# UV FILTERS AS COMMON ORGANIC WATER CONTAMINANTS: A TOXICOLOGICAL STUDY OF SELECTED UV FILTERS ON *DAPHNIA MAGNA*, A MONITORING STUDY OF SELECTED OKLAHOMA LAKES, AND THE DEVELOPMENT OF AN UNDERGRADUATE ENDOCRINE DISRUPTION AUTHENTIC RESEARCH LAB.

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Abstract:

UV filters are added to a number of personal care products to mitigate damage to underlying surfaces and several studies have identified their presence of in a variety of water compartments in the environment. UV filters have demonstrated endocrine disrupting potential in vertebrate models, but few studies have addressed their effects on resident aquatic invertebrates. The acute and chronic effects of the UV filters avobenzone, dioxybenzone, homosalate, OMCN octisalate, and oxybenzone on *D. magna* were assessed. Only avobenzone was acutely toxicity at $LC_{50}$ 0.74 mg/L. A potential hormetic effect on reproduction was noted in dioxybenzone, homosalate, octisalate, and oxybenzone (LOEC 0.75, 0.075, 0.0019, 0.7 mg/L). Male neonates, a potential indication of endocrine disruption, were identified in avobenzone, homosalate, and oxybenzone tests (LOEC 0.004, 0.6, and 5 mg/L). Environmental monitoring of UV filters is limited, especially in recreational lake areas. The current work identified the presence octisalate, homosalate, oxybenzone, and OMCN in selected US lakes using GC/MS. A seasonal and spatial effect on UV filter concentration was noted for the detected UV filters, but only octisalate, homosalate, and oxybenzone were found to be significant. Hazard quotients (HQ) were calculated using the maximum environmental concentration from this study and LOEC from our toxicological study and previous studies. HQ's for detected UV filter were well below 1, indicating the tested UV filters are not likely occurring in environmentally relevant concentrations. Many anthropogenic compounds such as pharmaceuticals and insecticides have demonstrated potential for endocrine disruption. Despite this knowledge, teaching this concept in undergraduate labs is not common. A research system using the invertebrate *D. magna* and the pesticide fenoxycarb is presented that demonstrates endocrine disruption, alleviates the complications of vertebrate models, and engages students in an authentic research experience. The system has been implemented in a small class containing a variety of declared major with 90% of the students showing proficiency in procedures and conceptual knowledge.

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# CHAPTER I

## INTRODUCTION

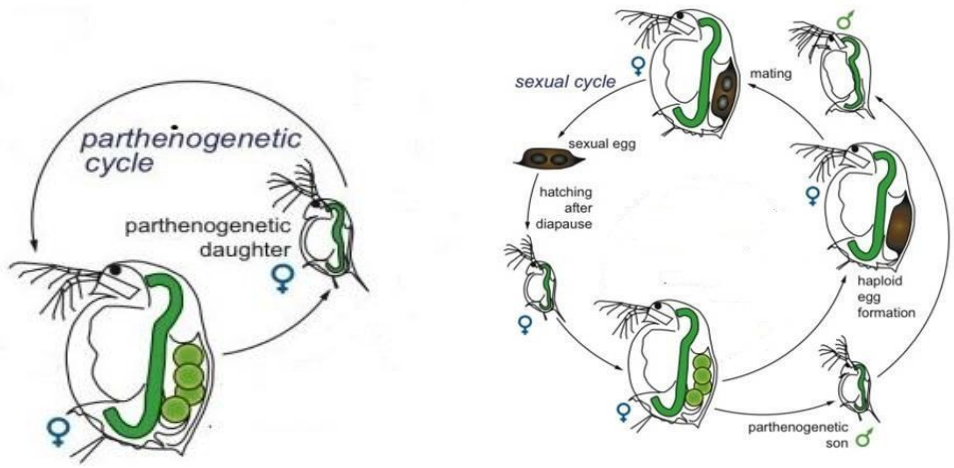
As the human population grows and the demand for anthropogenic compounds increase, the deposition and detection of these chemicals into surface waters have become more prevalent (Brausch, 2011). These chemicals are primarily released into the water supply by wastewater discharge and direct interaction (bathing and recreation). One group of anthropogenic compounds of interest to this study is ultraviolet (UV) filters often added to lotions, soaps, sunscreens, and plastics. UV filters work to absorb solar radiation and reduce the levels that reach the material the product is designed to protect. UV filters added to lotions and sunscreens help mitigate the potential UV damage to skin and prevent the formation of cancerous and pre-cancerous growths. UV filters added to plastics help reduce photodegradation of either the material in the container or the container itself. Recent studies have identified 11 common UV filters lakes and rivers that occurred in the ng/l range (Cuderman, 2007 and Fent, 2008). Although it has been demonstrated that UV filters contaminate the environment, research is limited regarding the effects of these chemicals on resident populations and the concentration at which they occur.

Although the likelihood that UV filters are occurring in aquatic environments at concentrations high enough to cause acute toxicity is low, the potential to cause chronic effects is unknown. Several UV filters have demonstrated the ability to disrupt the endocrine systems of vertebrates; reproductive effects were demonstrated in human receptor studies (estrogenic and thyroid), fathead minnows, frogs, rainbow trout, and Japanese medaka (Diaz-Cruz, 2009). Such chemicals, frequently referred to as endocrine disrupting chemicals (EDC's), can inhibit or promote the expression of hormonal actions by interacting with hormone receptor, binding to the hormone itself, or alerting other portions of the hormone signaling pathway. They tend to show effects on populations in concentrations much lower than those observed in acute toxicity. These endocrine disrupting compounds (EDC's) have been shown to affect a wide range of organisms, such as aquatic invertebrates, insects, fish, amphibians, and humans (Rodriguez, 2007).

Governmental and international agencies such as the United States Environmental Protection Agency (EPA) and the Organization for Economic Co-operation and Development (OECD) have implemented testing protocols in response to the mounting evidence of the ubiquitous nature of EDC's (Wang, 2005 and Tatarazako, 2007). These protocols typically include acute and sub-lethal testing on aquatic invertebrates, amphibians, rodents, and human receptors. The majority of studies pertaining to potential EDC's has been focused on vertebrate models, presumably due to their higher degree of correlation with humans (deFur, 2004). Another hindrance to the work with aquatic invertebrates was the limited knowledge of the endocrine systems in these organisms. Recent understanding of crustacean endocrinology and the realization that these organisms may serve as model organisms to identify EDC's have prompted an increase in studies focusing on crustacean models such as *Daphnia* (Tatarazako, 2003; deFur, 2004; and Lampert, 2006).

*Daphnia magna* is an aquatic crustacean species of critical importance to aquatic ecosystems because it serves as a primary consumer of algae which impacts water quality and is a major constituent in aquatic food chains. Daphnia reproduce primarily by cyclic parthenogenesis, an

asexual process, whereby the females of the species produce varying numbers of female offspring under normal environmental conditions (Barker, 1985 and Herbert, 1987). These parthenogenic females are diploid clones of the mother and are recognized by a variety of phenotypic traits. Daphnia females tend to be larger with short first antennae length and a pointed rostrum. The onset of varied environmental (stress) conditions such as colder temperatures, decreased photoperiods, food scarcity, and crowding can stimulate pathogenic males capable of sexual reproduction (Hobaek, 1990). Males are able to fertilize special haploid eggs to produce specialized structures called epiphia. Epiphia, or resting eggs, are encased in a protective layer that allows them to survive in the environment (Kleiven, 1992) presumably to allow the species to survive in winter or drying conditions. When environmental conditions return to normal, a female emerges from the epiphium. An overview of the *D. magna* life cycle under normal and stress conditions is provided in Figure 1. The environmental conditions that lead to the conversion from the asexual to the sexual reproducing stage had been well documented.




**"Normal" Conditions** **"Stress" Conditions**

Figure 1. *D. Magna* Life Cycle under Normal and Stress Conditions

Since the discovery of methyl farnesoate as the hormone responsible for the production of males, it has been identified in over 30 crustacean groups including *Daphnia*, although the specific receptor responsible for its binding has not been determined (LeBlanc, 2007). Methyl farnesoate is a terpenoid chemically related to the insect hormone, juvenile hormone III (JHIII); the primary difference being that methyl farnesoate does not contain a 3-sided ether group or epoxide found on juvenile hormone III (Laufer, 1992). Since its discovery as the male determinative hormone, chemical analogues such as kinoprene, fenoxycarb, and pyriproxyfen have been shown to induce the production of males (Haeba, 2008). Studies involving methoprene have produced conflicting results as to its ability to stimulate male production. Despite the appearance of parthogenically-produced males under test conditions, no evidence exists to demonstrate that methyl farnesoate induced males can fertilize sexual females, suggesting male production and sexually reproducing, epiphial-carrying females might be controlled by a different mechanism (Kim, 2006). While the testing of pesticides for *daphnid* toxicity is common, the majority of personal care products (PCP's) remain untested. Interestingly, many UV filters have ester functional groups coupled with aliphatic side chains or rings, which are the primary functional groups that are required for methyl farnesoate analogs to be effective (Hirakawa, 2005).

*Daphnia magna* has been adopted by several international organizations, including the OECD, as a model organism for aquatic toxicity testing. Its high reproduction rate, inexpensiveness, ease of handling, sensitivity, and multiple clearly defined endpoints make it a good model organism (LeBlanc, 1999 and OECD, 2004). Endpoints used to assess endocrine disruption have typically included fecundity, larval development, size, age of first reproduction, and molting abnormalities. One more recent endpoint that has gained interest in the determination of a chemical's ability to mimic methyl farnesoate is sex ratio (Tatarazako, 2007). This simple endpoint can determine methyl farnesoate agonists or mimics by an increase in male offspring under environmental conditions favorable to female reproduction. One proposed mechanism of action is that the agonist, along with

the natural hormone, binds to the cellular receptors that leads to signal transduction and the eventual cellular effects leading to male development (Laufer, 1992). Likewise, antagonists of methyl farnesoate could lead to an increase in females under conditions favorable to male production by blocking the attachment of the natural hormone to the cell receptor site thereby limiting male production.

Prior to 2002, comprehensive studies of organic wastewater contaminants (OWC) were nonexistent. The first national survey assessed the contamination in high-risk streams and found OWC's including pharmaceuticals, flame retardants, hormones, and other personal care products were ubiquitous (Koplin, 2002). Since that study, many studies have been conducted both in other types of water sources and on other types of contaminants. Anthropogenic compounds have now been detected in nearly all compartments of the water cycle, including rivers, lakes, estuaries, and oceans (Peck, 2006). Most of the studies focus on contaminants in association with wastewater treatment plants and not on implication of direct inputs during high use times. The first preliminary risk assessment, based on limited data, for UV filters indicated that environmental risk could not be ruled out for 2 (4-methylbenzylide camphor and Ethyl- methoxycinnemate) of the 5 chemicals tested (Fent, 2010). The current risk assessments to determine the dangers of ambient levels of sunscreen products in surface waters are, at best, incomplete and in need of further study. Furthermore, most environmental testing has only focused on municipal sewage effluent. For these compounds, direct sources to the aquatic systems may exist where no treatment occurred. For example, UV filters are used extensively around many lakes that have high recreation.

EDCs have become a current and substantial environmental concern that should be included in undergraduate laboratory studies in classes such as biology, environmental science, physiology, or undergraduate research courses. The inherit concerns of conducting an endocrine disruption lab include expense, complexity, space limitations, time limitations, and invasiveness in vertebrate models. Finally, there has been a call to include research experiences in undergraduate courses to

provide students with authentic experience, increase critical thinking, and promote lasting learning (AAAS, 2011).

Given the deposition of UV filters in water sources and lack of sufficient research and understanding about their ability to disrupt invertebrate physiology, this study's primary objectives include:

1. Assess the acute toxicity of the selected UV filters on *Daphnia magna* by determining the 24 and 48 hour $LC_{50}$ value with immobility as an endpoint
2. Assess the chronic effects of the selected UV filters on *Daphnia magna* with number of offspring, average day of first brood production, and alteration of sex ratios as endpoints in a 21 day study
3. Determine the levels of the selected UV filters in various Oklahoma lake environments and compare to toxicological endpoints (Objective 1 and 2)
4. Develop an undergraduate lab to teach the concepts of endocrine disruption and research techniques using a *Daphnia* model organism

# CHAPTER II

## ACUTE AND CHRONIC EFFECTS OF SIX COMMON UV FILTERS ON *DAPHNIA MAGNA*.

**Preface:** This chapter is prepared for submission to Ecotoxicology


### Abstract

Organic UV filters are added to a variety of personal care products and plastic structures to protect underlying surfaces. Numerous environmental studies have identified the presence of UV filters in wastewater and surface waters in the low ng/L to µg/L range; however, limited studies have been completed to assess the acute and chronic effects UV filters have on resident aquatic populations such as *Daphnid* species. The current study examined the acute and chronic effects of avobenzone, dioxybenzone, homosalate, octisalate, octylmethoxycinnamate (OMCN), and oxybenzone to the model organism *Daphnia magna*. Avobenzone was the only UV filter that demonstrated acute toxicity under experimental conditions- $LC_{50}$ value of 0.74 (0.41, 0.94) mg/L. Chronic exposure produced male neonates at LOEC values of 0.004 mg/L (avobenzone), 5 mg/L (oxybenzone), and 0.6 mg/L (homosalate) suggesting these chemicals may act as endocrine disruption compounds (EDC') for *D. magna*. Delays in first brood production

were noted in the highest tested concentrations for avobenzone (LOEC 0.22 mg/L), OMCN (LOEC 0.03 mg/L), and oxybenzone (LOEC 10 mg/L). The average number of surviving neonates was reduced at the highest test concentrations for each UV filter. An apparent hormetic effect of mean neonate per surviving adult was noted in homosalate (LOEC 0.075 mg/L), OMCN (LOEC 0.0019 mg/L), and (LOEC 0.7 mg/L). To our knowledge, this is the first study to identify UV filters as potential EDC's in *D. magna* with male production and as a measured endpoint.

**Introduction**

Ultraviolet (UV) filters work by absorbing solar radiation and reduce the levels that reach the underlying material; UV filters are often added to lotions, soaps, and sunscreens to help mitigate the potential UV damage to skin and prevent the formation of cancerous and pre-cancerous growths (Fent, 2010; Liu, 2010; and Rodriguez, 2015). Additionally, they are added to plastics and foams to help reduce photodegradation of either the material in the container or the container itself (Zenker, 2008, Diaz-Cruz, 2009, and Tsui, 2014). These chemicals are frequently released into the water supply by wastewater discharge and direct interaction such as bathing and recreational activities (Bratkovics, 2011 and Tsui, 2015). UV filters have been identified in all compartments of the water system across a variety of geographical locations. Several UV filters were identified in untreated wastewater in a variety countries in the low µg/l range (Balmer, 2005; Li, 2007; and Rodil, 2008). Wastewater treatment procedures appear to generally eliminate some UV filters as wastewater effluent studies identified selected UV filters in lower concentrations than the intake waters (Balmer, 2005; Rodil, 2008 and Fent, 2010). Several recent studies have identified 11 common UV filters in lakes and rivers that occurred in the ng/l range (Fent, 2008 and Cuderman, 2007). Some studies have focused on high use recreational areas where direct input of UV filters is expected to be the highest. A study of Greece recreational and bathing waters identified 4 common UV filters in the low ng/L range (Goikas, 2004). Studies of South Carolina (US) beaches, Oklahoma (US) lake recreational areas and Canary Islands beaches

identified several common UV filter in ranges between 1 and 2000 ng/L (Bratkovis, 2011; Layton, 2015; and Rodriguez, 2015). A multi-country study revealed similar concentrations that have been previously reported, except for 2 UV filters occurring between 4 and 6 µg/l in Hong Kong and lower levels of UV filters in artic samples (Tsui, 2015). UV filters have also been identified a sediment samples in the low ng/g range (Jeon, 2006 and Baron, 2013). Although it has been demonstrated that UV filters contaminate aquatic environments, research is limited regarding the effects of these chemicals on resident populations and the concentration at which they occur.

Several UV filters have demonstrated the ability to disrupt the endocrine systems of vertebrates; reproductive effects were demonstrated in human receptor studies (estrogenic and thyroid) (Schlumphf, 2001 and Schlecht, 2004) fathead minnows (Fent, 2008), rats (Hofkamp, 2008), frogs (Kunz, 2004), rainbow trout, and Japanese medaka (Coranado, 2008). The majority of studies pertaining to potential endocrine disruption chemicals (EDC's) has been focused on vertebrate models, presumably due to their higher degree of correlation with humans (deFur, 2004). Recent understanding of crustacean endocrinology and the realization that these organisms may serve as model organisms to identify EDC's have prompted an increase in studies focusing on crustacean models such as *Daphnia* (Tatarazako, 2003; deFur, 2004; and Lampert, 2006).

*Daphnia magna* is an aquatic crustacean species of critical importance to aquatic ecosystems because it serves as a primary consumer of algae which impacts water quality and is a major constituent in aquatic food chains. Daphnia reproduce primarily by cyclic parthenogenesis, an asexual process, whereby the females of the species produce varying numbers of female offspring under normal environmental conditions (Barker, 1985; Herbert, 1987). These parthenogenic females are diploid clones of the mother and are recognized by a variety of phenotypic traits (Kleiven, 1992). Daphnia females tend to be larger with short first antennae length and a pointed rostrum. The onset of varied environmental (stress) conditions such as colder

temperatures, decreased photoperiods, food scarcity, and crowding can stimulate parthogenic males capable of sexual reproduction (Ingle, 1937; Stross, 1969; Kleiven, 1992; Tatarazako, 2007 and Pietrzak, 2010). The presence of the hormone methyl farnesoate (MF) signals the production of males which are able to fertilize special haploid eggs to produce specialized structures called epiphia (Hobaek, 1990). Epiphia, or resting eggs, are encased in a protective layer that allows them to survive in the environment presumably to allow the species to survive in winter or drying conditions. When environmental conditions return to normal, a female emerges from the epiphium. The environmental conditions that lead to the conversion from the asexual to the sexual reproducing stage have been well documented.

*Daphnia magna* has been adopted by several international organizations, including the Organization for Economic and Cooperation and Development (OECD), as a model organism for aquatic toxicity testing. Its high reproduction rate, inexpensiveness, ease of handling, sensitivity, and multiple clearly defined endpoints make it a valuable model organism (LeBlanc, 1999; OECD, 2004 and Lampert, 2006). Endpoints used to assess endocrine disruption have typically included fecundity, larval development, size, age of first reproduction, and molting abnormalities. One more recent endpoint that has gained interest in the determination of a chemical's ability to mimic methyl farnesoate is sex ratio (Tatarazako, 2007). This simple endpoint can determine methyl farnesoate agonists by an increase in male offspring under environmental conditions favorable to female production.

Although the likelihood that UV filters occur in aquatic environments at concentrations that would cause acute toxicity is low, the potential to cause chronic effects, which are often triggered at much lower concentrations, is unknown. Governmental and international agencies such as the United States Environmental Protection Agency (EPA) and the Organization for Economic Co-operation and Development (OECD) have implemented testing protocols in response to the mounting evidence of the ubiquitous nature of EDC's (Snyder, 2003 and

Tatarazako, 2007). These protocols typically include acute and sub-lethal testing on aquatic invertebrates, amphibians, rodents, and human receptors. While the testing of pesticides for daphnid toxicity is common, the majority of personal care products (PCP's) remain untested. A preliminary risk assessment of the UV filters on *Daphnia magna* identified 48-hour acute toxicities ($LC_{50}$) of 0.56, 0.29, 1.9 mg/L for 4- methylbenzylidene camphor(4-MBC), octylmethoxycinnamate (OMCN), and oxybenzone (BP-3), respectively (Fent, 2010). More recently a study identified $LC_{50}$ values of 1.67 (BP-3), 0.57 (OMCN), 3.61 (3-benzilydene camphor- 3 BC), 0.8 (4-MBC) mg/L on *D. magna* 48 hour toxicity and chronic effects of decreased fecundity and shorter length in adults for concentrations of OMCN above 0.04 mg/L and concentrations of 3-BC and 4-MBC above 0.1 mg/L (Sieratowicz, 2011). However, the majority of commonly used UV filters remain untested. Interestingly, many UV filters have ester functional groups coupled with aliphatic side chains or rings, which are the primary functional groups that are required for methyl farnesoate analogs to be effective (Hirakawa, 2005). Due to these structural similarities, chronic testing using male production as an endpoint is important.

Given the deposition of UV filters in water sources and lack of sufficient research and understanding about their effects on crustaceans, this study's primary objectives is to assess the acute toxicity of the selected UV filters avobenzone, dioxybenzone, octyl methoxycinnamate (OMCN), homosalate, octisalate, and oxybenzone (BP-3) on *Daphnia magna* by determining the 48 hour $EC_{50}$ value with immobility as an endpoint. Additionally, selected UV filters will be assessed for the ability to chronically effect the average number of offspring, the average first day of reproduction, and alter sex ratios of *Daphnia magna* in a 21 day study.

## Methods

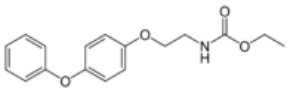
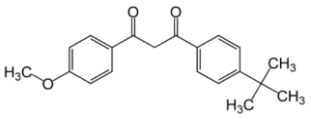
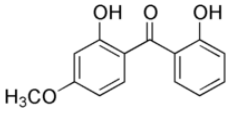
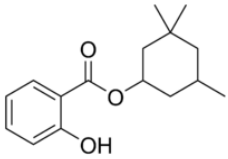
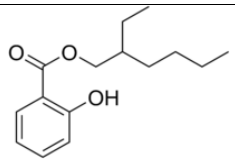
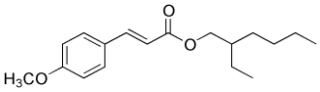
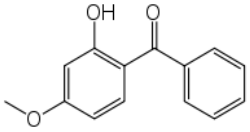
### *Chemicals*

Reagent grade (97%-100% pure) UV filters (avobenzone, dioxybenzone, octyl methoxycinnamate (OMCN), homosalate, octisalate, and oxybenzone) were purchased from VWR (Sugar Land, TX). Table 1 provides a complete test reagent list with relevant chemical data. Stock solutions of each analyte were produced from neat material. All additional solvents and reagents were pesticide grade or better.

### *Organisms*

The cladoceran *Daphnia magna* served as the test organism. Although smaller cladocerans are more common in most lake environments due to fish predation, *D. magna* is well studied physiologically and its toxicity protocols are well established. The *Daphnia* population was reared for several months in 1 L glass jars at a density of between 30-40 *Daphnia*/ L in dechlorinated tap water (hardness 140-250 mg/L and pH between 6 and 9) under conditions that favored female offspring production (20 C with a 16/8 hour light and dark cycle) to ensure the viability of the population. Cultures were fed daily with the 2.5 ml of the algae *Selenastrum capricornutum* ($3.7 \times 10^{7}$ cells/ ml) and 1.5 ml of yeast- trout chow (YTC) three times a week, both purchased from Aquatic BioSystems (Fort Collins, CO). Water changes were performed weekly by emptying two-thirds of the old water and replacing the volume with fresh dechlorinated water just prior to feeding. Frequent water changed prevented fouling on the jars in most instances; if fouling occurred the jar was replaced with a new reproduction jar. Offspring were eliminated regularly to control the overall population size.

**Table 1. Chemical Data for Selected UV filters**

| Chemical | Structural Formula | CAS Number | Mol. Weight (g/mol) | Purpose |
|---|---|---|---|---|
| Methyl farnesoate |  | 10485-70-8 | 250.38 | Natural Hormone |
| Fenoxycarb |  | 72490-01-8 | 301.34 | Insecticide Positive Control |
| Avobenzone |  | 73056-09-1 | 310.39 | UV Filter |
| Dioxybenzone |  | 131-53-3 | 244.25 | UV Filter |
| Homosalate |  | 118-56-9 | 262.36 | UV Filter |
| Octisalate |  | 118-60-5 | 250.33 | UV Filter |
| Octyl methoxycinnamate (OMCN) |  | 5466-77-3 | 290.40 | UV Filter |
| Oxybenzone (BP-3) |  | 131-57-7 | 228.24 | UV Filter |

*Acute toxicity studies*

Acute toxicity tests were adapted from the parameters established by the Organization of Economic Co-Operation and Development test guideline 202 (OECD, 2008). Each UV filter was tested independently. Since the literature suggested that most UV filters were not acutely toxic at environmentally relevant concentrations, upper limit tests were set at 50% of estimated water solubility. If acute toxicity was identified at the upper limit, range finding experiments were performed to allow the final test to be performed at concise levels. Acute test concentrations are summarized in Table 2. For each treatment, 4 replicate experimental units were established. Each experimental unit consisted of five *D. magna* neonates, less than 24 hours old, placed into a 50 ml beaker containing 20 ml of dechlorinated tap water. UV filters were introduced into the water using no more than 0.1 ml acetone carrier per L of test water. The amount of acetone for each treatment for a particular UV filter remained constant, but acetone amounts varied between UV filters. Solvent controls (n=4), matching the maximum amount of solvent per UV filter, were conducted for each test. Daphnia were not fed during the test and immobilization, checked at 24 and 48 hours, was used as an endpoint to determine mortality Immobilization was measured by visual exam and was defined as any *Daphnia* that cannot swim after gentle agitation of the test beaker; movement of the antennae that produced no locomotion counted as immobilized.

*Chronic Studies: Reproduction and sex-ratio tests*

Reproduction and sex ratio tests were adapted from the parameters established by the Organization of Economic Co-Operation and Development test guideline 211 (OECD, 2004). Each experimental unit consisted of a single female neonate, less than 24 hours old in a small glass jar containing 100 ml of dechlorinated tap water. Each experimental unit was replicated 10 times for each treatment. Treatments included solvent controls, and 5 concentrations of each toxicant. UV filters were introduced into waters in a similar manner to the acute studies, but the

maximum acetone amounts were between 25 and 63 µl. Avobenzone was tested at a total of seven concentrations in response to significant male production all of the initial test concentrations.

The highest concentration for the chronic tests was set at 20% water solubility for each analyte as determined by a variety of estimation software if acute toxicity was not noted at that level. Range finding experiments were used to determine appropriate testing levels when appropriate. Chronic test concentrations are summarized in table 2.

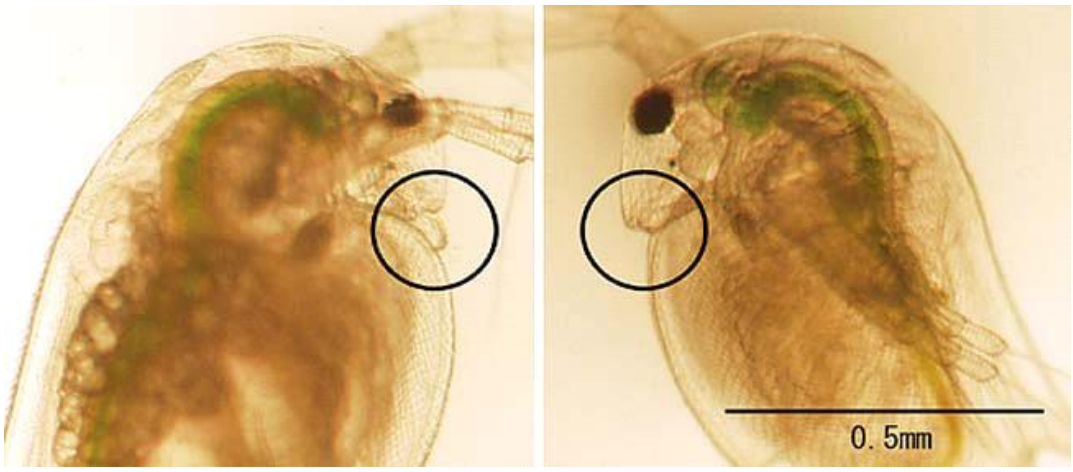
**Table 2. UV Filters Nominal Test Concentrations of Acute and Chronic Toxicity Tests.**

| UV Filter | *D. magna* Acute Immobilization Test Concentrations (mg/L) | *D. magna* Reproduction Test Concentrations (mg/L) |
|---|---|---|
| Avobenzone | 2, 1.5, 1, 0.75, 0.45 | 0.22, 0.11, 0.06, 0.03, 0.016, 0.008, 0.004 |
| Dioxybenzone | * (26) | 12, 6, 3, 1.5, 0.75 |
| Homosalate | * (2) | 1.2, 0.6, 0.3, 0.15, 0.075 |
| OMCN | * (0.8) | 0.03, 0.015, 0.0073, 0.0037, 0.0019 |
| Octisalate | * (8.5) | 1.8, 0.9, 0.45, 0.22, 0.11 |
| Oxybenzone | * (10) | 6, 3, 1.5, 0.75,0.38 |

* UV filter not acutely toxic at test limit

*Daphnia* were fed with the algae *S.* capricornutum ($3.7 \times 10^7$ cells/ ml) 5 times a week and with YTC 3 times a week. Test waters were renewed 3 times a week with the adult being transferred to the new container via large bore pipette. Parameters of pH and oxygen concentration were checked weekly to make sure they remained within accepted ranges. Offspring were removed, counted and stored in ethanol for later male/female analysis. Sex was

determined by the length of the first antennae; the first antennae, located under the rostrum, is more pronounced in males than females. (See Figure 1).The test was performed for 21 days, normally time enough for the reproduction of at least three broods of offspring under control conditions.




**Male** **Female**

Figure 1. Sex determination by length of first antennae in *Daphnia magna* (Tatarazako, 2004).

*Statistical Analysis*

UV filters that demonstrated acute toxicity were analyzed by probit analysis and $LC_{50}$ values were identified with 95% confidence intervals (SPSS). Chronic samples were subjected the Shapiro-Wilk test ($p \leq 0.05$) and the Levene test to determine normality and homogeneity respectively. Each endpoint that met the normality and homogeneity tests were subjected to ANOVA followed by Dunnett's test to determine significant differences between treatments and controls; otherwise, the nonparametric Kruskal-Wallis test was performed. Chi square analysis was used to determine if the mean number of males produced per treatment was significantly different from controls. The no observed effects concentration (NOEC) and/or the lowest observed effects concentration (LOEC) were define based on the highest concentration not

significantly different from controls (NOEC) and the lowest concentration that was different from controls (LOEC). Significance was defined as $p \leq 0.05$.

## Results

### Acute Toxicity

At the highest treatment level, 50% of water solubility, there was less than 10% acute effects based on immobility noted for dioxybenzone, homosalate, octisalate, OMCN, and oxybenzone and effects were not significant from controls. However, for avobenzone, 100% acute toxicity occurred at the high test concentration (2 mg/L) resulting in further testing. The acute toxicity results for avobenzone are summarized in Figure 2. The $LC_{50}$ value was calculated to be 0. 74 (0.41, 0.94) mg/L using probit analysis.

### Chronic Toxicity

#### *Male production*

Solvent controls, Dioxybenzone, OMCN, and Octisalate treatments produced no males during the course of the 21-day reproductive study (NOEC 3, 0.03, and 0.22 mg/L respectively). Males were identified at a low frequency (1.57%) only in the highest surviving homosalate concentration (LOEC = 0.6 mg/L). The three highest concentrations of oxybenzone produced males (LOEC 5 mg/L); however, the 2.5 mg/L concentration was not statistically significant. Males were produced in significant levels in all avobenzone concentration (LOEC= 0.004 mg/L) in what appear to be a dose dependent manner. Avobenzone male production occurred at similar levels to the positive control fenoxycarb; albeit with higher concentrations necessary. A summary of the percentage of males produced per chemicals concentration is summarized in Table 3

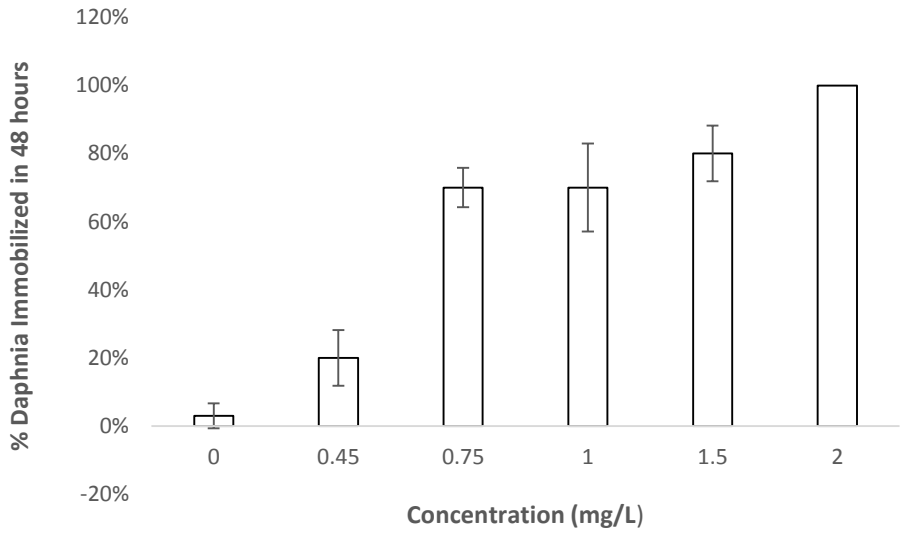



Figure 2. Percentage of *D. magna* immobilized when exposed to various concentrations of Avobenzone for 48 hr. Percent immobilized represent the mean of 4 replicates ± SD.

**Table 3. Male Production by Concentration in Fenoxycarb, Avobenzone, Homosalate, and Oxybenzone**

| Fenoxycarb (mg/L) | % males | Homosalate (mg/L) | % males | Avobenzone (mg/L) | % males | Oxybenzone (mg/L) | % males |
|---|---|---|---|---|---|---|---|
| #0.00025 | 10.2* | 0.075 | 0 | #0.004 | 4.8* | 0.7 | 0 |
| 0.0005 | 55.8* | 0.15 | 0 | 0.008 | 18.1* | 1.4 | 0 |
| 0.001 | 55.5* | 0.3 | 0 | 0.016 | 37.5* | 2.5 | 0.27 |
| 0.002 | 87.9* | #0.6 | 1.57* | 0.03 | 79.6* | #5 | 3.6* |
| 0.004 | 89.0* | | | 0.06 | 85.8* | 10 | 12.7* |
| | | | | 0.11 | 88.7* | | |
| | | | | 0.22 | 92.2* | | |

* Indicates significant difference from the control ($p \leq 0.05$) using chi square analysis
# Indicates LOEC

*Time to First Brood Production*

Dioxybenzone, homosalate, and octisalate had no significant effect on the average first day of reproduction compared to the control for all concentrations that did not exhibit mortality (NOEC = 0.75, 0.075, and 0.22 mg/ L). Significant delays in first brood production were identified at the highest concentration of avobenzone, OMCN, and oxybenzone (LOEC = 0.22, 0.3, and 6 mg/L), the only treatment for each UV filter that showed significant delays. A summary of the mean time for first brood production is summarized in Table 4.

*Mean Neonates per Surviving Adult*

Effects on the mean number of offspring were noted with each UV filter; some significantly reduced the mean number of neonates, while other significantly increased the mean number of neonates. Avobenzone, in general, decreased the average number of neonates in the concentration above the LOEC 0.016 mg/L, although on the 0.22 mg/L was the only other concentration found to be statistically significant. Octisalate reduced the mean number of offspring in the 2 lowest concentrations (LOEC 0.11 mg/L). The remaining UV filters tended to decrease average offspring number at upper concentrations and increase offspring number at lower ranges compared to solvent controls. Dioxybenzone concentrations produced significant effects on the number of neonates, 1.5 mg/L (LOEC) decreasing the number of neonates and 0.75 (LOEC) mg/L showing a significant increase in offspring production. Homosalate produced effects similar to dioxybenzone with the lower concentration (LOEC 0.075 mg/L) stimulating increases in average offspring and upper concentrations (LOEC 0.3 mg/L) reducing fecundity. The highest OMCN concentration significantly decreasing neonate production (LOEC 0.03 mg/. The two lowest OMCN treatments resulted in a significant increase in offspring production (LOEC 0.0019 mg/L). Oxybenone produced significant decreases in offspring production (LOEC 10 mg/L), while increases in the number of neonates increased in the remaining concentrations

(LOEC 0.7 mg/L). A summary of the mean number of neonates per surviving adult is summarized in Table 4.

*Chronic Mortality*

For test validity, no control could produce more than 20% mortality during the test period. Control mortality never exceeded 10 %. No significant chronic mortality was observed in any avobenzone or oxybenzone treatments. Chronic mortality was observed in the upper concentrations for each of the other four analytes. Dioxybenzone produced chronic mortality that appears to follow a dose response curve; on average, mortality occurred on day 7 at 12 mg/L, day 11 at 6 mg/L, and day 15 at 3 mg/L (LOEC). Homosalate and OMCN demonstrated chronic mortality of in the upper 2 test concentration (LOEC 0.6 and 0.015 mg/L), but the timing of death appeared to be random. Octisalate was chronically toxic to *D. magna* in all treatment groups (LOEC 0.11 mg/L). The highest 3 octisalate concentrations produced mortality in all adults between day 3-8, while mortality was delayed in other treatment groups with no clear pattern based on concentration. The percentage of surviving adults for each treatment is summarized in table 4.

## Discussion

Avobenzone exhibited the highest acute toxicity for *D. magna* among the six UV filters tested with the NOEC being approximately 10% of water solubility. For the remaining UV filters, acute toxicity was very low with high concentrations near 50% water solubility not causing any significant lethality. Two of our acute studies showed unexpectedly low toxicity as compared to previous literature (Fent, 2010 and Sieratowicz, 2011). The OMCN maximum concentration tested (0.8 mg/L) resulted in no immobility while previous studies reported $LC_{50}$ values at 0.29 and 0.57 mg/L (Fent,

**Table. 4 Results of a 21 Day *D. magna* Chronic Toxicity Test Exposed to Six common UV Filters**.

| Chemical | Concentration (mg/L) | First day of Brood (mean + SD) | Neonates per Surviving Adult (mean + SD) | Percentage of Surviving Adults |
|---|---|---|---|---|
| Avobenzone | Solvent Control | 8.67 (± 1.22) | 61.7 (± 13.7) | 90% |
| | 0.004 | 8.2 | 62.7 (± 10.6) | 100% |
| | 0.008 | 9.3 | 58.2 (± 9.55) | 90% |
| | 0.016 | 8.8 | 48.1 (± 9.25)** | 90% |
| | 0.03 | 9 (± 1.41) | 44.9 (± 12.6) | 100% |
| | 0.06 | 9.22 (± 1.20) | 53.0 (± 5.07) | 90% |
| | 0.11 | 8.3 (± 1.00) | 51.9 (± 9.36) | 90% |
| | 0.22 | 11.5 (± 1.90)* | 38.3 (± 9.75)*** | 100% |
| Dioxybenzone | Solvent Control | 8.7 (± 0.48) | 76.3 (± 7.66) | 100% |
| | 0.75 | 8.6 (± 0.97) | 117 (± 15.0)*** | 100% |
| | 1.5 | 8.8 (± 1.30) | 50.2 (± 23.8)** | 90% |
| | 3 | 9 (± 0.00) | 17 | 10% |
| | 6 | X | X | 0% |
| | 12 | X | X | 0% |
| Homosalate | Solvent Control | 7.6 (± 0.97) | 95.6 (± 7.49) | 100% |
| | 0.075 | 7 (± 0.00) | 121 (± 8.90)*** | 100% |
| | 0.15 | 7.38 (± 0.52) | 117 (± 8.54)*** | 100% |
| | 0.3 | 7.4 (± 0.52) | 64.5 (± 8.82)*** | 80% |
| | 0.6 | 7.4 (± 0.55) | 38.8 (± 10.4)*** | 50% |
| | 1.2 | X | X | 0% |
| OMCN | Solvent Control | 7.6 (± 0.97) | 95.6 (± 7.49) | 100% |
| | 0.0019 | 7.56 (± 0.53) | 120 (± 14.7)** | 90% |
| | 0.0037 | 8.25 (± 0.46) | 124 (20.9)** | 80% |
| | 0.0073 | 9.33 (± 1.32) | 102 (± 20.1) | 90% |
| | 0.015 | 9 (± 0.00) | 108 (± 8.43) | 60% |
| | 0.03 | 14 (± 2.74)* | 24.0 (± 11.7)*** | 60% |
| Octisalate | Solvent Control | 8.67 (± 1.22) | 61.7 (± 13.7) | 90% |
| | 0.11 | 10 (± 0.00) | 38.0 (± 8.53)** | 60% |
| | 0.22 | 9.25 (± 1.50) | 27.3 (± 14.4)** | 40% |
| | 0.45 | X | X | 0% |
| | 0.9 | X | X | 0% |
| | 1.8 | X | X | 0% |
| Oxybenzone | Solvent Control | 8.7 (± 0.48) | 76.3 (± 7.66) | 100% |
| | 0.7 | 7 (± 0.00) | 172 (± 12.3)*** | 100% |
| | 1.4 | 7.4 (± 0.84) | 132 (± 35.3)*** | 100% |
| | 2.5 | 7 (± 0.00) | 150 (± 21.0)*** | 100% |
| | 5 | 7.2 (± 0.63) | 106 (± 31.6)* | 100% |
| | 10 | 11.4 (± 2.00)* | 26.0 (± 13.8)*** | 80% |

* Indicates significant difference from the control ($p \leq 0.05$)

** Indicates significant difference from the control ($p \leq 0.01$)

*** Indicates significant difference from the control ($p \leq 0.001$)

X represents chronic mortality

2010 and Sieratowicz, 2011). Similarly for oxybenzone, our highest concentration tested (10mg/L) resulted in no acute effect and our limited chronic mortality while previous studies reported $LC_{50}$ values between 1.67 and 1.9 mg/L (Fent, 2010 and Sieratowicz, 2011). Differences in reported toxicities could be a result of variation in experimental protocol such as the volume of test water used, or inaccurate dosing among studies given how close the values are to water solubility.

The production of males by some UV filters is not unreasonable since several anthropogenic compounds have shown the ability to increase male production (Olmstead, 2003). Specifically, several studies have indicated that some UV filters have endocrine disruption potential in a variety of vertebrates and invertebrates species (Schlumpf, 2001; Kunz, 2006; and Schmitt, 2008). The few studies that have assessed the chronic effects of UV filters on Daphnia have focused on alteration in reproductive capacity and body length as measurable endpoints (Sieratowicz 2011 and Pablos, 2015), but male production can be a valuable indicator. Increases in male production under conditions favorable for female reproduction either indicate avobenzone, homosalate, and oxybenzone initiate stress conditions for *Daphnia* or that the UV filters may be interfering with the endocrine system by mimicking the effects of MF, the hormone needed for male production. Previous studies have indicated that most MF agonists typically decrease *Daphnia* reproduction rates along with producing high percentages of males (Tatarazako, 2003 and Oda, 2005). Although it is difficult to specifically determine if the effects are endocrine disruption or environmental stress, the results demonstrated in homosalate and oxybenzone, seem to be more indicative of the general pattern of stress since the male ratio is low and in some cases the UV filters stimulate an increase in reproductive rates. Avobenzone appears to more closely follow the previous observation of endocrine disrupting MF agonists with a high male ratio accompanied by a general decrease in mean number of offspring. In addition, effects were noted at 0.004 mg/L, much lower than the $LC_{50}$ value of 0.6 mg/L, further suggestion a

specific mode of action rather than a stress response. To our knowledge, this is the first study to report the production of males with exposure to avobenzone in what appears to be an endocrine disrupting mechanism.

Higher chronic test concentrations with surviving *Daphnia* had a negative impact on neonate production for all UV filters. Avobenzone generally had no significant effect on neonate production in the majority of the test concentrations, aside from 0.22 and 0.016 mg/L. Octisalate had an overall negative impact on neonate production in the two concentrations that did not result in chronic mortality (LOEC 0.11 mg/L). This suggests that further studies are needed at lower concentrations to determine a NOEC for Octisalate. Dioxybenzone, homosalate, OMCN, and BP-3 each showed significant increases in neonate production (LOEC 0.75, 0.075, 0.0019, 0.38 mg/L respectively). Previous studies have shown that BP-3 (NOEC 0.2 mg/L) and OMCN (NOEC 0.015 mg/L) had no effect on *Daphnia* reproduction in a 21 day test, despite noting increases in reproductive rates for 4-MBC; observed differences in reproduction could be attributed to different experimental procedures, most notably a smaller amount of test median and the feeding schedule which consisted of fewer feedings per week (Pablos, 2015). Exposure of invertebrates to some UV filters have resulted in increased reproduction rates. 4-MBC resulted in increased reproduction in *D. magna* (Pablos, 2015) and in the snail *Potamopyrgus antipodarium* when exposed to 3-BC and 4-MBC (Schmitt, 2008). The features of the treatments for the UV filters than demonstrated decreases in neonates at the upper ranges and increases at lower ranges appear to show a hormetic effect. Hormesis typically includes some key elements such as a biphasic distribution, a dose-response pattern, and a stimulation that doesn't typically exceed two times the control (Calabrese, 2002). Dioxybenzone, homosalate, OMCN, and BP-3 chronic results generally follow that pattern, indicating the response is hormetic in nature which may include possible endocrine disruption.

UV filters do not appear to cause substantial delays in the timing of first brood production, especially since delays were only noted in the highest test concentrations for avobenzone, OMCN, and oxybenzone. These findings are similar to a previous study which identified no significant delays in first brood production for OMCN and oxybenzone (Sieratowicz, 2011).

Chronic endpoints selected for this study appear to disproportionally useful in determining chronic effects on *D. magna*. Delays in first brood production appeared to be the least sensitive endpoint with only two treatments showing significant effects. The most sensitive endpoint appears to be the number of neonates produced since the majority of treatments induced or reduced the number of neonates. Although males were produced in few treatments, male production remains a valuable endpoint for determination of chemicals that mimic the effects of MF needed for male production in *D. magna*.

A recent study of US Lakes identified the environmental levels of four of the six tested UV filter at 0.04 µg/L OMCN, 0.7 µg/L octisalate, 1.3 µg/L homoslate and 1.8 µg/L oxybenzone at high use beach sites during peak seasons (Layton, 2015). Additional world-wide studies identified the highest levels of the tested UV filters at 4 µg/L OMCN, 5.4 µg/L BP-3, 2.8 µg/L homoslate, 0.177 µg/L dioxybenzone at tests sites around Hong Kong (Tsui, 2014), and 0.3 µg/L avobenzone at South Carolina, US beaches (Bratkovics, 2011). Most river samples, non- beach lake samples, and waste water effluent samples produced lower concentrations of UV filters compared to high use recreational areas (Balmer, 2005, Fent, 2010, Kameda, 2011). Given that peak environmental levels of UV filters are between 7 and 4000 times less concentrated than the effects concentration reported in this study, it is unlikely that these 6 UV filters are occurring in concentrations high enough to produce either acute or chronic effects to aquatic invertebrates. However, to date there is limited environmental monitoring available so maximum environmental

concentrations are likely not known. Additionally, the EDC capabilities of avobenzone suggest that more detailed chronic evaluations such as full life cycle assessments may be warranted.

## Conclusion

The present study was designed to reconfirm or provide initial toxicological data for six UV filters. In general, UV filters were not found to be acutely toxic at the tested concentrations with the exception of avobenzone. UV filters under the current study parameters appear to have relatively little effect on the timing of first brood development; however, most UV filters appear to significantly affect the average number of neonates either by producing an inhibitory effect which only occurred at the upper limits of the test concentration or a stimulatory hormetic effect at the mid to lower concentrations. Half of the UV filters tested produced males under conditions that would typically result all female neonates. It is more likely that male production in avobenzone is a result of endocrine disruption due to its similarities to the positive control group and to other studies. Although significant acute toxicity was noted for avobenzone and chronic toxicity was noted in the µg/L range, UV filters most likely do not pose considerable environmental risk to *D. magna* since several aquatic studies have identified environmental concentrations in the ng/L range for most UV filters (Zenker, 2008 and Layton, 2015 unpublished study). Further studies are needed to confirm these initial findings that should include analytical confirmation of test waster concentrations as opposed to nominal concentrations. Since most of the formulations of sunscreen lotions contain combinations of several UV filters, other studies are needed to identify possible synergistic, toxicological relationships that might exist between different UV filters.

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# CHAPTER III

# THE OCCURRENCE OF SIX COMMON UV FILTERS IN SELECTED OKLAHOMA LAKES

**Preface:** This chapter is prepared for submission to Archives of Environmental Contamination and Toxicology.


## Abstract

Organic UV filters, used in sunscreens, other personal care products and commercial products, may be deposited into aquatic ecosystems by wastewater treatment plants and direct inputs from recreational activities. However, few monitoring studies have identified the levels of UV filters in high use recreational waters, especially in the United States. This study examines the occurrence of the UV filters avobenzone, dioxybenzone, homosalate, Octyl methoxycinnamate (OMCN), octisalate, and oxybenzone (BP-3) in six US lakes in both high use recreational areas and areas not expected to have major direct input from July to October, 2012. Avobenzone and dioxybenzone were not identified above detection limits in any sampling. Mean concentrations of octislate (301 ng/L), homoslate (537 ng/L), and oxybenzone (605 ng/L) at beach areas were higher in the month of July corresponding with the highest use times; mean concentrations decreased by around 50% in September samples and further diminished into October. UV filters were found in lower

concentrations in offsite locations. Hazard quotients (HQ) based on toxicity to *Daphnia magna* were well below one, suggesting that the 4 detected UV filters are not likely to pose significant hazard to aquatic organisms even at the highest reported levels. This study demonstrates that high use recreational areas result in UV filter deposition into lakes ecosystems, but it is unlikely that they are occurring in concentrations high enough to be to be toxic to resident aquatic species.

## Introduction

Ultraviolet (UV) filters are substances that absorb UV radiation from the sun to protect underlying materials. Organic UV filters tend to be a diverse class of anthroogenic compounds that typically have at least one aromatic group with hydrophobic properties (Giokas, 2004). Aside from typically being added in combinations in sunscreen products, they are also components in personal care products such as lotions, cosmetics, and shampoos; they are also added to a variety of plastics and aquatic foams to prevent photodegradation of the underlying materials. Currently there are 16 UV filters in the United States and 27 UV filters in the European Union that are licensed for use in sunscreen formulations, although they are disproportionally used (Reisch, 2015).

UV filters are deposited into the environment primarily through wastewater or by direct input activities such as recreation or bathing. There is potential for the amounts of UV filters deposited into the environment to become more prevalent with the increase of products sun protection factor (SPF) rating in response to concerns over skin cancers. Previous studies have identified common UV filters in lakes, rivers, wastewater treatment plants (WWTP), and salt water sources between the low ng/L and the low µg/L range

(Giokas, 2004; Poiger, 2004; Balmer, 2005; Cuderman, 2007; Fent, 2008; Rodil, 2008; Liu, 2010; Bratkovics, 2011). Some UV filters have also been known to accumulate in sediments and sludge due to the lipophilic nature (Zhang, 2011; Baron, 2013; and Tsui, 2015). Several monitoring studies have focused on releases from WWTP. These studies have generally demonstrated increases in UV filter concentration with a higher plant service population, high use season (summer months), and in influents versus effluents suggesting that WWTP are a significant source of environmental UV filter deposition (Balmer, 2005; Langford, 2008; Rodil, 2008; Fent, 2010; Kameda, 2011 and Tsui, 2015). Despite the potential for higher levels of contamination during recreation and boating resulting in direct release to water bodies, only a few studies have addressed this potential. Monitoring of beach areas has mostly focused on salt-water beach areas and have generally demonstrated an increase in UV filter concentration in high use times and locations (Langford, 2008; Bratkovics, 2011; Tashiro, 2013; and Sandoka, 2015). Additional monitoring is needed to identify levels of UV filters in fresh water samples at high-use recreational areas. To date, most environmental monitoring has been conducted in European Union countries and Japan, although sparse studies have been conducted worldwide. To date, limited monitoring has been conducted in the United States: a study of South Carolina beaches (Bratkovics, 2011) and waters associated with WWTP in New York and Los Angeles (Tsui, 2015). Additional studies are needed to determine the occurrence of UV filters in the United States both in high use recreational areas and ambient levels in low use areas.

UV filters are an environmental concern because they have been shown to be estrogenic in fish, frogs, and human receptor studies (Diaz-Cruz, 2009). More recent

studies have focused on resident aquatic invertebrate populations such as *Daphnia magna*. Limited testing of 48 hour acute toxicity in *D. magna* have revealed $LC_{50}$ values in the low mg/L (0.29- 1.9) range, and chronic toxicity associate with decreases in growth and reproduction rates between 40 and 100 µg/L for 4 of the most common UV filters (Fent, 2010 and Sieratowicz, 2011). A more recent study found that 6 tested common UV filters either enhanced or reduced reproductive rates in *Daphnia* (between 1.9 and 6000 µg/L nominal) and that avobenzone (LOEC= 4 µg/L), homosalate (LOEC= 600 µg/L), and oxybenzone ( 150 µg/L) stimulated the production of male *Daphnia* under conditions only expected to produce females (Layton, 2015 unpublished). The production of males might indicate the potential for endocrine disruption by the aforementioned UV filters. However, due to the lack of targeted environmental monitoring, it is unclear if the relative high concentrations required to cause an effect frequently occur in the environment.

The goal of this study is to determine the potential for contamination of six common filters at high-use recreation areas within lakes. Our study sites, lakes in eastern Oklahoma, USA, included bodies of water that are frequented by recreational users and sites relatively removed from direct input to test the localized impact that occurs in a recreational area. This study will provide preliminary information of the occurrence of selected UV filters within the local lake environments in the United States. Environmental concentrations were compared to previously reported effective concentrations for aquatic organisms to investigate the hazard posed to aquatic wildlife.

## Methodology- Environmental Analysis

*Chemicals*

Reagent grade (97%-100% pure) UV protectants were purchased from VWR (Sugar Land, TX). Table 1 provides a complete list with relevant chemical data. A stock solution of each analyte was produced from neat material for analytical purposes. All additional solvents and reagents were pesticide grade or better.

*Sample Locations*

Lake officials and patrons were consulted to identify high population lake areas throughout eastern Oklahoma such as swimming beaches and party coves where the deposition of UV filters would likely be the greatest. Eastern Oklahoma represented an ideal location for testing as it provided a high density of recreational lakes. Popular recreation areas were selected on six lakes (Fort Gibson, Grand, Kaw, Keystone, Oologah, and Skiatook) listed in Figure 1. Data was collected on the recreational features of each site such as boat launches, camp sites and RV spaces from each lake office. Two samples were collected from each lake per sampling run. One sample was collected from designated recreational beach areas and the second sample from an area not designated for swimming that was at approximately 500 m from the recreational area. Sampling occurred three times from July 2012 through October 2012. The first sampling coincided with the 4$^{th}$ of July weekend to represent a period of time where direct deposition of UV filters from swimming and leaching from aquatic foams and structures would be at a maximum. The second set of samples was collected in early September from each location for comparison to a time period with moderate, non-peak activity. The final samples were collected

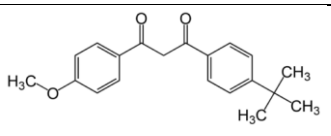
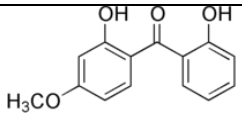
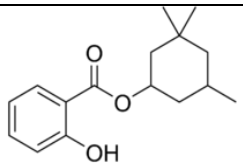
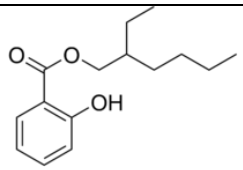
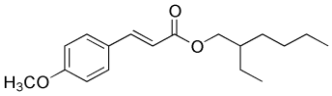
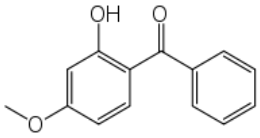
in late October to represent a period of relative inactivity with levels of UV filters at lower levels due to the reduction of direct input from recreational sources.

*Sampling Protocol*

GPS coordinates were recorded upon arriving at the beach sampling sites, and the number of patrons in the water or directly on the beach was recorded prior to sample collection. Accidental sample contamination was minimized by not applying sunscreens or personal care products suspected of containing UV filters. Gloves were also worn prior to opening the sample bottle and remained on until the sample bottles were sealed. Glass bottles were used as collection devices and washed with soap, triple rinsed with deionized water, and acetone rinsed, followed by a final deionized water rinse prior to collection. Beach test areas were visually divided into quadrants. Approximately 250 ml water samples were collected in random locations from each quadrant of depths of at least a meter by submerging an amber 1-liter collection bottle within 5 cm of the water surface. Bottles were filled to capacity to reduce the evaporation of the target chemicals into the headspace of the sampling bottle. Surface water was intentionally obtained to capture non aqueous phase liquids that may have risen to the water surface. Samples were kept on ice at approximately 4° C before returning to the lab for extraction and extraction occurred within 48 h to reduce the risk of degradation and evaporation. GPS coordinates were obtained and used as a reference point for offsite testing.

Offsite distances, approximately 500 meters, were determined by a pedometer program, and GPS coordinate data was collected once the desired distance was achieved. Precautions against accidental sample contamination were employed. Sample sites were selected from the closest accessible location that could realistically be reached and still provide at least a 1 meter water depth. Upon site selection, samples were collected as previously described obtaining water across an approximate 10-meter area.

**Table 1. Relevant chemical data for selected UV filters.**

| Chemical | Structural Formula | CAS Number | Mol. Wt. (g/mol) | Purpose |
|---|---|---|---|---|
| Avobenzone |  | 70356-09-1 | 310.39 | UV Filter |
| Dioxybenzone |  | 131-53-3 | 244.25 | UV Filter |
| Homosalate |  | 118-56-9 | 262.36 | UV Filter |
| Octisalate |  | 118-60-5 | 250.33 | UV Filter |
| Octyl methoxycinnamate (OMCN) |  | 5466-77-3 | 290.40 | UV Filter |
| Oxybenzone (BP-3) |  | 131-57-7 | 228.24 | UV Filter |

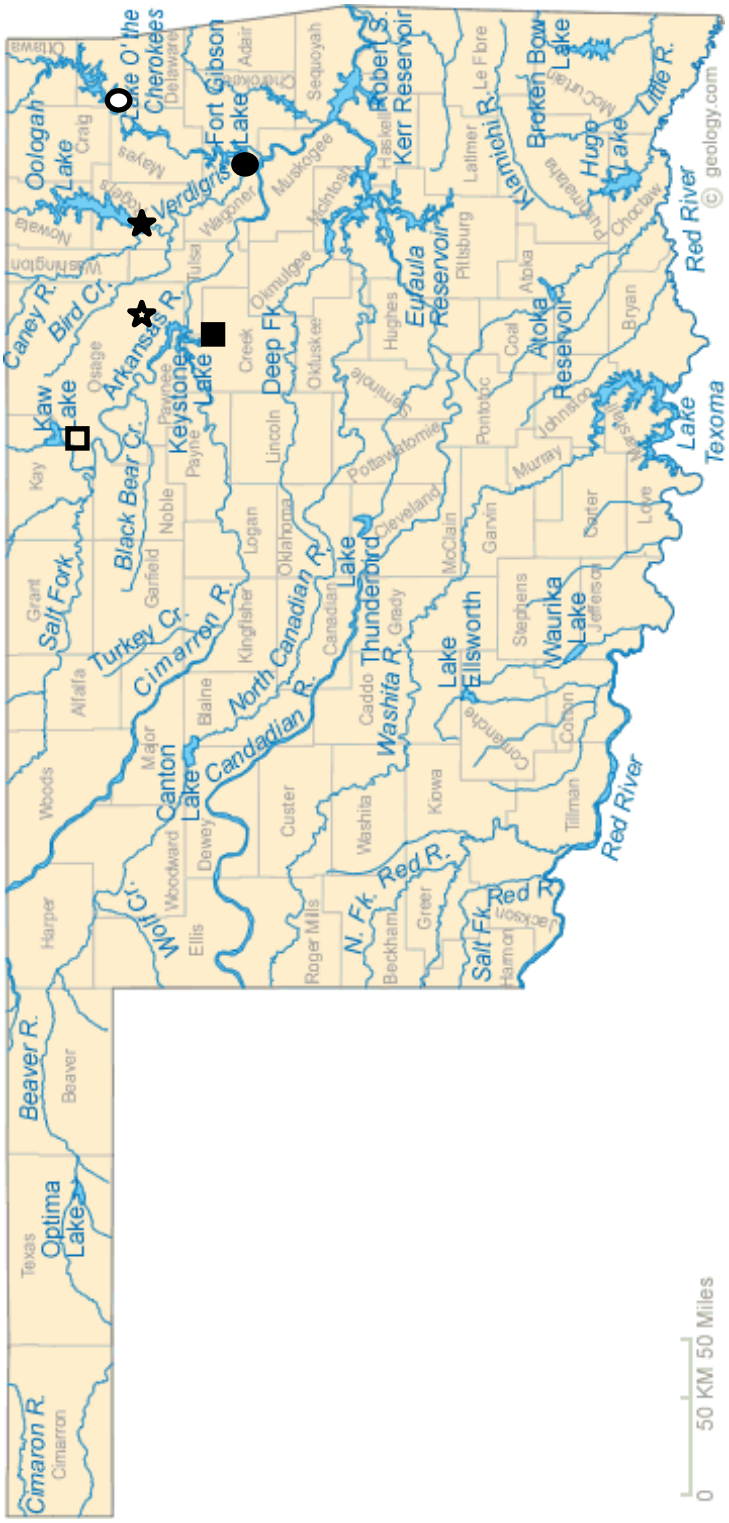



Figure 1. Oklahoma Lake Sampling sites. Fort Gibson Lake, Taylor's Ferry North ● ; Grand Lake, Disney Recreation Area ○ ; Kaw Lake, Sandy Beach □ ; Keystone Lake, Salt Creek Cove ■ ;Oolagah Lake, Hawthorn Bluff ★; Skiatook Lake, Tall Chief Cove ☆

*Extraction Protocol*

Extraction protocols were adapted from the EPA Method 3510C- Separatory Funnel Liquid-Liquid Extraction (EPA, 1996). Lake samples were added to a 2L separatory funnel with 50 µl of the surrogate p-terphenyl. Dichloromethane ($MeCl_2$), 60ml, was added to the sampling bottle to collect any residual target compounds and then emptied into the separatory funnel. The funnel was shaken for 2 minutes and the solvent was allowed to settle to the bottom of the separatory funnel. The solvent layer was carefully drained from the funnel into a 250 ml flask through a Whattman-41 filter containing anhydrous sodium sulfate ($Na_2SO_4$) to absorb any residual water drained from the funnel. The extraction protocol was repeated 2 more times to produce an approximate volume of 200 ml of $MeCl_2$ after rinsing the sodium sulfate and filter into the flask.

Extracts were evaporated to 3-5ml in the TurboVap II using heat (40°C) and a stream of nitrogen. Extracts were quantitatively transferred to 10 ml test tubes using ethyl acetate as a rinse agent, evaporated to near dryness under a stream of nitrogen, (approximately 0.3 ml) and quantitatively transferred to 2-ml GC vials with a resulting final volume of 1ml in ethyl acetate. Internal standards (deuteruated- PAHs at 200 ng each) were added to each sample. Vials were stored in the freezer to prevent degradation of the UV filters prior to analysis in the GC-MS.

Sunscreen products are found in numerous everyday personal care products, clothing, plastics, and instruments. In order to prevent sample contamination from the experimenter, latex gloves were worn in all phases of the analysis process and clothing exposure was kept to a minimum. Personal care products were not applied during elution of samples and all laboratory equipment that might contact the samples were washed with soap, triple rinsed with deionized water, and acetone rinsed, followed by a final deionized water rinse prior to collection.

*GC-MS Analysis*

Analysis was conducted on a 6850 Gas Chromatograph coupled with a 5975C mass spectrometer (Agilent, Palo Alto, CA, USA). Separation of analytes was obtained using a30 m x 0.25 mm HP-5 column (Agilent) and splitless injection. The inlet temperature was set to 280 °C and the column flow was 1.0 ml/min (37 cm/sec average velocity).

Analysis was conducted based on 3-ion SIM (Table 2). Electron ionization was used (70eV) as an ionization source. The source temperature was 230 °C and the quadropoles were 150 °C. Qualitative identity was based on retention time (±0.05m) and ratios of qualitative ions to the quantitative ion being within 20% of expected. Quantitation limits were set at the lowest level we could calibrate accurately and identify qualitative ions and was always greater than three times the method detection limit in water blanks.

**Table 2 GC/MS analyte retention time, target ions, and recovery efficiencies for selected UV filters**.

| Chemical | Retention Time (min) | Quantitation: Qualitative Ions | Spiked Recovery (% and SD) |
|---|---|---|---|
| Octisalate | 6.83 | 120: 138, 250 | 94.5% (+ 19.23%) |
| Homosalate | 7.39 | 138: 109, 262 | 88% (+ 10.71%) |
| Oxybenzone | 8.42 | 227: 151, 228 | 92.5% (+ 3.54%) |
| Dioxybenzone | 9.14 | 227: 121 | 95.75% (+ 3.3%) |
| OMCN | 10.22 | 178: 161, 290 | 93.25% (+ 6.85%) |
| Avobenzone | 12.75 | 310: 295, 309 | 76.5% (+ 8.23%) |

*Quality Control*

Laboratory control spikes were conducted to insure and measure accuracy and precision. Reagent water samples were prepared at a concentration of 500 ng/L (1500 and 30,000 ng/L for

dioxybenzone and avobenzone respectively) and analyzed as described for environmental samples. Surrogate standard p- tertphenyl $D_{10}$ (200 ng/ L) was added to all samples to monitor matrix interference and help indicate the accuracy of the method. Quantitation was performed using internal standards including anthracene $D_{10}$, phenanthrene $D_{10}$, chrysene $D_{10}$, and perylene $D_{10}$ (EPA, 2007).

During each sampling run, 2 field blanks were collected to determine the potential for contamination or sampling errors. Duplicate samples were collected from two random sites for each collection period to determine sample precision. Additionally, spiked samples and water blanks, prepared with deionized water, were used to test method accuracy and determine potential lab contamination.

*Environmental Hazard*

Hazard quotients (HQ) for the each UV filter detected in lake samples was calculated to provide a conservative estimate of hazard. HQs were calculated by dividing the highest measured environmental concentration (MEC) for each UV filter by the LOEC for a sensitive species (*Daphnia magna*). HQs resulting in a score of greater than 1 are considered potentially harmful, HQ's at 1 indicate that the chemical alone is not likely to cause effect, and HQ's below 1 represent that harmful effects are not likely (EPA, 2011).

## Results

*Sampling Locations*

All beach areas were readily accessible to patrons with all of them being closely associated with paved parking areas. As expected, the July sample run had considerably more patrons (21-135) at the time of sampling, followed by the September run (0-52) and finally the

October run in which no patrons were reported. A complete description of each sampling location and the number of patrons are provided in Table 3. No patrons were reported in the Kaw and Skiatook September sample because these locations were closed to the public at the time of sampling.

**Table 3. Lake sampling locations, including area usage description of facilities and number of observed patrons in the water or beach area at the time of sampling.**

| Lake | Site Location (GPS Coordinates) | Area Description | July Patrons | September Patrons | October Patrons |
|---|---|---|---|---|---|
| Fort Gibson | Taylor's Ferry North (35.93714571496316, -95.27791142463684) | 95 RV sites Camping Swim beach Boat launch | 35 | 12 | 0 |
| Grand | Disney Recreation Area (36.476549823075885, -95.0257408618927 | 80 RV sites Camping Swim beach Boat launch | 21 | 6 | 0 |
| Kaw | Sandy Beach (36.70015001943481, -96.90728902816772) | Swim beach Party cove | 135 | 0 | 0 |
| Keystone | Salt Creek Cove (36.13263678658701, -96.32594704627991) | 125 RV sites, Camping Swim beach Boat launch | 47 | 30 | 0 |
| Oolagah | Hawthorn Bluff (36.42678160247689, -95.67936301231384) | 56 RV sites Camping Swim beach Boat launch | 74 | 52 | 0 |
| Skiatook | Tall Chief Cove (36.32460813055351, -96.11306548118591) | Camping Swimbeach Boat launch | 58 | 0 | 0 |

*July samples*

Avobenzone and dioxybenzone were not detected at any of the sample locations in July. OMCN was only identified above detection limits at 2 beach areas (Kaw and Oologah) at 37.94 and 176.45 ng/L. Octisalate and homosalate were found in all July sampling locations. The mean concentration of octisalate was 301 ng/L at beach locations and 130 ng/L in offsite locations.

Homosalate mean concentrations were 537 ng/L at beach sites and 136 ng/L at offsite locations. Oxybenzone was recovered in all sample locations with the exception of the Kaw beach offsite location in mean concentration of 605 ng/L at beach locations and 57 ng/L in offsite locations. UV filter concentrations varied widely between testing sites. A summary of the detected concentrations of UV filters during the July sampling run is provided in Figure 2. Figures 5-8 describe the concentration ranges found for the most commonly detected UV protectants.

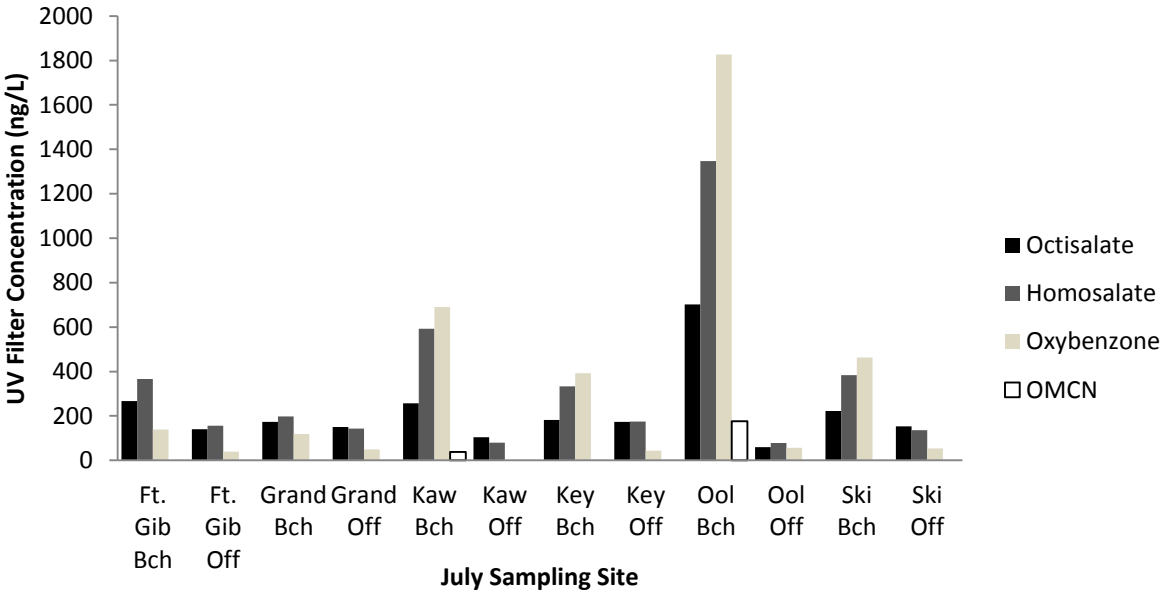



Figure 2. Concentrations of UV filter detected at sampling site locations in July 2012. Avobenzone and dioxybenzone were not found in concentrations above reporting limits.

*September Samples*

Avobenzone and dioxybenzone were not detected above the current study's reporting limits at any of the sample locations during the September run. Octisalate and homosalate were found in all locations except the Grand offsite. Octisalte mean concentrations of 154 ng/L at beach locations and 84 ng/L in offsite locations were recorded. Homosalate occurred at mean concentrations of 218 ng/L at beach sites and 72 ng/L at offsite locations. Oxybenzone was

recovered in only 1 offsite location (Keystone) at 44 ng/L and 4 beach locations a mean concentration of 200 ng/L. OMCN was only recovered from the Oologah beach location at 42.8 ng/L and the Skiatook offsite location at 36.2 ng/ L. A summary of the detected concentrations of UV filters during the September sampling run is provided in Figure 3.

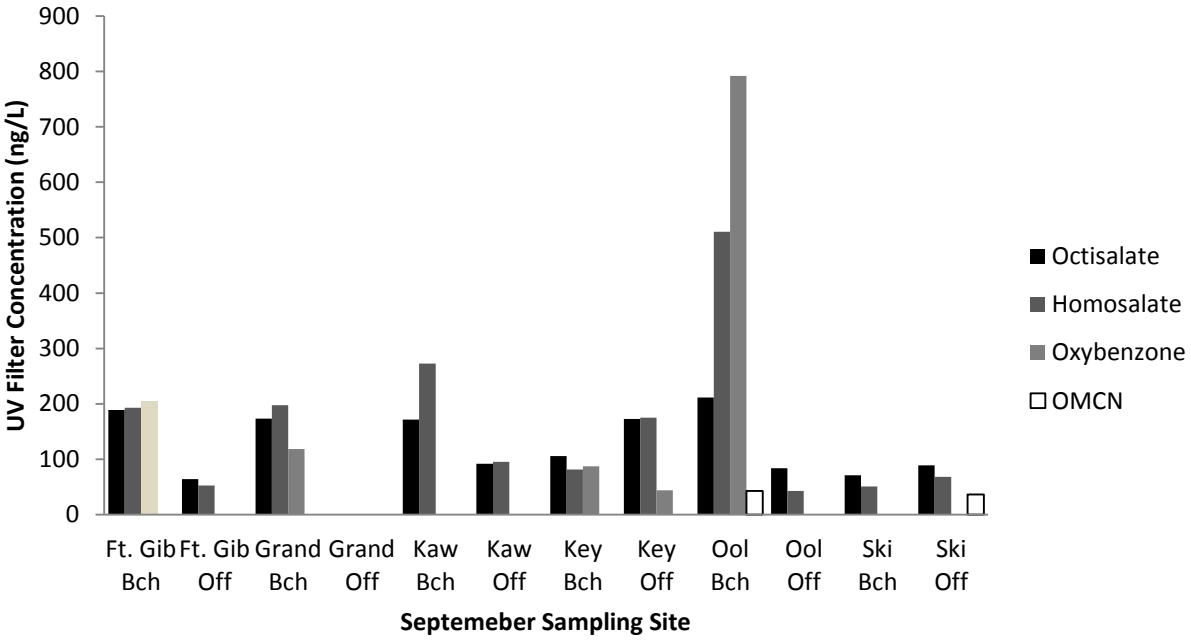



Figure 3. Concentrations of UV filter detected at sampling site locations in September 2012. Avobenzone and dioxybenzone were not found in concentrations above reporting limits.

*October Samples*

Avobenzone, dioxybenzone, homosalate, and OMCN were not detected above reporting limits at any of the sample locations during the October run. Octisalate was identified at 3 beach locations (Fort Gibson, Kaw and Skiatook) at an average concentration of 49 ng/L; it was not detected at any offsite location. Homosalate was detected at one offsite location (Kaw at 44 ng/L) and 5 beach locations averaging 114 ng/L. A summary of the detected concentrations of UV filters during the October sampling run is provided in Figure 4.

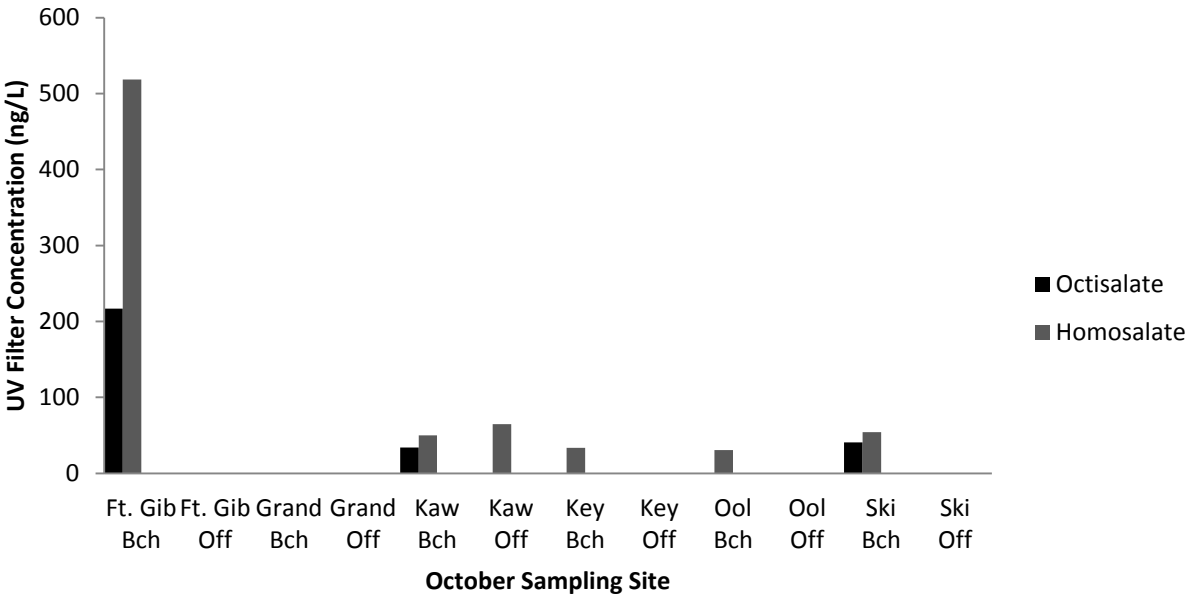



Figure 4. Concentrations of UV filter detected at sampling site locations in October 2012. Avobenzone, dioxybenzone, OMCN, and oxybenzone were not found in concentrations above reporting limits.

*Usage effects*

Significant differences in UV filter concentrations were noted for homosalate, octisalate, and oxybenzone in beach versus offsite locations and in sampling months using ANOVA ($p \leq 0.05$). OMCN could not be properly analyzed due to the low number of samples containing detectable levels. Box and whisker plots provided in Figure 5- 8 show the seasonal relationship between each detected UV filter in beach and offsite locations. The number of patrons (Table 3) in or around the beach areas for each sample was compared with the beach concentration of the three UV filters showing significant differences (Figure 9-11). A significant positive correlation was found between the number of patrons at a beach area and the concentration of UV filter for homosalate ($r = 0.65$, $p = 0.003$), octisalate ($r = 0.60$, $p = 0.008$), and oxybenzone ($r = 0.70$, $p = 0.001$) using Pearson's product moment correlation at $p \leq 0.05$. A regression analysis was performed on the data and coefficient of variation ($r^2$) values were calculated for each analyte to determine the amount of effect that could be associated between the variables.

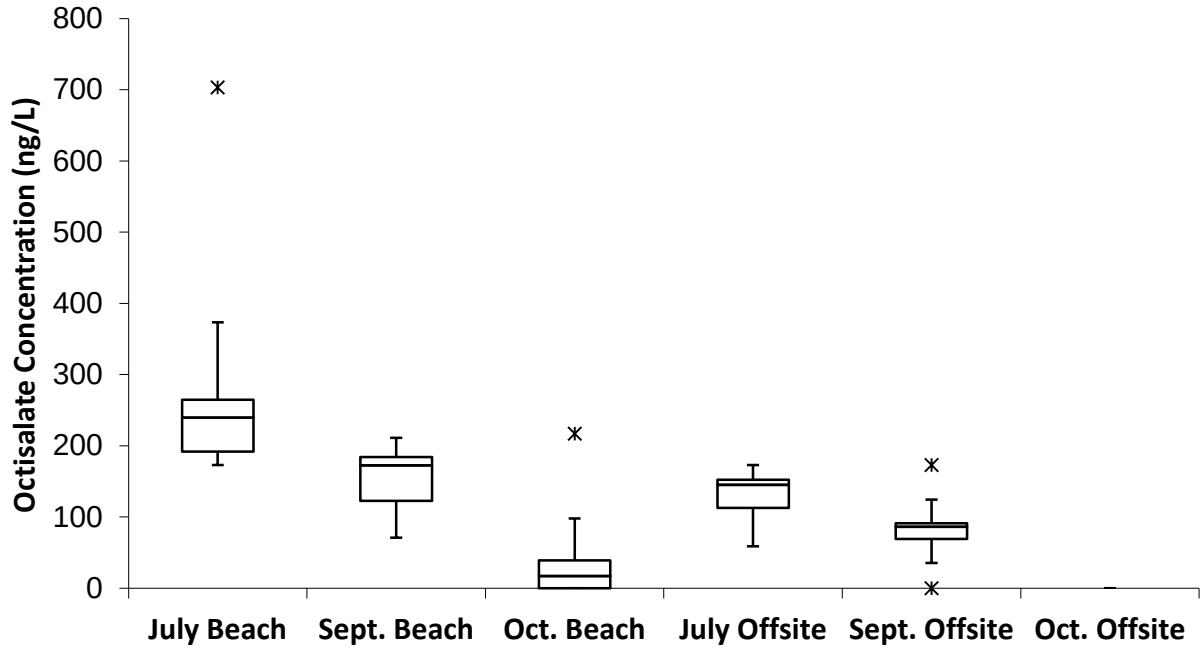



Figure 5 Box and whisker plots for octisalate for beach and offsite location in high use, moderate use and low/no use time. The box represents the 50th percentile of the data, while the upper and lower whiskers represent the upper and lower quartile. The horizontal line in the box represents the median and the asterisks represent outliers (1.5x above the upper or lower quartile).

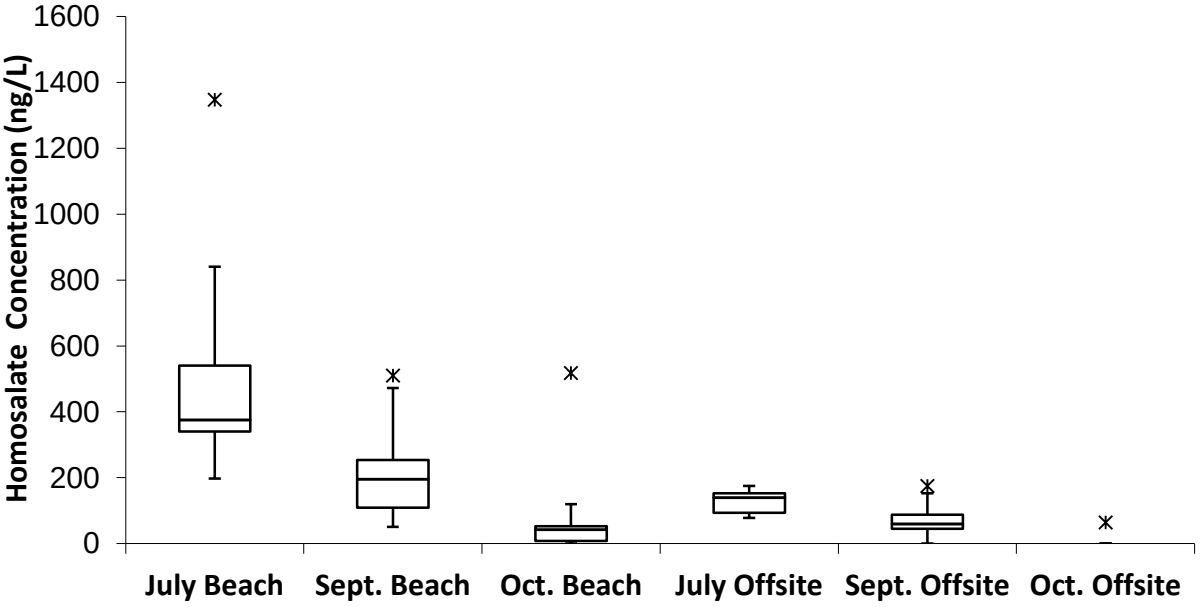



Figure 6 Box and whisker plots for homosalate for beach and offsite location in high use, moderate use and low/no use time. The box represents the 50th percentile of the data, while the upper and lower whiskers represent the upper and lower quartile. The horizontal line in the box represents the median and the asterisks represent outliers (1.5x above the upper or lower quartile).

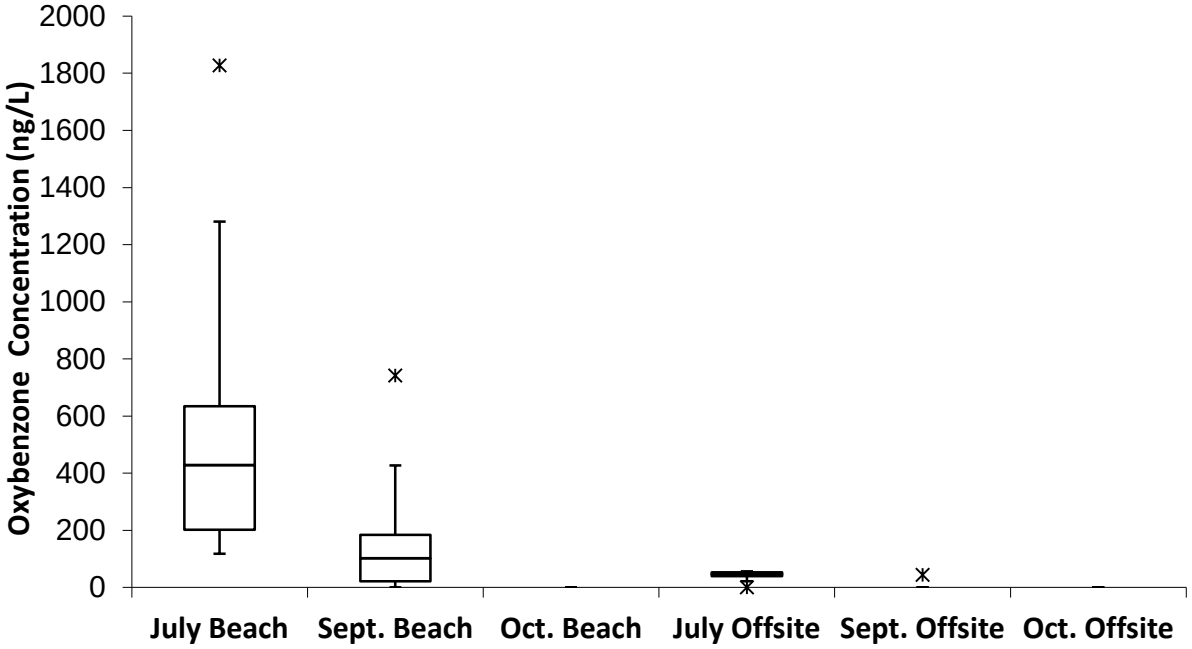



Figure 7. Box and whisker plots for oxybenzone for beach and offsite location in high use, moderate use and low/no use time. The box represents the 50th percentile of the data, while the upper and lower whiskers represent the upper and lower quartile. The horizontal line in the box represents the median and the asterisks represent outliers (1.5x above the upper or lower quartile).


Figure 8. Box and whisker plots for OMCN for beach and offsite location in high use, moderate use and low/no use time. The box represents the 50th percentile of the data, while the upper and lower whiskers represent the upper and lower quartile. The horizontal line in the box represents the median and the asterisks represent outliers (1.5x above the upper or lower quartile).

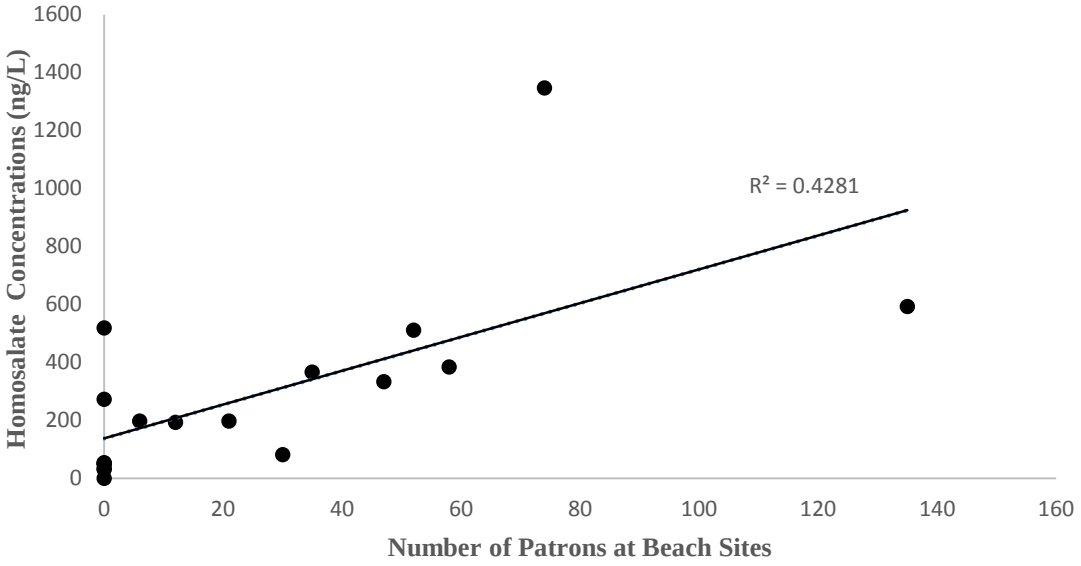



Figure 9. The concentration (ng/L) of homosalate compared to the number of patrons in the water or on the beach area during July, September, and October 2012 sampling. A simple linear regression line is included. $r^2$ is a measure of the coefficient of determination.

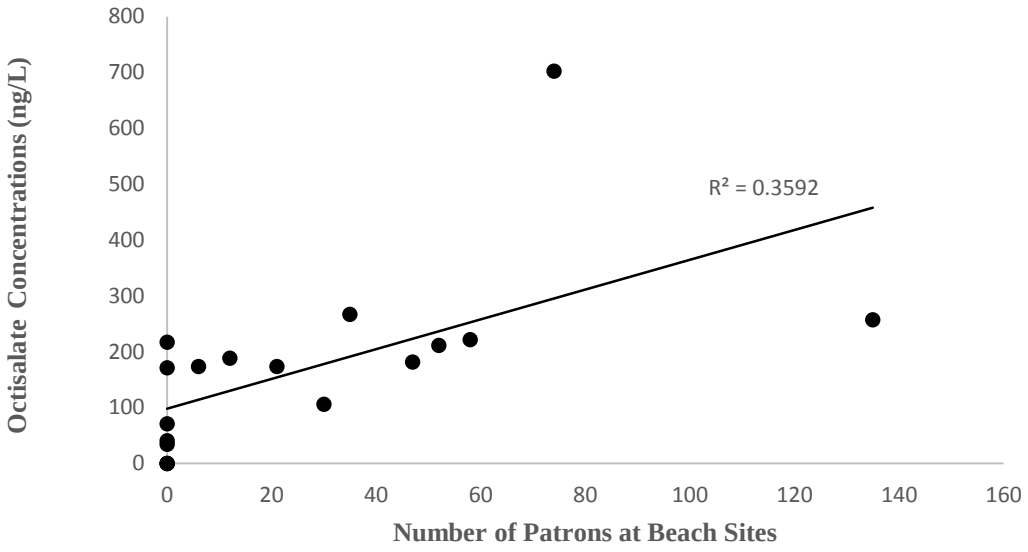



Figure 10. The concentration (ng/L) of octisalate compared to the number of patrons in the water or on the beach area during July, September, and October 2012 sampling. A simple linear regression line is included. $r^2$ is a measure of the coefficient of determination.

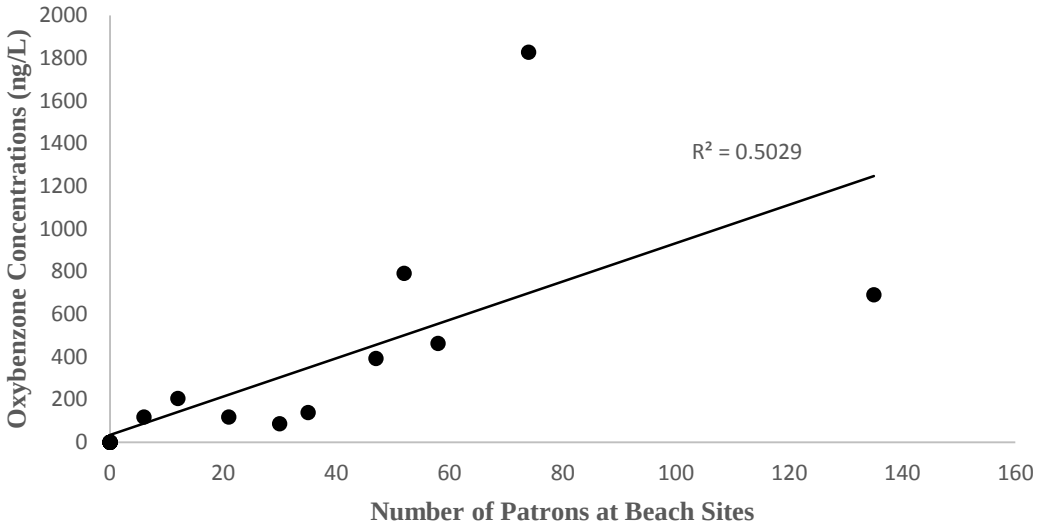



Figure 11. The concentration (ng/L) of oxybenzone compared to the number of patrons in the water or on the beach area during July, September, and October 2012 sampling. A simple linear regression line is included. $r^2$ is a measure of the coefficient of determination.

*Environmental risk calculation.*

Hazard quotients were calculated for each UV filter from the MEC for any location in this study with the exception of dioxybenzone and avobenzone, which were not identified in any location. UV filter HQ values ranged between 0.004 and 0.006; complete data for HQ calculations are provided in Table 4.

**Table 4. The HQ for each UV filter calculated from the MEC (µg/L) from the current study and LOEC (µg/L) of UV Filters on *D. magna* from previous studies.**

| UV filter | MEC | LOEC | HQ |
|---|---|---|---|
| Homosalate | 1.3[b] | 300[b] | 0.0043 |
| OMCN | 0.18[b] | 30[b] | 0.0060 |
| Octisalate | 0.70[b] | 110[b] | 0.0064 |
| Oxybenzone | 1.8[b] | 500[a] | 0.0036 |

a = (Bratkovics, 2011) b= Layton, 2015

**Discussion**

Avobenzone and dioxybenzone were not detected in any samples regardless of the timing of the sampling run. The lack of detection is not unexpected for dioxybenzone after a considerable review of local sunscreen formulations found that dioxybenzone was absent in all products examined. Its absence from products makes it unlikely to be found in the local environment. The absence of dioxybenzone from product formations is likely because of its difficulty in solubilizing (Reisch, 2005). Similar studies of coastal waters in South Carolina and Korean freshwater samples did not detect the presence of dioxybenzone in any of their sampling sites (Jeon, 2006 and Bratkovics, 2011). In addition, the absence of avobenzone could be a result of higher quantitation limits. Avobenzone was more difficult to quantify with the GC/MS and had a 30x higher detection limit. However, the quantitatition limit of 300 ng/L is still over 13-fold below reported LOEC values (Layton, 2015).

Of the UV filters detected, OMCN tended to be least frequently detected as it was identified in only 4 of all of the locations and times tested. It occurred at an average of 31 ng/L, which also corresponded to the highest number of patrons in all but one instance. However, since it was only detected in 3 beach sites and 1 offsite, no meaningful statistical relationship could be determined. Similar levels of OMCN were reported at Norwegian coastal areas (Langford, 2008). OMCN was detected in only one offsite location (Skiatook/September run). OMCN levels in the Skiatook offsite could have been elevated because of the presence of a boat ramp which may have increased the chemical levels from patrons' recreational activities; patrons may have been more inclined to be in the boat ramp area because the Skiatook beach area was closed by the September run due to low water levels. Lower levels of OMCN in all locations may be attributed to its ability to accumulate in sediment and resident organisms. A study of Swiss aquatic ecosystems identified OMCN in levels between 49.2 to 172.5 ng/g in all animal tropic levels tested which

included aquatic invertebrates and fish, as well as the least concentrated UV filter detected in water samples (Fent, 2010).

Oxybenzone was regularly detected in beach locations when patrons were present and absent when patrons were absent. It is difficult to attribute the same pattern to offsite locations because there are different levels of opportunities for usage via boat ramps and swimming among the offsite locations tested. For instance, the offsite location for Kaw was relatively inaccessible to patrons because it was down a steep ledge to the lake and as such there was no oxybenzone detected, whereas the Skiatook site had a boat ramp which patrons would more regularly frequent. Oxybenzone showed considerable seasonal change as it was not detected in any sample in the October run while some of the UV filters persisted. The lack of its detected presence at locations without patrons and its absence in the final run suggests it poorly persists in water for extended durations. Oxybenzone (38.99 - 1826.96 ng/L) occurred in similar levels to that detected in Slovinian rivers and lakes between 32 and 114 ng/L (Cudderman, 2007), at 10 and 2013 ng/L on South Carolina beaches (Bratkovics, 2011), and Swiss lakes at 2 and 125 ng/L; similarly, oxybenzone was shown to degrade seasonally from high to low use times (Poiger, 2004).

Octisalate and Homosalate were the most consistently detected UV filters in the sites sampled. Octisalate occurred in all but 1 sampling site in the July and September at a mean of 167 ng/L and occurred in 3 beach locations in October at a mean concentration of 49 ng/L., Few studies have looked for octisalate in environmental samples. Homosalate occurred in all but 1 sampling site in the July and September at a mean of 239 ng/L and occurred in six sampling locations in October at a mean of 67 ng/L. Detected levels of homosalate, with the exception of the October Ft. Gibson beach sample, seem to be consistent with a previous environmental study of Slovinian rivers between 165 and 345 ng/L (Cudderman, 2007). The October Ft. Gibson beach

sample showed the third highest concentration of homoslate (518 ng/L) and octisalate (217 ng/L) in all samples tested. The reason for this seemingly elevated level of these UV filters is unknown and may have been from and isolated event.

In general, the UV filters dissipated from the July to the October run which corresponded with the high to low use time. The seasonal decrease in UV filter concentration is consistent with previous studies of Norwegian (Langford, 2008) and Japanese (Sandoka, 2015) coastlines. The amount of UV filter detected decreased by 50-60% for the 4 detected UV filters between the July and September run. OMCN and Oxybenzone were not detected in the October run, but the octisalate and homosalate were still present at approximately 30% of recorded levels in the July run. As expected, sampling sites tended to have higher UV filter concentration with increases in the number of patrons. This supports that recreational activities are a significant source of UV filter deposition into local lake environments. The most obvious exception to this general pattern was at the Oologah sampling site which produced, by far, the highest recorded UV filter concentrations for octisalate, homosalate, and oxybenzone in both the July and September runs despite having nearly half the number of recorded patrons as the Kaw site in July. This discrepancy could be general error in making a count at one isolated time; perhaps collecting daily use data on the day of sampling could provide a more accurate count. The Oologah sampling site has the potential to have more prolonged UV filter deposition because of the higher number of recreational features such as RV and camping sites than the Kaw location. The most likely explanation of this phenomenon is the physical geography of the Oologah sampling site. The swimming beach is formed in a narrow inlet with a smaller surface area exposed to the body of the lake which may reduce the dissipation of the UV filters into waters adjacent to the swimming area. Additionally, there is a buoy that separates the swimming beach from the main lake which may reduce UV filter exchange.

The Grand beach offsite in the September run produced no detectable levels of any of the UV filters despite the corresponding beach area having significant levels of analytes. It is conceivable that the UV filters were diluted below detection limits, but that does not fit the general pattern of reduction observed in most other samples.

UV filter HQ values ranged between 0.0036 and 0.0064. This data can be combined with other physical and environmental factors to aid in a complete risk assessment of UV filters to resident aquatic populations. The preliminary HQ data seems to suggest that the UV filters concentrations detected in high use lake areas in not likely to pose a risk to resident populations since all values were below 1. Although similar studies used a different calculation technique adopted from the European Chemicals Bureau, oxybenzone and OMCN were found to pose no to low significant risk or risk could not be ruled out in most test locations (Fent, 2010 and Rodriguez, 2015). Another study identified a medium to high risk for oxybenzone and OMCN using a different evaluation method combined with probalistics (Tsui, 2014). Despite higher MEC for oxybenzone and OMCN in the aforementioned study, they alone cannot account for the difference in the perception of risk in the previous studies and are most likely to be differences in the methods of calculating the HQs. One of the main features of these alternate calculation methods is to include a safety factor of 100 for NOEC from chronic data and a safety factor or 1000 for NOEC acute data. The current study's HQs were calculated from chronic concentrations and even with a safety factor of 100, still do not exceed a HQ of 1 with the exception of avobenzone. In addition, HQ were calculated at worst case scenarios and given the significant decrease in UV filter concentrations due to seasonal and dilution effects, risk is even less likely for prolonged periods of time. The HQ values for homosalate, and octisalate are the first to be reported to our knowledge. Similar and more in depth studies are needed to confirm the HQ findings and complete a complete risk assessment of UV filters to aquatic invertebrates.

## Conclusion

The current study identified 4 common UV filters in ranges between 30 and 1827 ng/L in six lakes US lakes. Homosalate, octisalate and oxybenzone were consistently present in the majority of sampling sites. Concentrations of UV filters were higher in high-use recreation areas and dissipated in offsite test sites most likely due to dilution and sorption. A seasonal relationship was demonstrated as concentrations almost uniformly declined in the low or no use test times as expected. In pairing our previous unpublished UV filter toxicological data with the environmental concentrations detected in this study, it appears unlikely that UV filters, even in the areas of highest concentration, are occurring at environmentally relevant concentrations to *D. magna.* Future studies should focus on determining the environmental levels of previously unidentified UV filters that are used in sunscreen formulations to help further understand their ambient levels and potential consequences to resident aquatic populations.

# CHAPTER IV

## ENGAGING UNDERGRADUATES IN THE SCIENTIFIC PROCESS: EXPLORING INVERTEBRATE ENDOCRINE DISRUPTION

**Preface:** This chapter has been accepted for publication in The American Biology Teacher as Engaging Undergraduates in the Scientific Process: Exploring Invertebrate Endocrine Disruption on June 17th, 2015 for publication in spring 2016.


**Abstract**

Engaging students in the process of science to increase learning and critical thinking has become a key emphasis in undergraduate education. Introducing environmental topics, such as the effects of endocrine disrupting chemicals, into undergraduate courses offers a new means to increase student engagement. *Daphnia magna* can serve as a model organism for endocrine disruption, and its ease of handling, rapid reproduction rate, and clearly defined endpoints make them useful in short-term, student research projects. The concept of endocrine disruption can be tested through a 21-day reproductive study of *D. magna* exposed to varying concentrations of the pesticide fenoxycarb. Students will observe an altered reproduction rate and increased production of male offspring. This research system allows students to formulate hypotheses, set up experiments, analyze data, and present results leading to a greater appreciation and interest in science.

**Key Words**

Endocrine disruption, *Daphnia magna*, Inquiry-based learning, fenoxycarb, undergraduate research

**Why study endocrine disruption?**

Endocrine disrupting chemicals (EDCs) can inhibit or promote the expression of hormonal actions by interacting with hormone receptors, binding to the hormone itself, or altering other portions of hormone-signaling pathways. Several studies identified the potential for endocrine disruption from naturally occurring compounds in the environment, but the idea that anthropogenic compounds could act as EDCs gained national attention from a position statement produced by a group of concerned scientists known as the "Wingspread" Statement (Bern, 1992). Since then, EDCs have been well documented and shown to affect a wide range of organisms, such as aquatic invertebrates, insects, fish, amphibians, and humans in concentrations much lower than lethally toxic levels (Rodriguez, 2007). These chemicals are primarily released into the water supply by wastewater discharge and direct deposition such as recreation and agricultural runoff. The majority of studies pertaining to potential EDCs have been focused on vertebrate models, presumably due to their higher degree of correlation with humans. However, recent understanding of crustacean endocrinology and the realization that these organisms may serve as model organisms to identify EDCs have prompted an increase in using crustacean models such as *Daphnia magna* (Tatarazako, 2003; deFur, 2004; and Lampert, 2006). The potential for environmental contaminants to cause endocrine disruption frequently causes widespread public interest, for example bisphenol *a* in water bottles, suggesting that the topic will be exciting to students.

**Why use *Daphnia?***

*D. magna* is an aquatic crustacean species of critical importance to aquatic ecosystems because it serves as a primary consumer of algae which impacts water quality and is a major constituent in aquatic food chains. *Daphnia* reproduce primarily by cyclic parthenogenesis, an asexual process, whereby the females of the species produce varying numbers of female offspring under normal environmental conditions. These parthenogenic females are diploid clones of the mother. Environmental stress such as colder temperatures, decreased photoperiods, food scarcity, and crowding can stimulate the production of males capable of sexual reproduction (Hobaek, 1990). Males are able to fertilize special haploid eggs to produce specialized structures called epiphia. Epiphia, or resting eggs, are encased in a protective layer that allows them to survive in the environment presumably to allow the species to survive in winter or drying conditions. When environmental conditions return to normal, a female emerges from the epiphium. Males and females can be distinguished by a variety of phenotypic traits. Specifically, *Daphnia* females tend to be larger with short first antennae length and a pointed rostrum as noted in Figure 1. The onset of poor environmental conditions stimulates the release of a hormone that brings about the production of males. Since the discovery of methyl farnesoate as the hormone responsible for the production of males, it has been identified in over 30 crustacean groups including *Daphnia*, although the specific receptor responsible for its binding has not been determined (LeBlanc, 2007). Methyl farnesoate is a terpenoid chemically related to the insect hormone, juvenile hormone III (JHIII) (Laufer, 1992b). Studies have identified several anthropogenic chemical analogues of methyl farnesoate such as the insecticides kinoprene, fenoxycarb, and pyriproxyfen that can induce the production of males (Haeba, 2008). Fenoxycarb in particular has demonstrated the potential to produce males in minute concentration in the low µg/L range (Oda, 2007). *D. magna* has been adopted by several international organizations,

**Figure 1. Sex Determination by Length of First Antennae in *Daphnia magna* (Tatarazako, 2004)**


**Male** **Female**

including the Organization of Economic Co-Operation and Development (OECD), as a model organism for aquatic toxicity testing. Its high reproduction rate, inexpensiveness, ease of handling, sensitivity, and multiple clearly defined endpoints make it a good model organism. As part of this project, a *Daphnia* culture was maintained in a college teaching laboratory with low input of supplies or time.

Endpoints used to assess endocrine disruption have typically included reproductive rate, larval development, size, age of first reproduction, and molting abnormalities. One more recent endpoint that has gained interest in the determination of a chemical's ability to mimic methyl farnesoate is sex ratio (Tatarazako, 2007). This simple endpoint can determine methyl farnesoate agonists that mimic the natural hormonal effects, leading to increased male production under environmental conditions favorable to female production. One proposed mechanism of action is that the agonist, along with the natural hormone, binds to the cellular receptors that lead to signal transduction and the eventual cellular effects leading to male development (Laufer, 1992a).

## Goals and Objectives

EDCs have become a current and substantial environmental concern that should be included in undergraduate laboratory studies in classes such as biology, environmental science, physiology, or undergraduate research courses. Further, there has been a call to include research in undergraduate courses to provide students with authentic experience, increase critical thinking, and promote lasting learning (AAAS, 2011). Therefore, we suggest a laboratory exercise to demonstrate and learn the concept of endocrine disruption that alleviates the majority of inherent concerns by using the invertebrate model organism *D. magna.* The objective of the experiment the students conduct is to determine if endocrine disruption is occurring following exposure to a pesticide. Through the proposed activities, the goal for the instructor is to:

1. Engage students in the process of science which includes hypothesis formation, data collection, and analysis of results.

2. Expose students to endocrinology and illustrate the complex changes that can occur if the endocrine system is disrupted.

3. Aid students in interpreting the meaning of data using simple forms of statistical analysis such as mean, standard deviation, and t-tests.

## Teacher Laboratory Preparation

### *Chemicals and Solutions*

Fenoxycarb (CAS number 72490-01-8) powder, purchased from VWR (Sugar Land, TX), was used to produce stock solutions dissolved in acetone. Neat material was used to produce a primary stock of 1 million µg/ L, followed by a secondary stock diluted to 25, 000 µg/ L, a

concentration suitable for the production test waters. All stock solutions were stored in the freezer to help conserve the reported concentration.

*Daphnia*

*Daphnia*, purchased from Aquatic Biosystems (Fort Collins, CO), were reared in glass covered 1 L glass jars containing dechlorinated tap water produced by filtering tap water through a charcoal filter followed by 24 hours of bubbling using a common fish tank pump. Charcoal filters sold for drinking water are adequate, or bottled spring water could be used for ease. Environmental conditions favorable for the production of females included temperatures between 20-24C, pH between 6 and 9, 16/8 hour light to dark cycle, and low population density, between 40-50 *Daphnia* per jar. Cultures were fed daily with the 2 ml of the algae *Selenastrum capricornutum* ($3.7 \times 10^{7}$ cells/ ml) and 1 ml of yeast- trout chow- YTC three times a week; both purchased from Aquatic BioSystems (Fort Collins, CO). Alternately, both *Daphnia* and appropriate food sources such as *Spirulina* can be purchased individually or as a kit from common biological supply companies such as Carolina (Burlington, NC). Cultures were refreshed weekly by pouring off three-quarters of the culture and refilling to appropriate levels with fresh culture water. Cultures were reared for several weeks to ensure proper acclimation to the environmental parameters. Prior to the beginning of the experiment, several adult *Daphnia* were isolated into a separate jar to obtain enough neonates less than 24 hours old needed to initiate the experiment. *Daphnia* were transferred between jars using a large bore pipette (10 ml) connected to a fast release pump to minimize the risk of physical damage.

**Student Introductory Activities**

Prior to the beginning of the investigation, students were provided with the necessary background activities to increase the effectiveness of the investigation; these activities included a

review of the scientific process, required readings in primary peer-reviewed literature, and research ethics. To begin the investigation, students were given an introduction to fenoxycarb and the OECD test 211 Daphnia Reproduction Test and asked to brainstorm the background information needed to conduct an appropriate study concerning the potential for fenoxycarb to produce toxicological effects on *Daphnia* populations. The ensuing discussion and instructor guidance resulted in the following student research background topics: *Daphnia* life cycle and ecology, test parameters, previous studies of fenoxycarb, chemical nature of fenoxycarb, endocrine disruption, and *Daphnia* as a model organism. Small groups of students were assigned to research each component, which was followed by an informal presentation of that research to the class.

*Daphnia* from reproducing jars were used to practice transferring *Daphnia* via pipette to petri dishes for observation of the length of the female first antennae using a stereomicroscope. A second period of sex determination was conducted on ethanol euthanized *Daphnia* that were previously exposed to fenoxycarb producing a mix of males and females to observe.

**Student Laboratory Methods**

*Initial Set-up*

Experiments were adapted from the OECD test guideline 211- *Daphnia* 21-day reproduction test (OECD, 2004). The secondary stock was used to produce test waters in 1 L volumetric flasks in concentrations of 2 µg/L, 1 µg/L, 0.5 µg/L, and 0.25 µg/L fenoxycarb as described in Table 1. Modifications of transfer amounts may need to be adjusted based on primary stock concentrations. Previous studies have shown these concentrations to produce a range of effects on reproductive rates, day of first reproduction, and male/female sex ratios in *D.*

*magna* (Oda, 2007; Layton, 2012 unpublished). For each concentration, 100 ml (± 5ml) of test solution was dispensed into 10 separate glass containers, followed by 0.2 ml of *S. capricornutum* ($3.7 \times 10^7$ cells/ ml) and 0.1 ml of YTC. One *D. magna*, less than 24 hours old, was transferred to each container via pipetting. An acetone solvent control was set up under the same conditions; afterward, all jars were covered with glass.

**Table 1. Instructions for Producing Fenoxycarb Test Water Concentrations**

| Secondary Stock Concentration (c1) | Transfer volume (v1) | Final Volume (v2) | Final Water Concentration (c2) |
|---|---|---|---|
| 25, 000 µg/ L | 80 µL | 1L (1,000,000 µL) | 2 µg/ L |
| 25, 000 µg/ L | 40 µL | 1L (1,000,000 µL) | 1 µg/ L |
| 25, 000 µg/ L | 20 µL | 1L (1,000,000 µL) | 0.5 µg/ L |
| 25, 000 µg/ L | 10 µL | 1L (1,000,000 µL) | 0.25 µg/ L |

*Data Collection & Experimental Maintenance*

Daily observations of mortality, irregularities in behavior, and number of offspring were documented on record sheets modified from the OECD as seen in Table 2. Offspring were counted and euthanized for later sex determination by pipetting the *Daphnia* into vials containing methanol. Each container was fed *S. capricornutum* 5 times a week and YTC 3 times a week corresponding to water changes when appropriate. Water changes are meant to maintain the target concentration of fenoxycarb at reported levels and should be conducted at least 2 to 3 times a week. The parameters of dissolved oxygen concentrations (no less than 3mg/L), pH (between 6 and 9), and temperature (between 20-24C) were checked weekly in 1 test jar just before and 1 jar just after a water change for quality control.

Euthanized *Daphnia* sex determination was made by stereomicroscope observation. Daphnia were pipetted into drops on a petri dish and manipulated with dissection probes to maneuver them onto their sides. Males were identified by the presence of a prominent first antennae located just beneath the beak-like projection or rostrum as illustrated in Figure 1.

**Endpoint and Statistical Analysis**

The mean and standard deviation of the number of offspring and the number of days until the first reproduction were calculated for each control and treatment concentration; T-tests were used to determine if the number of offspring in each fenoxycarb concentration differed significantly from the control. Percentages of the number of males and females were calculated per concentration and compared to the control; the appearance of any males under the test conditions indicates potential endocrine disruption. The upper fenoxycarb concentrations in this lab produced around 90% males, 10% males in the lower fenoxycarb concentration, and 0% males in the control. Several forms of graphical data can be generated from the endpoint statistics such as histograms or dose response curves.

## Outcomes of Instructional Activity

This research project has been successfully implemented into an introductory college course and has shown substantial promise as a learning system for the scientific process, endocrine disruption, and statistical data interpretation. A group of 10 freshmen at a Kansas community college, with no formal research training or prior knowledge of *Daphnia* life cycles or male to female determination were enrolled in a research

**Table 2. *Daphnia* Reproduction and Parent Mortality Data Sheet**

Experiment Number: Date Started: Medium: Type of Food: Test Chemical: Concentration:

| Day | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Medium Renewal (check) | | | | | | | | | | | | | | | | | | | | | | | | |
| Food provided (check) | | | | | | | | | | | | | | | | | | | | | | | | |
| pH* | | | | | | | | | | | | | | | | | | | | | | | New | |
| | | | | | | | | | | | | | | | | | | | | | | | Old | |
| O2 (mg/l)* | | | | | | | | | | | | | | | | | | | | | | | New | |
| | | | | | | | | | | | | | | | | | | | | | | | Old | |
| Temp (°C)* | | | | | | | | | | | | | | | | | | | | | | | New | |
| | | | | | | | | | | | | | | | | | | | | | | | Old | |
| Number of live offspring ** | | | | | | | | | | | | | | | | | | | | | | | | Total |
| Jar 1 | | | | | | | | | | | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | | | | | | | | | | | |
| 3 | | | | | | | | | | | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | | | | | | Total | |

* Pick one new and one old jar a week to check pH, oxygen, and temperature. Record the reading in the appropriate cell.

** Record any adult mortality with an M in the appropriate cell.

methods class as part of a scholarship requirement or on a volunteer basis. The self-reported student majors included nursing, chemistry, dental hygiene, elementary education, biology, and neuroscience. Students were evaluated throughout the process for the quality of initial research, introductory activities, completion of methods, *Daphnia* sex determination, selection of appropriate statistical measures, and making relevant conclusions. The final project required students to produce a written paper in the form of a peer reviewed journal and give a group oral presentation. Students were assessed using the rubric in Figure 2. After the conclusion of the lab procedures, 9 of the 10 students were adept at transferring, experimental set-up, and sex determination of *Daphnia*. The one student not demonstrating adequate skills in the lab was most likely related to poor attendance as opposed to the difficulty of the lab procedures. Students demonstrated the ability to research acceptable sources, formulate hypotheses, reach valid conclusions, and present their findings in both written and oral forms; 90% of the students earned an A (60%) or B (30%) for the project as a whole. The successful implementation and completion of the project given the students' lack of background knowledge and diverse majors indicate the lab can serve as a meaningful exercise to teach research based skills and principles of endocrine disruption to a diverse set of students. Informal discussions with students and high levels of participation and commitment outside of class hours suggest the project promoted interest with the students.

**Figure 2. *Daphnia*/Fenoxycarb Endocrine Disruption Rubric**

| Project Component | Incomplete 0 points | Poor 4 points | Marginal 6 points | Good 8 points | Excellent 10 points | Grade |
|---|---|---|---|---|---|---|
| **Research** | No research or research without references | Research not complete and not thorough or inaccurate | Research mostly complete but not thorough or some inaccuracies | All research complete but not thorough or few inaccuracies | All research complete, thorough, and accurate | |
| **Background Activities/ Readings** | No background activities or preparation | Few background activities complete and preparation for activities not evident | Most background activities complete but preparation for activities was not always evident | All background activities complete but preparation for activities was not always evident | All background activities complete on time with significant evidence of preparation | |
| **Project Goals including hypothesis** | No input provided for project goals or hypotheses | Little significant input provided for project goals or hypotheses | Some input in setting project goals and hypotheses; improper hypotheses based on research | Major input in setting project goals and hypotheses; improper hypotheses based on research | Major input in setting project goals and hypotheses; hypothesis proper based on research | |
| **Procedures Methods** | No procedures completed | Few assigned procedures completed | Several procedures completed. | Most procedures completed effectively and responsibly and/or no substitutes were found to cover absences | All procedures completed effectively and responsibly or substitutes were found to cover rare absences | |
| ***Daphnia* Sex Determination** | Did not complete sex determination | Mistakes regularly observed in sex determination | Some mistakes observed in sex determination | Mistakes rarely observed in sex determination | No observed mistakes in sex determination | |
| **Graphical / Statistical Data** | No statistical or graphic data provided | Neither Statistics nor graphs appropriate for findings | Either statistics or graphs were not appropriate for data | Statistics and graphs appropriate for data; few reporting errors | Statistics and graphs appropriate for data; no reporting errors | |
| **Conclusions** | No conclusion of results provided. | Conclusions were not appropriate based on the data. | Conclusions were appropriate based on the data, but overstated, and data was not compared to findings from prior studies | Conclusions were appropriate based on the data but were overstated, or data was not compared to findings from prior studies | Conclusions were appropriate based on the data and were not overstated; data was compared to prior studies | |
| **Project Component** | **Incomplete 0 points** | **Poor 6 points** | **Marginal 9 points** | **Good 12 points** | **Excellent 15 points** | |
| **Written Presentation of Project** | No paper submitted or paper submitted without references or plagiarized. | Paper not written clearly or concisely, has significant errors or not all sections of the paper included | Paper not written clearly, has some errors; Includes Abstract, Introduction, Methods, Results, Discussion and Reference sections | Paper written clearly, concisely, with some errors; Includes Abstract, Introduction, Methods, Results, Discussion and Reference sections | Paper written clearly, concisely, with no significant errors; Includes Abstract, Introduction, Methods, Results, Discussion and Reference sections | |
| **Oral Presentation of Project** | No participation in the oral presentation of the project | Presentation was not well practiced; little evidence of knowledge about the project | Presentation was not well practiced; some knowledge of the project evident, but not complete; lacks the ability to answer audience questions | Presentation was well practiced; knowledge of the project evident; inability to accurately answer audience questions | Presentation was well practiced; knowledge of the project evident; accurately answers audience questions | |

Final Grade _____/100

# CHAPTER V

## GENERAL CONCLUSIONS

This dissertation shows that the selected UV filters rarely exhibit acute toxicity to *D. magna* even at 50% water solubility with the exception of avobenzone which demonstrated acute toxcicity at $LC_{50}$ 0.74 (0.41, 0.94) mg/L. Average day of first brood production was not a sensitive indicator of chronic toxicity for the tested UV filters. The most sensitive endpoint was effects on mean number of neonates per adult. The highest chronic test concentrations for the six UV filters tested reduced fecundity in diverse levels between 0.03 and 10 mg/L. Significant chronic mortality was observed in the upper test range for octisalate and dioxybenzone. Dioxybezone, homosalate, OMCN, and oxybenzone enhanced the mean number of neonates in middle to low test concentrations which appear to be a hormetic effect. Exposure to avobenzone, homoslate, and oxybenzone stimulated significant production of male neonates; however, avobenzone produced males in all concentrations at rates similar to the positive control fenoxycarb. It appears that avobenzone could be acting as an endocrine disrupting compound by mimicking the effects of the crustacean male producing hormone methyl farnesoate. This is the first study to demonstrate male production as a chronic effect of UV filter exposure in *D. magna*. Male production may be a useful chronic endpoint to show potential endocrine

disruption for UV filters. Further studies are needed, on *D. magna* and other test organisms, to identify potential toxicological effects of other UV filters commonly added to personal care products.

This dissertation shows that four of the six tested UV filters were identified in tested lakes in various concentrations ranging from low ng/L to low µg/L, which is similar to other monitoring studies. Average UV filter concentrations were significantly higher in beach areas compared to offsite areas and peaked in July, which corresponds to high use human recreational activities. Concentrations were reduced in September samples and occurred in the lowest levels in October corresponding to no/low use times. A spatial and seasonal effect on UV filter environmental concentration was observed. Additionally, average UV filters' concentrations increased with average number of patrons. These findings indicate seasonal high use recreational beach areas as major anthropogenic sources of UV filter contamination. Despite their association with wastewater treatment plants and coastal areas, this is the first monitoring study to identify UV filter concentrations for homosalate, octisalate, OMCN, and oxybenzone in U.S. lakes.

Comparison of the maximum environmental concentration of the four detected UV filters to LOEC values, resulted in hazard quotients well below one indicating that UV filters are not likely occurring in the environmentally relevant levels that would induce toxicological effects even on a sensitive organism such as *D. magna*. Effects are even less likely than the calculated value due to the conservative nature of the calculation method. Further monitoring studies are needed to determine MEC values in high use beach areas for other approved UV filters. Since mixtures of UV filters regularly occur in monitoring studies, additional toxicological studies are needed to determine potential synergistic effects these UV filters may have on aquatic organisms at environmentally relevant concentrations.

UV filters, like other anthropogenic compounds, have demonstrated potential endocrine disruption in *D. magna* in this study. This phenomenon should be emphasized in undergraduate education to enhance the understanding of the concept as an example of environmental effects on cell signaling. A 21-day reproduction study of *D. magna* exposed to the pesticide fenoxycarb provides an effective research system that explores endocrine disruption while enhancing research skills. The project has been successfully implemented in a small class of freshman and sophomores with diverse majors. Ninety percent of the students (n= 10) showed proficient understanding of the concept of endocrine disruption and effective research skills. This dissertation advances the knowledge of the toxicological effects of UV filters on *D. magna*, identifies the occurrence and distribution of 4 UV filters in US lakes, determines a preliminary hazard estimation of environmentally relevant concentrations of 4 common UV filters, and introduces a simple and effective research system that can advance student learning of the process of science and endocrine disruption.

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