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The physiological consequences of exposure to chronic, sublethal waterborne nickel in rainbow trout (*Oncorhynchus mykiss*): exercise *vs* resting physiology

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Summary

In rainbow trout (*Oncorhynchus mykiss*), following chronic (42 day) exposure to both 384 μg Ni l^{-1} and 2034 μg Ni l^{-1}, Ni accumulation was greatest in the gill, kidney and plasma, with the plasma as the main sink for Ni. Indeed, trapped plasma analysis revealed that extensive loading of Ni in the plasma accounted for substantial percentages of accumulated Ni in several tissues including the liver and heart. Accumulated Ni in the gill and kidney was less dependent on plasma Ni concentration, suggesting a more intracellular accumulation of Ni in these tissues.

We present evidence for a clear, persistent cost of acclimation to chronic, sublethal Ni exposure. Chronic (40–99 day) exposure to sublethal waterborne Ni (243–394 μg Ni l^{-1}; ~1% of the 96 h LC_{50}) impaired the exercise physiology, but not the resting physiology, of rainbow trout. Ni acted as a limiting stressor, decreasing maximal rates of oxygen consumption ($\dot{M}_{O_2,max}$) during strenuous exercise in trout exposed for 34 days to sublethal Ni. This drop in high-performance gas exchange was attributed mainly to a reduction in relative branchial diffusing capacity (D_{rel}) caused by thickening of secondary lamellae. Morphometric analysis of the gills of chronically exposed fish revealed overall swelling of secondary lamellae, as well as hypertrophic respiratory epithelia within secondary lamellae. Additionally, contraction of the lamellar blood pillar system and narrowing of interlamellar water channels occurred, possibly contributing to decreased high-performance gas exchange. Decreased aerobic capacity persisted in fish previously exposed to nickel despite a clean-water exposure period of 38 days and an almost complete depuration of gill Ni, suggesting that extrabranchial mechanisms of chronic Ni toxicity may also be important.

Chronic impairment of such a dynamically active and critical organ as the gill may depress the overall fitness of a fish by impairing predator avoidance, prey capture and migration success with obvious environmental implications.

Key words: nickel toxicity, acclimation, exercise, gill, *Oncorhynchus mykiss.*

Introduction

Mechanistic investigations of a waterborne toxicant acting both acutely and chronically on aquatic fauna provide valuable information about the toxicant. Studies investigating the toxic mechanism of an environmental contaminant presented to an aquatic organism in an acutely lethal fashion generally yield a diagnostic cause of death. Using the most often studied freshwater teleost, the rainbow trout (*Oncorhynchus mykiss)*, this type of acute diagnostic study has been conducted for many trace metals, including Cu (Laurén and McDonald, 1986), Al (Playle et al., 1989), Ag (Wood et al., 1996), Mo (Reid, 2002) and Ni (Pane et al., 2003). These studies provide valuable information for modeling the acute toxicity of trace metals by incorporating a component of the unique physiological impact of each toxicant (e.g. the biotic ligand model, or BLM; see Di Toro et al., 2001 and Paquin et al., 2002 for detailed explanations of this model).

An obvious limitation of this acute diagnostic approach, however, is that the concentrations used in these studies are often environmentally unrealistic. In the case of acute waterborne Ni exposure, adverse effects on gill ultrastructure and respiration in freshwater fish have been investigated using concentrations of 3200 μg Ni l^{-1} (Hughes and Perry, 1976; Hughes et al., 1979; rainbow trout), 11 700 μg Ni l^{-1} (Pane et al., 2003a; rainbow trout), 10 700 μg Ni l^{-1} (E. F. Pane, A. Haque and C. M. Wood, submitted; rainbow trout) and 14 000 μg Ni l^{-1} (Nath and Kumar, 1989; *Colisa fasciatus*). These acute concentrations are far higher than concentrations of Ni found in contaminated freshwaters (typically <500 μg l^{-1}; Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998). Additionally, the information gained typically comes from animals completely out of balance with their environment and fighting a losing battle with a toxicant.

Chronic, sublethal exposure, however, allows one to investigate steady-state conditions that exist between an

aquatic organism and a toxicant and the processes of acclimation leading to this steady state. When applied to a general target tissue, the acclimation phenomenon can be divided temporally into three phases: (1) the 'shock', or damage, phase, during which the morphology and physiology of the target tissue are disturbed, (2) a defense phase, during which tissue-specific responses are mounted in an attempt to decrease the rate of influx or accumulation of the toxicant and (3) a recovery phase, during which compensation and repair occur to restore perturbed physiological processes and increase resistance to the toxicant (McDonald and Wood, 1993).

In the present study, we concentrated on the third phase and examined the physiology of juvenile and adult rainbow trout following 40–99 days of exposure to sublethal concentrations of waterborne Ni. Initially, a concentration of 2034 μg Ni l^{-1} was used as a screening tool to gauge the impact of a relatively high (partially lethal) chronic concentration. This concentration is 13% of the 96-h LC_{50} for juvenile trout in the same Hamilton city tap water (Pane et al., 2003) and 6% of the 96-h LC_{50} for adult trout (Segner et al., 1994). While such a concentration is probably greater than Ni concentrations measured in the most heavily contaminated industrial sites (Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998), it served as a reference point, as we know of only two other studies that have examined the effects of chronic waterborne Ni exposure on freshwater fish. Pickering (1974) assessed the reproductive effects of chronic Ni exposure, while Calamari et al. (1982) examined the kinetics of Ni accumulation. Neither study investigated physiological mechanisms.

Most of the experiments detailed herein were conducted at concentrations between 243 μg Ni l^{-1} and 394 μg Ni l^{-1}. These concentrations fall within the range of Ni concentrations found in watersheds heavily impacted by mining and industrial activity (Chau and Kulikovsky-Cordeiro, 1995; Eisler, 1998), and the values are only approximately 2% and 1% of the 96-h LC_{50} values for juvenile and adult trout, respectively. These concentrations are entirely sublethal, and our goal was to extensively characterize the physiology of rainbow trout chronically acclimated to this range of waterborne Ni concentrations. Wilson et al. (1994) conducted similar chronic, sublethal exposures with rainbow trout and Al and showed increased energy expenditures associated with exposure. Therefore, we also set out to document whether similar costs of acclimation occurred during chronic, sublethal Ni exposure.

Materials and methods

Experimental animals

Juvenile (10–50 g) and adult (200–350 g) *Oncorhynchus mykiss* Walbaum were purchased from Humber Springs Trout Farm, Orangeville, ON, Canada. Fish were acclimated for at least two weeks to aerated, flowing dechlorinated Hamilton tap water from Lake Ontario at 12–14°C and fed *ad libitum* several times weekly with commercial trout pellets. Water composition was: Ca^{2+} ~1 mmol l^{-1}, Mg^{2+} ~0.2 mmol l^{-1}, Na^{+} ~0.6 mmol l^{-1}, Cl^{-} ~0.8 mmol l^{-1}, SO_4^{2-} ~0.25 mmol l^{-1}, titratable alkalinity to pH 4.0 ~1.9 mmol l^{-1}, background Ni ~4 μg l^{-1}, dissolved organic carbon (DOC) ~3 mg l^{-1}, total hardness (as $CaCO_3$) ~140 mg l^{-1} and pH ~ 7.9–8.0. Fish were starved at least 24 h prior to and throughout all experiments.

Chronic exposure conditions

In all exposures, Ni was delivered as $NiSO_4.6H_2O$ by gravity feed from a concentrated stock solution in a flow-through set-up with dechlorinated Hamilton tap water. Three exposure regimes were used: (1) juvenile trout (20–50 g) were exposed to either control, 384 μg Ni l^{-1} or 2034 μg Ni l^{-1} for 42 days, (2) adult trout (200–350 g) were exposed to either control or 243 μg Ni l^{-1} for 40 days and (3) juvenile trout (10–20 g) were exposed initially to either control or 394 μg Ni l^{-1} for 99 days, followed by 38 days of exposure (of both groups of fish) to clean water. In all experiments, fish were fed 1% of their body mass daily. The composition of the food was: crude protein ~40%, crude fat ~11%, crude fiber ~3.5%, Ca ~1.0%, P ~0.85%, Na ~0.45% and Ni ~3.86 mg kg^{-1} dry mass. Water samples for analysis of dissolved Ni were taken every other day, 0.45-μm filtered, acidified with trace metal grade HNO_3 (Fisher Scientific, Nepean, ON, Canada) and analyzed for dissolved Ni by graphite furnace atomic absorption spectrophotometry (GFAAS; 220 SpectrAA; Varian, Palo Alto, CA, USA) against certified atomic absorption standards (Fisher Scientific). Atomic absorption values were normalized to an independent reference standard (Fisher Scientific) interspersed at every 10 samples. The accepted recovery limits of this reference standard were 90–110%.

Sampling protocols – Experiment 1

On days 12 and 24 of the exposure, critical swimming speed (U_{crit}) was determined in fish exposed to either control, 384 μg Ni l^{-1} or 2034 μg Ni l^{-1}. The evening before the swim trial, fish were transferred in groups of five (all from the same exposure tank) to a large (~150 liter) swim respirometer and left overnight in clean, flowing dechlorinated Hamilton city tap water. Fish were then swum in clean water at 1-h intervals, increasing water speed by 7 cm s^{-1} during each interval, until exhaustion (as determined by each fish being impinged on the rear screen of the respirometer and refractive to physical stimulation). Fork length was measured to the nearest 0.1 cm, and individual U_{crit} values were calculated according to the formula of Brett (1964):

$$U_{crit} = \mathbf{V}_f + \left(\frac{T}{t} * \delta\mathbf{V} \right) \quad (1)$$

where U_{crit} is measured in cm s^{-1}, t is the time increment (in min), T is the time spent at the final speed ($\mathbf{V}_f$) before exhaustion, and $\delta\mathbf{V}$ is the speed increment in cm s^{-1}. Individual U_{crit} values were then converted to body lengths per second (*BL* s^{-1}).

On day 42, fish in all three treatments were euthanized by

an overdose of MS-222 and placed on ice. A blood sample was then taken by caudal puncture and, following brief centrifugation (14 000 ***g*** for 1 min), the plasma was frozen in liquid nitrogen and stored at –80°C for later analysis. Tissues surgically removed to measure Ni concentration included the gills, heart, liver, stomach, intestine, kidney and white muscle. After tissues were digested at 60°C for 48 h in trace metal grade 1 mol l^{-1} HNO_3, the digest was homogenized by vortexing, centrifuged at 14 000 ***g*** for 10 min and the supernatant diluted with double-distilled water for Ni analysis by GFAAS as described above.

Prior to all analyses, plasma samples were sonicated on ice for 5 s at 5 W (Microson; Misonix Inc, Farmingdale, NY, USA) to ensure homogeneity. Plasma [Na^+], [Ca^{2+}] and [Mg^{2+}] were determined by flame atomic absorption spectrophotometry (FAAS; 220FS SpectrAA; Varian), while plasma [Cl^-] was measured by the mercuric thiocyanate method (Zall et al., 1956). Plasma [Ni] was determined by GFAAS as described above. Plasma protein was determined using Bradford reagent (Bradford, 1976) and bovine serum albumin standards (Sigma-Aldrich, St Louis, MO, USA). Plasma total ammonia and lactate concentrations were determined enzymatically (glutamate dehydrogenase/NADP and L-lactate dehydrogenase/NADH, respectively; Sigma-Aldrich). Prior to analysis, plasma for lactate was deproteinized in two volumes of 6% perchloric acid (Milligan and Wood, 1986). Plasma cortisol was determined using an ^{125}I radioimmunoassay (ICN Biomedicals, Montreal, QC, Canada) with radioactivity measured by γ counting (Minaxi γ; Canberra-Packard, Meriden, CT, USA).

Sampling protocols – Experiment 2

On day 40, adult trout (200–350 g) chronically exposed to either control or 243 μg Ni l^{-1} were anesthetized with 0.075 g l^{-1} of MS-222 (neutralized with NaOH; pH 8.0) and fitted with indwelling dorsal aortic catheters (Soivio et al., 1972). During surgery, the anesthetic solution irrigating the gills of chronically Ni-exposed fish was spiked with $NiSO_4.6H_2O$ to yield an Ni concentration comparable to that to which these fish had been chronically exposed. Post surgery, fish were transferred to individual darkened Plexiglas chambers (3 liter) served with a water flow of 100 ml min^{-1} and continuous aeration and allowed to recover for 48 h prior to sampling on day 42. Boxes housing chronically Ni-exposed fish received a comparable Ni solution delivered from a stock solution by gravity flow as described above.

After recovery, control and experimental fish (*N*=9; both treatments) were sampled once for the various parameters shown in Table 1. The sampling protocol closely followed that of Wood et al. (1996), as described in Pane et al. (2003a). Each fish was sampled as follows: ventilation rate was counted visually and then water samples from in front of the mouth of each fish were filtered (0.45 μm) and analyzed for dissolved Ni by GFAAS as described above. Unfiltered water samples were then taken for inspired O_2 tension (P_{IO_2}) and inspired pH (pH_I).

Table 1. *Arterial blood gases, acid–base balance, hematological and ventilatory parameters, and water balance in control and chronically acclimated adult rainbow trout (243 μg Ni l^{-1}; 42 days)*

	Control	Ni-exposed
Arterial P_{O_2} (Pa_{O_2}) (torr)	109.4±2.4	111.5±4.1
Arterial P_{CO_2} (Pa_{CO_2}) (torr)	2.63±0.16	2.43±0.20
Arterial pH (pHa)	7.867±0.015	7.856±0.020
HCO_3^- (mmol l^{-1})	8.89±0.54	7.81±0.59
Hematocrit (%)	22.14±2.83	24.18±2.12
Hemoglobin (g dl^{-1})	5.68±1.02	5.93±0.56
MCHC (g Hb ml^{-1} RBC)	0.27±0.02	0.25±0.01
Plasma protein (g dl^{-1})	1.44±0.11	1.22±0.19
Plasma total ammonia (μmol l^{-1})	28.1±3.8	47.4±13.8
Plasma cortisol (ng ml^{-1})	41.6±14.1	44.5±16.4
Plasma lactate (mmol l^{-1})	0.78±0.17	0.86±0.28
White muscle water content (%)	77.9±0.7	77.2±0.9
Plasma water content (%)	96.0±0.2	96.7±0.4
Red blood cell water content (%)	66.9±0.5	67.1±0.6
Ventilation rate (breaths min^{-1})	73.2±5.5	73.7±4.8

Values are means ± 1 S.E.M. (*N*=6–10). Blood (and plasma) was sampled *via* indwelling dorsal aortic catheters. There were no significant differences between control and exposed fish in any parameter measured. MCHC, mean cellular hemoglobin concentration. 1 kPa=7.5 torr.

Blood (1 ml) was drawn anaerobically *via* the arterial catheter into an ice-cold, Li-heparinized (50 i.u. ml^{-1}; Sigma-Aldrich), gas-tight Hamilton syringe for analysis of arterial blood pH (pHa), O_2 tension (Pa_{O_2}), plasma total CO_2 (Ca_{CO_2}), hematocrit (Ht), blood hemoglobin (Hb) and plasma concentrations of lactate, protein, cortisol, total ammonia and water content. Plasma was separated by centrifugation at 14 000 ***g*** for 1 min, and erythrocytes were reserved for determination of water content.

At the end of the experiment, fish were euthanized with an overdose of MS-222, and a piece of gill tissue (approximately 50 filaments) was trimmed off the central portion of the second gill arch on the left side of the fish, wrapped in foil, frozen in liquid nitrogen and stored at –20°C for later analysis of gill [Ni]. Additionally, a sample of white muscle was taken for determination of water content.

Analytical methods – Experiment 2

For the analyses of pHa, Pa_{O_2}, water pH_I and P_{IO_2}, we used Radiometer electrodes and meters, similar to those used by Wood et al. (1988), thermostatically set to the experimental temperature. Hb was determined by the colorimetric cyanmethemoglobin method (Sigma-Aldrich reagents). Plasma for Ca_{CO_2} was obtained by centrifuging whole blood (5000 ***g*** for 30 s) in ammonium-heparinized microhematocrit tubes in duplicate. Ht was measured directly from the tubes, while Ca_{CO_2} was analyzed on true plasma using a Corning 965 CO_2 analyzer (Corning Life Sciences, Acton, MA, USA). Plasma protein, total ammonia, lactate and cortisol

concentrations were determined as described above. Water content of plasma, erythrocytes and white muscle was determined by pre- and post-weighing samples after drying to a constant mass in a 70°C oven.

Calculations – Experiment 2

Calculations of $P_{a\mathrm{CO_2}}$ and plasma HCO_3^- based on measured pHa and $C_{a\mathrm{CO_2}}$ were identical to those described in Playle et al. (1989) using the Henderson–Hasselbach equation and values for CO_2 solubility (αCO_2) and apparent pK (pK′) at the appropriate temperature from Boutilier et al. (1984). Mean cellular hemoglobin concentration (MCHC) was calculated as the ratio of simultaneous measurements of Hb to Ht in whole blood samples and is expressed as g Hb ml^{-1} of red blood cells (RBC).

Sampling protocols – Experiment 3

On days 0 (initial control), 9 and 34 of chronic Ni exposure (394 μg Ni l^{-1}) and on day 38 of subsequent exposure to clean water, oxygen consumption of swimming fish was measured using a variation of a technique described in Wilson et al. (1994). Briefly, fish (N=9; both treatments) were transferred the night before an experiment to small Blazka-type swim respirometers (~3.2 liter) served overnight with a water flow of ~300 ml min^{-1} and an orientation velocity of 15 cm s^{-1} (approximately 1 *BL* s^{-1}). Temperature control was achieved throughout the experiment by submersing the respirometers in a wet table receiving a constant flow of water. The overnight acclimation temperature was 15°C and, over the 5 h needed to complete the respirometry experiment, the temperature rose to 16.5°C due to increased thermal output by the respirometers at greater r.p.m. This temperature increase, however, was consistent across all respirometry trials involving both control and treated fish and therefore should not have contributed greatly to differences in oxygen consumption between treatments. On days 9 and 34, respirometers housing experimental fish were served with a comparable Ni concentration delivered from a stock solution by gravity flow as described above.

At each water velocity (increments of 5 cm s^{-1}; Wilson et al., 1994), oxygen consumption was determined using a variation on a closed respirometry technique. At the start of each hour, immediately following a water velocity increase, the respirometers were opened to flowing water for 20 min to allow for near saturation of water with oxygen. After 20 min, the respirometers were sealed and an initial water sample was taken for partial pressure of oxygen (P_{O_2}) followed 40 min later by a final water sample. The process was continued until each fish was exhausted.

Immediately following exhaustion, fish were killed with an overdose of MS-222, weighed to the nearest 0.01 g, and fork length measured to the nearest 0.1 cm for calculation of individual U_{crit} values as described above. On day 34 of Ni exposure and day 38 of clean water exposure, a gill sample was quickly removed and analyzed for Ni as described above.

At all times, oxygen consumption ($\dot{M}_{O_2,max}$) was calculated according to the formula:

$$\dot{M}_{O_2} = \frac{(\alpha O_2)\,(\Delta p)\,(V)}{(M)\,(t)} \qquad (2)$$

where αO_2 is the solubility co-efficient of oxygen in water at the experimental temperature (Boutilier et al., 1984), ΔP is the difference in partial pressures between initial and final water samples, V is the volume of water, M is the mass of the fish and t is the time interval in hours. The periodic opening of the system to flowing water kept the partial pressure of oxygen in the water above 100 torr (13.3 kPa) at all times. Oxygen consumption rates were corrected for 'blank' oxygen consumption by the experimental apparatus in the absence of fish. Oxygen was typically consumed by the apparatus at a rate of 1.7–3.2 torr h^{-1}, depending on the individual respirometer. These values were approximately 15–30% of the lowest rates of oxygen consumption (~11 torr h^{-1}) measured at the lowest swimming speed tested (15 cm s^{-1}).

For each fish, the log of oxygen consumption was plotted against swimming speed (in *BL* s^{-1}; see Fig. 1). The regression line of each fish was extrapolated back to 0 *BL* s^{-1} to yield basal oxygen consumption ($\dot{M}_{O_2,basal}$) and out to the individual U_{crit} value of each fish (see Fig. 1), at which point oxygen consumption was taken to be $\dot{M}_{O_2,max}$ (Wilson et al., 1994). Aerobic scope for activity was calculated as the difference between $\dot{M}_{O_2,basal}$ and $\dot{M}_{O_2,max}$. Only fish yielding a significant linear regression (P<0.05) of the log of oxygen consumption *vs* swimming speed were included in the analysis.

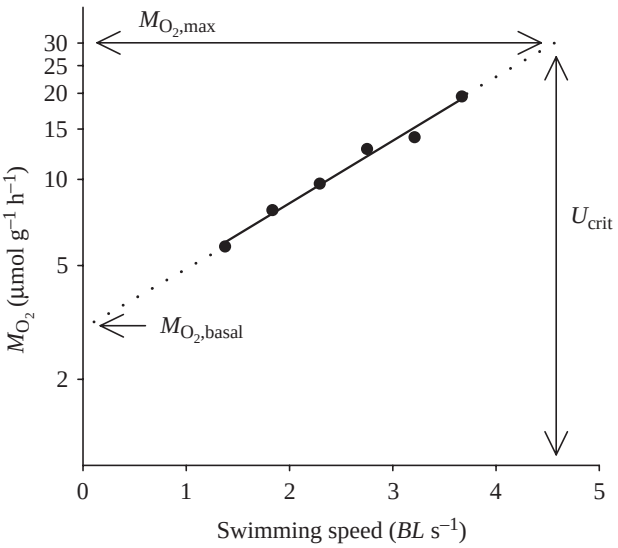

Fig. 1. Example of a regression between the log of oxygen consumption ($\dot{M}_{O_2}$) and swimming speed for an individual juvenile rainbow trout, showing the extrapolations (broken lines) leading to the determination of $\dot{M}_{O_2,max}$ and $\dot{M}_{O_2,basal}$. The solid line represents swimming speeds within the range in which oxygen consumption was measured.

Gill morphometric analysis – Experiment 3

After 69 days of Ni exposure, gills from control and experimental fish (N=5; both treatments) were fixed for light microscopic examination of morphometrics. Fish were netted and immediately euthanized by a blow to the head. A large section of filaments (approximately 50) was cut away from the second gill arch on the left side of the head, rinsed quickly with 0.1 mmol l^{-1} Sorenson phosphate buffer (Hayat, 1981) and placed in neutral buffered formalin (NBF) for 1 h. The NBF was adjusted to pH 7.5 with NaOH and vacuum filtered (0.45 μm). After 1 h, individual filaments were placed in fresh NBF for 24 h at 4°C and then placed in tap water overnight at 4°C. Filaments were then dehydrated in a graded alcohol series and embedded (one filament per block) in Spurrs resin (Hayat, 1981).

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 Richardson's stain (Richardson et al., 1960). Sections were examined and digitally captured with a Leica DM IRBE inverted microscope. Digitally captured images were adjusted for contrast only using Adobe Photoshop 6.0 software.

Determination of the various volume ratios and blood–water diffusion distances (BWDD: see Table 2) closely followed techniques outlined by Hughes and Perry (1976) and Hughes et al. (1979). Briefly, a six-lined anisotropic Merz grid was laid over images of sections magnified 715×, and point counts were used to estimate relative volumes within secondary lamellae (for details, see Hughes et al., 1979). A typical section included a portion of an individual filament body with 30–40 secondary lamellae. Measurements included the volume of the lamellar region (V_{LR}), as defined by the area between the body of the filament and the distal tips of the lamellae, the volume of the secondary lamellar tissue (V_{SL}), the volume of epithelial tissue lying outside the blood pillar system (V_{OPS}) and the volume of the pillar system (V_{PS}). The ratios tabulated 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}). The portion of the lamellar region occupied by the pillar system (V_{PS}/V_{LR}) was then calculated by:

$$V_{PS}/V_{LR} = [1 - (V_{OPS}/V_{SL})](V_{SL}/V_{LR}) . \quad (3)$$

BWDD were determined at the same magnification using the Merz grid to randomize the measurement points (Wilson et al., 1994). Distances 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).

Each fish (N=5 per treatment) was assigned a mean value for each parameter based on a total of approximately 200 point counts per individual using three or four fields of view per section on two or three sections per fish (Hughes et al., 1979).

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

$$D_{rel} = (S/\mathrm{BWDD})_{exp}/(S/\mathrm{BWDD})_{con} , \quad (4)$$

given that diffusing capacity is directly proportional to the surface area available for gas exchange (S) and inversely proportional to BWDD. 'Exp' and 'con' refer to experimental and control fish, respectively. S was estimated by counting the intersections of the test grid with the lamellar surface using the magnification and statistical details given above. Estimation of S assumes 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). Because the reference area used (a Merz grid) was the same for both control and experimental fish, a direct comparison can be made between the two treatments, yielding relative diffusing capacities and a comparison of relative surface areas available for gas exchange.

Table 2. *Morphometric measurements of the gills of control and chronically Ni-exposed juvenile rainbow trout (394 μg Ni l^{-1}; 69 days)*

	Control	Ni-exposed
V_{SL}/V_{LR}	0.550±0.015	0.635±0.017*
V_{OPS}/V_{SL}	0.497±0.015	0.650±0.016*
V_{PS}/V_{LR}	0.276±0.007	0.223±0.013*
Blood–water diffusion distance (μm)	3.319±0.135	3.658±0.106
D_{rel}	–	0.897

Values are means ± 1 S.E.M. (N=5). 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 the percent volume of the lamellar region occupied by tissue lying within the pillar system. D_{rel} is an index of relative diffusing capacity (see Materials and methods and equations 3, 4 for details).

Statistical analyses

Data are presented as means ± S.E.M. (N=number of fish). Where appropriate (Figs 2–5), experimental means (at two Ni concentrations) were compared with control means using a one-way analysis of variance (ANOVA) with a two-sided Dunnett's *post-hoc* multiple comparison test. When only one Ni concentration was used (Figs 6, 8), experimental means were compared with control means by an unpaired two-tailed Student's *t*-test. Additionally, where appropriate (Fig. 6), time-dependent responses of both control and experimental fish were tested against respective time 0 values by a one-way ANOVA with a two-sided Dunnett's *post-hoc* multiple comparison test. The slopes and intercepts of group regression equations (Fig. 7) were compared as described by Zar (1984). Statistical significance in all cases was accepted at P<0.05.

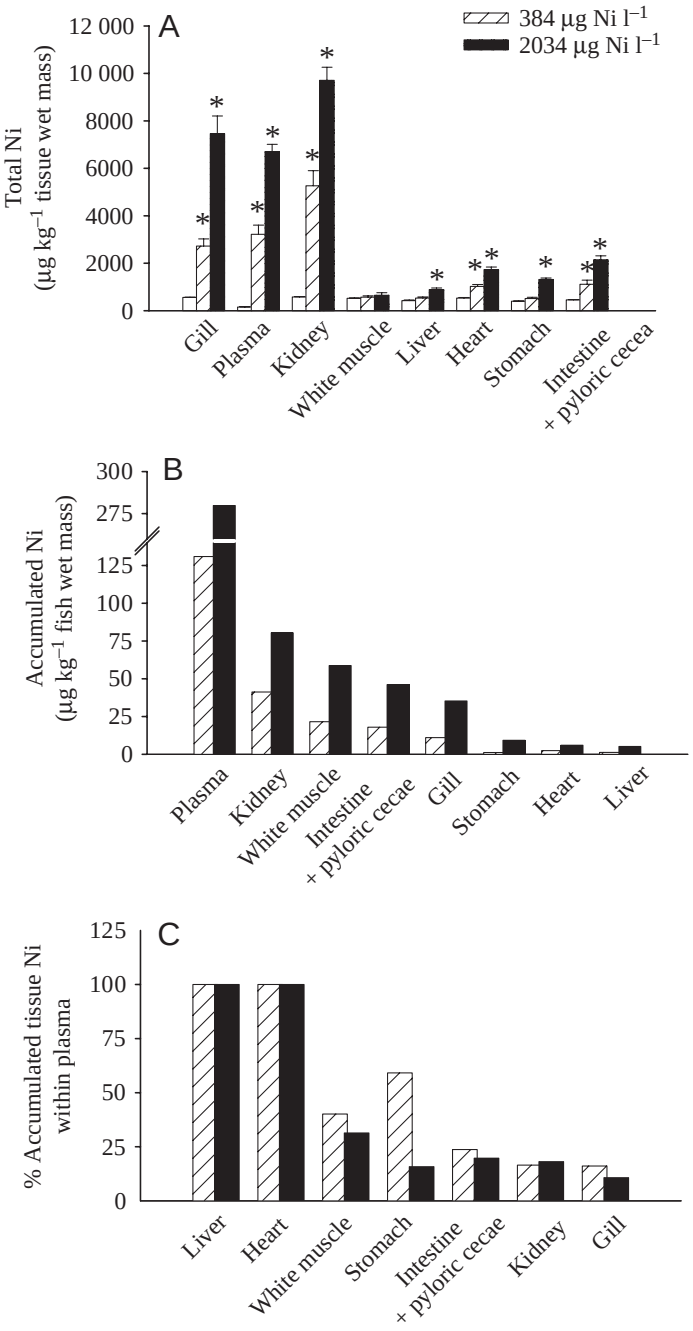

Fig. 2. (A) Ni concentrations in various tissues of juvenile rainbow trout following 42 days of exposure to either control (open bars), 384 μg Ni l^{-1} (hatched bars) or 2034 μg Ni l^{-1} (filled bars). Values are means ±1 S.E.M. (N=6–10). Asterisks indicate significant difference (P<0.05) from control mean by a one-way ANOVA with a two-sided Dunnett's *post-hoc* multiple comparison test. (B) Relative distribution of accumulated Ni among eight tissues in a hypothetical 1-kg rainbow trout. Accumulated Ni in each tissue (μg Ni kg^{-1} tissue; see A) was multiplied by that tissue's relative proportion of total body mass to normalize individual tissue Ni burdens to a hypothetical 1-kg fish. Therefore, normalized accumulation is expressed as μg Ni kg $fish^{-1}$ for each tissue. (C) Percentage of accumulated tissue Ni burdens that can be explained purely by Ni within the blood plasma perfusing each tissue (see Discussion for details).

Results

Chronic exposure to the range of 243–394 μg Ni l^{-1} resulted in no mortality, while chronic exposure to 2034 μg Ni l^{-1} resulted in 33% mortality. Mortality in control fish was 7%. Tissue Ni burdens after 42 days of exposure to either control, 384 μg Ni l^{-1} or 2034 μg Ni l^{-1} are given in Fig. 2. In fish exposed to either Ni concentration, accumulation in the plasma, gill and kidney was statistically significant when compared with control fish and markedly greater than in other tissues. Ni concentrations in these three tissues were 21.2-, 4.9- and 9.1-fold above control levels, respectively, in fish exposed to 384 μg Ni l^{-1} and 44.1-, 13.3- and 16.7-fold above control levels, respectively, in fish exposed to 2034 μg Ni l^{-1}. These plasma Ni concentrations were 8.4-fold and 3.3-fold higher than the waterborne exposure concentrations in the two series, respectively. White muscle did not accumulate Ni at either exposure concentration. Fish exposed to 384 μg Ni l^{-1} accumulated Ni significantly in the heart and intestine (1.9- and 2.4-fold above controls, respectively), while fish exposed to 2034 μg Ni l^{-1} accumulated significantly elevated amounts of Ni in the liver, heart, stomach and intestine (2.1-, 3.2-, 3.3- and 4.7-fold above control levels, respectively; Fig. 2).

Chronic exposure to 384 μg Ni l^{-1} had no impact on plasma ion concentrations (Fig. 3). This low concentration of Ni did not appear to induce any markedly deleterious effects on any measured parameter in resting fish (cf. Figs 4, 5; Table 1). Table 1 presents the results of a more detailed analysis of the effects of chronic, low-level (243 μg Ni l^{-1}) Ni exposure on resting, cannulated rainbow trout. There were no significant differences between control and Ni-exposed fish with respect to 15 different blood gas, acid–base, hematological, stress, water balance and ventilatory parameters. In this experiment, the gill Ni burden of chronically Ni-exposed fish was increased

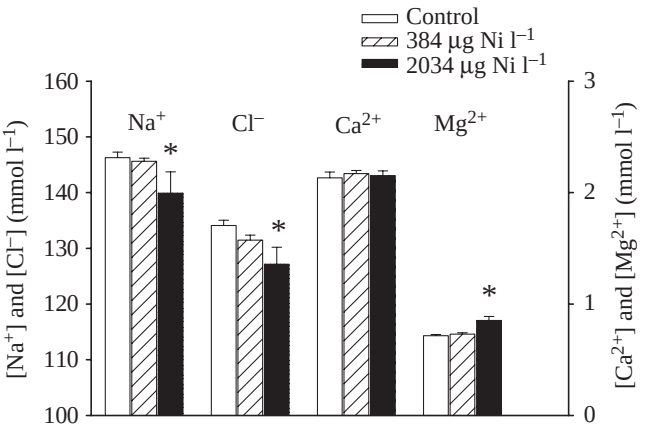

Fig. 3. Plasma ion concentrations (Na^+, Cl^-, Ca^{2+} and Mg^{2+}) of juvenile rainbow trout following 42 days of exposure to either control (open bars), 384 μg Ni l^{-1} (hatched bars) or 2034 μg Ni l^{-1} (filled bars). Values are means ±1 S.E.M. (N=6–10). Asterisks indicate significant difference (P<0.05) from control mean by a one-way ANOVA with a two-sided Dunnett's *post-hoc* multiple comparison test.

by approximately 3.2-fold over that of control fish (2035±527 *vs* 642±26 μg kg^{-1} wet mass).

Plasma concentrations of Na^+, Cl^-, Ca^{2+} and Mg^{2+} in juvenile rainbow trout (30–50 g) exposed for 42 days to 2034 μg Ni l^{-1} were only slightly affected (Fig. 3) despite the marked effects of exposure to this concentration on certain hematological parameters and swimming performance (Figs 4, 5) and the fact that this Ni concentration produced 33% mortality over 42 days. Although the reductions in plasma Na^+ and Cl^- were statistically significant in fish exposed to 2034 μg Ni l^{-1} (Fig. 3), losses of these two ions from the plasma were only 4% and 5%, respectively. Plasma [Ca^{2+}] was well conserved, and plasma [Mg^{2+}] was actually elevated.

While plasma protein, total ammonia, cortisol and lactate concentrations were very similar in control fish and fish exposed to 384 μg Ni l^{-1}, exposure to 2034 μg Ni l^{-1} had a marked impact on both plasma protein and total ammonia concentration, with these two parameters being significantly increased by 29% and 200%, respectively (Fig. 4A,B). Additionally, although the changes were not statistically significant, plasma lactate concentration was elevated at this higher Ni concentration and plasma cortisol was suppressed (Fig. 4C,D).

The discrepancy between the effects of these two chronic Ni concentrations (384 μg Ni l^{-1} *vs* 2034 μg Ni l^{-1}) was further evidenced by measurements of U_{crit}. U_{crit} in fish exposed to the higher Ni concentration (2034 μg Ni l^{-1}) was markedly reduced by 42% and 35% after 12 days and 24 days of exposure, respectively (Fig. 5). Exposure to the lower Ni concentration (384 μg Ni l^{-1}) resulted in slight (~7%), but not statistically significant, decreases of U_{crit} on both sampling days (Fig. 5).

In contrast to the lack of effects found in resting fish, chronic Ni exposure had a significant impact on oxygen consumption patterns when fish were exercised. After 34 days of exposure to 394 μg Ni l^{-1}, treated fish exhibited a significantly lower maximal oxygen consumption rate ($\dot{M}_{O_2,max}$; 33%) and aerobic scope for activity (38%; Fig. 6B,C). These trends persisted even after 38 days of exposure of both groups of fish to clean water, with the reduction in aerobic scope remaining statistically significant (Fig. 6C). Basal oxygen consumption rates ($\dot{M}_{O_2,basal}$) were not changed throughout the exposure regime (Fig. 6A). Additionally, U_{crit} values were not significantly changed, despite a tendency towards reduced values (~6–10% lower) in the Ni group at all times after the initial control (Fig. 6D; cf. Fig. 5).

Group regressions of the log of oxygen consumption rate *vs* swimming speed are shown for both groups of fish swum on days 0 and 34 in Fig. 7A and 7B, respectively. The slopes and intercepts of the two regressions on day 0 were essentially identical (Fig. 7A), while the slope of the regression line for Ni-exposed fish on day 34 was significantly lower than its control counterpart (Fig. 7B; $P<0.05$). The group regression

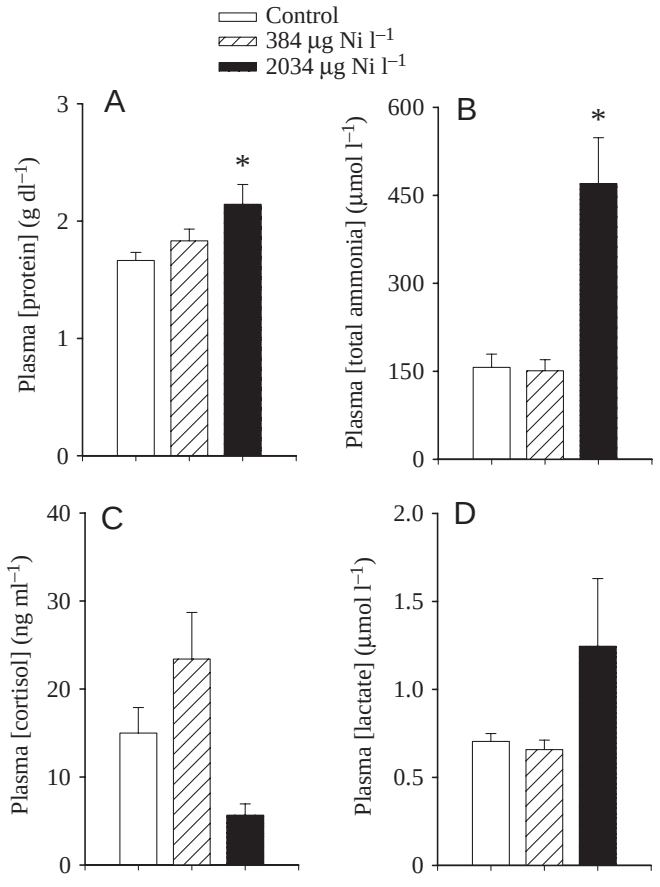

Fig. 4. Plasma stress indicators in juvenile rainbow trout following 42 days of exposure to either control (open bars), 384 μg Ni l^{-1} (hatched bars) or 2034 μg Ni l^{-1} (filled bars). Values are means ±1 S.E.M. (N=6–10). Asterisks indicate significant difference ($P<0.05$) from control mean by a one-way ANOVA with a two-sided Dunnett's *post-hoc* multiple comparison test.

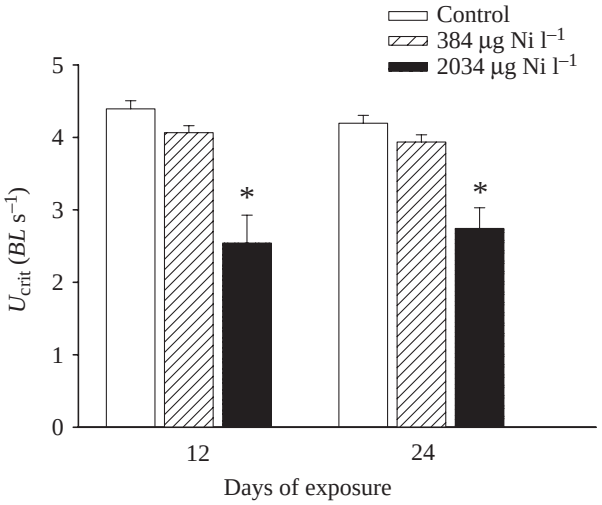

Fig. 5. Critical swimming speed (U_{crit}) of juvenile rainbow trout following 12 days and 24 days of exposure to either control (open bars), 384 μg Ni l^{-1} (hatched bars) or 2034 μg Ni l^{-1} (filled bars). Values are means ±1 S.E.M. (N=6–10). Asterisks indicate significant difference ($P<0.05$) from control mean by a one-way ANOVA with a two-sided Dunnett's *post-hoc* multiple comparison test. *BL* = body lengths.

lines showed little change in $\dot{M}_{O_2,basal}$ (note the log scale on the *y*-axis), and the intercepts in Fig. 7B were not significantly different from one another. $\dot{M}_{O_2,max}$, however, was noticeably reduced and, correspondingly, so was aerobic scope for activity (cf. Fig. 6A–C).

Morphometric analysis of gills from control and experimental fish after 69 days of exposure revealed significant Ni-induced changes in the ultrastructure of secondary lamellae (Table 2). The percentage of the lamellar region occupied by secondary lamellae (V_{SL}/V_{LR}), and the percentage of secondary lamellae occupied by tissue outside the pillar system (blood channels; V_{OPS}/V_{SL}) increased significantly in Ni-exposed fish by 15.4% and 30.9%, respectively (Table 2). Additionally, the percentage of the lamellar region occupied by the pillar system (V_{PS}/V_{LR}) decreased significantly by 19.4%. Although elevated by slightly more than 10%, blood–water diffusion distance (BWDD) in experimental fish was not significantly different from that of control fish. These Ni-induced changes to the lamellar ultrastructure are illustrated by the light micrographs in Fig. 8.

The persistence of significantly reduced scope for aerobic activity in Ni-exposed fish following exposure to clean water (Fig. 6C) can be contrasted with the almost complete depuration of gill Ni burden in these fish. Fig. 9 shows the near return of gill Ni to control levels in fish previously exposed to Ni (99 days) followed by 38 days of exposure to clean water. Although the gill burden of fish previously exposed to Ni was still significantly elevated, the Ni burden of these fish was only 36% higher than that of control fish (593±39 μg kg^{-1} *vs* 436±9 μg kg^{-1}). The gill burden of fish previously exposed to Ni after exposure to clean water falls towards the higher end of typical background gill Ni concentrations and was similar to that of control trout from both experiment 1 (560±10 μg kg^{-1}; see Fig. 2) and experiment 2 (642±26 μg kg^{-1}; see Results above). In comparison, the gill burden in Ni-exposed fish after 34 days (3434±530 μg kg^{-1}) was approximately seven times higher than that of control fish (Fig. 9).

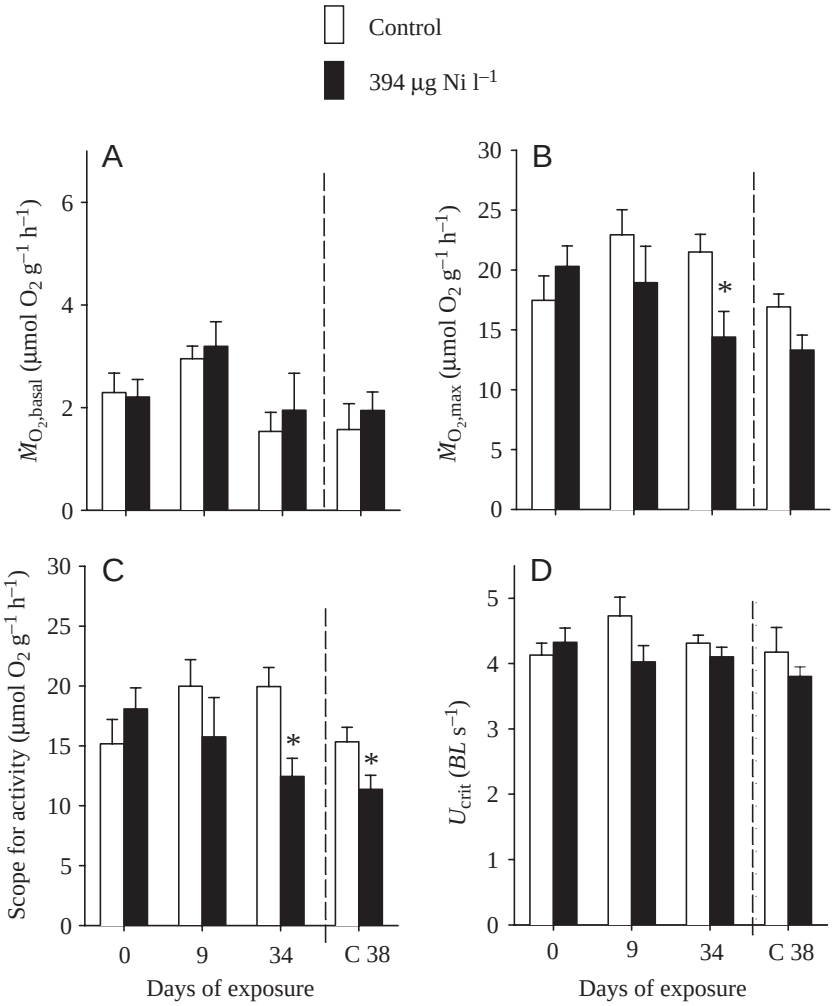

Fig. 6. Aerobic swimming performance in juvenile rainbow trout exposed to 394 μg Ni l^{-1}. (A) Basal oxygen consumption rate ($\dot{M}_{O_2,basal}$). (B) Maximal oxygen consumption rate ($\dot{M}_{O_2,max}$). (C) Aerobic scope for activity. (D) Critical swimming speed (U_{crit}). Values are means ±1 S.E.M. (N=5–8). Asterisks indicate significant difference (P<0.05) from simultaneous control mean by an unpaired two-tailed Student's *t*-test. Bars to the right of the broken vertical line represents measurements made after 38 days of exposure to clean water (C 38). *BL*, body lengths.

Discussion

Following both chronic (Fig. 2A,B) and acute (Pane et al., 2003) Ni exposure, the plasma was the main sink for Ni. Using tissue Ni accumulation data (Fig. 2A) and the relative proportion of total body mass represented by each tissue or body fluid (using values for rainbow trout from Hogstrand et al., 2003), tissue-specific Ni burdens were calculated to estimate internal distribution of accumulated Ni within certain tissues of a hypothetical 1-kg rainbow trout following chronic Ni exposure to 384 μg Ni l^{-1} and 2034 μg Ni l^{-1} (Fig. 2B). The plasma accumulated more than three times as much Ni as any other tissue at both exposure concentrations, followed by the kidney, white muscle, intestine and gill. Note that although white muscle was the third largest Ni sink, this poorly vascularized tissue did not significantly accumulate Ni (Fig. 2A).

Given such high amounts of plasma Ni, one might speculate that a large portion of Ni accumulated in tissues may be a function of vascularization, especially in tissues that are highly vascularized. Fig. 2C plots the percentage of accumulated Ni in each tissue that can be explained simply by accounting for the degree of vascularization, using estimates of salmonid ^{125}I plasma space values for each tissue from Olson (1992). At both exposure concentrations, all of the Ni accumulated by the liver

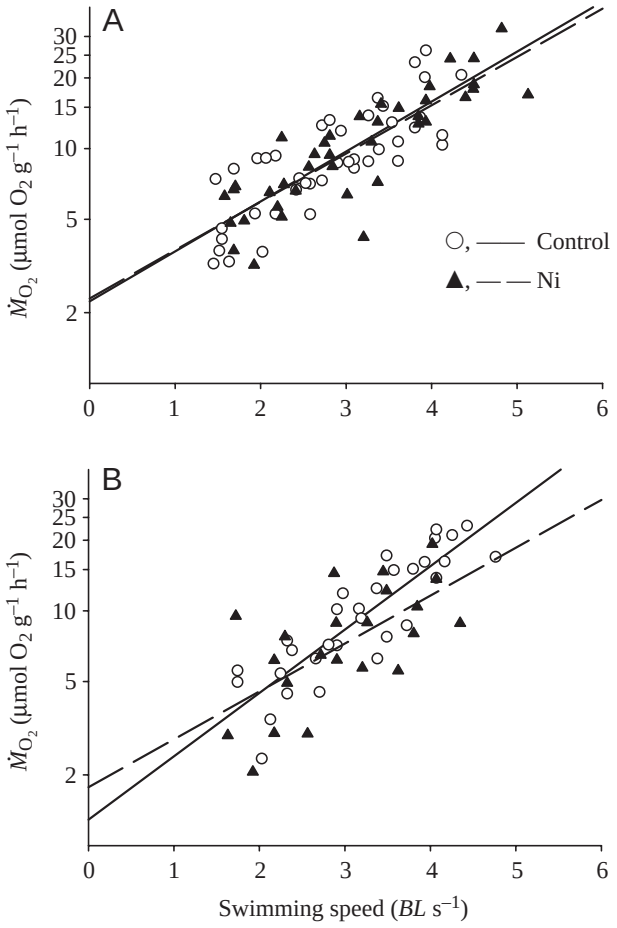

Fig. 7. Group regressions of the log of oxygen consumption rate ($\dot{M}_{O_2}$) against swimming speed for which *BL* represents body lengths. (A) Day 0 (initial sampling). The slopes of the two group regression lines are not significantly different. Group regression equations were y=0.213x+0.349, r=0.809 (control) and y=0.206x+0.361, r=0.853 (Ni), where y=log $\dot{M}_{O_2}$ and x=swimming speed. (B) Day 34 of Ni exposure. The slope of the regression line in the Ni group is significantly different (P<0.05) from that of the control fish. Group regression equations were y=0.270x+0.111, r=0.869 (control) and y=0.204x+0.249, r=0.644 (Ni). *BL*, body lengths.

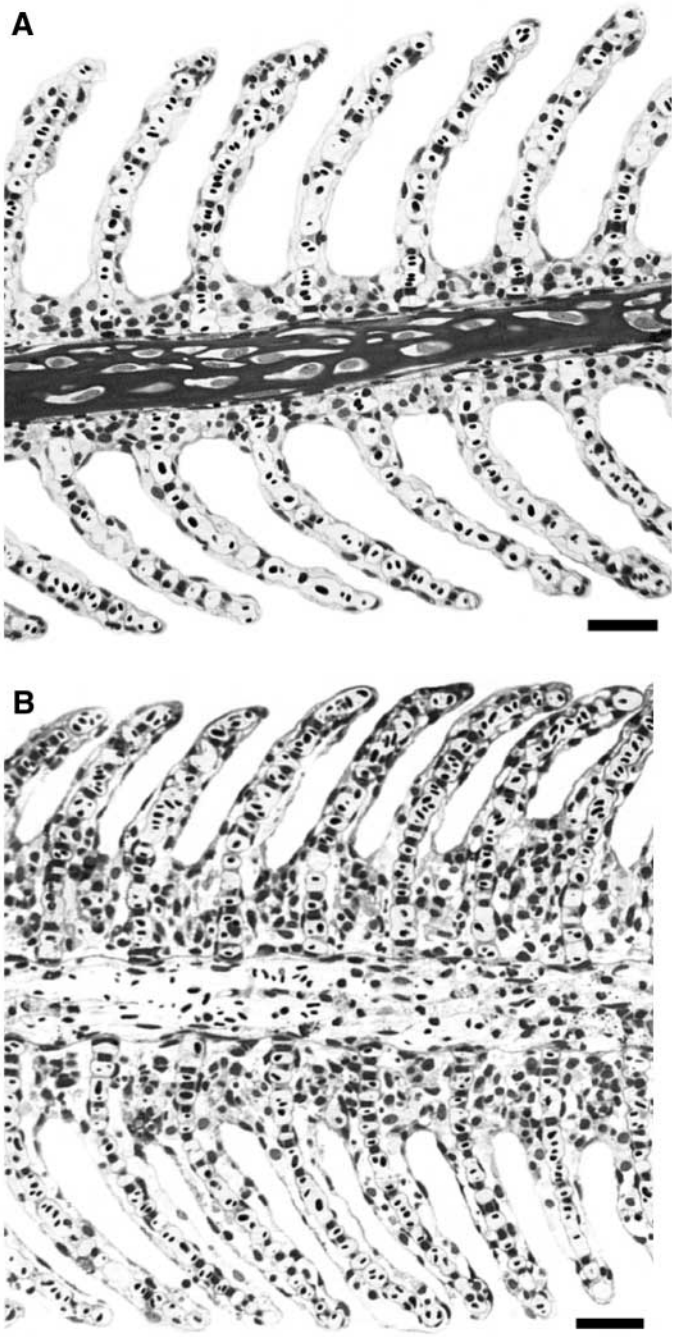

Fig. 8. Light micrographs of the secondary lamellar structure of gills from (A) control or (B) Ni-exposed fish (394 μg Ni l^{-1}; 69 days). Note the thickened lamellae and decreased interlamellar water space in the treated gill (B). Sections are 1 μm thick, stained with Richardson's stain. Scale bars, 25 μm.

and heart was present in the blood perfusing these tissues, as was a substantial portion of Ni in the white muscle (31–40%). Interestingly, the two tissues with markedly higher overall Ni burdens (kidney and gill) had the lowest percentage of Ni burden that could be explained by vascularization and are the tissues in most intimate contact with either the exposure water (gill) or the urine (kidney). While the exposure water is obviously high in Ni, it is also assumed that the Ni concentration of the urine is elevated during waterborne Ni exposure, given that renal clearance is the primary excretory mechanism of bloodborne Ni (Eisler, 1998; USEPA, 1986). Although the distribution of Ni among various ligands within the blood plasma of fish is poorly understood, the relative affinity of mammalian serum albumin for Ni determines the extent of Ni capable of crossing biological membranes bound to low-molecular-mass ligands (USEPA, 1986; Kasprzak, 1987). The present data suggest that Ni is not easily accessing the interstitial space (and the intracellular compartment) and is primarily being retained in the blood plasma, perhaps bound to either serum albumin or as another protein complex.

After 42 days of exposure, Ni concentrations in the gill and kidney were approximately equilibrated with plasma Ni at both

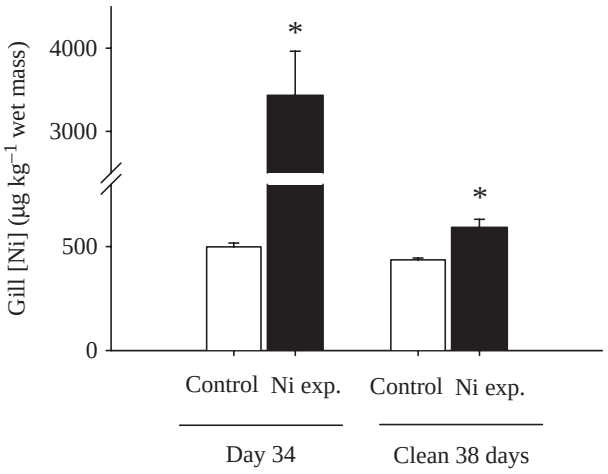

Fig. 9. Gill Ni concentrations in juvenile rainbow trout. Ni-exposed fish were exposed to Ni for 99 days, followed by 38 days in clean water. Values are means ± 1 S.E.M. (N=6–9). Asterisks indicate significant difference (P<0.05) from simultaneous control mean by an unpaired two-tailed Student's t-test.

exposure concentrations (384 μg Ni l^{-1} and 2034 μg Ni l^{-1}; Fig. 2A). A similar phenomenon occurred following 120 h of acute, high-concentration Ni exposure (Pane et al., 2003), although in that case Ni concentrations in the gill and kidney were equilibrated with those in both the plasma and the exposure water. During chronic Ni exposure, however, plasma concentrations exceeded Ni concentrations in the exposure water (see Results). Additionally, during acute Ni exposure, plasma Ni concentrations increased linearly with time over 120 h of exposure (Pane et al., 2003). What is not known during chronic exposure, however, is whether the high plasma Ni seen after 42 days represents a plateau (homeostatically regulated level) or simply a point during a time course of slowly but continually increasing plasma Ni concentrations.

Exposing rainbow trout chronically to a relatively high Ni concentration (2034 μg Ni l^{-1}) provided insight into the mode of chronic toxicity. Clearly, ionoregulatory disruption is far less important than respiratory toxicity under these conditions (Figs 3, 4). Despite substantial mortality and signs of respiratory distress in resting fish exposed to 2034 μg Ni l^{-1} (Fig. 4), plasma ion disturbances were minimal (Fig. 3). These results agree well with those of acute Ni studies in which respiratory toxicity is very pronounced while ionoregulatory disturbance is not substantial (Pane et al., 2003).

The respiratory effects of chronic, very low-level Ni exposure were quite subtle and were only unmasked by strenuous aerobic exercise (Figs 6, 7). The following discussion of Ni-induced limitation of aerobic swimming performance focuses on the gill as a key site of toxic action underlying the observed reductions in maximal oxygen utilization rates. In support of this specific focus on the gill during chronic Ni exposure are three pieces of evidence: (1) acute respiratory toxicity occurs exclusively at the gill and involves no bloodborne or systemic component (E. F. Pane, A. Haque and C. M. Wood, submitted); (2) chronically, white muscle, which contributes to aerobic swimming at speeds close to U_{crit}, did not significantly accumulate Ni at either chronic concentration used (Fig. 2A); and (3) significant ultrastructural alterations to the branchial epithelium were observed, consistent with diffusive limitations of high-performance gas exchange (Table 2; Fig. 8).

Within the context of the rainbow trout gill, the connection between decreased available surface area for diffusion and decreased maximal oxygen exchange capacity has been well established. It is thought that at times of maximal oxygen usage, the gills are fully perfused with blood and the system is diffusion limited rather than perfusion limited (Daxboeck et al., 1982; Duthie and Hughes, 1987). Accordingly, small decreases in gas exchange capacity may not be detected at rest or at lower swimming speeds (Duthie and Hughes, 1987) but may become important as the intensity of exercise increases (Nikl and Farrell, 1993). Indeed, this phenomenon clearly applies during strenuous exercise following chronic Ni exposure. At or near U_{crit}, maximal oxygen consumption rates ($\dot{M}_{O_2,max}$) in the present study decreased by 33.0% (Figs 6B, 7). An alternative explanation of the data in Figs 6B, 7, that swimming became more efficient in treated fish at higher swimming speeds, is inconsistent with our unquantified observations that treated fish consistently relied more frequently on erratic burst swimming near U_{crit}.

The 33% decrease in $\dot{M}_{O_2,max}$ was consistent with a 10.3% decrease in the relative diffusing capacity (D_{rel}) of experimental fish (Table 2). This decrease in D_{rel} was driven more by increased blood water diffusion distance (BWDD) in experimental fish (Table 2) rather than a distinct decrease in available lamellar surface area (S) (from equation 4, D_{rel} is directly proportional to S and inversely proportional to BWDD). Because it incorporates both S and BWDD, D_{rel} is a more comprehensive parameter than either S or BWDD taken alone and is a better morphometrically determined approximation of the efficiency of oxygen transfer from the water to the blood across the branchial epithelium (Hughes and Perry, 1976; Hughes et al., 1979).

The observed decrease in D_{rel} corresponded well with a slight swelling of the secondary lamellae in chronically Ni-exposed fish, as evidenced by significantly increased V_{SL}/V_{LR} (15.4%; Table 2; Fig. 8). The most prominent Ni-induced change in branchial ultrastructure was swelling of the lamellar epithelial layer, as indicated by a 30.9% increase in V_{OPS}/V_{SL} (Table 2; Fig. 8). In the gills of Ni-exposed fish, lifting of the lamellar epithelium from the blood channel system appeared more frequently than in the lamellae of control fish (E. F. Pane, personal observation), and the increases in V_{SL}/V_{LR} and V_{OPS}/V_{SL} appeared to be driven more by hypertrophy, or cell swelling, than a hyperplastic increase in cell number (Fig. 8). Mallat (1985) cited both epithelial lifting and hypertrophy among common lesions associated with metal exposure, with epithelial lifting being the most common response. Additionally, hypersecretion of mucus and hyperplasia were identified as common defense mechanisms. In the present

study, hyperplasia was not evident, while hypertrophic pavement cells were commonly observed in fish subjected to chronic low-level Ni exposure (Fig. 8).

Ni-induced edema in the lamellar epithelium of the gill has been documented during acute exposure to high concentrations of Ni in several earlier studies. Nath and Kumar (1989) reported extensive hypertrophy of the respiratory epithelium leading to separation from the pillar system in the gills of *Colisa fasciatus* acutely exposed (96 h) to approximately 14 000 μg Ni l^{-1}. Additionally, marked increases were observed in V_{SL}/V_{LR} and V_{OPS}/V_{SL} (57% and 49%, respectively) following only 3 days of exposure of rainbow trout to 3200 μg Ni l^{-1} (Hughes and Perry, 1976; Hughes et al., 1979). Such profound acute swelling within the delicate respiratory surface is presumably the cause of marked Ni-induced disturbances in blood gases and acid–base balance, such as those observed by Pane et al. (2003) in trout acutely exposed to 11 700 μg Ni l^{-1}. These authors observed a linear decrease in arterial oxygen tension with time (96 h) to less than 35% of control values, with a twofold increase in carbon dioxide tension and a concomitant respiratory acidosis, suggestive of a substantial limitation of branchial diffusive capacity. Indeed, in a separate study by Pane et al. (E. F. Pane, A. Haque and C. M. Wood, submitted), rainbow trout acutely exposed to 10 700 μg Ni l^{-1} experienced a 46.4% increase in V_{SL}/V_{LR} as well as significantly increased V_{OPS}/V_{SL}, BWDD and lamellar width. Additionally, Hughes and Perry (1976) examined the relative contributions to Ni-induced edema of both tissue and non-tissue (lymphoid) spaces, concluding that overall lamellar swelling was due to swelling in both epithelial components.

In mammals, Ni is considered a moderate contact allergen (Kligman, 1966), and it is possible that lamellar swelling in fish may be an inflammatory response. Although leukocyte infiltration of lamellar blood channels and vasodilation have occasionally been observed in acutely exposed fish (E. F. Pane, A. Haque and C. M. Wood, submitted), the contraction of the pillar system seen during chronic Ni exposure (decreased V_{PS}/V_{LR}; Table 2) argues against a chronic inflammatory response. An alternative suggestion proposed by Mallat (1985) is that intraepithelial fluid may result not from blood exudates but from the overlying freshwater medium; this is particularly relevant in the case of freshwater fish that are continually faced with the osmotic challenge of water absorption from a very hypoosmotic medium.

The decreases in $\dot{M}_{O_2,max}$ and D_{rel} observed during Ni exposure are qualitatively similar to the findings of Duthie and Hughes (1987), who surgically reduced the available gill surface area in trout and reported decreases in $\dot{M}_{O_2,max}$ that almost exactly corresponded (i.e. a 1:1 ratio) to the decreases in available surface area. Additionally, following 34 days of exposure to sublethal Al at moderately low pH, an approximate 1.2:1 ratio was found between these two variables in rainbow trout (Wilson et al., 1994). According to Duthie and Hughes (1987), however, percent decreases in $\dot{M}_{O_2,max}$ greater than percent decreases in available lamellar surface area suggest some extra-diffusional limitations, such as perfusion or convection limitations. In the present study, the ratio of decreased $\dot{M}_{O_2,max}$ (33%) to D_{rel} (10.3%) was approximately 3.2:1. Despite the conventional belief that the branchial vascular network is relatively resistant to waterborne irritants, a significant contraction of the lamellar vasculature was observed following chronic Ni exposure (Table 2) and may have contributed to the increased negative impact on maximal oxygen consumption rates. Additionally, the decreased interlamellar water space in chronically exposed fish (see Fig. 8B) would decrease the volume of water flowing over the respiratory surface and impose a ventilatory convective limitation also capable of impairing high-performance oxygen uptake at the gill.

Furthermore, the 3.2:1 ratio of the present study suggests that some extra-branchial mechanisms such as muscle, erythrocytic or renal impairment may be responsible for some portion of decreased aerobic capacity in Ni-exposed fish (see above). Although Ni did not accumulate significantly in white muscle, muscle ammonia concentrations, for example, were not measured. Therefore, we cannot entirely exclude the possibility that Ni-induced perturbation of swimming performance was mediated through elevated muscle ammonia concentrations, as is the case with acute exposure of brown trout to sublethal copper and low pH (Beaumont et al., 2003). The latter effect is caused by a significant ammonia-induced depolarization of the resting membrane potential of muscle fibers (Beaumont et al., 2000). Plasma ammonia in rainbow trout chronically exposed to 384 μg Ni l^{-1}, however, was not significantly elevated (Fig. 4), suggesting that ammonia is not a mediating factor of reduced swimming performance at this low concentration. Plasma ammonia was substantially elevated, however, following chronic exposure to 2034 μg Ni l^{-1}. Unfortunately, we did not measure Ni in the red muscle.

Given such high (comparable to gill) concentrations of Ni in the plasma (blood) and kidney (Fig. 2) following chronic exposure, we also cannot entirely dismiss the possibility that these two tissues may contribute to the limitation of high-performance aerobic function in Ni-exposed fish. Chronically impaired hemoglobin would cause such a decline, as might chronic renal damage, due to the importance of renal handling of water and electrolytes during exercise (Wood and Randall, 1973).

It must also be considered, given the persistence of impaired aerobic capacity several weeks after the removal of Ni from the exposure water, that some degree of extrabranchial limitation of exercise performance may have been due either to the persistence of extrabranchial accumulated tissue Ni, to specific organ damage that persisted after the Ni exposure period, or to some combination of both. Unfortunately, we can only speculate at this point in time, as the kinetics of tissue Ni handling and specific organ function during Ni exposure and depuration remain to be tested. Additionally, there is the possibility that decreased aerobic performance in both the presence and absence of direct toxicant insult may be

secondary to Ni-induced impairment of physiological function at a higher level of organization than that of an individual organ. Although Ni is both immunogenic (Barchowsky et al., 2002) and carcinogenic (Costa, 1991) in mammalian systems, such effects of Ni in fish are currently unknown.

In summary, we present evidence of a clear cost of acclimation to chronically sublethal Ni exposure in terms of subtle alterations to the branchial ultrastructure and reduced aerobic swimming performance. In the classic terms of Brett (1958), regarding environmental contaminants and aerobic metabolism, Ni acted as a 'limiting stressor' at 34 days of exposure by limiting oxygen exchange with the environment during high demand, thereby reducing $\dot{M}_{O_2,max}$ (Figs 6B, 7B). 'Loading stress', or increased costs of day-to-day living, was not seen, as $\dot{M}_{O_2,basal}$ remained unchanged throughout Ni exposure and subsequent exposure to clean water (Figs 6A, 7A,B). It is also evident that the cost of acclimation to Ni is not a transient phenomenon. To some degree, chronically depressed aerobic capacity persisted for 38 days post Ni-exposure (Fig. 6C), despite an almost complete depuration of gill Ni burden (Fig. 9). Because we only measured oxygen consumption rates and gill Ni burdens after the extended depuration period, we can only speculate as to the possible causes of long-lasting effects of Ni even after fish were returned to clean water.

Chronic impairment of such a dynamically active and critical organ is likely to depress the overall fitness of a fish (Wood, 2001) with obvious environmental implications. In the context of chronic sublethal Ni exposure (394 μg Ni l^{-1}), reduced maximal oxygen consumption could compromise fitness by possibly impairing both predator avoidance and prey capture. Additionally, possible impairment of migratory success is particularly relevant to salmonids returning to freshwater spawning streams.

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