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DETERMINING THE IMPACTS OF ENVIRONMENTAL CONTAMINANTS TO ZEBRA MUSSELS USING GENETIC BIOMARKERS

by

Nicklaus James Neureuther

A Thesis Submitted in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

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ABSTRACT

DETERMINING THE IMPACTS OF ENVIRONMENTAL CONTAMINANTS TO ZEBRA MUSSELS USING GENETIC BIOMARKERS
by

Nicklaus James Neureuther

The University of Wisconsin-Milwaukee, 2016
Under the Supervision of Professor Rebecca Klaper, PhD

Persistent legacy contaminants and emerging chemicals of concern continue to be a threat to the function and health in the Great Lakes Areas of Concern (AOCs). While chemical monitoring programs traditionally sample water and sediment, these studies can only provide information of the type and level of contamination within an (AOC). This being said, information on the biological impacts to the biota are needed to measure impairments of chemical exposure, to support remediation efforts in their ability to eventually restore AOCs. To accomplish this, I proposed to measure chemical exposure using molecular biomarkers from *D. polymorpha* (Pallas, 1771), more commonly known as the zebra mussel. This species has been successfully used as a bioindicator of contamination in the Great Lakes and in Europe due to it being an invasive species and having the physiological qualities of being a sessile filter feeder. These unique physiological properties, in addition to the zebra mussel already having a library of gene expression biomarkers known to be critical in relation to stress and detoxification, including the genes: GST, AHR, P-gP and HSP70, made this organism an obvious choice. Working in conjunction with the already

established chemical monitoring program, the NCCOS NOAA Mussel Watch Program (MWP), the goal was to test these genomic biomarkers to see how robust they could be as an indicator of exposure in conjunction with chemical data from the field. We demonstrated that in an aquatic environment that these genes of exposure revealed a significant relationship with the legacy contaminants polychlorinated bi-phenols (PCBs) and polycyclic aromatic hydrocarbons (PAHs) and the emerging contaminants, 4 nonylphenol (4NP) and triclocarban (TCC) over a gradient of contamination and that these results were affected by length of exposure. Likewise, AOCs are dynamic environments containing complex mixtures of contaminants, which tend to co-correlate, making it difficult to parse out effects of exposure from single contaminants. To investigate individual chemical and gene expression relationships further, zebra mussels were exposed to environmentally relevant levels of TCC under laboratory conditions. This study confirmed my field results that the gene GST could be a potential biomarker of TCC. As a whole, these two studies demonstrated that using the zebra mussel as not only a bioindicator of contamination but as a biomonitor of exposure using gene expression biomarkers could be an effective tool used by monitoring programs to help gauge restoration success.

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This work is dedicated to my wife Carolyn Neureuther for her unwavering support of this endeavor, my daughter Emmy, and in loving memory of my mother Joy

The weeping willow bends but never breaks

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LIST OF ABBREVIATIONS

4NP	4 Nonylphenol
AHR	Aryl Hydrocarbon Receptor
AOC	Area of Concern
CEC	Contaminates of Emerging Concern
GST	Glutathione S. Transferase
HSP 70	Heat Shock Protein 70
MWP	Mussel Watch Program
OC	Organic Contamination
P-gP	P. Glycoprotein
PAH	Polycyclic Aromatic Hydrocarbon
PCBs	Polychlorinated Biphenyls
PPCP	Pharmaceuticals and Personal Care Products
RTqPCR	Real-time Quantitative PCR
TCC	Triclocarban

ACKNOWLEDGMENTS

This work was accomplished with support from the Milwaukee School of Freshwater Sciences and funding from the Great Lakes Restoration Imitative through NOAA's NCCOS Mussel Watch Program. I would also like to thank all the members of the Klaper lab, mostly my Advisor Rebecca Klaper and Nicholas Niemuth for their mentorship and to The Great Lakes Genomics Center for technical support. Lastly, I would like to recognize Timothy Michael Schierenbeck for lending his expertise in GIS and Dylan Olsen for lending his time and SCUBA experience.

CHAPTER 1: INTRODUCTION

Chemical Contamination in the Great Lakes

Contaminants such as polychlorinated biphenyls (PCBs), heavy metals e.g. mercury, lead, and pesticides including DDT have been banned but are still prevalent in the Great Lakes (Kimbrough et al., 2013). There are 31 Areas of Concern (AOCs) in The Great Lakes, designated by the Great Lakes Water Quality Agreement (1978). This is due to their degradation under the influence of anthropogenic contamination (U.S.EPA., 1998; IJC 1978). The ability for these toxics to adsorb to particulates and algae, are the cause of sediment contamination which has become a potential route of exposure which bioaccumulates up the food chain (Cho et al., 2004; Jacobs et al., 2004; Schwartz et al., 1983; Van der Oost et al., 2003; Walters et al., 2010). While the use of many of these pollutants has drastically decreased or been eliminated, they are still measured at levels that can impact wildlife and human health as evidenced by fish consumption advisories (Bence et al., 2008; Jacobson et al., 1984; Jones and De Voogt, 1998; Li et al., 2009; Schwartz et al., 1983). In addition to these legacy persistent pollutants, contaminants of emerging concern (CECs) which include polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers, (PBDEs), as well as personal care products and pharmaceuticals (PPCPs), are making their way into the aquatic ecosystems, due to their high volume use, runoff (PAHs) or incomplete removal at wastewater treatment plants (PPCPs) (Blair et al., 2013; Klecka et al., 2010; Lee et al., 2012). Typically, concentrations of PPCPs are relatively low, though some have

been shown to have deleterious effects on wildlife in part because of chronic low dose exposures they cause sub-lethal effects such as endocrine disruption (Crago and Klaper, 2012; Villeneuve etl., 2016)

Biomarkers

Chemical monitoring programs are essential in providing evidence for risk assessment and determining restoration initiatives. Directly measuring contaminants in organisms provides evidence not only of contamination, but also the potential for exposure to organisms (De Luca-Abbott et al., 2005; Johns, 2011; Kimbrough et al., 2014). However, chemical monitoring, even within organisms, only provides evidence of the presence of chemicals and not their effects on the health of the biota.

Biomarkers are a way to integrate chemical monitoring with the impacts of contaminants on an organism. The National Academy of Science/National Research Council (NAS/NRC), defines the term “biomarker” as an induced disruption in cellular biochemical processes, structures, or functions, in response to a xenobiotic compound, that can be measured in a biological sample (NRC, 1987). Most importantly, these can be used as a tool, which can provide evidence of exposure as well as an organism’s health. As an example, many environmental investigations have used enzyme ethoxyresorufin –O-deethylase (EROD) activity to demonstrate the impacts of organo-chlorine contamination (Arcand-Hoy and Metcalfe, 1999; Ku et al., 2014; Liang et al., 2013). Other types of stress can be measured using biomarkers. For instance oxidative stress, resulting from reactive oxygen species (ROS), a by product of detoxification

which can result in lipid peroxidation, resulting in DNA damage, which can be analyzed by measuring the anti-oxidant enzymes superoxide dismutase and catalase (Riva et al., 2010; Rocher et al., 2006).

More recently, the quantification of messenger RNA (mRNA) biomarkers, which code for proteins important for particular biochemical pathways are being used as an early indicator of organismal stress in laboratory and environmental studies (Châtel et al., 2014; Hook et al., 2006; Navarro et al., 2011; Venier et al., 2006). Using genes involved in the detoxification of xenobiotic substances provides an indication of the instigator or disruption and in some cases can be linked to specific contaminants. For example, investigators routinely measure vitellogenin (VTG), an egg yolk protein precursor, to analyze the effects of endocrine disruption in male fish and the gene metallothionein (MT) is recognized at the European level as a biomarker for heavy metal contamination in fish and invertebrates (Amiard et al., 2006; Harries et al., 1997). Additionally, monitoring changes in an organism at a molecular level can allow researchers to detect early on the effects of an environmental and potentially mitigate population level impacts (Klaper and Thomas, 2004; Thomas and Klaper, 2004; Van der Oost et al., 2003). .

There are some issues in the use of genomic biomarkers for environmental monitoring. For instance, gene expression monitoring only provides a snapshot in time and consequently researchers can miss the optimal period that causes changes in key biomarker expression. Additionally, the species that a researcher may want to use needs to be found living in or near the

particular location of study. Lastly, RNA can easily degrade and therefore requires special handling. This can be a hindrance in remote field conditions where access to liquid nitrogen or refrigeration is not easily accessible.

The goal of this research is to investigate the impacts of toxics in relation to genomic biomarkers of toxicity and stress in order to enhance chemical monitoring capabilities. In the studies described herein, I used *Dreissena polymorpha* (Pallas, 1771), which is commonly used as a bioindicator of environmental chemical contamination (Binelli et al., 2008; Binelli and Provini, 2003; De Jonge et al., 2012; Kimbrough et al., 2013; Kraak, 1997; Minier et al., 2006). Since its introduction into the Great Lakes, the zebra mussel quickly comprised a dominant proportion of the biomass inhabiting the Great Lakes benthos (Johnson and Carlton, 1996; Nalepa and Schloesser, 1993; Schloesser et al., 1996). The NOAA Mussel Watch Program (MWP), an already established monitoring program, using native clams and oysters to monitor coastal marine environments, seized the opportunity to use this invasive species to monitor contamination in twenty-three core sampling sites for over 150 contaminants throughout the Great Lakes basin for over two decades (Kimbrough et al., 2013). Zebra mussels retain several unique properties, including a sessile nature, an ability to accumulate toxics, and are an invasive species on a global scale (Bruner, K., Fisher, S. & Landrum, 1994).

The second chapter describes using caged zebra mussels in the Niagara River AOC for five and ten weeks of exposure. The main goals for this project were to answer the questions: will genomic biomarkers correlate to specific

contaminants measured in the zebra mussel tissue to identify the chemical or chemicals that are having the greatest impact on organismal health including legacy and emerging contaminants? Additionally, I wanted to test how the length of exposure will affect the expression of these genes? Lastly, is gene expression different in free-living *in situ* mussels harvested from within the Niagara River versus caged mussels?

Complex mixtures of contaminants measured in the Niagara River environmental study made it difficult to differentiate which chemicals were having effects on the zebra mussels. For example, many of the organic contaminants measured in the first study were similar in structure and were found to statistically correlate with each other, which is not uncommon. As a result, identifying which chemical was having the effect; it was necessary to single out a chemical and test it against the genes of interest under laboratory conditions. The third chapter describes a second lab-based study, which investigated the relevance of the anti-microbial agent Triclocarban (TCC). In the environmental study TCC correlated significantly with the gene GST, representing 64% of the variability in the data. However, its effect was not clear in the environmental mixture study and there is only a small amount of information about this compound in the literature. Also, TCC is a high volume, down the drain compound that also has an estimated logarithmic octanol/water partition coefficient (log K_{ow}) of 4.9, this level of lipophilicity is generally high enough to bioaccumulate in the environment (Halden, 2014; Halden and Paull, 2005). Most importantly, this compound was relatively undetected in water ways due to insufficient testing methods and is now

estimated to be quantifiable in 60-90% of U.S. streams (Halden, 2014). All of this evidence pointed to TCC as being a candidate for further investigation. As a matter of fact, the FDA recently banned TCC, in addition to 18 other anti-microbial agents, as an ingredient in hand soaps and sanitizers due to its ineffectiveness and effects on human health in regard to hormones and bacterial resistance (FDA, 2016).

Collectively, these two studies contribute to the mounting evidence that genomic biomarkers can have a substantial role in monitoring the health of aquatic communities impacted by historic and emerging anthropogenic contamination; and as a consequence they will help to advance the zebra mussel as a global model organism for monitoring freshwater ecosystems.

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CHAPTER 2: Monitoring Exposure and Impacts of Toxics in The Great Lakes Using Genomic Biomarkers of *Dreissena polymorpha* in Conjunction With The Contaminant Monitoring of the NOAA Mussel Watch Program

Under Review:

Journal of Great Lakes Research

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Abstract

Toxics continue to threaten the health and function of Great Lakes Areas of Concern (AOCs). Although chemical monitoring provides some data on impairments, biological information on potential impacts to biota are needed to assess the need and success of remediation. The goal of this project was to integrate molecular biomarkers related to stress and detoxification with chemical data to better understand correlations between gene expression and exposure. Caged zebra mussels were used to monitor specific pollutants, levels of contamination, and exposure using mRNA biomarkers critical to the processes of detoxification including, P-gP, GST, AHR and HSP70 extracted from gill tissue. Seven mid-sized creeks were evaluated using these metrics which were measured at five and ten week time periods. Additionally, *in situ* native zebra mussels were harvested to assess conditions in the Niagara River. This project was run in conjunction with NOAA's NCCOS Mussel Watch Program, which monitors chemical pollution in the Great Lakes. Watershed wide, PCBs and PAHs were the major pollutants, though chemicals of emerging concern (CECs) were measured as well. Overall, contamination was found to be greater in the tributaries then in the Niagara River. The gene expression analysis resembled these results, and although the in situ mussels within the river were not useful predictors of contamination, the caged mussels were. The contaminated streams at five weeks of exposure showed that all genes were down regulated from control. AHR and GST significantly correlated with legacy contaminants PCBs and PAHs, and the CECs 4 Nonylphenol and Triclocarban over a gradient

of contamination, suggesting that these biomarkers have potential for environmental monitoring of exposure to these chemicals.

Keywords

Zebra mussels, biomarkers, Niagara River, legacy contaminants, emerging contaminants, gene expression

Introduction

Toxic substances have burdened the Great Lakes and its tributaries for decades. Legacy contaminants such as mercury, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyl (PCBs) in addition to a host of other organochlorine contaminants (OC) including pesticides have been monitored and identified as a persistent threat to aquatic environments (Kuntz and Warry, 1983;Ulbrich and Stahlmann, 2004). In addition to legacy contaminants, new classes of pollutants referred to as contaminants of emerging concern (CECs) have recently emerged as a threat to the Great Lakes ecosystem. CECs are a broad assortment of toxins that include wastewater contaminants such as pharmaceuticals, hormones, and steroids as well as antibacterial agents, pesticides and brominated flame-retardants among many others (Klecka et al., 2010).

A majority of the harbors and associated tributaries in the Great Lakes have contamination from legacy contaminants and an associated degradation of biota in these areas (Adrians et al., 2002). These contaminants impact the health of benthic organisms, and can bioaccumulate up the food web leading to

deformities in wildlife, and lead to human health concerns, most commonly through fish consumption advisories (Jacobson et al., 1984; Schwartz et al., 1983). (Arcand-Hoy and Metcalfe, 1999; Diggins et al., 1993; Fox, 2001; Van der Oost et al., 2003). Of these sites, 31 have been designated Areas of Concern (AOCs) by the International Joint Commission in the US-Canada Great Lakes Water Quality Agreement (1987). Monitoring the status of the benthos is critical to the function and well-being of the AOCs and are a priority for managers (Grapentine, 2009). Chemical monitoring programs have been critical to listing and delisting AOCs for the reason that chemical contamination is the origin for many of the beneficial use impairments (BUIs) assigned to an AOC. However chemical monitoring data only provide evidence of the presence of a chemical and not the effects of these chemicals on the health of the biota. In addition a single measure of contamination may not accurately represent exposures of organisms in the ecosystems due to variability over time.

Biomarkers are a way to integrate chemical monitoring with information as to the potential impacts of contaminants on an organism. The National Academy of Science/National Research Council (NAS/NRC), defines the term “biomarker” briefly stated here, as an induced disruption in cellular or biochemical processes, structures, or functions, in response to a xenobiotic compound, that can be measured in a biological sample (NRC, 1987). The expression of genes that are associated with these disrupted processes can be used as a biomarker providing an indication of exposure and potential effects of exposure to a variety of chemical stressors (Klaper and Thomas, 2004; Marinković et al., 2012; Navarro

et al., 2011). Since the expression of a gene is an early indicator of an exposure taking place, being able to measure specific genes that are associated with key pathways, could be effective in determining physiological effects further downstream e.g. behavior, growth and reproduction (Lam, 2003; Schirmer et al., 2010). Genes involved in the detoxification of xenobiotic substances for example are currently being used to demonstrate exposure to organic contaminants. For example, catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), HSP70, aryl hydrocarbon receptor (AHR), P glycoprotein (P-gP), and metallothionein (MT), isoforms of the cytochrome genes CYP1A1 and P4501A have been shown to be indicative of organochlorine (OC) and polycyclic aromatic hydrocarbon (PAH) exposure (Châtel et al., 2014) (Mandal, 2005) (Fisher et al., 2006). Moreover, these genes of exposure and organismal stress have been used across species, in vertebrates such as rainbow trout (*Oncorhynchus mykiss*), swordtail fish (*Xiphophorus helleri*), fathead minnow (*Pimephales promelas*) and invertebrates such as blue mussel (*Mytilus edulis*) and blood worms (*Chironomous riparius*) (Crago and Klaper, 2012; Hook et al., 2006; Lacroix et al., 2014; Liang et al., 2013; Marinković et al., 2012).

Gene expression biomarkers are not without issues however. For instance, gene expression provides information on a snapshot of time and researchers may miss an exposure time period or the course of experiments may not match the time period that shows the greatest impact on the expression of a particular gene. Also, as with other monitoring studies, the target species of

interest can only be studied where it can be found geographically, which impacts the ability to draw conclusions across a range of environmental conditions. The best species for monitoring have a broad distribution and therefore can be used over a broad range of conditions.

In this experiment we examined the use of zebra mussels, *Dreissena polymorpha* (Pallas, 1771), as a monitoring tool and studied the reliability of gene expression of caged mussels in monitoring sites and their relationship to chemical data and time. The zebra mussel, is a native of the Ponto-Caspian basin and is invasive worldwide but because of its distribution has been used in the monitoring of chemical exposures in lakes and rivers in Europe and the Laurentian Great Lakes, which makes it such a favorable organism to use for monitoring freshwater ecosystems (Carrasco et al., 2008; Johns, 2011; Kimbrough et al., 2013; Riva et al., 2008). *D. polymorpha* are sessile filter feeders, and are capable of accumulating excessive amounts of toxics (Bruner, K., Fisher, S. & Landrum, 1994). They are also relatively easy to sample due to their abundance in many freshwater systems in North America and Europe, where special permitting for collection is not required. Since the accidental introduction of the zebra mussel into the Great Lakes in the late 1980s, rapid growth and fecundity have made this species a dominant portion of the biomass in the Great Lakes (Johnson and Carlton, 1996) (Schloesser et al., 1996). The NOAA Mussel Watch Program (MWP) seized the opportunity to use this invasive starting nearly two decades ago to monitor contamination in twenty-three core

sampling sites for over 150 contaminants throughout the Great Lakes basin (Kimbrough et al., 2013).

The site chosen for this study was the upper Niagara River AOC (Fig. 1). The Niagara River is a Great Lakes connecting channel that flows north from Lake Erie 36km, over the Niagara Falls, continuing 22km into Lake Ontario. Additionally, the river is physically separated in the middle by Grand Island with an American side and a Canadian side of the river. The portion of the Niagara River governed by the United States has been plagued by inputs of toxic substances from industry, municipalities and hazardous waste sites while the Canadian side of the river is relatively clean and served as a control (Allan et al., 1983; The Niagara River Secretariat, 2007). There are approximately seven mid-sized creeks that contribute toxics to the main stem of the Niagara River, some having different chemical signatures. For this reason, we selected the Niagara AOC as an ideal site to serve as a microcosm to demonstrate the usefulness of molecular biomarkers in the Great Lakes to examine how gene expression may predict exposure to different chemical signatures. We sampled zebra mussel tissue for a vast grouping of bioaccumulated legacy contaminants and CECs from each of the seven creeks and in the main trunk of the Niagara River. Furthermore, we monitored the expression of several gene expression biomarkers that indicate general stress to the health of the zebra mussel. Gene expression was monitored as a biomarker for several genes associated with known contaminants (Table 1). In a broad overview of a common pathway of detoxification, it is recognized that PAHs for instance, are ligands known to bind

to AHR, which induces phase II enzymes such as GST in response to oxidative stress. GST's ability to make the reduced product more water soluble will enable it than to be transported out of the cell, mediated by P-gP and thus reducing its toxicity. Lastly, if damage does occur it is widely accepted that HSP70 will repair and refold damaged proteins (Châtel et al., 2012a; Hankinson, 1995; Mandal, 2005; Singer et al., 2005) (Table 1). Analyzing the mRNA transcripts of these genes should permit us to make inferences in to the health of the zebra mussel in relation to chemical contaminants.

The goal of this study was to test how well biomarkers of stress and xenobiotic metabolism in the gill of the zebra mussel correlate to toxic compounds in the Great Lakes AOCs to provide a reference of exposure and potential impacts to the already well established chemical monitoring program. Preliminary studies using whole mussel tissue were shown to be less sensitive and therefore tissue from the gill of the zebra mussel was chosen for this study. Also, the gill, due to its location at the interface of the aqueous environment is therefore the first organ to react to an environmental insult (Binelli et al., 2015). Moreover, this tissue has been successfully used and reproduced in many studies to show changes in gene expression (Binelli et al., 2011; Lacroix et al., 2014).

Mussels were harvested from the outer breakwater at Buffalo Harbor Light, which is located about 1 km southeast of the water intake for Buffalo, NY. The mussels were relocated to locations within the Niagara AOC that were estimated to have varying levels and types of contaminants near or at the mouths of seven of its

tributaries on the U.S. side. Two cages of mussels were deployed and recovered, one at five weeks and one at ten weeks of exposure and analyzed for both chemical accumulation and molecular biomarkers, and also to examine how the length of exposure impacts gene expression and the correlation between biomarkers and gene expression. Finally, data were examined to analyze how clean the river sites in the Niagara were based on gene expression biomarkers versus smaller creek sites in comparison to actual chemical data.

Materials and Methods

Mussel caging and experimental design

Zebra mussels *D. polymorpha* (*Pallas, 1771*) were collected in June 2014, by SCUBA from harvest site NR1 (Fig 1) in Lake Erie off the coast of Buffalo, New York and put in coolers with site water. Zebra mussels were then distributed into vinyl dipped steel mesh (10" x 9.75" x 9") torpedo cages and placed in the tributaries of interest (Fig. 1). Two separate cages were attached to a sampling station, a frame constructed of black steel pipe to keep the cages from sinking into the sediment and to anchor them in the current. In each location time 0 was considered the initial sampling point having collected 20 mussels to provide a baseline, cages were than sampled at five weeks and ten weeks for chemical and gene expression analysis. In situ zebra mussels were also collected directly from sites NR4, NR5, NR6, and NR9 by SCUBA divers in the main branch of the Niagara River (Fig.1). Site NR9 served as the control site due to predetermined low levels of contamination. Twenty zebra mussels were immediately extracted

from the control site for time 0. Additionally, twenty mussels were extracted from each sampling location at five and ten week intervals. For each individual mussel sample gills were separated from whole mussel tissue and preserved in RNAlater™ (Ambion, Austin, TX) per manufacturers instruction, put on dry ice and sent overnight to the University of Wisconsin-Milwaukee, School of Freshwater Sciences where samples were immediately stored in a -80 freezer. The map (Fig. 1) made to reference sampling locations was created using ArcGIS Desktop: Release 10. Redlands, CA: Environmental Systems Research Institute.

In situ mussel experiment

In conjunction to the caged mussel experiment, zebra mussels living within the upper Niagara River, which will be referred to as "native mussels" were harvested from several locations, NR1, NR4, NR5, NR6 (Fig. 1) by divers from the U.S. Army Corp of Engineers in June of 2014. In each of these locations mussels were taken for chemical analysis and gills were removed from twenty samples per site for gene expression analysis.

Chemical Analysis

Sub samples of mussels were rinsed with site water to remove debris then placed into one-gallon sealed plastic bags. Samples were then put on water ice and shipped to TDI-Brooks International, Inc. for tissue chemical analysis (See Tables 4, 5, and 6 for a full list) Whole mussels were rinsed and shucked; soft tissue removed, weighed, homogenized and the pooled sample from each site

weighed and frozen in a -20 freezer. 15g of wet weight tissue were chemically dried with Hydromatrix®. The mixtures are extracted with dichloromethane using a Dionex Accelerated Solvent Extractor (ASE200). A portion of the mixture was purified using alumina/silica gel column chromatography and gel permeation column (GPC)/ high performance liquid chromatography (HPLC). After HPLC, the eluents were reduced to 0.5 ml and analyzed for PAHs, PCBs, pesticides, PBBs and PBDEs by gas chromatography (McDonald et al., 2006). An aliquot of the mussel homogenate was sent to AXYS Analytical Services Ltd. (B.C. Canada) for the analysis of CECs. AXYS followed EPA method 1694 for analysis of all CECs.

RNA extraction and quantitative RT-PCR analysis

Samples were thawed on water ice, and excess RNAlater was blotted from the gill with a sterile Kimwipe per manufacturer's instructions. Ribonucleic acid (RNA) was extracted using 200ul of TRIzol (Thermo Scientific, Wilmington, DE) per sample and a Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA) using an on-column deoxyribonuclease (DNAse) treatment. The concentration of total RNA was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and absorption checked at 260/280nm and 260/230nm. Samples were also evaluated for RNA quality on an Agilant 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription of 100ng of RNA was used to make complimentary DNA (cDNA) using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA).

Gene expression was quantified using the iTaq Universal SYBR Green Supermix 20ul protocol (Bio-Rad, Hercules, CA) for selected genes associated with oxidative and general stress as well as xenobiotic metabolism that had been shown to be related to chemical exposures (see Table 1). Due to the fact that the zebra mussel's genome has not yet been sequenced it is not considered a "model" organism. Few studies have investigated gene expression in the zebra mussel and therefore, limited transcripts are available.

Real time quantitative polymerase chain reactions were run on a StepOnePlus™ RT-PCR system (Applied Biosystems, Foster City, CA) using the following protocol: 1) cycle at 95°C for 10min, and 2) 40 cycles of 95°C for 15s and 60°C for 30s. All qRT-PCR reactions were run in duplicate. The real-time quantitative PCR data were first processed through the Miner program, which allowed us to extract meaningful cycle threshold (CT) values from the raw fluorescence data produced by the StepOnePlus™(Zhao and Fernald, 2005). The data driven normalization algorithm (NORMA-gene) developed by Heckmann et al., (2011) was used to determine differences in gene expression among sites. This program estimates a normalization factor by averaging replicates of all target genes. It has been shown that the normalization factor improves by increasing the number of genes used, however a minimum of five genes are needed to reach optimization and that is the number of target genes used in this study. Gene expression for mussels from sampling sites was normalized to the control and then $\log_2$ transformed to determine the relative fold expression levels.

Statistical Analysis

The chemical data set was $\log_2$ transformed in order for it to be in the same scale as the gene expression data to make functional inferences. A bivariate Pearson Correlation was used to find correlations among the variables in the chemical data set. Although collinearity of our chemical variables was a common occurrence in the dataset, due to the amount of chemical data a Stepwise linear regression was used initially to parse out individual chemicals of interest. Furthermore, single linear regressions were used in validating the findings of the Stepwise regressions and relationships between the chemical data and gene expression data. All these analyses were performed in SPSS™ (24).

The Shapiro-Wilk test was used to test for normality and variance was tested using Sigma plot™ (12.5). Significant differences ($p < 0.05$) between the five and ten-week time periods and control site versus sampling stations were measured using a Students T-test. Nonparametric data were analyzed using the Mann-Whitney rank sum test and a Kruskal Wallace One Way ANOVA with a Dunn's method post hoc test was used to assess differences among the Niagara River Tributaries.

Results

Chemical Analysis

Analyses of the chemical data show that PCBs and PAHs were the main contaminants in the upper Niagara River and its tributaries. Additionally, these contaminants were found to be at a lower concentration in the main branch of the river in comparison to the creeks feeding into it. Also, the CECs, 4 Nonylphenol (4NP), a surfactant used in detergents, pesticides and paints, and Triclocarban (TCC), a commonly used ant-microbial agent, were found to be chemicals of interest due to their concentrations correlating with genes of interest and were generally found at higher levels in the creeks as well (Table 2)

Furthermore, Pearson correlation analysis showed that the individual PCB congeners and the PAHs correlated with each other. Therefore, all 83 PCB congeners and 81 PAHs were summed (individual PCBs and PAHs summed can be found in the supplementary data) and a Pearson correlation was computed to assess the relationship between the summed PCBs and summed PAHs. In fact, a significant positive relationship did exist between the two variables, $r = 0.702$, $n = 12$, $p < 0.05$. The scatterplot (Fig 2) encapsulates the results.

Stepwise Regression Analysis

To assess relationships between the contaminants measured in the tributaries, and the mRNA biomarkers of interest, stepwise-multiple regression analyses were performed. The results from the model infer that there is a significant association between the chemicals methylanthracene, heptachlor-epoxide, PCB 101 and carbazole and GST, explaining 96% of the variance ($p < 0.05$, $df = 11$,

R^2 = .961) Additionally, the gene HSP70 was correlated with PCB 8 explaining approximately 42 % of the variance ($p < 0.05$, df = 11, R^2 = .426). Last, the gene AHR was correlated with PCBs congeners 178, 92, and 44, which explains approximately 92 % of the variance ($p < 0.05$, df = 11, R^2 = .924).

Gene expression of caged mussels as a predictor of chemical contamination

As many of the chemicals correlated with each other, individual linear regressions were used to further examine if associations did exist between genes and contaminants. Due to collinearity of PCBs, these chemicals were summed and analyzed with each gene as were PAHs, additionally a few CECs were shown to correlate with the gene expression, although these chemicals correlated to legacy contaminants as well.

The summed PCB congeners correlated significantly with GST: R^2=0.411, $p < 0.05$, df=11, F=6.994, and AHR: R^2=0.456, $p < 0.05$, df=11, F=8.401 (Fig.3). The genes P-gP and HSP70 were not significant $p > 0.05$. Summed PAHs correlated significantly with GST: R^2=0.717, $p < 0.05$, df=11, F=5.397), and AHR: R^2=0.358, $p < 0.05$, df=11, F=5.570) (Fig. 3). The genes P-gP and HSP70 were not found to correlate significantly $p > 0.05$. 4 Nonylphenol (4NP) and Triclocarban (TCC) classified, as CECs were the only two CECs that correlated significantly with genes monitored here. 4NP associated significantly with GST: R^2=0.394, $p < 0.05$, df=11, F=6.510, and HSP70: R^2=0.450, p<. 05, df=11, F=8.190) (Fig 4). The genes P-gP and AHR were not significant $p > 0.05$. Lastly, TCC showed a significant relationship with GST: R^2= 0.65, $p < 0.05$, df=11, F=17.602. P-gP, AHR, and HSP70 were not significant $p > 0.05$. Linear regressions calculated

with the data from the ten week data exhibited no significant correlations ($p > 0.05$)

Relationship of gene expression to time of exposure

All four genes, P-gP, GST, AHR, and HSP70 showed significant down regulation when measured at the 5 week and 10 week time point, in comparison to the control site $p < 0.05$, except HSP70 at Smokes Creek, which was not significantly downregulated in comparison to the control $p > 0.05$ (Fig. 5).

Additionally, significant differences occurred between the measurements of expression at the five-week time point in relation to the expression measured at the ten week time point in the creeks. Tonawanda Creek was the only sampling site where all of the genes were down regulated in response to the time lapse (Mann Whitney Rank Sum: GST: $U = 0$, df 39, $p < 0.05$; P-gP: $U = 0$, df 39, $p < 0.05$; AHR: $U = 38$, df 39, $p < 0.05$; HSP70: $U = 4$ df 39, $p < 0.05$) (Fig. 5). In one case, the HSP70 was expressed approximately 2.5 fold less than the five week time point.

Next, at Two Mile Creek the genes GST, P-gP and HSP70 were all significantly expressed less (Mann Whitney Rank Sum: GST: $U = 33$, df, 39, $p < 0.05$; P-gP: $U = 4$, df 39, $p < 0.05$; HSP70: $U = 8$, df 38, $p < 0.05$)(Fig. 5). In reference to the earlier time point, these genes at Two Mile Creek have the largest disparity in expression, the largest being P-gP, with an almost four fold decrease. At Scajaquada Creek, the HSP70 was down regulated while; the AHR was upregulated (Mann Whitney Rank Sum: HSP70: $U = 0$, df 39, $p < 0.05$; AHR: $U = 18$, df =39, $p < 0.05$) (Fig. 5). At Ellicott Creek, HSP70 was significantly

decreased while GST significantly increased upregulated (Mann Whitney Rank Sum: HSP70: $U = 4$, df 39, $p < 0.05$; GST: $U = 57$, df 39, $p < 0.05$) (Fig. 5). Next, at Cayuga Creek, we saw significant down regulation of GST (Mann Whitney Rank Sum: GST: $U = 52$, df 39, $p < 0.05$) (Fig. 5). Lastly, at Smokes Creek, no significant changes in gene expression were documented ($p < 0.05$, Fig. 5).

Gene expression between tributaries

To see if there was a differentiation amongst just the tributaries in relation to gene expression, we performed a Kruskal Wallace One Way ANOVA with a Dunn's post hoc test. We found that in three out of the four genes of interest, P-gP, GST, and AHR showed a significant trend. For instance, the expression of these genes was significantly less expressed in Scajacquada Creek in reference to the others. P-gP expression was significantly lower (Kruskal Wallace One Way ANOVA: $X^2 = 54.22$, df 6, $p < 0.05$) and a Dunn's post hoc showing that Creeks Gill, Tonawanda, Smokes, Two Mile and Ellicott were all significantly different in expression ($p < 0.05$ (Fig. 6). Also, GST expression was lower as well, (Kruskal Wallace One Way ANOVA: $X^2 = 70.037$, df 6, $p < 0.05$) and a Dunn's post hoc showing that Creeks Gill, Cayuga, Tonawanda, Smokes, and Two Mile were all significantly different in expression ($p < 0.05$) (Fig. 7). Lastly, AHR continued the trend (Kruskal Wallace One Way ANOVA: $X^2 = 32.391$, df 6, $p < 0.05$) and a Dunn's post hoc revealed that Creeks Gill, Cayuga, Tonawanda, Smokes, and Ellicott were all significantly different in expression from Scajacquada ($p< 0.05$) (Fig. 8).

In situ monitoring of gene expression and its relation to chemical contamination

Results from the in situ experiment to evaluate gene expression of the native zebra mussels living in the Niagara River sites revealed a pattern at NR6, NR5 and NR1. For example, GST, AHR, and P-gP were all significantly different $p < 0.05$, while the HSP70, was not $p > 0.05$ (Fig.1). Additionally, NR4 was considered to be an outlier, due to the fact that all four of the genes measured at this site were not significantly different from the control $p > 0.05$. Furthermore, any correlations of gene expression in regards to the river sites relative to the chemical data using linear regression calculations were not found to be statistically significant $p > 0.05$.

Discussion

Chemical contamination varied significantly across watersheds and gene expression correlated with the level of contamination. Not surprising, due to their persistence and historic abundant usage, the legacy contaminants, PCBs and PAHs were the most prevalent chemicals found in the Niagara AOC (Table 2). Contamination in the creeks was greater than sites sampled in the Niagara River. For example, in the Niagara River sites, the sum of PCBs measured was between 133.09 ng/g and 316.28 ng/g (Table 2). In relation to sum PCBs in the creeks which were measured between 451.15 ng/g and 4428.18 ng/g (Table 2). Additionally, sum PAHs in the Niagara River were measured to be 864.438 ng/g and 5820 ng/g (Table 2). Comparatively, the creeks had samples ranging from 1808 ng/g to 48407.91 ng/g (Table 2).

The expression of genes GST and AHR correlated significantly with PCBs and PAHs (Fig. 3). It is not unusual that these two genes would respond to this

type of contamination, for the weight of evidence exists to confirm that AHR activity is linked to OC contamination as well as GST to concentrations of PAHs in the environment (Lam, 2009; Mandal, 2005). In fact, the strongest association we recorded was GST to the sum PAHs that explained 75 percent of the variation in the data.

CECs were sampled at concentrations much less than the legacy contaminants (Table 2) and our analysis showed that 4NP and TCC significantly correlated to the expression of our genes of interest as well. For instance, 4NP showed a significant correlation to the genes HSP70 and GST. In this case, the evidence is analogous to other 4NP exposures to invertebrates, confirming that there is an association between concentrations of 4NP and expression of genes HSP70 and GST (Lee and Choi, 2006). Additionally, the correlation of TCC to GST expression was significant explaining approximately sixty-five percent of the variation. However, data regarding gene expression in relation to TCC is severely lacking, and these results warrant verification in a lab study to confirm this significant correlation.

In addition to the significant correlations between our genes of interest and chemicals measured, a pattern emerged revealing that almost all of the genes of interest measured in caged mussels from the creeks were down regulated in comparison to control (Fig. 5). Genes can be down regulated for several reasons, though a common response to environmental provocation is an impulse pattern of regulation. For instance, levels of mRNA transcripts tend to spike, sustain levels for a period of time, and then transition to a state comparable to

original levels (Yosef and Regev, 2011). For this study, it is possible that we missed the critical period for when the genes were initially expressed and that the synthesized proteins had already been processed to help the organism through the exposure, our study having sampled near or at the end of the pulse.

Time was also a significant factor when monitoring gene expression biomarkers. For instance, at ten weeks there were no significant correlations among the genes investigated and chemical contamination that correlated with the genes at five weeks. One plausible explanation for these effects is that the caged mussels could be shutting down protein production and metabolism due to the cumulative impacts of contamination such as through an unfolded protein response. If this were the case, it would make sense that the gene HSP70 was the only gene not correlated with the other genes of interest (Table 3), for it would be in the beginning stages of refolding damaged proteins, while translation of other proteins would be shutting down (Haynes et al., 2007).

Additionally, there were some significant gene expression changes between the five and ten week time points (Fig. 5). However, no apparent pattern could be inferred. For instance, a trend could be seen at Tonawanda Creek, evidence pointing to significant down regulation in all the genes measured, though this did relate to the chemical data. Although some of the genes were significantly changing at the later time points, it is possible that gene expression could be too sensitive to be a predictor of chemical contaminants in a long term chronic exposure. This evidence as a whole, suggests that monitoring gene expression at the five week time period is more preferable than the ten

week time period to be a predictor of the effects of contaminate exposure on an organisms health.

Furthermore, one of the goals of this study was to attempt to rank the tributaries based on the gene expression data. When comparing just the tributaries, we found that there was a significant trend, showing that, strictly based on the gene expression data; the site sampled in Scajacquada Creek was the most affected. For instance, in three of the four genes of interest, AHR, P-gP and AHR, we found that Scajacquada Creek values of these genes were significantly down regulated in relation to five out of the six other sites sampled (Fig. 6,7,8). It would be difficult to show that this was strictly because of levels of chemical contamination, for gene expression can be effected by environmental factors such as water temperature, depth and general water quality standards such as the amount of dissolved oxygen. To improve the strength of sampling data in future studies, some of these physical parameters should be measured to help account for trends in the gene expression data set.

The results from the in situ study demonstrated that sampling already present zebra mussels do not provide an effective way to predict chemical contamination using these genes. This study showed that no significant relationships existed between these samples of native mussels and chemical contamination. Additionally, although chemical contamination in the Niagara River was generally low and in this respect, the sites were comparable, the gene expression from the populations of native mussels in relation to gene expression showed the sites to be dissimilar. Previous studies have demonstrated that

cellular stress was undetected in the chronically exposed “native” mussels in comparison to caged mussels in the prescience of PAHs (Lacroix et al., 2015).

Conclusion

This study found a significant relationship between specific genes of detoxification and chemical contaminants measured in the Niagara River AOC**.** The results support GST and AHR as short term biomarkers for exposure to legacy contaminants, specifically PCBs and PAHs. This study also shows that the CECs, TCC and 4NP, although difficult to separate out, may also be causing impacts on aquatic invertebrates as they also correlate with GST and HSP70 gene, which are associated with oxidative stress and protein damage. This work merits additional studies in a laboratory environment to investigate effects of individual chemicals and optimal time points to monitor these effects in zebra mussels. Overall, the zebra mussel is known to be a robust instrument for chemical monitoring in North America and Europe. However, this utility could be significantly enhanced to a dual role using molecular biomarkers of chemical exposure.

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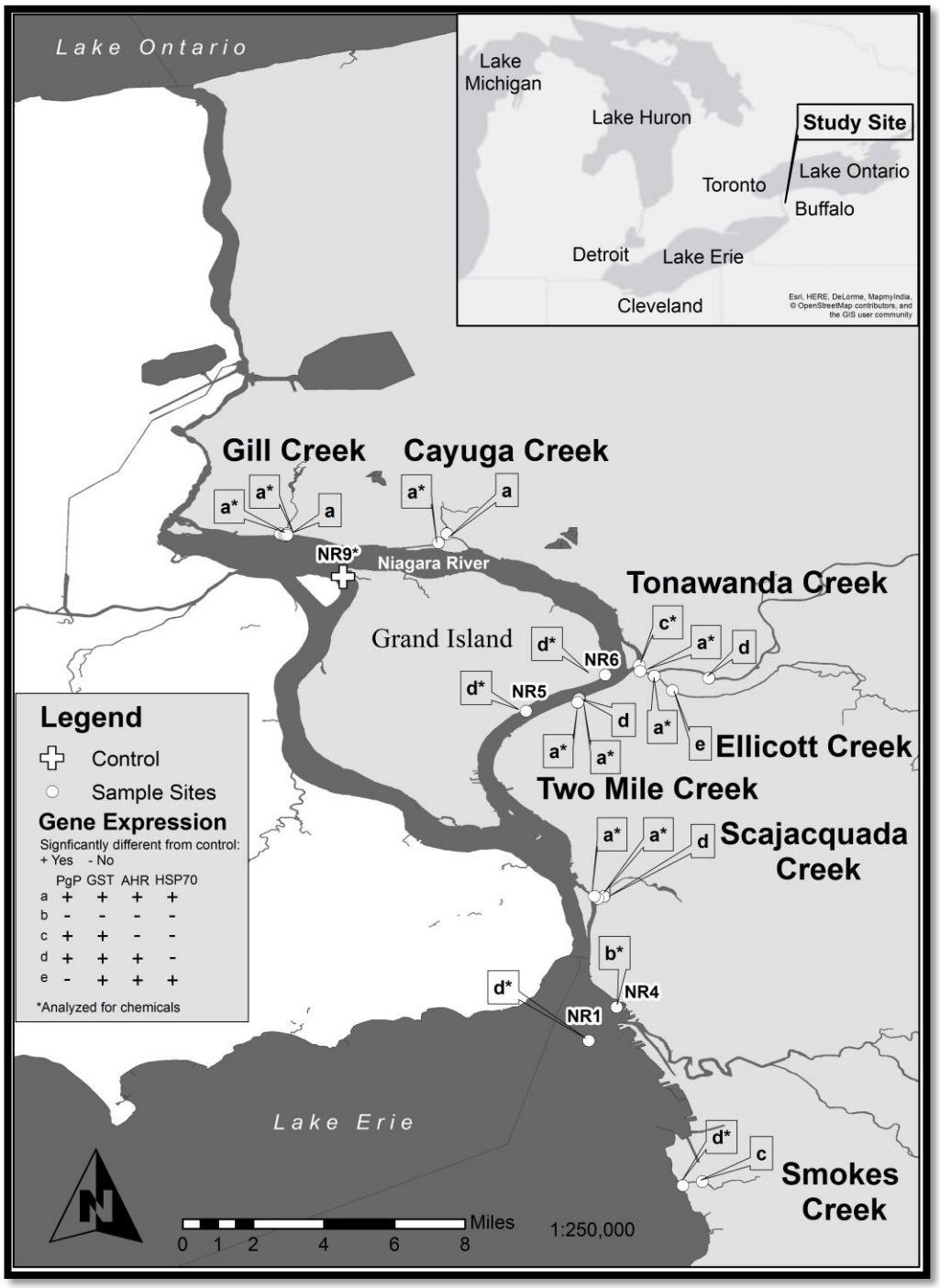

Figure 1. In situ sites and caged mussel sampling locations. Letters in Text boxes represent sampling sites and their corresponding gene expression patterns from the control site $p < 0.05$.

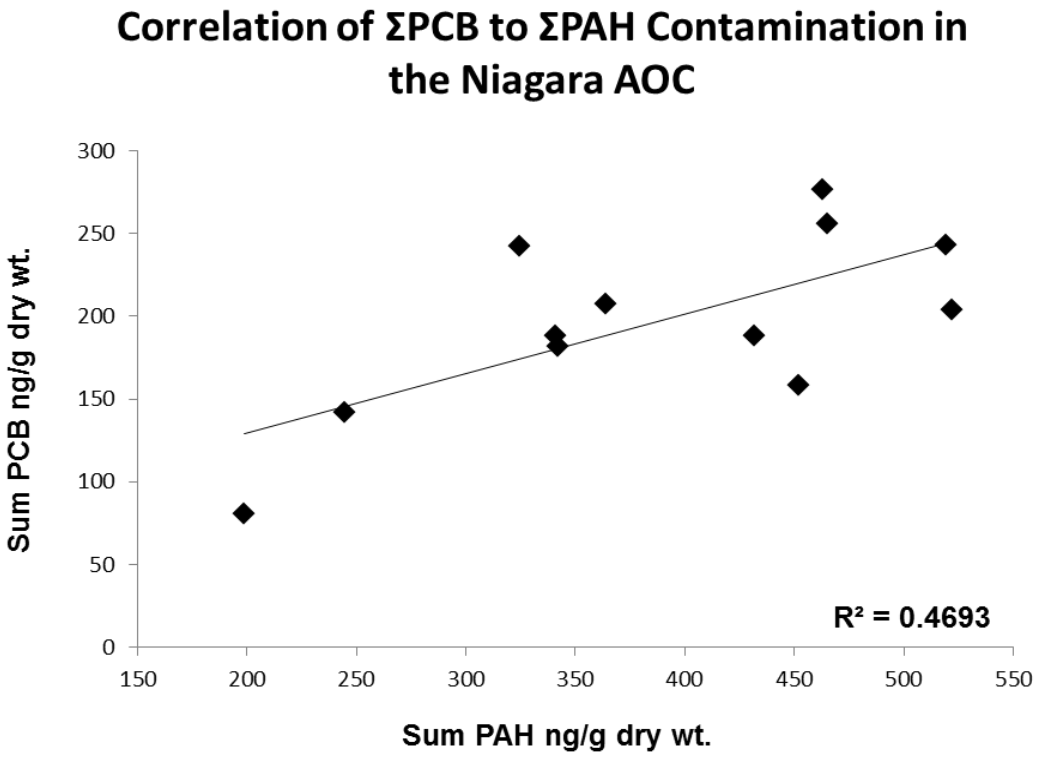

Figure 2. Linear regression representing a significant correlation between sum PAH and Sum PCB concentrations (n=12) $p < 0.05$, $R^2=0.4693$

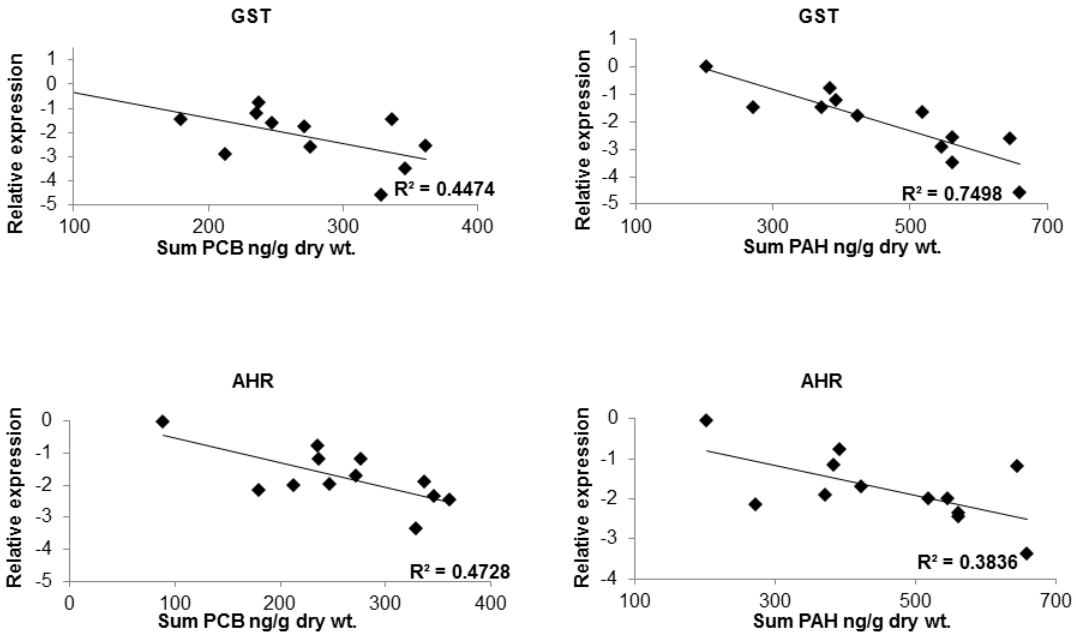

Figure 3. Correlations of Legacy Contaminants to gene expression for caged mussels from the 12 sites of study with chemical data. Gene expression is log_2 transformed, and fold change relative to control. The chemical data is likewise log_2 transformed to calculate linear regressions.

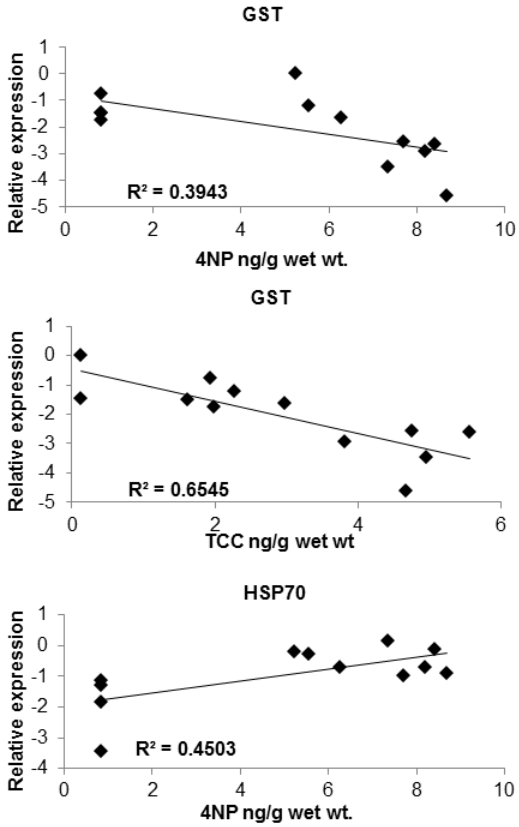

Figure 4. Correlations of 4NP and TCC to gene expression for caged mussels from 12 study sites. Gene expression is log_2 transformed, and fold change relative to control. The chemical data is likewise log_2 transformed to calculate linear regressions.

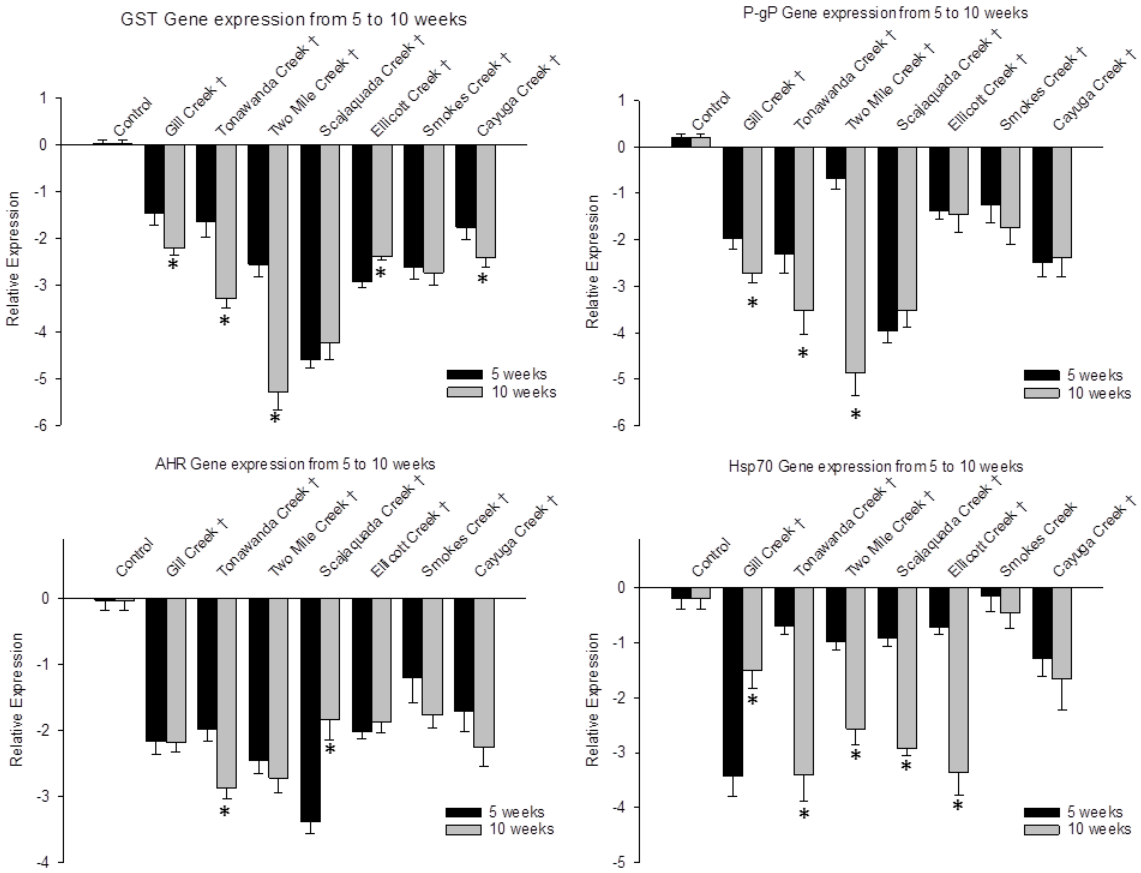

Figure 5. Levels of GST, P-gP, AHR and HSP70 in the gill of the zebra mussels collected at five and ten weeks relative to control. Data shown in figure are from one sampling site per creek to represent pattern of significant down regulation in creeks to the control site. (†): Creeks significantly different from control at five and ten week intervals ($p < 0.05$). (*): Significantly different changes in gene expression from five week to ten-week values ($p < 0.05$)

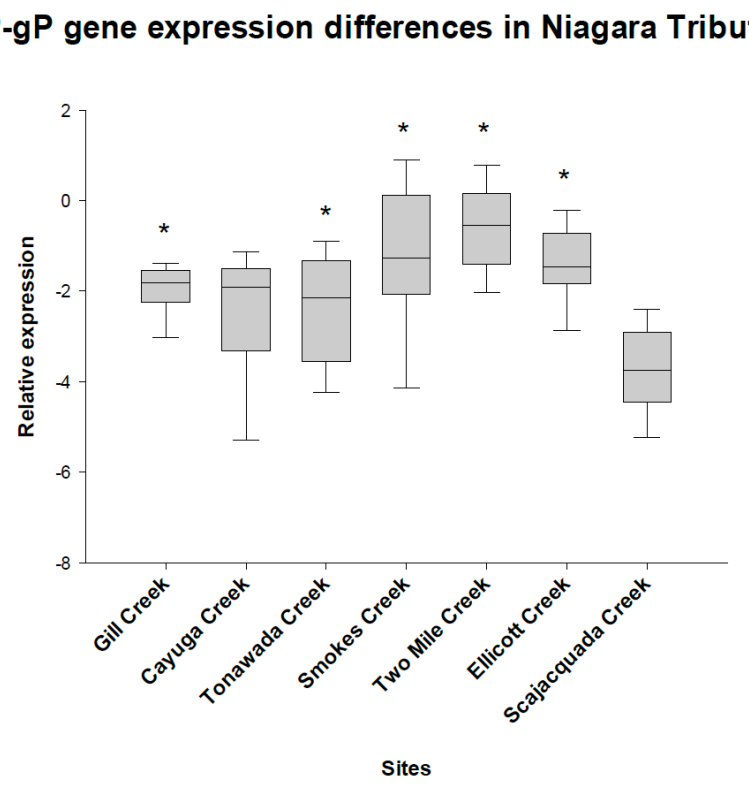

Figure 6. P-gP gene expression differences in Niagara Tributaries. (*) Represent significant differences in gene expression ($p < 0.05$) in relation to Scajacquada Creek.

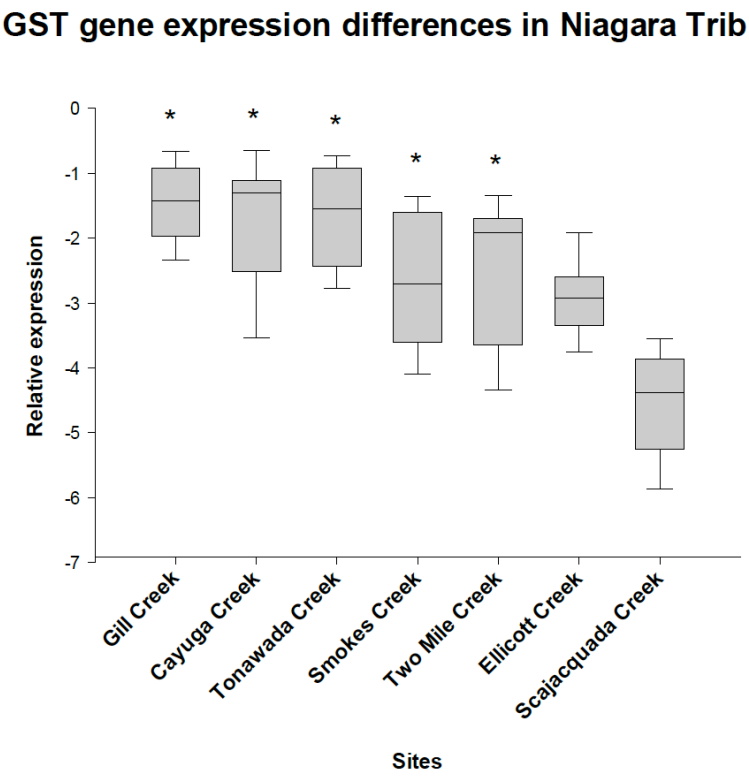

Figure 7. GST gene expression differences in Niagara Tributaries. (*) Represent significant differences in gene expression ($p < 0.05$) in relation to Scajacquada Creek.

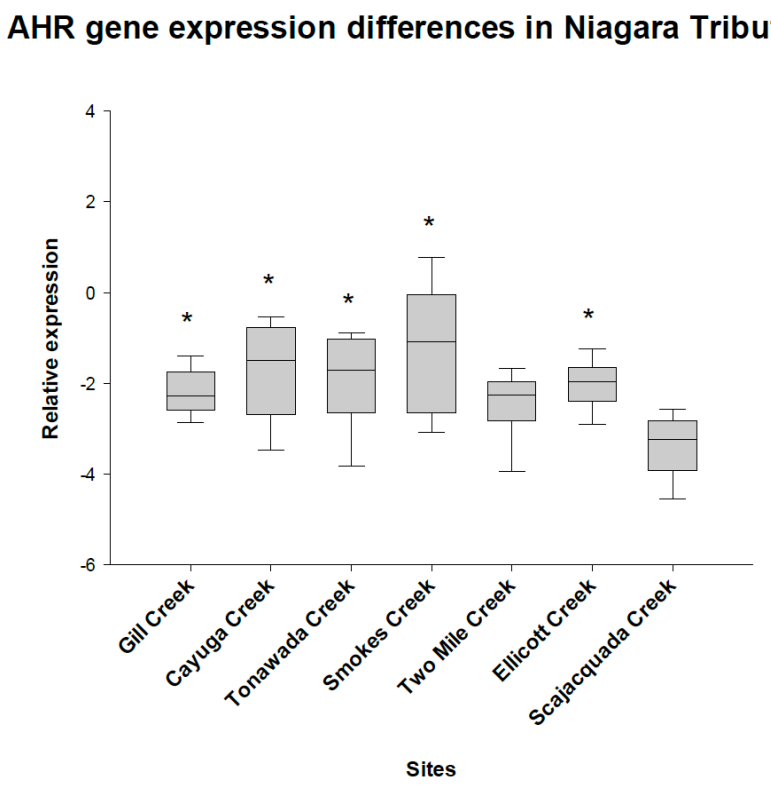

Figure 8. AHR gene expression differences in Niagara Tributaries. (*) Represent significant differences in gene expression ($p < 0.05$) in relation to Scajacquada Creek.

Table 1. Primer sequences (5'-3') used in qRTPCR

Target Gene	Forward primer	Reverse primer	Accession number
18s Ribosomal RNA (18s)	TCGATGGTACGTGATATGCC	CGTTTCTCATGCTCCCTCTC	L33452
Ribosomal protein (S3)	CAGTGTGAGTCCCTGAGATACAAG	AACTTCATGGACTTGGCTCTCTG	AJ517687
P-glycoprotein (P-gP)	CACCTGGACGTTACCAAAGAAGATATA	TCACCAACCAGCGTCTCATATTT	AJ506742
Heat-shock protein 70 (HSP70)	GCGTATGGACTTGATAAGAACCTCA	GAACCCTCGTCGATGGTCA	EF526096
Glutathione S-transferase (GST)	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTAGTCCGC	EF194203
Aryl-Hydrocarbon Receptor (AHR)	ATCACAGCGATGAGCCTCAG	AGACAGCATTGCGAGGTCAC	DQ159188

Table 2. Sites analyzed for Chemical Analysis. Sum PAH and PCB concentrations (ng/g dry wt.) the level of quantification reported at .1ng/g based on 10g of mussel tissue. 4NP and TCC concentrations (ng/g wet wt.) level of quantification reported at 1.2 ng/g based on 2.5g of mussel tissue.

River Sites	Σ 83 PCBs	Σ 81PAHs	4NP	TCC
NR9	133.09	864.438	36.9	<LOQ
NR6	316.28	1919.801	NS	NS
NR5	202.47	1414.69	NS	NS
NR4	282.8	5820.13	NS	NS
NR1	261.33	1431.68	30.1	<LOQ
Creek Sites				
Gill Creek				
GL01	451.15	1808.91	18	<LOQ
GL03	3310.06	6791.45	33.2	2.09
Cayauga Creek				
CY01	1410.42	10921.7	39.7	2.97
Tonawanda Creek				
TW00	921.95	8569.68	45.9	3.82
TW01	1025.98	28168.756	76.7	6.93
Ellicott Creek				
EL01	691.21	45434.36	293	13.1
Two Mile Creek				
TM00	965.73	9072.61	38.8	3.36
TM01	4428.18	45914.683	207	26.1
Scajacquada Creek				
SC00	3632.48	39342.7	163	30.1
SC01	2559.97	75063.8	410	24.5
Smokes Creek				
SM01	1819.96	48407.91	343	46.2

(NS)=No Sample (River sites chemical analysis of 4NP and TCC limited to NR9 and NR1)

<LOQ: below Limit of Quantification

Table 3. Significant correlations measured using a Pearson Correlation across genes of interest. **. Correlation is significant at the 0.01 level (2-tailed)

Gene		P-gP	GST	HSP70	AHR
P-gP	Pearson Correlation	1	.742**	0.05	.778**
	Sig. (2 tailed)		0.006	0.987	0.003
	N	12	12	12	12
GST	Pearson Correlation	.742**	1	-0.218	.827**
	Sig. (2 tailed)	0.006		0.496	0.001
	N	12	12	12	12
HSP70	Pearson Correlation	0.05	-0.218	1	0.228
	Sig. (2 tailed)	0.987	0.496		0.476
	N	12	12	12	12
AHR	Pearson Correlation	.778**	.827**	0.228	1
	Sig. (2 tailed)	0.003	0.001	0.476	
	N	12	12	12	12

Table 4. Individual PCB congeners summed for analysis

ΣPCB Congeners				
PCB1	PCB46	PCB99	PCB167	PCB205
PCB7_9	PCB47_48_75	PCB101_90	PCB169	PCB206
PCB8_5	PCB49	PCB105	PCB170_190	PCB209
PCB15	PCB52	PCB107	PCB172	
PCB16_32	PCB56_60	PCB110_77	PCB174	
PCB18	PCB66	PCB114_131_122	PCB176_137	
PCB22_51	PCB70	PCB118	PCB177	
PCB24_27	PCB74_61	PCB126	PCB178	
PCB25	PCB77	PCB128	PCB180	
PCB26	PCB81	PCB129_126	PCB183	
PCB28	PCB82	PCB136	PCB185	
PCB29	PCB83	PCB138_160	PCB187	
PCB31	PCB84	PCB141_179	PCB189	
PCB33_53_20	PCB85	PCB146	PCB191	
PCB40	PCB86	PCB149_123	PCB194	
PCB41_64	PCB87_115	PCB151	PCB195_208	
PCB42_59_37	PCB88	PCB153_132_168	PCB196_203	
PCB43	PCB92	PCB156_171_202	PCB199	
PCB44	PCB95	PCB158	PCB200	
PCB45	PCB97	PCB166	PCB201_173_157	

Table 5. Individual PAHs summed for analysis

ΣPAHs				
Decalin	C1-Fluorenes	C4-Fluoranthenes/Pyrenes	C1-Dibenzo[a,h]anthracene	Benzo(b)fluorene
C1-Decalin	C2-Fluorenes	Naphthobenzothiophene	C2-Dibenzo[a,h]anthracene	
C2-Decalin	C3-Fluorenes	C1-Naphthobenzothiophene	C3-Dibenzo[a,h]anthracene	
C3-Decalin	Carbazole	C2-Naphthobenzothiophene	Benzo[g,h,i]perylene	
C4-Decalin	Anthracene	C3-Naphthobenzothiophene	2-Methylnaphthalene	
Naphthalene	Phenanthrene	C4-Naphthobenzothiophenes	1-Methylnaphthalene	
C1-Naphthalenes	C1-Phenanthrenes_Anthracenes	Benz[a]anthracene	2,6-Dimethylnaphthalene	
C2-Naphthalenes	C2-Phenanthrenes_Anthracenes	Chrysene	1,6,7-Trimethylnaphthalene	
C3-Naphthalenes	C3-Phenanthrenes_Anthracenes	C1-Chrysenes	1-Methylfluorene	
C4-Naphthalenes	C4-Phenanthrenes_Anthracenes	C2-Chrysenes	4-Methyldibenzothiophene	
Benzothiophene	Dibenzothiophene	C3-Chrysenes	2/3-Methyldibenzothiophene	
C1-Benzothiophene	C1-Dibenzothiophenes	C4-Chrysenes	1-Methyldibenzothiophene	
C2-Benzothiophene	C2-Dibenzothiophenes	Benzo[b]fluoranthene	3-Methylphenanthrene	
C3-Benzothiophene	C3-Dibenzothiophenes	Benzo[k]fluoranthene	2-Methylphenanthrene	
C4-Benzothiophenes	C4-Dibenzothiophenes	Benzo(a)fluoranthene	2-Methylanthracene	
Biphenyl	Fluoranthene	Benzo[e]pyrene	4/9-Methylphenanthrene	
Acenaphthylene	Pyrene	Benzo[a]pyrene	1-Methylphenanthrene	
Acenaphthene	C1-Fluoranthenes_Pyrenes	Perylene	3,6-Dimethylphenanthrene	
Dibenzofuran	C2-Fluoranthenes_Pyrenes	Indeno[1,2,3-c,d]pyrene	Retene	
Fluorene	C3-Fluoranthenes_Pyrenes	Dibenzo[a,h]anthracene	2-Methylfluoranthene	

Table 6. Pharmaceuticals and Personal Care Products analyzed

PPCPs	
10-hydroxy-amitriptyline	Furosemide
13C12-Triclosan	Gemfibrozil
13C3-Ibuprofen	Glipizide
13C6-Triclocarban	Glyburide
13C-D3-Naproxen	Hydrochlorothiazide
2-Hydroxy-ibuprofen	Hydrocodone
Albuterol	Hydrocortisone
Alprazolam	Ibuprofen
Amitriptyline	Meprobamate
Amlodipine	Metformin
Amphetamine	Methylprednisolone
Atenolol	Metoprolol
Atorvastatin	Naproxen
Benzoylecgonine	Norfluoxetine
Benztropine	Norverapamil
Betamethasone	Oxycodone
Bisphenol A	Paroxetine
Cimetidine	Prednisolone
Clonidine	Prednisone
Cocaine	Promethazine
Codeine	Propoxyphene
Cotinine	Propranolol
D11-Glipizide	Ranitidine
D3-Glyburide	Sertraline
D5-Warfarin	Simvastatin
D6-Bisphenol A	Theophylline
D6-Gemfibrozil	Trenbolone
DEET	Trenbolone acetate
Desmethyldiltiazem	Triamterene
Diazepam	Triclocarban
Dioxin	Triclosan
Enalapril	Valsartan
Fluocinonide	Verapamil
Fluticasone propionate	Warfarin

CHAPTER 3: Triclocarban and Gene Expression Biomarkers of Exposure in *Dreissena polymorpha*

Abstract

Complex mixtures of contaminants in aquatic environments can cause varying degrees of organismal stress and effects to health. One method used to measure these effects are by using gene expression biomarkers. Evidence from a previous field study in the Niagara River Area of Concern (AOC) revealed that GST, AHR, P-gP and HSP70 were significantly down regulated in Niagara's tributaries, and correlated with levels of contamination by PCBs, PAHs and emerging contaminants such as Triclocarban. However, as many of the chemicals were co-located, it was difficult in the field to attribute gene expression to any one individual chemical. Nevertheless, a significant relationship between GST and the antibacterial agent Triclocarban (TCC; 3, 4, 4'-trichlorocarbanilide) was found. Based on this finding, we attempted to validate this relationship by exposing zebra mussels to environmentally relevant levels of TCC. Messenger RNA was isolated from the gill of the zebra mussel and analyzed using quantitative real time reverse transcriptase polymerase chain reaction. Results demonstrate that after seven days, the expression of the genes GST, AHR and P-gP, were significantly upregulated in a dose dependent manner at concentrations of TCC at 50 ng/L, 100 ng/L, though at 200 ng/L, while still upregulated from control expression was reduced in relation to the other treatments. Furthermore, Additional investigations of the temporal changes in the expression of GST, P-gP, and AHR linked to downstream physiological

effects are warranted to increase the efficacy of the use of these select genetic biomarkers.

Introduction

Mixtures of anthropogenic pollutants in aquatic ecosystems create difficult scenarios for timely and cost-effective remediation, restoration and successive monitoring efforts. Toxic substances such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) are persistent pollutants and are only two of the numerous historic persistent priority contaminants found globally in surface waters and sediments. Although many of these persistent contaminants have been banned for several decades, they continue to be measured at levels of concern in aquatic ecosystems (Bence et al., 2008; Jones and De Voogt, 1998). Novel techniques that allow the chemical quantification of emerging persistent contaminants are increasingly being developed, enabling us to conclude that long term, low level exposures of these chemicals are having effects on organisms. Pharmaceuticals and personal care products (PPCPs) are a new subset of compounds which exhibit the chemical structure associated with historic persistent pollutants. Examples include over the counter prescriptions, hormones, antibiotics and antimicrobials (Blair et al., 2013; Klecka et al., 2010).

The abundant use of well-known anti-microbial agents such as triclosan (TCS), which is a common ingredient in several consumer products such as detergents, bar soaps, deodorants and similar household cleaning products (Perencevich et al., 2001), has been found to have lethal effects on aquatic biota

like algae and invertebrates, and has reproductive effects on fish (Dann and Hontela, 2011).

Triclocarban (TCC; 3, 4, 4'-trichlorocarbanilide), found in similar products, is closely related to TCS and only differs slightly in chemical structure. Although the risks associated with TCS are better known, there is a need for assessments of lesser-known anti-microbial agents such as TCC. Triclocarban was first developed over 60 years ago and more recently has been found in aquatic environments in concentrations thought to potentially have effects on aquatic species, based on estimates modeled with TCS(Chalew and Halden, 2009; Halden, 2014). Still lab based studies of TCC have shown depleted levels of aggression in fat head minnows (*Pimephales promelas*) and

This recent detection is, in part, due to advances in analytical techniques using liquid chromatography electrospray ionization mass spectrometry (LC/ESI/MS) that is now able to measure concentrations of TCC as low as 3.0 ng/L (Halden and Paull, 2004; Sapkota et al., 2007). TCC has been recorded in surface waters in the United States as high as 6.75 µg/L, although mean and median ranges are more readily documented between 0.1 and 0.2 µg/L (Halden and Paull, 2005, 2004). Adsorption of TCC to sediments has been measured at 748 ng/g to 2633 ng/g in China's Shijing River and in sediment cores from Jamaica bay, New York, USA at a concentration of 24 mg/kg at depths of 2-3 centimeters and a concentration of 1mg/kg in surface sediment (Miller et al., 2008; Zhao et al., 2010)

TCC has an estimated logarithmic octanol/water partition coefficient (log K_{ow}) of 4.9 and its level of lipophilicity is generally high enough to be considered as being able to bioaccumulate and persist in the environment (Halden, 2014; Halden and Paull, 2005). Bioaccumulation of TCC in benthic organisms has been confirmed in multiple studies. For example, in a caged snail study, levels of TCC that accumulated in tissue were measured at 299 ng/g (wet weight) from a stream in North Texas, USA. Additionally, in zebra mussel, tissue analyzed in a caged mussel study from a tributary of the Niagara River, TCC at was quantified at a concentration of 48.6 ng/g (wet weight) (Coogan and La Point, 2008; Neureuther et al.). This data illustrates that TCC is able to adsorb to sediment and bioaccumulate in the tissue of aquatic organisms in the environment, making these concentrations relevant to use for this laboratory investigation.

The presence of chemical contamination in the tissues of aquatic organisms is a more relevant indicator of the potential exposure and impacts of environmental contamination and these impacts are useful gauges of contamination and progress of remediation efforts. *D. polymorpha* or zebra mussel (Pallas, 1771), for example, has been used in the United States and Europe as a biological tool for monitoring the contamination of aquatic ecosystems due to specific favorable qualities such as: its geographic range, filtration rate, ability to withstand accumulated toxicants and sessile nature (Contardo-Jara et al., 2009; Johns, 2011; Kimbrough et al., 2008; Riva et al., 2008). However, chemical exposure and accumulation alone are not enough to

determine the possible impacts, especially when not all chemical contaminants act the same.

The National Academy of Science/National Research Council (NAS/NRC), defines the term “biomarker” as a disruption in cellular biochemical processes, structures, or functions, induced in response to a xenobiotic compound, that can be measured in a biological sample (NRC, 1987). Examples of biomarkers used for monitoring aquatic environmental quality are prolific. In zebra mussels specifically, when exposed to PCBs, PAHs and metals, biomarkers measuring, proteins, DNA adducts as well as oxidative stress have been used to show the deleterious effects of exposure to zebra mussels (Châtel et al., 2012; Faria et al., 2009; Michel and Vincent-Hubert, 2012).

Gene expression biomarkers of organism health and chemical exposure have several advantages over traditional biomarkers. For instance, using small quantities of sample, an investigator can analyze several different molecular endpoints at once from either an individual or a specific tissue and determine molecular pathways that are perturbed in response to a chemical substance (Thomas and Klaper, 2004). The sensitivity of molecular biomarkers enables the detection of a chemical perturbation that is causing changes in an organism at an early stage. As a result, these tools may be used as an early warning system to detect exposure before adverse impacts occur and in response, risk management could take place, mitigating potential population level impacts to sensitive species (Van der Oost et al., 2003). Lastly, genetic biomarkers have been proven to be indicative of specific chemical-induced organismal stress as

they have been linked to different types contamination (Châtel et al., 2014; Crago and Klaper, 2012; Navarro et al., 2011). Collectively, genetic biomarkers can be invaluable for the early detection and validation of specific chemical exposure and subsequent adverse outcomes.

For this investigation zebra mussels were evaluated to determine their response to the emerging contaminant TCC to compare to the results of our previous study from the Niagara River AOC, where we found TCC correlate with gene expression. Many AOCs contain a complex mixture of historic and newly recognized, persistent contaminants making it difficult to tease out which toxics are having the greatest impacts on organisms living within them. TCC correlated with many other contaminants and therefore, it was difficult to determine if it was having an impact.

In the present study, effects of environmental levels of TCC on zebra mussels were explored in the laboratory using biomarkers of general stress: glutathione S-transferase (GST), aryl hydrocarbon receptor (AHR), p-glycoprotein (P-gP) and heat shock protein 70. GST is a phase 2 metabolizing enzyme, which reduces glutathione (GSH), increasing its solubility and in turns its excretion rate. This is in addition to its role in the redox cycle where it lessens the impacts of reactive oxygen species (ROS)(Binelli et al., 2009). The aryl hydrocarbon receptor (AHR) is part of one of the most commonly studied metabolic pathways of detoxification. Historically referred to as the dioxin pathway, the study of the expression of AHR has long been linked to the detoxification of anthropogenically sourced chemical contamination and was

recently shown to be induced in zebra mussel gills when exposed to PAHs (Beischlag et al., 2008; Châtel et al., 2012). Just as important is P-gP which is induced specifically to efflux xenobiotics out of the cell in a process which is ATP dependent (Faria et al., 2011). Lastly, HSP70 is induced to refold damaged proteins and as a chaperone in relation to stress defense (Contardo-Jara and Wiegand, 2008).

Generally, TCC is greatly underrepresented in terms of studies assessing effects to aquatic organismal health in relation to legacy contaminants as well as a closely related chemical TCS. In addition, this is the only investigation of TCC exposure to *Dreissena polymorpha*, which makes this study highly relevant due to *D. polymorph's* status as a tool for environmental monitoring.

Materials and Methods

Zebra Mussel Collection, Maintenance and Care

Approximately five hundred zebra mussels were harvested from a depth of 2-3 meters, from the outer harbor of Milwaukee, WI on Lake Michigan, in the first week of May 2016. The mussels were placed in coolers with site water and taken directly to the laboratory's environmental chamber at the UWM School of Freshwater Sciences. The mussels were lightly cleaned of silt and debris and introduced into a thirty-gallon aquarium filled with 25 gallons of dechlorinated tap water. The aquaria were maintained with a photoperiod of 10 hours light and 14 hours dark, constant temperature (18±1 ˚C) and oxygenation (>90% saturation). Mussels were fed 800 ml of freshwater algae, *Selenastrum capricornutum* (Printz, 1914), with a density of 4.9 $*10^6$ per ml daily. Water was changed daily

for three weeks to depurate mussels of any xenobiotic contamination present in the harbor, which had accumulated in the mussel tissue as per (Binelli et al., 2009). Next, specimens with a similar shell length (20 ± 2 mm) were selected and acclimatized in 8 L glass tanks filled with 5 L of dechlorinated water. These animals were kept in the same conditions described above for a week and only mussels with reattached byssi were used in the experiment.

Experimental Design

Triclocarban (TCC; 3, 4, 4'-trichlorocarbanilide; 99% purity; CAS# 101-20-2) and DMSO (dimethylsulfoxide) were purchased from Sigma Aldrich, St. Louis MO. TCCs have a low solubility in water, and therefore three stock solutions were prepared in 100% DMSO and kept in the dark at 4 ˚C. Tanks were dosed for each treatment to yield a final concentration of 200 ng/L, 100 ng/L and 50 ng/L of. Three replicate tanks of 20 individuals were created for each treatment, this exposure was estimated to cause target tissue concentrations of 50 ng/g ww, 100 ng/g ww and 250 ng/g ww after seven days, based on an exposure of TCS to zebra mussels by Riva et. al.,(2012). The control tanks were exposed to 100 µL of DMSO. These treatments were chosen based on data of environmental concentrations found in mussel tissue from the Niagara River AOC (Neureuther et al., 2016, in review) and similar concentrations representative of TCC in the aquatic environment.

Water for the exposure experiments was changed daily for seven days, and TCC was reintroduced with each water change. Mussels were fed daily, 100 mL of freshwater algae, one hour before the water change to ensure exposure

took place through the water only. After seven days, 10 zebra mussels were removed from each tank and the gills were removed and flash frozen with liquid nitrogen and stored at -80 ˚C until gene expression analysis could take place. Tissue from the remaining 10 mussels in each tank (~1.5 g per replicate) was collected and stored at -20 ˚C and then shipped next day air to AXYS Group (BC, Canada) for TCC bioaccumulation analysis. AXYS Group followed EPA method 1694, which uses liquid chromatography – tandem mass spectrometry (LC/MS-MS) for analysis of TCC in zebra mussel tissues. The data set was from AXYS showed that the concentration of TCC in mussel tissue from 30 individuals in the 200 ng/L treatment was 823.33 ± 108 ng/g ww (Fig. 5), 100 ng/L treatment 440.66 ± 36 ng/g ww (Fig. 2), and 50 ng/L treatment 215 ± 42 ng/g ww (Fig. 5). Two of the three DMSO controls had trace amounts of TCC above the limit of quantification (LOQ). These concentrations were shown to be (n=30) 1.95 ± 0.12 ng/g ww, the LOQ for TCC being 1.82 ng/g ww. Because, these mussels were harvested from the environment and depurated for three weeks to remove contamination, it is possible that these trace amounts of TCC may be artifacts of environmental exposure.

RNA extraction and quantitative RT-PCR analysis

Frozen samples were thawed on ice and total ribonucleic acid (RNA) was extracted using 200 µL of TRIzol (Thermo Scientific, Wilmington, DE) per sample and a Direct-zol™ RNA MiniPrep kit (Zymo Research, Irvine, CA) using an on-column deoxyribonuclease (DNAse) treatment per manufacturer's instructions. The concentration of total RNA was quantified using a Nanodrop 1000

spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and absorption ratios were checked for potential contamination. Samples were also evaluated for RNA quality on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse transcription of 100 ng of RNA was used to make complementary DNA (cDNA) using the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, CA).

Gene expression was measured using the iTaq Universal SYBR Green Supermix 20 µL protocol (Bio-Rad, Hercules, CA) for selected genes related to general stress as well as genes that have been previously associated with chemical exposures in environmental studies of zebra mussel gills. Specifically, evidence that was citedin our previous study from the Niagara River AOC (Neureuther et. al. in review) where GST, AHR, P-gP and HSP70 (table 1) were found to be significantly down regulated in relation to control in the environment and GST was found to correlate significantly with the chemical TCC.

Real time quantitative polymerase chain (qRT-PCR) reactions were run on a StepOnePlus™ RT-PCR platform (Applied Biosystems, Foster City, CA) using the following protocol: 1) cycle at 95°C for 10min and 2) 40 cycles of 95°C for 15s and 60°C for 30s. All qRT-PCR reactions were run in duplicate. The real time quantitative PCR data results were analyzed using the ΔCt method.

Statistics

The Kolmogorov-Smirnov and Levene's test were used to check data for normality and homogeneity of variance; also outliers were identified and removed, defined as any data outside 1.5 times the interquartile range. A two-

way nested ANOVA was applied to test for treatment and tank effects. There was not a significant tank effect found in any of the treatments ($p > 0.05$). In addition to the two way nested ANOVA, a Tukey's post-hoc test was used to identify significant differences between groups ($p < 0.05$). All statistical analyses were performed using SPSS v24.0. Armonk, NY: IBM Corp.

Results

The exposure of TCC lasted seven days and mussel behavior was normal across treatments based on visual evidence of mussels siphoning. During the experiment, no mussel mortality was recorded.

Chemical Analysis in tissue and bioaccumulation of TCC

The concentrations found in the mussel tissue in this experiment were higher than the values measured in the zebra mussel tissue from our previous field experiment in the Niagara River AOC. However, the concentrations that were achieved across treatments are similar to measurements of TCC found in snail tissue from a stream in Texas (Coogan and La Point, 2008), showing that this study's results are still environmentally relevant.

Gene Expression Biomarkers affected by TCC Exposure

Observed changes in mRNA expression were found to be significantly different from controls for the genes GST, AHR and P-gP throughout all levels of treatment, although not for the gene HSP70 ($p > 0.05$) (Fig. 9,10,11,12). Two-way nested ANOVA showed GST expression was significantly up regulated from control in all treatments the average and standard error being: 50 ng/L = 0.96 ±

0.15, 100 ng/L = 2.17 ± 0.37, 200 ng/L = 2.07 ± 0.12 ($p < 0.05$, df=3, F=12.456) (Fig. 1). Furthermore, treatments were significantly different from each other from the 50 ng/L to 100 ng/L concentration treatments ($p < 0.05$), but not between 100 ng/L and 200 ng/L concentration treatments ($p > 0.05$). P-gP expression was also significantly up-regulated across 50 ng/L, 100 ng/L and 200 ng/L treatments when compared to the controls under two-way nested ANOVA analysis the average and standard error being: 50 ng/L = 1.53 ± 0.19, 100 ng/L = 1.95 ± 0.25, 200 ng/L = 1.25 ± 0.13 ($p > 0.05$, df=3, F=7.465) (Fig. 2). Additionally, P-gP expression in the 50 ng/L, 100 ng/L were not statistically different from each other ($p > 0.05$), however in relation to the 100 ng/L treatment P-gP was significantly lower from the 200ng/L treatment ($p < 0.05$). AHR expression was significantly up regulated when compared to controls in all treatments under two-way nested ANOVA analysis the average and standard error being: 50 ng/L = 2.16 ± 0.16, 100 ng/L = 1.81 ± 0.25, 200 ng/L = 0.76 ± 0.19 ($p > 0.05$, df=3, F=39.024) (Fig. 3). Differences amongst treatments in relation to AHR expression showed no difference between 50 ng/L and 100 ng/L concentration treatments ($p > 0.05$). However, expression of AHR to TCC concentration in the 200 ng/L treatment was less when compared to the 50 ng/L and 100 ng/L concentration treatments ($p < 0.05$).

Discussion

The results of this study reveal that accumulation of TCC in the tissue of zebra mussels influence the expression of genes GST, P-gP and AHR that are critical for organisms coping with contamination and stress. Evidence from our

previous study in the Niagara River AOC showed historic, persistent contaminants such as PCBs up to 4428 ng/g dry weight, and PAHs in excess of 75063 ng/g dry weight, were significantly higher in magnitude, than TCC measured in zebra mussel tissue 46.8 ng/g w.w. However, we measured a significant correlation of TCC to the expression of GST. The current study demonstrates that this contaminant could be partially responsible for the molecular responses seen in the field (Neureuther et al.) Previous studies have demonstrated the difficulty of linking contaminants to biological impacts, as many organisms in the environment are exposed to a mixture of contaminants and contamination by one chemical often correlated with another (Christiansen et al., 2014).

The expression of the genes GST, P-gP and AHR in all treatments was significantly induced after seven days. As seen in other invertebrate studies, GST is induced when exposed to TCC (Han et al., 2016). Also, similar anti-bacterial agents, triclosan for instance, have been reported to affect zebra mussels GST enzymatic activity (Binelli et al., 2011). The induction of GST in relation to TCC and TCS in this study and other studies in the literature provide evidence that toxicity may be related to oxidative stress from the metabolism of these antibacterial agents. In addition to GST induction, P-gP and AHR expression were also significantly upregulated in relation to the control. Although TCC studies of this nature are in its infancy, one study that explores organism exposures to triclosan confirm that after seven days, P-gP expression is upregulated in the swordtail fish (Liang et al., 2013). An additional study

observed TCC to be a moderate agonist of AHR in human breast cancer cells (Tarnow et al., 2013).

All of the genes tested in this study revealed a hormetic response, referred to as a u-shaped dose response (Calabrese and Baldwin, 2001). In this study, the genes were upregulated in response to lower doses of TCC, but in the highest dose of TCC there is a reduction in gene expression in relation to other treatments (Fig. 9,10,11). It is difficult to speculate why this occurs; however, evidence of a similar effect was seen in the expression of GST and P-gP in triclosan exposures. For instance, after seven days of yellow catfish exposure to triclosan, mRNA levels of GST from liver tissue were measured from the highest concentration of triclosan and were found to be significantly reduced while the opposite was observed at the three lower concentrations tested (Ku et al., 2014). Additionally, relative expression of P-gP after seven days also showed the same pattern in swordtail fish (Liang et al., 2013).

In comparison to our previous study, it was revealed that bioaccumulation of TCC after seven days of exposure instigated a significant up-regulation in the expression of genes related to organismal stress, showing that gene expression monitoring is temporally sensitive. In reference to the Niagara River AOC field study, GST expression had a significantly negative correlation over a gradient of TCC concentrations after five weeks of exposure and no correlation at all after 10 weeks. We hypothesize that this could have been the result of a physiological adaptation to the chemical, or the result of sampling post transcription, the proteins already having been synthesized. In addition to the results from this lab

study, mounting evidence would lead us to believe that when monitoring using genomic biomarkers, earlier sampling would be more revealing as to when the organism is being affected by an environmental insult.

Conclusion

The results of this laboratory study revealed that environmentally relevant concentrations of TCC will bioaccumulate in zebra mussel tissue and these levels of TCC have a statistically significant effect on the organism and expression of the biomarkers of stress and chemical detoxification. These results complement findings from the previous study in the Niagara River AOC, which helps to demonstrate that these markers could be of interest when monitoring effects of TCC, especially where dynamic mixtures of unknown amounts of chemicals are found. Additionally, further exposure studies, using the evidence collected here, could help to to evaluate specific functions of genes used for detoxification in zebra mussels to reveal whole mechanisms of detoxification. Moreover, it seems that even low concentrations of TCC can influence health of zebra mussels on a molecular scale and it would be of great use to investigate the relationships of gene expression biomarkers to downstream endpoints of exposure, such as correlations with enzymes (e.g. protein production) and physiological manifestations (e.g. tumors, neoplasms, fecundity, size or condition factor). Restoration and monitoring of freshwater systems are becoming a global priority, and the zebra mussel, although an invasive species could have a significant role to perform in the detection and evaluation of not only historic contaminants but emerging contaminants as well.

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Table 7. Primer sequences (5’-3’) used in qRTPCR

Target Gene	Forward primer	Reverse primer	Accession number
18s Ribosomal RNA (18s)	TCGATGGTACGTGATATGCC	CGTTTCTCATGCTCCCTCTC	L33452
P-glycoprotein (P-gP)	CACCTGGACGTTACCAAAGAAGATATA	TCACCAACCAGCGTCTCATATTT	AJ506742
Heat-shock protein 70 (HSP70)	GCGTATGGACTTGATAAGAACCTCA	GAACCCTCGTCGATGGTCA	EF526096
Glutathione S-transferase (GST)	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTAGTCCGC	EF194203
Aryl-Hydrocarbon Receptor (AHR)	ATCACAGCGATGAGCCTCAG	AGACAGCATTGCGAGGTCAC	DQ159188

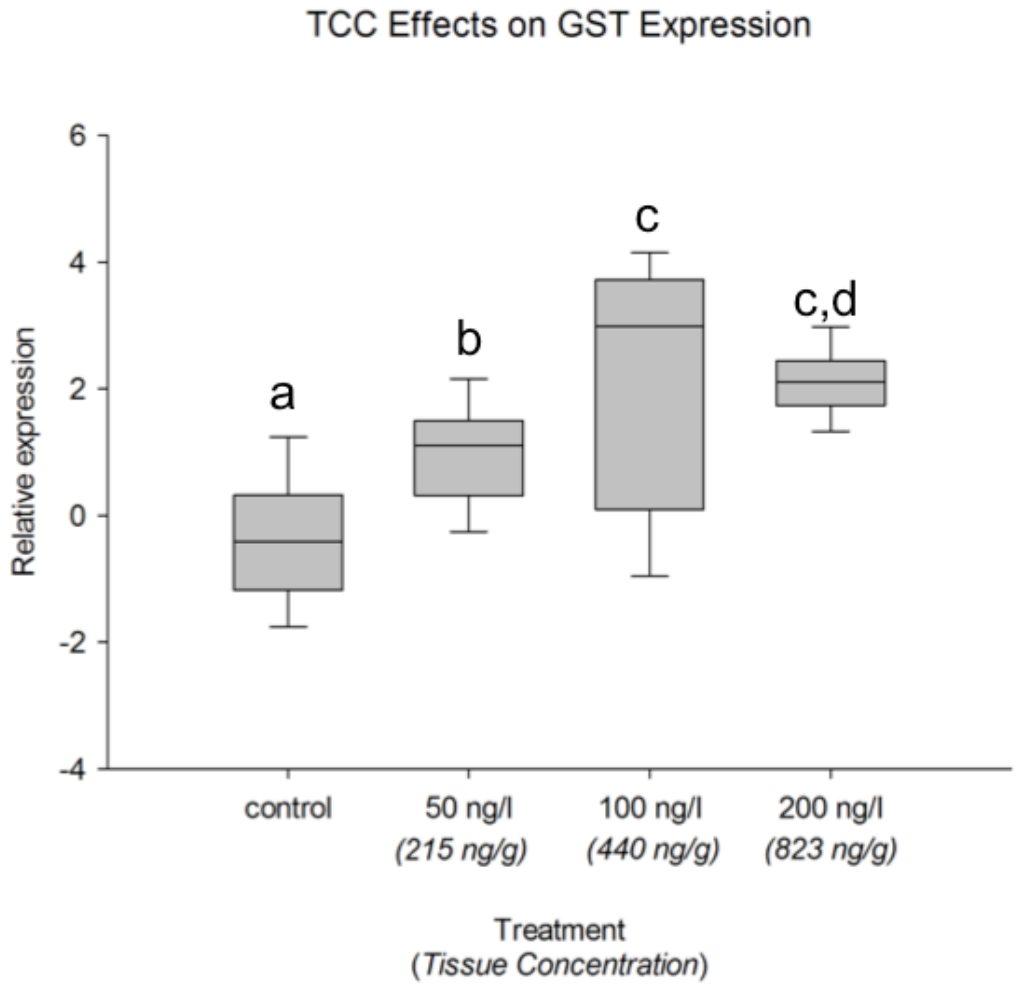

Figure 9. GST expression after seven days of exposure to TCC.

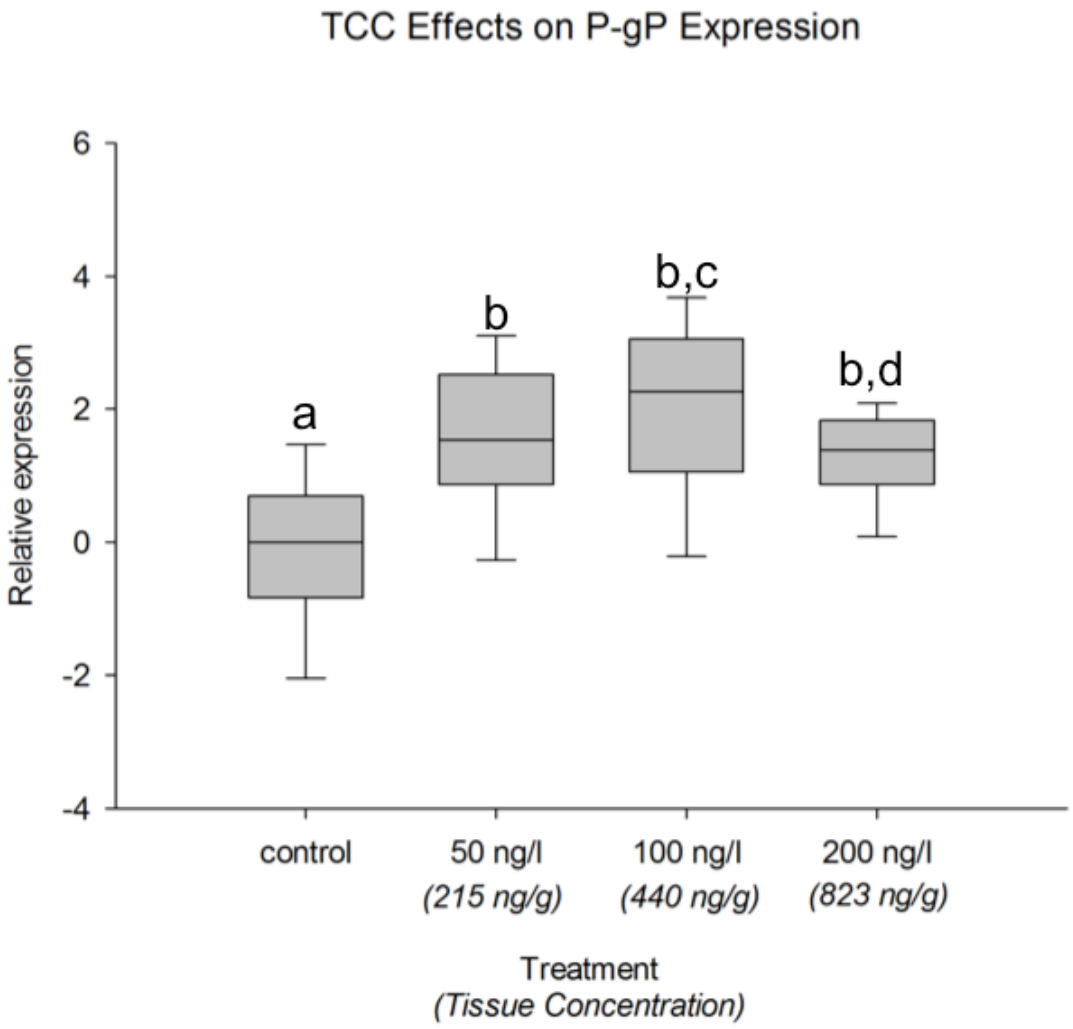

Figure 10. P-gP expression after seven days of exposure to TCC.

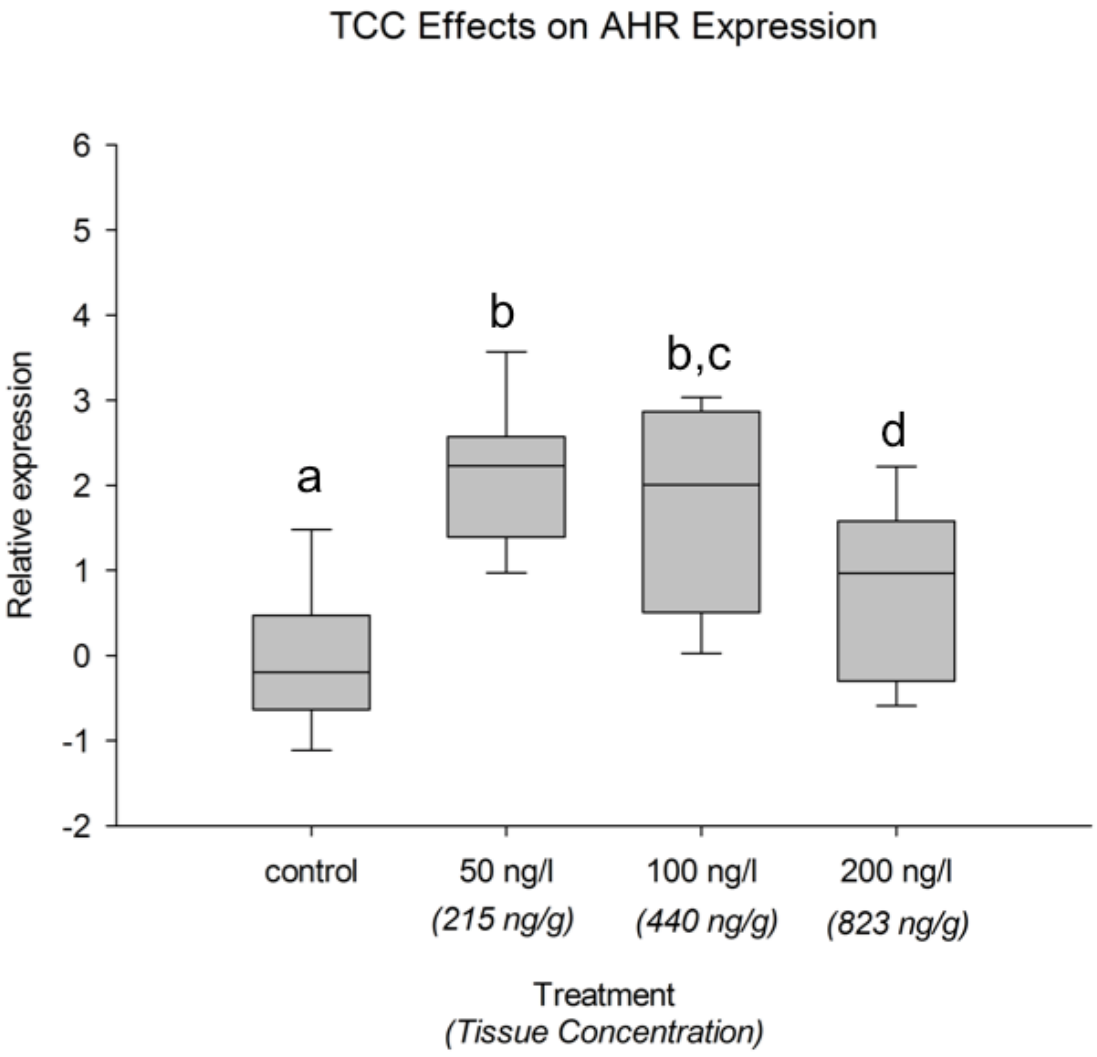

Figure 11. AHR expression after seven days of exposure to TCC.

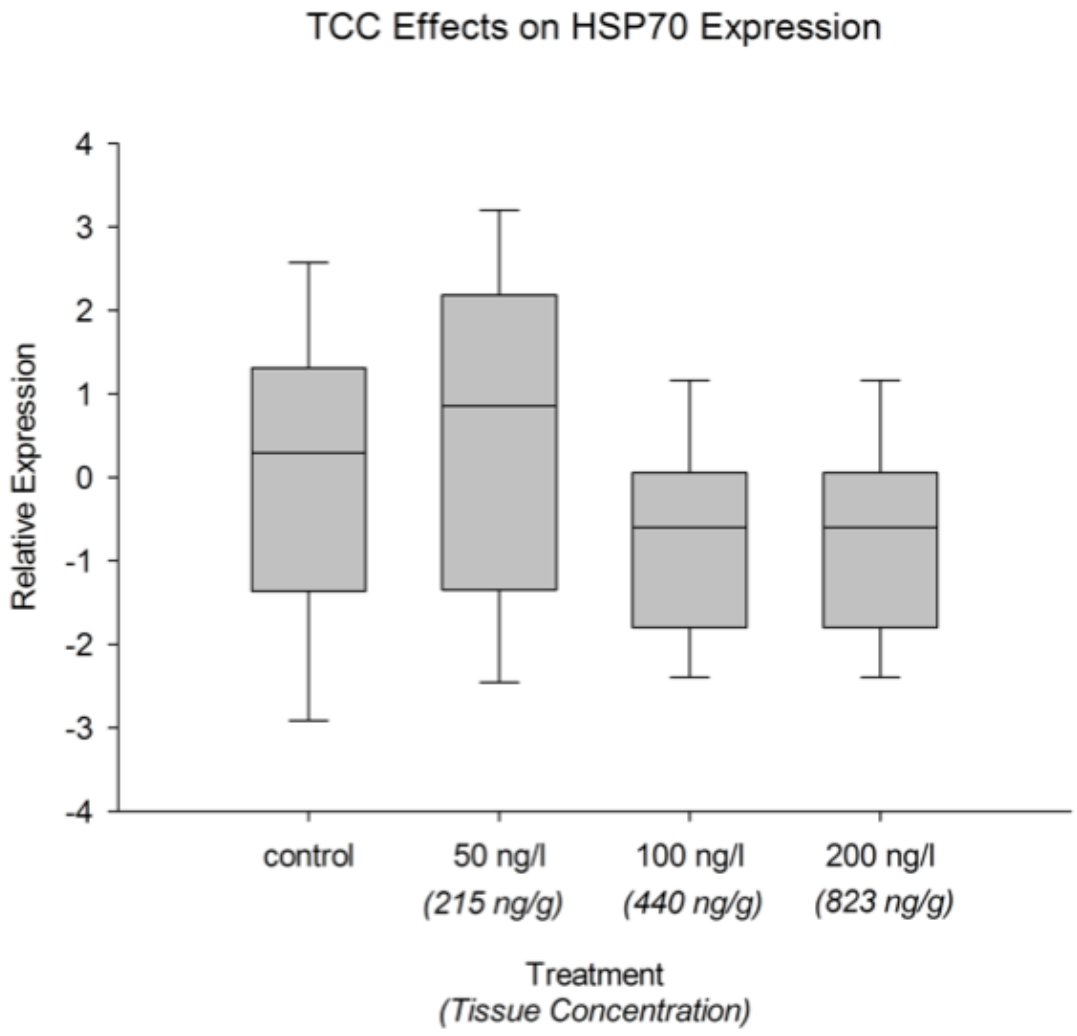

Figure 12. HSP70 expression after seven days of exposure to TCC.

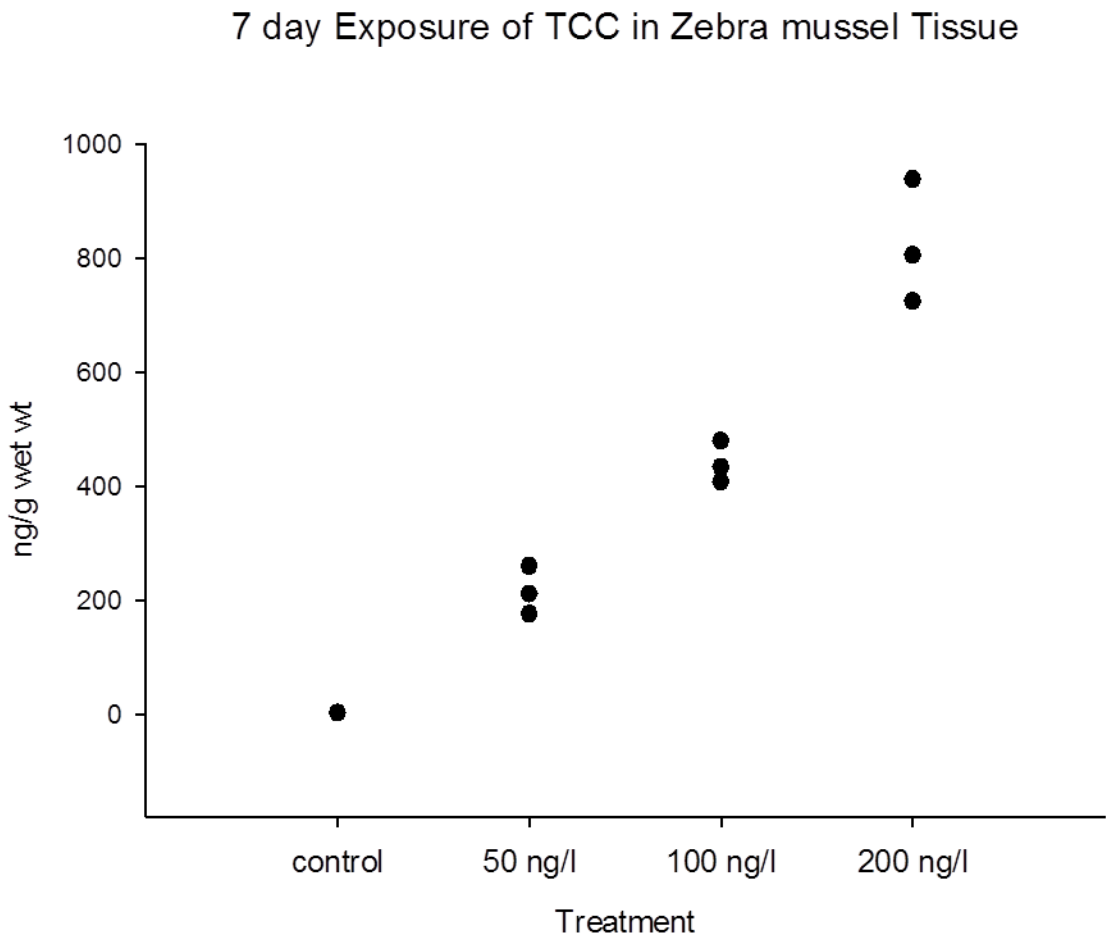

Figure 13. Bioaccumulation of Triclocarban in the tissue of mussel after seven days of exposure, three replicates per treatment

Chapter 4: Conclusion and Discussion

The purpose of this research was to investigate the impacts of toxics in relation to genomic biomarkers of stress and toxicity in the invasive species *D. polymorph* to determine how well they predicted exposure to specific chemicals and the dose response relationship. By furthering the development of genomic biomarkers, this research could help to enhance the capacity of the zebra mussel as more than just a bioindicator of contamination, but a monitor of exposure and potential effects as well.

The first study was an environmental investigation using caged zebra mussels in the Niagara River AOC for five and ten weeks of exposure. The main goals for this project were to answer the questions: will genomic biomarkers correlate to specific contaminants measured in the zebra mussel tissue to identify the chemical or chemicals that are having the greatest impact on organismal health including legacy and emerging contaminants? Additionally, to test how does the length of exposure affect the expression of these genes? Lastly, to investigate if gene expression would be different in free living *in situ* mussels harvested from within the Niagara River versus caged mussels?

The results from our field study in the Niagara River revealed that aquatic environments like many of the Great Lakes AOCs contain a complex mixture of chemical contamination, and that many of these contaminants are co-correlated, making the analysis of determining relationships between individual contaminants and these genes of exposure a challenging process.

Nevertheless, several conclusions could be drawn from the data collected from this environmental study.

First, expression of the genes GST and AHR significantly correlated to the legacy contaminates PCBs and PAHs, supporting the evidence that these could potentially be used as biomarkers of exposure for these chemicals in general. Additionally, that CECs measured in quantities far lower in magnitude than the legacy contaminates, were also significantly correlated to GST as well as HSP70 that was not correlated to PCBs or PAHS and explained 65% of the variability in the expression of GST.

Second, in addition to the results from the regressions calculated, we hypothesize that time is a significant factor when monitoring using gene expression biomarkers. Time had a significant effect on these regressions in that our significant results were calculated from data collected at five weeks of exposure, and that after ten weeks no significant correlations were found. Additionally, in almost all cases, our genes of interest showed a significant pattern of down regulation in the caged mussels placed in the tributaries after five and ten weeks of exposure. This evidence was contrary to other environmental studies which typically show induction of genes to exposure, evidence that either our samples were likely collected after the organisms mRNA expression peaked in an effort to deal with detoxification of the contamination. In addition to down regulation being seen as a general trend, a temporal pattern could not be shown to explain the variation in gene expression measured between the time periods.

Collectively, this evidence showed that sampling gene expression biomarkers at an earlier time point is more revealing of exposure.

Last, the results from the in situ study verified that sampling zebra mussels, already present in the Niagara River did not provide an effective way to predict chemical contamination using these genes. This investigation provided no evidence that significant relationships existed between *in situ* zebra mussels and chemical contamination despite there being variation in expression across locations with associated differences in gene expression. Once again, these results imply that time and lengths of exposure are factors when monitoring gene expression biomarkers.

The third chapter was initiated to investigate individual chemicals to gene expression due to co-correlation of contaminants found in our environmental study. A preliminary investigation using PCB aroclor 1242 was our first exposure; however no significant results were measured. Moving forward, a laboratory investigation of the anti-microbial agent Triclocarban was initiated to verify the discovery that bioaccumulated TCC in the tissue of zebra mussels from the Niagara River had significantly correlated to the gene GST. After an exposure for seven days at exposures of 50 ng/L, 100 ng/L and 200 ng/L, we found GST to be significantly induced in addition to the genes AHR, and P-gP as well. This confirms the possibility that TCC, although low in concentration could be having a realistic toxic effect on the zebra mussels in the environment and that GST could be a biomarker of TCC exposure.

Future Directions

The body of work presented here in these studies displays the relevance of using molecular biomarkers to monitor contaminant exposure in the environment and the need to advance the development of these markers. Furthermore, environmental monitoring of freshwater ecosystems using genetic biomarkers in the zebra mussels could be a powerful tool not only as a bioindicator of contamination, but as a sentinel organism of exposure and eventually effects as well. While this study only exhibits a handful of biomarkers that indicated potential exposure, future studies should correlate markers of exposure to more tangible downstream physiological effects like growth or reproduction. As a result, the joining of effects biomarkers to genomic markers of exposure could help to detect early insults of environmental exposure, heading off effects at a population or ecosystem level. Additionally, being able to monitor these effects in an invasive species that has almost no sampling restrictions, high fecundity and resides in freshwater on nearly a global scale could be used as a sentinel organism to protect more sensitive species. Additionally, as the technology accelerates, it is only a matter of time until genomic sequencing is as economical as it is expedient. Having a fully sequenced zebra mussel genome would hopefully reveal not only individual sequences, but help to unveil whole mechanisms of toxicity. Until then, next generation sequencing could be a way to discover mRNA sequences to measure global changes in gene expression in relation to chemical exposures as well. We tried this method unsuccessfully, however further research into next generation sequencing has promise to expand our library of biomarkers. The benefits of which, would greatly enhance the

ability of researchers to distinguish gene responses to different classes of chemicals. This would be very useful in places like Great Lakes AOCs, where contaminants reside as complex mixtures, and being able to tease out the effects of individual chemical components on the biota is essential for managers to accomplish restoration goals and initiatives.