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The Effects of Urban Contaminants on Neutrophils of Fathead Minnows (Pimephales promelas)

Joshua Gordon
jgordon4115@gmail.com

The Effects of Urban Contaminants on Neutrophils of Fathead Minnows

(*Pimephales promelas*)

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Joshua Gordon

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Thesis Committee:
Marina Cetkovic-Cvrlje, Chairperson
Satomi Kohno
Heiko L. Schoenfuss
Nathan Bruender

Abstract

Contaminants of emerging concern (CECs), including personal care products, pharmaceuticals, industrial agents and agricultural runoff, have shown adverse effects on reproduction and behavior in aquatic species, such as fathead minnow. Since the reproductive system and the immune system are tightly linked, it is critical to investigate effects of CECs on the immune system. An innate immunity, which is characterized by a specific cell type, neutrophils, represents an important branch of fish immune system. A decrease in innate immune functions could lead to an increase of infections, with a consequent impact on fish survival. We developed a neutrophil functional assay (myeloperoxidase degranulation assay, MPO), and a quantitative measurement of neutrophil-specific mRNA abundance (*myeloid-specific peroxidase*, *elastase 2* and *NADPH oxidase*) by the reverse-transcription quantitative polymerase chain reaction (qPCR). Anterior kidneys, as a main source of fish neutrophils, were analyzed by the MPO assay and the qPCR after a 96-hour flow-through exposure of male adult fathead minnows containing 8 individual compounds, which are commonly detected urban CECs, and their mixture. Chemical concentrations were based on those found in over 500 water samples collected by the United States Geological Survey as a part of the Great Lakes Restoration Initiative. A significant increase in degranulation was found in the preliminary experiments using estrogenic compounds, estrone and bisphenol A in particular concentrations. Urban CECs, such as ibuprofen, sulfamethoxazole and the urban mixture, increased the degranulation of neutrophils. Fexofenadine showed a significant increase in both degranulation and *mpo* mRNA abundance. 5-methyl-1H-benzotriazole was the only CEC to show a decrease in degranulation. Observed impact of studied CECs on the innate immune system might provide a novel insight in the ecotoxicology and expand our knowledge of CECs' influence on the innate immune system of aquatic species.

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Chapter 1: Introduction

Contaminants of emerging concern (CECs), consisting of chemicals, pharmaceuticals, personal care products, industrial agents and hormones that have previously not been detected or have newly found effects, are ubiquitous in aquatic systems all over the world. With the increase in detection at low levels in surface waters, these chemicals are requiring additional consideration on how water quality affects aquatic ecosystems. Defined as "any synthetic or naturally occurring chemical or any microorganism that is not commonly monitored in the environment but has the potential to enter the environment and cause known or suspected adverse ecological and/or human health effects," by the United States Geological Survey (USGS), CECs are already known to have adverse effects on behavior (Saaristo, Craft, Lehtonen, & Lindström, 2009; Schoenfuss et al., 2016; Schultz et al., 2011; Weinberger & Klaper, 2014; Wibe, Rosenqvist, & Jenssen, 2002), development (Brown, Adams, Cyr, & Eales, 2004; Xia, Zheng, & Zhou, 2017), reproduction (Flippin, Huggett, & Foran, 2007; Liang et al., 2014), physiology (Lee, Barber, & Schoenfuss, 2014; Schoenfuss et al., 2016; Schultz et al., 2011) and resource allocation (Lee et al., 2014; Wang, Guo, Chen, Sun, & Fan, 2017).Their mechanism of action is proposed to be associated with aryl hydrocarbon receptor activation (Andreasen et al., 2002; Hu, Sorrentino, Denison, Kolaja, & Fielden, 2007; Meucci & Arukwe, 2006) and endocrine disruption (Liang et al., 2014; Wang et al., 2017; Wibe et al., 2002).

To address this rising issue of human activity and growth contaminating aquatic systems with potentially harmful chemicals, 11 federal agencies initiated the Great Lakes Restoration Initiative (GLRI). Introduced in 2010, the GLRI analyzed over 290 water and 80 bottom-sediment

samples of the surrounding Great Lakes' tributaries for CEC presence. The results showed the presence of 28 chemicals in 30% of total samples (Elliott et al., 2017; Lee et al., 2012). CECs can be separated into two main categories - agricultural or urban, with chemicals not being exclusive to either category. Agricultural contaminants, including pesticides, hormones and fertilizers, represented those commonly found in samples collected at sites where the surrounding land is designated for agricultural use. Urban contaminants include those commonly found in developed areas, such as pharmaceuticals, industrial byproducts and personal care products. Urban CECs have become a special concern, because of the financial limitations to remove all chemicals from waste water treatment plants (Perkins et al., 2017). Chemicals observed at urban sites not only showed higher concentrations of chemicals than at agricultural sites, but had a more diverse mixture of chemicals (Elliott et al., 2017).

The immune system is a network of organs, cells and proteins specialized to protect the host from foreign entities. There are two distinct branches of the immune system in respects to vertebrates, the innate (natural) and adaptive (acquired) immunity. Innate immunity is the organisms' first line of defense, responsible for the initial recognition and attack of foreign molecules, as well as activation and recruitment of other, more specialized immune cells. Anatomical barriers provide the first defense base of the innate immune system. Epithelial surfaces block the entry of most pathogens, creating a physical layer. This includes the skin, and mucosal surfaces of the respiratory, gastrointestinal and genitourinary tracks. Complementing the physical barriers, these surfaces also contain so-called chemical barriers, including the acidic pH, and numerous enzymes and anti-microbial peptides and proteins, such as lysozyme

and defensins, that contribute in destruction of pathogens (Kindt, Goldsby, Osborne, & Kuby, 2013). If anatomical and chemical barriers fail, there is an assembly of innate immunity cells. Specifically, neutrophils are the most abundant innate immune cell and the first cell to migrate to the scene of inflammation (Kindt et al., 2013). Immune cells can be categorized by their explicit functions. Phagocytes are cells that ingest pathogens through endocytosis to degrade pathogens, including neutrophils, macrophages and dendritic cells. Phagocytosis, after destruction of pathogen, allows the presentation of antigens by the major histocompatibility complex molecules (MHCs). Antigen presenting cells (APCs), including macrophages and dendritic cells, digest pathogens through a process of phagocytosis/endocytosis, and present them as antigenic peptides by MHC to activate the adaptive immune system. Polymorphonuclear leukocytes (PMNs), or granulocytes, are white blood cells, characterized by multi-lobulated nucleus and the presence of cytosolic granules. Granules are specialized vesicles that contain proteins, released through a process called degranulation, responsible for the allergic responses, inflammation and the destruction of pathogens. PMNs include neutrophils, basophils, and eosinophils.

Innate immune cells provide a non-specific immune response, due to the promiscuity of their receptors, and an array of pathogens they recognize. Innate immune cells possess receptors, pattern recognition receptors (PRRs), that recognize pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs), toll-like receptors (TLRs) being the most renowned (Kindt et al., 2013). PAMPs are conserved microbial features, such as carbohydrates (lipopolysaccharides, mannose), nucleic acids (dsRNA, DNA), and a broad

range of surface proteins from flagellin to heat shock proteins (Akira, Uematsu, & Takeuchi, 2006). DAMPs are host cell stress signals or intracellular molecules that recruit the innate immune system to lyse and/or clean up damaged cells.

Innate immune cells are responsible for the inflammatory response. Neutrophils are the first cell type to arrive at the site of inflammation. Cytokines, such as interleukin 1, interleukin 6 and tumor necrosis factor alpha are released by innate immune cells as a response to PAMPs and DAMPs recognition. These cytokines cause vasodilation and increase permeability of blood capillaries, allowing an increased fluid leakage as well as the extravasation of immune cells and other molecules to the site of inflammation. Among the serum molecules pooling to the inflammatory site is a group of proteins that make up the complement system. The complement system consists of 30-40 serum proteins, pro-enzymes, designated C1-C9. The three pathways of the complement system activation, classical, alternative and lectin, all produce the same final product, the membrane attack complex (MAC). MAC forms a transmembrane channel that causes the loss of membrane integrity, resulting with cell lysis (Kindt et al., 2013).

In contrast to the innate immune system, the adaptive immune system is antigen-specific. The adaptive immune cells, lymphocytes – T and B cells, are responsible for the memory response that allows for a stronger response to recognized antigens in the future exposures. To initiate an adaptive immune response, T cells need to be activated by antigens processed and presented in the context of MHC by an APC. Once activated, T cells differentiate into effector and memory cells. Effector T cells have a wide range of functions, from helping

(activating) other cells (T helpers), regulating immune responses (regulatory T cells), to killing infected host cells (cytotoxic T cells). In contrast to T cells, B cells can directly recognize antigens, which causes activation into effector and memory B cells. Effector B cells release the antibodies that assist the innate immunity by marking pathogens for phagocytosis, a process called opsonization (van Kesse, Bestebroer, & van Strijp, 2014).

Even though mammals and teleost have been diverging for 450 million years, much of the immune system is conserved between them (Carradice & Lieschke, 2008; Lieschke, Oates, Crowhurst, Ward, & Layton, 2001; Nonaka & Smith, 2000). The main lymphoid organs in teleost consist of the anterior (head) kidney, thymus, and the spleen (Zapata, Diez, Cejalvo, Gutiérrez-de Frías, & Cortés, 2006). Whereas bone marrow is the lymphoid organ responsible for hematopoiesis in mammals, the anterior kidney exhibits this function in teleost (Traver et al., 2003). Since fish do not have lymph nodes, the secondary lymphoid organs crucial for providing an environment for innate-adaptive immunity interactions, the anterior kidney and the spleen in teleost replace that role allowing the presentation of antigens to the cells of adaptive immunity (Iliev, Thim, Lagos, Olsen, & Jørgensen, 2013).

The innate immune response, in comparison to adaptive, is considered as critical for fish, due to the limitations of the adaptive immunity. It was not until animals became homeothermic that they acquired higher adaptive immunity specialization (Tort, Balasch, & Mackenzie, 2003). Innate immunity in teleost can also be divided into three categories, comparable to other vertebrates, consisting of anatomical, chemical, and cellular barriers. Anatomical barriers include flakes, skin mucus and gills. Besides trapping pathogens, skin mucus

contains a repertoire of antimicrobial peptides, enzymes and lectins; comparable to mammalian chemical barriers (Alexander & Ingram, 1992; Ellis, 2001; Hellio, Pons, Beaupoil, Bourgougnon, & Gal, 2002). Considering cellular immunity, all innate immune cell types present in mammals are also found in zebra fish (Traver et al., 2003). The main phagocytic cells are also neutrophils and macrophages. Dendritic cells and macrophages exhibit APC functions, however, neutrophils have shown to upregulate MHC when stimulated, assisting adaptive immunity in antigen presentation in Atlantic salmon (Iliev et al., 2013).

The key components of inflammation are present in teleost, when using mammals as a baseline (Grayfer & Belosevic, 2012). The main difference is there is no systemic raise in body temperature as a response to inflammatory process, since teleost are poikilothermic. Instead of this physiological reaction, fish move toward warmer water (Gräns, Rosengren, Niklasson, & Axelsson, 2012). The complement system in fish is also similar to the mammals'. However, it is suggested that complement plays a more prominent role in the innate immunity in teleost (Nonaka & Smith, 2000; Sunyer, Zarkadis, Sahu, & Lambris, 1996) since there are three isotypes of C3 component, the driving factor of the complement system, while in mammals there is only one (Sunyer et al., 1996).

Neutrophils are the primary innate immunity cell defensive mechanism of vertebrates against bacterial, viral and fungal infections, and are also the main effector of inflammation (Havixbeck, Rieger, Wong, Hodgkinson, & Barreda, 2016). Teleost neutrophils possess the same morphological and physiological characteristics as mammals' neutrophils, except in their circulatory concentrations. In mammals, neutrophils represent 50-70% of total leukocytes

(Kindt et al., 2013), while in healthy teleost (*Carassius auratus*), they represent around five percent of white blood cells. However, once a fish is exposed to a pathogen, neutrophil counts increase to 50% of blood cells (Havixbeck et al., 2016).

Neutrophils possess numerous specialized functions, one of them being degranulation. They contain primary (azurophilic), secondary (specific), and tertiary granules, characterized by a specific assortment of proteins. The primary granules contain enzymes responsible for generation of the reactive oxygen species (ROS) during degranulation. One of these enzymes is myeloperoxidase (MPO). MPO is a neutrophil-specific enzyme that is only expressed in the primary granules (Amanzada et al., 2011). It produces hypochlorous acid and superoxide molecules from hydrogen peroxide, generated by NADPH oxidase during a respiratory burst (Hampton, Kettle, & Winterbourn, 1998). Elastase 2 (ELANE), or neutrophil elastase, is another neutrophil-specific enzyme found in primary granules. ELANE and MPO's products, released during degranulation process, exhibit direct antimicrobial properties. Secondary granules' most prominent component is lysozyme, released to destroy the cell wall of gram-positive bacteria. In addition, NADPH oxidase, a multi-component electron-transfer complex, is located in secondary granules that fuse with phagosomes during phagocytosis. NADPH catalytic, electron-transfer portion, is a membrane-bound flavohemoprotein cytochrome b_{558}, a heterodimer made up of two subunits, $p22^{phox}$ and $gp91^{phox}$ (also known as NOX2), that is responsible for the production of hydrogen peroxide (Bylund, Brown, Movitz, Dahlgren, & Karlsson, 2010). Tertiary granules contain cathepsin and collagenase that target extracellular protein structures.

Neutrophil function is essential for the normal development and survival of an animal population (Segal, 2005). A decrease in neutrophil counts (neutropenia), or neutrophil disorders, make the host highly susceptible to infections, while over-activation of neutrophils leads to an oxidative stress of surrounding tissues that can induce acute and chronic inflammation (Wheeler, Martin, & Lawrence, 2013).

It has been shown that an array of exogenous chemicals, including the industrial compounds, act as aryl hydrocarbon receptors (AhR) activators (Billiard et al., 2002; Meucci & Arukwe, 2006; Muusse, 2015). AhRs are nuclear hormone receptor complexes consisting of a heat shock protein 90 (hsp90), prostaglandin E synthase 3 (p23) heterodimer, and a AhR interacting protein (AIP). Once a ligand (AhR activator) binds to the receptor, there is a conformation change, where AIP disassociates, creating an active state. Once activated, the complex heterodimer is transported into the nucleus where it interacts with specific promoter sequences on DNA, directly influencing gene expression. It has been shown that AhR-activating compounds influence the immune system (Hanieh, 2014; Stockinger, Hirota, Duarte, & Veldhoen, 2011). Specifically, neutrophils have been shown to infiltrate the infected tissues in influenza A-infected mice exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a strong activator of AhRs, (Teske, Bohn, Regal, Neumiller, & Lawrence, 2005). Another study showed that influenza A-infected mice exposed to TCDD had a significant increase in interferon gamma (IFN-γ), driven by excessive expression of IFN-γ in phagocytes, (Neff-LaFord, Teske, Bushnell, & Lawrence, 2007). IFN-γ is a cytokine that plays a critical role in inflammation and is known to mediate tissue damage when over-expressed (Geiger et al., 1994; Laskin, Fakhrzadeh, & Laskin,

2001; Toyonaga et al., 1994). However, it has been shown that neutrophils are not directly stimulated by AhR activators, but affected through TCDD-induced Th17 differentiation. (Nakahama et al., 2013).

Endocrine disrupting compounds (EDCs) are a category of chemicals, defined by the EPA as “an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process” (United States Environmental Protection Agency). In the past two decades, researchers have clearly shown the effects of EDCs on the innate immune system (Bartoskova et al., 2013; Milla, Depiereux, & Kestemont, 2011; Tellez-Bañuelos, Santerre, Casas-Solis, Bravo-Cuellar, & Zaitseva, 2009). EDCs, such as a xenoestrogen bisphenol A (BPA), have shown to increase expression of oxidative species, causing oxidative stress of the host immune cells (Jin, Chen, Liu, & Fu, 2010). Another study looking at BPA and nonylphenol (NP), confirmed EDCs causing oxidative stress, but also showed altered expression in TLR pathway molecules (Xu, Yang, Qiu, Pan, & Wu, 2013). Multiple EDCs have been suggested to inhibit macrophage function, by inhibiting a MyD88-independent TLR4 signaling pathway (Ohnishi, Yoshida, Igarashi, Muroi, & Tanamoto, 2008). C3 has shown expression sensitivity to xenoestrogens in rats (Heikaus, Winterhager, Traub, & Grümmer, 2002).

Contamination with pharmaceuticals is becoming a larger concern because of expansions of developed areas. Developed to sustain a biologically active state, pharmaceuticals do not degrade rapidly. High concentrations of accumulated pharmaceuticals

have been found at waste water treatment plants before being expelled into waterways (Kostich, Batt, & Lazorchak, 2011). Various pharmaceuticals, such as sulfa drugs and tetracyclines, commonly used antibiotics, have been discovered as AhR activators through a large scale screening of AhR biomarker expression (Hu et al., 2007), have shown estrogenic effects (Kang, Choi, Kim, & Kim, 2006). Some pharmaceuticals directly affect immune pathways. For example, ibuprofen, a frequently used anti-inflammatory drug, affects neutrophil function by inhibiting prostaglandin production through inhibition of cyclooxygenase activity.

Fathead minnows have been used as a toxicological model since the 1950s (Ankley & Villeneuve, 2006), and has recently emerged as a model organism in immunotoxicology (Thornton et al., 2017). Since fathead minnows exhibit ability to tolerate a broad range of environmental variables, survivability in a laboratory setting and ubiquitous presence in the waters of North America (Isaak, 1961), they represent the optimal model organism for studying the effects of water contaminants of the Great Lakes.

Chapter 2: Aim and Hypothesis

There is a gap in currently available information regarding the immunotoxic potential of CECs toward aquatic organisms. Due to the fact that aquatic animals live their entire lives in water being chronically exposed to CECs, the immunotoxic effects of CECs are even of greater concern than for terrestrial or semi-terrestrial species. Therefore, there is a critical need to obtain basic data on the acute effects of complex urban mixtures, and their discrete components, on the innate immune system of fathead minnow. As neutrophils are the first innate immune cells that contribute to the host protection, they have been chosen as the target cells in this study. While the fathead minnow is a widely-accepted model organism in aquatic toxicological studies, there are no available adequate research tools to study immune parameters in that organism. Thus, the general aim of this research is to A) develop tools, specifically primers, and modify an existing functional assay, that will allow for the quantitative assessment of neutrophil-specific gene expression and neutrophil function, respectively, and B) to use these tools for an assessment of the impact of acute exposures to complex urban mixtures, and their discrete components, on the innate immunity of fathead minnows.

Based on literature findings on effects of particular pharmaceuticals on innate immunity of other species, it was hypothesized that:

1. Exposures to anti-inflammatory compounds will decrease neutrophil function in fathead minnows.
2. Exposures to aryl hydrocarbon receptor-active compounds will increase neutrophil function in fathead minnows.

3. Exposures to antibacterial compounds will decrease neutrophil function in fathead minnows.
4. Exposures to serotonin-norepinephrine reuptake inhibitors will decrease neutrophil function in fathead minnows.

To test these hypotheses, several specific objectives were developed:

1. Define an optimal lymphoid organ for neutrophil evaluation in fathead minnow.
2. Generate/optimize PCR primers that would allow quantification of mRNA abundance of the following neutrophil-specific genes in fathead minnow – myeloperoxidase (*mpo*), NADPH oxidase (*nox2*) and elastase 2 (*ela2*).
3. Develop myeloperoxidase-based (MPO) assay for an assessment of neutrophil degranulation function in an individual fathead minnow.
4. Test effects of the exposures to individual urban CECs on the mRNA abundance of neutrophil-specific genes of fathead minnow.
5. Test effects of the exposures to individual urban CECs on the function of fathead minnow's neutrophils using MPO assay.
6. Test effects of the exposures to complex urban mixtures on the mRNA abundance of neutrophil-specific genes of fathead minnow.
7. Test effects of the exposures to complex urban mixtures on the function of fathead minnow's neutrophils using MPO assay.

Chapter 3: Materials and Methods

3.1. Animals

Adult male fathead minnows (*Pimephales promelas*), 3-month of age, were purchased from Environmental Consulting and Testing (Superior, WI), and maintained at Aquatic Toxicology Laboratory at St. Cloud State University (SCSU) under the approval of the SCSU Institutional Animal Care and Use Committee (IACUC). Fish were kept in 20-gallon tanks, fed twice a day with a brine shrimp, blood worm mix, exposed to 16:8 light to dark cycle, and treated for 96-hours using a flow through system. All procedures performed on alive fish were approved by SCSU IACUC (#8-107, Schoenfuss).

3.2. Experimental Design

Fish were exposed to 9 different exposures, containing 8 individual compounds and one mixture. Five groups of fish per exposure, six animals per group, were exposed at different concentrations for 96-hours using a flow through system. Each exposure consisted of a control, ultra-low, low, medium, and high concentration treatment. Control fish were exposed to SCSU well water. Medium treatment fish were exposed to environmentally relevant concentrations of compounds (Appendix, Table 8.1.) (Elliott et al., 2017), whereas low and ultra-low treatment fish were exposed to 1/10 and 1/100 concentrations of environmentally relevant concentrations, respectively. High treatment fish were exposed to concentrations ten times higher than environmental concentrations.

3.3. Collection of Lymphatic Organs

Fathead minnows were caught using a net and immediately euthanized by immersion in 8% w/v tricaine methanesulfonate (MS-222) buffered with equal parts sodium bicarbonate. Whole spleens and approximately 1/10 of the anterior kidney were isolated and preserved into 1.5 ml tubes with 500 µL of RNA later (#AM7020, Invitrogen, Thermo Fisher Scientific, Waltham, MA) for gene expression analyses. The rest of the anterior kidney was dissected into 50-mL tubes containing 12 mL of Hank's Balanced Salt Solution (HBSS) without calcium, magnesium and phenol red (HBSSwo; #21-022, Corning, Corning, NY). Kidneys were pooled into their appropriate treatment 50-mL tubes with HBSS for a neutrophil isolation and a functional test. Tissues collected in RNA later were incubated at 4°C overnight and stored at -80°C until use.

3.4. RNA Extraction and Purification

Total RNA was extracted by an acid guanidinium thiocyanate-phenol-chloroform method (Green, 2012). RNA later-preserved tissue was homogenized with 700 µL of lysis reagent (1.9 M guanidinium thiocyanate, 12 mM sodium citrate, 0.24% (w/v) sodium N-lauroyl sarcosine, 95 mM sodium acetate, 50 mM β-mercaptoethanol) and a 6 mm metal bead using a Retsch MM400 bead mill (Haan, Germany) at a frequency of 30 per second for 40 seconds. Homogenates were incubated at room temperature for 5 minutes, and 140 µL of chloroform was added to a homogenate. After 3 minutes incubation, the samples were centrifuged for 15 minutes at 12,000 x g at 4°C.

The aqueous layer was purified on a column with silica membranes (Epoch, Sugar Land, TX) using solutions of SV total RNA isolation system (Promega, Madison, WI), following a

manufacture's protocol of miRNeasy Mini isolation system (Qiagen, Germantown, MD). An aqueous layer was transferred into a new 1.5 mL tube, and mixed with 1.5 volumes of 100% ethanol (#2716, Decon Labs inc., King of Prussia, PA). The mixture was transferred into a column and centrifuged at 10,000 x g for 20 seconds at room temperature. A flow through was discarded. Seven hundred µL of RNA wash solution (65 mM potassium acetate, 10 mM Tris-HCl, 60% ethanol) was added to the column and centrifuged at 10,000 x g for 20 seconds, flow through being discarded. The same procedure was repeated with 500 µL of SV RNA wash solution, twice, for 15 seconds and 2 minutes, respectively. A washed column was transferred into a new 1.5 mL tube and RNA eluted with 30 µL of diethyl pyrocarbonate- (DEPC; #97062, Amresco, VWR, Radnor, PA) treated ultra-pure water and a centrifuge at 10,000 x g for 1 minute at room temperature. Ultrapure water was obtained from a Millipore Synergy UV-R water system (MilliQ; Loveland, CO). RNA samples were stored at -80°C.

3.5. RNA Quality Check using Electrophoresis

Each RNA quality was evaluated using agarose gel electrophoresis (Masek, Vopalensky, Suchomelova, & Pospisek, 2005). Two µl of RNA was mixed with ten µL of RNA Loading mix (4.35% glycerol, 1.45 mM EDTA, 0.036% bromophenol blue, 85.5% formamide, 10x SYBR Green-II (#S7564, Invitrogen, Thermo Fisher Scientific), and heat denatured at 65-70°C for 10 minutes. Immediately after, the sample was placed directly on ice for 1 minute and spun down. A denatured RNA was run on a 1.5% agarose gel in TAE (Tris-acetate-EDTA) buffer at 100 V for 25 minutes. The gel was visualized with an Aplegen Omega Lum G imaging system Gel Company

(San Francisco, CA). RNA was evaluated with band densities of 28S and 18S ribosomal RNA (rRNA), and 1:1 illumination between the 28S and 18S rRNA bands was considered satisfactory.

3.6. DNase Treatment

RNA was treated with DNase to remove genomic DNA contaminations, that could lead to unwanted amplification during reverse-transcription polymerase chain reaction (RT-PCR), using TURBO DNA-*free* kit (#AM1907, Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. RNA extracts were diluted to <200 ng/µL with DEPC treated ultra-pure water. DNase master mix was made (per reaction; 2 µL 10x TURBO DNase buffer, 0.4 µL TURBO DNase) and 2.4 µL was added to 17.6 µL of RNA sample. Samples were briefly vortexed and incubated at 37°C for 30 minutes, and DNase was inactivated by adding the inactivation reagent. The samples were incubated at room temperature for 5 minutes, intermittently mixing by flicking to disperse reagent. Samples were centrifuged for 2 minutes at 10,000 x g, and approximately 18 µL of purified RNA extract was transferred to a new tube. Purified RNA was stored at -80°C.

3.7. DNA Contamination Check

A quantitative PCR (qPCR) without RT reaction was run using purified RNA extract as a template to check for any germinal DNA contamination on CFX96 touch real-time PCR detection system (Bio-Rad, Hercules, CA). A primer pair, forkhead box protein L2 (*foxl2*) that has been designed and optimized without an intron, was used to check for germinal DNA contamination (Table 1). Ultra-pure water and a subcloned PCR product of *foxl2* were used as a positive and negative control, respectively.

3.8. cDNA Synthesis

Using prepared RNA (2 µg) as templates, cDNA was synthesized in 15 µL of reaction, after heat-denaturing RNA at 70°C for 10 minutes, using High Capacity cDNA Reverse Transcription Kit (#4368814; Applied Biosystems, Thermo Fisher Scientific) according to the manufacturer's protocol. A reaction mixture was incubated for 10 min at 25°C, for 120 min at 37°C and for 5 minutes at 85°C. The cDNA was stored at -20°C.

3.9. Primer Design and Optimization

Although fathead minnow genome has been sequenced (Burns et al., 2016), the annotation has not been completed (Saari, Schroeder, Ankley, & Villeneuve, 2017). However, neutrophil-specific sequence, such as myeloid-specific peroxidase (*mpx*), neutrophil elastase (*ela2*) and NADPH oxidase (*nox2*) have been published, based on the annotation of 20 immune genes for the assessment of toxicological effects on gene expression in fathead minnow (Jovanović, Anastasova, Rowe, & Palić, 2011) (Table 1). To normalize target gene expressions, three housekeeping genes were evaluated for best stability, which were ribosomal protein L8 (*rpl8*), hypoxanthine phosphoribosyltransferase 1 (*hprt1*) and TATA box binding protein (*tbp*) (Table1). Three housekeeping genes were used to find the most stable housekeeping gene for normalization. NormFinder was used to find the most stable housekeeping gene or a housekeeping gene combination (Andersen et al., 2004).

Table 3.1. Primer sequences for quantitative RT-PCR amplification

		Sequence	**Position**	**Tm**	**GenBank Accession #**
ela2	Forward	316	ATCGTGCATGAGAACTGGGA	66.2	DT350430
	Reverse	593	ATGAGGTTGGTCACGAGGTT		
foxl2	Forward	569	TTAACGTGAAAGGCTTCACC	62	(Saari, Schroeder, Ankley, & Villeneuve, 2017)
	Reverse	668	CTCATGCCGTTGTAAGAGTT		
hprt1	Forward	507	ATCTGTCCACACTCACAGGA	64	DT085800
	Reverse	647	TCCTCTTCACCAGCAAACTG		
mpx	Forward	466	TGTCTGCAACAACAGGAGGA	64	DT092840
	Reverse	746	TGCTTGGTGAGTTGGGTGTA		
nox2	Forward	94	CGGCATCAATGCGTTTCTCT	-	DT188783
	Reverse	351	TAGGCCACCAGTTTGTGGAA		
rpl8	Forward	375	CCCACAATCCTGAGACCAAG	64	AY919670
	Reverse	473	TTGTCAATACGACCACCACC		
tbp	Forward	792	CATTCGATTAGAGGGCCTGG	62	(Saari, Schroeder, Ankley, & Villeneuve, 2017)
	Reverse	861	CCTGGGAAATAACTCTGGTTCA		

PCR primers were obtained from Eurofins Scientific (Louisville, KY), and their annealing temperatures optimized in qPCR with a temperature gradient from 60-70°C using CFX96 real-time PCR system. A specificity of each qPCR reaction was confirmed by analyzing a melting temperature of each amplicon and sequencing a representative amplicon. Since a primer pair for *nox2* had non-specific amplification, it was excluded from a further analysis.

Each qPCR product was sub-cloned and sequenced using pGEM-T Easy Vector System (#A3610; Promega, Madison, WI) according to manufacturer's protocol. Plasmid DNA was isolated using Wizard Plus Minipreps DNA Purification System (Promega) followed by a polyethylene glycol (PEG) precipitation. Plasmid sequence was obtained from Eurofins using the standard Sanger cycle sequencing method. To use cloned plasmid as standard samples for

qPCR, PEG precipitated plasmid was serially diluted with a dilution buffer (5 ng/µl tRNA in Tris-EDTA buffer, pH 8.0) from 10^8 through 10^1 copy/µL.

3.10. Quantitative Polymerase Chain Reaction

In comparison with samples of unknown "copies," through PCR starting concentrations can be quantified. qPCR was run using a homemade SYBR Green reaction mix containing 20 mM Tris-HCl (pH 7.75), 50 mM KCl, 3 mM MgCl2, 0.5% Glycerol, 0.5% Tween-20, 0.5x SYBR Green-I (#S7563, Invirtogen), 0.2 mM dNTP mix (Takara Bio, Mountain View, CA) and 0.01 U/µL Ampli Taq Gold (Applied Biosystems, Thermo Fisher Scientific) with 0.2 µM each primer and 1/25 volume of template. Samples were briefly centrifuged and each sample was loaded onto a Hardshell PCR plate (#HSP9655, Bio-Rad) in triplicates of 15 µL each with Microseal 'B' seals (#MSB1001, Bio-Rad). Thermocycler was set at two-step amplification and melting curve protocol (1 cycle at 95°C for 5 minutes; 40 cycles at 95°C for 15 seconds, optimal annealing temperature (see Table 1) for 45 seconds, plate read; 95°C for 10 seconds; melting curve 65°C to 95°C in 0.5°C increments, 5 seconds and plate read at each temperature; end). All data and starting concentration calculations were generated using CFX Manager 3.1 software (Bio-Rad). For samples to be compared, cDNA synthesis has to be created at the same time, with the same RT master mix and run using the same qPCR master mix.

3.11. Statistical Analysis

All statistical analysis was performed using JMP software by SAS Institute. Arcsin transformation was used for analyzing degranulation data sets. A distribution analysis was performed using a goodness of fit test to check for normality. Parametric data were analyzed

by ANOVA followed by Dunnett's test with control if ANOVA was positive. Non-parametric data were analyzed using Kruskal-Wallis test followed by Steel method if Kruskal-Wallis test was positive.

3.12. Neutrophil Isolation

Kidneys were extracted from 5-6 fish per sample, pooled in a 50-mL tube containing 5 mL of HBSSwo, and homogenized with a tissue grinder using 10-15 strokes. The obtained single cell suspension was filtered through a 40-µm nylon strainer and centrifuged for 15 minutes at 250 *x* g at room temperature with no brake. The supernatant was discarded, and pellet resuspended in 6 mL of HBSS without calcium and magnesium, with phenol red (#21-021, Mediatech-Cellgro, Corning), 50 µl of that suspension was used for determination of total cell counts and cell viability by hemocytometer using a Trypan blue (TB; #17-92E, Lonza, Basal, Switzerland) exclusion method. The suspension was laid over 5 ml of lymphocyte separation medium with the specific gravity of 1.078 g/mL (Lymphocyte separation media 1078, #25-072, Mediatech-Cellgro, Corning) in a 15-mL tube, and centrifuged for 30 min at 400 *x* g with no brake. Cells formed at the gradient interface were removed, resuspended in HBSSwo, and centrifuged for 15 minutes at 400 *x* g with no brake. Final cell suspension was prepared in 1 mL of HBSSwo and used for determination of total cell counts and cell viability by Trypan blue exclusion method. Finally, the appropriate number of cells was used for cytochemical staining and neutrophil degranulation (MPO) functional assay.

3.13. Neutrophil Degranulation Functional Assay

A degranulation assay, that measures myeloperoxidase (MPO) exocytosis from neutrophils primary granules (Palić, Andreasen, Menzel, & Roth, 2005), was performed after the particular CEC (or CEC's mixture) treatment, on a day when all fish exposed to such a CEC were sacrificed, and their neutrophils isolated from anterior kidneys, as described in the Neutrophil isolation chapter. The MPO assay was run in a 96-well flat-bottomed microtiter plates. Contaminant treatment groups consisted of the control, ultra-low-, low-, medium-, and high-contaminant concentrations. The MPO assay contained three different treatments of the same sample: control (cells lysed to assess complete enzyme presence in the sample), background (assess any background enzyme release without the stimulation; cells kept in Hank's Balanced Salt Solution with calcium and magnesium without phenol red (HBSSw; #21-023, Mediatech-Cellgro, Corning), and stimulated treatment (assessment of degranulation by a stimulation with calcium ionophore), each run in triplicates. The stimulated wells were loaded with 75 µL of HBSSw and 50 µl of 5 µg/mL calcium ionophore (#C7522, Sigma-Aldrich), background wells contained 125 µL of HBSSw, and control wells contained 125 µL of 0.02% hexadecyltrimethylammonium bromide (CTAB; #H5882, Sigma-Aldrich). Then, 25 µL of cells at the concentration of 2 x 10^7 cells/mL were added into each well. The plate was incubated at room temperature for 20 minutes. After incubation, 100 µL of room temperature 3,3',5,5'-tetramethylbenzidine (TMB) (#T4319, Sigma-Aldrich) was added to all wells, stopping the reaction after 2 minutes by adding 25 µL of 1 N sulfuric acid. The plate was centrifuged at 600 x g for 2 minutes. 200 µL of the supernatant from each well was transferred to a new 96-well

plate. Absorbance was read at 405 nm using a GeneMate microtiter plate spectrophotometer. The percent release of MPO was calculated using the following formula:

$$\%\, released = \frac{\left(OD_{stimulated} - OD_{background}\right)}{OD_{control} - OD_{background}}$$

3.14. Cytospin and Cytochemistry

Cells isolated as described in Neutrophil isolation chapter were diluted in HBSSwo with 10% fetal bovine serum to 10^6 cells/mL. Two hundred µL of each sample was loaded into a cytospin column, with Shandon filter cards from Thermo Scientific. Samples we spun in a Cytospin3 (Shendon, Thermo Scientific), at 500 rpm for 5 minutes. Slides were laid to dry for 30 minutes before staining. Hemacolor staining kit from Harleco (#65044, Millipore) was used to stain the nucleus and cytoplasm of isolated cells for cell type differentiation, allowing for typical neutrophil's lobulated nucleus morphology to be observed. Sudan Black B Staining System from Sigma-Aldrich (#380B-1KT) was used to stain primary granules containing MPO for neutrophil semi-quantification. Slides were analyzed under a light microscope at 1000 x for neutrophil differentiation; 100 counted cells on each stained slide. Cells were considered as neutrophils based on their multi-lobulated nucleus morphology (Hemacolor staining) or their granules stained black (Sudan Black staining).

Chapter 4: Results

4.1. Defining Lymphoid Organ as a Neutrophil Source in Fathead Minnow

Preliminary experiments were performed in order to determine the type of the lymphoid organ that can be used as a source of neutrophils, and to establish a reproducible isolation procedure for obtaining the adequate number of viable neutrophils in fathead minnow.

Spleen and anterior kidney were considered as the candidate lymphoid organs. Neutrophil content was evaluated by Sudan black staining of cytospin preparation of cells, obtained from the spleen and kidney of fathead minnows via lymphoprep gradient purification. Figure 1. shows that kidneys exhibited a significantly higher neutrophil purity of 62.8 ± 6.5 % compared to 10.5 ± 6.2 % obtained from the spleens. Figure 2. shows a representative image of neutrophil, obtained either by staining with (A) hemacolor or (B) Sudan black post-gradient isolation. Typical neutrophil nucleus morphology, indented and/or lobulated, with a moderate nucleus to cytoplasm ratio, can be seen after the hemacolor staining of cytospin cell preparation (Figure 2.A), while brownish-black staining in the cytoplasm, specific for neutrophil granules, can be observed by Sudan black staining (Figure 2.B).

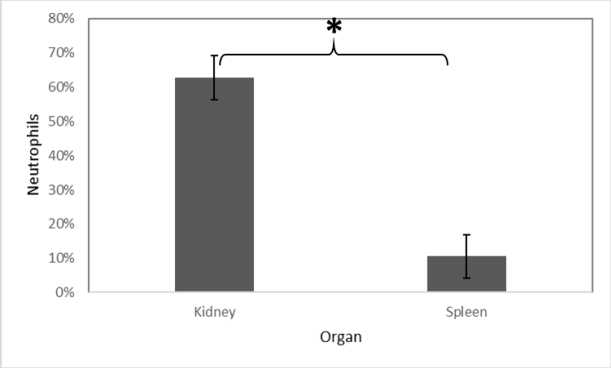

Figure 1. Neutrophil content (%) evaluated in cytospin preparation of cells post-isolation of kidney and splenic cells of fathead minnow. Neutrophils were detected using Sudan black staining, with a sample size of n=5 for both organs. Statistical significance represented by *, p=0.01.

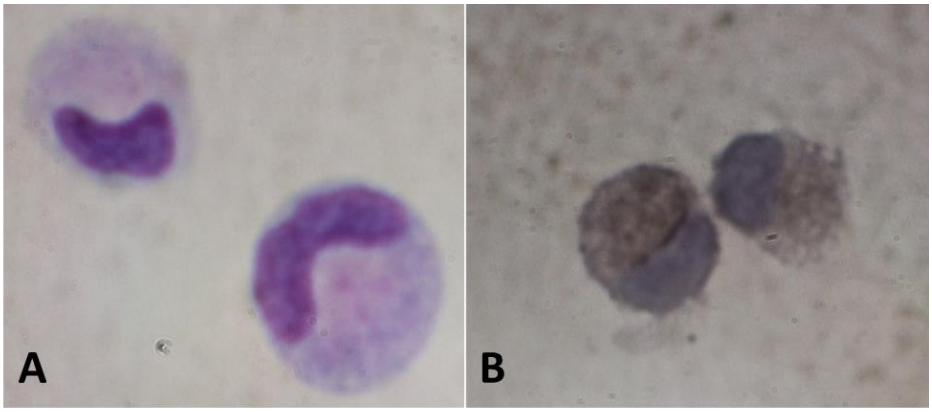

Figure 2. Neutrophils of fathead minnow obtained from the kidney by staining with (A) hemacolor and (B) Sudan black (1000x magnification).

After figuring out the neutrophil content in cytospin preparation of lymphoid organs of fathead minnows, the expression of *mpo* gene, a crucial neutrophil-specific gene of interest in our study, was assessed in the kidneys versus spleens. The results, shown in Figure 3., point out that the anterior kidney of fathead minnows has significantly higher *mpo* mRNA abundance compared to the spleen, confirming the anterior kidney of fathead minnow as the optimal lymphoid organ for assessing neutrophils.

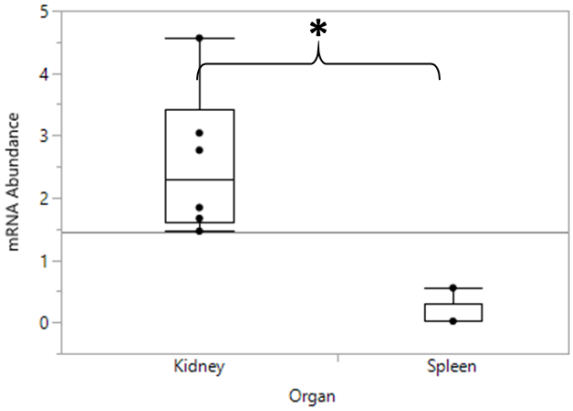

Figure 3. *mpo* mRNA abundance in kidneys and spleens of fathead minnows. Sample size of kidney and spleen groups were six and five, respectively; *mpo* mRNA abundance was normalized using gene expression of *hprt1*. Statistical significance (p=0.003) represented by *.

4.2. Neutrophil Isolation from Kidneys of Fathead Minnow

Next question was whether neutrophil isolation from anterior kidney would deliver consistent, reproducible results regarding the number, viability and purity of neutrophils, and whether one kidney (obtained from a single fish) would provide sufficient number of neutrophils for further testing. The consistently visible layer of cells on the gradient, in conjunction with the cell numbers, were obtainable by pooling the kidneys from six fish. The results in Figure 4. were obtained from eight experiments, with six kidneys pooled per sample; the kidney cell suspension pre-gradient averaged 61.7 ± 12.2 x 10^6 cells, and cell count recovered post-gradient averaged 23.3 ± 4.2 x 10^6 cells (Figure 4A). These cells recovered post-gradient exhibited 91.1 ± 3.8 % viability and 62.7 ± 3.9 % neutrophil purity (Figure 4B).

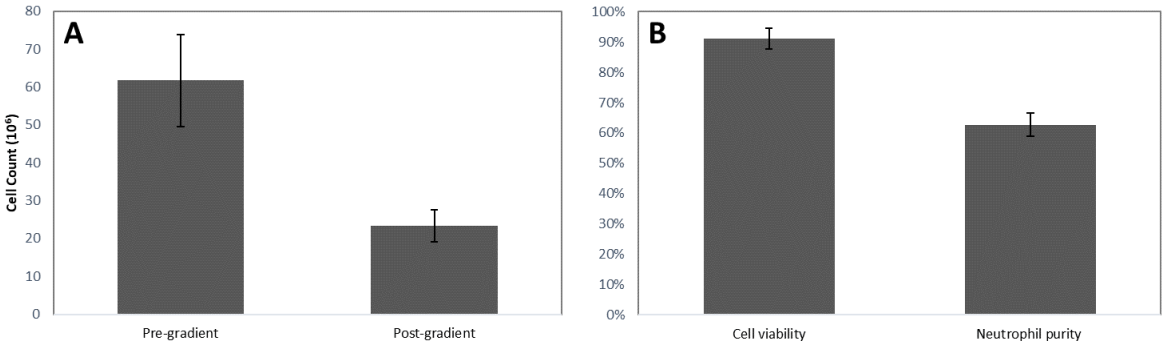

Figure 4. Assessment of neutrophil isolation quality: cell counts, cell viability and neutrophil yield. (A) Cell counts obtained before the gradient (Pre-gradient) and after the gradient (Post-gradient) by hemocytometer and 0.1% trypan blue staining. (B) Cell viability and neutrophil purity evaluated post-gradient by 0.1% trypan blue and Sudan black staining, respectively; expressed as percentage. Sample size of eight for each assessment.

4.3. Myeloperoxidase Assay for Assessment of Neutrophil Function

Further experiments were performed in order to establish an assay for evaluation of neutrophil function. A rapid, direct myeloperoxidase (MPO) assay (originally developed by Palić et al., 2005) was considered. Each individual experimental trial (labeled from 1 to 8) was performed on isolated neutrophils obtained from six pooled kidneys of non-treated fathead minnows (Figure 5.). The calculated percentage of degranulation showed a range from 27.4 % to 37.2 % among those eight trials, with an average degranulation of 31.5 ± 4.7 % (Figure 5).

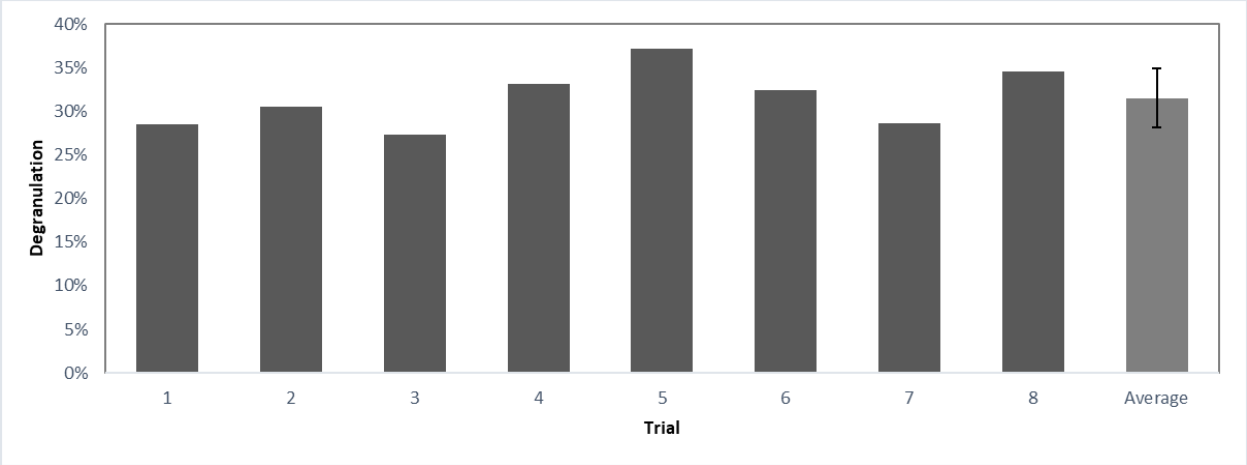

Figure 5. Preliminary myeloperoxidase (MPO) functional assay trials presented as percentage of degranulation. Each MPO assay (labeled from 1 to 8) was performed on neutrophils isolated from six pooled fathead minnow kidneys; last column represents an average degranulation percentage ± SEM of 8 trials.

The effects of exposures to estrone and BPA, known xenoestrogen, were tested in MPO assay. Single cell suspensions from kidneys, obtained after the purification, exhibited 60.3 ± 2.7 % and 67.4 ± 3.5 % neutrophil purity, with 97.8 ± 0.9 % and 96.8 ± 1.6 % cell viability for estrone and BPA exposures, respectively. Exposures to estrone showed a significant increase in degranulation compared to the control in the high-concentration ($p=0.0084$) treatment groups, while BPA exposures showed an increase of degranulation in the low- ($p=0.0041$) and high-concentration ($p=0.0275$) treatment groups, compared to the control.

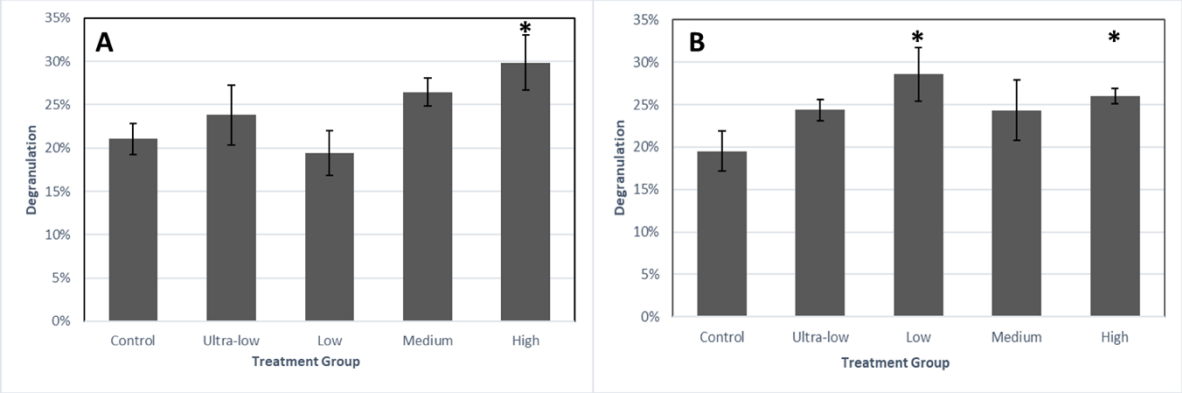

Figure 6. The effect of estrogenic compounds on neutrophil degranulation isolated from fathead minnow kidneys. (A) Estrone and (B) bisphenol A(BPA) exposures to kidneys. Sample size per treatment group for estrone and BPA MPO assay are pseudo-replicates of three. Significant difference from control (Estrone; High-concentration, p=0.0084)(BPA; Low-concentration, p=0.0041; High-concentration, p=0.0275) represented by *****.

4.4. Effects of Urban CEC Exposures on Neutrophil Function and *mpo* mRNA Abundance

The effects of urban CECs on fathead minnow neutrophils were tested by assessing neutrophil function and *mpo* mRNA abundance in anterior kidneys of fathead minnows acutely exposed over the period of 5 days to the following drugs: 5-methyl-1H-benzotriazole, desvenlafaxine, fexofenadine, fluoranthene, ibuprofen, metformin, sulfamethoxazole, triclosan and a mixture of all the individual drugs (urban mixture). The anterior kidney obtained from each exposed and control fish was divided in a such way that about 1/10 of a sample was prepared for *mpo* mRNA abundance assessment, while the other 9/10 was pooled with other kidney samples from the same experimental group and used for MPO functional assay (degranulation assay). Thus, all the results for *mpo* mRNA abundance were obtained from the individual fish and then summarized, while results of MPO assays were obtained from a 5-6 anterior kidney pooled samples and assessed in a triplicate (pseudo-triplicates).

4.4.1. *5-methyl-1H-benzotriazole*

The kidney cell suspensions obtained after the purification via lymphoprep gradient exhibited 71.6 ± 3.6% neutrophil purity and 94.5 ± 3.7% cell viability. Exposures to 5-methyl-1H-benzotriazole of different concentrations showed a significant decrease in neutrophil degranulation for the medium-concentration treatment ($p<0.001$) compared to the control. There was no significance found for *mpo* mRNA abundance in the kidneys of exposed fish.

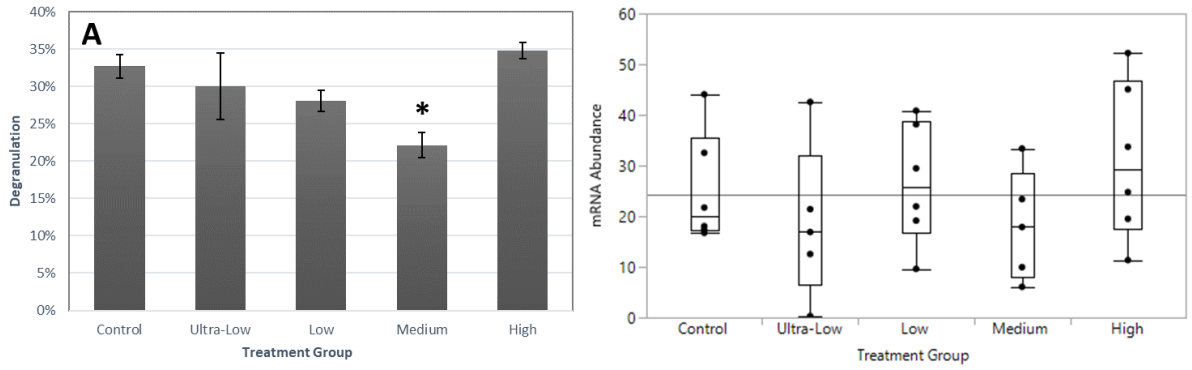

Figure 7. The effects of 5-methyl-1H-benzotriazole exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is a pseudo-replicate of three. Sample size per treatment group for *mpo* mRNA abundance is n=6, except for ultra-low (n=5) and medium treatment group (n=5); *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundances of *hprt1* and *tbp*. Significant difference from control ($p<0.001$) represented by *****.

4.4.2. *Desvenlafaxine*

Single cell suspensions from kidneys obtained after the purification exhibited 73.6 ± 3.4 % neutrophil purity and 95.4 ± 1.3 % cell viability. Desvenlafaxine exposures of different concentrations showed no significant difference in neutrophil degranulation when compared to the controls. The *mpo* mRNA abundance was not different in the kidneys of exposed and control fish.

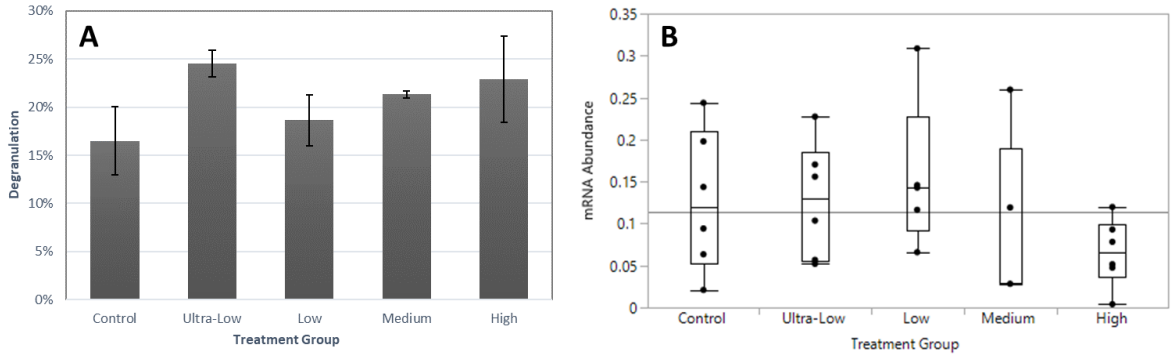

Figure 8. The effects of desvenlafaxine exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is a pseudo-replicate of three. Sample size per treatment group for *mpo* mRNA abundance is n=6, except for low (n=5) and medium treatment group (n=5); *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundance of *rpl8* and *hprt1*.

4.4.3. *Fexofenadine*

The kidney cell suspensions, collected after the neutrophil isolation from kidneys in the experiments where fish was exposed to fexofenadine, exhibited 73.5 ± 2.9 % neutrophil purity and 96.1 ± 2.0 % cell viability. Exposures to different concentrations of fexofenadine showed a significant increase in neutrophil degranulation for the low-concentration ($p=0.0094$) and high-concentration treatment ($p=0.011$), compared to the control. Low-concentration treatment group also showed a significant increase compared to control ($p=0.0001$) for *mpo* mRNA abundance in the kidneys of exposed fish.

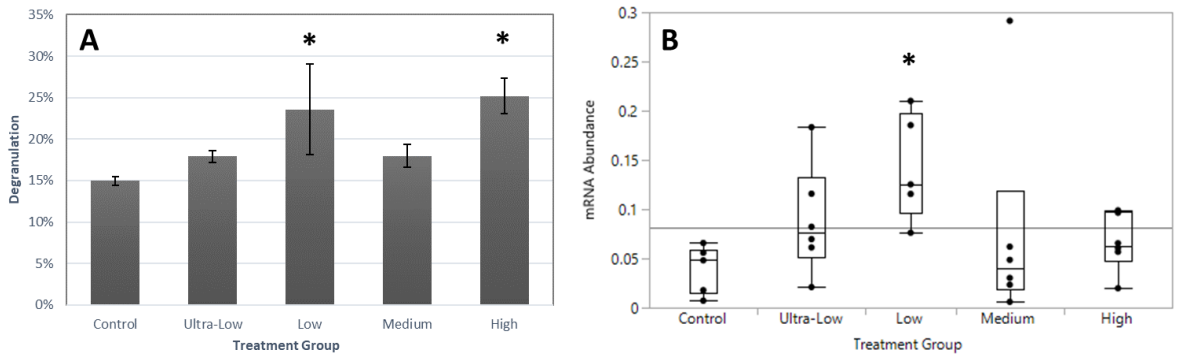

Figure 9. The effects of fexofenadine exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is a pseudo-replicate of three, except for control (n=2). Sample size per treatment group for *mpo* mRNA abundance is n=6, except for low-dose group (n=5). *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundance of *hprt1* and *tbp*. Significant difference from control (MPO; Low-concentration, p=0.0094; High-concentration, p=0.0001) (*mpo*; Low-concentration, p=0.005) represented by *****.

4.4.4. *Fluoranthene*

The purified kidney cell suspensions, obtained from fish exposed to fluoranthene and their respective controls, exhibited 96.2 ± 1.9 % cell viability. The purity of samples was not determined in this experiment. Exposures to fluoranthene of different concentrations showed no significant difference in neutrophil degranulation compared to the control. Due to collection error, *mpo* mRNA abundance could not be assessed.

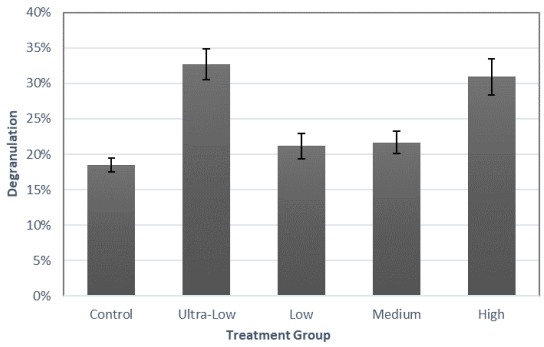

Figure 10. The effects of fluoranthene exposures on kidney neutrophils of fathead minnows on neutrophil degranulation. Sample size per treatment group for MPO assay is pseudo-replicate of three.

4.4.5. *Ibuprofen*

Isolated kidney neutrophils in this exposure group exhibited 73.3 ± 2.2 % purity and 98.0 ± 0.7 % viability. Different concentration exposures of ibuprofen showed a significant increase in neutrophil degranulation for the low treatment ($p=0.0059$) and high treatment ($p=0.0191$), compared to the control. The *mpo* mRNA abundance in the kidneys of ibuprofen-exposed fish did not show any significant differences when compared to the control.

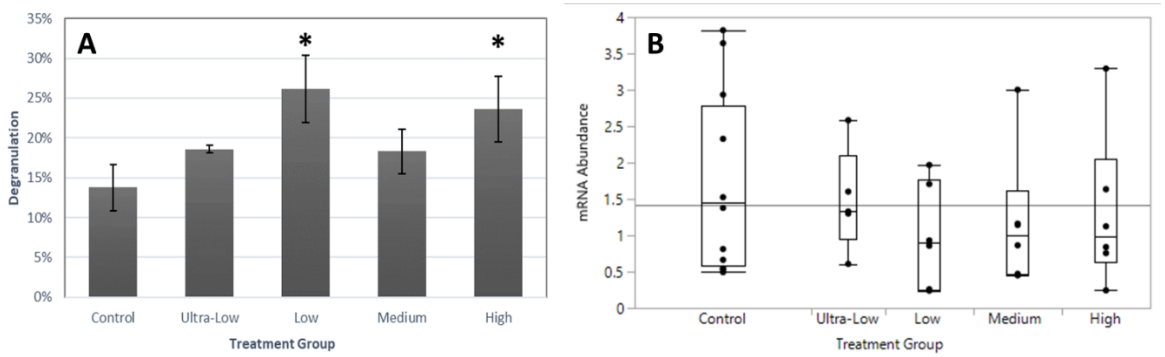

Figure 11. The effects of ibuprofen exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is a pseudo-replicate of three, except for control (n=2). Sample size per treatment group for *mpo* mRNA abundance is n=6, except for control (n=12) and ultra-low treatment group (n=5); *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundance of *rpl8* and *tbp*. Significant difference from control (MPO; Low-concentration, p=0.0059; High-concentration, p=0.0191) represented by *.

4.4.6. *Metformin*

The purified kidney cell suspensions, obtained from fish exposed to metformin and their respective controls, exhibited 97.0 ± 1.8 % cell viability. Neutrophil purity was not assessed for this dataset. Exposures to metformin of different concentrations showed no significance when compared to the control for neutrophil degranulation. There was no significance found in the *mpo* mRNA abundance in the kidneys of exposed fish.

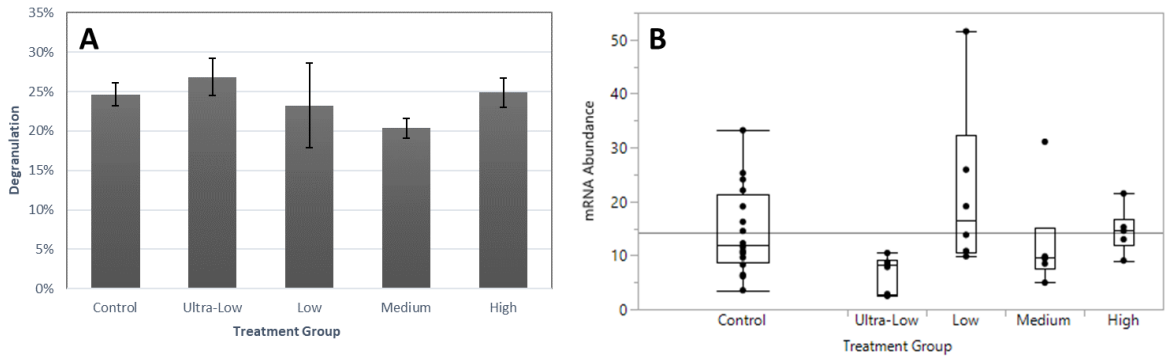

Figure 12. The effects of metformin exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is pseudo-replicate of three. Sample size per treatment group for *mpo* mRNA abundance is n=6, except for control (n=16); *mpo* mRNA abundance was normalized using mRNA abundance of *rpl8*.

4.4.7. *Sulfamethoxazole*

Sulfamethoxazole exposures of different concentrations, when compared to the control, showed a significant difference in high treatment group (p=0.0168) for the neutrophil functional assays. Neutrophil purity was 72.1 ± 2.4 % and viability 97.1 ± 1.0 %. There was also no significant difference found in the *mpo* mRNA abundance in the kidneys of exposed fish compared to controls.

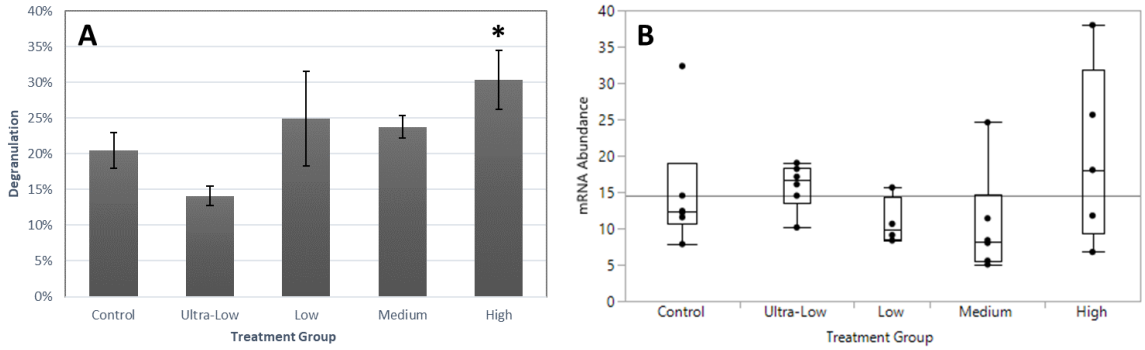

Figure 13. The effects of sulfamethoxazole exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is pseudo-replicate of three, except for ultra-low (n=2) and medium treatments (n=2). Sample size per treatment group for *mpo* mRNA abundance is n=6, except for low (n=4) and high treatment group (n=5); *mpo* mRNA abundance was normalized using mRNA abundance of *tbp*. Significant difference from control (MPO; High-concentration, p=0.0168) represented by *****.

4.4.8. *Triclosan*

The kidney neutrophils, obtained after the isolation from the kidney cell suspensions, exhibited 70.3 ± 5.0 % neutrophil purity and 96.9 ± 3.0 % cell viability. MPO assays of fish neutrophils exposed to different concentrations of triclosan were not different from controls. No significant difference was found in the *mpo* mRNA abundance in the kidneys of exposed fish compared to controls.

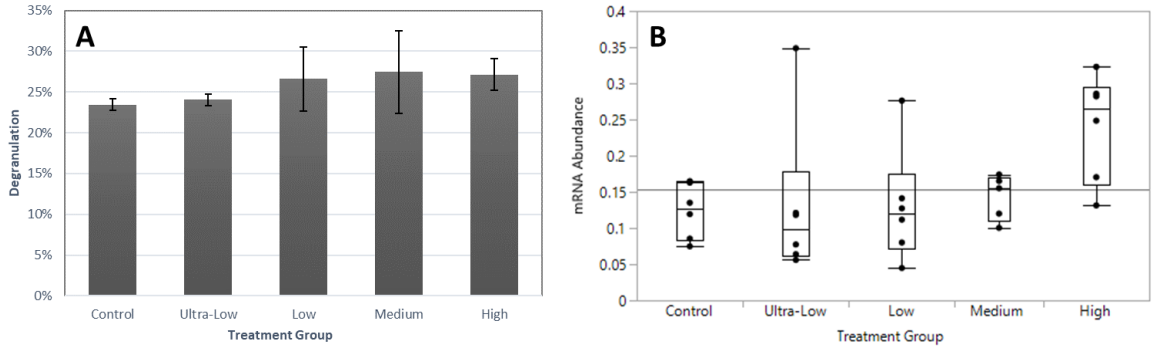

Figure 14. The effects of triclosan exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is pseudo-replicate of three. Sample size per treatment group for *mpo* mRNA abundance is n=6, except for medium-dose (n=5); *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundance of *tbp*.

4.4.9. *Urban mixture*

Kidney single cell suspensions exhibited 76.0 ± 3.0 % neutrophil purity and 97.1 ± 1.2 % cell viability post purification over the gradient. Out of all urban mixtures exposures of different concentrations, only the ultra-low treatment group showed a significant increase (p=0.0062) in MPO assay compared to the control. There was no significant difference found in the *mpo* mRNA abundance in the kidneys of exposed fish.

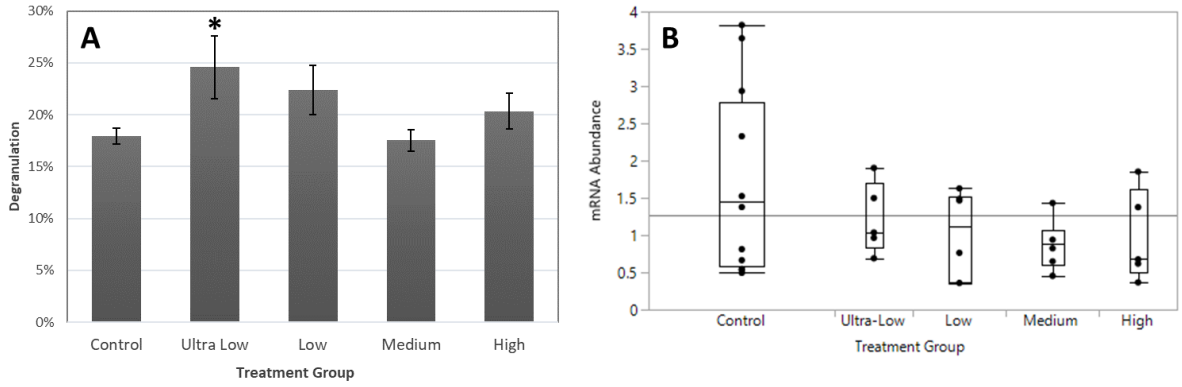

Figure 15. The effects of urban mixture exposures on kidney neutrophils of fathead minnows. (A) Degranulation and (B) *mpo* mRNA abundance in the kidneys. Sample size per treatment group for MPO assay is pseudo-replicate of three. Sample size per treatment group for *mpo* mRNA abundance is n=6, except for control (n=12) and ultra-low treatment group (n=5); *mpo* mRNA abundance was normalized using the geometric mean of mRNA abundance of *rpl8* and *tbp*. Significant difference from control (p=0.0062) represented by ***.**

In conclusion, this study found, based on the high number of neutrophils detected by Sudan black staining, as well as by high mRNA abundance of neutrophil-specific *mpo* gene, that the anterior kidney is the optimal lymphoid organ for assessing neutrophils in fathead minnows. Preliminary experiments of neutrophil isolation showed desirable cell counts, viability and neutrophil purity post lymphoprep gradient separation. The consistent results of neutrophil degranulation were obtained by MPO assay, validating its use in assessing neutrophil function. Although not all intended PCR primers were usable (*nox2* and *ela2*), *mpo* PCR primer set was successfully confirmed for quantification of *mpo* mRNA abundance. These test methods were then used to assess the effects of exposures to a complex urban mixture, as well as the individual compounds it is comprised of.

Exposures to particular concentrations of urban CEC mixture, and some of its individual compounds, such as fexofenadine, ibuprofen, and sulfamethoxazole, increased degranulation

of neutrophils, while exposure to 5-methyl-1H-benzotriazole decreased it. Interestingly, only one drug – fexofenadine, used in medium concentration, increased mRNA abundance of *mpo* compared to the control group. Following drugs, desvenlafaxine, fluoranthene, metformin and triclosan, did not affect either neutrophil function nor *mpo* mRNA abundance. There were no dose-response changes observed in MPO assays/*mpo* mRNA abundance for any of the tested urban CECs. Fexofenadine was the only drug that induced both an increase in degranulation as well as *mpo* mRNA abundance. The results obtained in our study do not support any of the proposed hypotheses.

Chapter 5: Discussion

5.1 General Discussion

The progressive conversion of land use into urbanized areas brings a larger concern for investigation of fresh water contaminations. CECs have already been found at alarming concentrations around the Great Lakes' tributaries (Elliott et al., 2017), and North America's river systems (Bradley et al., 2017). Many of these compounds found in urbanized runoff, may have effects on local organisms that have yet to be studied. Common use of pharmaceuticals, developed to maintain a biologically active state, become concentrated and expelled into rivers (Kostich et al., 2011).

To assess how urbanized runoff could potentially affect the innate immune system of aquatic organism, specifically the neutrophils of a model organism, the fathead minnow, research tools for studying neutrophils needed to be developed. Firstly, it was asked which lymphoid organ was the optimal organ for assessing neutrophils, the "first responder" and most abundant cell of the innate immune system. After the optimal lymphoid organ was addressed, an assay for a reliable assessment of neutrophil function needed to be developed and optimized. To evaluate proteins involved in neutrophils ability to defend its host from foreign entities, PCR primers were optimized for the evaluation of granule-specific proteins. Once these research tools were optimized, they were used to assess the effects of urban mixtures, and their individual compounds, on neutrophil function (degranulation) and mRNA abundance of granule-specific genes.

Cells isolated from the anterior kidney of fathead minnow showed a tenfold increase in mRNA abundance of neutrophil-specific gene *mpo* compared to spleen. Also, kidneys exhibited significantly higher neutrophil purity in comparison to spleens. These results confirmed that the anterior kidney is the optimal lymphoid organ for an assessment of neutrophils in fathead minnows. Our results agree with previously published data regarding the expression level of *mpo* (Jovanović et al., 2011) as well as the high neutrophil content in the anterior kidney of fathead minnow (Palić, Andreasen, Frank, Menzel, & Roth, 2005).

Preliminary assessment of neutrophil isolation from anterior kidney of fathead minnows, based on the procedure developed by Palić, Andreasen, Frank, Menzel, & Roth showed consistent results for cell viability and neutrophil purity (2005). The purity was evaluated by MPO-specific Sudan black staining of neutrophils, isolated using a gradient and processed by cytospin. This study showed a post-gradient cell viability of 91.1 ± 3.8% and a neutrophil purity of 62.7 ± 3.9%, which is similar to previously published data of 95.4 ± 1.1% cell viability and 72.0 ± 7.9% purity (Palić, Andreasen, Frank, et al., 2005). Our intention was to obtain a sufficient number of neutrophils post isolation from an anterior kidney of a single fish. However, we were not able to obtain it. Thus, six kidneys were pooled in order to get the number of cells necessary for performing a degranulation assay, confirming Palic's previous observation (Palić, Andreasen, Menzel, et al., 2005). Using the calcium ionophore method for stimulation of control (not-treated) fathead minnow neutrophils, we obtained 31.5% degranulation, compared to 47.7% observed by Palić, Andreasen, Menzel, & Roth (2005).

Overall, preliminary results showed that the protocols developed to isolate and assess neutrophil function provided reproducible results comparable to published ones.

Women are thought to have a more active immune system, which can lead to a stronger immune system (response), as well as to a higher prevalence of autoimmune diseases (Jacobson, Gange, Rose, & Graham, 1997). Estrogen is often linked to this reasoning. Estrogen has been shown to increase neutrophil infiltration (Chung et al., 2017; Plackett, Deburghraeve, Palmer, Gamelli, & Kovacs, 2016), recruitment (Robinson, Hall, Nilles, Bream, & Klein, 2014) and degranulation (Chiang, Parthasarathy, & Santanam, 2004) in mammals. As part of our preliminary study in control not-treated fathead minnow, not only did estrone show a significant increase in neutrophil degranulation, but the xenoestrogen BPA as well. This can give some confidence when comparing neutrophil function in fish with published findings on neutrophil function in mammals.

Due to the limitations of sample collection and use of pseudo-replicates, only one conclusion can be made from the results of the neutrophil myeloperoxidase assay. Similar protocols used for bovine (Quade & Roth, 1997) and human neutrophils (Mengazzi, Zabucchi, Knowles, Cramer, & Patriarca, 1992) allowed for the use of total MPO, based on the assumption that it reflected the total cell counts of a particular sample. However, in our case, total MPO between the treatment groups could not be assessed, as a single anterior kidney was always divided between the sample for RNA extraction and a sample for neutrophil isolation. In order to preserve the cell viability for MPO assay, the kidneys were not weighted. Thus, the total cells per kidney could not be accurately obtained.

Selected PCR primers for assessing neutrophils were chosen based on a variety of variables. Ideally, the selected genes not only had to be specific for neutrophils, but also have published PCR primers. Jovanovic & Palic developed an assortment of PCR primers for evaluation of general immune proteins, as well as neutrophil-specific proteins of fathead minnows, such as myeloperoxidase, NADPH oxidase and elastase 2 (neutrophil elastase) (2011). These three target genes were selected for our study, as they represent neutrophil's granules-specific genes. Myeloperoxidase produces the high amount of ROSs, thus assessing its expression could give insight on how environmental pollutants affect the mRNA abundance of gene that encodes such an important enzyme involved in ROS production. In addition, *mpo* mRNA abundance was assessed in parallel with the neutrophil functional assay, that evaluated degranulation based on released MPO. Although MPO assay (degranulation) and *mpo* mRNA abundance were run in parallel, the analysis of each test is exclusive and cannot be connected. Literature shows that in *mpo*-deficient humans, degranulation is significantly increased in the neutrophils collected and isolated from healthy adults (Dri, Cramer, Menegazzi, & Patriarca, 1985). NADPH oxidase fuels myeloperoxidase's ROS production by producing hydrogen peroxide, which alone is used by neutrophils to degrade exogenous entities. Elastase 2, one of the three proteases produced by neutrophils, is used to kill bacteria, degrade biological toxins and convert chemokines to more potent chemoattractants (Pham, 2006). Although the PCR primer sequences for all the mentioned genes were published, only one PCR primer set turned out to be usable under our study laboratory condition. During initial development and testing of PCR primers for NADPH oxidase, more than one product was produced, making this primer

set not usable for quantification. Elastase 2 PCR primer pair, during initial testing, did not demonstrate high enough mRNA abundance of elastase 2 to allow a reliable quantification. This shows that even published PRC primers need to be tested for reproducibility. Low mRNA abundance of elastase 2 compared to *mpo* mRNA abundance could suggest that myeloperoxidase is expressed at higher level than elastase 2 in neutrophils of fathead minnows.

Although at the start of this study there were three possible genes of interest, involved in degranulation or the elimination of pathogens, only one (*mpo*) gene could be successfully assessed. Gene mRNA abundance of *mpo* could be different because of two possibilities. Firstly, an increase or decrease in *mpo* mRNA abundance could be observed from a change in cell population in the tissue. Each CEC treatment has the potential to increase or decrease neutrophil numbers either directly or indirectly through other physiological means, affecting the total presence of *mpo* mRNA. The second scenario could be that the CEC treatment affects the mRNA abundance of *mpo* in an existing neutrophil population, without perturbation in cell numbers. If both scenarios were to happen simultaneously, the result could have masked their individual effects.

5.2. Urban CEC Exposures

When studying the effects of CECs' exposures, no significant difference was found for either neutrophil function or *mpo* mRNA abundance in desvenlafaxine, fluoranthene, metformin and triclosan exposures compared to controls.

The function of desvenlafaxine is to increase the amount of serotonin and epinephrine in a biological system. Serotonin, often linked to the central nervous system as a

neurotransmitter, controlling mood, sleep and appetite, has a second life as a peripheral hormone (Walther & Bader, 2003). One of the leading theories is that serotonin interrupts the function of MPO, acting as a scavenger of ROSs (Huether, Fettkötter, Keilhoff, & Wolf, 1995). However, serotonin inhibition was reported in 2010 to only be seen in lymphocytes, not in neutrophil isolates (Prachařová, Okénková, Lojek, & Číž, 2010).

Fluoranthene is an AhR-active compound. Besides the direct metabolism of xenobiotics, AhRs are involved in immune responses. AhR-deficient mice have a reduced rate of cell proliferation and different morphology (Ma & Whitlock, 1996). Many studies on AhRs and neutrophils do not show a direct response, but suggest an indirect response through other cells' AhR response. During influenza infection, a TCDD treatment showed neutrophilia in the lungs of mice, but the mechanism of AhR-mediated neutrophilia did not involve elevated levels of neutrophil chemoattractants, adhesion molecules, delayed apoptosis, or vascular damage (Teske, Bohn, Hogaboam, & Lawrence, 2008).

Metformin helps control type 2 diabetes through decreasing production of glucose by the liver, non-competitively inhibiting the redox shuttle enzyme mitochondrial glycerophosphate dehydrogenase (Madiraju et al., 2014). Metformin increases activation of AMPK, which has been shown to have anti-inflammatory effects through inhibition of pro-inflammatory cytokines and transcription factors (Cameron et al., 2016).

Originally used as an additive in medical devices for its ability to inhibit bacterial growth in wounds (Ming, Nichols, & Rothenburger, 2007), triclosan is now used mainly in consumer products. Although triclosan has been shown to be tolerated by humans (DeSalva, Kong, & Lin,

1989), there has been an association with triclosan levels in urine and aero-allergenic/food sensitization (Savage, Matsui, Wood, & Keet, 2012).

5.2.1. *5-methyl-1H-benzotriazole*

Exposure of fathead minnows to a medium concentration of 5-methyl-1H-benzotriazole (M1HB) showed an inhibitory effect on neutrophil degranulation. No significant differences were found following M1HB exposure for *mpo* mRNA abundance between the treatment groups and controls.

M1HB is an anti-icing agent used by commercial airlines, and an industrial anti-corrosive used for copper and brass. Literature on M1HB effects on the immune system is lacking; in respect to the innate immune system, no citation could be found. Some speculations can be made from the research linking HIV-1 reactivation by benzotriazole treatment through the inactivation of STAT5 (Bosque et al., 2017). STAT5 is a transcription factor, activated by cytokine signal transduction involved in activation of regulatory genes controlling cell growth/death, proliferation and differentiation. In respect to neutrophils, STAT5 is responsible for the maturation and differentiation of granulocyte progenitor cells. In STAT5-/- mice, the mice were unable to maintain homeostasis of neutrophils, resulting in neutrophilia (Fiévez et al., 2007). In our case, it could be speculated that M1HB affected maturation of neutrophils; these "immature granulocytes" would not be able to release MPO post stimulation of degranulation. Since MPO is produced at all stages of neutrophil maturation (Naeim et al., 2013), this would add to the "total" MPO in the testing wells, and would have no effect on neutrophil purity observed by Sudan black staining.

5.2.2. *Fexofenadine*

Post fexofenadine exposure, medium and high treatment groups showed a significant increase in degranulation compared to the control group. The medium treatment group also showed a significant increase in *mpo* mRNA abundance compared to the control group.

Fexofenadine is a commonly used anti-histamine, known as H1-receptor antagonist, which acts by blocking histamine binding to H1 receptor. By supressing histamine-induced stimulation through H1 receptor, fexofenadine inhibits the allergic response caused by allergens. Blocking histamine interaction with its H1 receptor has many effects on the immune system, such as decreasing migration of immune cells and cytokine production (Shimizu et al., 2004). In respect to neutrophils, Benbarek et al. found that supraphysiological doses of histamine showed an increase in production of superoxide anion and/or hydroxyl radicals in horse neutrophils, while H1-receptor antagonists decreased it (1999). Azelastine, a second generation H1-antagonist, showed a decrease in production of three ROSs, superoxide anion radical, hydrogen peroxide and hydroxyl radical, in human neutrophils (Akamatsu, Miyachi, Asada, & Niwa, 1991). Studies have shown an inhibitory effect of H1-antagonist on oxidative burst on isolated neutrophils of humans (Akamatsu et al., 1991; Mikawa et al., 1999), but degranulation has not been assessed. Besides these inhibitory effects of H1-antagonist, other studies have shown controversial data regarding the effects of histamine and histamine antagonist inhibition on neutrophil function (Číž & Lojek, 2013), even suggesting that certain H1-inhibitors interact with intracellular increase of calcium (Mikawa et al., 1999). Our data appear to agree with these studies.

5.2.3. *Ibuprofen*

Low- and high-concentration treatment ibuprofen groups showed a significant increase in degranulation compared to the control. There was no significant difference found between treatment groups for *mpo* mRNA abundance.

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) used to treat inflammation through the inhibition of prostaglandin production by decreasing cyclooxygenase activity. An increase in prostaglandins leads to increase in blood flow and white blood cell recruitment to afflicted tissues. It was suggested that NSAIDs reduce inflammation by inhibiting neutrophil activation (Altman, 1990). Another NSAID, piroxicam, inhibited three functions of human neutrophils *in vitro*, aggregation, lysosomal enzyme release and superoxide production (Abramson S, 1989). In the same study, ibuprofen exhibited an inhibition of neutrophil aggregation and lysosomal enzyme release. Although all NSAIDs share the commonality of inhibiting prostaglandin production, by individual compounds showing different results of neutrophil inhibition supports the claim of non-prostaglandin mechanisms of anti-inflammatory effects.

Described effects of ibuprofen on neutrophil function have been researched in mammals. The finding in this study, about an increase of neutrophil function, does not support data obtained in mammals about its inhibition. However, direct comparison is impossible, not only because of different species that were studied, but also because of assessment of different neutrophil functions.

5.2.4. *Sulfamethoxazole*

The high-concentration exposure group of sulfamethoxazole showed a significant increase in degranulation compared to the control. There was no significant difference found between treatment groups for *mpo* mRNA abundance.

Sulfamethoxazole is a bacteriostatic antibiotic commonly used for urinary tract infections and bronchitis, which prevents the growth of gram negative and positive bacteria by interfering with the bacteria's ability to synthesize folic acid and DNA.

Very few studies have investigated sulfamethoxazole effects on the immune system in any organism. Sulfamethoxazole is often paired with trimethoprim, another antibiotic, in medical practice and in research studies. Trimethoprim is suggested to be responsible for the found effects (Bjornson, McIntyre, Harvey, & Tauber, 1986). Sulfamethoxazole has been shown to inhibit neutrophil hydrogen peroxide production in humans, without affecting other neutrophil functions (Anderson, Grabow, Oosthuizen, Theron, & Van Rensburg, 1980).

5.2.5. *Urban mixture*

As stated at the beginning of the discussion chapter, CECs are being found at alarming rates in aquatic environments. Although testing the individual compounds of the environmental mixture is useful, it is known that these CECs exist together in a particular environment and that they interact in a complex way (Binderup et al., 2003). The urban mixture, which contained all the individual compounds at four different concentrations, was assessed in this study. The exposure to urban mixture showed a significant increase in degranulation for the ultra-low treatment group when compared to the control. No significant difference was found for *mpo*

mRNA abundance. Of all the significant effects on neutrophil function observed in this study, beside BTA, there is a common trend of increased degranulation in neutrophils of fathead minnows. The mixture of these compounds does not seem to interact in a way that biologically amplifies the effects of individual chemicals.

5.3. Adverse Outcome Pathways

Following the trend of a statistically significant increase in degranulation post CEC exposures, this change in neutrophil function could lead to generally compromised health status of the organism being affected. Upon stimulation of neutrophil degranulation, pro-inflammatory cytokines are released alongside the classical granules in preformed, distinct secretory vesicles (Pellmé et al., 2006). Pro-inflammatory cytokines IL-6, IL-12, and CXCL2 are suggested to be stored within tertiary granules (Denkers, Del Rio, & Bennouna, 2003). Not only do neutrophil granule contents protect the host from pathogens, but can also cause oxidative damage to surrounding tissues. Damaging surrounding tissues, specifically epithelial cells, can cause those cells to release pro-inflammatory cytokines (Stadnyk, 1994), escalating the inflammatory process. Pairing indirect oxidative damage with cytokine production from neutrophils, an increase in degranulation could lead to increased acute or chronic inflammation. Inversely, decreasing neutrophil function may decreases inflammation, yet it is equally as threating to the host. A decrease in degranulation can be compared to the human diseases, Chediak-Higashi syndrome (CHS) and neutrophil-specific granule deficiency (SGD). As neutrophils are mainly responsible for the initial clearance of pathogens and debris, it is not surprising that CHS and SGD drastically increase the risk of infection of afflicted host (Kaplan, De

Domenico, & Ward, 2008; McIlwaine, Parker, Sandilands, Gallipoli, & Leach, 2013). The immune system is a finely balanced system; a disturbance in this balance, by increasing or decreasing neutrophil function, can be catastrophic to the host.

Chapter 6: Conclusion

Disturbing neutrophil function through urban CEC exposure could have potentially harmful effects on aquatic organisms. Even though exposures to these urban compounds in our experimental setting lasted only 96 hours, a significant increase in degranulation was found in the preliminary experiments using exposures to particular concentrations of estrogenic compounds (estrone and BPA) as well as urban CECs, such as, ibuprofen, sulfamethoxazole and the urban mixture. Fexofenadine exposure showed a significant increase in both degranulation and *mpo* mRNA abundance. BTA was the only CEC to show a decrease in degranulation. Finding significant effects on degranulation and *mpo* mRNA abundance in fish acutely exposed to pharmaceuticals suggest that functional and gene expression studies of MPO might serve as an early endpoint for evaluation of CECs' effects on neutrophils of fathead minnows.

Immunotoxicology in fish is a relatively new field of study and is rarely used in ecotoxicology risk assessments. With the immune system being a diverse, sophisticated network, present in almost all tissues, the immune system is exposed to exogenous chemicals as much or even more than most other systems of an organism. One of the main challenges of immunotoxicology is how to assess toxicants ability to stimulate or inhibit immune function, and if these effects even have adverse outcomes. As of now, there is no aquatic immunotoxicology tests that shows a response to all chemicals that shows an effect on different aspects of the immune system (Rehberger, 2017). There are still major gaps in the assessment of toxicants on immune function, concealing the larger picture. With potentially

toxic chemicals being found in aquatic systems at higher concentrations, assessing immune function should be a focus for future ecotoxicology studies.

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Appendix

Table A.1. Compound classes and their environmentally relevant concentrations.

Compound	Class	Sub-class	Environmentally relevant concentration (ng/L)
5-methyl-1H-benzotriazole	industrial	AhR active	6680
Bisphenol A	plasticizer	ER active	600
Desvenlafaxine	pharmaceutical	Antidepressant	583
Estrone	pharmaceutical	ER active	24
Fexofenadine	pharmaceutical	antihistamine	1,000
Metformin	pharmaceutical	Antidiabetic	1,210
Sulfamethoxazole	pharmaceutical	antibiotic	559
Fluoranthene	PAH	AhR active	0.1
Triclosan	personal care products	Antibacterial	0.5
Ibuprofen	pharmaceutical	Anti-inflammatory	440

Table A.2. Myeloperoxidase assay summary.

Treatment	Estrone	BPA	BTA	Desvenlafaxine	Fexofenadine	Fluoranthene	Ibuprofin	Metfomin	Sulfamethozaxole	Triclosan	URM
Ultra Low											↑
Low		↑			↑		↑				
Medium			↓								
High	↑	↑			↑		↑		↑		