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Interactions of waterborne and dietborne Pb in rainbow trout, *Oncorhynchus mykiss*: Bioaccumulation, physiological responses, and chronic toxicity

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ABSTRACT

In Pb-contaminated environments, simultaneous exposure to both waterborne and dietborne Pb is likely to occur. This study examined the potential interactive effects of these two pathways in juvenile rainbow trout that were exposed to Pb in the water alone, in the diet alone, and in combination for 7 weeks. The highest waterborne Pb concentration tested (110 $\mu g\,L^{-1}$) was approximately equivalent to the 7-week LC20 (97 $\mu g\,L^{-1}$) measured in a separate trial, while the lowest was a concentration often measured in contaminated environments (8.5 $\mu g\,L^{-1}$). The live diet (10% daily ration on a wet mass basis) consisted of oligochaete worms (*Lumbriculus variegatus*) pre-exposed for 28 days to the same waterborne Pb concentration, and the highest dietary dosing rate to the trout was 12.6 μg Pb g $fish^{-1}$ day^{-1}. With waterborne exposure, whole body Pb burden increased to a greater extent in the worms than in the fish. Nonetheless, in trout waterborne exposure still resulted in 20–60-fold greater Pb accumulation compared to dietborne Pb exposure. However, combined exposure to both waterborne and dietborne Pb reduced the whole body accumulation extensively at waterborne Pb > 50 $\mu g\,L^{-1}$, with similar antagonistic interaction in liver and carcass (but not gill or gut) at a lower threshold of 20 $\mu g\,L^{-1}$. Growth effects in trout were minimal with marginal reductions in the dietborne and combined exposures seen only at 110 $\mu g\,L^{-1}$. Chronic Pb exposure reduced lipid and carbohydrates level in the worms by 50% and 80% respectively, while protein was unchanged, so growth effects in trout may have been of indirect origin. After 7 weeks, Ca^{2+} homeostasis in the trout was unaffected, but there were impacts on Na^+. Blood Na^+ was reduced in waterborne and dietborne exposures, while gut Na^+/K^+ ATPase activities were reduced in waterborne and combined exposures. This study is the first, to our knowledge to examine the interaction of waterborne and dietborne Pb exposure in fish. While physiological impacts of Pb were observed in both worms and fish, higher concentrations of dietborne Pb actually protected fish from waterborne Pb bioaccumulation and these effects. The impacts of metals on diet quality should not be neglected in future dietborne toxicity studies using live prey.

1. Introduction

Fish can accumulate Pb from the water and diet (Crespo et al., 1986; Rogers et al., 2003; Rogers et al., 2005; Alves and Wood, 2006). Mager (2012) has provided a comprehensive review of the pathophysiology of Pb exposure in fish. Acute waterborne Pb exposures produced ionoregulatory effects by significantly reducing Ca^{2+} influx and Ca^{2+}ATPase activity in rainbow trout (Rogers and Wood, 2004). In addition, Na^+ and Cl^- uptake and branchial Na^+/K^+ ATPase (NKA) activity were inhibited (Rogers et al., 2005). However, neurotoxicological (Mager et al., 2010; Rademacher et al., 2005) and hematological effects (Dawson, 1935; Hodson et al., 1977) are usually the primary mechanisms of action in the chronic exposure. Rainbow trout exposed to Pb at13 $\mu g\,L^{-1}$ for 32 weeks exhibited significant increases in red blood cell numbers, decreases in red blood cell volumes, and d-aminolevulinic acid dehydratase activity, an enzyme that catalyses the formation of porphobilinogen from aminolevulinic acid in the heme synthesis pathway (Hodson et al., 1977). Therefore, fish exposed chronically to waterborne Pb may

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be at risk of anemia (Dawson, 1935). Prolonged Pb exposure can also produce adverse effects on pituitary function, gonadosomatic index, and oocyte growth (Ruby et al., 2000), and cause neurological disorders, and scoliosis (Holcombe et al., 1976) in freshwater fish.

Metal toxicity may also occur via the gastrointestinal tract as dietary sources for fish may become contaminated from polluted water and/or sediments. Meyer et al. (2005) and DeForest and Meyer (2015) have provided detailed reviews of dietary metal toxicity to aquatic organisms. Crespo et al. (1986) demonstrated morphological alterations of the intestinal brush border of rainbow trout that were orally administered with Pb in an artificial diet (10 μg Pb g fish^{-1} day^{-1}). These were associated with an impairment of intestinal Na^+ and Cl^- absorption. Fish fed natural diets containing metals (As, Cd, Cu, Pb, and Zn) from contaminated sites have reduced feeding activity, growth, and survival rates (Farag et al., 1994; Woodward et al., 1994; Woodward et al., 1995). While this could reflect the impact of any or all of the metals in the diet, it is notable that trout fed Pb-contaminated artificial diet (commercial pellet food spiked with Pb nitrate) up to 520 μg g diet^{-1} at a daily 2.5% ration (i.e. 13 μg Pb g fish^{-1} day^{-1}) did not have the same chronic effects (Alves et al., 2006). This phenomenon was similarly observed for another metal, in which up to 500 μg Cd g^{-1} artificial diet at a daily 2% ration (i.e. 10 μg Cd g fish^{-1} day^{-1}) produced no effects on growth in trout after exposure for 4 weeks (Franklin et al., 2005). However, when oligochaete worms (*Lumbriculus variegatus*) at much lower Cd concentrations (up to 30 μg g diet^{-1}) were used as the dietary source at a daily 3.5% ration (i.e. only 1.05 μg Cd g fish^{-1} day^{-1}), a significant growth reduction of 50% was observed (Ng and Wood, 2008). The contradiction in effects was possibly caused by the difference in metal binding between natural and artificial food, thus affecting the assimilation or absorption of metals by the predator (Harrison and Curtis, 1992). It is also possible that the presence of higher calcium in the pellet food than in the worms protects against the toxicity of Pb and Cd (Franklin et al., 2005; Alves and Wood, 2006). In view of this, natural diets should be used in dietborne studies, not only to simulate the natural conditions, but also to provide early warning of toxic effects for metal risk assessment. However, the impact of metal exposure on the nutrient content of the prey has not always been taken into consideration in previous metal studies. This question will be addressed in the present study.

Under field conditions, aquatic animals can be exposed to metals from both routes of exposure (waterborne + dietborne). In recent years, there has been a growing interest in the toxicity of waterborne mixtures in the regulatory perspective (e.g. Mebane et al., 2012; Alsop and Wood, 2013; Clemow and Wilkie, 2015; Van Genderen et al., 2015; Niyogi et al., 2015). However, to our knowledge, studies on the interaction of exposure pathways are rare, especially for non-essential metals such as Pb. For essential metals, there is some evidence of a homeostatic interaction between the two routes of uptake. For example, in trout, waterborne Cu uptake contributed the majority of the body's Cu accumulation under Cu-deficient conditions while dietary Cu contributed more at high dietary levels of Cu (Kamunde et al., 2002). Furthermore, pre-exposure to waterborne Ni down-regulated the gastrointestinal uptake of dietary Ni into the plasma and red blood cells (Chowdhury et al., 2008). There was also interaction of waterborne and dietborne toxicity of Zn in *Daphnia* (Evens et al., 2012); the contribution of dietary Zn to reproductive inhibition gradually decreased with increasing waterborne Zn concentrations. To understand the ecological and physiological effects under field conditions, where simultaneous exposure to both waterborne and dietary Pb is likely to occur, it is crucial to study if there is interaction of the two exposure routes with respect to Pb accumulation and toxicity. If such interactions occur, they should be taken into account in regulatory frameworks such as the Biotic Ligand Model (BLM) (Paquin et al., 2002; Niyogi and Wood, 2004).

A recent review (DeForest and Meyer, 2015) noted that "exposure of test organisms to matched water-borne and diet-borne metal concentrations is perhaps the most relevant for evaluating the protectiveness of water-borne metal guidelines" but that this had rarely been done. In the present study, this approach was adopted. We employed the oligochaete *Lumbriculus variegatus* as a live food source for juvenile rainbow trout. These worms have been used successfully in a number of dietary toxicity studies (Hansen et al., 2004; Mount et al., 2006; Ng and Wood, 2008; Erickson et al., 2010). In the wild, they may obtain contaminants from water or sediment (Hansen et al., 2004; Erickson et al., 2010), and therefore may be a potential source of dietary Pb to their natural predators.

To understand possible interactive effects on sublethal toxicity, bioaccumulation, and physiology, juvenile rainbow trout were exposed to Pb at a range of concentrations in the water alone, in the diet alone, and in combination for a period of 7 weeks. In the former, the trout were fed uncontaminated worms. In the latter two tests, worms that had been exposed for 28 days to the relevant waterborne Pb concentration (i.e. the same as in the water to which the fish were exposed) were used as the diet. Survival, growth, tissue Pb burdens, enzymatic activities, and ion concentrations in the blood and whole body were employed as endpoints. Nutrient concentrations in the worms were also examined to look at possible indirect effects of the Pb-exposed diet on growth. This study is the first, to our knowledge to examine the interaction of waterborne and dietborne Pb exposure in fish, and the first to simulate field conditions of matching exposures where the predator and prey both experience the same waterborne Pb concentration. As such, it will provide an insight into risk assessment of non-essential metals when animals are simultaneously exposed via the waterborne and dietborne pathways.

2. Material and methods

2.1. Experimental organisms

2.1.1. Rainbow trout

Juvenile rainbow trout (*Oncorhynchus mykiss*, average size = 2–4 g) were obtained from the Humber Springs Trout Hatchery (Orangeville, ON, Canada). They were acclimated to 13 °C in dechlorinated City of Hamilton tap water from Lake Ontario [pH 7.9, DOC: 2.5 mg L^{-1}, Na^+: 0.7 mM, Ca^{2+}: 1.0 mM, water hardness: 140 mg L^{-1} as $CaCO_3$, Pb: below detection limit (0.3 μg L^{-1})], in flow-through holding tanks for 2 weeks before experiments started. The tanks were aerated and illuminated under a12 h light:12 h dark photoperiod. Fish were fed commercial salmon fry pellets (Nelson's Silver Cup fish feed, Murray, Utah, U.S.A.) at a body ration of 1–2% daily during acclimation. Procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the McMaster University Animal Research Ethics Board (AUP 09-04-10).

2.1.2. Oligochaete worms

Lumbriculus variegatus were purchased from Aquatic Foods (Fresno, CA, U.S.A.) and acclimated in a holding tank of flow-through dechlorinated City of Hamilton tap water (the same water chemistry as above). The tank was aerated and subjected to the same photoperiod and ambient conditions as for the fish. The worms were fed Nutrafin® MAX tropical fish flakes (Rolf C. Hagen Inc., Baie d'Urfé, QC, Canada) twice every week.

2.2. Pb exposure

2.2.1. Experiment 1: waterborne exposure—LC/EC value determination and effect on growth in rainbow trout

This experiment served as a range-finder to detect the potential sublethal effects of waterborne Pb by determining the effective concentration (EC) on growth of rainbow trout over 7 weeks. An acute lethal concentration (96-LC50) for Pb in juvenile rainbow trout (1.04 mg Pb L^{-1}; Rogers et al., 2003) was previously determined in the same water chemistry and was used as a guide in the selection of Pb concentrations. The nominal Pb concentrations in the treatments were 0 (control), 4, 10, 20, 80, 240 and 800 μg L^{-1}. Pb concentrations were achieved through dripping of a concentrated stock (as $Pb(NO_3)_2$, acidified with 0.02% HNO_3) from a Mariotte bottle at a rate of 1 ml min^{-1} into the header tank that simultaneously received water from a large flowing-water tank at 1 l min^{-1}. Fish were held in black 20-L tanks receiving 250 ml min^{-1} fresh water from the header tanks. Each treatment consisted of duplicate tanks with 10 fish per tank (20 fish per treatment, average starting weight of 4 g). Fish tanks were aerated and each contained two pieces of PVC pipe (10 cm long, 6 cm in diameter) for shelters. The pH in all the control and exposed tanks remained at 7.8–8.0 throughout the experiment. Fish were fed live, unexposed *L. variegatus* at a ration of 10% body weight per day in a single meal. In all treatments and at all times the worms were completely and rapidly consumed by the fish within 2–3 min. Feces were siphoned from the tanks daily. The quantity of worms was adjusted daily if mortalities occurred. Individual fish weights were measured weekly for determination of the specific growth rates and adjustments of the feeding rations. To measure individual fish weight, a tank of fish was gently poured into a dark bucket with a lid. Subsequently, fish were netted one by one, gently blotted to remove excess water and placed in a beaker on a scale. After the weight was recorded, the fish were transferred back to the original housing tank. The total time of air exposure was approximately 5 s. The bucket and beaker were cleaned between tanks. The experiment lasted for 7 weeks.

Specific growth rate (SGR, % body weight change per day) of the trout was calculated using the following formula:

$$SGR(\%d^{-1}) = 100\% \times [(LnW_2 - LnW_1)/(T)]$$

where W_2 and W_1 are average weights of fish in each tank at the start and end of each growth period respectively. T is the interval of time in days. Biomass was also calculated in the standard fashion for regulatory science (USEPA, 2008; Ng et al., 2010) from the total weight of fish at the end of exposure divided by the number of fish at the start of the experiment.

2.2.2. Waterborne exposure to worms—a dietary source of Pb for trout

A preliminary waterborne Pb exposure was performed to determine the time course of Pb accumulation in *L. variegatus*. Worms were exposed to waterborne Pb alongside the flow-through system used in the waterborne fish exposure described above (Section 2.2.1). Approximately 10 g of worms were placed into a 500-ml tank (duplicate tanks per each concentration) which received 50 mL min^{-1} of fresh water with Pb at nominal concentrations of 0, 4, 10, 20, 80, 240 and 800 μg L^{-1}. The worms were fed crushed fish flakes twice per week during the 7-week exposure.

Since our preliminary result showed that worms did not attain a stable level of Pb even at the highest exposure concentration after 7 weeks (Supplementary Fig. S1), a second exposure was conducted for 28 days only, which is a period used in standard testing protocols for freshwater invertebrates by the U.S. EPA (EPA, 1994). This exposure was used to generate the Pb-loaded worms to be used in weeks 1–4. Experimental conditions were similar to those in the first exposure but the nominal Pb concentrations were 0, 8.5, 20, 60 and 110 μg L^{-1}. These were the same concentrations that the fish would experience in the waterborne/dietborne Pb interaction Experiment 2 (Section 2.2.3). After 28 d, the worms were transferred to a 5 °C cold room in static tanks (with aeration and the same waterborne Pb concentration) for storage because our preliminary results showed that relocation to a cold room maintained a constant whole body Pb burden in the worms for at least 4 weeks and it did not affect their survival. Worms were not fed once transferred to the cold room. During the storage period, worms were sampled once per week (3 pools of 5–10 worms) to monitor whole body Pb burden. Worms stored in the cold room were used as the Pb-contaminated diet for the waterborne/dietborne Pb interaction study (Experiment 2) from weeks 1–4. They were rinsed with clean water before feeding to the fish. A second batch of worms was exposed along with the fish at the start of interaction study (Experiment 2), in order to prepare Pb-loaded worms for the fish diet from weeks 5–7. The exposure conditions were the same, but worm tanks received water directly from the outflows of the fish tanks, so the worms were exposed to the same concentrations as those for the fish. Again, at 28 d, these worms were relocated to the cold room for storage and prepared as the fish food for weeks 5–7. Remaining worms were rinsed, sampled and stored for nutrient analyses.

2.2.3. Experiment 2: waterborne/dietborne Pb interaction exposure in rainbow trout

The waterborne Pb concentrations for the 7-week waterborne-dietary interaction experiment were 0, 8.5, 20, 60 and 110 μg L^{-1}. The lowest Pb exposure concentration of 8.5 μg L^{-1} was chosen based on the environmentally realistic levels observed in aquatic monitoring data (Demayo et al., 1982; Mager, 2012), and is slightly above chronic criteria values in several jurisdictions (EPA, 1980; Environment Canada, 2007). The other treatments were based on the results of Experiment 1 (the waterborne trout exposure described in Section 2.1). Therefore 20 μg L^{-1} approximated the 7-week LC_{01}, 60 μg L^{-1} approximated the 7-week LC_{10}, whereas the highest waterborne Pb level, 110 μg L^{-1}, approximated the 7-week LC_{20}.

The experiment consisted of three exposure scenarios. Rainbow trout were exposed to either: (1) waterborne Pb at 0. 8.5, 20, 60, or 110 μg L^{-1}; (2) dietary Pb in the form of worms pre-exposed for 28 d to waterborne Pb at 0, 8.5, 20, 60, or 110 μg L^{-1}; (3) both waterborne and dietborne Pb (called combined exposure hereafter) at 0, 8.5, 20, 60, or 110 μg L^{-1}. The fish in the control or waterborne only treatment were fed clean/unexposed worms whereas fish in the dietborne only or combined exposures were fed Pb-contaminated worms which had been pre-exposed for 28 days to the same waterborne Pb concentration as the trout. For example, fish exposed to 20 μg Pb L^{-1} were fed worms that had also been pre-exposed to 20 μg Pb L^{-1}. All three treatment groups were fed 10% weight ration each day as a single meal. For this experiment, each treatment consisted of duplicate tanks housing 12 fish per tank (24 fish per treatment) with an average starting weight of 2 g.

Survival was recorded daily and individual weights of the fish were determined weekly. The fish weighing regimen was the same as previously described in Section 2.2.1. All fish was euthanized in neutralized 0.25 g L^{-1} MS-222 (tricaine methanesulfonate, Syndel Laboratories, Nanaimo, BC, Canada) at the end of the 7-week exposure. The fish were rinsed in clean water, then whole blood, gills, liver, kidney and carcass were collected and stored at −80 °C for later analysis.

2.2.4. Water sampling, tissue digestion and metal analysis

Water samples were taken throughout the exposures, 2–3 times during the first week, then 1–2 times per week during the remain-

der of the experiment. Sampling times ranged from 2 h to 8 h post-feeding. Both total Pb and dissolved Pb fractions were measured. Dissolved Pb was obtained by passing the water samples through an Acrodisk 0.45 μm in-line-syringe-tip filter (Pall Canada Ltd., Saint-Laurent, QC, Canada). All samples were acidified to 1% with trace metal grade HNO_3 immediately after collection and analyzed along with the certified reference materials (TM-24.3, TM-25.3; National Water Research Institute, Environment Canada, Burlington, ON, Canada) using a Graphite Furnace Atomic Absorption Spectrometer (GFAAS, Varian Spectra AA-20 with graphite tube atomizer [GTA-110], Varian Instruments, Mulgrave, Australia). Pb agreement was always within 20%. The detection limit (0.3 $\mu g\,L^{-1}$) was determined by using multiple standards in the low range and examining the standard deviation of the lowest concentration of the calibration line relative to zero.

Dissolved Na^+, Ca^{2+} and Mg^{2+} levels in the water were also analysed after acidification and 1% lanthanum chloride (w/v) was added for Ca^{2+} and Mg^{2+} analyses. They were measured using a Flame Atomic Absorption Spectrometer (FAAS, Varian Spectra-220FS, Varian Instruments, Mulgrave, Australia). Water samples were also collected through 0.45-μm filters into 40-ml pre-washed clear closed-top VOA vials (Unid International Technology Inc., Coquitlam, BC, Canada) and acidified to 1% HNO_3 for dissolved organic carbon (DOC) measurements. DOC was analysed on a total organic carbon (TOC) analyzer (Shimadzu TOC-VCPH, Mandel Scientific Company Inc., Guelph, ON, Canada). Standards were made from certified potassium hydrogen phthalate (Nacalai Tesque Inc., Kyoto, Japan). Before analysis, the TOC analyzer sparged all samples for 3 min to remove inorganic carbon. A dissolved organic carbon standard was also checked every 20 samples.

Fish tissue/blood and whole body of *Lumbriculus* were digested in 1 N and 8 N HNO_3 respectively at 60 °C for 48 h, diluted with de-ionized water, and assayed for Pb accumulation on the GFASS, using the same certified reference materials as described earlier. As all tissues were harvested, weighed, and analyzed for trout, whole body Pb accumulation could be calculated. Acidified fish tissues were also measured for Ca^{2+} (carcass) and Na^+ levels (blood), and worm tissues for both ions, on the FAAS.

2.2.5. Enzyme activities in the fish gill and gut

2.2.5.1. Na^+/K^+ ATPase (NKA) activity. Samples of gill or gut (n = 4-10 per treatment concentration) were thawed immediately prior to assay and kept on ice throughout. About 500–800 μl homogenization buffer (0.1% w/v sodium deoxycholate, 150 mM sucrose, 10 mM EDTA and 50 mM imidazole) was added to each sample (gut average weight: 0.1 g; gill average weight: 0.04 g). It was then homogenized and supernatant was collected for analysis after centrifugation at 1200 g for 3 min at 4 °C. The assay method was modified from McCormick (1993) and run in duplicate. The ouabain-sensitive hydrolysis of adenosine triphosphate (ATP) is enzymatically coupled to the oxidation of nicotinamide adenine dinucleotide (reduced form, NADH) which is directly measured at 340 nm in a microplate reader (SpectraMAX-340PC, Menlo Park, CA, U.S.A.) at 20 s intervals for 15 min. ATP in solution A was 3.5 mM and ouabain in solution B was 0.7 mM. All other chemicals and procedures followed the original protocol. Adenosine diphosphate (ADP) was used as the standard. Activities (expressed per mg protein) have been reported as control activity (no ouabain) minus ouabain-treated activity. Protein concentrations were measured at 595 nm with Bradford Reagent using bovine serum albumin as a standard (Sigma-Aldrich, St. Louis, MO, USA).

2.2.5.2. Ca^{2+} ATPase activity. The same gill and gut homogenates (n = 4–10 per treatment concentration) from the NKA assay were used. The assay method was modified from Vajreswari et al. (1983) who measured the inorganic phosphate released after ATP was added to the medium. Each sample and standard was run in duplicate. All procedures followed the original protocol except the incubation was carried out at 30 °C for 30 min and the tubes were put on the ice bath for 10 min before the colour reagent was added. The standard was inorganic phosphate ($NaHPO_4$) diluted in deionized water. The absorbance of the samples was measured at 820 nm in the microplate reader.

2.3. Nutrient content of worms

Protein, lipid, carbohydrates, Ca^{2+} and Na^+ concentrations in worm whole body samples were measured at the end of the 28-d Pb exposures. All nutrient assays were run in triplicate with about 0.3 g worms in each pool

2.3.1. Protein

For the protein analysis, worms were thawed and mixed with 10 x volumes of 0.5 M NaOH. Samples were incubated at 37 °C for 30 min and centrifuged at 1500 g for 10 min. The supernatant was diluted and protein concentration was measured by the Bradford reagent.

2.3.2. Lipid

The lipid analysis method was modified from that used by previous investigators (Barnes and Blackstock, 1973; Christie, 1982; Lauff and Wood, 1996). Lipid was extracted by homogenizing the worms in 10 x volumes of a chloroform and methanol (2:1) mixture to the worms. The slurry was then centrifuged and the lowest chloroform layer was removed and evaporated to dryness in nitrogen. The sulpho-phospho-vanillin method (Barnes and Blackstock, 1973) was applied to measure the lipid content at 520 nm. Canola oil was used as the standard.

2.3.3. Carbohydrates

Lactate was determined by the lactate dehydrogenase (LDH) and NAD^+ method (Gutmann and Wahlefeld, 1974). Worms were homogenized in 10 x volumes of 8% perchloric acid and 1 mM EDTA. After centrifugation, about 970 μl glycine/NAD^+ (1.7 mg NAD^+ ml^{-1} glycine) buffer was added into 20 μl supernatant and absorbance was read at 340 nm. Then 15 units LDH were added into each tube and absorbance was measured again after 45 min. Change in absorbance in the samples was compared with that of an L+ lactic acid standard.

Glycogen and glucose concentrations were determined by the method of Kepler and Decker (1984). Worms were homogenized in 10× volumes of acetate buffer (pH 5). About 700 μl was taken for digestion of glycogen by amyloglucosidase (50 units) at 37 °C. About 300 μl from the same sample was left undigested. Glucose-6-phosphate dehydrogenase (50 units) (pH 7.5, in 11.9 mM ATP, 2.5 mM NAD^+, 2.5 M triethanolamine hydrochloride, 0.05 M magnesium sulphate) was added to each undigested or digested sample for reaction. Then hexokinase (5 units) was added to the mixture and samples were measured at 340 nm after 30 min. Absorbance in the undigested samples is proportional to the concentration of free glucose (A), whereas absorbance in the digested samples is proportional to the total concentration (B) of glycogen and free glucose. Thus glycogen concentration is B-A. Glucose and glycogen standards were used in this assay and digestion efficiency was 93.7 ± 3.4%. Lactate, glycogen and glucose concentrations are presented individually, as well as summed for total carbohydrate concentration (lactate + glycogen + glucose).

2.4. Calculation of LC/EC values and statistical analysis

Unless otherwise noted, data have been expressed as means ± 1 standard error, and all concentrations are reported on a wet weight

Table 1
Experiment 1: Lethal concentrations (LC) with 95% confidence intervals (CI) of Pb to rainbow trout in the waterborne exposure. Values were calculated by ToxCalc™ software, based on the measured dissolved Pb concentrations, and mortality recorded. Due to the variability of the data at 7 weeks, 95% CI were not provided by the program.

	96 h ($\mu g\,L^{-1}$) (95% CI)	7 week ($\mu g\,L^{-1}$)
LC10	304.3 (197.4–392.9)	55.6
LC20	357.7 (249.9–455.6)	96.9
LC50	487.3 (374.9–633.3)	280.2

basis. The LC or EC values were calculated from observed responses and measured dissolved Pb concentrations using ToxCalc—Toxicity Data Analysis Software v5.0.32 (Tidepool Scientific Software, McKinleyville, CA, U.S.A.), with protocol EPAA 91-EPA/600/4-90/027F and with *Oncorhynchus mykiss* indicated as the test species. Data were first tested for normality and equality of variance (SigmaStat 3.5). If the normality or variance tests failed, data were transformed (log, ln, square root) and analyses were reapplied. If normality still failed, ANOVA on ranks were performed. For parametric data, differences among treatments or among concentrations within treatments were tested using One-Way ANOVA or Two-Way ANOVA, followed by Tukey's Multiple Comparison tests. For non-parametric data, differences among treatments were tested by Kruskal-Wallis One-Way ANOVA on Ranks, followed by Dunn's Pairwise Comparison tests. Linear or non-linear regression analyses were performed using SigmaPlot 10.0. SGR (%) data were first arcsine-transformed before statistical analysis. Pairwise comparisons were performed using Student's two-tailed *t*-test. A significance level of $p < 0.05$ was used throughout.

3. Results

3.1. Water chemistry

Mean measured concentrations of Pb were reasonably close to nominal values (Supplementary Table S1), so for convenience, nominal values have been used to refer to treatments in the text, Tables, and Figures. The mean absolute deviation from nominal values was 10.5 ± 2.3%. In the waterborne Pb treatments, dissolved Pb accounted for 90.2 ± 1.6% of total Pb in the water (Supplementary Table S1). Note that 0.45 μm filtration provides an operational definition of the dissolved fraction which may depend on the filter type used. In the dietborne only treatment, the dissolved waterborne Pb level was slightly elevated only in the highest dietary concentration treatment (average concentration: 0.69 $\mu g\,L^{-1}$), while waterborne Pb was below detection limits (0.3 $\mu g\,L^{-1}$) in all other control and dietary Pb treatments. Concentrations of Ca^{2+} Na^{+}, Mg^{2+} and DOC in all water samples were 1.4 ± 0.0 mmol L^{-1}, 0.7 ± 0.0 mmol L^{-1}, 0.3 ± 0.0 mmol L^{-1}, and 2.5 ± 0.0 mg C L^{-1} respectively (all SEMs were less than 0.05). In light of the high water turnover, there were no detectable increases in DOC concentrations with time after feeding.

3.2. Experiment 1: waterborne exposure – LC/EC values and effects on growth in rainbow trout

Mortality in the control treatment was less than 10%. The LC values are listed in Table 1. Unexpectedly, all fish exposed to 800 μg Pb L^{-1} died in the first week of the experiment. In general, longer exposure increased the toxicity of Pb. Specifically, 96 h LC_{20} and 7-week LC_{20} values of waterborne Pb to rainbow trout were 358 $\mu g\,L^{-1}$ and 96.9 $\mu g\,L^{-1}$ respectively, whereas 96 h LC_{50} and 7-week LC_{50} of waterborne Pb to rainbow trout were 487 $\mu g\,L^{-1}$ and 280 $\mu g\,L^{-1}$, respectively.

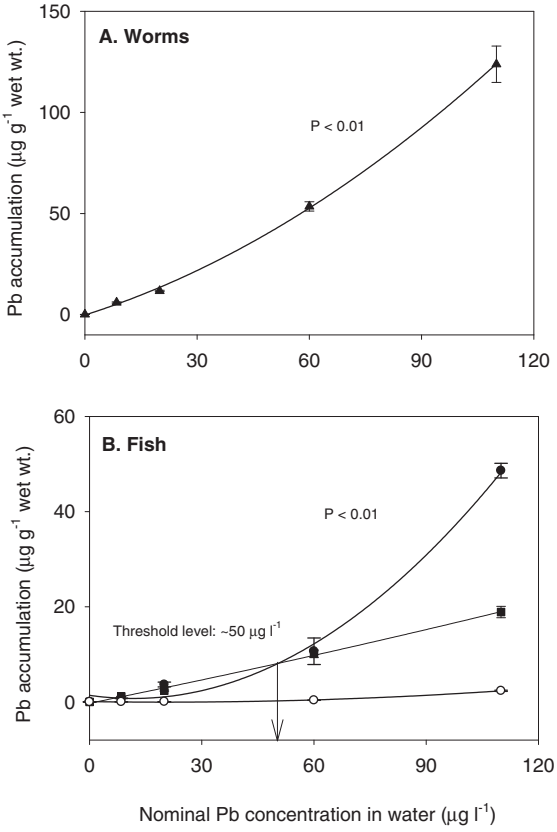

Fig. 1. Experiment 2. Pb accumulation ($\mu g\,g^{-1}$ wet wt.) in (Panel A) worms after 4 weeks and (Panel B) whole body of rainbow trout (lower panel) via waterborne (solid circles), dietborne (open circles) or combined exposures (solid squares) for 7 weeks. Accumulation in combined exposures was lower than from waterborne exposure alone after external Pb concentration passed the threshold level of approximately 50 $\mu g\,L^{-1}$. Regressions relationships were tested for statistical significance at $P < 0.05$.

Waterborne Pb had no effects on SGR over each week ($P > 0.05$, Supplementary Table S2). Overall SGR values (49 d) for control, 4, 10, 20, 80 and 240 $\mu g\,L^{-1}$ treatments were 2.1 ± 0.1, 2.2 ± 0.3, 2.9 ± 0.3, 2.2 ± 0.2, 1.8 ± 0.2, 2.2 ± 0.4% d^{-1} respectively. Biomasses of fish between control and Pb treatments were not significantly different at the beginning or end of the 7-week exposure (data not shown).

3.3. Waterborne exposure to worms—a dietary source of Pb for trout

The first and second batches of worms that were prepared for the dietary source of Pb had similar Pb levels, therefore they were pooled for calculating the average whole-body Pb concentration after 28-d exposure and subsequent storage at 5 °C. Mortality of *Lumbriculus* did not increase with increasing exposure concentration, and as concentration increased, Pb accumulation at 28 d increased proportionally or in a slight exponential relationship ($P < 0.01$, Fig. 1A, $r^2 = 0.99$). Specifically, worms accumulated 0.1 ± 0.02, 6.0 ± 0.3, 11.7 ± 0.4, 53.5 ± 2.3, 123.8 ± 9.0 $\mu g\,g^{-1}$ wet wt. in control, 8.5, 20, 60 and 110 $\mu g\,L^{-1}$ treatments respectively. These are equivalent to 0.6 ± 0.1, 35.2 ± 1.7, 68.9 ± 2.1, 314.7 ± 13.5 and 728.2 ± 52.9 $\mu g\,g^{-1}$ dry wt. when 83% of total weight as water is taken account (Mount et al., 2006). The Bioconcentration Factor from water (BCF = Pb concentration in worms on a wet weight basis/Pb concentration in water) tended to increase slightly with increasing Pb concentrations, from 704 to 1126 L kg^{-1}

Table 2
Experiment 2. Bioconcentration factor on a wet weight basis (BCF, L kg^{-1}), biomagnification factor on a wet weight basis (BMF) and bioaccumulation factor on a wet weight basis (BAF, L kg^{-1}) in the worms and trout at different concentrations of Pb exposure. BCF is calculated from the accumulation in the body divided by waterborne Pb concentration in the waterborne exposure alone. BMF is a unitless ratio calculated from the accumulation in the fish divided by accumulation in the worms in the dietborne exposure alone. BAF is calculated from the accumulation in the fish divided by waterborne Pb concentration in the combined exposure. Mean ± SE. The variability of BMF in fish cannot be calculated because there is variability of Pb concentrations in both fish and worms from the dietborne exposure, therefore only the mean BMF is presented. Significant differences between concentrations were evaluated by One-Way ANOVA, followed by Tukey Multiple Comparison test. Means not sharing the same upper case letter were significantly different ($P < 0.05$). No statistical analysis can be performed for BMF.

Pb concentration in water (μg l^{-1})	Worms	Fish		
	BCF	BCF	BMF	BAF
8.5	704 ± 34^{A}	89 ± 5^{A}	0.003	135 ± 3^{AB}
20	586 ± 17^{A}	183 ± 26^{B}	0.005	120 ± 6^{A}
60	892 ± 39^{B}	178 ± 46^{AB}	0.007	169 ± 13^{B}
110	1126 ± 82^{B}	442 ± 14^{C}	0.002	172 ± 11^{B}

at waterborne Pb concentrations from 8.5 to 110 μg L^{-1} respectively (Table 2).

3.4. Experiment 2: waterborne/dietborne Pb interaction exposure in rainbow trout

At the Pb concentrations tested, waterborne, dietborne or combination treatments did not cause mortality; survival in all treatments was comparable to the control (≥90%). In the waterborne only treatment, fish accumulated from 0.003 to 48.6 μg Pb g^{-1} wet wt. in the whole bodies; accumulation increased progressively with increasing Pb in the water ($P < 0.01$, $r^2 = 0.99$, Fig. 1B). On an absolute basis, the accumulation rate in fish was less than 40% of that in worms at the same waterborne concentration (Fig. 1A, B). For example, at 110 μg L^{-1}, the fish BCF was 441.9 L kg^{-1}, whereas the worm BCF was 1126 L kg^{-1} (Table 2).

When fish consumed Pb-contaminated worms, a pattern of far smaller increase of Pb in fish from the dietborne treatment alone than from the waterborne treatment alone was observed ($P < 0.01$, $r^2 = 0.99$, Fig. 1B). The Biomagnification Factor from food (BMF = concentration in fish on a wet weight basis/concentration in worms on a wet weight basis) was only 0.002 at 110 μg L^{-1} (Table 2) Waterborne Pb at the same concentration contributed to a much higher extent to accumulation compared to dietborne Pb alone (a difference of 20–60 fold).

When fish were simultaneously exposed to waterborne and dietborne Pb (combination treatment), whole body Pb accumulation was comparable or slightly higher than from the waterborne treatment alone at the two lower concentrations. However the pattern changed at the two higher concentrations where lower Pb accumulation was observed in the combined treatment than in the waterborne alone treatment. The crossover threshold was about 50 μg Pb L^{-1} (Fig. 1B). Thus, waterborne and dietborne Pb interact on Pb accumulation; the effect was additive at the lower concentrations and antagonistic at the higher concentrations. At the highest exposure concentration, the Bioaccumulation Factor (water and food) (BAF = concentration in fish on a wet weight basis from combined treatment/concentration in water) was 172 L kg^{-1} at 110 μg L^{-1}, in contrast to the BCF of 442 L kg^{-1} in the waterborne exposure alone at this same concentration (Table 2). Generally, as waterborne Pb concentration increased, BCF in worms and fish increased, while BMF and BAF fluctuated (Table 2).

Among all tissues examined, tissue Pb concentrations (μg Pb g^{-1}, wet weight) followed this order: carcass ~ gill > liver ~ gut

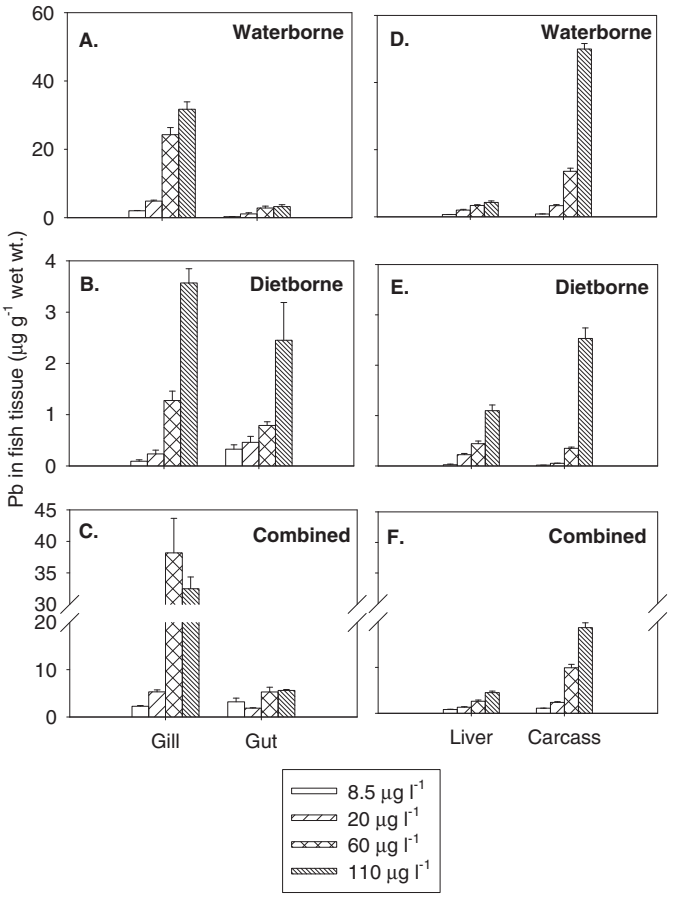

Fig. 2. Experiment 2. Tissue-specific distribution of Pb (μg g^{-1} wet wt.) in gill, gut, liver and carcass of rainbow trout after waterborne (Panels A and D), dietborne (Panels B and E) or combined exposures (Panels C and F) for 7 weeks. Significant difference ($P < 0.05$) among concentrations and exposures was tested by One-Way ANOVA or Kruskal-Wallis One-Way ANOVA on Ranks followed by Tukey's Multiple Comparison or Dunn's Pairwise Comparison test. Statistical results are presented in Supplementary Tables S4.

(Fig. 2, Supplementary Table S4). Waterborne exposure resulted in a higher accumulation (10-fold or higher) than dietborne exposure in all tissues except in gut which accumulated similar amounts of Pb from both treatments (Fig. 2A, B, D, E, Supplementary Table S4). Pb concentrations in tissues increased with increasing Pb in the water and this was mostly significant when exposure concentration was 60 μg L^{-1} or higher (Fig. 2A, D, Supplementary Table S4). The antagonistic interaction of waterborne and dietborne exposure seen in the whole body Pb accumulation (Fig. 1B) was manifested at the tissue level as well. The liver had slightly lower Pb in the combined treatment than in the waterborne alone treatment at 20 and 60 μg L^{-1} ($P < 0.05$, Fig. 2D, F, Supplementary Table S4). A similar effect was observed in carcass at 20, 60 and 110 μg L^{-1}, but it was more extensive ($P < 0.05$, Fig. 2D, F, Supplementary Table S4). Pb accumulation was reduced by 27 – 63% (combined versus waterborne treatment) and higher exposure concentration resulted in a higher magnitude of antagonistic effect. No interaction was observed in the gill where Pb accumulation was similar between combined and waterborne treatments. In the gut there was also no interaction observed since the combined treatment resulted in a higher Pb concentration than the waterborne treatment alone (Fig. 2A, B, C, Supplementary Table S4).

Despite the increase in Pb accumulation with increasing Pb exposure concentrations in the waterborne, dietborne and combined experiments, SGR did not differ significantly among the four exposure groups (and the control) over the 7-week exper-

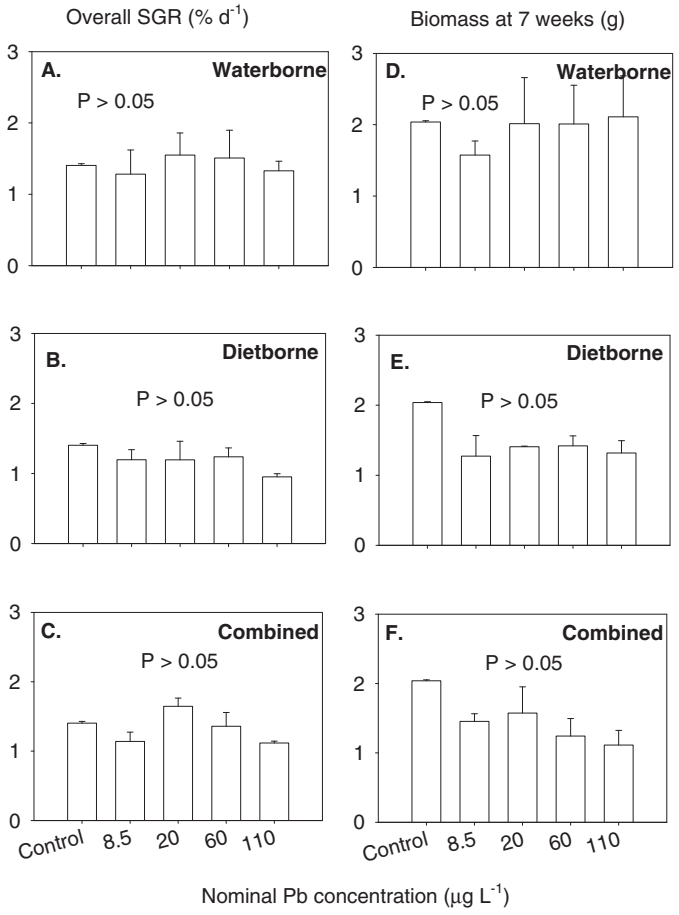

Fig. 3. Experiment 2. Overall SGR (49 d, % d^{-1}) and biomass (g) of rainbow trout after 7 weeks of (Panels A and D) waterborne, (Panels B and E) dietborne or (Panels C and F) combined exposures. Significant difference among concentrations was tested by One-Way ANOVA, followed by Tukey's Multiple Comparison test at $P < 0.05$.

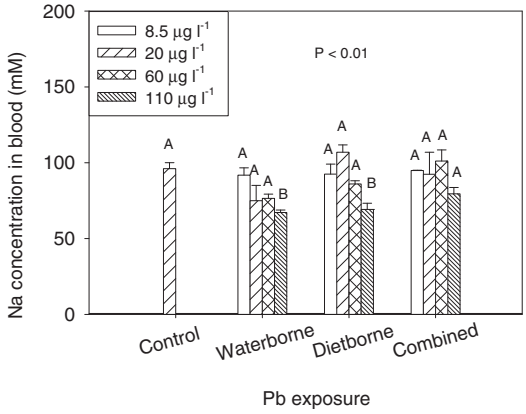

Fig. 4. Experiment 2. Concentration of Na^+ in the blood of rainbow trout (µmol g^{-1} wet wt.) after waterborne, dietborne or combined exposures for 7 weeks. Significant difference was tested by One-Way ANOVA, followed by Tukey's Multiple Comparison test. Different letters indicate significant difference at $P < 0.05$.

iment (Supplementary Table S3, Fig. 3A–C). SGR at each week was not significantly different among different routes of exposure or concentrations (Supplementary Table S3), similar to the first waterborne experiment (Section 3.2). However trends for growth inhibition (approximately 20–25%) were seen the highest exposure concentrations for both dietborne and combination treatments, Similarly, biomass at 7 weeks was not affected by the waterborne only treatment, but trends of lower biomass were observed in the Pb-exposed fish from both the dietborne treatment alone (30.4–37.6% reduction) and the combined treatment (22.8–45.4% reduction). These were not statistically significant (Fig. 3D–F).

Ca^{2+} concentration in the carcass of the fish after 7 weeks of exposure did not vary among treatments, ranging from 235 to 285 µmol g^{-1} wet wt. in the control and Pb treatments (Supplementary Fig. S2). Ca^{2+} ATPase activity (gill: 0.8–2.5 µmol ADP mg^{-1} protein h^{-1}; gut: 1.0–2.8 µmol ADP mg^{-1} protein h^{-1}) was also not impacted by Pb at any concentrations or exposure pathway (Supplementary Fig. S3).

Na^+ concentration in the whole blood was lower by 28.0 – 30.1% in both the waterborne and dietborne Pb treatments at the highest Pb exposure level ($P < 0.01$, Fig. 4). However, when the fish were exposed to Pb through both routes, Na^+ concentration did not differ significantly amongst the different exposure concentrations (Fig. 4). In the waterborne alone exposure, there was an apparent inverse relationship between NKA activity in the gill and exposure concentrations, although it was not statistically significant ($P = 0.10$, Fig. 5A). A stronger inverse relationship which was significant was observed in the gut (Fig. 5D). Dietborne alone exposure did not have any significant impact at all on NKA activity in either tissue (Fig. 5B, E). In the combined exposure, the same inverse relationship for NKA was observed in the gut (Fig. 5C, F).

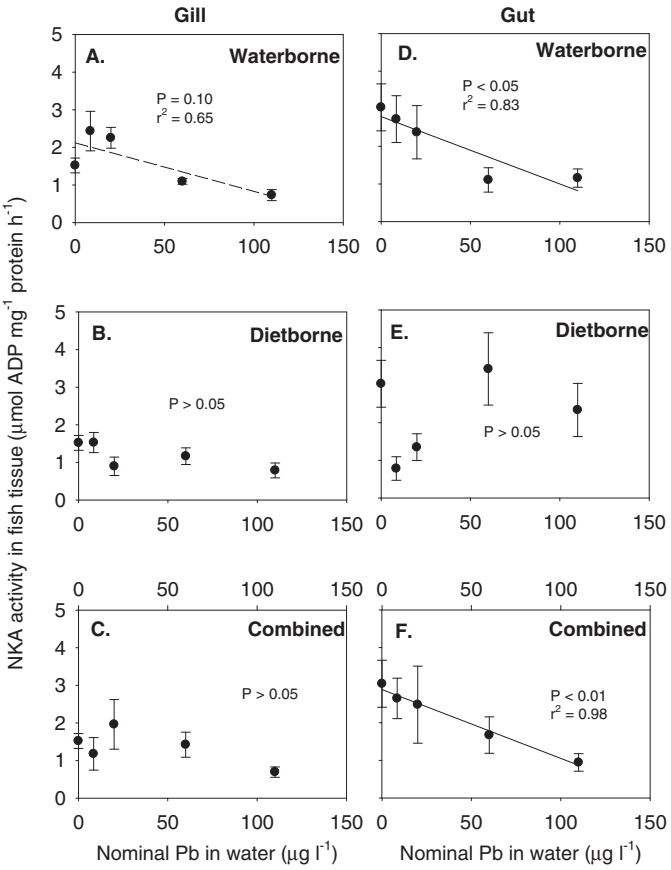

Fig. 5. Experiment 2. Na^+/K^+ ATPase (NKA) activity (µmol ADP mg^{-1} protein h^{-1}) in gill and gut of rainbow trout after waterborne (Panels A and D), dietborne (Panels B and E) or combined exposures (Panels C and F). Regression was tested for statistical significance at $P < 0.05$. Solid line indicates significance whereas dashed line indicates non-significance but $r^2 > 0.60$, $P \leq 0.10$.

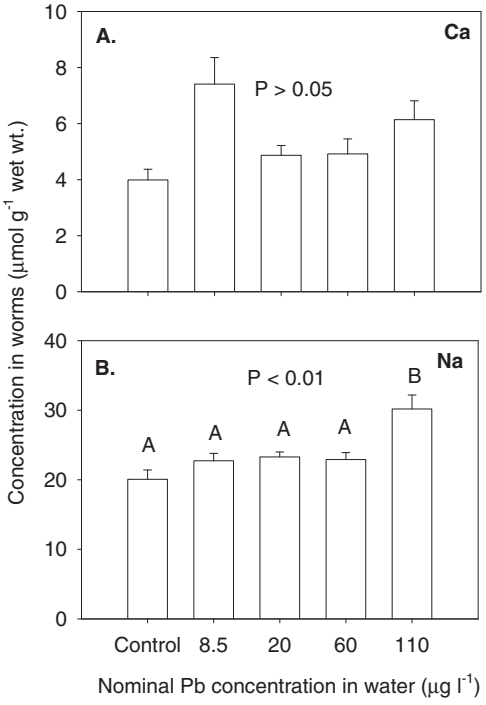

Fig. 6. Concentration of (A) Ca^{2+} and (B) Na^{+} in the worms ($\mu mol\ g^{-1}$ wet wt.) after exposure to different concentrations of Pb for 28 days. Significant difference was tested by One-Way ANOVA, followed by Tukey's Multiple Comparison test. Different letters indicate significant difference at $P < 0.05$.

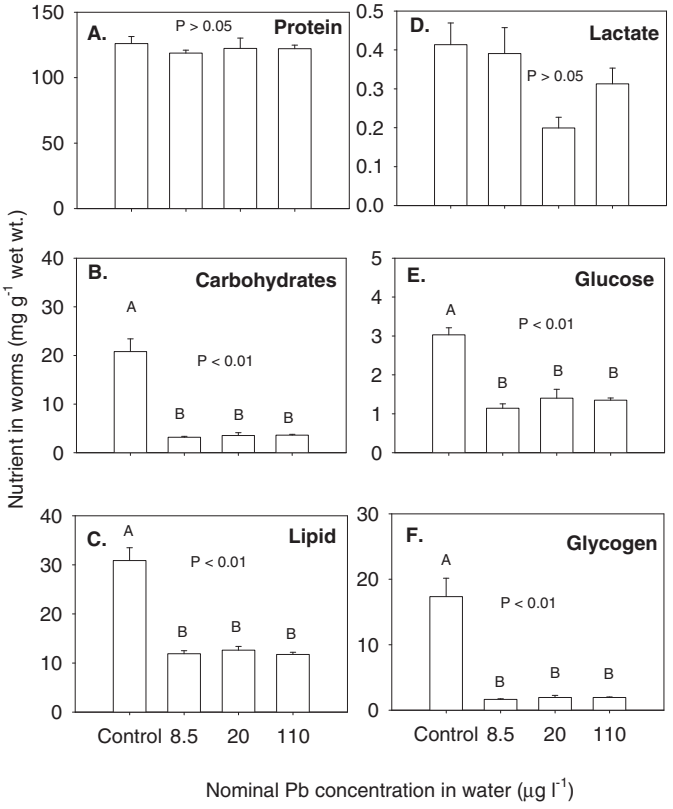

Fig. 7. Nutrient contents (A. Protein; B. Carbohydrates; C. Lipid) in the worms ($mg\ g^{-1}$ wet wt.) after exposure to different concentrations of Pb for 28 days. Worm samples from the 60 $\mu g\ L^{-1}$ treatment were lost. The right-hand panels (Panel D, E and F) are the breakdown results for different carbohydrates. Significant difference was tested by One-Way ANOVA, followed by Tukey's Multiple Comparison test. Different letters indicate significant difference at $P < 0.05$.

3.5. Experiment 2: effect of waterborne Pb on nutrient content of worms

Besides having a direct effect on the fish, Pb may have also had indirect effects on the fish diet by altering the nutrient composition of the worms. Although waterborne Pb exposure for 28 days had no significant effects on Ca^{2+} concentration in the worms (Fig. 6A), it did significantly increase the Na concentration at 110 $\mu g\ L^{-1}$ (Fig. 6B). In general, the protein concentration of worms was unaffected by Pb (Fig. 7A), but carbohydrates and lipid levels were greatly reduced by waterborne Pb exposure, even at the lowest exposure concentration of 8.5 $\mu g\ L^{-1}$ ($P < 0.01$, Fig. 7B, C). For carbohydrates, glucose and glycogen were reduced starting at the lowest Pb treatment, although lactate concentration was similar at all Pb exposure levels (Fig. 7D, E, F). The protein, carbohydrates and lipid content of worms from the 60 $\mu g\ L^{-1}$ treatment could not be measured due to loss of samples.

4. Discussion

4.1. Regulatory and environmental relevance

The rainbow trout is generally considered to be one of the more metal-sensitive freshwater fish species, and therefore is often used in toxicity tests (Besser et al., 2007; Teather and Parrott, 2006). The trout in the present study appeared to be more acutely sensitive than a previous study, which recorded a 96 h-LC50 approximately twice that observed presently in the same water chemistry (Rogers et al., 2003). Rogers et al. (2003) surveyed a number of other rainbow trout acute toxicity studies at a range of fish sizes and water hardnesses, and in all, the LC50 values were again higher than those of the present study. The present 7-week LC20 value (97 $\mu g\ L^{-1}$; Table 1) for waterborne Pb compares favourably with a recent summary of unpublished chronic toxicity data for trout (J. Chowdhury, in Wang et al., 2014) where data were normalized to a common hardness (102.4 $mg\ L^{-1}$ as $CaCO_3$).

Nevertheless, both acute and chronic toxicity species sensitivity distributions (SSDs) for waterborne Pb, as with other metals, are dominated by invertebrates such as snails and daphnids at the lower end, whereas fish, including trout, populate the higher end of the SSDs. For example, waterborne Pb up to 240 $\mu g\ L^{-1}$ for 7 weeks in the present study had no effects on growth or biomass of juvenile trout (Supplementary Table S2), yet the 30 d EC_{50} for growth in the snail *Lymnaea stagnalis* was <4 $\mu g\ L^{-1}$ (Grosell et al., 2006). There are very few data for dietary Pb, but as with other metals, invertebrates appear to be more sensitive than fish (DeForest and Meyer, 2015). For example, *Hyalella azteca* exhibited a LOEC of 3.5 $\mu g\ L^{-1}$ when dietary Pb exposure was combined with waterborne Pb exposure, versus 16 $\mu g\ L^{-1}$ for waterborne exposure alone (Besser et al., 2005), whereas we observed no effects in trout up to far higher Pb concentrations. Furthermore, for the water hardness of the present tests (140 $mg\ L^{-1}$ as $CaCO_3$), the current USEPA hardness-adjusted water quality criteria (acute: 114 $\mu g\ L^{-1}$; chronic: 4.5 $\mu g\ L^{-1}$, U.S. EPA (EPA, 1980) and the Canadian Water Quality Guidelines) chronic only: 6.3 $\mu g\ L^{-1}$, CCME (Environment Canada, 2007) are both sufficient to protect rainbow trout in fresh water from both waterborne and dietary Pb toxicity.

Data on dietborne metal toxicity to aquatic animals are sparse, but the available information suggest that acute toxicity is rarely observed; however chronic toxicity, when referenced to the waterborne concentration to which the prey were exposed, may be comparable or occasionally greater than waterborne toxicity for some metals (DeForest and Meyer, 2015). As noted in the Introduction, natural diets with biologically incorporated metals in the prey

appear to be most effective in this regard. However, to date there is as yet no evidence of this for Pb (DeForest and Meyer, 2015), so this situation was the motivation for the natural diet approach used in the present study.

Several reviews on dietary metal toxicity (Clearwater et al., 2002; Meyer et al., 2005; DeForest and Meyer, 2015) have concluded that the dosing rate (i.e. the product of metal concentration in the food times daily ration) is a more meaningful metric than metal concentration alone, and that natural diets are more relevant than spiked commercial diets. Note that in particular that the present study used live prey with biologically incorporated Pb and matching waterborne and dietborne exposure concentrations at levels which mimicked realistic environmental conditions for contaminated sites. For example, *L. variegatus* in the highest exposure concentration of our study accumulated levels of Pb in their bodies which were either similar to or lower than levels reported for the benthic invertebrates of the Coeur d' Alene River, Idaho (Farag et al., 1998; Farag et al., 1999). The dietary dosing rate was measured in the present study, and ranged from ~0.6 μg Pb g fish^{-1} day^{-1} up to ~12.6 μg Pb g fish^{-1} day^{-1} at the highest exposure concentration. The highest rate is therefore comparable to that used in a study (Alves et al., 2006) where trout were fed a Pb-contaminated artificial diet (commercial pellet food spiked with Pb nitrate) at a dosing rate of ~13 μg Pb g fish^{-1} day^{-1} without detrimental effect on growth. It also appears to be higher than several other studies reporting no effects of dietary Pb on growth in fish (Hodson et al., 1978; Mount et al., 1994; Alves and Wood, 2006; Boyle et al., 2010).

In the second experiment, prey delivering the highest dosing rate (~12.6 μg Pb g fish^{-1} day^{-1}) caused no lethality in the predator (trout) and at most had marginal effects on growth (Fig. 3), as discussed subsequently. These prey were exposed to the highest waterborne Pb levels, around 110 μg L^{-1} (Supplementary Table S1) a level that approximated the 7-week waterborne LC20 (97 μg L^{-1}) for the predator in the first experiment (Table 1). There were clearly no lethality or growth effects in trout when prey were exposed to the second highest Pb concentration, about 60 μg L^{-1}, approximating the 7-week waterborne LC10 (Fig. 3), and delivering ~5.3 μg Pb g fish^{-1} day^{-1}. The combination of waterborne and dietary Pb did not change this result. Furthermore, as discussed subsequently, changes in the nutritional value of the prey occurred, associated with Pb exposure, such that any effects occurring in the predator could have been of indirect origin.

In the present study, using One-Way ANOVA with multi-comparison testing, there were no significant growth effects, as shown in Fig. 3. Furthermore,Two-Way ANOVA (treatment pathway x exposure concentration) for SGR in each week, or over the whole 49-day period also yielded no significant differences. This may reflect the fact that there were four treatments (control, waterborne, dietborne, combined), and four exposure concentrations in three of the treatments and only two tank replicates per treatment-exposure combination, which greatly reduced the power for detecting specific differences. However, a much less conservative approach, simple pairwise comparisons of the 0–49 day SGR values between experimental and control treatments revealed significant inhibitory effects on growth for both dietborne and combination treatments, but only at the highest Pb exposure concentration, around 110 μg L^{-1} (Figs. 3B, C; Supplementary Table S3). A preliminary report of this same data set cited by DeForest and Meyer (2015) used a different statistical approach based on cumulative growth and concluded that there were significant inhibitory effects for the dietborne treatment only. Regardless of the computational approach, the inhibitions seen in the current study with both high Pb treatments were in the order of 20–25%, just at the threshold for "biological significance" defined by DeForest and Meyer (2015). Clearly a study with greater replication will be required to clarify the effects of dietary Pb on growth, alone or in combination with waterborne Pb.

In this regard, the study of Erickson et al. (2010), which employed 4 tank replicates, is instructive. The authors fed Pb-contaminated *L. variegatus* to juvenile trout, fathead minnow, and channel catfish for 30 days at a variable but apparently higher ration (15–34% per day). Pb concentrations in the worms were almost identical to that used in the highest exposure of the present study, so the dosing rate was about 1.5–3.4 fold higher. Erickson et al. (2010) reported no lethality or growth inhibition in the fish. However only dietary Pb exposure was assessed, and not the combination with waterborne Pb exposure.

In contrast to the present study, growth of the trout fed Cd-contaminated oligochaetes at a much lower dosing rate (1.05 μg Cd g fish^{-1} day^{-1}) was reduced by about 50% after a month's exposure (Ng and Wood, 2008). This difference suggests that trout are more sensitive to dietborne Cd than to dietborne Pb, similar to the difference for waterborne sensitivity to these two metals, or alternately, that the nutritional quality of the worms was differentially impacted by the two metals.

4.2. Accumulation of Pb

Both rainbow trout and *L. variegatus* accumulated greater amounts of Pb with increasing Pb concentration in the water or food (Fig. 1A, B; Supplementary Fig. S1). The accumulation rate of Pb from water was higher for oligochaete worms than for rainbow trout, resulting in higher BCF values, in accordance with the review of McGeer et al. (2003) who surveyed literature data to evaluate relationships between chronic exposure and bioaccumulation of Cd, Cu, Zn, Pb, Ni and Ag. All 66 species from algae, insects, arthropods, gastropods, mussels to fish displayed a log–log positive relationship between internal Pb burden and exposure concentration (McGeer et al., 2003). The oligochaete and trout in this study also have similar BCFs compared to other studies (mean: 598 L kg^{-1}, McGeer et al., 2003). However our study demonstrated a positive relationship between BCFs and exposure concentration and a more or less independent BAF (Table 2), in contrast to the inverse relationships reported by McGeer et al. (2003). In addition, the BCF is assumed to be a ratio at equilibrium after chronic exposure. However, the body burden of the prey in this study was not at equilibrium because the worms were harvested at 4 weeks whereas accumulation kept increasing through to 7 weeks (and likely beyond; Supplemental Fig. S1). We have no information as to whether equilibrium was achieved in trout as the body Pb burden was only measured at the end of the 7 weeks (Fig. 1B), though in previous studies with Pb-contaminated artificial diets, body burdens had generally stabilized before this time (Alves et al., 2006; Alves and Wood, 2006). Future research should address this question of how important complete equilibration is with respect to relevance to real-world field situations. In agreement with Alves et al. (2006) and Alves and Wood (2006), Pb retention from the diet was very low in the present study, ranging from 0.07% at the lowest dietary exposure to 0.40% at the highest dietary exposure over 49 days.

The waterborne exposure was the major pathway for Pb accumulation in trout, compared to the dietary pathway of worms held at the same Pb concentration for 28 d. The difference in whole body Pb accumulation between the two routes was 20–60-fold at our tested concentrations (Fig. 1B). The BMF was extremely low (Table 2). In general, metals exhibit low bioavailability to the gut through dietborne exposure because metals may be largely bound to subcellular fractions of the prey (e.g., cellular debris and inorganic granules) which cannot be assimilated (Wallace and Lopez, 1996, 1997). In addition, chronically exposed worms may store a greater fraction of their metal burden in the inorganic granules than the unpolluted worms, resulting in a lower bioavailability of metals

to the predator on a percentage basis (Wallace et al., 1998). Therefore, the role of waterborne exposure may be even higher when the concentration is higher and the contaminated prey has developed resistance to the metal. However, the present results demonstrate that the story is complicated by simultaneous exposure via water and diet.

Combined exposure to both waterborne and dietborne Pb reduced the whole body accumulation in trout extensively, relative to waterborne Pb exposure alone, when external Pb concentration was greater than a threshold of ~50 μg L^{-1} (Fig. 1B). To our knowledge, this study is the first to show interaction of Pb from the two uptake routes in rainbow trout. Whole body Pb accumulation was reduced up to 61% (i.e. BAF/BCF = 0.39) at 110 μg Pb L^{-1}. Previous studies have demonstrated an antagonistic interaction between gastrointestinal and waterborne uptake of essential metals e.g., Cu, Ni and Zn in rainbow trout (Kamunde et al., 2002; Niyogi et al., 2006; Chowdhury et al., 2008). For essential metals, regulation of uptake is important to maintain proper body functions and metabolism whereas for non-essential metals, control of uptake may be relevant for reducing toxicity in the fish. Chowdhury et al. (2008) suggested that pre-exposure to a metal from one exposure pathway down-regulates gene expression of transporters in target organs of the other exposure pathway. In this regard it is interesting that elevated dietary Ca^{2+}, an essential metal, down-regulates both Cd and Zn uptake at the gills of trout, as well as the uptake of Ca^{2+} itself (Baldisserotto et al., 2004; Franklin et al., 2005; Niyogi et al., 2006). However, for the non-essential metal Cd, dietary exposure did not decrease waterborne Cd uptake (Baldisserotto et al., 2005).

An alternative explanation for the present results may relate to excretion rates. Similar antagonistic interaction of Pb accumulation occurred at the tissue level of rainbow trout, but the threshold was at a lower waterborne concentration, 20 μg Pb L^{-1} (Fig. 2, Supplementary Table S4). Combined exposure resulted in a significantly lower accumulation at liver and carcass but not at gill or gut, compared with waterborne exposure alone. Gill and gut are the organs for uptake and transport of metals to other organs whereas liver and carcass (which includes kidney) are the major organs for detoxification and elimination of metals (De Boeck et al., 2004). A lower accumulation in carcass and liver may imply a faster elimination of Pb from the combined exposure. In order to better understand the interactive mechanisms occurring during combined exposures, uptake of Pb via the gill and gut, as well as gene expression of candidate transporters, and elimination rates through various routes should be quantified in future investigations.

On an absolute basis, Pb concentrations from waterborne and combined exposures were much higher in gills and carcass than in other tissues, presumably reflecting the major uptake (gills) and storage sites (carcass, containing bone, skin, scales, and kidney; Alves and Wood, 2006; Alves et al., 2006) respectively (Fig. 2, Supplementary Table S4). In contrast, Pb concentrations from the dietborne exposures were much more uniform amongst tissues. Waterborne exposure was clearly the major player in accumulation for gill (9–20 fold higher), liver (4–35 fold higher) and carcass (20–80 fold). Even in the gut tissue, concentrations from waterborne exposures were higher than from dietborne exposures, but these two sources appeared to be additive in the combined exposures (Supplementary Table S4).

4.3. Physiological effects

Blood Na^+ levels were reduced by the highest concentrations of Pb during both waterborne and dietborne exposures (Fig. 4). Note that due to the small size of the fish, whole blood rather than plasma Na^+ was measured, explaining the lower absolute values than obtained from traditional plasma measurements. Inhibitions of NKA activity were significant at the gut but not the gills for both the waterborne and combined exposures, and not significant at either site for the dietborne exposure (Fig. 5). Overall these observations are in accord with some but not all previous studies. Rogers et al. (2005) reported that acute exposure to waterborne Pb at even higher levels (259 and 497 μg L^{-1}) reduced Na^+ influx rate at the gills and slightly increased its efflux rate in rainbow trout; branchial NKA activity was also reduced, an effect which was correlated with Pb accumulation. Again in trout, Crespo et al. (1986) found that chronic dietary exposure to Pb in an artificial diet at a dosing rate (10 μg Pb g $fish^{-1}$ day^{-1}) similar to the highest used in the present study significantly inhibited intestinal Na^+ and Cl^- uptake *in vitro*, as well as intestinal NKA activity. On the other hand, Alves and Wood (2006), again using a similar dosing rate with an artificial diet, found exactly the opposite, an increase in intestinal NKA activity, while Alves et al. (2006) reported unchanged branchial Na^+ influx rates in a similar dietary Pb exposure trial. The present study is the only one to report that waterborne Pb exposure inhibits intestinal NKA activity. The apparent protective effect of the combined exposure on blood Na^+ regulation (Fig. 4) may relate to replenishment by the higher concentration of Na^+ in the worms exposed to 110 μg L^{-1} (Fig. 6B).

When presented acutely, Pb is generally taken up via the same mechanism as Ca^{2+}, through voltage-dependent, lanthanum-sensitive apical Ca^{2+} channels in the fish gill, resulting in a blockade of basolateral Ca^{2+}ATPase, a reduction in branchial Ca^{2+} uptake, and a depression of internal Ca^{2+} concentrations (Rogers et al., 2003; Rogers et al., 2005). Similarly, at the gut, Pb appears to compete with Ca^{2+} for uptake pathways (Alves and Wood, 2006). Therefore we expected that in the present chronic exposures there would have been a disruption in Ca^{2+} uptake and homeostasis in Pb-exposed trout, as seen during acute waterborne exposures. However, there were no impacts of Pb on either Ca^{2+} homeostasis (Ca^{2+} in carcass; Supplementary Fig. S2) or activities of Ca^{2+} ATPase in the gill and gut (Supplementary Fig. S3), suggesting that these effects are compensated during chronic exposures

4.4. Indirect effects on prey quality

Pb may have impacts on the diet which indirectly affect the fish physiologically or ecologically. Most dietborne studies have examined direct effects of metals, but have rarely considered indirect effects. However, a few notable exceptions have reported changes in nutrient and ionic composition of *L. variegatus* associated with various metal treatments (Hansen et al., 2004; Mount et al., 2006; Erickson et al., 2010). In the present study, the effects were quite marked (Figs. 6 and 7). Commercial fish pellets (e.g., Nelson's Silver Cup trout chow) normally have about 40–42% and 10–16% dry wt. of protein and lipid respectively. In our study, the control oligochaete had higher protein content and similar lipid as the pellets and comparable nutrient levels as *L. variegatus* in the other cited studies. However after 28 days of Pb exposure, these oligochaetes had about 80% less carbohydrates (50% less glucose, 88% less glycogen) and 50% less lipid than the control, and at the highest Pb concentration, 50% more Na^+. Protein and lipid are important components for fish growth (Jauncey, 1982; Watanabe, 1982). The indirect effects of Pb through reduction of the nutritional value of the worms may have contributed to the slightly lower growth of fish observed in the dietborne and combined treatments. Conversely, as noted earlier, the high Na^+ content of the worms may have helped protect internal Na^+ homeostasis of Pb-exposed trout in the face of Pb-induced disruption of Na^+ uptake. The indirect effects of Pb on fish diet quality should not be neglected in future dietborne toxicity studies using live prey. In future studies, it will also be informative to investigate the mechanism(s) behind the changes in worm composition with Pb exposure.

4.5. Perspectives

In the context of risk assessment, the present results demonstrate that dietborne Pb exposure does not cause any significant impacts on toxicity (ecological or physiological) or Pb bioaccumulation in juvenile rainbow trout, at least at low concentrations (i.e. prey loaded at waterborne concentrations up to 60 $\mu g L^{-1}$). However when combined exposure occurs and waterborne concentration is increased, dietborne exposure may in fact protect against bioaccumulation from the waterborne exposure, and against at least one pathophysiological effect of waterborne exposure (disturbed Na^+ homeostasis). Importantly, the indirect effects of Pb exposure on the nutritional value of the prey may be considerable, and could contribute to marginal growth inhibition of the predator at higher Pb exposure concentrations. Therefore, it is essential to incorporate dietborne Pb exposure into risk assessment and consider it together with waterborne exposure. More research is required to understand the implications of our results for the mechanistic framework of biotic ligand models (BLM) and the interactive mechanism(s) of waterborne and dietborne pathways in metal exposure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2016.06.007.

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