THE EFFECTS OF THE EMERGING MARINE POLLUTANT OXYBENZONE ON THE METABOLLIC PHYSIOLOGY OF EMBRYO-LARVAL ZEBRAFISH

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of the Emerging Marine Chemical Pollutant Oxybenzone on the Metabolic Physiology of Embryo-Larval Zebrafish

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This project analyzed the effects of the emerging marine pollutant commonly known as oxybenzone. Oxybenzone is a chemical ingredient commonly used in sunscreen lotions, as well as other hygienic products. There is growing concern that oxybenzone can cause adverse effects in exposed invertebrate and vertebrate organisms, such as estrogenic endocrine effects and negatively altered growth or survival. Embryo-larval fish are expected to be particularly sensitive at this early life stage. They are likely to be impacted by oxybenzone exposure as their developmental processes are likely to be perturbed, potentially causing long term sub-lethal effects that compromise the potential for growth, survival, and reproduction during the life-span of the impacted organism. If these potentially physiologically hindering effects are occurring in wild coastal populations, it may raise a risk of a possible population decrease to those organisms that are exposed to impactful levels of oxybenzone. At present, there is a lack of studies analyzing the effects of oxybenzone on fish at environmentally relevant concentration levels. My project aims to address this knowledge gap by exposing early life stages of zebrafish (*Danio rerio*) to increasing levels of oxybenzone and measuring their altered oxygen consumption and

metabolic rates as a result of exposure. The levels chosen for testing encompass environmentally relevant concentration ≤10 μg/L (i.e ≤10ppb). The effects of oxybenzone exposure on fish were tested using multi-well micro-respirometery for oxygen consumption rates. More detailed analysis of the physiological effects will also be determined by performing whole-genome transcriptomics analysis (RNA sequencing or RNA-seq) on the zebrafish in the future. Together these analyses will provide information on the effects at a whole organism level, from effects on respiration and metabolism, to RNA sequencing effects at the genome level. The results of my project will determine whether fish living in oxybenzone polluted areas are at risk of adverse physiological effects.

DEDICATION

It is my genuine gratefulness and warmest regard to dedicate this work to my beloved family and friends for listening to me constantly babble on with excitement about my experiments and encouraging me even though they had no idea what I was talking about.

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CHAPTER I

INTRODUCTION

Background

Almost half of global coastal and marine ecosystems are considered to be impacted by anthropogenic activities or extreme climatic events. These include effects from excessive pollution, nutrient inputs (causing hypoxia zones), overfishing and global climate change (Halpern et al., 2008). Recent concern has focused attention on the near ubiquitous presence of man-made synthetic chemicals, such as: pharmaceuticals, personal care products, veterinary medicines, agrochemicals and biocides in the aquatic environment (Arnold, Brown, Ankley, & Sumpter, 2014; Daughton & Ternes, 1999). National monitoring surveys show the environmental 'footprint' of such synthetic compounds to reflect their usage, with pharmaceuticals, personal care products and biocides (or PBs) dominating in aquatic ecosystems (Focazio et al., 2008; Kolpin et al., 2002) as well as in drinking water and in wastewater (Loraine and Pettigroce, 2006; Balmer et al. 2005; Centers for Disease Control and Prevention (CDC), 2003]. The stability of coastal ecosystems has been suffering in recent years due to increased anthropogenic involvement in coastal territories where aquatic and marine organisms live. These human involvements in coastal regions include physical as well was chemical factors that are declining the overall population and biodiversity health such as overfishing and bycatch, plastic/debris and chemical pollution, and a wide array of other anthropogenic activities in these areas has impacted populations of animals to the point where they are in jeopardy of population crashes (Pawar et al. 2016). It has been shown that these anthropogenic factors are the root cause of some species population decrease or loss due to a study that showed once depleted

populations have population regrowth attributed to the reduction of human interference and exploitation with decrease in activities such as those listed above (Lotze, 2011). Coastal ecosystems are already under a large amount of pressure from anthropogenic causes due to their conditions becoming unstable due to indirect human impacts on the environment such as ocean acidification, sea surface temperature rise, overexploitation by humans, disease, decreasing biodiversity due to extinction and genetic degradation, habitat fractioning and loss, marine debris and pollution, invasive species, and altered salinity all directly or indirectly related to anthropogenic activity. (Hughes, 2003; Pawar 2016). The consequences of widespread pollution are a threat to the health of the entirety of coastal ecosystems as well has human communities in these areas that rely of on coastal ecosystems such as commercial coastal and marine fisheries (Islam & Tanaka, 2004). The stability of ecosystems are already under threat by a combination of these factors and are becoming more of a concern because of the questionable and unknown impacts of emerging contaminants in ecosystems that have yet to be studied.

The chemical of interest for this study, known as oxybenzone, is a chemical that is considered to be an emerging contaminant. The impacts of this chemical in the environment have not been extensively studied, especially at an environmentally realistic level. It is unknown what the emerging contaminant oxybenzone does to vertebrate organisms at environmentally realistic levels.

Emerging contaminants in coastal ecosystems

An emerging contaminant is a new class of chemical pollution due to increased concern of substances that have been newly discovered to be present in ecosystems as a result of human use that are seen to be as potentially harmful for ecosystems. An emerging contaminant can be defined as chemicals used for human use that are becoming a growing concern due to their new presence in ecosystems outside of human contact. The most prevalent of emerging contaminants are those endocrine disrupting compounds used in the pharmaceutical industry for prescription drugs as well as personal care and hygiene products (Inam et. Al, 2015). Pharmaceuticals themselves are the grand majority of these emerging contaminants and people are concerned due to their unknown impacts on local ecosystems and wildlife. Pharmaceutical products such as those in prescription drugs are beginning to end up in local waterways such as rivers and streams that are in close proximity to anthropogenic activity and are making their way into the water that not only inhabits local wildlife but also the water that people are drinking. There have been studies that have shown that female estrogens in birth controls are being found in coastal environments. Estrogens found in municipal waters have been studied and have been linked to fish populations of feminization of male fishes and eventual collapse (Kidd, 2007). Male fish in areas with a notable concentration of estrogens in the water have been found to have female sexual organs. A whole lake study conducted at the Experimental Lakes Area in northwestern Ontario Canada found that fat-head minnows subjected to chronic exposure to 17α-ethylnylestridol, a synthetic estrogen, feminized male fish into producing the precursor to egg yolk protein, vitellogenin, leading to the development of female gonads in males. The female production of reproductive tissue (such as oocytes) in males (or feminization) ultimately led to the extinction of the species in the lake as the sexual reproductive function of male fat-head

minnows had become impeded and sexual reproduction was no longer possible to keep population replenishment of the specie could no longer continue (Kidd, 2007). This is likely due to birth control user's bodies excreting these chemicals into septic systems where they eventually end up in water ways and local aquatic and marine environments. The effects of synthetic estrogens are of importance due to the estrogenic effects that oxybenzone has on organisms. The unknowns of emerging contaminants are of great concern because we are unaware of what these chemical pollutants may be doing to the physiology of not only local wildlife but humans as well; because water treatment plants are not built to rid of individual molecular compounds in water. It is highly possible that emerging contaminants are a huge underlying threat towards aquatic and marine ecosystems that has not yet been uncovered. Local wildlife is living with constant exposure towards the contaminants suspended within the water that these organisms are continuously intaking into their bodies. The physiological impacts of emerging contaminants are widely unknown as most of them have not been adequately studied to understand if there is direct correlation of anthropogenically manufactured chemicals on water dwelling organisms. Environmental monitoring studies also show man-made synthetic chemicals to bio-concentrate in various piscine species (Brooks et al., 2005; Gaw, Thomas, & Hutchinson, 2014; Ramirez et al., 2009), and also accumulate across trophic levels with detectable body burdens measured in various invertebrate and vertebrate species inhabiting polluted coastal ecosystems (Dodder et al., 2014). A recent survey of fish inhabiting freshwater/estuarine ecosystems showed body-burdens to be highly dependent on local exposure to man-made pollutants via contaminated waters (Du et al., 2016). As a result, coastal ecosystems are particularly vulnerable due to their close proximity to human habitation and industrialization. The consequences of widespread pollution is also a

threat human communities due to a loss of ecosystem 'services', such as fisheries, commerce and recreation (Daily et al., 1997).

In this project, I am studying the effects of the emerging man-made pollutant, oxybenzone, on fish metabolism. Oxybenzone is an active ingredient in sunscreen lotions and is considered an 'emerging' pollutant as its impacts on the environment have not been extensively studied. In particular, its effects on the physiology of fish is not known. To address this knowledge gap, my project will use zebrafish (*Danio rerio*) as a model organism, and study the effects of exposure to environmentally relevant concentrations of oxybenzone (spanning from 0.1 – 10 µg/L). Specifically, I will study the effects of oxybenzone exposure on embryo-larval zebrafish metabolism using two approaches: 1) measure whole-organism metabolic rate using multi-well microrespirometry to quantify oxygen consumption; and 2) use whole-genome transcriptomics (RNA-sequencing or RNA-seq) analysis to quantify effects on the molecular biology (i.e. gene expressions) of exposed fish. The successful completion of my project will provide new information on the effects of oxybenzone on the physiology of fish at environmentally relevant concentration ranges.

Presence of oxybenzone in aquatic ecosystems

Oxybenzone is an active chemical ingredient in common sunscreens and hygiene products that are in widespread use. Oxybenzone is UV filtering chemical is used as a UV filtering chemical to protect the skin from harmful UVA and UVB rays that have the potential to damage the skin that have the potential to damage the skin due to prolonged sun exposure. The concentration of oxybenzone in coastal areas has been reported to be at its highest in areas with high beach use and tourism (Bratkovics, 2015). Oxybenzone concentrations in coastal areas have been found to be as high as ~10ppm in areas that are frequented by frequent beach tourism and high human activity (Wood, 2018). In the U.S Virgin Islands, oxybenzone concentrations in

water samples ranged from 75ppb to 1400 ppb with an average of 250ppb and 0.8 to 19.2 ppb in the Hawaiian Islands (Downs et al., 2015). However these concentrations seem to be the more extreme as the environmental levels of these chemicals can span over orders of magnitude, from ~0.001 ppb to ≥1 ppb, which in some cases are close to (or exceed) intended therapeutic or biological-effect concentrations (Kolpin et al., 2002; Petrie, McAdam, Scrimshaw, Lester, & Cartmell, 2013). Other studies have concluded oxybenzone concentrations similar to these concentrations of approximately 1ppb-10ppb worldwide (Woods, 2018). Appendix A shows concentrations of oxybenzone in coastal ecosystems in different coastlines around the world. It has also been concluded in these studies that concentrations of oxybenzone are the most prominent on shorelines. Sampling that occurred closer to shorelines had a trend of higher oxybenzone concentrations in the water samples than samples that were taken further from the shoreline (Downs et al., 2015; Bargar et al., 2015; Bratkovics and Sapozhinikova et al.,2015). These sampling trends may further indicate that areas with high beach tourism have higher oxybenzone concentrations in the water due to the higher levels of oxybenzone present in the water in the same areas where beach tourism is focused. The near ubiquitous presence of man-made synthetic in the environment has raised concern for adverse human and wildlife health effects though inadvertent exposures (Arnold et al., 2014). The environmental levels of these chemicals can span over orders of magnitude, from ~0.001 µg/L to ≥1 µg/L, which in some cases are close to (or exceed) intended therapeutic or biological-effect concentrations (Kolpin et al., 2002; Petrie et al., 2013).

Ecotoxicity of oxybenzone

In areas of high oxybenzone concentrations causing adult coral bleaching and death (Danovaro et al., 2008). Coral larvae have been observed to exhibit reduced growth and fecundity (Downs, et al., 2015). Coral cell LC50 for coral cells for seven different species ranged from 139 to 3100 ppb (Downs et al., 2008). The observed levels of oxybenzone in the U.S Virgin Islands is well beyond he calculated LC50 for coral cells and quickly approaching lethal levels in Hawaii likely causing the bleaching and/or cell death of coral in these areas where higher concentrations of oxybenzone is prevalent (DiNardo JC, 2018). Laboratory toxicological studies have shown oxybenzone to also act as a weak estrogen, causing endocrine disruption. A bioassay analyzed the endocrine disrupting effects of oxybenzone on male juvenile rainbow trout and male Japanese meduka as well as the reproductive success of female Japanese meduka. The bioassay found that the estradiol components of oxybenzone induced the production of a glycolipo-phosphopeptide, vitellogenin (precursor for the egg yolk protein) in both of these species of fish in males; as well as a significant decrease the in the number of eggs/females each day observed by the female Japanese meduka (Coronado et al., 2008). Oxybenzone has been shown to impact both invertebrate and vertebrate species at high levels. However, there is a lack of studies showing the impacts of oxybenzone on vertebrate species with concentrations that have been found in the natural environment. There needs to be an assessment of laboratory studies on fish for an accurate reflection of negative repercussions of the commonly used chemical ingredient. There needs to be scientific understand to know the implications that oxybenzone is having on the metabolic physiology of organisms in current time. My study aims to achieve showing these effects on a physiological level as well as a genomic level.

Objectives of the project

This project aims to study the effects of a marine pollutant of emerging concern on the metabolic physiology of fish. The physiological effects of a chemical ingredient commonly used in sunscreens, oxybenzone, will be studied at environmentally relevant concentrations. At present, there are a lack of studies assessing the effects of oxybenzone exposure on fish at environmentally realistic levels. My central hypothesis is that exposure of a model fish species, the zebrafish (*Danio rerio*), to environmentally realistic levels of oxybenzone will cause subtle metabolic effects that will result in changes of oxygen consumption (or metabolic) rate. Changes in oxygen consumption rates relative to a control (un-exposed) group of fish will indicate stress and a likely compromise of activities that are dependent on the aerobic capabilities of an organism, i.e. swim performance, foraging for food, predator evasion and fecundity. Assessing the effects of oxybenzone on the metabolism of fish will involve measuring the metabolic rate (i.e oxygen consumption rate) of embryo/larval zebrafish at environmentally realistic concentrations, making the results of this study highly relevant for understanding the adverse effects of oxybenzone on aquatic ecosystems. The zebrafish is a well-studied animal model and is representative of other aquatic (fresh water and marine) fish species. Furthermore, zebrafish are also used as a sentinel organism for aquatic toxicity testing (such as in toxicity tests performed by the U.S. Environmental Protection Agency).

CHAPTER II

METHODS

This project aims to test the metabolic effects of oxybenzone (a commonly used sunscreen ingredient) on embryo-larval life-stages of zebrafish (*Danio rerio*). In order to do so we will expose zebrafish for up to 12 days to a solvent control (0.01% DMSO) and various environmentally relevant exposure concentrations of oxybenzone (≤ 10 µg/L) to determine the metabolic physiological effects of chronic exposure of larval fish to oxybenzone. My key research methods are detailed below.

Rotifer culture for zebrafish feeding

Rotifers cultures purchased from Reed Mariculture were raised in the lab in order to provide an in-house live feed for our zebrafish. The rotifers were maintained in 5 gallons buckets containing saltwater at 22 ppt salinity. Rotifers were harvested for zebrafish feeding once/day. Four liters of culture water was filtered through a 53 µm screen to isolate rotifers within the culture to collect for feeding. Rotifers were reconstituted from the filter into 20ml solution and an average of 300 ul of the rotifer solution were fed to each beaker of larval zebrafish. A final concentration of 100,000 rotifers/mL was fed to zebrafish

Rotifers were fed concentrated (Cell count = ~68 billion per ml) Nanno 3600 algae from Reed Mariculture *(Nannochloropsis sp.)* Reed Mariculture Nanno 3600 algae was diluted in a beaker system reservoir at approximately 0.0015mL/L. A final concentration of approximately 102,000,000 algae cells/mL. The algae culture was continuously moved into the rotifer culture using a peristaltic pump operating at 1mL/minute. Figure 1 shows the set-up of the peristaltic pump with the algae culture reservoirs for the rotifer culture.

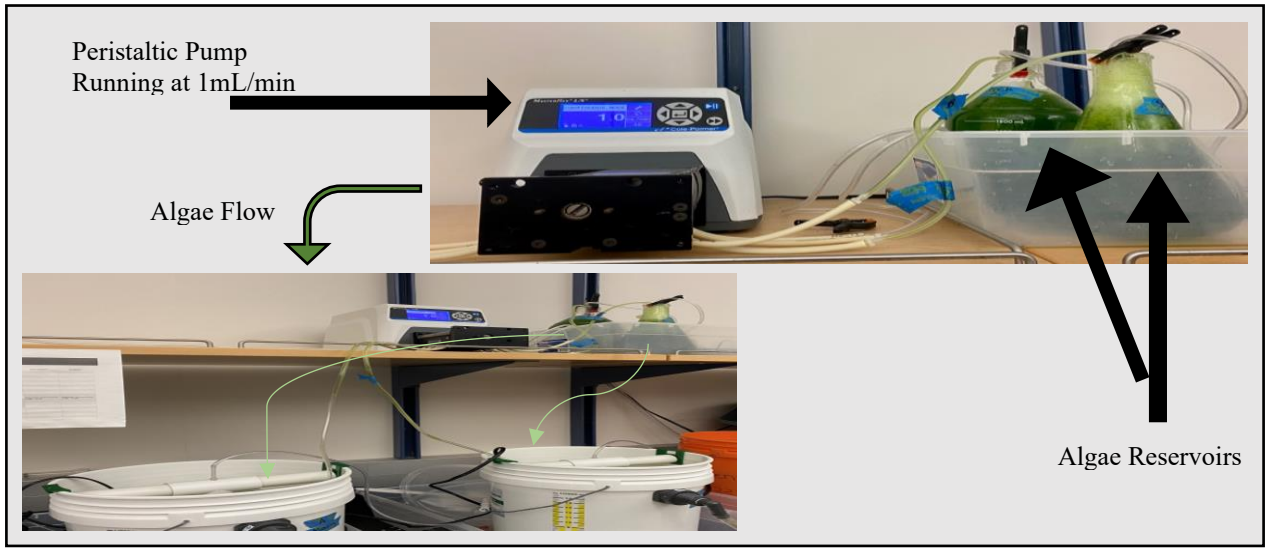

Figure 1. Peristaltic Pump and Algae Reservoirs. Tubing connects the algae reservoirs to the rotifer culture buckets at a rate of 1ml/min via peristaltic pump.

Zebrafish culture and exposure

The solvent control (0.01% DMSO) exposure study was repeated twice with different methods of water exchange in order to ensure adequate water quality and data collection for our future exposure trials. Each trial involved the use of two 1-liter beakers, with approximately 50 embryo larval zebrafish maintained in each at a water temperature of ~27°C.

a. First solvent control exposure trial: This trial involved a semi-static renewal exposure design (i.e. 50% of exposure aquaria was replaced twice a day in order to maintain water quality and ensure the high % survival of fish). Therefore, an exchange of 500 mL for per beaker was done by preparing a 0.01% DMSO solution (100 uL DMSO was spiked into 1 Liter of culture water). Water quality parameters were monitored at least twice a day, and included measuring ammonia and nitrate levels in water.

b. Second solvent control exposure trial: We found that the semi-static renewal was an inefficient system for the purposes of our study due to higher concentrations of ammonia that we could not control, as we saw a massive die off by day 5 with the semi static renewal. As a result, the solvent control trial was repeated with a flow through system to

ensure quality data collection and healthy individuals. In a flow-through system, the exposure water is delivered continuously at a fixed flow rate. A 4-liter solvent stock reservoir of 0.1% (100 microliters/liter) was set up. The bottle was connected to a peristaltic pump operating at 1mL/minute. Water exited a beaker through a small tube connected to a pipette tip. The effluent from each beaker drained into the lab sink. This system was found to be much more efficient at lowering concentrations of nitrogenous waste compounds.

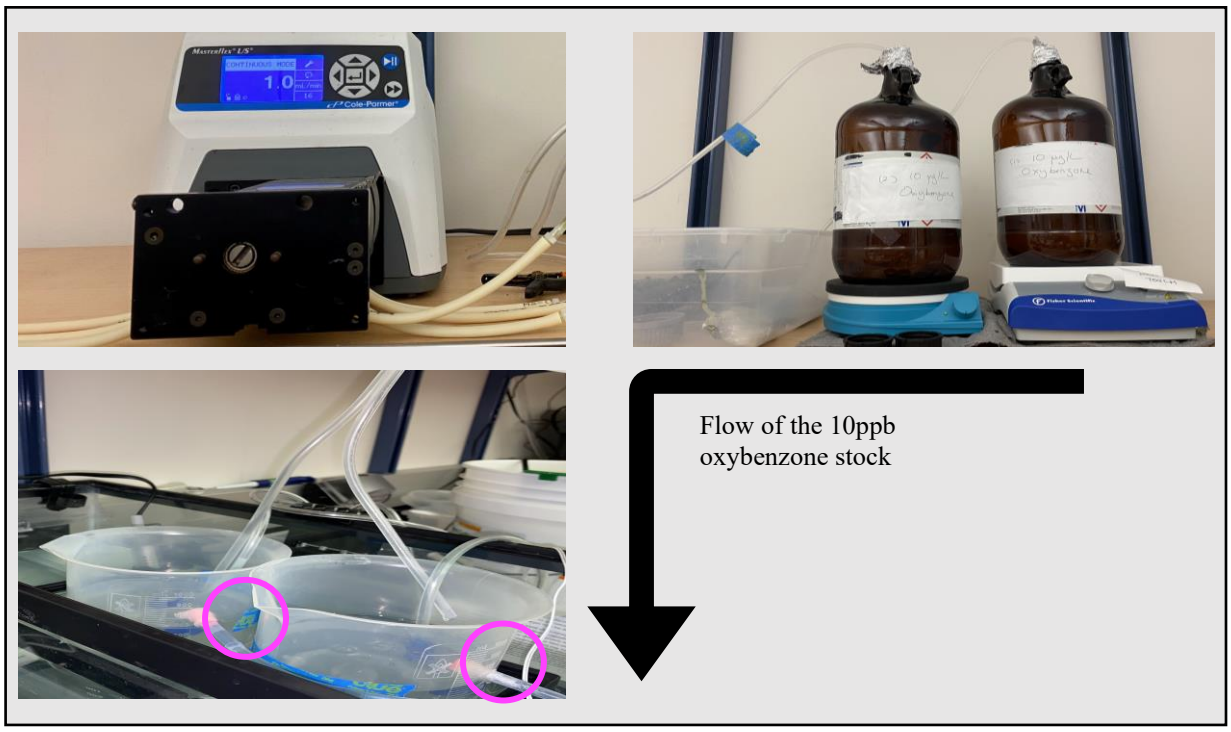

Figure 2. Oxybenzone Top off System. Flow-through aquaculture system with the amber bottle reservoirs with the 10ppb concentration of oxybenzone flowing into the zebrafish beakers at 1ml/min with small tubing. Overflow in the beakers will flow out of tubing covered with a filter (pink) into a pipe connected to a sink.

c. 10ppb exposure trial: From our solvent control trials, we learned that the flow through system provided a cleaner environment for the fish to live in. As a result, we used the amber stock bottles to have a continuous flow through reservoir at 10ppb for the duration of the exposure. The stock bottles were refilled daily at a concentration of 100ul (of 100ppm oxybenzone & DMSO)/L of water from the Sea Life Facility, as this water had

optimum parameters for fish to live in. The 100ppm oxybenzone & DMSO allowed for the oxybenzone to properly dissolve and dilute in the stock bottles at the once the bottles were refilled. After the stock bottles were refilled water samples were taken to ensure proper concentrations of oxybenzone in the stock. The stock bottle reservoirs of 10ppb oxybenzone concentration water was pumped into the zebrafish beakers at 1ml/min as a flow through system. In addition, in order to maintain proper oxybenzone concentrations in the zebrafish culture water semi-static 50%water changes were done twice a day in order to replace any oxybenzone molecules that may have stuck to the plastic or broken down as well as keep water quality parameters at optimum levels.

i. Oxybenzone Dosing at 10ppm

   Solvent control trial of DMSO was at a concentration of 0.01%

   (Dilution factor =10,000).

   To dose the zebrafish at a concentration of 10ul/L the stock needs to be $10\mu L \times 10{,}000 = 1000{,}000\mu L$ $(or\ 100ppm)$. The 1000ppm oxybenzone is diluted to 100ppm in DMSO. To dose the stock into the stock bottles, 100uL of the 100ppm oxybenzone in DMSO solution is aliquoted into 100,000uL (1,000mL) of Sea Life Facility water for the chronic exposure of oxybenzone to the embryo-larval zebrafish.

Micro-Respirometry Methods

Prior to the beginning of micro respirometry data collection, the silicon cover used is soaked in water for 30 minutes prior to the experiment to enhance its sealing capabilities. The oxygen consumption rates of the zebrafish were measured using a 500uL micro-respirometer (from Loligo systems). To run the trials, the program is run as Administrator and the sample interval is. Set to “fastest” so that the reading is as accurate as possible. The local atmospheric pressure is set to hectopascals, the room temperature is inserted into the trial settings, chamber volume is set to 500 and the oxygen units are set to % air saturation. Before measuring the oxygen consumption of the larval zebrafish, the micro-respirometer was calibrated with a solution consisting of 10% sodium sulfide in order to get a control reading of 0% oxygen. When the low oxygen levels have stabilized the values are saved to the system as a low oxygen control group for the trial. The system is then calibrated a second time to get a control group for a higher reading of oxygen. Aerated deionized water is placed into the wells of the micro-respirometer and measured on the software to calculate the control for oxygen levels in the water before the fish are added. When the readings for high oxygen control has stabilized the oxygen and temperature values are saved for the control. Once this has been done the larval zebrafish are added to all of the well plates that already contain the aerated deionized water. Before the fish are put into the wells, the oxygen concentration is changed to ug/L. The first and last rows of the micro-respirometry well plate are left dry in order to obtain more accurate readings from the well plates containing aerated water and larval fish. The second row is filled with the aerated deionized water with no larval fish present to serve as a blank. Larval fish are present in the rest of the rows. After the fish were placed a silicon cover and a heavy block was placed over well-

plate in order to maintain a tight seal. In addition to the silicon and heavy block, a binder was placed once the well plate as well in order to further enhance the tightness of the seal as well as reduce the amount of light getting into the plate. Upon the placement of the seal, the oxygen level inside of the well plates were continuously watched until the oxygen readings began to drop. Once a drop in oxygen levels had begun to be observed, the oxygen consumption measurements were recorded for a time period of 15 minutes. Following the 15-minute trial, the fish were culled humanely by cold stunning and weighed for calculation of oxygen consumption rate. When the fish were weighed following humane culling them, they were placed on a scale with tweezers with as little water externally touching them a possible so that the wet weight of the larval zebrafish was still as accurate as possible.

RNA extraction and quantification

Total RNA was extracted from whole larval fish. The RNA was extracted using a commercial kit (NucleoSpin RNAXS). Prior to RNA extraction, the larval fish were humanely euthanized in an ice bath. Once the fish are euthanized, the larval fish homogenized in a lysis solution using a hand-held automated pestle. Subsequent RNA extraction steps were as per the instructions detailed in the RNA extraction kit as well as a more comprehensive protocol written by the advisor. The process involves completely isolating and eluting highly pure RNA from the rest of the fish and debris so that the samples can be adequately sequenced an analyzed. The concentration of the total RNA extracted was quantified using a spectrophotometer (Cytation5 plate reader). Once the total RNA concentration was measured, RNA samples were stored at -80C until further analysis by RNA sequencing (as a service contract to TAMU AgriLife genomics facility). Once the data was analyzed by Agrilife, we use the software (created by Biobam known as omicsbox). Omicsbox was used to analyze the RNA data for alterations in the

genetic coding for responses to chronic exposure of oxybenzone. We decided for the purposes for this UGR project that the RNA data would be used for a later publication due to time constraints.

Oxybenzone Water Analysis

Oxybenzone water analysis was done daily to ensure proper concentrations of oxybenzone in the water during the embryo-larval zebrafish exposure trial. The concentrations of oxybenzone in the water was analyzed by running samples through the Aligo systems Mass Spectrometer. Collection of water samples for oxybenzone concentration analysis were done by extracting 990μl of the water sample and a 10 μl spike of d10-caramazipine. The spike of the d10-crabamazipine was added so that the mass-spectrometry readings of the oxybenzone concentrations in the water samples could be more accurate. We took samples after 24 before and after water change.

CHAPTER III

RESULTS

10 ppb oxybenzone exposure data was compared to the solvent control trials as well as previous timepoints within the same 10ppb trial. Chronic exposure to oxybenzone over time was shown to have negative effects on the oxygen consumption of the zebrafish. However, although effects when exposed to oxybenzone did see an increase in mass specific metabolic rate, the data was not statistically significant.

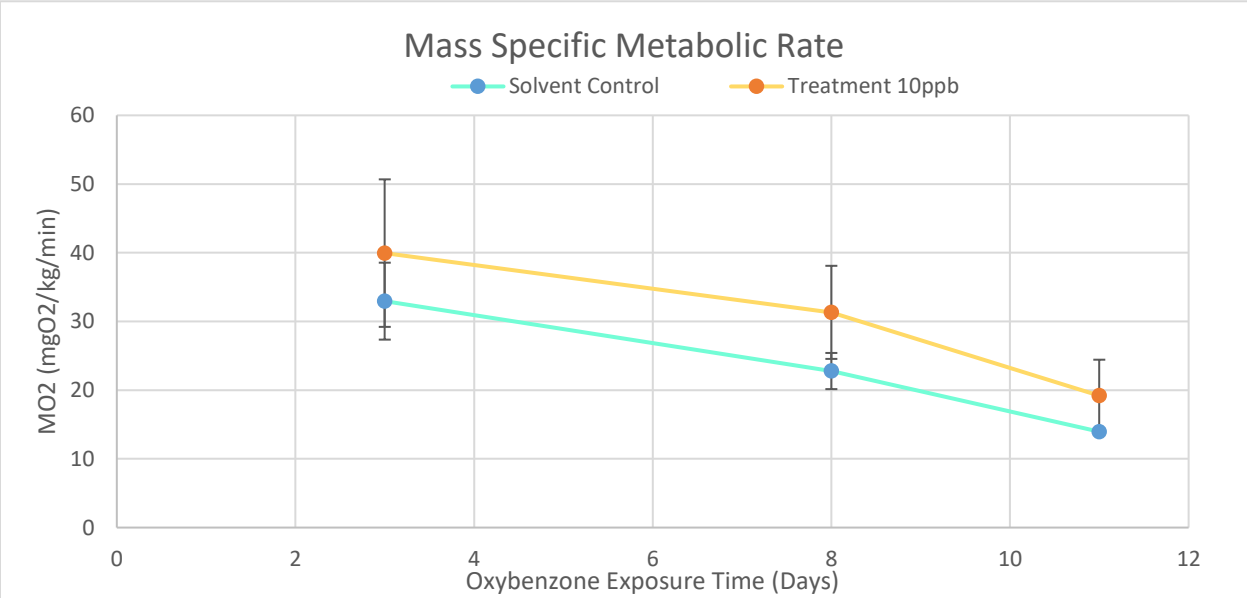

Figure 3. Mass Specific Metabolic Rate of fish in the Solvent Control Trial vs. Treatment 10ppb Oxybenzone Trial. Line graph shows the trend of increased metabolic rate of fish in the 10ppb trial. However, error bars show it may not be statistically significant.

Even though the data indicates a lack of statistical significance between the metabolic rates between the solvent control and the oxygen consumption, there does appear to be a trend that the fish exposed to 10ppb of oxybenzone had a slightly higher metabolic rate. At present,

there are plans to repeat this study and record metabolic rates in a larger cohort of fish, and to record the oxygen consumption over a much longer time duration (i.e around 12 hours vs. minutes done in this current trial)

CHAPTER IV

DISSCUSSION

Study Optimization

Feed for Zebrafish

Upon the beginning of conducting the solvent control trials the zebrafish were originally going to be fed Paramecium, as this was the main staple invertebrate that was fed to zebrafish in previous physiological toxicology studies conducted. These low yields of paramecium made it very difficult to feed the zebrafish experiment specimen due to limitations in being able to concentrate the Paramecium cell count before feeding. However, there were major issues with producing enough Paramecium culture to adequately feed the rotifers; as well as the Paramecium culture harboring nitrogenous wastes that were harmful for the zebrafish. These factors were detrimental for past conducted experiments due to these issues of low yield and high ammonia. Rotifers were be used in place of Paramecium due to their easy care and high yields. Upon switching to rotifers, the increase in cell count/volume of water was incredibly large. The culture methods of the rotifers can be found in the methods section. However, we still have issues with the rotifers causing high ammonia. Within the rotifer culture itself, chloromax was added to the culture to suppress any ammonia spikes. Other methods of keeping the ammonia concentrations in the zebrafish culture will be listed in the section below.

Culture Systems for Controlling Ammonia and Fish Mortality

Upon reaching the end of our first solvent control trial, it was realized that the exposure trial would need to be repeated due to the high amount of mortality of zebrafish (as caused by high ammonia). The use of the semi-static renewal system alone for the zebrafish was failing to keep the ammonia concentrations low. In addition, we had realized that we were feeding the zebrafish a much higher concentration of rotifers than we had originally believed that they needed at this life stage, which was also causing ammonia spikes in the system that the zebrafish could not handle, and caused high percentages of mortality.

Once we concluded our second solvent control trial in order to fix the perceived problems of the first trial, we saw a significant decrease in mortality. The flow-through method implemented in the second control trial showed significant decreases in mortality as well as ammonia concentrations with the newly implemented systems. However, this new flow through system did have a flaw with the outflow of water. Fish would get caught in the outflow hose from the beaker and as a result would end up in the pipes connected to the sink. This issue was later resolved by putting a 53micron filter on the pipes to prevent the fish from flowing out of their culture water. This system was incredibly effective in reducing fish mortality as we saw a 58.33% decrease in fish mortality between the two systems (Figure 3).

More details on the set up and methodology of the semi-static and flow-through systems can be found in their respective methods section.

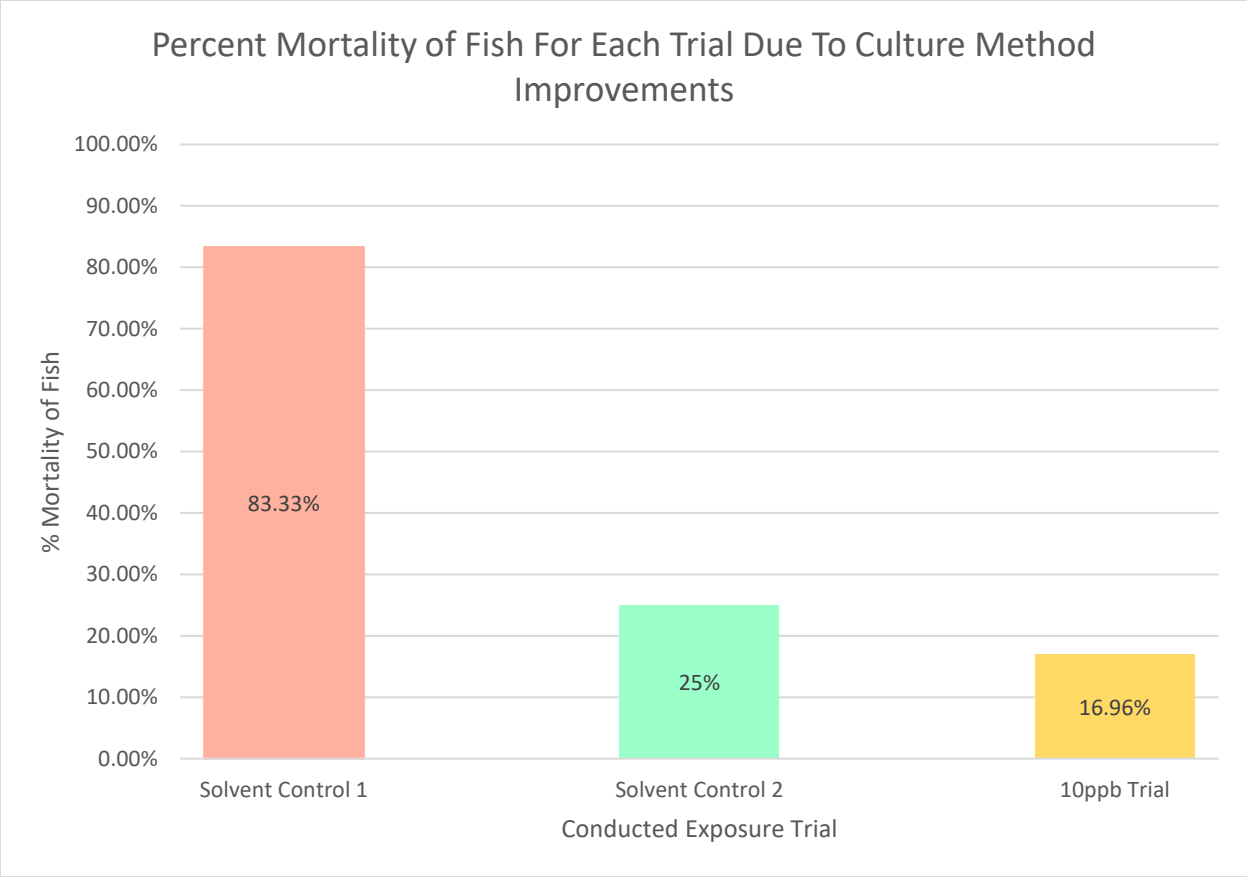

Figure 4. Percent Mortality of Zebrafish for Each Trial Due To Culture Method Improvements. A significant decrease in the percentage of culture system related deaths between the semi-static renewal method and the flow-through system is observed by the two solvent control trials. The decrease in the percentage of mortality between solvent control 2 and the exposure trial is hypothesized to be the implementation of both the flow through system and the semi-static renewal, even though the semi-static renewal was only intended to replace oxybenzone molecules that had degraded in the water.

Maintaining Proper Oxybenzone Concentrations

Water samples taken from the exposure water as well as the from the stock bottle analyzed by liquid chromatography and tandem mass spectrometry (LC-MS/MS) revealed that oxybenzone concentration levels decreased from the time the culture stock was initially dosed into the water. We hypothesize that the oxybenzone molecules were either degrading in the water over time or adsorbing to potential binding sites in the plastic tubing and beakers that comprised the zebrafish culture system. As a result, we began the expose the system to oxybenzone a few days before the fish arrived so as to minimize this factor. In addition, we optimized the culture's

experimental design by incorporating the flow through system to flush ammonia out of the culture water to keep these levels to a minimum as well as semi-static renewal in order to replace the oxybenzone concentration lost over the previous 24 hours. Implementing these two designs together was incredibly beneficial for the overall experimental design. This was seen by being able to keep the culture stocks at approximately 50-60% of the intended 10ppb oxybenzone concentration (Figure 4). The implementation of the two water replacement methods together also unintentionally kept ammonia levels so low that they were undetectable; which therefore decreased fish mortality by another 8.04% (Figure 3).

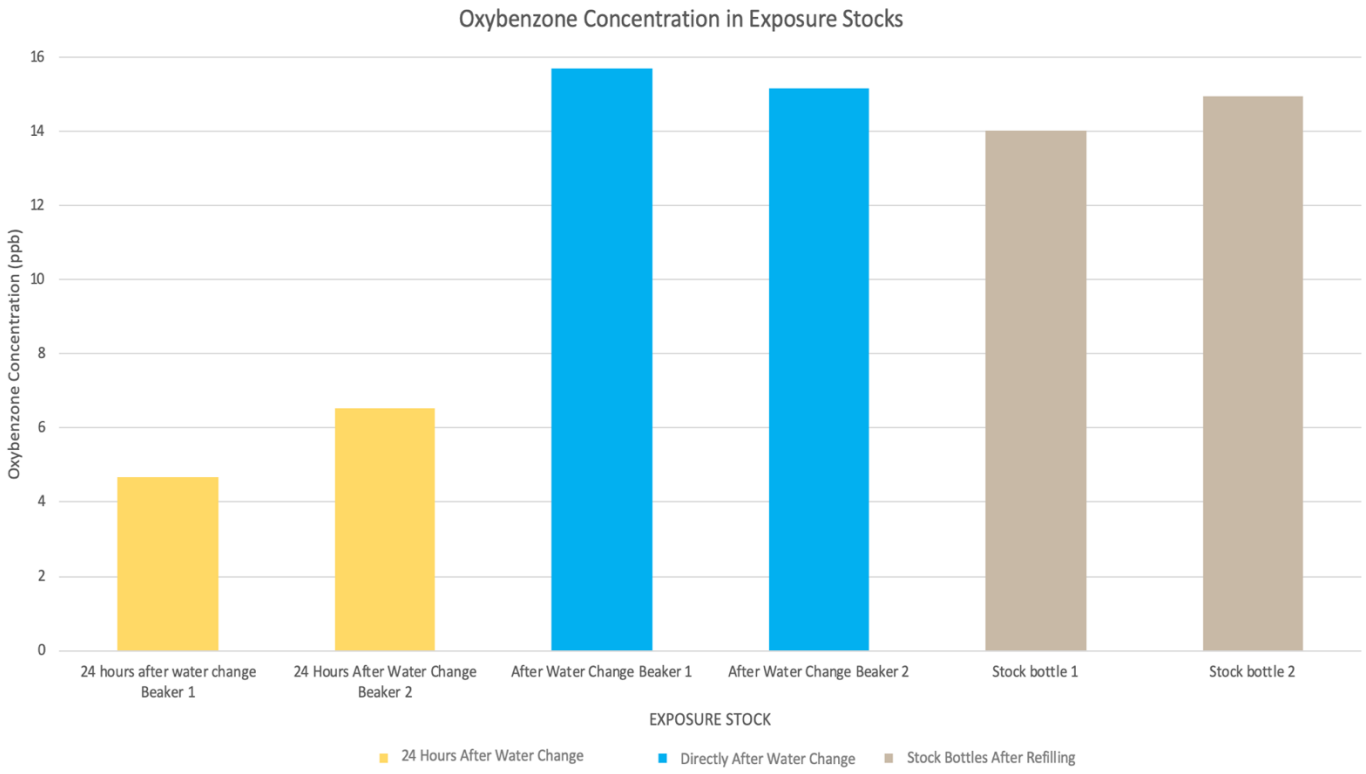

Figure 5. Water Samples Taken to Determine Oxybenzone Concentration in the Stock Water. This graph shows the possible degradation of the oxybenzone chemical after 24 hours in the stock water for the zebrafish seen by the drop-in concentration 24 after a water change.

Analytical Method Changes

Weight Collection

Weights of fish were collected in order to determine their mass specific oxygen consumption for every zebrafish for analysis. It was quickly realized that the method of weight collection was deeply flawed. Before optimizing the weight collection, weight of the fish was collected before they were put in the micro-respirometer for oxygen consumption rate measurements were conducted. This was an issue because fish had to be weighed while still alive. This caused many errors the in-weight data due to excess water on the scale, as well as them perishing in the micro-respirometer and causing the data for that fish to become unusable. After realizing this, fish weights were taken after cold-stunning fish after the respirometry experiment data collection was over, so that extra water surrounding the animal causing a false increase in weight could be minimized.

Respirometry Experiment Length

For this study, the microrespirometer readings were taken over only a 15-minute period. Once the data was collected and analyzed, it became apparent that the time period in which the studies were conducted was not enough time to collect efficient data. It was seen that data collected during this time period had too much noise to be reliable due to the short data collection time. For the future of this study, the data collection period has been extended to an overnight time period of approximately 20 hours for reliable data collection.

Metabolic Effects of Oxybenzone at 10 ppb vs Solvent Control

The results of the comparison between the respiration data rates of the solvent control and the treatment of semi-chronic oxybenzone exposure at 10ppb did see an effect but it was not considered statistically significant due to errors and noise in the data.

Physiologically this trend of an increased metabolic rate does indicate an added stressor towards the zebrafish, and could be explained by an adaptive response exhibited by the embryo-larval zebrafish due to the exposure of oxybenzone at 10ppb to increase their metabolic rate.

CHAPTER V

CONCLUSION

After the comprehensive efforts to optimize the exposure design of the toxicology trials of oxybenzone effects on zebrafish, key methodology changes were implemented. These included the change in zebrafish diet from paramecia to live rotifers, change of dosing from semi-static renewal only, to a combination of semi-static renewal and flow through dosing, and placing the 53 μm mesh filter at the outflow to each beaker.

These changes made marked improvements in the survival success of zebrafish (i.e. from 83% mortality to 17% mortality only). In the most recent exposure trial oxybenzone was found to have a trend of an increased metabolic rate, but was not statistically significant.

This project will continue be further studied in order to find an answer with reliable data by taking all of the optimized methodology and putting it into action. Additional trials are planned to improve upon the micro-respirometry analysis. These improvements will include acquiring respirometry data for longer, i.e. few hours vs. few minutes, to allow sufficient time to detect changes in oxygen consumption rates. In addition, RNA-sequencing analyses will also be performed to assess the effects of oxybenzone exposure on the whole-organism transcriptome of exposed early life-stage zebrafish.

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APPENDIX

Appendix A.
Concentrations of Oxybenzone in Coastal Areas Around the World (Elizabeth Wood, 2018)\

SITE	SAMPLING LOCATION	OXYBENZONE CONCENTRATION
CHINA		
Hong Kong (Tsui et al., 2017)	4 locations; wet and dry seasons	12.9 - 31.9 ng L-1
JAPAN		
Okinawa (Tashiro and Kameda, 2013)	Shallow water 300-600m from beach	0.4 - 3.8 ng L-1
HAWAII (DOWNS ET AL., 2015)		
Oahu Island; Maunalua Bay May 2011	4 sites; 35cm depth	Detectable >100 ng L-1; below quantitative range of 5 µg L-1
Maui Island: Kapalua Bay May 2011	1 site; 35cm depth Often >500 swimmers	1 site: 19.2 µg L-1 Detectable >100 ng L-1; below quantitative range of 5 µg L-1
PALAU (BELL ET AL., 2017)		
Palau: Jellyfish Lake water. Jan 2016	8 samples. Tourist location: visited by tens of thousands of people. Marine lake water	4.12 - 10.2 ng L-1 in 3 samples. Not detectable in 4 samples; present but not measurable in 1 sample.
Palau: Jellyfish Lake, from inlet by tourist dock: 8 samples Jan 2016.	8 samples. Marine lake water by tourist dock	4.99 - 5.36 ng L-1 in 2 samples. Not detectable in 6 samples
Palau: Outside Jellyfish Lake near outside dock: Jan 2016.	4 samples. Not a swimming area	Not detectable in any of 4 samples
Palau: Ngermeuangel Lake water. Jan 2016.	8 samples. Marine lake water. Intended as control site away from tourist areas	4.4 to 18.5 ng L-1 at 3 sites; not detected at 9 sites
Palau: lagoon outside entrance to Ngermeuangel Lake: Jan 2016.	4 samples. Not a swimming area	Not detectable in any sample
Palau: Ocean outside Lighthouse Reef	4 samples: control site	Not detectable in any sample
US VIRGIN ISLANDS: CARIBBEAN SEA		
Trunk Bay 2007 (Downs et al., 2015)	Shallow water adjacent to reef. 180 people in water prior to sampling	580µg L-1 - 1.395 mg L-1
Trunk Bay June 2013. (Bargar et al., 2015)	2 samples at shoreline, 1 sample 30m from offshore island at 1m depth	1,943 – 4,643ng L-1
Trunk Bay June 2014. (Bargar et al., 2015, Fig 3 data supplied by Bargar)	shoreline at <1m depth	6,073 ng L-1
Trunk Bay June 2014	60m from shore at <1m depth	1,416 ng L-1
Trunk Bay June 2014	120m from shore at <1m depth	363 ng L-1
Trunk Bay June 2014	220m from shore at <1m depth	116 ng L-1
Trunk Bay June 2014	220m from shore at c 3m depth	0 ng L-1
Hawksnest Bay 2007 (Downs et al., 2015)	Shallow water. 230 people in water prior to sampling	75 - 95 µg L-1
Caneel Bay 2007	Shallow water. 17 swimmers in 48hr prior to sampling	Not detectable
SOUTH CAROLINA: NORTH WESTERN ATLANTIC		
Folly Beach S Carolina 2010 (Bratkovics and Sapozhnikova, 2011)	4 sites. Shallow water 1.5-2m from water line	10 – 2,013 ng L-1
S Carolina local Beach Front Park (Bratkovics et al., 2015)	Shallow water	max 2,200 ng L-1
CANARY ISLANDS: EASTERN ATLANTIC (SÁNCHEZ RODRIGUEZ ET AL., 2015)		
Gran Canaria Island May to Oct 2011	3 'semi-enclosed' beaches at 1 - 1.5m depth	12.7 – 3,316 ng L-1
Gran Canaria Island May to Oct 2011	3 'open' beaches at 1 - 1.5m depth	<1.4 - 182.6 ng L-1

The chart shown in Appendix A from the 2018 paper by Elizabeth Wood was a huge contributor to the decision that we would test a oxybenzone concentration of 100ppb. The chart shows oxybenzone concentrations on various coastlines in multiple locations around the world. This alone shows that oxybenzone as an emerging pollutant is a worldwide phenomenon and should be a cause for concern.