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A FTIRM study of the interactive effects of metals (zinc, copper and cadmium) in binary mixtures on the biochemical constituents of the gills in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

We employed Fourier Transform Infrared Microspectroscopy to examine, in situ, the effects of waterborne Cu, Cd and Zn, alone and in binary mixtures, during acute exposure on the integrity of major lipid and protein constituents of the gill of a model teleost species, rainbow trout (*Oncorhynchus mykiss*). Our findings demonstrated that acute exposure to metals, both individually and in binary mixture, resulted in the degradations of various components of proteins and lipids in the gill tissue. Generally, when comparing the effects of individual metals, Cu was found to induce the maximum adverse effects followed by Cd and Zn, respectively. Among the binary metal-mixture combinations, Cu and Cd produced additive effects on the degradation of major proteins and lipid moieties, whereas the co-exposure of Zn with Cd or Cu elicited ameliorative effects, indicating antagonistic (less than additive) interactions between Zn and Cd or Cu in the rainbow trout gill. Overall, the present study demonstrates that FTIRM can be a useful tool to gain novel mechanistic insights into the biochemical changes induced by metals in the fish gill, which could influence the overall toxicity of metals to fish.

1. Introduction

The gill is an important multifunctional organ in fish. It performs several important physiological functions such as gaseous exchange, acid-base balance, nitrogenous waste excretion, and osmoregulation. In addition, the gill is also the site of toxic action of pollutants in fish, including metals. Since fish gill is in continuous contact with water contaminated with metals, it is often the first organ to respond to metal exposure (Wood, 2001; McDoanld and Wood, 1993). Exposure of fish to waterborne metals at elevated concentrations can induce structural changes in the gill. The structural changes occur mainly as various types of pathophysiological responses such as lamellar fusion, edematous swelling, necrosis, hypertrophy and hyperplasia. These adverse responses may lead to respiratory and osmoregulatory malfunctions, and may ultimately cause fish death (Wendelaar Bonga and Lock, 1992; Lauren, 1991; Mallatt, 1985). Apart from these pathophysiological effects, waterborne metal exposure can also result in changes in the biochemical and molecular composition of the biomolecules such as proteins and lipids, which are all essential components of all biological systems in the gill tissue of fish.

Zinc (Zn), cadmium (Cd) and copper (Cu) often co-occur in many metal-impacted aquatic environments (ATSDR, 2004; UNEP, 2010). All of these metals cause acute toxicity in fish mainly by disrupting ion transport pathways in the gill, which eventually causes loss of ionic balance in the body. For example, Zn and Cd are known to share uptake routes in the fish gill (Ca transport pathways), and the acute toxicity of both of these metals to fish occurs as a result of the disruption of Ca homeostasis (Hogstrand et al., 1996; Niyogi and Wood, 2004). In contrast, the uptake of Cu in the fish gill is known to occur mainly via Na transport pathways, and thus the acute toxicity of Cu to fish has been attributed to the disruption of Na homeostasis (Grosell and Wood, 2002). Numerous studies have investigated the toxic effects of each of these metals in fish, however information regarding their interactive effects in mixtures is relatively limited. In fact, most studies on the interactions of these metals in fish have been based on metal uptake and/or accumulation as endpoints (Kamunde and Macphail, 2011; Komjarova and Blust, 2009; Niyogi et al., 2015). Currently, very little is known about the underlying mechanisms of interactions of metals in

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mixture, by which they may elicit antagonistic or synergistic toxicity to fish.

Over the last few years, Fourier infrared transform spectroscopy (FTIR) has emerged as a viable technique to examine the biochemical changes induced in various biological organisms as a result of contaminant exposure. FTIR utilizes the absorption of infrared radiation by a biological sample to evaluate changes in organic macromolecules. This is made possible because each biomolecule vibrates at a characteristic frequency or wavelength. The changes in the peak and absorption intensities of these biomolecules can thus be used to characterize biochemical and molecular changes in samples (Marcelli et al., 2012). FTIR is a sensitive and non-invasive technique that allows the examination of tissues in their natural state with little or no chemical pre-treatment (Davis and Mauer, 2010). To date, the studies that have utilized FTIR to study metal-induced biochemical changes in fish gills have essentially based on single metal exposure such as Zn and As (Palaniappan et al., 2010; Palaniappan and Vijayasundaram, 2009). However, in the natural environment, metals do not exist singly, but rather as complex mixtures. The interactions of metals in mixture could influence their uptake, as well as their internal handling, resulting in less-than-additive, or more-than-additive toxicity in exposed organisms (Daka and Hawkins, 2006). At present, there is a general lack of knowledge on the interactive effects of metals in mixtures on the biochemical components (e.g., proteins and lipids) of vital organs of the fish such as the gill. In addition, the previous studies (Palaniappan et al., 2010; Palaniappan and Vijayasundaram, 2009) that focused on the effects of metals on the composition of biomolecules in fish gill were conducted using bulk FTIR spectroscopy. This approach provided valuable information on biochemical effects of metals in tissues, but it is not particularly useful in providing spatially resolved information on the changes in the distribution of these functional groups following metal exposure.

One of the modern tools used for the site-specific risk assessment of metals in the aquatic environment is the biotic ligand model (BLM) (Di Toro et al., 2001; Niyogi and Wood, 2004; Paquin et al., 2000). The underlying assumption of this model is that toxicity in aquatic organisms occurs as a result of metal accumulation at the physiological site of toxic action (i.e. the biotic ligand). In fish, the gill acts as the biotic ligand, and the BLM assumes that the short-term (3–24 h) critical metal accumulation at the gill results in toxicity to fish (Niyogi and Wood, 2004). The BLM has been successfully employed by United States Environmental Protection Agency (USEPA) and is currently being evaluated by European Union (EU) to derive water quality criteria for single metals such as Cu, Zn and Ni (Rüdel et al., 2015; USEPA, 2007). Since metals often co-exist in the natural environment including freshwater systems, there has been a growing interest in further developing the BLM approach for predicting acute toxicity of metals in mixture to freshwater fish (Balistrieri and Mebane, 2014; Santore and Ryan, 2015). This would ultimately provide a more holistic protection of the aquatic life against metal pollution. With that in mind, the main aim of this research, therefore, was to use FTIR coupled with microscopy (FTIRM) to enhance our understanding of the toxic interactions of metals in mixture in fish gills following short-term exposure (24 h) to acute concentrations (96 h LC_{50s}) of Zn, Cd and Cu. Specifically, the objectives of this research were two-fold: (i) to characterize the effects on vital macromolecules (proteins and lipids) in the gills of rainbow trout (*Oncorhynchus mykiss*) exposed to acute waterborne Zn, Cd or Cu concentrations, singly and in binary mixtures, and (ii) also to examine the relative changes in the spatial distribution of proteins and lipids in the gills of *O. mykiss* under the same exposure conditions described in objective (i). We hypothesized that since the uptake and acute toxicity of waterborne Zn and Cd occur mainly via common pathways in fish (Niyogi and Wood, 2004), the mixture of Zn and Cd would elicit less than additive effects in the gill. Similarly, we also assumed that since the mechanisms of Cu uptake and acute toxicity in fish differ to that of Zn or Cd (Niyogi and Wood, 2004), the mixture of Zn and Cu, or Cu and Cd would produce additive or more than additive effects in the fish gill.

2. Materials and methods

The experimental protocol described in this study was in compliance with the Canadian Council for Animal Care Guidelines and was approved by the Animal Care Committee of the University of Saskatchewan.

2.1. Animal model

Juvenile rainbow trout (*O. mykiss*) weighing approximately 100 g were purchased from the Lucky Lake Fish Farm (Saskatoon, Saskatchewan) and reared at the Aquatic Toxicology Research Facility (ATRF) of the University of Saskatchewan. A photoperiod cycle of 16 h light:8 h dark and a water temperature of 12 ± 1 °C were employed for fish maintenance. Fish were fed with commercial diets once daily at a ratio of 2% body weight. The measured Zn, Cu and Cd concentrations in the fish diet were 124.4 ± 7.8 μg/g dry weight, 8.58 ± 2.7 μg/g dry weight, and 0.5 ± 0.1 μg/g dry weight, respectively ($n = 3$ for each measurement). Prior to the beginning of the experiment, the fish were acclimated to the exposure water (dechlorinated Saskatoon City tap water; $Ca^{2+} = 44$, $Mg^{2+} = 18$, $Na^{+} = 26$, $K^{+} = 3$, $Cl^{-} = 11$, $SO_4^{2-} = 50$, hardness = 160, alkalinity = 110 (both as $CaCO_3$), dissolved organic carbon (DOC) = 2.5 (all in mg/L), pH = 7.9) (Driessnack et al., 2016) for 21 d. All the fish used in the experiments were fasted for 24 h prior to their use in the experiment and also during the experimental period to prevent the deterioration of water quality through the degradation of fish diet and waste products.

2.2. Experimental procedure

The experimental treatments consisted of seven groups: (i) control (no added metals in the exposure water), (ii) Zn (1 mg/L), (iii) Cu (100 μg/L), (iv) Cd (20 μg/L), (v) Zn (1 mg/L) and Cd (20 μg/L), (vi) Zn (1 mg/L) and Cu (100 μg/L), and (vii) Cu (100 μg/L) and Cd (20 μg/L). Fish were exposed individually to nominal concentrations of waterborne metals for 24 h in 20 L of exposure water under static non-renewal exposure condition. Each experimental exposure was replicated three times. The exposure chambers were kept in a temperature-controlled water bath to maintain the temperature of the exposure water at 12 °C and fish were constantly supplied aeration throughout the exposure period. Metal concentrations were added as metallic salts of $ZnSO_4{\cdot}7H_20$, $CdNO_3{\cdot}4H_2O$ and $Cu(NO_3)_2{\cdot}5H_20$ (all purchased from Sigma-Aldrich, MO, USA) for Zn, Cd and Cu respectively. For this study, the concentrations of metals employed are similar to the 96 h median lethal concentration (LC_{50}) of each individual metal in rainbow trout, as reported by Alsop et al. (1999), Taylor et al. (2000), and Niyogi et al. (2008) under similar water chemistry conditions as used in the present study. Water samples were collected at the beginning and at the end of the exposure. They were filtered using a 0.45 μm nylon syringe filter (Nalgene, NY, USA), acidified with 0.2% HNO_3 (Trace metal grade; VWR, ON, Canada) and subsequently stored at 4 °C until analysis.

The dissolved metal concentrations for the exposure were verified using a graphite furnace atomic absorption spectrometer (AAnalyst 800, Perkin Elmer, CT, USA). The measured concentrations of each metal in the exposure waters were within ± 10% of the nominal concentrations (data not shown). At the end of the exposure period, fish were euthanized with an overdose of neutralized Tricaine Methanesulfonate (MS-222; Syndel Laboratories Ltd., Canada) and gill baskets were dissected out, rinsed thoroughly in deionized water for 10 s, and blotted dry. Gill tissues were thereafter placed in tissue cassettes, fixed in 10% neutral buffered formalin (VWR, ON, Canada) for 24 h. Following fixation, gill tissues were cryoprotected in 30% sucrose solution for 48 h. The tissues were then placed in cryomolds and flash frozen in optimal cutting temperature (OCT) medium (Fisher Scientific,