could also not be calculated for the Kittanning sample location because this location was not analyzed as part of the surface water study. Table 15, Bioconcentration Factors (BCF) for BPA Using Individual Surface Water Results, shows the ranges of BCFs by location and species. In order to calculate BCFs for all locations and to determine what may be more representative BCFs for the entire Pittsburgh area, BCFs were also calculated using an average of all of the surface water results from Chapter 3. The average value used for calculations was 2.9 ppt, which included all six positive results from the seven analyses total (Ford City had two positive results). The non-detectable result from Harmarville was assumed to be zero for averaging purposes. These calculations are included in Table 16, Bioconcentration Factors (BCF) for BPA Using Average Surface Water Results From All Locations. Calculations performed using the average of the surface water results determined BCFs to be much lower than the localized calculations. This implies that the general Pittsburgh

area does not have a regional problem with bioconcentration effects of BPA in fish tissue, but that specific communities within the Greater Pittsburgh Area may have a higher risk than others, influenced by what industry is in the vicinity.

Table 15. Bioconcentration Factors (BCF) for BPA Using Individual Surface Water Results

Location	Number of Samples	Number of Positive Results	Mean Concentration (pg/g or ppt by weight)	BCF for Shad	BCF for Alewife	BCF for Small Mouth Bass
Freeport	22	14	12.9	0.2-111.1	66.7	5.6-25.0
Ford City	18	14	11.0	3.4	2.4-10.9	3.1-22.7
Braddock	1	1	11.5	N/A[(a)]	N/A[(a)]	14.4
Monessen	4	4	37.3	N/A[(a)]	N/A[(a)]	14.8-142.9
Springdale	7	5	7.9	N/A[(a)]	N/A[(a)]	0.7-10.0
Total	52	38	7.9-37.3	0.2-111.1	2.4-66.7	0.7-142.9
(a) Species not sampled at this location.						

Table 16. Bioconcentration Factors (BCF) for BPA Using Average Surface Water Results From All Locations

Location	Number of Samples	Number of Positive Results	Mean Concentration (pg/g or ppt by weight)	BCF for Shad	BCF for Alewife	BCF for Small Mouth Bass
Freeport	22	14	12.9	0.05-23.0	13.8	1.1-5.2
Ford City	18	14	11.0	1.4	1.0-4.5	1.3-9.4
Braddock	1	1	11.5	N/A[(a)]	N/A[(a)]	4.0
Monessen	4	4	37.3	N/A[(a)]	N/A[(a)]	0.7-3.1
Springdale	7	5	7.9	N/A[(a)]	N/A[(a)]	0.5-6.9
Harmarville	3	3	61.2	N/A[(a)]	N/A[(a)]	1.2-41.4
Kittanning	3	3	5.4	N/A[(a)]	N/A[(a)]	0.1-3.1
Total	58	44	5.4-61.2	0.05-23.0	1.0-13.8	0.1-41.4
(a) Species not sampled at this location.						

4.3.3 Statistical Results for BPA Analysis

Univariate statistical analysis determined that there were no significant correlations between BPA concentration and length or weight. There was also not a significant relationship between gender and BPA. A significant relationship between species and BPA concentration was found

(p = 0.03) and a borderline significant relationship was found between BPA concentration and location (p = 0.06). Table 17, Relationships Between BPA and Fish Characteristics from Preliminary Univariate Analysis**,** contains p-values and test methods utilized for univariate analysis of BPA concentrations. These results were further investigated utilizing Subject Specific Random Effects statistical models.

Table 17. Relationships Between BPA and Fish Characteristics from Preliminary Univariate Analysis

Characteristic	p-value	Test
Length	0.4629	Spearman's Rank Correlation
Weight	0.8419	Spearman's Rank Correlation
Species	**0.0314**	Kruskal-Wallis
Gender	0.1873	Wilcoxon Mann Whitney
Location	0.0613	Kruskal-Wallis

After model selection, location and gender were initially found to be significant predictors of BPA concentration when using all seven sample locations as determined using the Subject Specific Random Effects Model. However, the location and gender factors could not be placed into a model together because of collinearity errors, so they were placed into separate models. Although species was not found to be a significant predictor during the step-wise model selection process, species was selected to remain in both models because of results from the univariate analysis.

Table 18, The BPA Location Model**,** lists the results of the location model statistics. Harmarville was found to produce significantly higher results than all of the other locations. This is particularly interesting because Harmarville was the only surface water sample to produce non-detectable results.

As described, gender was also found to be a significant predictor of BPA concentration. Table 19, The BPA Gender Model, lists the results of the gender model statistics. Males were found to have a significantly lower BPA concentration than females from these locations.

Table 18. The BPA Location Model

Parameter			*Parameter Estimate*	*95% Confidence Limits*		*Standard Error*	*Pr > ChiSq*
Location (baseline='Braddock'; n=1)	*Ford City*	8	1.269692	-34.5712	37.11059	18.28651	0.945
	Freeport	2	-697427	-35.6994	35.55991	18.17873	0.997
	Harmarville	***3***	***49.66***	***10.80591***	***88.51409***	***19.82388***	***0.012***
	Kittanning	3	-.143333	-4.99742	32.71076	19.82388	0.757
	Monessen	4	25.735	-1.88531	63.35531	19.19439	0.180
	Springdale	7	-.891428	-1.86333	30.08047	18.35335	0.748
Species (baseline='Alewife'; n=14)	*Shad*	5	2.126917	-2.94078	17.19462	7.687743	0.782
	Sm Mouth Bass	9	6.179099	-.034173	20.39237	7.251802	0.394

Table 19. The BPA Gender Model

Parameter			*Parameter Estimate*	*95% Confidence Limits*		*Standard Error*	*Pr > ChiSq*
Gender (baseline='Female'; n=8)	***Male***	**8**	***-13.5959***	***-0.70724***	***-0.484558***	***4.648728***	***0.003***
Species (baseline='Alewife'; n=14)	*Shad*	5	-1.3366	-0.802331	7.129131	4.31933	0.757
	Sm Mouth Bass	9	4.525412	-0.558123	12.60895	4.124328	0.273

The significant results are different from the univariate analysis. This is because the Subject Specific Random Effects Model adjusts for other covariates while the univariate analysis only considers one factor at a time. After adjusting for gender and location, species no longer showed a significant effect on BPA concentrations. The effect that was seen in the univariate analysis may actually have been caused by gender or location, not species.

Table 20. BrdU Analyses Results

Fish	1st BrdU Raw Analysis	2nd BrdU Raw Analysis	Average BrdU Raw Analysis	1st BrdU Corrected Analysis	2nd BrdU Corrected Analysis	Average BrdU Corrected Analysis
1	0.402	0.310	0.333	10.55	12.73	11.64
2	1.683	1.029	1.192	189.02	69.55	129.29
3	0.553	1.375	1.169	14.64	42.97	28.80
4	0.771	0.500	0.568	8.19	7.30	7.75
5	0.696	0.662	0.671	19.01	14.77	16.89
6	1.009	0.837	0.880	58.06	43.69	50.87
7	0.552	1.082	0.949	15.97	30.12	23.04
8	1.607	2.073	1.957	61.94	90.60	76.27
9	1.509	1.698	1.651	35.70	38.70	37.20
10	0.949	0.000	0.237	21.32	0.00	10.66
11	0.279	0.468	0.421	21.32	29.36	25.34
12	1.255	-0.073	0.259	61.35	-9.71	25.82
13	1.031	1.091	1.076	32.38	37.72	35.05
14	1.268	1.316	1.304	25.40	18.90	22.15
15	1.794	1.989	1.940	103.13	105.81	104.47
16	1.777	1.877	1.852	176.74	184.06	180.40
17	1.548	1.499	1.512	60.76	63.41	62.09
18	1.829	1.352	1.471	105.24	97.09	101.16
19	-0.043	0.310	0.222	-2.37	17.94	7.78
20	0.750	1.047	0.973	14.88	21.99	18.44
21	1.210	1.769	1.629	110.89	124.42	117.65
22	1.524	1.171	1.260	145.94	127.20	136.57
23	1.450	1.411	1.421	47.80	50.21	49.00
24	1.189	0.747	0.857	67.04	35.77	51.41
25	0.000	0.064	0.048	0.00	1.65	0.82
26	0.249	0.886	0.727	14.74	54.55	34.64
27	1.333	1.490	1.451	33.28	52.33	42.80
28	0.726	1.280	1.141	78.58	108.35	93.47
29	1.014	1.673	1.508	20.39	27.61	24.00
30	1.370	1.806	1.697	61.54	66.20	63.87
31	0.453	0.458	0.457	14.91	17.50	16.20
32	1.176	1.218	1.207	34.41	33.72	34.07
33	1.675	1.093	1.238	111.49	84.04	97.77
34	0.442	0.837	0.738	11.61	19.63	15.62
35	0.853	0.691	0.732	28.38	25.05	26.71
36	0.632	0.170	0.285	26.83	6.27	16.55
37	0.414	0.604	0.556	10.64	16.80	13.72
38	1.078	0.760	0.839	38.37	26.58	32.47
39	1.155	0.690	0.806	31.92	18.10	25.01
40	0.804	1.478	1.309	26.23	71.31	48.77
41	1.280	1.522	1.462	94.32	94.67	94.49
42	0.555	0.557	0.557	83.78	95.88	89.83
43	0.795	0.434	0.525	28.67	23.54	26.10
44	0.959	0.774	0.821	72.13	47.38	59.75
45	1.632	1.446	1.492	168.32	125.07	146.70
46	1.323	1.054	1.122	132.52	80.27	106.40
47	0.306	1.756	1.393	12.05	48.06	30.06
48	1.025	0.457	0.599	21.22	14.57	17.90
49	1.292	1.090	1.140	109.08	306.53	207.80

Table 20 Continued

Fish	1st BrdU Raw Analysis	2nd BrdU Raw Analysis	Average BrdU Raw Analysis	1st BrdU Corrected Analysis	2nd BrdU Corrected Analysis	Average BrdU Corrected Analysis
50	1.277	1.978	1.803	47.52	61.22	54.37
51	0.400	2.247	1.785	6.96	40.63	23.80
52	1.176	1.974	1.774	51.96	50.86	51.41
53	0.980	0.881	0.905	22.33	39.32	30.83
54	1.921	0.653	0.970	140.83	34.51	87.67
55	1.077	1.386	1.309	59.63	73.11	66.37
56	0.664	1.881	1.577	300.76	325.39	313.08
57	2.114	2.137	2.131	253.27	94.68	173.97
58	2.006	2.275	2.208	28.30	53.37	40.83

4.3.4 Descriptive Results of Raw BrdU Data

Raw BrdU data was analyzed alongside of a known estradiol standard (3 ppb) as well as a known ethinyl estradiol standard (3 ppb), in order to compare results from the samples to determine their relative estrogenicity. Table 20, BrdU Analyses Results, lists all the results from the BrdU analyses, including the average raw results from the two BrdU analyses. Figure 29, Graph of Average Raw BrdU Data Compared to Estradiol Control, is a graph of the average raw data plotted as a fold increase over the untreated MCF-7 control cells. The estradiol and ethinyl estradiol standards had a very similar response, therefore only one was included on the graph for comparison purposes. Two of the three Harmarville samples were greater than the estradiol control, exhibiting a very strong estrogenic response from this area.

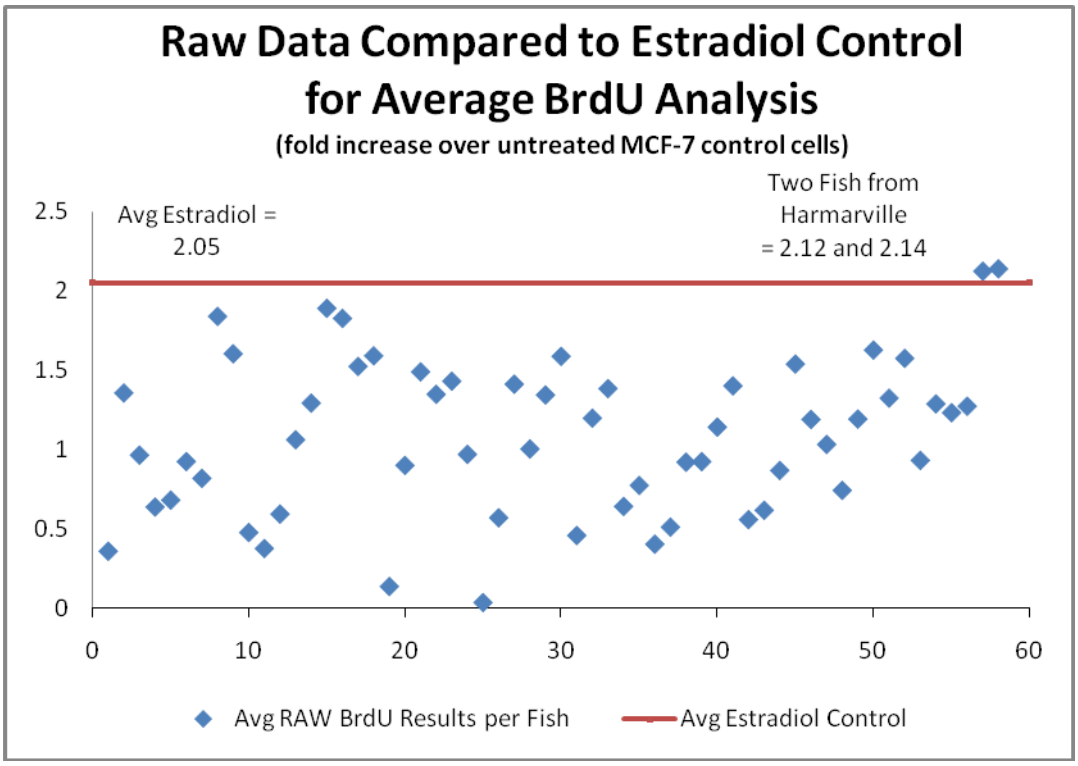


Figure 29. Graph of Average Raw BrdU Data Compared to Estradiol Control

4.3.5 Statistical Results for BrdU Analysis

Univariate statistical analysis showed no significant correlations between average BrdU analysis results and any of the fish characteristics. There were borderline significant relationships for length ($p = 0.06$) and species ($p = 0.07$). Table 21, Relationships Between Average BrdU Analysis Results and Fish Characteristics from Preliminary Univariate Analysis, lists all p-values and test methods utilized for univariate analysis of BrdU data. These results will be further investigated utilizing the Subject Specific Random Effects Model.

Table 21. Relationships Between Average BrdU Analysis Results and Fish Characteristics from Preliminary Univariate Analysis

Characteristic	p-value	Test
Length	0.0550	Spearman's Rank Correlation
Weight	0.1308	Spearman's Rank Correlation
Species	0.0715	Kruskal-Wallis
Gender	0.9770	Wilcoxon Mann Whitney
Location	0.3761	Kruskal-Wallis

After model selection, location and species were found to be significant predictors of BrdU analysis results. Although length was not found to be a significant predictor during the step-wise model selection process, it was selected to remain in the model because of results from the univariate analysis in order to be consistent with methods employed for BPA analyses. However, the length factor caused collinearity errors and could not be incorporated into the BrdU analysis model. Table 22, The BrdU Model, contains BrdU model statistics. Shad exhibited significantly lower estrogenicity when compared to the alewife. Harmarville was found to produce significantly higher results than all of the other locations. This is particularly interesting because Harmaville was also the significant location for BPA concentration and produced strong estrogenic potential for raw BrdU analytical comparisons.

Table 22. The BrdU Model

Parameter			*Parameter Estimate*	*95% Confidence Limits*		*Standard Error*	*Pr > ChiSq*
Location (baseline='Braddock'; n=2)	*Ford City*	6	-17.11418	-117.0978	82.86944	51.01299	0.737
	Freeport	4	3.853815	-95.54053	103.2482	50.71233	0.939
	Harmarville	***6***	***116.2051***	***7.815732***	***224.5945***	***55.30173***	***0.036***
	Kittanning	6	1.870021	-106.5194	110.2594	55.30173	0.973
	Monessen	8	5.044485	-99.90309	109.9921	53.54567	0.925
	Springdale	14	-18.37425	-118.7233	81.97482	51.19945	0.720
Species (baseline='Alewife'; n=28)	***Shad***	***30***	***-54.43011***	***-96.46374***	***-12.3964***	***21.44613***	***0.011***
	Sm Mouth Bass	58	-29.85474	-69.50482	9.795345	20.23	0.140

The significant results of the Subject Specific Random Effects Model are different from the univariate analysis. This happens because the model adjusts for other covariates while the univariate analysis only considers one factor at a time. The discrepancy is also likely because it was necessary to use the average BrdU analysis results, rather than the individual repeated measures for the univariate analysis. The model allows for the use of repeated measures, thus increasing the data set and providing more statistical power. Refer to Table 20, BrdU Analyses Results, for a listing of the individual corrected results utilized from the repeated measures analyses.

4.3.6 Correlation between BPA and BrdU Results

BPA concentration and average BrdU analysis results were tested using the Spearman's Rank Correlation test. BPA concentration and average BrdU analysis results were significantly correlated ($r = 0.2793$, $p = 0.0337$). See Figure 30, Graph of Average Corrected BrdU Data Compared to BPA Concentration, for a graph of the correlation.

4.4 CONCLUSIONS AND PUBLIC HEALTH SIGNIFICANCE

The statistical methods employed in this chapter discovered that location and gender have predictor effects on BPA concentration. Males had lower BPA concentrations than females. The Harmarville location had significantly higher BPA concentrations than all six other sampling sites. This is interesting because it was the one site that had non-detectable surface water BPA

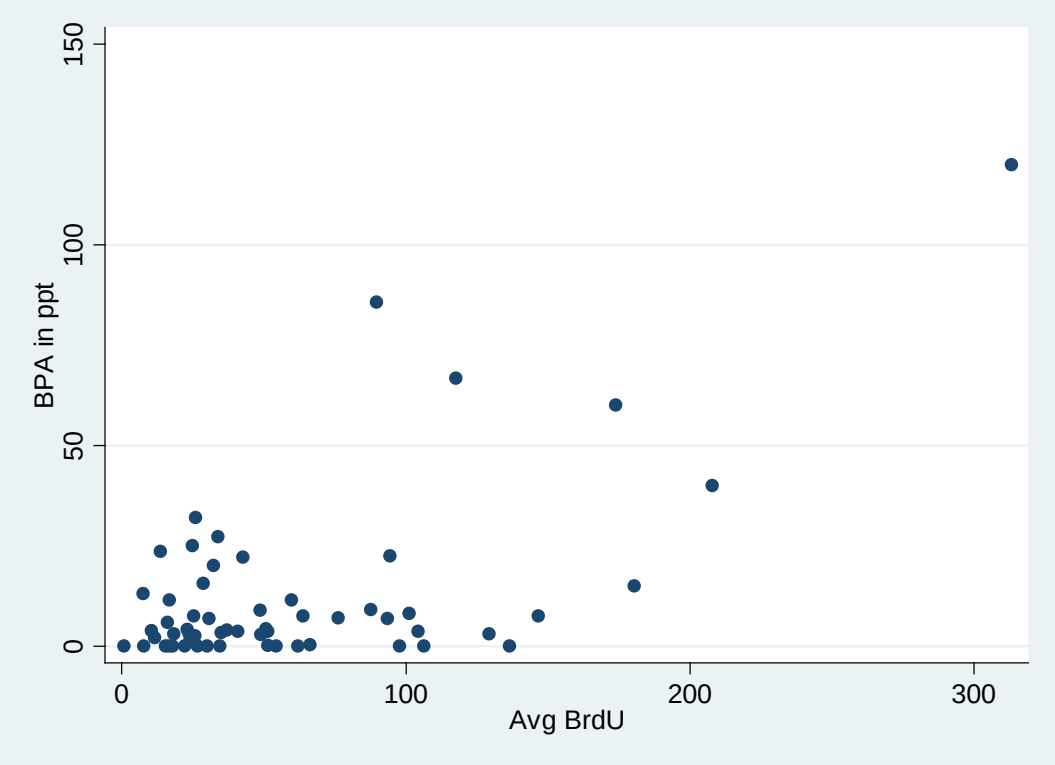


Figure 30. Graph of Average Corrected BrdU Data Compared to BPA Concentration

results in Chapter 3. That is probably most easily explained by the fact that only one surface water sample was obtained and analyzed. More accurate readings would have been available with a larger study. The statistical methods for BrdU analysis showed that location and species had predictor effects on estrogenicity of the samples. Shad had exhibited significantly lower estrogenicity when compared to the alewife. It was determined that this was not due to the size (as determined by weight or length measurements) of the fish.

The Harmarville sample site was of particular interest in all facets of this study. This location had higher results for raw BrdU analysis results, as well as significantly higher results for both BPA concentration and MCF-7 cell proliferation when compared to all six other sample sites during the statistical analyses of corrected results. It is interesting that the sample results

produced with the highest BPA concentrations also provided the greatest estrogenic response, leading to the conclusion that BPA may be associated with the increased MCF-7 cell proliferation. This correlation is statistically supported by the results of the Spearman's Rank Correlation, which described a significant relationship between BPA concentration and average BrdU analysis results.

Upon further investigation of the sample location, it was determined that there are epoxy paint manufacturing facilities located near the Harmarville sample site (both upstream and downstream). There is also a plastic bottling facility located less than two miles downstream of the sample location and within the same lock and dam pool. There is a good likelihood that fish exposed downstream could travel and be caught at an upstream location. These plants do not have direct permitted discharges into the Allegheny River according to the United States Environmental Protection Agency's (EPA) public database. They do, however, have discharges into tributaries that flow into the Allegheny River [159]. The proximity to manufacturing facilities which utilize BPA in their products could account for the significantly higher results found at the Harmarville location.

BPA has an Octanol-Water Partition Coefficient (K_{ow}) of 3.32, indicating that its lipophilicity will allow it to bioconcentrate in exposed fatty tissues [33]. Exposed fish will bioconcentrate BPA via oral and gill exposure routes and therefore have the potential to cause the bioaccumulation of BPA throughout the food chain. This concept has not been generally accepted as it is believed that BPA will be conjugated in the gastrointestinal tract quickly enough not to allow for bioconcentration [33]. In our research, we tested fish brains; not as a pathway to human exposure, but to study the bioconcentration properties of BPA. Brain tissue has a high lipid content, and would therefore be likely to have a higher potential to bioconcentrate

chemicals than fish flesh. Analytical methods for testing fish tissue have been available since the 1990s, however, they have mostly focused on the edible tissues of fish, leaning towards a method to quantify exposure [63]. We have proven that BPA can be bioconcentrated in the brain tissue of fish.

Although there are no specific criteria for determining if a chemical is likely to bioaccumulate in the environment, the EPA generally uses a BCF factor of >1000 as determining a chemical to be a persistent environmental hazard [160]. None of the BCFs calculated in this study are anywhere near that range, and are at least a factor of 10 lower than that. This would lead to the conclusion that BPA is not a strongly persistent chemical. However, the European Union considers that any chemical with a BCF >100 has the potential to bioaccumulate in the environment and is therefore a danger to the environment [161, 162].

BPA has a log K_{ow} equal to 3.32, indicating that it will bioconcentrate [27]. Experimental studies have calculated the expected BCF for BPA to be 68 [161]. Using the log K_{ow} of 3.32 and a general BCF equation derived for a variety of fish species and chemicals, the calculated (or expected) BCF for BPA is exactly 100 from octanol-water partitioning alone, indicating that BCFs lower than this are not really bioconcentrating [163]. The observed BCFs reported in this chapter are comparable to both expected values.

Expected values for parabens should be theoretically similar to BPA but because the log K_{ow} values for parabens are slightly lower than BPA, it is expected that they would have a weaker ability to bioconcentrate in the fish tissue. Log K_{ow} values reported for parabens increase with increasing chain length: below 3 for methyl and ethyl paraben, just around 3 for propyl paraben, and are greater than 3 for butyl paraben [104, 105, 107, 108]. However, they might still be expected to bioconcentrate if the chemicals had been detectable in the surface water. In our

study, only methyl paraben was found at consistent concentrations across all locations. This analyte has the lowest log K_{ow}, and therefore the least likelihood to be bioconcentrated. It is not surprising that we were unable to detect parabens in extracts from brain tissue of the fish from these locations after analyzing the surface water locations also.

Environmental concentrations of BPA found in this study (maximum of 120 pg/g and average 16 pg/g) may be at biologically relevant concentrations. Median estradiol values in the human body range from 100 – 1000 pmol/L (50 – 250 pg/mL) for the average woman of child-bearing age [164, 165]. Assuming that the density of human blood and fish brains are relatively similar, these concentrations are within the same magnitude of each other. BPA is known *in vitro* to be approximately 10,000 t imes weaker than estradiol [13]. However, this study determined that fish with the highest levels of BPA (from Harmarville) also exhibited significant estrogenic effects in the BrdU assay, at levels equivalent to the estradiol controls. This suggests a few possibilities that cannot be determined from this study: BPA may not be the only contributor to the estrogenicity of the fish extract, BPA may have an additive/synergistic effects in combination with unknown chemicals present in the sample, or BPA is more potent than originally thought.

Usually bioconcentration studies are employed to determine exposure pathways to humans. However, this is not the case with a study on fish brains because they are rarely a food source by the general American public, although some local anglers and foreigners do consume the whole fish. Fish brains were selected for this study because they are high in fat content, and therefore would be a good site for lipophilic chemicals to choose concentration. Although this study may not be illustrating an exposure pathway to humans, it could be modeling what is happening in the human body. It is impossible to test if BPA is bioaccumulating in the brains of

human subjects because of ethical issues, but there may be a true public health need to investigate the idea further. There is evidence of higher prevalence rates in psychological and learning disorders, like autism, in children, which may or may not be due to an environmental concern [166]. EDCs or xenoestrogens, such as BPA, may be to blame for their methods of endocrine disruption or simply because of their toxic ability to bioaccumulate in areas of the brain affecting the learning centers.

It is possible for BPA to be concentrating in the brains of fish by one of two different mechanisms: directly entering the bloodstream then crossing the blood-brain barrier or by axonal transport. All vertebrates are known to have a blood-brain barrier which helps to keep toxic agents out of the brain [167]. Axonal transport is the normal physiological process for the transport of dissolved chemicals along nerve axons, thus circumventing the protection of the blood-brain barrier [168-170]. It is unknown at this time which mechanism is causing the BPA to enter the brains of fish. BPA has already been found in human blood and proven to cross the maternal-fetal placental barrier [13, 87]. If it is determined that BPA is crossing the blood-brain barrier, it could have grave public health consequences. Current research in neuroendocrinology is focusing efforts on the effects of estrogens and xenoestrogens on the brain and other central nervous system functions. Certain areas of the brain, particularly the hypothalamus and pituitary gland, control synthesis and secretion of molecules which regulate endocrine function of the body. For example, the pituitary gland secretes Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH), which directly target the testes and ovaries [171]. Interference with the normal function of this delicate hormonal balance could prove detrimental at some stages of development. Estrogen is known to play a large role in the areas of cognition and memory and is suspected to be have the ability to mitigate conditions such as Alzheimer's and

Parkinson's [172]. Studies have found BPA to have antagonistic effects on spine synapses for both estradiol in female monkeys and testosterone in male rats [173]. It is unclear at this time what effect BPA may have on the natural levels of estrogen in the human brain.

There is a need to continue the study the bioconcentration and bioaccumulation properties of BPA and parabens. The paraben data from this study is weak because ethyl, propyl and butyl parabens were not consistently detected in surface water samples from Chapter 3. It is not sufficient to say that parabens do not bioconcentrate in fish tissue when we cannot be sure if parabens were present in the water source to begin with. More research needs to be completed to correlate the results of chemical analyses with MCF-7 cell proliferation assays for estrogenic potential, to continue to determine causal relationships. Further investigation should be made to determine what public health effect on humans, if any, is resulting from BPA bioconcentrating in areas around epoxy paint and plastic manufacturing plants. Clearly, more research is needed to determine the effects of BPA on the human brain.

5.0 OPINION OF POLICY AND REGULATIONS REGARDING PARABENS AND BISPHENOL A

With regard to any new research developments, regulation and policy should be reviewed to determine whether or not there are adequate protective margins being employed by the government to safeguard the public from an established hazard. Parabens and Bisphenol A (BPA), as well as other Endocrine Disrupting Compounds (EDCs), have been a topic of political debate for the last few years.

5.1 POLICY AND REGULATION REGARDING PARABENS

5.1.1 Summary of Regulatory Actions for Parabens

The European Commission (EEC) placed restrictions on parabens for the use of preservatives: products are not to exceed 0.4% w/w for one ester and 0.8% for mixtures of esters. This restriction is not applicable if the use of parabens in the product was for something other than as a preservative [136, 137]. Dietary administration studies *in vivo* calculated No-Observed-Adverse-Effect-Level (NOAEL) for methyl and ethyl paraben at 1000 mg/kg bw/day and a Lowest-Observed-Adverse-Effect-Level (LOAEL) of 10 m g/kg bw/day for propyl paraben [139]. Toxicological data has led the EU Scientific Committee on Food to establish an

Acceptable Daily Intake (ADI) for total parabens at 10 mg/kg bw/day [139]. The 2005 opinion that methyl and ethyl parabens can be safely used up to maximum concentrations of 0.4% w/w remains unchanged, but more data was requested for propyl, butyl and isobutyl parabens [139].

In the United States, the FDA has limited the use of heptyl paraben to a maximum level of 12 ppm in fermented malt beverages and 20 ppm in noncarbonated soft drinks and fruit-based beverages [141]. No other paraben derivatives have restricted uses in the United States.

5.1.2 Pros and Cons of Using Parabens

Parabens are used as preservatives in many hygiene products. They are very effective at reducing bacterial growth and therefore extending the shelf-life of products. P arabens are relatively inexpensive, easy to use in both water and oil based products and are generally well tolerated. Parabens are also used as food preservatives because of their antifungal properties, and are therefore present in edible coatings on produce to extend the shelf life of agricultural products [110, 111].

Parabens were found to be estrogenic during *in vitro* testing [127]. Darbre et. al. reported parabens accumulated in human breast tumours, although this study is considered to be controversial [132]. Spanish researchers detected the formation of three new halogenated transformation products (for each parent paraben) when parabens were exposed to chlorinated tap water, making a total of five by-products for each parent paraben. They were identified as bromo- and bromochloro-parabens, because of the traces of bromine also found in tap water [117]. This formation takes place within minutes, allowing dermal and possibly ingestion routes of exposure to be realistic while bathing. Formation of brominated by-products occurred when bromine was present in the tap water in only minimal amounts. This research group also found

the presence of di-chlorinated forms of methyl and propyl parabens in raw sewage for the first time [117]. These by-products could be instrumental in the process of determining any dangers of using parabens, possibly more so that the endocrine disrupting potential of the parent parabens themselves.

5.1.3 Alternatives to the Use of Parabens

There are a number of alternatives to parabens. Mikrokil PCC may be used for skin care and hair care products; Cosmocil CQ can be used in contact lens solutions; and Biovert is a new preservative that mimics the natural enzyme, lacto peroxidase, found in tears, saliva and breast milk [174]. The addition of diazolidinyl urea to sodium benzoate and potassium sorbate can be a combination that offers a promising alternative to parabens, as well [175]. Sodium Hydroxymethylglycinate is a potential alternative to parabens, however research as to the safety and effectiveness is still being performed [176]. Other alternatives include certain essential oils for use in oil-based products (will not effectively preserve water-based products), potassium sorbate (for mold and yeast only, not bacteria), and vitamins [176].

5.1.4 Recommendations for Parabens

In the work reported here, only methyl paraben was detected in all surface water samples, propyl and butyl parabens were only detected in one location, and ethyl was not detected in any. Paraben concentrations were not found in significant amounts at any location. Parabens were not detected in extracts from fish brain tissue in any sample, and therefore could not be determined to bioconcentrate in the environment from this study. Consequently, only environmental

exposure to methyl paraben seems possible in the Greater Pittsburgh Area water supply and this compound remains unregulated at this time.

Therefore, it is my opinion that the current regulatory stance on parabens remains adequate for unregulated environmental usage and disposal/discharge. However, it would be prudent to continue further investigation into the effects of discharges near locations where parabens and paraben-containing products are manufactured, as this was not accounted for in our study of the Greater Pittsburgh Area. Manufacturing locations for parabens could lead to localized public health consequences specific to those areas, as it has been indicated in previous literature reviews suggesting that parabens do have the potential to cause endocrine disruption.

5.2 POLICY AND REGULATIONS REGARDING BISPHENOL A

5.2.1 Summary of Regulatory Actions for BPA

Although BPA remains a hot topic for debate among regulatory agencies, little action has been taken to remove BPA from the market. The maximum tolerated dose of BPA was determined to be 1000 mg/kg bw/day and the EPA calculated reference dose is 50 μg/kg/day, while a NOAEL has yet to be found because adverse responses have always been detected in even the lowest administered doses [13]. In 2008, the National Toxicology Program reported a subpanel critique of the data available on BPA exposure and potential low-dose health effects. They concluded that there is credible evidence available to show that low doses of BPA can cause specific effects, but that the low dose effects of BPA have not been established as reproducible findings [95]. In the United States, though, most withdraws have been market driven, meaning that

companies have voluntarily phased out BPA-containing products in order to promote positive customer relations [96]. Some local and state governments have started to propose regulations and enforce them, but as of yet, nothing has been legislated on a federal government level.

Canadian government officials have chosen to withdraw products containing BPA from their market. Prior to this decision, the Canadian governments respected limits for BPA of 0.1 mg/L for drinking water resulting from contact with BPA containing packaging [97]. A Provisional Tolerable Daily Uptake (PTDU) for BPA from food was established to be 25 μg/kg bw/day [97]. The European Scientific Committee on Food set its tolerable daily intake to 50 μg/kg bw/day in 2006 and exposure to BPA from migration out of food packaging was set at 0.6 mg/kg [97].

The current opinion of the United States government is that BPA is safe to use and the benefits far outweigh the risks. The U.S. Food and Drug Administration (FDA) and the National Toxicological Program believe that current research provides some concern as to the public health effects of BPA on the brain and behavior of infants and children, however the FDA is not recommending the discontinuation of the use of BPA at this time [177]. The EPA has considered addressing environmental concerns about BPA in its Bisphenol A Action Plan started in early 2010, but resulted in deferring to the FDA as the primary regulator because the majority of human exposure to BPA comes directly from food packaging, not drinking water [12, 178].

5.2.2 Pros and Cons of Using BPA

BPA is used in the manufacturing of epoxy resins and polycarbonate plastics, which are used for food packaging, metal can linings, and dental filings, among other things. Polycarbonate plastic is selected for use because of its high durability, resistance to shattering, transparency, light

weight and resistance to heat [179]. The manufacturing of polycarbonate plastics is a large worldwide industry, creating many jobs with a huge socioeconomic impact. Public health improvements since the development of BPA-containing materials have been significant and have included the reduction of food contamination from metal cans, the extension of the shelf life of canned goods and an alternative to mercury amalgam dental composite fillings [12, 180].

BPA is known to exhibit estrogenic and antiandrogenic properties [13]. BPA is known to mimic estrogen at low doses and impedes the binding of testosterone and thyroid hormone to their associated receptors at higher doses [25]. This classifies BPA as an endocrine disruptor. BPA is also known to bind to persistent organic pollutants, such as dioxin and polychlorinated biphenyls (PCBs) causing longer exposures to BPA and increasing the environmental risk [25].

5.2.3 Alternatives to the Use of BPA

There are alternative products on the market available for use. Some alternatives to BPA-containing polycarbonate plastics are High Density Polyethylene (HDPE) plastics, polypropylene plastics, and Polyethylene Terephthalate (PET) plastics. All of which are considered non-carcinogenic and have not been established to have endocrine disrupting potential at this time [181]. Other less attractive alternatives are glass, aluminum and stainless steel containers, which tend to be heavy and cumbersome, plus glass is easily damaged. An alternative to the BPA-containing epoxy resins used for metal can liners is Oleoresin, a mixture of oil and resin found in various plants. However, Oleoresin cannot be used in combination with acidic foods [181, 182]. Products made with paperboard combined with aluminum and Low Density Polyethylene (LDPE) are also available alternatives [181].

5.2.4 Recommendations for BPA

The research generated by this dissertation supports earlier reports suggesting that BPA will be present in United States surface water media and that it will bioconcentrate to low degrees in the environment [27, 38, 39]. As discussed in Chapter 4, our observed BCFs were within the ranges established by experimental studies reported in the literature (BCF = 68) and calculated/expected values based on the log K_{ow} of 3.32 (BCF = 100) [161, 163]. However, the EPA generally uses a BCF factor of > 1000 as determining a chemical to be a persistent environmental hazard [160]. The European Union considers that any chemical with a BCF > 100 has the potential to bioaccumulate in the environment and is therefore a danger to the environment [161, 162]. Of the BCFs calculated in this study most were below 100, with only two species at two locations reaching above 100, leading to the conclusion that BPA is not a strongly persistent chemical in the Greater Pittsburgh Area river system and water supply.

However, this new research has suggested that BPA is capable of crossing the blood-brain barrier in fish. There remains a difficult public health dilemma then to answer the question as to whether this occurrence could happen in humans as well. It is known that humans are exposed to BPA through oral exposure routes from food packaging, and that BPA has been positively detected in blood serum and found to cross the maternal-fetal placental barrier [13, 87]. Determining if BPA is concentrating in human brain tissue will be an insurmountable ethical task that can only be overcome by relying on *in vivo* animal and *in situ* environmental studies such as this one. There could also be profound public health implications if this phenomenon lurks unnoticed.

Therefore, because of this new question, paired with the known fact that BPA is an endocrine disruptor that exhibits both estrogenic and antiandrogenic effects, it is recommended

that stricter regulations be enforced on BPA until such time when research questions about BPA can be answered [13]. Unfortunately, it would be difficult to remove BPA from the market entirely. Movements could be made to ban the use of BPA-containing materials in all instances where the risk to public health from BPA exposure is less than the benefit to public health from using BPA-containing materials. For example, because there are adequate alternatives for plastics, BPA could be banned from use for the manufacturing of plastic bottles, electronic equipment, epoxy paint and the like in the luxury market economy. However, because the use of BPA-containing materials for medical equipment and dental fillings may have greater public health benefits than the risk from BPA exposure, it may not be necessary to remove this source of exposure at this time. An adequate alternative for metal can linings to be used with acidic foods has not really been established either, so it would not be realistic to remove this exposure from the market at this time. Although the EPA deferred to the FDA as the primary regulator for BPA, mostly due to exposure coming directly from food ingestion, it would not hurt to enforce environmental regulatory limits on discharges from manufacturing plants that are producing BPA-containing plastics and epoxy resins.

Removing part of the exposure to BPA by enforcing regulations on industry could reduce environmental levels of BPA, and therefore reduce the risk of endocrine disruption, although admittedly it would not likely reduce exposure by a significant amount. While research studies and regulators are considering BPA to be a safe chemical and environmental levels are to be of no concern, it is easy to overlook that a known phenomena of EDCs is their ability to have additive or synergistic effects when present with other EDCs [13, 15]. While we may not be able to remove all exposure to BPA from the market at this time, any reduction in exposure could have profound effects by removing unknown hazards associated with the mixture of BPA with

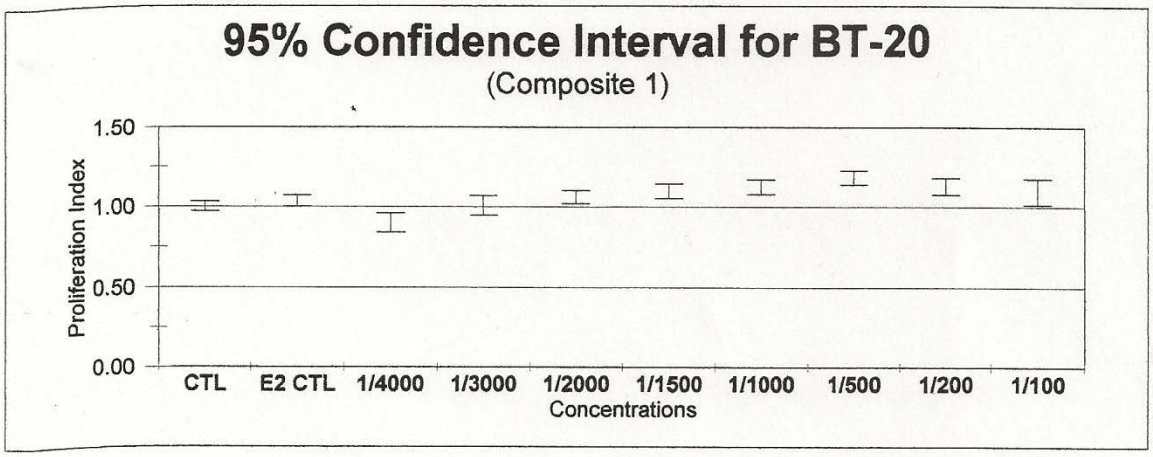
other chemicals, hazards of which have yet to be discovered and adequately studied. These precautionary regulatory actions are necessary and well established by sound science in the case of BPA.

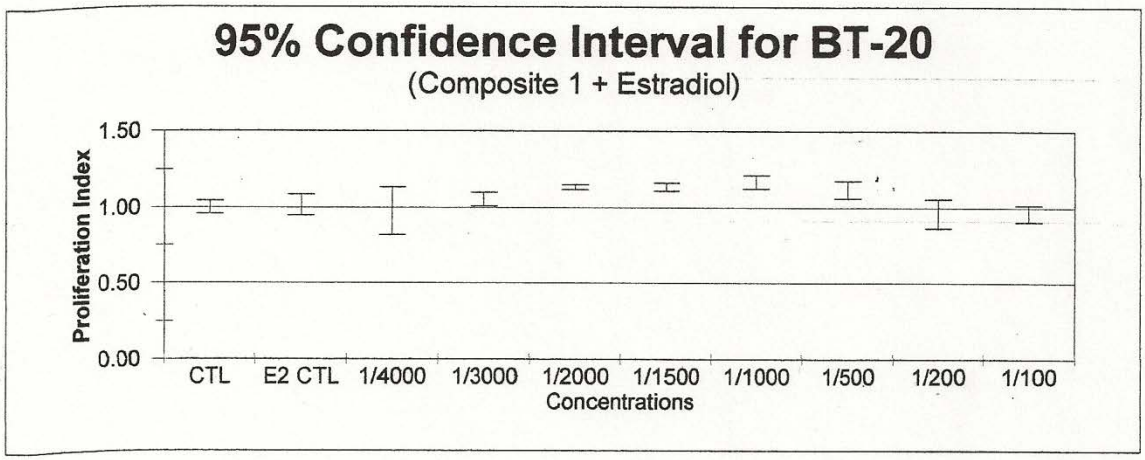
5.3 CONCLUSIONS

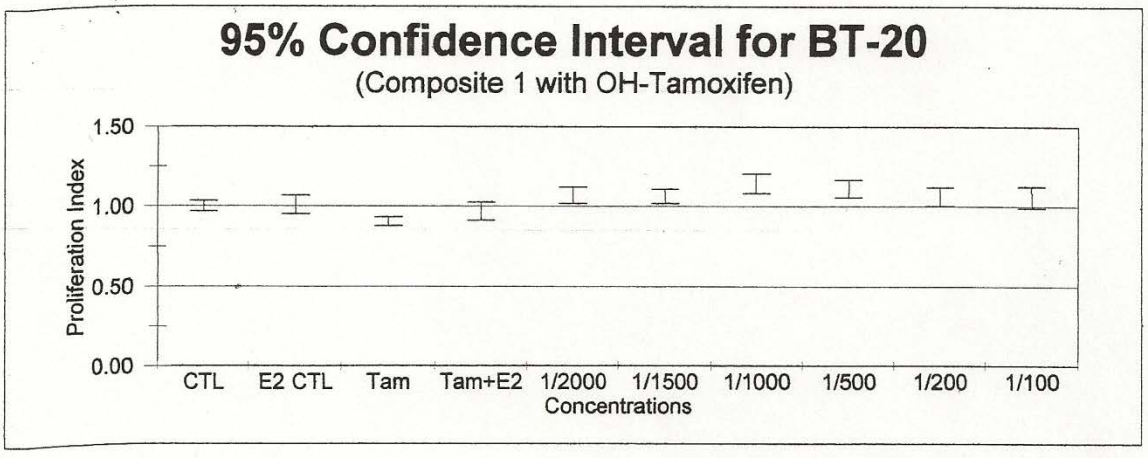
In summary, this dissertation and the research it describes supported all previous available reports leading to the conclusion that parabens are safe to remain on the market and not a significant environmental concern. In particular, there does not seem to be any need for concern over paraben levels detected in the Greater Pittsburgh Area river system and water supply. The BPA portion of this dissertation research, however, was in agreement with previous literature as to the bioconcentration tendencies of BPA; but new implications regarding the public health consequences stemming from the effect of BPA on brain tissue may require some re-evaluation of concern as to the significance of BPA transport and fate in the environment around Pittsburgh and elsewhere.

APPENDIX A: ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR BT-20

A.1 BT-20 COMPOSITE 1







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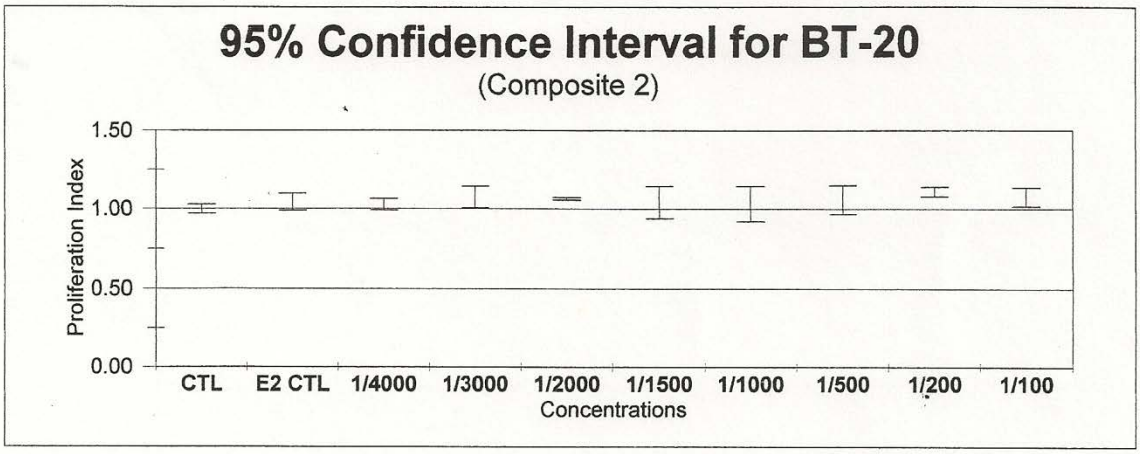

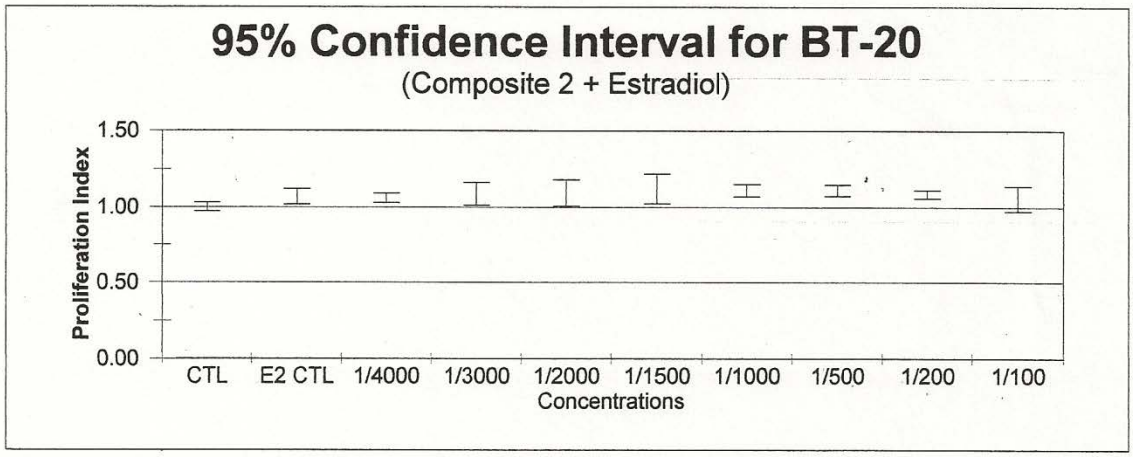

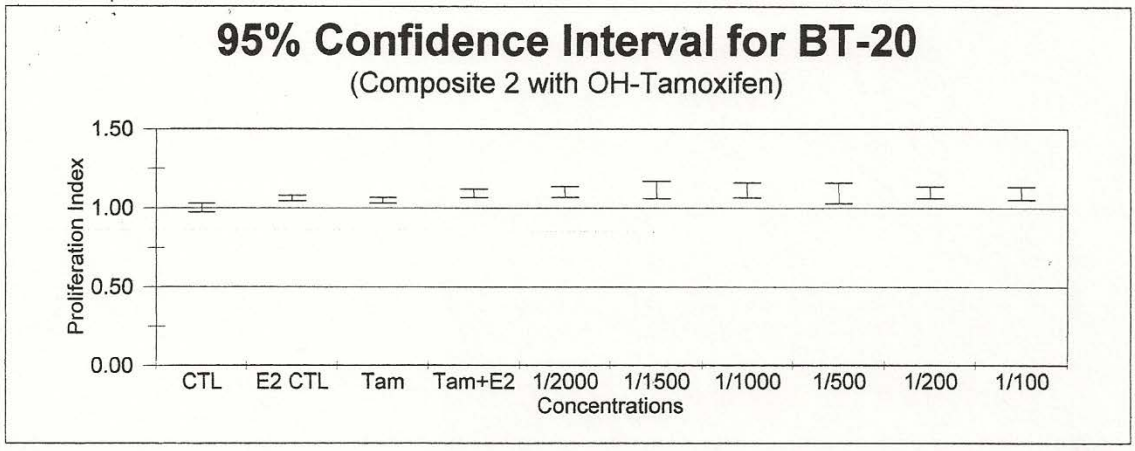

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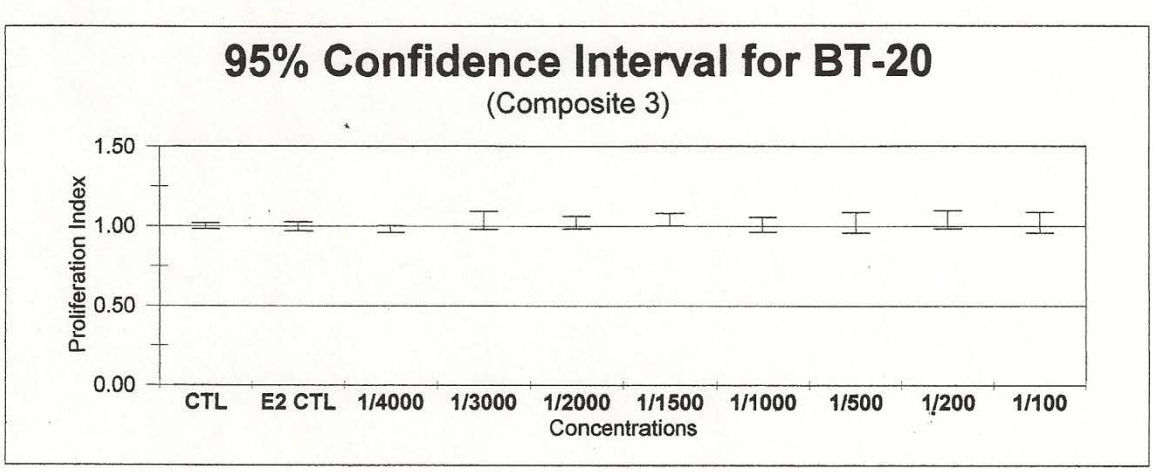

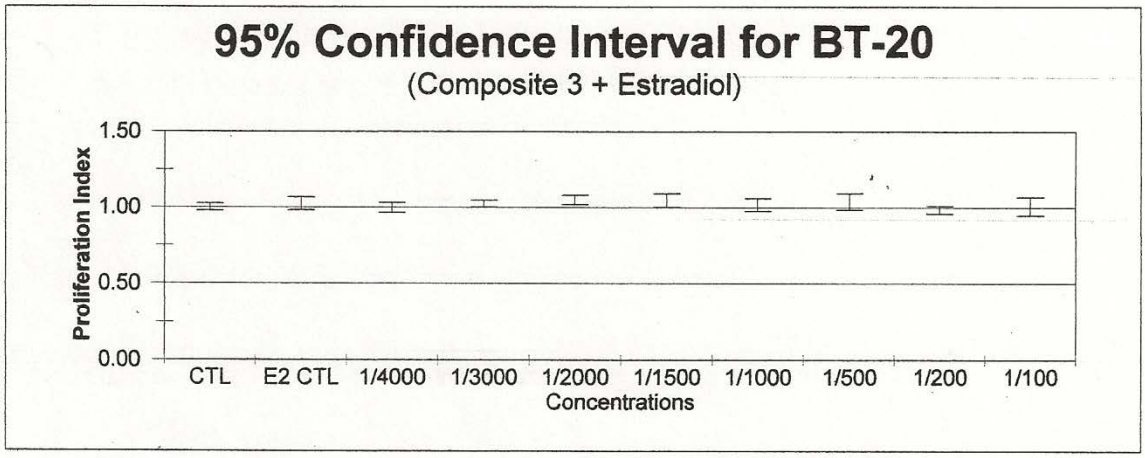

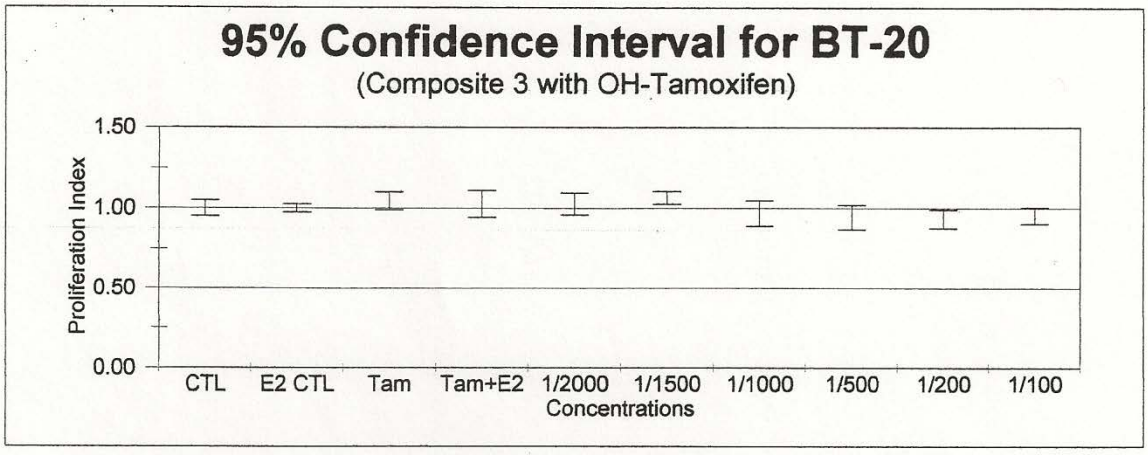

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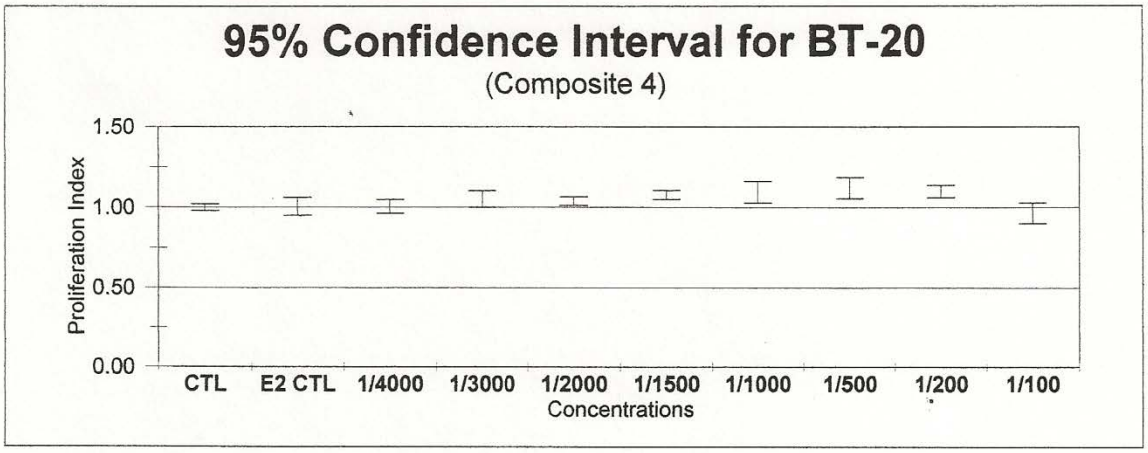

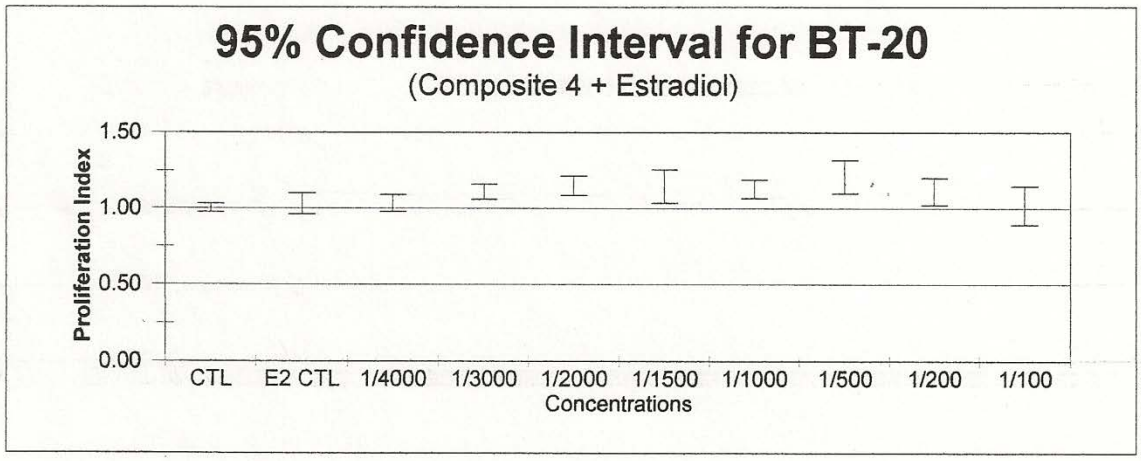

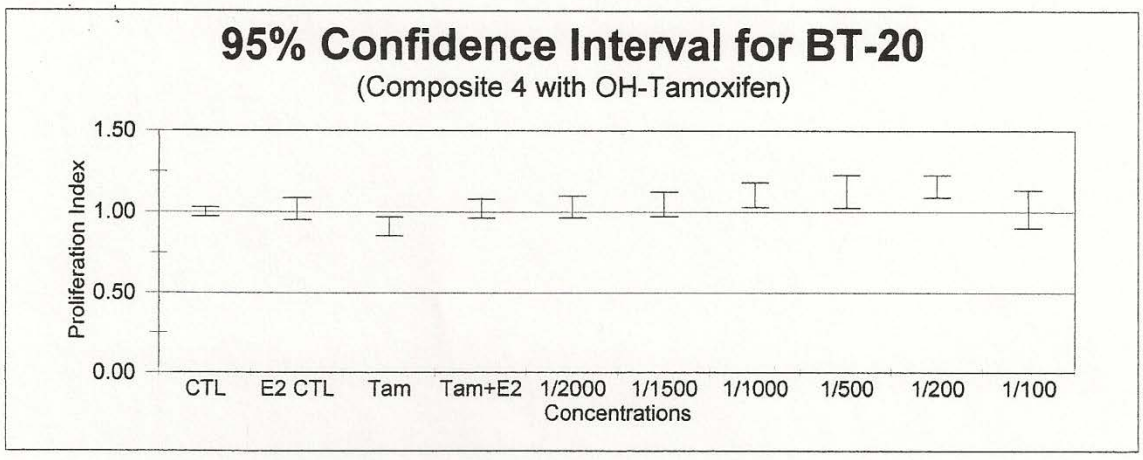

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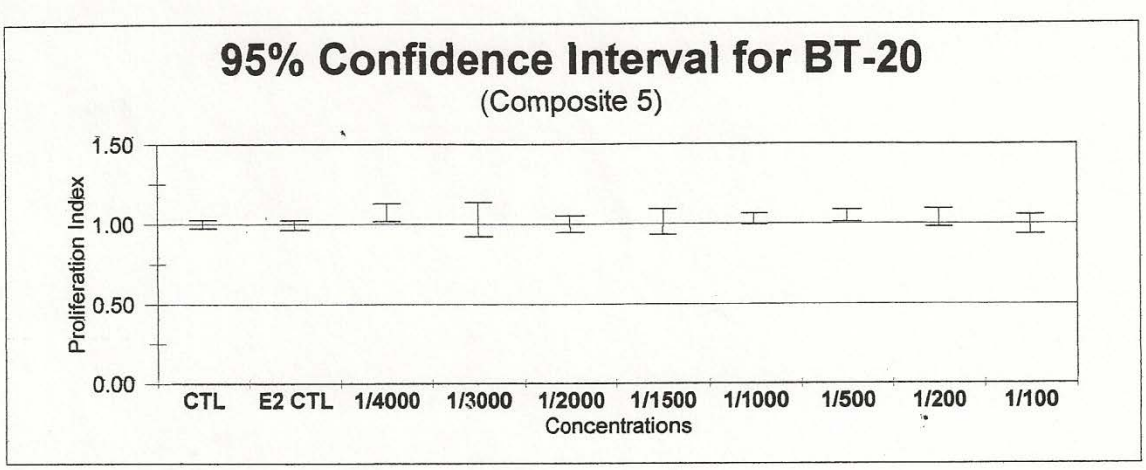

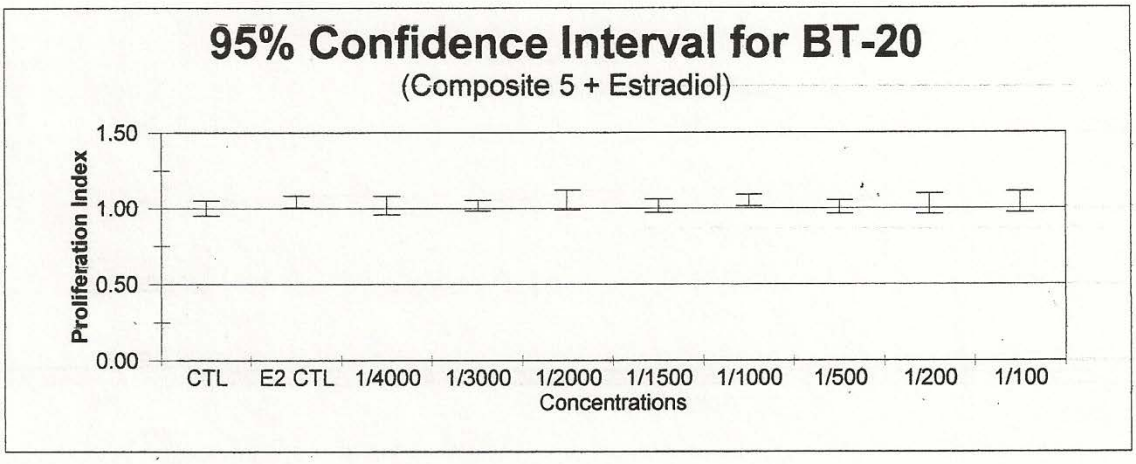

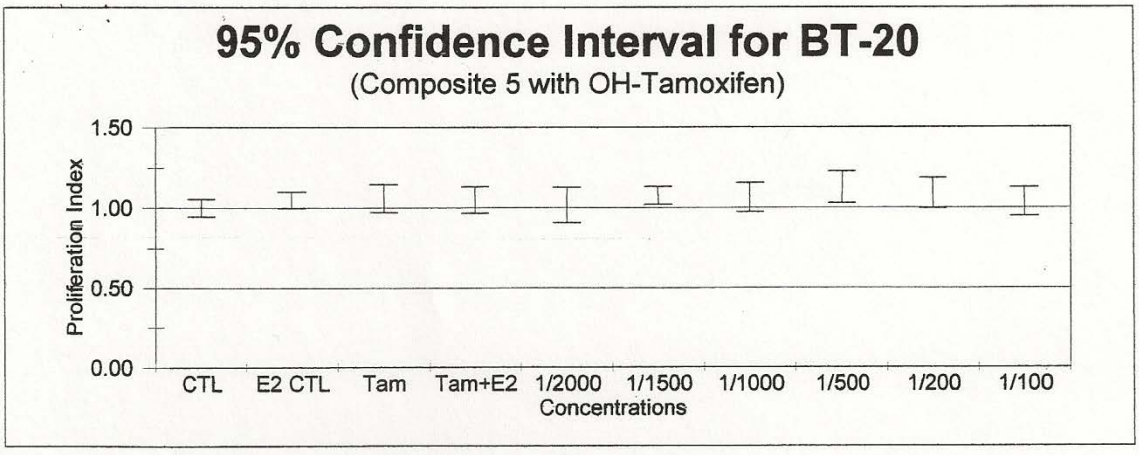

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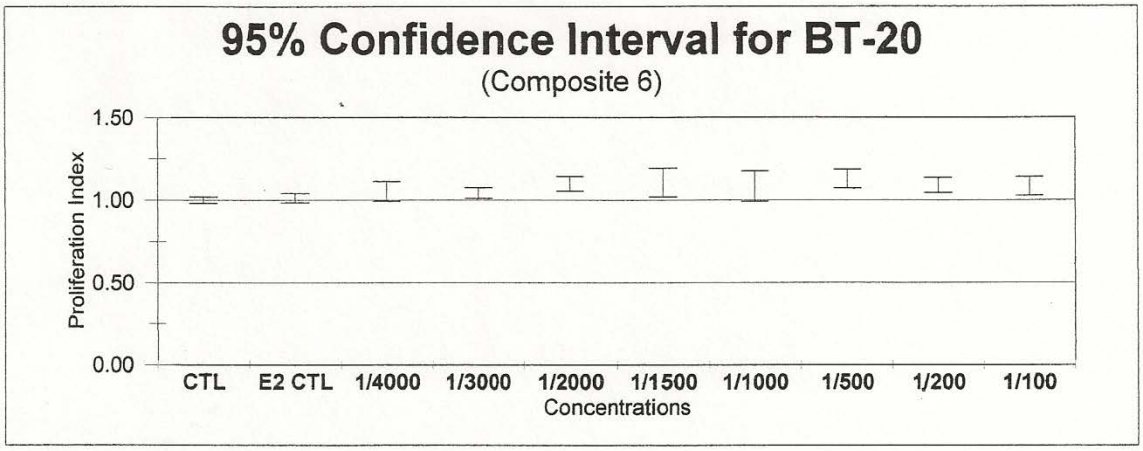

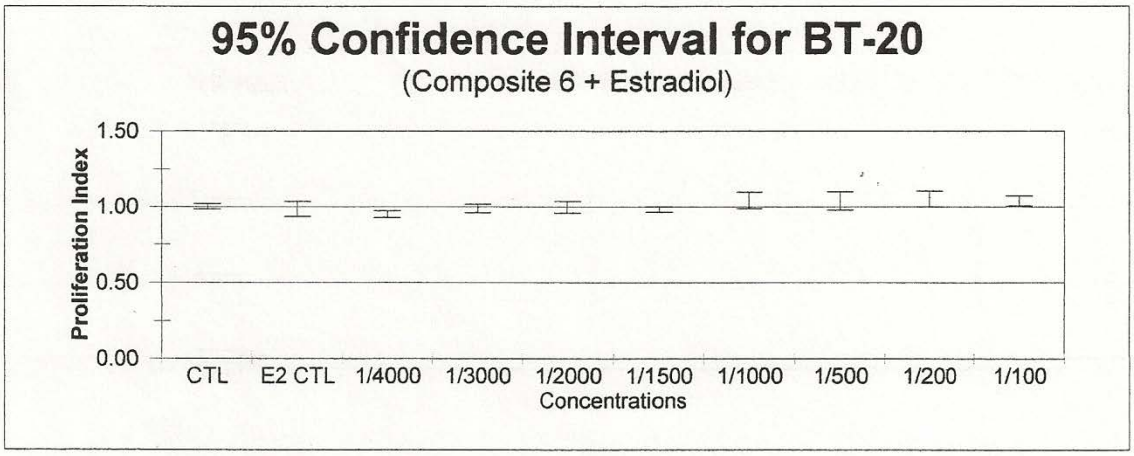

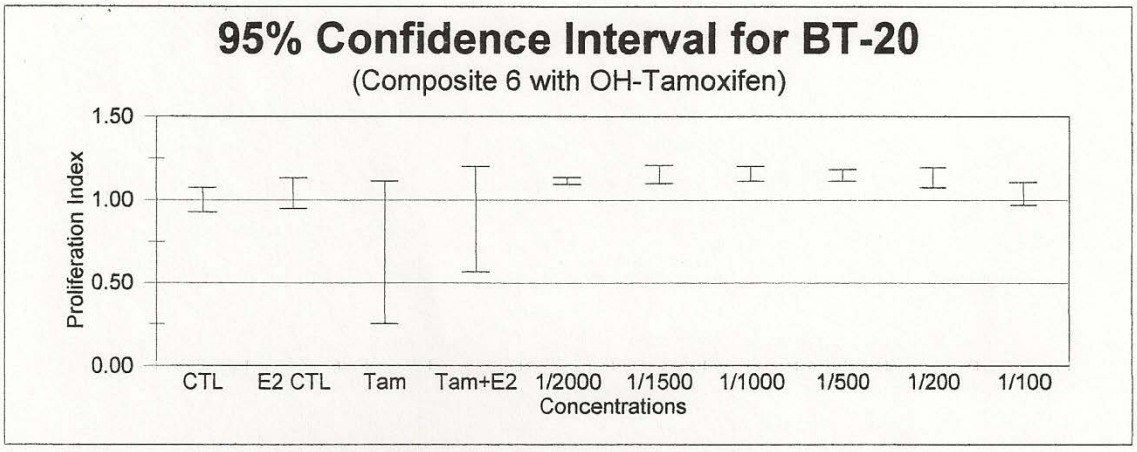

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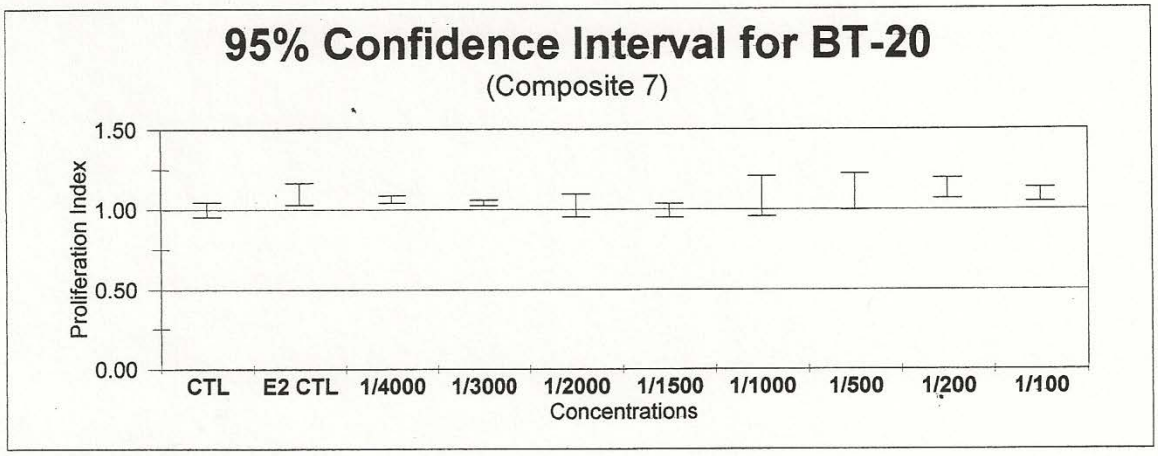

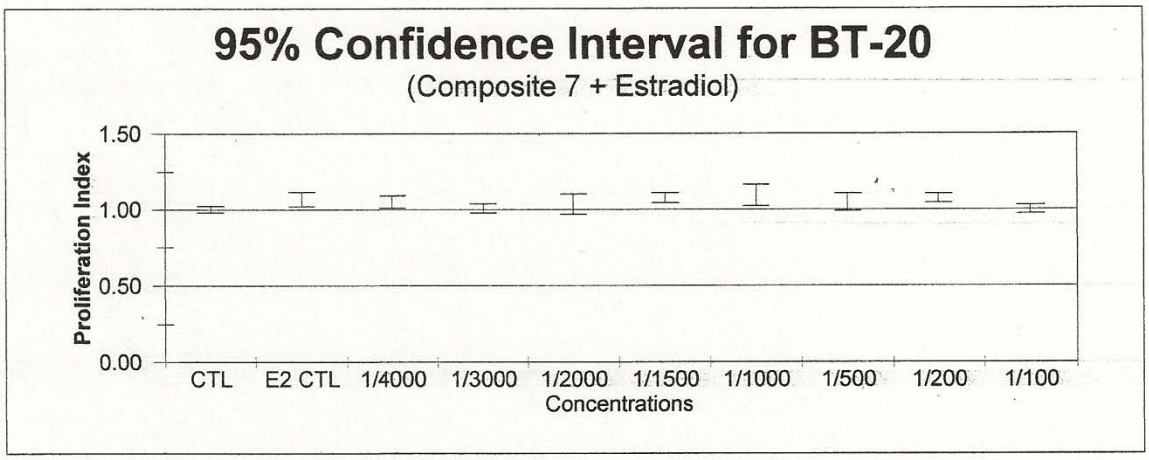

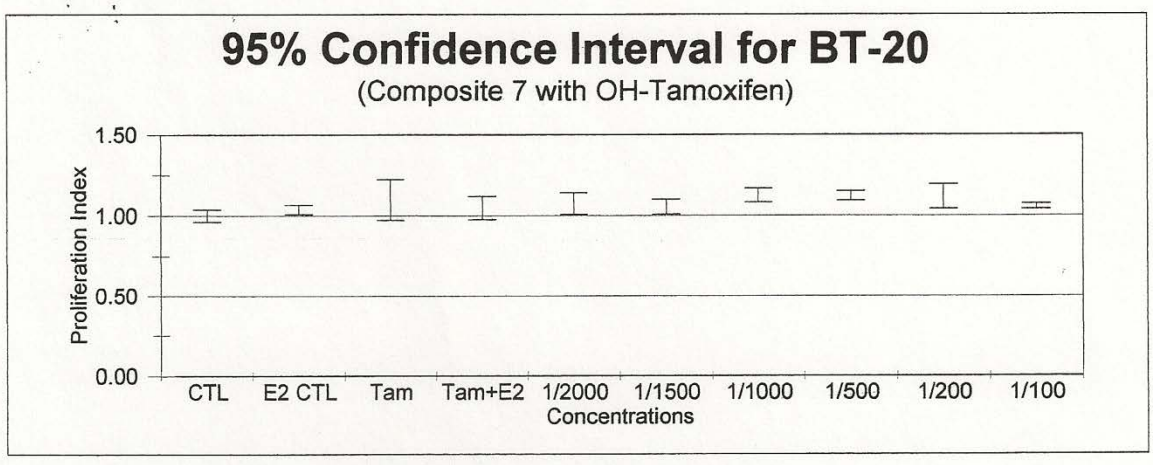

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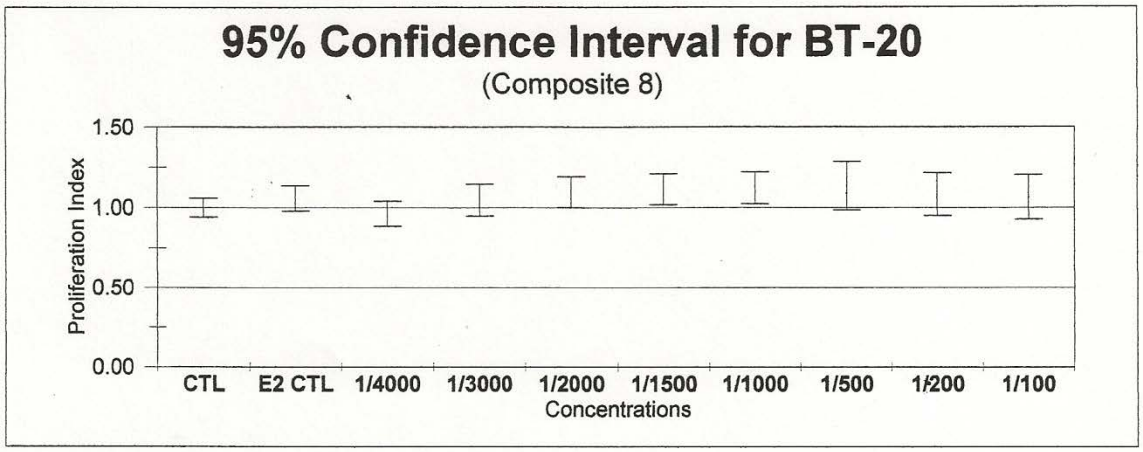

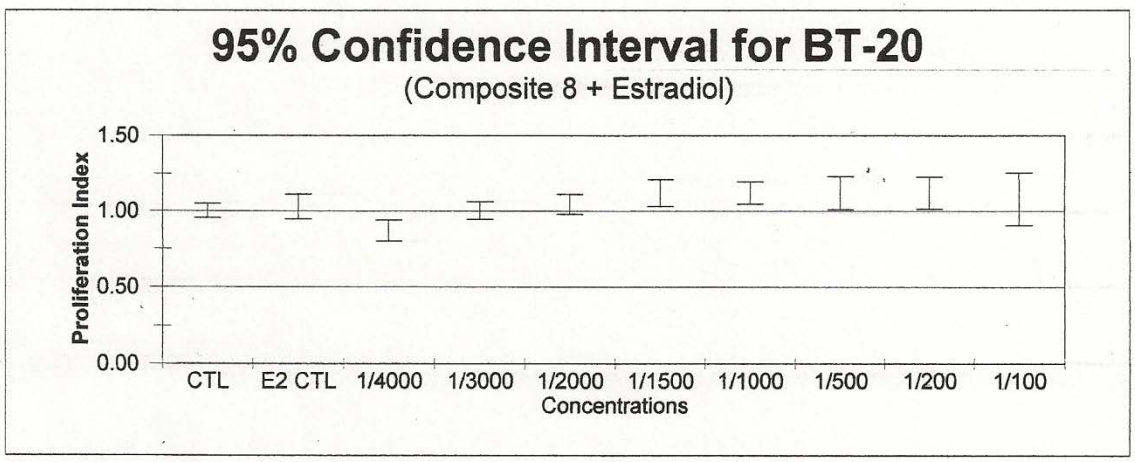

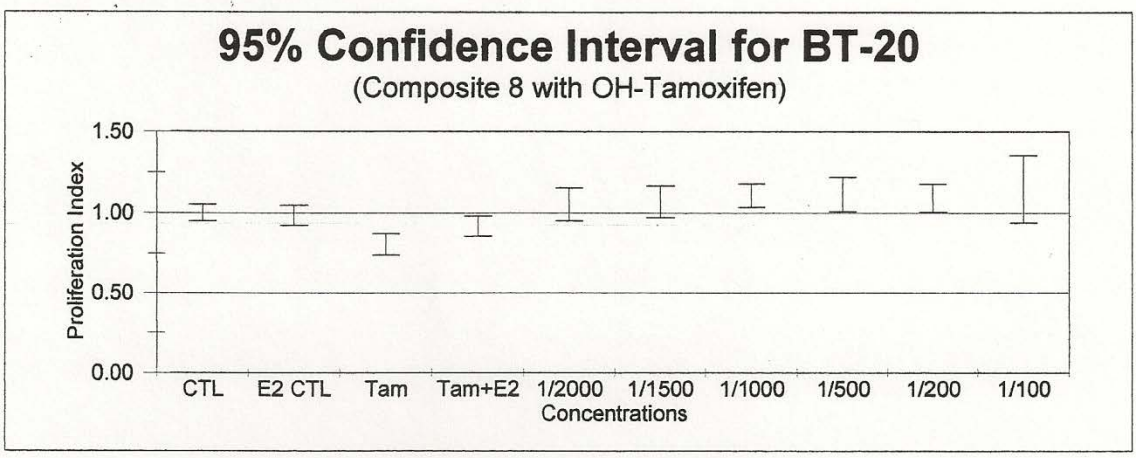

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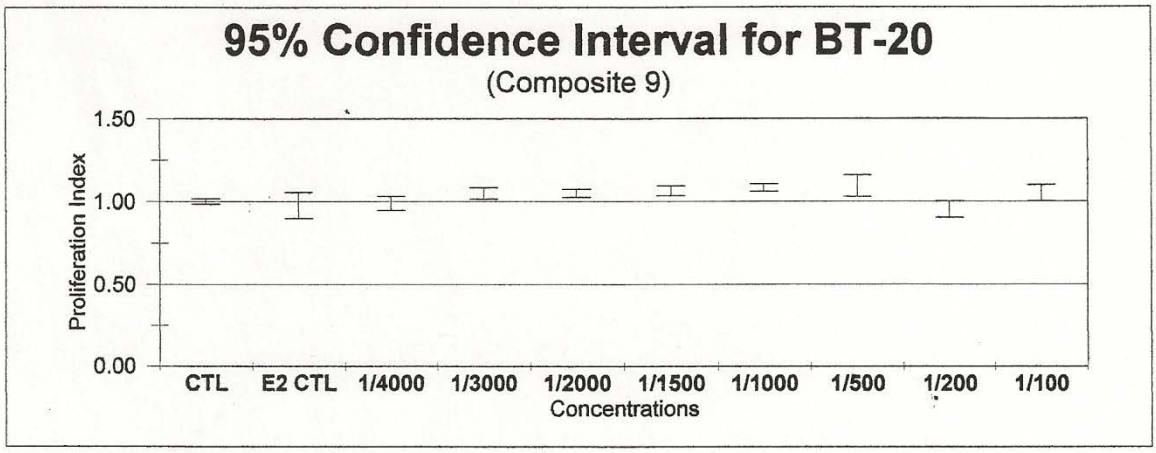
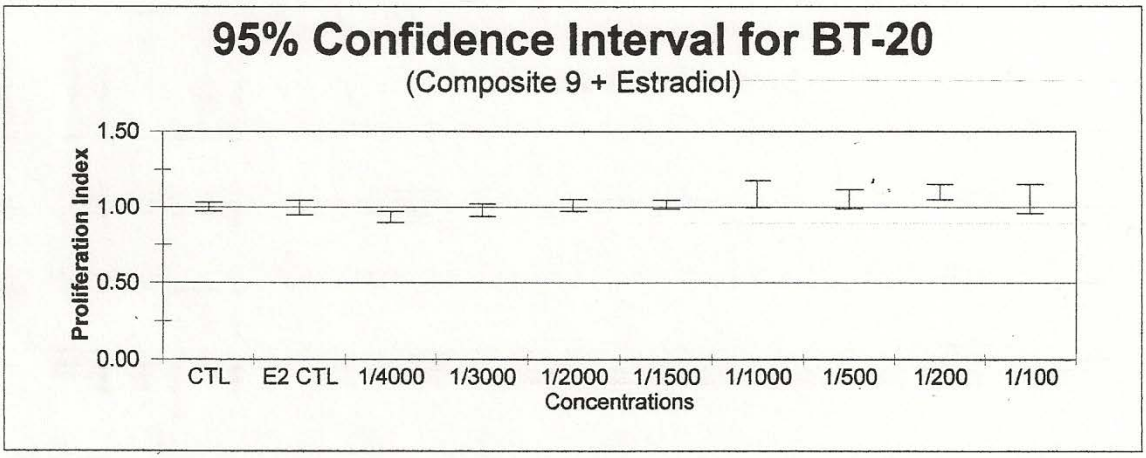
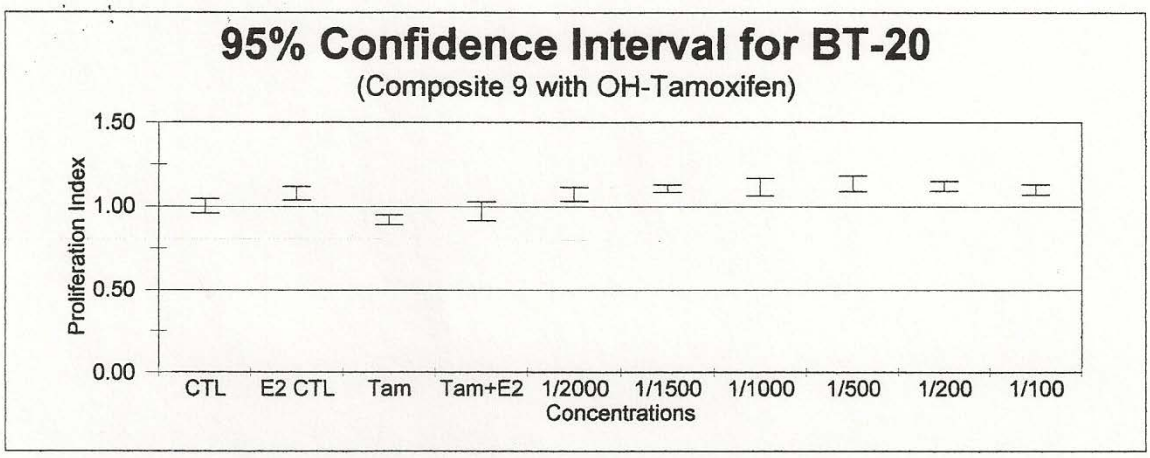
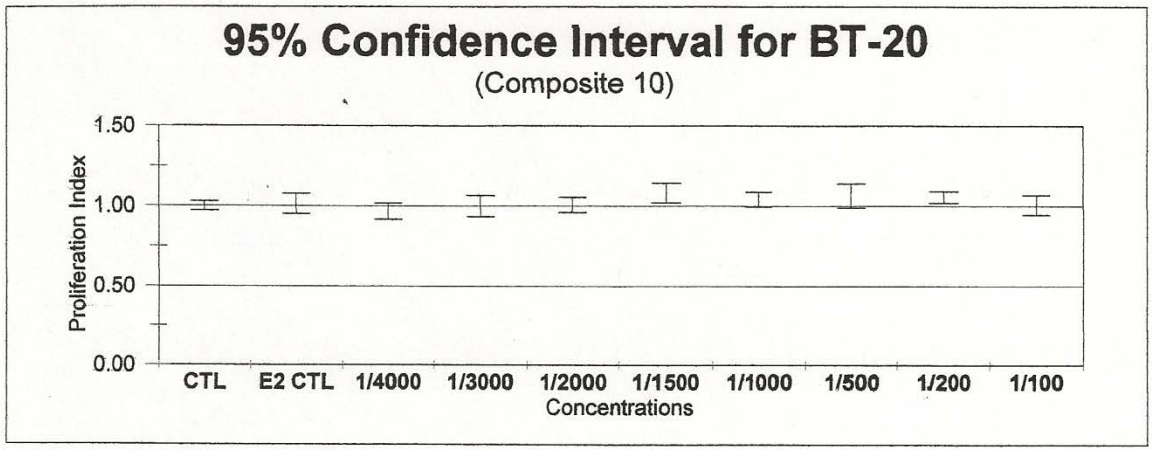
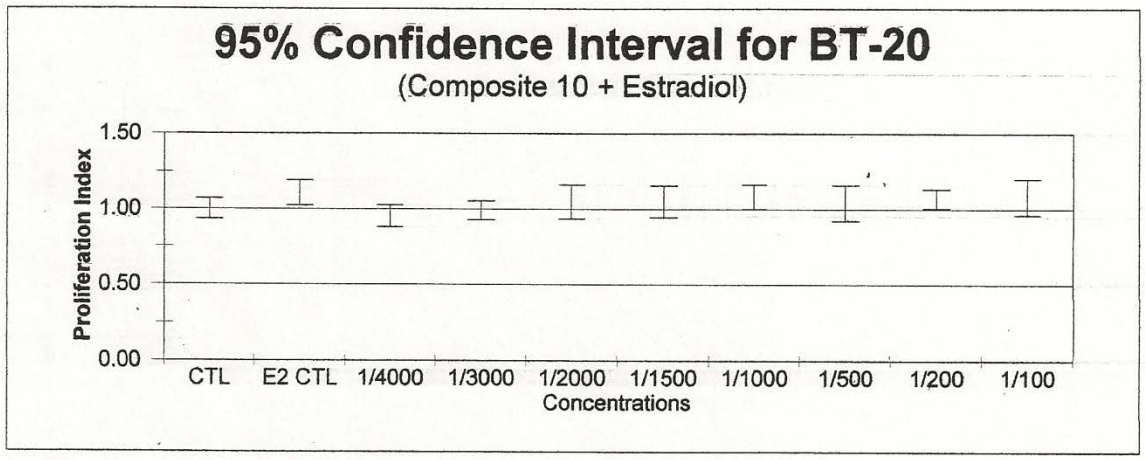


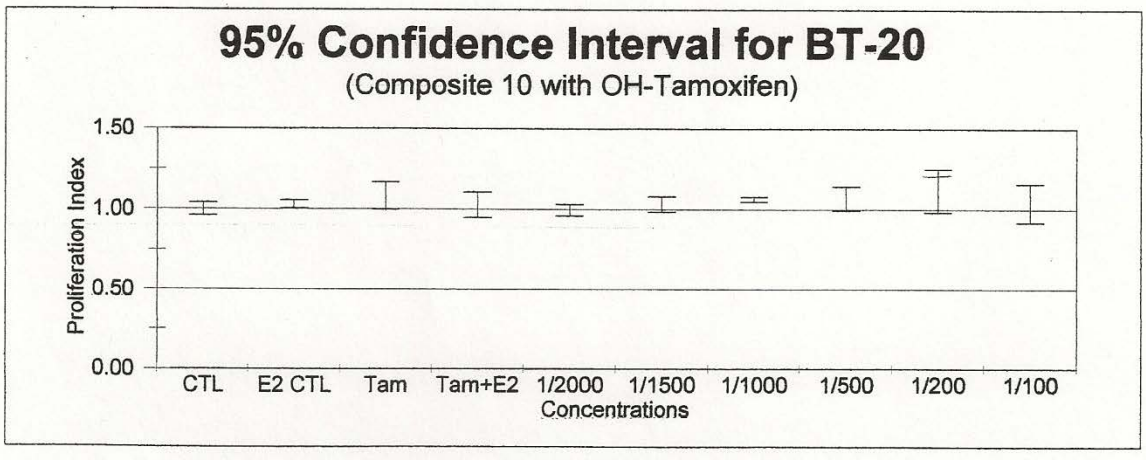




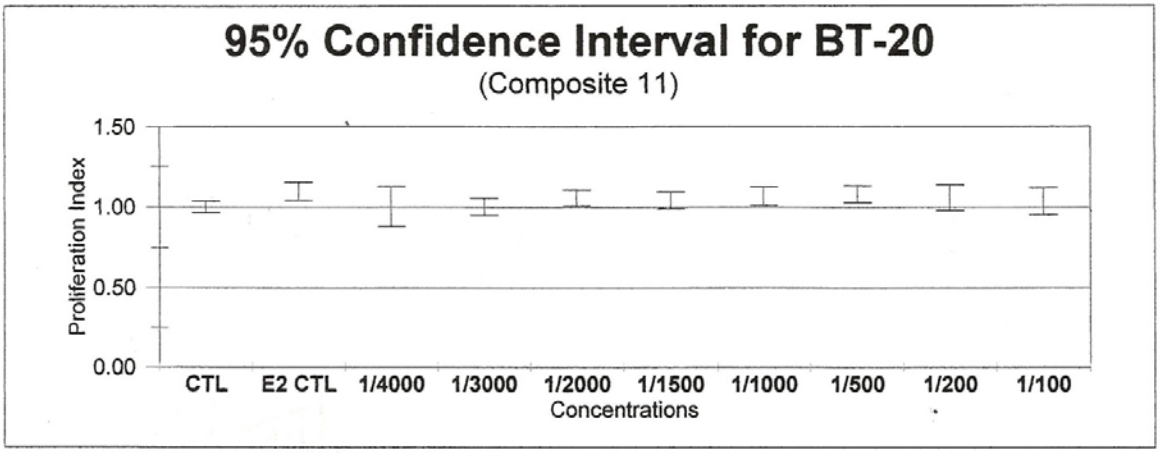
A.10 BT-20 COMPOSITE 10

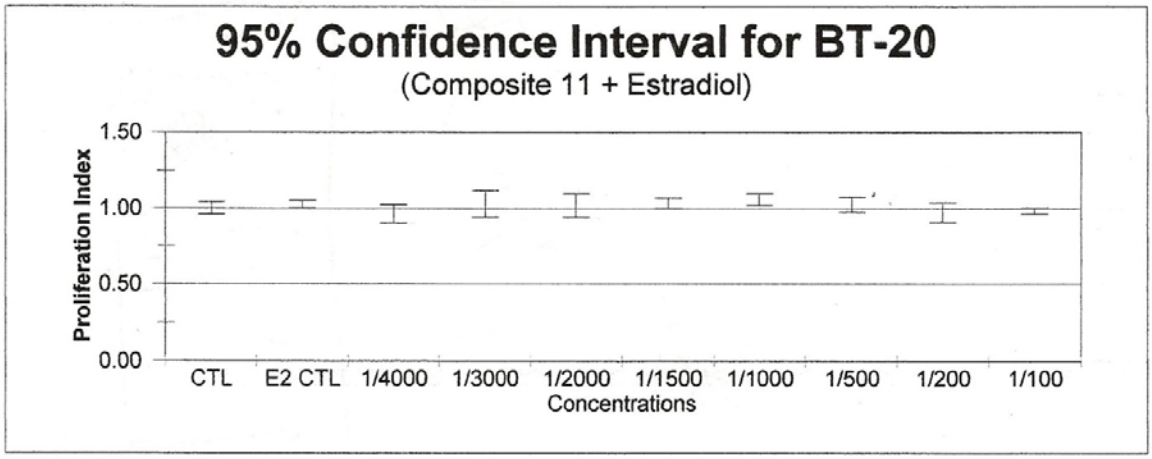


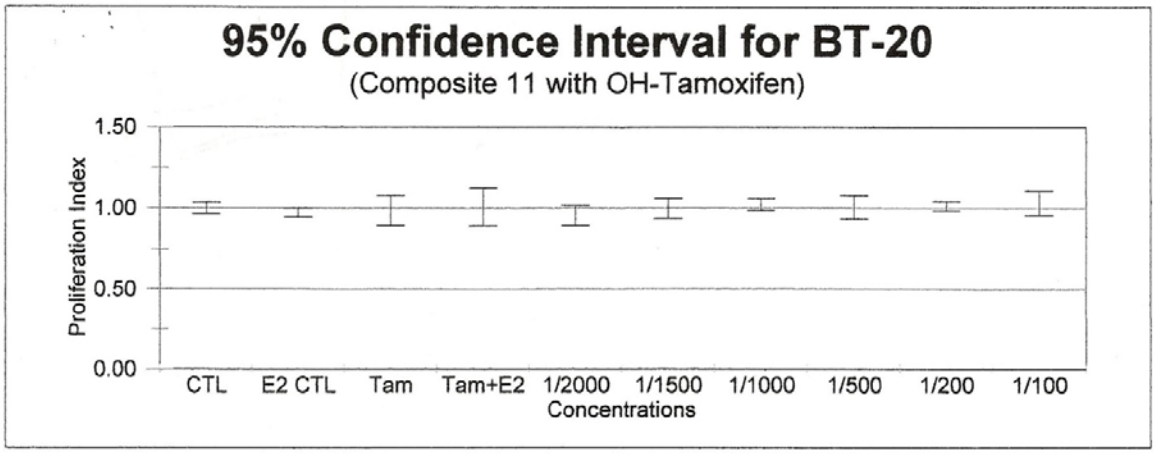




A.11 BT-20 COMPOSITE 11

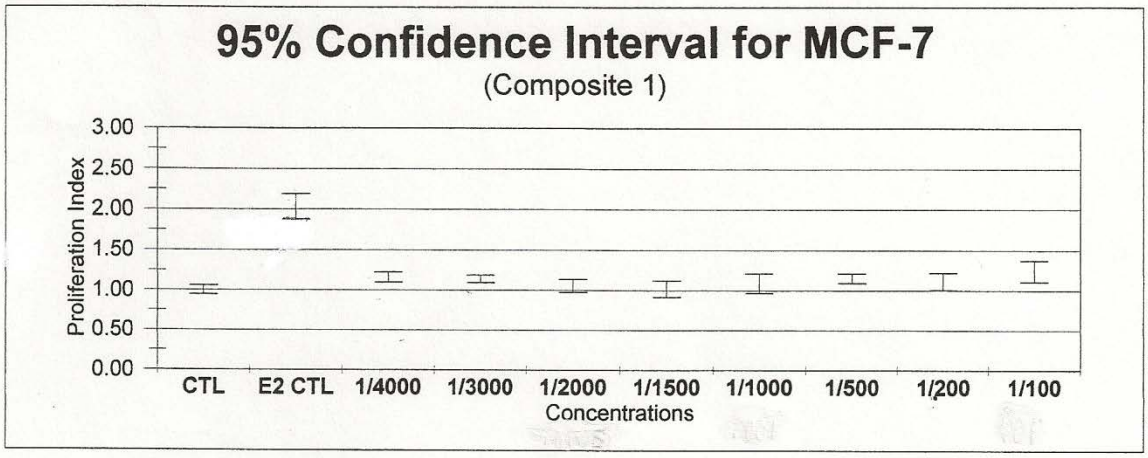


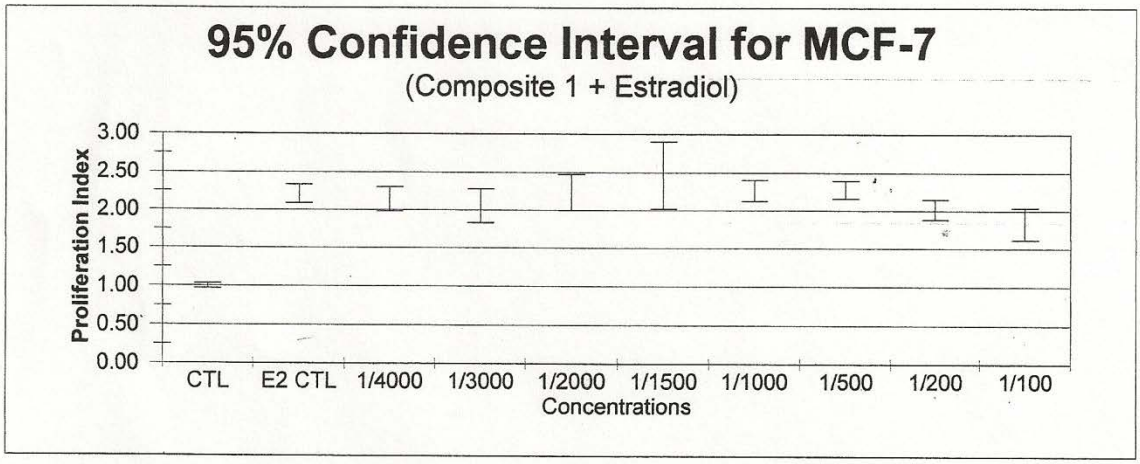


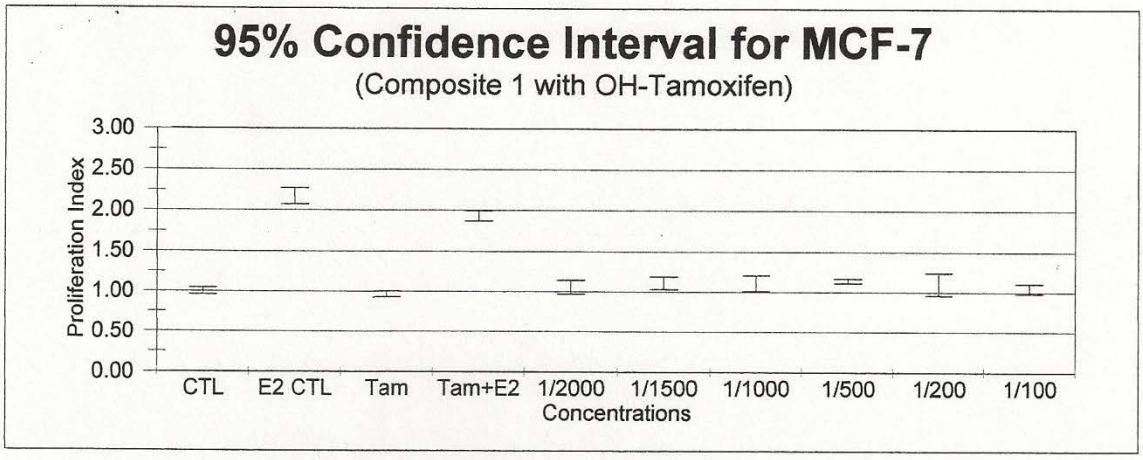


APPENDIX B: ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR MCF-7

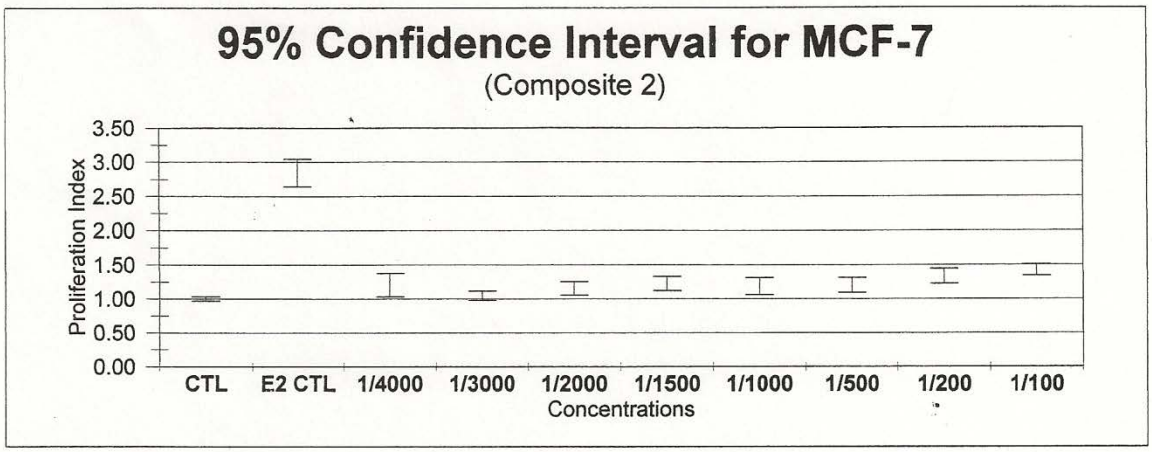
B.1 MCF-7 COMPOSITE 1

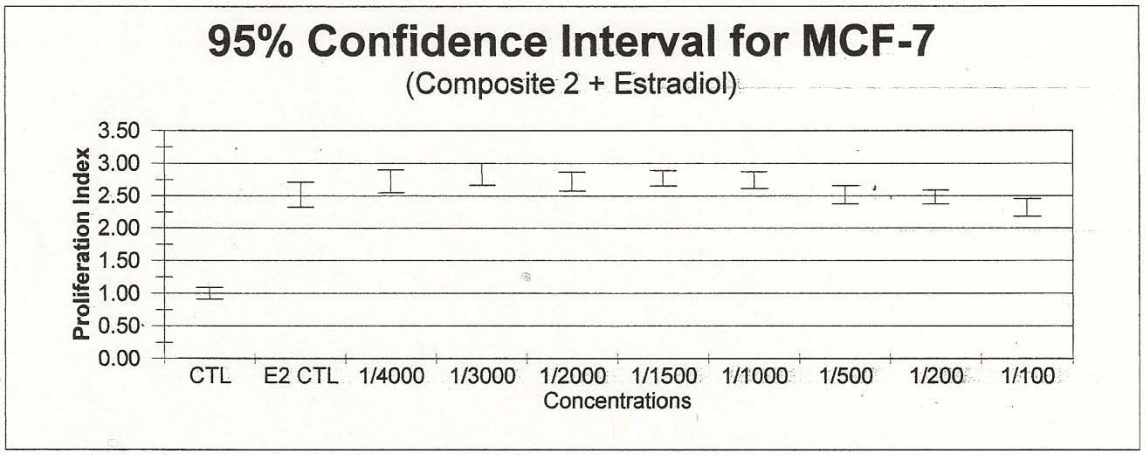


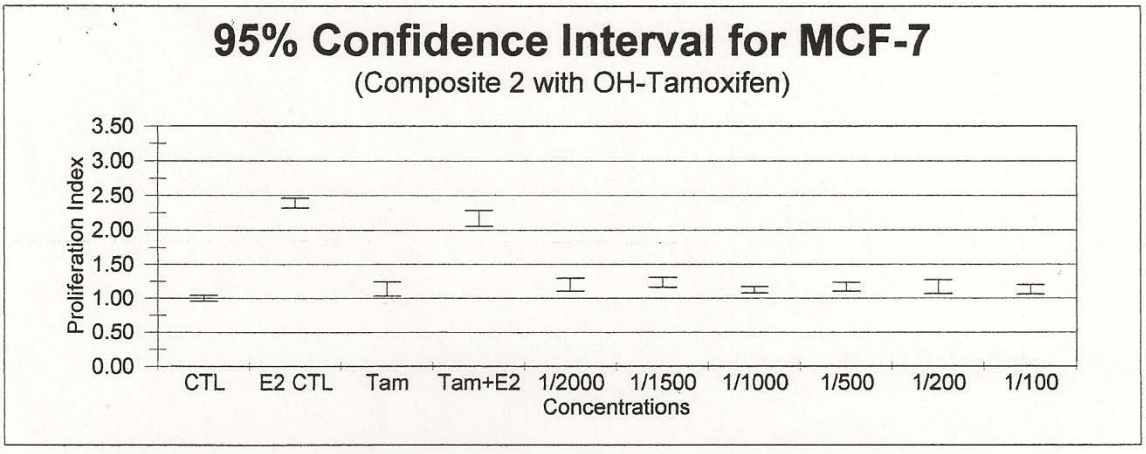




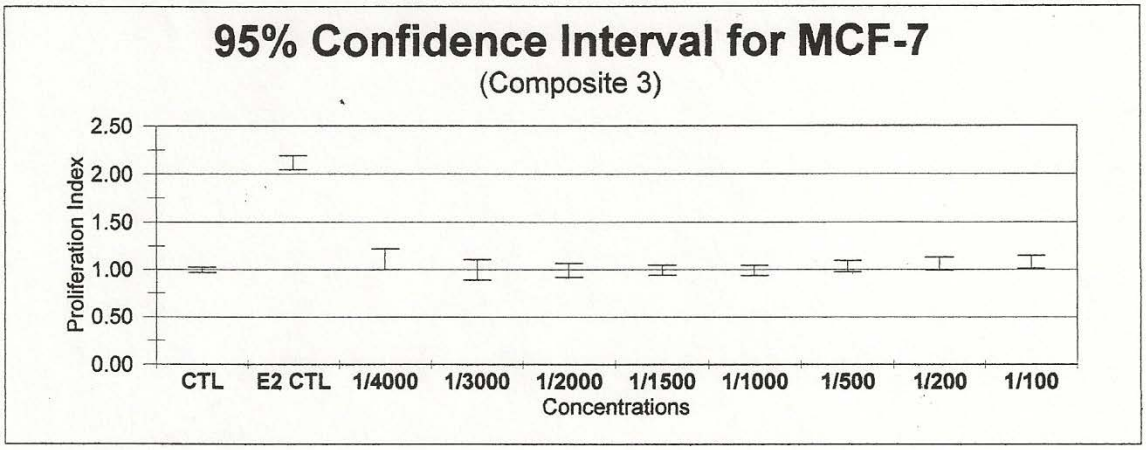
B.2 MCF-7 COMPOSITE 2

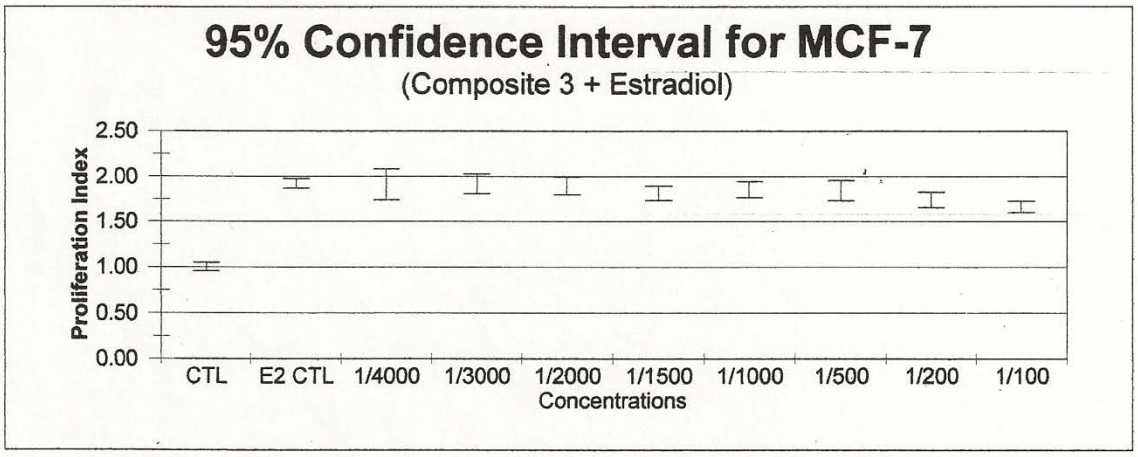


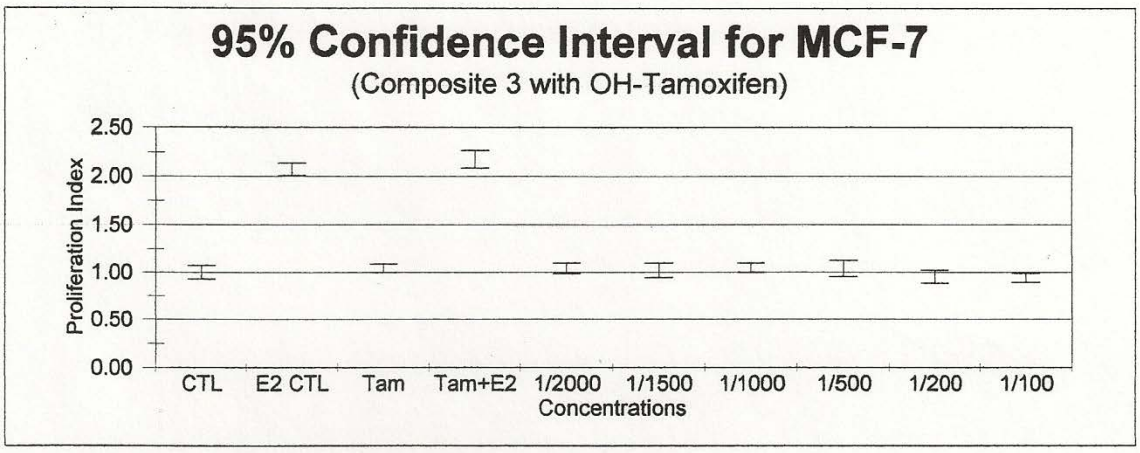




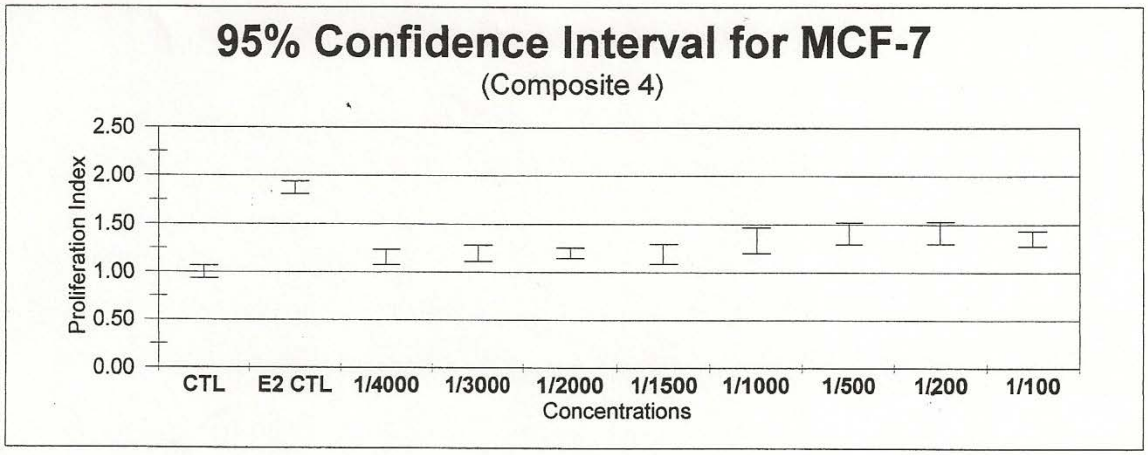
B.3 MCF-7 COMPOSITE 3

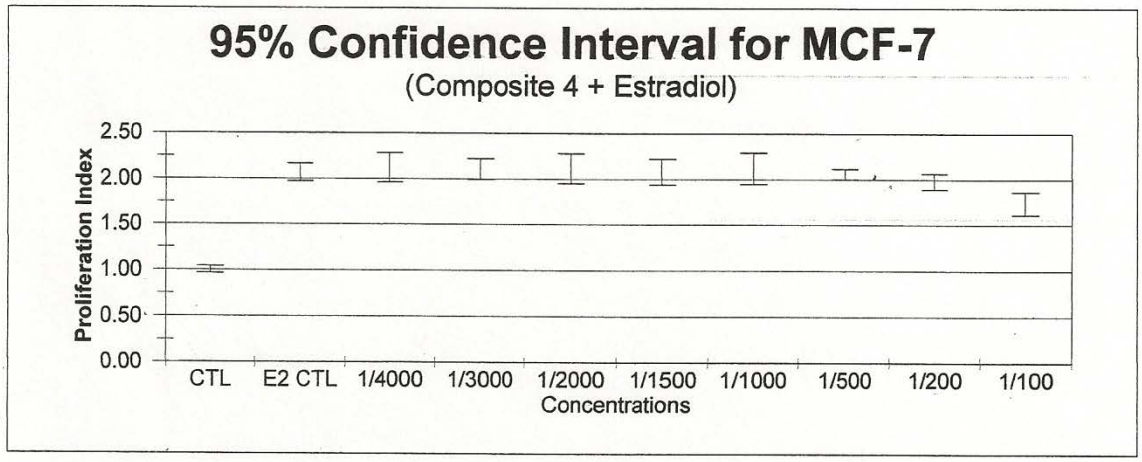


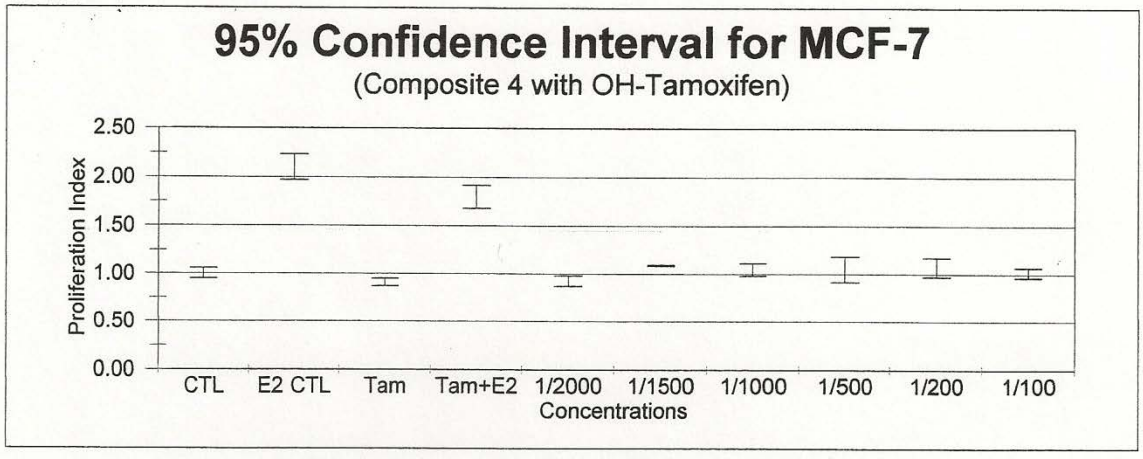




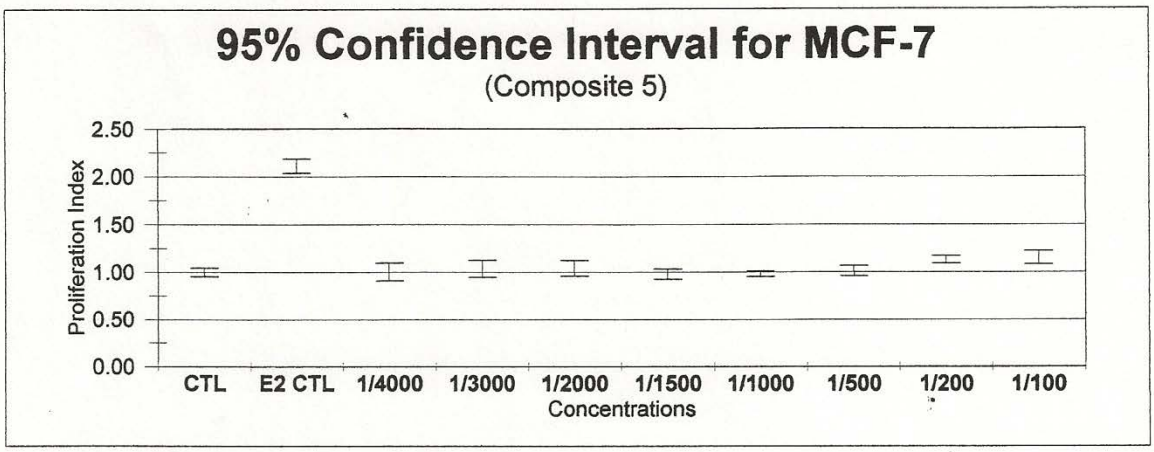
B.4 MCF-7 COMPOSITE 4

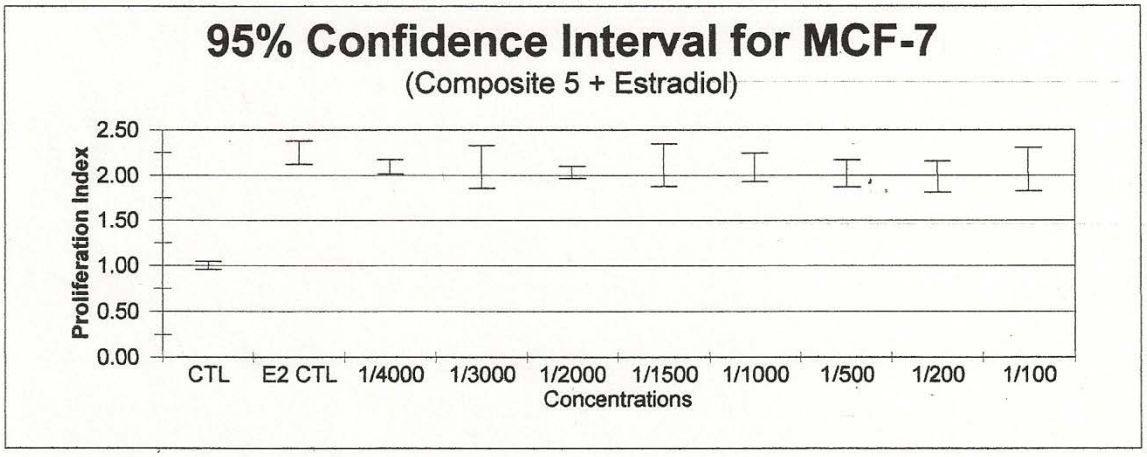


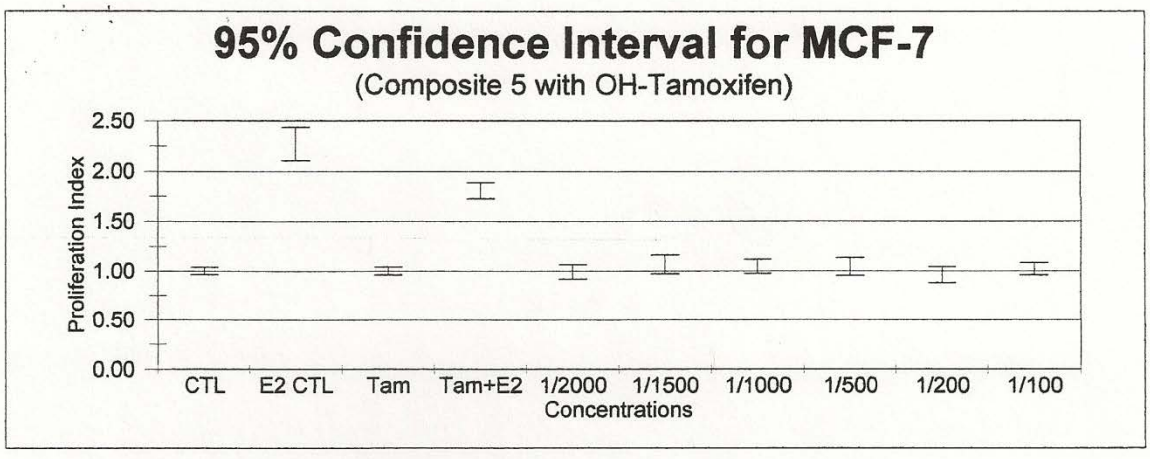




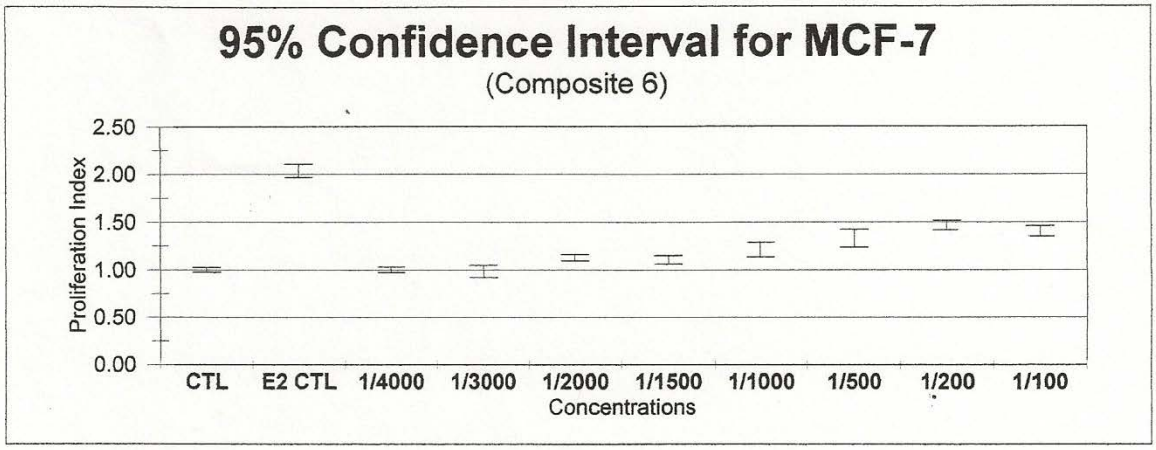
B.5 MCF-7 COMPOSITE 5

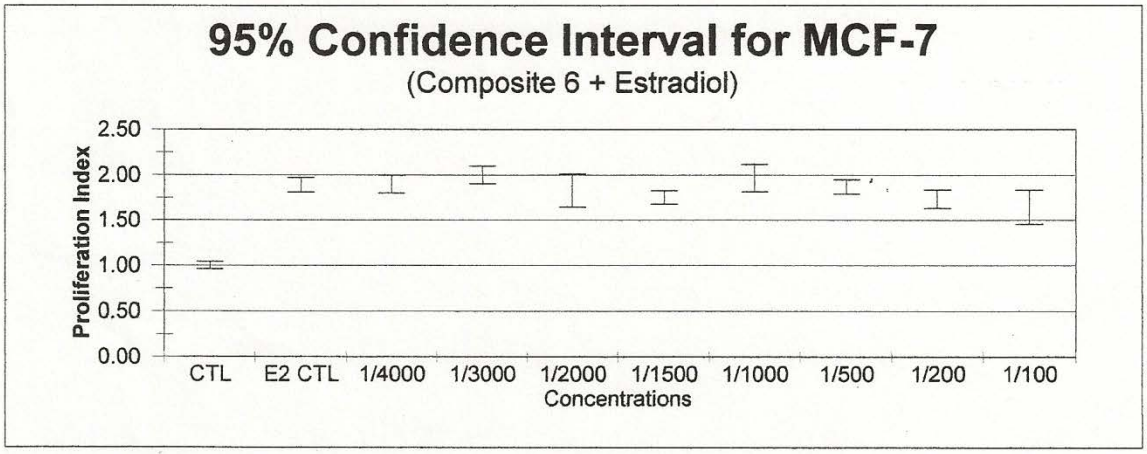


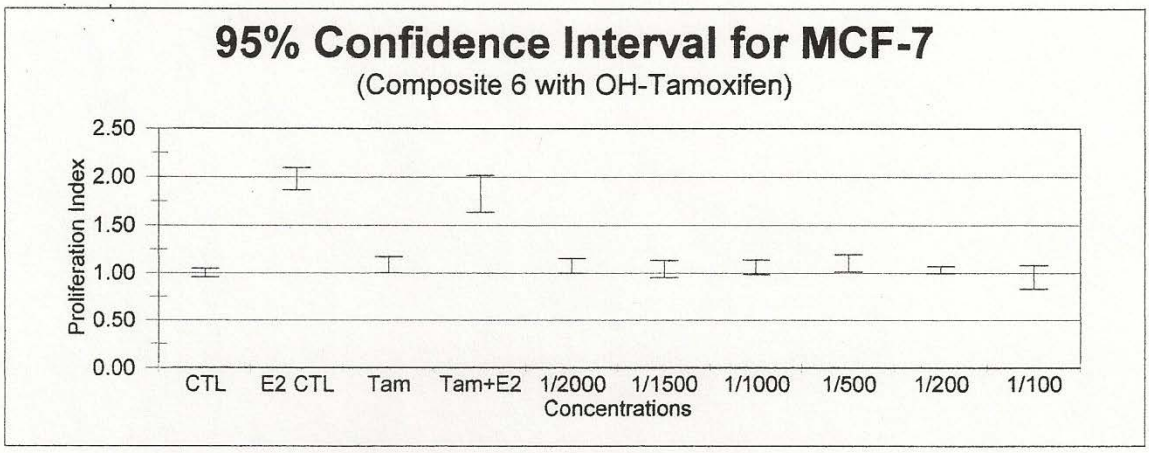




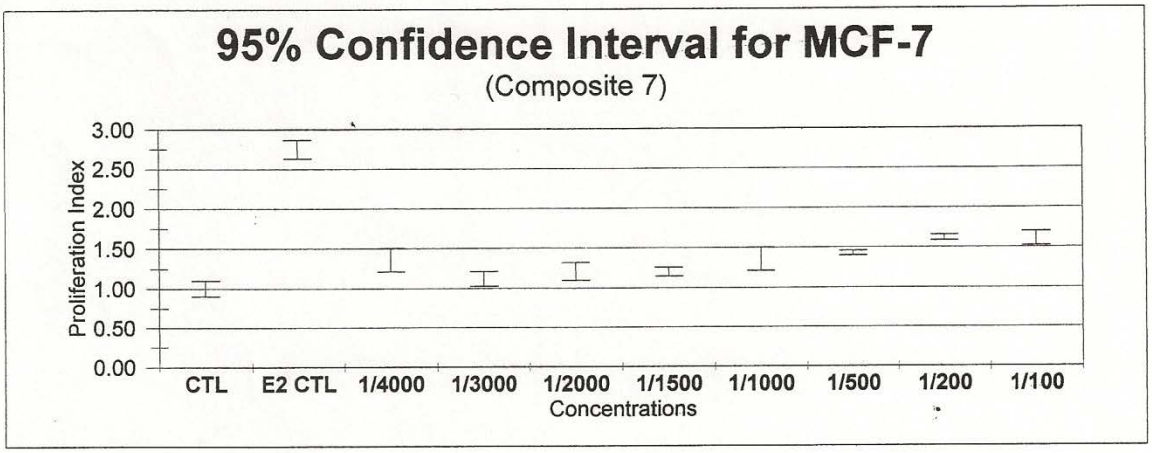
B.6 MCF-7 COMPOSITE 6

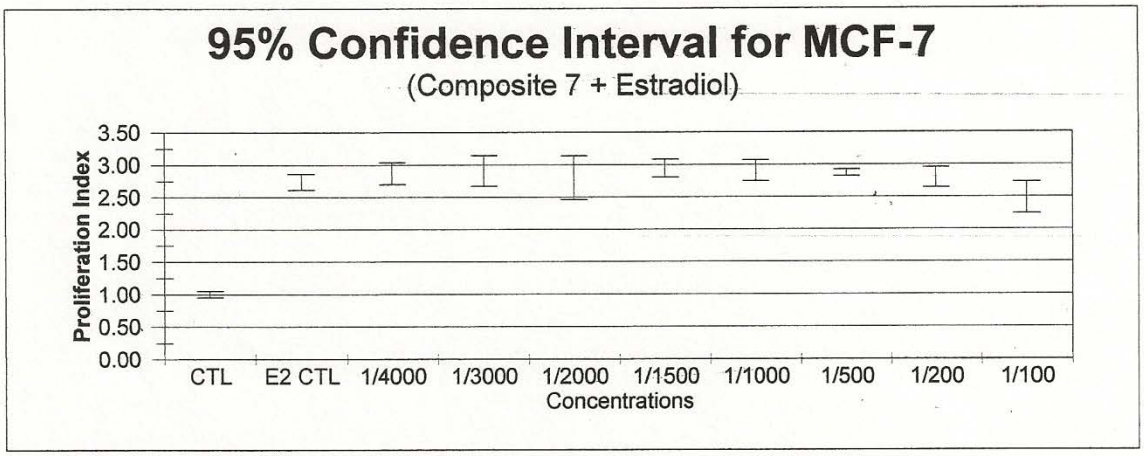


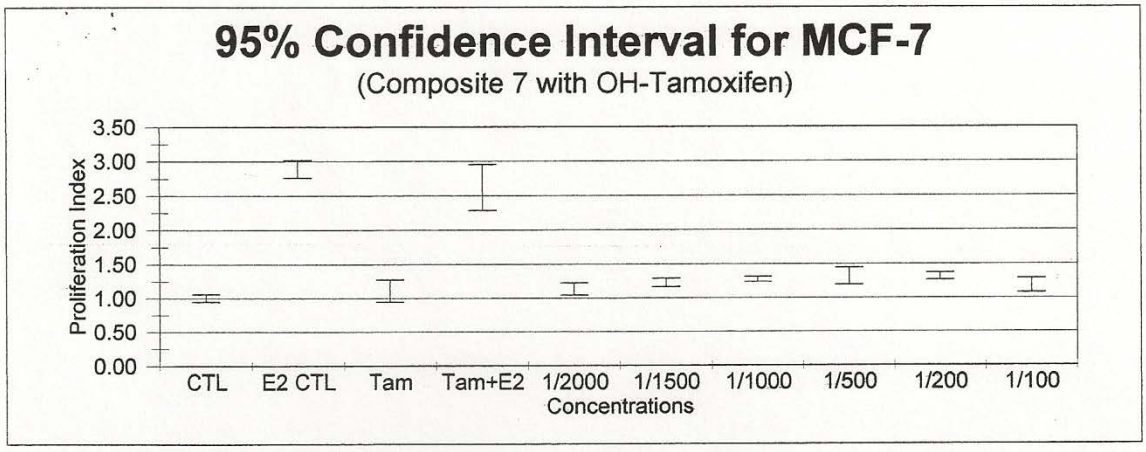




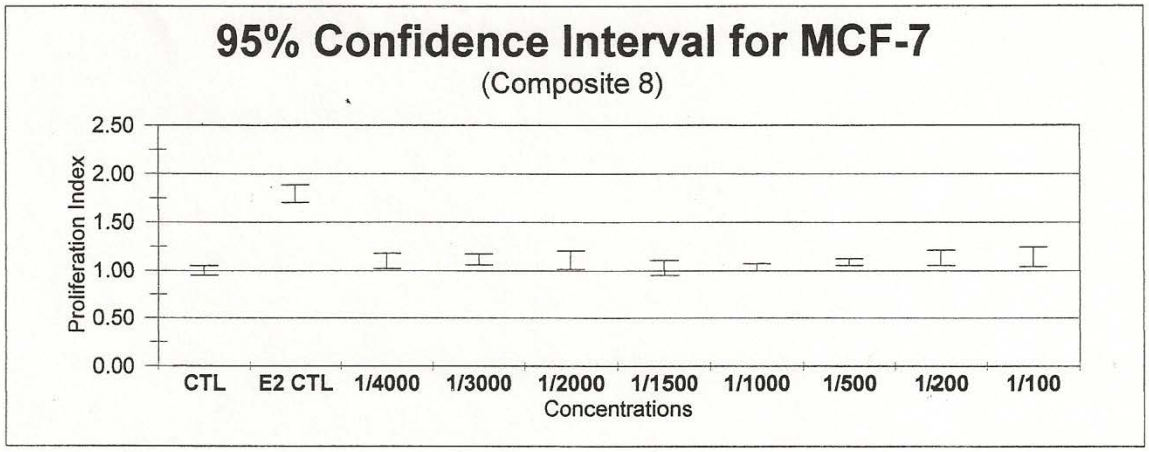
B.7 MCF-7 COMPOSITE 7

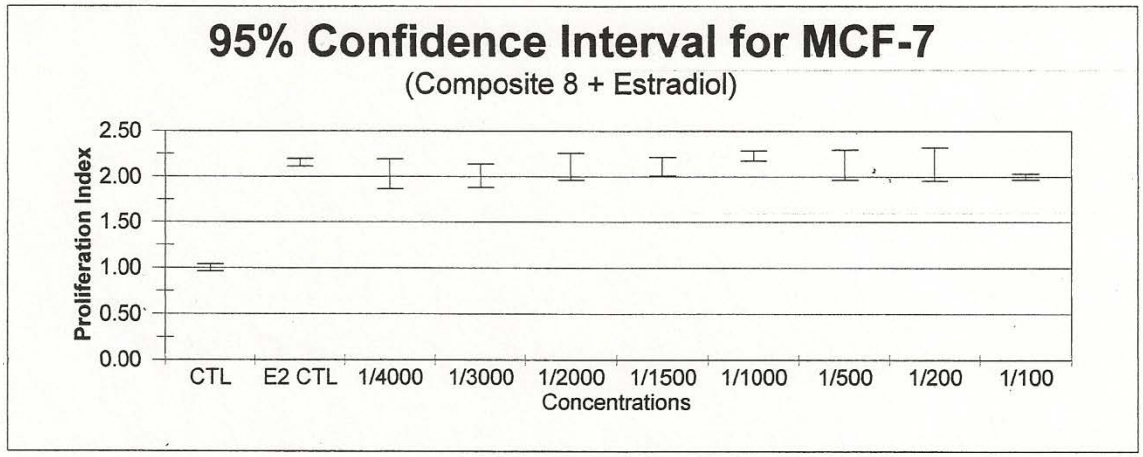


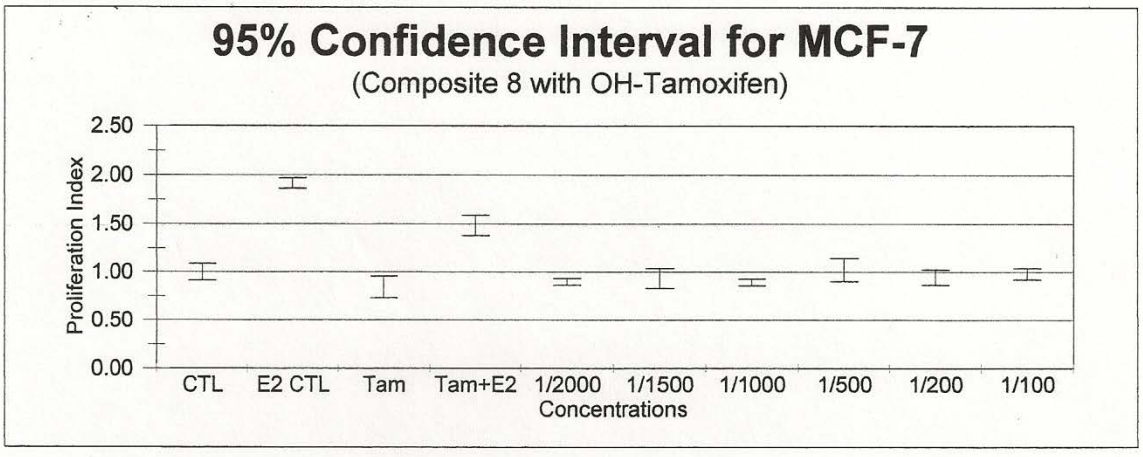




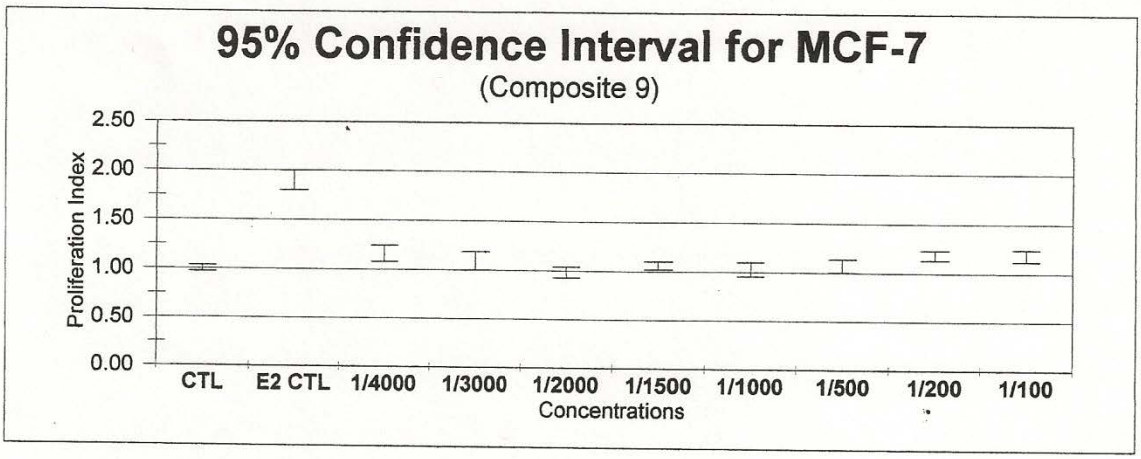
B.8 MCF-7 COMPOSITE 8

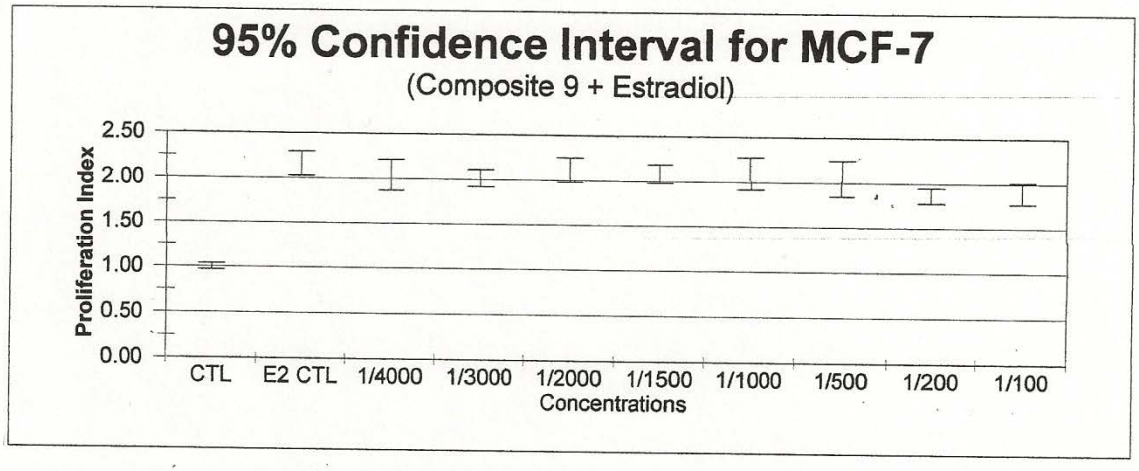


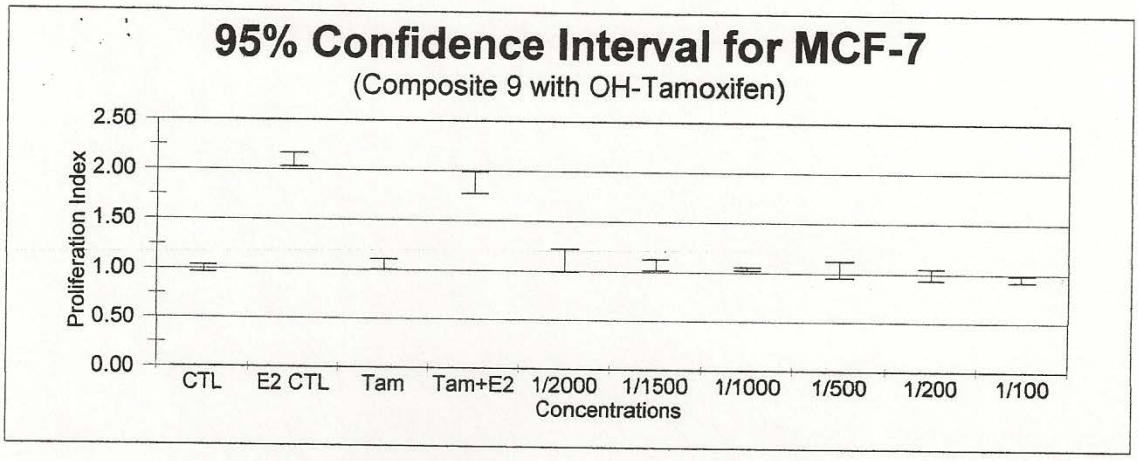




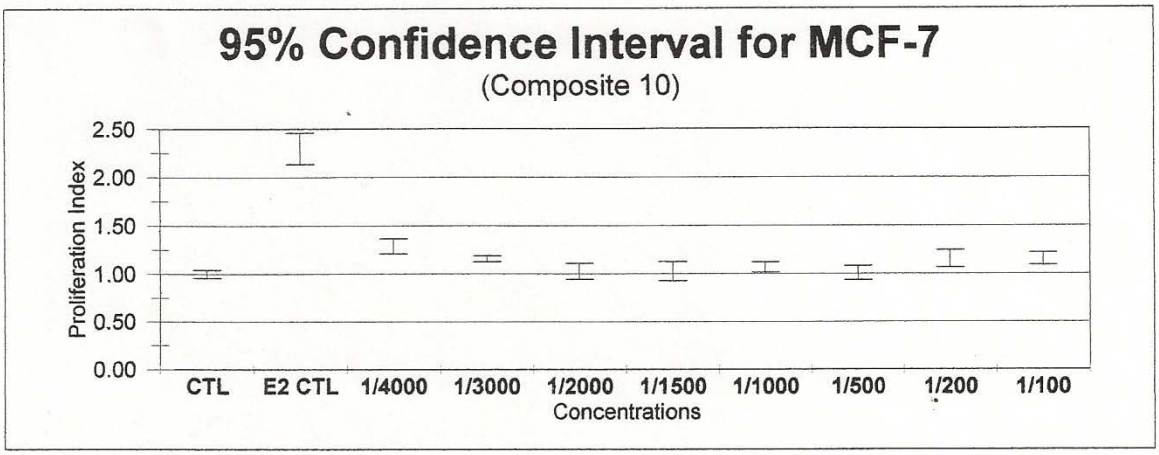
B.9 MCF-7 COMPOSITE 9

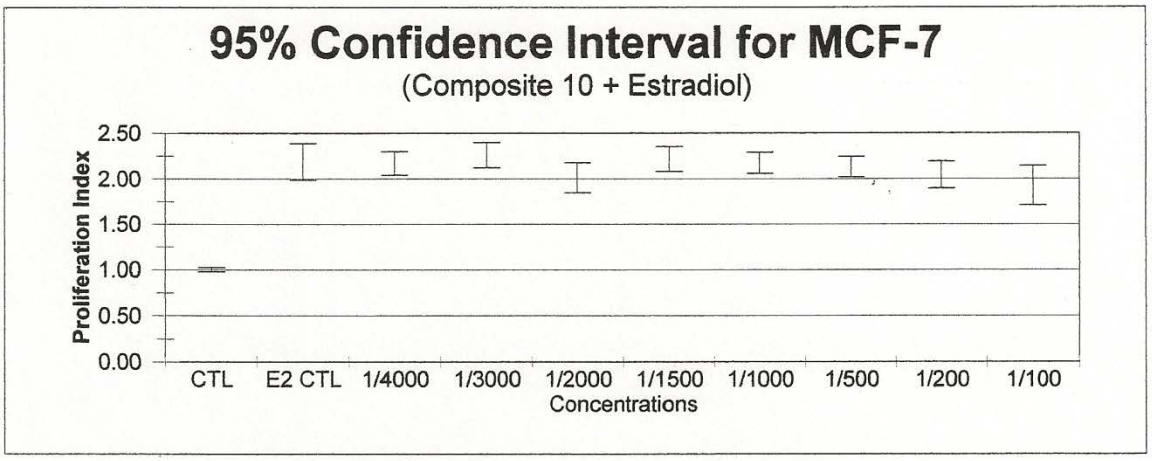


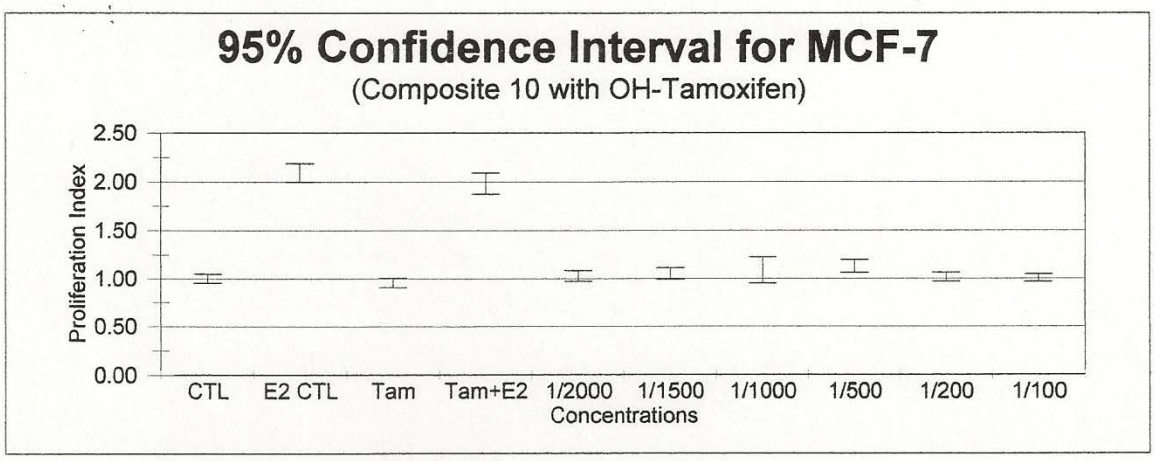




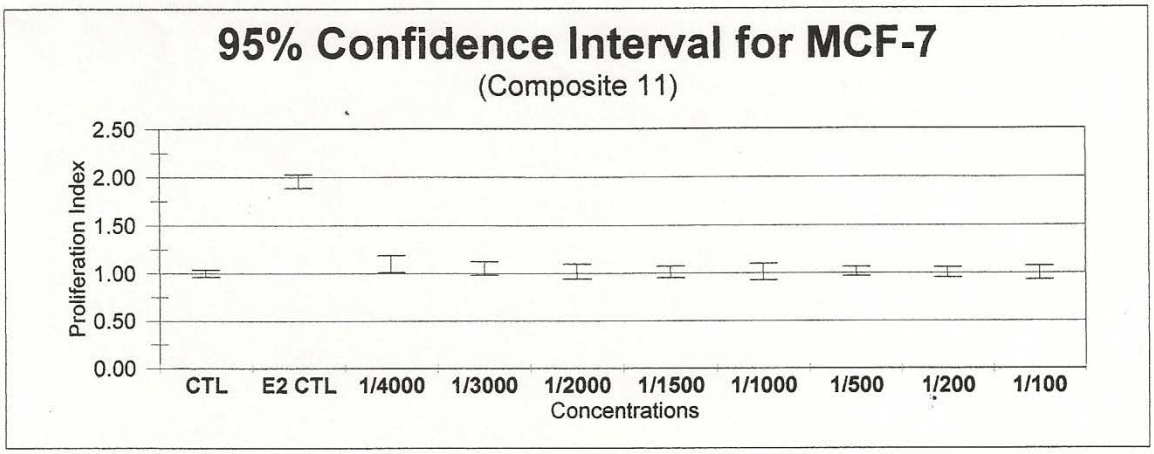
B.10 MCF-7 COMPOSITE 10

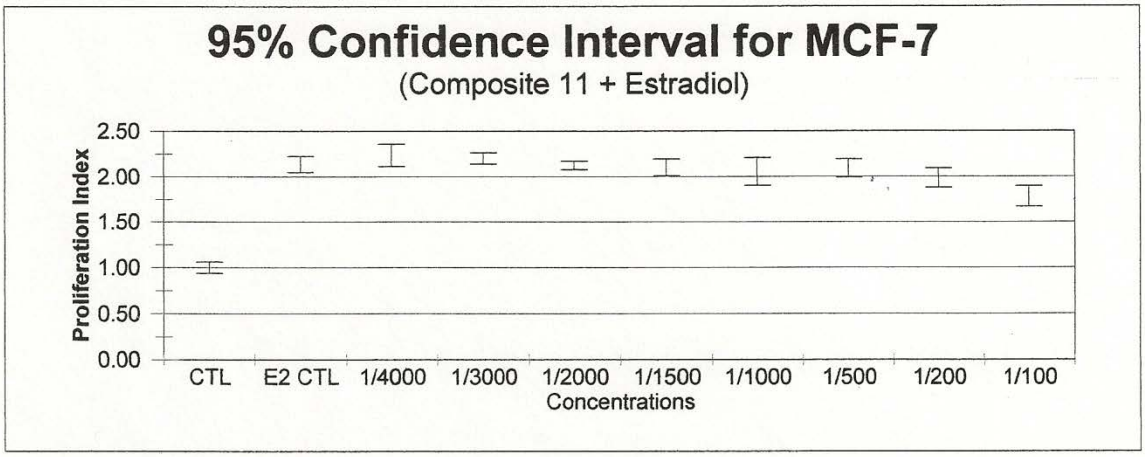


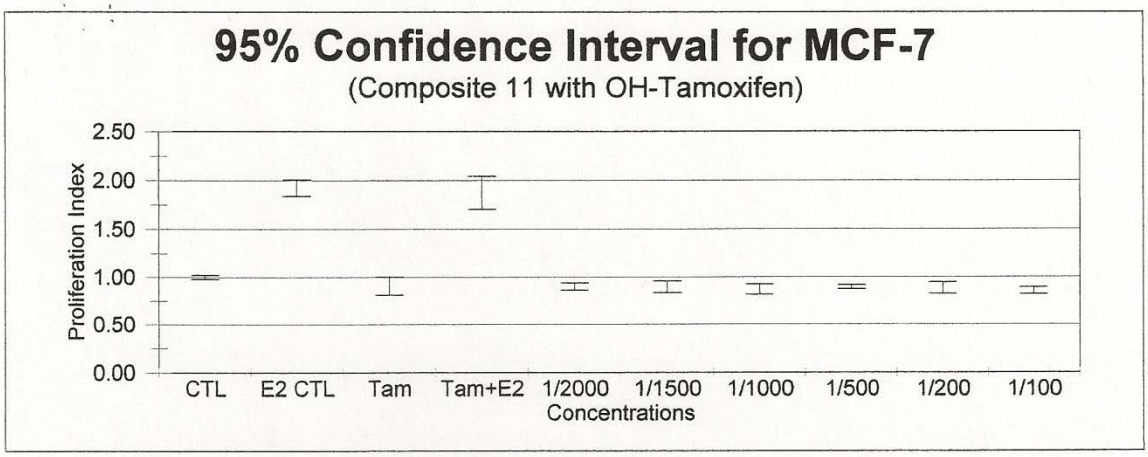




B.11 MCF-7 COMPOSITE 11

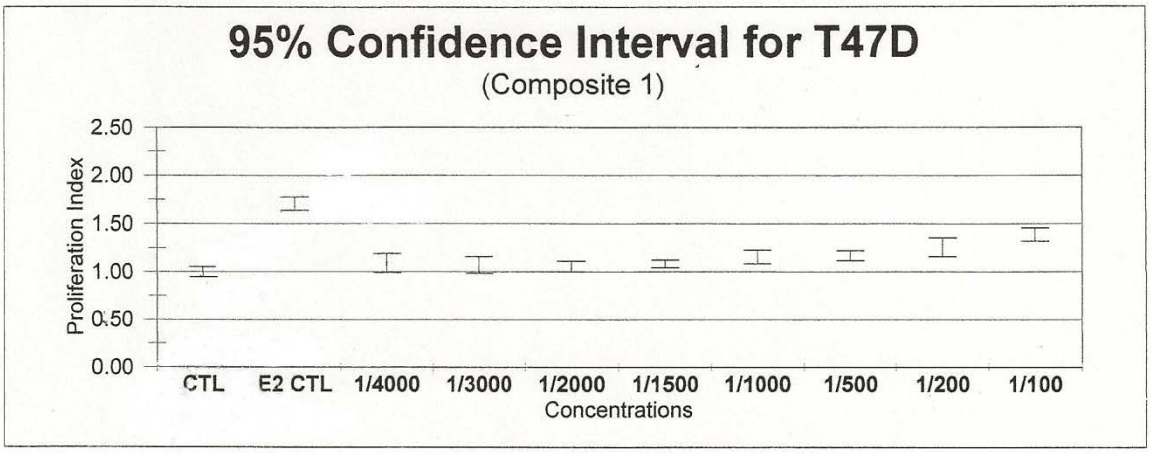


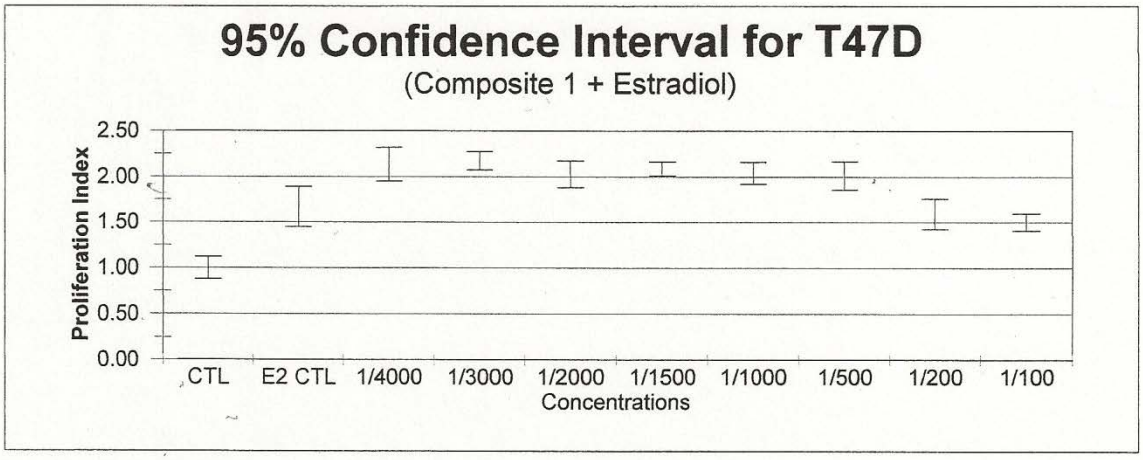


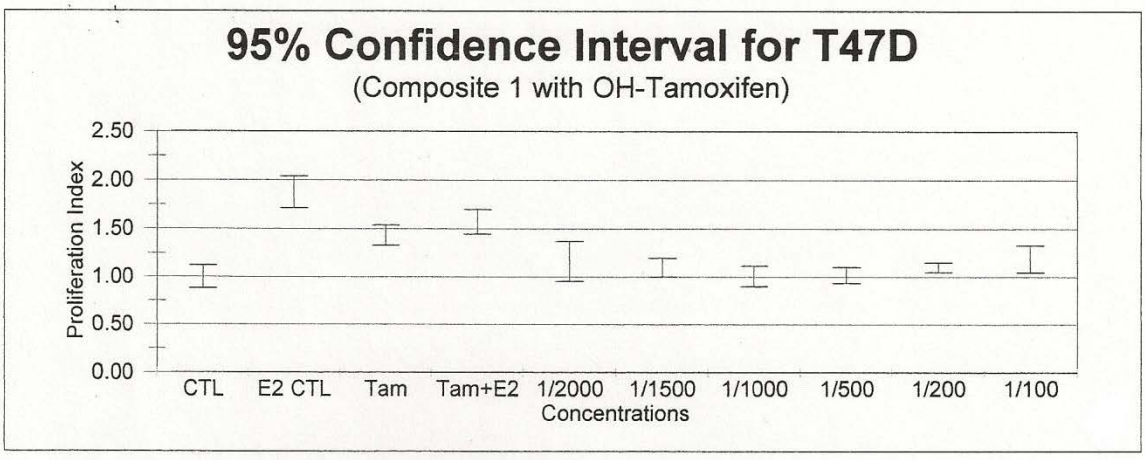


APPENDIX C: ERP MEAN AND 95% CONFIDENCE INTERVAL PLOTS FOR T47D

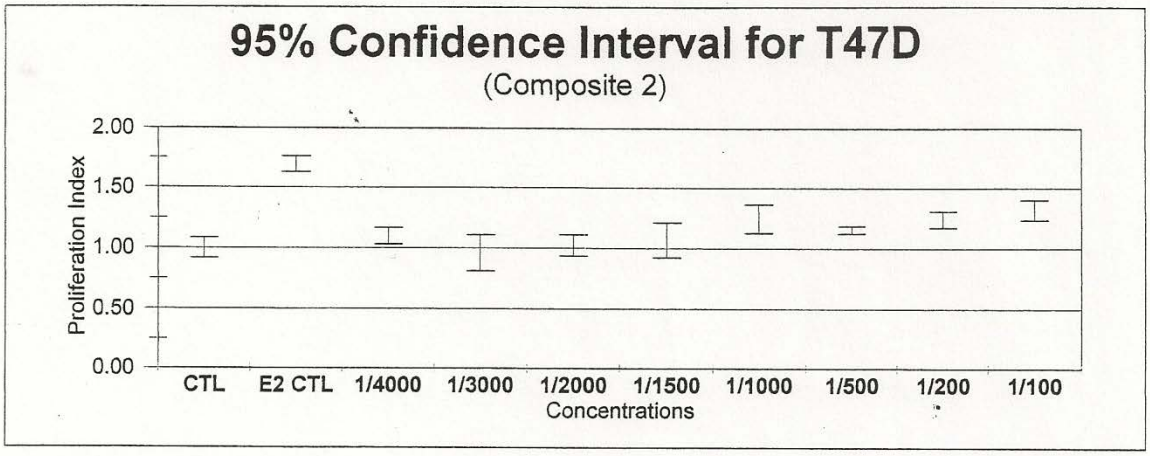
C.1 T47D COMPOSITE 1

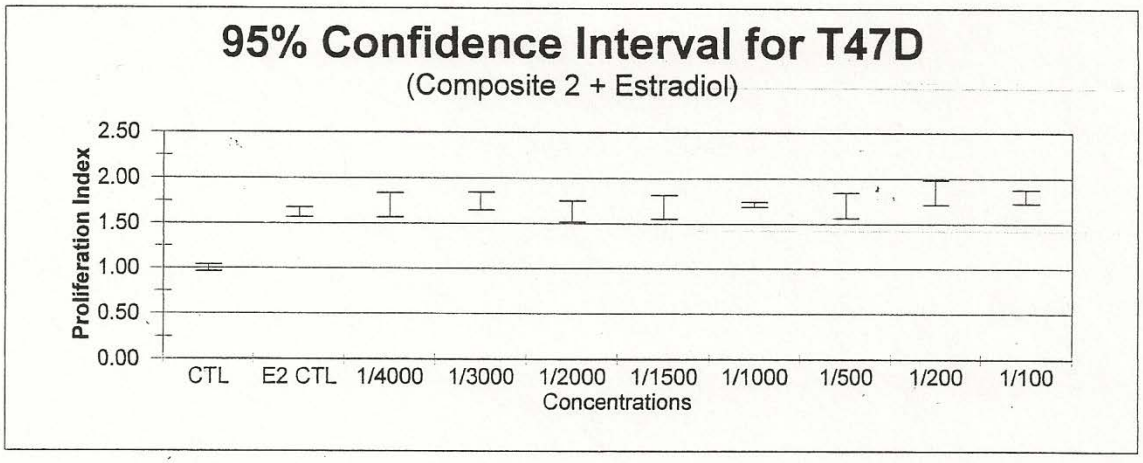


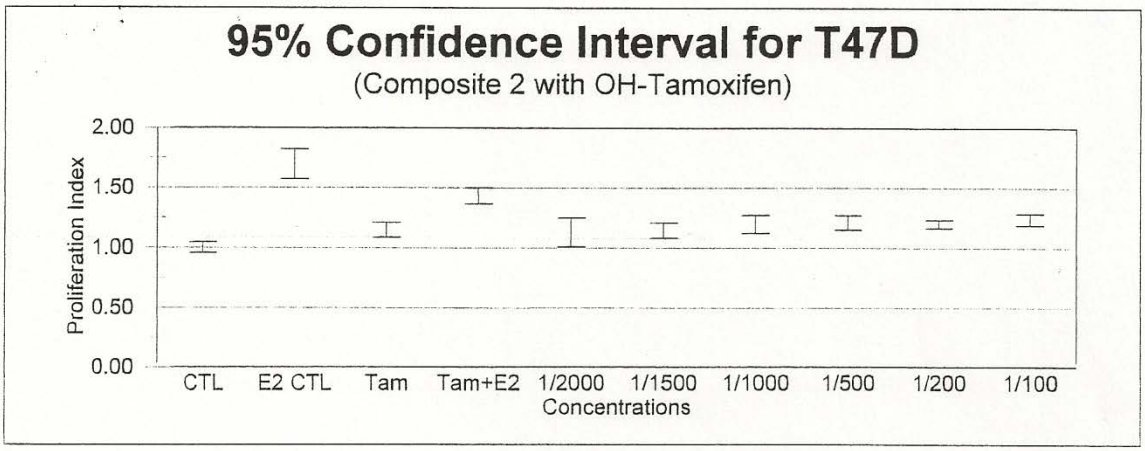




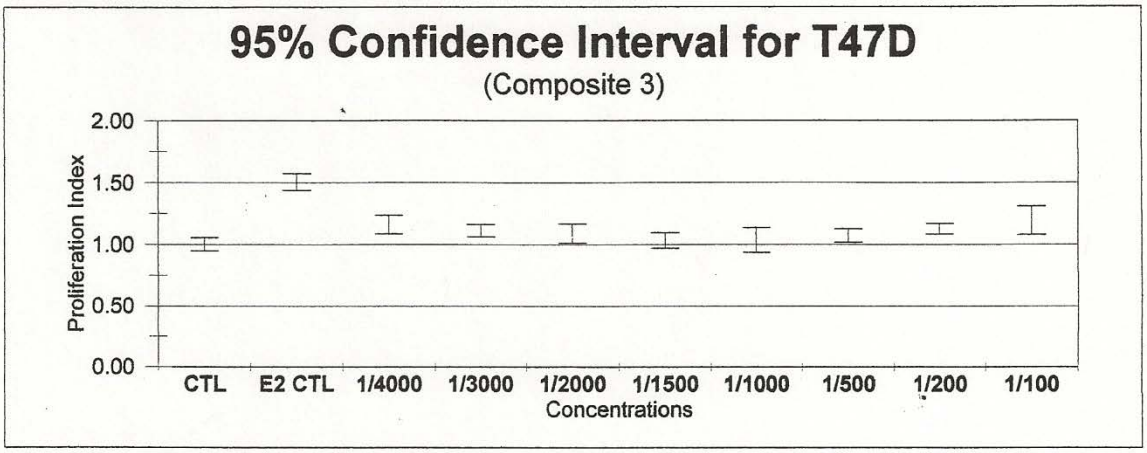
C.2 T47D COMPOSITE 2

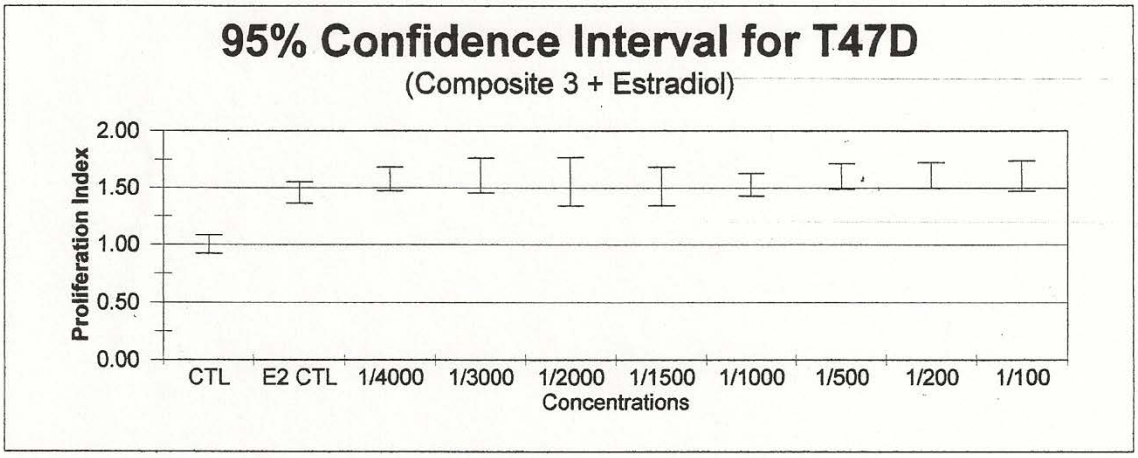


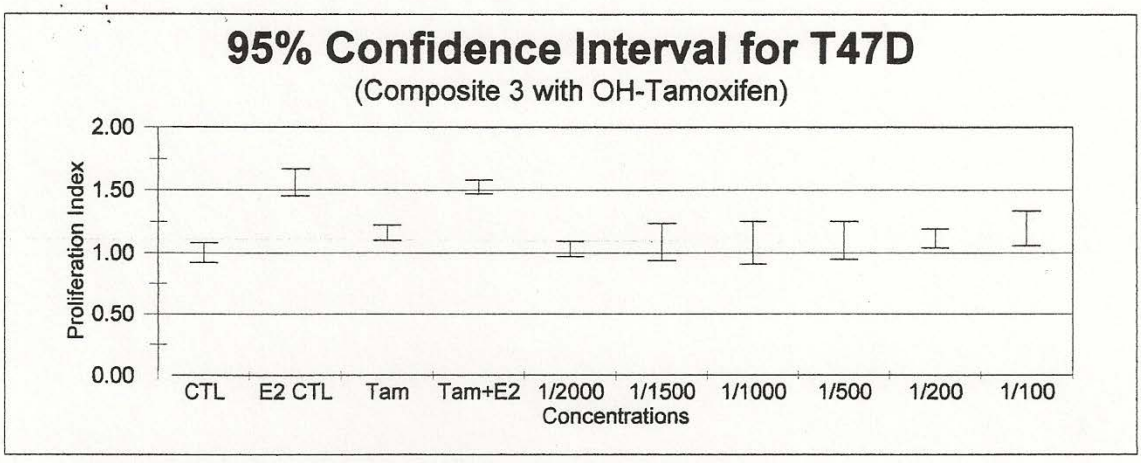




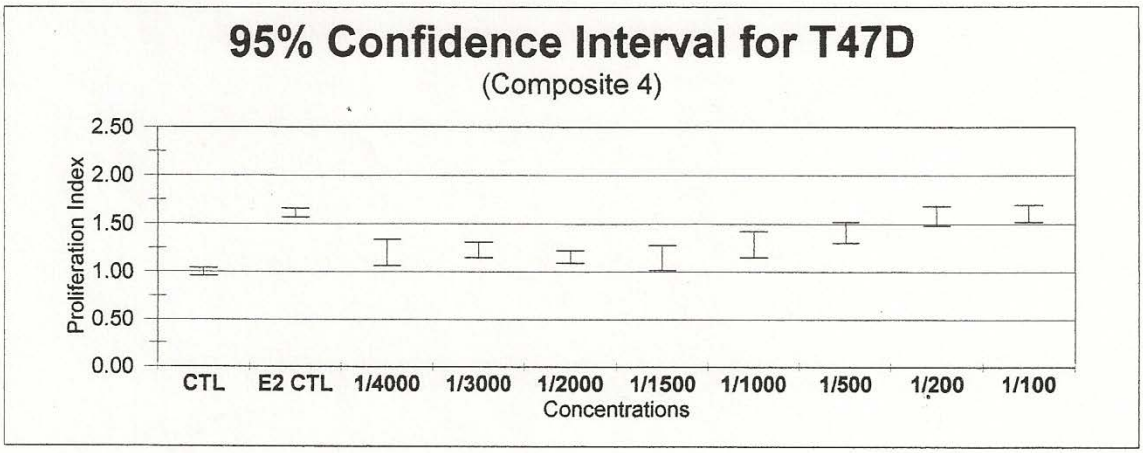
C.3 T47D COMPOSITE 3

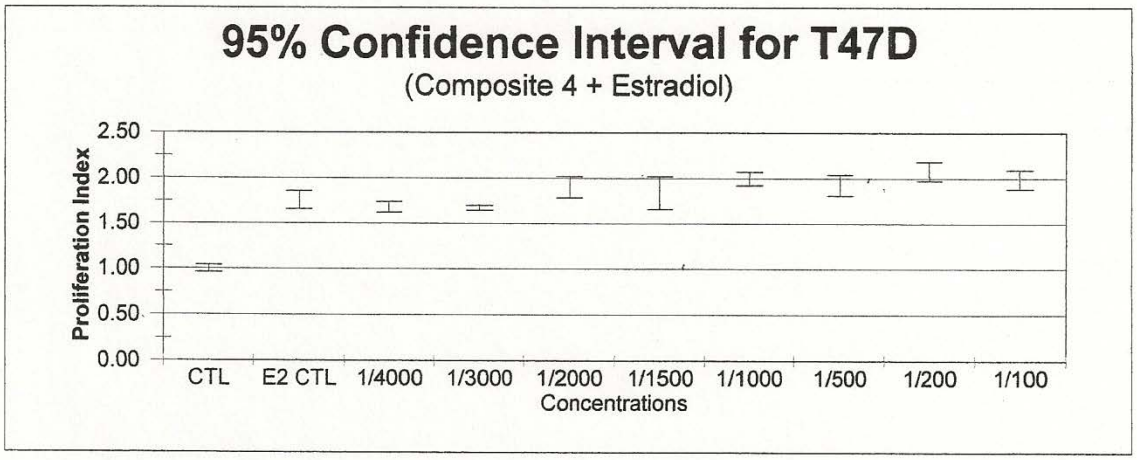


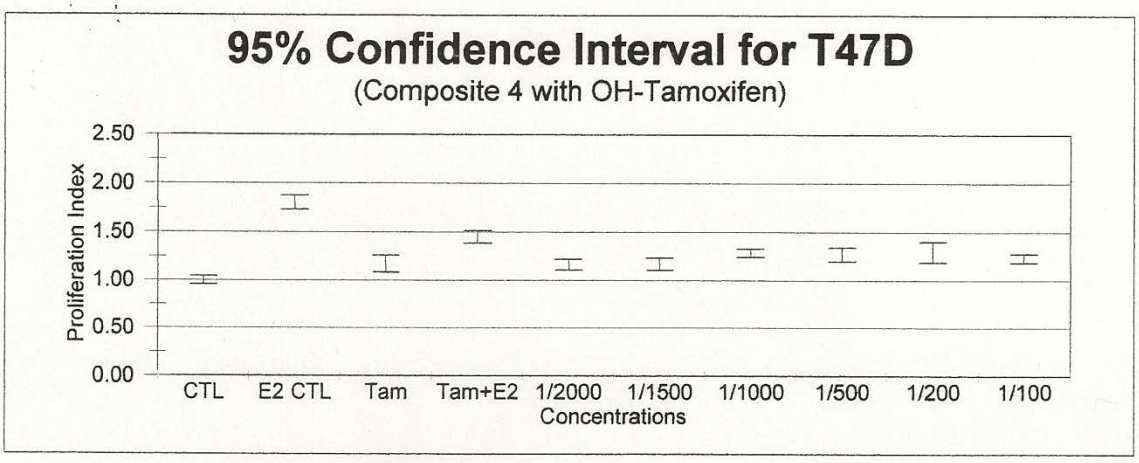




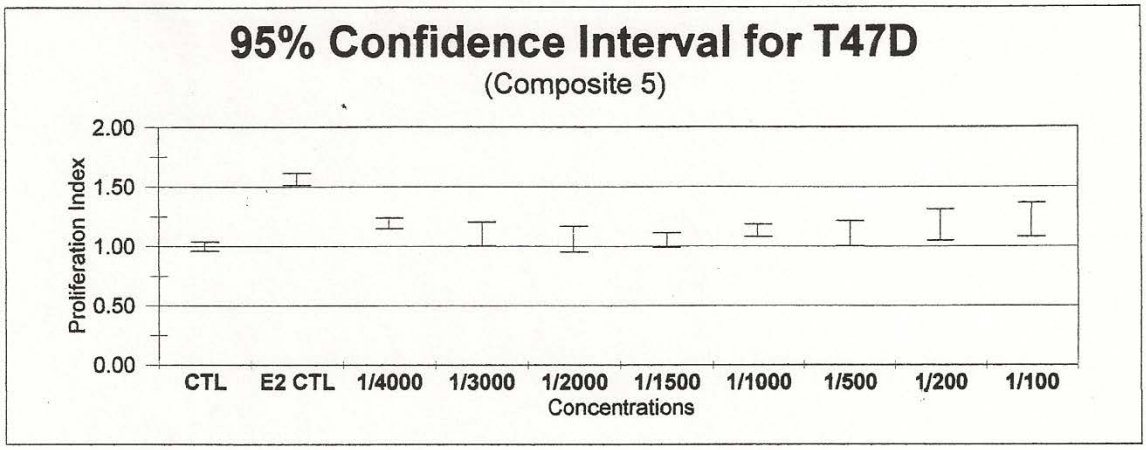
C.4 T47D COMPOSITE 4

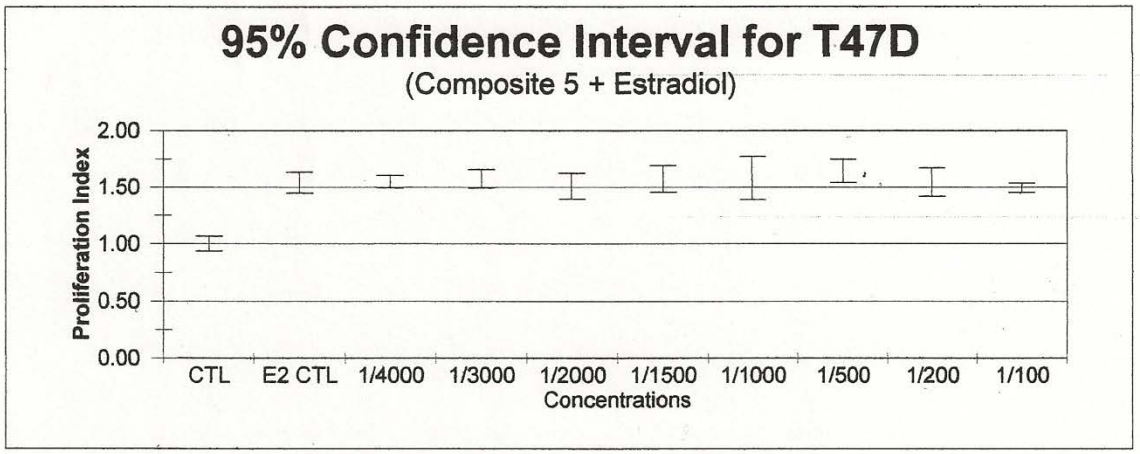


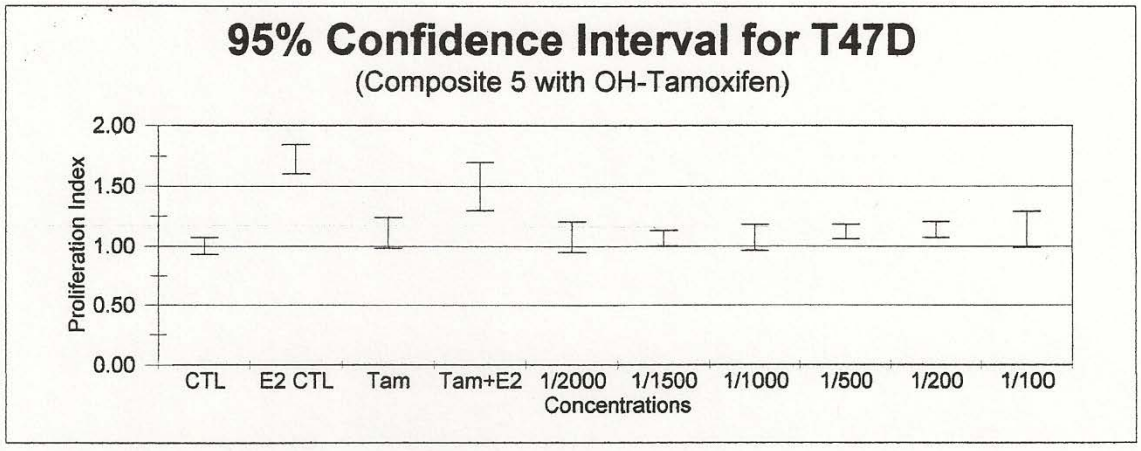




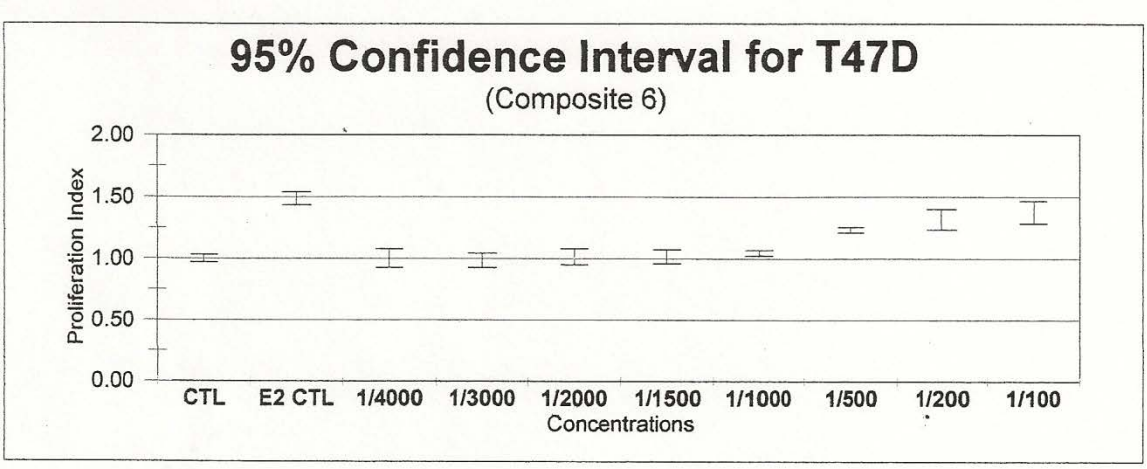
C.5 T47D COMPOSITE 5

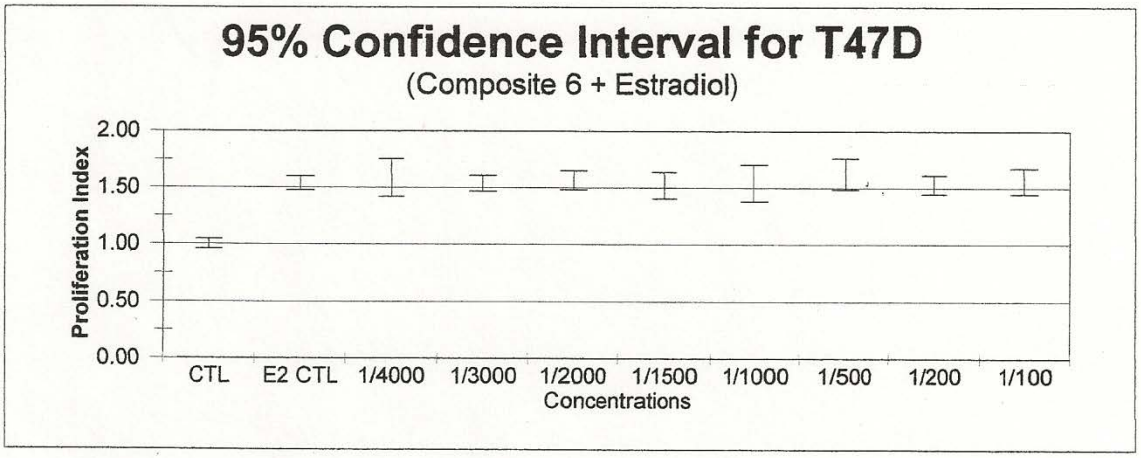


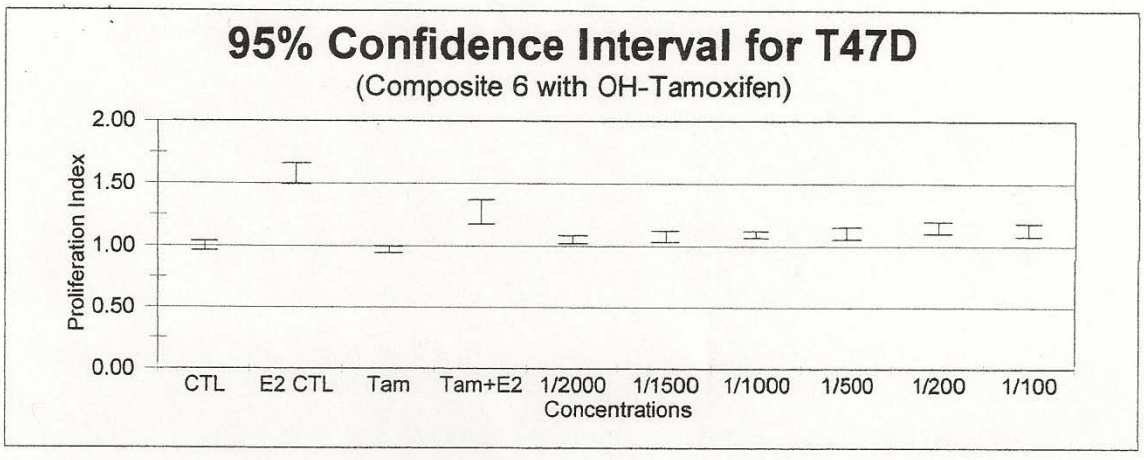




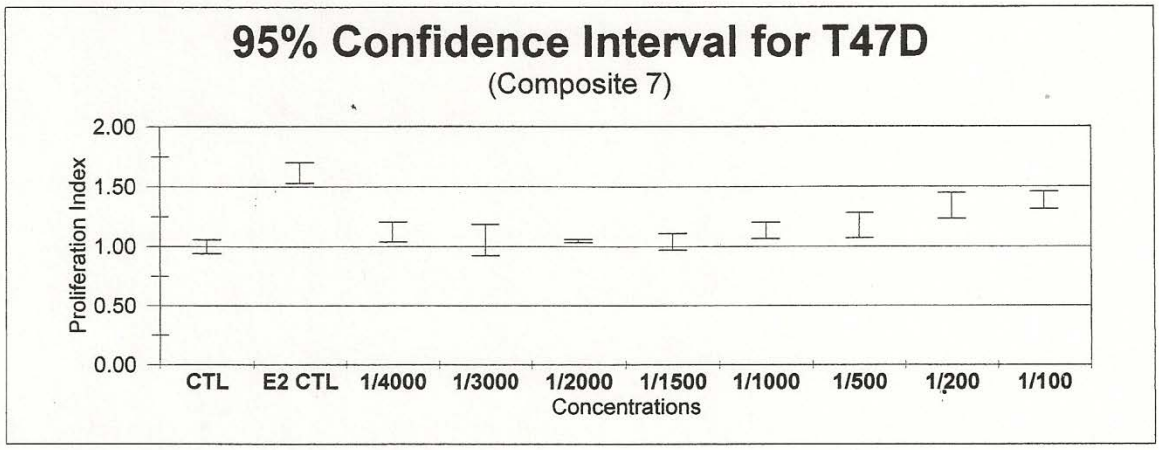
C.6 T47D COMPOSITE 6

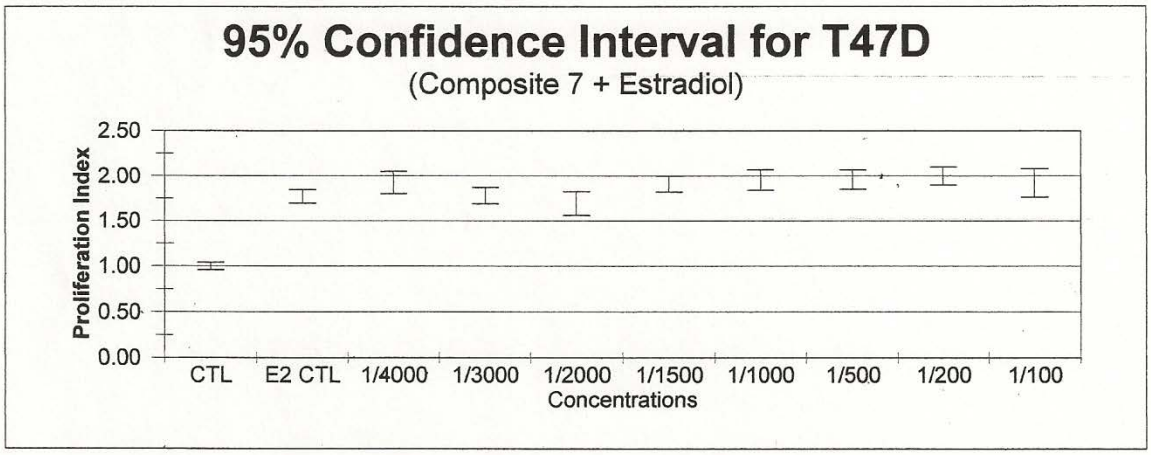


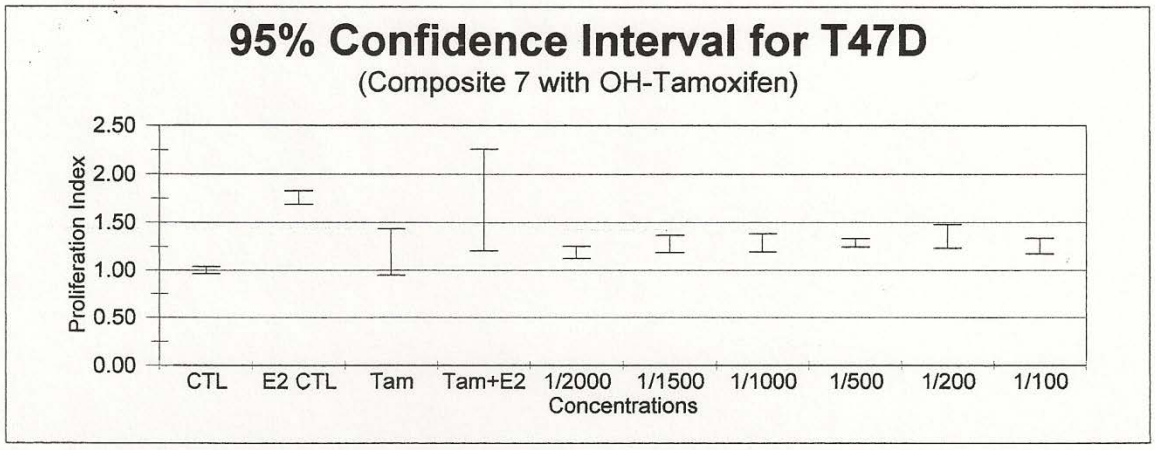




C.7 T47D COMPOSITE 7







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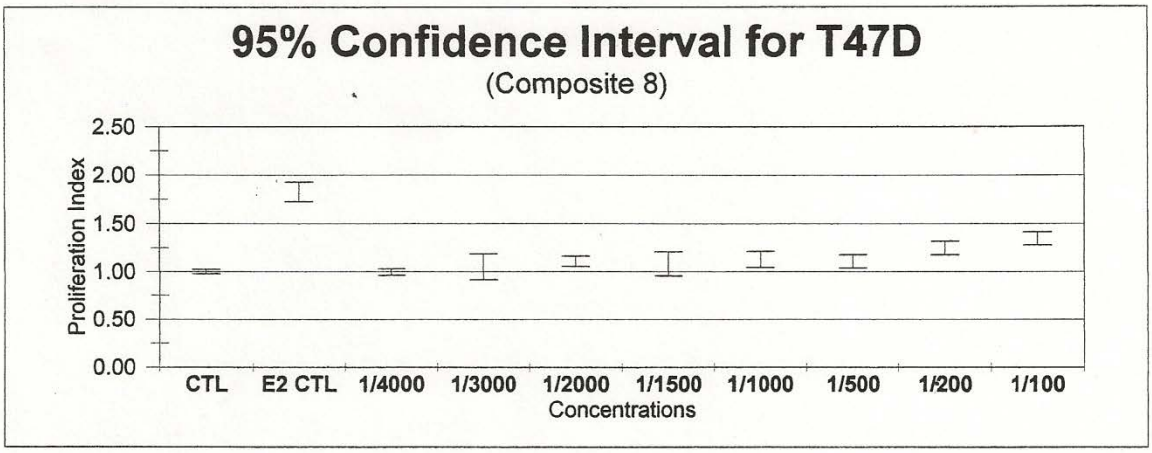

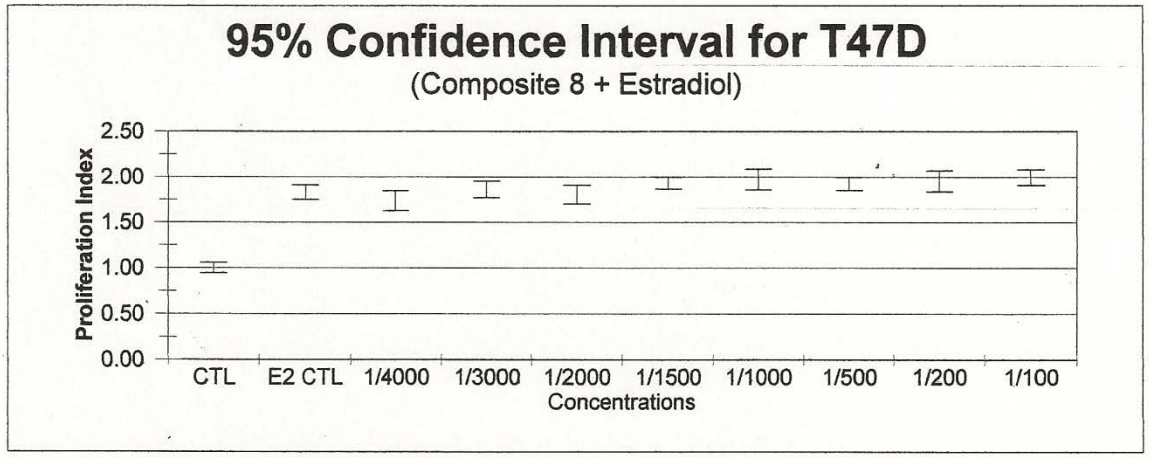

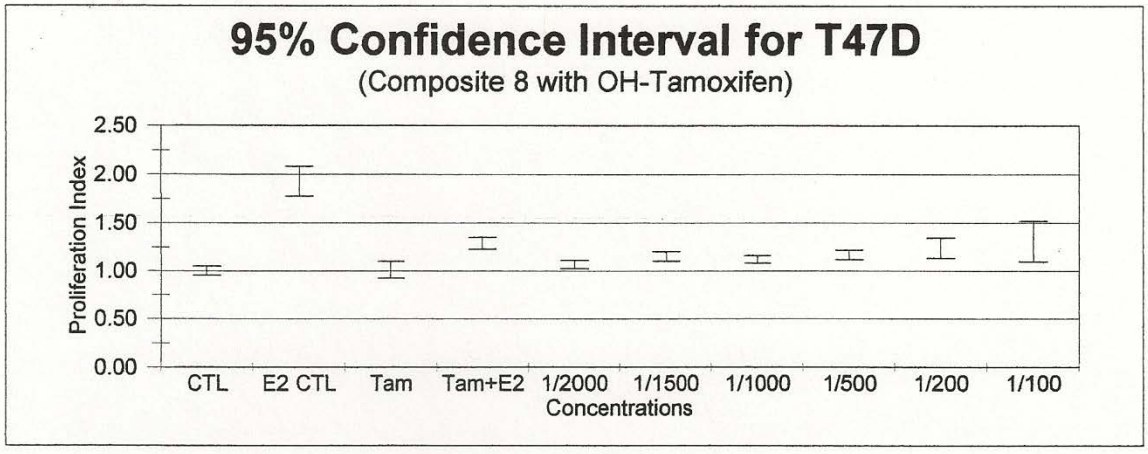

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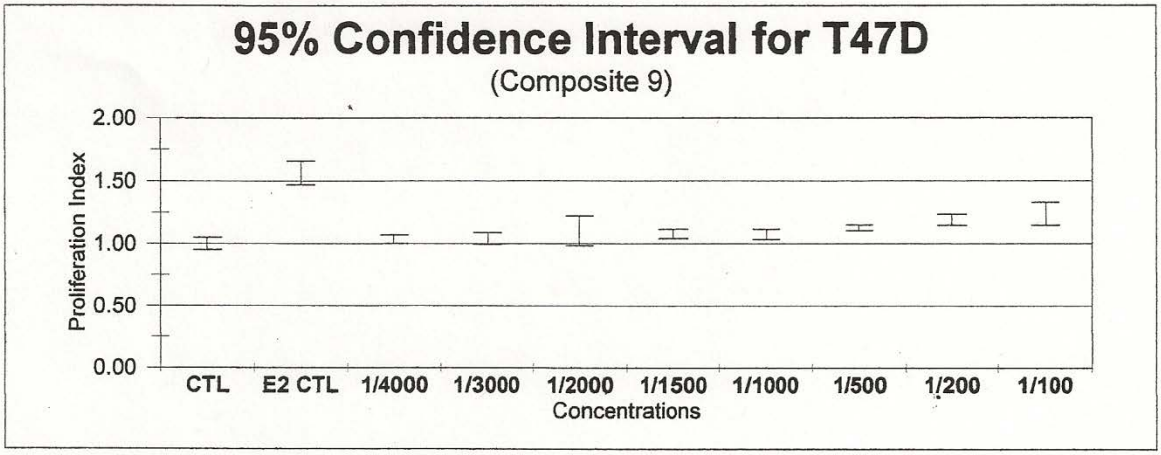

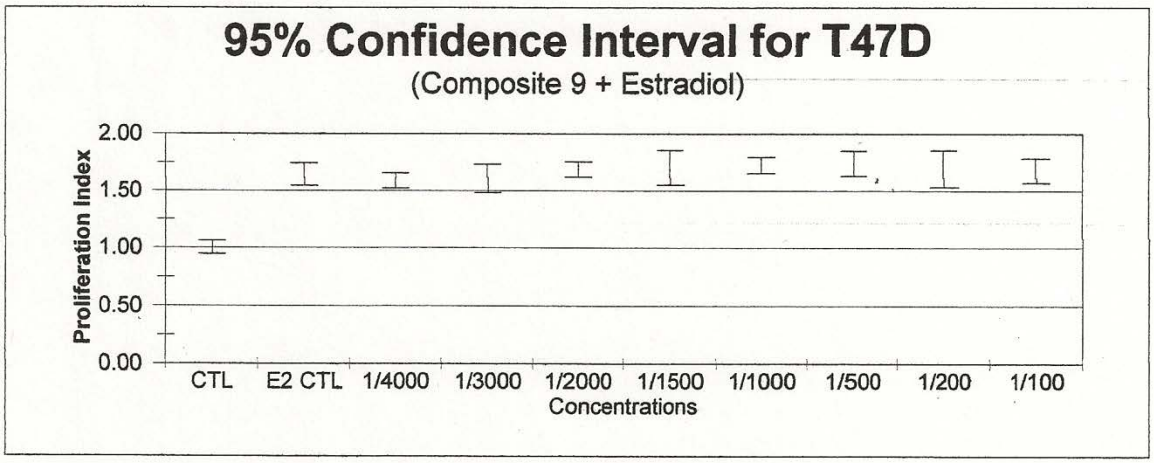

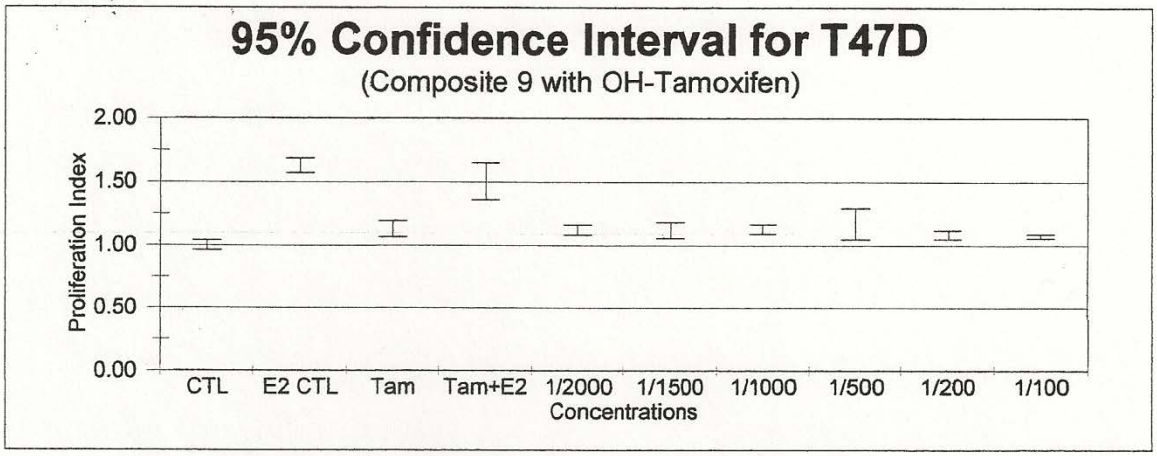

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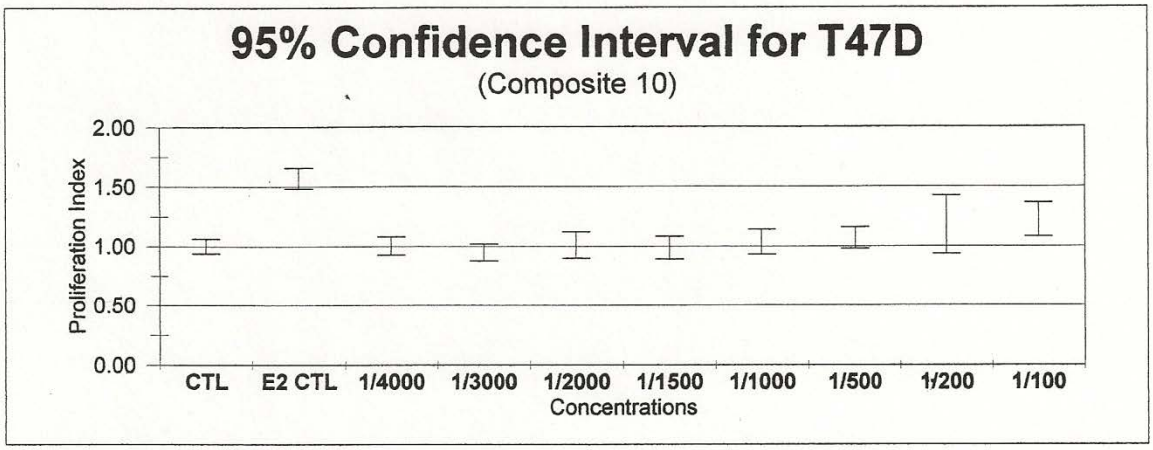

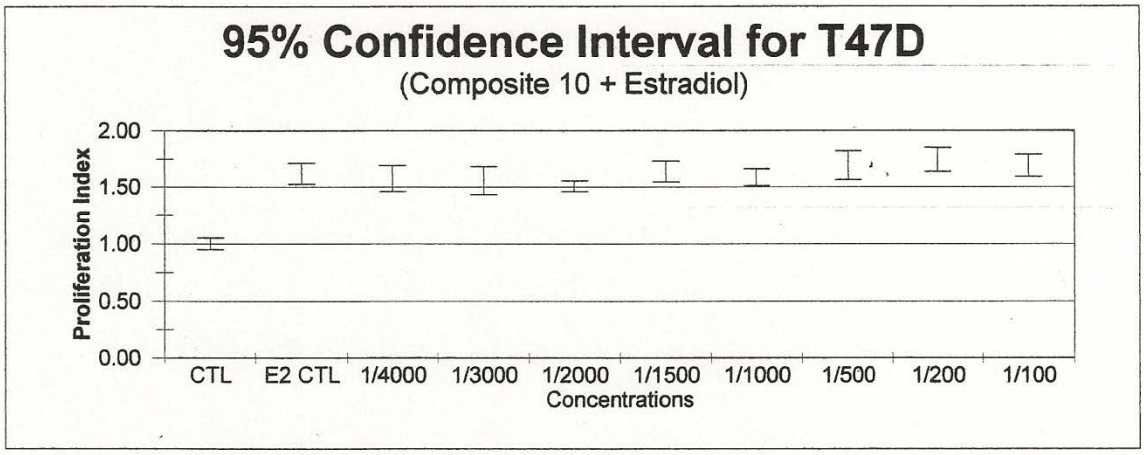

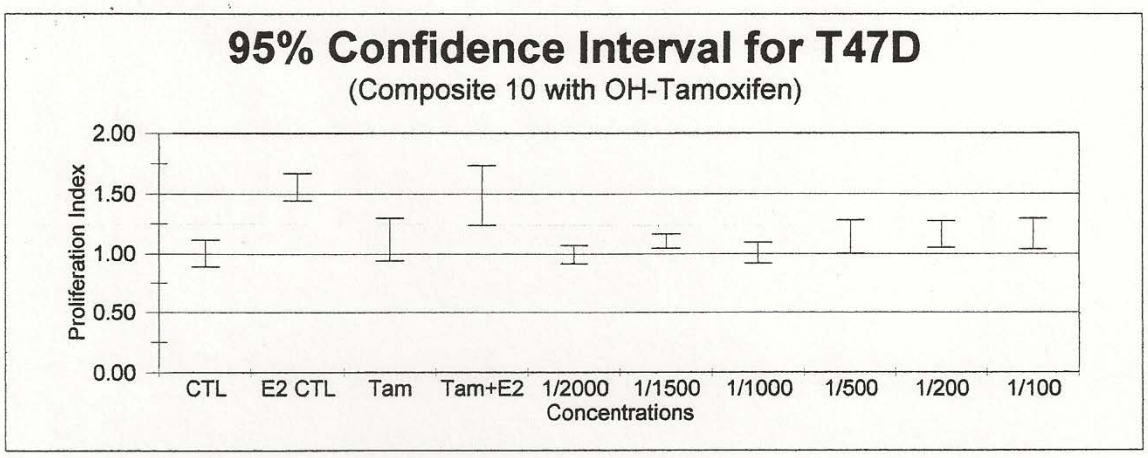

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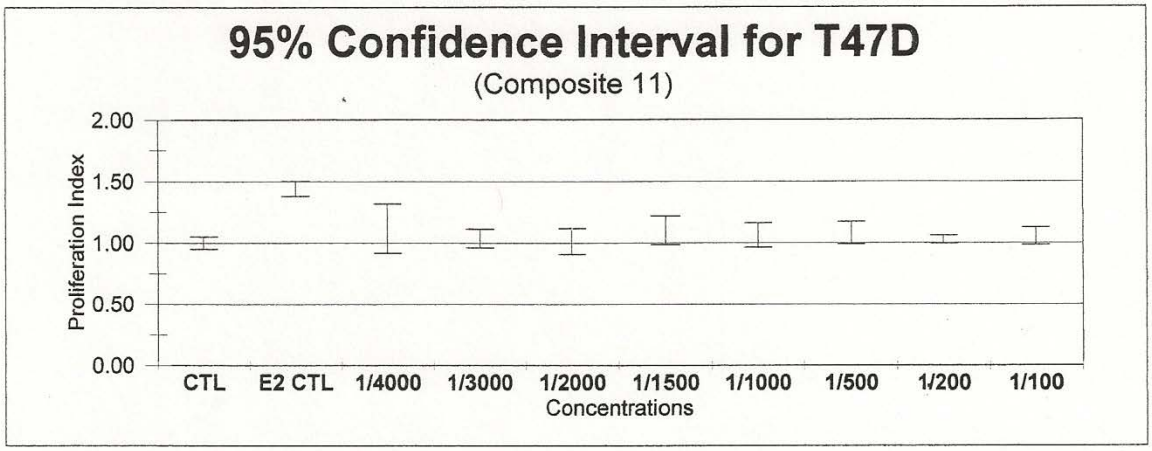

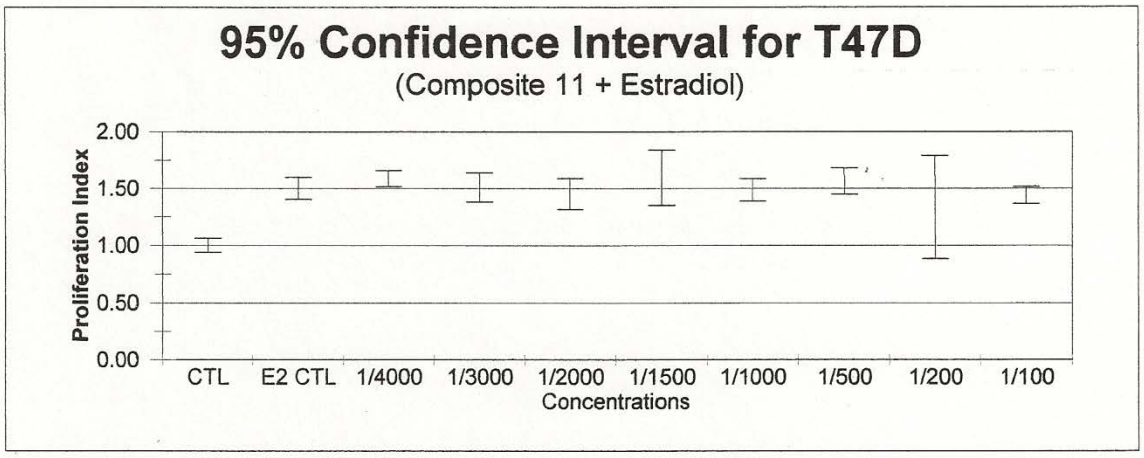

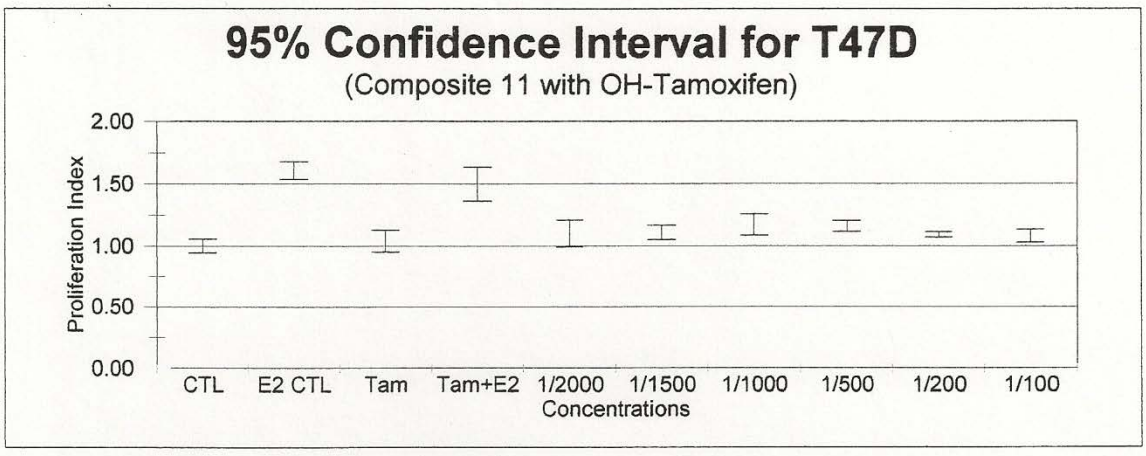

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