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Mechanistic analysis of acute, Ni-induced respiratory toxicity in the rainbow trout (*Oncorhynchus mykiss*): an exclusively branchial phenomenon

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Abstract

In moderately hard Lake Ontario water (~140 mg L^{-1} as $CaCO_3$) waterborne Ni (9.7–10.7 mg Ni L^{-1}) is acutely toxic to adult rainbow trout (*Oncorhynchus mykiss*) exclusively via branchial mechanisms. Ventilation in resting trout (evaluated using a ventilatory masking technique) was adversely affected, as ventilation rate (V_R), ventilation volume (V_G), opercular stroke volume (V_{SV}) and resting oxygen consumption ($\dot{M}O_2$) were all increased, and oxygen extraction efficiency (U%) decreased over 48 h of Ni exposure. Extensive gill Ni accumulation (41-fold over control levels) during 82 h of waterborne Ni exposure resulted in marked ultrastructural damage to the respiratory epithelium of the gill, including swelling of the secondary lamellae evidenced by changes to both the lamellar region (increased secondary lamellar tissue volume (V_{SL}/V_{LR})), and to the secondary lamellae themselves (increased volume of tissue lying outside the pillar system (V_{OPS}/V_{SL})). Additionally, decreased lamellar height and increased lamellar width indicated a reduction in lamellar surface area available for gas diffusion. The relative diffusing capacity of experimental fish was only 59% of that of control fish. Infusion of Ni into the blood, achieving a similar time course and magnitude of plasma [Ni] elevation to that during waterborne exposure, failed to elicit any signs of respiratory toxicity typically diagnostic of acute, high level waterborne Ni exposure. Infusion of Ni into the blood for 96 h resulted in only minor accumulation of Ni in the gill, suggesting that acute Ni-induced respiratory toxicity is related to accumulation of high levels of Ni in the gill from the water. Additionally, infusion of Ni into the bloodstream led to significant extrabranchial Ni accumulation only in the kidney. White muscle, heart, liver, stomach, and intestine did not significantly accumulate Ni following infusion into the bloodstream and trapped plasma analysis revealed that, with the exception of the kidney, a substantial portion of Ni accumulated in tissues following infusion could be accounted for by extracellular (blood-bound) Ni.

Keywords: Nickel; Acute; Rainbow trout; Respiratory; Gill

1. Introduction

Recently, the acute toxic mechanism of waterborne Ni has been elucidated in the rainbow trout (*Oncorhynchus mykiss*) (Pane et al., 2003). At high concentrations (~11 mg Ni L^{-1}, or 33% of the 96 h

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LC_{50} for adult trout), Ni acts as a respiratory toxicant, causing a marked, time-dependent decrease in arterial oxygen tension, an increase in arterial carbon dioxide tension and a subsequent respiratory acidosis. Ionoregulatory disturbance is minimal. Although the details surrounding the mechanism of respiratory toxicity were not thoroughly investigated, the physiological effects observed were presumed to result from impairment of gas exchange between the animal and the water caused by a diffusive limitation at the gill (Pane et al., 2003).

Several previous studies have indirectly implicated the gill as a key organ in acute Ni toxicity. Most recently, Meyer et al. (1999) determined that the best predictor of acute toxicity to fathead minnows (*Pimephales promelas*) was gill Ni burden, rather than any abiotic factor (i.e. calculated free ion activity or free aquo ion concentration), and therefore advocated a biotic ligand modelling (BLM) approach (e.g. DiToro et al., 2001) for the prediction of waterborne Ni toxicity. Using morphometric analyses, Hughes and co-workers (Hughes and Perry, 1976; Hughes et al., 1979) quantified a nickel-induced deleterious effect on the theoretical gas exchange capacity of the rainbow trout gill. Following 3.5 days of exposure to $3.2\,mg\,Ni\,L^{-1}$, the estimated diffusing capacity of the gill decreased by 85% and the harmonic mean thickness of the water/RBC barrier more than doubled (Hughes et al., 1979). Nath and Kumar (1989) also reported massive damage to the gill architecture of the tropical freshwater perch (*Colisa fasciatus*) following exposure to approximately $14\,mg\,Ni\,L^{-1}$. Ultrastructural damage to the gill included hypertrophy of respiratory and mucous cells, epithelial lifting, necrosis, hyperplasia of the respiratory epithelium, fusion of adjacent lamellae, and lamellar clubbing. There seems little doubt, therefore, that the gill is a major target organ of waterborne Ni.

In the study by Pane et al. (2003), however, a significant negative correlation was observed between plasma Ni concentration and arterial oxygen tension, suggesting that acute Ni toxicity may also be, to some extent, a hematological phenomenon. It has been established that both the gill and the blood can be sites of toxic action of waterborne respiratory toxicants. Aluminum (Al) at moderately low pH, for example, binds to the branchial epithelium, increasing the blood–water diffusion distance, and causing an overall swelling of the secondary lamellae and thickening of the epithelium (Wilson et al., 1994). Nitrite (NO_2^-), on the other hand, causes extensive oxidation of erythrocytic haemoglobin to methemoglobin, increasing blood O_2 affinity while greatly decreasing blood O_2 capacity, thereby impairing blood oxygen handling and transport (Jensen et al., 1987, 1993).

The primary objective of the present study, therefore, was to further investigate the details surrounding the site(s) and mechanism(s) of toxic action of waterborne Ni. Given two possible sites of toxic action of Ni (the branchial epithelium and the blood), and the general hypothesis that the gill was the primary site, we used three experimental approaches to critically evaluate the physiological functioning of the gill during waterborne Ni exposure. The first was to characterise ventilatory function during acute waterborne Ni exposure by physically separating prebranchial and postbranchial water by means of a specialised van Dam-style ventilation box and a masking technique developed by Davis and Cameron (1971). This approach was used with success by Walker et al. (1988) to diagnose the mechanism of respiratory toxicity of Al at low pH in the brook trout (*Salvelinus fontinalis*). Hypothetically, Ni-induced limitation of gas diffusion at the gill would be evidenced by increased ventilatory effort concomitant with decreased efficiency of oxygen extraction from the water and an increase in oxygen consumption rate.

The second approach was to quantify, using morphometric techniques similar to those used by Hughes and co-workers (Hughes and Perry, 1976; Hughes et al., 1979), possible ultrastructural gill damage during acute waterborne Ni exposure. Theoretical estimates of gas exchange capacity could then be directly related to empirical measurements generated by the in vivo ventilatory techniques described above.

Finally, bypassing the gill and infusing Ni directly into the blood of fish kept in clean (Ni-free) water allowed us to load the blood with very high levels of Ni and avoid the confounding effects of branchial Ni accumulation from the water. The relative contributions of waterborne Ni and blood-bound Ni could then be evaluated by examining the extent, if any, of respiratory toxicity elicited solely by blood-bound Ni. Our goal with infusion of Ni into the blood was to create a plasma Ni profile very similar to that shown by Pane et al. (2003) in trout exposed acutely to waterborne

Ni in identical water quality. Having done so (see Fig. 2A), we present, where appropriate, data from waterborne Ni-exposed trout of Pane et al. (2003) to facilitate direct comparison with respiratory parameters measured during acute Ni infusion.

Furthermore, by comparing the accumulation of Ni in various tissues following acute arterial Ni infusion with accumulation patterns following acute waterborne Ni exposure (Pane et al., 2003), we hoped to shed light on some possible mechanisms whereby the rainbow trout regulates and excretes Ni in a tissue-specific manner.

2. Materials and methods

2.1. Experimental animals

Adult rainbow trout for use in ventilation (150–250 g) and infusion (200–350 g) experiments were purchased from Kinmount Fish Farm, Kinmount, Ont., and Humber Springs Trout Farm, Orangeville, Ont. Fish were acclimated for at least 2 weeks to aerated, flowing dechlorinated Hamilton tap water (of moderate hardness) from Lake Ontario at 12–14 °C and fed ad libitum several times weekly with commercial trout pellets. The composition of the food was: crude protein ≅ 40%, crude fat ≅ 11%, crude fibre ≅ 3.5%, calcium ≅ 1.0%, phosphorus ≅ 0.85%, sodium ≅ 0.45%, and Ni ≅ 3.86 mg kg^{-1} dry weight. Water composition was (in mM) Ca^{2+} ≅ 1, Mg^{2+} ≅ 0.2, Na^{+} ≅ 0.6, Cl^{-} ≅ 0.8, SO_4^{2-} ≅ 0.25, titratable alkalinity to pH 4.0 ≅ 1.9, background Ni ≅ 2 μg L^{-1}, dissolved organic carbon (DOC) ≅ 3 mg L^{-1}, total hardness (as $CaCO_3$) of approximately 140 mg L^{-1} and pH 7.9–8.0. Fish were starved 72 h prior to and throughout all experiments.

2.2. Ventilation experiments

2.2.1. Surgical procedures and exposure conditions

In order to measure ventilatory parameters on resting rainbow trout, fish were surgically fitted with an oral mask using a technique developed by Davis and Cameron (1971). In several preliminary experiments, an indwelling dorsal aortic cannula was also implanted prior to masking surgery following the technique of Soivio et al. (1972). Arterial oxygen tension (Pa_{O_2}) was then measured on masked fish (using blood drawn into an ice-cold, Li-heparinized Hamilton syringe, Radiometer oxygen cells and electrodes thermostatted to the correct temperature, and a Cameron oxygen meter) until a suitable masking procedure was developed that consistently produced normal values of Pa_{O_2} in masked fish (90–115 Torr). The details of the final procedure are outlined below.

Fish were anaesthetised with MS222 (0.075 g L^{-1} neutralised with NaOH) and placed on an operating table with anaesthetic solution irrigating the gills. A mask was made from a latex surgeon's glove. The glove was sliced open with the fingers and part of the thumb removed. The open thumb-hole was then placed over the fish's snout and sewn tightly around the mouth with 2.0 silk sutures placed every 2 mm, ensuring that the fish had full range of movement for opening and closing the mouth. Generally, approximately 40 sutures were needed per fish and each surgery typically lasted 20 min. The fish was then transferred to a large (8 L) van Dam style ventilation box, where several restraints prevented undue movement of the fish (see Fig. 1 of Davis and Cameron, 1971). First, the latex mask was stretched across a partition in the box and sealed tightly in place using a plexiglass restraining ring and nylon screws and wingnuts. Secondly the fish was slipped into smaller, open-ended restraining box (7 cm wide by 7 cm high by 25 cm long) that was mounted inside the larger ventilation box so as to prevent thrashing movement (the smaller restraining box also served to temporarily trap expired water, to mix it, and to prevent mixing with the bulk water of the posterior chamber). Additionally, a crossbar on the restraining ring prevented excessive forward movement. The overall effect was to have the snout of the fish extending past the partition of the box which formed a tightly sealed latex barrier between the water in the chamber anterior to the buccal cavity and the water in the chamber posterior to the opercular cavity. Once arranged, all water appearing in the posterior chamber must necessarily have passed through the mouth and over the gills. To ensure that this was the case, the ventilation boxes were fitted with a standpipe in each chamber, and water flow in excess of the ventilation volume was piped into the anterior chamber. When these standpipes were levelled precisely, the pulsatile overflow collected from the posterior standpipe was ventilated water (i.e. water

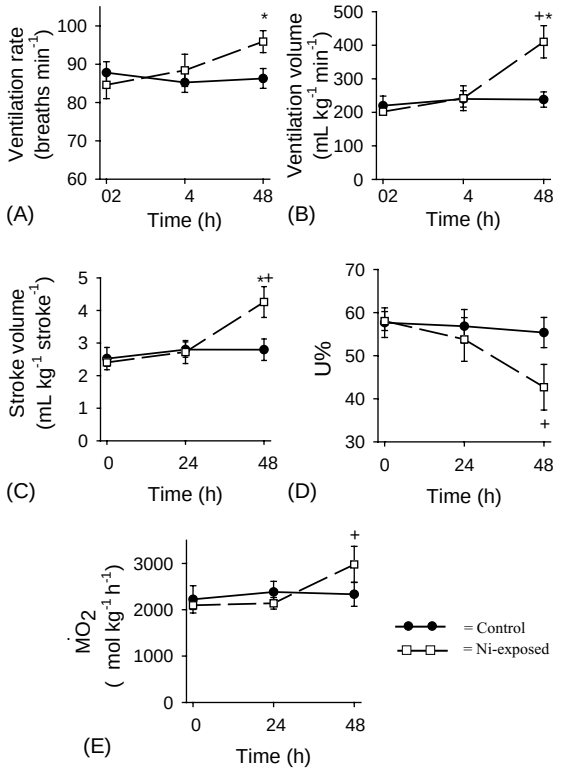

Fig. 1. Ventilatory parameters from adult rainbow trout exposed for 48 h to either control or 9.7 mg Ni L^{-1}. Data are expressed as mean ± 1 S.E.M. (n = 6–8). Asterisk (*) indicates significant difference ($P < 0.05$; two-tailed Student's t-test) from respective control mean; (+) indicates significant difference ($P < 0.05$; one-way ANOVA plus two-sided Dunnett's post hoc test) from time 0 h mean: (A) ventilation rate (V_R); (B) ventilation volume (V_G); (C) opercular stroke volume (V_{SV}); (D) oxygen extraction efficiency (U%); (E) resting oxygen consumption rate ($\dot{M}O_2$).

passed over the gills by each ventilatory stroke of the fish).

Once the fish and its latex mask were sealed into the box, the standpipes were arranged overnight so as to create approximately 5 cm of positive head pressure to facilitate ventilation and recovery. In the morning, the mask was checked visually for tears or gaps, and the standpipes adjusted to create 5 cm of negative head pressure to test for leaks. If the mask was leak-free and could hold 5 cm of head pressure for ten seconds or greater, the standpipes were levelled carefully, and the fish was observed for several minutes to ensure normal ventilation. The fish was then allowed several hours to recover before sampling or Ni exposure.

At all times, boxes were served with approximately 250 mL min^{-1} of water flowing into the anterior chamber. Boxes were painted black and covered with black plastic lids. Control water was served directly from an aerated head tank, while water to the Ni exposure boxes was routed (gravity-fed) through a mixing chamber before feeding the individual exposure boxes. Water temperature was 15.0 ± 0.5 °C. Immediately following a control (0 h) sampling, $NiSO_4 \cdot 6H_2O$ was added by gravity feed from a concentrated stock solution stored in a light-shielded stock bottle, into the vigorously aerated mixing chamber. Additionally, at this time, the mixing chamber and all individual exposure boxes were spiked with stock solution to bring them up to a measured dissolved Ni concentration of 9.7 mg Ni L^{-1}. Water samples were taken daily, 0.45-μm filtered, acidified with trace metal grade HNO_3, and analysed for dissolved Ni by graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian, Australia) against certified atomic absorption standards (Fisher Scientific). Only fish whose mask remained intact and functional for a full 48 h were included in the data set.

2.2.2. Sampling protocols

At 0 (control), 24 and 48 h of exposure, both groups of control (n = 6) and experimental (n = 8) fish were sampled using the following protocol. Firstly, ventilated water volume was collected for 3 min (three replicates of 1 min each) in pre-weighed vials without opening the lid to the box or otherwise disturbing the fish, as ventilation volume is extremely sensitive to disruption (Davis and Cameron, 1971). In the next 3 min, ventilation rate was counted visually (again, three replicates of 1 min each) by carefully opening the lid of the box from behind the fish and observing opercular movements.

Water samples for partial pressure of oxygen in expired water (P_EO_2) were then taken from the posterior chamber by means of two pieces of PE 160 tubing (Clay Adams) mounted on the inside of the interior, open-ended restraining box, 1–2 cm behind the posterior edge of the operculum. The two pieces of tubing were joined and run outside the box. To sample the expired water, a 1 mL Hamilton syringe (without the plunger) was attached to the tubing and allowed to fill by gravity at a rate of 1 mL min^{-1}. When the barrel was full, ten drops of water were allowed to overflow

and the plunger was carefully replaced so as to exclude air bubbles. The partial pressure of oxygen in the expired water (P_EO_2) was then measured as described above for Pa_{O_2}.

Water samples were then collected from in front of the fish's mouth in the anterior chamber with a one mL Hamilton syringe and the P_IO_2 (oxygen tension of inspired water) was measured as described above. P_IO_2 ranged from 145 to 155 Torr.

2.2.3. Calculations

Ventilation volume (V_G) (mL min^{-1} kg^{-1}) was calculated by dividing the volume of water collected in 1 min from the standpipe draining the posterior water chamber by the fish weight, and ventilatory stroke volume (V_{sv}) (mL kg^{-1} $stroke^{-1}$) was calculated by dividing the ventilation volume by the ventilation rate (strokes min^{-1}). Oxygen utilisation efficiency (U%) was calculated by the formula:

$$U\% = \frac{P_IO_2 - P_EO_2}{P_IO_2} \times 100 \quad (1)$$

Oxygen consumption rate ($\dot{M}O_2$) (μmol kg^{-1} h^{-1}) was calculated by the Fick principle using the formula:

$$\dot{M}O_2 = (P_IO_2 - P_EO_2) \times \alpha O_2 \times V_G \quad (2)$$

where αO_2 is the solubility co-efficient of oxygen in water at the experimental temperature (Boutilier et al., 1984).

2.3. Ni infusion and waterborne Ni experiments

2.3.1. Surgical procedures and exposure conditions

Adult rainbow trout were anaesthetised with MS222 (0.075 g L^{-1} neutralised with NaOH) on an operating table with anaesthetic solution irrigating the gills, surgically fitted with indwelling dorsal aortic catheters (Soivio et al., 1972), transferred to individual darkened plexiglass boxes (3 L) served with a water flow of 100 mL min^{-1} and continuous aeration, and allowed to recover for 48 h. After recovery, fish were divided randomly into four treatments—waterborne control (WC), waterborne Ni-exposed (WN), infused control (IC), and Ni-infused (IN).

Boxes receiving either clean water (WC, IC, and IN) or Ni solution (WN) were served via gravity feed as described above with a similar spiking procedure using a concentrated stock of $NiSO_4{\cdot}6H_20$ to yield a measured dissolved Ni concentration of 10.7 mg Ni L^{-1}. Water samples were taken daily and analysed for dissolved Ni as described above.

Infusates were delivered through dorsal aortic cannulae via a peristaltic pump at a rate of approximately 0.6 mL h^{-1} so as not to exceed 3 mL kg^{-1} h^{-1}, thereby avoiding complications of volume regulation (Grosell et al., 2001). The control infusate was 140 mM NaCl, while the Ni infusate was 140 mM NaCl spiked with $NiSO_4{\cdot}6H_2O$ to deliver approximately 0.24 μmol Ni kg^{-1} h^{-1}. This infusion rate was determined from preliminary experiments designed to yield a linear time course for the increase of plasma Ni which would best replicate the linear time course of plasma Ni accumulation during acute waterborne Ni exposure (Pane et al., 2003).

2.3.2. Sampling protocols

After 82 h of exposure, waterborne control and waterborne Ni-exposed fish (WC and WN) were killed by a blow to the head for morphometric analysis of gill condition. Infused fish (IC and IN) were blood sampled at 0 (initial control), 24, 48, 72, and 96 h, and arterial oxygen tension (Pa_{O_2}) was measured as described above. Plasma lactate concentration was measured on infused fish as an additional indicator of respiratory status. Plasma for lactate determination was separated by centrifugation of whole blood (drawn through the dorsal aortic cannula) at 13,000 × g for 1 min, immediately deproteinized in two volumes of ice cold 6% perchloric acid, frozen in liquid nitrogen, and stored at −20 °C until analysis. Lactate was measured enzymatically (L-lactate dehydrogenase/NADH at 340 nm; Sigma–Aldrich). Additionally, Ni concentration was determined in plasma aliquots from Ni-infused fish (IN) by GFAAS. Prior to Ni analysis, plasma aliquots were sonicated on ice for 5 s at 5 W (Microson; Misonix Inc., Farmingdale, NY).

2.3.3. Morphometric analysis

Immediately following the 82 h Pa_{O_2} sampling point in the waterborne treatments (WC and WN), two large chunks of filaments (~30–40 filaments each) were trimmed off the central part of the second gill arch on the left side of the fish. One set of filaments was wrapped in aluminum foil, placed in liquid nitrogen, and stored at −20 °C for later

analysis of gill Ni concentration, while the other set of filaments was immediately placed in ice-cold 2% gluteraldehyde in a 0.05 Sorenson's phosphate buffer (Hayat, 1981) for 90 min. The gluteraldehyde solution had been adjusted to pH 7.40 with 0.1 M NaOH and to an osmolarity of 325 mOsm with sucrose, and then vacuum filtered (0.22 μm). After 90 min of fixation, individual filaments (5–6) were separated under fixative and transferred to fresh 2% gluteraldehyde overnight at 4 °C. Filaments were then rinsed in 0.1 mM phosphate buffer, post-fixed in 1% osmium tetroxide, rinsed in phosphate buffer, dehydrated in a graded ethanol series, and embedded in Epon resin. One filament was embedded in each resin block.

Tissue blocks were oriented along the axis of the gill filament to allow for longitudinal (saggital) sectioning of the filament. Thick sections (1 μm) were cut with a Reichert Jung Ultracut microtome (Vienna, Austria) and stained with toluidine blue. Sections were examined and digitally captured with a Zeiss Microsystems LSM microscope. Digitally captured images were adjusted for sharpness and contrast only using Metamorph and Adobe Photoshop 6.0 software.

Among the morphometric parameters measured were various volume ratios and blood–water diffusion distances following closely techniques outlined in Hughes et al. (1979) (also see Pane et al., 2004). Briefly, a six-lined anisotropic Merz grid was laid over images of sections magnified approximately 1000×, and point counts were used to estimate relative volumes within secondary lamellae (see Hughes et al. (1979) for details). The volumes tabulated were: the volume of the entire lamellar region (V_{LR}) including tissue and water spaces, the volume of secondary lamellae (V_{SL}), and within the secondary lamellae—the volume of epithelial tissue outside the pillar system (V_{OPS}), and the volume of tissue within the pillar system (V_{PS}).

The ratios subsequently calculated were: the portion of the lamellar region occupied by secondary lamellae V_{SL}/V_{LR}, and the portion of the secondary lamellae occupied by tissue outside the pillar system (blood channels) V_{OPS}/V_{SL}. That portion of the lamellar region occupied by the pillar system, V_{PS}/V_{LR}, was then calculated by:

$$\frac{V_{PS}}{V_{LR}} = \left(1 - \frac{V_{OPS}}{V_{SL}}\right)\left(\frac{V_{SL}}{V_{LR}}\right) \quad (3)$$

Blood–water diffusion distances (BWDD) were measured at the same magnification using a Merz grid to randomise the points from which measurements were made (Wilson et al., 1994), and were measured from the intersection of the grid with the lamellar epithelium to the nearest erythrocytic surface. If the path between the epithelial intersection and the nearest erythrocyte crossed an empty blood channel, that measurement was discarded (Hughes et al., 1979).

Additionally, a relative diffusing capacity (D_{rel}; Hughes et al., 1979) was calculated as:

$$D_{rel} = \frac{(S/\text{BWDD})\text{exp}}{(S/\text{BWDD})\text{con}} \quad (4)$$

given that diffusing capacity is directly proportional to gas exchange area S and inversely proportional to BWDD. The scripts "exp" and "con" refer to experimental and control fish, respectively. The relative surface areas available for gas exchange (S) were estimated by counting the intersections of the test grid with the lamellar surface using the magnification and statistical details provided above. The analysis is based on the fact that the number of intersections per reference unit area of a randomly oriented test grid with a particular surface in a two-dimensional section is proportional to the surface area of that structure per unit volume (Underwood, 1970).

Additionally, lamellar heights, widths, and "perimeter" were measured. Perimeter is another means of estimating the relative surface area available for diffusion and was calculated according to the formula:

$$\text{Perimeter} = 2 \times (h - [0.5 \times w]) + (0.5 \times \pi \times w) \quad (5)$$

where h is the lamellar height and w is the lamellar width (Wilson et al., 1994). While this calculation makes the simplifying assumption that all lamellae are uniform in shape, it provides a useful estimate of lamellar surface area.

Because morphometric variation within fish is greater than between fish (Hughes et al., 1979), a large number of measurements were made per individual ($n = 5$ fish per treatment). Each fish was assigned a mean value for each parameter. For determination of volume ratios (V_{SL}/V_{LR}, V_{OPS}/V_{SL}, and V_{PS}/V_{LR}), a total of approximately 400 point counts was used for each fish using three fields of view per section, on two

sections per filament, two filaments per fish (Hughes et al., 1979). From the same fields, approximately 600 measurements of BWDD were made per fish. For determination of lamellar height, 20 lamellae were measured per section, two sections per filament, three filaments per fish. For lamellar width, three measurements (basal, central, and distal portions) were made per lamellae, 20 lamellae per section, two sections per filament, three filaments per fish (Wilson et al., 1994).

2.3.4. Tissue Ni concentrations

After 96 h of infusion, control (IC) and Ni-infused (IN) fish were killed by a blow to the head, and the gills were removed, frozen in liquid nitrogen, and stored at −20 °C for analysis of gill Ni concentration, similar to the treatment of gills from waterborne Ni-exposed (WN) and control (WC) fish. Additionally, the heart, spleen, liver, stomach, intestine, kidney, and a piece of white muscle were removed from infused fish, frozen in liquid nitrogen, and stored at −20 °C. After tissues were digested at 60 °C for 48 h in 1 N HNO_3 (Fisher Scientific; trace metal grade), the digest was homogenised by vortexing, centrifuged at 13,000 × *g* for 10 min and the supernatant diluted with double distilled water for analysis of Ni by GFAAS.

2.4. Statistical analyses

All measured and calculated values are presented as mean ± 1 standard error of the mean (S.E.M., *n*: number of fish). In time course experiments (Figs. 1 and 2), time-dependent responses in both control and experimental groups were tested against their respective 0 h values by a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison test. If there were only two treatments (control and Ni-exposed; i.e. Fig. 1), each experimental mean was compared to its simultaneous control mean by an unpaired two-tailed Student's *t*-test. Where each time point had four treatment means (Fig. 2B and C), all means at each time point were compared with a one-way ANOVA with a Bonferroni post hoc multiple comparison test. In all other cases (Fig. 4; Table 1), experimental means were compared to control means using an unpaired two-tailed Student's *t*-test (Table 1), or by a one-way ANOVA with a two-sided Dunnett's post hoc multiple comparison test (Fig. 4). Statistical significance in all cases was accepted at $P < 0.05$.

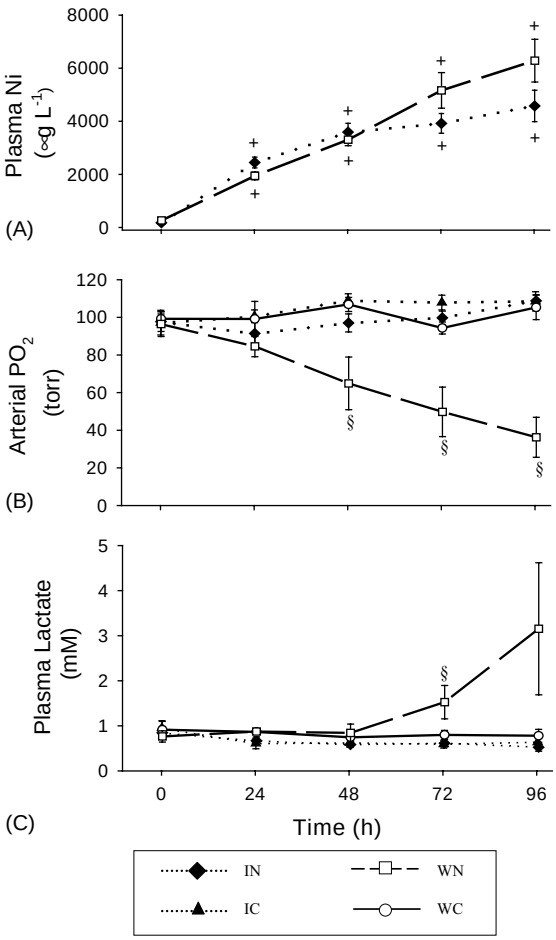

Fig. 2. Time course of plasma [Ni], arterial oxygen tension (Pa_{O_2}), and plasma [lactate] in adult rainbow trout acutely exposed to Ni either via infusion into the dorsal aorta (IN) at a rate of 0.24 μmol kg^{-1} h^{-1}, or via the water (WN) to a concentration of 11.7 mg Ni L^{-1}. Waterborne data are from Pane et al. (2003). Data are expressed as mean ± 1 S.E.M. ($n = 6$–8); (+) indicates significant difference ($P < 0.05$; one-way ANOVA plus two-sided Dunnett's post hoc test) from time 0 h mean; (§) indicates significantly different ($P < 0.05$; one-way ANOVA plus Bonferroni post hoc test) from all other simultaneous treatment means: (A) plasma [Ni]; (B) arterial oxygen tension (Pa_{O_2}). Time courses are included for both infused controls (IC) (140 mM NaCl infusate) and waterborne controls (WC). (C) Plasma [lactate]. Time courses are included for both infused controls (IC) (140 mM NaCl infusate) and waterborne controls (WC).

Table 1
Morphometric measurements of the gills of adult rainbow trout acutely exposed for 82 h via the water to either control (WC) or 10.7 mg Ni L^{-1} (WN)

	Control (WC)	Ni-exposed (WN)
V_{SL}/V_{LR}	0.521 ± 0.007	0.763 ± 0.051*
V_{OPS}/V_{SL}	0.606 ± 0.024	0.701 ± 0.018*
V_{PS}/V_{LR}	0.205 ± 0.018	0.223 ± 0.007
Blood–water diffusion distance (BWDD) (μm)	8.051 ± 0.118	10.190 ± 0.520*
D_{rel}	1.000	0.592
Lamellar height (μm)	225.92 ± 16.62	158.69 ± 11.50*
Lamellar width (μm)	22.81 ± 0.71	26.71 ± 1.33*
Lamellar perimeter (μm)	464.85 ± 33.52	332.08 ± 23.26*

Mean ± 1 S.E.M. ($n = 5$). Asterisk (*) indicates significant difference ($P < 0.05$; two-tailed Student's t-test) from respective control mean. V_{SL}/V_{LR} is the percent volume of the lamellar region (lying between the body of the filament and the distal tips of the lamellae) occupied by secondary lamellae. V_{OPS}/V_{SL} is the percent volume of the secondary lamellae occupied by tissue lying outside the pillar (blood channel) system, while V_{PS}/V_{LR} is that percent volume of the lamellar region occupied by tissue lying within the pillar system. D_{rel} is an index of relative diffusing capacity. Lamellar perimeter is an estimate of lamellar surface area (see Section 2 and Eqs. (1)–(3) for details).

3. Results

3.1. Ventilatory parameters

Acute waterborne Ni exposure had a marked impact on ventilatory parameters in the rainbow trout. Ventilation rate (+13%), ventilation volume (+103%), and ventilatory stroke volume (+52%) were all significantly increased relative to the control treatment after 48 h of exposure to 9.7 mg Ni L^{-1} (Fig. 1A–C). Additionally, there was a decrease in oxygen utilisation (−26%) and an increase in oxygen consumption rate (+42%) at 48 h. (Fig. 1D and E). The latter effects were significant relative to the time 0 values.

3.2. Ni infusion versus waterborne Ni exposure

Fig. 2A shows good agreement between the time courses (96 h) of plasma Ni accumulation following infusion at a rate of 0.24 μmol Ni kg^{-1} h^{-1} and following acute, high level waterborne Ni exposure (11.7 mg Ni L^{-1}, data from Pane et al. (2003)). Over 96 h of infusion, the concentration of Ni in the plasma increased roughly 30-fold, and the values were not significantly different between waterborne exposure and infusion treatments.

Infusion of the blood of rainbow trout with very high levels of Ni clearly had no effect on selected respiratory and metabolic parameters (Fig. 2B and C). Fig. 2B shows no effect of blood-bound Ni on arterial oxygen tension, in sharp contrast to the severe effects of waterborne Ni, which caused a marked decline in Pa_{O_2}. Additionally, plasma lactate (Fig. 2C) was unchanged by Ni infusion, despite being significantly increased during waterborne Ni exposure (Fig. 2C).

3.3. Morphometric analysis

Acute (82 h) Ni exposure via the water (10.7 mg Ni L^{-1}) significantly altered the branchial ultrastructure of experimental fish (Fig. 3; Table 1). Prominent lamellar swelling was evidenced by significant increases in the volume of the lamellar region occupied by secondary lamellae (V_{SL}/V_{LR}) and the volume of secondary lamellae occupied by epithelial tissue lying outside the pillar system (V_{OPS}/V_{SL}). These two parameters were increased by 46.4 and 15.8%, respectively. The integrity of lamellar blood channels was apparently unaffected by Ni exposure, as the volume of the lamellar region occupied by tissue within the pillar system (V_{PS}/V_{LR}) was not significantly different from control levels (Table 1). Blood–water diffusion distance (BWDD) was significantly elevated (+26.6%) in experimental fish, while the overall relative diffusing capacity of experimental fish was only 59.2% of the diffusing capacity of control fish (Table 1).

Lamellar height and width of experimental fish were significantly decreased (−29.8%) and increased (+17.1%), respectively, following acute Ni exposure (Table 1). Estimation of lamellar perimeter of

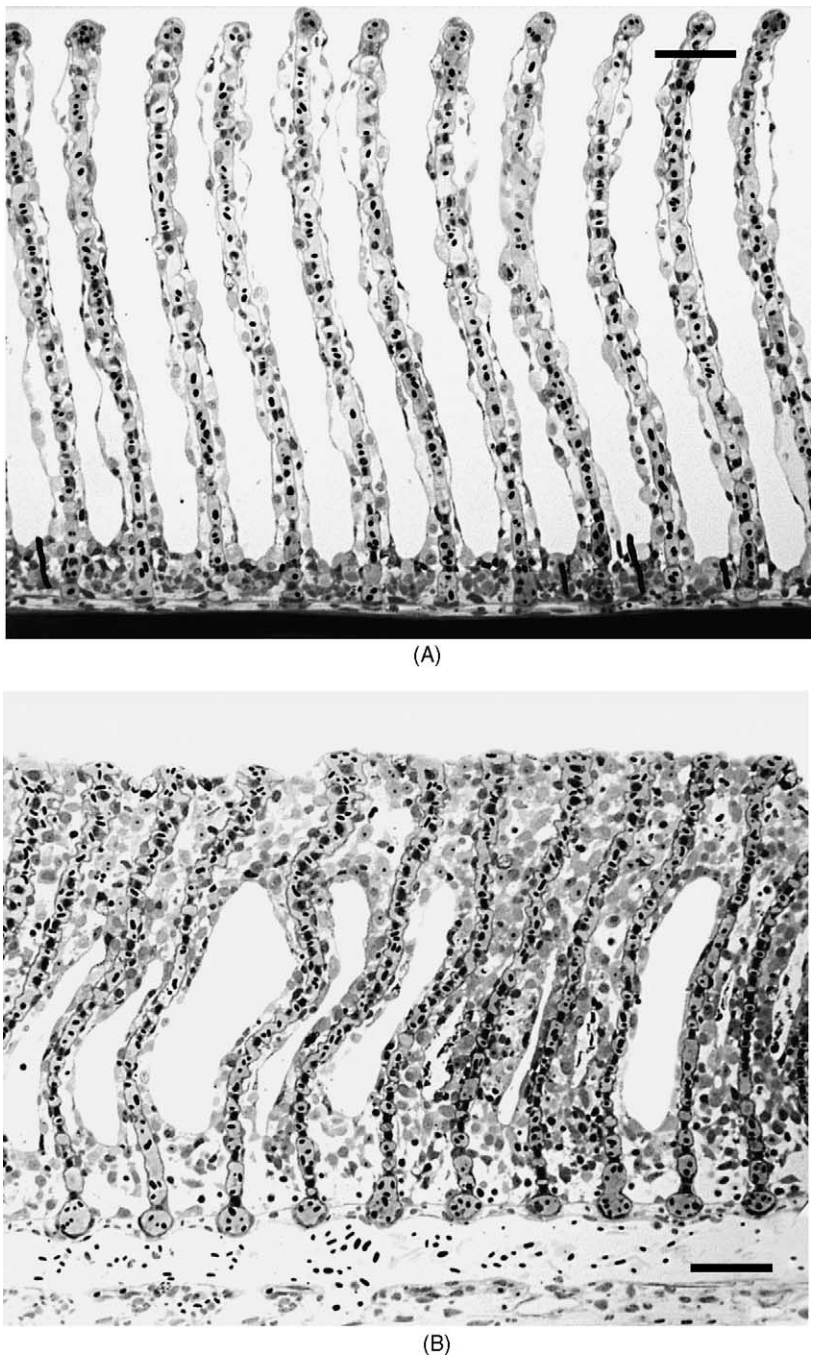

Fig. 3. Light micrographs of the secondary lamellar structure of gills from (A) waterborne control (WC) or (B) waterborne Ni-exposed fish (WN) (10.7 mg Ni L^{-1}; 96 h). Lamellae are completely fused in the distal portion and partially fused in the central and proximal regions (B). Sections are 1 μm, stained with Richardson's stain. Scale bars are 50 μm.

experimental fish yielded a value that was 28.6% lower than the control value (Table 1).

3.4. Tissue Ni burdens

Tissue Ni burdens following 96 h of Ni infusion are given in Fig. 4, along with parallel data from Pane et al. (2003) from fish exposed to waterborne Ni. It should be noted, however, that the gill Ni burdens from waterborne Ni-exposed fish (WN) are part of the current data set and are very close to those reported by Pane et al. (2003), while all other tissue burdens from waterborne Ni-exposed fish are from Pane et al. (2003). As the background levels of Ni were very similar in the two control groups (WC and IC) in all tissues, the control mean shown for each tissue is a combination of the two control means.

The terminal gill Ni burdens of rainbow trout exposed by the two different methods (waterborne and infusion) were markedly different from one another despite very similar plasma Ni concentrations (Fig. 4). While Ni-infused fish did not significantly accumulate Ni in white muscle, heart, liver, stomach, or intestine, as compared to tissue levels in control fish, levels of Ni in the gill of Ni-infused fish were significantly elevated, relative to controls, by 4.6-fold. In contrast, after waterborne Ni exposure, levels of Ni in the gill were 41.5 times higher than those of the control. Ni accumulation in the kidney was significantly elevated by 9.3-fold following both Ni infusion and waterborne exposure, with almost identical mean tissue burdens following both treatments.

4. Discussion

4.1. Branchial toxicity

Previously, our laboratory determined that waterborne Ni is an acute respiratory toxicant (Pane et al., 2003). The most obvious conclusion to be drawn from the present study is that the target organ of this acute respiratory toxicity is exclusively the gill. Bypassing the gill and infusing Ni directly into the blood did not adversely affect respiration in resting rainbow trout (Fig. 2B and C), despite the fact that Ni infusion caused substantial Ni loading in the plasma and organs other than the gill. Indeed, following 96 h of infusion,

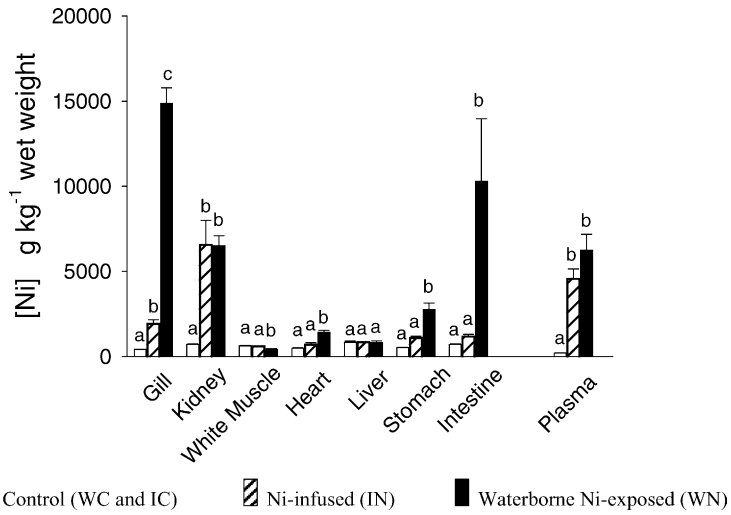

Fig. 4. Terminal tissue Ni burdens in adult rainbow trout acutely exposed to Ni either via infusion into the dorsal aorta (IN) at a rate of 0.24 μmol kg^{-1} h^{-1}, or via the water (WN) to a concentration of 11.7 mg Ni L^{-1}. Waterborne data for all tissues except gill are from Pane et al. (2003). Both waterborne control (WC) and infused control (IC) (140 mM NaCl infusate) tissue means were combined for simplicity. Data are expressed as mean ± 1 S.E.M. ($n = 6$–8). Bars not sharing the same letter are significantly different. Plasma Ni concentrations after 96 h of both treatments are given for reference (see Fig. 2A).

levels of Ni in the plasma (Figs. 2A and 4) and the kidney (Fig. 4) were very high and on par with those found following acute waterborne Ni exposure (Pane et al., 2003). Note that gill accumulation following infusion was markedly different than that following waterborne exposure (Fig. 4).

In the current study, we measured only plasma Ni and not blood Ni levels, and cannot, therefore, completely disregard the possibility that plasma Ni is completely excluded from erythrocytes during loading via infusion. It seems unlikely, however, that this is the case, given a 30-fold increase in plasma Ni levels (Fig. 2A). Regardless of the extent of erythrocytic Ni loading, however, Ni does not appear to affect gas exchange across the erythrocytic membrane, or any component of oxygen handling in the blood of rainbow trout. Despite the profound increase in plasma Ni in infused fish, arterial oxygen tension (Pa_{O_2}) was perfectly conserved after 96 h (Fig. 2B).

Accumulation of Ni in the gill of rainbow trout was almost 10 times higher following waterborne Ni exposure than following infusion of Ni into the blood (Fig. 4), and subsequently, arterial Pa_{O_2} was markedly impacted only during waterborne Ni exposure (Fig. 2B). Respiratory toxicity elicited by blockade of gas exchange appears to be dependent on a critical gill Ni burden that was apparently not reached during blood Ni infusion. In fact, a substantial portion of the Ni accumulated in the gill during Ni infusion was not actually incorporated into gill cells, rather it was an artifact of blood-bound Ni "trapped" within the branchial blood space. This explanation is supported by both the high degree of vascularization of the gill and the high plasma Ni concentration caused by Ni infusion (Fig. 4). From the work of Olson (1992), who estimated the plasma ^{125}I space of the gill of salmonids to be 12%, and from our plasma Ni data, we calculated that a minimum of 33% of newly accumulated gill Ni following Ni infusion was present simply as blood-bound Ni. A similar calculation using the data of Pane et al. (2003) yielded a value of only 4% for the gills of fish acutely exposed to waterborne Ni. Because we used plasma Ni values, any appreciable erythrocytic Ni content in the larger blood space would contribute still further to this trapped blood effect. Backloading of Ni from the blood into gill cells, therefore, appears to account for only a portion of branchially-accumulated Ni.

4.2. Morphological effects of acute waterborne Ni exposure

The specific toxic mechanism of waterborne Ni is to damage the delicate ultrastructure of the thin respiratory epithelium of the gill. Significant changes to the branchial ultrastructure given in Table 1 are indicative of an increased volume of lamellar epithelial tissue overlying the blood pillar system. These included increased V_{SL}/V_{LR}, V_{OPS}/V_{SL}, lamellar width, and BWDD, and decreased lamellar height and perimeter (Table 1). An increase in the volume of tissue overlying the pillar system impairs gas exchange function and leads to arterial hypoxemia and subsequent respiratory acidosis, such as observed during acute Ni exposure (Fig. 2B; Pane et al., 2003). Furthermore, like most waterborne toxicants (as reviewed by Mallat, 1985), Ni did not significantly alter the pillar system. Although occasional lamellar hemorrhaging was observed in extreme instances (data not shown), the volume of lamellar tissue occupied by the pillar system (V_{PS}/V_{LR}) in experimental fish was not significantly different from that of control fish (Table 1). This, coupled with the lack of respiratory toxicity observed during Ni loading by infusion (Fig. 2B and C), further implicates the respiratory epithelium as the specific site of waterborne Ni-induced damage responsible for marked hypoxemia.

Increased epithelial tissue volume can be driven both by hyperplasia and hypertrophy of lamellar epithelial cells, and both are cited by Mallat (1985) as common responses to waterborne irritants. Proliferation of mucous cells within the central and distal portions of secondary lamellae, in conjunction with hypersecretion of mucus and subsequent sloughing of mucus-bound metal, are common defences against metal exposure (Mallat, 1985). Indeed, hyperplastic mucous cells were observed within the secondary lamellae following acute Ni exposure (data not shown), and could partly explain the increase in volume of epithelial tissue.

Hypertrophy of respiratory (pavement) cells can also contribute to swelling of the respiratory epithelium. Indeed, modestly hypertrophic pavement cells caused subtle increases in the two morphometric parameters V_{SL}/V_{LR} and V_{OPS}/V_{SL} following chronic, low-level Ni exposure in rainbow trout (Pane et al., 2004). Extensive hypertrophy observed in the present

study was severe enough to frequently cause epithelial lifting, whereby the respiratory epithelial detached from the basal lamina lying between the epithelium and the underlying pillar cell system. Again, such a response is common during severe toxicant exposure (Mallat, 1985), and was also observed by Nath and Kumar (1989) upon exposing the tropical freshwater perch (*Colisa fasciatus*) to waterborne Ni at a fairly comparable level of 14 mg Ni L^{-1}. To what extent this response within the gills of rainbow trout is driven by an allergic reaction to Ni is currently unknown. Ni is a moderate contact allergen in humans (Kligman, 1966), and the contribution of the immune system during acute, high-level Ni challenge in teleosts deserves further investigation.

Given the extent of hypertrophy of the lamellar epithelium observed in the present study, it is surprising that we saw no ionoregulatory disturbance (but comparable respiratory disturbance) in our previous study with trout exposed to a virtually identical level of waterborne nickel for a similar period (Pane et al., 2003). Certainly, hypertrophy is often indicative of a disruption of cellular homeostasis which might in turn disrupt active transport, and the observed epithelial lifting and occasional hemorrhaging would intuitively suggest an increased diffusive permeability of the gills. However, structure does not always reflect physiology, especially fine structure which can undergo subtle changes (e.g. in the fixative) once the tissues are removed form the organism.

4.3. Ventilatory response and transfer of oxygen at the gill

The ultrastructural damage observed in fish exposed acutely to Ni via the water (Table 1) results in two pathologies that can readily explain the decreased oxygen extraction by the gill (U%; Fig. 1D). One is the significant elevation of the blood to water diffusion distance (BWDD; Table 1), resulting in a physical barrier to oxygen diffusion from the water to the blood. The other is a reduction in interlamellar water channel space (increased lamellar width and decreased lamellar height; Table 1) and a concomitant ventilatory convective limitation. Subsequently, to offset reduced U%, a hyperventilatory response was mounted (increased V_G; Fig. 1B), driven more by an increase in V_{SV} (74%; Fig. 1C) than by increased V_R (13%; Fig. 1A). This pattern of small V_R increase and large V_{SV} increase is typical of rainbow trout exposed to hypoxia (Davis and Cameron, 1971), and likely reflects the lower energetic cost of increasing V_{SV} as compared to increasing V_R (Perry and Wood, 1989). A response of very similar magnitude was seen in brook trout acutely challenged with waterborne Al and low pH (Walker et al., 1988).

An additional cause of reduced U% at increased V_G stems from changes in gill musculature needed to reduce the resistance to water flow over the gill. Such changes physically constrain increased anatomical dead space and further reduce oxygen extraction from the water during hyperventilation (Hughes, 1966; Holeton and Randall, 1967; Cameron and Cech, 1970). Given that 5–15% of $\dot{M}O_2$ is the cost of ventilation in resting fish (Cameron and Cech, 1970), and resting $\dot{M}O_2$ increases with hyperventilation (Perry and McDonald, 1993), it is not surprising that resting $\dot{M}O_2$ was significantly increased at 48 h of Ni exposure (Fig. 1E).

4.4. Tissue Ni accumulation—infusion versus waterborne exposure

Comparison of tissue Ni levels in rainbow trout following acute infusion (Fig. 4) with those accumulated during acute waterborne Ni exposure (Pane et al., 2003) yields several interesting points. It has been well documented that following both acute and chronic waterborne exposure, Ni accumulation in the kidney is marked (Pane et al., 2003; Calamari et al., 1982). Mean kidney Ni burden following infusion was very similar to that accumulated during acute waterborne Ni exposure (Fig. 4). Given that plasma Ni levels were very similar, also, in these two studies, the data suggest that renal Ni concentration is proportional to plasma (blood) Ni level. A trapped plasma analysis for the kidney revealed that only approximately 18% of newly accumulated Ni following infusion can be accounted for by blood-bound Ni, and that similarly, 29% of newly accumulated Ni following acute waterborne Ni exposure (Pane et al., 2003) can be accounted for by blood-bound Ni. These calculations imply that upto 80% of Ni in the kidney of rainbow trout may be accumulated within renal cells, an important finding with respect to possible acute and chronic Ni-induced disruption of renal function. Accordingly,

this is an active area of research within our laboratory.

Ni in human blood is bound to three carriers, one of which is the low molecular weight amino acid histidine (Abdulwajid and Sarkar, 1983). Blood-bound Ni, therefore, is presumably subject to high rates of ultrafiltration in the glomeruli of the kidney and one would expect high levels of Ni in the urine concomitant with high blood levels. Indeed, single bolus injections of Ni in mammals are quickly and almost exclusively cleared via the kidney and renal clearance is the primary excretory route of blood-bound Ni (USEPA, 1986; Eisler, 1998). The physiological implications of such marked accumulation of Ni in the kidney remain to be investigated.

Renal Ni distribution patterns, then, are different from those of the gill, or intestine, where the pattern is one of major contribution by blood-bound Ni following infusion versus only minor contribution following acute waterborne Ni exposure. The data for gill Ni accumulation strongly suggest that branchial Ni must be either cellurarly incorporated or surficially bound during aqueous exposure. Additionally, these data suggest that urine Ni concentrations would be similar between the two treatments.

In contrast, the accumulation patterns in the stomach, and intestine (plus pyloric cecae) were markedly different depending on the route of exposure. The extent of accumulation following waterborne exposure greatly exceeded that from infusion. For example, via the water, the stomach and intestine accumulated 8.1 and 33.8 times more Ni than control fish, respectively, but only 2.1 and 1.7 times more than control fish when Ni was delivered as an infusate (Fig. 4). Olson (1992) estimated salmonid ^{125}I plasma space of the stomach and intestine (plus pyloric cecae) to be 3 and 5%, respectively, again with the caveat that erythrocytic Ni would contribute still further to overall tissue burden. It would take only 14% vascularization of the stomach and intestine to account for all of the increase in Ni burden in these tissues of Ni-infused fish, and it can be calculated that a minimum of 17 and 48% of the newly accumulated Ni of the stomach and intestine, respectively, can be explained by blood-bound Ni. Similar calculations using data from rainbow trout acutely exposed to waterborne Ni (Pane et al., 2003) yield values of only 6 and 4%, respectively, for these two tissues. As plasma (blood) Ni levels were very similar in the two studies, the data suggest that exposure to high levels of waterborne Ni caused either stress-induced drinking, or marked “stripping” of plasma Ni—predominantly by the intestine. It should be noted that acute waterborne Ni exposure did not cause significant Ni accumulation in either the liver or the bile (Pane et al., 2003), suggesting that biliary excretion is a relatively unimportant pathway in trout and that intestinal Ni is not likely due to hepatic clearance.

5. Conclusions

In summary, we present evidence that the site of acute waterborne Ni toxicity is exclusively the gill. In resting rainbow trout, extensive gill Ni accumulation and ultrastructural damage during acute waterborne exposure were consistent with marked effects on ventilation. By extension, acutely perturbed ventilation is consistent with hypoxemia, hypercarbemia, and respiratory acidosis observed earlier by Pane et al. (2003) during acute Ni exposure. Additionally, the present data provide physiological, mechanistic explanations for the toxicological finding of Meyer et al. (1999) that gill Ni burden is a constant predictor of acute toxicity, even across a wide range of water hardness. Accordingly, this combination of physiological and toxicological data should facilitate modelling of acute respiratory toxicity by a mechanistically based metal-ligand binding approach (i.e. the biotic ligand model, or BLM; DiToro et al. (2001)), previously attempted only with ionoregulatory toxicants such as Ag or Cu.

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References

Abdulwajid, A.W., Sarkar, B., 1983. Nickel-sequestering renal glycoprotein. Proc. Natl. Acad. Sci. U.S.A. 80, 4509–4512.

Boutilier, R.G., Heming, T.A., Iwama, G.K., 1984. Physico-chemical parameters for use in fish respiratory physiology. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 10A. Academic Press, London.

Calamari, D., Gaggino, G.F., Pacchetti, G., 1982. Toxicokinetics of low levels of Cd, Cr, Ni and their mixture in long-term treatment on *Salmo gairdneri* Rich. Chemosphere 11, 59–70.

Cameron, J.N., Cech, J.J., 1970. Notes on the energy cost of gill ventilation in teleosts. Comp. Biochem. Physiol. 34, 447–455.

Davis, J.C., Cameron, J.N., 1971. Water flow and gas exchange at the gills of rainbow trout, *Salmo gairdneri*. J. Exp. Biol. 54, 1–18.

DiToro, D.M., Allen, H.E., Bergman, H.L., Meyer, J.S., Paquin, P.R., Santore, R.C., 2001. Biotic ligand model of the acute toxicity of metals. 1. Technical basis. Environ. Toxicol. Chem. 20, 2383–2399.

Eisler, R., 1998. Nickel hazards to fish, wildlife, and invertebrates: a synoptic review. US Geological Survey, Biological Resources Division, Biological Science Report 1998–2001.

Grosell, M., McGeer, J.C., Wood, C.M., 2001. Plasma copper clearance and biliary copper excretion are stimulated in copper-acclimated trout. Am. J. Physiol. 280, R796–R806.

Hayat, M.A., 1981. Fixation for Electron Microscopy. Academic Press, New York.

Holeton, G.F., Randall, D.J., 1967. The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of the rainbow trout. J. Exp. Biol. 46, 317–327.

Hughes, G.M., 1966. Morphometrics of fish gills. Resp. Physiol. 14, 1–25.

Hughes, G.M., Perry, S.F., 1976. Morphometric study of trout gills: a light microscopic method suitable for the evaluation of pollutant action. J. Exp. Biol. 64, 447–460.

Hughes, G.M., Perry, S.F., Brown, V.M., 1979. A morphometric study of effects of nickel, chromium and cadmium on the secondary lamellae of rainbow trout gills. Water Res. 13, 665–679.

Jensen, F.B., Andersen, N.A., Heisler, N., 1987. Effects of nitrite exposure on blood respiratory properties, acid–base and electrolyte regulation in the carp (*Cyprinus carpio*). J. Comp. Physiol. B 157, 533–541.

Jensen, F.B., Nikinmaa, M., Weber, R.E., 1993. Environmental perturbations of oxygen transport in teleost fishes: causes, consequences and compensations. In: Rankin, J.C., Jensen, F.B. (Eds.), Fish Ecophysiology. Chapman & Hall, London.

Kligman, A.M., 1966. The identification of contact allergens by human assay. III. The maximization test: a procedure for screening and rating contact sensitizers. J. Invest. Dermatol. 47, 393–409.

Mallat, J., 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can. J. Fish. Aquat. Sci. 42, 630–648.

Meyer, J.S., Santore, R.C., Bobbitt, J.P., DeBrey, L.D., Boese, C.J., Paquin, P.R., Allen, H.E., Bergman, H.L., DiToro, D.M., 1999. Binding of nickel and copper to fish gills predicts toxicity when water hardness varies, but free-ion activity does not. Environ. Sci. Tech. 33, 913–916.

Nath, K., Kumar, N., 1989. Nickel-induced histopathological alterations in the gill architecture of a tropical freshwater perch, *Colisa fasciatus* (Bloch, *Colisa fasciatus* (Bloch & Schn.). Sci. Tot. Environ. 80, 293–299.

Olson, K.R., 1992. Blood and extracellular fluid volume regulation: role of the rennin–angiotensin system, kallikrein–kinin system, and atrial natriuretic peptides. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 12B. Academic Press, London.

Pane, E.F., Richards, J.G., Wood, C.M., 2003. Acute waterborne nickel toxicity in the rainbow trout (*Oncorhynchus mykiss*) occurs by a respiratory rather than ionoregulatory mechanism. Aquat. Toxicol. 63, 65–82.

Pane, E.F., Haque, A., Goss, G.G., Wood, C.M., 2004. The physiological consequences of exposure to chronic, sublethal waterborne Ni in the rainbow trout (*Oncorhynchus mykiss*): exercise versus resting physiology. J. Exp. Biol. 207, 1249–1261.

Perry, S.F., Wood, C.M., 1989. Control and co-ordination of gas transfer in fishes. Can. J. Zool. 67, 2970–2991.

Perry, S.F., McDonald, D.G., 1993. Gas exchange. In: Evans, D.H. (Ed.), The Physiology of Fishes. CRC Press, Boca Raton.

Soivio, A., Westman, K., Nyholm, K., 1972. Improved method of dorsal aorta catheterization: hematological effects followed for three weeks in rainbow trout (*Salmo gairdneri*). Finn. Fish Res. 1, 11–21.

Underwood, E.E., 1970. Quantitative Stereology. Addison-Wesley, London.

USEPA, 1986. Health assessment document for nickel and nickel compounds. EPA Report 600/8-83/012FF, 460 pp.

Walker, R.L., Wood, C.M., Bergman, H.L., 1988. Effects of low pH and aluminum on ventilation in the brook trout (*Salvelinus fontinalis*). Can. J. Fish. Aquat. Sci. 45, 1614–1622.

Wilson, R.W., Bergman, H.L., Wood, C.M., 1994. Metabolic costs and physiological consequences of acclimation to aluminum in juvenile rainbow trout (*Oncorhynchus mykiss*). 2. Gill morphology, swimming performance, and aerobic scope. Can. J. Fish. Aquat. Sci. 51, 536–544.