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Assessing Toxicity of Endocrine Disrupting Compounds in Wastewater for Water Reuse

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1.0. Introduction

1.1. Background

As indicated by University of California Berkeley professor Dr. David Sedlak in his book *Water 4.0*, "If water is the essential ingredient of life, then water supply is the essential ingredient of civilization." While water is undoubtedly crucial, one-fifth of the population in the world currently lives in areas of physical water scarcity (UN 2015).

As both world population and industrial development increase, water consumption also increases. When combined with localized drought, these elements have led to predictions that there will be 1.8 billion people living in absolute water scarcity and nearly 70 percent of the world could be experiencing water-stressed conditions by 2025 (UN 2015). Currently, many water treatment plants rely on water intake from reservoirs or aquifers; however, under water-stressed conditions, the reliability of reservoirs or aquifers could be jeopardized. To supplement water resources, water reuse may be utilized when confronted with water scarcity.

1.2. Water Reuse

Recycling treated wastewater as a drinking water source may be necessary to meet future drinking water needs. Water reuse, or water recycling, can be classified in two different

categories: indirect and direct. Indirect reuse implies utilizing water downstream from a community's wastewater discharge. In many countries, indirect water reuse is employed to augment drinking water supplies (Le-Minh *et al.* 2010). On the other hand, direct reuse involves treated wastewater being sent directly to the point of use, typically for non-potable purposes (Dishman *et al.* 1989).

In the midst of water scarcity, direct reuse of wastewater effluent as potable water may be required. To accomplish this, wastewater treatment plants would require retrofitting for direct potable reuse. The quality of the reuse water must ensure safety, especially for public consumption purposes.

1.3. Endocrine Disrupting Compounds

There are an estimated 70,000 compounds with endocrine disrupting potential (Gillesby *et al.* 1998). EDCs are typically found in personal care products, pharmaceuticals, and pesticides (Snyder *et al.* 2003), and can potentially cause detrimental health effects if consumed. Studies show EDCs have the potential to affect normal reproduction (Colborn *et al.* 1993, Rupnik *et al.* 2011). However, little is known about the way in which EDCs affect humans (Rupnik *et al.* 2011). Moreover, as documented by Falconer *et al.* (2006), due to lack of scientific knowledge and concerns expressed by the public, further assessment about the risks of EDCs to humans should be conducted.

Disconcertingly, EDCs are currently found in wastewater effluents (Snyder *et al.* 2003). In fact, current wastewater treatment plants are not designed to remove EDCs from water (Lemanik *et al.* 2007), nor are there maximum contaminant limits for personal care products or pharmaceuticals in drinking water (Snyder *et al.* 2003). To recycle wastewater for drinking water purposes, a more complete understanding of the harmfulness of EDCs is required. Further, a

benchmark for EDCs in reuse water should be established. The toxicity of select EDCs was evaluated to further understand the adverse impact of EDCs in wastewater effluents. The results of the evaluation are documented herein.

1.4. Selection of Endocrine Disrupting Compounds for Study

Within a wastewater treatment plant in Oklahoma, several EDCs were found within the effluent, including sucralose, amoxicillin, estrone, caffeine, triclosan, and atrazine, among others. This particular wastewater treatment plant employed primary and secondary clarifiers, biological nutrient removal, and post-aeration with ultraviolet disinfection. The EDCs and the respective concentrations within the wastewater effluent are reported in Table 1.

Table 1. Occurrence of EDCs in Wastewater Treatment Plant Effluent

Compounds	Occurrence of Compounds (parts per trillion) (ng/L)
Sucralose	49000
Amoxicillin	4600
Acesulfame-K	4100
Estrone	130
Caffeine	60
Triclosan	43
Atrazine	16

Based on the effluent data from the wastewater treatment plant in Oklahoma and the EDC classification that was present, five compounds were selected for this particular study: amoxicillin, estrone, triclosan, atrazine, and acetaminophen. Although acetaminophen was not found in this particular wastewater discharge, the compound was chosen because of its recent popularity in pharmaceutical products such as Dayquil® and Tylenol®. Sucralose and caffeine were not selected because of previous studies regarding their toxicity and removal from wastewater (Heberer 2002, Mawhinney *et al.* 2011, Soh *et al.* 2011).

Amoxicillin, or $C_{16}H_{19}N_3O_5S$, is a common pharmaceutical compound. Amoxicillin is a penicillin antibiotic used to treat illness such as pneumonia, ear infection, and tonsillitis. In a study conducted by Andreozzi *et al.* (2004), algal assays were utilized to show amoxicillin as non-toxic to eukaryotic organisms. However, little is known about the toxicity of the compound to ecosystems and human health.

Estrone, or $C_{18}H_{22}O_2$, is a hormone and a steroid. The compound is a known female carcinogen. Additionally, the compound is thought to potentially cause anorexia, nausea, vomiting, and other health effects among men (OSHA 2016).

Triclosan, or $C_{12}H_7Cl_3O_2$, is a common antibacterial soap and a household and personal care product. Triclosan has shown irreversible toxic effects to DNA at concentrations above 0.25 mg/L, and it also poses a potential risk to the environment (Ciniglia *et al.* 2005). Triclosan has little acute toxicity to estuarine organisms, but has a potential to possess chronic, sub-lethal, or metabolite toxicity (DeLorenzo *et al.* 2008).

Atrazine, or $C_8H_{14}ClN_5$, is an herbicide. Among the compounds within this study, atrazine is the most researched, and therefore, atrazine is the most regulated. In fact, atrazine is regulated under water statutes from the United States Environmental Protection Agency (US EPA). More specifically, the US EPA has placed a maximum contaminant level for atrazine at 3 parts per billion. The US EPA has also declared that atrazine has relatively low acute toxicity and is not likely a human carcinogen (EPA 2016).

Acetaminophen, or $C_8H_9NO_2$, is a pharmaceutical product found in brand name products such as Tylenol® and DayQuil®. The compound is often utilized to treat the common cold, flu, and allergies, as well as to ease pain and decrease fever. The Food and Drug Administration emphasizes that customers strictly adhere to the dosage recommendations for acetaminophen

because of the toxicity of the compound. More specifically, ingesting amounts of 4,000 mg in a 24-hour period acetaminophen can cause liver damage (FDA 2016).

1.5. Microtox Assay

The objective of this study was to assess the relative acute cytotoxicity of the aforementioned five EDCs in wastewater effluent for the purpose of direct reuse. The Microtox assay was chosen for the cytotoxicity assessment. Microtox is an *in vitro* test that inversely correlates luminescence and toxicity. For this experimental purpose, the naturally luminescent marine bacterium *V. fischeri* was used because the bacteria naturally emit light as a result of their metabolic processes. The assay functions on the principle that exposure to a toxic substance inhibits the amount of light emerging from the bacteria as a function of the degree of toxicity of the substance. In other words, as the toxicity of an EDC increases, the luminescence of the bacteria decreases, and vice versa. Therefore, by measuring the amount of light inhibited by the bacteria, a relative toxicity of compounds can be determined.

2.0. Procedures

2.1. Laboratory Procedures

Analytical grades of the five selected EDCs were purchased from Sigma Aldrich (St. Louis, MO), and the Microtox® test system was purchased from Modern Water Inc. (New Castle, DE). Stock solutions of each EDC were prepared in a sodium chloride buffer solution (i.e., diluent provided in the Microtox® kit, in order to maintain a pH between 6 and 8, an innocuous range for *V. fischeri* according to Modern Water). For the EDCs with low solubility limits (particularly atrazine and estrone), extra measures were taken to achieve higher concentrations that inhibit light from the bacteria. Although not preferred when compared to a sodium chloride buffer, a dimethylsulfoxide (DMSO) solution was utilized to dissolve atrazine

and estrone at higher concentrations. DMSO was selected because of the low acute toxicity of DMSO (IC_{50} of 54,900 ppm) to *V. fischeri* and the adequacy of DMSO as a solvent (Jennings *et al.* 2001). After the compounds were dissolved in the DMSO, the solution was mixed into a sodium chloride buffer (1:10) to achieve a desired concentration. Furthermore, the standard curve for estrone and atrazine was adjusted by adding proportional DMSO to draw a more accurate representation of the relative toxicity of the compounds.

The toxicity of the selected EDCs was also assessed in wastewater collected from the Westside Wastewater Treatment Plant (Fayetteville, AR) after primary treatment. Toxicity was first compared between wastewater and EDC-spiked wastewater. The spiked wastewater contained 1 ppm of each of the five selected EDCs. Concentrations of 1 ppm were considered an appropriate upper limit of EDCs found in wastewater for the toxicity tests, as determined by the Oklahoma wastewater treatment plant effluent. The spiked wastewater was then treated using a membrane bioreactor (MBR) and samples were taken throughout a 24-hour treatment process. These samples were taken from both the anoxic and aerobic tanks in the secondary treatment process and tested for toxicity. Lastly, the cytotoxicity of wastewater effluent was also assessed.

For quality control, a pH meter was periodically used to check the acidity of solutions. Then the stock solutions were pipetted into a 96 well u-bottom culture plate (Corning, Inc., Corning, NY) and serially diluted. The reagent vials of *V. fischeri*, initially freeze-dried at -20 °C, were reconstituted with 1 mL of ultra-pure water, which is provided within the Microtox® kit as Reconstitution Solution. After pipette mixing, a portion of the reagent solutions were aliquoted and diluted from 100,000 bacteria/µL to 2,000 bacteria/µL as the stock solution. The reagent solutions were then pipetted into a white wall, flat bottom 96 well plate (Thermo Fischer Scientific, Waltham, MA). Each plate well received 50 µL of solution; therefore, each well

contained 100,000 bacteria per well. When not in use, the remainder of the reagent was placed on ice and removed from light to increase preservation time of the bacteria (luminescent sensitivity of the reagent only remains for approximately one to two hours). Once both the EDCs and bacteria were prepared in their respective plates, the EDC solutions were transferred from the u-bottom plate to the white wall, flat bottom plate using a multi-channel pipette.

Three replicates of each EDC solution and positive and negative controls were employed for quality control purposes. After completing the transfer of the EDC solutions to the bacteria using multi-channel pipettes, the plates were immediately taken to a BioTek Synergy H1 microplate reader (BioTek, Winooski, VT) with a pre-established protocol. The luminescence of the bacteria was monitored over an exposure interval of 30 minutes. The bacteria were generally not monitored for a prolonged period due to bacterial luminescence inconsistency in trials longer than one hour.

2.2. Data Analysis

Luminescence data were analyzed utilizing a standard curve with varying bacteria counts per well. For each new reagent vial, a new standard curve was created due to variances in bacteria luminescence across vials. Additionally, because the Microtox test measures relative toxicity, luminescence results were only compared within one reagent vial for accuracy.

To assess the toxicity of the tested EDCs, concentrations of each EDC that inhibited 50% (IC_{50}) of the bacterial luminescence after 15 minutes of exposure were determined. Based on the IC_{50} value, a toxicity unit of each EDC was calculated based on the Empirical Toxicity Scale, a measure approved by the European Community Commission (Persoone *et al.* 1993). The Empirical Toxicity Scale as presented in Table 2 and the toxicity units are derived from Equation 1 below.

$$Toxicity\ Units = \frac{1}{IC_{50}} * 100 \qquad \text{EQ. 1}$$

Table 2. Empirical Toxicity Scale

Toxicity Units (TU)			Classification
=		0	Non-Toxic
0	≤	1	Weakly Toxic
1	≤	10	Toxic
1	≤	100	Very Toxic
1	≤	∞	Extremely Toxic

To check the accuracy of the experimental procedure before proceeding with the toxicity testing of the selected EDCs, a compound with a known toxicity unit (zinc sulfate with a toxicity unit between 10 and 50) was tested using the Microtox assay. The experimental procedure produced a toxicity unit of 43.3, which falls within the acceptable range, and verified the accuracy of the laboratory methods.

Statistical analyses were utilized to determine correlations between toxicity data sets. More specifically, wastewater toxicity sampled from aerobic and anoxic tanks during the laboratory treatment process was compared using a paired t-test. Another paired t-test evaluated if non-spiked wastewater is equally toxic as EDC-spiked wastewater. P-values were calculated to gauge the strength of all t-tests performed.

3.0. Results and Discussion

The cytotoxicities of amoxicillin, estrone, triclosan, atrazine, and acetaminophen were first assessed individually through the Microtox assay. The relationship between the concentration of acetaminophen exposed to *V. fischeri* and the percentage of light inhibited of the bacteria after 15 minutes of exposure is shown in Figure 1. As displayed in Figure 1, acetaminophen yielded an IC_{50} value of 3504 parts per million (ppm). Graphs similar to Figure 1 for the other four selected compounds are presented in the appendix.

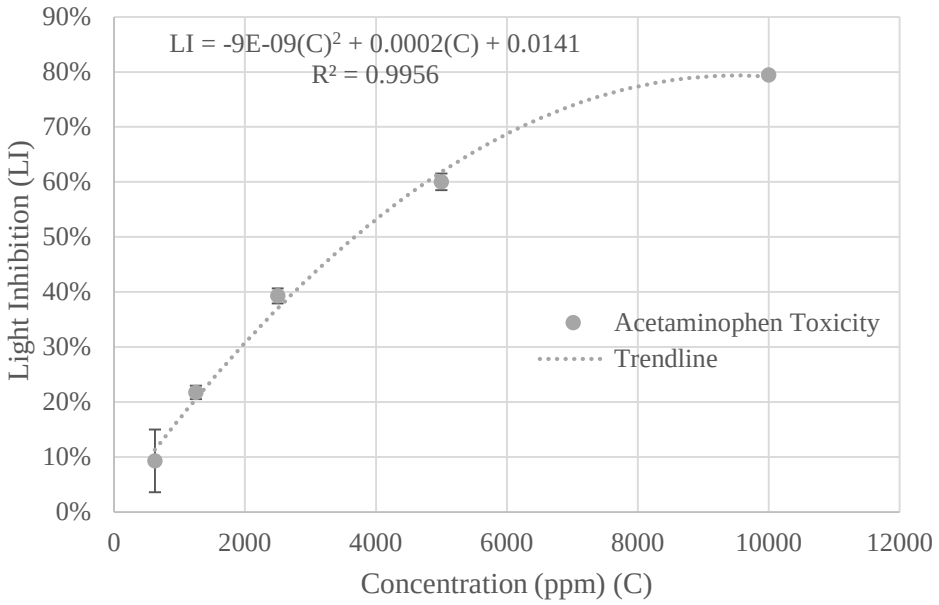

Figure 1. The relationship between the concentration of acetaminophen exposed to *V. fischeri* for 15 minutes and the percentage light inhibited by the bacteria as a result of the compound.

Toxicity can also vary as a function of the amount of time the bacteria are exposed to toxic environments. Although *V. fischeri* only remains sensitive for one to two hours, some compounds demonstrated amplified toxicity even over a short interval. Acetaminophen, as displayed below in Figure 2, did not intensify in toxicity during the 30-minute interval. However, amoxicillin and triclosan did inhibit an additional 22% and 28% of bacterial luminescence after 30 minutes of exposure, respectively. Graphs for the relationship between exposure time and bacteria luminescence for the other four chosen compounds (namely, amoxicillin, triclosan, estrone, and atrazine) are accessible in the appendix.

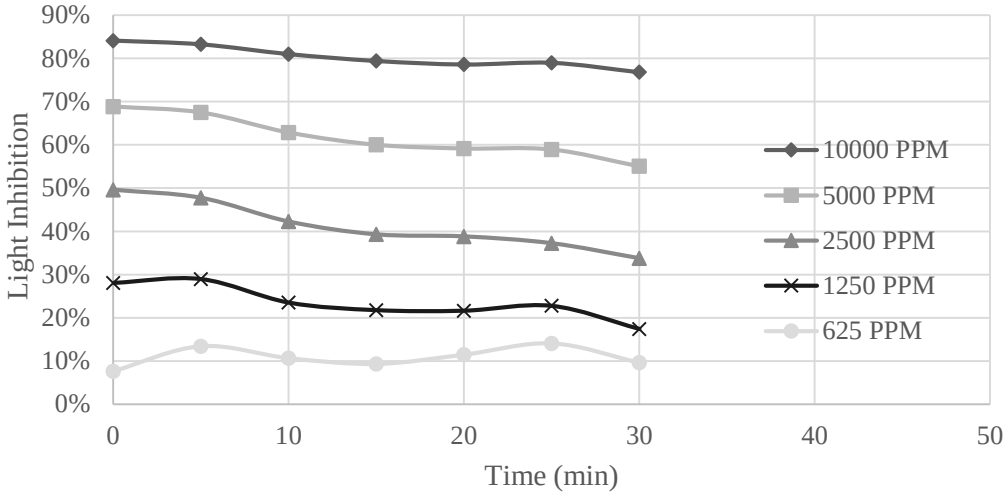

Figure 2. The luminescence response of *V. fischeri* over 30 minutes of exposure to acetaminophen at select concentrations.

The compilation of the toxicity units for each of the five selected EDCs is presented in Table 3. From highest toxicity unit to least toxicity unit, the compounds ranked in the following order: triclosan, estrone, atrazine, amoxicillin, acetaminophen. Estrone and atrazine were dissolved in a DMSO solution. Therefore, a direct comparison in toxicity units between these two compounds with the others must be taken with caution. Though DMSO has a low toxicity unit of 54,900 ppm to *V. fischeri*, an extra variable was introduced that may have caused a discrepancy.

Table 3. Toxicity Units of EDCs

Compound	Toxicity Units	IC_{50} (ppm)
Acetaminophen	0.029	2776
Triclosan	8.00	12.5
Amoxicillin	0.042	2401
Estrone	1.73	57.8
Atrazine	0.36	277.8

Due to the relative luminescence intensity, Microtox acute toxicity assay is only beneficial when comparing to compounds with known toxicities. Consequently, a comparison between compounds of known toxicity and the five selected EDCs was performed. As shown in Figure 3, triclosan has a similar cytotoxicity to formaldehyde, estrone and atrazine have cytotoxicities in a range between phenol and chloroform, and amoxicillin and acetaminophen have cytotoxicities in a range between chloroform and ethanol.

Based on information published by the US Department of Health and Human Services, formaldehyde has shown to have high acute toxicity on rats and rabbits (US DHHS 1993). In fact, formaldehyde is likely carcinogenic to humans (Gupta *et al.* 1982). With triclosan possessing a toxicity unit similar to formaldehyde from the Microtox assay, triclosan raises concerns for directly reusing wastewater as drinking water.

Chloroform, according to Figure 3, has a slightly less cytotoxicity than atrazine, estrone, and triclosan. Yet, chloroform has shown capability of producing cancer in rat kidneys (Jorgenson *et al.* 1985). Ethanol has even shown to promote tumor progression or to cause gonadal atrophy with prolonged exposure (Yirmiya *et al.* 2002, Gavaler *et al.* 1980), yet ethanol is less cytotoxic to *V. fischeri* than the five EDCs studied herein. Therefore, the presence of all five of the tested EDCs (namely, atrazine, estrone, acetaminophen, triclosan, and amoxicillin) in wastewater effluents is a concern, even at small concentrations. As the EDCs tested within this study possess similar toxicity units to compounds with detrimental health effects to humans, further study into the health effects of EDCs is warranted. Although cytotoxicity was confirmed for these selected EDC compounds, the IC_{50} for all compounds are in the parts per million range, which is several orders of magnitude higher than the concentrations in wastewater effluent. This

indicates that the cytotoxicity of EDCs to *V. fischeri* may be insignificant at concentrations typically found in wastewater effluents.

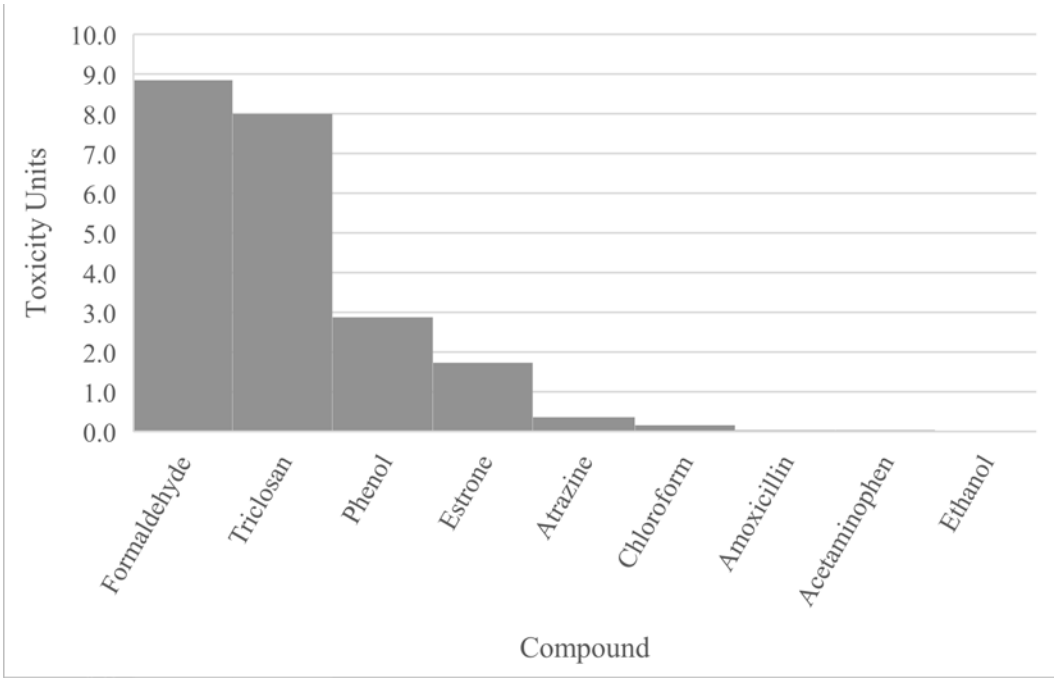

Figure 3. A comparison between acute toxicity of selected EDCs and other common compounds based on the Empirical Toxicity Scale.

As observed in Figure 4, the wastewater, both spiked and non-spiked, was sampled before the wastewater treatment process began. The relative toxicities of spiked and non-spiked wastewater to *V. fischeri* are nearly identical as the exposure time to *V. fischeri* increases. The results show the addition of EDCs at 1 ppm did not induce additional cytotoxicity in wastewater.

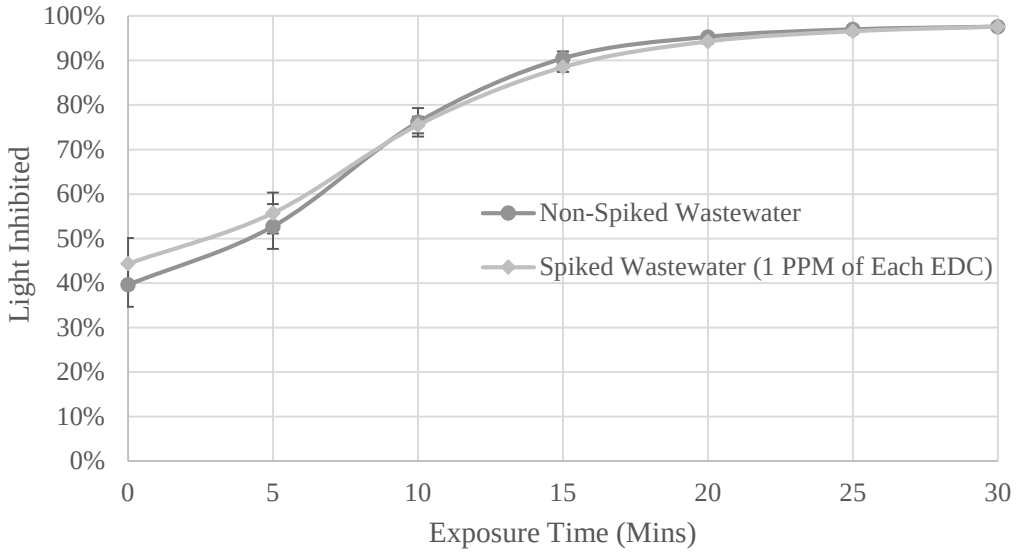

Figure 4. A toxicity comparison of EDC-spiked and non-spiked wastewater. Luminescence of *V. fischeri* was monitored over a 30-minute time interval.

The toxicity of EDC-spiked wastewater was also tested from samples taken during the treatment process. The treatment process included an anoxic tank, aerobic tank, and a MBR. In Figure 5, a filtered sample from each of the anoxic and aerobic tanks was taken after 4, 8, 12, and 24 hours of treatment. The luminescence of the bacteria was recorded after 15 minutes of exposure to the wastewater and recorded as a function of the time that the samples were taken in the treatment process.

A paired t-test was performed to examine whether non-spiked wastewater is equally toxic to *V. fischeri* as wastewater spiked with 1 ppm of each of the selected EDCs. A p-value of 0.288 was determined, which establishes considerable statistical support for the null hypothesis—wastewater spiked at a total concentration of 5 ppm of EDCs has equal toxicity as non-spiked wastewater.

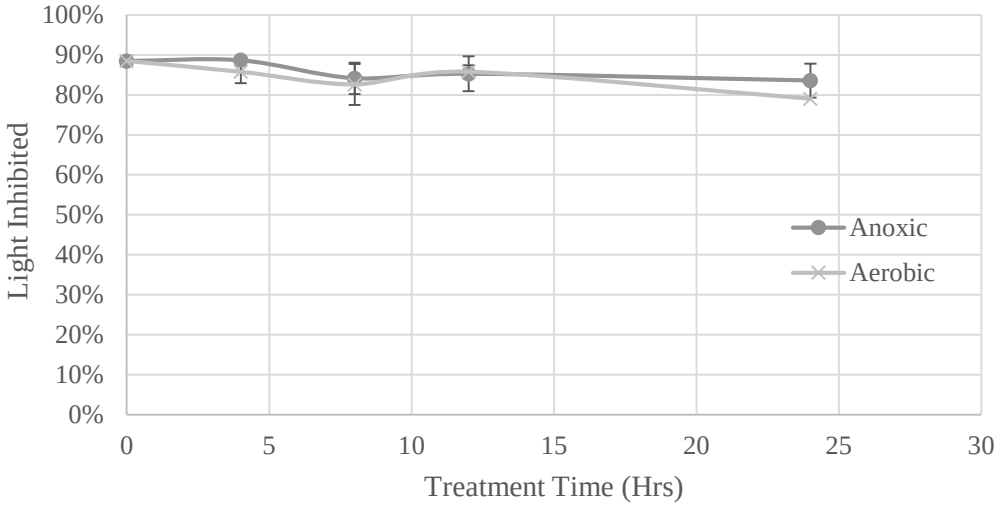

Figure 5. EDC-spiked wastewater was treated for a period of 24 hours. Over this time, the wastewater in the anoxic and aerobic tanks was sampled and later tested for cytotoxicity. After 15 minutes of exposure to EDCs, the luminescence of *V. fischeri* was measured for each of the samples taken from the treatment process.

A slight reduction in cytotoxicity of the wastewater was observed as the treatment process continued (Figure 5). However, even after 24 hours of treatment time the spiked wastewater still inhibited about 80% of light emitted from the bacteria. A paired t-test was conducted on the toxicity of the wastewater in the anoxic and aerobic tanks. The light inhibition of the anoxic tank falls between -0.26% and 2.78% of the light inhibition of the aerobic tank with 99% confidence. The t-test yielded a p-value of 0.03. Assuming a level of significance of 0.05, the mean toxicity in the anoxic and aerobic tanks are not equivalent.

To further assess the toxicity of EDCs without toxicity caused by the wastewater itself, EDC-spiked non-wastewater solutions were created at various concentrations. A sodium chloride buffer solution was spiked with triclosan, acetaminophen, atrazine, and amoxicillin in equivalent concentrations. These solutions were exposed to *V. fischeri* over a period of 30 minutes as the luminescence was monitored.

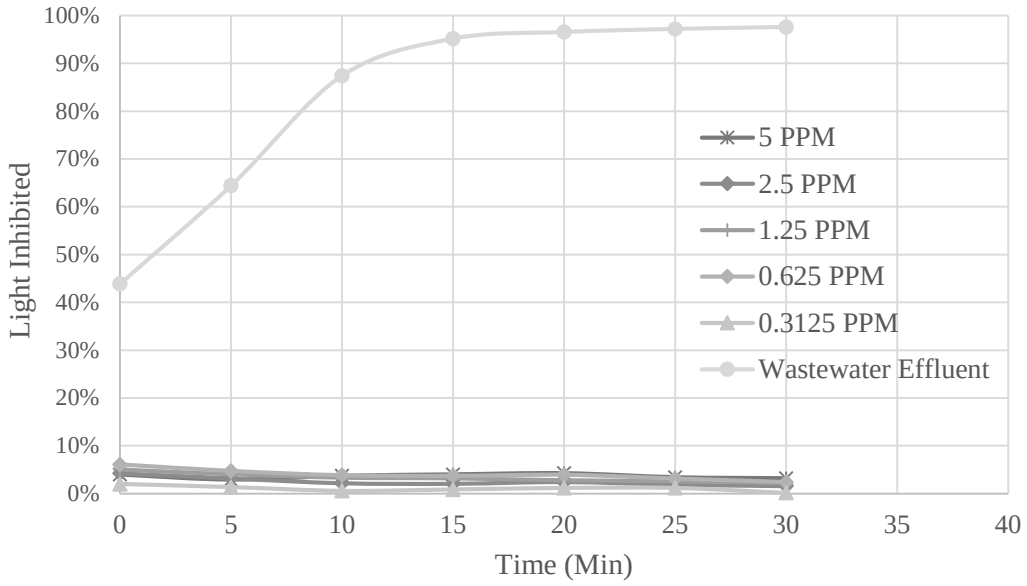

Figure 6. A toxicity comparison between a buffer spiked with various equivalent concentrations of triclosan, acetaminophen, amoxicillin, and atrazine (estrone was excluded due to solubility restraints) and wastewater effluent from a 24-hour treatment process. The light inhibition of *V. fischeri* was measured after 15 minutes of exposure to the EDC solutions and wastewater effluent.

Compared to the toxicity of wastewater effluent shown in Figure 6, the EDC samples (spiked at 5 ppm of each included compound) showed significantly less toxicity. The EDC-spiked water inhibited less than 10% of light from *V. fischeri*, while EDC-spiked wastewater effluent inhibited as much as 98% of light. Therefore, the toxic effects of other constituents (non-EDCs) from the laboratory MBR wastewater effluent in the study was far greater than the toxicity caused by the EDCs alone.

4.0. Conclusions

Based on the toxicity units of the five tested EDCs, the cytotoxicity of endocrine disruptors is variable. From most to least toxic, the toxicities of the selected EDC compounds rank in the following order: triclosan, estrone, atrazine, amoxicillin, acetaminophen. Additionally, based on the Empirical Toxicity Scale, triclosan and estrone classify as toxic;

amoxicillin, acetaminophen, and atrazine categorize as weakly toxic. Of the five EDCs assessed, the toxicity of the compounds can be compared to a range of other compounds, from formaldehyde to ethanol.

While the studied EDCs are characterized as toxic or weakly toxic, the concentrations of EDCs required to inhibit bacterial luminescence substantially exceeded the concentrations found in wastewater. In fact, the IC_{50} of all the selected EDCs were several orders of magnitude higher than the concentrations of EDCs found in the Oklahoma wastewater treatment effluent. Therefore, the acute toxicity of EDCs to *V. fischeri* is insignificant at concentrations typically found in wastewater.

Wastewater has shown significant cytotoxicity, however, based on this research the primary source of cytotoxicity in wastewater is not credited to EDCs. Rather, the cytotoxicity to the bacteria is likely caused by other contaminants within wastewater. After the secondary treatment process, the wastewater still showed significant toxicity. In fact, 24 hours of treatment only decreased toxicity by less than 10% compared to the untreated wastewater. Therefore, the laboratory MBR was inadequate at removing cytotoxicity from wastewater.

While a relative idea of the toxicity of these compounds can be determined, the scope of this paper only covers the toxicity of five EDCs to one strain of marine bacterium. Hence, more studies are needed to fully assess the toxicity of the selected compounds, as well as other EDCs. Specifically, further research regarding the mutagenicity and genotoxicity should be conducted, such as the Ames Fluctuation Test and the Comet Assay, respectively. More cytotoxicity assays utilizing alternative strains of bacteria should also be considered.

5.0. Acknowledgements

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Appendix

A.1. Triclosan

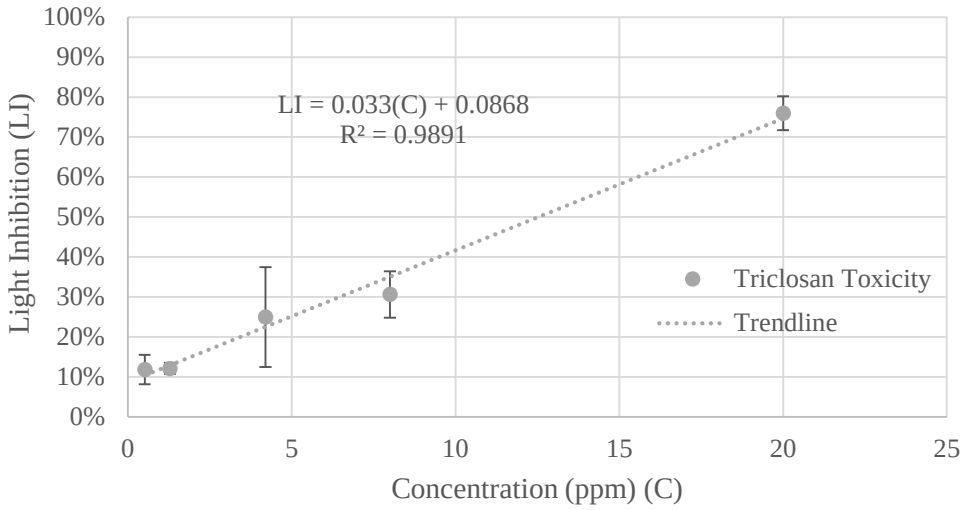

Figure A1. Correlation between percent of light inhibited of *V. fischeri* after 15 minutes of exposure and the concentration of triclosan exposed to the bacteria.

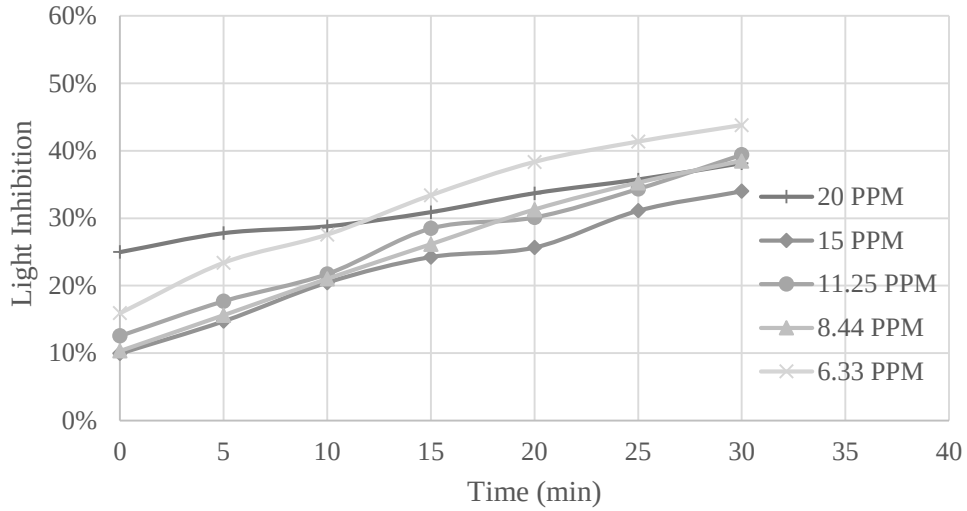

Figure A2. Percent light inhibited of *V. fischeri* over 30 minutes of exposure to a 1:1.5 serial dilution of triclosan.

A.2. Estrone

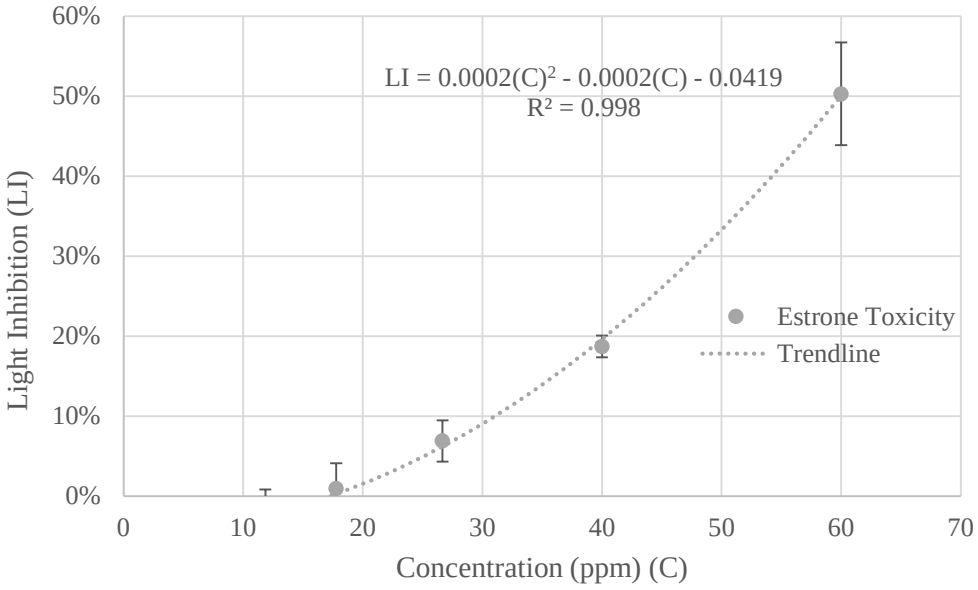

Figure A3. Percent of light inhibited of *V. fischeri* after 15 minutes of exposure to increasing concentrations of estrone in a DMSO solution.

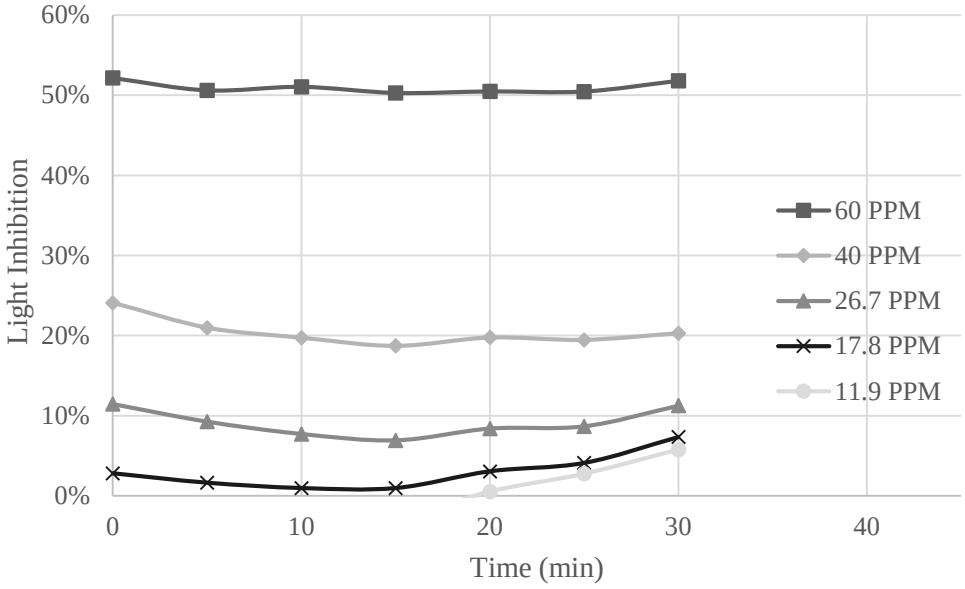

Figure A4. The effect of luminescence of *V. fischeri* over 30 minutes of exposure to estrone at different concentrations in a DMSO solution.

A.3. Atrazine

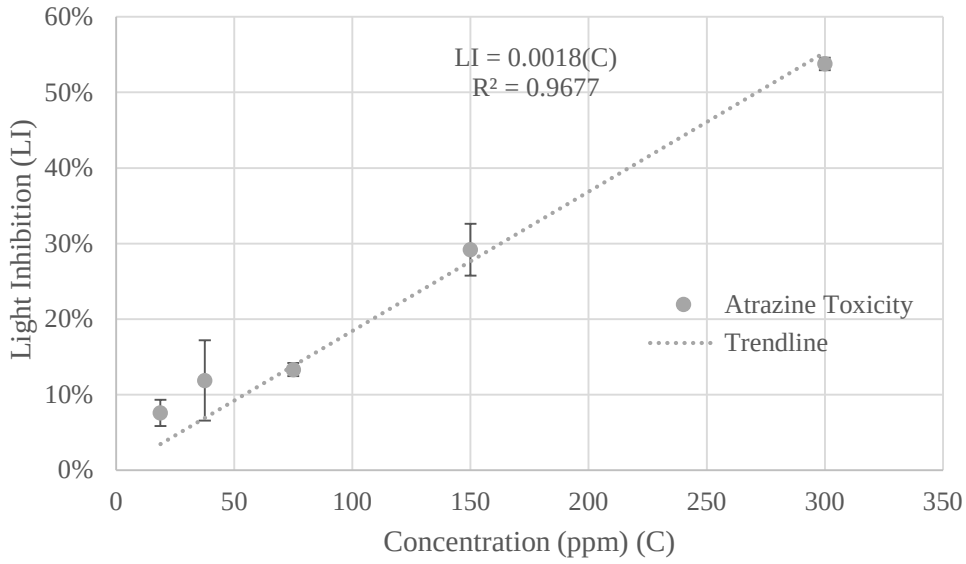

Figure A5. The relationship between light inhibited of *V. fischeri* after 15 minutes of exposure to various concentrations of atrazine presented to the bacteria.

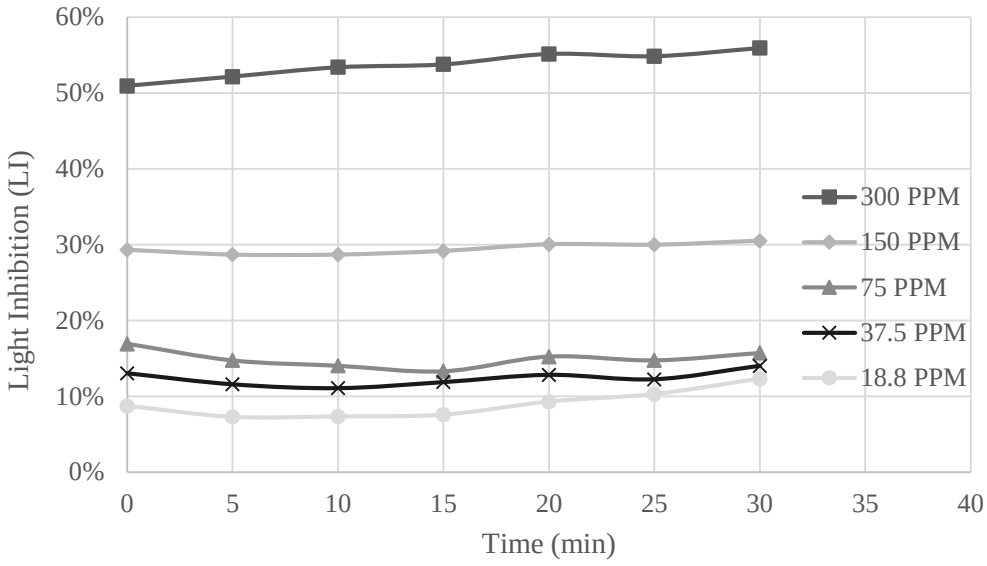

Figure A6. Percent of light inhibited of *V. fischeri* as a function of time with respect to the concentration of atrazine exposed to the bacteria.

A.4. Amoxicillin

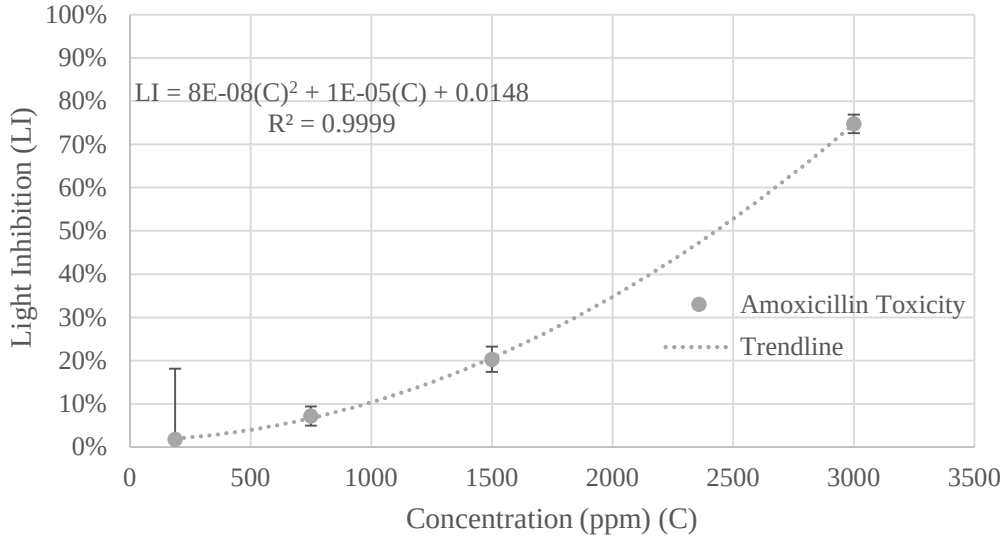

Figure A7. Percent of light inhibited of *V. fischeri* after 15 minutes of exposure as it relates to the concentration of amoxicillin in contact with the bacteria.

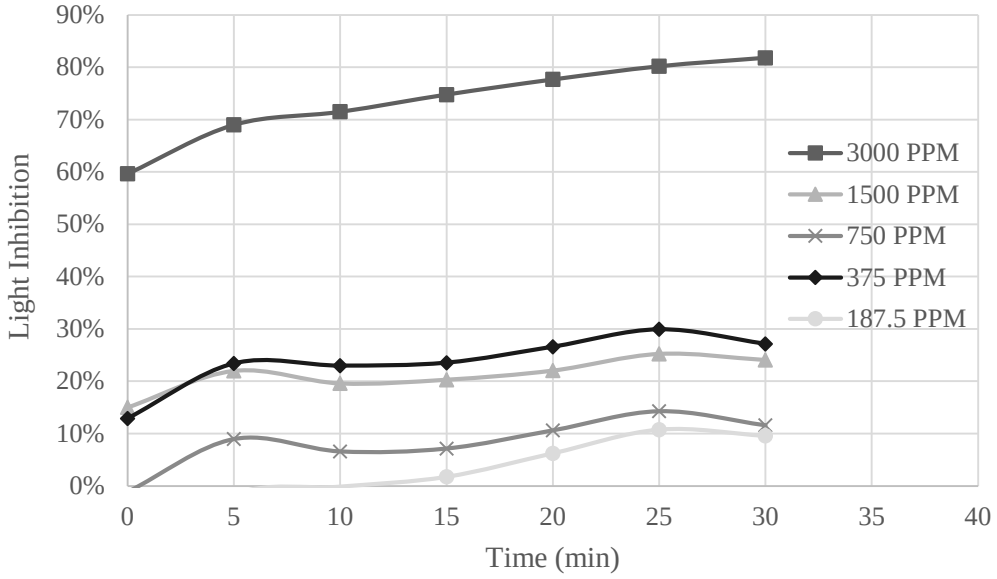

Figure A8. Luminescence of *V. fischeri* over 30 minutes of exposure to amoxicillin at various concentrations.