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Fish olfaction: a biosensor for anthropogenic contaminants

by

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Abstract

Anthropogenic contaminants can impair olfactory responses to natural odorants. In fishes, these impairments may be used as a metric of sub-lethal toxicity. My studies aimed to determine the effects of two contaminant sources on fish olfaction as measured by electro-olfactography (EOG). The effects of treated municipal reuse water and oil sands process affected water were examined in two fish species, goldfish (*Carassius auratus*) and rainbow trout (*Oncorynchus mykiss*). Both contaminant sources were found to decrease olfactory responses to odorants during acute (30 min) and long term (60 and 7 d) exposures. My studies also aimed to identify a novel class of odorants (nucleosides). It was determined that nucleosides are detected, as are the nucleobases that comprise part of their structure. Overall the focus of my thesis was to investigate olfactory toxicity in fishes and to characterize a new class of odorants for future studies.

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List of abbreviations

- 17,20-β-pregnen-3-one: 17,20-βP
- Carbon filtered reuse: CF/MF reuse
- Electro-olfactogram: EOG
- G-protein coupled receptor: GPCR
- Litres: L
- Membrane filtered reuse: MF reuse
- Millilitres: mL
- Millivolts: mV
- Minutes: min
- Napthenic Acid: NA
- Novel contaminant mixture: NCM
- Oil sands process affected water: OSPW
- Olfactory Receptor: OR
- Olfactory sensory neuron: OSN
- Ozonated oil sands process affected water: O3OSPW
- Prostaglandin F2α: PGF2α
- Seconds: s
- Taurocholic Acid: TChA
- Ultraviolet light and hydrogen peroxide filtered reuse: UV/H2O2/MF reuse
- Vomeronasal receptor class 1: V1R

- Vomeronasal receptor class 2: V2R
- Waste water treatment plant: WWTP
- Personal care products and pharmaceuticals: PCPPs

Chapter 1: Introduction

Fish olfaction

Olfaction is an important sensory system used by fish to gather information about their surroundings and is used to guide behaviors such as mating, homing/ migration, and predators / prey avoidance / attraction. The olfactory epithelium of fishes is comprised of two bilaterally paired structures, known as olfactory rosettes, contained within olfactory chambers (Appendix A; Appendix B)[46]. In the fish species I have studied, goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*), the olfactory chamber is covered by a flap of skin with two openings (nares), one anterior through which water enters the chamber and one posterior through which water exits the chamber (Appendix A). Olfactory rosettes are comprised of multiple lamellae which are folded together (Appendix B). Olfactory lamellae contain olfactory sensory neurons (OSNs), which occur at different concentrations on different lamellae within the rosette [42].

Three classes of OSNs have been identified in fishes: ciliated, microvillus and crypt; ciliated and microvillus being named after the appearance of their projected structure. Olfactory receptors also have specific g-protein coupled receptors associated with them, with more than 100 GPCRs occurring in the fish olfactory system [79]. The GPCR super-family can be subdivided into three sub-families which include olfactory receptors (OR), and vomeronasal receptor class one and two (V1R and V2R, respectively), each associated with the OSN sub-families. Ciliated OSNs express ORs, which express the G-protein G-$_{\alpha olf}$, microvillus OSNs

express V2Rs, which express the G-proteins G-$\alpha_{,o}$, G-$_{\alpha,q,}$ and G-$_{\alpha,i-3}$, and crypt OSNs appear to express both V1Rs and V2Rs, which express the G-proteins G-$\alpha_{,o}$ and G-$_{\alpha,q}$ [40].

For an olfactory response to occur in fishes, water with odorants or other molecules/ions passes through the olfactory chamber, over the olfactory rosette, and to the GPCRs [46]. If an agent binds a receptor, a G-protein subunit may dissociate and affect the activity of one of two secondary messenger systems: one which stimulates phospholipase C leading to the production of inositol triphosphate, or; one which stimulates adenylyl cyclase leading to the production of cyclic AMP [102]. Secondary messengers can activate a Na^+ or Ca^{2+} permeable conductance in cation channels, resulting in an increase in free intracellular Na^+ or Ca^{2+}. The change in membrane conductance affects voltage, which in turn may activate a second conductance of Cl^-, a cation, or K^+, leading to the generation of generator potential. A strong enough generator potential at the apical end of an OSN may result in an action potential. Changes in OSN membrane potential can be measured using an extracellular recording technique referred to as electro-olfactogram (EOG) by Ottoson [78, 88].

Following the generation of the action potential, the electrical signal travels along axons extending from OSNs into glomeruli at the base of the olfactory bulb [46]. Multiple OSN axons form a bundle which enters into singular glomeruli for a convergence of one signal. Mitral cells within the olfactory bulb form a synapse with the glomeruli and convey this signal into the brain for further processing.

This processing of the signal leads to behavioral or physiological responses related to olfactory detection of odorants. Should the generator potential not be high enough to activate an action potential, olfactory signaling to the olfactory bulb would not occur and therefore neither would the processing of the olfactory signal. Inhibition of the signaling pathway at any point causes a decrease in olfactory ability, and therefore a decrease in information being gathered via olfaction [118].

The electro-olfactogram (EOG)

The EOG is a measure of the combined generator potentials of groups of OSNs within the recording area, with the recording area defined loosely as the region under and around the micro-electrode used in the recording [78]. In simpler terms, EOGs are a measure of the change in many cells' membrane potentials, resulting from an efflux of ions that occurs during the aforementioned binding of odorants to ORs. To measure these generator potentials, an electrode composed of Na+/Cl- and gelatin is placed in close proximity to lamellae within the olfactory rosette (Appendix C) [7]. A second electrode is placed either on the body of the fish or in the water bath containing the fish, and the potential at this electrode is also measured. The EOG is a difference between the generator potential of a group of OSNs within the recording area of the electrode and the reference electrode. Signals are amplified using an amplifier and digitized using a computer interface [7]. The differential EOG is displayed as a negative peak in the baseline, returning to baseline upon cessation of olfactory stimulation. The magnitude of the peak

correlates to the strength of the generator potential, with decreased generator potentials resulting in decreased peak magnitudes. If generator potentials of a group of OSNs are decreased then the action potentials will also be decreased, resulting in a disruption of the information sent for processing. The EOG provides information regarding detection of odorants via the generator potential; however it does not provide information on disruption of the olfactory signaling pathway.

Measuring the EOG

As previously stated the EOG is measured as the difference of the peak amplitude from the pre-odorant baseline. This measure has been used traditionally as it represents the maximal response evoked from all cells in the recording area. This is in contrast to electro-encephalogram recordings that measure sum action potentials and use area under the peak to determine the response. EOGs are not measured using the area under the curve as the slow return to baseline may occur over a large amount of time (several seconds) thus making it impractical to use this measurement [104]. This slow return to baseline may be due to odorants remaining in the olfactory cavity and stimulating ORs for a second time; however it is also possible that this slow return to baseline is due to signals from other cells which have been shown to evoke responses due to changes in the micro-environment as a result of a release of ions during the depolarization events that occur during odorant binding [7, 104].

Odorants

To date five odorant classes have been shown to be detected in fishes: amino acids, steroids, prostaglandins, bile salts, and nucleotides [46, 118]. Not all fish are able to detect each class of odorant and the sensitivity of the olfactory tissue to specific odorants differs between species [117]. An example of this is with prostaglandins: rainbow trout do not appear to detect them, while goldfish and Atlantic salmon (*Salmo salar*) can at a threshold of 10^{-8} and 10^{-11} M, respectively [110, 115]. Specific behaviors are associated with the detection of some odorants, examples including feeding behaviors such as food searching and biting exhibited by the detection of the amino acid L-alanine and mating behaviors such as chasing of females associated with male goldfish upon the detection of various pheromones [108, 110, 126, 127]. Below I have described the detection thresholds and behaviors associated with the odorants used in my thesis.

Amino acids

L-alanine

Olfactory detection of L-alanine was first observed in catfish during a study conducted by Suzuki and Tucker [114]. The study used EOG recordings to demonstrate the detection of six amino acids and determined their threshold concentration for detection. It was shown that channel catfish (*Ictalurus punctatus*) can detect L-alanine at dilutions as low as 10^{-8} M, and that detection occurred in a concentration dependent manner. The steriochemistry of odorant

molecules also plays a role in detection, as the L forms are more stimulatory than D forms [47, 48]. Hara et al. found that L-alanine was also detectable by rainbow trout (*Oncorhynchus mykiss*), with the detection threshold the same as that found by Suzuki and Tucker [47]. It was again demonstrated that the L- isomer was more stimulatory than its D- isomer. Hara demonstrated that detection of amino acid structures was impacted by the positioning of both the amino and carboxyl group, with OSN responses the greatest when both groups are in the α position [48]. Behavior evoked in the presence of L-alanine is known to include feeding behaviors such as snapping, biting and increased searching in multiple fish species [13, 34, 66, 126, 127]. Studies investigating olfactory detection of L-alanine suggest that it is an important olfactory cue related to food searching. Decreased detection of L-alanine may result in decreased ability to find prey organisms, resulting in malnourishment and decreased fitness.

L-serine

Multiple studies have shown that L-serine represents a potent olfactory stimulus for multiple fish species [47, 53, 62, 93, 94, 109] and while most behavioral studies have focused on salmonids, behavioral responses to its introduction have been demonstrated as avoidance [53, 93]. L-serine was first identified as a potential odorant for salmonids in a study conducted by Idler et al., in which they attempted to determine the components of a human hand rinse that were associated with avoidance behavior [53]. The study examined the willingness of salmonids to continue migration via a ladder when human hand rinse or

compounds extracted from human hand were introduced to the ladder. They found that hand rinse released in the ladder resulted in fish stopping at the base of the ladder, as well as increasing rapid movements, or returning downstream. Fish would eventually climb the ladder; however only after the pulse of hand rinse had passed. Of all compounds extracted and tested from human hand rinse, L-serine was the only compound to elicit the same behaviors in the fish at a similar dilution (10^{-6} M). The researchers identified L-serine as an odorant associated with mammalian predators. Hara et al. recorded EOG responses by rainbow trout to L-serine and determined that it was detectable down to a concentration of 10^{-8} M. L-serine was also shown to be detected by channel catfish at a dilution of 10^{-7} M [18]. Rehnberg and Schreck used a y-maze test, in which fish were placed at the base of a y-shaped trough and allowed to swim up one arm with or without the presence of an odorant, to show that rainbow trout avoided L-serine[93]. Hara et al. observed lake whitefish (*Coregonus clupeaformis*) and goldfish behavior post introduction of L-serine in a behavioral trough. After the introduction of L-serine, swimming behavior changed from end to end swimming to small circular swimming and searching along the trough wall. Decreases in L-serine detection may result in decreased ability of fishes to detect mammalian predators, and as such become easier targets.

Prostaglandin

Prostaglandin F2α

The pheromone prostaglandin F2α (PGF2α) has been shown to be an olfactory stimulus in multiple fish species and is associated with both behavioral and physiological responses [65, 80, 110]. The EOG thresholds determined were between 10^{-7} and 10^{-11} M (Table 1-1). The pheromone PGF2α is a potent olfactory stimulant released by female goldfish to evoke mating behavior in male goldfish [110]. Laberge et al. demonstrated that lake whitefish and brown trout increased locomotor activity after the introduction of PGF2α; however rainbow trout did not display the same increase [65]. When PGF2α was released by female Atlantic salmon it was shown to evoke increased milt production in males [80]. The EOG and behavior data regarding PGF2α suggests that it relays important sensory information that relates to mating in multiple fish species. Decreases in PGF2α detection may result in male fish not performing mating behaviors or increasing milt production. If such changes persisted in a field setting, decreases in fish populations could occur.

Steroid

17α, 20β-dihydroxy-4-pregnen-3-one

17α, 20β-dihydroxy-4-pregnen-3-one (17, 20-βP) was identified by Sorensen et al. as a steroidal pheromone detected via olfaction by goldfish[108]. The detection threshold determined by EOG recordings from male, female, and gonadally

regressed male goldfish was 10^{-13} M. Male goldfish exposed to 17,20-□P were shown to have increased milt production as well as increased mating success [113]. This data suggests that 17,20-□P is an important olfactory cue to goldfish mating. Decreases in 17,20-□P detection may result in fish not performing mating behaviors or increasing milt production. If such changes persisted in a field setting, decreases in fish populations could occur.

It should be noted that odorants such as steroids require odorant binding proteins to reach ORs within the olfactory tissues of terrestrial animals due to the change of phase for these highly volatile compounds, they being present in air and ORs being in an aqueous environment [129]. This is not likely the case for these odorants when interacting with the fish olfactory system as the steroids is released into and detected in an aqueous environment, and no such proteins have been described in fish olfactory tissue.

Bile salt

Taurocholic Acid

Taurocholic acid (TChA) was first described as an odorant in Arctic char (*Salvelinus alpines*) and grayling (*Thymallus thymallus*) with a mean detection threshold of 6.3 x 10^{-8} by Doving et al. [25]. Goldfish and rainbow trout detect TChA with concentration thresholds of 10^{-8} and 10^{-10} M, respectively [33, 109]. Giaquinto et al. suggest that TChA functions as a pheromone in rainbow trout [33]. TChA represents an important odorant to salmonids evoking mating

behaviors. Decreases in its detection may result in fish not performing courtship behaviors, and potentially decreasing fish populations.

Olfactory toxicity of fishes

The olfactory tissue of fishes is vulnerable to contaminants due to its highly exposed nature. To date, olfactory toxicology studies have primarily focused on the effects of pH, metals, and pesticides [22, 36, 49, 54, 56, 62, 81, 82, 84, 101]. As there are multiple OSN classes, not all of which use the same mechanisms to evoke olfactory responses, it is likely that different forms of contaminants affect fishes' ability to detect different classes of odorants. My thesis focuses on the effects of organic contaminant mixtures from two sources: one including personal care products and pharmaceuticals (PCPPs) and pesticides; a second containing a mixture of naphthenic acids (NAs). To date no studies have focused on the effects of PPCPs or NAs on olfaction; however studies examining the effects of pesticide mixtures on EOG responses exist. Atlantic salmon exposed to a pesticide mixture for 30 min displayed decreased EOG responses to both L-serine and PGF2α. The mixture contained equal parts of simazine and atrazine (1 μg/L) and decreased L-serine and PGF2α EOGs by 51 and 70% respectively[82]. The effects of the mixture were similar to those of a 30 min exposure to 2 μg/L simazine, which decreased EOG responses to L-serine and PGF2α by 50 and 72% respectively. Tierney et al. examined the effects of an environmentally realistic pesticide mixture, exposing juvenile rainbow trout to a mixture containing dimethoate, simazine, methamidophos, diazinon, chlorpyriphos, endosulphan, malathion,

atrazine, linuron, and parathion for 96 h [121]. Total concentrations of pesticides of 0.186 and 1.01 μg/L reduced L-serine evoked EOGs by 14 and 42% respectively. Contaminant mixture studies are not well represented in the literature; however it is important that we better understand the effects of contaminant mixtures as they represent exposures scenarios that are more likely encountered by wild fish populations.

Studies examining the effects of contaminant mixtures on behavior provide important information relating to ecological impacts on fish that may encounter similar exposure scenarios in the wild. Behaviors with obvious survival relevance, e.g. homing, migration, mating, and predator/prey detection, often rely on the detection of chemosensory cues via olfaction. Contaminants within the environment that disrupt the detection and perception of odorants alter a fish's ability to gather this information, and this may negatively affect survival. For fish to avoid contaminants, they must be able to detect them and also associate their presence with negative effects. Avoidance of contaminants such as pesticides and metals has been shown to occur in multiple fish species [16, 17], however some contaminants are not detectable and other contaminants are not perceived as harmful and so do not result in avoidance behaviors [32, 38], and may even result in attraction [5, 122]. The herbicide nicosulfuron is an example of a contaminant which evokes attraction (in goldfish), although there is no known benefit to this exposure [100]. Not only is it important to understand behaviors associated with these contaminants, but also behaviors associated with natural odorants during

and after exposure to contaminants, as these may be altered. My thesis focused on behavior studies involving exposure to a contaminant mixture containing both PCPPs and pesticides; however behavior data relating to these types of contaminant mixtures is limited. Tierney et al. monitored time spent in the inflow zone of a flow through system by adult zebrafish (*Danio rerio*) when presented with a pulse of tank water (control), low, medium, or high concentrations of a pesticide mixture containing glyphosate, dicamba, mecoprop and 2,4-D. Zebrafish presented with the high concentration (875 ng/L) spent more time in the inflow zone than controls during the first min following the pulse introduction, demonstrating an attraction behavior. Tierney et al. also measured time spent in the inflow zone upon the introduction of the amino acid L-alanine, a food odorant, after a 96 h exposure to the herbicide mixture. Zebrafish exposed to all concentrations of the mixture spent more time in the inflow zone during the second and third minutes post introduction than controls, and those exposed to the medium concentration continued this behavior into the fourth minute post introduction. Teather et al. measured the distance travelled in two min by Japanese medaka (*Oryzias latipes*) fry at 3 weeks post-hatching after an exposure to the pesticides azinphos-methyl, chlorothalonil, and endosulfan as well as a mixture of all three beginning at fertilization and ending seven days post-hatch [116]. Fry exposed to the mixture containing a total concentration of 0.18 μg/L were shown to have decreased distance travelled compared to all other exposure groups and controls.

Fish olfaction as a biosensor

To use fish olfaction as a biosensor both detection via OSNs and changes in behavior associated to contaminants were integrated, allowing us to identify concentrations of contaminants within waters which may be harmful to fishes. This has been reviewed by Tierney and two methods for this application were suggested: concentration responses to contaminants correlated to avoidance/ attraction responses, and reductions in EOG to natural odorants correlated to loss of behavioral responses to these same odorants[118]. Tierney et al. demonstrated that studies investigating the concentration responses to contaminants and avoidance/ attraction responses have a general trend suggesting that contaminants are detected at lower concentrations than those evoking avoidance behaviors. Correlations between loss of odorant detection via OSNs and behavioral responses to these same odorants are less predictable. Small decreases in OSN detection of odorants may still result in loss of behavioral responses to these odorants. One example of this is a study conducted by Tierney et al. where exposures to pesticides resulted in decreases in behavioral preference to the amino acid L-histidine at concentrations lower than those that decreased EOG responses to the same odorant [123]. The use of such tests with both individual contaminants and contaminant mixtures could help identify specific contaminants and their concentrations within mixtures which elicit negative effects in both olfactory and behavioral endpoints. This may help with the development of other technologies, such as those involved in treatment of contaminated water, by

demonstrating the contaminants and their concentrations that are of potential concern to fishes.

Thesis objectives

My thesis focused on four main objectives: 1) determining EOG concentration response relationships for odorants or organic contaminant mixtures ; 2) determining the effects of long and short term exposures to organic contaminant mixtures on EOGs; 3) determining the behavioral effects of introduction and exposure of contaminant mixtures on olfactory mediated behaviors; and 4) characterizing a novel class of odorants. Objective l was accomplished by recording EOGs to the odorants discussed above (L-alanine, L-serine, PFG2α and 17,20-βP), and treated reuse in goldfish and odorants discussed above (TChA and L-serine), and OSPW in rainbow trout. Objective 2 was accomplished by recording EOGs to odorants during 30 min and 60 d exposures to treated reuse water in goldfish and 30 min and 7 d exposures to OSPW in rainbow trout. Objective 3 was accomplished by tracking goldfish movement to determine changes in distance travelled during two scenarios: 1) after the introduction of a contaminant mixture pulse; 2) after exposure to a contaminant mixture and upon the introduction of the food odorant L-alanine. Objective 4 was accomplished by recording EOG concentration response curves to adenosine, adenine, hypoxanthine, and guanosine, and also performing a cross adaptation study using these compounds. Overall this work aimed at investigating the effects of contaminant mixtures on olfaction and olfactory mediated behavior, while also providing evidence of a novel class of odorants.

Tables

Species	Threshold concentration (M)	Source
Goldfish	10^{-9}	Sorensen et al. 1988
Atlantic salmon	10^{-11}	Moore. 1996
Salmo trutta (brown trout) Whitefish	10^{-10} 10^{-8}	Laberge and Hara. 2003
Salvelinus alpinus (Arctic char)	10^{-11}	Sveinsson and Hara. 2000
Goldfish *Cyprinus carpio*(common carp) *Zacco temmincki* (dark chub) *Zacco platypus (*pale chub*)* *Misgurnus anguillicaudarus (*loach*)* *Pleccoglossus altivelis (*ayu*)*	10^{-7*}	Shoji et al. 1994

Table1.1. Threshold concentrations for the detection of PGF2α. * only one concentration was tested.

Chapter 2: Determining sub-lethal toxicity of treated reuse water as measured by olfactory impairment to natural odorants in goldfish (*Carassius auratus*)

Introduction

The growing global population has increased the demand on the limited potable water supply [4, 68]. In an attempt to conserve potable water resources, the use of treated municipal effluent, or final effluent wastewater (FE wastewater) for industrial, agricultural and urban development / landscaping practices is increasingly viewed as an acceptable alternative [3]. In regions where potable water is scarce, use of FE wastewater for groundwater recharge as well as for potable uses has been approved [3, 68]. The use of FE wastewater for such practices raises concern as this water contains three forms of contaminants: microbes and pathogens, synthetic organic compounds, and heavy metals. The release of FE wastewater to the environment poses a risk for species whose habitat may become contaminated, such as fishes. This study will focus on the effects of synthetic organic compounds on goldfish olfaction, and as such will only cover background related to synthetic organic contaminants.

Gold Bar Waste Water Treatment Plant

The Gold Bar WWTP was the source of treated reuse water. This WWTP is responsible for treating 310 mega liters of municipal effluent daily from the city

of Edmonton [26]. Water enters the treatment facility and begins pretreatment in aerated grit tanks used to remove large inorganic solids. It then enters clarifying ponds for primary treatment where heavy organic solids sink to the bottom and light organic solids are skimmed off of the top. During this phase liquid effluent and solid waste are separated and treated individually. The effluent, now referred to as primary effluent, moves on to large bioreactors for secondary treatment. Preexisting microorganisms within the effluent multiply and use dissolved organic matter, including contaminants, as a source of nutrients. The primary effluent then flows into a second set of bioreactors which involve modifications allowing microorganisms to remove excess phosphorus and ammonia. The effluent is now referred to as secondary effluent and enters ponds for final clarification. These ponds use rakes to remove bacterial mats known as floc that remain after the bioreactors. If the treated effluent is to be returned to the North Saskatchewan, it is disinfected using a high intensity ultra-violet light. Of the water that goes through the Gold Bar WWTP, 5% is diverted from UV disinfection and passes through a membrane filtration process referred to as effluent polishing. The membranes are composed of highly porous synthetic strands that are able to remove bacteria and other microorganisms. This 5% of diverted effluent is high grade process water, or reuse water (MF reuse), that is intended for industrial applications (e.g. cooling and steam production at oil sand processing plants) throughout Alberta.

The use of MF reuse for such applications comes with concerns regarding its toxicity (defined as the ability of a substance to harm an organism or tissues

within it [28]), should it be accidentally released. Use of MF reuse in agricultural and urban landscaping practices would lead to an immediate release into the surrounding environment and increases concentrations of pharmaceutical and personal care products (PPCPs) and endocrine disrupting compounds (EDCs) within receiving waters. The use of MF reuse in industrial applications may also result in release to the environment should complications arise from transportation of the water or should it be used in steam processes.

Wastewater toxicity

Studies examining toxicity of wastewater to date have focused on EDCs. These compounds impart their effects in one of five ways: binding to hormone receptors and changing protein synthesis or mitosis; interfere with binding of hormones with membrane receptors; altering steroidogenesis; interfering with the synthesis of hormones; and alteration of ion flux across a membrane. EDC classes include estrogens, antiestrogens, antiprogestins, andtiandrogens and heavy metals. Studies with multiple fish and amphibian species have shown that exposure to EDCs at concentrations found in wastewater or to wastewater containing EDCs was linked to increased intersex individuals, decreased number of males, and retarded development in juveniles [30, 67, 91, 112, 125, 133].

While EDCs are the current front runner as the cause of toxicity associated with wastewater, many other compounds exist within this chemical mixture. Wastewater contains a suite of chemical contaminants including analgesics, pesticides, stimulants, anticonvulsants, antipsychotics, anti-lipidemics, and more.

A study conducted by Kerr et al. aimed to examine the estrogenic effects within water from the Gold Bar WWTP [60]. Screening the effluent for 60 different organic contaminants, Kerr et al. identified nine different PPCPs and eight pesticides, with the pesticides occurring on a seasonal basis during the summer and winter. Kerr et al. only examined the MF reuse for compounds that were potential endocrine disruptors; however a more recent analysis, which used methods to determine acid extractable herbicides and PPCPs, showed MF reuse contains at minimum 75 different organic contaminants all at concentrations of ng/L (Table 2.1).

A major problem associated with studying the toxicity of wastewater is its highly diverse chemical composition from one geographic location to the next. While some compounds such as caffeine and acetominophine are present in the majority of wastewater effluents, even these commonly occurring compounds are present in variable concentrations from one location to the next [1, 11, 29, 60, 133]. Little is understood regarding the synergistic effects of compounds found within wastewater and so studies must be conducted that examine not only whole effluent toxicity, but also various combinations of commonly occurring contaminants at concentration ranges at which they are found.

Ultraviolet light and hydrogen peroxide treatment of reuse water

Ultraviolet light and hydrogen peroxide treatment (UV/H_2O_2/MF reuse) is one of the treatments investigated in study. The filtration process using UV/H_2O_2 is an example of an advanced oxidation process, a new class of treatment methods

currently being investigated for the treatment of wastewaters containing organic contaminants. The process begins with UV irradiation causing the photolysis of H_2O_2 molecules, creating two hydroxyl radicals (OH-). Hydroxyl radicals are highly reactive compounds that when reacted with organic compounds are able to oxidize compounds by two methods: 1) removal of hydrogen atoms or 2) by binding to structures at points where double bonds exist, removing the need for the double bond [57]. Aside from oxidation via hydroxyl radicals, the process also has the ability to breakdown organic contaminants using photolysis via UV irradiation alone.

UV/H_2O_2 treatment decreases concentrations of organic contaminants such as atrazine, humic acid, and the poly aromatic hydrocarbons flurorene, phenanthrene, and acenaphthene [8-10, 130]. Factors that impact the effectiveness of the UV/H_2O_2 treatment include the concentration of H_2O_2, pH, and bicarbonate concentrations [10]. All of these factors impact the availability of hydroxyl radicals, which directly relates to the rates of oxidation of organic contaminants. It should be noted that UV/H_2O_2 treatment simply changes chemical structures of compounds and does not eliminate all forms of organic compound from the matrix [10, 57]. Beltran et al. used gas chromatography mass spectrometry to identify 33 byproducts occurring as a result of UV/H_2O_2 treatment of water containing the poly-aromatic hydrocarbons fluorine, phenanthrene, and acenaphthene. As wastewater effluent contains many different organic contaminants occurring at concentrations in the ng/L range, it is likely that the number of byproducts occurring from UV/H_2O_2 treatment will be

numerous, however due to low concentrations of reactants, the concentration of products should be at or below ng/L concentrations, depending on the contaminant. Typically such concentration ranges are below those associated with toxicity [6, 56, 101, 118].

Fish olfaction as a measurement of MF reuse and toxicity

As processes that involve the use of MF reuse may result in its release into the environment, a result of the process itself or spillage during shipping, it is important that we understand the impacts on species , especially those with habitats that may be compromised. It is also important to determine if the treatment processes that have been applied to MF reuse are sufficient, or if further treatment is required to lower its toxicity. The use of fish olfaction to study contaminants at and below the μg/L has been well established [22, 36, 49, 101, 119, 121, 123]. Behaviors associated with fish olfaction such as freezing responses post-introduction of the odorants L-alanine and L-serine, have also been studies in both ecological and laboratory settings and have proved valuable in determining sub-lethal toxicity concentrations for multiple forms of contaminants [46, 77, 87, 134].

Aims/ Hypothesis

This study aimed to use fish olfaction as a measure of sub-lethal toxicity of MF reuse and two forms of further treated MF reuse. The first form of treatment used granular activated carbon, which was previously shown to remove many organic contaminants, and will be identified as CF/MF reuse. The second treatment used

the UV/H_2O_2 process previously described, and will be referred to as UV/H_2O_2/MF reuse. This study also aimed to identify potential olfactory toxicity of a novel contaminant mixture (NCM) containing components commonly found in WWTP effluents. The aims of this study were to answer six questions:

1. Could goldfish detect MF reuse, CF/MF reuse, UV/H_2O_2/MF reuse, and an NCM in a concentration dependent manner?

2. Did a 30 min exposure to MF reuse, CF/MF reuse, UV/H2O2/MF reuse, or an NCM cause a decrease in EOGs evoked by natural odorants?

3. Did a 60 d exposure to MF reuse, CF/MF reuse, or UV/H_2O_2/MF reuse cause a decrease in EOGs evoked by natural odorants?

4. Did the introduction of a pulse of MF reuse, CF/MF reuse, UV/H_2O_2/MF reuse or an NCM result in a change in goldfish behavior?

5. Did a 30 min exposure to MF reuse, CF/MF reuse, UV/H_2O_2/MF reuse, or an NCM result in a change in goldfish behavior post-introduction of a natural odorant?

6. Did a 60 d exposure to MF reuse, CF/MF reuse, or UV/H2O2/MF reuse result in a change in goldfish behavior post-introduction of a natural odorant?

I hypothesized that EOGs evoked from goldfish using increasing concentrations of MF reuse, CF/MF reuse, UV/H2O2/MF reuse, and an NCM would occur in a

concentration dependent manner. This was hypothesized as compounds within MF reuse and its treatments have been shown to resemble polycyclic organic structures, such as steroids, that have been associated with fish olfaction in the past [63, 109]. I hypothesized that goldfish exposed to all forms of reuse water or an NCM would experience altered EOG responses, regardless of the odorant, during a 30 min exposure [6, 101, 119]. Exposures of 60 d to MF reuse and its treatments were hypothesized to alter EOGs evoked by all odorants during the first 10 d of exposure, however EOGs evoked by all odorants were expected to return at 11 d into the exposure and any time point past this. This was hypothesized as 7 d exposures to copper have been shown to affect EOGs to natural odorants less so than 30 min exposures [6, 101].

Behavior as measured by distance travelled was expected to change as a result of the introduction of one of MF reuse, CF/MF reuse, UV/H2O2/MF reuse, and an NCM. This was expected as the polycyclic compounds within MF reuse and its treatments may resemble compounds associated with mating behavior, and these may cause increased movement [20, 110]. As it was expected that olfaction would be impaired by a 30 min exposure to MF reuse, CF/MF reuse, UV/H2O2/MF reuse, or an NCM, it was logical to expect that distance traveled post-introduction of L-alanine would not decrease due to a lack of ability to detect the odorant. This same logic was applied to distance travelled by goldfish exposed to MF reuse, CF/MF reuse, or UV/H2O2/MF reuse for 60 d when behavior experiments were conducted. No decreases in distance travelled post-introduction were expected to occur post introduction of the odorant during the first 10 d of exposure, however

as olfaction returned over time so would behavioral responses associated with it, i.e. decreases in distance travelled as a response to L-alanine introduction. The hypotheses were based on studies that have previously investigated sources of organic contamination and its effects on olfaction, which show that above μg/l concentrations, organic contaminants have the ability to decrease EOGs evoked in fish species [21, 54, 82, 118-121]. While most contaminants within MF reuse occur in the ng/L range, it is hypothesized that the contaminants would act either additively or synergistically to impair olfaction. The rationale here is that fish OSNs appear to be sensitive to all synthetic and natural compounds introduced to water [118].

Table 2.1

Contaminant		Concentration in MF reuse (ng/L) (n=1)		Concentration in Aquatics Facility water (ng/L) (n=1)
1,7-Dimethylxanthine	ND	120	ND	129
13C12-Triclosan (% Recovery)		109		107
13C2-15N-Acetaminophen (% Recovery)		141		134
13C2-Erythromycin-H2O (% Recovery)		82.6		80.4
13C3-Caffeine (% Recovery)		129		125
13C3-Ibuprofen (% Recovery)		95.7		83.8
13C3-N15-Ciprofloxacin (% Recovery)		36.3		60
13C3-Trimethoprim (% Recovery)		60.8		81.3
13C6-Sulfamethazine (% Recovery)		77		99.9
13C6-Sulfamethoxazole (% Recovery)		92.9		89
13C6-Triclocarban (% Recovery)		67.5		73.3
13C-D3-Naproxen (% Recovery)		78.2		85.4
2-Hydroxy-ibuprofen	NDR	192	ND	172
Acetaminophen	ND	30	ND	32.3
Azithromycin		13.5	ND	3.23
Bisphenol A	ND	999	ND	1080
Caffeine	ND	30		101
Carbadox	ND	10.6	ND	3.23
Carbamazepine		511		30.4
Cefotaxime	ND	93.4	ND	29.9
Ciprofloxacin		156	ND	12.9
Clarithromycin		434	ND	3.23
Clinafloxacin	ND	119	ND	39.8
Cloxacillin	ND H	6	ND H	6.46
d10-Carbamazepine (% Recovery)		142		128
D11-Glipizide (% Recovery)		79.1		72.1
D3-Glyburide (% Recovery)		72.9		64
D5-Fluoxetine (% Recovery)		69.1		79.7
D5-Warfarin (% Recovery)		55.6		91.6
D6-Bisphenol A (% Recovery)		49.2		48.6

D6-Gemfibrozil (% Recovery)		101		107
D6-Thiabendazole (% Recovery)		61.6		76.6
Dehydronifedipine		44	ND	1.29
Digoxigenin	ND	581	ND	207
Digoxin	ND	12	ND	12.9
Diltiazem		34.2		0.658
Diphenhydramine		38.7	ND	1.29
Enrofloxacin	ND	6	ND	6.46
Erythromycin-H2O		97.1	ND	4.95
Flumequine	ND	3.29	ND	3.23
Fluoxetine	ND	3	ND	3.23
Furosemide	NDR	140	ND	86.1
Gemfibrozil		57.3	ND	3.23
Glipizide	ND	12	ND	12.9
Glyburide		5.1	ND	4.52
Hydrochlorothiazide		107	ND	21.5
Ibuprofen	ND	30	ND	32.3
Lincomycin	ND	6	ND	6.46
Lomefloxacin	ND	40.5	ND	9.29
Miconazole	ND	3	ND	3.23
Naproxen		26.2	ND	6.46
Norfloxacin	ND	82.1	ND	33.8
Norgestimate		149		77.8
Ofloxacin		39.3	ND	3.78
Ormetoprim	ND	1.2	ND	1.29
Oxacillin	ND H	6	ND H	6.46
Oxolinic Acid	ND	2.99	ND	1.52
Penicillin G	ND H	20	ND H	21.5
Penicillin V	ND	6	ND	6.46
Roxithromycin		1.24	ND	0.646
Sarafloxacin	ND	33.6	ND	32.3
Sulfachloropyridazine	ND	5.73	ND	3.23
Sulfadiazine	ND	3	ND	3.23
Sulfadimethoxine		4.01	ND	1.15
Sulfamerazine	ND	2.23	ND	1.29
Sulfamethazine	ND	4.15	ND	1.62
Sulfamethizole	ND	1.2	ND	1.29
Sulfamethoxazole		195	ND	1.29
Sulfanilamide	ND	30	ND	32.3
Sulfathiazole	ND	3.28	ND	3.23
Thiabendazole		19.2	ND	3.23

Triclocarban	ND	6	ND	6.46
Triclosan	ND	120	ND	129
Trimethoprim		68.2	ND	3.23
Tylosin	ND	12	ND	12.9
Virginiamycin	ND	52.7	ND	10.1
Warfarin	ND	3	ND	3.23

Table 2.1. Concentration of contaminants found within MF reuse. A sample of MF reuse and aquatics facility water was obtained on July 18th 2012 and sent to AXYS analytical services. The table shows concentrations of contaminants found in each sample using two methods: an acid extractable herbicide method and a pharmaceutical and personal care method. ND indicates concentrations detected below the reportable level while H indicates estimated concentrations.

Materials and Methods

Fish

Goldfish were obtained from Mt. Parnell Fisheries (Mercersburg, PA) and held at 18°C in flow-through aquaria at the University of Alberta Aquatics Facility.

Chemicals

Chemicals used in the construction of electrodes (NaCl, KCl, and gelatin), as well as sodium bicarbonate (Na_2CO_3), were obtained from Fischer Scientific (Toronto, ON). Chemicals used to construct the NCM (gemfibrozil, mecoprop, carbamazepine, diclofenac and caffeine) were all obtained from Sigma (Mississauga, ON). The anesthetic, tricaine methanosulfate, was obtained from Syndell (Vancouver, BC).

MF reuse

MF reuse water was obtained from Gold Bar WWTP, Edmonton Alberta, Canada. MF reuse water was created by passing final effluent through hollow fiver membrane ultrafilration using a Zenon ZeeWeed 500 with a pore size of 0.04 μm.

CF/MF reuse

MF reuse water obtained from Gold Bar WWTP was passed through granular activated carbon (GAC), particle size 0.1-0.3 mm, at a flow rate of 1.0 L/min. This flow rate allowed for a retention time >15 min, allowing wastewater to interact with the GAC. The filter unit containing the GAC was constructed from

polyvinyl chloride (PVC) piping and had a length of 1.22 m and an outer diameter of 15.0 cm. The GAC filter was capped at both ends by glass wool followed by PVC diffusers.

UV/H_2O_2/MF reuse

MF reuse water obtained from the Gold Bar WWTP was passed through an electronic dosing apparatus that dosed the water with 20 mg/L H2O2. The MF reuse then passed through a UV filter dosing at 1000 mJ/cm^2. The MF reuse flow rate through both the dosing apparatus and UV filter at a flow rate of 5.6 L/min [60].

NCM

A secondary approach to examining the effects of chemical classes within reuse water involved examining the effects of an NCM. Contaminants that occurred at the highest concentration for each class during the study conducted by Kerr et al. were selected as representative contaminants [60] which resulted in the following mixture: the herbicide MCPP (Mecoprop®); the antiepileptic agent carbamazepine; the hypolipodemic agent gemfibrozil; the non-steroidal anti-inflammatory (NSAID) drug diclofenac; and the stimulant caffeine [60]. The concentration of each contaminant reflected the total concentration of all members within its class and was as follows: mecoprop= 29.7 µg/L; carbamazepine= 103.6 µg/L; gemfibrozil= 11.5 ; diclofenac= 1.61 µg/L; caffeine= 0.095 µg/L.

Solvent Control

To control for effects that may be attributed to methanol used to dissolve both carbamazepine and gemfibrozil, a solvent control containing 11.6 μL/L of methanol was created and used during 30 min EOG and behavior experiments.

Electro-olfactogram experiments

Electro-olfactograms

Differential EOG responses were recorded using electrodes made of Ag/Ag-Cl inside of a borosilicate glass capillary tube embedded in 2% Agar/ 1M NaCl plug [27]. Capillary tubes were pulled using an electrode puller to achieve a tip diameter of 25μm, then trimmed using forceps to achieve a diameter of 100μm, and flame polished before being filled. All signals were AC coupled, with filtering occurring between 0.1-1000 Hz. Signals were amplified 1000×. All recording was monitored and acquired using a PowerLab 4/25 and Chart5 software (ADInstruments, Colorado Springs). Goldfish were anaesthetized by immersion in 200 mg/L tricaine solution, and anesthesia was maintained by placing goldfish in a water bath containing water at the same temperature as their tank and flowing a 100 mg/L tricaine solution over the gills. The covering of the right nares was surgically removed and a recording electrode was placed on the third lamellae from the right of the olfactory rosette; a reference electrode of the same composition was placed on the body of the goldfish or within the water bath. Olfactory epithelium was perfused with background water for 10 min and odorants and contaminants were delivered in 2 s pulses. If goldfish did not have EOGs evoked by at least 2 odorants or if EOGs evoked were less than 0.5 mV in

magnitude, the electrode was repositioned. A maximum of 3 repositions was allowed per fish, and if conditions were not met on the third reposition, the fish was not used. Odorant pulses were given every 2 min for concentration response curves, and for 30 min exposures, and every 5 min for goldfish tested during the 60 d exposure (Appendix E). Fluid delivery was maintained at 6 mL/min for both background water and odorants. To quantify odorant responses, EOG (olfactory neuron response) peak responses were taken as the maximum change compared to the pre-pulse baseline.

Concentration dependent responses

Increasing concentrations of the odorants L-alanine, L-serine, PGF2α, and 17,20-βP or contaminant mixtures, of MF reuse, CF/MF reuse, UV/H2O2/MF reuse or a NCM were delivered to the olfactory epithelium in 2 s pulses, with 2 min being left between each pulse (Appendix E). This time period is known to allow the generator potentials of the OSNs to return to baseline [7]. EOG responses were determined as the mean of three evoked responses.

30 min exposures

After the 10 min acclimation period, EOG responses to 2 s pulses of L-serine, L-alanine, and 17,20-βP, were evoked every 2 min (Appendix E). After 30 min background water was switched to one of MF reuse, CF/MF reuse, UV/H2O2/MF reuse or a NCM, and EOG responses were measured as previously stated. After

30 min of exposure, background water was returned and EOGs were again measured for 30 min post-exposure.

60 d exposures

Goldfish were housed at the Gold Bar WWTP in the same manner described by Kerr et al.[60]. This exposure period was chosen at random by collaborating researchers. Briefly, goldfish were acclimated for 7 d to dechlorinated municipal water before exposures to MF reuse, CF/MF reuse, and UV/H2O2/MF reuse. Goldfish were collected throughout the 60 d exposure and returned to the University of Alberta where EOG recordings were taken. Background water used during EOGs matched that used in the exposure for each goldfish. EOGs responses were determined as the mean of three evoked responses. During the first exposure (fall 2011), EOGs were evoked using L-alanine, PGF2α, and 17,20-βP. During the second exposure (spring 2012), EOGs were evoked using L-alanine and 17,20-βP. During the third exposure (summer 2012), EOGs were evoked using L-alanine, L-serine, and 17,20-βP. Odorants were changed between exposure periods due to the inability of PGF2α to evoke EOGs in control after 30 d during the 1st exposure.

Behavioral Experiments

Introduction of a contaminant

These experiments were conducted using methods from Tierney et al. 2011[122]. Briefly, 2 L beakers were filled with dechlorinated municipal tap water and

goldfish were placed inside the beaker and left to acclimate for 30 min. An inflow line that was secured to the wall of the beaker delivered a 20 mL injection of one of MF reuse, CF/MF reuse, UV/H2O2/MF reuse, novel odorant mixture, dechlorinated municipal water control or solvent (ethanol) control. Goldfish behavior was recorded using video surveillance positioned above the arena over the 30 min acclimation and 10 min post injection. Behavior was analyzed as distance travelled and this was determined using EthoVision XT (Noldus, Netherlands).

30 min and 60 d exposures

Experiments were conducted as stated above, using methods from Tierney et al. 2011 [122]. Briefly, 2 L beakers were filled with one of MF reuse, CF/MF reuse, UV/H2O2/MF reuse, a NCM, a decholrinated municipal water control or solvent (ethanol) control and goldfish were placed inside the beaker and left to acclimate for 30 min. The inflow line delivered a 20 mL injection of 10^{-3} M L-alanine, resulting in a final concentration within the beaker of 10^{-5}M. Goldfish behavior was recorded using video surveillance positioned above the arena over the 30 min acclimation and 10 min post injection. Behavior was analyzed as distance travelled and this was determined using EthoVision XT (Noldus, Netherlands).

Statistical Analysis

Differences in EOG responses evoked by increasing concentrations of odorants and contaminant mixtures were determined using one-way repeated measures

(RM) ANOVA with Holm-Sidak post hoc tests. Differences between : different contaminant mixtures at the same concentration; EOG responses evoked throughout the 30 min and 60 d exposures; and distance travelled pre- and post-exposure to a contaminant mixture or L-alanine, were determined using two-way RM ANOVA with Holm-Sidak post hoc tests. Differences in distance travelled between exposure groups during a 60 d exposure to treated reuse water were determined using a three-war ANOVA with Holm-Sidak post hoc tests. All statistical analysis was conducted using Sigma Plot 11.0 (Systat, Chicago, Illinois).

Results

EOG concentration response curves to odorants

L-alanine and L-serine evoked EOGs in a concentration-dependent manner (L-alanine mean values, in mV= 10^{-11} mean = 1.88 ± 1.36; 10^{-9} mean = 2.95 ± 1.64; 10^{-7} mean = 3.52 ± 2.26; $F_2 = 6.01$, p=0.01;L-serine: 10^{-12} mean = 0.56 ± 0.21; 10^{-10} 0.61 ± 0.23; 10^{-8} mean = 0.65 ± 0.28; 10^{-6} mean = 0.86 ± 0.42; 10^{-5} mean = 1.66 ± 0.51; $F_4 = 15.0$, $p < 0.01$) (Figure 2.1A, Figure 2.2A,B,C). L-alanine-evoked EOGs by concentrations of 10^{-7} and 10^{-9} M were increased by 87 and 57% respectively compared to those evoked by 10^{-11} M (p=0.02; p=0.04, respectively). L-serine evoked EOGs of 10^{-5}, 10^{-6}, 10^{-8}, and 10^{-10} M were increased by 200, 54, 16, and 8.9% respectively compared to those evoked at 10^{-12} M (p<0.01; p<0.01; p<0.01; p<0.01, respectively). However, L-alanine appears to be a more potent odorant than L-serine in goldfish, as L-serine EOGs were only statistically

elevated at 10^{-5} M while those evoked by L-alanine were statistically elevated at 10^{-9} M.

The pheromone 17,20-βP evoked EOGs in a concentration dependent manner (17,20-βP: 3×10^{-12} mean, in mV = 1.90 ± 1.54; 3×10^{-10} mean = 1.84 ± 1.57; $3x10^{-8}$ mean = 4.55 ± 1.30; F_2= 96.9, $p<0.01$) (Figure 2.1B, Figure 2.2D,E,F). The 17,20-βP evoked EOGs of $3x10^{-8}$ and 3×10^{-10} M were increased by 140 and 150%, respectively, compared to those evoked by 3×10^{-12} M ($p<0.01$; $p<0.01$, respectively). The pheromone PGF2α appears to have evoked EOGs in a concentration dependent manner numerically, however the changes between concentrations were not found to be statistically different from each other (PGF2α: 3×10^{-13}mean in mV= 1.88 ± 0.88; 3×10^{-11}mean = 2.58 ± 2.09; 3×10^{-9} mean = 3.04 ± 1.05; one-way RM ANOVA: $F_{2,13}$ = 0.95, p = 0.43) (Figure 2.1B, Figure 2.2 G,H,I). The PGF2α evoked EOGs at 3×10^{-11} M were numerically greater than those evoked by 17,20-βP at 3×10^{-10} M, suggesting that PGF2α is a more potent odorant than 17,20-βP.

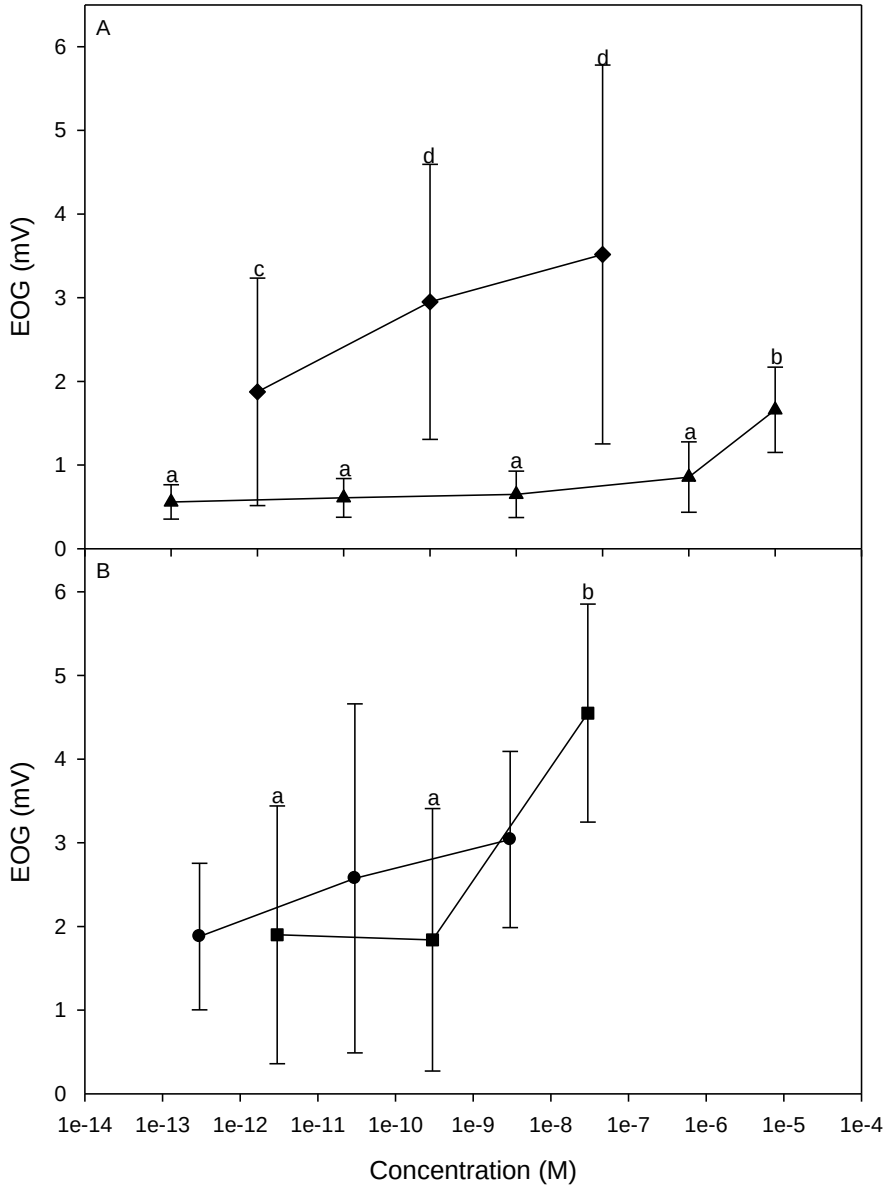

Figure 2.1. Concentration dependency for A) the amino acids ▲ L-serine (n =7) and ◆ L-alanine (n =13) and B) the pheromones ■ 17,20-βP (n =5) and ● PGF2α (n = 5). These graphs display EOGs evoked by increasing concentrations of odorants presented to goldfish.

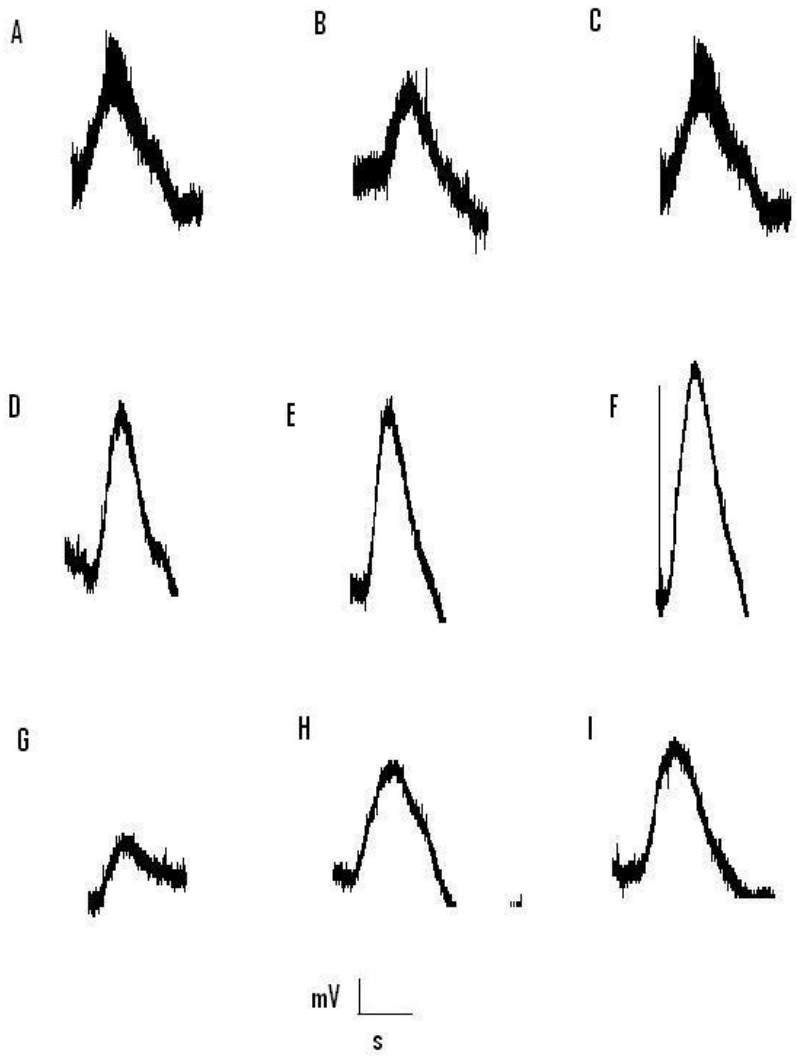

Figure 2.2. Sample traces for concentration dependent responses. A, B, C are EOG traces evoked by l-alanine at concentrations of 10^{-12}, 10^{-10}, and 10^{-8} M, respectively. D, E, F are EOG traces evoked 17,20-βP at concentrations of 3 x 10^{-11}, $3x10^{-9}$, and $3x10^{-7}$ M respectively. G, H, I are EOG traces evoked by PGF2α at concentrations of 3 x 10^{-12}, $3x10^{-10}$, and $3x10^{-8}$ M respectively. The legend indicates measurement of time and peak amplitude.*EOG concentration response curves to contaminant mixtures*

The EOGs evoked by increasing concentrations of MF, CF/MF, and UV/H_2O_2/MF reuse occurred in a concentration-dependent manner while those evoked by a NCM did not (MF reuse: 100% mean, in mV = 7.46 ± 3.11; 50% mean = 6.02 ± 2.07; 10% mean = 2.85 ± 0.73; 1% mean = 2.32 ± 0.59; 0.1% mean = 1.18 ±0.46; CF/MF reuse: 100% mean = 4.96 ± 1.96; 50% mean = 4.55 ± 1.80; 10% mean = 2.26 ± 1.14; 1% mean = 2.07 ± 0.92; 0.1% mean = 1.54 ± 0.85; UV/H_2O_2/UV reuse: 100% mean = 5.81 ± 2.98; 50% mean = 3.75 ± 2.38;10% mean = 1.47 ± 0.52; 1% mean = 1.05 ± 0.71; 0.1% mean = 0.86 ± 0.53; $F_{4,139}$ = 63.7, $p < 0.01$; $F_{4,139} = 63.7$, $p < 0.01$; Figure 2.3). However, if NCM is analyzed on its own, concentration-dependence is seen ($F_4 = 7.26$, $p < 0.01$) (Figure 2.4). The reason for the lack of significance is likely that the NCM EOGs were much lower. EOG responses were lowest at a 0.001% dilution of all forms of treated reuse, and greatest at 50 and 100% dilutions. In general, EOG magnitudes can be ordered MF reuse > CF/MF or UV/H_2O_2/MF reuse> NCM (100% dilutions means, in mV: 7.46 ± 3.11; 4.96 ± 1.96; 5.81 ± 2.98; 1.11 ± 0.26 mV, respectively; $F_{3,139} = 9.68$, $p < 0.01$; Figure 2.3).

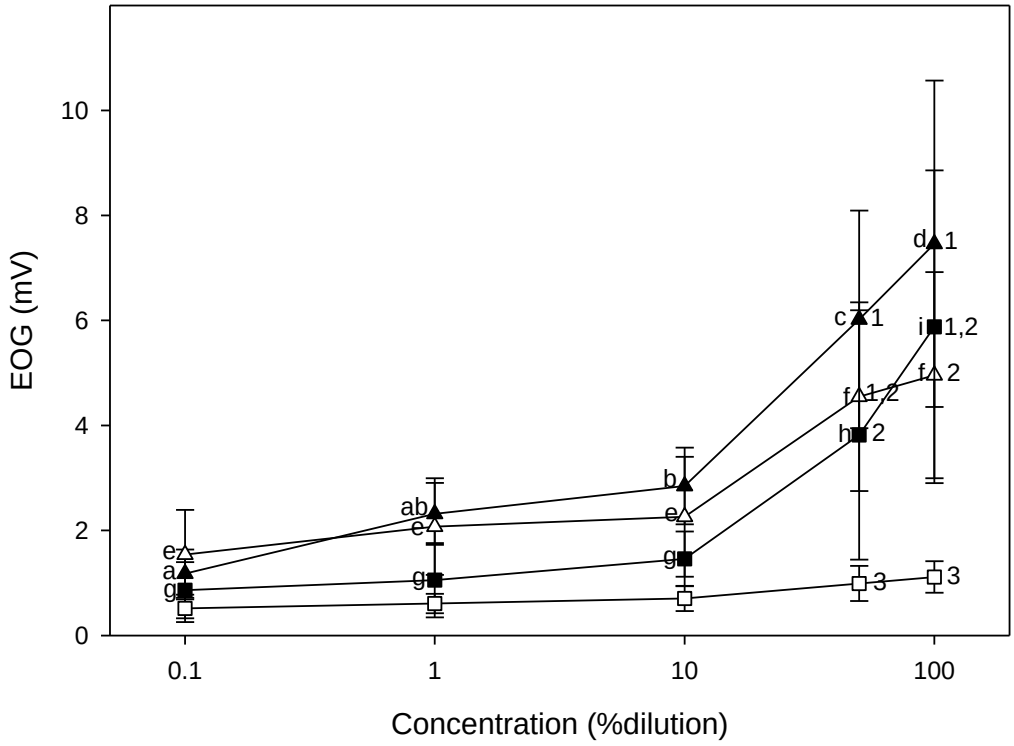

Figure 2.3. Concentration response curves to ▲MF reuse (n = 7), △CF/MF reuse (n = 8), ■UV/H2O2/MF reuse (n = 8) and a □NCM (n = 6). This graph displays EOGs evoked by increasing concentrations of treated reuse and NCM presented to goldfish.

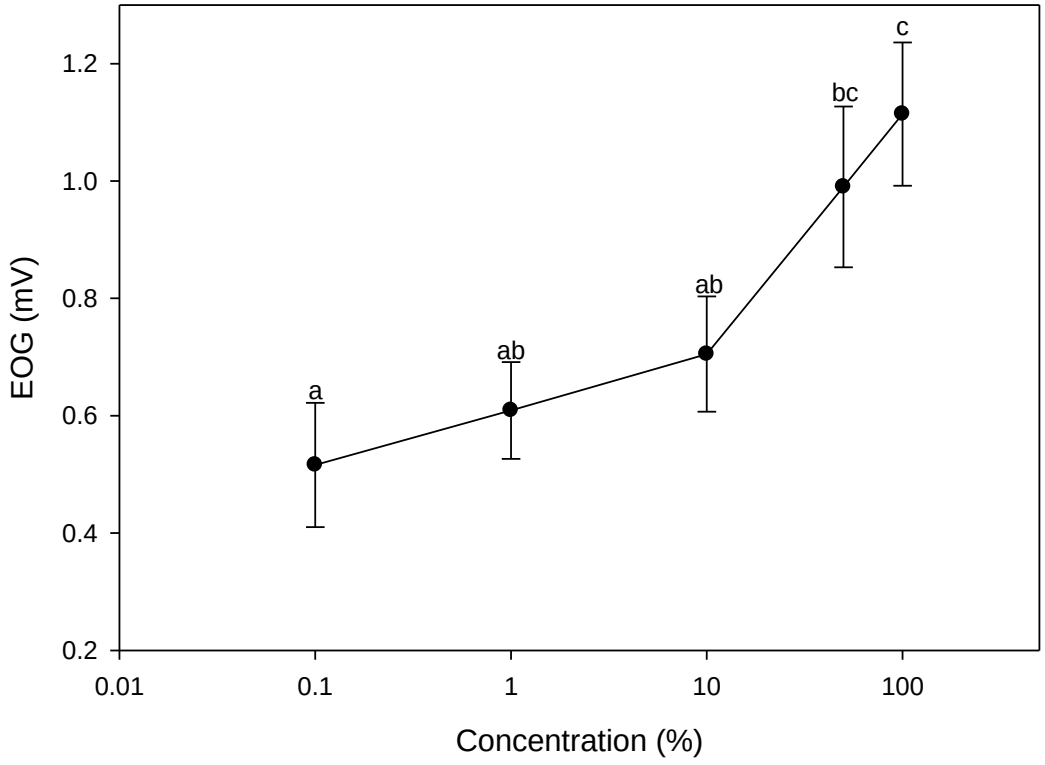

Figure 2.4. Concentration response curve to a NCM (n = 6). This graph displays EOGs evoked by increasing concentrations of an NCM presented to goldfish.

EOG responses to L-alanine during a 30 minute exposure to treated reuse water

L-alanine-evoked EOGs were compared to both pre-exposure and control EOGs throughout a 30 min exposure to MF, CF/MF, UV/H_2O_2/MF reuse, NCM (0.1 and 100%) and a solvent control. Pre-exposure EOGs did not differ between exposure groups (F_6= 1.41, p=0.23;Figure 2.5). The EOGs evoked from MF reuse exposed goldfish were decreased on average by 72% compared to pre-exposure throughout the 30 min exposure ($F_{9,425}$ = 13.7, p < 0.01; Table 2.2). EOGs evoked by CF/MF reuse and NCM (0.1 and 100%) exposed goldfish were numerically decreased by an average of 33 and 46 respectively. throughout the exposure compared to pre-exposure; however they did not differ statistically. UV/H_2O_2/MF reuse and solvent control exposed goldfish had EOGs that were numerically increased by an average of 22 and 63%, respectively, when compared to pre-exposure throughout the 30 min exposure. EOGs evoked by 0.1% NCM exposed goldfish increased on average by 47% from pre-exposure. All other exposure groups returned to pre-exposure EOG values. Exposure groups did not differ compared to controls on average throughout the exposure ($F_{6,425}$ = 2.33, p = 0.05). EOGs evoked by MF reuse exposed goldfish at 20 and 26 min into the exposure were decreased by 82 and 83%, respectively, compared to controls ($F_{54,425}$ = 1.79, p < 0.01; p = 0.04, 0.01, respectively). No other exposure groups differed from controls at specific time points during the exposure (Table 2.2). In general, MF reuse-exposed

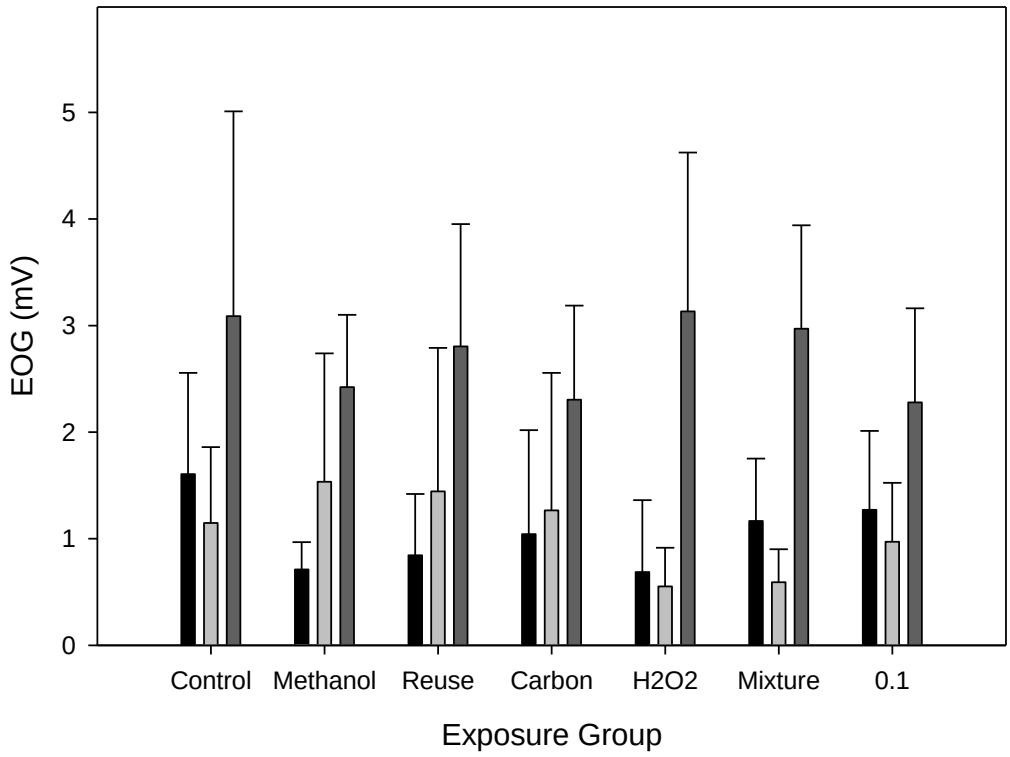

Figure 2.5 Pre-exposure EOG responses before a 30 min exposure to treated reuse water or a NCM. No difference was found in pre-exposure EOGs evoked by ■ L-alanine, ■ L-serine and ■ 17,20-□P.

Table 2.2

Odorant	Exposure group	Time	Comparison with pre-exposure (p-value)	Comparison with control (p-value)
L-alanine	MF reuse	2	0.02	0.19
		8	0.02	0.26
		14	0.01	0.14
		20	0.01	0.05
		26	0.01	0.01
		32	0.68	0.96
		38	0.72	0.96
		44	0.55	0.89
		50	0.59	0.56
	CF/MF Reuse			
		2	0.62	0.57
		8	0.67	0.66
		14	0.67	0.58
		20	0.68	0.43
		26	0.57	0.46
		32	0.80	0.98
		38	0.69	0.99
		44	0.71	0.81
		50	0.78	0.58
	UV/H2O2/MF Reuse	2	0.63	0.81
		8	0.57	0.88
		14	0.58	0.94
		20	0.44	0.85
		26	0.52	0.85
		32	<0.1	0.46
		38	<0.1	0.44
		44	<0.1	0.31
		50	0.08	0.67
	NCM	2	0.56	0.69
		8	0.57	0.66
		14	0.43	0.48
		20	0.51	0.39
		26	0.57	0.15
		32	0.91	0.96
		38	0.98	0.97

	0.1% NCM	44	0.97	0.75
		50	0.95	0.53
		2	0.95	0.75
		8	0.77	0.85
		14	0.81	0.47
		20	0.97	0.64
		26	0.95	0.41
		32	0.94	0.97
		38	0.35	0.99
		44	0.26	0.83
		50	0.20	0.74
	Solvent Control	2	0.25	0.66
		8	0.23	0.66
		14	0.09	0.46
		20	0.27	0.64
		26	0.27	0.80
		32	0.30	0.86
		38	0.22	0.93
		44	0.35	0.86
		50	0.24	0.85
17,20-βP	MF reuse	4	<0.01	<0.01
		10	<0.01	<0.01
		16.00	<0.01	<0.01
		22.00	<0.01	<0.01
		28.00	<0.01	<0.01
		34.00	0.68	0.12
		40	0.47	0.08
		46	0.61	0.02
	CF/MF Reuse	4	0.02	0.05
		10	0.01	<0.01
		16.00	0.03	0.02
		22.00	0.00	<0.01
		28.00	0.03	0.03
		34.00	0.33	0.51
		40	0.55	0.29
		46	0.83	0.16
	UV/H2O2/MF Reuse	4	<0.01	<0.01
		10	<0.01	<0.01
		16.00	<0.01	<0.01

		22.00	<0.01	<0.01
		28.00	<0.01	<0.01
		34.00	0.95	0.27
		40	0.93	0.21
		46	0.92	0.18
	NCM	4	0.21	0.17
		10	0.05	0.01
		16.00	<0.01	0.01
		22.00	0.03	<0.01
		28.00	0.22	0.06
		34.00	0.99	0.44
		40	1.00	0.22
		46	1.00	0.09
	0.1% NCM	4	0.95	0.31
		10	0.93	0.24
		16.00	0.99	0.35
		22.00	1.00	0.25
		28.00	0.96	0.65
		34.00	0.95	0.60
		40	0.92	0.26
		46	0.14	0.14
	Solvent Control	4	0.67	0.28
		10	0.73	0.06
		16.00	0.06	0.04
		22.00	0.58	0.05
		28.00	0.63	0.09
		34.00	0.95	0.50
		40	0.96	0.37
		46	0.89	0.14
L-serine	MF reuse	6	0.80	0.80
		12	0.91	0.91
		18	0.91	0.91
		24	0.92	0.92
		30	0.93	0.93
		36	0.90	0.90
		42	0.93	0.93
		48	0.93	0.93
	CF/MF Reuse	6	0.99	0.93

		12	0.97	0.90
		18	0.95	0.98
		24	0.74	0.99
		30	0.96	0.86
		36	0.89	1.00
		42	0.91	0.82
		48	0.99	0.61
	UV/H2O2/MF Reuse	6	0.21	0.90
		12	0.24	0.93
		18	0.10	0.90
		24	0.22	0.91
		30	0.03	0.80
		36	0.20	0.99
		42	0.32	0.75
		48	0.03	0.52
	NCM	6	0.99	0.82
		12	0.97	0.95
		18	0.86	0.89
		24	0.96	0.90
		30	0.57	0.93
		36	1.00	0.99
		42	0.99	0.88
		48	0.99	0.68
	0.1% NCM	6	0.92	0.79
		12	0.88	0.81
		18	0.96	0.89
		24	0.82	0.64
		30	0.75	0.78
		36	0.85	0.99
		42	0.86	0.64
		48	0.78	0.68
	Solvent Control	6	0.99	0.79
		12	0.99	0.80
		18	0.93	0.88
		24	0.90	0.79
		30	0.74	0.74
		36	0.97	0.98
		42	0.96	0.82

		48	0.99	0.60

Table 2.2. Two-way Anova results for 30min reuse water exposures. This table shows the specific timepoints during the exposure that differ from either pre-exposure or control. If p is less than 0.05 then a significant difference was detected.

goldfish experienced larger decreases in L-alanine evoked EOGs than any other exposure groups.

EOG responses to L-serine during a 30 min exposure to treated reuse water

L-serine evoked EOGs were compared to both pre-exposure and control EOGs throughout a 30 min exposure to MF, CF/MF, UV/H_2O_2/MF reuse, NCM (0.1 and 100%) and a solvent control. Pre-exposure EOGs did not differ between exposure groups ($F_6 = 1.13$, $p = 0.36$; Figure 2.5). Exposure groups did not differ statistically from pre-exposure groups or controls throughout the 30 min exposure ($F_{8,371} = 1.84$, $p = 0.07$; $F_{6,371} = 1.12$, $p = 0.37$, respectively; Figure 2.6B). MF reuse and 0.1% NCM, exposed goldfish were numerically decreased from pre-exposure by an average of 33 and 22%, respectively, throughout the 30 min exposure. CF/MF and UV/H2O2/MF reuse and 100% NCM exposed goldfish were numerically increased from pre-exposure by an average of 20, 63, and 25% respectively throughout the 30 min exposure. MF and solvent control exposed goldfish were numerically increased by an average of 47 and 36%, respectively, compared to controls throughout the 30 min exposure; while goldfish exposed to UV/H_2O_2/MF reuse increased on average by 28%. All exposure groups returned to pre-exposure and control values during the recovery period. No exposure groups differed from controls at any specific time point during the exposure ($F_{48,371} = 0.76$, $p = 0.87$; Table 2.2). In general MF reuse, exposed goldfish experienced larger decreases in L-serine evoked EOGs than any other exposure group.

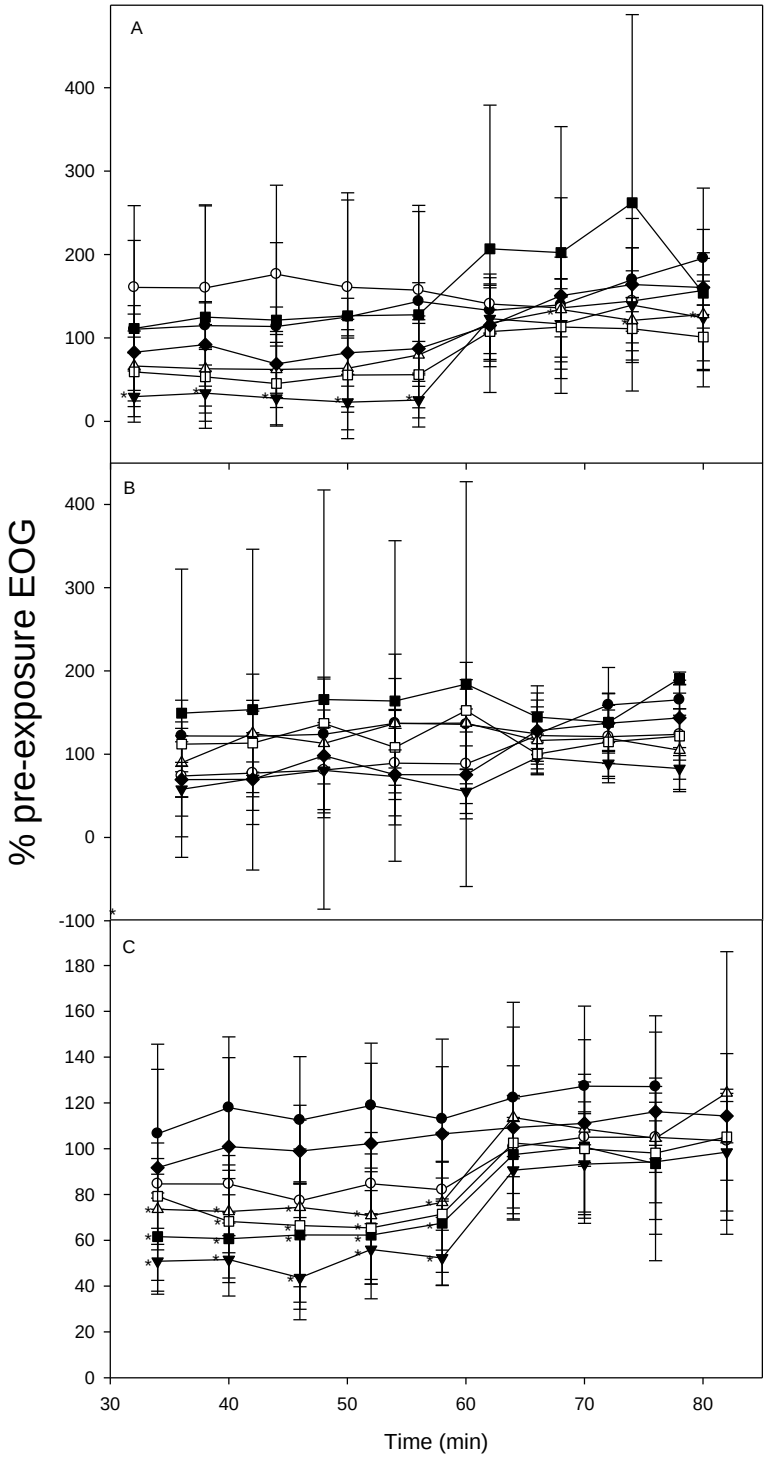
A
B
C
% pre-exposure EOG
Time (min)
400
300
200
100
0
-100
180
160
140
120
100
80
60
40
20
30
40
50
60
70
80

Figure 2.6

Figure 2.6. EOG responses during a 30 min exposure to treated reuse water and a NCM. A) EOG responses to L-alanine during a 30 min exposure to ▼MF reuse (n = 8), △CF/MF reuse (n = 8), ■UV/H2O2/MF reuse (n = 8), □ a NCM (n = 6), ◆0.1%NCM (n = 6), ○ solvent control (n = 5), or a ●dechlorinated water control (n =6); B) EOG responses to L-serine during a 30 min exposure to ▼MF reuse (n = 6), △CF/MF reuse (n = 9), ■UV/H2O2/MF reuse (n = 8), □ a NCM (n = 6), ◆0.1%NCM (n = 6), ○ solvent control (n = 5), or a ●dechlorinated water control (n = 5); C) EOG responses to 17,20-□P during a 30 min exposure to ▼MF reuse (n = 9), △CF/MF reuse (n = 9), ■UV/H2O2/MF reuse (n = 8), □ a NCM (n = 6), ◆0.1%NCM (n = 6), ○ solvent control (n = 5), or a ●dechlorinated water control (n = 5). Significant differences from pre-exposure EOG values are denoted with an asterisk.

EOG responses to 17, 20-βP during a 30 min exposure to treated reuse water

The 17,20-βP-evoked EOGs were compared to both pre-exposure and control EOGs throughout a 30 min exposure to MF, CF/MF, UV/H_2O_2/MF reuse, NCM (0.1 and 100%) and a solvent control. Pre-exposure EOGs did not differ between exposure groups ($F_6 = 0.63$, $p = 0.70$) (Figure 2.5). MF, CF/MF and UV/H2O2/MF reuse and 100% NCM exposed goldfish 17,20-βP-evoked EOGs were decreased by an average of 49, 26, 37 and 30%, respectively, compared to pre-exposure EOGs throughout the 30 min exposure ($F_{8,414} = 22.8$, $p < 0.01$; Figure 2.6). Both solvent control and 0.1% NCM exposed goldfish, 17,20-βP-evoked EOGs appeared to be unaffected. In MF, CF/MF and UV/H2O2/MF reuse and 100%NCM exposed goldfish, 17,20-βP-evoked EOGs were decreased by an average of 55, 35, 63 and 38%, respectively, compared to control EOGs throughout the 30 min exposure ($F_{6,414} = 5.34$, $p < 0.01$) (Table 2.2). In solvent control exposed goldfish, 17,20-βP-evoked EOGs were decreased numerically by an average of 27% compared to control EOGs throughout the 30 min exposure. All exposure groups 17,20-βP-evoked EOGs returned to pre-exposure and control values during the recovery period (Figure 2.6, Table 2.2). Time points that differed significantly from pre-exposures and controls can be found in table 2.2 (overall: $F_{48,414} = 1.47$, $p = 0.03$). In general, MF reuse exposed goldfish experienced the largest decreases in EOGs, though they were comparable to those of CF/MF and UV/H2O2 reuse and 100% NCM exposed goldfish, while 0.1% NCM and solvent control exposed goldfish EOGs were not affected.

EOG responses to amino acids and pheromones over 30 and 60 d exposures

Changes to EOG responses evoked by the AAs L-alanine and L-serine and the pheromones PGF2α were measured during exposure to MF, CF/MF and UV/H^2O_2/MF reuse throughout three different seasons (fall 2011, spring 2012, and summer 2012). L-alanine and 17,20-βP were measured during each of the time periods while L-serine was only measured during summer 2012 and PGF2α was only measured during fall 2011. Differences in EOGs evoked by the AAs and pheromones are described below.

Overall, EOGs evoked by L-alanine and 17,20-βP decreased due to exposure to treated reuse water; however, recovery was observed in some exposure groups, depending on the season. L-serine and PGF2α trends were not similar between exposure groups, and could not be compared between seasons as they were only collected during the summer and fall.

EOG responses to L-alanine, PGF2α, and 17,20-βP during a 30 d exposure to treated reuse water: Fall 2011

Goldfish exposed to UV/H_2O_2/MF reuse experienced the largest decreases in EOGs at 7 and 30 d for all odorants, while those exposed to CF/MF reuse saw initial decreases at 7 d that began to recover by 30 d into the exposure. MF reuse exposed goldfish did not follow a simple pattern as explained above, with L-alanine and PGF2α EOGs decreasing over time; while those evoked by 17,20-βP were not affected. Sample traces are shown in figure 2.8.

L-alanine-evoked EOGs at 7 and 30 d into the exposure did not differ (Figure 2.6A, Table 2.3). L-alanine-evoked EOGs in goldfish exposed to MF reuse decreased numerically at d 30 compared to 7 d by 50%, while those evoked by goldfish exposed to CF/MF reuse increased numerically at 30 d compared to 7 d by 55%. L-alanine-evoked EOGs in goldfish exposed to UV/H_2O_2/MF reuse were decreased by 50% (Figure 2.7A, Table 2.3).

PGF2α-evoked EOGs at 7 and 30 d into the exposure did not differ (Figure 2.6B, Table 2.3). PGF2α-evoked EOGs in goldfish exposed to both MF and UV/H2O2/MF reuse decreased numerically at 30 d compared to 7 d by 41 and 23%, respectively (Figure 2.7B, Table 2.3). PGF2α-evoked EOGs in goldfish exposed to CF/MF reuse increased numerically at 30 d compared to 7 d by 90%.

17,20-βP-evoked EOGs at 7 and 30 d into the exposure did not differ (Figure 2.6C Table 2.3). The 17,20-βP-evoked EOGs in goldfish exposed to CF/MF reuse increased numerically at 30 d compared to 7 d by 39% (Figure 2.7C, Table 2.3).

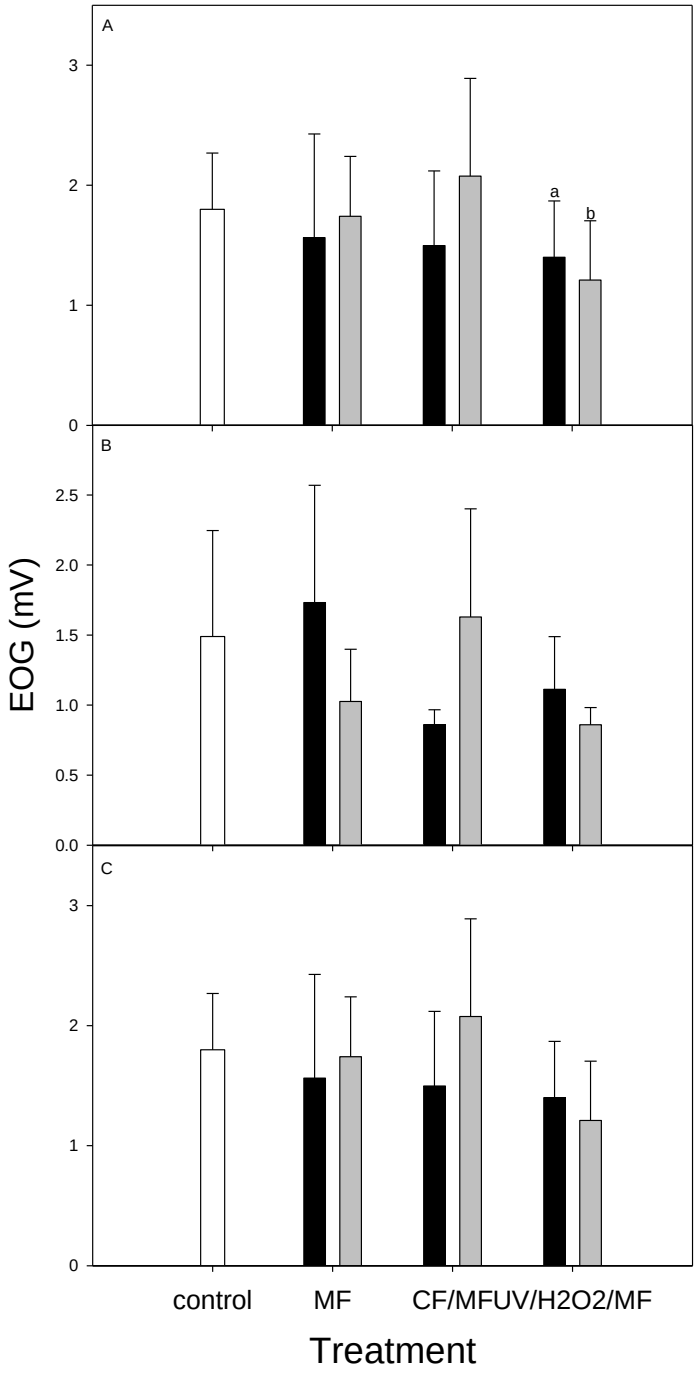

Figure 2.7.

Figure 2.7. EOG responses during a 30 d exposure to treated reuse water during fall 2011. A) EOG responses to L-alanine at ■ 7 and ■ 30 ds into exposures to MF reuse (n = 3; n = 3, respectively), CF/MF reuse (n = 3; n = 8, respectively), UV/H2O2/MF reuse (n =4; n =2, respectively) and controls (n = 5); B) EOG responses to PGF2α at ■ 7 and ■ 30 ds into exposures to MF reuse (n = 2; n = 8, respectively), CF/MF reuse (n = 4; n = 6, respectively), UV/H2O2/MF reuse (n = 3; n = 4, respectively) and controls (n =4); C) EOG responses to 17,20-βP at ■ 7 and ■ 30 ds into exposures to MF reuse (n = 5; n = 8, respectively), CF/MF reuse (n = 3; n = 8, respectively), UV/H2O2/MF reuse (n =4; n =5, respectively) and controls (n = 5). Significant differences within exposure groups are denoted by different letters.

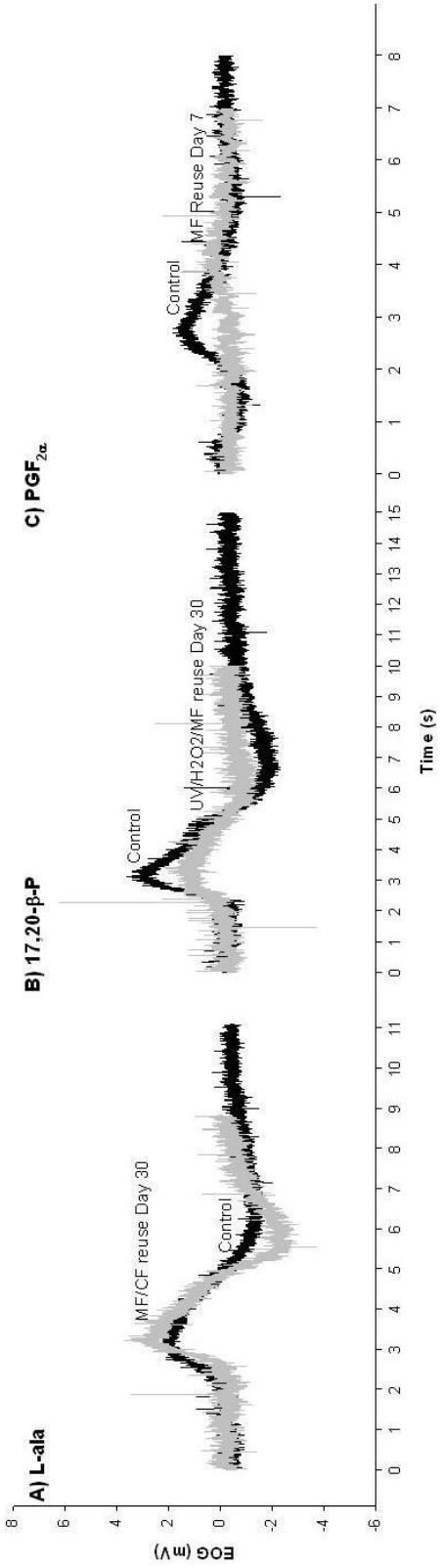
A) L-ala
MF/CF reuse Day 30
Control
B) 17,20-β-P
Control
UV/H2O2/MF reuse Day 30
C) PGF2α
Control
MF Reuse Day 7
EOG (mV)
Time (s)

Figure 2.8

Figure 2.8. Sample traces of EOGs collected during 30 d exposure to treated reuse water. Red traces are those of exposed fish, while those in black are those of controls collected during the same time points. A) Comparison of L-alanine evoked EOGs between MF/CF reuse exposed fish at 30d and controls. B) Comparison of 17,20-βP evoked EOGs between UV/H_2O_2/MF reuse exposed fish at 30d and controls. C) Comparison of PGF2α evoked EOGs between MF reuse exposed fish at 7d and controls.

Table 2.3

Odorant	Reuse Treatment	Comparison	t	p
L-alanine	MF reuse	control vs 7d	0.44	0.68
		control vs 30d	1.4	0.23
		7d vs 30d	1.4	0.23
	CF/MF reuse	control vs 7d	1	0.34
		control vs 30d	0.51	0.62
		7d vs 30d	-1.2	0.25
	UV/H_2O_2/MF reuse	control vs 7d	0.78	0.46
		control vs 30d	1.2	0.29
		7d vs 30d	5	<0.01
17,20-bP	MF reuse	control vs 7d	0.49	0.64
		control vs 30d	0.19	0.85
		7d vs 30d	-0.48	0.64
	CF/MF reuse	control vs 7d	0.74	0.49
		control vs 30d	-0.62	0.55
		7d vs 30d	-1.1	0.3
	UV/H_2O_2/MF reuse	control vs 7d	1.2	0.27
		control vs 30d	1.8	0.11
		7d vs 30d	0.59	0.58
PGF2α	MF reuse	control vs 7d	-0.36	0.74
		control vs 30d	1.5	0.18
		7d vs 30d	-1.9	0.09
	CF/MF reuse	control vs 7d	1.6	0.15
		control vs 30d	-0.28	0.79
		7d vs 30d	-1.9	0.09
	UV/H_2O_2/MF reuse	control vs 7d	0.78	0.47
		control vs 30d	1.6	0.15
		7d vs 30d	1.3	0.25

Table 2.3. T-test results for 30d reuse water exposures. Goldfish exposed to UV/H_2O_2/MF reuse display a significant difference between EOGs evoked at 7 and 30 d into the exposures. All other comparisons were not significantly different.

EOG responses to L-alanine and 17,20-βP during a 60 d exposure to treated reuse water: Spring 2012

MF reuse exposed goldfish appeared to recover responses to L-alanine over time while those to 17,20-βP were decreased throughout the exposure. CF/MF reuse exposed goldfish did not experience the same initial decrease in L-alanine-evoked EOGs experienced by other groups during the 1-20 d period; however these EOGs decreased after this period and showed no sign of recovery. CF/MF reuse exposed goldfish did not display a general trend for 17,20-βP-evoked EOGs. UV/H2O2/MF reuse exposed goldfish experienced an initial decrease in EOGs evoked by both L-alanine and 17,20-βP; however, by the end of the 60 d period recovery of these EOGs was apparent.

When L-alanine-evoked EOGs were compared at the same time points during the exposure, no statistical difference was found between goldfish exposed to MF, CF/MF and UV/H_2O_2/MF reuse and controls ($F_{6,69} = 1.43$, $p = 0.22$; Figure 2.7A). MF reuse exposed goldfish differed numerically from controls throughout the exposure, with EOGs decreased compared to controls at 1-20 d into the exposure by 24% ; however, EOGs were increased at 21-40 d into the exposure by 110% compared to controls. CF/MF reuse exposed goldfish had L-alanine EOGs that were numerically decreased at 41-60 d into the exposure by 47% compared to controls; however, L-alanine EOGs were numerically increased at 21-40 d into the exposure by 38%, compared to controls. UV/H_2O_2/MF reuse exposed goldfish differed numerically from controls throughout the exposure, EOGs decreased at

1-20 d into the exposure by 54.% , compared to controls. No difference was found between goldfish exposed to MF, CF/MF and UV/H_2O_2/MF reuse when compared at the same time points or across time points within the same exposure group ($F_{4,55}$ = 1.76, p = 0.15; Figure 2.9A). It should be noted that controls experienced a large decrease in EOGs to L-alanine at 21-40 d into the exposure that cannot be explained, but does account for some groups numerically increased at this time point.

When 17,20-βP-evoked EOGs were compared at the same time points during the exposure, no statistical difference was found between goldfish exposed to MF, CF/MF and UV/H_2O_2/MF reuse and controls ($F_{3,41}$ = 1.12, p = 0.36; Figure 2.7B). MF reuse exposed goldfish differed numerically from controls at 1-20 and 21-40 d into the exposure, with decreases of 33 and 43%, respectively. CF/MF exposed goldfish differed numerically from controls at 21-40 d into the exposure, with a decrease of 31%. UV/H_2O_2/MF reuse exposed goldfish differed numerically from controls with EOGs decreased by 61.7% at 1-20 d into the exposure and increased by 27.5% at 21-40 d into the exposure. No difference in 17,20-βP-evoked EOGs were found between exposure groups within the same time period or within the same exposure group across time points ($F_{4,51}$ = 1.18, p = 0.34; Figure 2.9B).

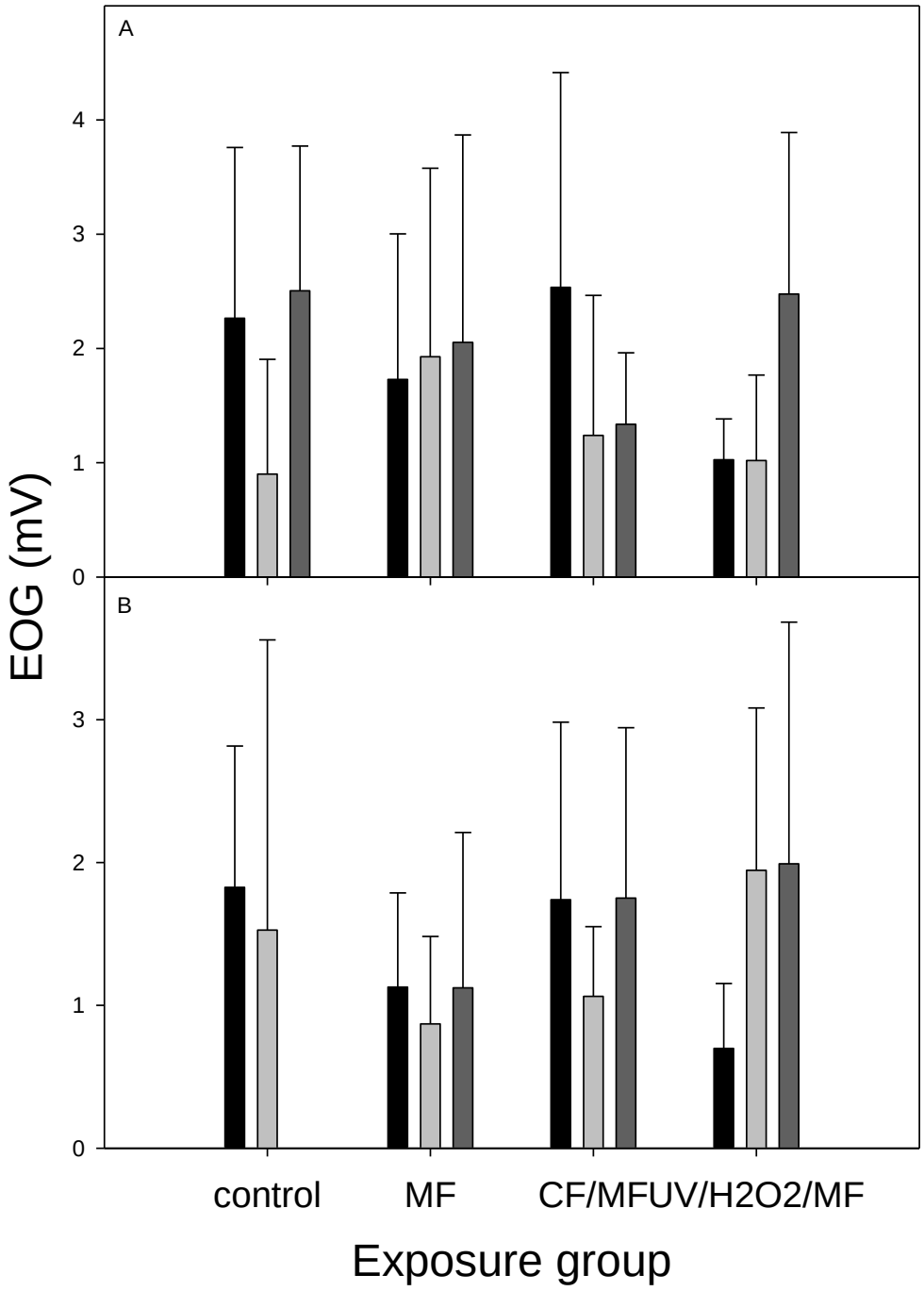

Figure 2.9.

Figure 2.9. EOG responses during a 60 d exposure to treated reuse water during spring 2012. A) EOG responses to L-alanine at ■ 1-20, ■ 21-40, and ■ 41-60 ds into the exposures to MF reuse (n = 5; n = 4; n = 9, respectively), CF/MF reuse (n = 5; n = 5; n = 10, respectively), H2O2/MF reuse (n = 4; n = 5; n = 9, respectively), and controls (n = 4; n = 5; n = 5, respectively); B) EOG responses to 17,20-□P at ■ 1-20, ■ 21-40, and ■ 41-60 ds into the exposures to MF reuse (n = 5; n = 5; n = 7, respectively), CF/MF reuse (n = 7; n = 6; n = 9, respectively), H2O2/MF reuse (n = 4; n = 4; n = 5, respectively), and controls (n = 3; n = 8, n = 1 (not included) respectively).

EOG responses to L-alanine during a 60 d exposure to treated reuse water: Summer 2012

A 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse result in decreased EOG responses to L-alanine-, L-serine-, and 17,20-βP-evoked EOGs. MF reuse exposed goldfish had the largest decreases, while those exposed to CF/MF reuse displayed the smallest decreases. L-serine appeared to be the odorant that was least affected by the exposures, while L-alanine appeared to be the most affected.

A 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse, decreased EOGs evoked by L-alanine compared to controls at the same time points ($F_6 = 3.29$, $p < 0.01$; Figure 2.8A). In MF reuse exposed goldfish EOG responses to L-alanine were statistically decreased at 21-40 and 41-60 d into the exposure by 71 and 60%, respectively, compared to controls (Figure 2.8A,). CF/MF reuse exposed goldfish EOG responses to L-alanine were statistically decreased at 21-40 and 41-60 d into the exposure by 70 and 80%, respectively, compared to controls; however, EOGs at 1-20 d into the exposure were numerically decreased by 20% compared to controls. UV/H_2O_2/MF reuse exposed goldfish EOG responses to L-alanine decreased statistically at 1-20, 21-40, and 41-60 d into the exposure by 60, 65, and 47%, respectively, compared to controls.

MF, CF/MF and UV/H_2O_2/MF reuse exposed goldfish had decreased L-alanine-evoked EOGs between time points during the exposure ($F_{4,70} = 5.64$, $p < 0.01$). MF reuse exposed goldfish had L-alanine EOGs at 21-40 d into the exposure that were statistically decreased by 61% compared to those evoked at 1-21 d into the

exposure ($p < 0.01$); while those evoked at 41-60 d into the exposure were numerically decreased by 33% compared to 1-20 d into the exposure, and numerically increased by 72% compared to 21-40 d into the exposure. CF/MF exposed goldfish had L-alanine EOGs at 21-40 and 41-60 d into the exposure that were statistically decreased by 48 and 55% respectively compared to those evoked at 1-20 d into the exposure ($p = 0.07$, 0.09, respectively). UV/H_2O_2/MF reuse exposed goldfish had L-alanine EOGs at 1-20 and 21-40 d into the exposure that were statistically decreased by 57 and 48%, respectively, compared to those evoked at 41-60 d into the exposure ($p = 0.02$, < 0.01, respectively); while those evoked at 21-40 d into the exposure were numerically decreased by 48% compared to those at 41-60 d.

L-alanine-evoked EOGs differed between exposure groups at the same time points during a 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse ($F_{4,70} = 5.64$, $p < 0.01$). UV/MF reuse exposed goldfish had statistically decreased L-alanine EOGs at 1-20 d into the exposure by 62 and 50% compared to MF and CF/MF reuse exposed goldfish, respectively (p: < 0.01, 0.02, respectively), while those evoked from CF/MF exposed goldfish were numerically decreased at 1-20 d into the exposure by 24% compared to those exposed to MF reuse. CF/MF reuse exposed goldfish had L-alanine EOGs at 41-60 d into the exposure that were statistically decreased by 61% compared to goldfish exposed to UV/H2O2/MF reuse ($p < 0.01$); while those evoked by MF reuse exposed goldfish were numerically increased by 94 and 24% compared to CF/MF and UV/H2O2/MF reuse exposed goldfish.

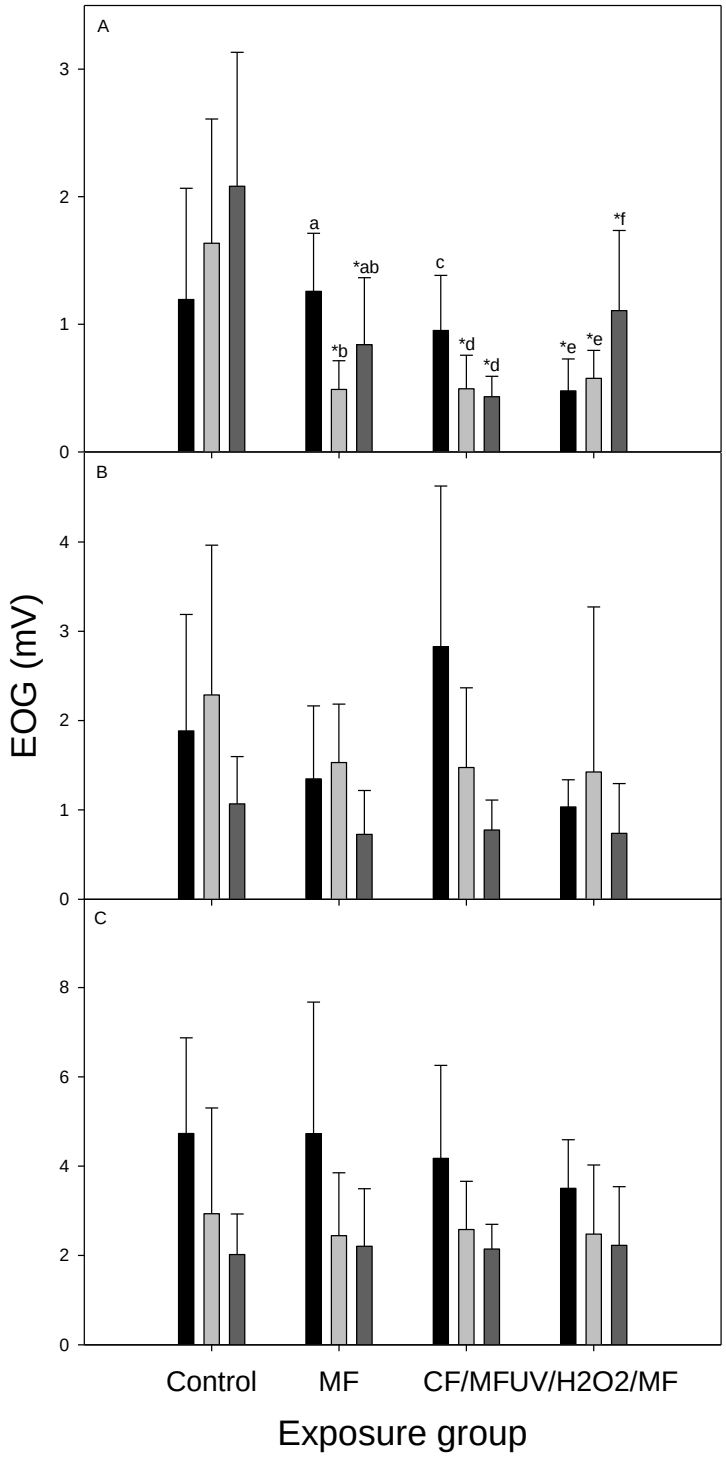
A
B
C
EOG (mV)
a
*ab
*b
c
*d
*d
*e
*e
*f
Control
MF
CF/MFUV/H2O2/MF
Exposure group

Figure 2.10

Figure 2.10. EOG responses during a 60 d exposure to treated reuse water during spring 2012. A) EOG responses to L-alanine at ■ 1-20, ■ 21-40, and ■ 41-60 ds into the exposures to MF reuse (n = 4; n = 10; n = 9, respectively), CF/MF reuse (n = 9; n = 11; n = 6, respectively), H2O2/MF reuse (n = 8; n = 7; n = 7, respectively), and controls (n = 7; n = 6; n = 3, respectively); B) EOG responses to L-serine at ■ 1-20, ■ 21-40, and ■ 41-60 ds into the exposures to MF reuse (n = 9; n = 8; n = 8, respectively), CF/MF reuse (n = 8; n = 11; n = 6, respectively), H2O2/MF reuse (n = 6; n = 9; n = 7, respectively) and controls (n = 7; n = 9, n = 4, respectively); C) EOG responses to 17,20-□P at ■ 1-20, ■ 21-40, and ■ 41-60 ds into the exposures to MF reuse (n = 9; n = 9; n = 9, respectively), CF/MF reuse (n = 9; n = 11; n = 7, respectively), H2O2/MF reuse (n = 7; n = 9; n = 7, respectively), and controls (n = 6; n = 8, n = 4 respectively). Differences from control are denoted with an asterisk and differences within groups between time points are denoted with different letters.

EOG responses to L-serine during a 60 d exposure to treated reuse water: Summer 2012

A 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse, did not result in statistically decreased EOGs evoked by L-serine compared to controls at the same time points ($F_{6,91}$ = 1.40, p = 0.22; Figure 2.10B). MF reuse exposed goldfish had L-serine evoked EOGs at 1-20, 21-40, and 41-60 d into the exposure that were decreased numerically by 29, 33, and 32%, respectively, compared to controls. CF/MF reuse exposed goldfish had L-serine evoked EOGs at 21-40, and 41-60 d

into the exposure that were numerically decreased by 36 and 27% respectively compared to controls, while those evoked at 1-20 d into the exposure were numerically increased by 50% compared to controls. UV/H_2O_2/MF reuse exposed goldfish had L-serine evoked EOGS that 1-20, 21-40, and 41-60 d into the exposure that were numerically decreased by 45, 38, and 31%, respectively, compared to controls.

L-serine-evoked EOGs did not differ statistically within exposure groups between time points ($F_{4,71} = 2.06$, $p = 0.10$; Figure 2.10B). MF reuse exposed goldfish had L-serine EOGs at 41-60 d into the exposure were numerically decreased by 53% compared to those at 21-40 d into the exposure; while those evoked at 41-60 d into the exposure were also numerically decreased by 46% compared to those evoked at 1-20 d into the exposure. CF/MF reuse exposed goldfish had L-serine EOGS at 21-40 and 41-60 d into the exposure that were numerically decreased by 48 and 73%, respectively, compared to 1-20 d into the exposure, while those evoked at 41-60 d into the exposure were also numerically decreased by 47% compared to those evoked at 21-40 d into the exposure. UV/H_2O_2/MF reuse exposed goldfish had L-serine EOGs at 1-20 and 41-60 d into the exposure that were numerically decreased by 27 and 48%, respectively, compared to those evoked at 21-40 d into the exposure, while those evoked at 41-60 d into the exposure were also numerically decreased by 28% compared to those evoked at 1-20 d into the exposure.

L-serine-evoked EOGs did not differ statistically between exposure groups at the same time points during a 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse ($F_{4,71}$ = 2.06, p = 0.10; Figure 2.10B). MF reuse and UV/H_2O_2/MF reuse exposed goldfish had L-serine EOGs at 1-20 d into the exposure that were numerically decreased by 52 and 63%, respectively, compared to those exposed to CF/MF reuse; while those evoked by UV/H_2O_2/MF reuse exposed goldfish were also numerically decreased by 23% compared to MF reuse exposed goldfish.

EOG responses to 17α,20β-20-dihydroxy-pregnen-3-one during a 60 d exposure to treated reuse water: Summer 2012

A 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse, did not result in statistically decreased EOGs evoked by 17,20-□P compared to controls at the same time points ($F_{6,94}$ = 0.30, p= 0.93; Figure 2.10C). UV/H_2O_2/MF reuse exposed goldfish had 17,20-βP-evoked EOGs at 1-20 into the exposure that were numerically decreased by 26 % compared to controls.

17,20-βP-evoked EOGs did not differ statistically within exposure groups between time points ($F_{4,76}$ = 0.40, p = 0.81; Figure 2.10C). MF reuse exposed goldfish had 17,20-βP-evoked EOGs at 21-40 and 41-60 d into the exposure were numerically decreased by 48 and 53% compared to those evoked at 1-20 d into the exposure. CF/MF reuse exposed goldfish had 17,2-βP-evoked EOGs at 21-40 and 41-60 d into the exposure that were numerically decreased by 38 and 49%, respectively, compared to those at 1-20 d into the exposure. UV/H_2O_2/MF reuse exposed goldfish had 17,20-βP-evoked EOGs at 21-40 and 41-60 d into the

exposure that were numerically decreased by 29 and 36%, respectively, compared to those evoked at 1-20 d into the exposure.

17,20-βP-evoked EOGs did not differ statistically between exposure groups at the same time points during a 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse ($F_{4,76}$ = 0.40, p = 0.81; Figure 2.10C). UV/H2O2/MF reuse exposed goldfish had 17,20-βP-evoked EOGs at 1-20 d into the exposure that were decreased by 26%, compared to MF reuse exposed goldfish.

EOG responses to L-alanine and 17α,20β-20-dihydroxy-pregnen-3-one between two seasonal 60 d exposures to treated reuse water

Exposure to treated reuse during the summer results in larger decreases in L-alanine-evoked EOGs compared to those in the spring; however, 17,20-βP EOGs were generally decreased in the spring compared to summer even in controls suggesting this effect was not due to exposure to treated reuse. The EOGs evoked using L-alanine and 17,20-βP in goldfish during the spring and summer differed depending on the season. L-alanine-evoked EOGs by goldfish exposed to MF, CF/MF, and UV/H2O2/MF reuse during the spring were statistically increased on average by 160, 150, and 150%, respectively, compared to those evoked during the summer ($F_{1,40}$ = 7.54, p < 0.01; $F_{1,45}$ = 18.5, p < 0.01; $F_{1,39}$ = 8.97, p < 0.01, respectively; p-values: < 0.01, < 0.01, < 0.01, respectively). L-alanine-evoked EOGs in control goldfish during the spring were increased numerically by 21% compared to those evoked during the summer ($F_{1,29}$ = 0.37, p = 0.55). The 17,20-βP-evoked EOGs in goldfish exposed to MF, CF/MF, and UV/H2O2/MF reuse

and controls during the summer were statistically increased on average by 48, 92, 130 and 140%, respectively, compared to those evoked during the spring ($F_{1,43}$ = 15.6, $p < 0.01$; $F_{1,48}$ = 15.2, $p < 0.01$; $F_{1,35}$ = 6.65, $p = 0.02$; $F_{1,24}$ = 5.6, $p = 0.03$, respectively; p-values: < 0.01, < 0.01, 0.02, 0.03, respectively).

Behavioral Endpoints

Treated reuse or a NCM did not evoke a behavioral response in goldfish. Exposures to treated reuse or NCM of 30 min and up to 60 d do not impact a behavioral response evoked by L-alanine, with decreases in distance travelled post-introduction occurring in both control and exposure groups.

Behavioral response to the introduction of a pulse of treated reuse water or NCM

The introduction of a pulse of MF, CF/MF or UV/H2O2/MF reuse or NCM or solvent control did not change distance travelled post-introduction compared to controls ($F_{5,143}$ = 2.06, $p = 0.08$; Figure 2.11). During the introduction of pulses of MF, CF/MF or UV/H2O2/MF reuse or NCM, solvent control or dechlorinated water (control), distance travelled post-introduction was statistically decreased on average by 16% compared to pre-introduction distance travelled ($F_{1,143}$ = 37.6, $p < 0.01$; $p < 0.01$).

Behavioral response to a pulse of L-alanine after a 30 min exposure to treated reuse water or an NCM

The introduction of a pulse of L-alanine post exposure to MF, CF/MF or UV/H2O2/MF reuse or NCM or solvent control did not result in a different

response than was displayed by controls ($F_{5,167}$ = 1.65, p= 0.16) (Figure 2.12). Goldfish exposed to MF, CF/MF or UV/H2O2/Mf reuse or NCM, solvent control, or dechlorinated water (control) had distance travelled post-introduction of L-alanine that was statistically decreased on average by 22% respectively compared to pre-introduction distance travelled ($F_{1,167}$ = 57.3. p < 0.01; p < 0.01).

Behavioral response to a pulse of L-alanine during a 60 d exposure to treated reuse water

The introduction of a pulse of L-alanine throughout the 60 d exposure to MF, CF/MF, or UV/H_2O_2/MF did not result in a different response than controls throughout the exposure. The introduction of a pulse of L-alanine resulted in an average decreased distance travelled post exposure of 15% compared to pre-exposure distance travelled ($F_{1,223}$ = 7.56, p < 0.01; Figure 2.13).

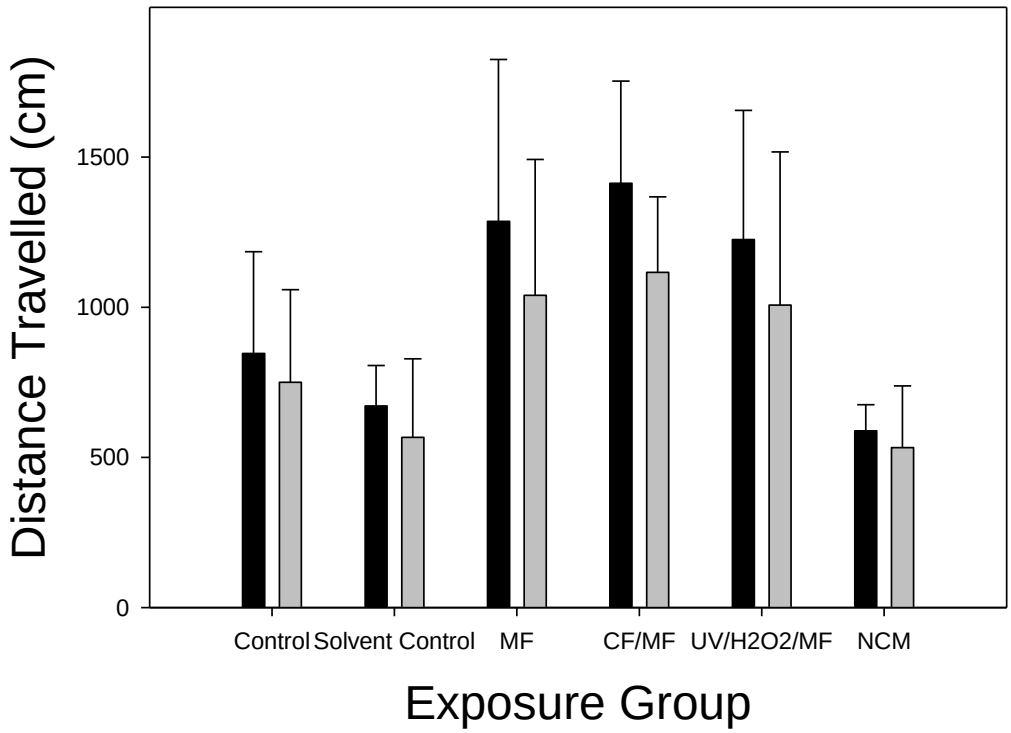

Figure 2.11. Behavioral response to the introduction of a contaminant pulse. Distance travelled ■ pre- vs. ■ post-exposure by goldfish presented with a pulse of MF reuse (n = 8), CF/MF reuse (n = 8), UV/H202/MF reuse (n = 8), a NCM (n = 16), a solvent control (n = 13) or dechlorinated municipal water (control) (n = 19) over time.

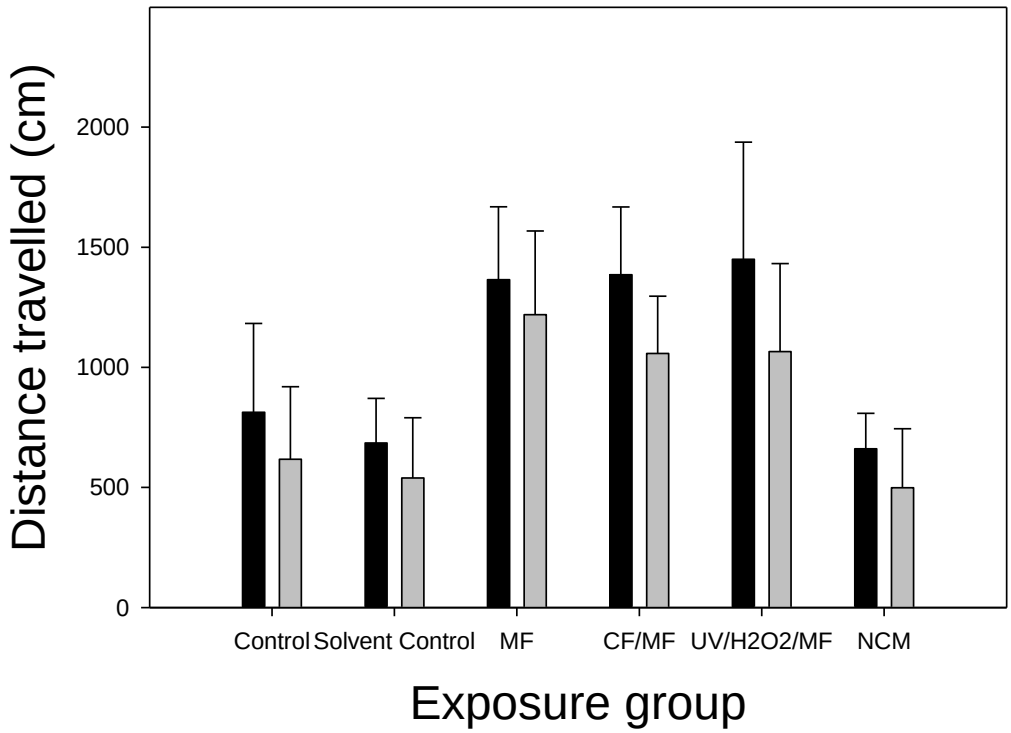

Figure 2.12. Behavioral responses to the introduction of a pulse of L-alanine post 30 min exposure to a contaminant source. Distance travelled ■ pre- vs. ■ post-exposure by goldfish exposed to MF reuse (n = 8), CF/MF reuse (n = 8), UV/H202/MF reuse (n = 8), a NCM (n = 22), a solvent control (n = 22) when compared to goldfish exposed to dechlorinated municipal water (control) (n = 19).

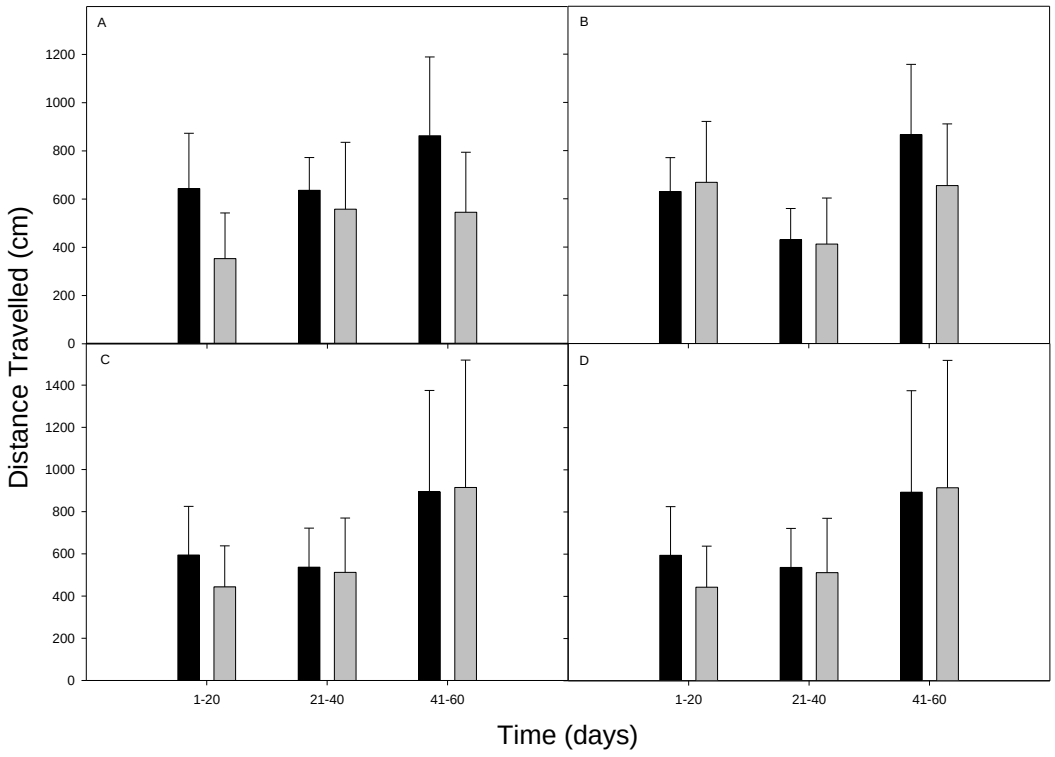

Figure 2.13 Behavioral responses to the introduction of a pulse of L-alanine during a 60 d exposure to treated reuse water. Distance travelled ■ pre- vs. post-exposure by goldfish exposed to A) dechlorinated municipal water (control) (n = 7; n = 7; n = 5); B) MF reuse (n = 11; n = 10; n = 9); C) CF/MF reuse (n = 12; n = 9; n = 10); D) UV/H2O2/MF reuse (n = 12; n = 10; n =10).

Discussion

Goldfish olfaction is able to detect the contaminants found within treated reuse water. EOGs evoked by goldfish using both natural odorants and contaminant mixtures were seen to occur in a concentration-dependent manner. In general, L-alanine and 17,20-βP-evoked EOGs at 10^{-6} M evoked the greatest responses from all odorants and concentrations tested; however, 100% MF reuse-evoked EOGs were greater than both of the aforementioned odorants. The 100% CF/MF and UV/H_2O_2/MF reuse-evoked EOGs were greater than 10^{-6} M 17,20-βP evoked EOGs, and were within the same range as those evoked by 10^{-6} M L-alanine. This ability to detect contaminants within treated reuse suggests that detection of natural odorants may be impacted by their presence.

EOG concentration dependency to amino acids and pheromones

L-serine and L-alanine evoked EOGs in a concentration dependent manner similar to that described in rainbow trout by Hara et al. and Sorensen et al. [47, 109]. L-serine evoked EOGs were similar in magnitude to those evoked in Coho salmon (*Oncorhynchus kisutch*) [6]. L-alanine-evoked EOGs in goldfish were greater than those evoked in rainbow trout [47], suggesting that L-alanine is a more suitable odorant than L-serine to test olfactory toxicity in goldfish. Concentration dependency evoked by 17,20-βP resembled those reported in goldfish by Sorensen et al. [109]; $3x10^{-6}$ M 17,20-βP-evoked EOGs were 270% greater than those evoked using 10^{-5} M L-serine. Concentration dependency to PGF2α

resemble those reported in goldfish by Sorensen et al.[110]; $3x10^{-8}$ M PGF2α-evoked EOGs were 183% greater than those evoked using 10^{-5} M L-serine.

EOG concentration response curves to treated reuse water and a NCM

Aside from the point of discharge at the Gold Bar WWTP it is unlikely that fish will encounter concentrations of treated reuse at 100 or 50% dilutions; however, responses to treated reuse at a 1% dilution were similar to those evoked by all odorants at concentrations used during 30 min exposures (Figure 1; Figure 2). As all contaminants were present at or below the μg/L concentration in 100% dilutions, detection of 1% dilutions suggests that contaminants in the ng/L concentration range may evoke behavioral responses.

Detection of chemicals may allow fish to determine potentially harmful areas and avoid them [35, 51]. Detection can also be problematic, however, as fish may be attracted to certain contaminants, as has been shown with some pesticide mixtures [122], placing them in environments which may be harmful. Treated reuse water not only contains detectable contaminants, but has been in contact with human skin (e.g. through showering), potentially containing amino acids such as L-serine and L-alanine that have been shown to evoke olfactory responses at dilutions as low as 10^{-8} M [47]. Amino acids evoke behaviors such as feeding and predator avoidance, so exposure to high concentrations of amino acids may cause confusion for fish. For example, feeding behavior is associated with L-alanine, and an increase in its concentration within the environment may result in food searching behavior in a region where there is no prey. This would be energetically

costly to fish and may impact their ability to survive if they cannot properly search for areas containing prey. An artificially high L-alanine concentration may also serve to mask the scent of actual prey.

Effects of treated reuse water on EOGs

EOG responses to all odorants were impacted by exposure to treated reuse or NCM. Exposure to MF reuse resulted in the largest decreases in EOGs evoked by all odorants during 30 min exposures; while exposure to UV/H_2O_2/MF reuse resulted in the largest decreases in EOGs evoked by all odorants during 30 and 60 d exposures. EOGs appear to be the least impacted by exposure to treated reuse or NCM; while L-alanine evoked EOGs appear to be the most impacted. Seasonal effects of are seen during 60 d exposures with summer exposures resulting in larger decreases to L-alanine-evoked EOGs during the summer compared to those during the spring. Recovery of EOGs was seen in all odorants during 30 min and 60 d exposures, and does not appear to be affected by the season during which the exposure occurred. Overall this suggests that fish exposed to treated reuse water may have trouble detecting natural odorants, and so olfactory mediated behaviors may also be affected.

Effects of treated reuse water on EOGs evoked by amino acids

L-alanine- and L-serine evoked EOGs were decreased during 30 min and 60 d exposures to treated reuse water; however, L-alanine-evoked EOGs appeared to be more affected than those evoked by L-serine. No other studies have

investigated the effects of treated reuse water on olfactory toxicity; however, a comparison can be made with a contaminant that has been well studied in regards to olfactory toxicity, copper. During 30 min exposures to MF, CF/MF, and UV/H_2O_2/MF reuse, L-alanine-evoked EOGs were decreased on average by 72.1, 33.0 and 22.4%, respectively; while Atlantic salmon (*Salmo salar*) exposed to 0.2 and 2.2 μg/L copper solution experienced 50 and 90% decreases to L-alanine-evoked EOGs, respectively, during a 5 min exposure. L-serine-evoked EOGs were decreased on average by 49.7% during exposure to MF reuse and increased on average by 20.3 and 63.4% during a 30 min exposure to CF/MF and UV/H_2O_2/MF reuse, respectively; while Coho salmon exposed to 10 μg/L copper experienced decreased L-serine-evoked EOG by 50%. These comparisons suggest that treated reuse is less toxic to olfactory receptors than copper during a 30 min exposure (at a concentration range of 10-100μg/L), and that treated reuse takes much longer than copper to affect olfactory receptors than copper. These data also suggest that the UV/H_2O_2/MF treatment may remove components from the reuse water that stimulate olfactory neurons; both MF and CF/MF treatments do not appear to be as effective. Regardless, as L-alanine- and L-serine evoked EOGs returned to pre-exposure values post-exposure; the data suggest that no permanent damage to the receptors associated amino acid odorant detection occurred.

L-alanine-evoked EOGs did not decrease in comparison to controls during fall and spring exposures to treated reuse water; however, summer exposures resulted in decreases compared to controls throughout the 60 d exposure. Goldfish exposed to MF, CF/MF, and UV/H202 reuse experienced average decreases

throughout the exposure of 50, 58, and 54% respectively. L-serine evoked EOGs were also decreased in comparison to controls during the summer exposures with MF, CF/MF and UV/H2O2 reuse exposures resulting in average decreases of 50, 66, and 70%, respectively. No other studies have conducted 60 d exposures to investigate olfactory toxicity; however data on 96 h exposures to a pesticide mixture do exist. Tierney et al. found that 96 h exposure to a pesticide mixture containing dimethoate, simazine, methamidophos, diazinon, chlorpyriphos, endosulphan, malathion, atrazine, linuron and parathion with a total concentration of 13.9 μg/L was able to reduce L-serine evoked EOGs in rainbow trout by 47%. This concentration is double the total contaminant concentration found in MF reuse, however the decrease in amino acid evoked EOGs are not merely double. This comparison suggests that an increased number of contaminants at lower concentrations had less effect than fewer contaminants at greater concentrations. These results also suggest that seasonal components of treated reuse water have the ability to impair OSNs used to detect both L-alanine, and this will be discussed in greater detail further on.

As goldfish have the ability to detect all forms of reuse water it is possible that chemicals within reuse water are stimulating olfactory neurons. Exposure of olfactory tissue to a constant barrage of compounds that bind ORs may result in receptor down regulation. This had previously seen to occur during short term 30 min cross adaptation studies using amino acids [17]. If the neurons that are being stimulated also detect amino acids, it is possible that reuse chemicals compete with L-alanine and L-serine and so reduce their ability to evoke responses. These

two AAs were found to share a receptor within the olfactory tissue of rainbow trout, and this receptor can detect short chain amino acid structures and compounds similar to L-alanine and L-serine that contain substituted functional groups [15, 16, 93]. Substitution of the amino or carboxylic acid group of L-alanine resulted in decreased binding of L-alanine the receptor(s) in channel catfish; however, these same compounds inhibited the binding of L-alanine to receptors within olfactory tissue. Inhibition of L-alanine binding by β-alanine was no different than that occurring during an L-alanine saturation of the tissue [16]. Inhibition of L-serine in the presence of substituted forms of L-alanine was shown to be much less effective than the inhibition of L-alanine, β-alanine acclimation only resulting in a 20% decrease in L-serine binding. This information allows us to speculate upon two potential mechanisms by which L-alanine and L-serine evoked EOGs are being decreased during a 30 min exposure.

First, there may be competitive binding for the receptor(s) at their orthosteric site(s). Previous studies have shown that antibiotics, such as cycloserine, have the ability to bind receptors associated with L-alanine [92, 99]. Compounds which contain the same carbon backbone and substituted functional groups as L-alanine, such as D-alanine and β-alanine, can act as inhibitors of L-alanine detection by channel catfish [16]. Therefore it is possible that short chain carbon structures occurring as byproducts from microbial degradation of contaminants prior to MF, CF/MF, or UV/H_2O_2/MF filtration may be decreasing the detection of L-alanine (via competition). Removal and decomposition of these structures by CF/MF and

UV/H2O2/MF filtration may have decreased the concentrations of short chain carbon structures which resemble L-alanine (within treated reuse). This would explain why 30 min exposures to MF reuse resulted in larger decreases in EOGs evoked by L-alanine and L-serine when compared to CF/MF and UV/H2O2/MF reuse exposures.

Second, it is possible that contaminants within reuse water interacted with L-alanine at the carboxylic acid or α-amino site, which could have caused structural changes in olfactory receptors that alter odorants' ability to bind them. The functional groups of L-serine and L-alanine which have been identified as crucial to binding with their shared receptor are the carboxylic acid group and α-amino group [17, 94]. When comparing L-serine and L-alanine structures, it must be noted that L-serine contains a polar hydroxyl group on the third carbon and L-alanine does not. The distance of the hydroxyl group from other functional groups of L-serine makes it an easier target for substitution as there is less interference caused by the charge of surrounding functional groups. Substitutions made at this hydroxyl group may not result in decreases in binding of L-serine to the shared receptor or perhaps may result in binding of the receptor in the same manner as L-alanine; however, this has yet to be studied. Interactions between contaminants and L-alanine are likely to occur at the carboxylic acid terminal and will likely result in decreases in the substituted L-alanine compounds acting as inhibitors as has previously been described above. Further studies investigating the changes to the structures of L-alanine and L-serine in the presence of contaminants should be conducted to determine if this mechanism of action is possible. Analysis of L-

alanine- and L-serine-like compounds using GC/MS have previously been described by Bruch et al. and these methods could be used to determine the presence and quantity of substituted L-alanine and L-serine structures [16].

Allosteric modification of receptors and non-specific effects on OSNs may have also occurred. Allosteric inhibition of amino acid receptors has previously been reported in channel catfish [70]. Li et al. found that OSNs that appear to detect multiple amino acids contain multiple binding sites, and when one of these sites is bound, modification of other sites occurs. As L-alanine appears to be more affected than L-serine during both 30 min and 60 d exposures, it is possible that allosteric modification of the L-alanine binding site occurred. A non-specific effect that may be affecting olfactory responses to amino acids may be increases of ions, such as Ca^{2+}, which alter the concentrations of intracellular calcium and may result in interference with secondary messenger systems that use cation channels [89].

Effects of treated reuse water on EOGs evoked by 17,20-βP

17,20-βP-evoked EOGs were decreased during both 30 min and 60 d exposures to treated reuse water. During a 30 min exposure to treated reuse water, EOGs evoked by the pheromone 17,20-βP were seen to decrease during a exposure to MF, CF/MF, and UV/H2O2/MF reuse, average decreases of 52, 30 and 39% occurring respectively. To date studies have focused on the pheromone PGF2α, however, PGF2α is a monocyclic pheromone, unlike 17,20-βP which is a polycyclic pheromone, and so mechanism(s) of action by which contaminants

decreased EOGs to PGF2α may differ with those of 17,20-βP. Also, no previous studies have examined the effects of treated reuse water on olfactory toxicity; however, studies on pesticides do exist. During 30 min exposures to 10 μg/L atrazine, 5 μg/L diazinon, 10 μg/L carbofuran, and 2 μg/L simazine, PGF2α responses in Atlantic salmon were decreased by 44, 49, 33, 28%, respectively [82-84, 131]. A 30 min exposure to a mixture of 1 μg/L atrazine and 1 μg/L simazine decreased PGF2α-evoked EOGs in Atlantic salmon by 30% [82]. The concentrations of atrazine, diazinon and carbofuran used in these experiments are comparable to the total concentration of contaminants found in MF reuse water; however EOGs were not decreased to the same magnitude, suggesting that multiple contaminants at this concentration were better able to decrease 17,20-βP-evoked EOGs than singular contaminants. A notable comparison can be made with potential to recover olfactory responses post-exposure. Recovery of PGF2α-evoked EOGs after a 30 min exposure to 1 μg/L diazinon in rainbow trout took 4.5 h, and only resulted in EOGs being 80% of the pre-exposure, while goldfish exposed to MF reuse for 30 min had 17,20-βP-evoked EOGs that returned to 97% of pre-exposure within 22 min post-exposure. This short period of recovery suggests that no permanent damage occurred to OSNs that detected 17,20-βP. In the study conducted by Kerr et al., it was demonstrated that CF/MF removal of cyclic carbon contaminants, such as pesticides, was not complete in reuse water [60]. The pesticides 2,4-D, dicamba, MCPA, and mecoprop had percent removal values of 58, 50, 45, and 42%, respectively, indicating that these components of reuse persist post-filtration. As cyclic organic contaminants have previously been

shown to impact fishes' ability to detect pheromones [83, 84, 131], it is possible that the aforementioned compounds and others of similar composition may cause the decreases seen in 17,20-βP evoked EOGs, possibly again acting as competitive agonists.

Throughout the 2012 summer exposure, EOGs evoked by 17,20βP decreased across all exposure groups including controls. These results suggest that contaminants within treated reuse water that have the ability to impair olfactory detection of the pheromone 17,20-βP persist regardless of treatment processes. Current data regarding the contents of tap water suggests that synthetic progestins such as norethindrone and levonorgestrel are non-detectable; however, progesterone itself is present at ng/L concentrations [64]. Studies examining the contents of FE wastewater have shown the presence of progesterones, progestins, and progesterone receptor antagonists at ng/L concentrations [63]. In two studies conducted by Sorensen et al. it was demonstrated that progesterone and progesterone derivatives have the ability to evoke EOG responses in male, female, and gonadally regressed male goldfish at concentrations in the ng/L range [109, 111]. As Sorensen et al. showed that progesterone and progesterone derivatives are both detectable and their detection can be inhibited by 17,20-βP, and so it can be assumed that these compounds are able to bind to ORs used to detect 17,20-βP. These contaminants present themselves as potential competitive inhibitors for ORs associated with 17,20-βP; however, data is lacking concerning the concentration at which inhibition occurs. As 17,20-βP is detectable at a concentration of 10^{-13} M (approximately 0.33 ng/L) and progesterone and its

derivatives were detectable at concentrations as low as 10^{-12} M, it is likely the presence of progesterone and its derivatives at low concentrations will impact the ability of fish to smell 17,20-βP. This information leads me to believe that progesterone and progesterone derivatives exist within MF, CF/MF and UV/H2O2/MF reuse at concentrations that are able to impair ORs associated with 17,20-βP. Flow-through tanks may provide a steady exposure to progesterone, and as such may result in a decrease in expression of the OR that they bind (OR expression will be down-regulated with constant exposure, as seen in goldfish during cross adaptation to PGF2α [110]).

Seasonal effects of treated reuse on EOG responses

When reuse water from the Gold Bar WWTP was screened for 68 potential xenobiotics by Kerr et al., it was found that 8 pesticides were present during the spring and summer (pooled samples from April-July of 2005); however, these pesticides were not present during the winter sampling period (pooled samples from January-April of 2006) [60]. The study also reported that contaminants measured throughout all seasons were highly variable in concentration between sampling dates. Studies investigating grey water pre-treatment have shown that variability between urban sources are high, even when types and amounts of PCPP used at each source was held constant [135]. I began the spring exposure period in April 2012 and ended it in May 2012 and began the summer exposure period in July 2012 and ended it in September 2012, so it is possible that pesticide concentrations within the reuse water increased throughout summer months that

were unmeasured by Kerr et al. I began fall exposure periods in September of 2011 and ended in October 2012, another set of dates untested by Kerr.et al. but with the potential to contain pesticides during the August and September time periods. Currently chemical analysis of treated reuse water is ongoing and data is unavailable to confirm this theory.

Edmonton does have an urban pesticide program in place for 2013 which describes pesticide use throughout the city on a monthly basis. The 2013 pest management programs including pesticide use-activity plan showed that pesticides used to control weeds were not implemented on school grounds until July and August. Pest control, which may include pesticides, for rodents began in May and continues throughout September, while pest control for insect pests of trees began in May and continues on through August. Pest control for yellow jackets, which may include pesticide use, began in June and continues on throughout September, while human pathogens at outdoor water sports events were only controlled for during June and July. This plan does not indicate the amount of pesticide used; however, one can assume that this would vary depending on the year and amount of pests present. The pest management programs including pesticide use-activity plan suggests that pesticides within urban waterways will increase during July and August and that different pesticides will be present during August and September when compared to April, May, and June. These increases and changes in pesticides present may account for the decrease in EOG seen during the summer exposure but not during the spring and fall sampling periods.

Previous studies have shown that pesticides are able to reduce EOG responses in a dose dependent manner, with increasing pesticide concentrations causing larger EOG reductions [54, 56, 84, 119, 121]. Decreases in L-alanine- and 17,20-βP-evoked EOG compared to controls during 60 d exposures appear to follow a seasonal trend that corresponds to the city's use of pesticides. EOGs evoked by L-alanine and 17, 20-βP were not statistically decreased from controls during the fall or spring exposure periods, but were statistically decreased during the summer exposure. This seasonal effect on EOGs may be at least partially explained by the seasonal introduction of pesticides.

Detoxification enzymes may prevent damage to olfactory tissue

During our study, it was not observed that any goldfish were completely anosmic. Increased expression of olfactory receptors as a result of contaminant exposure may have occurred; however, data can neither confirm nor refute this hypothesis. Our lab did however use qPCR to determine the presence of the detoxification enzymes cytochrome p450 (CYP) 1A and 3A as well as GST α, θ, and π within olfactory rosettes. Appendix E shows that increased expression of CYP 1A was observed in goldfish exposed to treated reuse. Goldfish exposed to CF/MF reuse displayed a 2-fold increase in CYP 1A mRNA expression compared to controls, while goldfish exposed to both MF reuse and UV/H2O2/MF reuse displayed a 4-fold increase in CYP 1A mRNA expression. Detoxification enzymes in the CYP 1A class are responsible for the breakdown of xenobiotics within vertebrates and have previously been found in the olfactory epithelium of topminnows exposed to

benzo [a] pyrene [86, 106]. The comparable increase in CYP 1A mRNA in goldfish exposed to both MF reuse and UV/H2O2/MF reuse suggests that compounds that elicit an increase in CYP 1A detoxification enzymes were still present post treatment in UV/H2O2/MF reuse at or near the same concentration as those found in MF reuse. These compounds may be the reason that olfactory impairment did not differ between goldfish exposed to these treatments.

Effects of exposure to a NCM

In order to identify if olfactory effects could be attributed to contaminant classes found in reuse water, a mixture containing the major classes was created (NCM). No such previous work had been conducted; however, I hypothesized that there would be greater reductions to EOGs evoked by amino acids and pheromones due to the high concentration of the contaminants used to represent each class. I did not see this, as EOGs evoked by both amino acids and pheromones during an exposure to a NCM were not as affected as those during an exposure to MF reuse. The 0.1% NCM, in which the contaminants were 10-fold higher than the concentration at which they exist in MF reuse, did not cause significant decreases in EOG responses to either amino acids or pheromone. This suggests that other components found within reuse water are able to impair detection of these odorants via olfaction. This also means that some of the reuse water components we analyze for may be unrelated to toxicity, or at least neurotoxicity.

Behavioral responses to the introduction of a pulse of treated reuse or NCM

Goldfish do not appear to respond to the introduction of treated reuse or a NCM. This was not expected as previous studies found fish avoidance and attraction to contaminants, both behaviors requiring increased movement [5, 51, 52, 122]. Decreases in distance travelled post-introduction of a pulse of treated reuse or a NCM, as well as during introductions of a solvent control and dechlorinated municipal water, were observed during our study. These results suggest that goldfish did not respond behaviorally to the introduction of treated reuse or a NCM, but they did respond to the stimulus (water addition). This has been found before in water-exposed zebrafish [105]. It should be noted that the arena used for this testing was not a preference/ avoidance trough, and perhaps the decrease in movement occurred as goldfish had no place to move away from the contaminant source.

Effects of an exposure to treated reuse or a NCM on behavioral responses to L-alanine

Behavioral responses to L-alanine have previously been described as a searching behavior in cyprinids and so I expected to see an increase in distance travelled post-introduction of L-alanine in control goldfish; however, the EOG results previously presented (i.e. reduced detection of L-alanine) suggested that this same behavior could be reduced or absent in goldfish exposed to treated reuse or a NCM for 30 min, or in goldfish exposed to treated reuse over a 60 d period [39]. This was not observed, as a decrease in distance travelled post-introduction of L-

alanine in controls and all exposed goldfish was seen. No difference was observed between controls and exposed goldfish after the introduction of L-alanine, suggesting that a 30 min exposure to MF, CF/MF, UV/H2O2/MF reuse, a NCM, or a solvent control do not impair the behavioral response to L-alanine. This same result was obtained from goldfish throughout the 60 d exposure to treated reuse water. A study conducted by Hamdani et al. suggested other behaviors associated with L-alanine introduction including snapping and mouth opening [39]. They also suggested that three different searching behaviors exist, including swimming to the surface, bottom food searching, and exploring. As distance travelled does not seem to be affected by exposure to contaminant sources, I suggest that a more detailed analysis of the behavior be conducted to determine if a difference exists within each of the individual categories outlined by Hamdani et al. This analysis may give more detailed description of changes in behavior as a result of exposure to a contaminant source.

Conclusions

Goldfish were able to detect treated reuse water and a NCM in a concentration-dependent manner. This suggests that contaminants found in each have the ability to affect olfaction. A 30 min exposure to both treated reuse and a NCM resulted in decreased EOG responses; however, the responses returned, suggesting again that contaminants within both sources have the ability to bind receptors associated with natural odorants. A 60 d exposure to treated reuse water resulted in a decreased ability to detect L-alanine, suggesting that compounds found within

reuse water are able to impair olfaction by damaging OSNs. Increases in CYP 1A mRNA expression within olfactory tissue may be part of the reason why we do not see complete loss of olfaction during a 60 d exposure. Behavioral experiments do not suggest any change in behavior as a result of introduction or exposure to treated reuse water or a NCM over 30 min period. Behavioral experiments also do not suggest that a change in behavior associated with L-alanine occurred during a 60 d exposure.

Chapter 3: Determining sub-lethal toxicity of oil sands process affected water as measured by olfactory impairment to natural odorants in rainbow trout (*Oncorynchus mykiss*)

Introduction

The Athabasca oil sands located in northern Alberta are the world's second largest heavy oil deposit and contain an estimated 1.3 billion barrels of oil. Of those 1.3 trillion, 173 billion have been deemed recoverable, i.e. they can be recovered using open pit mining because the overhead (amount of soil, rock, etc) does not exceed 50m [12, 72]. The component of the oil sands which is sought after is known as bitumen, a viscous substance that is bound to sand particles and once extracted is used to manufacture synthetic crude oil. To extract bitumen from the oil sands an alkaline hot water extraction process is used, a process that causes naphthenates within the oil sands to act as a surfactant and help remove bitumen from sand particles. The extraction process uses approximately 1 m^3 of water per ton of oil sands; however, due to water recycling an average of 0.3-0.4 m^3 is used to extract each barrel of oil [76].

Oil sands process-affected water

The water used in the extraction process, known as oil sands process-affected water (OSPW), becomes contaminated with sand, clay, unrecoverable bitumen, organic compounds such as naphthenic acids (NAs), sulphate, ammonia, aromatic hydrocarbons, and metals such as lead, copper, nickel, chromium and zinc [12,

72]. OSPW is toxic to fish, mammals, plants, zooplankton and bacteria, and as such is under a no release policy [12, 19, 23, 24, 38, 50, 73, 132]. OSPW is stored in large tailings ponds intended to stop release into groundwater and the close by Athabasca river; however NA associated with OSPW have been identified in tributaries of the Athabasca [97]. Deposition of compounds found within OSPW occurs via air and water and has been shown to occur up to 80 km from tailings pond sites [59]. Currently the Athabasca oil sands have yielded over one billion m^3 of OSPW from the Syncrude operation alone, with numbers from Suncor's operation not being available [12, 19]. Reclamation of the water contaminated with OSPW is required by all companies prior to termination of their land lease, and as such investigation into the cause of OSPW toxicity and methods on how to treat it is highly important. While OSPW contains high concentrations of ammonia and aromatic hydrocarbons, current literature suggests that its toxicity is not due to increases in these compounds but instead increases in NAs [73, 132].

Naphthenic acids

Naphthenic acids are a highly diverse group of alkyl-substituted aliphatic and alicyclic carboxylic acids that can be described by the formula $C_nH_{2n+Z}O_2$, where 2n is the number of carbon atoms and Z is a negative number that indicates the number of hydrogen atoms lost during ring formation [14]. In their simplest form, NAs resemble fatty acids; however, they can also be mono-, di-, tri-, and tetra-cyclic. NAs have a wide range of chemical properties, boiling points ranging from 250-350°C and molecular weights ranging from 200-700 g/mol. The dissociation

constants of NAs are similar to those of carboxylic acids, in the order of 10^{-5} to 10^{-6}. Different sources of OSPW have highly variably types of NAs within them, and until recently, discerning which NAs were present in different sources of OSPW was not possible. However, a new method developed by Rowland et al. that uses gas chromatography × gas-chromatography- time of flight- mass spectrometry can determine structures and quantities of individual NAs within OSPW [98].

Whole OSPW toxicity

Multiple studies have explored OSPW toxicity to a variety of aquatic organisms and have shown that OSPW toxicity varies greatly between sources. MacKinnon and Boerger used the Microtox assay to show that acidifying OSPW could reduce its toxicity [12]. The Microtox assay measures changes in luminescence emitted by the luminescent bacteria *Vibrio fischeri* that occur after an exposure to contaminants at varying concentrations for 20 min, decreased luminescence from pre-exposure correlating with mortality. They determined that the concentration inhibiting 20% luminescence (IC_{20}) was 43% OSPW; however this result should be interpreted cautiously as an IC_{20} was chosen arbitrarily due to the inability to achieve a concentratin inhibiting 50% luminescence (IC_{50}) . In contrast, these researchers found that 96 h exposures caused mortality in 50% of the test groups (LC_{50}) for a fish species (rainbow trout) and an invert (water flea, *Daphnia magna*) were far lower, at 7 and 2%, respectively. This suggests bacteria are far more resistant to OSPW toxicity. Munkittrick and Power determined the

correlation of effect concentrations associated with Microtox and LC_{50} of rainbow trout, fathead minnows (*Pimephales promelas*), and daphnia [85]. They showed that correlations between Microtox and lethality tests were high for complex contaminant mixtures, such as municipal effluents, industrial wastes, and organic contaminants, and low for mixtures containing metals and inorganics. While waste waters associated with oil production generally had a high correlation between lethality tests and the Microtox assay, all studies examining OSPW observed that Microtox was a less sensitive measure than lethality testing. A study conducted by Toor et al. showed that rainbow trout exposed to a Syncrude OSPW sample containing 73 mg/L NAs had an 96 h LC_{50} of 67% (v/v) [124]. Since many OSPW toxicity studies have linked toxicity to NA concentration, studying NA toxicity without other components of OSPW (e.g. metals) is of interest in the scientific community [73, 132].

NA toxicity

Studies on the toxicity of NAs have focused on a myriad of species, but due to the large amount of OSPW created in the extraction process that must one day be remediated, have also begun studying the ways in which OSPW can be treated to lower NAs and toxicity. A study conducted by Madill et al. used the Microtox assay to show that OSPW toxicity can be attributed to NAs, as fractions of OSPW containing NAs evoked no luminescence [73]. Further confirmation that NAs are a major contributor to OSPW toxicity had previously been demonstrated by Wong et al., who showed that filtration of OSPW using granular activated carbon

removed NAs and the toxicity to OSPW [132]. Specifically, Wong et al. exposed rainbow trout to OSPW before and after filtration with granular activated carbon, and found survival rates of 20, 50, and 45% pre-treatment and 97, 100, and 99% post-treatment, respectively [132]. Studies conducted by Dokholyan and Magomedov determined the 10 day LC_{50} for NAs was 25 mg/L for two month old chum salmon (*Oncorhynchus ketachum*), 50 mg/L for two month old kutum (*Rutilus kutum*) and roach (*Rutilus rutilus*), and 2 year old Russian sturgeon (*Acipenser gueldenstaedtii*), and 75 mg/L for two year old roach and round goby (*Neogobius melanostomus*) [23, 24]. These data together indicate that NA toxicity is in the mg/L range and can be influenced by life stage and species. These researchers also determined that the zooplankton (*Nephargoides maeoticus*) could only tolerate NAs concentrations of 0.15 mg/L and as such suggested this be the maximum allowable concentration of NAs within waters (note: these data indicate that NAs may exert indirect toxicity to fishes by killing their food source). At the sub-organismal level, NA toxicity has also been recorded. For example, a study by Hagen et al. showed immune function of goldfish (*Carassius auratus*) exposed to > 5 mg/L NAs had increased expression of pro-inflammatory cytokines and decreased ability to control infections of the trypanosome parasite *Trypanosoma carassii* [38].

In sum, toxicity associated with OSPW is in part attributable to the NA concentration, as studies that have focused on NA removal have shown that OSPW post-NA removal is less toxic than the NA fraction. Invertebrates are more

sensitive to NAs than larger vertebrates; however, NA concentrations found within OSPW from Alberta tailings ponds are toxic to multiple fish species.

Ozonation of OSPW reduces toxicity and increases biodegradability of NAs

Detoxification of OSPW is essential for companies such as Syncrude and Suncor, which are required to return their leased land to conditions that meet or exceed those in which they acquired them. Ozonation is one method that is currently showing promise for decreasing OSPW toxicity by breaking down and oxidizing NAs [31, 103]. Gamal El-din et al. showed that an 80 mg/L ozone treatment resulted in a 64% decrease in acid extractable organics, a group of compounds that includes NAs, oxy-NAs and other acid organics [31, 37]. A study by Scott et al. showed decreased toxicity of ozonated OSPW (O3OSPW) in comparison to OSPW as determined using the Microtox assay [103]. In their study, the NA concentration in OSPW was 59 mg/L, and this had an IC_{20} of 23% (v/v %). After 20 min of ozonation, the IC_{20} was increased to 31% (v/v %), and after 50 min, it was <100% (v/v %). The elimination of toxicity at 50 min came as a surprise as the NA concentration was still 20 mg/L, and so results showed that toxicity of NAs did not mean that NAs had to be completely removed or destroyed. Gamal El-din et al. also showed reduced toxicity using the Microtox assay that agreed with results found by Scott et al., with 150 mg/L ozonation resulting in an IC_{20} <100%. He et al. showed that fathead minnow development was less affected by O3OSPW than OSPW, and activated carbon filtered OSPW [50]. Not only was hatching success at >120 h greater in O3OSPW-exposed fish than those exposed

to OSPW, incidences of hemorrhaging, pericardial edema, and spinal malformations did not differ from control. He et al. determined that gene transcripts (mRNA) for enzymes involved in detoxification, specifically CYP 3A, GST and SOD were increased in comparison to controls in embryos exposed to OSPW but not in those exposed to O3OSPW. It was also seen that reactive oxygen species were present in higher concentrations in embryos exposed to OSPW in comparison to those exposed to O3OSPW. A study by Garcia-Garcia et al. showed a decrease in impact on the mammalian immune system by O3OSPW compared to OSPW [32]. Exposure of adult mice to OSPW containing 100 mg/L NAs and O3OSPW containing 25 mg/L NAs via gavagei showed that ozonation of OSPW abolished OSPW's ability to alter cytokine gene expression.

Studies investigating methods to reduce NA concentrations within OSPW have determined that toxicity is decreased by lowering the overall NA concentration. Gamal El-din et al. used the carbonaceous biochemical oxygen demand as a measure of biodegradation and found an increase in 8 mg/L to 25 mg/L O_2 after OSPW was treated with 148 mg/L ozone, suggesting increased biodegradation. Martin injected OSPW, 54% O3OSPW and 73% O3OSPW samples with microbes endogenous to OSPW tailings ponds at concentrations found in the ponds, and reported that microbial biodegradation of OSPW increased if ozonation was applied [74]. Martin et al. found that degradation rates differed between NAs with different Z numbers post ozonation, which agrees with

findings by Han et al.'s upon examination of commercial and synthetic NAs (Table 3.1) [41].

Olfactory toxicity due to OSPW and O3OSPW exposre

Fish olfaction has been previously used to study sub-lethal effects of contaminants including pesticides [35, 40, 42, 43] and metals [22]. The remediation of OSPW tailing ponds will likely result in its return to aquatic environments, and this would allow fish to be exposed to OSPW. Olfaction is essential to behaviors such as foraging, predator avoidance, homing/migration and mating, and can be impacted by contaminants at and below μg/l concentrations [46, 77, 87, 134]. It is therefore important to study the impacts of contaminants found within OSPW and O3OSPW on fish olfaction as its return to waters containing fish may impact olfactory mediated behaviors.

Study Objectives and Hypothesis

My study aimed to use fish olfaction as a measure of sub-lethal toxicity in rainbow trout exposed to OSPW and O3OSPW. Rainbow trout were used in these experiments as opposed to goldfish as they are a more ecologically relevant species to the region where OSPW contamination of water is likely to occur. I hypothesized that rainbow trout would be able to detect both OSPW and O3OSPW, as NAs within the mixture have similar structures to natural odorants, and polycyclic contaminants such as those found in pesticides have previously been found to be detectable [119, 120]. I also hypothesized that both 10% OSPW

and 10% O3OSPW would result in a altered EOGs throughout a 30 min exposure; however, the decrease would cease immediately post exposure as has been seen with exposure to other organic contaminants, e.g. those found in pesticides and organic contaminant mixtures. I hypothesized that EOGs from 10% OSPW and 10% O3OSPW exposed rainbow trout would be altered after a 7 d exposure, as these concentrations of organic contaminants (0.5-4 mg/L) have previously been found to cause lasting decreases in EOG post 7 d exposure. While current literature suggests that ozonation has the potential to reduce toxicity of OSPW, I do not think that olfactory tissue will be able to cope with such high concentrations of organic contaminants, regardless of the source and structure. My hypotheses were based on studies that have previously investigated sources of both organic and metallic contamination and their effects on olfaction, which show that above mg/L concentrations both metals and organic contaminants decrease EOGs evoked by TChA and L-serine in teleost fish species [22, 36, 49, 101, 119, 121, 123].

My overall objectives were to answer the following three questions:

1. Can rainbow trout detect OSPW and O3OSPW via olfaction?

2. Does a 30 min exposure to 1% OSPW, 10% OSPW, or 10% O3OSPW cause a decrease in EOGs evoked by natural odorants?

3. Does a 7 d exposure to 1% OSPW, 10% OSPW, or 10% O3OSPW cause a decrease in EOGs evoked by natural odorants?

	Fold increase in 50% microbial biodegradation in comparison to OSPW	
Z number	**54% ozonated OSPW**	**73% ozonated OSPW**
-2	**2.5**	**3.3**
-4	**1.5**	**1.6**
-6	**1.4**	**1.5**
-8	**1.9**	**2.5**
-10	**NA**	**NA**
-12	**NA**	**NA**

Table 3.1. Residual NA microbial biodegradation kinetics adapted from Martin et al. (2010)[74]. Microbial biodegradation of NAs to reach 50% of the initial concentration was seen to increase in OSPW samples treated with both 54 and 73% ozonation.

Behavior endpoints were also tested for rainbow trout exposed to OSPW; however this work was conducted by other members in the lab and was not a component of my thesis work. Behavior data was unavailable at this time as it was being used to construct the three dimensional behavior analysis previously discussed in chapter 2.

Materials and Methods

Fish

Rainbow trout eggs were obtained from Raven Brood Trout Station (Caroline, Alberta) and hatched during December 2011 at the University of Alberta. Rainbow trout were acclimated to flow through tanks containing 15°C dechlorinated municipal water from the city of Edmonton. Rainbow trout were kept on a 16:8 light: dark photoperiod and were fed twice daily. Experiments were approved by the University of Alberta Animal Care Committee (#7301003) and followed the Canadian Council of Animal Care guidelines.

OSPW and O3OSPW

Non-aged OSPW and O3OSPW containing 40mg/L and 5mg/L NAs respectively were obtained from Syncrude (Mildred Lake samples, Alberta, Canada).

Na+ / Cl- control

Concentrations of Na+ and Cl- were determined in OSPW samples as these have previously been identified as major ionic constituents contributing to the salinity

of OSPW [58]. Sodium concentrations were measured using atomic absorption spectrophotometry (AAS), and chloride concentrations were determined using a Pharmacia Biotech Ultrospec3000 UV/Visible Spectrophotometer at an optical density of 480 nm [128]. Samples used in analysis were diluted seven parts sodium nitrate to one part OSPW or O3OSPW to stay within detection limits. Standard curves for both sodium and chloride (Figure 1, Figure 2) were used to determine concentrations in concentrated and diluted samples of OSPW and O3OSPW.

The sodium chloride control contained sodium and chloride concentrations similar to those found in OSPW (Table 3.3). To create the control water, NaCl and $NaSO_4$ were added to dechlorinated municipal water. Sodium and chloride concentrations were measured and accounted for in the dechlorinated municipal water to ensure concentrations did not exceed those found in OSPW.

	Chloride		Sodium	
	µM	mg/L	µM	mg/L
H_20	239	8.47	51	8.207
1% OSPW	309	10.95		57.74
10% OSPW	3090	100.95		577.4
100% OSPW	30900	1009.5		5774

Table 3.3. Concentrations of Chloride and Sodium found in dilutions of OSPW.

Concentration response curves

Odorants

Concentration response curves for odorants were conducted using the same methods described in Chapter 2. The odorants used in these concentration response curves were L-serine and taurocholic acid (TChA).

OSPW, O3OSPW, and NaCl control

Concentration responses curves to OSPW, O3OSPW, and the NaCl control were conducted in the same manner as those for reuse water treatments in Chapter 2.

EOG responses to dilutions of 100, 50, 10, 1, 0.1, 0.001% of OSPW, O3OSPW, and the NaCl control were recorded in triplicate from each fish. EOGs were recorded from lowest concentration to highest concentration.

Exposures

To determine the effects of a 30 min exposure, OSPW was diluted to 1 and 10%, and O3OSPW was diluted to 10% of the original concentration using dechlorinated municipal water. Exposures followed methods for 30 min exposures previously described in chapter 2A. 10^{-4} M L-serine and 10^{-5} M TChA were used as odorants throughout the exposure.

To determine the effects of 7 d exposure to OSPW or O3OSPW, rainbow trout were placed in 20 ga tanks containing one of aquatic facility water (control), a 1%

OSPW solution, a10% OSPW solution, a 10% O3OSPW solution, or a solution containing Na+ and Cl- concentrations equivalent to those found in a 10% OSPW solution (sodium and chloride control). Static renewal of 50% of the total tank volume began 24 h after the start of exposure and was performed daily. Exposure tanks were placed in a flow through water bath to maintain a temperature of 14°C and photoperiods and feeding cycles remained the same as previously stated (University of Alberta, Edmonton).

Electro-olfactograms

EOGs were measured from olfactory sensory neurons using methods described by Hara and Evans [27] and equipment described by Sorensen, Hara, and Stacey [109], previously described in chapter 2. Briefly, fish were anesthetized using a 150 mg/L tricaine methanesulfonate solution (Syndell, Vancouver, BC). Upon cessation of opercular pumping, fish were transferred to a water bath containing 15°C water and the anesthesia was delivered over the gills at a concentration of 75 mg/L. The olfactory rosette was exposed by surgically removing the covering of the nares, and olfactory tissue was perfused with dechlorinated municipal tap water delivered through a Teflon feed at a rate of 7 mL/min. Ag-AgCl electrodes filled with gelatin containing 0.6% (m/V) NaCl were used to record differential EOGs. One electrode was placed slightly adjacent to the raphe close to the surface of the lamellae which was third from the bottom of the olfactory rosette and the second electrode was placed in the water bath. Following electrode placement, tissue was allowed to acclimate to background water before pulsing of odorants

commenced. A computer controlled solenoid valve was used to control the input of the background water feed, switching of the valve delivered a 2 s pulse of odorant or contaminant from a second line. EOGs were amplified 100× using a DAM50 differential amplifier (World Precision Instruments, Sarasota, FL) and signals were digitized for 10 s after. Peak height was compared to the pre-pulse baseline to quantify odorant or contaminant responses.

Statistical Analysis

Concentration response curves

Responses to all odorants and contaminants were presented as the recorded EOG values (mV). Differences among concentrations of the odorants, L-serine and TChA, were tested using a one-way repeated measure (RM) analysis of variance (ANOVA). Differences among concentrations of OSPW, O3OSPW and NaCl control were tested using a one-way RM ANOVA followed by Holm-Sidak post-hoc test. Differences between OSPW, O3OSPW, and NaCl control concentrations were tested using a two-way RM ANOVA followed by Holm-Sidak post-hoc test.

For each fish in 30 min exposures, exposure and post-exposure EOG values were determined as the percent of the average of the first three pre-exposure EOG values. Differences between controls and exposure groups were tested using a two-way RM ANOVA followed by Holm-Sidak post-hoc test. The percent change was also determined between controls and exposed fish, when a significant difference was found. The percent change was calculated using the

formula: % change = 100 - (% pre-exposure EOG exposed / % pre-exposure EOG control) * 100.

For each fish in 7 d exposures, EOG values were determined as the average of the first three responses recorded and expressed as mV. Differences between controls and exposure groups were tested using a one-way RM ANOVA followed by Holm-Sidak post-hoc test.

Results

Na+ / Cl- analysis

A linear regression model was fitted to the spectrophotometer measurements taken from known, nominal chloride concentrations. The equation of the line was y = 0.0021 + 0.007x, with y being the optical density and x the concentration in mg/L; the equation R^2 value was 0.994 (Figure 3-1). A linear regression model was fitted to atomic absorption spectrophotometer measurements taken from known Na+ concentrations. The equation of the line was y = 0.019 + 0.096x, with y being the absorbance and x the concentration in mg/L, with an R^2 value of 0.997 (Figure3-2).

The 1% dilutions of OSPW and O3OSPW were found to be within the range of the standard curve, and so these dilutions were used to determine the amount of Na+ and Cl- to be included in the control. 1% OSPW and 1% O3OSPW had OD (480) of 0.033 and 0.030, respectively, and emission values of 0.807 and 0.618, respectively. As 1% OSPW was found to have a higher Na+ concentration, its

values were used to create the NaCl control. Results were extrapolated to create a control representative of 10% OSPW, and as such contained 101 mg/L Cl- and 578 mg/L of Na+.

Concentration response curves for odorants

L-serine and TChA evoked EOGs in a concentration-dependent manner, with EOGs increasing with increasing odorant concentrations ($F_{3,55} = 15.7$, $p < 0.01$; Figure 3.3). EOGs evoked by 10^{-3} and 10^{-5} M L-serine (mean = 9.23 ± 1.64 mV, 3.06 ± 0.62 mV, respectively) were statistically increased by 550 and 110%, respectively, compared to those evoked by 10^{-7} M (mean = 1.43 ± 0.38 mV; p = 0.01, < 0.01, respectively) (Figure 3.3). EOGs evoked by concentrations of 10^{-3} and 10^{-5}M (mean= 9.73 ± 0.81 mV, 5.97 ± 0.57 mV, respectively) were statistically increased by 560, 480, and 310%, respectively, compared to those evoked at 10^{-9} M (mean= 1.47 ± 0.55 mV; p = < 0.01, < 0.01, < 0.01, respectively) (Figure 3.3).

L-serine and TChA-evoked EOGs compared at the same concentrations differed with each other ($F_{,355} = 15.7$, $p < 0.01$; Figure 3.3). TChA-evoked EOGs at 10^{-5} M were statistically increased by 110% compared to L-serine-evoked EOGs at the same concentration ($p < 0.01$), while TChA-evoked EOGs at 10^{-7} M were numerically increased by 28% compared to L-serine-evoked EOGS at the same concentrations. These results suggest that TChA is a slightly more potent odorant than L-serine for rainbow trout.

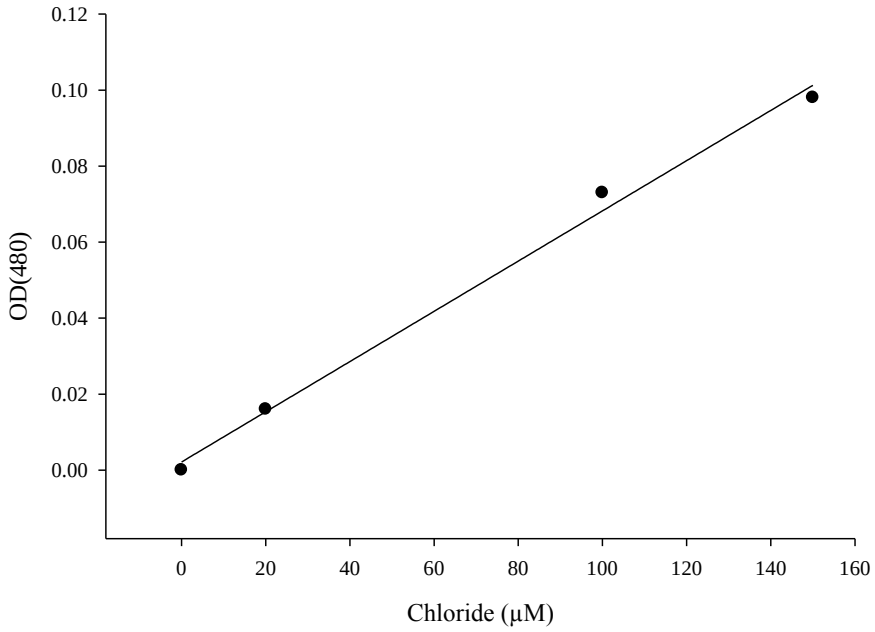

Figure 3.1. Cl- standard curve as determined using a spectrophotometer with an optical density of 480nm. Single readings of standards containing 20µM, 50 µM, 100 µM and 150 µM chloride were measured. The equation of the line is y= 0.0021 + 0.007x, and has an r^2= 0.994.

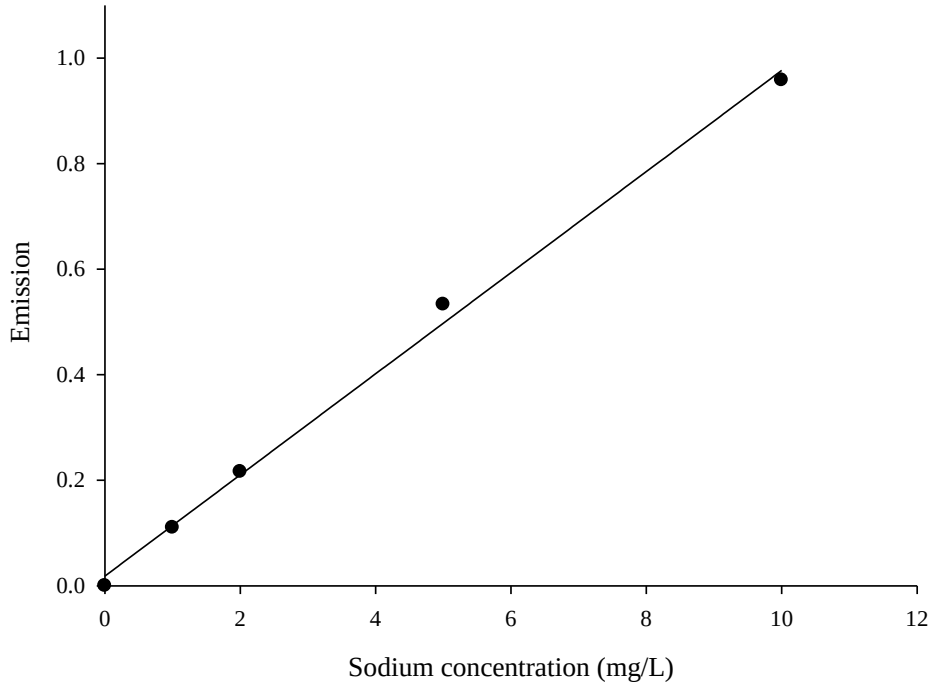

Figure 3.2. Na+ standard curve determined by atomic absorption spectrophotometry. Single readings from sodium solutions with known concentrations of 1, 2, 5 and 10 mg/L were measured. The equation of the line is $y = 0.019 + (0.096)\ x$, $r^2 = 0.997$.

Concentration Response Curves for OSPW, O3OSPW, and Sodium and chloride control

OSPW-evoked EOGs increased in a concentration dependent manner, with EOGs increasing with increasing concentrations of NAs ($F_{5,35} = 71.2$, $p < 0.01$; Figure 3.4). EOGs evoked from rainbow trout using OSPW containing 40, 20 and 4 ppm NAs (mean= 6.37 ± 1.66 mV, 5.44 ± 1.65 mV, 3.33 ± 1.04 mV, respectively) were statistically increased by 910, 770 and 430% respectively compared to those evoked by OSPW containing 0.004 ppm NA (mean= 0.63 ± 0.28 mV; p = 0.01, 0.01, <0.01, respectively), while those evoked by OSPW containing 2 and 0.4 ppm NAs (mean = 1.60 ± 1.11 mV , 1.21 ± 0.54 mV, respectively) were numerically increased by 160 and 92%, respectively, compared to those evoked by OSPW containing 0.004 ppm NA.

O3OSPW-evoked EOGS increased in a concentration dependent manner, with EOGs increasing with increasing concentrations of NAs ($F_{5,34} = 247$, $p < 0.01$) (Figure 3.4). The EOGs evoked from rainbow trout using O3OSPW containing 5, 2.5, 0.5 and 0.25 NAs (mean = 6.68 ± 0.26mV, 5.28 ± 0.57 mV, 2.35 ± 0.53 mV, 1.52 ± 0.41 mV, respectively) were statistically increased by 1300, 1000, 400, and 230% compared to those evoked by O3OSPW containing 0.0005 ppm NA (mean = 0.47 ± 0.23 mV) (p = 0.01, 0.01, <0.01, <0.01); those evoked by O3OSPW containing 0.05 ppm NA (mean=0.74 ± 0.29 mV) were numerically increased by 58% compared to those evoked by OSPW containing 0.0005 ppm NA.

Concentrations of Na+ and Cl- matched to concentrations found in dilutions of OSPW evoked EOGs in a concentration-dependent manner, with EOGs increasing with increasing concentrations of Na+ and Cl- ($F_{5,34} = 51.3$, $p < 0.01$) (Figure 3.4). The NaCl control evoked EOGs at a dilution used to represent Na+ and Cl- concentrations found in OSPW containing 40, 20, 4, and 2 ppm NAs (mean = 15.5 ± 4.40 mV, 11.0 ± 2.99 mV, 6.24 ± 1.53 mV, 4.84 ± 1.51 mV, respectively) were statistically increased by 760, 510, 250 and 170% compared to those evoked by Na+ and Cl- concentrations found in OSPW containing 0.0004 ppm NAs (mean = 1.81 ± 0.35 mV) ($p = 0.01, < 0.01, < 0.01, 0.04$, respectively); those evoked by Na+ and Cl- concentrations found in OSPW containing 0.4 ppm NA (mean = 2.30 ± 1.05 mV) were numerically increased by 27% compared to those evoked by Na+ and Cl- concentrations found in OSPW containing 0.0004 ppm NA.

EOGs evoked by similar % dilutions of OSPW, O3OSPW, and the NaCl control differed between groups ($F_{10,106} = 13.6$, $p < 0.01$; Figure 3.5). The 100% dilutions of OSPW and NaCl control were previously expressed as 40 ppm NAs, and decreasing dilutions correspond to previously mentioned decreases in NAs. 100% dilutions of O3OSPW were previously expressed as 5 ppm NAs and decreasing dilutions correspond to previously mentioned decreases in NAs. EOGs evoked by the NaCl control at 100, 50, 10, and 5% dilutions were statistically increased by 59, 51, 47 and 67%, respectively, compared to EOGs evoked by OSPW at the same dilutions (p values $< 0.01, < 0.01, < 0.01, < 0.01$, respectively); NaCl control EOGs evoked by 1 and 0.01% dilutions were numerically decreased by 47

and 66%, respectively, compared to those evoked by OSPW at the same dilutions. EOGs evoked by the NaCl control at 100, 50, 10, and 5% dilutions were statistically increased by 57, 52, 62 and 69% respectively compared to EOGs evoked by O3OSPW at the same dilutions (p values < 0.01, < 0.01, < 0.01, < 0.01, respectively); NaCl control EOGs evoked by 1 and 0.01% dilutions were numerically decreased by 68 and 75%, respectively, compared to those evoked by O3OSPW at the same dilutions. These results suggest that EOGs evoked by OSPW and O3OSPW do not depend on the concentration of NA within the sample, but may depend on concentrations of ions such as Na+ and Cl-.

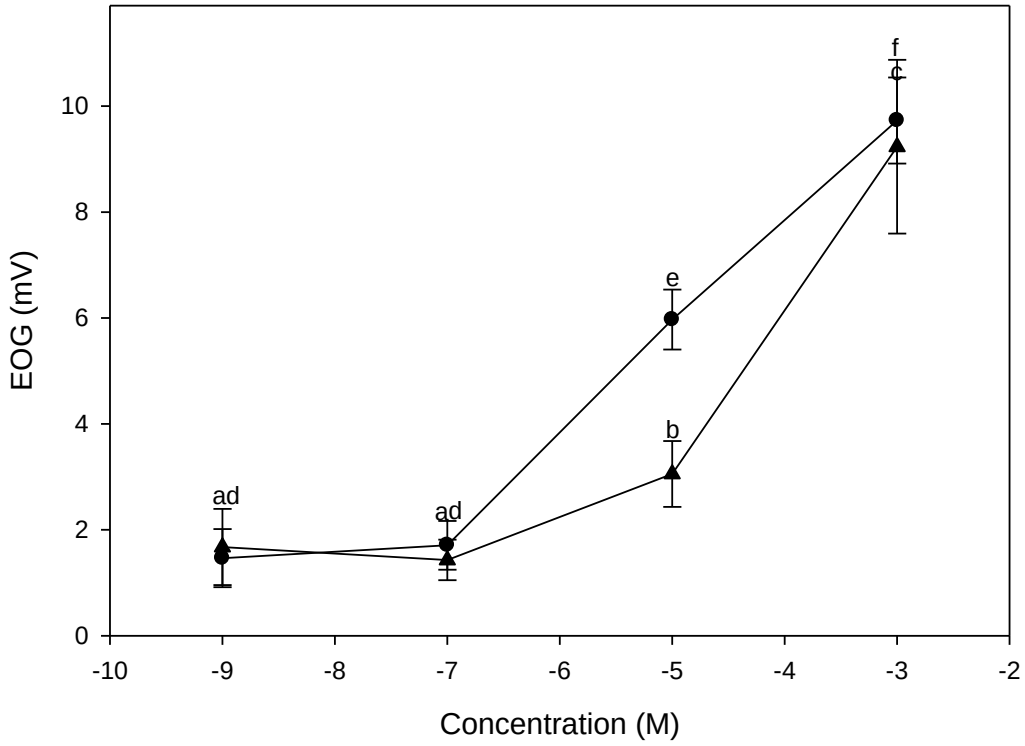

Figure 3.3. Concentration response curves for ▲L-serine (n=8) and ● TChA (n=7). EOGs were evoked by increasing concentrations of L-serine or TChA. Differences in EOGs between concentrations are denoted by different letters.

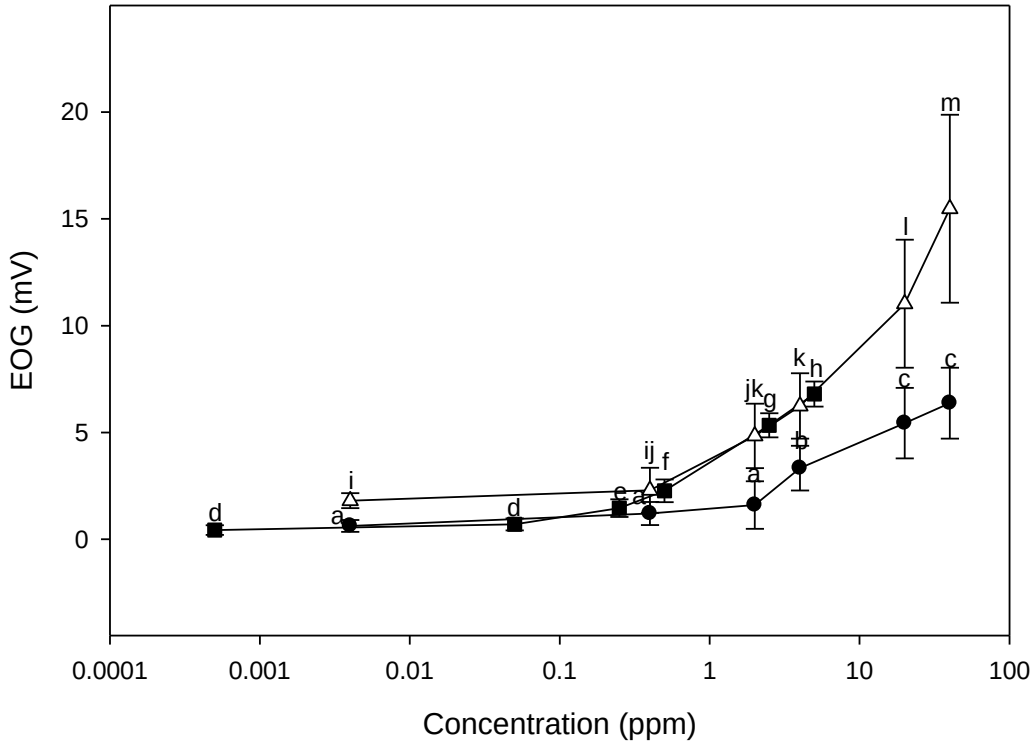

Figure 3.4. Concentration response curves for ●OSPW (n=6), ■O3OSPW (n=6), and the △NaCl control (n=7). EOGs were evoked by increasing concentrations of OSPW, O3OSPW, or the NaCl control. Differences between increasing concentrations were denoted using different letters.

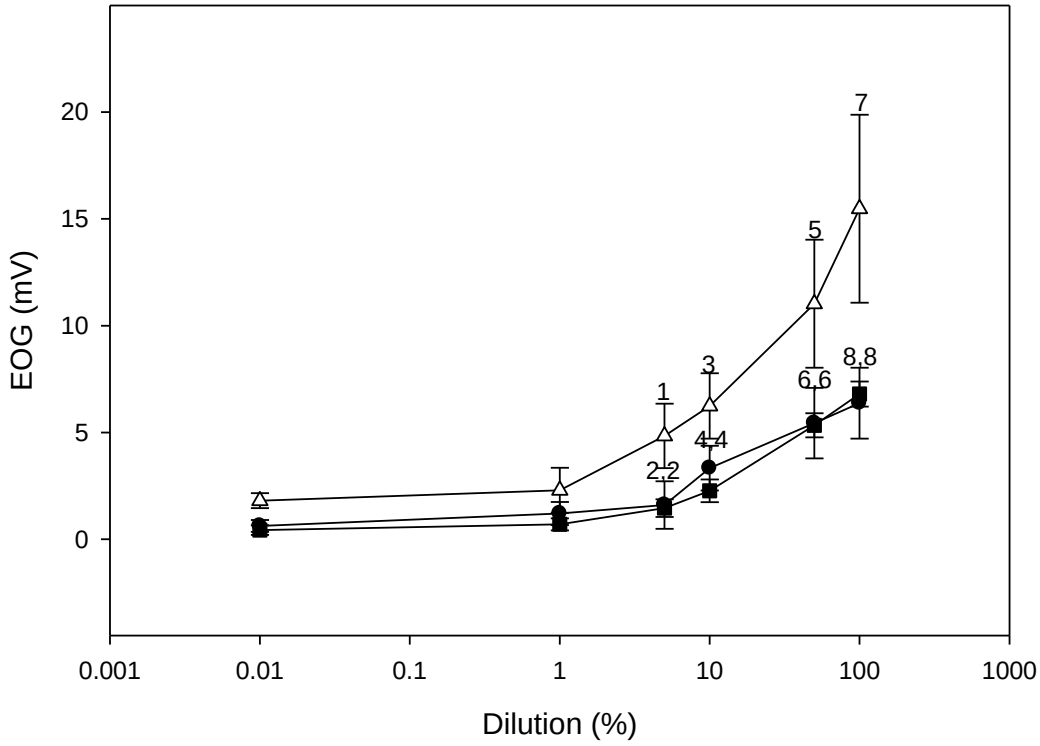

Figure 3.5. Concentration response curves for EOGs evoked by ● OSPW (n=6), ■O3OSPW (n=6), and the △NaCl control (n=7). EOGs were evoked by increasing concentrations of OSPW, O3OSPW, or the NaCl control. Differences between OSPW, O3OSPW, and the NaCl control are denoted using different numbers.

30 min exposures

L-serine

L-serine-evoked EOGs were compared to both pre-exposure and control EOGs throughout a 30 min exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl control. Pre-exposure EOGs did not differ between groups to be exposed to 10% OSPW, 1% OSPW, 10% O3OSPW, the NaCl control, or dechlorinated water (control) (means, in mV = 1.96 ± 0.64, 2.25 ± 1.08, 1.33 ± 0.54, 1.56 ± 0.43; 2.27 ± 1.51, respectively; $F_{4,35} = 1.80$, $p = 0.15$; Figure 3.6).

The EOGs evoked by 10% O3OSPW exposed rainbow trout were statistically decreased on average by 60% compared to pre-exposure throughout the 30 min exposure ($F_{11,338} = 1.96$, $p < 0.01$; Figure 3.7, Table 3.2). EOGs evoked by all other exposure groups returned to pre-exposure values during the recovery period.

The EOGs evoked by 10% O3OSPW exposed rainbow trout were statistically decreased on average by 72% compared to controls throughout the 30 min exposure ($F_{40,314} = 1.99$, $p < 0.01$) (Figure 3.7, Table 3.2). The EOGs evoked by 10% OSPW, 1% OSPW and the NaCl control exposed rainbow trout were numerically decreased on average by 42, 33 and 32%, respectively, compared to controls throughout the 30 min exposure (Figure 3.7, Table 3.2). All exposure groups returned to control values during the recovery period.

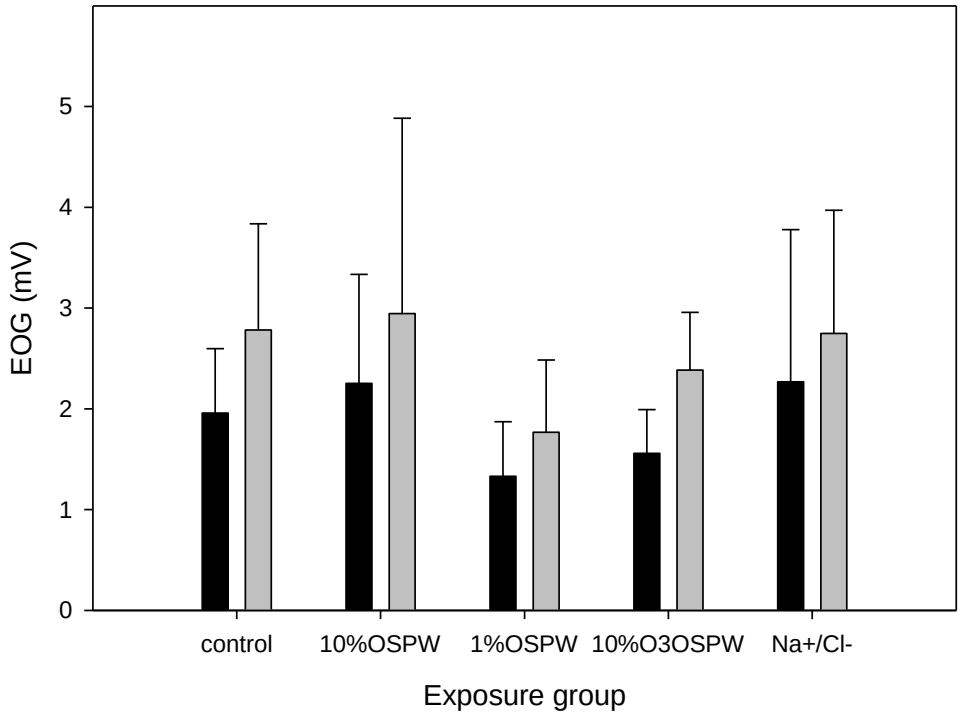

Figure 3.6. Pre-exposure EOGs evoked using ■ L-serine and ■ TChA. No differences were found between groups that would be exposed to 10% OSPW (n=6), 1% OSPW (n=11), 10% O3OSPW (n=7), the NaCl control (n=6) and dechlorinated water (control) (n=6).

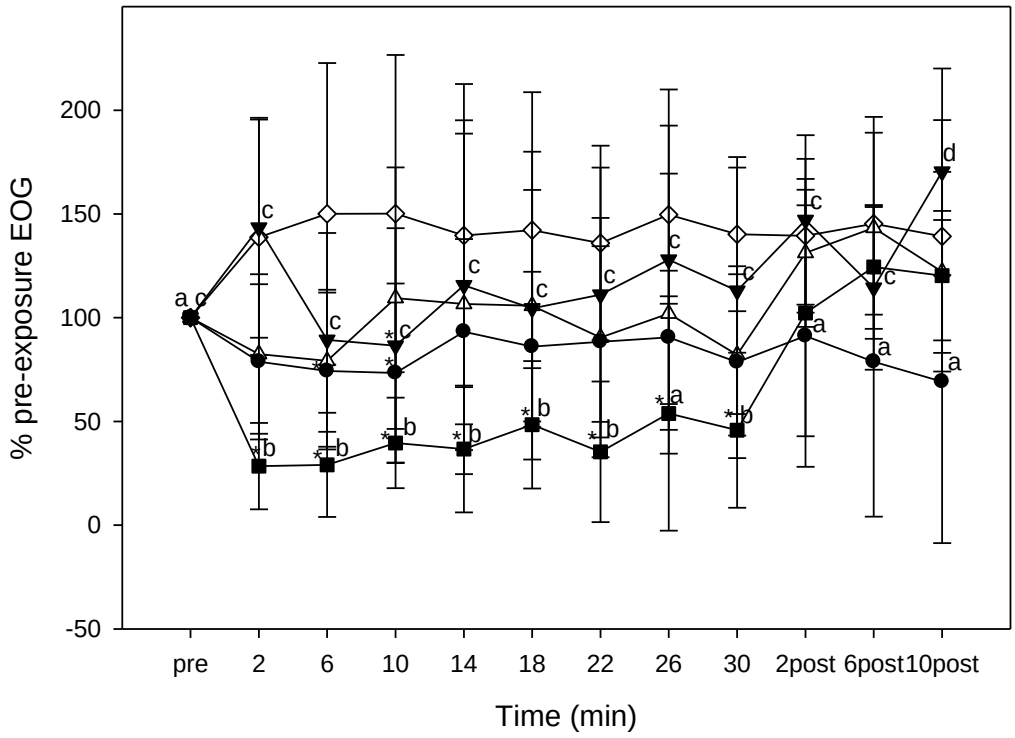

Figure 3.7. Effects of a 30 min exposure to ●10% OSPW (n=6), ▼1% OSPW (n=11), ■10% O3OSPW (n=7), △the NaCl control (n=6) and ◇dechlorinated water (control) (n=6) on EOGs evoked by L-serine. EOGs evoked from fish exposed to 10% O3OSPW are decreased during exposure; however return to pre-exposure values post-exposure. Differences between pre-exposure and EOGs evoked throughout the exposure were denoted using different letters and asterisk denotes differences from controls within that time point.

Table 3.2

Odorant	Exposure Group	Time point	Comparison with pre-exposure (p-value)	Comparisons with controls at the same time point (p-value)
L-serine	10% OSPW	2	0.95	0.15
		6	0.91	0.04
		10	0.67	0.02
		14	0.97	0.36
		18	0.97	0.18
		22	0.95	0.11
		26	0.96	0.06
		30	0.96	0.26
		2 post	0.94	0.52
		6 post	0.97	0.15
		10 post	0.98	0.67
	1% OSPW	2	0.99	0.44
		6	0.96	0.05
		10	0.71	0.02
		14	0.98	0.31
		18	0.99	0.20
		22	0.84	0.10
		26	0.95	0.19
		30	0.99	0.28
		2 post	0.14	0.90
		6 post	0.66	0.85
		10 post	0.01	0.77
	10% O3OSPW	2	0.01	< 0.01
		6	0.01	< 0.01
		10	0.02	< 0.01
		14	0.02	0.01
		18	0.02	0.01
		22	0.03	0.00
		26	0.13	0.01
		30	0.02	0.01
		2 post	0.35	0.59
		6 post	0.90	0.85
		10 post	0.43	0.99

	NaCl control	2	0.97	0.11
		6	1.00	0.06
		10	0.99	0.22
		14	0.99	0.26
		18	0.99	0.21
		22	1.00	0.14
		26	0.92	0.19
		30	0.96	0.26
		2 post	0.65	0.78
		6 post	0.22	0.94
		10 post	0.96	0.99
TChA	10% OSPW	4	0.45	0.03
		8	0.37	0.01
		12	0.45	0.02
		16	0.39	0.02
		20	0.45	0.03
		24	0.45	0.02
		28	0.48	0.03
		4 post	0.85	0.77
		8 post	0.96	0.48
		12 post	0.46	0.93
	1% OSPW	4	0.99	0.21
		8	0.98	0.14
		12	0.99	0.11
		16	0.99	0.25
		20	0.99	0.33
		24	0.98	0.14
		28	0.98	0.16
		4 post	0.18	0.99
		8 post	0.11	0.70
		12 post	0.01	0.25
	10% O3OSPW	4	0.09	0.01
		8	0.03	< 0.01
		12	0.25	0.03
		16	0.15	0.01
		20	0.09	0.01
		24	0.08	< 0.01

		28	0.14	0.02
		4 post	0.28	0.97
		8 post	0.87	0.58
		12 post	0.60	0.93
	NaCl control	4	1.00	0.21
		8	1.00	0.06
		12	1.00	0.15
		16	1.00	0.25
		20	0.99	0.23
		24	1.00	0.22
		28	1.00	0.09
		4 post	0.06	0.94
		8 post	0.01	0.71
		12 post	0.01	0.58

Table 3.2. Comparisons between exposure groups and pre-exposure or control EOG values throughout the 30 min exposure. Significance is denoted be p-values less than 0.5.

Taurocholic Acid

TChA-evoked EOGs were compared to both pre-exposure and control EOGs throughout a 30 min exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl control. Pre-exposure EOGs did not differ between groups to be exposed to 10% OSPW, 1% OSPW, 10% O3OSPW, the NaCl control, or dechlorinated water (control) (mean, in mV = 2.95 ± 1.05, 1.77 ± 0.72, 2.39 ± 0.57; 2.75 ± 1.22, respectively; $F_{4,35} = 1.47$, $p = 0.24$; Figure 3.6).

The EOGs evoked by 10% O3OSPW exposed rainbow trout were statistically decreased on average by 40% compared to pre-exposure throughout the 30 min exposure ($F_{10,314} = 1.99$, $p < 0.01$) (Figure 3.8, Table 3.2). The EOGs evoked by 10% OSPW exposed rainbow trout were numerically decreased on average by 26% compared to pre-exposure throughout the 30 min exposure (Figure 3.8, Table 3.2). EOGs evoked by goldfish exposed to 1% OSPW and the NaCl control were statistically increased on average by 50.3 and 50.5% respectively compared to pre-exposure during the recovery period ($F_{40,314} = 1.99$, $p < 0.01$; Figure 3.8, Table 3.2). EOGs evoked by all other exposure groups returned to pre-exposure values during the recovery period.

The EOGs evoked by 10% OSPW and 10% O3OSPW exposed rainbow trout were statistically decreased on average by 58 and 48%, respectively, compared to controls throughout the 30 min exposure ($F_{40,314} = 1.99$, $p < 0.01$) (Figure 3.8, Table 3.2). The EOGs evoked by 1% OSPW and the NaCl control exposed rainbow trout were numerically decreased on average by 24 and 29%,

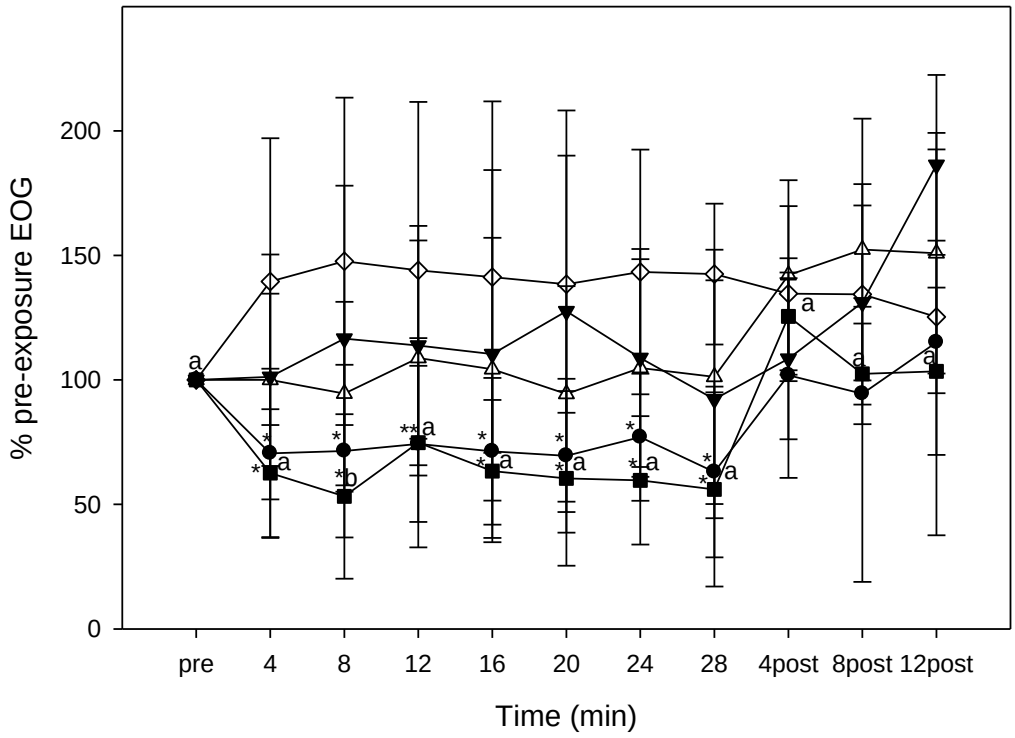

Figure 3.8. Effects of a 30 min exposure to ●10% OSPW (n=6), ▼1% OSPW (n=11), ■10% O3OSPW (n=7), , △the NaCl control (n=6) and ◇dechlorinated water (control) (n=6) on EOGs evoked by TChA. 10% OSPW and 10% O3OSPW decrease TChA EOGs throughout the exposure; however recovery does occur post exposure. Differences between pre-exposure and EOGs evoked throughout the exposure were denoted using different letters and asterisk denotes differences from controls within that time point.

respectively, compared to controls throughout the 30 min exposure (Figure 3.8, Table 3.2). All exposure groups returned to control values during the recovery period.

In general, a 30 min exposure to 10% O3OSPW results in the largest decreases in both L-serine and TChA-evoked EOGs. Decreases in EOG magnitudes, for both L-serine and TChA-evoked EOGs, during 30 min exposures can be ordered as follows: 10% O3OSPW > 10% OSPW > NaCl control > 1% OSPW.

7 d exposures

L-serine

L-serine-evoked EOGs were compared to controls and between exposure groups after a 7 d exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl control. L-serine EOGs evoked by rainbow trout exposed to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl control were not statistically different from controls ($F_{4,35}$ =1.06, p = 0.39; Figure 3.8). EOGs evoked by rainbow trout exposed to 10% OSPW or the NaCl control (mean, in mV = 1.33 ± 0.63, 1.40 ± 0.59, respectively) were numerically decreased by 26 and 22% respectively compared to controls (1.80 ± 0.63 mV) after a 7 d exposure. L-serine EOGs evoked by rainbow trout exposed to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl did not differ statistically between groups ($F_{4,35}$ =1.06, p = 0.39; Figure 3.8). EOGs evoked by rainbow trout exposed to 10% O3OSPW, or the NaCl

control were numerically increased by 31, and 26%, respectively, compared to those evoked by rainbow trout exposed to 10% O3OSPW.

Taurocholic Acid

TChA-evoked EOGs were compared to controls and between exposure groups after a 7 d exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, or the NaCl control. EOGs evoked by rainbow trout exposed to 10% OSPW (mean = 1.25 ± 0.33 mV) were statistically decreased by 49% compared to controls (mean = 2.39 ± 0.93 mV) after a 7 d exposure ($F_{4,38} = 4.53$, $p < 0.01$; $p < 0.01$; Figure 3.8). EOGs evoked by rainbow trout exposed to 1% OSPW or the NaCl control (mean, in mV = 1.63 ± 0.44, 1.44 ± 0.45, respectively) were numerically decreased by 32 and 40% respectively compared to controls after a 7 d exposure. EOGs evoked by rainbow trout exposed to 10% O3OSPW were statistically increased by 81% compared to those evoked by rainbow trout exposed to 10% OSPW after a 7 d exposure ($F_{4,38} = 4.53$, $p < 0.01$; $p = 0.04$; Figure 3.8). EOGs evoked by rainbow trout exposed to 1% OSPW were numerically increased by 33 compared to those evoked by rainbow trout exposed to 10% OSPW after a 7 d exposure.

In general, a 7 d exposure to 10% OSPW resulted in the largest decreases in both L-serine and TChA-evoked EOGs. Decreases in L-serine-evoked EOG magnitudes can be ordered as follows: 10% OSPW > NaCl control > 10% O3OSPW > 1% OSPW. Decreases in TChA-evoked EOG magnitudes can be ordered as follows: 10% OSPW > NaCl control > 1% OSPW > 10% O3OSPW.

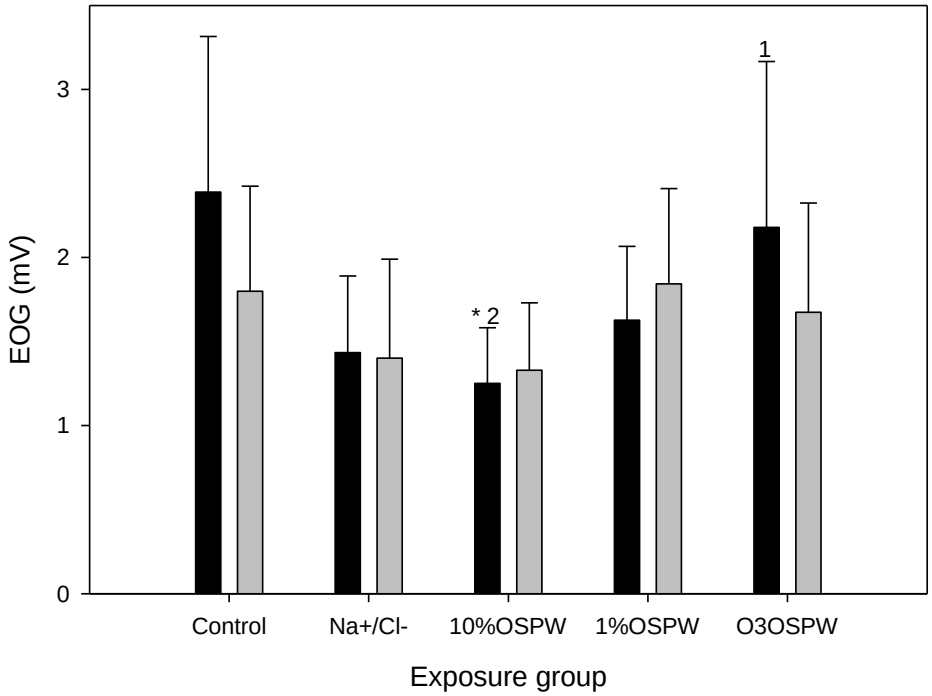

Figure 3.9. Effects of a 7 d exposure to 10% O3OSPW (n=7), 10% OSPW (n=6), 1% OSPW (n=11), the NaCl control (n=6) and dechlorinated water (control) on electro-olfactograms (EOG) evoked by ▒ L-serine and ■ TChA. EOGs evoked from fish exposed to 10% OSPW are the most effected with respect to both odorants. Differences between exposure groups are denoted by different numbers and differences between exposure groups and controls are denoted by asterisk.

Discussion

Rainbow trout were able to detect OSPW and O3OSPW in a concentration dependent manner. This finding is important, as detection may allow rainbow trout to avoid regions contaminated with either OSPW or O3OSPW. I hypothesized that OSPW and O3OSPW would evoke EOGs in rainbow trout; however, I did not expect it to do so in a concentration dependent manner as Tierney et al. did not see a concentration-dependent increase in EOGs with increasing concentrations of other cyclic organic contaminants [37, 38]. I am confident in these findings as L-serine and TChA concentration response curves recorded from the same rainbow trout also occurred in a concentration-dependent manner. EOGs evoked by natural odorants were also decreased as a result of exposure to OSPW and O3OSPW, however recovery occurred immediately after a 30 min exposure. Overall, my data suggest that OSPW and O3OSPW contain compounds (most likely NAs) that act as odorants with the ability to compete with other odorant molecules for olfactory receptors (ORs).

Detection of OSPW and O3OSPW

Concentrations of NAs within OSPW are eight-fold greater than those in O3OSPW at the same dilution (v/v); however, EOGs evoked by OSPW and O3OSPW did not differ at the same dilution factors. These data suggest that NA species within O3OSPW, that are detectable via olfaction, occur at a higher concentration than in OSPW. Martin et al. showed that tetra- and mono-cyclic NA concentrations increased post-ozonation while di- and tri-cyclic NA concentrations decreased. I suggest that mono- and tetra-cyclic NAs may bind

ORs, while di- and tri-cyclic NAs may not [74]. This would account for the lack of difference in EOGs at the same dilutions of OSPW and O3OSPW, as OSPW contained an eight-fold higher concentration of NAs, meaning there were still concentrations of mono and tetra cyclic NAs that may be comparable to those in O3OSPW.

The lack of difference in EOGs at the same dilutions of OSPW and O3OSPW may also be attributed to changes in conductivity; however, the EOGs evoked by the NaCl control were greater than those evoked by both OSPW and O3OSPW at 1% dilutions and above, and detection again occurred in a concentration dependent manner. Anderson et al. showed that conductivity between OSPW and O3OSPW does not differ greatly, suggesting that ion concentrations within the two is quite similar at the same dilution [2]. EOGs evoked by the NaCl control were greater than those evoked by OSPW and O3OSPW at the same dilutions, which suggests that perhaps NAs within OSPW and O3OSPW decreased the ability of ions within both mixtures to evoke EOG. It should be noted however that decreases in L-serine and TChA EOGs seen during 30 min exposures to 10% OSPW and 10% O3OSPW give weight of evidence that components of OSPW are in fact binding olfactory receptors. This is further supported as 30 min exposures to 1% OSPW did not result in the same magnitude of decrease in either L-serine or TChA-evoked EOGs when compared to 10% OSPW exposures, and the NaCl control did not result in the same decreases as either 10% OSPW or 10% O3OSPW, suggesting that rainbow trout are in fact detecting NAs within the

mixture. It should also be taken into consideration that other ions such as calcium were not present in this control, which may explain the high responses to the NaCl control. Calcium and positively charged metals such as copper have previously been shown to both evoke and suppress olfactory responses [6, 22, 55], suggesting that should these ions be included in the NaCl control, EOGs may not have been at such high magnitudes. The magnitude of the NaCl EOGs is likely attributed to changes in membrane polarization in all cells in the olfactory epithelium, hence the large potentials measured.

30 min exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, and the NaCl control

Exposure to both 10% OSPW and 10% O3OSPW resulted in decreased EOGs evoked by L-serine and TChA. There are no previous OSPW olfactory toxicology studies, but a comparison can be made to a standard toxin, copper. Copper is a contaminant that has been well characterized to affect fish olfaction [22, 36, 43, 49, 62, 101] and so I elected to compare results to effects of copper on EOGs evoked by both L-serine and TChA. L-serine-evoked EOGs in rainbow trout exposed to 10% OSPW and 10% O3OSPW for 30 min were decreased by 16.2 and 59.5%, respectively, a decrease that is comparable to the 30 and 50% decreases seen in L-serine-evoked EOGs by Coho salmon (*Oncorhynchus kisutch*) exposed to 2 and 5 μg/L $CuCl_2$, respectively, for 30 min [6]. TChA-evoked EOGs by rainbow trout exposed to 10% OSPW and 10% O3OSPW were decreased by 26.1 and 40.1%, respectively, decreases that are comparable to the 33% decrease seen in TChA-evoked EOGs in Coho salmon exposed to 10 μg/L

$CuCl_2$ for 30 min. NA concentrations in 10% OSPW and 10% O3OSPW were 4.0 and 0.50 mg/L, respectively, and these concentrations are 400- and 10-fold greater than concentrations of copper that resulted in similar decreases to L-serine, and 400- and 50-fold greater than concentrations of copper that resulted in similar decreases in TChA. Recovery of EOGs was also seen to occur more quickly in OSPW and O3OSPW exposed rainbow trout than copper exposed fish, as rainbow trout L-serine- and TChA-evoked EOGs returned immediately post exposure to OSPW and O3OSPW, while Coho salmon L-serine- and TChA-evoked EOGs did not return for 90 min follow Cu exposure. These comparisons suggest that OSPW and O3OSPW at 100 μg/L NA concentrations are less toxic to olfactory tissue than copper at 1-10 μg/L concentrations (Cu appears >10-fold more toxic than NAs).

An interesting comparison is between 1% OSPW and 10% O3OSPW, as they have similar NA concentrations (0.40 and 0.50 μg/L, respectively), yet evoke EOGs at different magnitudes, and differ in their ability to decrease olfactory detection of both L-serine and TChA. A study by Anderson et al. found that mono- and tert-NAs were increased in concentration and di- and tri-NAs were decreased in concentration in O3OSPW compared to OSPW [2]. A study by Pérez-Estrada et al. on the effects of ozonation on NAs showed that the composition of NAs within OSPW and O3OSPW differed, as ozonation removed many of the alkyl groups attached to the cyclic portions of NAs within OSPW [90]. As rainbow trout were shown to detect 10% O3OSPW using olfaction and detection of both L-serine and TChA returned immediately during the recovery

period by rainbow trout exposed to 10% O3OSPW, I suggest that alkyl groups that have been removed from NAs and the remaining cyclic carbon structures were able to bind L-serine and TChA ORs during a 30 min exposure. L-serine ORs in rainbow trout have been shown to detect L-alanine and some of its derivatives, all of which are two or three chain carbon structures with a carboxylic acid group and an amino group[17] . The structure of TChA is a four ringed carbon structure which resembles steroids such as estrogen, and as such its OR may be able to detect other four ringed carbon structures [45]. The NAs found within OSPW all contain an alkyl group with a carboxylic acid terminal, which may be removed during ozonation, and a base mono or polycyclic carbon ring structure [14]. Martin et al. showed that NA with Z numbers of -8, which are tetra cyclic ring structures similar to that of TChA, increase in concentration after the ozonation process [74]. These two components may interact with both L-serine and TChA ORs during a 30 min exposure to O3OSPW; however, the alkyl groups are not removed from NAs in 1% OSPW, and this may contribute to their lack of effect on both OSN families.

7 d exposure to 10% OSPW, 1% OSPW, 10% O3OSPW, and the NaCl control

A 7 d exposure to 10% OSPW resulted in decreased L-serine- and TChA-evoked EOGs compared to those evoked in rainbow trout exposed to decholorinated water control and 10% O3OSPW. Specifically, L-serine- and TChA-evoked EOGs were reduced by 26 and 47% compared to controls. Previous studies examining 96 h and 7 d exposures have shown that mixtures of organic

contaminants, as well as individual organic contaminants within the μg/l concentration, also have the ability to impair olfactory detection of both L-serine and TChA as measured by EOG in multiple teleost fish species [56, 101, 119, 121]. Specifically, Coho salmon exposed to 0.625 μg/L chlorpyrifos had L-serine-evoked EOGs that were decreased by 25%, and those exposed to 2.5 μg/L had TChA-evoked EOGs that were decreased by 55% [101]. Rainbow trout exposed to a pesticide mixture previously described in chapter 2 with a total concentration of 10.1 μg/L experienced 41% decreases in L-serine-evoked EOGs. OSPW does not appear to be as toxic as individual pesticides or mixtures, as NAs within OSPW are at much greater concentrations than pesticides the pesticides used in the aforementioned studies, yet evoked similar EOG decreases in the same time period.

Concentration dependent EOG reductions

Decreases in L-serine and TChA-evoked EOGs occurred in a concentration dependent manner in rainbow trout exposed to OSPW. These results suggest that olfactory tissue was able to adapt to the NAs within OSPW at concentration of 0.40 mg/L but not at a concentration of 4.0 mg/L during a 30 min and 7 d exposure. It should be noted, however, that L-serine and TChA evoked EOGs were still greater in 1% OSPW than 10% O3OSPW; as previously mentioned this was likely due to the composition of NAs within the mixture.

Ozonation and detoxification

Structural modifications of the NAs within OSPW may affect their biodegradability by detoxification enzymes within the olfactory epithelium of rainbow trout. There is evidence that cytochrome p450 (CYP) 1A and 3A exist within the olfactory tissue, and both of these detoxification enzymes can breakdown polycyclic organic molecules [106] (Appendix 2-1). While a study by He et al. showed increases in CYP 1A and 3A within the liver do not occur as a result of exposure to O3OSPW, it could be suggested that, due to its highly exposed nature, olfactory tissue may experience increases of these enzymes in the presence of both OSPW and O3OSPW [50]. It is possible that up-regulation of CYP 1A and 3A occurred to protect olfactory tissue in rainbow trout exposed to NA concentrations within a range of 0.40-0.50 mg/L, and resulted in the recovery of L-serine and TChA EOGs during a 7 d exposure. This up-regulation may also have occurred in rainbow trout exposed to 4.0 mg/L NAs; however, the NA concentration may have been too high for these detoxification enzymes to be effective. Together these findings suggest that OSPW is more toxic to olfactory tissue than O3OSPW at the same concentrations during a 7 d exposure.

It should be noted that the EOG decreases were found at concentrations below the LC_{50} for two-month old salmon reported by Dokholyan and Magomedov [9], and likely close to that found by MacKinnon and Boergers, who suggested a 96h LC_{50} for rainbow trout was at a 7% dilution of OSPW (no NA concentration given) [12, 24]. Together these results suggest that O3OSPW is less toxic to rainbow trout

olfaction during a 7 d exposure, and may indicate that ozonation is a suitable treatment for OSPW.

Conclusions

My study shows that rainbow trout have the ability to detect OSPW and O3OSPW in a concentration-dependent manner that is unrelated to ozonation. This finding, combined with findings that a 30 min exposure to 10% OSPW, 1% OSPW, and O3OSPW, decreased L-serine and TChA EOGs, and with findings that during a 7 d exposure to 10% OSPW, 1% OSPW and 10% O3OSPW, OSPW exposures decreased L-serine and TChA EOGs while O3OSPW did not, suggests that the composition of NAs within solution plays an important role in its ability to disrupt olfaction. Alkyl groups removed during ozonation and tetra cyclic NAs / tetra cyclic organic structures may be able to bind ORs associated with L-serine and TChA during a 30 min exposure; however, their increased biodegradability may allow tissue to adapt to their presence during a 7 d exposure. Concentrations of NAs within OSPW at the mg/L range appear harmful to ORs associated with L-serine and TChA, but concentrations of NAs within OSPW at the μg/L range have as great of an effect. Furthermore, NAs associated with O3OSPW were less harmful to olfaction than those associated with OSPW at μg/L concentrations during exposures of 7 d.

Chapter 4: Olfactory detection of nucleosides and nucleobases by goldfish (*Carassius auratus*)

Introduction

Nucleotides have previously been identified as chemosensory cues in fishes. The 5´-nucleotides adenosine-5´-monophosphate, adenosine-5´-diphosphate, adenosine-5´-triphosphate (ATP), citidine-5´monophosphate, guanosine-5´-monophophate, inosine-5´-monophosphate (IMP) and uridine-5´-monophosphate have all been identified as chemosensory cues that can be detected via gustatory receptors in fishes [44]. Many compounds, for example amino acids, that can be detected by gustatory receptors can also be detected by olfactory receptors (ORs) [47, 75]. Currently ATP and IMP have been shown to evoke responses in the olfactory bulb when presented together to olfactory tissue of zebrafish (*Danio rerio*) [69]; however, no evidence has been collected regarding receptor binding or specificity.

Nucleotides, the building blocks of nucleic acids such as DNA and RNA, can be divided into three components: a phosphate group, a sugar group, and a nucleobase [61]. Together, the sugar group and nucleobase are identified as a class of compounds known as nucleosides. Nucleotides differ in with respect to the number of phosphate groups present, with substitutions of mono-, di-, and tri-phosphate groups occurring. Sugar groups, including pentose, ribose, or deoxyribose, also vary between nucleotides. The nucleobase components are classified as either purines such as adenine or guanine, or pyrimidines, which includes cytosine, uracil, or thymine.

My study used electro-olfactography to demonstrate that goldfish (*Carassius auratus*) olfactory ORs can detect the nucleotide adenosine. Adenine, the nucleobase associated with adenosine, was also tested to demonstrate that neither the sugar or phosphate groups were needed for olfactory detection. Specificity of the receptor that may detect adenosine and/ or adenine was also investigated by measuring electro-olfactogram (EOG) responses to hypoxanthine, a derivative of adenine, and guanosine, a nucleoside containing the nucleobase guanine and a ribose sugar, during cross adaptation experiments.

Materials and Methods

Electro-olfactograms

Concentration response curves

Concentration response curves to the purine nucleosides, guanosine and adenosine, and the purine derivatives, adenine and hypoxanthine were measured as previously described in chapter 2, following methods of Evans and Hara [27]. Each odorant was tested at a concentration of 10^{-5}, 10^{-7}, and 10^{-9} M.

Cross adaptation

Cross adaptations were conducted as described by Sorensen et al [110]. Briefly, fish olfactory tissue was perfused with dechlorinated municipal water (background water) for 10 min before evoking EOGs using 10^{-5} M L-serine, adenine, adenosine, guanosine, hypoxanthine, or a background water control. Background water was then switched to one of adenine, adenosine, guanosine, or

hypoxanthine at a concentration of 10^{-5} M. Background adaptation was given for 30 min or until EOGs evoked by the adapting odorant, at a concentration of $2.0x10^{-5}$ M, were equal to those of background water pulses before adaptation.

Statistical Analysis

Concentration response curves were analyzed using one-way ANOVA followed by Holm-Sidak post hoc tests. Cross adaptation results were analyzed using one-way ANOVA followed by Holm-Sidak post hoc test.

Results

Concentration response curves

EOG responses to adenosine and hypoxanthine both occurred in a concentration dependent manner (adenosine: 10^{-9} M mean, in mV = 0.68 ± 0.43; 10^{-7} M mean = 0.77 ± 0.44; 10^{-5} M mean = 1.06 ± 0.40; $F_{2,37} = 6.28$, $p < 0.01$; hypoxanthine: 10^{-9} M mean = 0.33 ± 0.20; 10^{-7} M mean = 0.76 ± 0.33; 10^{-5} M mean = 0.73 ± 0.47; $F_{2,36} = 8.89$, $p<0.01$; Figure 4-1). EOGs evoked by 10^{-7} and 10^{-9} M adenosine were decreased by 27 and 36%, respectively, compared to those evoked by 10^{-5} M ($p = 0.03$, 0.02, respectively). EOGs evoked by 10^{-9} M hypoxanthine were decreased by 57 and 55% compared to those evoked by 10^{-5} and 10^{-7} M, respectively ($p = 0.02$, 0.03, respectively).

EOG responses to adenine and guanosine did not occur in a concentration dependent manner (adenine: 10^{-9} M mean = 0.79 ± 0.63; 10^{-7} M mean= 0.44 ± 0.33; 10^{-5} M mean = 0.95 ± 0.51; $F_{2,31} = 2.77$, $p = 0.09$; guanosine: 10^{-9} M mean =

0.56 ± 0.31; 10^{-7} M mean = 0.43 ± 0.31; 10^{-5} M mean = 0.64 ± 0.39; $F_{2,31} = 2.77$, $p = 0.09$; Figure 4-1).

Cross-adaptation responses

Cross adaptation of olfactory tissue to the aforementioned nucleosides and nucleobases revealed a relationship between the detection of adenosine, adenine and hypoxanthine (Figure 4.2). Adaptation of olfactory tissue to adenosine numerically decreased adenine- and hypoxanthine-evoked EOGs by 57 and 36%, respectively, compared to pre-adaptation (Figure 4.2 B). Adaptation of olfactory tissue to adenine numerically decreased adenosine-evoked EOGs by 17.7% compared to pre-adaptation (Figure 4.2A). Adaptation of olfactory tissue to hypoxanthine statistically decreased adenosine-evoked EOGs by 43% ($F_{3,23} = 13.6$, $p < 0.01$; $p < 0.01$) and numerically decreased adenine EOGs by 27% compared to pre-adaptation (Figure 4.2D). Post-adaptation EOGs did not differ statistically from pre-exposure EOGs in all groups, and EOGs post-adaptation appeared to return at pre-exposure magnitudes. Cross adaptation using guanosine did not appear to affect the EOGs evoked by adenosine, adenine, or hypoxanthine during or after the adaptation period when compared to pre-adaptation (Figure 4.2C).

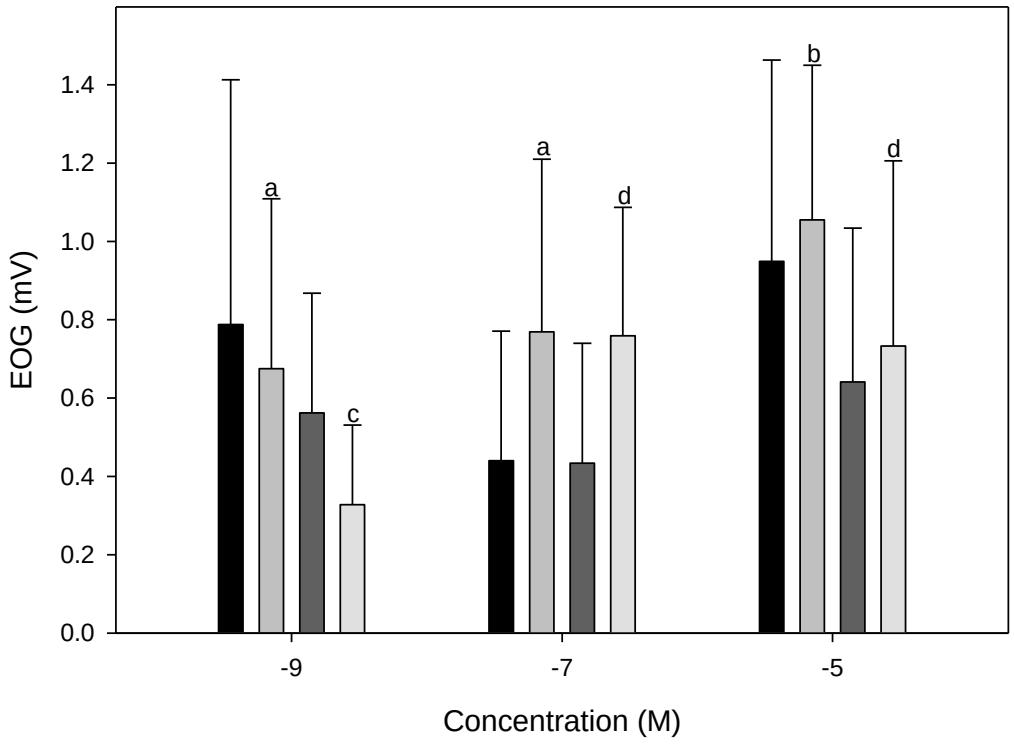

Figure 4.1. Concentration response of olfactory sensory neurons as measured by electro-olfactography (EOG) evoked by increasing concentrations of purine nucleosides, ■ guanosine and ■ adenosine, and purine derivatives, ■ adenine and ■ hypoxanthine. EOGs evoked by adenine and hypoxanthine occurred in a concentration dependent manner. Differences in EOGs evoked by different concentrations are denoted by different letters.

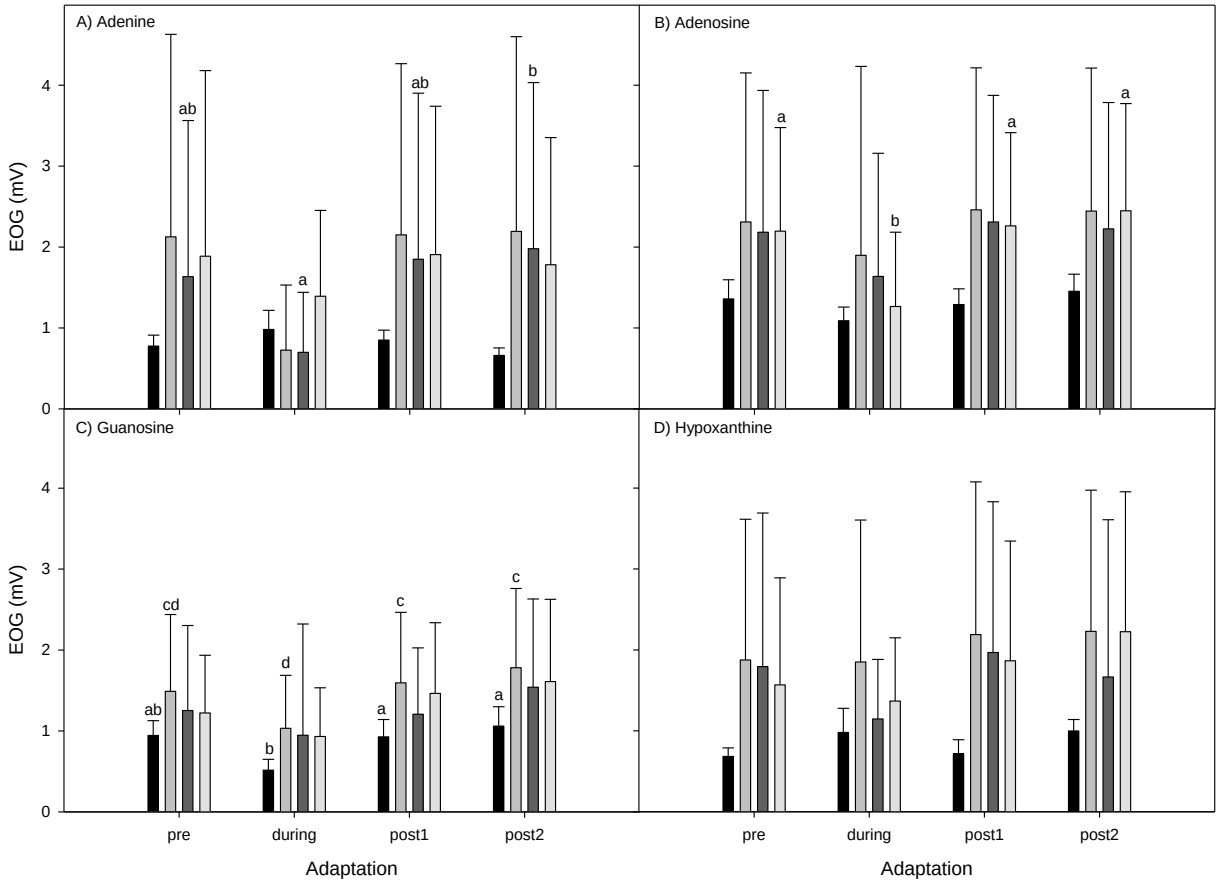

Figure 4.2. Cross adaptation of purine nucleosides, a nucleobase, and a purine derivative. A) Adaptation of olfactory tissue to adenine (n= 8); B) Adaptation of olfactory tissue to adenosine (n= 6) increased responses to adenine post-adaptation ; C) Adaptation of olfactory tissue to guanosine (n = 6); D) Adaptation of olfactory tissue to hypoxanthine (n = 6). The data suggests that adenosine and hypoxanthine share a receptor. Differences within groups are denoted by different letters.

Discussion

Goldfish are able to detect the nucleosides adenosine and guanosine, as well as the nucleobase adenine, and the purine derivative hypoxanthine; however, only adenosine and hypoxanthine appear to evoke EOGs in a concentration dependent manner.

Concentration response results and cross-adaptation results taken together suggest that adenosine has a receptor that is specific to its detection; however, both adenine and hypoxanthine were able to compete with binding of this receptor. An adaptation to adenosine decreased adenine-evoked EOGs, suggesting that the presence of the sugar molecule is required for complete binding of the receptor, and that the presence of the nucleobase alone may only result in partial binding.

The adaptation to either adenosine or hypoxanthine resulted in large decreases to the other has an interesting implication to the potential specificity of a nucleoside based olfactory receptor. Hypoxanthine is the nucleobase present in inosine, the nucleoside portion of IMP, a nucleotide that has previously been shown to evoke olfactory responses at the olfactory bulb of zebrafish [69]. The ability of hypoxanthine adaptation to reduce adenosine EOGs by 43.3%, while adenosine adaptation was only able to reduce hypoxanthine EOGs by 35.8%, suggests that perhaps the receptor present may detect both adenosine and inosine; however, inosine may be the dominant ligand for the receptor. This finding also suggests that only the nucleoside structure, not the entire nucleotide may be required for binding at the olfactory receptor. As nucleosides associated with ATP and IMP

are quite similar in structure (Appendix F), it can be suggested that the presence of the oxygen group on the hypoxanthine and inosine molecules may play a role in the binding specificity of a nucleotide-based olfactory receptor.

While guanosine appears to act as an odorant, it does not do so in a concentration dependent manner, and my results do not suggest that it shares a receptor with the other compounds tested. This is likely due to binding specificity at the receptor level being related to the nucleobase present. As guanosine has an amine group that extends from its six atom ring structure (Appendix F), it is likely that it is unable to share a receptor with those that do not contain this substitution, and so may require its own receptor. Yet the absence of a concentration response relationship, suggests that it is not the primary ligand for the receptor that it was able to bind.

Summary of major findings

- Goldfish OSNs detect MF, CF/MF, UV/H_2O_2/MF reuse and a NCM in a concentration-dependent manner.
- A 30 min exposure to MF, CF/MF, UV/H_2O_2/MF reuse and a NCM decreased EOG responses to L-alanine, L-serine, and 17,20-βP. EOGs evoked during these exposures are not as reduced as much as those during exposures to copper and pesticides, suggesting that many contaminants at low concentrations do not impair olfaction to the same extent as individual contaminants at higher concentrations.
- A 60 d exposure to MF, CF/MF and UV/H_2O_2/MF reuse decreased EOG responses to L-alanine and 17,20-□P. These reductions are comparable to those seen in lower concentrations of individual pesticides during 7 day exposures; however this indicates again that mixtures of contaminants at low concentrations do not impair olfaction to the same extent as individual contaminants at higher concentrations.
- Distance travelled by goldfish post introduction of a pulse of MF, CF/MF, UV/H_2O_2/MF reuse and a NCM did not differ from controls introduced to a pulse of dechlorinated water. Avoidance and attraction to contaminants has previously been demonstrated, responses which involved increased distance travelled. This may indicate that goldfish do not perceive treated reuse or a NCM as harmful or beneficial.

- Distance travelled by goldfish exposed to MF, CF/MF, UV/H_2O_2/MF reuse and a NCM decreased in the same manner as controls when a pulse of L-alanine was introduced to the environment. The lack of change in response indicates that fish still respond to L-alanine, regardless of the presence of treated reuse or a NCM.
- Rainbow trout OSNs detect OSPW, O3OSPW, and a Na+/Cl- solution in a concentration-dependent manner.
- A 30 min exposure to 10% O3OSPW decreased EOG responses by rainbow trout to L-serine and TChA throughout the exposure. EOGs evoked during these exposures were not as reduced as those during exposures to copper and pesticides, suggesting that NAs at higher concentrations do not impair olfaction to the same extent as individual contaminants at lower concentrations.
- A 7 day exposure to 10% OSPW decreased EOG responses by rainbow trout to L-serine TChA. These reductions are comparable to those seen in lower concentrations of individual pesticides during 7 day exposures; however, this indicates again that a mixture of NAs at comparable concentrations to pesticides do not impair olfaction to the same extent.
- Hypoxanthine and adenosine evoked EOG responses in goldfish in a concentration dependent manner, while adenine and guanosine did not.
- A cross adaptation with adenosine decreased EOGs to hypoxanthine and adenine during exposure. A cross adaptation with adenine decreased EOGs to adenosine. A cross adaptation with hypoxanthine decreased

EOGs to adenosine and adenine. These findings suggest that all three of the aforementioned compounds share (an) olfactory receptor(s).

- A cross adaptation to adenosine, adenine, or hypoxanthine does not affect guanosine-evoked EOGs. A cross adaptation to guanosine does not affect adenosine-, adenine-, or hypoxanthine-evoked EOGs. These findings suggest that guanosine does not share a receptor with any of the other compounds tested.

Troubleshooting EOG acquisition

Often times while conducting EOGs problems with the technique will arise. Below I have outlined potential problems that may occur while trying to record EOGs and have included methods by which to determine the problem. It should be noted that these methods have been written in the order by which they should be used.

1. Baseline is unresponsive and does not appear to be recording.

   -Ensure amplifier is turned on.

   -Ensure gelatin within electrodes has actually set, if it has not and is still liquid often times the fluid will leak out and the electrode holder simply contains an empty glass capillary tube.

   -Blow on the recording electrode while the reference electrode is in the water bath. If unresponsive, replace electrode. If baseline remains unresponsive, repeat this test, however this time blow on the reference electrode and leave the recording electrode in the water bath. If no prepared electrodes are response, likely missing NaCl in gelatin and so electrodes should be refilled with gelatin solution.

   -Examine electrode holders. If the silver pellet present in the bottom of the holder appears to be white instead of silver, likely a salt build up that can be washed out with deionized water. This build up can be avoided be rinsing holders after each use. If the silver pellet appears brown and scaly, likely that the electrode holder is no longer of use and should be replaced.

   -Turn off channels going to headstage and use test button on amplifier to

ensure amplifier is working. Pressing the test button should produce a sine wave that oscillates between 0.5mV above and 0.5mV below the baseline. If this does not appear, replace batteries in amplifier or change location that amplifier is plugged into.

-Remove connections to headstage and place fingers on each connector of headstage. Tap one finger repeatedly on headstage connection, this should produce small peaks in the baseline.

-Examine all wires used in connections for frays or cuts. If present, replace wiring.

-Unplug all wiring, and rewire entire setup. Replace wires that you feel may be contributing to the problem. Also investigate connection from amplifier to the data collection device, as many programs are designed to have a baseline appear regardless of input being present or not.

2. Baseline is thick or noisy (random large spikes occurring repeatedly).

   -Ensure electrode is not cupping. Cupping is a term used to describe when gelatin has pulled back from the tip of the electrode. Gelatin should be slightly protruding from electrode tip.

   -Look under microscope to identify aspects of prep that may be causing noise. If nares covering is not completely removed, movement of this tissue can cause noise in baseline. Remove any excess tissue that may be in the olfactory chamber. If bubbles are forming where fluid delivery is entering the olfactory chamber, likely that air is present in delivery line. Remove air from fluid delivery lines by increasing flow rate or flicking the

delivery line with your finger.

-Check grounding of all components within setup. Improper grounding will lead to noise.

-Determine if electrical use in building has changed. Often times change in draw on electricity, especially due to construction in building, may result in noise.

-Remove any external hard drives that are connected to the computer being used in data acquisition. Unlike hard drives in the computer itself, external hard drives are often not shielded and will result in noise in baseline.

3. Baseline is responsive, however EOGs are not being evoked upon delivery of odorant.

   -Examine olfactory chamber for excess mucus. This can result from the electrode touching the olfactory rosette and irritating it or from contaminants within odorant delivery lines.

   -Examine electrodes for cupping or gelatin that is not set.

   -Replace electrodes or electrode holders.

   -Attempt recording from another fish as current fish may be anosmic. Anosmia can arise due to water quality in holding tank. Ensure tank is clean and perform water chemistry to ensure calcium or ammonia concentrations are not above normal.

   -Remake odorant solutions. If this still does not appear to resolve the

issue, remake odorant stock solutions. Note, many odorants will degrade over time and as such stock solutions should be made weekly.

4. Fish waking up during recording process.

   -Examine anesthetic delivery. Operculum should be open, however water should not be moving too quickly through this opening. If this is the case, decrease the flow rate by shutting the delivery valve slightly. If operculum are closed, increase the flow rate by opening the delivery valve. Note, the anesthetic source tank should have a minimum volume that it does not fall below. Should it fall below this volume the flow rate of anesthetic will be decreased.

   -Examine the expiration date of tricaine methanosulfate. If it is expired it will not be as potent and should be discarded.

   -Increase concentration of stock used to make tricaine methanosulfate anesthetic solutions.

   -Check water temperature. If temperature is higher than the animal is acclimated to, likely they are metabolizing the anesthetic faster than normal. Speak with aquatics facility staff about water source temperature should this be the case.

   -Remake stock solution of tricaine methanosulfate used in creation of anesthetic.

Future Directions

Chapter 2

To demonstrate that EOGs are in fact a measure of electrical potentials generated by OSNs a compound that inhibits the ability of odorants to act on the olfactory pathway should be used. Forskolin activates the adenylate cyclase pathway that is associated with the detection of many odorants, and has been demonstrated as a compound that can be used to inhibit and even abolish EOGs to multiple odorants [71]. I suggest in future that forskolin be applied to the olfactory tissue after concentration dependent response experiments and 30 min exposures to demonstrate that the EOGs measured are actually a measure of OSN activity and not other cells within the olfactory epithelium having electrical potentials evoked.

One obstacle that arises during the study of contaminant mixtures, as stated by Tierney et al., is the lack in ability to derive a mechanism through which toxicity occurs [118]. Studies upon individual contaminants allow us to determine specific contaminants that correlate to reduction in EOG values for specific odorants. When dealing with contaminant mixtures there may be multiple compounds acting additively, synergistically or antagonistically to alter olfaction. Adding to the complexity of the contaminant mixture found within reuse water is the high variability in composition over short periods of time. The mixture is known to change in composition not only on a seasonal basis but on a daily basis (personal communication Arvinder Singh). To gain a better understanding of the effects

caused by this mixture, studies must be conducted looking at individual components of the mixture.

To identify contaminants within reuse water that have the ability to impair olfaction, we must first identify which contaminants have the ability to evoke olfactory responses using EOG. Concentration response curves must be determined for individual contaminants and a maximum response concentration must be identified. Once the maximum response concentration is identified, a cross adaptation study similar to that described by Sorensen et al. should be conducted [109]. Should a decrease in EOG to a specific odorant or odorant class occur during the cross adaptation and then return post-cross adaptation, it is likely that the contaminant present during the adaptation is able to bind the receptor or is causing modifications to the receptor to prevent binding of the odorant. Should a decrease in EOG to a specific odorant or odorant class occur during the cross adaptation and no return is seen post-cross adaption, it is likely that the contaminant present during the adaptation is able to cause cellular damage to the olfactory sensory neuron or is able to bind irreversibly to the receptor, resulting in a permanent loss in receptor function. As the duration of the cross adaptation may result in increases or decreases in impairment, different durations of cross adaptation should be used. Exposures which lasted 30 min on the EOG rig were quite similar to this procedure and have already shown that up to 50% inhibition was caused by contaminants within treated reuse water in concentration in the μg/L range. A second experiment that would need to be conducted would be to conduct a cross adaptation study as previously described; however, in this

experiment multiple combinations of contaminants, which were shown to reduce EOG responses to natural odorants, would be used to determine if synergistic effects were occurring.

To improve results obtained from the 60 d exposure, I suggest two modifications: 1) reconstructing the contaminant mixture found in reuse water based on averages of contaminants found within each season, and 2) recording EOGs from the same goldfish at different time points during the exposure.

To recreate the contaminant mixture found in reuse water I suggest collecting treated reuse water samples three times each week and analyzing them for a broad spectrum of contaminants. As contaminants in reuse water have been shown to fluctuate over time (personal communication Arvinder Singh), an average should be determined for each contaminant during each season. The contaminant mixture could then be reconstructed using these average concentrations for each season, and exposure could be conducted at the University of Alberta. Static renewal of the contaminant mixture would occur on a daily basis, allowing for the application of carbon filtration and UV/H2O2 treatment of the mixture each day. Using this method would keep contaminants within the mixture at a constant concentration, controlling for variance seen throughout exposures.

By using the same goldfish to record from at each time point, variation between individuals at different time points will be removed. This was the method I had wanted to use during exposures; however returning goldfish to the Gold Bar WWTP was not an option due to daily time constraints. As can be seen in the

error presented in concentration response curves and 30 min exposures, not all goldfish were impacted in the same manner by the presence of contaminants. By identifying each fish with a fin clip and repeatedly measuring them at set intervals, it would decrease the variation seen by testing different individuals along the time course. This would also allow a staggering of the exposures, allowing for measurements at precise time points during the exposure as opposed to a range of time points during each exposure.

The current method used to detect changes in behavior has two flaws: 1) lack of 3 dimensional properties and 2) small sample size that does not account for personality. The first flaw may have contributed in the failure to detect behavioral changes as measured by distance travelled, as changes in depth are not factored into the analysis. This may cause problems as food searching behaviors such as those previously described by Valentincic et al. [126] often involve changes in depth. The second flaw may have contributed in the failure to detect behavioral changes as Shamchuk and Tierney [105] have shown that fish exhibit personalities that affect their behaviors. With the small sample size used, an accurate representation of these personalities was not achieved. As a three dimensional assessment of behavior is quite complex and methods to analyze this data are still being constructed I suggest the behavior experiments be conducted again, this time using a preference/ avoidance trough and a larger sample size. This method allows fish to discriminate between a contaminated area and non-contaminated area and allows them to make a choice between the environments, if they can detect a difference. This method would allow one to better determine if a

behavioral response to the introduction of contaminants exist (as it allows for movement out of the contaminated zone), unlike the test which I conducted.

Chapter 3

To further identify the ability of rainbow trout to detect NAs within OSPW and O3OSPW, commercially available NAs, as well as NAs extracted from both OSPW and O3OSPW, should be tested for their ability to evoke EOGs. Both OSPW and O3OSPW could also be filtered using granular activated carbon, as was carried out by Wong et al. [132], and water post-filtration could be tested for its ability to evoke olfactory responses. By testing a dilution series of this water it could be determined whether or not the compounds being detected are organic in nature or if detection of OSPW and O3OSPW is simply occurring due to changes in conductivity.

The NaCl control used in my experiments needs to be better characterized to reflect total ion and mineral concentrations within OSPW and O3OSPW. To do so I would suggest following methods outlined by Sonthalia et al. [107] and Rogers et al. [96], who determined concentrations of ions within OSPW [95, 107]. The saltwater control used by Anderson et al. [2] could also be used as a conductivity control. This control was similar to the one I used except for the inclusion of Ca+.

Chapter 4

To determine receptor specificity of the aforementioned receptor, EOG cross adaptations should be conducted using ATP, IMP, adenosine, and inosine. An EOG cross adaptation study using these compounds may allow us to determine if

ATP or IMP is the dominant ligand for the receptor, and also if the phosphate groups are required for their detection.

Conclusion

The goal of my thesis was to develop fish olfaction as a biosensor for anthropogenic contaminants. I was able to show that two fish species have olfactory responses that are evoked by anthropogenic contaminant mixtures, and also that these contaminant mixtures are able to impair olfaction. I was also able to identify two novel classes of odorants, nucleosides and nucleobases. Further refinement of the model is needed however, specifically in regards to behavioral assays as was discussed in future directions. My research has laid the ground work to develop fish olfaction as a biosensor of anthropogenic contaminants; however the model does need further refinement.

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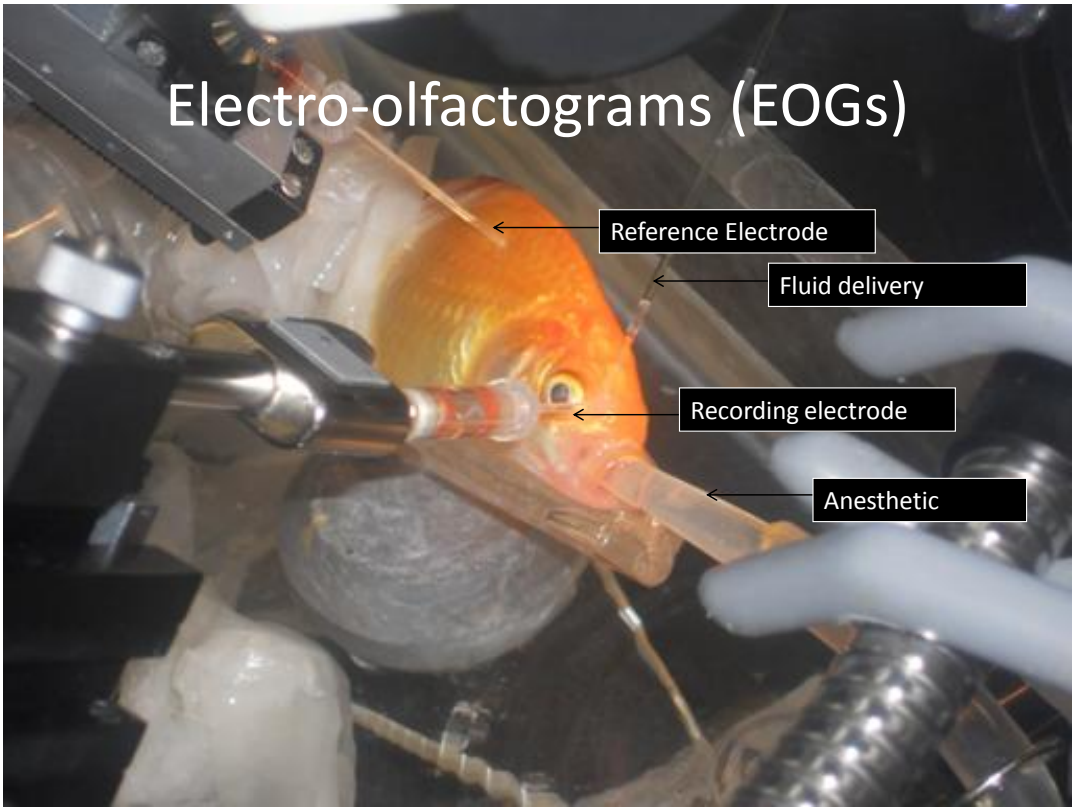
Appendix A

Page 179 has been removed due to copyright restrictions. This page contained a rendering of the nares of fish from Tierney, ed. *Chapter 23: Olfaction in aquatic vertebrates*. Handbook of olfaction and gustation (3rd edition): Modern perspectives. 2013, Doty RL, ed. Wiley & Sons.

Appendix B

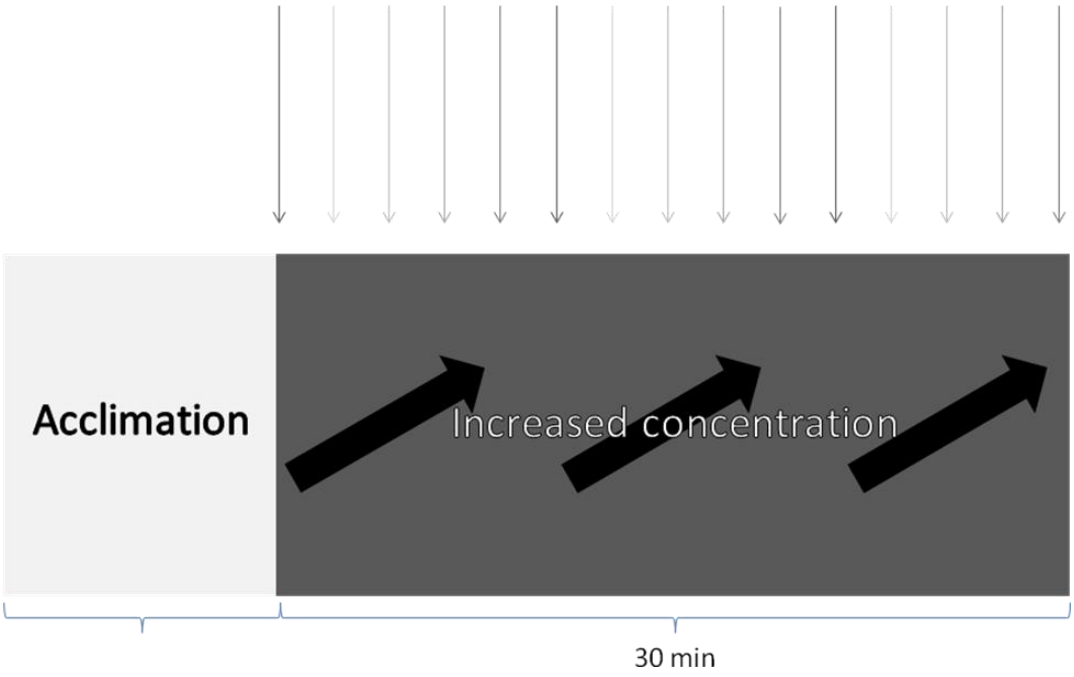
Page 180 has been removed due to copyright restrictions. This page contained a photograph of the olfactory rosette and was obtained from Baldwin and Scholz. 2005. An in vivo measure of peripheral olfactory function and sublethal neurotoxicity in fish. Techniques in Aquatic Toxicology, Volume 2.

Appendix C

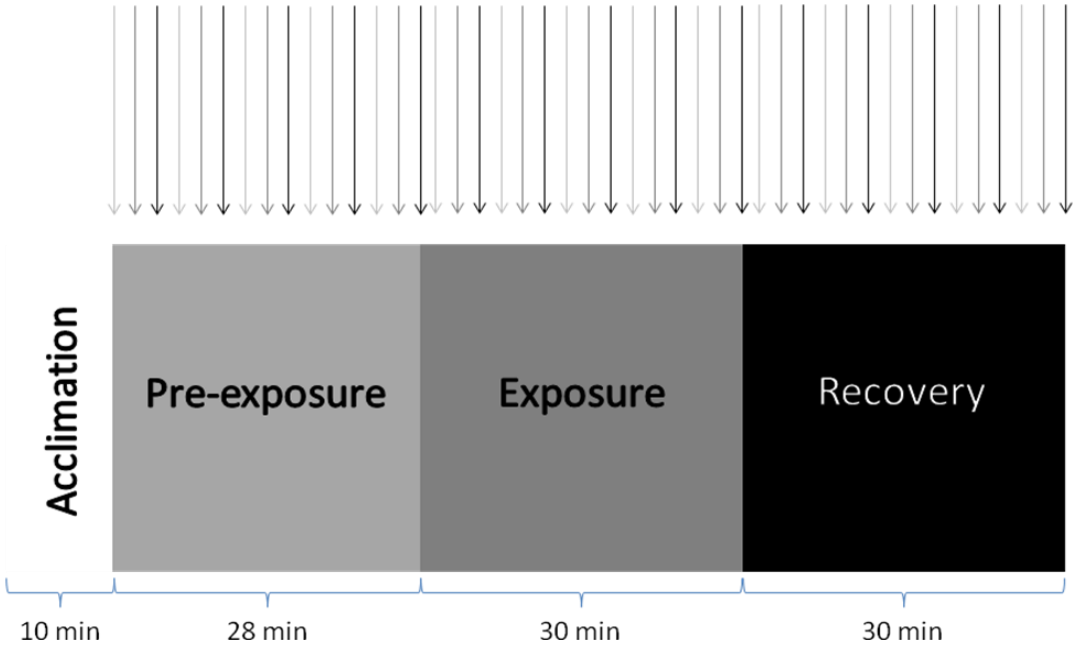

Electro-olfactograms (EOGs)
Reference Electrode
Fluid delivery
Recording electrode
Anesthetic

Appendix D

Concentration Dependent Response

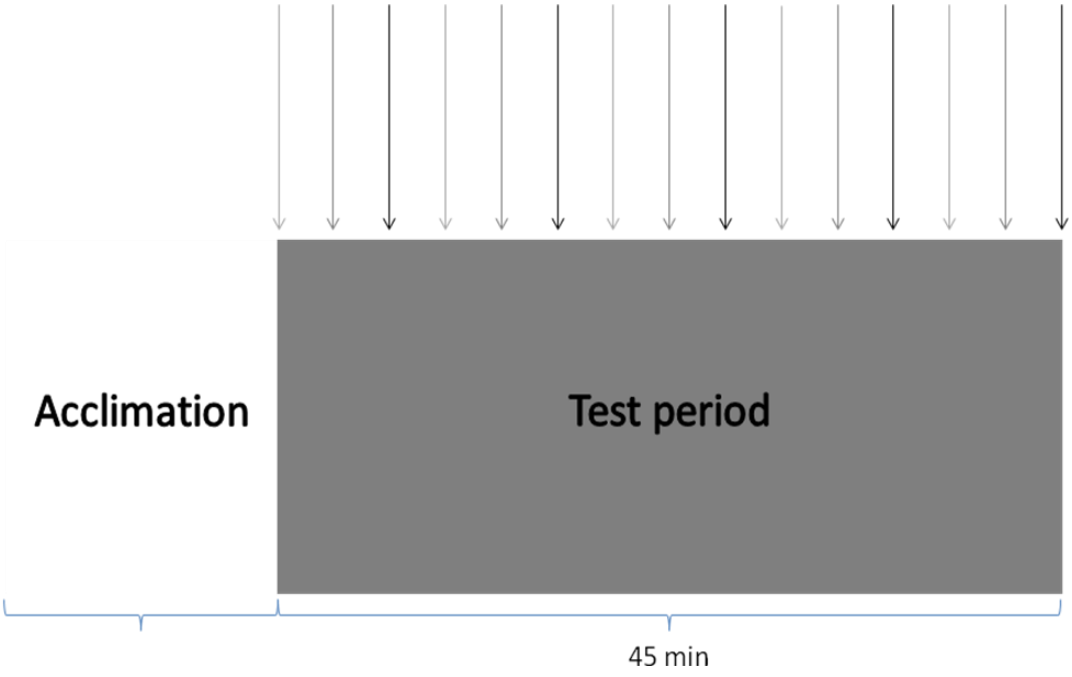


30 min exposure

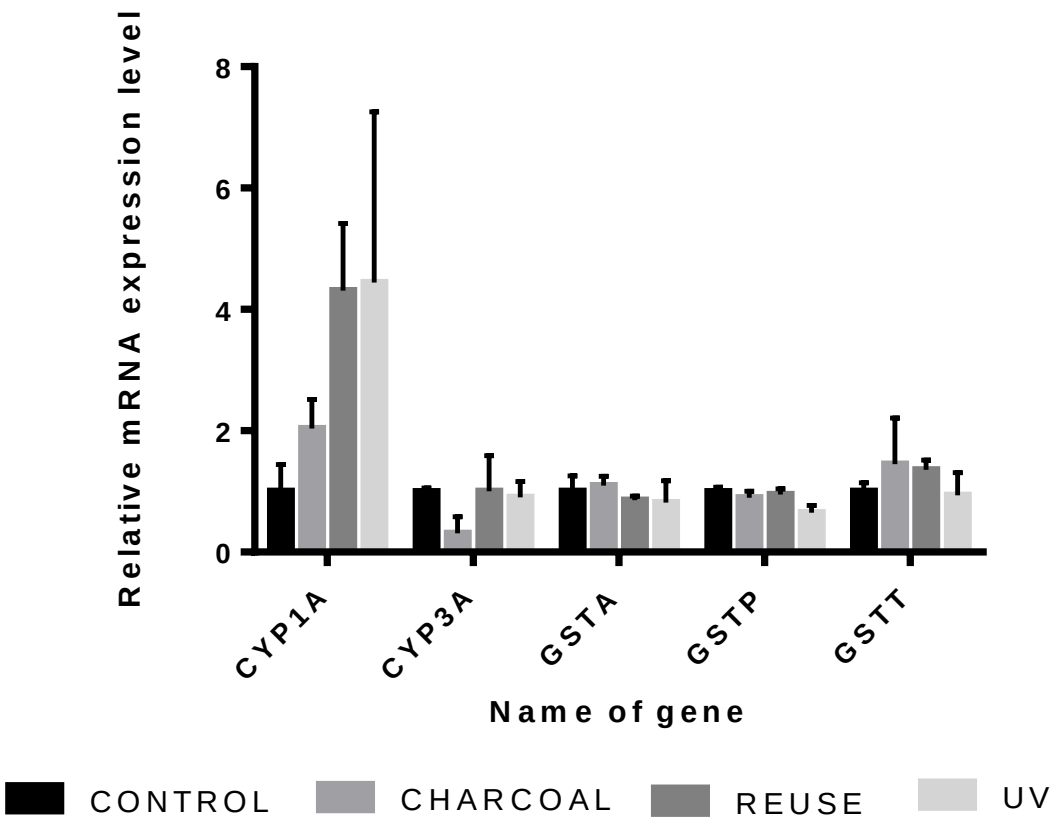


Appendix D(continued)

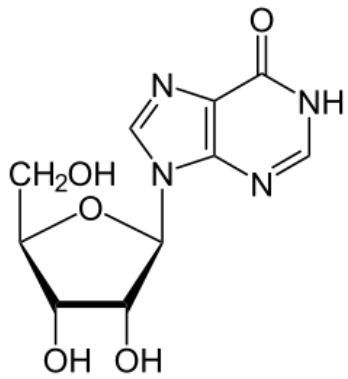
Long term exposure



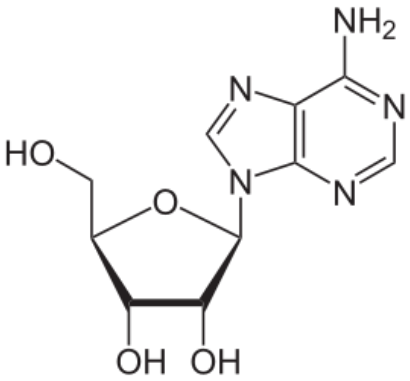
Appendix E


Relative mRNA expression level
8
6
4
2
0
CYP1A
CYP3A
GSTA
GSTP
GSTT
Name of gene
CONTROL
CHARCOAL
REUSE
UV

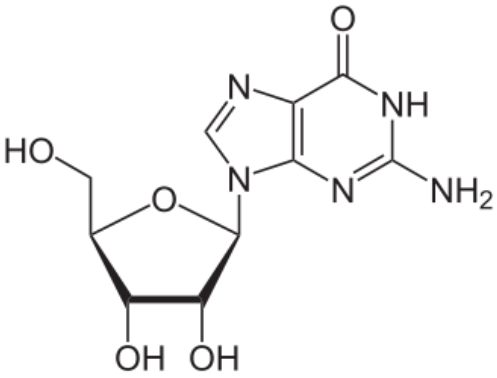
Appendix F



Inosine



Adenosine



Guanosine