Interacting Effects of Tributyltin oxide (TBTO) and Diethylstilbestrol (DES) on the African Clawed Frog, *Xenopus laevis*

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I hereby recognize and pledge to fulfill my responsibilities, as defined in the Honor Code, and to maintain the integrity of both myself and the college community as a whole.

Pledge: _____

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ABSTRACT

The effects of organotin compounds on the endocrine system of amphibians are still largely unknown. However, recent research suggests that tributyltin may have the potential to block, at environmentally relevant concentrations, xenobiotic estrogens in amphibian models (Micael et al., 2007; Santos et al., 2006). This study was undertaken in order to examine the effects of exposure to binary mixtures of tributyltin oxide (TBTO) and the synthetic estrogen diethylstilbestrol (DES) on sexually mature male *Xenopus laevis*. The frogs were injected with no (untreated control), 0.667 mg TBTO, 0.667 mg DES, and 0.667 mg TBTO + 0.667 mg DES, and left for an exposure period of 10 days. Frogs treated with tributyltin oxide alone exhibited concentrations of vitellogenin similar to that of the untreated controls. DES treatments elevated vitellogenin concentrations above those of controls and those treated with TBTO. Treatment with both TBTO + DES resulted in vitellogenin concentrations intermediate to those of the other groups. These results suggest that tributyltin antagonistically inhibits the vitellogenin expression of diethylstilbestrol, possibly through the involvement of P450 aromatase or the suppression of gene expression.

INTRODUCTION

Over the past few decades, research has revealed that many anthropogenic compounds released into the environment impair the proper function of the endocrine system (Kloas and Lutz, 2006). These so-called ‘endocrine-disrupting chemicals’ (EDCs) alter normal developmental and reproductive steps in a wide range of vertebrates. Either by mimicking or blocking the action of endogenous hormones at receptor sites, endocrine disruptors augment normal gland-cell communication (Colborn and Celment, 1992; Pelissero et al., 1991). Even at

low-level exposures, these exogenous compounds have the ability to reverse sexual orientation, cause deformities of reproductive organs, and diminish reproductive potential of species (Kloas and Lutz, 2006).

Among the most studied of these endocrine disruptors are those substances that mimic the action of the steroid hormone estrogen. Estrogenic chemicals such as atrazine, 17β-estradiol, diethylstilbestrol, and alkylphenol compounds cause feminization in a number of fish, mollusks, and amphibian species (Rasmussen and Korsgaard, 2004; Hayes et al., 2003; Jobling et al., 2003; Kinneberg et al., 2003; Palmer and Palmer, 1995). However, only a few studies have focused on the potential of EDCs to masculinize an organism. Imposex, the occurrence of male genitalia in female neogastropods exposed to tributyltin (TBT), is the best-documented example (Santos et al., 2006; Morcillo and Porte, 2000; Gibbs and Bryan, 1986).

Endocrine disruptors, particularly TBT, accumulate in surface waters and bottom sediments were they persist for long periods of time (Kloas and Lutz, 2006). Aquatic biota, especially fish, mollusks, and amphibians, are then subjected to these endocrine disruptors directly and indirectly. Amphibians rely on osmotic transfer between their epithelial tissue and the surrounding water for the uptake of oxygen and vital ions, therefore making them particularly vulnerable to chemically contaminated waters. As a consequence, endocrine disrupting chemicals have been implicated in the decline of amphibian populations worldwide (Hayes et al., 2003). A variety of factors, including environmental conditions, pH, temperature, salinity and presence of chemicals are influential in the sex determination of amphibians (Reeder et al., 1998). However, for amphibians the effects of exposure to TBT are virtually unknown.

Tributyltin

Since the early 1970's tributyltin (TBT) has been used extensively in antifouling paints to prevent the settlement and growth of marine organisms on submerged structures. TBT is widely accepted as the most effective biocide ever devised for use on boats (Bosselmann, 1996). In the shipping industry alone the use of organotin antifoulants saves ~$5.7 billion per annum in fuel and repainting costs (Evans, 1999). Despite the economic and immediate environmental benefits, TBT contamination causes deleterious and lethal effects to many non-target organisms. The International Maritime Organization (IMO) signed legislation to entirely ban the use of organotins in anti-fouling paint by January 1, 2008 (Nehring, 2001). Despite the IMO's efforts, a large percentage of countries (~ 75%) have still not ratified the agreement. Other regulatory efforts to reduce the use of organotins in the shipping industry have met with limited success.

Tributyltin is a ubiquitous and persistent member of the organotin family. It is composed of three alkyl groups attached to a tin (Sn) atom (Figure 1). The presence of tin enables the compound to be readily absorbed by other particles forming complex aggregates that settle into bottom sediments. As a result, degradation processes are slow. TBT has a half-life of a year to decades when deposited in sediments (Evans, 1999).

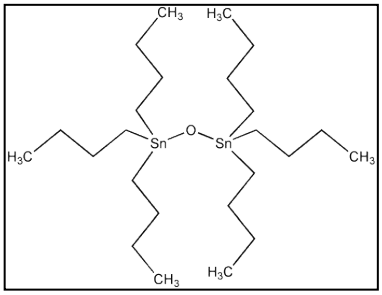


Figure 1: Chemical structure of bis (tributyltin) oxide *(www.pesticideinfo.org)*

Bivalves are highly susceptible to the negative effects of deposited TBT compounds. Exposure causes abnormal shell growth in oysters, reduced scope for growth, and reproductive

failure (Alzieu, 1991; Widows and Page, 1993). Low-level exposure causes imposex in over 150 snail species throughout the world (Fent, 1996). Accessory Sex Organs (ASO) including, sperm duct, seminal vesicle, external sperm groove, and penis, have all been identified in females exposed to concentrations of TBT (Oberdorster and McClellan-Green, 2002). These observations suggest that tributytin is an effective chemical in the masculinization of neogastropods.

However, current research is inconsistent regarding the biochemical pathway of Tributyltin (TBT). There are several proposed mechanisms of actions. One hypothesis is that TBT induces the release of peptide hormone Penis Morphogenic Factor (PMF) to abnormally high levels (Oberdorster and McClellan-Green, 2002). However, there is no evidence that PMF exists. Another hypothesis states that aromatase activity is inhibited by the presence of TBT, which results in elevated testosterone and decreased estradiol levels (Oberdorster and McClellan-Green, 2002). TBT-dosed snails not exhibiting imposex have a ~52% reduction in aromatase activity to those of untreated mud snails. Furthermore, exogenous estrogens have the capacity to block the masculinizing effect of tributyltin. The study concluded that a dietary co-exposure to a binary mixture of both TBT and estrogen-ethinylestradiol (EE2) at 25 and 100 ng TBT/g completely blocked the growth of accessory sex organs in zebra fish (Santos et al., 2006). These findings highlight the need for further research on tributyltin, especially as to the combined effects of contaminates present in the environment.

Diethylstilbestrol

Diethylstilbestrol (DES) is one such contaminant in the water column of aquatic ecosystems worldwide. Originally developed in the late 1940's to prevent premature birth, it was not until nearly a decade later that DES was discovered to cause significant detrimental effects

on embryonic development in humans and animals (Palmer and Palmer, 1995). The progeny of women taking DES have reproductive organ dysfunction, reduced fertility, abnormal pregnancies, and increased occurrences of vaginal clear-cell adenocarcinomas (Palmer and Palmer, 1995).

Unlike estrogen-ethinylestradiol (EE2), a similar synthetic estrogen, DES is metabolized at a much slower rate once present in the body. As a consequence, diethylstilbestrol bioaccumulates in the fatty tissues of organisms. Current research confirms the capacity of DES to induce the production of vitellogenin (Vtg) in male organisms over that of similar endocrine disrupting chemicals (Yamauchi et al., 2000). Once in the body, diethylstilbestrol induces characteristics of feminization (vitellogenesis) in a wide range of oviparous vertebrates, most notably the African Clawed Frog, *Xenopus laevis* (Palmer and Palmer, 1995).

Vitellogenin

Since diethylstilbestrol acts estrogenically, the production of vitellogenin (Vtg) is an ideal biomarker in accessing DES-containing binary mixtures. Vitellogenin is a large serum phospholipoglycoprotein responsible for egg yolk production in oviparous (egg laying) and ovoviviparous vertebrates (Heppell et al., 1995). Estrogens, both exogenous and endogenous, are the primary stimulus that activates the synthesis of vitellogenin in the liver (Palmer and Selcer, 1996). After being produced in the liver, vitellogenin is transported to the oocytes via the circulatory system and is incorporate into egg yolk (Heppell et al., 1995). Vitellogenin is of particular importance to developing organisms, such as *X. laevis*, since it serves as the metabolic energy source (Palmer et al., 1998). Maturing females naturally have elevated levels of estrogens circulating in their blood, thus the production of vitellogenin is normal. However, the presence of

this estrogen-inducible protein (vitellogenin) in sexually mature male *X. laevis* can be taken as strong evidence of exposure to endogenous or exogenous estrogens or estrogen mimics (Heppel et al., 1995).

Xenopus laevis

The African Clawed Frog, *X. laevis*, has long been a model for vertebrate endocrine studies as it is particularly sensitive to the effects of estrogens (Palmer et al. 1998). Furthermore, the amphibian is completely aquatic, making it a representative model for exposure studies to tributyltin (TBT) and diethylstilbestrol (DES), as they are compounds that are primarily found in aquatic ecosystems. *Xenopus* is also very easy to obtain and rear in the lab.

Injection Studies and Hypothesis

This study was undertaken in order to determine whether or not tributyltin oxide (TBTO) acts as an endocrine disrupting chemical in the African Clawed Frog, *X. laevis.* The secondary objective of the study was to examine the effects of co-exposure to binary mixtures of TBTO and the synthetic estrogen DES. By injecting the frogs with varying dosages of the TBTO, DES, and TBTO +DES, the chemicals were readily present in the bloodstream. I hypothesized (1) that tributyltin oxide would not induce the production of vitellogenin. Furthermore, I hypothesized (2) that in sexually mature male *Xenopus* low does exposure to tributyltin oxide would inhibit the production of vitellogenin when co-exposed to diethylstilbestrol.

MATERIAL AND METHODS

Animals

Sexually mature male African clawed frogs, *X. laevis*, were acquired from Xenopus 1 Inc (Ann Arbor, Michigan) and housed in 10-gallon aquaria with 4 gallons of dechlorinated tap water (pH 6.8 ± 0.3) maintained at room temperature (23 +/- 1 ^{o}C). Initial stock populations consisted of 40 frogs split between two aquaria. In order to acclimate the frogs to laboratory conditions they were placed under a blanket (~ 24 hour dark cycle) until the time of experimental use. The dechlorinated water was regularly changed every four days. Frogs were fed every other day with dry *Xenopus* brittle.

Chemicals

Bis (tributyltin) oxide 96%, 17β Estradiol, and Diethylstilbestrol (DES) were obtained from Sigma-Aldrich Chemical Corporation, St. Louis, MO. Polyclonal rabbit anti-*Xenopus* vitellogenin was a gift from Dr. Kyle Selcer, Department of Biology, Duquesne University. Goat Anti-Rabbit IgG (H+L) and TMB Peroxidase EIA Substrate Kit were purchased from Bio-Rad Laboratories Inc., Hercules, CA. All remaining chemicals were obtained from Allegheny College Biology Department.

Note: Phosphate Buffered Saline (PBS) or mineral oil was used as the solvent for the concentrations of Bis (tributyltin) oxide, 17βEstradiol, and DES.

Supplies

The Allegheny College Biology Department provided all supplies used throughout the study.

Procedure

Pilot Study

The rationale of this study was to establish a level of sublethality to bis (tributyltin) oxide. Once determined, appropriate concentrations could be derived and applied to future studies. *X. laevis* were randomly selected from the stock population, injected with various doses of the chemical, and placed into 6.6L plastic containers filled partially with dechlorinated tap water. Each container housed two specimen exposed to the same treatment. The initial treatments consisted of 20 mg and 10 mg bis (tributyltin) oxide. Frogs were injected with 200 uL (20 mg) and 100 uL (10 mg) of TBTO [100 mg TBTO/ mL PBS]. The concentration and dosages used were based on the findings of Oberdorster and McClellan-Green (2002). Nevertheless, all frogs administered the initial dose died within 24hrs of exposure. Therefore, the dosages of bis (tributyltin) oxide for the subsequent treatment groups were injected with 100 uL (1 mg) [10 mg TBTO/ mL PBS] and 100 uL (0.1 mg) [1 mg TBTO/ mL PBS]. Observations were made over a 24hr period, post-injection. Frogs that perished were removed from their containers immediately upon discovery. Treatment groups exhibiting no mortality were recorded as exposed to sublethal concentrations.

Experimental Study 1

Mature male frogs were randomly chosen from the stock population, two at a time, and injected with various amounts of bis (tributyltin) oxide through the lymph cavity of the lower

back. The dosages consisted of 200 μL (0.2 mg), 100 μL (0.1 mg), and 10 μL (0.01 mg) bis (tributyltin) oxide [1 mg TBTO/ mL PBS]. 17β Estradiol (with a concentration of 3 mg/ mL PBS) and Phosphate Buffered Saline served as the positive and negative controls. Each treatment group was assigned to a 6.6L plastic container filled with 4L de-ionized tap water. Exposure lasted 10 days, at which point, 5 cc of MS222 (with a concentration of 3 mg/ mL PBS) was used to euthanize all the frogs in accordance with Allegheny College's Animal Care Guidelines. The legs were then removed with scissors in order to extract a blood sample from the femoral artery. Blood samples were collected in 1.5mL microcentrifuge test tubes and centrifuged for 5-10 minutes at 12,000 rpm. The blood plasma (serum) was then decanted and placed into a 1.5 mL microcentrifuge tube. Both serum and blood samples were stored in a refrigerator at -20 ^{o}C until Enzyme Linked Immunosorbent Assay (ELISA) analysis.

Experimental Study 2

This study was conducted with the intention of determining the combined effect of the chemicals tributyltin oxide (TBTO) and diethylstilbestrol (DES). Mature male frogs were randomly selected from the stock population and injected with various amounts of bis (tributyltin) oxide [3 mg TBTO/ mL mineral oil] and/ or Diethylstilbestrol [3 mg DES/ mL mineral oil]. The dosages consisted of 200 μL (0.667 mg) TBTO, 200 μL (0.667 mg) TBTO + 200 μL (0.667 mg) DES, and 200 μL (0.667 mg) DES. These dosages were based on the range of sublethal concentrations obtained in the pilot study. Untreated animals served as control. The sample size for each treatment consisted of 3 frogs. After administering the injections, each treatment group was assigned to a 6.6L plastic container filled with 4L de-ionized water. Exposure lasted for a period of 10 days, at which point, the animals were pithed in accordance

with Allegheny College's Animal Care Guidelines. The legs were then surgically removed with scissors in order to extract an adequate blood sample from the femoral artery. Blood samples were collected with 1.5mL test tube and centrifuged for 5-10 minutes at 12,000 rpm. The blood plasma (serum) was then decanted and placed into a 1.5 mL microcentrifuge tube. Both serum and blood samples were stored in a refrigerator at -20 ^{o}C until Enzyme Linked Immunosorbent Assay (ELISA) analysis.

ELISA Protocol

Through the use of an Enzyme Linked Immunosorbent Assay (ELISA), vitellogenin present in the serum of samples was relatively quantified (Appendix 1). Phosphate-buffered saline (PBS, 8.0g NaCl, 0.2g KH2PO4, 2.2g Na2HPO4.7HSO, 0.2g KCl, 1L dH20, pH 7.2) was used to dilute serum samples to concentrations of 1:100, 1:200, 1:400, 1: 800, and 1: 1600 (serum: PBS). After the serum dilutions had been prepared, 100uL of each serial dilution (including replicates) and phosphate buffer (PBS) were added to the microtiter plates. Plates were covered with parafilm, and incubated at room temperature for 2 hr in a shaker at low speed. After the serum was given sufficient time to bind to the wells, the excess was flicked into the sink. PBS was then used to wash the plates 4 times.

"Blotto" blocking buffer (5g non-fat dry milk in 100mL of PBS) was then prepared to adhere to the unbound sites of each well. A 100uL volume of the "blotto" was added to each well before the microtiter plates were covered with parafilm and incubated in a shaker at low speed for 30 minutes at room temperature. The plates were then washed with PBS up to 4 times (if necessary). 1^{O} Rabbit anti-Xenopus vitellogenin was prepared. 5uL aliquot of 1^{O} Antibody was added to 5uL of dH20 and vortexed. 10uL of the resulting solution was added to 30mL of

blotto and vortexed. An aliquot of 100uL of the 1^{O} Rabbit anti-Xenopus vitellogenin antibody dilution was then added to each of the wells before the microtiter plates were placed into a refrigerator at 4^{o}C.

After a full day (~24hrs) at $4\ ^{o}$C, the plates were washed 6 times. The goat anti-rabbit IgG (2^{O} Antibody) was then prepared by adding 10uL of 2^{O} Antibody to 10mL of "blotto" buffer solution and vortexing. 100uL of the 2^{O} Antibody was then added to each well, covered with parafilm, and incubated at room temperature for 2 hr. The plates were then washed with PBS 4 times. A Bio-Rad TMB Peroxidase Substrate Kit (Bio-Rad Laboratories) was used to produce a developing agent. 1mL of solution B was added to 9mL of solution A before being vortexed. Immediately following which, a 100uL aliquot of the resulting developer agent was added to each well of the plates, creating a color change. Once the plates had incubated for 90 minutes 1 NH_2SO_4 (486.1mL dH_2O + 13.9mL H_2SO_4) was used to stop the reaction. A plate reader (colorimeter) was used to measure the absorbance of color differences at a wavelength of 450nm for each plate. The intensity of color is positively correlated with levels of vitellogenin in the serum.

Statistical Analysis

Statview (Cary, NC) statistical software was used for all statistical analysis. One-way ANOVA was utilized in determining if any differences existed between treatments and dilutions. Differences were then evaluated for statistical significance by using a Student-Newaman-Keuls Test. The null hypothesis was rejected at a $p \leq 0.05$.

RESULTS

Sublethality to TBTO

Dosages of 20 and 10 mg TBTO [3 mg TBTO/ mL PBS] (Table 1) caused 100% mortality. At a dosage of 1 mg TBTO, half of the test population perished (Table 1). A dosage of 0.1 mg TBTO [3 mg TBTO/ mL PBS] was not lethal (Table 1). Therefore, the sublethal concentration of *X. laevis* to TBTO can be approximated within the range of 1-0.1mg TBTO [3 mg TBTO/ mL PBS].

TBTO effects on vitellogenin

Except for the TBTO-dosed animals, all treatment groups of *Xenopus* exhibited significantly altered ($p \leq 0.05$) absorbencies in comparison with the untreated control (Fig. 2, Table 2). TBTO-treated and control groups displayed similar concentrations of serum vitellogenin at the end of 10-day exposure period (Table 2, Fig. 2), no statistical difference could be ascertained. The DES-dosed group displayed the highest concentration of vitellogenin production, with a significant decrease observed in the combined DES + TBTO treatment (Figure 2). Mortality was 0% over the course of the exposure period.

Note* One frog of the DES treatment group lost pigmentation in its right eye, seemingly rendering the individual blind.

Table 1: Effects of tributyltin oxide (TBTO) on survivorship of *Xenopus laevis* over a 24 hr period post-injection. Each treatment group contained two animals. Tributyltin oxide was administered a concentration of 3 mg TBTO/ mL PBS. * LD_{50} was experienced at a dosage of 1 mg TBTO.

Lethality Observation for *Xenopus laevis*	
Dosage (TBTO)	Mortality (%)
20 mg	100
10 mg	100
1 mg	*50
.1 mg	0

Table 2: One-way ANOVA for relative mean absorbance of vitellogenin between all treatments groups.

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Treatment	3	30.280	10.093	44.168	<.0001	132.504	1.000
Residual	116	26.509	0.229				

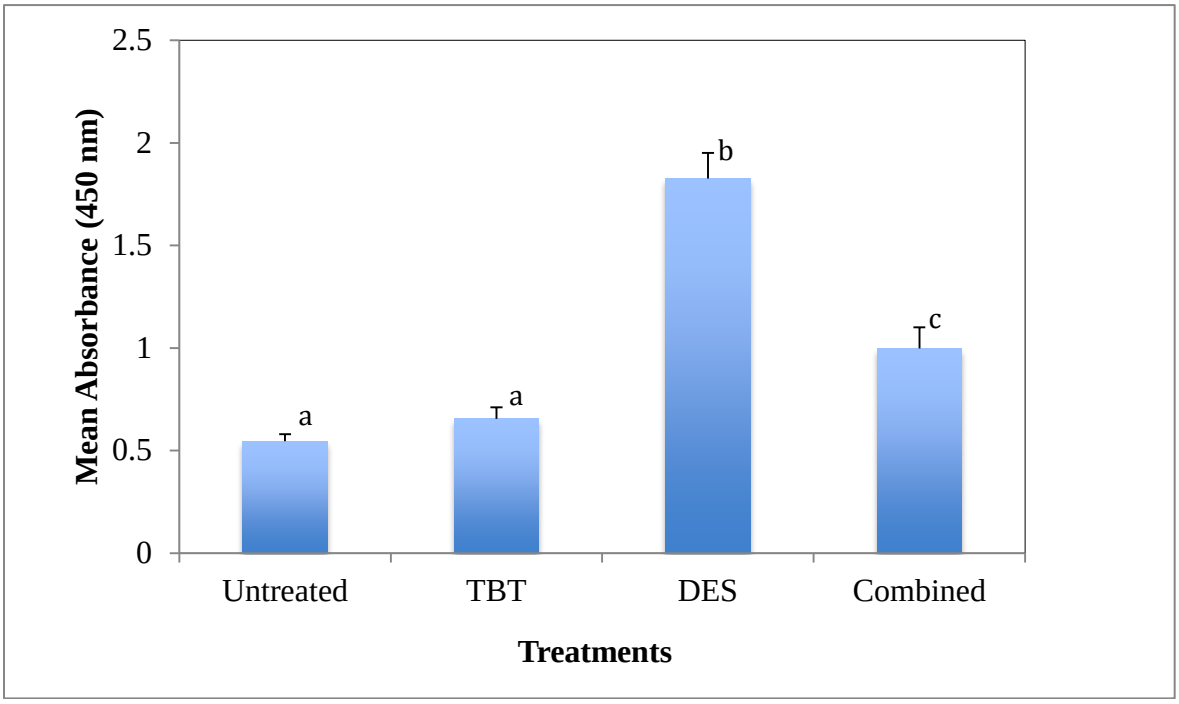


Figure 2: Comparison of absorbance (± S.E.M) of serum samples taken from African Clawed Frogs, *Xenopus laevis*, exposed to 0.667 mg TBTO, 0.667 mg DES, and 0.667 mg TBTO + 0.667 mg DES (Combined) for 10 day period. Notice the suppression of absorbency in the combined treatment, in comparison to the DES treatment alone. Histograms with different letters are significantly different from one another.

DISCUSSION

Tributyltin and other organotin compounds (TPT) cause masculinizing effects, most notably imposex, in a number of aquatic organisms (Santos et al., 2006; Morcillo and Porte, 2000; Gibbs and Bryan, 1986). As a result, tributyltin is one of few chemicals found in the environment to act as an androgen. However, because the mechanism(s) by which tributyltin acts is/are still unknown and the effects varied, organisms must be studied on a species-by-species basis.

TBTO as a xenobiotic estrogen

The hypothesis that tributyltin oxide (TBTO) would not cause induction of vitellogenin was supported by the present study. Frogs treated with a concentration of TBTO exhibited similar concentrations of vitellogenin in their sera when compared to those of the untreated controls (Fig. 2). Therefore, it is plausible that tributyltin oxide does not act as a xenobiotic estrogen in sexually mature male amphibians. More research is still needed to determine if tributyltin oxide causes masculinization in *X. laevis.*

Inhibition of vitellogenin

Additionally, *X. laevis* treated with diethylstilbestrol (DES) generally exhibited dramatically higher levels of vitellogenin in their sera than animals treated with a binary mixture of TBTO + DES (Figure 2). These finding support the hypothesis that TBTO has the capacity to block the estrogenic effects of DES. However, tributyltin oxide did not entirely inhibit the expression of the vitellogenin-inducing estrogen diethylstilbestrol, which perhaps may be

explained by the differing rates of metabolic breakdown. Evidence suggests that the degradation of diethylstilbestrol in the body is far slower than that of tributyltin oxide (Xueping et al., 2004).

In a similar set of studies conducted on zebrafish, tributyltin blocked the production of vitellogenin resulting from exposure to the synthetic estrogen-ethinylestradiol (EE2) (Micael et al., 2007; Santos et al., 2006). These studies were conducted using binary mixtures of TBT +EE2, not TBTO + DES. The concentrations of TBT were directly administered to the fish through their diets, while exposure to the synthetic estrogen EE2 was through immersion.

Based on the similarity of the endocrine system of fish and amphibians (Kloas and Lutz, 2006) one explanation to the findings observed in this study is the following. Hormones, both estrogen and androgen, play an important role in the reproductive and developmental processes of amphibians and fish (Kloas and Lutz, 2006; Santos et al., 2006; Heppell et al., 1995; Palmer and Palmer, 1995). In female Japanese flounder, TBT exposure induces masculinization, and suppresses the expression of the P450 aromatase gene (Shimasaki et al., 2003). Aromatase P450, also known as Cyp 19, is responsible for catalyzing the conversion of androgens to oestrogens (Toda et al., 2001). Shimasaki et al. (2003) suggest that TBT-induced masculinization is primarily the result of P450 aromatase inhibition or the suppression of its gene expression. One additional hypothesis is that TBT induces the release of peptide hormone Penis Morphogenic Factor (PMF) to abnormally high levels, which results in the masculinization of organisms (Oberdorster and McClellan-Green, 2002). However, to date there is no evidence that PMF exists. Therefore, it is possible that *X. laevis* exposed to TBTO may be experiencing similar aromatase inhibition as experienced by fish.

DES, like many other estrogens, induces the synthesis of vitellogenin in amphibians as a consequence of circulating estrogens in the blood stream (Palmer and Palmer, 1995).

Vitellogenin is an egg-yolk precursor protein that is produced in the liver via the binding of estrogens to localized receptor sites (Palmer and Selcer, 1996). Once present in the body, DES agonistically binds to the estrogen receptors, eliciting the transcription of estrogen-induced proteins (vitellogenin) (Palmer and Palmer, 1995). Furthermore, DES may inhibit transhyretins (TTRs) that are responsible for a major part of thyroid hormone-protein binding in *Xenopus* (Yamauchi et al., 2000). Thyroid hormones play an integral role in sexual and developmental processes in amphibians.

Taken as a whole, these studies on the effects of TBT and DES support the findings of present work. Diethylstilbestrol acts as an agonist to induce vitellogenin production, while TBTO most likely inhibits armotase activity resulting in elevated levels of testosterone in the blood of the circulatory system.

Although the mechanism by which TBT acts in fish and amphibians is still controversial, the findings of this study illustrate an important point. The results suggest that feminization of sexually mature male *X. laevis* resulting from low level diethylstilbestrol exposure can be partially blocked if the frogs are co-exposed to an equivalently low level of TBT.

Limitations of the study

The use of injection versus immersion is still a controversial issue in the exposure of aquatic organisms to chemicals, particularly endocrine disruptors. Since all *X. laevis* were injected with specific dosages, the chemical treatment was readily incorporated into the blood of the circulatory system. This exposure technique has been criticized for its environmental relevance. Direct exposure would only occur in the presence of an epidermal abrasion. Therefore, the results of this study may have limited application.

Additionally, the untreated male *Xenopus* had relatively little vitellogenin present in their blood (Figure 2). In order to evaluate the ability of tributyltin oxide to act as a xenobiotic androgen, the testes of frogs should be weighed both pre- and post-exposure. Tributyltin to date is still the only endocrine disruptor documented to cause the translocation and enlargement of male genitals onto female organisms (Santos et al., 2006; Morcillo and Porte, 2000; Gibbs and Bryan, 1986).

Finally, due to the fact that a vitellogenin standard could not be obtained, all treatment absorbencies of this study are relative.

Follow-up studies

This experiment supports the theory that tributyltin oxide has the potential to block the vitellogenin-inducing effects of diethylstilbestrol, perhaps by antagonistically binding to the estrogenic receptor sites of the liver. However, it is not yet known if tributyltin oxide acts as a xenobiotic androgen in the amphibian model. Therefore, in a follow-up study female *Xenopus*, particularly pre-metamorphic, should be used. Females naturally have an high level of circulating estrogens in their sera, which means that vitellogenin should be produced in the untreated control group. Furthermore, the estrogenic response of females is more rapid and intense (Palmer and Palmer, 1995). The effects of tributyltin oxide exposure alone can then be assessed accurately in amphibians. If tributyltin oxide were identified as an androgenic substance, then the next experiment naturally would be to determine the mechanism of action through which the compound acts.

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Appendix 1: Calculations of Tributyltin oxide (TBTO) and Diethylstilbestrol (DES) Treatments

Stock solution:

a) $\frac{1000\text{ mg}}{1000\text{ mL}} = \frac{10\text{ mg}}{10\text{ mL}}$ $\frac{3\text{ mg of TBTO}}{10\text{ mL PBS}} = \frac{3\text{ mg of DES}}{10\text{ mL Mineral}} = 0.3\text{ ppm}$

Treatments:

a) **0.2 mg (TBTO or DES)** = 200 uL injection

$$\frac{3\text{ mg}}{10\text{ mL}} = \frac{0.2\text{ mg}}{X}$$

b) **0.1 mg (TBTO or DES)** = 100 uL injection

$$\frac{3\text{ mg}}{10\text{ mL}} = \frac{0.1\text{ mg}}{X}$$

c) **0.01 mg (TBTO or DES)** =10 uL injection

$$\frac{3\text{ mg}}{10\text{ mL}} = \frac{0.01\text{ mg}}{X}$$

Appendix 2: Vitellogenin Enzyme-Linked Immunosorbent Assay (ELISA) Protocol

(from Dr. K Selcer laboratory Duquesne University)

Phosphate Buffered Saline (PBS): for 1L

8.0 g. NaCl
0.2 g KH2PO4
2.2 g Na2HPO4.7H20 (dibasic)
0.2 g KCL
bring up to 1L with distilled water; pH to 7.2

Serum Dilutions: Prepare as follows; vortex to mix well between serial dilutions

		Antigen	Buffer
#1s	1:100	5 uL Serum	+ 495 uL PBS
#2s	1:200	200uL #1s	+ 200uL PBS
#3s	1:400	200uL #2s	+ 200uL PBS
#4s	1:800	200uL #3s	+ 200uL PBS
#5s	1:1600	200uL #4s	+ 200uL PBS

1. Add 100 uL of each serum antigen dilution to a designated well on the microtiter plate.

2. Incubate plate for 2 hours on a low speed shaker at room temperature to allow antigen to bind to plastic wells.

3. Wash 4 times with PBS:
   - use a 500 mL squirt bottle
   - remove between rinses by flicking over the sink, then firmly blotting wells on paper towels

4. Make blocking buffer “Blotto” (5% milk in PBS) (make fresh every time)
   - 5 g non-fat dry milk in 100 mL PBS

5. Add 100 uL of blocking buffer to each well. Incubate for 30 min on low speed shaker at room temperature to allow milk protein to block unbound sites on the plastic. Save extra blotto for making 1° Ab (below)

6. Wash 4 times with PBS (if necessary)

7. Prepare 1° antibody (Rabbit Anti-Xenopus Vitellogenin; Rabbit #1, Selcer Lab)
    - add 5 uL of 1° Ab to 5 **uL** dH20 (vortex)
    - add 10 **uL** of above to 30 **mL** blotto: vortex

8. Add 100 uL of 1° Ab (#498) to each well and incubate overnight in refrigerator covered with saran wrap.

9. Remove 1° Ab
    - flick into sink
    - wash 6 times with PBS and firmly blot on paper towels

10. Prepare 2° Ab (Goat Anti-Rabbit IgG, Bio-Rad)
    - add 10 uL 2° Ab to 10 mL blotto; vortex (final dilution is 1: 1000)

11. Add 100 uL of 2° Ab to each well and incubate 2 hours at room temperature on a low speed shaker.

12. Remove 2° Ab
    - flick into sink
    - wash 4 times with PBS and firmly blot on paper towels

13. Turn on microtiter plate reader and allow it to warm up for 15min. Set wavelength to 655 nm.

14. Prepare developer (Bio-Rad TMB Peroxidase Substrate Kit)
    - add 1 mL solution B to 9 mL solution A; vortex
    - immediately add 100 uL of developer to each well
        - incubate 1 hr 45 min5 min. at room temp

15. Add 1 N H_2SO_4 to stop the reaction (100 uL to each well)
    (486.1 mL dH_2O + 13.9 mL H_2SO_4)
    - read at 450 nm

Appendix 3: Raw Data of the Absorbance at 450nm for Saline (negative control) Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
Saline	1	1	A(100)	0.206
Saline	1	1	B(200)	0.194
Saline	1	1	C(400)	0.181
Saline	1	1	D(800)	0.171
Saline	1	1	E(1600)	0.184
Saline	1	2	A(100)	0.406
Saline	1	2	B(200)	0.302
Saline	1	2	C(400)	0.326
Saline	1	2	D(800)	0.296
Saline	1	2	E(1600)	0.725
Saline	1	3	A(100)	0.302
Saline	1	3	B(200)	0.286
Saline	1	3	C(400)	0.181
Saline	1	3	D(800)	0.197
Saline	1	3	E(1600)	0.402
Saline	2	1	A(100)	0.178
Saline	2	1	B(200)	0.16
Saline	2	1	C(400)	0.183
Saline	2	1	D(800)	0.133
Saline	2	1	E(1600)	0.261
Saline	2	2	A(100)	0.293
Saline	2	2	B(200)	0.265
Saline	2	2	C(400)	0.243
Saline	2	2	D(800)	0.551
Saline	2	2	E(1600)	1.094
Saline	2	3	A(100)	0.912
Saline	2	3	B(200)	0.232
Saline	2	3	C(400)	0.248
Saline	2	3	D(800)	0.218
Saline	2	3	E(1600)	0.945

Appendix 4: Raw Data of the Absorbance at 450nm for 0.01 mg TBT Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
0.01 mg TBT	1	1	A(100)	0.265
0.01 mg TBT	1	1	B(200)	0.208
0.01 mg TBT	1	1	C(400)	0.203
0.01 mg TBT	1	1	D(800)	0.155
0.01 mg TBT	1	1	E(1600)	0.159
0.01 mg TBT	1	2	A(100)	0.369
0.01 mg TBT	1	2	B(200)	0.305
0.01 mg TBT	1	2	C(400)	0.48
0.01 mg TBT	1	2	D(800)	0.226
0.01 mg TBT	1	2	E(1600)	0.213
0.01 mg TBT	1	3	A(100)	0.341
0.01 mg TBT	1	3	B(200)	0.234
0.01 mg TBT	1	3	C(400)	0.21
0.01 mg TBT	1	3	D(800)	0.251
0.01 mg TBT	1	3	E(1600)	0.66
0.01 mg TBT	2	1	A(100)	0.273
0.01 mg TBT	2	1	B(200)	0.237
0.01 mg TBT	2	1	C(400)	0.18
0.01 mg TBT	2	1	D(800)	0.141
0.01 mg TBT	2	1	E(1600)	0.17
0.01 mg TBT	2	2	A(100)	0.471
0.01 mg TBT	2	2	B(200)	0.348
0.01 mg TBT	2	2	C(400)	0.377
0.01 mg TBT	2	2	D(800)	0.325
0.01 mg TBT	2	2	E(1600)	0.273
0.01 mg TBT	2	3	A(100)	0.384
0.01 mg TBT	2	3	B(200)	0.233
0.01 mg TBT	2	3	C(400)	0.242
0.01 mg TBT	2	3	D(800)	0.266
0.01 mg TBT	2	3	E(1600)	0.742

Appendix 5: Raw Data of the Absorbance at 450nm for 0.1 mg TBT Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
0.1 mg TBT	1	1	A(100)	0.268
0.1 mg TBT	1	1	B(200)	0.199
0.1 mg TBT	1	1	C(400)	0.204
0.1 mg TBT	1	1	D(800)	0.169
0.1 mg TBT	1	1	E(1600)	0.197
0.1 mg TBT	1	2	A(100)	0.33
0.1 mg TBT	1	2	B(200)	0.344
0.1 mg TBT	1	2	C(400)	0.275
0.1 mg TBT	1	2	D(800)	0.269
0.1 mg TBT	1	2	E(1600)	0.216
0.1 mg TBT	1	3	A(100)	0.371
0.1 mg TBT	1	3	B(200)	0.327
0.1 mg TBT	1	3	C(400)	0.208
0.1 mg TBT	1	3	D(800)	0.247
0.1 mg TBT	1	3	E(1600)	0.29

Appendix 6: Raw Data of the Absorbance at 450nm for 0.2 mg TBT Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
0.2 mg TBT	1	1	A(100)	0.298
0.2 mg TBT	1	1	B(200)	0.336
0.2 mg TBT	1	1	C(400)	0.303
0.2 mg TBT	1	1	D(800)	0.224
0.2 mg TBT	1	1	E(1600)	0.448
0.2 mg TBT	1	2	A(100)	0.255
0.2 mg TBT	1	2	B(200)	0.24
0.2 mg TBT	1	2	C(400)	0.543
0.2 mg TBT	1	2	D(800)	0.197
0.2 mg TBT	1	2	E(1600)	0.2
0.2 mg TBT	1	3	A(100)	0.238
0.2 mg TBT	1	3	B(200)	0.284
0.2 mg TBT	1	3	C(400)	0.259
0.2 mg TBT	1	3	D(800)	0.455
0.2 mg TBT	1	3	E(1600)	0.778
0.2 mg TBT	2	1	A(100)	0.171
0.2 mg TBT	2	1	B(200)	0.169
0.2 mg TBT	2	1	C(400)	0.185
0.2 mg TBT	2	1	D(800)	0.139
0.2 mg TBT	2	1	E(1600)	0.178
0.2 mg TBT	2	2	A(100)	0.253
0.2 mg TBT	2	2	B(200)	0.243
0.2 mg TBT	2	2	C(400)	0.286
0.2 mg TBT	2	2	D(800)	0.211
0.2 mg TBT	2	2	E(1600)	0.212
0.2 mg TBT	2	3	A(100)	0.329
0.2 mg TBT	2	3	B(200)	0.179
0.2 mg TBT	2	3	C(400)	0.21
0.2 mg TBT	2	3	D(800)	0.226
0.2 mg TBT	2	3	E(1600)	0.434

Appendix 7: Raw Data of the Absorbance at 450nm for Estradiol (positive control) Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
Estradiol	1	1	A(100)	0.214
Estradiol	1	1	B(200)	0.184
Estradiol	1	1	C(400)	0.15
Estradiol	1	1	D(800)	0.127
Estradiol	1	1	E(1600)	0.174
Estradiol	1	2	A(100)	0.414
Estradiol	1	2	B(200)	0.322
Estradiol	1	2	C(400)	0.305
Estradiol	1	2	D(800)	0.224
Estradiol	1	2	E(1600)	0.228
Estradiol	1	3	A(100)	0.295
Estradiol	1	3	B(200)	0.267
Estradiol	1	3	C(400)	0.254
Estradiol	1	3	D(800)	0.144
Estradiol	1	3	E(1600)	0.817
Estradiol	2	1	A(100)	0.257
Estradiol	2	1	B(200)	0.23
Estradiol	2	1	C(400)	0.218
Estradiol	2	1	D(800)	0.147
Estradiol	2	1	E(1600)	0.167
Estradiol	2	2	A(100)	0.368
Estradiol	2	2	B(200)	0.35
Estradiol	2	2	C(400)	0.364
Estradiol	2	2	D(800)	0.271
Estradiol	2	2	E(1600)	0.569
Estradiol	2	3	A(100)	0.287
Estradiol	2	3	B(200)	0.317
Estradiol	2	3	C(400)	0.283
Estradiol	2	3	D(800)	0.235
Estradiol	2	3	E(1600)	0.673

Appendix 7: Raw Data of the Absorbance at 450nm for Phosphate Buffered Saline (PBS) Treatment After the Addition of 1 NH_2SO_4

Treatment	Replicate	Dilution	Absorbance
PBS	1	A(100)	0.061
PBS	1	B(200)	0.091
PBS	1	C(400)	0.069
PBS	1	D(800)	0.064
PBS	1	E(1600)	0.121
PBS	2	A(100)	0.121
PBS	2	B(200)	0.095
PBS	2	C(400)	0.09
PBS	2	D(800)	0.136
PBS	2	E(1600)	0.126
PBS	3	A(100)	0.146
PBS	3	B(200)	0.105
PBS	3	C(400)	0.075
PBS	3	D(800)	0.093
PBS	3	E(1600)	0.188
PBS	1	A(100)	0.103
PBS	1	B(200)	0.084
PBS	1	C(400)	0.059
PBS	1	D(800)	0.08
PBS	1	E(1600)	0.08
PBS	2	A(100)	0.118
PBS	2	B(200)	0.221
PBS	2	C(400)	0.13
PBS	2	D(800)	0.517
PBS	2	E(1600)	0.517
PBS	3	A(100)	0.389
PBS	3	B(200)	0.106
PBS	3	C(400)	0.085
PBS	3	D(800)	0.155
PBS	3	E(1600)	0.847

Appendix 8: Raw Data of the Absorbance at 450nm for Untreated Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
Untreated	1	1	A(100)	0.564
Untreated	1	1	B(200)	0.482
Untreated	1	1	C(400)	0.371
Untreated	1	1	D(800)	0.465
Untreated	1	1	E(1600)	0.521
Untreated	1	2	A(100)	0.811
Untreated	1	2	B(200)	0.462
Untreated	1	2	C(400)	0.32
Untreated	1	2	D(800)	0.31
Untreated	1	2	E(1600)	0.718
Untreated	2	1	A(100)	0.448
Untreated	2	1	B(200)	0.47
Untreated	2	1	C(400)	0.386
Untreated	2	1	D(800)	0.494
Untreated	2	1	E(1600)	0.497
Untreated	2	2	A(100)	0.504
Untreated	2	2	B(200)	0.402
Untreated	2	2	C(400)	0.307
Untreated	2	2	D(800)	0.272
Untreated	2	2	E(1600)	0.728
Untreated	3	1	A(100)	0.822
Untreated	3	1	B(200)	0.716
Untreated	3	1	C(400)	0.633
Untreated	3	1	D(800)	0.649
Untreated	3	1	E(1600)	0.768
Untreated	3	2	A(100)	1.095
Untreated	3	2	B(200)	0.648
Untreated	3	2	C(400)	0.452
Untreated	3	2	D(800)	0.404
Untreated	3	2	E(1600)	0.653

Appendix 9: Raw Data of the Absorbance at 450nm for Diethylstilbestrol (DES) Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
0.667 mg DES	1	1	A(100)	3.382
0.667 mg DES	1	1	B(200)	2.922
0.667 mg DES	1	1	C(400)	2.123
0.667 mg DES	1	1	D(800)	1.555
0.667 mg DES	1	1	E(1600)	1.916
0.667 mg DES	1	2	A(100)	2.929
0.667 mg DES	1	2	B(200)	2.561
0.667 mg DES	1	2	C(400)	1.736
0.667 mg DES	1	2	D(800)	1.556
0.667 mg DES	1	2	E(1600)	1.725
0.667 mg DES	2	1	A(100)	1.52
0.667 mg DES	2	1	B(200)	1.353
0.667 mg DES	2	1	C(400)	1.263
0.667 mg DES	2	1	D(800)	1.143
0.667 mg DES	2	1	E(1600)	1.022
0.667 mg DES	2	2	A(100)	1.765
0.667 mg DES	2	2	B(200)	1.023
0.667 mg DES	2	2	C(400)	0.858
0.667 mg DES	2	2	D(800)	0.881
0.667 mg DES	2	2	E(1600)	0.84
0.667 mg DES	3	1	A(100)	2.892
0.667 mg DES	3	1	B(200)	2.644
0.667 mg DES	3	1	C(400)	2.144
0.667 mg DES	3	1	D(800)	1.935
0.667 mg DES	3	1	E(1600)	2.018
0.667 mg DES	3	2	A(100)	2.377
0.667 mg DES	3	2	B(200)	2.016
0.667 mg DES	3	2	C(400)	1.489
0.667 mg DES	3	2	D(800)	1.589
0.667 mg DES	3	2	E(1600)	1.621

Appendix 10: Raw Data of the Absorbance at 450nm for Tributyltin (TBT) Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
0.667 mg TBT	1	1	A(100)	0.893
0.667 mg TBT	1	1	B(200)	0.69
0.667 mg TBT	1	1	C(400)	0.414
0.667 mg TBT	1	1	D(800)	0.456
0.667 mg TBT	1	1	E(1600)	0.485
0.667 mg TBT	1	2	A(100)	0.854
0.667 mg TBT	1	2	B(200)	0.41
0.667 mg TBT	1	2	C(400)	0.444
0.667 mg TBT	1	2	D(800)	0.348
0.667 mg TBT	1	2	E(1600)	0.679
0.667 mg TBT	2	1	A(100)	1.768
0.667 mg TBT	2	1	B(200)	0.726
0.667 mg TBT	2	1	C(400)	0.493
0.667 mg TBT	2	1	D(800)	0.469
0.667 mg TBT	2	1	E(1600)	0.564
0.667 mg TBT	2	2	A(100)	1.528
0.667 mg TBT	2	2	B(200)	0.744
0.667 mg TBT	2	2	C(400)	0.433
0.667 mg TBT	2	2	D(800)	0.496
0.667 mg TBT	2	2	E(1600)	0.693
0.667 mg TBT	3	1	A(100)	0.805
0.667 mg TBT	3	1	B(200)	0.69
0.667 mg TBT	3	1	C(400)	0.496
0.667 mg TBT	3	1	D(800)	0.481
0.667 mg TBT	3	1	E(1600)	0.547
0.667 mg TBT	3	2	A(100)	0.945
0.667 mg TBT	3	2	B(200)	0.613
0.667 mg TBT	3	2	C(400)	0.466
0.667 mg TBT	3	2	D(800)	0.461
0.667 mg TBT	3	2	E(1600)	0.523

Appendix 11: Raw Data of the Absorbance at 450nm for Diethylstilbestrol (DES) + Tributyltin (TBT) Treatment After the Addition of 1 NH_2SO_4

Treatment	Frog	Replicate	Dilution	Absorbance
DES (0.667 mg) + TBT (0.667 mg)	1	1	A(100)	1.862
DES (0.667 mg) + TBT (0.667 mg)	1	1	B(200)	1.475
DES (0.667 mg) + TBT (0.667 mg)	1	1	C(400)	1.311
DES (0.667 mg) + TBT (0.667 mg)	1	1	D(800)	1.462
DES (0.667 mg) + TBT (0.667 mg)	1	1	E(1600)	1.423
DES (0.667 mg) + TBT (0.667 mg)	1	2	A(100)	1.524
DES (0.667 mg) + TBT (0.667 mg)	1	2	B(200)	1.003
DES (0.667 mg) + TBT (0.667 mg)	1	2	C(400)	1.131
DES (0.667 mg) + TBT (0.667 mg)	1	2	D(800)	1.338
DES (0.667 mg) + TBT (0.667 mg)	1	2	E(1600)	1.058
DES (0.667 mg) + TBT (0.667 mg)	2	1	A(100)	0.527
DES (0.667 mg) + TBT (0.667 mg)	2	1	B(200)	0.407
DES (0.667 mg) + TBT (0.667 mg)	2	1	C(400)	0.351
DES (0.667 mg) + TBT (0.667 mg)	2	1	D(800)	0.328
DES (0.667 mg) + TBT (0.667 mg)	2	1	E(1600)	0.377
DES (0.667 mg) + TBT (0.667 mg)	2	2	A(100)	0.58
DES (0.667 mg) + TBT (0.667 mg)	2	2	B(200)	0.247
DES (0.667 mg) + TBT (0.667 mg)	2	2	C(400)	0.288
DES (0.667 mg) + TBT (0.667 mg)	2	2	D(800)	0.24
DES (0.667 mg) + TBT (0.667 mg)	2	2	E(1600)	0.337
DES (0.667 mg) + TBT (0.667 mg)	3	1	A(100)	1.324
DES (0.667 mg) + TBT (0.667 mg)	3	1	B(200)	1.134
DES (0.667 mg) + TBT (0.667 mg)	3	1	C(400)	1
DES (0.667 mg) + TBT (0.667 mg)	3	1	D(800)	1.063
DES (0.667 mg) + TBT (0.667 mg)	3	1	E(1600)	1.084
DES (0.667 mg) + TBT (0.667 mg)	3	2	A(100)	1.175
DES (0.667 mg) + TBT (0.667 mg)	3	2	B(200)	0.873
DES (0.667 mg) + TBT (0.667 mg)	3	2	C(400)	0.794
DES (0.667 mg) + TBT (0.667 mg)	3	2	D(800)	0.74
DES (0.667 mg) + TBT (0.667 mg)	3	2	E(1600)	0.9

Appendix 12: Raw Data of the Absorbance at 450nm for Phosphate Buffered Saline (PBS) Treatment After the Addition of 1 NH_2SO_4

Treatment	Replicate	Dilution	Absorbance
PBS	1	A(100)	0.409
PBS	1	B(200)	0.332
PBS	1	C(400)	0.129
PBS	1	D(800)	0.607
PBS	1	E(1600)	0.121
PBS	2	A(100)	1.849
PBS	2	B(200)	1.352
PBS	2	C(400)	1.113
PBS	2	D(800)	0.947
PBS	2	E(1600)	1.357
PBS	1	A(100)	1.232
PBS	1	B(200)	1.46
PBS	1	C(400)	0.751
PBS	1	D(800)	0.933
PBS	1	E(1600)	1.294
PBS	2	A(100)	1.649
PBS	2	B(200)	1.837
PBS	2	C(400)	0.997
PBS	2	D(800)	0.827
PBS	2	E(1600)	1.966