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

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Abstract

Through emission processes, palladium (Pd) particulates from industrial sources are introduced into a range of ecosystems including freshwater environments. Despite this, research on Pd-induced bioaccumulation, uptake, and toxicity is limited for freshwater fishes. Unlike other metals, there are currently no regulations or protective guidelines to limit Pd release into aquatic systems, indicating a global absence of measures addressing its environmental impact. To assess the olfactory toxicity potential of Pd, the present study aimed to explore Pd accumulation in olfactory tissues, olfactory disruption, and oxidative stress in rainbow trout (*Oncorhynchus mykiss*) following waterborne Pd exposure. Olfactory sensitivity, measured by electro-olfactography, demonstrated that Pd inhibits multiple pathways of the olfactory system following 96 h of Pd exposure. In this study, the concentrations of Pd for inhibition of olfactory function by 20% (2.5 μg/L; IC20) and 50% (19 μg/L; IC50) were established. Rainbow trout were then exposed to IC20 and IC50 Pd concentrations in combination with varying exposure conditions, as changes in water quality alter the toxicity of metals. Independent to Pd, increased water hardness resulted in decreased olfactory perception owing to ion competition at the olfactory epithelium. No other environmental parameter in this study significantly influenced Pd-induced olfactory toxicity. Membrane-associated Pd was measured at the olfactory rosette and gill following exposure; however, this accumulation did not translate to oxidative stress as measured by the production of malondialdehyde. Our data suggest that 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 provide novel insights into the olfactory toxicity in fish following Pd exposure. *Integr Environ Assess Manag* 2024;20:1407–1419. © 2024 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

KEYWORDS: Dissolved organic carbon; Electro-olfactography; pH; Water hardness

INTRODUCTION

Fishes rely on optimal olfactory perception to mediate a range of crucial life-history behaviors including foraging, homing, mating, and predator avoidance (Klaschka, 2009). The olfactory epithelium comprises a variety of olfactory sensory neurons (OSNs) that are in direct contact with surrounding waters. Chemical compounds (odorants) within the water bind to specified receptor proteins at one of multiple 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 odor, resulting in cell-specific odorant detection (Hansen et al., 2003). Microvillous OSNs perceive food cues, ciliated OSNs detect social cues, and crypt cells recognize sex pheromones (Hamdani & Døving, 2007). Research involving freshwater fishes has demonstrated that 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). Combined, these studies demonstrated that properly functioning olfaction is therefore critical to organismal health and survival.

A variety of contaminants are known to impair fish olfactory function, including metals (Tierney, 2016). Research on waterborne metals such as aluminum, cadmium, copper, mercury, nickel, and zinc demonstrates their inhibitory effects on fish olfaction (Dew et al., 2016; Tierney et al., 2010). Change in odorant perception can occur by direct binding

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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 OSN 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 OSN, which can be measured through the presence of oxidative stress (Razmara et al., 2021; Tilton et al., 2008). Resultant disruption of the olfactory system may lead to maladaptive behavioral responses and potential adverse outcomes at the organism or population level.

Physical and chemical conditions in the natural environment, including water hardness, organic matter concentrations, and pH, play a vital role in metal speciation and subsequent toxicity thresholds to aquatic organisms (Pinheiro et al., 2021). It is well established that hardness cations, such as Ca^{2+} and Mg^{2+}, compete with metal ions for physiologically sensitive binding sites on the animal (Smith et al., 2015). Additionally, pH affects metal speciation through the formation of inorganic complexes, leading to alterations in bioavailability (de Paiva Magalhães et al., 2015). The dissolved fraction of organic matter, typically measured as dissolved organic carbon (DOC), forms organic–ionic complexes that restrict metal bioavailability and uptake (Di Toro et al., 2001). Few studies have investigated palladium (Pd) speciation and toxicity thresholds in aquatic environments, so it is unclear how these parameters influence Pd toxicity in freshwater fish.

The limited investigation and monitoring of Pd to date have shown increasing concentrations of Pd in multiple components of the environment, likely due to anthropogenic deposition (Ravindra et al., 2004). Regular environmental sampling remains scarce; however, measured waterborne Pd concentrations are generally in the low µg/L range (Fortin et al., 2011). Currently, 82% of global Pd demand is attributable to the automobile industry, where Pd comprises approximately 72% of the metal mixture used in vehicle exhaust catalyst (VEC) manufacturing (Johnson Matthey, 2021). Palladium is extracted from mineral deposits alongside other platinum group metals (PGMs) and is often obtained as a co-product of nickel or copper ores. It is widely used in the production of VECs because of its catalytic and heat-resistant properties. Abrasions from chemical, mechanical, and thermal wear result in Pd emission into the surrounding environment over the lifetime of a vehicle (Bencs et al., 2003). Detection of Pd in rural areas including those 200 km away from primary emitters and in fresh mountain snowfall demonstrates the mobility and dispersion of Pd once it enters the natural environment (Moldovan et al., 2007; Rauch et al., 2005; Reimann & Niskavaara, 2006).

Pollution of Pd ultimately reaches aquatic habitats, but the implications of such contamination for aquatic organisms are unclear. Owing to its high relative mobility and solubility, Pd is the most bioavailable metal in its group (Colombo, Monhemius, et al., 2008). Roughly 50% of VEC-emitted Pd occurs in its mobile and soluble, ionic form (Moldovan et al., 2002), which, when released into the natural environment, complexes with environmental ligands including chlorides, sulfides, polysulfides, cyanide, and organic acids (Colombo, Oates, et al., 2008; Dahlheimer et al., 2007; Mountain & Wood, 1988; Mulholland & Turner, 2011; Šebek et al., 2011). Accumulation in soil and dust along roadways enhances the transport of Pd into aquatic ecosystems (Ek et al., 2004).

Uptake and bioaccumulation of Pd have been demonstrated in aquatic organisms including *Anguilla anguilla* (Sures et al., 2001; Zimmermann, Von Bohlen, et al., 2005), *Barbus barbus* (Sures et al., 2005), and *Danio rerio* (Chen et al., 2015). Chronic exposures in fish indicate that PGMs accumulate in the liver, which is the main site for toxicant detoxification (Zimmermann, Von Bohlen, et al., 2005), and at the gills (Zereini & Wiseman, 2015). Studies that investigated the effects of PGM bioaccumulation concluded that Pd is more concentrated than other PGMs at all measured tissues. Nonetheless, the physiological implications of Pd bioaccumulation remain unknown.

The present study explored 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 by a history of olfactory toxicity investigation using metal contaminants (Dew et al., 2016; Hara et al., 1976; Lari et al., 2018; Razmara et al., 2019; Wolf & Rumsey, 1985). The objectives of this study were to (1) establish 96 h Pd-induced olfactory toxicity thresholds (20% and 50% inhibition) in rainbow trout, (2) understand how the chemical parameters of varying water hardness, pH, and DOC affect olfactory toxicity after exposure to Pd, (3) assess accumulation of Pd in water-facing (olfactory and gill) and internal (liver) tissues, and (4) assess oxidative stress in olfactory tissue following Pd exposure.

MATERIALS AND METHODS

Animal husbandry

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 and immediately transferred to the Aquatic Research Facility at the University of Lethbridge. Fish were stocked at a density of 5 g/L in static-renewal holding tanks. Water used throughout this experiment, herein referred to as processed cold water (PCW), was created from City of Lethbridge water using chemical, biological, and physical filtration including dechlorination, large particle removal, buffering, ultraviolet sterilizing, and bioinoculation. Fish were fed twice daily with 1.5 mm trout chow pellets (EWOS) and left to acclimate to holding conditions for a minimum of two weeks prior to being used in experimentation. Fish were held under 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, 93.1 ± 1.1% saturation; 0.01 ± 0.01 mg ammonia/L;

0.004 ± 0.003 mg nitrite/L; 2.1 ± 0.5 mg nitrate/L; 2.04 ± 0.2 mg DOC/L; pH, 8.24 ± 0.1; and hardness 142 ± 3.2 mg/L as $CaCO_3$.

Palladium preparation and analysis

Palladium (II) nitrate solution (in 5% HNO_3) was purchased from Inorganic Ventures at a certified concentration of 1002 ± 6 µg/mL (99.9%–100.0% Pd purity; Inorganic Ventures). Palladium solution was stored at 4 °C in the dark until use. Palladium concentrations in tank water were measured at the start (i.e., prior to fish or Pd addition), Day 1 (i.e., 24 h following fish and Pd addition), and at the end (i.e., 96 h from fish and Pd addition) of the experiment using graphite-furnace atomic absorption spectrometry (GFAAS; GTA120; Agilent Technologies). Water samples were collected and immediately acidified to 1% 12 N HNO_3 (TraceSelect grade; Sigma Aldrich) 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) was used as a run standard and tested for quality assurance/quality control (QA/QC) verification. Further, the calibration curve replotted every 20 samples with mean QA/QC recovery within the accepted ±10% error. The method detection limit (MDL) was measured to be 5 µg Pd/L.

Establishment of 96 h Pd-induced olfactory toxicity thresholds

To assess Pd-induced olfactory toxicity thresholds, fish were exposed to a dilution series. As no current studies describe Pd toxicity thresholds for rainbow trout, a range-finder study was conducted to narrow down the inhibitory concentrations (ICs) range. A 96 h IC assay was conducted to determine Pd concentrations required to inhibit olfactory function by 20% and 50%, elucidating the 96 h IC20 and IC50. In this experiment, fish were exposed to a nominal dilution series of 0, 20, 50, 100, and 200 µg/L of Pd for 96 h. Static-renewal exposure systems used one fish per tank in 15 L of solution. 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 h light:dark photoperiod and dissolved oxygen was maintained above 90% saturation. To allow for adequate Pd solution equilibrium (as established through preliminary experimentation, not published), 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). Fish were anesthetized using 120 mg/L tricaine methanesulfonate (TMS; AquaLife) and buffered to pH 7.4 using 360 mg/L $NaHCO_3$ (Fisher Scientific). Anesthesia was considered complete when no further movement was observed from the operculum. Upon anesthetization, fish were wrapped in a 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. To obtain strong readings on the olfactory rosette, a glass microelectrode recording probe was placed to the right of the median raphe at the third lamella approximately at 1/3 distance from the base and 1/2 lamella width, as measured from the anterior naris. A reference microelectrode was placed on the skin between the naris and the eye. The measured EOG amplitude was determined by subtracting the reference signal from the recording signal. Prior to collecting data, fish were allowed a 5-min acclimation period upon completion of probe and line placement. Following acclimation, odorants were delivered in a randomized order to negate odor habituation a minimum of three times in 3-s pulses with a recovery time of two minutes between odorant deliveries.

To determine if Pd preferentially targets a specific subtype of OSN, receptor-specific odorants were utilized in this study. The activity at microvillous and ciliated OSNs was respectively measured using 10^{-4} M L-alanine (CAS #56-41-7; Fisher Scientific) and TCA (CAS #345909-26-4; Sigma-Aldrich). Additional blanks, containing only clean water and no odorants, were used to confirm that odorant responses were from receptor-specific odorants. The resultant EOG signal was amplified as described by Razmara et al. (2019) and calculated using the difference between baseline and maximum amplitudes. The 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 that responses were from live olfactory tissue and not subject to external interference. The IC20 and IC50 Pd values were interpolated and used for subsequent water quality manipulation experiments.

Alterations to Pd toxicity under varying water hardness, pH, and DOC

To investigate the impact of water hardness, pH, and DOC from dissolved humic acid solution on Pd-induced olfactory toxicity, each of the three water quality characteristics was independently manipulated. A total of 27 treatment groups of fish ($n = 6$) were exposed to predetermined IC20 (10 µg Pd/L) and IC50 (42.5 µg Pd/L) olfactory impairment values in addition to a control (0 µg Pd/L) for 96 h. Prior to exposure, fish were allowed an acclimation period to manipulated water over 72 h to mitigate shock risk and solution instability. Daily 50% water changes were completed with the 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). In terms of

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experimental conditions, a 16:8 h light:dark photoperiod and dissolved oxygen above 90% saturation were maintained. 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 1. It is important to note that hardness, pH, and DOC were manipulated in independent experiments to mitigate resource challenges inherent in a multiplicative, full-factorial design. Water hardness was decreased by the addition of Millipore double-distilled water (EMD Millipore) and increased through the addition of equal parts $CaSO_4$ (CAS #7778-18-9; Fisher Scientific) and $MgSO_4$ (CAS #7487-88-9; Fisher Scientific). Since PCW water had a basic pH, 12 N HCl (CAS #7647-01-0; ACS reagent grade; EMSURE) was added to reduce the pH to neutral and acidic pH levels. Addition of humic acid (CAS #1415-93-6; Alfa Aesar) to PCW yielded our DOC stock solution. The DOC stock solution was used within 48 h of creation, maintained at 4 °C, and wrapped in aluminum 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 the surface, middle, and bottom of each stock container to ensure that complete solution equilibrium was met, and no fluctuation occurred between water changes.

A subset of tanks per treatment ($n = 3$) was randomly selected prior to exposure, 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 Day 1 (i.e., 24 h after fish and Pd addition) and the end (i.e., 96 h after fish and Pd addition) of experimentation. Additional daily measurements were taken from hardness and pH stock solutions. Dissolved organic carbon stock was measured after creation. Water samples for analysis of DOC concentrations were filtered using 0.45 µm cellulose acetate membrane filters (VWR), acidified to pH 2 with 12 M HCl, and analyzed using high-temperature catalytic oxidation with a Shimadzu TOC-L CPH, as described by Zhou et al. (2023). Water quality during each experiment is reported in Table 1.

Bioaccumulation

Fish utilized in the water quality manipulation study were also subsequently used for assessments of Pd bioaccumulation at the olfactory organ, gill, and liver. After the EOG assay, fish were euthanized using buffered TMS solution (240 mg/L TMS [AquaLife] and 720 mg/L $NaHCO_3$ [Fisher Scientific]) 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 preweighed 15 mL Falcon tubes (Fisher Scientific). Tissues were subjected to a digestion protocol, following the procedure described by Lindh et al. (2019). In summary, tissues were 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) and held at 4 °C until GFAAS analysis. The tissue quantification protocol for GFAAS was the same as that outlined in section Palladium preparation and analysis. Currently, no tissue matrix CRM exists that contains Pd. However, to assess the accuracy of the digest protocol, DOLT-4 (5 mg dogfish liver; National Research Council Canada) was digested and analyzed alongside known metal protocols to ensure complete digestion and metal recovery (94% mean metal recovery) for

TABLE 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 mean ± SD	8.24 ± 0.1	8.05 ± 0.1	8.50 ± 0.1	6.04 ± 0.1	7.04 ± 0.2	8.06 ± 0.2	8.17 ± 0.2
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

Abbreviations: DOC, dissolved organic carbon; PCW, processed cold water; SD, standard deviation.

unknown Pd samples. Normalized tissue Pd concentration was calculated as:

$$\frac{\text{absorbance} \times \text{total digest volume}}{\text{dried sample weight}}.$$

To assess the bioaccumulation potential of Pd in specific fish tissues, a concentration factor (CF) was calculated, where C_{tissue} is the mean Pd concentration within a specified tissue (μg/kg) and C_{water} is the mean measured true Pd concentration in water (μg/L) ($CF = C_{tissue}/C_{water}$).

Oxidative stress

Another subset of olfactory rosettes from fish used in the water quality manipulation studies was harvested after the EOG assay and immediately stored in −80 °C until use. The 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 the thiobarbituric acid-reactive substances Parameter Assay Kit (Catalog #KGE013; R&D Systems). Using a Bicinchoninic Acid Kit (Catalog #SK3021; Bio Basic) to quantify protein content, MDA contents were normalized to protein concentration. Both kits were run according to the provided manufacturer protocol.

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 homogeneity of variance using Shapiro–Wilks and Bartlett tests, respectively. When test assumptions were violated and data transformations failed to meet 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 data set. Outliers were removed only if the resulting statistical significance was not altered, but their removal led to improving parametric test assumptions. The remaining Pd concentrations that fell below MDL, but had nominal Pd addition, were replaced by a calculated value using the Kaplan–Meier model from the survival (Therneau, 2020) package. Effect sizes were calculated as η^2 where applicable using the effectsize (Ben-Shachar et al., 2021) 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 data set was created by 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 odorant type and Pd treatment regarding olfactory inhibition in the series dilution. 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 analysis of variance (ANOVA) was conducted to compare the effect of manipulated water hardness and Pd treatment on olfactory dysfunction. The same test was used to assess for mitigatory effects of DOC on Pd-induced olfactory impairment. Both tests 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 compared using one-way PERMANOVAs for all tissue types. A permutational post hoc test was carried out after 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 considered a priori to be statistically significant at $\alpha = 0.05$.

RESULTS

Palladium analysis

The quantified concentrations of Pd in experimental tank samples revealed that measured metal from the testing solution was <50% (Table 2). When both time points were combined, the average percent difference from nominal and measured Pd was 26% and 46% for 9.7 and 42.5 μg Pd/L treatments, respectively. Upon comparing Pd concentrations in solution at 24 h with those taken at 96 h, significantly less Pd was measured at the latter time point.

TABLE 2 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

$Pd_{nominal}$ (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%

Note: Asterisks denote a significant Pd concentration difference between sample times (*$p \leq 0.05$, $n = 42$).
Abbreviation: Pd, palladium.

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Establishment of 96 h Pd-induced olfactory toxicity thresholds

Our observations suggest that 96 h waterborne Pd exposure led to equal impairment in microvillous (L-alanine stimulated) and ciliated (TCA stimulated) OSNs (data not shown). To determine Pd concentrations for use in the water quality manipulation study, inhibitory estimations were performed using a combined odorant response, as Pd is assumed to be a broad OSN inhibitor. Rainbow trout showed a 20% decrease in their ability to detect odorants compared to the control at a concentration of 16.2 ± 4.4 μg/L Pd after 96 h of exposure. Furthermore, we observed 50% impairment in fish olfaction when measured against the control treatment after 96 h of exposure to 42.8 ± 6.0 μg/L Pd. These measurements can be described as the concentrations at which rainbow trout olfaction was impaired by 20% (IC20) and 50% (IC50) (Figure 1).

Alterations to Pd toxicity under varying water hardness, pH, and DOC

In water samples, the measured Pd concentrations for nominal IC20 and IC50 treatments were 2.5 and 19 μg/L, respectively, across studies. 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 dependent on water hardness. However, varying water hardness alone resulted in an additional decrease in olfactory perception ($F_{2,43} = 5.633$, $p = 0.007$, effect size $\eta^2 = 0.08$; Figure 2B). The effect size suggests that water hardness had a moderate inhibitory effect on olfactory perception. Compared to soft water, fish olfaction was decreased in very hard water by 70% and 83% in IC20 and IC50 Pd treatments, respectively. Further, no mitigatory effects on the olfactory system were observed, resulting in acute Pd exposure reducing olfaction in both treatments regardless of the environment as demonstrated by the observed medium effect on olfaction ($F_{2,43} = 49.259$, $p < 0.001$, $\eta^2 = 0.62$; Figure 2A).

Varying pH did not alter Pd-induced olfactory toxicity. There was no mitigatory or worsening effect between pH 6 and 8, resulting in continued olfactory disruption caused only by Pd addition (pseudo-$F_{2,42} = 36.976$, $p < 0.001$; Figure 3). Further, there was no main effect of pH on olfaction.

The addition of up to 5.1 mg/L DOC resulted in no olfactory mitigation when combined with waterborne Pd, resulting in continued drastic reduction of olfactory perception due to Pd ($F_{2,42} = 13.281$, $p < 0.001$, $\eta^2 = 0.37$; Figure 4). Further, there was no main effect of DOC on fish olfaction.

Bioaccumulation

Acute exposure to waterborne Pd resulted in metal accumulation at tissues in direct contact with the exterior environment. The quantity of Pd measured in each tissue is reported as dry weight. The olfactory rosette accumulated significant amounts of Pd when exposed to IC20 (pseudo-$F_{1,12} = 37.412$, $p = 0.001$; Figure 5A) and IC50 (pseudo-$F_{1,12} = 21.466$, $p = 0.001$; Figure 5A) treatments. Further, fish exposed to IC50 levels of Pd had significantly higher metal concentrations in olfactory tissue compared to those exposed to the IC20 treatment (pseudo-$F_{1,12} = 16.451$, $p = 0.001$; Figure 5A). The amount of Pd at the olfactory rosette ranged from 1.2 to 2.6 μg/g and from 5.4 to 25.9 μg/g in the IC20 (16.2 ± 4.4 μg/L Pd) and IC50 (42.8 ± 6.0 μg/L Pd) treatments, respectively. This translates to a mean increase of 714% in Pd at the olfactory rosette when exposed to Pd at IC50 versus IC20 levels.

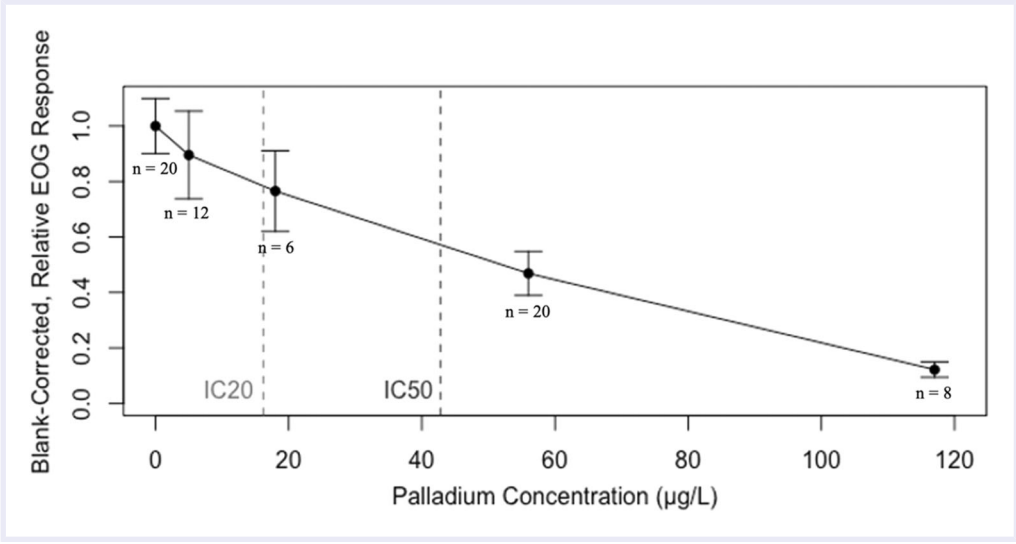

FIGURE 1 Blank-corrected, relative EOG response of rainbow trout after 96 h exposure to varying Pd treatments and the resultant mean predicted inhibitory concentration causing impairment of olfactory function at 20% and 50% using measured Pd concentrations (error bars ± 1 SEM). EOG, electro-olfactography; IC, inhibitory concentration; Pd, palladium; SEM, standard error of mean

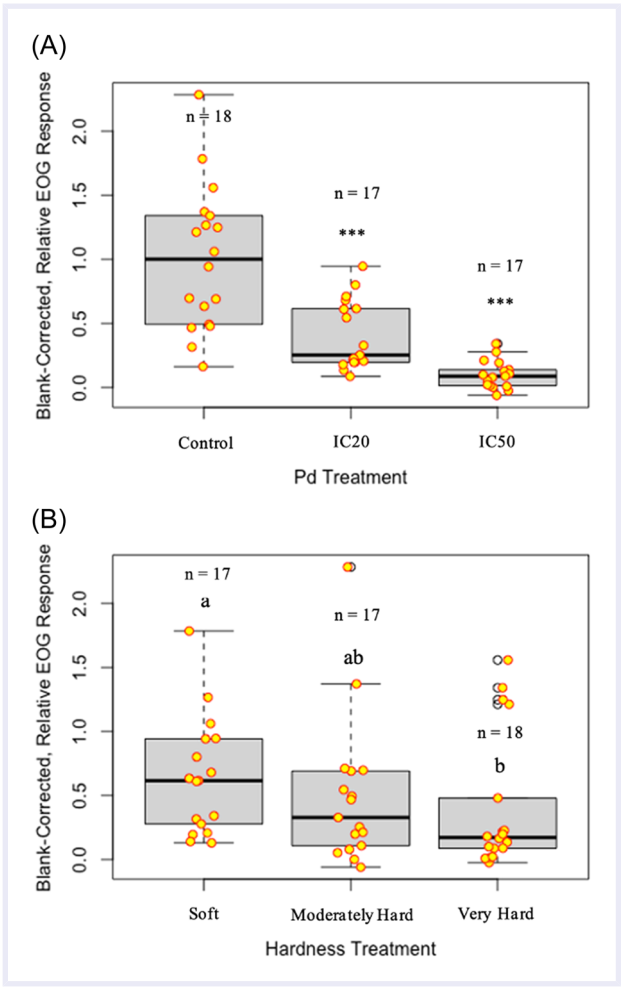

FIGURE 2 Effect of Pd (Panel A) and water hardness (Panel B) on rainbow trout olfaction shown as the relative EOG response to L-alanine following 96 h exposure. Asterisks show significant differences between Pd treatment groups and the control (***$p < 0.001$, IC20 = 2.5 μg/L, IC50 = 19.0 μg/L). Lowercase 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 $CaCO_3$). The shaded box area represents the interquartile range, the line within the box represents the median, and the whiskers represent the minimum and maximum values. EOG, electro-olfactography; IC, inhibitory concentration; Pd, palladium

Similarly, we found significantly more Pd in the gill when fish were exposed to IC20 (pseudo-$F_{1,40} = 67.635$, $p = 0.001$; Figure 5B) and IC50 (pseudo-$F_{1,40} = 39.805$, $p = 0.001$; Figure 5B) Pd treatments compared to the control. 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 from 3.6 to 28.1 μg/g in IC20 and IC50 treatments, respectively. No Pd was detected in liver samples for any treatment (Figure 5C).

Calculated tissue-specific CF values are presented in Table 3. The CF values at the gill and the liver were approximately five and six times higher, respectively, following exposure to the IC20 Pd treatment compared to IC50 levels. All measured tissues took up measurable

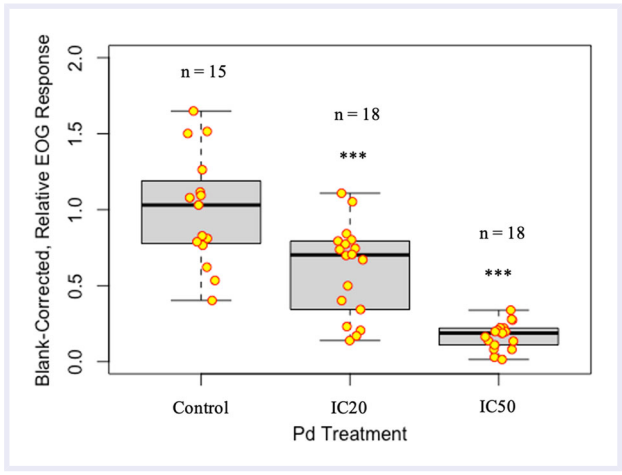

FIGURE 3 Effect of Pd at varying pH on rainbow trout olfaction shown as the relative EOG response to L-alanine following 96 h exposure. Asterisks show significant differences between Pd treatment groups and the control (***$p < 0.001$, IC20 = 2.5 μg/L, IC50 = 19.0 μg/L). The shaded box area represents the interquartile range, the line within the box represents the median, and the whiskers represent the minimum and maximum values. EOG, electro-olfactography; IC, inhibitory concentration; Pd, palladium

amounts of Pd. Moreover, the highest Pd CF was found in gills exposed to the IC20 Pd treatment (2207), while the lowest CF was found in livers exposed to the IC50 Pd treatment (13). Unlike the other two tissues, CF values within the olfactory rosette remained consistent between Pd treatment groups.

Oxidative stress

There was no significant change in MDA contents at any Pd treatment following protein concentration normalization (Figure 6).

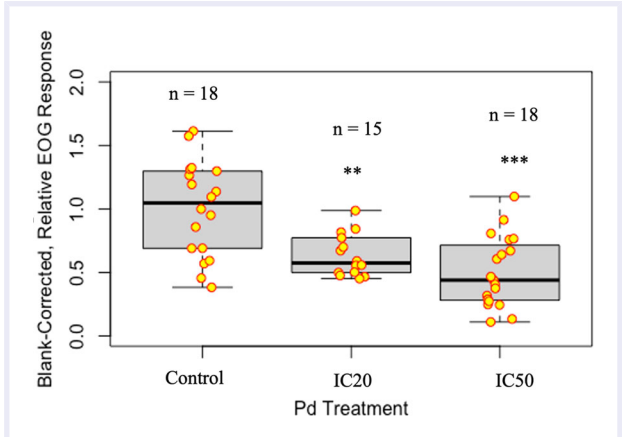

FIGURE 4 Effect of Pd at varying DOC concentrations on rainbow trout olfaction shown as a relative EOG response to L-alanine following 96 h exposure. Asterisks denote significant differences between Pd treatment groups and the control (**$p \leq 0.01$, ***$p < 0.001$, IC20 = 2.5 μg/L, IC50 = 19.0 μg/L). The shaded box area represents the interquartile range, the line within the box represents the median, and the whiskers represent the minimum and maximum values. DOC, dissolved organic carbon; EOG, electro-olfactography; IC, inhibitory concentration; Pd, palladium

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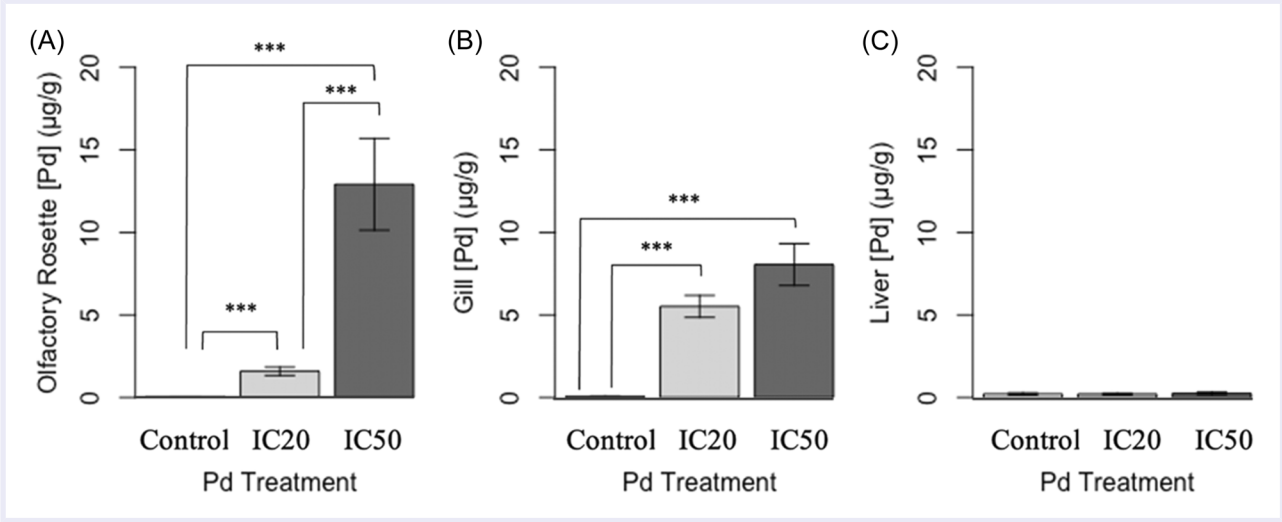

FIGURE 5 Palladium (Pd) bioaccumulation in (A) the olfactory rosette ($n = 7$) (B) the gill ($n = 21$), and (C) the liver ($n = 21$) of rainbow trout following 96 h waterborne exposure. Asterisks denote significant differences between Pd treatment groups (***$p \leq 0.001$, error bars ± 1 SEM). IC, inhibitory concentration; SEM, standard error of mean

DISCUSSION

Palladium analysis

Some of the published research on Pd toxicity reports nominal rather than measured exposure concentrations (Khangarot & Das, 2009; Zimmermann, Messerschmidt, et al., 2005). This practice can lead to misconceptions regarding the Pd concentrations required to elicit toxic effects, as the true concentrations are often much lower than those initially introduced into the system. This study demonstrates the importance of reporting measured values, as measured Pd in waterborne exposures were below nominal values as shown in Table 2. This observation corroborates previous studies that found measured Pd concentrations to be consistently lower than nominal concentrations despite tank and material conditioning prior to start of exposure (Zimmermann & Sures, 2018). Although the percent metal loss presented here (50%–75%) seems to be high, other laboratory experiments have consistently reported 75%–90% loss of Pd within 24 h (Cobelo-Garcia et al., 2007). Our water sampling revealed a significantly lower Pd concentration at 96 h when compared to 24 h for the 42.5 µg/L treatment shown in Table 2. Nonetheless, the mean measured concentrations between sampling times during the IC50 Pd treatment showed a fluctuation of only 3 µg/L.

TABLE 3 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	2207	424
Liver	85	13
Olfactory rosette	634	680

Abbreviations: CF, concentration factor; IC, inhibitory concentration; Pd, palladium.

The high percent loss of Pd in aquatic experiments is likely related to shifts in chemical speciation. The most common species of Pd present in freshwater environments at pH values explored in this study is $Pd(OH)_2$, which shows the greatest affinity for surfaces including glass, plastics, and silicone (Colombo, Oates, et al., 2008; 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 (Zereini & Wiseman, 2015), and yet no resolution has been provided at this time for maintaining Pd concentrations in solution.

Establishment of 96 h Pd-induced olfactory toxicity thresholds

Lack of reliable data is one reason why Pd toxicity in aquatic organisms remains largely unknown (Zereini & Alt, 2006). Use of IC*x* values 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 (Figure 1). 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 (method used to determine the appropriate concentration range of a substance to be tested

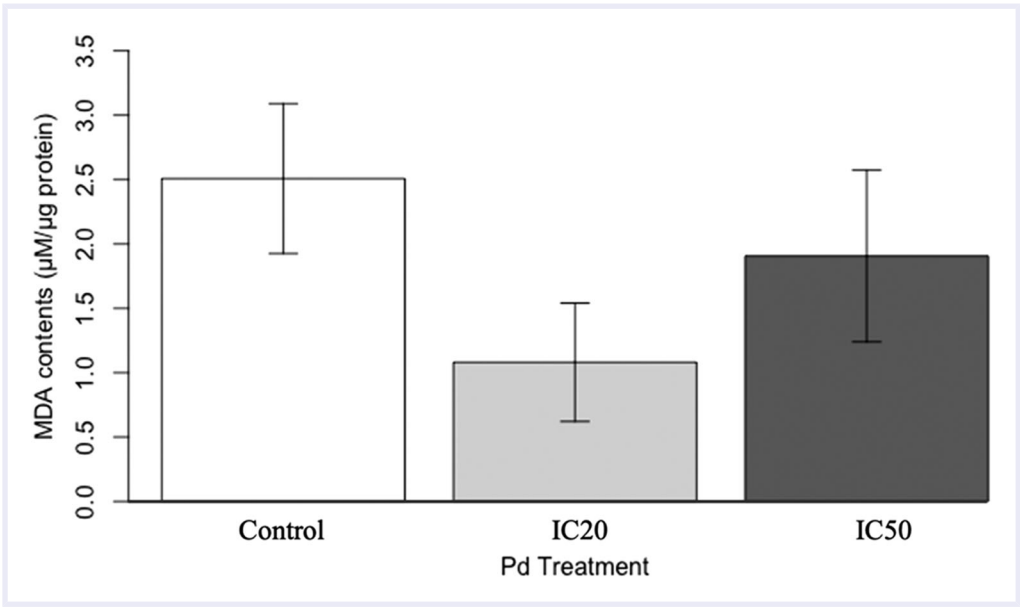

FIGURE 6 Investigation of cellular injury measured as lipid peroxidation from levels of malondialdehyde in the olfactory rosette ($p > 0.05$, $n = 6$, error bars ± 1 SEM). IC, inhibitory concentration; SEM, standard error of mean

in an experiment) data to align with other work that uses sublethal effective concentrations to inform development of protective guidelines. However, it should be noted that low-percentage effect concentrations (e.g., IC20 values) are preferred over IC50s in the development of water quality guidelines (Australian and New Zealand Guidelines, 2018; Canadian Council of Ministers of the Environment, 2007).

In contrast to some metals such as Cu (Razmara et al., 2019) and Ni (Dew et al., 2014), exposure of rainbow trout to Pd resulted in equal signal reduction in both OSN types assessed. Differential responses were not observed when TCA (which specifically stimulates ciliated OSNs) and L-alanine (which specifically stimulates microvillous OSNs) were administered at staggered intervals into the olfactory chamber of fish. Therefore, Pd can be classified as a broad OSN inhibitor as it does not specifically target a subset of OSNs. Our data highlight the environmentally relevant risk that Pd could pose. This study is a first step toward understanding Pd toxicity at a sublethal level in cold-freshwater fishes.

Selective influence of water hardness on alterations to Pd toxicity

The current study demonstrates minimal impacts of individual water quality parameters on the concentration thresholds to metal-induced olfactory impairment. Although the water quality variables that we tested provided no protection against Pd-induced olfactory toxicity, increasing water hardness caused additional olfactory dysfunction in rainbow trout. As seen with other metals in olfactory toxicity studies, the addition of Ca^{2+} and Mg^{2+} offered no protection from Pd-induced olfactory impairment. It is likely that Ca exerts its own inhibitory effect on fish olfaction in hard water conditions (Dew et al., 2012). In contrast to other metals, Pd toxicity may be lower in soft water than hard water. In fact, when compared against 62 other metals, Pd was the only metal in which *Hyalella azteca* lethality was lower for a water hardness of 18 mg/L compared to 124 mg/L as $CaCO_3$ (Borgmann et al., 2005). Although the chemical mechanisms underlying Pd behavior in varying water hardness conditions are unclear, the results shown in Figure 2 cannot solely be explained by the individual effects of Ca and Pd on the olfactory epithelium.

Most natural freshwater systems have a pH of 6–9 (Finlay & Bogard, 2022), with the optimal value for aquatic organisms ranging from pH 6.5–8 (Health Canada, 2015; USEPA, 2017). Our results present no evidence of an interaction between pH and Pd-induced olfactory toxicity, as shown in Figure 3. Although a small pH range was tested during this study, our results are corroborated by the chemical behavior of Pd in freshwater systems. Recent studies have concluded that dissolved Pd content is positively correlated with alkaline water conditions up to pH 8 (Liu et al., 2020). Further, Pd lipid transfer from the environment has been demonstrated in the pH range of 7–8, indicating Pd mobility to tissues in this pH range (Zimmermann et al., 2003). The effect of Pd-induced olfactory toxicity, as demonstrated in Figure 3, corroborates these findings. While metals in their ionic forms are more toxic to aquatic organisms due to increased presence of free ions, the ionic form of Pd does not dominate as a species until $pH < 2$, a range much too acidic to support rainbow trout (Colombo, Oates, et al., 2008). Thus, our finding of no mitigatory or worsening effect on 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; Kennedy et al., 2012; McGeer et al., 2002; Wood et al., 2011). Unlike research on other metals, our results failed to demonstrate amelioration of toxicity in the presence of DOC (Figure 4). This finding is

supported by Zimmermann et al. (2002), who observed that humic substances increase the solubility of Pd in water. In the same study, researchers concluded that the occurrence of Pd in a single oxidation state ($Pd(OH)_2$) may account for this particular metal having a greater propensity to bioaccumulate when organic compounds are present. This is the first study, to our knowledge, to manipulate humic acid as DOC to test for Pd interaction with fish olfaction. Our results show that the same pattern of trace metal toxicity observed from other metals in solution cannot be directly applied to understanding Pd toxicity. Thus, varying types of aromatic DOC matter should be explored to obtain a more accurate estimate of Pd toxicity in solution.

Bioaccumulation

Palladium accumulates in the olfactory rosette of fish exposed for 96 h to concentrations as low as 2.5 μg/L (Figure 5A). As the olfactory system is in direct contact with surrounding waters, Pd accumulation in olfactory tissue is unsurprising. Exposure studies using other trace metals have confirmed similar results (Razmara et al., 2021; Sloman et al., 2003); however, no other study, to our knowledge, has explored Pd bioaccumulation in olfactory tissue. While unable to provide a bioconcentration factor (as system equilibrium was not determined), calculated CF values support Pd accumulation within the target tissue (Table 3) (Petoumenou et al., 2015). Although the underlying molecular mechanisms have yet to be established for Pd-impaired olfaction, it is likely that membrane-associated Pd at the olfactory epithelium is at least partially responsible for the functional toxicity observed in rainbow trout.

Previous bioaccumulation studies have established Pd bioavailability to aquatic organisms (Ek et al., 2004; Zimmermann et al., 2002). Similar to the olfactory rosette, the gill surface is highly susceptible to metal binding, and the presence of target ligands in gills makes them an important focus 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 (Figure 5B). The EU Registration, Evaluation, Authorisation and Restriction of Chemicals (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). Based on these guidelines, our results indicate that Pd accumulates in rainbow trout gills ($CF = 2207$) (Table 3). Although dissimilar in experiment duration, species, and Pd addition, Sures et al. (2005) reported a gill CF of >2000, thereby providing additional support for the moderately bioaccumulative nature of Pd. Our study demonstrates that 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, 2005; Zimmermann, Von Bohlen, 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 nonsignificant Pd accumulation at the liver compared to control fish, as shown in Figure 5C, 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, Von Bohlen, et al., 2005), mollusks (Sures & Zimmermann, 2007), and *B. barbus* (Sures et al., 2005), all of which accumulated significant Pd over a range of 28–42 days exposure. 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 (Fortin et al., 2011; Odiyo et al., 2005). Our approach recognizes that short-term bioaccumulation is an important parameter to consider in understanding the effects of Pd on aquatic organisms.

Oxidative stress

To explore the mechanism of Pd toxicity in rainbow trout olfaction, it is crucial to understand the role, if any, that reactive oxygen species formation and oxidative stress play. Currently, no available data exist on oxidative stress response in fishes following Pd exposure. However, nano-Pd 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 Figure 6. It is likely that the protection offered by antioxidant defenses at the peripheral olfactory system was sufficient to block LPO production (Tilton et al., 2008). 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 result in reduced olfactory acuity. However, it remains unknown if the olfactory system can recover following acute Pd exposure.

Besides 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 our findings, Razmara et al. (2021) ruled out oxidative stress as a mechanism for olfactory dysfunction after acute waterborne Cu exposure. However, in the same study, transcriptional regulation of neuroregeneration and immune system pathways were demonstrated to change following trace metal exposure. Evaluation of secondary messengers involved in olfactory signal transduction pathways was also shown to explain olfactory reduction in salmonids following acute Cu exposures (Wang et al., 2013). Although beyond the scope of this study, bioinformatics, gene expression, and transcriptomics (reviewed in Tierney et al., 2010) are informative molecular and biochemical indicators of olfactory toxicity

and should be applied in future Pd toxicity studies to elucidate the mechanism of olfactory impairment in fishes.

CONCLUSION

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 that acute Pd exposure significantly reduces rainbow trout olfaction, the mechanism of action is yet to be defined. It is clear from our study that Pd is bioavailable to exterior-facing tissues such as the olfactory rosette and gills, as Pd was shown to interact with both membrane types during the 96 h exposure. Olfactory impairment can influence foraging, mating, and homing behaviors, potentially resulting in population-level effects. Notably, we observed no significant effects of pH and DOC on fish olfaction. Further, of the water quality parameters tested, water hardness was the only one that additionally impacted fish olfaction. This study demonstrated limited findings for the effects of water quality on Pd-induced olfactory toxicity in fish. Nonetheless, water quality plays a vital role in accurate risk assessment and guideline development for the protection of aquatic life as observed in other trace metal toxicity studies. Therefore, water quality should be considered in the evaluation of Pd toxicity as many additional parameters remain untested. Our study contributes to the evaluation of Pd toxicity in fish and highlights the importance of continued research into this study area.

AUTHOR CONTRIBUTION

Carolyn Simonis: Conceptualization; data curation; investigation; methodology; writing—original draft; writing—review and editing. **Lauren Zink**: Conceptualization; investigation; methodology; writing—review and editing. **Sarah E. Johnston**: Investigation; writing—review and editing. **Matthew Bogard**: Resources; writing—review and editing. **Gregory G. Pyle**: Conceptualization; writing—review and editing.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data, associated metadata, and calculation tools are available upon request from corresponding author Lauren Zink (zink@uleth.ca).

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