INDIVIDUAL EFFECTS OF PLATINUM GROUP ELEMENTS (PALLADIUM AND PLATINUM) ON RAINBOW TROUT OLFACTION

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DEDICATION

This thesis is dedicated to my parents Lynne and Stephen, and sister Jennifer, who have supported me unconditionally for the entirety of my academic pursuits.

ABSTRACT

The platinum group elements (PGEs) palladium (Pd) and platinum (Pt) are introduced to aquatic ecosystems through emissions processes from industrial applications. Despite this, little is understood about the toxicity of these metals to aquatic organisms, especially fishes. Considering ambient concentrations of PGEs are relatively low in natural systems, the use of sensitive endpoints such as olfaction is ideal in informing the development of ecological guidelines and risk assessments. We can conclude from the present study that Pd is toxic to rainbow trout olfaction near environmentally relevant concentrations, whereas Pt is not. Through the manipulation of water quality parameters, it is clear water hardness plays a role in olfactory acuity. Further investigation on Pd-induced olfactory toxicity demonstrated bioaccumulation of Pd at the olfactory organ, supporting the bioavailability of waterborne Pd. As PGE contamination continues to increase, understanding ecological impacts remains critical to protect aquatic life from these contaminants of emerging concern.

PREFACE

The candidate is the primary author of chapters 1–4. The candidate conducted all experiments and primarily analyzed data in chapters 2 and 3. Dr. Greg Pyle contributed to experimental design and data interpretation of chapters 2 and 3, while editing chapters 1–4 and provided funding for this research. Lauren Zink provided scientific input, guidance, and training for chapter 2. Dr. Sarah Ellen Johnston and Dr. Matthew Bogard provided guidance, writing, and DOC quantification for chapter 2.

The research contained in this document required ethics approval which was sought and obtained by the University of Lethbridge Animal Welfare Committee (protocol #1914).

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LIST OF ABBREVIATIONS

%SAT	percent saturation
AAS	atomic absorption spectrometry
AChE	acetylcholinesterase
ANOVA	analysis of variance
AO	adverse outcome
ARF	aquatic research facility
AWC	animal welfare committee
BCF	bioconcentration factor
BLM	biotic ligand model
$CaCO_3$	calcium carbonate
cAMP	cyclic adenosine monophosphate
$CaSO_4$	calcium sulfate
CCME	Canadian Council of Ministers of the Environment
CF	concentration factor
CO	carbon monoxide
CO_2	carbon dioxide
CRM	certified reference material
DOC	dissolved organic carbon
DOLT-4	dogfish liver certified reference material for trace metals
EC16	16% effect concentration
EC50	50% effect concentration
ECCC	Environment and Climate Change Canada
EOG	electro-olfactography
ETV ICP-OES	electrothermal vaporization inductively coupled plasma-optical emission spectrometry
EU REACH	European Union Registration, Evaluation, Authorisation and Restriction of Chemicals
GFAAS	graphite-furnace atomic absorption spectrometry
HC	Hydrocarbon
HISS-1	Marine sediment certified reference material for trace metals
IC	inhibitory concentration
IC20	20% inhibitory concentration
IC50	50% inhibitory concentration
ICP-MS	inductively coupled plasma-mass spectrometry
ICP-OES	inductively coupled plasma-optical emission spectrometry
IP_3	inositol triphosphate signaling molecule
IQR	interquartile range
KE	key event
KM	Kaplan Meier
LC50	50% lethal concentration

LEP-OES	liquid electrode plasma optical emission spectrometry
LOEC	lowest observed effect concentration
LPO	lipid peroxidation
MDA	malondialdehyde
MDL	method detection limit
$MgSO_4$	magnesium sulfate
MIE	molecular initiating event
MOA	mechanism of action
$NaHCO_3$	sodium bicarbonate
NOEC	no observed effect concentration
NOM	natural organic matter
NO_x	nitrogen oxides
NSERC	Natural Sciences and Engineering Research Council of Canada
OB	olfactory bulb
OE	olfactory epithelium
OSN	olfactory sensory neuron
OSP	olfactory signalling pathways
OST	olfactory signal transduction
PCW	processed cold water
Pd	palladium
$Pd(OH)_2$	palladium hydroxide
PERMANOVA	permutational multivariate analysis of variance
PGE	platinum group element
Pt	platinum
QA/QC	quality assurance/quality control
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
SLRS-6	River Water Certified Reference Material for Trace Metals
SOP	standard operating protocol
SSD	species sensitivity distribution
TBARS	thiobarbituric acid reactive substances
TCA	taurocholic acid
TMS	tricaine methanesulfonate
TOC	total organic carbon
USEPA	United States Environmental Protection Agency
UV	ultraviolet
VEC	vehicle exhaust catalyst
WQG	water quality guideline

CHAPTER 1: Introduction

1.1 Fish olfactory system

1.1.1 Olfactory anatomy and physiology

Akin to other animals, fish use many senses to navigate their environment. The ability to detect chemosensory cues within their surroundings is essential for enacting vital behaviours related to survival, growth, and reproduction (Klaschka, 2009). Olfaction provides invaluable information for fishes that other sensory functions cannot, including environmental perception over long distances (Tierney et al., 2010). A range of behaviours are mediated through fish olfaction, including conspecific/mate detection, contaminant avoidance, foraging for food, homing, and predator evasion (Olivares & Schmachtenberg, 2019). As an example, it is well established that many salmonid species use olfactory information to locate their natal streams through homing migrations (Dittman & Quinn, 1996). Further, chemical alarm cues released by conspecifics (e.g., disturbance cues) or predators (e.g., kairomone) can inform on the presence of a nearby threat (Wisenden, 2014). Detection of this odourant type can trigger stereotypical antipredator behaviours and fright responses, including avoidance, freezing, and skittering. Other researchers revealed fish can detect and subsequently enact avoidance responses to specific waterborne contaminants (Giattina et al., 1982; Hidaka & Tatsukawa, 1989; Tierney, 2016; Lari & Pyle, 2017). Additionally, fish perceive amino acids in their environment as food and initiate feeding behaviours (Hara, 2006). Lastly, sex pheromones from conspecifics lead to courtship movements and other behavioural responses that facilitate reproductive processes (Stacey, 2003; Kasumyan, 2004). These examples demonstrate olfactory-mediated chemosensory

behaviours are ecologically relevant responses that directly influence a fish's overall health and fitness.

Located on the dorsal side of the snout in most teleost fish are two narial openings that lead into the olfactory chambers, which store the primary olfactory organs. Each opening consists of an anterior inlet and posterior outlet divided by a prominent nasal flap (Kumari, 2008). Water flows into the chamber through the inlet nostril and exits via the outlet, resulting in information perception from the moving surrounding environment (Laberge & Hara, 2001). The paired peripheral olfactory organ is aptly named the olfactory rosette for its oval rose-like shape and petal-like projections called lamellae. The multi-lamellar folding in the rosette structure maximizes available surface area, allowing for increased olfactory sensory activity between the sensory epithelium and surrounding aquatic environment (Hansen & Zielinski, 2005). The epithelium of the olfactory rosette has various receptor proteins embedded within to detect a multitude of odourants. Upon reaching a stimulus threshold, olfactory sensory neurons (OSNs) deliver environmental messages to the olfactory bulb (OB) through thin axons, which get further propagated to higher centres within the brain for processing (Hamdani & Døving, 2007). The propagation of signals from chemical odourant to an electrical signal is called an olfactory signal transduction (OST) pathway.

There are at least three distinct subtypes of OSNs which are responsible for detecting different odorant molecules. These are microvillous sensory neurons that detect food cues from amino acids with short dendrites and ample microvillae; ciliated sensory neurons with long dendrites and few cilia which perceive social cues such as bile acids and are integral to migration and alarm responses from conspecifics or predators; and crypt cells that use both cilia and microvillae to detect sex pheromones (Hamdani & Døving,

2007) (Fig 1.1). In addition to OSNs, there are many assisting cells within the olfactory epithelium (OE) including supporting cells, goblet cells, globose basal cells, horizontal basal cells, and ensheathing cells (Kasumyan, 2004; Su & He, 2010). If the olfactory system is irreparably damaged, fish cannot enact behaviours essential for growth, reproduction, and survival.

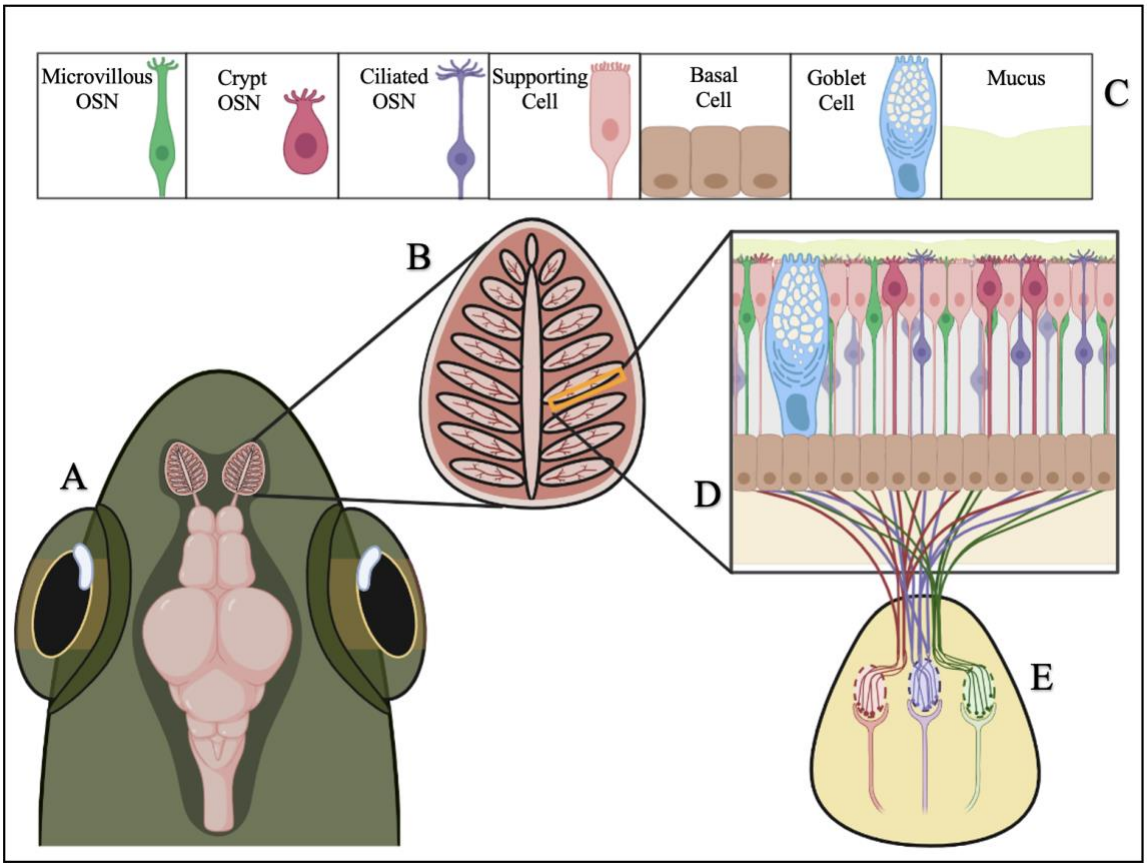

Figure 1.1. Anatomical diagram of a fish olfactory system. A) Dorsal view of fish head with localization of the exposed brain and olfactory rosette pair; B) Morphology of the olfactory organ; the olfactory rosette with arranged lamellae; C) Cell legend for the olfactory epithelium (OE) of lamellae; D) OE matrix; E) Olfactory bulb organization.

1.1.2 Metal interaction with fish olfactory epithelium

A variety of contaminants are known to impair fish olfactory function, including metals (Tierney, 2016). Research on waterborne metals such as aluminium, cadmium,

copper, mercury, nickel, and zinc demonstrated their inhibitory effects on fish olfaction (Tierney et al., 2010; Dew et al., 2016). Change in odourant perception can occur by direct binding of metal contaminants to epithelial receptor protein sites at OSNs (Tierney et al., 2010). During this interaction, metals can effectively block ion channels within the OST including sodium and calcium channels (Florea & Büsselberg, 2006). Additionally, OSNs are subject to functional impairment through epithelial damage, or dysregulation of genes at the cellular level within the OST (Tilton et al., 2008; Razmara et al., 2021). Resultant disruption of the olfactory system by trace metals may lead to maladaptive behavioural responses and potential adverse outcomes at the organism or population level for fishes.

1.2 Platinum group elements

The platinum group elements (PGEs) play a vital role for many industries in the production of modern technologies. This metal group remains attractive for their rarity and high value, in addition to beneficial physical and chemical properties (Rao & Reddi, 2000). The PGE group consists of six metallic elements: platinum, palladium, rhodium, ruthenium, osmium, and iridium. Of the six members, platinum (Pt) and palladium (Pd) remain in highest demand and thus are a primary focus for PGE research. For this thesis, mention of PGEs refers only to Pt and Pd unless otherwise described.

1.2.1 Global occurrence and Canadian abundance

Characterized by highly siderophile properties, the vast majority of PGEs are naturally found in the earth's core (Rauch & Peucker-Ehrenbrink, 2015). Compared to other elements, natural environmental quantities are exceedingly low at 0.015-0.010 mg/kg (Komendova, 2020). Natural phenomena such as volcanic events and continental crust erosion bring PGEs to the earth's surface where they enter their natural biogeochemical

cycle (Mitra & Sen, 2017). Once at the surface, PGEs commonly occur in alluvial deposits in their pure elemental forms, as alloys, as tellurides/bismuthotellurides, as arsenides in copper and nickel deposits, or as sulfides (Oberthür et al., 2003; Hutchinson & Kinnaird, 2005; Dare et al., 2011). Mining operations primarily source PGEs from alluvial deposits due to their proximity to the surface.

The bulk of PGE extraction occurs in South Africa (53.1%), Russia (27.1%), and Canada (7.1%), with the remaining 12.7% distributed amongst other countries (JM, 2021; Natural Resources Canada, 2022a). Mining efforts in Canada occur in Ontario, Manitoba, and Quebec, where PGE extraction equals approximately 1 million troy ounces per year (Natural Resources Canada, 2022b). Ontario leads extraction efforts with one PGE-dedicated mine. The remaining thirteen mines that recover PGEs as co-products are in Ontario (10), Quebec (2), and Manitoba (1) (Natural Resources Canada, 2021). PGE extraction in Canada is dominated by palladium (69.1%) and followed by platinum (25.7%) (Natural Resources Canada, 2022b). Ranking third for global PGE deposits, Canada plays an essential role in the worldwide production of these rare metals.

1.3 PGE emission sources

1.3.1 Vehicle exhaust catalysts

The automobile industry introduced vehicle exhaust catalysts (VECs) in response to newly developed global emission standards in the 1970s and 1980s (Palacios et al., 2000; Moldovan et al., 2002; Dubiella-Jackowska et al., 2009; Rauch & Peucker-Ehrenbrink, 2015). This industry is undoubtedly the largest user of PGEs (Fig. 1.2). To provide increased surface area for efficient removal of toxicants, a honeycomb shape is applied to VEC design (Moldovan et al., 2002). The catalyst interior requires metals resistant to heat,

mechanical, and chemical fluctuations conducive to VECs function. The resistant and catalytic properties of PGEs result in their use as the interior coating for these exhaust emission control devices.

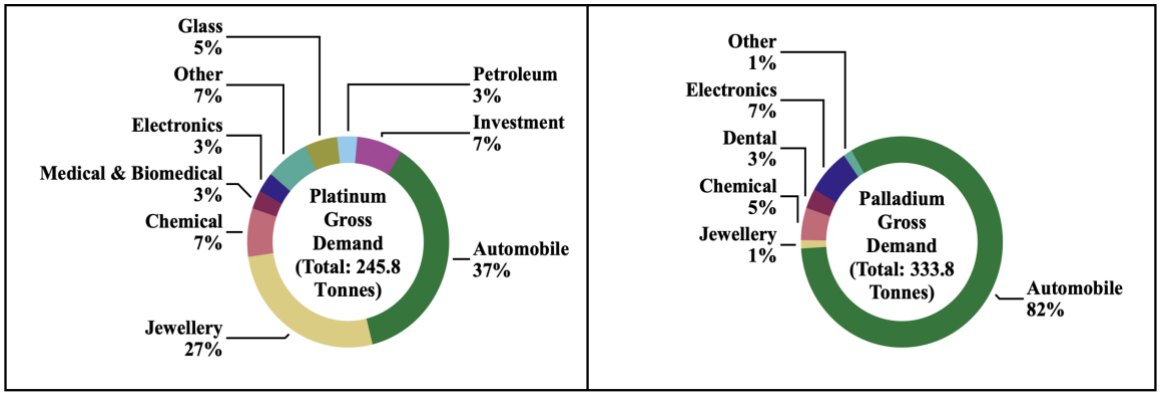

Figure 1.2. Yearly estimates of PGE demand in tonnes in various applications as a 6-year average (2016-2021) (Data source: JM, 2021).

The high redox potential of PGEs results in their use as the active components in VECs. By catalyzing redox reactions, harmful toxicants are transformed into their less dangerous derivatives prior to exiting the vehicle's exhaust (Pyrzynska, 2015). Converted exhaust gases include carbon monoxides (CO), nitrogen oxides (NO_x), and hydrocarbons (HC). As a reductive and oxidative catalyst, Pt simultaneously reduces NO_x groups to N_2 and oxidizes HC and CO to CO_2 and H_2O whereas Pd is an oxidative catalyst which performs the latter reaction (Twigg, 2006). The optimized removal of air pollutants using VECs assist in achieving reduced global emission standards.

Although VECs have positively impacted air quality, their use has led to an unprecedented increase of PGEs in the environment. PGE emission from VECs results from constant heat, mechanical, and chemical stressors (Bencs et al., 2003). Other less-resistant metals form connection points within the device, causing mechanical and chemical abrasions further contributing to PGE release. Since PGEs are applied as a coating rather

than to the entire device, new VECs (0 – 20 000 km) emit increased quantities of metals during use compared to their older counterparts (30 000 – 80 000 km) (Moldovan et al., 2002). Moreover, engine malfunction and higher driving speeds may also increase PGE emission rates (Helmers, 1997; Helmers & Kümmerer, 1999; Moldovan et al., 2002).

The Johnson Matthey PGM market report (2021) stated demands from the automobile industry annually totalled 91.6 tonnes Pt and 274.8 tonnes Pd for VEC production over a 6-year average. Overall, this industry uses 60% of all PGEs, with 82% of Pd and 37% of Pt demand allocated for VEC production. Interior VEC coating is primarily composed of Pd (72%), with additional Pt (20%) and other elements (8%) claiming the remainder of the mixture (JM, 2021). Moreover, a study by Palacios et al. (2000) found when catalytic material is crushed and subsequently analyzed, Pd recovery is most abundant. It is estimated that ~ 40% of PGEs are lost to emissions during an automobile's lifetime, resulting in the largest source emission of these metals (Helmers, 1997).

1.3.2 Anthropogenic PGE uses

The unique properties of PGEs include a high melting point, high corrosion resistance, mechanical strength, catalytic activity, and ductility (Ravindra et al., 2004; Fortin et al., 2011; Sobrova et al., 2012; Pawlak et al., 2014; Savignan et al., 2021). As powerful catalysts, many electrical and chemical industries commonly use PGEs in products. Second to the automobile industry, Pt demand (23% of total Pt use) remains high for jewellery production due to its durability and extravagance (JM, 2021). These sectors, however, are believed to be negligible in their contribution to PGE emissions. Jewellery is

generally recycled, and PGE use in these products are unlikely to wind up contributing to anthropogenic contamination (Rauch & Peucker-Ehrenbrink, 2015).

Mining, processing, and refinement activities are contributing sources of PGE emissions. South Africa, Russia, and Northern Europe are important regional sources for PGE emissions during these processes, as studies show elevated PGE concentrations in these locations (Rauch & Peucker-Ehrenbrink, 2015). Although Canada ranks third for PGE production, lack of environmental sampling may explain an absence of connection to Canadian extraction efforts as a contributing source of PGE emissions. Global emissions from mining, processing, and refining activities are estimated to exceed 5% of total PGE production, equating to 9 and 10 metric tonnes for Pt and Pd, respectively (Reimann & Niskavaara, 2006; Rauch & Peucker-Ehrenbrink, 2015).

The medical industry makes use of PGEs in a variety of applications. Cancer-treating drugs including cisplatin (cis-diammine-dichloro-platinum[II]) and carboplatin (diamine[1,1-cyclobutanedicarboxylato] platinum[II]) use Pt as its active component since it inhibits cellular division (Fortin et al., 2011). Soluble Pt excreted from cancer patients is found in medical treatment facility and household effluent ranging from low ng/L to high μg/L (Kummerer et al., 1999; Lenz et al., 2007; Vyas et al., 2014; Ghafuria et al., 2018). Documents show cardiac biomedical devices such as pacemakers, defibrillators, and catheters also contain Pt (Fortin et al., 2011). Additionally, alloys containing Pd are commonplace in dentistry; however, as the price of Pd rises they are increasingly replaced by less-expensive alternatives (Wataha, 2002).

Sewage and waste contribute to PGE emissions in urban areas, with industrial zones acting as an additional source. An example of a secondary source, waste and sewage discharge can release PGEs into natural aquatic environments. Municipal waste,

agricultural sewage sludge, and wastewater treatment plants are all documented sources for PGE emissions (Helmers & Kümmerer, 1999; Ek et al., 2004a; Stüben & Kupper, 2006; Elshkaki, 2013; Westerhoff et al., 2015; Vriens et al., 2017; Birke et al., 2018). For example, in Munich, Germany 1.3 kg of Pt is released into local rivers every year from wastewater treatment plants (Laschka & Nachtwey, 2000). Although VECs are the largest PGE emission source in urban locations, Rauch et al. (2006) described low traffic industrial areas that measured an abundance of PGEs in the surrounding environment. Thus, industrial emissions also contribute to PGE accumulation in urban areas.

Total PGE deposition in the northern hemisphere is estimated to be about 9-20 (Pt) and 20-50 (Pd) metric tonnes per year distributed amongst a range of sources (Rauch et al. 2005). With many activities utilizing PGEs in urban areas, it remains challenging to determine the exact source for area-specific PGE contamination. Some studies successfully calculated abundance ratios based on PGE source proximity; however, identifying the exact source remains elusive (Rauch & Peucker-Ehrenbrink, 2015). Although emission sources are mainly restricted to urban areas, the rural discovery of PGEs provides evidence that anthropogenic activities largely contribute to global surface contamination. Much of what we know about key PGE emission sources remains uncertain, including that from VECs (Zereini & Wiseman, 2015). Despite four decades of research, PGE emissions and fate within the environment continue to be largely unknown.

1.3.3 Environmental transportation of PGEs from anthropogenic sources

In addition to primary PGE sources, secondary sources can also elevate environmental levels of contamination. Emissions from VECs are predicted to remain in close proximity to the original deposition site as they are typically bound to particulate

matter in roadside environments (Morton et al., 2001; Ravindra et al., 2004; Morcelli et al., 2005; Bencs et al., 2006; Dubiella-Jackowska et al., 2009). In other studies, however, researchers documented PGE concentrations remaining higher than natural background levels at a great distance away from these areas, supporting widespread distribution. For example, areas that surround industrial, mining, processing, and refinement plants also find scattered PGE particulate as far as 200 km away (Reimann & Niskavaara, 2006). As such, these metals are thought to disperse over wide areas due to their high affinity for atmospheric particles.

Using atmospheric trajectory modeling, PGE ratios, and anthropogenic trends, it was discovered that PGE deposition in non-adjacent locations to primary emitters occurs through long-range atmospheric transport. For example, PGEs were identified in Central Greenland and European mountain ranges, in addition to fresh snow fall (Barbante et al., 2001; Barbante et al., 2004; Rauch et al., 2005; Moldovan et al., 2007). Despite the greatest risk for environmental PGE contamination remaining within roadside locations, urban areas, and those surrounding extraction-related activities, secondary sources pose a realistic emission source into remote ecosystems.

1.4 Environmental fate of PGEs

1.4.1 Distribution of PGEs in the environment

Prior to their use in anthropogenic activities, the natural biogeochemical cycle of PGEs had limitations due to rarity, low reactivity, solubility, and mobility (Mitra & Sen, 2017). The increased presence of PGEs within surface environments by humans have necessitated researchers to revise their understanding of the biogeochemical processes that drive transportation, distribution, and ecological fate of these metals. Upon environmental

deposition, PGEs become available for transportation to new environments or for transfer into biota (Fig. 1.3).

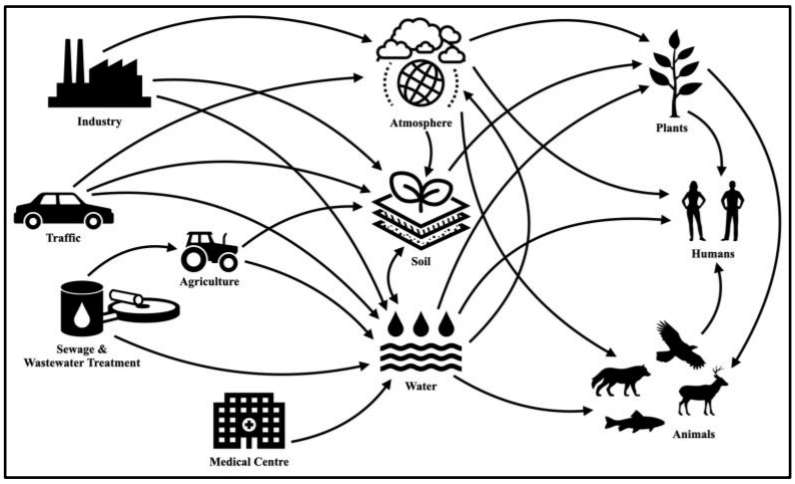

Figure 1.3. Fate diagram displaying PGE prime emissions sources and distribution pathways within the environment.

Much of the available field analytical efforts were in detecting PGEs within soil and dust near primary emitters. Particulate PGEs are commonly found in roadside environments where they exist in their inert metallic forms (Moldovan, 2007). However, environmental parameters can transform PGEs into their mobile forms, resulting in transport from roadside soil and dust environments (Zereini & Alt, 2006; Zereini et al., 2007; Jackson et al., 2010; Pawlak et al., 2014; Liu et al., 2015; Komendova, 2020). Although more concentrated within urban centres, non-urban areas still accumulate PGEs, with rural areas ranging from <0.3-218 μg/kg Pt and 0.1-656 μg/kg Pd, while urban areas vary from <0.5-2240 μg/kg Pt and <0.5-432 μg/kg Pt in soil matrices (Savignan et al., 2021). The highest reported Pt concentrations are 1730 μg/kg in dust (Wichmann & Bahadir, 2015) and 333 μg/kg in soil (Morton et al., 2001). Additionally, the highest reported Pd concentrations are 998 μg/kg in dust (Spada et al., 2012) and 191 μg/kg in soil (Zereini et al., 2007).

Other critical environmental areas, including aqueous environments, have received fewer analytical efforts. Of all six elements classified as PGEs, Pd is the most soluble, mobile, and bioavailable resulting in a greater likelihood to be transported to new environments compared to other PGE group members (Moldovan, 2007; Colombo et al., 2008; Haus et al., 2010). Further, a study by Aruguete et al. (2020) demonstrated VEC-associated Pd bound strongly to chloride and cyanide in road salt, which increased its environmental deposition and transport. This kind of inorganic complexation leads to increased Pd mobility. In general, measured PGE concentrations are in the low μg/L range for the water column, and μg/kg range for sediment (Fortin et al., 2011). The highest reported concentrations in freshwater are 10 μg/L and 3.3 μg/L (Odiyo et al., 2005), and sediment are 64 μg/kg and 57 μg/kg (Prichard et al., 2008) for Pt and Pd respectively. Sewage effluent can achieve higher PGE concentrations than other water sources due to its urban location and can reach concentrations nearing those found in soil and dust.

1.4.2 Chemical behaviour and transformation

Although frequently released as larger metal particulate, approximately 10% of Pt emitted from VECs is soluble compared to 50% of Pd (Zereini et al., 2001; Moldovan et al., 2002; Wiseman & Zereini, 2009). Some of the available elemental PGEs remain in soil as they undergo oxidation transformations and form insoluble compounds (Savignan et al., 2021). With lower solubility, Pt is much more likely than Pd to remain in soil and road dust in addition to other abiotic locations. Nonetheless, sources such as medical facility effluent have the potential to directly deposit soluble Pt into both non-natural and natural water bodies.

Various physico-chemical interactions impact solubility, mobility, complexation, and bioavailability of Pt and Pd. Similar to other metals, redox potential (Eh), pH, natural

organic matter (NOM), and the presence of complexing agents all influence the chemical behaviour of PGEs within the environment (Fortin et al., 2011). The release of PGEs within natural deposits is enhanced from high Eh, low pH, high concentrations of chloride ions, and NOM (Bertine et al., 1996; Bowles and Gize, 2005; Cobelo-Garcia et al., 2007; Dahlheimer et al., 2007). Additionally, Auge & Legendre (1994) found mobility increases when PGEs are complexed with oxides.

In non-saline aquatic environments, Pt(II) and Pd(II) are the dominant species (Mountain & Wood, 1988; Gammons, 1996). Typically, oxidation does not readily occur in aquatic environments; however, oxidation and thus solubility of PGEs can be increased in the presence of NOM, sulfur, and chloride (Ek et al., 2004a; Fortin et al., 2011). Upon oxidation from their natural elemental forms, PGEs readily form complexes with a multitude of elements, including chlorides, sulfides, polysulfides, cyanide, and organic acids (Nachtigall et al., 1996; Lustig et al., 1998; Mountain &Wood, 1988; Dahlheimer et al., 2007; Colombo et al., 2008; Mulholland & Turner, 2011; Šebek et al., 2011). Despite the increased investigation efforts for these emerging contaminants, many links connecting speciation and bioavailability in aquatic environments remain undiscovered.

1.5 PGE effects on living organisms

1.5.1 Analytical methods for determining PGEs in biological matrices

Environmental sampling of PGEs remains challenging for many reasons. Analytical efforts require measurements from instruments with high sensitivity to obtain accurate estimates (Bencs et al., 2003; Moldovan, 2007). Additionally, interference-free methods are necessary to detect low background levels and decern natural vs. anthropogenic presence of PGEs (Ely, 1999; Godlewska-Żyłkiewicz & Malejko, 2015). The challenge

faced in acquiring accurate environmental concentrations has led to an ongoing knowledge gap in PGE measurements for both field and in-lab quantification.

There are a variety of acceptable methods for detecting trace levels of PGEs. Arguably, inductively coupled plasma-mass spectrometry (ICP-MS) is the most sensitive technique for ascertaining low PGE levels (Rao & Reddi, 2000). Other techniques include atomic absorption spectrometry (AAS), inductively coupled plasma-optical emission spectrometry (ICP-OES), liquid electrode plasma optical emission spectrometry (LEP-OES), and electrothermal vaporization inductively coupled plasma-optical emission spectrometry (ETV ICP-OES) (Feichtmeier & Leopold, 2015; Komendova, 2020). Additional methods such as adsorptive stripping and differential pulse voltammetry can only detect Pt due to the irreversibility of electrode processes required for Pd measurement (Georgieva & Pihlar, 1997; Feichtmeier & Leopold, 2015).

1.5.2 Bioavailability and bioaccumulation

Awareness of the ongoing and increasing deposition of PGEs within the environment has triggered enhanced investigation efforts into how these metals may impact various species. However, publications are limited for the uptake, absorption, and adsorption of PGEs within biota. At a time where ecological reporting is scarce for PGEs, the inclusion of biomonitoring is a powerful tool to help predict relationships between environments and the organisms living within them (Orecchio & Amorello, 2015).

Plants are crucial bioindicators in PGE biomonitoring efforts (Ayrault et al., 2006; Feichtmeier & Leopold, 2015; Zechmeister et al., 2015). Studies demonstrate PGE concentrations found in plant material range from 0.09 ng/g to 14.6 μg/g, with Pd content noticeably higher than Pt (Feichtmeier & Leopold, 2015). Although this concentration

range exceeds that reported in soil samples, it is possible PGE dust on top of plant material was included in the total analysis leading to extremely high concentrations. Of the plant species surveyed to accumulate PGEs, not all have root systems. Detection of PGEs within non-rooting plants (e.g., moss and lichens) must result from atmospheric deposition, further demonstrating PGE distribution can be spread beyond the site of metal introduction (Conti & Cecchetti, 2001). Although plants with root systems (e.g., grasses) take up proportionally more PGEs compared to those without roots, both groups can be used to reflect current environmental concentrations owing to their functionality as PGE bioindicators (Pawlak et al., 2014).

There is scarce reporting of PGE bioaccumulation in terrestrial animals despite the known increase of PGE concentrations within soil and plant material. Currently only two studies, both of which used raptors, investigate PGE uptake, concentration, and distribution in field collected animals. Taken together, they show PGE accumulation in the ng/g range within blood, egg, liver, kidney, and feathers (Jensen et al., 2002; Ek et al., 2004b). Further, a mobility gradient of Pd>Pt was demonstrated through higher relative concentrations of Pd within the select samples. In addition to plants, birds can be useful bioindicators to assess the level of metal contamination originating from their food source and environment by examining feathers (Burger, 1993). By integrating temporal and spatial exposure routes, metals can be internally deposited within feather structure, or externally attached (Markowski et al., 2013). By analysing metal content in field collected feathers, researchers can estimate bioavailability and ecological presence of PGEs.

Like other contaminants, PGEs released to the environment eventually enter aquatic ecosystems. Of the available data on bioaccumulation and uptake of PGEs within aquatic animals, reports are generally split between marine and freshwater environments. Despite

a focus on freshwater for this thesis, it remains important to include marine studies to make up for the absence of information on freshwater areas and organisms. The previously shown bioavailability of soluble PGEs in field collected samples presents the opportunity for bioaccumulation within a range of aquatic organisms. Field studies confirm PGE accumulation increases in aquatic vertebrates and invertebrates in the following order: Pd>Pt (Moldovan et al., 2001; Essumang et al., 2008; Essumang, 2010). It should however be noted that metal concentrations in water were not measured within collection areas. Thus, these field assessments cannot be used to estimate species-specific uptake rates of each metal.

Available in-lab studies on uptake and bioaccumulation implement a variety of PGE material in their exposures including crushed VEC particulate, salts, and pre-dissolved solutions. Species of interest range from invertebrates (Rauch & Morrison, 1999; Moldovan et al., 2001; Sures et al., 2005) to crustaceans (Wren & Gagnon, 2014), mollusks (Zimmermann et al., 2002; Singer et al., 2005; Zimmermann et al., 2005a; Sures & Zimmermann, 2007; Osterauer et al., 2009; Mulholland & Turner, 2011; Ruchter & Sures, 2015; Brand et al., 2019), and fish (Sures et al., 2001; Zimmermann et al., 2004; Zimmermann et al., 2005b; Osterauer et al., 2009; Chen et al., 2015). Whole body measurements are standard for PGE bioaccumulation in invertebrates, whereas various organs and tissues in crustaceans, mollusc, and fish are typically assessed. In chronic PGE exposures to fishes, the liver and kidney are shown to accumulate PGEs (Zimmermann et al., 2005b). Accumulation at these organs is unsurprising as the liver is the main site for toxicant detoxification and the kidney excretes toxicants Further, researchers demonstrated PGEs accumulated at the gills owing to the presence of waterborne PGEs within the test environment (Zimmermann et al., 2015). Studies which investigated the effects of PGE

bioaccumulation concluded Pd is more concentrated than Pt at all measured areas. Few studies found opposite effects, where Pt was found at select tissues to a greater extent than Pd (Sures et al., 2005; French & Turner, 2008). However, in these instances, Pt and Pd were delivered in their inert forms and in non-equivalent concentrations and should be accepted with reservation.

An additional key parameter which influences uptake and bioaccumulation in aquatic systems is water quality. Using *Desulfovibrio desulfuricans* bacteria, Yong et al. (2002) tested a pH range of 2-7 and revealed the maximum biosorption capacity was at pH 2. Further, only pH values below 3.5 resulted in significant Pd uptake. These are expected results considering pH-dependent speciation graphs for Pd demonstrate the free-ionic form (Pd^{2+}) dominates at and below pH 2. To our knowledge, no other researcher has investigated the effects of pH on PGE uptake. Water hardness is an additional important parameter to consider when investigating water quality and its effects on metal bioaccumulation and uptake. It is well established that there is an ameliorative relationship between increased hardness and resultant metal toxicity, including uptake. As essential cation (e.g., Ca^{2+} and Mg^{2+}) concentration increases, contaminant metal concentrations are outcompeted for binding sites (Smith et al., 2015). However, to date there are no published studies which explore hardness in the context of PGE bioaccumulation. The last environmental variable which may influence PGE uptake is dissolved organic carbon (DOC). A fraction of organic carbon, DOC attenuates metal uptake by increasing the amount of organic material for ions to complex with, resulting in less-bioavailable metal species (Di Toro et al., 2001). In two separate studies, Zimmermann et al. (2002, 2005a) demonstrated PGE accumulation within zebra mussels was increased when combined with the addition of humic acid expressed as DOC. However, contradictory conclusions were

drawn in a separate study with the same organism, which showed the presence of humic substances reduced Pd bioaccumulation while having no effect on Pt uptake (Sures & Zimmerman, 2007). Differences may be attributed to PGE form used in the investigation (e.g., ground VEC material vs. soluble liquid), but it remains inconclusive as many less-understood factors may contribute to bioaccumulation and uptake of PGEs in aquatic animals.

1.5.3 PGEs as aquatic toxicants

The vast majority of PGE-toxicity research is focused on aquatic toxicity. Toxicological information remains limited, with much of the available data exploring yeasts (Frazzoli et al., 2007), microalgae (Fujiwara et al., 2008; Fortin et al., 2011), invertebrates (Biesinger & Christensen, 1972; Borgmann et al., 2005; Khangarot & Das, 2009; Okamoto et al., 2015), annelids (Khangarot, 1991; Veltz et al., 1996), and molluscs (Osterauer et al., 2009). Very little focus has been on complex aquatic organisms such as fishes (Ferreira & Wolke, 1979; Osterauer et al., 2009; Chen et al., 2015). Additionally, most toxicological studies report nominal exposure concentrations as opposed to measuring PGEs in solution, leaving knowledge gaps in our understanding of environmental relevance and risk.

Metal toxicity is known to vary according to factors including but not limited to exposure concentration, time, and physico-chemical parameters (Ferreira & Wolke, 1979; Khangarot, 1991; Farago & Parsons, 1994; Veltz et al., 1996). Thermally tolerant animals that can survive in an extensive range of temperatures (from 4°C to 20°C), such as annelids, confirm an increase in PGE toxicity as a function of increased temperature (Veltz et al., 1996). This observation is not surprising, as an increase in temperature results in increased

metabolism and expected uptake of contaminant within the exposed organism. Currently, only one water quality parameter has been explored in the investigation of PGE toxicity. A study by Borgmann et al. (2005) exposed the invertebrate *Hyalella azteca* to sixty-three metals, including Pt and Pd, and assessed resulting toxicity in soft and hard water conditions. Results from this investigation conclude the toxicity of sixty-two metals is diminished in hard water, including Pt. It is generally accepted that as water hardness increases, metal toxicity decreases (Pagenkopf, 1983), thus this result is expected. In contrast, Pd was the only metal of the study in which the opposite was observed with a greater toxicity in hard water. No explanation was provided in this study, or in follow up research, as to why Pd toxicity increased in hard water. However, palladium on calcium carbonate ($Pd/CaCO_3$) is widely used in catalytic systems including VECs where Pd complexes are reduced by $CaCO_3$ (Ballesteros-Soberanas et al., 2022). We can hypothesise this relationship persists in aqueous systems owing to higher Pd toxicity in hard as opposed to soft water due to an increase of bioavailable Pd complexes or species. Nonetheless, additional research is needed to further explore Pd toxicity in relation to varying water hardness.

The relative toxicity of Pt and Pd remains under debate. Although it is widely agreed upon that Pd emissions have a greater potential to be toxic in aquatic environments than Pt due to higher availability, uptake rates, and bioaccumulation; toxicity ranking remains inconclusive. Some studies conclude Pt is more toxic of the two, while others reveal opposite results (Table 1.1). Contradictions between studies may be due to the absence of non-standardized conditions in addition to differences in exposure duration, concentrations, and endpoints. Nonetheless, our current understanding of PGE toxicity remains underdeveloped, incomplete, and inadequate.

Table 1.1. PGE toxicity to aquatic animals.

Species	Endpoint	PGE	Concentration (μg/L)	Exposure Duration	Reference
Tubifex tubifex	Immobilization	Pt	EC50 = 95	24 h	(Khangarot, 1991)
Tubifex tubifex	Immobilization	Pt	EC50 = 86	48 h	(Khangarot, 1991)
Tubifex tubifex	Immobilization	Pt	EC50 = 61	96 h	(Khangarot, 1991)
Tubifex tubifex	Immobilization	Pd	EC50 = 237	24 h	(Khangarot, 1991)
Tubifex tubifex	Immobilization	Pd	EC50 = 142	48 h	(Khangarot, 1991)
Tubifex tubifex	Immobilization	Pd	EC50 = 92	96 h	(Khangarot, 1991)
Lumbriculus variegatus	Survival	Pt	LC50 = 397 to 30 000	96 h	(Veltz et al., 1996)
Cypris subglobosa	Immobilization	Pt	EC50 = 95	48 h	(Khangarot & Das, 2009)
Cypris subglobosa	Immobilization	Pd	EC50 = 195	48 h	(Khangarot & Das, 2009)
Hyalella azteca	Survival	Pt	LC50 = 131 to 221	7 d	(Borgmann et al., 2005)
Hyalella azteca	Survival	Pd	LC50 = 570 to >1000	7 d	(Borgmann et al., 2005)
Daphnia magna	Reproduction	Pt	EC16 = 14	21 d	(Biesinger & Christensen, 1972)
Daphnia magna	Reproduction	Pt	EC50 = 82	21 d	(Biesinger & Christensen, 1972)
Daphnia magna	Survival	Pt	LC50 = 520	21 d	(Biesinger & Christensen, 1972)
Daphnia magna	Immobilization	Pt	EC50 = 140	48 h	(Okamoto et al., 2015)
Daphnia magna	Survival	Pd	LC50 = 14	48 h	(Zimmermann et al., 2017)
Daphnia magna	Survival	Pd	LC50 = 25	24 h	(Zimmermann et al., 2017)
Daphnia magna	Survival	Pt	LC50 = 157	48 h	(Zimmermann et al., 2017)
Daphnia magna	Immobilization	Pd	EC50 = 19	24 h	(Zimmermann et al., 2017)

Daphnia magna	Immobilization	Pd	EC50 = 13	48 h	(Zimmermann et al., 2017)
Daphnia magna	Immobilization	Pt	EC50 = 276	24 h	(Zimmermann et al., 2017)
Daphnia magna	Immobilization	Pt	EC50 = 110	48 h	(Zimmermann et al., 2017)
Marisa cornuarietis	Heart Rate	Pt	LOEC = 0.1	14 d	(Osterauer et al., 2009)
Danio rerio	Heart Rate	Pt	LOEC = 0.1	96 h	(Osterauer et al., 2009)
Danio rerio	Survival	Pd	LC50 = 292.6	96 h	(Chen et al., 2015)
Danio rerio	Hatching rate	Pd	IC50 = 181.5	72 h	(Chen et al., 2015)
Oncorhynchus kisutch	Survival	Pt	LC50 = 15 500	24 h	(Ferreira Jr & Wolke, 1979)
Oncorhynchus kisutch	Survival	Pt	LC50 = 5 200	48 h	(Ferreira Jr & Wolke, 1979)
Oncorhynchus kisutch	Survival	Pt	LC50 = 2 500	96 h	(Ferreira Jr & Wolke, 1979)

1.6 Environmental regulations

1.6.1 Toxicology models and protective guideline creation

Over the years, increased waste production and the introduction of new contaminants has exponentially risen along with resource exploitation and technological development. Ongoing demand for risk assessment and environmental evaluation can prove difficult as each year there is not only more quantities of known pollutants within aquatic ecosystems, but the introduction of novel contaminants or contaminants of emerging concern such as PGEs (Sousa et al., 2018). Predictive modeling, however, provides the necessary tools for high predictability and reliability while conserving the unnecessary exploitation of resources and animals (Villeneuve et al., 2014). Using toxicology models can guide current and future PGE research directions in obtaining data that can be used towards the creation of risk assessments and government guidelines.

Governing committees create regulatory standards to protect organisms from harmful contaminant exposure. Currently, criteria guidelines for organismal protection against PGEs are absent globally for any matrix, including freshwater. Proper risk assessments remains futile for PGEs from lack of suitable data, including fate and effects within the environment (Fortin et al., 2011; Bengtsson, 2019). Regulatory communities require more quality data to make formal recommendations for guideline development, including water quality guidelines (WQGs). Until that time, the risk PGEs pose to aquatic life remains largely unclear.

1.6.2 Biotic ligand model

Another useful approach used in regulatory development and risk assessment in aquatic systems is the biotic ligand model (BLM). Built on foundational work of the free-ion activity model (Morel, 1983; Campbell, 1995) and gill surface interaction model

(Pagenkopf, 1983) the BLM framework describes toxicity as the point when a metal-biotic ligand complex reaches critical concentration (Di Toro et al., 2001). Researchers have used this predictive model to demonstrate the vital role water quality plays in metal toxicity and bioavailability, adding support to the argument that total aqueous metal concentration is not the only important factor (Mebane et al., 2020). Understanding competing cations in addition to organic matter and inorganic ligand complexation at the site of action results in a better understanding of metal toxicity thresholds at a specified biotic ligand (Di Toro et al., 2001).

BLMs consider the influence of site-specific water criteria on contaminant toxicity and are commonly used by regulatory communities in the development of environmental policies such as WQGs (Väänänen et al., 2018). While not yet implemented globally in the development of water protection policies, BLMs are used within the establishment and revisions of WQGs in some governing bodies such as the EU, USA, and Canada (Adams et al., 2020). Additionally, although proposed as a framework to move away from gill-based toxicity, many current working BLMs use gills as the biotic ligand when evaluating fish. Thus, although considered an effort to increase protection to aquatic life, established gill-based BLMs may not be inclusive when estimating trace metal toxicity to other biotic ligands such as olfactory tissue.

As increased investigation efforts focus on contaminants of emerging concern including PGEs, the use of predictive modeling is essential. By implementing predictive toxicological modeling tools such as BLMs, risk assessment and regulatory development can be centred on variables which are historically difficult to measure in the field. Direct linkages spanning biological levels of organization including molecular, cellular, organ, and organism responses can be confidently connected within a controlled laboratory setting

and applied to real-world ecosystems. In the estimation of PGE toxicity to a range of species, the two models can work together to assess the risk PGEs pose in various aqueous environments. As with all complicated concepts, there is not a one size fits all solution for toxicological remediation and these models aim to exploit that.

1.6.3 Use of rainbow trout in ecotoxicology research

Rainbow trout (*Oncorhynchus mykiss*) is an established representative cold-freshwater fish for ecotoxicological research. This fish species is readily available, distributed globally, is sensitive to environmental contaminants, and an extensive toxicology database has been assembled for this species (Wolf & Rumsey, 1985). Further, studies revealed metal-induced olfactory sensitivity using rainbow trout including Cd (Sloman et al., 2003), Cu (Hara et al., 1976), Ni (Dew et al., 2016), and Hg (Hara et al., 1976). When investigating data-poor metals, such as Pt and Pd, selecting the right model organism for the study is imperative. Thus, rainbow trout is the ideal species for investigating Pt- and Pd-induced olfactory toxicity.

1.7 Research objectives

Although naturally rare, Pt and Pd are classified as contaminants of emerging concern due to their widespread use in industry and in common devices such as VECs. Additionally, environmental concentrations of these metals are expected to rise annually along with continued anthropogenic use. Despite this knowledge, little information exists on PGE toxicity and bioavailability leading to difficulty in assessing aquatic health when PGEs are introduced to the environment. Using olfaction, direct linkages can be made between PGE exposure and valuable life history traits to determine if PGE contamination poses a realistic risk of toxicity in freshwater fish. The broad goal of this study is to explore

Pt- and Pd- induced olfactory toxicity in rainbow trout. To this end, the central thesis objectives are as follows:

1. Establish threshold concentrations for Pt and Pd required to inhibit olfactory function in rainbow trout
2. Understand how water quality affects metal toxicity compared to baseline through varying levels of DOC, pH, and water hardness
3. Investigate the mechanism for PGE-induced olfactory system disruption

1.8 References

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CHAPTER 2: Effects of water quality on palladium-induced olfactory toxicity and bioaccumulation in rainbow trout (*Oncorhynchus mykiss*)

This thesis aims to explore olfactory perception in rainbow trout when separately exposed to two contaminants of emerging concern: palladium (Pd) and platinum (Pt). To this end, the current chapter explores effective waterborne concentrations of Pd required to elicit olfactory reduction by 20% (IC20) and 50% (IC50). In this research chapter we manipulated a range of water quality parameters to measure worsening, ameliorating, or neutral effects on the Pd-exposed olfactory system when placed in varying environments. Further, the mechanism of olfactory dysfunction following Pd exposure was broadly investigated through oxidative stress and tissue-specific bioaccumulation measurement.

This is a manuscript-style thesis written for the University of Lethbridge based on submission requirements and regulations. As such, content repetition can be noted within the introduction, materials, and methods sections of research chapters. This chapter is formatted to be submitted to *Integrated Environmental Assessment and Management*.

Contribution of Authors: I designed, performed, collected, analyzed the data, and wrote the manuscript. Lauren Zink provided scientific input, guidance, and training. Dr. Sarah Ellen Johnston and Dr. Matthew Bogard provided guidance and quantified DOC content in addition to providing writing concerning DOC quantification methods. Dr. Gregory Pyle provided direction, guidance, scientific input, supervision, comments/assisted writing, and funding for this research.

2.1 Abstract

Through emission processes, palladium (Pd) particulate from industrial applications is introduced into a range of ecosystems including freshwater environments. Despite this, Pd-induced bioaccumulation, uptake, and toxicity has been scarcely explored in freshwater fishes. To assess the toxicity potential of Pd, the present study aimed to explore olfactory disruption in rainbow trout (*Oncorhynchus mykiss*) following waterborne Pd exposure. Olfactory acuity measured by electro-olfactography (EOG) demonstrated Pd inhibits the entire olfactory system following 96 h exposure. Considering no protective guidelines exist, measured Pd concentrations required to inhibit olfactory function by 20% (2.5 μg/L; IC20) and 50% (19 μg/L; IC50) were established. Rainbow trout were further exposed to IC20 and IC50 Pd concentrations in combination with varying environmental conditions. As water hardness increased olfactory perception decreased, owing to additional ion interference at the olfactory epithelium. No environmental parameter in this study had a significant interaction with Pd-induced olfactory toxicity. Lastly, the production of malondialdehyde (MDA) as a lipid peroxidation (LPO) by-product was not detected, thus oxidative stress at the target ligand was deemed to not play a role in olfactory dysfunction. In contrast, significant levels of membrane associated Pd was measured at the olfactory rosette and gill. Our data suggest Pd is toxic to rainbow trout via waterborne contamination near field measured levels. This study further demonstrated Pd bioavailability and uptake at water-adjacent tissues, adding to our collective understanding of the toxicological profile of Pd. Taken together our results are the first to explore olfactory toxicity in fish following Pd exposure.

2.2 Introduction

Fishes rely on optimal olfactory perception to mediate a range of crucial life-history behaviours including foraging, homing, mating, and predator avoidance (Klaschka, 2009). The olfactory epithelium comprises a variety of olfactory sensory neurons (OSNs) which are in direct contact with surrounding waters. Odourants within the water bind to specified receptor proteins at one of at least three OSN subtypes where, if a threshold potential is met, an electrical signal is sent to the brain for information processing (Laberge & Hara, 2001). The three most researched types of OSNs found in the fish olfactory epithelium are microvillous, ciliated, and crypt cells (Hansen & Zielinski, 2005). Each cell type is stimulated by a distinct odour, resulting in cell-specific odourant detection (Hansen et al., 2003). Microvillous OSNs perceive food cues, ciliated OSNs detect social cues, and crypt cells recognize sex pheromones (Hamdani & Døving, 2007). Work in freshwater fishes has demonstrated microvillous cells respond to amino acids, such as L-alanine, whereas ciliated cells react to bile acids, such as taurocholic acid (TCA) (Dew et al., 2014). A variety of contaminants are known to impair fish olfactory function, including metals (Tierney, 2016). Resultant disruption of the olfactory system may lead to maladaptive behavioural responses and potential adverse outcomes at the organism or population level.

Automobile traffic causes environmental contamination with a variety of metals including palladium (Pd). Widely used in the production of vehicle exhaust catalysts (VECs) for its catalytic abilities and resistance to internal fluctuation, Pd extraction has soared in recent decades. Currently 82% of global Pd demand is allocated to the automobile industry, comprising a 72% majority in the metal mixture used in VEC manufacturing (JM, 2021). Unsurprisingly, environmental detection of Pd has risen over the decades and is chiefly attributed to anthropogenic VEC effluent (Ravindra et al., 2004). Abrasions from

chemical, mechanical, and thermal wear over continued vehicle use result in Pd emission into the surrounding environment (Bencs et al., 2003). Detection of Pd in rural areas including those 200 km away from primary emitters and in fresh mountain snowfall demonstrate the mobility and dispersion of Pd once it enters surface environments (Rauch et al., 2005; Reimann & Niskavaara, 2006; Moldovan et al., 2007). Consistent environmental sampling remains scarce, however measured waterborne Pd concentrations are generally in the low μg/L range (Fortin et al., 2011).

It is estimated that 50% of VEC emitted Pd is in its soluble form (Moldovan et al., 2002). Although Pd detection is highest in soil and dust along roadways, its chemical behaviour results in likely transformation to more mobile and soluble forms, bringing Pd into aquatic ecosystems (Ek et al., 2004). Palladium readily forms complexes through interactions with environmental ligands including chloride, sulfides, polysulfides, cyanide and organic acids (Mountain & Wood, 1988; Dahlheimer et al., 2007; Colombo et al., 2008a; Mulholland & Turner, 2011; Šebek et al., 2011). In addition to its high mobility and solubility compared to other platinum group elements (PGE), studies have shown Pd is the most bioavailable metal in its group (Colombo et al., 2008b). Further, Pd uptake and accumulation has been demonstrated by *Anguilla anguilla* (Sures et al., 2001; Zimmermann et al., 2005) and *Barbus barbus* (Sures et al., 2005) supporting bioavailability of Pd to aquatic vertebrates. To our knowledge, only one preceding study has investigated toxic endpoints in fish following Pd exposure (Chen et al., 2015). This investigation used zebrafish (*Danio rerio*) as a model species to explore Pd-induced morphology changes in embryonic development and its related molecular mechanisms. Although important, this study provides limited knowledge to the toxicity profile of Pd, specifically to the development of risk assessments. Despite increased research efforts into the toxicity and

mechanism of action (MOA) of Pd, our understanding of Pd-induced toxicity is largely fragmented, and conclusive links have yet to be made. As many knowledge gaps exist surrounding the level of Pd toxicity, it remains a priority to develop a functional understanding of Pd in aquatic ecosystems and the ecological risk Pd contamination may pose.

Increased surface Pd via anthropogenic deposition has led to investigators classifying this metal as a contaminant of emerging concern (Mitra & Sen, 2017). However, there exist no risk assessment or guidelines for the protection of aquatic life. The biotic ligand model (BLM) was developed to predict metal toxicity at a specific biological ligand in varying environmental conditions and best extrapolate laboratory results to the field (Di Toro et al., 2001; Paquin et al., 2002). The gill-based BLM has been implemented in many environmental management guidelines for metals such as copper (EU, 2007; USEPA, 2007; ECCC, 2021). By providing site-specific water quality criteria based on ambient conditions, ecologically relevant WQGs can be applied to different biological ligands such as the olfactory epithelium. It is generally understood that water quality parameters including water hardness, natural organic matter, and pH play a vital role in metal toxicity thresholds to aquatic organisms (Pinheiro et al., 2021). These variables however do not provide the same level of protection to all biological ligands and investigated tissues are shown to be more sensitive than others (Mebane et al., 2020). Consequently, what may be deemed a safe environment when quantifying metal effects on one ligand (e.g., gills) may still prove detrimental to the organism of concern.

The main goal of this research was to explore olfactory disruption in rainbow trout (*Oncorhynchus mykiss*) following waterborne Pd exposure. Rainbow trout are a well-established cold-freshwater model species for ecotoxicological research backed with a

history of olfactory toxicity investigation using metal contaminants (Hara et al., 1976; Wolf & Rumsey, 1985; Dew et al., 2016; Lari et al., 2018; Razmara et al., 2019). The objectives of this study were: 1) to establish 96 h Pd-induced olfactory toxicity thresholds in rainbow trout, 2) understand how the physico-chemical parameters of varying water hardness, pH, and dissolved organic carbon (DOC) affect olfactory toxicity after acute exposure to Pd, and 3) investigate fundamental toxicity for Pd-induced olfactory system disruption.

2.3 Materials and methods

2.3.1 Test animals

Juvenile rainbow trout (n = 278, standard length = 10.8 ± 1.7 cm, weight = 19.5 ± 8.9 g (mean ± SD)) were obtained from Sam Livingston Fish Hatchery (Calgary, Canada) and immediately transferred to the Aquatic Research Facility (ARF) at the University of Lethbridge (UofL). Fish were stocked at a density of 5 g/L in static-renewal holding tanks supplied with processed cold water (PCW). Lab-ready PCW was created from city water using both mechanical (dechlorinated, large particle removal, buffered, and UV sterilized) and biological (bio-inoculated media) filtration. Fish were fed twice daily with 1.5 mm trout chow pellets (EWOS, Canada) and let acclimate for a minimum of two weeks prior to being used in experiment. Holding conditions maintained a 16:8 light:dark photoperiod with measured water quality as follows (mean ± SD; n=24): temperature, 12.0 ± 0.1 °C; dissolved oxygen, 8.4 ± 0.1 mg/L; ammonia, 0.01 ± 0.01 mg/L; nitrite, 0.004 ± 0.003 mg/L; nitrate, 2.1 ± 0.5 mg/L. Experiments were reviewed and approved by the UofL Animal Welfare Committee (AWC) (protocol #1914).

2.3.2 Preparation of test solutions

Palladium (II) nitrate solution (in 5% HNO_3) was purchased from Inorganic Ventures (99.9-100.0% Pd purity; Inorganic Ventures, Canada) at a concentration of 1002 ± 6 μg/mL. Pd solution was stored at 4 °C in dark lighting until use. No changes were made to the Pd solution and was added directly into experimental tanks in pre-determined quantities following fish addition.

2.3.3 Experimental design

To assess Pd-induced olfactory toxicity, fish were exposed to a dilution series. As no current literature describes Pd toxicity thresholds to rainbow trout, broad preliminary ranges were explored to obtain an ideal dilution series for inhibitory concentrations (IC). A 96 h IC assay was conducted to discover Pd concentrations required to inhibit olfactory function by 20% and 50%; in other words the 96 h IC20 and IC50. In this experiment, fish were exposed to a nominal dilution series of 0 μg/L, 20 μg/L, 50 μg/L, 100 μg/L, and 200 μg/L of Pd for 96 h. Static-renewal exposure systems used one fish per tank in 15 L of solution to comply with optimal biomass limits. To maintain ammonia levels below 0.02 mg/L, 50% of the total water volume was replaced daily with respective treatment solutions in each exposure vessel. Additionally, fish were not fed during exposure periods to maintain tolerable organic waste by-product levels and to ensure no dietary exposure. Fish were held at a 16:8 light:dark photoperiod and dissolved oxygen never measured below 90% saturation. To allow for adequate Pd solution equilibrium, treatment solutions were created at least 12 h prior to water changes. Olfactory activity was measured using electro-olfactography (EOG) as described by Lari and Pyle (2017) and the UofL AWC standard operating protocol (SOP) #A14. Metal contaminants can be classified as broad or targeted

OSN inhibitors. Thus, receptor-specific odourants were selected to investigate OSN sub-population targeting after acute exposure to Pd. In this study, microvillous and ciliated OSN activity were measured using 10^{-4} M L-alanine (Fisher Scientific, Canada) and taurocholic acid (TCA; Sigma-Aldrich, USA) respectively. Using EOG derived calculations, the IC20 and IC50 Pd values were used for subsequent water quality manipulation experiments.

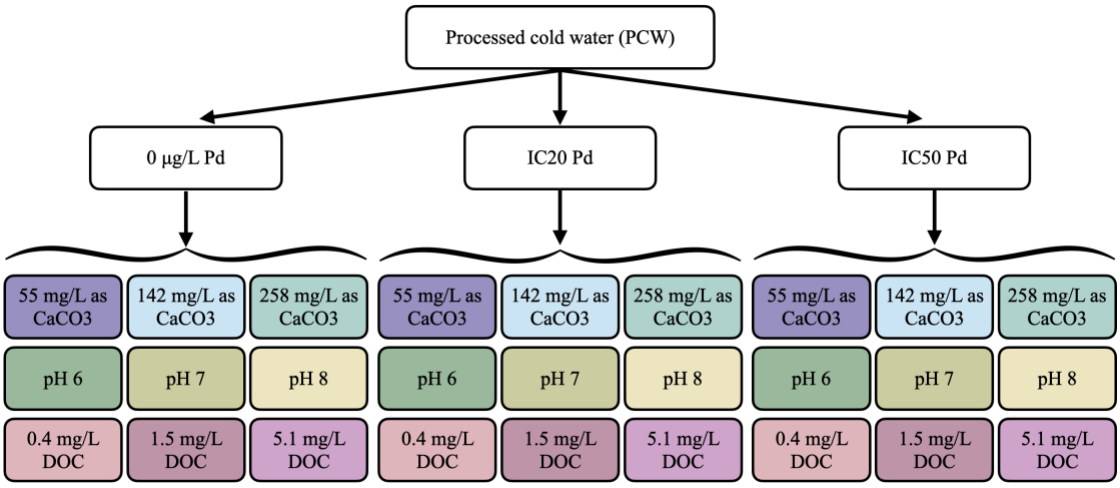

Figure 2.1. An illustrated diagram of the 27 exposure groups used when studying the effects of water quality parameters and their interaction with Pd on rainbow trout olfaction.

To investigate the impact of water hardness, pH, and DOC from dissolved humic acid solution on Pd-induced olfactory toxicity, 27 total treatment groups of fish (n = 6) were exposed to predetermined IC20 (10 µg/L) and IC50 (42.5 µg/L) olfactory impairment values in addition to a control (0 µg/L) for 96 h as shown in Fig. 2.1. Prior to exposure start, fish experienced an acclimation period to manipulated water over 72 h to mitigate shock risk and solution instability. Daily 50% water changes were completed with same solution replacement during both acclimation and exposure periods. Fish were fed once a day during the acclimation period using 1.5 mm trout chow pellets but not during exposure (EWOS, Canada). Experimental conditions maintained a 16:8 light:dark photoperiod and dissolved

oxygen was always above 90% saturation. Following the 96 h exposure, fish olfactory activity was measured using EOG. Each Pd treatment was represented in the following water quality parameters: soft water (55 mg/L as $CaCO_3$), hard water (142 mg/L as $CaCO_3$), very hard water (258 mg/L as $CaCO_3$), acidic pH (pH 6.02), neutral pH (pH 7.04), basic pH (pH 8.24), no DOC (0.4 mg/L), low DOC (1.5 mg/L), and high DOC (5.1 mg/L) as shown in Table 2.1. Water hardness was decreased by cutting PCW with Millipore (EMD Millipore, USA) and increased through the addition of equal parts $CaSO_4$ (Fisher Scientific, USA) and $MgSO_4$ (Fisher Scientific, USA). Since PCW maintained a basic pH, 12N HCl (ACS reagent grade, EMSURE, USA) was added to reduce pH to acidic and neutral levels. Addition of humic acid (Alfa Aesar, USA) to PCW created our DOC stock solution. The DOC stock solution was used within 48 h of creation, maintained at 4°C, and wrapped in aluminium foil to prevent photodegradation. To allow for sufficient equilibration time in all water quality manipulated solutions, salts or acids were added a minimum of 72 h prior to use and agitated using an air stone. Measurements were taken at full, half, and low water content in each stock container to ensure complete solution equilibrium was met, and no fluctuation occurred between water changes.

2.3.4 Palladium and water quality analyses

Palladium concentrations in tank water were measured at the start (i.e., prior to fish or Pd addition), beginning (i.e., 24 h following fish and Pd addition) and end (96 h from fish and Pd addition) of the experiment using graphite-furnace atomic absorption spectrometry (GFAAS) (GTA120 GFAAS, Agilent Technologies, USA). Water samples were collected and immediately acidified to 1% 12 N HNO_3 (TraceSelect grade; Sigma Aldrich, Canada) and held at 4 °C until required for analysis. Every 10 samples a certified

reference material (CRM) (in 10% HCl, 99.3-100.7% certified Pd value; Inorganic Ventures, USA) was used as a run standard and tested for QA/QC verification. Further, the calibration curve re-plotted every 20 samples with mean QA/QC recovery within the accepted ± 10% error. Method detection limit (MDL) for Pd was measured to be 5 µg/L.

Despite pre-conditioning of tanks and materials, Pd adsorption and precipitation within the test system was still prevalent as is reported in other Pd toxicological exposures (Cobelo-Garcia et al., 2007). At this time, no mitigatory strategies have been presented. Although preliminary tests were completed with the aim of improving the amount of Pd remaining in solution, efforts were futile. Pre-conditioning was practiced nonetheless to present the best possible test system, with Pd quantification after water addition and prior to metal addition to rule out leached exposure from the tank system.

A subset of tanks per treatment (n=3) was randomly selected prior to exposure start where temperature, dissolved oxygen, conductivity, ammonia, nitrate, and nitrite were measured from the same tanks daily. Water hardness and alkalinity were measured from the subset of tanks at the beginning (i.e., 24 h after fish and Pd addition) and end (i.e., 96 h after fish and Pd addition) of experimentation. Additional daily measurements were taken from hardness and pH stock solutions. DOC stock was measured after creation. Water samples from DOC stock were filtered with a 0.45 µm pore size using 25 mm, cellulose acetate membrane filters (VWR, USA). Samples were analyzed using high temperature catalytic oxidation with a Shimadzu TOC-L CPH on filtered (0.45 µm) and acidified to pH 2 with 12 M HCl. Samples were sparged for 5 minutes with CO_2-free air to remove inorganic carbon prior to analyses. Concentrations were quantified using a 5-point standard curve and three out of seven injections with a standard deviation less than 0.1 mg/L. Water quality during each experiment is reported in Table 2.1.

Table 2.1. Measured water quality in exposure tanks (n = 32) and stock solution (pH, n = 17; water hardness, n =17; DOC, n=3).

	PCW	Soft	Very Hard	Acidic pH	Neutral pH	Low DOC	High DOC
Temperature (°C) Mean ± SD	11.9 ± 0.2	12.0 ± 0.1	12.0 ± 0.3	12.3 ± 0.1	12.3 ± 0.1	12.2 ± 0.1	12.2 ± 0.3
Dissolved Oxygen (%SAT) Mean ± SD	95.1 ± 3.3	101.0 ± 3.8	101.3 ± 3.7	103.1 ± 3.6	101.8 ± 3.8	97.5 ± 4.5	96.5 ± 2.8
Conductivity (µS/cm) Mean ± SD	333.5 ± 9.3	144.1 ± 10.5	539.7 ± 9.4	408.3 ± 9.5	404.8 ± 9.0	329.5 ± 10.1	332.1 ± 8.7
Ammonia (mg/L) Mean ± SD	0.15 ± 0.1	0.11 ± 0.2	0.16 ± 0.1	0.19 ± 0.1	0.12 ± 0.1	0.15 ± 0.1	0.13 ± 0.2
Nitrite (mg/L) Mean ± SD	0.003 ± 0.0	0.003 ± 0.0	0.002 ± 0.0	0.003 ± 0.0	0.002 ± 0.1	0.003 ± 0.0	0.004 ± 0.1
Nitrate (mg/L) Mean ± SD	0.8 ± 0.7	1.3 ± 0.5	1.7 ± 0.3	1.1 ± 0.8	0.9 ± 0.3	1.3 ± 0.7	1.2 ± 0.4
pH Median (range)	8.24 (8.14 – 8.33)	8.05 (7.98 – 8.17)	8.50 (8.38 – 8.57)	6.04 (5.92 – 6.21)	7.04 (6.91 – 7.30)	8.06 (7.92 – 8.26)	8.17 (8.06 – 8.36)
Hardness (as mg/L $CaCO_3$) Mean ± SD	142 ± 3.2	55.1 ± 6.6	257.7 ± 7.2	147.4 ± 3.4	147.9 ± 3.4	143.4 ± 4.6	144.9 ± 4.3
Alkalinity (mg/L) Mean ± SD	123 ± 3.9	40.9 ± 4.7	121.8 ± 4.9	2.5 ± 1.1	9.5 ± 5.4	124.6 ± 5.1	122.3 ± 4.8
DOC (mg/L) Mean ± SD	0.4 ± 0.1	-	-	-	-	1.5 ± 0.1	5.1 ± 0.3

2.3.5 Electro-olfactogram (EOG) assay

Following the 96 h exposure to clean or Pd contaminated solution, fish were anaesthetized using 120 mg/L tricaine methanesulfonate (TMS; AquaLife, Canada) and buffered to pH 7.4 using 360 mg/L $NaHCO_3$ (Fisher Scientific, USA). Anesthetization was assumed to be complete upon ceased movement from the operculum. Olfactory response was measured using the neurophysiological assay, electro-olfactography (EOG). Upon anesthetization, fish were wrapped in damp paper towel to prevent drying and placed under a dissecting scope to reveal the primary olfactory organ, the olfactory rosette. Surgical forceps were used to extract the intranarial septum, which separates the anterior and posterior opening of the naris and partially covers the olfactory rosette. Fish were immediately placed within the perfusion trough where a grounding clip was clamped onto the tail to remove any outside electrical interference and the gill perfusion line was inserted into the mouth to maintain viability of the fish throughout the assay.

The olfactory perfusion line was placed directly above the naris, allowing for solution to flow over the rosette. For olfactory cue makeup, a 10^{-4} M solution of TCA (CAS #345909-26-4; Sigma-Aldrich, USA) or L-alanine (Fisher Scientific, Canada) was made fresh daily in PCW. Additional blank cues contained only PCW to verify odourant responses. To obtain strong readings on the olfactory rosette, a glass microelectrode recording probe was placed at the third lamella, as measured from the bottom right of the rosette, approximately at 1/3 distance from base and ½ lamella width. An additional glass microelectrode reference probe was placed on the skin above the naris parallel to the eye to remove electrical interference from the fish. Prior to collecting data, fish were subject to a 5-minute acclimation time upon completion of probe and line placement. Following acclimation, cues were delivered in a randomized order to negate odour habituation a

minimum of three times in three second pulses with a recovery time of two minutes between cue deliveries. PCW constantly perfused into the olfactory chamber to ensure olfactory tissue did not dry out during acclimation and between cue deliveries. Olfactory responses from cues were amplified as described by Razmara et al. (2019) and calculated using the difference between baseline and maximum amplitudes. True OSN response from a cue was determined by subtracting blank responses from the calculated cue response. This technique was also performed on euthanized fish to ensure responses were from live olfactory tissue and not subject to external interference.

2.3.6 Oxidative stress and bioaccumulation assays

Following the EOG assay, fish were euthanized using buffered TMS solution (240 mg/L TMS (tricaine methane sulfonate, AquaLife, Canada) and 720 mg/L $NaHCO_3$ (Fisher Scientific, USA)) and select tissues were harvested for further investigation. Using information provided by preliminary bioaccumulation exploration, the gill basket (n=21), olfactory rosette (n=7), and liver (n=21) were isolated and placed in pre-weighed 15 mL Falcon tubes (Fisher Scientific, USA). Tissues were then oven-dried at 60 °C until a constant weight was reached. Prior to acidification, an additional weight measurement was taken to obtain dry weight. Room temperature tissue samples were acidified to 6% using 12 N HNO_3 (TraceSelect grade; Sigma Aldrich, Canada) and held at 4°C until GFAAS analysis. Tissue quantification protocol for GFAAS was the same as is outlined in section 2.3.4. Currently, there exists no tissue matrix CRM which contains Pd. However, to assess the accuracy of the digest protocol, DOLT-4 (5 mg dogfish liver; National Research Council Canada, Ottawa, ON, Canada) was digested and analyzed alongside known metal protocols to ensure complete digest and metal recovery (94% mean metal recovery) for unknown Pd samples. Normalized tissue Pd concentration was calculated as follows:

((absorbance*total digest volume)/dried sample weight). To best represent environmental conditions and determine if Pd leads to functional impairment of rainbow trout olfaction, bioaccumulation was measured as absorbed and adsorbed membrane associated Pd. Neither metal speciation nor phase was measured in this study. Therefore, it is unknown if equilibrium was achieved between the contaminant in solution and organism resulting in reporting concentration factor (CF) rather than bioconcentration factor (BCF). Tissue specific CFs were calculated to compare uptake capacities between tissues where C_{tissue} is mean Pd concentration within a specified tissue (μg/kg) and C_{water} is the mean measured true Pd concentration in water (μg/L) ($CF = C_{tissue}/C_{water}$).

A subset of olfactory rosettes were harvested following the EOG assay and immediately stored in -80°C until use. Presence of lipid peroxidation (LPO) was used to assess oxidative stress via lipid structure alterations within fish olfactory tissue (n = 6) following the 96 h exposure. Creation of LPO results in the formation of malondialdehyde (MDA) which was measured using thiobarbituric acid reactive substances (TBARS) Parameter assay kit (catalogue #KGE013; R&D Systems, USA). Using a Bicinchoninic Acid Kit (catalogue #SK3021; Bio Basic, Canada) to quantify protein content, MDA contents were normalized to protein concentration. Both kits were run according to the provided manufacturer protocol.

2.3.7 Statistical analyses

All statistical analyses were performed in R (R Core Team, 2020; version 4.0.0 Arbor Day). Experimental data were tested for normality and homogeny of variance using Shapiro-Wilks and Bartlett tests, respectively. When test assumptions were violated and data transformations failed to reclaim parametric assumptions, a permutational multivariate

analysis of variance (PERMANOVA) was performed using the adonis2 function in the vegan (Oksanen et al., 2020) package. Permutations (n= 4999) measured the absolute distance between data for two-way and one-way PERMANOVA designs using "Manhattan" or "Bray" dissimilarity indexes respectively. When a PERMANOVA yielded significant main effect results without an interaction, a *post hoc* permutation analysis was conducted using the pairwiseAdonis (Martinez Arbizu, 2017) package. Outliers were considered for removal if detected using the interquartile range (IQR) rule. The rstatix (Kassambara, 2021) package was applied and data points were considered an outlier if a datum was more than 1.5 * IQR above the third quartile or below the first quartile of each dataset. Outliers were removed only if the resulting statistical significance was not altered, but their removal led to improving parametric test assumptions. Remaining Pd concentrations that fell below MDL, but had known Pd addition, were replaced by a dataset-specific value which was calculated by the Kaplan Meier (KM) model from the survival (Therneau, 2020) package.

To estimate the relative EOG response to individual stimuli (L-alanine and TCA) at varying Pd concentrations in a series dilution, a blank corrected relative EOG stimulus-response dataset was created through dividing the control response (mean control cue response - blank response) by blank corrected cue specific values. A two-way PERMANOVA was applied to test the interaction between odourant type and Pd treatment regarding olfactory inhibition in the series dilution. To find the no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC), a one-way analysis of variance (ANOVA) followed by a Dunnett's *post hoc* test was applied. Using data from the series dilution, the drc (Ritz et al., 2015) package was applied to estimate IC20 and IC50 Pd values.

A two-way ANOVA was conducted to compare the effect of manipulated water hardness and Pd treatment to olfactory dysfunction. The same test was used to assess for mitigatory effects of DOC to Pd-induced olfactory impairment. Both were followed by Tukey's Honest Significant Difference *post hoc* tests. To test if pH altered olfactory changes induced by Pd, a two-way PERMANOVA was completed followed by a permutational *post hoc* test.

Bioaccumulation of Pd was measured using one-way PERMANOVAs for all tissue types. A permutational *post hoc* test followed each analysis to compare tissue specific Pd concentrations between treatments. Protein-corrected MDA contents in the olfactory rosette were compared across Pd treatments by a one-way ANOVA. To compare measured Pd concentrations at varying sample times, a two-way PERMANOVA was used followed by a permutational *post hoc* analysis. All analyses were determined *a priori* to be statistically significant at $\alpha = 0.05$.

2.4 Results

2.4.1 Olfactory response of OSN sub-classes in rainbow trout following Pd exposure

There were no measured differences between olfactory response from stimuli (L-alanine and TCA) at any Pd treatment (Fig. 2.2). Our observations suggest 96 h waterborne Pd exposure led to equal impairment in microvillous (L-alanine stimulated) and ciliated (TCA stimulated) OSNs resulting in non-specific, broad OSN dysfunction. Although stimuli did not influence olfactory perception across treatments, Pd was found to be acutely toxic to olfactory function (pseudo-$F_{4,56} = 8.58$, $p < 0.001$, $r^2 = 0.37$; Fig. 2.2).

To determine Pd concentrations for use in subsequent studies, inhibitory estimations were calculated using a combined odourant response, as stimulants were

considered equal. Rainbow trout exposed to 16.2 ± 4.4 µg/L Pd over 96 h saw a 20% decrease in their ability to detect odourants compared to control. Additionally, we observed a 50% impairment to fish olfaction when measured against the control treatment after 96 h exposure to 42.8 ± 6.0 µg/L Pd. These measurements can be described as the concentrations in which rainbow trout olfaction was impaired by 20% (IC20), and 50% (IC50) respectively (Fig.2.3).

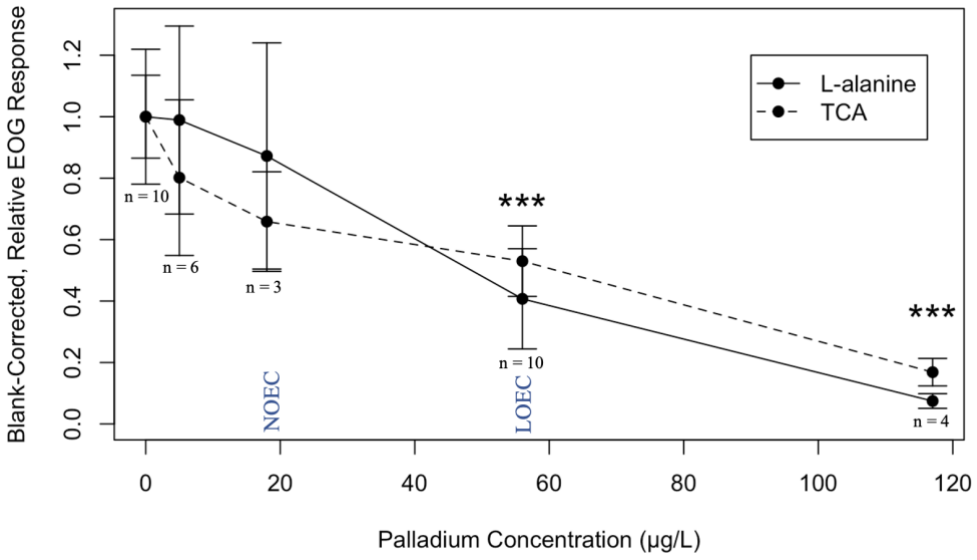

Figure 2.2. Blank-corrected, relative EOG response of rainbow trout to L-alanine (stimulates microvillous OSNs) and TCA (stimulates ciliated OSNs) for each measured Pd treatment after 96 h ($p > 0.05$, error bars ± 1 SEM). Asterisks denote significance of Pd treatment from control (***$p < 0.001$). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) are denoted by their respective acronyms.

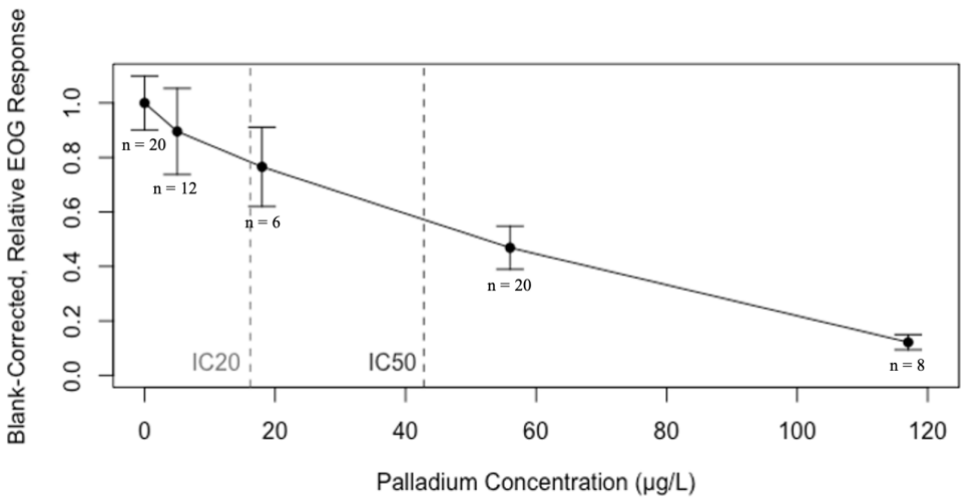

Figure 2.3. Blank-corrected, relative EOG response of rainbow trout after 96 h exposure to varying Pd treatments and resultant mean predicted inhibitory concentration to impair olfactory function at 20% and 50% using measured Pd concentrations (error bars ± 1 SEM).

2.4.2 Water quality implications on Pd-induced olfactory toxicity

The Pd treatments used across the remaining studies are not new inhibitory concentrations per water quality variable, but rather represent Pd values which were calculated from control water conditions (Figure 2.3). Upon measuring Pd concentrations in exposure tanks, the IC20 and IC50 treatments were 2.5 µg/L and 19.0 µg/L respectively. Water samples from control tanks always measured below MDL. No environmental parameter tested in this study (water hardness, pH, DOC) altered olfactory response to L-alanine in control animals.

Acute olfactory toxicity following Pd exposure was not dependant on water hardness as there was no evidence of a statistical interaction ($F_{4,43} = 1.553$, $p = 0.204$; Fig. 2.4). Although no interaction effects to the olfactory system were observed, acute Pd exposure significantly decreased fish olfaction ($F_{2,43} = 49.259$, $p < 0.001$, $\eta^2 = 0.62$; Fig. 2.4A). Further, increasing the hardness of water led to an additional decrease in olfactory perception ($F_{2,43} = 5.633$, $p = 0.007$, $\eta^2 = 0.08$; Fig. 2.4B). Compared to soft water, fish

olfaction was reduced by 23% and 37% in moderately hard and very hard treatments respectively.

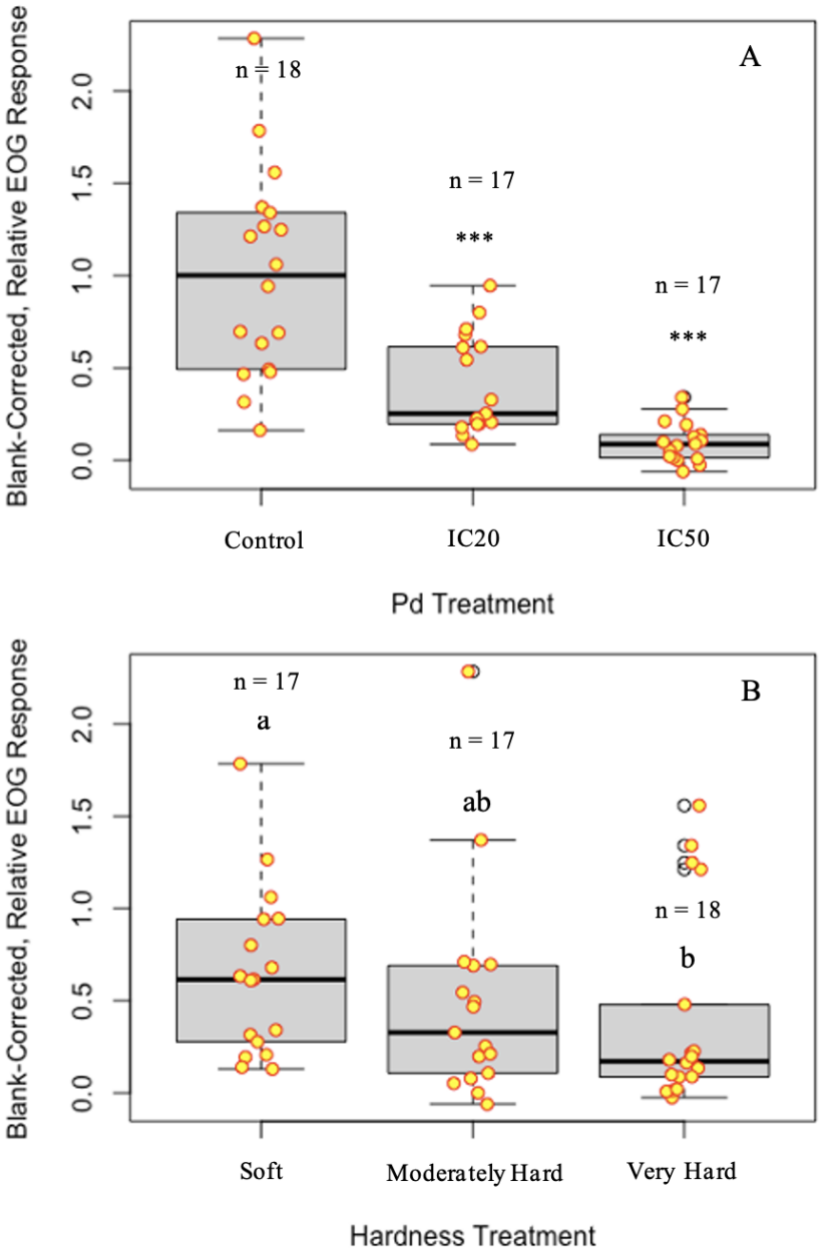

Figure 2.4. Effect of (A) Pd and (B) water hardness on rainbow trout olfaction shown as relative EOG response to L-alanine following 96 h exposure. Asterisks show significant differences between Pd treatment groups and the control (***$p < 0.001$, IC20 = 2.5 µg/L, IC50 = 19.0 µg/L). Lower-case letters denote significance between water hardness treatments ($p \leq 0.05$, soft = 55 mg/L as $CaCO_3$, moderately hard = 146 mg/L as $CaCO_3$, very hard = 258 mg/L as $CaCO_3$).

There was no interaction effect on olfactory toxicity between pH and Pd (pseudo-$F_{4,42}$ = 0.600, p = 0.661; Fig. 2.5). Moreover, there was no effect of pH on EOG response (pseudo-$F_{2,42}$ = 0.894, p = 0.430; Fig. 2.5) but there was evidence of continued olfactory disruption by Pd (pseudo-$F_{2,42}$ = 36.976, p < 0.001, r^2 = 0.616; Fig. 2.5).

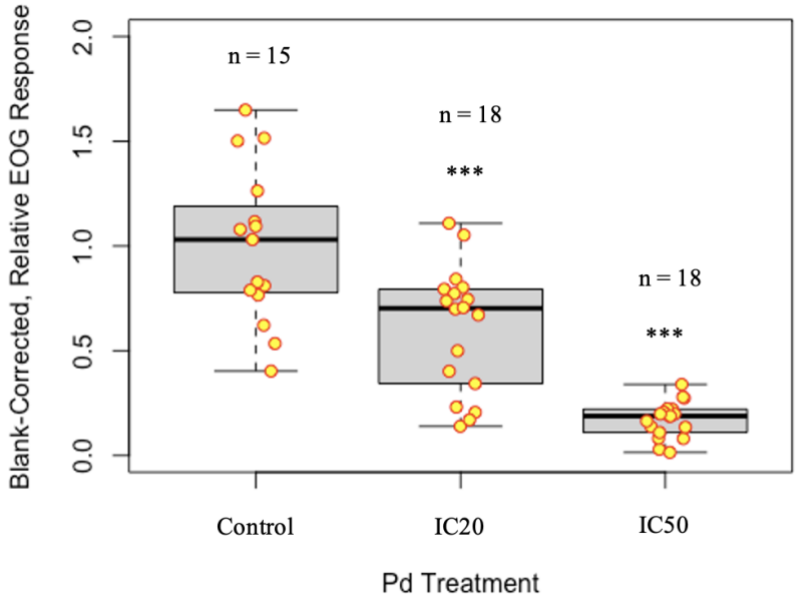

Figure 2.5. Effect of Pd in varying pH on rainbow trout olfaction shown as relative EOG response to L-alanine following 96 h exposure. Asterisks show significant differences between Pd treatment groups and the control (***p < 0.001, IC20 = 2.5 µg/L, IC50 = 19.0 µg/L).

The addition of up to 5.1 mg/L DOC resulted in no evidence of a statistical interaction when combined with waterborne Pd ($F_{4,42}$ = 0.821, p = 0.519; Fig. 2.6). However, acute Pd exposure led to reduced fish olfaction ($F_{2,42}$ = 13.281, p < 0.001, η^2 = 0.37; Fig. 2.6). Further, there was no effect of DOC on EOG response ($F_{2,42}$ = 0.198, p = 0.821; Fig. 2.6).

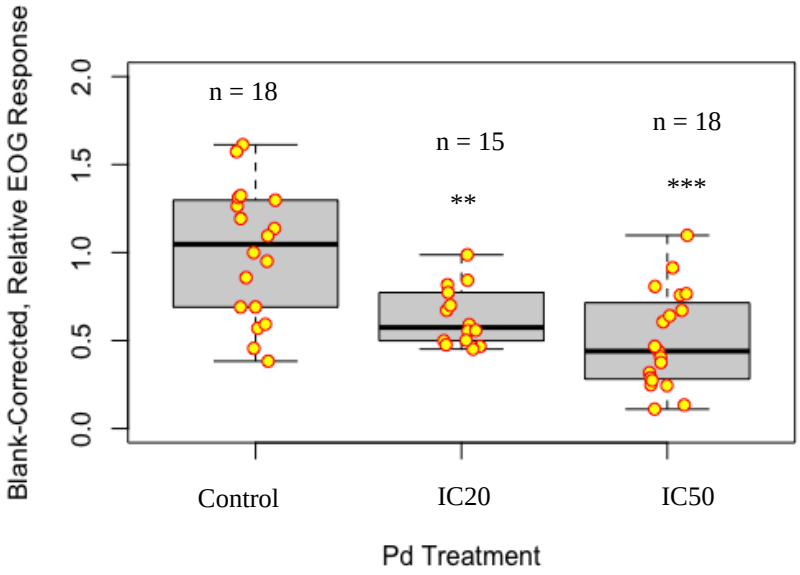

Figure 2.6. Effect of Pd in varying DOC concentrations on rainbow trout olfaction shown as relative EOG response to L-alanine following 96 h exposure. Asterisks denote significant differences between Pd treatment groups and the control (**p ≤ 0.01, ***p < 0.001, IC20 = 2.5 µg/L, IC50 = 19.0 µg/L).

2.4.3 Bioaccumulation

Acute exposure to waterborne Pd resulted in metal accumulation at tissues in direct contact with the exterior environment. The olfactory rosette accumulated significant amounts of Pd when exposed to IC20 (pseudo-$F_{1,12}$ = 37.412, p = 0.001, r^2 = 0.757; Fig. 2.7A) and IC50 (pseudo-$F_{1,12}$ = 21.466, p = 0.001, r^2 = 0.641; Fig. 2.7A) treatments. Further, fish exposed to IC50 levels of Pd experienced significantly higher metal concentrations in olfactory tissue as opposed to those exposed to the IC20 treatment (pseudo-$F_{1,12}$ = 16.451, p = 0.001, r^2 = 0.578; Fig. 2.7A). The amount of Pd at the olfactory rosette ranged from 1.2 to 2.6 µg/g and 5.4 to 25.9 µg/g in the IC20 and IC50 treatments respectively. We can translate these results to a mean 714% increase in Pd at the olfactory rosette when exposed to the IC50 level of Pd as opposed to the IC20.

Similarly, we found Pd in the gill when fish were exposed to IC20 (pseudo-$F_{1,40}$ = 67.635, p = 0.001, r^2 = 0.628; Fig. 2.7B) and IC50 (pseudo-$F_{1,40}$ = 39.805, p = 0.001, r^2 = 0.499; Fig. 2.7B) Pd levels. Unlike the olfactory rosette, there was no significant change in Pd accumulation at the gill between IC20 and IC50 treatments. Nonetheless, Pd levels in the gill ranged from 3.6 to 15.9 μg/g and 3.6 to 28.1 μg/g in respective IC20 and IC50 treatments. The liver was analyzed for Pd accumulation as the detoxifying organ of the fish, however no Pd was detected at any treatment (Fig. 2.7C).

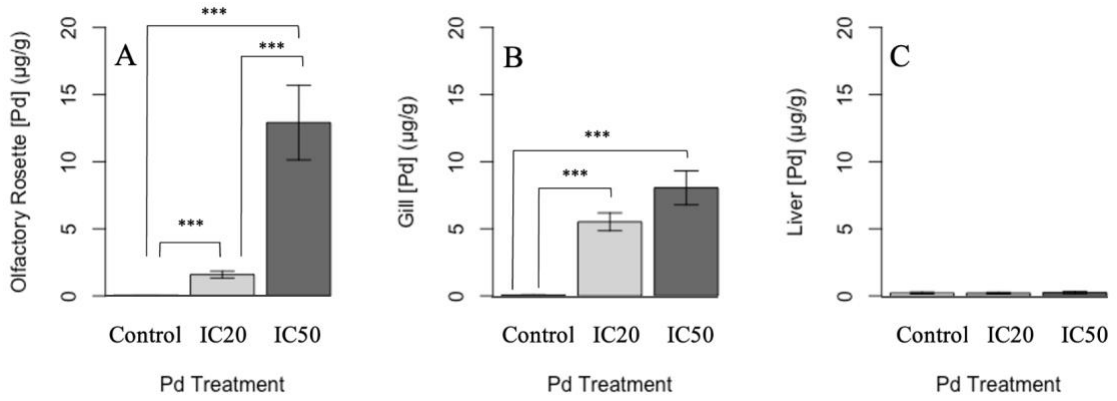

Figure 2.7. Palladium (Pd) bioaccumulation in (A) olfactory rosette (n = 7) (B) gill (n=21) and (C) liver (n = 21) of rainbow trout following 96 h waterborne exposure. Asterisks denote significant differences between Pd treatment groups (***$p \leq$ 0.001, error bars ± 1 SEM).

Calculated tissue-specific CF values are presented in Table 2.2. The CF value at gill and liver are approximately 5 and 6 times higher following exposure to the IC20 Pd treatment as opposed to IC50 levels. All measured tissues were shown to uptake Pd. Moreover, the highest Pd CF was found in gills exposed to the IC20 Pd treatment (2,207), while the lowest CF was found in liver experiencing the IC50 Pd treatment (13). Unlike the two other tissues, CF values within the olfactory rosette remained consistent between Pd treatment groups.

Table 2.2. Concentration factor (CF = C_{tissue}/C_{water}) of rainbow trout tissues following a 96 h exposure to Pd.

Tissue Type	CF (IC20 Pd)	CF (IC50 Pd)
Gill	2,207	424
Liver	85	13
Olfactory Rosette	634	680

2.4.4 Lipid peroxidation analysis

There was no significant change in MDA contents at any Pd treatment following protein concentration normalization ($F_{2,15}$ = 2.071, p = 0.161; Fig. 2.8).

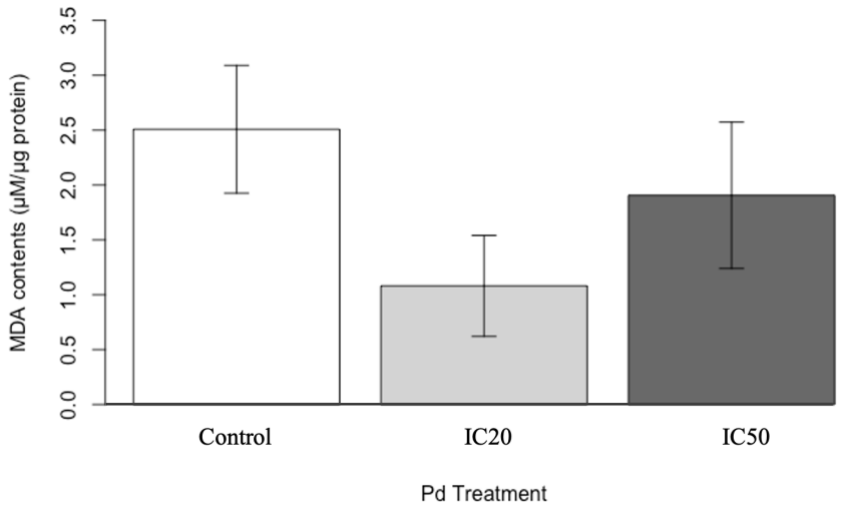

Figure 2.8. Investigation of cellular injury measured as lipid peroxidation (LPO) via levels of malondialdehyde (MDA) in the olfactory rosette (p > 0.05, n = 6, error bars ± 1 SEM).

2.4.5 Concentration of Pd in water

The quantified concentrations of Pd in water samples revealed measured metal from testing solution was < 50% (Table 2.3). When both time points were combined, the average percent difference from nominal and measured Pd was 26% and 46% for IC20 and IC50 Pd treatments respectively. Upon comparing Pd concentrations in solution at 24 h with those taken at 96 h, there was significantly less Pd measured at the latter time point.

Table 2.3. Mean Pd difference ($Pd_{quantified}$/ $Pd_{nominal}$) from water samples taken at 24 h and 96 h, including Pd concentration ratios ($Pd_{quantified96\ h}$/ $Pd_{quantified24\ h}$) over the 96 h exposure. Asterisks denote significant Pd concentration difference between sample times (*$p \leq 0.05$, n = 42).

$Pd_{nominal}$ (µg/L)	Percent difference (24 h)	Percent difference (96 h)	Ratio (96 h/24 h)
9.7	25%	27%	108%
42.5	50%	42%	*84%

2.5 Discussion

2.5.1 Olfactory response of OSN sub-classes in rainbow trout following Pd exposure

In contrast to some metals such as Cu (Razmara et al., 2019) and Cd (Williams et al., 2016), exposure of rainbow trout to Pd resulted in equal signal reduction at major OSN populations. Differential responses were not observed when TCA (specifically stimulates ciliated OSNs) and L-alanine (specifically stimulates microvillous OSNs) were delivered in staggered intervals to the olfactory chamber of fish as shown in Fig 2.2. Therefore, Pd can be classified as a broad OSN inhibitor as it does not specifically target a subset of OSNs. Other studies corroborate this finding and demonstrate that acute metal exposures can result in broad OSN impairment whereas chronic exposures can result in selective OSN dysfunction due to microvillous OSN recovery (Dew et al., 2014).

Since olfactory recovery was not explored in this study, we are unsure if select OSN populations can recover while in contact with waterborne Pd or following contaminant removal. Nonetheless, it is expected that generated action potential at both olfactory signalling pathways (OSPs) (cAMP-mediated and IP3-based) were reduced following Pd exposure (Hansen et al., 2003). Both OSPs use secondary messengers to activate sodium and calcium channels to propagate sensory information to the brain for processing (Laberge & Hara, 2001). It is known that metals are effective ion channel blockers and similar results

can be anticipated from Pd uptake (Florea & Büsselberg, 2006). Although not measured in this study, increased mucous production may also play a role in reduced olfactory perception following Pd exposure. Several studies have found certain metals such as Cu, Zn, Cd, Hg, can act as anti-acetylcholinesterase (AChE) contaminants which reduce OSN responsiveness in fishes due to increased mucous production (Frasco et al., 2005; Tierney et al., 2010). Further, a recent study by Ahmed et al. (2021) showed the addition of Pd(II) resulted in AChE inhibition in vertebrates demonstrating the likelihood of Pd being an anti-AChE contaminant. The exact MOA has yet to be defined for Pd-induced toxicity. Nonetheless, we can suggest from our results that Pd may induce a variety of effects at the olfactory epithelium leading to an overall reduced olfactory capacity.

To our knowledge, there are no studies which address Pd concentrations required to elicit effects in freshwater fishes. Lack of reliable data is one reason why Pd toxicity in aquatic organisms remains largely unknown (Zereini & Alt, 2006). Although NOEC and LOEC were determined in this study as shown in Fig 2.2, many ecotoxicologists agree these measures are outdated as they rely only on tested concentrations and can vary between experiments resulting in misled conclusions (Warne & Van Dam, 2008; Landis & Chapman, 2011; Jager, 2012). Instead, ICx enables researchers to estimate effective concentrations without the need for additional experimentation and animal use. The present study demonstrates that exposure to Pd in the low μg/L range can negatively impact olfactory perception in rainbow trout as shown in Fig 2.3. Environmental Pd levels can be difficult to ascertain due to the requirement of sensitive analytical equipment, lack of standardized water sampling protocols, and overall infrequent measurements (Zereini & Wiseman, 2015). Nonetheless, the effective concentrations observed in this study are predicted to overlap with levels found in select environments. Threshold concentrations of

Pd required to elicit 20% (IC20) and 50% (IC50) olfactory inhibition were calculated in this study using rangefinder data to align with other work that uses sublethal effective concentrations to inform protective guideline development. However, it should be noted that IC20 > IC50 for preferred endpoints in the current development of water quality guidelines (WQGs) in Canada (CCME, 2007). Our data highlight the environmentally relevant risk Pd could pose. This study is a first step in understanding Pd toxicity at a sublethal level in cold-freshwater fishes. Although olfaction provides a sensitive system in which to evaluate the toxicity potential of Pd, other areas must be investigated to fully understand Pd toxicity to aquatic organisms.

2.5.2 Water quality implications on Pd-induced olfactory toxicity

The current study demonstrates the importance of water quality consideration in establishing inhibitory concentration thresholds to metal-induced olfactory impairment. Although no interaction effects were observed, it is clear Pd-induced olfactory toxicity is different than metal toxicity to other tissues. Much attention is currently given to gill-based metal toxicity and although more sensitive than previous protective levels against lethality, the gill may not be the most sensitive environmentally relevant target ligand for many metals. We can reveal from the results of this study that increasing water hardness caused olfactory dysfunction in rainbow trout. As seen in previous metal-based olfactory research, the addition Ca^{2+} and Mg^{2+} offered no protection from Pd-induced olfactory impairment. It is likely Ca exerts its own inhibitory effect to fish olfaction when present in hard water conditions (Dew et al., 2012). Contradictory to other metals that have been tested, it must also be recognized that Pd toxicity may be mitigated in soft water as opposed to harder water. In fact, when compared against 62 other metals, Pd was the only metal in which

Hyalella azteca lethality decreased when combined with a water hardness of 18 mg/L versus 124 mg/L as $CaCO_3$ (Borgmann et al., 2005). Although no chemical explanation has been brought forth concerning Pd behaviour in varying water hardness conditions, the results shown in Fig 2.4 cannot solely be explained by the individual effects of Ca and Pd at the olfactory epithelium.

Prevailing environmental conditions in natural freshwater systems present a pH window of 4 to 9, with the optimal range for aquatic organisms falling within the pH 6.5 to 8 range (Health Canada, 2015; USEPA, 2017). Our results present no evidence of an interaction between pH and Pd-induced olfactory toxicity as shown in Fig 2.5. Although a small pH range was testing during this study, our results are corroborated by the chemical behaviour of Pd in freshwater systems. Recent studies have concluded dissolved Pd content is positively correlated with alkaline water conditions up to pH 8 (Liu at al., 2020). Further, Pd lipid transfer has been demonstrated in pH range of 7 to 8 (Zimmermann et al., 2003). The effect of Pd-induced olfactory toxicity, as demonstrated in Fig 2.5, corroborates with these findings. While metals in their ionic forms are more toxic to aquatic organisms by increased free ion presence, the ionic form of Pd does not dominate as a species until pH < 2; a range much too acidic to support rainbow trout (Colombo et al., 2008a). Thus, our finding of no mitigatory or worsening effect to fish olfaction was unsurprising given the lack of metal speciation change within the given pH range.

Metal toxicity typically decreases as aromaticity of organic matter increases, with high humic acid content linked to effective protection against metals such as Cu, Ag, and Pb to aquatic animals (Brown et al., 1974; McGeer et al., 2002; Wood et al., 2011; Kennedy et al., 2012). Dissimilar to other metal research, our study demonstrated the addition of dissolved humic acid solution did not protect fishes from Pd-induced olfactory toxicity as

no statistical interaction was observed between the two treatment types as shown in Fig 2.6. This finding may be explained by a result from Zimmermann et al. (2002) which observed humic substances increasing the solubility of Pd in water. In the same study, researchers conclude that the occurrence of Pd in a single oxidation state ($Pd(OH)_2$) may account for this particular metal to have an elevated bioaccumulation tendency when organic compounds are present. This was the first study to our knowledge which manipulated humic acid as DOC to test for Pd interaction to fish olfaction. Although our results demonstrate a difference between Pd-induced toxicity and other metals, the behaviour of Pd when combined with humic substances may result in metal accumulation at the target ligand as opposed to mitigatory effects on metal toxicity. However, there remains many other forms of allochthonous and autochthonous organic matter to be explored to confirm our findings.

2.5.3 Bioaccumulation

The present study provides a broad insight into the MOA for Pd-induced olfactory impairment and further supports Pd uptake in freshwater fish. Palladium accumulates in the olfactory rosette when exposed for 96 h to concentrations as low as 2.5 μg/L as shown in Fig. 2.7A. As the olfactory system is in direct contact with surrounding waters, Pd accumulation in olfactory tissue is unsurprising. Studies using other trace metals have confirmed similar results following waterborne exposures (Sloman et al., 2003; Razmara et al., 2021), however no other study to our knowledge has explored Pd bioaccumulation in olfactory tissue. While unable to provide a BCF as system equilibrium was not determined, calculated CF values support Pd accumulation within the target tissue for this study as demonstrated in Table 2.2 (Petoumenou et al., 2015). Although the underlying

molecular mechanisms have yet to be established for Pd impaired olfaction, it is clear membrane associated Pd at the olfactory epithelium is partially responsible for the functional toxicity observed in rainbow trout.

Previous bioaccumulation studies have established Pd bioavailability to aquatic organisms (Zimmermann et al., 2002; Ek et al., 2004). Similar to the olfactory rosette, the gill is highly vulnerable to metal binding and a common target ligand in the investigation of metal-based toxicity and bioaccumulation (Di Toro et al., 2001). Our findings demonstrate Pd accumulation at the gill in both Pd treatments as shown in Fig 2.7B. The EU REACH classifies a substance as bioavailable if it has a CF $\geq$ 2000, whereas Canada uses a threshold of CF $\geq$ 5000 (Gissi et al., 2015; Nendza et al., 2018). We can suggest from our results that Pd accumulates at rainbow trout gills as shown in Table 2.2. Although dissimilar in experiment duration, species, and Pd addition, Sures at al. (2005) also reveal a gill CF of >2000 thereby providing additional support for the moderately bioaccumulative nature of Pd. Our study demonstrates trace metal exposure can lead to higher Pd concentrations in freshwater fish tissues than what is present in surrounding waters.

The liver has previously been described as a target organ for Pd accumulation in freshwater organisms (Sures et al., 2001; Sures et al., 2005; Zimmermann et al., 2005). Functioning as a detoxifying organ, the liver was included in this investigation to increase our understanding of Pd mobilization within an organism following waterborne exposure. Although our findings conclude non-significant Pd accumulation at the liver compared to control fish as shown in Fig. 2.7C, the calculated CF demonstrates some level of Pd uptake. This discrepancy may be explained by the detection limit of GFAAS, as other studies report significance in the ng/g range. Investigations on tissue specific Pd uptake include eels (Sures et al., 2001; Zimmermann et al., 2005), mollusks (Sures & Zimmerman, 2007), and

Barbus barbus (Sures et al., 2005), all of which accumulated significant Pd over a range of 28- to 42-day exposures. Chronic bioaccumulation studies provide valuable information pertaining to the dynamic equilibrium between uptake and elimination. However, it is more likely that Pd will enter aquatic systems in pulse events at effective waterborne concentrations. Our approach recognizes short term bioaccumulation as an important parameter to acknowledge in understanding the effects of Pd on aquatic organisms.

2.5.4 Lipid peroxidation analysis

To explore the mechanism of Pd toxicity in rainbow trout olfaction, it is crucial to understand the role, if any, that ROS formation and oxidative stress have. There currently exists no available data on oxidative stress response in fishes following Pd exposure. However, nano-palladium research has demonstrated an oxidative stress response via elevated LPO levels in zebrafish gill, liver, and brain (Anila et al., 2021). Oxidative stress in olfactory epithelial membranes remained insignificant when measured as MDA formation from the creation of LPO as shown in Fig. 2.8. One suggestion is that the protection offered by antioxidant defences at the peripheral olfactory system was sufficient to block LPO production (Tilton et al., 2008). Another is that ROS was simply not produced by Pd exposure, which would also result in no LPO production. While olfactory epithelial injury may still play a role in olfactory disruption following Pd exposure, we can conclude from our results that it would not be caused by free radicals of olfactory cellular membranes. Our data suggest that Pd concentrations within the olfactory rosette results in reduced olfactory acuity. However, it remains unknown if the olfactory system can recover following acute Pd exposure.

Aside from this study there is no information pertaining to Pd effects on olfactory function in fish. However, it is well established that other metals, such as Cu, Cd, Ni, and Zn induce olfactory toxicity. Corroborating with our findings, Razmara et al. (2021) rules out oxidative stress as a mechanism for olfactory dysfunction after acute waterborne Cu exposure. However, transcriptional regulation of neuroregeneration and immune system pathways were demonstrated in the same study to change following trace metal exposure. Evaluation of secondary messengers involved in OST pathways were also shown to explain olfactory reduction in salmonids following acute Cu exposures (Wang et al., 2013). Although outside the scope of this study, use of bioinformatics, gene expression, and transcriptomics (reviewed in Tierney et al., 2010) are informative molecular and biochemical indicators of olfactory toxicity and should be applied to future Pd toxicity studies to elucidate the MOA for olfactory impairment in fishes.

2.5.5 Concentration of Pd in water

Some of the available published Pd toxicity research report nominal exposure values as opposed to measured concentrations. This practice presents the opportunity for misconception on Pd concentrations required to elicit toxic effects, as true concentrations are often much lower than what was originally added to the system. This study demonstrates the importance of reporting measured values, as measured Pd in waterborne exposures is not equal to nominal values as shown in Table 2.3. This observation corroborates with other studies which found measured Pd concentrations to be minimal despite tank and material conditioning prior to exposure start (Zimmermann & Sures, 2018). Although the percent metal loss presented here seems high varying between 50% and 75%, other laboratory experiments have consistently reported a 75% to 90% loss of Pd

beginning as early as the 24 h sampling time (Cobelo-Garcia et al., 2007). Our water sampling revealed a significantly lower Pd concentration at 96 h when compared to 24 h for the high treatment as shown in Table 2.3. Nonetheless, mean measured concentrations between sampled times at the IC50 Pd treatment resulted in a fluctuation of only 3 μg/L. Considering this disparity occurred only at higher Pd concentrations, metal uptake by fish can be expected to play a part as demonstrated by CF values presented in this study. Additionally, known precipitation processes may be augmented when more Pd is present in the test system (Cobelo-Garcia et al., 2007).

To understand the high percent loss demonstrated in aquatic systems, we must turn to Pd speciation. The most common species of Pd present in freshwater environments at pH values explored in this study is $Pd(OH)_2$ and unsurprisingly, it exhibits the greatest affinity for surfaces including glass, plastics, and silicone (Colombo et al., 2008a; Fortin et al., 2011). Although some Pd can theoretically be accounted for between nominal and measured concentrations in this study via fish uptake and precipitation, no attempt was made to quantify Pd loss to the system. Varying environmental parameters have been explored with the goal of improving or offering additional knowledge about Pd loss in static-renewal waterborne exposure systems, yet no resolution has been provided at this time for maintaining added Pd concentrations in solution.

2.6 Conclusions

Although recognized as a contaminant of emerging concern, Pd toxicity research remains underdeveloped at this time. The present study is the first to demonstrate Pd toxicity to olfactory function in fish. Although we found acute Pd exposure significantly reduces rainbow trout olfaction, the precise mechanism of action has yet to be defined. It

is clear from our study that Pd is bioavailable to exterior-facing tissues such as the olfactory rosette and gill, as Pd was shown to associate with both membrane types during the 96 h exposure. Further, of the water quality parameters tested, water hardness was the only one which additionally impacted fish olfaction. Nonetheless, this discovery corroborates with other trace metal toxicity to fish olfaction, supporting the vital role that water quality plays in accurate risk assessment and guideline development for the protection of aquatic life. Our study contributes to the evaluation of Pd toxicity in fish and highlights the importance of continued research into this study area.

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CHAPTER 3: Investigating olfactory toxicity potential of platinum in rainbow trout (*Oncorhynchus mykiss*)

This thesis aims to explore olfactory perception in rainbow trout when separately exposed to two contaminants of emerging concern: palladium (Pd) and platinum (Pt). In the previous chapter we demonstrated Pd-induced olfactory toxicity in rainbow trout, including bioaccumulation at target ligands and olfactory perception in manipulated environments following a 96 h waterborne exposure. The current chapter follows suit and explores the possibility of Pt as an olfactory toxicant. Levels of Pt contamination, both current, projected, and non-environmentally relevant are used as a guide to discern if Pt toxicity poses a realistic risk at this time in freshwater aquatic environments.

This is a manuscript-style thesis written for the University of Lethbridge based on submission requirements and regulations. As such, content repetition can be noted within the introduction, materials, and methods sections of research chapters. This chapter is formatted to be submitted to *Journal of Negative Results - Ecology & Evolutionary Biology*.

Contribution of Authors: I designed, performed, collected, analysed the data, and wrote the manuscript. Dr. Greg Pyle provided direction, guidance, scientific input, supervision, comments/assisted writing, and funding for this research.

3.1 Abstract

Platinum (Pt) is used in the production of many modern technologies including those within the automobile and medical industries. Metal particulate released from vehicle exhaust catalysts (VECs) and medical waste has resulted in an unprecedented high environmental presence of Pt compared to natural baseline concentrations. Processes such as runoff bring Pt to aquatic environments where its environmental and organismal impact remains unknown. To investigate the toxicity potential of Pt, rainbow trout (*Oncorhynchus mykiss*) olfaction was selected as a sensitive sublethal endpoint. Using a dilution series, fishes were nominally exposed to Pt concentrations ranging from 100 – 1,000 μg/L and olfactory acuity was measured using electro-olfactography (EOG). Crucial environmental information including but not limited to homing, foraging, mate detection, and predator evasion, is conveyed through olfaction. If impaired, fishes can no longer optimally perceive their environment which can result in ill-fated outcomes. Results from this study show Pt does not induce olfactory dysfunction following a 96 h waterborne exposure up to 1000 μg/L; a concentration that is orders of magnitude higher than what can be found in the environment. However, with limited supporting information more research is warranted to assess the potential of Pt as a freshwater toxicant.

3.2 Introduction

Because of its many exceptional physical and chemical properties, platinum (Pt) is integrated in modern technologies resulting in a total of over 200 tonnes used per year (JM, 2021). The introduction of Pt in vehicle exhaust catalysts (VECs) to reduce toxic exhaust fumes, and medical practices for cancer treatments, are understood to be significant Pt emission sources (Rauch & Peucker-Ehrenbrink, 2015). Consequently, this naturally rare metal is deposited into novel environments where it can accumulate within both terrestrial and aquatic areas (Zereini & Wiseman, 2015). Aqueous Pt concentrations are generally in the ng/L range but have been found to reach the μg/L range in run-off waters with the highest measured Pt concentration being 10 μg/L (Odiyo et al., 2005).

Typically emitted from VECs in its metallic form, Pt is relatively inert and poses no risk to flora and fauna (Moldovan, 2007). However, recent investigations revealed environmental variables can transform Pt into more mobile and soluble forms thereby transporting Pt to aquatic environments while also increasing the bioavailability and potential toxicity of the metal (Ek et al., 2004). Further, laboratory studies have demonstrated biological uptake and accumulation of Pt in aquatic organisms supporting Pt availability in solution (Rauch & Morrison, 1999; Sures et al., 2001; Zimmermann et al., 2005; Sures & Zimmermann, 2007; Osterauer et al., 2009). Few Pt toxicity studies have been completed at this time, with most investigating plants. Nonetheless, Pt toxicity was explored in a range of aquatic animals including arthropods (Biesinger & Christensen, 1972; Borgmann et al., 2005; Khangarot & Das, 2009; Okamoto et al., 2015; Zimmermann et al., 2017), annelids (Khangarot, 1991; Veltz et al., 1996), gastropods (Osterauer et al., 2011), and fish (Ferreira & Wolke, 1979; Osterauer et al., 2011) where effective Pt concentrations range from 60 μg/L – 30 000 μg/L. These studies focused on a variety of

endpoints including immobilization, reproduction, heart rate, and survival, demonstrating the possibility of adverse effects following Pt exposure. Despite increased research efforts, there remains limited knowledge on the toxic effects Pt may pose to aquatic animals. Additionally, few studies have produced data that are relevant to risk assessment or to inform the development of water quality guidelines (WQGs) for the protection of aquatic life. Currently, preferred endpoints for informing WQG are from sub-lethal exposures (Sigg, 2014).

Several studies have demonstrated the olfactory system can become impaired following trace metal exposure as the olfactory organ is in direct contact with surrounding waters (Tierney et al., 2010). A variety of olfactory sensory neurons (OSNs) are located in the olfactory epithelium and waterborne odourants bind to specified receptor proteins as each cell type is stimulated by a unique category of odourant (Hansen et al., 2003). An electrical signal is propagated to the brain for information processing if enough stimulus occurs at the receptor (Laberge & Hara, 2001). Of the available OSN classes, researchers have gathered the most information about microvillous, ciliated, and crypt cells (Hansen & Zielinski, 2005). Respectively, these OSNs perceive food cues, social cues, and sex pheromones (Hamdani & Døving, 2007). Studies using freshwater fish have demonstrated that amino acids, such as L-alanine, activate microvillous OSNs, and bile acids, such as taurocholic acid (TCA), bind to ciliated OSNs (Dew et al., 2014). Using distinct odourants, researchers can determine if a contaminant impedes a subtype of OSN or impair the entire system by broadly inhibiting multiple OSN subtypes. Resultant disruption of the olfactory system may lead to maladaptive behavioural responses and potential adverse outcomes at the organism or population level for a range of crucial life-history behaviours (Klaschka, 2009). Considering ambient Pt concentrations are relatively low in natural systems, the

sensitivity of olfactory-based toxicity endpoints may be useful for informing the development of ecologically relevant WQG for sublethal Pt exposures.

The objective of this study was to determine if Pt induces olfactory impairment in rainbow trout (*Oncorhynchus mykiss*). To this end a range of Pt concentrations, including those orders of magnitude higher than what is found in the environment, were used to assess olfactory acuity following a 96 h waterborne exposure. This experiment was performed to evaluate Pt toxicity and reveal if this contaminant of emerging concern poses a realistic risk to freshwater fish.

3.3 Materials and methods

3.3.1 Test animals

Juvenile rainbow trout (n = 584; standard length = 10.6 ± 2.4 cm; weight = 17.1 ± 13.2 g (mean ± SD)) were obtained from Sam Livingston Fish Hatchery (Calgary, Canada). Upon arrival to the Aquatic Research Facility (ARF) at the University of Lethbridge (UofL), fish were transferred to static-renewal holding tanks at a stocking density of 5 g/L supplied with processed cold water (PCW). Lab-ready PCW is created from city water using both mechanical (dechlorinated, large particle removal, buffered, and UV sterilized) and biological (bio-inoculated media) filtration. Holding conditions maintained a 16:8 h light:dark photoperiod with measured water quality as follows (mean ± SD; n=24): temperature, 12.0 ± 0.1 °C; dissolved oxygen, 8.4 ± 0.1 mg/L; ammonia, 0.01 ± 0.01 mg/L; nitrite, 0.004 ± 0.003 mg/L; nitrate, 2.1 ± 0.5 mg/L. Fish were fed a maintenance diet twice daily with 1.5 mm trout chow pellets (EWOS, Canada) and allowed to acclimate for a minimum of two weeks prior to being used in experiments. During acclimation, fish were treated with a parasiticide (PraziPro, Hikari, USA) to ensure olfactory-related parasites

were absent prior to experimentation. Experiments were reviewed and approved by the UofL Animal Welfare Committee (AWC) (protocol #1914).

3.3.2 Preparation of test solutions

Dissolved ammonium hexachloroplatinate in 10% HCl was purchased from SCP Science (99.2-100% Pt purity; SCP Science, Canada) at a concentration of 998 ± 4 μg/mL. Pt solution was stored at 4 °C in dark lighting until use. No changes were made to the Pt solution and was added directly into experimental tanks in pre-determined quantities following fish addition.

3.3.3 Platinum analyses

A measurable percent loss of quantified versus nominal concentrations is reported when using platinum group elements (PGEs) in aquatic toxicological exposures (Cobelo-Garcia et al., 2007). This same investigation revealed quantified Pt is typically ≥ 90% in conditions above pH 5. Although we did not anticipate extreme Pt loss to the system, all materials were pre-conditioned to Pt prior to experiment start to optimize Pt reteniton in water. In the present study, dissolved Pt was measured using graphite-furnace atomic absorption spectrometry (GFAAS) (GTA120 GFAAS, Agilent Technologies, USA). For QA/QC verification a certified run standard (in 10% HCl, 99.3-100% certified Pt value; Inorganic Ventures, USA) was tested every 10 samples with the calibration curve re-plotted every 20 samples with mean QA/QC recovery within the accepted ± 10% error. Water samples from a randomized subset of tanks were collected and immediately acidified to 1% HNO_3 (from 37% TraceSelect grade; Sigma Aldrich, Canada) at the beginning (i.e., 1 h after Pt addition) and end (i.e., 96 h following fish addition) of the experiment in addition to before and after each daily water change in the 96 h exposure. All samples were

refrigerated at 4 °C until required for analysis. Method detection limit (MDL) for Pt was measured to be 70 μg/L for a range between 0 and 1000 μg/L.

3.3.4 Experimental design

No previously described study investigates Pt toxicity thresholds to rainbow trout, thus a 96 h rangefinder was completed to explore olfactory thresholds to Pt. Olfactory activity was measured using electro-olfactography (EOG) as described by Lari & Pyle (2017) in addition to the UofL AWC standard operating protocol (SOP) #A14. In this study, microvillous and ciliated OSN activity were measured using 10^{-4} M L-alanine (Fisher Scientific, Canada) and taurocholic acid (TCA; Sigma-Aldrich, USA) respectively to investigate OSN sub-population binding following acute Pt exposure.

Fish were exposed individually in static-renewal exposure tanks containing 15 L of water. During the 96 h exposure period, 50% of the total water volume was replaced daily in each tank with the same treatment solution to keep ammonia levels below 0.02 mg/L. In addition to water changes, fish were not fed during the exposure to further minimize the accumulation organic wastes and to ensure exposure was only waterborne. Lastly, treatment solutions were created at least 12 hours prior to water changes to allow for Pt equilibrium within solution. Fish experienced a 16:8 light:dark photoperiod during exposures and water quality was measured daily and are outlined as follows (mean ± SD; n=12): temperature, 12.4 ± 0.6 °C; dissolved oxygen, 9.4 ± 0.2 mg/L; conductivity, 383.1 ± 10 μS/cm; hardness, 160.9 ± 2.4 mg/L as $CaCO_3$; alkalinity: 105.5 ± 9.5 mg/L as $CaCO_3$; pH, 7.98 ± 0.28, and dissolved organic carbon, 1.05 ± 0.27 mg/L.

3.3.5 Electro-olfactogram (EOG) assay

Olfactory response was determined using the EOG neurophysiological assay. Following the 96 h exposure to clean or contaminated PCW, fish were anaesthetized directly prior to use within the EOG using 120 mg/L tricaine methanesulfonate (TMS; AquaLife, Canada) and buffered to pH 7.4 using 360 mg/L $NaHCO_3$ (Fisher Scientific, USA). Anesthetization was achieved when opercular movements ceased. Once anaesthetized, individual fish were wrapped in a damp paper towel to prevent desiccation and placed under a dissecting scope. The intranarial septum, which separates the anterior and posterior opening of the naris, was removed using surgical forceps to expose the primary olfactory organ, the olfactory rosette. Fish were subsequently placed within the perfusion trough where a gill perfusion line was inserted into the mouth to maintain fish viability and a grounding line was clamped onto the tail to remove any electrical interference.

In the present study, 10^{-4} M of each odourant was used as an olfactory cue, which was made fresh daily in PCW. Additional blank cues contained only PCW to verify odourant responses. The glass microelectrode recording probe was placed at the third lamella from the bottom right of the rosette, approximately at 1/3 distance from base and ½ lamella width. On the same side of the fish, a glass microelectrode reference probe was placed on the skin above the naris parallel to the eye. Upon completion of probe placement, the olfactory perfusion line was placed directly above the naris allowing for solution to flow into the olfactory chamber. Fish were subject to a 5-minute acclimation time upon completion of probe and line placement. In a randomized order, each cue was delivered at minimum three times in 3 second pulses with a recovery time of 2 minutes between cue deliveries. During acclimation and between cue deliveries, PCW was constantly delivered

to the naris to ensure olfactory tissue did not dry out and to flush out any remaining olfactory stimulus from the previous delivery. Olfactory responses were amplified as described by Razmara et al. (2019) and calculated by the difference between baseline and maximum response amplitudes from cue stimulation. True OSN response from a cue was determined by subtracting blank responses from the calculated cue response. This neurophysiological technique was also performed on euthanized fish to ensure responses were from live olfactory tissue and not from external interference.

3.3.6 Statistical analyses

All statistical analyses were performed in R (R Core Team, 2020; version 4.0.0 Arbor Day). To calculate relative EOG responses to individual stimuli (L-alanine and TCA) at varying Pt concentrations in the rangefinder, a blank corrected relative EOG stimulus-response dataset was created by dividing the control response (mean control cue response - blank response) by blank corrected cue specific values. Permutational multivariate analysis of variance (PERMANOVA) was performed using the adonis2 function in the vegan (Oksanen et al., 2020) package. The number of permutations in all tests was 4999 and the "Manhattan" or "Bray" dissimilarity index was used to measure the absolute distance between data for two-way and one-way PERMANOVA designs respectively. A *post hoc* permutation analysis was conducted using the pairwiseAdonis (Martinez Arbizu, 2017) package when a PERMANOVA yielded significant main effect results without an interaction. A two-way PERMANOVA was applied to test the null hypothesis that there was no interaction effect between odourant type and Pt treatment. A one-way PERMANOVA was performed *post hoc* to the two-way to test the null hypothesis that Pt exposure resulted in no olfactory inhibition when compared to the control. Lastly, a two-

way PERMANOVA was applied to test the null hypothesis that there was no interaction effect between added Pt to the test system and sample time for recovered Pt in solution. Null hypotheses were rejected when significance (p) was ≤ 0.05.

3.4 Results

3.4.1 Olfactory response to odourants following acute platinum exposure

There was no evidence of a statistical interaction between EOG response from stimuli (L-alanine and TCA) at any Pt treatment (pseudo-$F_{3,16} = 1.024$, $r^2 = 0.095$, $p = 0.419$; Fig. 3.1). Further, no Pt treatment significantly altered rainbow trout olfactory response when compared to control animals (pseudo-$F_{3,16} = 2.1387$, $r^2 = 0.205$, $p = 0.129$; Fig. 3.1).

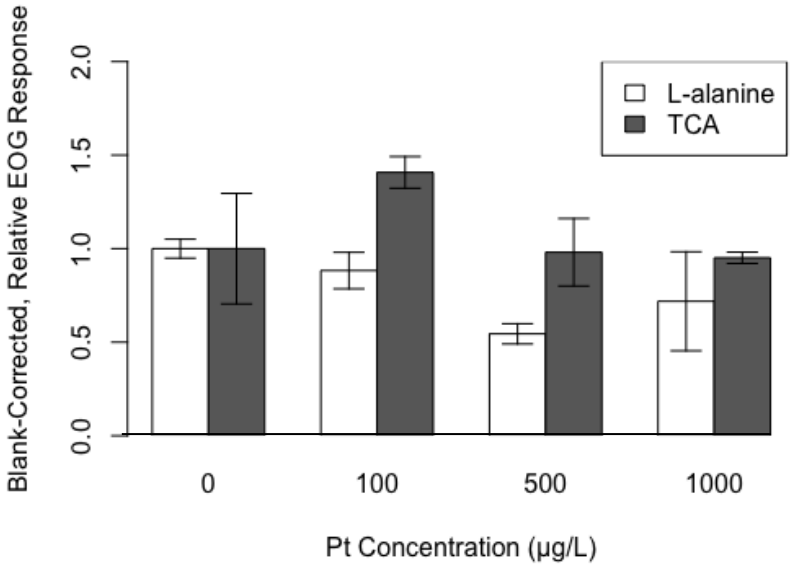

Figure 3.1. Blank-corrected, relative EOG response of rainbow trout to L-alanine (stimulates microvillous OSNs) and TCA (stimulates ciliated OSNs) following a 96 h nominal Pt exposure ($p > 0.05$, error bars ± 1 SEM, n = 3).

3.4.2 Platinum concentration in aqueous static-renewal test systems

Taken together, mean measured Pt from solution was 94% (Table 3.1). Although static-renewal test systems were subject to exposure fluctuations due to frequent water

changes, our study demonstrated no difference between Pt concentrations at the start (i.e., 1 h following fish and Pt addition) and end (i.e., 96 h following fish and Pt addition) of experiment.

Table 3.1. Percent difference between measured and nominal Pt concentrations ($Pt_{quantified}$/ $Pt_{nominal}$) from water samples taken at 1 h and 96 h, including mean percent difference between time points.

$Pt_{nominal}$ (µg/L)	Percent difference (1 h)	Percent difference (96 h)	Mean difference
0	___[na]	___[na]	___[na]
100	2%	2%	2%
500	3%	1%	2%
1000	31%	28%	30%

[na] Measured Pt levels at 0 µg/L was below the detection limit.

3.5 Discussion

Although studies on other trace metals, including Cu (Razmara et al., 2020), Cd (Dew et al., 2016), and Ni (Dew et al., 2014), report significant olfactory inhibition below 25 µg/L, our study demonstrates that Pt is not toxic to fish olfaction up to 1000 µg/L as shown in Fig 3.1. The direct mechanisms of action for metal-induced olfactory inhibition are not fully understood, however it is clear Pt did not interact with the olfactory epithelium. One suggestion for our findings is increased mucous production, as it is known to protect the olfactory organ from contaminants (Kasumyan, 2004). However, mucous quantities were not measured in this study and future investigations may choose to explore this in establishing threshold concentrations required to induce olfactory inhibition in fish.

Exploring specificity of OSN impairment informs a mechanistic understanding of olfactory inhibition, as not all waterborne contaminants act on the same OSN sub-population. When testing olfactory acuity using one odourant, contaminant effects on other

OSN classes may be missed, leading to incorrect conclusions on trace metal toxicity. By testing different OSN classes, we conclude that Pt is not toxic to fish olfaction using supra-environmental concentrations of dissolved Pt.

Lack of observed toxicity following Pt exposure up to 1000 μg/L is unsurprising given the Pt concentrations required to induce lethal and sublethal effects in a range of other aquatic organisms. In their 1979 study, Ferreira & Wolke (1979) conclude that the 96 h LC50 of Pt is 2.5 mg/L in coho salmon (*Oncorhynchus kisutch*). Although Pt toxicity was explored by Osterauer et al. (2011), they conclude no genotoxic responses in zebrafish (*Danio rerio*) following 96 h exposure up to 200 μg/L Pt. To our knowledge, no other Pt toxicity studies have been conducted using fish, however available data have demonstrated the high concentrations of Pt required to elicit toxic effects corroborate our findings.

With very limited published acute Pt toxicity studies on fish, our results must also be compared to available data on aquatic invertebrates. Annelid mobilization is impaired at 61 μg/L Pt at 96 h (Khangarot, 1991), whereas the LC50 ranges from 397 μg/L – 30 000 μg/L Pt at 96 h depending on water hardness (Veltz et al., 1996). Arthropod LC50 values range from 131 μg/L – 520 μg/L (Biesinger & Christensen, 1972; Borgmann et al., 2005; Zimmermann et al., 2017), whereas immobilization occurs at 95 μg/L – 276 μg/L depending on species and exposure duration (Khangarot & Das, 2009; Okamoto et al., 2015; Zimmermann et al., 2017). We must acknowledge that species, size, contaminant form, endpoint, exposure route and environment all contribute to understanding Pt toxicity and have yet to be consistent between studies. Although we did not find the threshold concentration to elicit Pt-induced olfactory toxicity in our study, we cannot conclude that Pt is not toxic to fish as we did not pursue higher concentrations. However, we can be confident that Pt is not toxic to fish olfaction at Pt levels currently found in the environment.

Our overall minimal Pt loss in solution was expected because Pt is considered the least reactive PGE (Cobelo-Garcia et al., 2007). Although we observed a decrease in measured Pt at the highest treatment, this was also demonstrated by Brand et al. (2019) who hypothesized that lack of Pt recovery in solution at higher concentrations is attributable to precipitation processes. Nonetheless, Pt concentrations remained relatively stable throughout the experiment despite daily water changes as shown in Table 3.1.

3.6 Conclusions

It is well understood that some metals are powerful olfactory toxicants to fish, resulting in olfactory disruption following exposure to trace metal concentrations (Tierney et al., 2010). In contrast, the present study revealed exposure to Pt at concentrations orders of magnitude higher than what can be found in the environment are not sufficient to induce olfactory inhibition. Although Pt toxicity was not demonstrated here, additional Pt investigation is warranted as risk assessments currently do not exist. Until supporting evidence can be produced in addition to increased environmental analysis, Pt contamination may be of environmental concern as Pt toxicity in aquatic organisms is not yet fully established.

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CHAPTER 4: Final Conclusions

4.1 General discussion and conclusions

The present thesis investigated two contaminants of emerging concern, palladium (Pd) and platinum (Pt), and their individual potentials to cause olfactory dysfunction in rainbow trout. There are limited published data on platinum group element (PGE) toxicity, thus it was important to investigate a biological ligand that is associated with apical endpoints while taking field knowledge into consideration. Being in direct contact with surrounding waters, disruptive effects can occur at the olfactory rosette when exposed to trace concentrations of metals. (Tierney et al., 2010). Further, olfactory-mediated behaviours are directly linked to those used in the derivation of risk assessments and water quality guidelines (WQGs) for the protection of aquatic life (CCME, 2007), both of which do not currently exist for any PGE (Ågerstrand et al., 2020). The first objective of this study was to establish threshold concentrations of Pd and Pt required to elicit olfactory inhibition following a 96 h waterborne exposure. Using the neurophysiological electro-olfactography (EOG) assay, a range of environmentally relevant to supra-environmental concentrations were selected for each metal to assess toxicity potential and compare our findings to current levels of freshwater contamination. We discovered that exposure to environmentally relevant Pd concentrations led to olfactory disruption in rainbow trout. Further, Pd does not target specific classes of olfactory sensory neurons (OSNs), but rather ciliated (stimulated by taurocholic acid (TCA); which mediates social behaviours) and microvillous (stimulated by L-alanine; which mediates food seeking behaviours) neurons were equally impaired. Based on our findings, we can conclude Pd is toxic to rainbow trout olfaction at environmentally relevant concentrations and that fish may be at risk of experiencing sub-

optimal olfactory perception in Pd-contaminated environments. The fact that Pd was toxic to fish olfaction at low μg/L ranges suggests that increased investigation into the development of WQGs for the protection of aquatic life is warranted. In contrast, the threshold concentration for olfactory dysfunction from Pt exposure was not discovered in this thesis. Nonetheless, we can conclude rainbow trout are not at risk to adverse chemosensory effects caused by Pt contamination, as it does not perturb olfactory function at concentrations exceeding 4,000x that currently found in freshwater environments (reviewed in Ruchter et al., 2015). To comply with animal ethics, it was determined that further investigation at higher concentrations was unwarranted as soluble field-levels of Pt are not expected to approach effective concentrations.

The second and third objectives of this thesis were explored only using Pd as a toxicant, as Pt toxicity was too low to be detected in this study. Physico-chemical parameters are known to influence metal toxicity by altering bioavailability and speciation (Pinheiro et al., 2021). The second objective in this thesis was to understand how water quality affects Pd-induced olfactory toxicity through varying levels of water hardness, pH, and dissolved organic carbon (DOC). By comparing our findings from the first part of this study to altered environmental conditions, we gained additional knowledge about Pd toxicity in fish.

As demonstrated in previous fish olfaction research, the addition of Ca^{2+} and Mg^{2+} to increase water hardness did not have mitigatory effects on olfactory toxicity, but rather exerted its own inhibitory effect to fish olfaction (Dew et al., 2012). Further, Borgmann et al. (2005) found Pd toxicity was heightened in hard water compared to soft water when exploring LC50 levels in *Hyalella azteca*. Taken together, data from the present study support these findings and showed very hard water was toxic to fish olfaction when

compared to soft water separate from Pd-induced olfactory disruption. Within a range of pH 6-9 our results failed to demonstrate altered Pd-induced olfactory disfunction. This was unsurprising given Pd is present primarily in its free ionic form when pH < 2, which is much too acidic to support freshwater fish (Colombo et al., 2008). Lastly, the addition of humic acid as DOC up to 5.1 mg/L did not protect fish against Pd-induced olfactory dysfunction. In their 2002 study Zimmermann et al. (2002) discovered Pd displays an elevated bioaccumulation tendency when organic compounds are present, which corroborates our findings.

The last objective in this study was to broadly investigate mechanisms for Pd-induced olfactory system disruption. The association of Pd at the olfactory rosette pointed to epithelial binding and/or channel blockages as a mechanism of action (MOA) for olfactory disruption. Uptake of Pd at tissues in direct contact with the surrounding environment was further demonstrated when Pd was measured at the gill. We were able to dismiss oxidative damage from lipid peroxidation (LPO) by the nonsignificant effect of Pd exposure on the formation of LPO product. Lack of elevated LPO levels at the olfactory rosette leads us to conclude that reactive oxygen species (ROS) formation did not damage the olfactory membrane and thus was not a cause for decreased olfaction.

Taken together, we can conclude from our findings that Pd induces olfactory toxicity in rainbow trout near environmentally relevant concentrations while Pt does not. Our results are not surprising, as Pd is more mobile, soluble, and bioavailable than Pt (Zereini & Wiseman, 2015). We know that PGE data is severely lacking in the area of aquatic ecotoxicology. Nonetheless, we can provide novel conclusions from our data on PGE toxicity in fish while highlighting the need for increased investigation on PGE toxicity with a focus on Pd.

4.2 Future research

There were a few shortcomings in the present study that may warrant investigation in future research. To begin, we did not quantity metal speciation in the exposure system. This robust analysis would require additional quantification of inorganic and organic ligands in addition to metal speciation (Sigg, 2014). Instead, metal speciated was estimated using available water quality models and from previous research which provided pH-based metal speciation curves and basic information pertaining to PGE-ligand interaction (Colombo et al., 2008; Šebek et al., 2011).

Graphite furnace atomic absorption spectrometry (GFAAS) is an efficient instrument to measure trace amounts of metal in solution. Following this study, we discovered Pd concentrations lower than our method detection limit (MDL) resulted in adverse olfactory effects. Based on preliminary research, levels of trace metal required to reach threshold concentrations for olfactory inhibition were above MDL which was why GFAAS was used in our study. This however did not account for the extreme percent loss of metal to the system that was found upon measuring exposure waters. The use of more sensitive analytical equipment such as inductively coupled plasma mass spectrometry (ICP-MS) would increase reporting accuracy in future studies through a lower instrument MDL.

In addition to thesis shortcomings, we noted complementary studies that would build upon our current understanding of PGE-induced olfactory toxicity. It may be useful to evaluate PGE mixtures as a toxicity model when conducting laboratory studies given primary emission sources deposit multiple metals or the addition of other contaminants in the field simultaneously. We did not discover Pt concentration thresholds required to elicit a toxic response in rainbow trout; however, the combination of Pt with other PGEs may yield different results. Although environmental variables were explored in context to

altering Pd toxicity, we did not explore all possible combinations of the levels of factors included in this study. As this was the first study to investigate PGE toxicity on fish olfaction, there remains many avenues yet to be explored. Olfactory recovery in addition to histology and gene expression at the olfactory rosette would improve our understanding the effects of Pd on olfaction at the cellular level.

4.3 Knowledge gaps and recommendations

There remain critical knowledge gaps in our understanding of Pd and Pt from a toxicological viewpoint. Without protective environmental quality guidelines and risk assessments for PGEs, adverse outcomes for aquatic life are possible in contaminated locations. Current knowledge gaps in PGE research in addition to recommendations for future studies are outlined below and should be taken into consideration to best advance the field of PGE toxicology.

First, reported field concentrations of PGEs remain scarce. Increased field sampling and subsequent analyses in both rural and urban areas is crucial in furthering our collective understanding of PGE toxicity and risk to aquatic organisms. Current toxicological laboratory studies cannot base methods on environmental relevance, as we only have a loose understanding of what that is. In urban areas, seasonal differences such as pulse events and their contribution to PGE contamination in freshwater remain elusive. Rural areas may also be subject to seasonal contamination through fresh snowfall or snowmelt events in mountain ranges as it is known PGEs are distributed in these areas (Barbante et al., 2001; Barbante et al., 2004; Rauch et al., 2005). Metal analysis remains difficult upon collecting field samples containing PGEs, as there is a lack of PGE integration into certified reference materials (CRM) requiring the creativity of researchers to find other acceptable

CRMs. Examples in Canada include SLRS-6, HISS-1, and DOLT-4 which currently do not contain any PGEs. This may explain the unequal effort given towards soil analysis compared to aqueous quantification, as it requires additional effort for researchers to find suitable CRMs for trace metal analysis in this matrix.

Second, is the need to understand the large percent loss observed during PGE exposures, specifically Pd. The ongoing reporting of significantly lower measured values compared to what was added to solution in PGE studies leads to inconsistencies when performing static, static-renewal, and non-static exposures. Although there has been a recent increase in kinetic studies aimed at mitigating this issue, no solution has been provided at this time. Many published PGE toxicology experiments are dated and do not report measured PGE concentrations, resulting in a lack of reproducibility and comparability within laboratory studies.

Third, increased research on PGE toxicity and bioaccumulation is critical to provide adequate data for proper risk analysis and guideline development. Despite the recent surge of uptake, bioaccumulation, and toxicity studies, general toxicity threshold concentrations for any PGE have yet to be established. Lack of measured exposure conditions and unreported physico-chemical parameters has contributed to contradictory conclusions as to the ranking of toxicity between Pd and Pt. The results of this thesis reveal the importance of reporting these concentrations to increase comparability in this research area. Of the limited studies investigating PGE toxicity in aquatic organisms, most focus on short-term exposures. Nonetheless, it is crucial to pursue chronic exposures when quantifying risk and developing formal environment guidelines. Chronic studies would provide useful information on environmentally relevant situations such as pulse and steady-state PGE influx in ecosystems near primary PGE emitters such as traffic-heavy, wastewater, sewer,

and processing plant locations. Further, studies focusing on uptake and bioaccumulation far outnumber those focused on toxicity, especially in aquatic vertebrates. During preliminary testing, threshold concentrations of Pd required to induce olfactory inhibition were close to those that caused lethality in rainbow trout. This finding suggests that it is highly plausible for biota to be at risk when combining under-reported field concentrations with our understanding of Pd mobility and solubility in aqueous environments. Incomplete understanding of PGE toxicity highlights the need for studies with aquatic organisms in which governmental guidelines are based. To develop WQGs in Canada, a minimum of three fish, three invertebrates, and one plant species are required to be investigated in which a range of effects data can be obtained (CCME, 2007). Using this information, a species sensitivity distribution (SSD) can be created where a hazard concentration of 95% species protection is calculated. Further, Alberta uses this information to prepare long- and short-term guidelines. Long-term guidelines are described where contaminant levels should result in negligible risk to apical endpoints, regardless of exposure length, whereas short-term guidelines are meant to provide protection from severe effects as organisms experience transient exposures (Government of Alberta, 2018). In addition to guideline development, risk assessors can use this information to understand the potentially affected fraction of species in contaminated areas.

4.4 Note on future PGE demand

The developed world is slowly creating options and incentives to promote transport methods that would not require vehicle exhaust catalysts (VECs), such as electric vehicles. Considering VECs are the primary emission source of PGEs into the environment, this change could lead to a decrease in PGE contamination and negate the need for robust

ecotoxicological research on these rare metals. Another promising addition contributing to sustainable transportation is hydrogen-powered vehicles. However, unlike electric vehicles, hydrogen fuel cells require the use of catalytic converters (JM, 2021). Similar to gas- and diesel-powered vehicles, auto-catalysts in these fuel cells require the resistant interior coating that PGEs provide. The continued demand for PGE coated catalytic converters poses the realistic future that PGE emission will continue to rise after the phasing out of gas/diesel-powered vehicles from hydrogen fuel cells. Additionally, it will take decades to phase out all fossil-fuel consuming vehicles from roads in developed countries. Unfortunately, converting to sustainable modes of transportation will take much longer in developing countries due to high costs. Thus, although it is plausible to reduce the number of primary PGE emissions sources in the upcoming decades in some areas, PGE contamination is expected to continue increasing on a global scale for the foreseeable future and toxicological research should follow suit.

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APPENDIX 1: SOP #A14

UNIVERSITY OF LETHBRIDGE ANIMAL WELFARE COMMITTEE STANDARD OPERATING PROCEDURE

Department: Biological Sciences

Title: Electro-olfactography of fish **SOP # - A14 Authors: L. Zink, G. Pyle & E. Stock AWC Approval**

Date: January 21, 2022

Purpose:

The purpose of an electro-olfactogram is to measure the olfactory acuity of a fish. This allows us to determine if exposure to specific toxicants can cause impairment to this vital sensory system.

1. Frequency of Procedure

As per approved protocol.

2. Person(s) Responsible

a. Trained laboratory research personnel

3. Documentation of Procedure

a. The removal of a fish from the Aquatic Research Facility to perform an EOG must be documented in the unit specific binder where the fish was housed.

4. Hazard Identification and Control

X Chemical Hazards (see SDS)
Hazard: MS-222, sodium bicarbonate, & KCl **Controls**: Gloves, lab coat, and safety glasses (as appropriate) must be worn during this procedure.

X Physical Hazards:
Hazard: probe holder half-cells need to be rinsed/filled via injection thus there is a risk of needle stick **Controls**: Proper handling of needles is required at all times. A sharps disposal container is required for proper disposal of syringes/needles. **Hazard:** Electrical cords and outlets **Controls:** Proper electrical grounding is required to ensure safe operation of equipment.

5. Specific Training Required

a. Anyone performing this procedure needs to be trained by a qualified individual and be given permission to perform EOG independently prior to proceeding without the direct supervision of a qualified individual
b. Discussion with qualified individual to determine the proper procedure for composing odourant solutions for each experiment to be completed
c. Biomethodology: Anaesthesia or Euthanasia of fish (refer to SOP B19)
d. IAUTP Part 1
e. IAUTP Part 2 (Fish)
f. WHMIS

6. Materials Required

a. Small cooler for transport (with lid)
b. Small dark plastic bucket
c. Buffered TMS (MS222) solution (concentration depends on fish used)
d. Odourant(s) (concentration and composition depends on experiment)
e. ddH20
f. 3M KCl
g. 3ml syringe
h. Paper towel
i. Surgical scissors and forceps
j. Gloves
k. Lab coat
l. Safety glasses (as appropriate)

7. Detailed Procedures

a. Prepare all of the necessary electrodes and solutions as appropriate.
b. Turn on the EOG rig (Figure 1).
c. Don gloves.
d. Complete steps to anaesthetize the fish as described in SOP #B19 "Anaesthesia or Euthanasia of Fish". Wrap the fish in wet (with processed water of the appropriate temperature) paper towel, making sure not to cover the tail fin and the head/gills.
e. Place the fish in the perfusion trough and insert the gill perfusion tube into its mouth.
f. Turn the valve on the gill perfusion tube to allow water to flow over the surface of the gills. The perfusion water is to maintain anesthesia, using the appropriate concentration for the species as listed in SOP #B19 "Anaesthesia or Euthanasia of Fish".
g. Adjust the odourant delivery system so the delivery tube is above one of the nares of the fish, this will ensure the sensitive olfactory tissue will not dry out.
h. Attach the grounding clip to the tail of the fish to remove any electrical interference from the fish during the recordings.
i. Looking through the dissection scope, position the fish so you can clearly see the olfactory chamber you will be measuring.
j. Assess appropriate depth of anaesthesia (stage 3) as described in SOP #B19 "Anaesthesia or Euthanasia of Fish". Using surgical forceps and/or scissors, remove the piece of soft tissue that separates the anterior and posterior opening of the naris, ensuring the naris is being continually perfused with the type of water in which the animal was exposed, less any added contaminant; rinse the naris with this water to remove any blood that may result (EOG will not read well if there is blood present on the olfactory tissue).
   Note: It may be easier to remove this tissue before placing the fish on the rig, providing that depth of anesthesia is confirmed and adequate before proceeding.
k. Rinse the internal portion of the electrode half-cells and holders (i.e. probe holders) with ddH20 and then fill with 3M KCl using a 3 ml syringe.
l. Place the reference and measurement electrodes, or probes (in half-cells and holders) into separate micromanipulators.
m. Attachthewiringassemblyfortheextracellulardifferentialheadstagetothegoldconnectpin of the half-cell.
n. Position the electrodes at the proper locations (Figure 2). The reference electrode should be placed gently on the surface of the skin, posterior to the olfactory chamber being used in the experiment; typically, this means place the probe on the skin midway between the eye and olfactory chamber (Figure 3A). Ensure that the reference electrode will be under the

flow from the odourant delivery line. The recording electrode should be placed in the naris, with the tip of the electrode making contact with the lamellae but not touching the lamellae tissue to the point of causing depression of the tissue at the point of contact , which provides good, repeatable results (Figure 3B, e.g., known sweet spots: for fathead minnows use the second posterior lamella, placing the probe over the lamella at 1⁄2 the distance of its length; for rainbow trout use the third lamella, placing the probe at 1/3 the distance of its length, proximal to the base). Ensure the tip of the electrode is not touching the surface of the lamellae and that the same position is used between fish.

o. Ensure the Powerlab and amplifier are turned on, start the Chart 5 software on the computer.
p. Begin recording of the signal from the fish. Adjust/replace the probes as needed to get a stable baseline.
q. Allow the olfactory chamber to acclimate for 5-10 minutes.
r. Add an odourant to the olfactory chamber using the odourant deliver system. Typically, a 3s pulse of odourant is used. Repeat a minimum of 3 times per odourant used with 2 minutes time between randomized, cue pulses.
s. At the end of the experiment the fish is removed from the apparatus and euthanized with an anaesthetic overdose (as stated in SOP #B19 “Anaesthesia or Euthanasia of Fish”).
t. Measure and record the weight and length of the fish.
u. Dispose of carcass in the fish carcass freezer in the ARF as per SOP A10.
v. Dispose of the anaesthetic buffered MS-222 solution down the regular drain.
w. Clean up EOG apparatus by disposing of probes and other sharps into the sharp’s container, disposing of any wastewater down the drain and wiping down any contaminated surfaces. Probe half-cells must be cleaned via syringe with plenty of ddH2O; no salts should remain on the rig components.
x. Remove and discard gloves.

Figure 1: EOG rig.

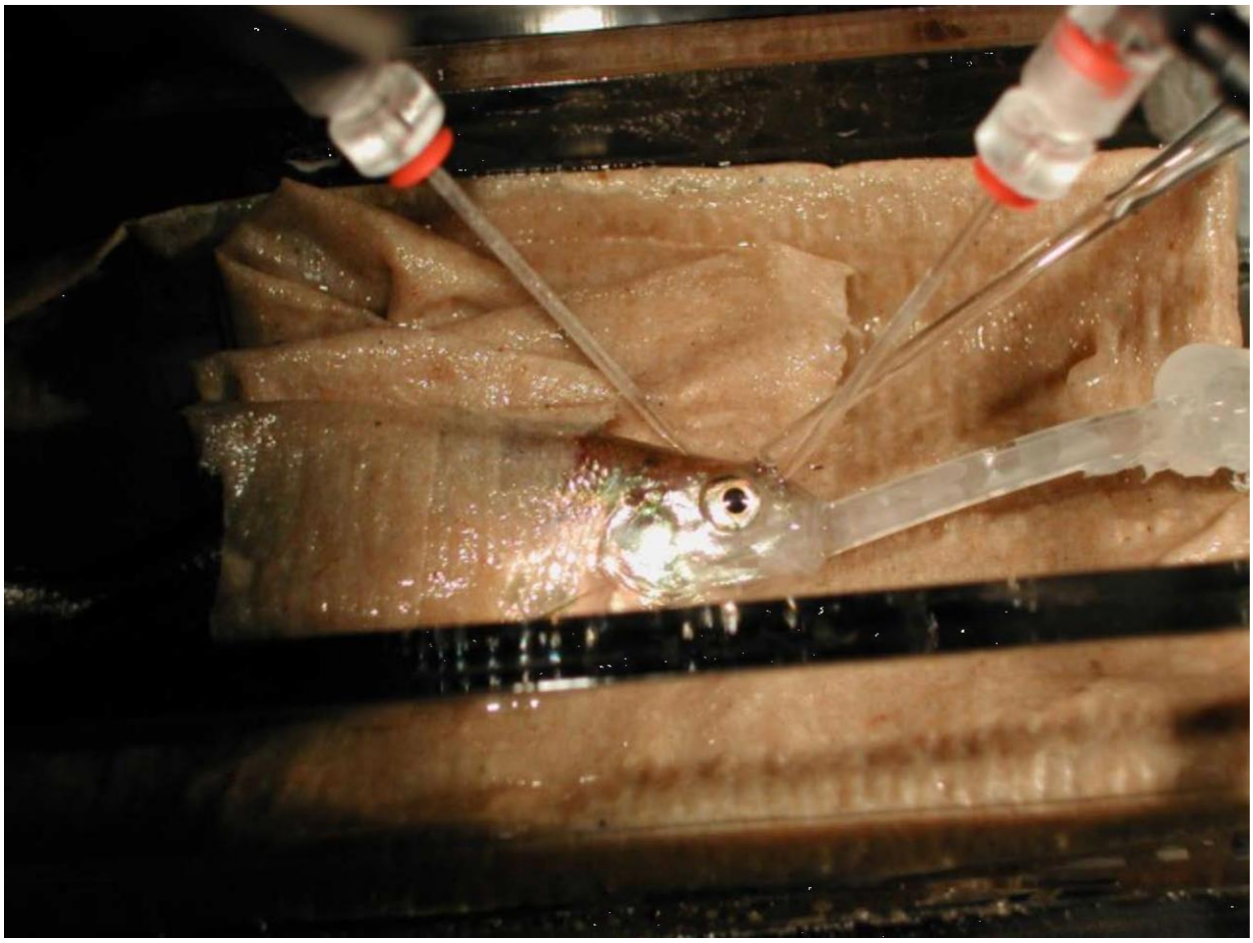

Figure 2: Placement of electrodes, stimulus deliver line, and gill perfusion tube.

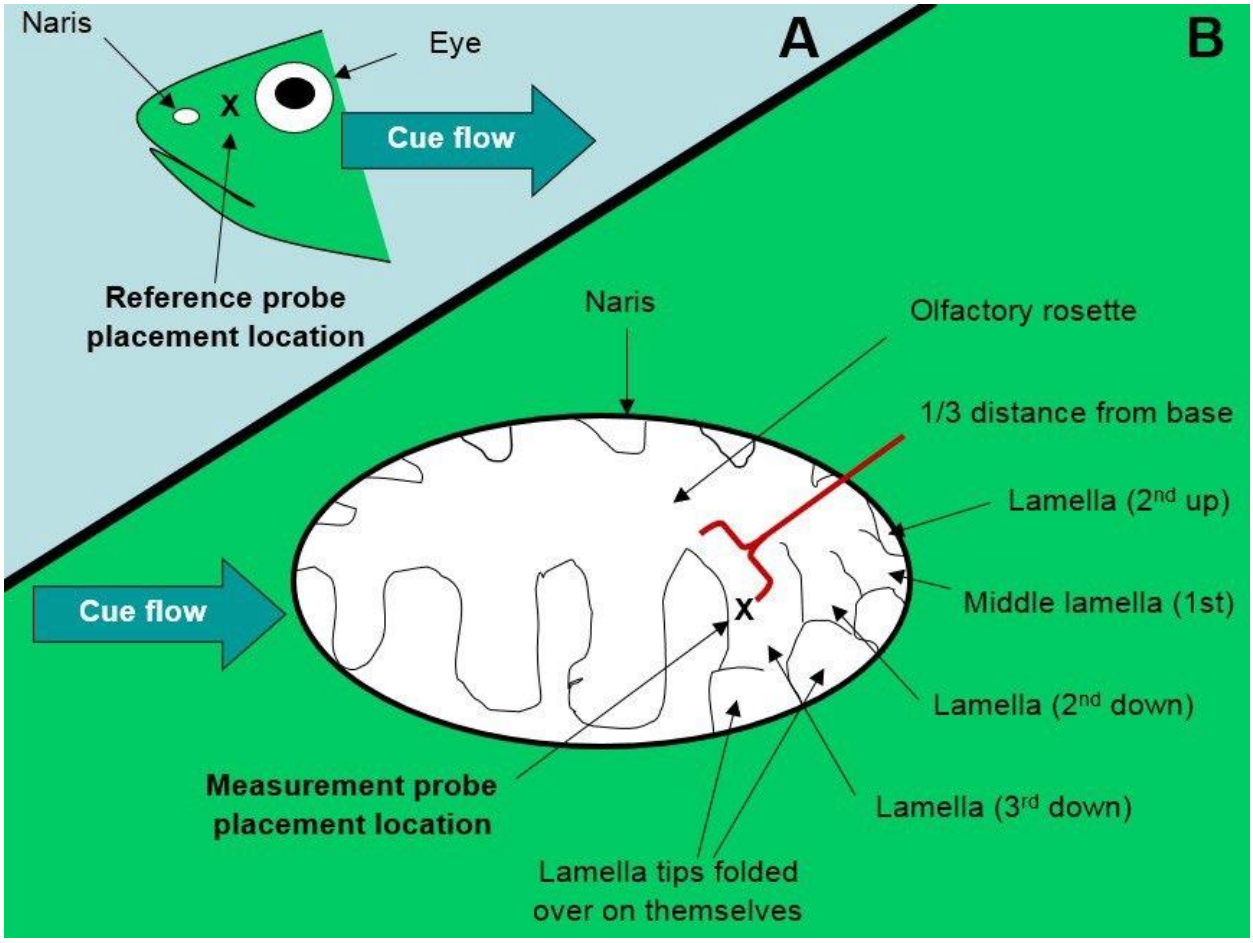

Figure 3: EOG probe placements in rainbow trout. A) reference probe location, B) zoom of naris with measurement probe placement in sweet spot.