ABSTRACT

Biological Responses from Contaminants Accumulated in Seafood Using an *In Vitro* Human Intestinal and Liver Co-Culture

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*In vitro* bioassays have been useful in predicting mechanisms of toxicity; however, conventional cell-based assays grown in monolayers are unavoidably poor models for human tissues due to the lack of complexity and physiological interplay observed *in vivo*. To address these limitations, the present study utilizes a combination of human intestinal and hepatic cells in a co-culture model. The purposes of these projects are to evaluate the differences between mono- and co-culture systems related to cytotoxicity and enzyme activity, and apply the co-culture model in the screening of seafood samples collected from the Galveston Bay. It was observed that the co-culture model had greater antioxidant enzyme activity compared to that of the monoculture, suggesting that hepatocytes grown in co-culture may be better suited to facilitate the expression of enzymes in response to xenobiotic metabolism in intestinal cells. This emphasizes the importance of adequate model selection to facilitate assessment of risk.

Biological Responses from Contaminants Accumulated in Seafood Using an *In Vitro* Human Intestinal and Liver Co-Culture

by

Grace E. Sutherland, B.S.

A Thesis

Approved by the Department of Environmental Science

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George P. Cobb, Ph.D., Chairperson

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# DEDICATION

To God, first and foremost, for his grace and mercy through this time, and for the many blessings I have been given. To my mother and father, for being my rock and my refuge, and for believing in me even when I do not believe in myself. To Doug, Erica, and Adam, who continue to set the bar higher and challenge me to go further. You, my family, are to whom I owe all my success. And finally, to the love of my life, Quinton Ross Cook, whose patience and unwavering support have made this degree possible.

# ATTRIBUTIONS

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Grace E. Sutherland – design of study, growth and maintenance of cellular cultures, performance of bioassays, statistical analysis, and writing of manuscript.

Marco E. Franco – writing of manuscript.

Sarah M. Willing – maintenance of cellular cultures and performance of bioassays.

Ramon Lavado – design of study, performance of bioassays, and statistical analysis.

# CHAPTER ONE

## Introduction

*In vitro* studies consist of biological processes or tests that occur outside of a living organism and are designed to occur in a laboratory setting, utilizing controlled experimental environments such as test tubes or culture dishes. *In vitro* bioassays are designed to be short-term assays that can provide mechanistic information of effects, while simultaneously being faster and less expensive than chronic assays *in vivo* (Klaassen and Watkins, 2015). *In vitro* assays can be designed at a cellular or biochemical level, and while they are more mechanistically-specific and less holistic than an *in vivo* model, which looks at the entire organism, they also do not require the sacrifice of large quantities of organisms. While *in vivo* models are ultimately the best surrogate for analyzing human toxicity, the issue of continual animal sacrifice on a large scale represents substantial financial and ethical challenges to scientists that conduct *in vivo* assays. The principles of the 3Rs, Replacement, Reduction and Refinement, are increasingly incorporated into legislations, guidelines, and practices regarding animal experimentation in order to safeguard animal welfare (Törnqvist et al., 2014). The 3Rs are currently incorporated as a key concept for humane use of animals in research into various important legislations in the European Union (EU) (EU, 2010) and they are also implicit in the respective Animal Welfare Acts in the United States (USA, 2012). For this reason, there has been a large push to better understand and enhance the use of *in vitro* assays that evaluate key biological pathways and molecular mechanisms.

*In vitro* methods are becoming more widely used, with their applicability for high-throughput testing, which enables less expensive, rapid screening of a vast number of chemicals and set testing priorities by predicting adverse health effects. Recent publications of frameworks such as the Adverse Outcome Pathway (AOP) framework and the Aggregate Exposure Pathway (AEP) framework established by the U.S. Environmental Protection Agency (EPA) have pushed for an enhanced focus on the use of *in vitro* assays to evaluate key biological pathways and molecular mechanisms linked to human or ecological health risks (EPA, 2003). The EPA-sponsored ToxCast program is a large-scale initiatives to evaluate *in vitro* testing methods and their ability to predict human toxicity (Kavlock et al., 2012). Phase I of the ToxCast program evaluated ~300 common pesticide active ingredients in an array of cell-free and cell-based assays. In Phase II, the chemical list was expanded to include chemicals used in consumer products, industrial processes, and pharmaceuticals.

While *in vitro* methods – classified as bioanalytical tools – have grown in popularity, they have their limitations. In monolayers, several limitations arise from the fact that a single layer of cells grown in a dish is an unavoidably poor model for human tissues. The artificial conditions in which cell culture takes place usually lacks the complexity and physiological interplay that occurs within a living organism. Key biological process such as cellular crosstalk and metabolism can be lost or overlooked when using a monoculture. Researchers have begun to address these limitations by developing an approach in which multiple cell types are cultured together.

These developing approaches for co-culture of multiple cell types or cultures of whole organs as slices or cell aggregates are collectively referred to as organotypic models.

Some of these models, such as organs-on-chips and three-dimension models, can become so complex and intricate that they lose their ability for high-throughput testing due to their increased cost and time requirements. An organ-on-a-chip is an advanced 3D tissue engineered construct that includes microfluidic systems to recreate physiological length scales, concentration gradients, and mechanical forces from fluid flow to replicate *in vivo* microenvironments (Bhise et al., 2014). This model has been adapted to several organs such as liver, kidney, heart, gut, breast, and blood vessels, and has the benefit of generating responses similar to those observed *in vivo* due to the complexity of the microenvironment produced. Unfortunately, that same complexity also limits this model in the application of high-throughput testing, as an increase in robustness leads to decreases in throughput.

Co-cultures on the other hand use two or more different cell types in the same system with the help of inserts. This model provides an extra level of complexity without losing the high-throughput capabilities. Co-cultures have been used to mimic different human systems, including the blood-brain barrier - culturing astrocytes and brain capillary endothelial cells together (Gaillard et al., 2001), lungs – culturing epithelial cell line Calu-3 with the endothelial cell line EA.hy926 together with macrophage-like THP-1 cells to mimic the bronchial barrier (Zhang et al., 2019), and skin – culturing keratinocytes over fibroblast-populated dermal matrices (Sriram et al., 2015). Co-cultures have also been used in replicating the digestive system, where intestinal cells are combined with other cells of different origin, including hepatic cells. This model is particularly important for humans, as the liver is the most metabolically-active organ, and responsible for the detoxification of xenobiotics. This digestive model has the potential to be applied to a variety of contaminants of concern, especially those found in food.

One example of the application of this model is to study metabolism and toxicities associated with chemical mixtures potentially found in food. The health benefits of a diet high in fish are well-known, including positive effects such as reduced coronary heart disease, antiarrhythmic results, and general improved heart function (Mozaffarian and Rimm, 2006). There are also significant health concerns associated with eating fish exposed to contaminants. Trophic transfer refers to the ability of some chemicals to move through a food web. This is concerning when coupled with biomagnification, or the ability of a compound to increase in concentration from one trophic level to the next (Rasmussen et al., 1990). There has been significant research on the human health effects of persistent organic pollutant and mercury exposures from fish consumption. However, there is little known about the risks of many other chemicals now routinely detected in fish. In response to known contaminant concerns, fish consumption advisories are already common in Texas. Currently, 28 advisories in effect (TDSHS, 2019) ban seafood consumption due to Mercury, Dioxins, Dieldrin, and Polychlorinated biphenyls (PCBs) content (TDSHS, 2017). However, there are many other chemicals present in aquatic ecosystems that pose possible health risks when ingested by humans. Additionally, aquatic systems are complex matrices, and so it is also unlikely that these compounds occur isolated, leading to the concern of mixture effects.

The movement towards greater utilization and development of *in vitro* systems is well documented. Continuation of these high-throughput systems is integral in the advancement of predictive models, while increasing the development of more complex systems such as co-cultures will increase accuracy and better mimic *in vivo* conditions. The purpose of this work is to utilize a well characterized co-culture model that recapitulates

first pass effect of human digestion to characterize toxicological effects associated with consumption of chemically contaminated seafood. This was done in two parts; first the effectiveness of the co-culture model was examined using well characterized seafood Standard Reference Material (SRM) from the U.S. National Institute of Standards and Technology (NIST), then the co-culture model was applied to evaluate seafood sampled from Galveston Bay. This exploratory study associated with human dietary exposure has the potential to provide information not just for seafood, but other areas of environmental public health that lack sufficiently detailed toxicology data and information.

*Objectives*

1. To determine differences of effect between monoculture and co-culture systems, and how results might change depending on which system is utilized. In order to do this, monocultures and co-cultures of intestinal and hepatic cells were used and exposed to different concentrations of fish and oyster tissue Standard Reference Material (SRM) after chemical extractions. Cytotoxicity and antioxidant enzyme responses were measured as a proxy for the formation and activity of reactive oxygen species (ROS) and the subsequent potential for oxidative stress.
    a. Hypothesis: It was initially hypothesized that cytotoxic and enzymatic responses to SRM would be lower in the hepatocytes grown in co-culture compared to hepatocytes grown in monoculture, since in the co-culture system there is a protective barrier element from intestinal cells.
2. To determine effects of pollutant mixtures present in seafood. Its safety was assessed in terms of cytotoxicity and antioxidant enzyme production as markers for oxidative stress. Determination of the main organochlorine pesticides (OCPs) and

polychlorinated biphenyl (PCBs) content in environmental fish and oyster samples collected from Galveston Bay was completed through analytical chemistry.

a. Hypothesis: The initial hypothesis was that fish and oyster extracts containing higher levels of PCBs and pesticides would induce greater toxicity and antioxidant enzyme activity.

# CHAPTER TWO

## Applicability of a Human Cell Co-Culture Model to Evaluate Antioxidant Responses Triggered by Chemical Mixtures in Fish and Oyster Homogenates

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*Abstract*

The accumulation of chemical compounds in fish tissue represents significant health concerns for seafood consumers, but little is known about the risks to human health associated with such substances. The identification of adverse biological responses upon exposure to contaminants has been facilitated by the development of *in vitro* systems resembling the human dietary pathway. The present study explores the applicability of an organotypic co-culture system, using intestinal (Caco-2) and hepatic (HepaRG) cell lines, to provide insight into the toxicity of chemical mixtures found in commercially available seafood. Chemical extractions were conducted utilizing fish and oyster standard reference material (SRM) from the U.S. National Institute of Standards and Technology (NIST). Cells were seeded in monoculture and co-culture systems and exposed to SRM extracts before measurements of cytotoxicity and antioxidant responses. Exposure to oyster extracts led to significant cell mortality in monocultures. HepaRG cells in monoculture expressed lower levels of glutathione peroxidase and superoxide dismutase than HepaRG cells in co-culture, upon exposure to both oyster and fish extracts. These observations illustrate the importance of organotypic co-culture models to explore biological responses that could be

otherwise difficult to evaluate in monocultures, and the adverse effects associated with the consumption of contaminated seafood

## *Introduction*

Seafood is known to be the source of many health benefits due to its high nutrient, protein and low-fat contents (Sidhu, 2003). However, fish are among the organisms with higher probability of exposure to environmental contaminants, raising significant health concerns associated with the consumption of contaminated seafood. Trophic transfer allows certain chemicals to move, bioaccumulate, and biomagnify through a food web, increasing their concentration from one trophic level to the next (Rasmussen et al., 1990). Some chemicals of concern associated with seafood include persistent organic pollutants (Fisk et al., 2001), methylmercury (Mason et al., 1996), microplastics (Farrell and Nelson, 2013), halogenated flame retardants (Law et al., 2009; Su et al., 2017), personal care products (Ramirez et al., 2009), and pesticides (Dromard et al., 2018; FAO, 2012; Weston et al., 2004).

In the early 2000's, toxicity testing was a significant matter in science with the development of frameworks highlighting the use of *in vitro* assays to evaluate key biological pathways and molecular mechanisms linked to human disease and exposure to contaminants (EPA, 2003). The EPA-sponsored ToxCast program (Kavlock et al., 2012), the Tox21 program (Krewski et al., 2009), and the European ACuteTox program (Clemedson, 2008) are specific examples of large-scale initiatives to evaluate *in vitro* testing methods and their ability to predict human toxicity.

*In vitro* bioassays have been useful in predicting mechanisms of toxicity; however, conventional cell-based cytotoxicity assays typically lack transport and metabolic

competence, and often overlook chemicals that require bioactivation. Many of these limitations arise from the fact that cells growing in monolayers are unavoidably poor models for human tissues because artificial *in vitro* conditions lack the complexity and physiological interplay associated with *in vivo* assays. To address these limitations, researchers have developed approaches for co-culture of multiple cell types or cultures of whole organs as slices or cell aggregates. Several *in vitro* models utilizing co-cultures have recently been reported, combining intestinal cells with other cells of different origin, such as neural, pancreatic, hepatic or monocytic cells (Castell-Auví et al., 2010; Rossi et al., 2012; Smith et al., 2018). These co-culture models have been demonstrated to have the ability to resemble organ systems, and allow for the detection of endpoints relevant to transport and metabolism of chemical compounds (Castell-Auví et al., 2010).

In the present study, the human intestinal adenocarcinoma cell line, Caco-2, and the hepatic cell line, HepaRG, were utilized. Caco-2 cells spontaneously differentiate *in vitro*, expressing several morphological and functional characteristics of mature small intestinal enterocytes (Rossi et al., 2012; Sambuy et al., 2005). The HepaRG cell line exhibits particularly unique qualities; confluent HepaRG cells can be differentiated into hepatocyte- and biliary-like cells (Cerec et al., 2007) and contrary to other human hepatic cell lines, including HepG2 cells, HepaRG cells maintain many liver-specific functions including expression of various cytochrome P450 enzymes, nuclear receptors and other cellular functions, such as membrane transport (Aninat et al., 2006; Guillouzo et al., 2007; Smith et al., 2018). Co-cultures of Caco-2 cells with hepatic cells have been previously reported in pharmacological studies (Castell-Auví et al., 2010; Rossi et al., 2012; Smith et al., 2018); however, the direct applicability of this co-culture model to evaluate the interplay between

intestinal and hepatic activity associated with exposure to bioaccumulated chemicals in fish and oyster homogenates is novel and, to the best of our knowledge, has not yet been investigated.

Besides utilizing a well characterized co-culture model that replicates human digestion to further characterize toxicological effects associated with consumption of contaminated seafood, the present study explores cytotoxicity and antioxidant enzyme responses as a proxy for the formation and activity of reactive oxygen species (ROS) and the subsequent potential for oxidative stress. The rationale for investigating these responses arises from the ability of many chemical compounds to undergo redox cycling and generate ROS (Di Giulio et al., 1989) and, especially in cell cultures, the fact that exposure to different pollutants often results in the expression of antioxidant enzymes due to the presence of ROS (Chung et al., 2007; Ghio et al., 2012; Kouadio et al., 2005; Li et al., 2003). This exploratory study associated with human dietary exposure has the potential to provide novel information not just for seafood consumption, but also to other areas of environmental and public health that lack sufficiently detailed toxicology data.

## *Materials and Methods*

### *Chemicals and Solutions*

Standard Reference Material (SRM) were obtained through the U.S. National Institute of Standards and Technology (NIST). The SRMs used in this study were obtained as powder-like tissue homogenates, prepared from American eastern oyster (*Crassostrea virginica*) and fillets of adult lake trout (*Salvelinus namaycush*) obtained from Lake Michigan. Specifically, the SRMs used in the study were 1566b-Oyster Tissue and 1947-

Lake Michigan Fish Tissue. The mass fraction values of chemical elements in tissue homogenates are summarized in Table 2.1. While the oyster SRM only certified mass fractions of elements, the fish SRM certificate of analysis included certified mass fraction values for several environmentally relevant compounds, summarized in Table 2.2. A certificate of analysis was provided with each SRM, and the full list of chemical compounds is publicly available through the NIST website.

Caco-2 cell line and Eagle's Minimum Essential Medium were obtained from ATCC (American Tissue Culture Collection HTB-37, Mannassas, VA). The differentiated cryopreserved NoSpin HepaRG cells, thawing, plating, and base HepaRG medium were purchased from Lonza Inc. (Allendale, NJ). L-glutamine, penicillin G, streptomycin, phosphate-buffered saline (PBS) and trypsin–EDTA were obtained from Gibco Life Technologies (ThermoFisher Scientific, Waltham, MA). HEPES salt (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) was obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from Atlas Biologicals (Fort Collins, CO). Deionized water (DI water) was obtained using a Milli-Q water purification system (Millipore, Billerica, MA).

Table 2.1. Certified Mass Fraction Values for Elements in NIST SRM 1566b Oyster and SRM 1947 Fish.

| **Element** | **Mass Fraction (mg/kg)** *SRM 1566b Oyster* | **Mass Fraction (mg/kg)** *SRM 1947 Fish* |
|---|---|---|
| Aluminum (Al) | 197.2 ± 6.0 | 0.732 ± 0.039 |
| Arsenic (As) | 7.65 ± 0.65 | - |
| Cadmium (Cd) | 2.48 ± 0.08 | - |
| Cobalt (Co) | 0.371 ± 0.009 | - |
| Copper (Cu) | 71.6 ± 1.6 | 0.411 ± 0.029 |
| Iron (Fe) | 205.8 ± 6.8 | 3.79 ± 0.42 |
| Lead (Pb) | 0.308 ± 0.009 | - |
| Manganese (Mn) | 18.5 ± 0.2 | 0.076 ± 0.004 |
| Mercury (total) (Hg) | 0.0371 ± 0.0013 | 0.254 ± 0.005 |
| Methylmercury (as mercury) | 0.0132 ± 0.0007 | 0.233 ± 0.010 |
| Nickel (Ni) | 1.04 ± 0.09 | - |
| Rubidium (Rb) | 3.26 ± 0.14 | 4.51 ± 0.09 |
| Selenium (Se) | 2.06 ± 0.15 | 0.475 ± 0.084 |
| Silver (Ag) | 0.666 ± 0.009 | - |
| Thorium (Th) | 0.0367 ± 0.0043 | - |
| Vanadium (V) | 0.577 ± 0.023 | - |
| Zinc (Zn) | 1424 ± 46 | 2.66 ± 0.08 |
| Total Metals | 1935.599 | 13.141 |

Table 2.2. Certified Mass Fraction Values for PCBs, pesticides, and PBDEs in NIST SRM 1947 Fish

| Compounds | Aproximate Mass Fraction (μg/kg) |
|---|---|
| ΣPCB Congeners | 1,471.88 |
| ΣPesticide | 1,173.13 |
| ΣPBDE | 126.17 |

*Chemical Extraction*

Fish and oyster SRM were extracted using pressurized liquid extraction (PLE), following the method described by Subedi et al. (2013) and Subedi and Usenko (2012). Briefly, a sub-sample of tissue homogenate (5g of fish tissue, 5g of oyster tissue) was dried and further homogenized with 40g of sodium sulfate. The homogenate was placed in a 100-

mL stainless steel body, and extracted with methylene chloride/ hexane (1:1) on an accelerated solvent extraction system (ASE 350 Dionex-Thermo Fisher Scientific, Waltham, MA), with extraction conditions of 100°C, 1500 psi, 5min static time and 290s purge time, and 75% flush volume. A laboratory blank sample was included at the end of each sample batch each day of extraction. This method has shown to have greater than 80% recovery of PCBs and other organic pollutants in both oyster and fish tissues; a list of these compounds can be found in the method section of Subedi et al. (2013) and Subedi and Usenko (2012). Extracts were then concentrated to dryness under a gentle stream of nitrogen using a TurboVapII (Biotage, Charlotte, NC), and stored at −20°C until cell dosing.

With an 80% recovery rate of chemical constituents in the SRM, oyster extracts contained approximately 1.55mg/mL of the total metals and elements listed under the specific SRM in Table 1. Similarly, fish extracts contained approximately 1.05mg/mL of the total metals and elements listed in Table 1, as well as 118μg/mL of PCB congeners, 94μg/ mL of pesticides, and 10μg/mL of PBDE (original concentrations from SRM are presented in Table 2). The highest dose tested in cell culture models was 1% of those concentrations. They were 15.5 μg/mL of metals and elements in the case of oyster SRM; 10.5 μg/mL of metals and elements, 1.18 μg/mL of PCB congeners, 0.94 μg/mL of pesticides and 0.1μg/mL of PBDEs in the case of fish SRM. With the decreasing concentrations of extracts, there is a proportional decrease in contaminants found in the percent of total exposure.

*Cell Culture*

Caco-2 cells were maintained, following manufacturer's instructions, at 37°C in a 5% $CO_2$ air atmosphere in ATCC-formulated Eagle's Minimum Essential Medium, supplemented with 1.78 g HEPES, 100 IU/mL penicillin and 100µg/mL streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). The differentiated cryopreserved HepaRG cells were thawed and plated as recommended by the manufacturer. The cell suspension supplemented with the appropriate amount of thawing and maintenance medium as defined in manufacturer's protocol were seeded in 24-well plates for biochemical studies and seeded in 96-well plates for cytotoxicity determination.

*Co-Culture Model*

When conducting co-culture assays, Caco-2 cells were seeded on polycarbonate filters (Transwell® inserts, 0.14 $cm^2$ area, 0.4µm pore diameter; Corning Inc., Corning, NY) and maintained for 5 days in medium supplemented with 10% FBS in both apical and basolateral compartments. Caco-2 cells were used for co-culture experiments after confluent layers were present on transwell inserts and were transferred to 24-well culture plates containing confluent differentiated HepaRG cells. The inserts contained Caco-2 media that had been dosed with chemical extracts at different concentrations, while the lower chamber held HepaRG base medium and supplement (Fig. 2.1). Dimethyl sulfoxide (DMSO) solvent controls were run in parallel.

*Exposure*

After being blown down to dryness, SRM extracts were resuspended in 50µL of DMSO, with subsequent dilutions occurring at 50% of the previous. Cells grown in

monoculture were exposed in 24-well plates containing 500µL of media and adding 5µL of resuspended extracts, and 96-well plates containing 100µL of media and adding 1µL of resuspended extracts, as to not exceed 1% of solvent exposure. Cells grown in co-culture were dosed in the same manner, with the lower chamber containing 500µL of HepaRG media and the insert containing 300µL of Caco-2 media. Dosing was conducted by adding 3µL of resuspended extract to the insert containing the Caco-2 media. Caco-2 cells grown on the transwell inserts were unable to cross the membrane (Castell-Auví et al., 2010), so any SRM exposure occurring in the HepaRG cells must have passed through the confluent Caco-2 cells. All cells were exposed for 48h, determined from the inability of detecting biomarker signals after 24h of exposure in preliminary studies.

*Cytotoxicity*

Cytotoxicity assays were directly conducted in 96-well plates for Caco-2 and HepaRG monocultures, and in 24-well plates for HepaRG cells in co-culture. Cell viability was measured through the application of a slightly modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-d-phenyltetrazolium bromide] assay. Briefly, 20% of a tetrazolium dye solution were added to each well, and plates were incubated for 4h. Viable cells reduce the tetrazolium dye by activity of NAD(P)H-dependent cellular oxidoreductase enzymes, which reduce the dye to insoluble formazan. The culture media was carefully removed for each well, and formazan crystals where then solubilized by adding a mixture of ethanol and DMSO (1:1). The resulting color was measured by absorption spectroscopy (BioTek SynergyTM H1, Winooski, VT) at 595nm

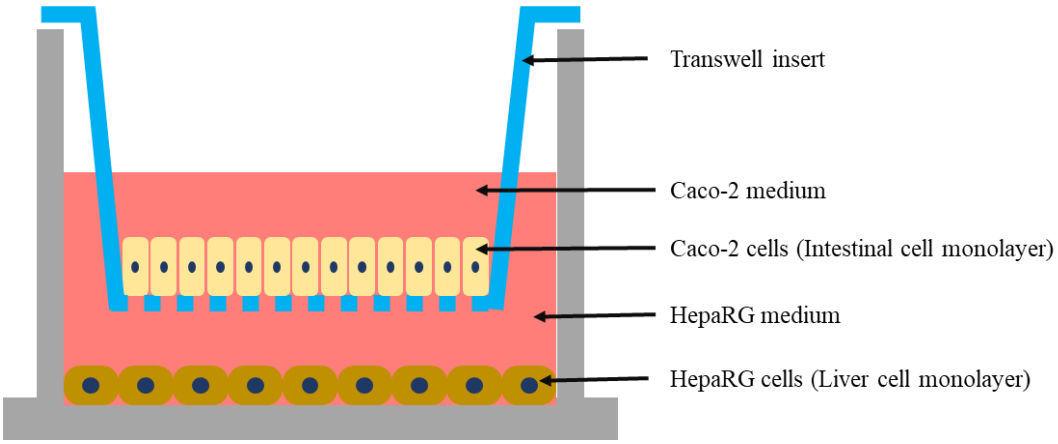


Figure 2.1. Schematic representation of the constructed *in vitro* co-culture system. Modified from Castell-Auví et al. (2010).

*Preparation of S9 Fractions From Cell Cultures*

S9 fractions were prepared as described by (Thibaut et al., 2009) with minor modifications. Cells were washed with PBS and detached from culture plates with 0.05% trypsin-EDTA (ethylenediamine tetra-acetic acid). Cells were then centrifuged at 300g for 5min at 4°C to separate cell pellet from remaining media. Supernatant was discarded, and pellet was resuspended in 100µL of cold homogenization buffer (100mM $KH_2PO_4/K_2HPO_4$ buffer pH 7.4, containing 100mM KCl, and 1mM EDTA). S9 fractions were obtained after centrifugation at 12,000g for 20 min at 4°C and immediately stored at −80°C. Protein concentrations were determined by the Coomassie Blue (Bradford assay) method using a commercial kit (Pierce Inc., Rockford, IL) and bovine serum albumin (BSA) as a standard. Briefly, 7µL of sample was mixed with 100µL of Coomassie Reagent solution (ThermoFisher Scientific, Waltham, MA) containing Coomassie Brilliant Blue G-250 dissolved in 95% ethanol and 85% phosphoric acid. Absorbance was read at 595nm and a standard curve (25, 125, 250, 500, 750, 1000 and 1500 µg/mL) was made with BSA.

*Measurement of Antioxidant Enzymes*

The activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) was measured in S9 fractions using commercial 96-well plate bioassay kits (Cayman Chemical, Ann Arbor, MI). The standard protocols for antioxidant enzyme measurements are publicly available through the Cayman Chemical website. Briefly, CAT activity was measured colorimetrically from the production of formaldehyde with the chromogen 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole at 540nm (Johansson and Borg, 1988). Activity is reported as μmol formaldehyde per minute per mg of protein. GPx activity was measured kinetically for 5min, from a coupled reaction with glutathione reductase, in which NADPH was oxidized to NADP+; absorbance was read at 340nm, and activity is reported as μmol per minute per mg of protein. Total amounts of SOD were quantified by the detection of superoxide radicals after 30min of incubation; absorbance was read at 450nm, and data are reported as nmol per minute per mg of protein.

*Statistical Analysis*

Statistical analyses were carried out using the SPSS v15.0 software package (SPSS Inc., Chicago, IL). Statistical differences between two treatment groups and more than two treatment groups were assessed using Student's t-test and one-way ANOVA, respectively. A p-value of less than 0.05 was considered statistically significant unless otherwise indicated. If an overall significance was detected, Tukey's multiple range tests were performed. Samples showing levels below the detection limits were considered as having 50% of the minimal values detectable only for statistical comparisons. All data were analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests.

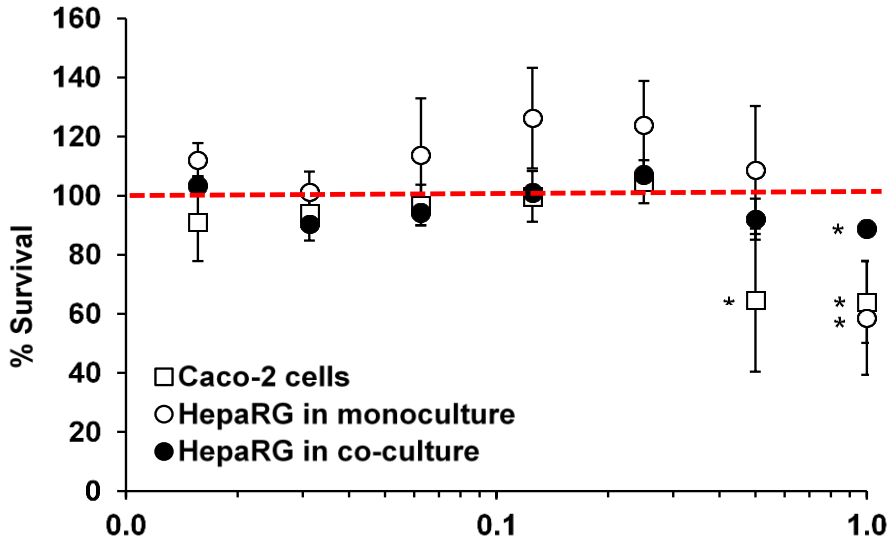
## *Results*

### *Cytotoxicity*

Exposure to oyster extracts (Fig. 2.2.A) led to significant differences in survival between cell lines and controls ($p<0.05$). The highest mortality for all three systems was observed at the highest concentration of oyster extract, denoted as 1% of exposure media. At this concentration, both monoculture systems were the most affected. For other concentrations, Caco-2 cells presented reduced survival (64.62%±24%) at 0.5% of exposure, while HepaRG cells in monoculture and co-culture were less compromised.

On the other hand, survival of cells in monoculture and in co-culture did not appear to be significantly compromised upon exposure to fish extracts (Fig. 2.2.B). No cell line experienced high levels of cytotoxicity, as survival remained above 80% for all concentrations. HepaRG cells in monoculture were observed to remain close to, and at high extract concentrations, above 100% survival; however, these cells presented slight decreases in survival when seeded in co-culture. Although survival of Caco-2 cells was the lowest at 1% of total exposure, these cells did not surpass 20% of mortality. Survival of the different cell lines at low concentrations of exposure were similar to the observations for oyster extracts, where HepaRG cells in monoculture were less compromised than Caco-2 cells and HepaRG from co-culture, even though survival was above 80% for all three systems. Due to the fact that the subsequent biomarkers are of sublethal nature, the higher concentrations of extract were not used in further analyses to prevent lethality from impacting results.

**A.**




**B.**

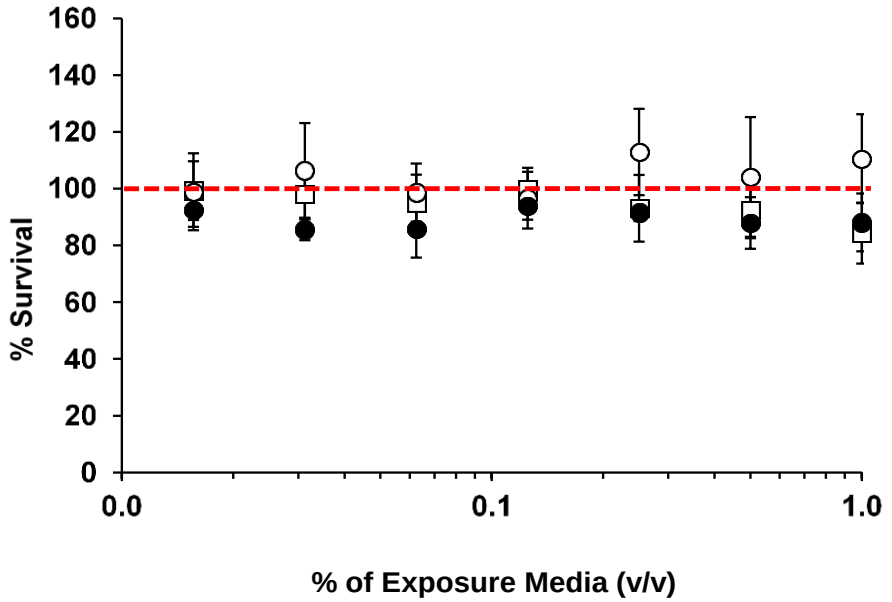



Figure 2.2. Cytotoxic response of the different cell lines: Caco-2 in monoculture, HepaRG in monoculture, and HepaRG in co-culture exposed to (A) oyster extracts and (B) fish extracts. Data are presented as mean ± standard deviation (n=10-12). Significant differences with the controls (unexposed and exposed to DMSO) are indicated as * ($p<0.05$; Student's t-test).

*Antioxidant Enzymes in Caco-2 Cells*

Caco-2 cells exposed to oyster extracts displayed significantly increased levels of CAT activity (Fig. 2.3.A), with approximately a 4-fold CAT induction observed, relative to the controls. However, no significant difference was observed in activity among concentrations of exposure. Similarly, when exposed to fish extracts (Fig. 2.3.B), most of the exposure concentrations yielded greater CAT activity compared to controls, with the only exception being at 0.06% exposure media. The highest CAT activity was observed at the lowest concentration of fish extract, denoted as 0.03% of exposure media.

When exposed to oyster extracts, Caco-2 cells were observed to display a dose-response relationship for GPx activity levels (Fig. 2.3.C), as these continued to increase when the percent exposure became higher. At the highest concentration, being 0.25% of exposure media, Caco-2 cells displayed approximately a 3.5-fold GPx induction relative to unexposed cells. When exposed to fish extracts however, GPx induction was more variable (Fig. 2.3.D). Only the highest concentrations of fish extracts of 0.25 and 0.5% exposure media displayed significantly higher GPx induction compared to that of controls ($p<0.05$).

SOD activity in Caco-2 cells displayed somewhat of a dose-response relationship when exposed to oyster extracts (Fig. 2.3.E). Significantly higher SOD activity is observed at the highest concentration of oyster extracts when compared to both controls and activity at the lowest concentration of exposure media. In contrast, when exposed to fish extracts, no dose-response relationship can be observed for SOD activity in Caco-2 cells (Fig. 2.3.F). Similar to oyster extracts, however, the greatest SOD activity can be observed at the highest concentration of exposure media, with more than 3-fold SOD induction compared to controls.

*Antioxidant Enzymes in HepaRG Cells*

*Catalase activity.* For HepaRG cells in co-culture, CAT activity did not appear to be significantly different across oyster extract concentrations (Fig. 2.4.A), the only exception being CAT activity at 0.06% of exposure media, which is twice that of controls; HepaRG cells in monoculture seemed to display dose-response reductions in CAT activity as total exposure increased.

Exposure to fish extracts (Fig. 2.4.B) led to differing CAT activity in HepaRG cells depending on which system they were grown in. HepaRG cells in co-culture exhibited the highest activity at the lowest concentration of fish extract, denoted as 0.03% of exposure media; however, CAT activity decreased in all other extract concentrations for the co-culture system. CAT activity in HepaRG cells in monoculture remained relatively constant across all treatments.

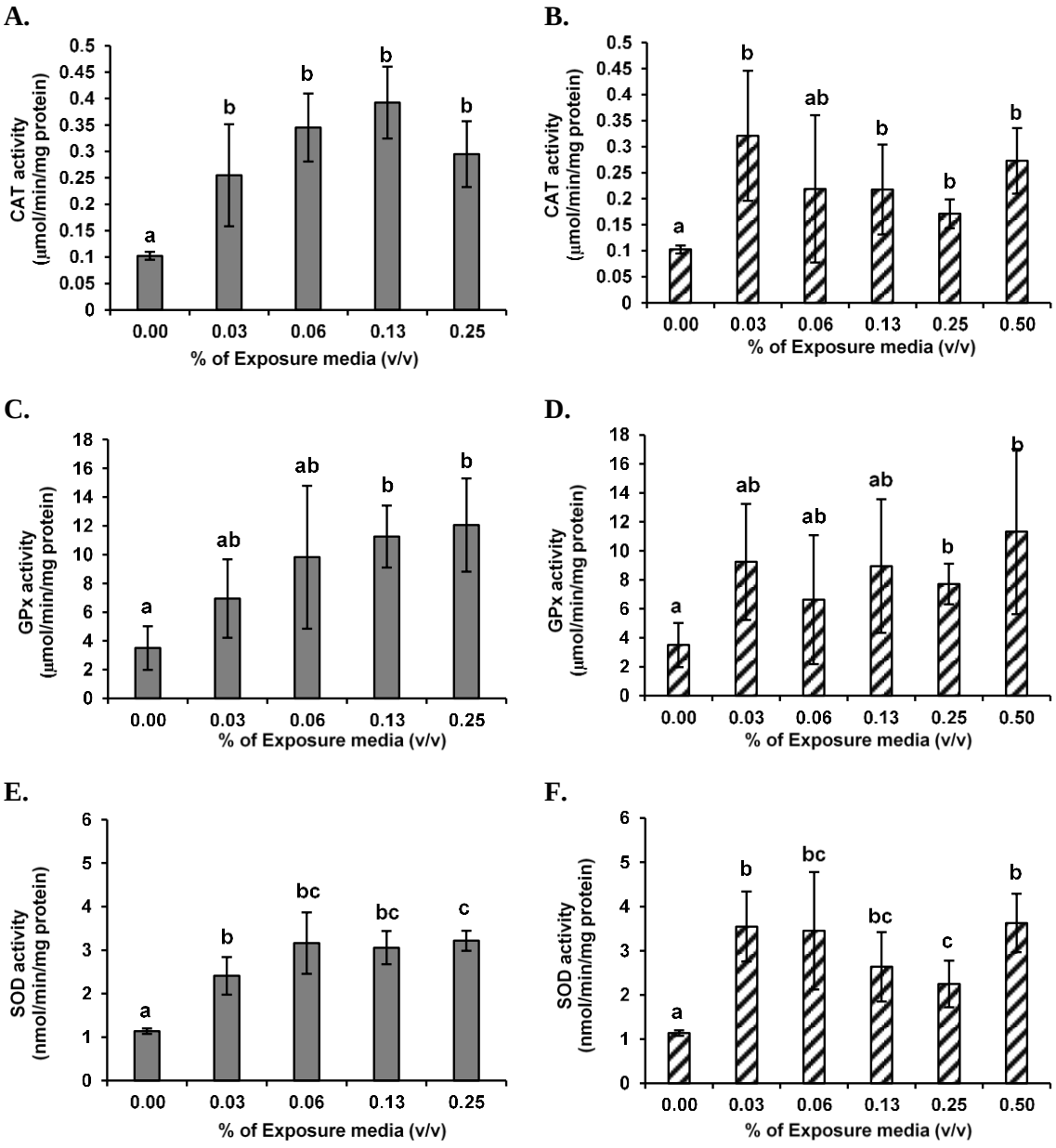



Figure 2.3. Catalase activity (A, B); glutathione peroxidase activity (C, D) and superoxide dismutase activity (E, F) determined in subcellular fractions of Caco-2 cells exposed to different concentrations of oyster (A, C, E) and fish (B, D, F) extracts (reported as mean ± standard deviation; n=6-8). Different letters indicate significant differences between exposure concentrations ($p<0.05$; One-way ANOVA).

*Glutathione peroxidase activity.* HepaRG cells grown in monoculture and co-culture exhibited relatively similar induction of GPx when exposed to oyster extracts (Fig. 2.5.A), with the exception of HepaRG cells at 0.06% total exposure and grown in co-culture, as approximately a 5-fold GPx induction was observed, relative to the controls. No other significant differences were observed between culture methods. HepaRG cells from monoculture maintained a relatively constant GPx activity (approximately twice that of unexposed cells) across all concentrations.

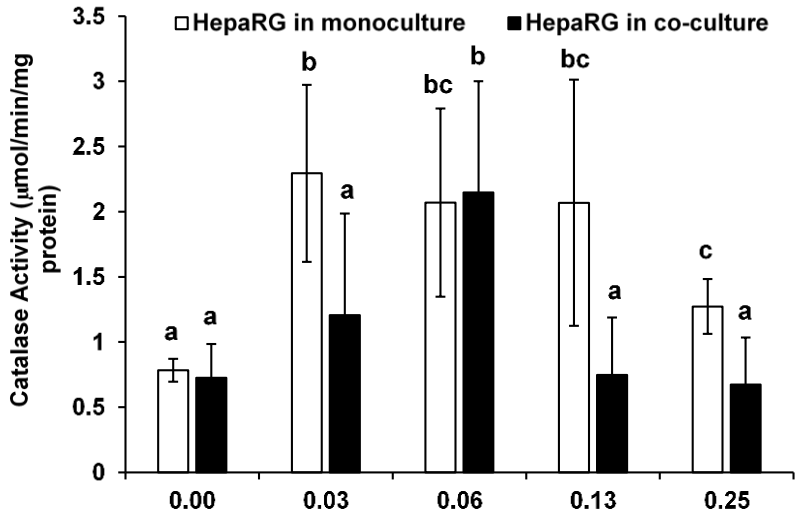
Exposure to fish extracts (Fig. 2.5.B) led to HepaRG cells from the co-culture model displaying higher GPx activity with respect to unexposed cells. Meanwhile, HepaRG cells in monoculture displayed lower enzymatic activity than when in co-culture, with high concentrations of fish extract leading to GPx levels lower than that of controls. HepaRG cells in monoculture did however surpass GPx levels from co-culture when exposed to 0.13% of exposure media, reaching 3-fold induction compared to controls.

*Superoxide dismutase activity.* Exposure to oyster extracts (Fig. 2.6.A) led to significant differences between culture types, where in some instances HepaRG cells grown in co-culture displayed SOD levels twice that of HepaRG cells from monoculture. Low SOD activity from HepaRG cells in monoculture was observed across treatments as these cells maintained a relatively constant SOD activity similar to that of unexposed cells. Furthermore, HepaRG cells from the co-culture model exhibited high SOD activity, with the levels almost reaching four times those of controls at 0.03 and 0.06% of exposure media.

Similarly, the activity of SOD observed in HepaRG cells grown in monoculture and exposed to fish extracts (Fig. 2.6.B) was significantly lower than the activity measured in

HepaRG cells in co-culture, with levels close to the limits of detection. While no distinctive patterns nor dose-response relationships were observed across all concentrations of fish extract, HepaRG cells from co-culture displayed an increased SOD activity compared to controls, with SOD reaching levels twice, and in some instances as high as three times, the basal activity of non-exposed cells.

**A.**




**B.**

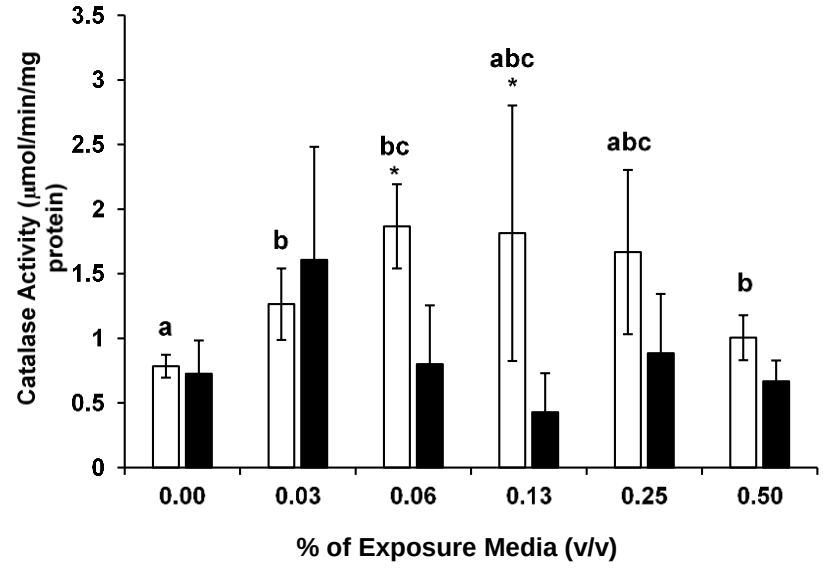



Figure 2.4. Catalase (CAT) activity determined in subcellular fractions of HepaRG cells exposed to different concentrations of (A) oyster and (B) fish extracts (reported as µmol/min/mg protein and as mean ± standard deviation) in monoculture and co-culture models. Significant differences with the controls (unexposed and exposed to DMSO) are indicated as * ($p<0.05$; Student's t-test). Different letters indicate significant differences between exposure concentrations ($p<0.05$; One-way ANOVA).

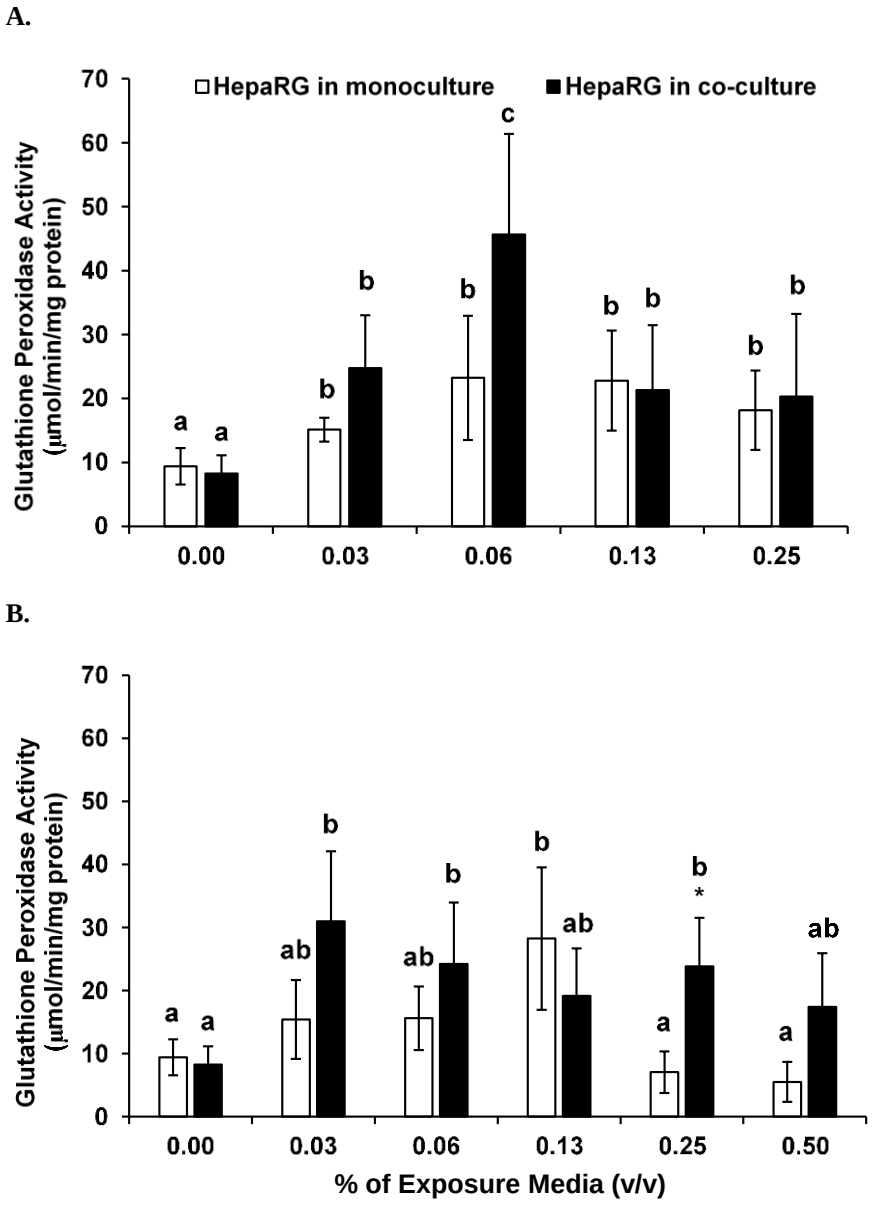



Figure 2.5. Glutathione peroxidase (GPX) activity determined in subcellular fractions of HepaRG cells exposed to different concentrations of (A) oyster and (B) fish extracts (reported as µmol/min/mg protein and as mean ± standard deviation; n=6-8) in monoculture and co-culture models. Significant differences with the controls (unexposed and exposed to DMSO) are indicated as * ($p<0.05$; Student's t-test). Different letters indicate significant differences between exposure concentrations ($p<0.05$; One-way ANOVA).

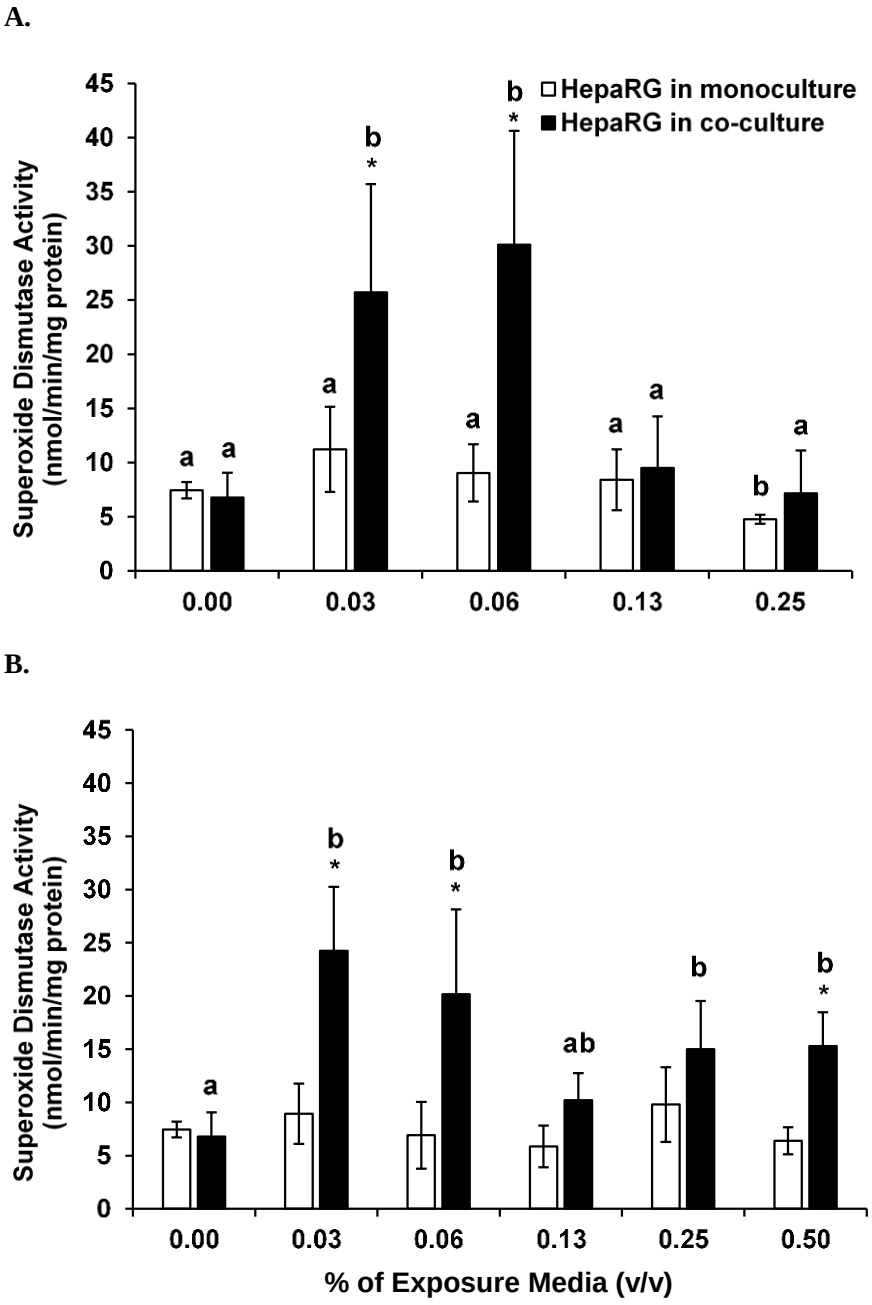



Figure 2.6. Superoxide dismutase (SOD) activity determined in subcellular fractions of HepaRG cells exposed to different concentrations of (A) oyster and (B) fish extracts (reported as nmol/min/mg protein and as mean ± standard deviation; n=6-8) in monoculture and co-culture models. Significant differences with the controls (unexposed and exposed to DMSO) are indicated as * ($p<0.05$; Student's t-test). Different letters indicate significant differences between exposure concentrations ($p<0.05$; One-way ANOVA).

## *Discussion*

In recent years, *in vitro* models have been widely utilized as alternatives to animal testing, leading to significant reductions of animal experimentation (Rusche, 2003). However, most *in vitro* approaches use individual cell lines that are unavoidably poor models for the complexity of *in vivo* conditions. While more sophisticated cell models exist, such as 3D culture systems (Griffith and Swartz, 2006; Kim et al., 2004), organs-on-chips (van der Meer and van den Berg, 2012), and spheroids (Mehta et al., 2012), these systems can be costly and labor-intensive. However, co-culture systems resemble the complexity of *in vivo* systems, while maintaining inexpensive high-throughput technology. The co-culture system that models human digestion and first pass metabolism through the utilization of intestinal enterocytes (Caco-2) and hepatocytes (HepaRG) has been utilized in previous studies (Castell-Auví et al., 2010; Smith et al., 2018) that have contributed to the foundation of co-culture systems. The use of specific end-points such as cytotoxicity and biomarkers like antioxidant enzymes (Yang and Lee, 2015) have also been extensively used to compare co-culture models to those of traditional monoculture layers of these cell lines.

Cytotoxicity is frequently used as a direct measurement of toxicity, and with many xenobiotics being hepatotoxic, it holds relevance to the current study (Niles et al., 2009; Rehberger et al., 2018). While higher mortality was observed in cells exposed to oyster extracts, the relatively high survival rates across all exposure treatments (fish and oyster extracts) may be the result of the metabolizing ability of cells, as toxicity is strongly influenced by biotransformation of xenobiotics (Thibaut et al., 2009). Comparisons between monocultures and co-cultures elucidate the increased levels of mortality in the

former, as cell monocultures exposed to high concentrations of SRM extracts experienced greater mortality than cells from the co-culture system. As seen in previous studies (Kasper et al., 2011), the reduced HepaRG mortality in the co-culture system may be due to a shielding effect from Caco-2 cells, which is absent in monocultures. In this context, the co-culture model appears to be less sensitive to experience toxicity.

Reactive oxygen species (ROS) can be deleterious to cells by way of DNA and protein damage (Simon et al., 2000), as well as being linked to effects on the liver, such as inflammation and even fibrosis (Poli, 2000). Organisms are equipped to cope with oxidative stress through a variety of different antioxidant enzymes. Catalase, glutathione peroxidase and superoxide dismutase are among the most important antioxidant enzymes protecting tissue from oxidative damage (Wijeratne et al., 2005), and their activity has been well documented in Caco-2 and HepaRG cells (Antherieu et al., 2013; Josse et al., 2008; Wijeratne et al., 2005). While these enzymes are commonly involved in the removal of ROS in most cell types, their levels of protection are likely to differ as the activity of these enzymes are organ- and cell-specific. Wijeratne et al. (2005) highlight the ability of the liver to express and maintain an encompassing suite of antioxidant enzymes due to its constant metabolic activity, allowing it to handle oxidative metabolism of a variety of substrates on a regular basis. On the other hand, intestinal cells can be overwhelmed by ROS, since this organ does not engage extensively in such metabolic processes, and therefore contains lower amounts of antioxidants.

In the present study, Caco-2 cells exposed to both fish and oyster extracts had significantly lower CAT activity than HepaRG cells grown in monoculture and in co-culture. These observations are in line with previous studies showing Caco-2 cells to

preferentially rely on GPx to degrade peroxides than on CAT (Wijeratne et al., 2005; Zodl et al., 2003), thus resulting in minimal to absent CAT activity from Caco-2 cells. Contrarily, HepaRG cells had higher CAT activity than Caco-2 cells after exposure to fish and oyster extracts, independently of their culture condition (monoculture vs co-culture). These observations are likely the result of liver cells generally being better equipped to metabolize xenobiotics (Antherieu et al., 2013; Antherieu et al., 2012).

Caco-2 cells appear to significantly express GPx upon exposure to SRM extracts, suggesting the expression of this enzyme to be a major antioxidant protection mechanism in intestinal cells. This relationship is not observed in HepaRG cells. A bell-shaped response can be seen for CAT, GPx, and SOD in HepaRG cells grown in co-culture when exposed to oyster SRM but does not display a dose-response trend when exposed to fish SRM. This may be the result of the metal content found in the oyster SRM that is absent from the fish SRM, as metals are known to be strong inducers of ROS production. On the other hand, HepaRG cells grown in monoculture exposed to oyster SRM displayed greater viability than in co-culture, indicating the possibility of a proliferation of HepaRG cells, similar to a hormesis effect where specific doses of SRM extract induced cellular division. This could potentially elucidate a link between viability and oxidative stress induction, being cell division a response to ROS production. However, since ROS was not directly measured, these conclusions requires further experimentation. The significantly lower GPx activity observed in HepaRG cells grown in monoculture compared to that of HepaRG cells grown in co-culture suggests that the activity of Caco-2 cells may support the formation of metabolites with the potential to cause GPx expression in HepaRG cells. Exposure of the cells seeded in the lower level of a co-culture model (HepaRG cells in this

study) to metabolites from the above layer (Caco-2 cells) has been observed in previous studies (Castell-Auví et al., 2010; Rossi et al., 2012), and support the observations in the present study. This has broad significance for *in vitro* experimentation as cells in monocultures loose the interaction with other cell types taking place in organ systems. If the ability of Caco-2 cells to modify the nature of the chemical compounds holds true, the activity of GPx in monocultures would be underestimated compared to the activity in more complex biological systems, however, further experimentation is required to support this explanation.

Additionally, in liver cells, the induction of GPx could be associated with an overexpression of SOD, as pointed out by Perez-Pertejo et al. (2008). This potential interaction between antioxidant enzymes may also be responsible for the observed SOD activity, as HepaRG cells in the co-culture system exhibited higher activity than HepaRG cells in monoculture. This suggests that hepatocytes grown in co-culture systems may be suited to facilitate the expression of GPx and SOD enzymes more efficiently, in response to xenobiotic metabolism in intestinal cells. As expressed by Rossi et al. (2012), Smith et al. (2018), and Castell-Auví et al. (2010), the intestinal-hepatic cell co-culture model is an excellent bioanalytical tool to further the concept of the 3Rs and the ability of mechanistically evaluate the effects of different stressors over biological organisms. The observed differences in the present study regarding cell mortality and expression of antioxidant enzymes between monocultures and co-cultures have broad significance in the investigation of mechanisms of toxicity taking place in organ systems, and given the necessity of developing *in vitro* models that resemble biological systems, the use of co-culture results are promising for the continued support of alternatives to *in vivo* testing.

## *Conclusion*

The present study demonstrated the importance of considering cell interactions in the development of *in vitro* systems to evaluate mechanisms of toxicity and support programs directed to evaluate adverse effects of environmental contaminants. While monocultures have been successfully applied in the fields of pharmacology and toxicology, the development of more complex systems may facilitate the understanding of whole organ systems, and their ability to cope with exposure to xenobiotics.

The utilization of *in vitro* systems with high-throughput capabilities is integral in the advancement of predictive models, and also represents suitable bioanalytical tools to support the 3R principle. Considering the complexity associated with choosing an appropriate cell culture method, such as mono- and co-culture systems, is fundamental for appropriate experimentation, as cell-based bioassays do not always respond in the same way. In some cases, and as demonstrated in the present study, co-culture systems may be better suited for certain bioassays than monocultures. Additionally, there is a need to better understand the effects of contaminant mixtures to which humans could be potentially exposed. Current experimentation aims to employ the described co-culture model to investigate the toxicity of chemicals bioaccumulated in seafood. In such cases, a co-culture system that uses intestinal and liver cells may be proven advantageous in the description of the risk brought by seafood consumption, and its impact on environmental and public health.

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# CHAPTER THREE

## Measurement of Oxidative Potential in a Human Cell Co-Culture Model After Exposure to Chemical Mixtures in Seafood from the Galveston Bay, Texas.

### *Introduction*

The safety of seafood in Galveston Bay has been an ongoing issue, with fish consumption advisories already in place. Estuarine habitats along the Gulf of Mexico provide critical nursery habitat for many ecologically, commercially, and recreationally important fisheries; however, there are ~1,400 industrial and municipal wastewater discharges into the Galveston Bay Watershed, accounting for ~60% of the wastewater discharge in Texas (TCEQ, 1995). This is of particular concern to the resident communities around Galveston Bay that more heavily utilize and rely on its natural resources. Additionally, there are regional economic impacts from fish contamination and subsequent health warnings and advisories, for both commercial and recreational stakeholders. This significant wastewater discharge and increasing number of contaminated bayous directly impact fish and invertebrate populations, ultimately threatening the Galveston Bay fisheries industry.

Many chemicals of concern biomagnify, leading to much higher concentrations in higher trophic level fish, like those favored by recreational fishermen. Recreational fishing in Galveston Bay accounts for $152.1 million of economic activity annually and contributes $87.2 million to the Texas economy (Ropicki et al., 2016). It is in this way that the seafood industry has more than just an environmental impact, but also a social and

economic influence. If compounds of concern are being stored in fish tissue and are accumulating in higher trophic fish – the same fish that are common targets for recreational fisherman and for human consumption – there should be a logical concern for those individuals consuming these contaminated fish.

Red drum (*Sciaenops ocellatus*) have been seen to exceed thresholds set by the Texas Department of State Health Services on total PCDD/PCDF, PCBs, and Mercury (Stunz and Robillard, 2011). Additionally, the Texas Department of State have issued advisories on all species of fish in the Houston Ship Channel for men and women of all ages to avoid consumption (DSHS, 2019), as well as an advisory for spotted seatrout (*Cynoscion nebulosus*) in the upper bay for women of childbearing age and children younger than 12 years old to avoid consumption, with an additional restriction of one meal per month for women past childbearing age and adult men (DSHS, 2013). However, economically disadvantaged communities that disproportionately rely on seafood as a main food source may not be able to afford abiding by these advisories. There is a need to better understand the risks of contaminants to commercial and recreational fisheries, specifically threats to the health of the humans that consume them.

*In vitro* bioassays have been useful in predicting mechanisms of toxicity, and are commonly used in the safety screening process for old and new chemicals (Kavlock et al., 2012). However, conventional monolayer cell-based assays typically lack the complexity associated with *in vivo* conditions, and often fall short in areas concerning transport and metabolism. This can lead to misinformed decisions concerning chemicals that require bioactivation. One solution to this problem of complexity is the use of a co-culture model, in which multiple cell types are cultured together. This model allows for several key

characteristics that occur *in vivo*, such as cellular crosstalk and metabolic activation of compounds. One such model that has recently been utilized in literature is a co-culture of intestinal cells and hepatic cells to characterize digestive activity (Castell-Auví et al., 2010; Rossi et al., 2012; Smith et al., 2018; Sutherland et al., 2019). These co-culture models have demonstrated the ability to resemble organ systems and allow for the detection of endpoints relevant to transport and metabolism of chemical compounds.

Knowing that co-cultures can provide more insight into biological effects related to metabolism and transport, the following chapter continues to utilize the same human co-culture model presented in chapter two (Sutherland et al., 2019), containing Caco-2 cells on transwell inserts, and HepaRG cells in the lower chamber. This model is used to examine effects associated with consumption of local seafood, sampled from Galveston Bay.

Red drum are popular game fish from Massachusetts to Mexico, with an average of 225,000 individuals caught by Texas anglers each year (Spiller, 2006). Red drum are regulated by local authorities, with a daily bag limit of three, and minimum-maximum slots of 20-28 inches for Texas anglers (TPWD, 2019); however, one red drum over the maximum length limit may be retained per year, if properly documented and affixed with a state regulations tag. Adults spawn in Gulf waters, but juveniles live in bays for the first few years of their life before returning to the Gulf. Adult red drum can live up to 40 years and weigh around 80 pounds (Spiller, 2006) feeding on other fish, shrimp, and crabs. This is an important piece of their life history because the longer a fish lives, the more time it has to accumulate chemicals and store contaminants in tissue – a concern for those who might eat this tissue.

Similar to the red drum is its relative, the black drum (*Pogonias cromis*), which is also a common target for anglers. Texas Hunting and Fishing Regulations prescribe a daily bag limit of five black drum per person, with minimum-maximum slots of 14-30 inches (TPWD, 2019). Black drum will spawn in either the bay or the Gulf, and usually weigh between 30 and 40 pounds as adults (TPWD, 2004a), feeding on small crabs, worms, mollusks, and other small fish. In addition to these two fish is the spotted seatrout, another common target for anglers. A minimum-maximum slot of 15-25 inches is required to keep a spotted trout, with a daily bag limit of 5 or 10 if it is caught south or north of highway 457 (TPWD, 2019). Males and females range from 19 to 25 inches long, respectively, and weigh two to three pounds on average, feeding on small crustaceans, shrimp, and smaller fish. Adults spawn in the coastal bays, and remain in this shallower water during the spring and summer, but move out to the Gulf as temperatures decline (TPWD, 2004b).

These characteristics in their life history make red drum, black drum, and spotted seatrout desirable targets for recreational fisherman in Texas. They simultaneously mark these species as concerning for human consumption, as all three fish species are piscivorous, and have the potential to accumulate chemicals of concern through trophic transfer. For these reasons, the three fish described above were used in the following study, along with the popular commercial invertebrate, the American oyster (*Crassostrea virginica*).

The United States produced $192 million worth of oysters in 2016, with 21% of that harvest coming from the Gulf of Mexico (NOAA, 2017). Oysters are filter feeders, eating plankton and any available matter in the water column. A single oyster can filter between 20 and 50 gallons of water every day (Weaver et al., 2018), making them

susceptible to bioaccumulation. In addition to human consumption, the American oyster is also a common food source for black drum (TPWD, 2004a), and is included in the following analyses.

The purpose of this study is to evaluate the interplay between intestinal and hepatic activity occurring in the co-culture model after exposure to bioaccumulated chemicals in fish fillets and oyster tissue. Many chemical compounds, including PCBs, undergo redox reactions that can generate reactive oxygen species (ROS) (Oakley et al., 1996), and by measuring the cytotoxicity and antioxidant enzyme responses, a greater understanding of the effects associated with human dietary exposure to these compounds can be gained. By further understanding these effects, a more thorough perspective can be taken in regard to risks associated with environmental and public health.

## *Materials and Methods*

### *Site Selection*

Fish were collected from three different regions within Galveston Bay, as depicted in Figure 3.1. Sampling site A was used as a reference site since it is farthest away from developed areas, and closer to the open Gulf. Site A is south of the Bolivar Peninsula, located off of the north jetty. Site B is south of Eagle Point, with large residential communities located bellow the sampling sight. Sampling site C is host to the traffic of shipping tankers and barges, and is located in the upper San Jacinto Bay, just north of state highway 146. This is where advisory 55 begins concerning fish from the Houston Ship Channel. North of site C is the Houston Ship Channel, which is lined with industrial plants,

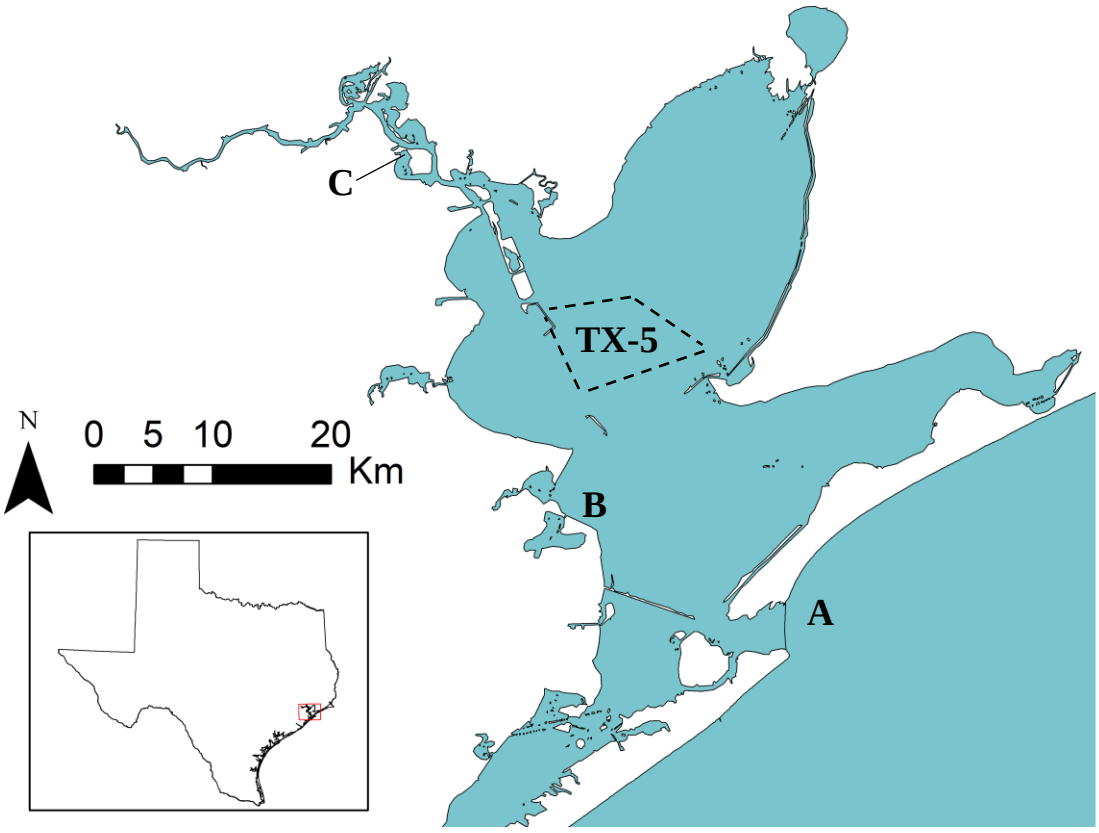

Figure 3.1. Map of sampling sites, where A, B, and C represent the locations for sampling fish, and TX-5 indicates the reef in which oysters were obtained.

such as refineries, petrochemical complexes, and oil storage facilities (King et al., 1987). In addition to the Houston Ship Channel and these industrial impacts are several Superfund sites containing toxic contaminants such as metals, volatile organic compounds (VOCs), dioxins/dibenzofurans, polycyclic aromatic hydrocarbons (PAHs), and polychlorinated biphenyls (PCBs) (TCEQ, 2017), located on connecting water ways like Buffalo Bayou and San Jacinto River. One site in particular is the San Jacinto River Waste Pits Superfund Site, in which 212,000 cubic yards of dioxin contaminated material are to be excavated, with an estimated cost of $115 million (Durant and Hubbard, 2018). Oysters were harvested from TX-5, north of site B. This area is considered a mixing zone, where fresh water and seawater meet and are continually blending.

*Sample Collection*

Three of the most important Texas coastal recreational game fish were sampled from differing parts of the bay; red drum (*Sciaenops ocellatus*), black drum (*Pogonias cromis*), and spotted seatrout (*Cynoscion nebulosus*), and one of the most popular commercial invertebrates, the American oyster (*Crassostrea virginica*) (Fig. 3.2). Fish were collected through the traditional method of hook and line during the months of October and November. Upon collection, fish were measured for length and weight (Table 3.1). The condition factor (CF) of the samples was calculated by dividing the weight from the length cubed. Following standard approved methods in the AVMA Guide to the Euthanasia of Animals (S6.2.2) (AVMA, 2013), two methods of euthanasia were utilized. Cervical transection followed by pithing was used for fish small enough to handle safely. For larger fish, manually applied blunt force trauma (cranial concussion via club) followed by pithing was the method used. Fish fillets and whole animal tissue for oysters were collected and kept on ice, until transferred to a -20 ºC freezer for storage until extraction.

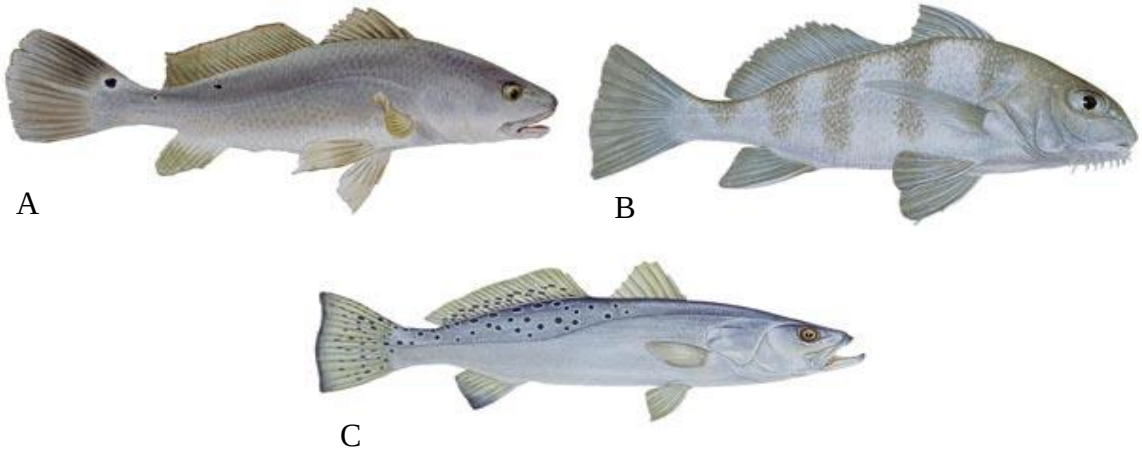


Figure 3.2. Morphological depiction (A) Red Drum, (B) Black Drum, and (C) Spotted Trout. Photo courtesy Texas Parks and Wildlife Department © 2006.

Table 3.1. Morphometric parameters of collected fish samples.

| Species | Sample ID | Sample Site | Size (cm) | Weight (g) | CF |
|---|---|---|---|---|---|
| Red Drum | RD01 | A | 92.71 | 5896.70 | 0.74 |
| | RD02 | B | 68.58 | 3175.14 | 0.98 |
| | RD03 | B | 67.95 | 3061.75 | 0.98 |
| Black Drum | BD01 | B | 40.64 | 907.18 | 1.35 |
| | BD02 | B | 38.10 | 725.75 | 1.31 |
| | BD03 | C | 38.10 | 680.39 | 1.23 |
| | BD04 | C | 40.01 | 816.47 | 1.28 |
| | BD05 | C | 38.10 | 680.39 | 1.23 |
| Spotted Trout | ST01 | A | 46.99 | 1360.78 | 1.31 |
| | ST02 | B | 39.37 | 566.99 | 0.93 |
| | ST03 | B | 41.91 | 680.39 | 0.92 |
| | ST04 | C | 50.80 | 1247.38 | 0.95 |
| | ST05 | C | 48.26 | 1043.26 | 0.93 |
| | ST06 | C | 45.72 | 907.18 | 0.95 |
| | ST07 | C | 48.26 | 1020.58 | 0.91 |
| | ST08 | C | 47.63 | 1020.58 | 0.94 |
| | ST09 | C | 44.45 | 907.18 | 1.03 |
| | ST10 | C | 44.45 | 680.39 | 0.77 |
| | ST11 | C | 44.45 | 907.18 | 1.03 |
| | ST12 | C | 44.45 | 793.79 | 0.90 |
| | ST13 | C | 44.45 | 793.79 | 0.90 |
| | ST14 | C | 43.18 | 907.18 | 1.13 |
| | ST15 | C | 40.64 | 589.67 | 0.88 |
| | ST16 | C | 41.91 | 635.03 | 0.86 |
| | ST17 | C | 41.91 | 566.99 | 0.77 |
| | ST18 | C | 40.64 | 566.99 | 0.84 |
| | ST19 | C | 40.64 | 589.67 | 0.88 |
| | ST20 | C | 39.37 | 498.95 | 0.82 |
| | ST21 | C | 41.28 | 680.39 | 0.97 |
| | ST22 | C | 38.74 | 566.99 | 0.98 |
| | ST23 | C | 41.28 | 589.67 | 0.84 |
| | ST24 | C | 38.74 | 566.99 | 0.98 |

*Chemistry*

Fish and oyster tissues were homogenized using a stainless-steel blender. Homogenates were analytically tested for the presence of 21 organochlorine pesticides (OCPs) and 32 PCBs (TestAmerica, Pittsburgh, PA). Chemicals were extracted using pressurized liquid extraction (PLE), following the method described by Subedi et al. (2013) and Subedi and Usenko (2012). Briefly, a sub-sample of tissue homogenate (10g of fish tissue, 5g of oyster tissue) was dried and further homogenized with 40g of sodium sulfate using a mortar and pestle. The homogenate was extracted with methylene chloride/ hexane (1:1) on an accelerated solvent extraction system (ASE 350 Dionex-Thermo Fisher Scientific, Waltham, MA), with extraction conditions of 100°C, 1500 psi, 5min static time and 290s purge time, and 75% flush volume. This method has shown to have greater than 80% recovery of PCBs and other organic pollutants in both oyster and fish tissues. Extracts were then concentrated to dryness under a gentle stream of nitrogen using a TurboVapII (Biotage, Charlotte, NC), and resuspended in 50µL of dimethyl sulfoxide (DMSO), with one dilution occurring at 50% of the previous.

*Cell Culture*

Caco-2 cells were maintained at 37°C in a 5% $CO_2$ atmosphere in Gibco-formulated Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12, supplemented with 100 IU/mL penicillin and 100µg/mL streptomycin, 0.5% gentamicin solution, and 10% heat-inactivated fetal bovine serum (FBS). Undifferentiated HepaRG cells were obtained from BioPredic International (Paris, France) (Lonza Walkersville Inc., Walkersville, MD) and grown as recommended by the manufacturer. Briefly, Gibco-formulated William's E Medium was supplemented with additives 710 for HepaRG growth medium (Lonza

Walkersville Inc., Walkersville, MD), and 5 mL of GlutaMAX to constitute HepaRG growth media. Undifferentiated HepaRG cells were maintained in this growth media for 28 days before undergoing differentiation. Gibco-formulated William's E Medium was supplemented with additives 720 for HepaRG differentiation medium (BIOPREDIC International), and 5 mL of GlutaMAX to constitute differentiation media. After 28 days of growth, cells were then cultured and maintained in this differentiation media for an additional 14 days. Culturing was conducted in 24-well plates for biochemical studies and in 96-well plates for cytotoxicity determination.

When conducting co-culture assays, Caco-2 cells were seeded on polycarbonate filters (Transwell® inserts, 0.14 $cm^2$ or 0.143$cm^2$ area for 24-well or 96-well plates, respectively, 0.4μm pore diameter; Corning Inc., Corning, NY) and maintained until confluent layers were present. Inserts were then transferred to culture plates containing confluent differentiated HepaRG cells. When using 96-well plates, Caco-2 cells were maintained in the transwell with 100μL of media and 1μL of resuspended extracts - as to not exceed 1% of solvent exposure – with the lower chamber containing 100μL of HepaRG differentiation media. When using 24-well plates, insert containing 300μL of Caco-2 media and 3μL of resuspended extract were contained in the upper chamber, while the lower chamber contained 500μL of HepaRG differentiation media. DMSO solvent controls were run in parallel. Biological and technical duplicates were included.

*Hepatocyte Differentiation*

Morphological changes to cell structure during the differentiation process can be visually identified, according to manufacturer's instructions. When reaching confluency, cells undergo progressive morphological changes as granular hepatocyte-like cells appear

(Fig. 3.3. A, B). Upon the addition of HepaRG differentiation media, hepatocyte-like cells organize into clusters of well-delineated trabeculae with many bright canaliculi-like structures (Fig. 3.3. C, D).

An indirect immunofluorescence assay (IFA) was used as a confirmatory test for hepatocyte differentiation of HepaRG cells (Fig. 3.4). The detection of two well characterized receptors in human hepatocytes was used for this confirmation. Glucose transporter 2 (GLUT2) is an integral plasma membrane glycoprotein of the liver and mediates facilitated bidirectional glucose transport. The asialoglycoprotein receptor 1 (ASGR1) binds to glycoproteins and transports them via a series of membrane vesicles and tubules to the lysosomes for degradation in hepatocytes. Both have been previously used for human hepatocyte characterization (Peters et al., 2016; Takeda et al., 1993).

Cells were grown and differentiated on collagen-coated chamber slides (Lab-Tek II, Thermo Scientific Nunc, Waltham, MA). After the differentiation step, they were washed three times with PBS pH 7.2 and fixed with 4% paraformaldehyde in PBS for 30 min. They were then permeabilized for 10 min with 0.1% Triton X-100 in PBS, blocked with 3% bovine serum albumin (BSA) in PBS and incubated with primary antibodies for 2 h at 25°C. The primary antibodies used were rabbit anti-human GLUT2 polyclonal antibody and rabbit anti-human ASGR1 polyclonal antibody, both from Invitrogen (Thermo Scientific). Both antibodies were diluted 1:100 in PBS with 0.1% Triton X-100. Subsequently, the coverslips were washed with PBS, and incubated with FITC-conjugated secondary antibodies (goat anti-rabbit IgG-FITC, dilution 1:50) for 1 h at 25°C in the dark. The slides were additionally stained with Texas Red-X Phalloidin (Thermo Scientific) for 30 min for actin detection. The coverslips were then air-dried and mounted using mounting

media with DAPI (Prolong Gold Antifade Mountant with DAPI, Invitrogen). Fluorescence was detected using a Brightfield Microscope Olympus IX-81, with DAPI-filter (375-415 nm excitation, 440-480 nm emission), GFP filter (450-490 nm excitation, 500-550 nm emission) and TRITC filter (530-560 nm excitation, 590-650 nm emission).

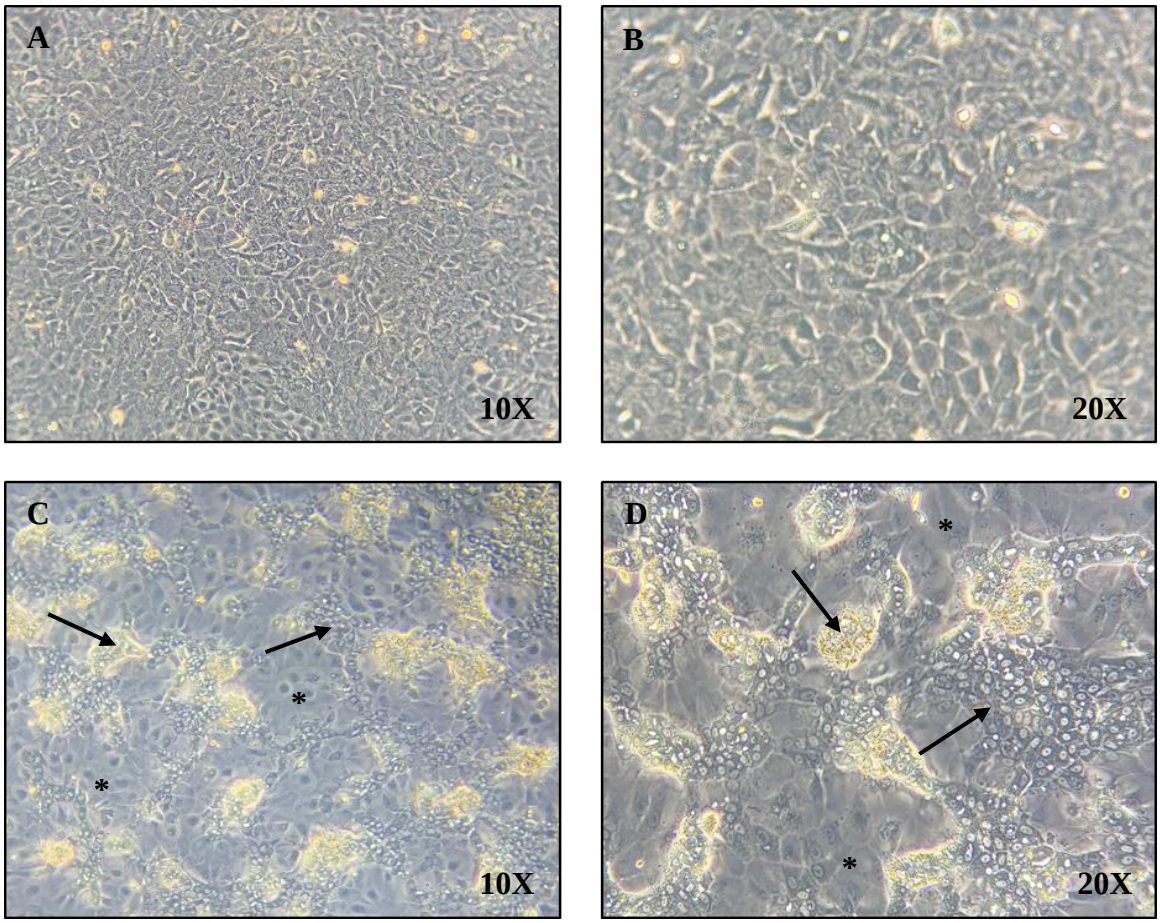


Figure 3.3. Light microscopy image showing HepaRG cells undifferentiated (A and B) and after the process of differentiation (C and D). After differentiation, the cells showed hepatocyte- (arrows) and biliary cell-like characteristics (asterisks).

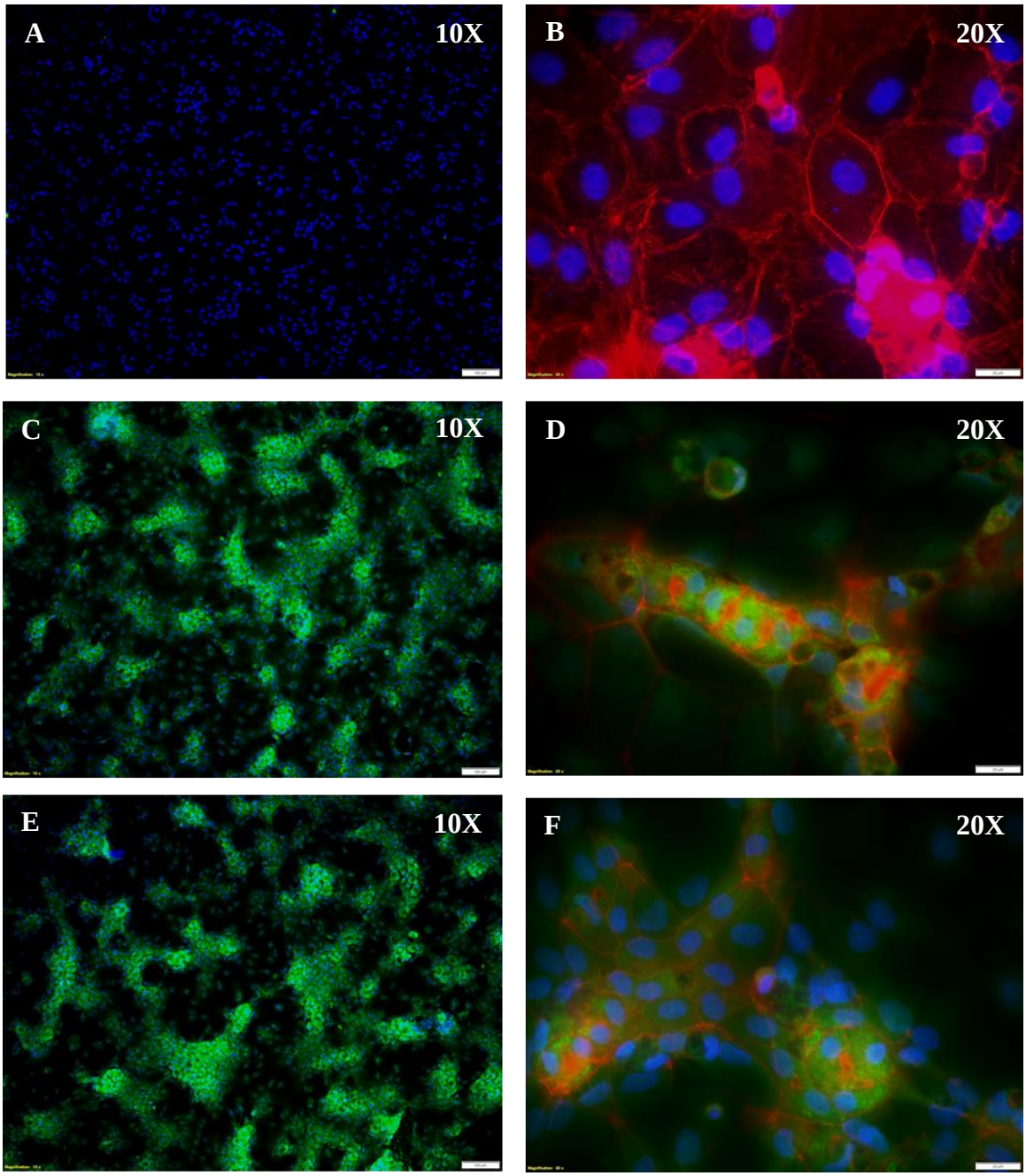


Figure 3.4. IFA microphotographs showing samples of differentiated HepaRG cells incubated with solvent control (A, B), anti-GLUT2 Ab (C, D) and anti-ASGR1 Ab (E, F) and secondary goat anti-rabbit IgG associated to FITC (green). Slides were stained with DAPI (blue), which stains nuclei and Texas Red Phalloidin (red), which stains actin. A, C and E were only stained with DAPI and FITC antibodies. E, D and F were stained with the three dyes.

*Cytotoxicity*

Cytotoxicity assays were conducted in 96-well plates. Cell viability was assessed after 48 hours of exposure to the selected extracts by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, 20% of a tetrazolium dye solution was added to each well 4h before the end of exposure. NAD(P)H-dependent cellular oxidoreductase enzymes reduce the tetrazolium dye to insoluble formazan. This occurs in viable cells. Upon termination of exposure, culture media was removed, a mixture of ethanol and DMSO (1:1) was added to each well to solubilize the formazan crystals, and the resulting color was measured at 595nm by absorption spectroscopy (BioTek SynergyTM H1, Winooski, VT).

*S9 Fractions and Antioxidant Enzymes*

S9 fractions were prepared as described above (Sutherland et al., 2019), using centrifugation and lysing of the cells. Briefly, cells were detached and collected from culture plates with 0.05% trypsin-EDTA (ethylenediamine tetra-acetic acid) and centrifuged at 300g for 5min at 4°C to separate the cell pellet from remaining media. The supernatant was discarded, and the cell pellet was resuspended in 100µL of cold homogenization buffer (100mM $KH_2PO_4/K_2HPO_4$ buffer pH 7.4, containing 100mM KCl, and 1mM EDTA). S9 fractions were obtained after centrifugation at 12,000*g* for 20 min at 4°C and immediately stored at −80°C. Protein concentrations were determined by the Coomassie Blue (Bradford assay) method using a commercial kit (Pierce Inc., Rockford, IL) and bovine serum albumin (BSA) as a standard.

*Measurement of Antioxidant Enzymes*

The activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) was measured in S9 fractions using commercially available 96-well plate bioassay kits (Cayman Chemical, Ann Arbor, MI). The protocols for antioxidant enzyme measurements are publicly available through the Cayman Chemical website. Briefly, CAT activity was measured colorimetrically from the production of formaldehyde, with activity reported as nmol formaldehyde per minute per milligram of protein. GPx activity was measured kinetically for 5 minutes, from a coupled reaction with glutathione reductase, in which NADPH was oxidized to NADP+; absorbance was read at 340nm, and activity is reported as nmol per minute per milligram of protein. SOD was quantified by the detection of superoxide radicals after 30 min of incubation; absorbance was read at 450nm, and data are reported as nmol per minute per milligram of protein.

*Statistical Analyses*

Statistical analyses were carried out using the SPSS v15.0 software package (SPSS Inc., Chicago, IL). Statistical differences between treatment groups was assessed using one-way ANOVA. A *p*-value of less than 0.05 was considered statistically significant. If an overall significance was detected, Dunnett's post-hoc tests were performed to determine difference from solvent controls. All data were analyzed prior to statistical analysis to meet the homoscedasticity and normality assumptions of parametric tests.

## *Results*

### *Chemical Analysis*

Muscle tissue from collected fish and oysters were analyzed for 32 PCBs and 21 OCPs, listed in tables 3.2-3.5 as µg/kg. Non-detects are represented with a dash and the minimum detection limit is abbreviated as MDL. Twelve dioxin-like PCB congeners were included in the analysis (PCB-77, 81, 105, 114, 118, 123, 126, 156, 157, 167, 169, and 189).

4,4'-DDE was found in all samples from all sites – the only exception being ST03. 4,4'-DDE was also the only pesticide found in spotted trout samples collected from sites A and B (ST01 & ST02, 0.7 and 0.34 µg/kg, respectively). Both the red drum and black drum sampled from site B (RD02-03 and BD01-02) also had the pesticide 4,4'-DDE detected in their tissues, at concentrations ranging from 0.23 to 0.62 µg/kg.

The highest concentration of total PCBs for spotted trout were found in samples ST11 and ST17 (65.65 and 46.19 µg/kg). These two samples were used for dosing cells to represent the higher concentrations in spotted trout. ST18 and ST20 were used for dosing as they had the lowest concentrations of total PCB accumulation from site C (6.4 and 4.14 µg/kg, respectively). ST08 was used for dosing to represent a medium accumulation of PCBs (33.89). Spotted trout and black drum collected from sites A and B (ST01-03 and BD01-02) had the lowest amount of PCB accumulation (0.68-2.63 µg/kg for spotted trout and 1.9 & 75.01 µg/kg for black drum) within their respective species. PCB-118 (a dioxin-like structure) was found in a majority of samples collected, the only exceptions being ST01-03 (collected at sites A and B) and OY03.

Table 3.2. Pesticide content in spotted trout samples.

| | Spotted Trout | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pesticide | ST01 | ST02 | ST03 | ST04 | ST05 | ST06 | ST07 | ST08 | ST09 | ST10 | ST11 | ST12 | ST13 | ST14 | ST15 | ST16 | ST17 | ST18 | ST19 | ST20 | ST21 | ST22 | ST23 | ST24 | MDL |
| 4,4'-DDD | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.6 | 0.32 |
| 4,4'-DDE | 0.7 | 0.34 | - | 1.9 | 3.1 | 3.7 | 1.3 | 7.1 | 1.2 | 5.1 | 9.4 | 1.7 | 2.7 | 1.3 | 1.7 | 1.5 | 3.2 | 2 | 2.5 | 0.55 | 4.8 | 3.9 | 2.3 | 2.2 | 0.15 |
| 4,4'-DDT | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.29 |
| Aldrin | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.23 |
| alpha-BHC | - | - | - | 0.32 | - | - | - | - | - | - | 0.66 | - | - | - | 0.21 | - | - | 0.36 | - | - | - | - | 0.31 | 0.28 | 0.18 |
| beta-BHC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.2 |
| cis-Chlordane | - | - | - | 0.28 | 0.76 | 0.5 | - | - | - | 1.3 | 1.9 | - | 0.6 | - | - | 0.5 | 0.52 | - | 0.37 | - | 1 | - | - | 0.37 | 0.18 |
| delta-BHC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.23 |
| Dieldrin | - | - | - | - | - | 1.3 | - | - | - | 1.9 | 1.8 | - | - | - | - | - | - | 0.68 | - | - | - | 1.3 | - | 0.98 | 0.18 |
| Endosulfan I | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.2 |
| Endosulfan II | - | - | - | 0.3 | - | - | - | 0.39 | - | - | 0.65 | - | - | - | 0.25 | - | - | - | - | - | - | - | - | 0.27 | 0.16 |
| Endosulfan sulfate | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.19 |
| Endrin | - | - | - | - | 1.1 | - | 0.63 | 3 | 0.61 | 2.4 | 4.9 | - | 1.1 | - | - | 0.72 | 2.3 | 0.69 | 1.1 | - | 3 | 1.9 | - | - | 0.29 |
| Endrin aldehyde | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.26 |
| Endrin ketone | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.26 |
| Lindane | - | - | - | 0.43 | - | 0.88 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.25 |
| Heptachlor | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.23 |
| Heptachlor epoxide | - | - | - | 0.54 | 0.64 | 0.63 | - | - | - | - | - | - | 0.66 | - | - | - | - | - | - | - | 0.67 | - | - | - | 0.19 |
| Methoxychlor | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.29 |
| Toxaphene | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 20 |
| trans-Chlordane | - | - | - | - | 1.1 | 0.93 | - | - | - | 1.6 | 2.8 | - | 0.82 | - | - | 0.63 | - | - | - | - | - | - | - | 0.55 | 0.17 |

Table 3.3. Pesticide content in red drum, black drum, and oyster samples.

| | Red Drum | | | Black Drum | | | | | Oysters | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pesticide | RD01 | RD02 | RD03 | BD01 | BD02 | BD03 | BD04 | BD05 | OY01 | OY02 | OY03 | OY04 | OY05 | OY06 | MDL |
| 4,4'-DDD | 0.63 | - | - | - | - | - | - | - | - | - | - | - | - | 0.36 | 0.32 |
| 4,4'-DDE | 1.4 | 0.56 | 0.23 | 0.49 | 0.62 | 2.3 | 0.86 | 1.7 | 0.54 | 0.54 | 0.64 | 0.32 | 0.43 | 0.54 | 0.15 |
| 4,4'-DDT | 1.5 | - | - | - | - | - | - | - | - | - | - | - | 1.6 | 0.76 | 0.29 |
| Aldrin | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.34 | 0.23 |
| alpha-BHC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.18 |
| beta-BHC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.2 |
| cis-Chlordane | - | - | - | - | - | 0.34 | 0.53 | - | - | - | - | - | - | - | 0.18 |
| delta-BHC | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.23 |
| Dieldrin | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.18 |
| Endosulfan I | 0.36 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.2 |
| Endosulfan II | - | - | - | - | - | - | - | - | - | - | - | - | 0.54 | - | 0.16 |
| Endosulfan sulfate | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.19 |
| Endrin | 0.48 | - | - | - | - | 1 | 0.39 | - | - | - | - | - | - | - | 0.29 |
| Endrin aldehyde | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.26 |
| Endrin ketone | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.26 |
| Lindane | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.25 |
| Heptachlor | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.23 |
| Heptachlor epoxide | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.19 |
| Methoxychlor | 1.2 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.29 |
| Toxaphene | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 20 |
| trans-Chlordane | 0.79 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.17 |

Table 3.4. PCB content in spotted trout samples.

| | Spotted Trout | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Congener | ST01 | ST02 | ST03 | ST04 | ST05 | ST06 | ST07 | ST08 | ST09 | ST10 | ST11 | ST12 | ST13 | ST14 | ST15 | ST16 | ST17 | ST18 | ST19 | ST20 | ST21 | ST22 | ST23 | ST24 | MDL |
| DCB | - | - | - | 0.94 | 0.93 | 0.95 | 1.2 | 1.3 | - | 1.5 | 2.4 | 0.88 | 2 | - | - | 1.2 | 2.3 | - | 1.6 | - | 1.9 | 1.1 | 1.1 | - | 0.62 |
| PCB-101 | - | 0.3 | - | 1.4 | 2.5 | 2.5 | 1.9 | 4.1 | 1.2 | 2.3 | 8.2 | 1.6 | 3.3 | 2.2 | 0.73 | 1.8 | 3.3 | 0.76 | 3 | 0.63 | 2.6 | 2 | 1.5 | 0.66 | 0.27 |
| PCB-105 | - | - | - | - | - | 0.39 | - | 0.5 | - | - | 1.4 | 0.29 | - | - | - | 0.28 | 0.84 | - | - | - | - | 0.37 | - | - | 0.28 |
| PCB-114 | - | - | - | - | 0.83 | - | - | - | - | - | 1.8 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.72 |
| PCB-118 | - | - | - | 0.96 | 1.7 | 1.5 | 1.4 | 2.5 | 0.98 | 1.4 | 5.6 | 1.3 | 1.8 | 1.7 | 0.64 | 1.4 | 3.4 | 0.73 | 2.2 | 0.42 | 1.7 | 1.6 | 1.2 | 0.38 | 0.29 |
| PCB-123 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.84 |
| PCB-126 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.6 |
| PCB-128 | - | - | - | - | - | - | 0.43 | 0.89 | - | 0.57 | 1.7 | 0.43 | 0.42 | - | - | 0.34 | 1.1 | - | 0.79 | - | 0.51 | 0.63 | 0.44 | - | 0.35 |
| PCB-138 | - | - | - | 1.2 | 2 | 1.5 | 1.4 | 2.9 | 1 | 1.8 | 6.4 | 1.9 | 2.2 | 2 | 0.94 | 1.5 | 5 | 1.1 | 3.2 | 0.58 | 2.3 | 2.5 | 2 | 0.84 | 0.38 |
| PCB-153 | 0.47 | 0.65 | 0.33 | 2.5 | 3.5 | 3.4 | 2.4 | 5.2 | 2.1 | 3 | 11 | 3.5 | 4 | 3.9 | 1.5 | 2.3 | 8.7 | 1.8 | 5.9 | 0.94 | 4.2 | 4.6 | 3.9 | 1.4 | 0.22 |
| PCB-156 | - | - | - | - | - | - | - | - | - | - | 0.74 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.51 |
| PCB-157 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.58 |
| PCB-167 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.85 |
| PCB-169 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.66 |
| PCB-170 | 0.39 | 0.32 | - | 0.9 | 1.1 | 0.97 | 0.92 | 1.5 | 0.53 | 1.1 | 3 | 1 | 1.5 | 0.9 | 0.57 | 0.76 | 2.8 | 0.57 | 1.6 | - | 1.6 | 1.5 | 1.2 | 0.57 | 0.2 |
| PCB-18 | 0.87 | - | - | - | - | - | - | - | - | - | 0.84 | - | - | - | - | - | - | - | 0.79 | - | - | - | 1 | - | 0.59 |
| PCB-180 | 0.55 | - | - | 1.4 | 1.6 | 1.5 | 1.3 | 2.4 | 0.82 | 1.8 | 5.3 | 1.6 | 2.3 | 1.5 | 0.77 | 1.2 | 5 | 0.81 | 2.6 | 0.53 | 2.7 | 2.2 | 1.9 | 0.87 | 0.45 |
| PCB-183 | - | - | - | - | - | - | - | - | - | - | 1.2 | - | - | - | - | - | 1.3 | - | 0.69 | - | 0.7 | - | - | - | 0.63 |
| PCB-184 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.22 |
| PCB-187 | 0.35 | 0.35 | 0.35 | 1.1 | 1.3 | 1.4 | 0.81 | 1.8 | 0.54 | 1.2 | 2.6 | 0.97 | 1.6 | 0.98 | 0.62 | 0.81 | 1.7 | 0.63 | 1.6 | 0.48 | 1.5 | 1.2 | 1.1 | 0.73 | 0.24 |
| PCB-189 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.45 |
| PCB-195 | - | - | - | - | - | - | - | - | - | - | 0.57 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 |
| PCB-206 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.43 |
| PCB-28 | - | - | - | - | - | - | - | 1.6 | - | - | 1.9 | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 |
| PCB-44 | - | - | - | 0.77 | 0.83 | 1.3 | 0.64 | - | - | - | - | 0.6 | 1.5 | 0.85 | - | 0.52 | 0.59 | - | 0.92 | - | 0.61 | 0.8 | 0.39 | 0.6 | 0.37 |
| PCB-49 | - | - | - | 0.69 | 0.78 | 0.97 | 0.47 | 1.9 | 0.53 | 1.1 | 3.5 | 0.45 | 1.6 | 0.93 | 0.55 | 0.87 | 0.96 | - | 1.2 | - | 1.1 | 0.75 | - | - | 0.41 |
| PCB-52 | - | - | - | 0.68 | 1.2 | 0.9 | 1.1 | 2.1 | 0.67 | 1.3 | 3.9 | 0.55 | 1.9 | 1 | - | 0.89 | 1 | - | 1.1 | - | 1.4 | 0.81 | - | - | 0.38 |
| PCB-66 | - | - | - | 0.47 | 0.95 | 0.92 | 0.81 | 1.6 | 0.36 | 0.85 | 3.6 | 0.53 | 1.4 | 0.98 | - | 0.81 | 1.1 | - | 0.91 | - | 0.9 | 0.66 | 0.51 | - | 0.33 |
| PCB-77 | - | - | - | 0.83 | - | - | - | - | - | 2.7 | - | 0.58 | - | 0.85 | - | - | - | - | - | 0.56 | - | - | 0.6 | 0.61 | 0.36 |
| PCB-8 | - | - | - | 3.3 | 6.4 | 4.2 | 4.1 | 3.6 | 1.9 | 5.2 | - | 2.7 | 6.6 | 2.2 | 6.9 | 8 | 7.1 | - | 2.7 | - | 6.9 | 4.2 | 3.4 | 3.5 | 0.84 |
| PCB-81 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.84 |
| PCB-87 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.24 |
| ΣPCB | 2.63 | 1.62 | 0.68 | 17.14 | 25.62 | 22.4 | 18.88 | 33.89 | 10.63 | 25.82 | 65.65 | 18.88 | 32.12 | 19.99 | 13.22 | 22.68 | 46.19 | 6.4 | 30.8 | 4.14 | 30.62 | 24.92 | 20.24 | 10.16 | 15.15 |

Table 3.5. PCB content in red drum, black drum, and oyster samples.

| | Red Drum | | | Black Drum | | | | | Oysters | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Congener | RD01 | RD02 | RD03 | BD01 | BD02 | BD03 | BD04 | BD05 | OY01 | OY02 | OY03 | OY04 | OY05 | OY06 | MDL |
| DCB | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.62 |
| PCB-101 | 0.38 | 0.44 | 1.1 | - | 0.44 | 1.2 | - | 0.89 | 0.67 | 0.68 | 0.46 | 0.56 | 0.57 | 0.26 | 0.27 |
| PCB-105 | - | - | - | - | - | - | 0.57 | - | - | - | - | - | - | - | 0.28 |
| PCB-114 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.72 |
| PCB-118 | 0.31 | 0.44 | 0.89 | 0.31 | 0.35 | 0.68 | 1.9 | 0.89 | 0.55 | 0.47 | - | 0.37 | 0.34 | 0.29 | 0.29 |
| PCB-123 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.84 |
| PCB-126 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.6 |
| PCB-128 | - | - | - | - | - | - | 0.52 | - | - | - | - | - | - | - | 0.35 |
| PCB-138 | 0.5 | 0.71 | 1.4 | - | 0.59 | 0.72 | 2.2 | 1.3 | - | - | - | - | - | - | 0.38 |
| PCB-153 | 1 | 1.4 | 3.1 | 0.47 | 0.84 | 1.2 | 3.3 | 2.4 | 1.3 | 1 | 0.72 | 0.79 | 0.93 | - | 0.22 |
| PCB-156 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.51 |
| PCB-157 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.58 |
| PCB-167 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.85 |
| PCB-169 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.66 |
| PCB-170 | 0.23 | 0.42 | 0.93 | 0.29 | - | 0.33 | 1.2 | 0.54 | - | - | - | - | - | - | 0.2 |
| PCB-18 | - | - | - | - | - | - | - | - | - | - | - | - | 0.73 | - | 0.59 |
| PCB-180 | - | 0.64 | 1.5 | - | - | 0.62 | 1.9 | 1 | - | - | - | - | - | - | 0.45 |
| PCB-183 | - | - | - | - | - | - | 0.73 | - | - | - | - | - | - | - | 0.63 |
| PCB-184 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.22 |
| PCB-187 | 0.44 | 0.67 | - | 0.37 | 0.6 | 0.52 | - | 0.84 | 0.46 | 0.34 | 0.22 | 0.33 | 0.31 | - | 0.24 |
| PCB-189 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.45 |
| PCB-195 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 |
| PCB-206 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.43 |
| PCB-28 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.5 |
| PCB-44 | - | - | - | - | 0.59 | 0.52 | 1.2 | - | - | 0.41 | - | 0.47 | 0.52 | 0.49 | 0.37 |
| PCB-49 | - | - | - | - | - | 0.71 | 1.8 | - | - | - | - | - | - | 0.42 | 0.41 |
| PCB-52 | 0.36 | 0.41 | 0.41 | 0.53 | - | 0.97 | 2.1 | - | 0.52 | 0.57 | - | - | 0.44 | 0.44 | 0.38 |
| PCB-66 | - | - | 0.36 | - | - | 0.58 | 1.2 | - | - | - | - | - | - | - | 0.33 |
| PCB-77 | 0.49 | - | 0.49 | - | - | - | - | - | 1 | - | - | - | 1 | - | 0.36 |
| PCB-8 | 0.81 | - | 3.5 | - | 1.6 | - | 1.8 | - | 1.4 | 0.93 | 0.94 | 1.2 | 0.84 | - | 0.84 |
| PCB-81 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.84 |
| PCB-87 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.24 |
| ΣPCB | 4.52 | 5.13 | 13.68 | 1.97 | 5.01 | 8.05 | 20.42 | 7.86 | 5.9 | 4.4 | 2.34 | 3.72 | 5.68 | 1.9 | 15.15 |

*Cytotoxicity*

Exposure to fish extracts (Fig. 3.5) did not lead to significant cell mortality, as survival remained above 80% for all fish samples. The highest mortality among fish samples was observed from the black drum extracts sampled at site B (BD01 and BD02), as well as from one of the red drums sampled at site B (RD03), where extract concentration was 1% of total exposure. On the other hand, extracts from the first two oyster samples (OY01 and OY02) led to significant cell death compared to that of controls, with 70% and 71% survival rates after exposure to OY01 at 1% and 0.5% of exposure media, respectively. HepaRG cells displayed a decrease in cell survival after exposure to all oyster extracts at 1% of exposure compared to that of controls. Cell survival increased when a majority of extracts (except ST03 and OY02) were diluted from 1% to 0.5% of exposure media.

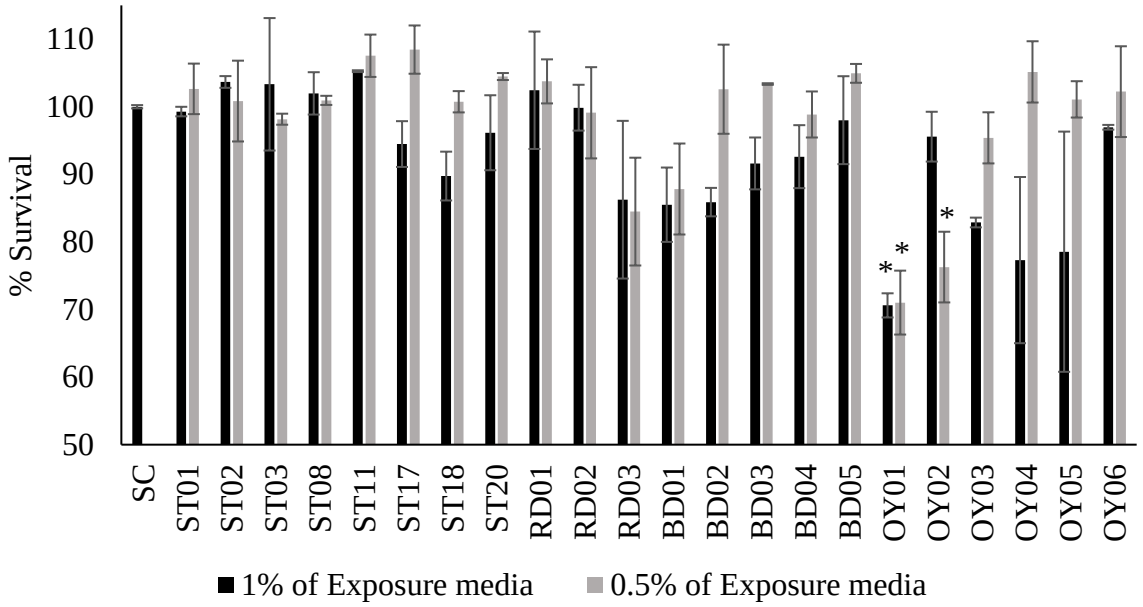


Figure 3.5. Cytotoxic response of HepaRG cells in co-culture exposed to seafood extracts at 1% and 0.5% dilutions. Data presented as mean ± standard deviation (n=2). Significant mortality (> 80% survival) is denoted with asterisk related to solvent control (SC).

*Antioxidant Enzymes*

CAT activity was not significantly different ($p<0.05$) for HepaRG cells exposed to seafood extracts at 1% of exposure media compared to controls (Fig. 3.6), with the exception of ST08 extracts, which caused a 3-fold induction of CAT activity in HepaRG cells. Exposure to 1% RD02 and ST03 extracts led to a lower CAT activity in HepaRG cells compared to solvent controls, indicating a suppression of the enzyme. Upon exposure to ST20 extract, GPx activity in HepaRG cells displayed a ~3-fold induction in cells compared to solvent controls (Fig. 3.7); however, no other significant difference was observed. HepaRG cells exposed to 1% of BD03 exposure media displayed significantly higher SOD activity compared to controls, with 2.5-fold induction (Fig. 3.8). 1% of ST03 exposure media led to a suppression of SOD activity compared to controls.

While exposure to 0.5% of extracts was examined, HepaRG cells did not display significantly different activity at this concentration compared to solvent controls. HepaRG cells did displayed a decreased response in CAT, GPx, and SOD activity when extracts went from 1% to 0.5% of exposure media across a majority of experimental treatments.

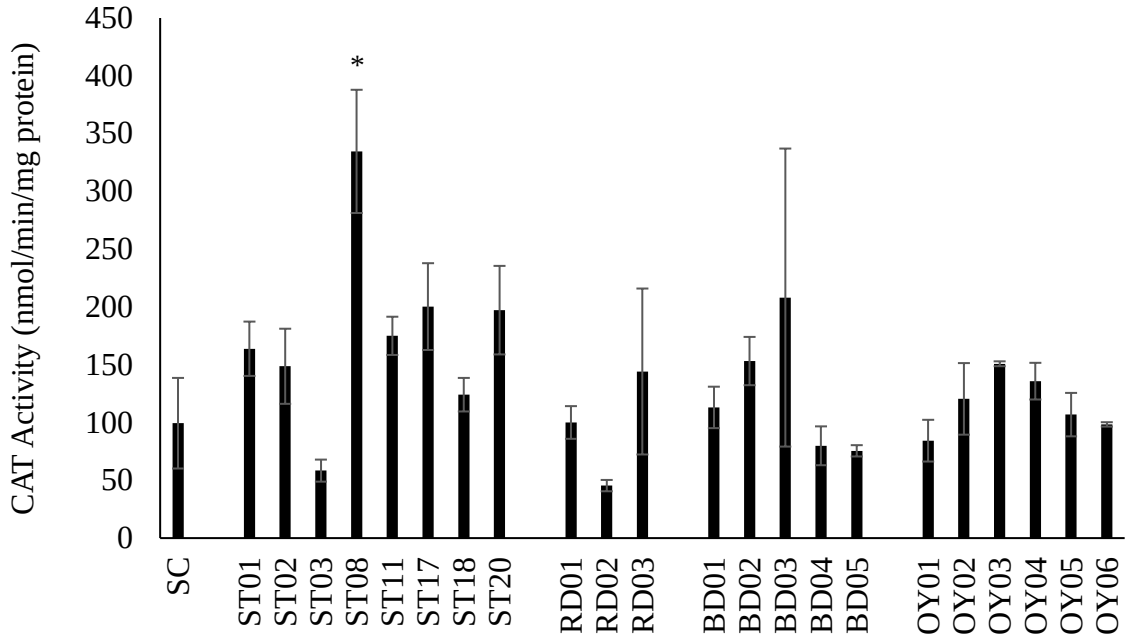


Figure 3.6. Catalase activity determined in subcellular fractions of HepaRG cells dosed in co-culture with 1% of exposure media. Data are reported as mean ± standard deviation (n=2-4). SC: Solvent control as 1% DMSO in exposure media. Significant differences between solvent control and sample extracts are shown with an asterisk ($p<0.05$; One-way ANOVA, Dunnett's post-hoc test).

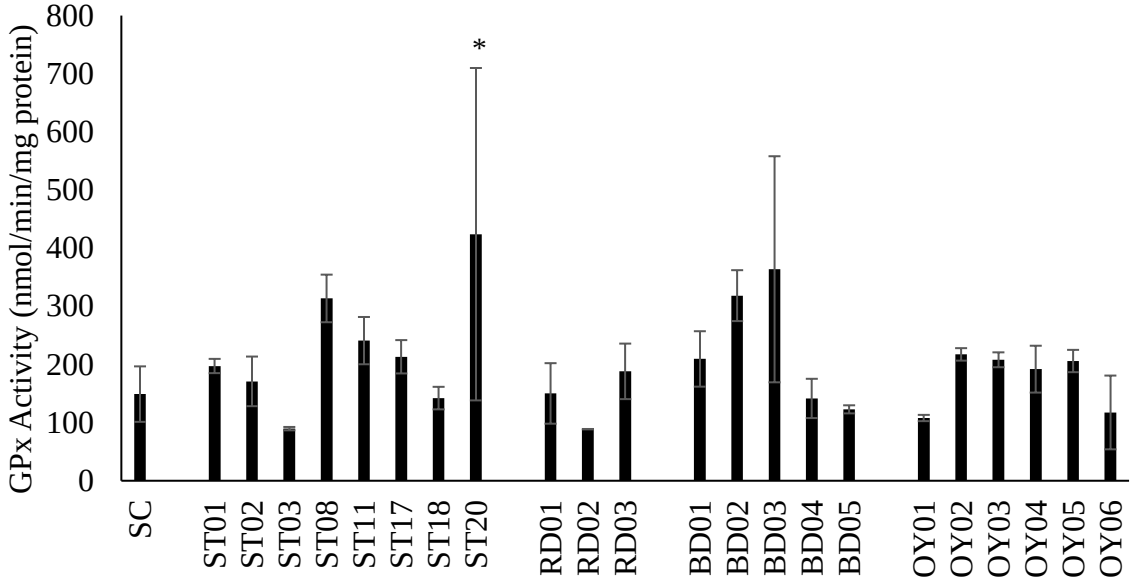


Figure 3.7. Glutathione peroxidase activity determined in subcellular fractions of HepaRG cells dosed in co-culture with 1% of exposure media. Data are reported as mean ± standard deviation (n=2-4). SC: Solvent control as 1% DMSO in exposure media. Significant differences between solvent control and sample extracts are shown with an asterisk ($p<0.05$; One-way ANOVA, Dunnett's post-hoc test).

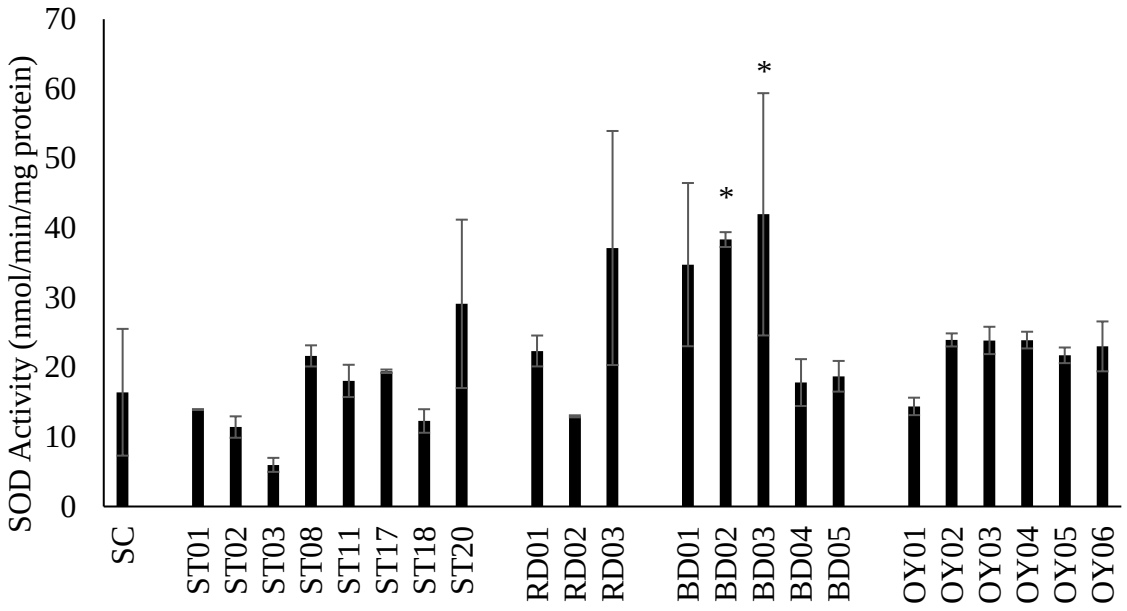


Figure 3.8. Superoxide dismutase activity determined in subcellular fractions of HepaRG cells dosed in co-culture with 1% of exposure media. Data are reported as mean ± standard deviation (n=2-4). SC: Solvent control as 1% DMSO in exposure media. Significant differences between solvent control and sample extracts are shown with an asterisk ($p<0.05$; One-way ANOVA, Dunnett's post-hoc test).

*Discussion*

Organochlorine pesticides (OCPs) are a group of chemicals that can accumulate in fatty tissue and are one of the most widely used pest control chemicals in the world. DDE is a common breakdown product of DDT, an OCP that is highly persistent in the environment, with a half-life of 150 years in aquatic systems (NPIC, 1999). While its use has been banned in the U.S. since 1972, DDT's persistence and previously wide applications would explain the presence of DDE in almost all samples collected in this study, regardless of site sampled. The other OCPs analyzed were more commonly detected in the spotted trout samples collected from site C, such as alpha-BHC, cis-Chlordane, Dieldrin, Endosulfan II, Endrin, Lindane, Heptachlor epoxide, and trans-Chlordane, which

is supported by fish advisories in this area (TDSHS, 2013). The concern associated with OCPs in fish tissue stems from the human health effects they cause from dietary exposure, as they are carcinogenic and neurotoxic in high doses, and can cause endocrine disruption and disproportion of thyroid hormones in low doses (Jayaraj et al., 2017). DDT and Lindane have also been seen to cause lipid peroxidation and SOD activity due to oxidative stress in mammalian cells (Koner et al., 1998), and the combination of lindane with dieldrin was demonstrated to have greater toxicity and ROS generation than when used separately (Sharma et al., 2010).

In addition to pesticides, PCBs were also seen to accumulate in the samples. Interestingly, the spotted trout collected from sites A and B had less accumulation of PCB congeners compared to spotted trout collected at site C, which had up to 25-fold (ST11) the concentration of PCBs compared to samples collected from sites A and B. This again is likely due to the fact that site C has more industrial impacts, as supported by Oziolor et al. (2018) who observed a concentration gradient in fish tissue between PCB contamination and distance from Buffalo Bayou and the industrial portion of the Houston Ship Channel. The highest accumulation of PCBs in black drum was also seen in samples that were collected from site C; however, red drum did not conform to this pattern, as sample RD03 originating from site B displayed higher accumulation of PCBs than several of the individuals sampled from site C. This is likely due to the fact that fish are not stationary animals, and often migrate or move around the bay making it difficult to determine the sources of contamination. On the other hand, oysters are stationary or fixed, suggesting the concentrations of accumulated compounds should not display large variability. However, the opposite was observed as PCB accumulation ranged from 1.9 to 5.9 µg/kg. This

difference in accumulation may be the result of microhabitat differences and the degree of contamination in such, as the sampling location was rather large, and individuals may experience different levels of exposure. To a lower extent, differences in metabolic processes related to size and age of individuals may have influenced bioaccumulation, though this deserves further experimentation as the majority of PCBs do not undergo biotransformation (Chu et al., 2000; Ferreira and Vale, 1998).

It is important to note that the sum of the minimum detection limits (MDLs) equates to 15.15 µg/kg, and considering the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) allow a daily PCB intake of 6 µg/kg per day (ATSDR, 2014), the findings presented in this study would be considered relatively conservative.

Cytotoxicity in HepaRG cells is a good indicator for hepatotoxic chemicals, as it is sensitive to chemical compounds that do or do not require metabolism (Guillouzo et al., 2007). The cell mortality observed for most oyster samples at 1% of exposure media may be due to an accumulated xenobiotic other than those measured, as these samples did not display significantly different accumulation in pesticide content or PCB congeners compared to fish samples. For instance, Neff et al. (1976) highlight the ability of fish to metabolize and excrete polycyclic aromatic hydrocarbons (PAH) more rapidly than oysters, while Lee (1985) found that oysters were slower than fish at metabolizing chemicals like Bis(tributyltin) oxide (TBTO), leading to greater accumulation of these compounds in the oyster species. However, PAHs and TBTO were not measured in the current study, and cannot be confirmed as the source of toxicity.

Antioxidant enzyme responses were used as a proxy for the formation and activity of ROS and the subsequent potential for oxidative stress. Many chemical compounds,

including PCBs, undergo redox reactions that can generate ROS (Oakley et al., 1996), which is concerning for humans because in human cells, ROS has been shown to cause DNA damage, alterations in gene expression, and carcinogenesis (Abdi and Ali, 1999; Ziech et al., 2011). The high GPx activity observed in HepaRG cells after exposure to 1% of ST20 may be the result of this sample having accumulated 0.56 µg/kg of PCB-77, which has a dioxin-like structure. PCBs with dioxin-like structures hold particular concern because they can cause a toxic effect at lower concentrations compared to non-dioxin-like PCBs (Giesy and Kannan, 2008). No other spotted trout sample analyzed had accumulated this particular congener, and while other samples had low levels of PCB-77 present in tissue, it is possible that ST20 has an optimal mixture of this congener with other chemicals of concern, yielding an induction of GPx activity.

Exposure to 1% and 0.5% of sample extracts induced similar trends in activity between CAT, GPx, and SOD, perhaps indicating that the mixture of chemicals in each extract elicits consistent ROS generation across all enzymes. Additionally, the differences between enzyme activity in 1% and 0.5% of exposure media for all three antioxidant enzymes measured demonstrated higher enzyme activity for the more concentrated extracts, and lower enzyme activity for the less concentrated extracts.

For several samples, exposure to extracts led to a decrease of enzyme activity in HepaRG cells. This may be the result of unknown chemicals suppressing activity. Karaca et al. (2014) observed a suppression of gene expression levels in CYP1A, glutathione-*S*-transferase 1 (GST1), and SOD in fish liver tissue from locations with high agricultural and industrial pollutants. While this study was done in fish tissue, Banudevi et al. (2006) found similar results, with rat liver displaying reduced CAT, GPx, glutathione reductase

(GR), and GST activity after exposure to Aroclor 1254 – a PCB mixture of noncoplanar congeners (Kodavanti et al., 2001) – while simultaneously the formation of hydrogen peroxide, hydroxyl radicals, and lipid peroxidation increased. If suppression of antioxidant enzymes is occurring, then detoxification of ROS is unable to take place, perhaps leading to greater oxidative stress than before; however, confirmation of this conclusion requires further experimentation.

While environmental mixtures can present unique challenges when attempting to determine effects, for both *in vitro* and *in vivo* systems, it is important to consider their environmental relevance. Fish and oyster tissue can accumulate an array of xenobiotics from their aquatic environments and understanding the mixture effects when characterizing risk is critical. Many chemicals are hepatotoxic, and metabolism can either mediate or accelerate these toxic effects, which is why it is important to have a metabolically robust model that can account for bioactivation. The co-culture system utilized has proven advantageous for the characterization of effects related to cytotoxic responses and ROS production in human hepatic cells.

# CHAPTER FOUR

## General Discussion and Conclusion

Bioanalytical tools, such as *in vitro* systems, have become commonplace, with monocultures successfully applied to fields of pharmacology and toxicology. These tools can provide an alternative for animal testing while maintaining reproducibility and high-throughput capabilities. While there are clear benefits to *in vitro* systems, they are not a replacement for *in vivo* experimentation. There are many aspects of whole-body organisms that cannot be replicated or recreated externally. However, the field of *in vitro* modeling has grown to account for some of these complexities. Consideration of cell interactions is critical in the development of *in vitro* systems, and with the help of established models such as the co-culture used presently, a greater understanding of biological effects similar to those occurring *in vivo* can be gained.

The functionality of such models has the potential to reach a multitude of diverse issues relating to not only environmental toxicology, but also human risk assessments. This study is just one application, utilizing a digestive co-culture to examine the biological effects and concerns associated with human consumption of seafood. It was observed that the mono- and co-culture systems did not produce the same responses when exposed to SRM extracts, and that the resulting high levels of GPx and SOD in co-culture may be indicative of this model being better suited to facilitate the expression of these enzymes in response to exposure to xenobiotics in intestinal cells. This provided evidence for the first hypothesis: cytotoxic and enzymatic responses to SRM were different in hepatocytes when

comparing monoculture and co-culture systems. Exposure of HepaRG cells in the lower level of the co-culture model to metabolites from the Caco-2 cells above resulted in differing cytotoxic and enzymatic responses, elucidating that in this case a co-culture model would be more appropriate for the description of biological effects. Therefore, this model was further utilized to examine environmental samples collected from Galveston Bay.

After collection of four economically and socially important seafood species, chemical analysis revealed higher concentrations of xenobiotics from samples collected in the upper San Jacinto Bay compared to those collected in southern parts of the bay. Spotted trout and black drum samples collected from site C had PCB burdens 2 to 25 times that of samples collected from site A and B. While advisories are already in place for this area, improved regulation and education is needed.

Exposure to these extracts led to varying degrees of antioxidant enzyme activity in hepatic cells, likely due to the chemical mixture contained in the extracts, with similar trends observed for CAT, GPx, and SOD. Additionally, a decrease in enzyme activity was observed when a decrease in concentration of exposure media occurred. In some cases, exposure to extracts led to lower levels of enzyme activity compared to controls, perhaps suggesting an inhibitory or suppressive chemical contained in the mixture. If proven true, this could be just as detrimental – if not more – to exposed cells, due to the lack of detoxifying agents available. For these reasons, the second hypothesis deserves further attention: fish and oyster extracts containing higher levels of PCBs and OCPs did not necessarily induce greater toxicity or antioxidant enzyme activity, possibly due to the mixture of unknown chemicals accumulated in tissue samples.

This exploratory study associated with human dietary exposure has demonstrated the different responses of mono- and co-culture systems, and the importance for choosing an appropriate *in vitro* model that maximizes similarities with *in vivo* conditions. The information gained from seafood sampling in Galveston Bay area supports current regulations of pollutants in designated areas of concern; however, further study in this area is needed to obtain a complete profile of accumulated contaminants in seafood. Seafood samples were shown to induce antioxidant enzyme activity of CAT, GPx, and SOD, signifying the potential of oxidative stress upon exposure to extracts; however, the results concerning suppression of enzymes and the question of unknown chemicals requires further experimentation. These descriptions may be advantageous in the determination of risk brought by seafood consumption, and its impact on environmental and public health.

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