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A nose-to-nose comparison of the physiological effects of exposure to ionic silver versus silver chloride in the European eel (*Anguilla anguilla*) and the rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Physiological mechanisms of silver toxicity (as silver nitrate) to the sensitive rainbow trout (*Oncorhynchus mykiss*) (96 h LC50: 10.2 μg silver l^{-1}, in soft, low chloride water) and the more tolerant European eel (*Anguilla anguilla*)(96 h LC50: 34.4 μg silver l^{-1}, in the same water) were investigated during acute exposure to silver, using concentrations varying from 3 to 22 μg silver l^{-1}. Silver was present either predominantly in the form of ionic silver, or in the form of silver chloride complexes ($AgCl_{aq}$). Inhibition of the branchial Na^+,K^+-ATPase enzyme activity and the active influx of Na^+ leading to net Na^+ loss were the key toxic effect in both species. In the rainbow trout, but not in the European eel, Cl^- influx was also impaired during silver exposure. However, even under control conditions, Cl^- influx was negligible in the eel. Water Cl^- clearly protected against the silver-induced physiological disturbance in rainbow trout, presumably by changing the speciation of silver from ionic silver to AgCl complexes. However, such a protective effect was not observed in the European eel. Differences in whole body Na^+ turnover rates between the two species (1.1% per day in the European eel versus 19% per day in the rainbow trout) together with the lack of effect of silver exposure on Cl^- homeostasis in the European eel are hypothesized to be the main reasons for the different silver tolerance observed in the two species. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Silver nitrate; Ionic silver; Silver chloride; Rainbow trout; *Oncorhynchus mykiss*; European eel; *Anguilla anguilla*; Ionoregulation; Physiological toxicity; Silver tolerance

1. Introduction

Silver is potentially one of the most toxic metals to freshwater fish: 96 h LC50 values under situations where most of the silver is present as the free

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silver ion (Ag^+) have been reported to be in the low μg l^{-1} range (Davies et al., 1978; Nebeker et al., 1983; LeBlanc et al., 1984). However, complexing agents such as Cl^- and dissolved organic matter (DOM) present in most natural fresh waters and thiosulfate, the major complexing agent in photographic industry effluent, have been reported to greatly reduce the toxicity of waterborne silver exposure (Janes and Playle, 1995; Hogstrand et al., 1996; Wood et al., 1996a,b; Galvez and Wood 1997; Erickson et al., 1998; McGeer and Wood 1998; Bury et al., 1999a,b).

Over the past few years, the physiological mechanisms of silver toxicity to freshwater fish have been investigated intensively (reviewed by Wood et al., 1999). In the rainbow trout, the sodium-potassium ATPase located at the basolateral membrane of the branchial epithelium seems to be the key site of toxicity (Morgan et al., 1997). This enzyme is responsible for extrusion of Na^+ in exchange for K^+ across the basolateral membrane of the cell into the extracellular fluid, creating a strong Na^+ gradient (Schou, 1973). In the branchial epithelium of freshwater fish, this transport is important in counteracting the diffusive loss of Na^+ to the hypo-osmotic freshwater environment. In rainbow trout, silver (at least if present as ionic silver) seems to enter the branchial epithelial cells via the proton ATPase-dependent Na^+ channel in the apical membrane (Bury and Wood 1999) and inhibit Na^+ transport via sodium-potassium ATPase across the gill epithelium in a non-competitive manner (Morgan et al., 1997; Webb and Wood 1998; Bury et al., 1999a,b). This inhibition of Na^+ influx is accompanied by a similar inhibition of Cl^- uptake. These reductions in influx result in a net loss of the major plasma ions which eventually leads to mortality by cardiovascular collapse (reviewed by Hogstrand and Wood, 1998).

The silver toxicity data concerning freshwater fish are limited to relatively few species-the rainbow trout being the most frequently studied. However, a comparison of toxicity to copper, which in many ways resembles silver in its toxic action, amongst different freshwater fish species reveals marked fish species-specific differences. For example, 96 h Cu LC50 values for adult rainbow trout are in the range of a few hundred μg Cu l^{-1} (reviewed by McDonald et al., 1989), whereas the corresponding LC50 value for the European eel is more than 4000 μg Cu l^{-1} (Grosell et al., 1998). Such differences could suggest different toxic mechanisms but could also be due to different test conditions and/or water quality, in different laboratories. The objective of the present study was to compare the silver tolerance and mechanisms of toxicity in the rainbow trout and the European eel under identical conditions. The key target of both silver and copper toxicity seems to be branchial Na^+ uptake, and since the European eel is more tolerant to copper than the rainbow trout, it was also expected to be more tolerant to silver.

In the freshwater rainbow trout, it has been shown conclusively that ambient Cl^- has a strong ameliorating effect on silver toxicity, presumably by changing the silver speciation from ionic silver to silver-chloride complexes (Hogstrand et al., 1996; Galvez and Wood 1997; McGeer and Wood 1998; Bury et al., 1999a,b.). However, a careful nose-to-nose species comparison of the protective effect of Cl^- on silver toxicity to fathead minnow vs. rainbow trout showed that Cl^- had no effect on silver toxicity in the former (Bury et al., 1999b). Consequently, an additional goal was to test whether the effect of Cl^- in reducing silver-induced physiological disturbances also applied to the European eel.

Na^+ and Cl^- influxes were studied in both rainbow trout and European eel under identical conditions during acute exposure to a range of silver concentrations at two different ambient Cl^- concentrations, 0.01 and 1.2 mM, resulting in predominantly ionic silver and silver-chloride complexes, respectively. The plasma concentration of Na^+ and Cl^- was measured at the end of the influx study as an indicator of accumulated silver-initiated osmoregulatory stress. The branchial activity of the Na^+,K^+-ATPase enzyme was measured as a terminal endpoint indicator of silver toxicity. To test whether chloride complexation of silver changed the bioavailability for accumulation, the silver concentrations in the gill filaments of both species were measured at the end of the experiments.

During exposure to copper, rainbow trout exhibit an increased Na^+ efflux rate in addition to the reduced Na^+ influx rate (Laurén and McDonald 1986), but silver exposure has been reported to have little or no effect on Na^+ efflux rate (Morgan et al., 1997; Webb and Wood 1998). Compared to most other freshwater teleost fish, the freshwater-adapted European eel has a remarkably low water and ion permeability (Kirsch, 1972). Freshwater adapted European eel can further reduce Na^+ permeability in response to acute transfer to very low ambient Na^+ concentrations (50 μM) by an increased branchial synthesis of wax-alcohols (Hansen et al., 1999). A similar response to silver exposure could counteract the anticipated reduced Na^+ influx and thus serve as an adaptive response. To test whether such a response could be one explanation for the expected higher silver tolerance in European eel, unidirectional Na^+ efflux and influx measurements were performed on eels during sublethal exposure to silver.

2. Materials and methods

2.1. Experimental fish

European eels (*Anguilla anguilla*) (45–75 g, mean 60 g) were caught in fyke nets in Roskilde Fjord (brackish water). All fish were yellow stage eels (adult, non-migrating). The eels were acclimated to laboratory conditions in aerated tapwater (14 ± 0.5°C) (see Table 1 for water chemistry) in 300 l PVC tanks (150 fish per tank) with a flow-through of 2 l min^{-1} for 30 days prior to further acclimation to experimental water. Juvenile rainbow trout (*Oncorhynchus mykiss*) (20–30 g, mean 25 g) were obtained from Reersø fish farm, Kalundborg, Denmark. The rainbow trout were acclimated to laboratory conditions in aerated tapwater (14 ± 0.5°C) (see Table 1 for water chemistry) for 3 days prior to subsequent acclimation to experimental water as described for the eels above. During the acclimation and the following experimental periods trout and eels were kept in separate tanks. Eels were fed fresh mussels and trout were fed commercial trout food once a day. The longer acclimation time to laboratory conditions for the wild-caught eels was chosen to ensure true acclimation. Commencement of feeding was used as an indicator of acclimation.

Table 1
Water chemistry prior to and during silver exposure

	Tapwater	'Low chloride water'	'High chloride water'
Na^+	1000 mM	50 μM	50 μM
Ca^{2+}	2600 μM	10 μM	10 μM
Mg^{2+}	–	<1μM	<1 μM
K^+	–	10 μM	1200 μM
Cl^-	16 μM	10 μM	1200 μM
DOC	–	1.3 mg l^{-1}	1.3 mg l^{-1}

2.2. Experimental design

Both eel and trout were acclimated in separate tanks to softwater (generated by large scale reverse osmosis) or softwater with 1.2 mM Cl^-, added as KCl from a concentrated stock solution via a peristaltic pump (see Table 1 for water chemistry) (14 ± 0.5°C) for 16 days prior to experiments. KCl was used to adjust Cl^- levels because we did not want to change Na^+ availability, and KCl at these levels is known to be well-tolerated by freshwater fish. Indeed, salmonids can tolerate up to 10 fold higher KCl concentrations without ill-effects (Wilkie et al., 1993). Acclimation to experimental water in the holding tanks was initiated by gradually increasing the flow of experimental water, while gradually decreasing the flow of tap water over 5 days. The final flow of experimental water was 0.7 l min^{-1} per tank.

2.3. Bioassay

96 h LC50 values for softwater-acclimated fish were determined for eels and trout separately. A total of eight fish (eel or trout) were transferred to each of five 25 l glass tanks supplied with a flow-through of 0.1 l min^{-1} softwater. The fish were allowed to recover from the transfer and to acclimate to the glass tanks for 24 h prior to toxicity testing. For both species, one tank served as control. In the other four tanks, silver exposure

was initiated by adding silver (as $AgNO_3$) from an acidified concentrated stock solution to instantly obtain the desired silver concentration. Addition of silver from stock solutions of different concentrations via a peristaltic pump ensured constant silver concentrations in the test tanks throughout the test period. Mortality was recorded and water samples were taken at 12-h intervals, acidified (1% HNO_3-trace metal analysis grade, Merck chemicals were used in all procedures) and stored at 4°C for later analysis of silver concentration. LC50 calculations were based on measured silver concentrations.

2.4. Na^+ and Cl^- influx

A total of 32 eels and 32 trout from both softwater and softwater with 1.2 mM Cl^- were transferred to four separate 25 l glass tanks (per water type) containing continuously aerated water of the same chemical composition as in the holding tanks (eight eels and eight trout $tank^{-1}$) and were allowed to settle overnight prior to addition of silver. All eight tanks (four containing softwater and four containing softwater with 1.2 mM Cl^-) were placed in a water bath with flow-through of temperature-controlled tapwater to maintain a constant temperature at 14 ± 0.5°C. In each group of four tanks (softwater and softwater with 1.2 mM Cl^-), one served as a control and silver (as silver nitrate) was added to the other three tanks to yield final measured concentrations of 2.7 ± 0.3, 9.1 ± 1.1 and 21.5 ± 2.8 μg silver l^{-1}, respectively. After 8 h of static exposure to silver, the water was renewed with water of the same composition including identical silver concentrations with addition of 2.2 μCi l^{-1} of ^{36}Cl (Amersham, specific activity 12.1 mCi g^{-1} Cl^-) and 12.5 μCi l^{-1} of ^{24}Na (RISOE isotope laboratory, specific activity 2.4 Ci g^{-1} Na^+), continuing the static silver exposure. Water samples for measurement of total [Ag], [Na^+], [Cl^-], ^{24}Na gamma radioactivity and ^{36}Cl beta radioactivity and thus specific activity of both ^{24}Na and ^{36}Cl were taken at 10, 30 min, 2, 4, 8, 16, 24 and 30 h after addition of the isotopes.

Thus for both species, flux measurements were started after 8 h of exposure to silver, but were ended at different times. Much longer measurement periods were required in eels because of their very low ionic influx rates. The trout were netted out of the exposure tanks after the 8 h of exposure to silver plus the additional 4 h of radioisotope exposure and the eels after the additional 30 h of radioisotope exposure. The fish were anesthetized in 0.1 g MS222, rinsed in radioisotope-free and silver-free water for 2 min, a blood sample was drawn from the caudal vessel, and plasma was obtained by centrifugation. Trout were killed by a blow to the head, eels by cutting the spinal cord. Samples of gill filaments where obtained by dissection and frozen in liquid nitrogen immediately and stored at −20°C for later analysis of Na^+,K^+-ATPase activity and silver concentration. The weighed fish were subsequently homogenized individually using a kitchen blender and subsamples (in triplicate) of the homogenate were prepared for analysis of both beta and gamma radioactivity.

2.5. Na^+ influx and efflux studies in European eel

Ten eels from the holding tanks were transferred to each of two different 80 l glass tanks, one containing softwater and one containing softwater with 1 mM Cl^-. The water of both tanks contained 5 μCi ^{22}Na l^{-1} (Amersham, specific activity; 303 Ci g^{-1} Na^+). The tanks were placed in a water bath with flow-through of temperature-controlled water to maintain a constant temperature of 14 ± 0.5°C. Water quality in the tanks was ensured by recirculating water (50 l min^{-1}) through biological filters. Fish were exposed to these conditions for 10 days prior to measurements of unidirectional Na^+ fluxes. Fish were not fed during the 10 days of ^{22}Na incubation which served to load the internal Na^+ pool with radiolabelled Na^+.

Individual eels were transferred to 0.6 l flux chambers (transparent PVC tubes) supplied with a flow-through of 60 ml min^{-1} of either soft water or soft water + 1 mM Cl^- and allowed to recover from handling for a minimum of 3 h. Water flow was terminated and 70 μCi ^{24}Na l^{-1} (RISOE isotope laboratory, specific activity 2.4 Ci g^{-1}

Na^+) was added to each flux chamber and allowed to equilibrate for 20 min. Water in the flux chambers was aerated continuously. After 0, 1 and 2 h, a 10 ml water sample was drawn from each flux chamber for a pre-exposure flux determination. Then silver was added from a concentrated stock to each flux chamber to achieve a final concentration of 3.9 ± 0.3 μg silver l^{-1}. Water samples (10 ml) were drawn from each flux chamber at 1 and 2 h after addition of silver. After 2 h of silver exposure, water flow to the flux chambers was re-established with addition of silver from a concentrated stock solution of $AgNO_3$ via a peristaltic pump resulting in the same final concentration of 3.9 ± 0.3 μg silver l^{-1}.

The process was repeated for additional flux measurements at 9–11 and 22–24 h of silver exposure. After 24 h, the eels were anesthetized (as above) and a blood sample was obtained from the caudal vessel and plasma was obtained by centrifugation. The eels were killed by cutting the spinal cord, homogenized as above, and subsamples of the homogenate were prepared (in triplicate) for counting of ^{22}Na radioactivity. A subsample of the homogenate was prepared for whole body Na^+ analysis as described below.

2.6. Analytical techniques

Gamma radioactivity from ^{22}Na and ^{24}Na in water samples from the Na^+ and Cl^- influx experiments (on both species) and the Na^+ influx and efflux experiment (on eels), and corresponding radioactivity in subsamples of homogenized fish, were counted at 1275 and 1370 KeV, respectively, with a Ge–Li gamma ray detector. Due to the short half-life of ^{24}Na (15 h), ^{24}Na radioactivity was counted within 36 h of obtaining the samples and was corrected to a constant time. Beta radioactivity from ^{36}Cl in water samples from the Na^+ and Cl^- influx experiment and in the corresponding subsamples of homogenized fish was determined using low background beta counters (Bøtter-Jensen and Nielsen, 1989) at more than ten half-lives of ^{24}Na (150 h) after obtaining the samples in order to avoid contamination by ^{24}Na beta-activity. For all ^{22}Na counting, ^{24}Na counting, and for ^{36}Cl counting in tissue samples obtained from trout, and in all water samples, radioactivity was determined with at least 99% counting accuracy (100%-($\sqrt{CPM}$ CPM^{-1} 100%)). Due to the very low Cl uptake rate in eels, tissues samples obtained from eels were determined with at least 97% counting accuracy.

The activity of Na^+,K^+-ATPase in control and silver exposed eels and trout was determined using the method of Holliday (1985). Gill filaments from one to two gill arches were homogenized in 3 ml of medium (250 mM sucrose, 6 mM Na_2-EDTA) at between 0 and 4°C with 20 strokes of a Teflon-in-glass homogenizer (Thomas Scientific, St. Laurent, Que., Canada). Phosphate liberated from ATP by Na^+,K^+-ATPase was measured in two media. The first medium contained optimal concentrations of all ions (100 mM Na^+; 30 mM K^+; 10 mM Mg^{2+}; 5 mM ATP and 20 mM imidazole; pH 7.2) and therefore the activity of all ATPases present was assayed. The second medium lacked K^+ (replaced with a equimolar concentration of Na^+) and contained 1 mM ouabain but was otherwise identical to the first medium. The second medium therefore assayed the activity of all ATPases except Na^+,K^+-ATPase. The reaction was carried out for 20 min at 37°C and was stopped by addition of ice-cold Bonting's color reagent (178 mM $FeSO_4$, 8.1 mM ammonium molybdate in 580 mM H_2SO_4). The concentration of inorganic phosphate (P_i) liberated was measured colorimetrically at 700 nm. The protein concentration in the homogenates was measured using the Bradford assay (Sigma kit no. B6916) with bovine serum albumin as a standard. The activity of Na^+,K^+-ATPase (expressed as μmol P_i mg^{-1} protein h^{-1}) was calculated from the difference in [P_i] between the two media.

Samples of gill filaments were prepared for analysis of silver concentration by adding approximately three times the volume of 1 N HNO_3 (trace metal grade, Merck Chemicals). The samples were left overnight at 75°C, vortexed and centrifuged. The supernatant was diluted appropriately prior to silver analysis on a graphite furnace atomic absorption spectroscope (Varian AA-1275 with GTA-9 atomiser) using a 10 μl injection volume, N_2 gas and standard operating conditions as documented by the manufacturer.

For whole body Na^+ analysis of eels from the unidirectional Na^+ flux experiments, a subsample of the whole body homogenate was digested in six times the volume of 10% HNO_3 for 48 h. The digest was centrifuged and a subsample of the supernatant was obtained for later Na^+ analysis. Na^+ concentrations in water samples, plasma samples and whole body digests were analysed using the atomic absorption spectrophotometer (Varian 1275). Cl^- concentrations in water and plasma samples were analysed using either coulometric titration (Radiometer CMT-10) or the colorimetric assay of Zall et al. (1956); the two methods were cross-validated.

2.7. Calculations and statistical evaluation

Water geochemical analysis was performed using the MINEQL+ computer program (Schecher and McAvoy, 1992) with the addition of the conditional equilibrium constants for Ag-DOC and H-DOC taken from Janes and Playle (1995).

The LC50 values were calculated by probit analysis (SPSS 6 computer package) and are reported with the 95% confidence intervals.

Na^+ and Cl^- influx rates in trout and eels (μmol kg^{-1} h^{-1}) were calculated as follows:

$$\text{Influx, } J_{in} = ((\text{CPM kg}^{-1})/\text{MSA})/t$$

where CPM are the final radioactivity (counts min^{-1}) of ^{22}Na or ^{36}Cl in the whole body homogenates, MSA is the mean specific activity of $^{24}Na^+$ or $^{36}Cl^-$ in the water over the entire experimental period and t is time (h).

Unidirectional Na^+ influx rates in eels in flux chambers were calculated as follows:

$$\text{Influx, } J_{in} = ((\text{CPM}_b - \text{CPM}_a)/(\text{MSA})(V/W \cdot t)$$

where CPM_b is the radioactivity (counts min^{-1}) of ^{24}Na in the water in the flux chamber at the end of a flux period (counts min^{-1} l^{-1}) and CPM_a is the radioactivity (counts min^{-1}) of ^{24}Na in the water in the flux chamber at the start of a flux period (counts min^{-1} l^{-1}), V is the volume of the flux chamber (l) and W is the weight of the fish (kg). MSA and t have the same meanings as above.

Unidirectional Na^+ efflux in eels in flux chambers was calculated as follows:

$$\text{Efflux, } J_{out} = -(\text{CPM}_b - \text{CPM}_a)/(i\text{MSA})(V/W \cdot t)$$

where CPM_b is the radioactivity (counts min^{-1}) of ^{22}Na in the water in the flux chamber at the end of a flux period (counts min^{-1} l^{-1}) and CPM_a is the radioactivity (counts min^{-1}) of ^{22}Na in the water in the flux chamber at the start of a flux period (counts min^{-1} l^{-1}). iMSA is the mean specific activity of internal $^{22}Na^+$ in the eels during the flux period. V, W, t have the same meanings as above.

Net Na^+ flux rates in the eels in flux chambers was calculated as follows:

$$\text{Net flux, } J_{net} = J_{in} + J_{out}$$

Repeated or prolonged radioisotope flux determinations may lead to significant internal accumulation of radioactivity. The efflux of this radioactivity during subsequent flux periods ('backflux') could influence the calculated J_{in} values. In no case was the internal specific activity of terminal plasma samples higher than 5% of the external specific activity, and correction for backflux, according to Maetz (1956), was not necessary.

Data are presented as mean ± S.E.M. (n) throughout. Data obtained from silver exposed fish, were compared to non-exposed control fish or to initial internal control values (during unidirectional Na^+ flux measurements in eel) using two-tailed t-tests, paired for unidirectional Na^+ flux measurements in eels, otherwise unpaired ($P < 0.05$), with appropriate Bonferroni correction procedures for multiple sample comparison.

3. Results

3.1. Bioassays

The 96 h LC50 values for silver were 10.2 μg l^{-1} (95% confidence interval; 9.83–11.34) for rainbow trout and 34.4 μg l^{-1} (95% confidence interval; 31.07–39.50) for European eel, determined in 'low chloride' softwater.

Table 2
Silver speciation according to MINEQL+ at the different silver concentrations used

Total (silver)	'Low chloride water' (10 μM Cl^-)				'High chloride water' (1200 μM Cl^-)			
	Ag^+	$AgCl_{aq}$	Ag-DOC	Others	Ag^+	$AgCl_{aq}$	Ag-DOC	Others
3.9[a]	3.3	0.1	0.5	–	1.0	2.2	0.5	0.2
2.7[b]	2.4	0.1	0.5	–	0.6	1.4	0.5	0.2
9.1[b]	8.4	0.2	0.5	–	2.5	5.5	0.5	0.6
21.5[b]	20.5	0.5	0.5	–	6.1	13.5	0.5	1.4
10.2[b]	9.5	0.2	0.5	–				
34.4[c]	332	0.7	0.5	–				

[a] Na^+ influx and efflux measurements in European eel.
[b] Na^+ and Cl^- influx in both rainbow trout and European eel.
[c] Bioassays conducted in the low chloride water. Numbers are μg silver l^{-1}.

3.2. Speciation analysis

The speciation of silver according to MINEQL+ at the different silver concentrations and KCl concentrations used in this study is summarized in Table 2. At the silver concentrations used in this study, the dominating form of silver in the 'low chloride water' was ionic Ag^+ and the dominating form of silver in the 'high chloride water' was $AgCl_{aq}$.

3.3. Ion influx-Na^+

Mean Na^+ influx rates were ≈450 μmol kg^{-1} h^{-1} in non-exposed rainbow trout and more than 20 times lower in the European eel, with a rate of 20 μmol kg^{-1} h^{-1}, at both ambient Cl^- concentrations (Fig. 1). Silver exposure for 8–12 h, even at the lowest concentration (2.7 ± 0.3 μg l^{-1}) reduced mean Na^+ influx in the rainbow trout by 67%, to 147 μmol kg^{-1} h^{-1}, independent of ambient Cl^- concentrations. At 9.1 ± 1.1 and 21.5 ± 2.8 μg silver l^{-1}, mean Na^+ influx in the rainbow trout was reduced to 29 and 21 μmol kg^{-1} h^{-1}, respectively, at the low Cl^- concentration and to 176 and 64 μmol kg^{-1} h^{-1}, respectively, at the higher Cl^- concentration. In the European eel, exposure to 2.7 ± 0.3 μg silver l^{-1} for 8–30 h did not affect Na^+ influx, but exposure to 9.1 ± 1.1 μg silver l^{-1} at the low Cl^- concentration did reduce the mean influx to 7 μmol kg^{-1} h^{-1}. Silver exposure at the highest concentration (21.5 ± 2.8 μg silver l^{-1}) caused a 50% inhibition of Na^+ influx in the eel, regardless of the ambient Cl^- concentration.

3.4. Ion influx-Cl^-

Mean control Cl^- influx at the low ambient Cl^- concentration in the European eel was only 0.05 μmol kg^{-1} h^{-1} compared to 116 μmol kg^{-1} h^{-1} in the rainbow trout. At the higher Cl^- concentration, the mean influx were 0.55 and 186

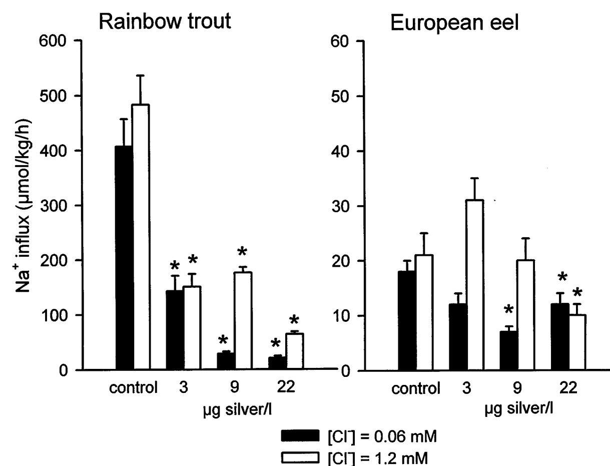

Fig. 1. Na^+ influx rates (μmol kg^{-1} h^{-1}) in rainbow trout and European eel exposed to a range of silver concentrations at two different ambient Cl^- concentrations for a total of 8–12 and 8–38 h respectively. Mean ± S.E.M. ($n = 8$). * indicates statistically significant difference from control ($P < 0.05$). Note the different scale on the Y-axis for trout versus eel.

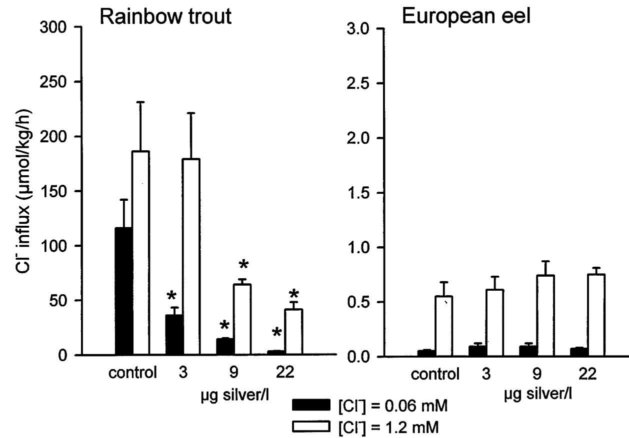

Fig. 2. Cl^- influx rates (μmol kg^{-1} h^{-1}) in rainbow trout and European eel. Note the different scale on the *Y*-axis for trout versus eel. Other details as in Fig. 1.

μmol kg^{-1} h^{-1} in European eel and rainbow trout, respectively (Fig. 2). Remarkably, the Cl^- influx in the European eel was not affected by silver exposure from 8– 30 h at either of the two ambient Cl^- concentrations. In contrast, silver exposure for 8–12 h at the low Cl^- concentration reduced Cl^- uptake to 36, 14 and 3 μmol kg^{-1} h^{-1} in the rainbow trout during exposure to 2.7, 9.1, 21.5 μg silver l^{-1}, respectively. No effect of silver at the higher Cl^- concentration was observed at exposure to the lowest silver concentration (2.7 μg silver l^{-1}), however, exposure to 9.1 and 21.5 μg silver l^{-1} reduced mean Cl^- influx to 64 and 41 μmol kg^{-1} h^{-1}, respectively.

3.5. Plasma Na^+ and Cl^- concentrations

The mean plasma Na^+ concentration in rainbow trout was significantly reduced only at the highest silver concentration in combination with the low ambient Cl^- concentration (Fig. 3), from 141 mM in the controls to 103 mM after 12 h of silver exposure. Similarly, 30 h of silver exposure at the low ambient chloride concentration caused reduced mean plasma Na^+ concentrations from 148 in the controls to 134 and 128 mM in the European eels exposed to 9.1 and 21.5 μg silver l^{-1}, respectively.

In agreement with the ion influx data, the mean plasma Cl^- concentration was reduced in the rainbow trout exposed to the two highest silver concentrations (9.1, 21.5 μg l^{-1}) at both ambient Cl^- concentrations (Fig. 4). At the low water Cl^- concentration, the resulting plasma concentration was 114 and 84 mM at the two silver concentrations, respectively, compared to the mean control value of 130 mM. At the high ambient Cl^- concentration, the relative reductions of mean plasma Cl^- concentrations were less pronounced, to 128 and 123 mM, respectively, compared to the mean control values of 134 mM. The mean plasma Cl^- concentrations of European eel acclimated to the low and the higher ambient Cl^- concentration were 88 and 97 mM, respectively, and in contrast to the rainbow trout, but in agreement with the eel Cl^- influx data, plasma Cl^- concentrations of European eel were not affected by silver exposure.

3.6. Na^+,K^+-ATPase activity

Interestingly, mean Na^+,K^+-ATPase activity in crude gill homogenates of non-exposed control European eel at both Cl^- concentrations were higher (0.74 μmol P_i mg^{-1} protein h^{-1}) than the corresponding rainbow trout gill homogenate: 0.65 and 0.39 μmol P_i mg^{-1} protein

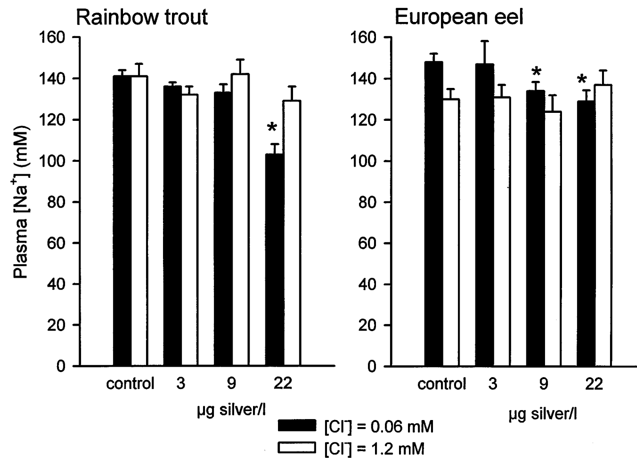

Fig. 3. Na^+ concentration (mM) in plasma from rainbow trout and European eel exposed to a range of silver concentrations at two different ambient Cl^- concentrations for a total of 12 and 38 h, respectively. Mean ± S.E.M. ($n = 8$). * indicates statistically significant difference from control ($P < 0.05$).

h^{-1} at the low and higher Cl^- concentration, respectively (Fig. 5)(difference not statistically significant). The 12 h of silver exposure caused inhibition of mean Na^+,K^+-ATPase activity to 0.15 and 0.0 µmol P_i mg^{-1} protein h^{-1} in rainbow trout at 9.1 and 21.5 µg silver l^{-1}, respectively, at the low ambient Cl^- concentration. The Na^+,K^+-ATPase activity in the rainbow trout was not affected at the high ambient Cl^- concentration. In the European eel, however, ambient Cl^- had no protective effect, and mean enzyme activity was reduced after 30 h of silver exposure to 0.18, 0.16 and 0.24 µmol P_i mg^{-1} protein h^{-1} at 2.7, 9.1 and 21.5 µg silver l^{-1}, respectively, regardless of Cl^- concentration.

3.7. Branchial silver accumulation

Neither of the two species showed statistically significant elevation of branchial silver concentration after silver exposure (Fig. 6). In both the rainbow trout and the European eel, however, there was a tendency for increased branchial silver concentration after exposure to the highest silver concentration (21.5 µg silver l^{-1}). For both species there were great variations in branchial silver accumulation and there was no difference between silver accumulation at the two different ambient chloride concentrations.

3.8. Na^+ influx and efflux in European eel

Unidirectional Na^+ efflux did not change during silver exposure. However, silver exposure (3.9 ± 0.3 µg silver l^{-1}) inhibited unidirectional Na^+ influx in European eel at both ambient chloride concentrations (Fig. 7).

Na^+ influx rates measured in European eel in the flux chambers were higher than when measured in the tanks; 41 versus 19 µmol kg^{-1} h^{-1}, respectively (compare Fig. 7 vs. Fig. 1) a differ-

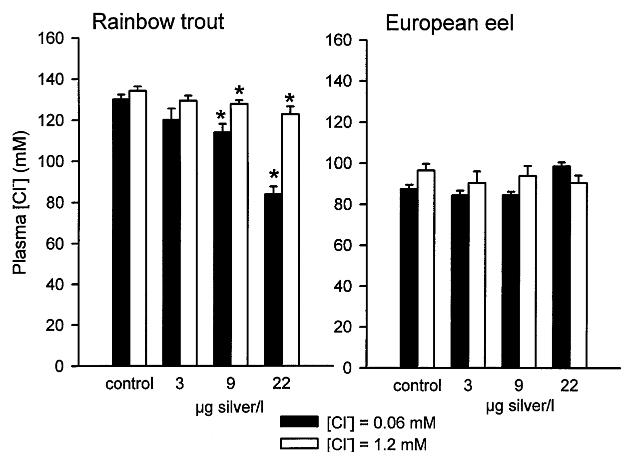

Fig. 4. Cl^- concentration (mM) in plasma from rainbow trout and European eel. Other details as in Fig. 3.

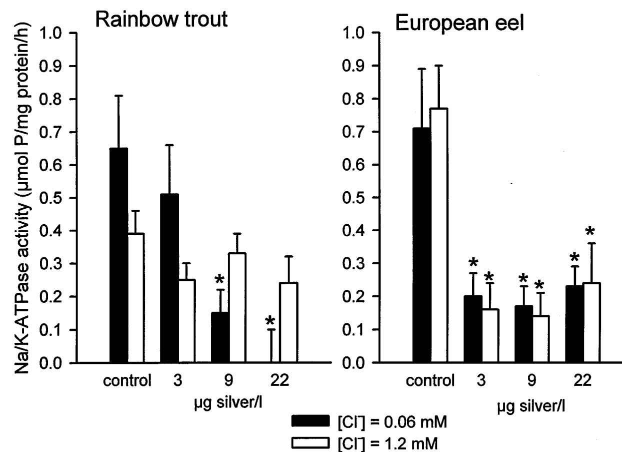

Fig. 5. Na^+,K^+-ATPase activity (μmol P_i mg^{-1} protein h^{-1}) in gill filament homogenates from rainbow trout and European eel exposed to a range of silver concentrations at two different ambient Cl^- concentrations for a total of 12 and 38 h, respectively. Mean ± S.E.M. ($n = 8$). * indicates statistically significant difference from control ($P < 0.05$).

ence likely associated with different stress. At both ambient chloride concentrations, Na^+ efflux exceeded the corresponding Na^+ influx leading to a net Na^+ loss during the control period and during most of the experimental period. The net Na^+ loss tended to be greater as the exposure was prolonged. Interestingly, the Na^+ influx was significantly reduced early (0–2 h of exposure) in eels exposed to silver at the high ambient chloride concentration and remained low throughout. At the low ambient chloride concentration, however, the effect of silver on Na^+ influx, was evident only after 9–11 and 22–24 h of exposure.

4. Discussion

4.1. Difference in silver tolerance

As water chemistry was identical during the trout and eel tests, the 3.4-fold difference in 96 h LC50 values found in the present study is a true species-specific difference in silver tolerance. This observed difference in tolerance may seem low compared to the variation in reported 96 h LC50 values for silver (as $AgNO_3$). In a recent review, Hogstrand and Wood, 1998 presented 96 h LC50 values for silver (as silver nitrate) between 5 and 70 μg silver l^{-1} in eight different freshwater fish species—a 14-fold difference in tolerance between the most sensitive and the most tolerant species. This 14-fold difference undoubtedly involves differences in toxicity due to factors other than inter-specific variation for example, differences in water chemistry between the different studies. Specifically, differences in the concentration of Cl^-, dissolved organic matter (DOM) and thiosulfate have been reported to greatly influence the toxicity of water-borne silver exposure (Janes and Playle, 1995; Hogstrand et al., 1996; Wood et al., 1996a,b; Galvez & Wood 1997; Erickson et al., 1998; McGeer and Wood 1998; Bury et al., 1999a,b).

4.2. Mechanisms of silver toxicity in rainbow trout and European eel

The mechanism of silver toxicity in rainbow trout is inhibition of both Na^+ and Cl^- influx, but in the European eel, only Na^+ influx is inhibited (Figs. 1 and 2). The key target of silver toxicity in freshwater rainbow trout was first reported to be inhibition of the branchial Na^+,K^+-ATPase by Morgan et al. (1997). This has been verified by Bury et al. (1999a) and McGeer and Wood (1998). This enzyme is the driving force for the active uptake of Na^+ needed by all freshwater fish to counteract the diffusive loss of Na^+ to the

surrounding hypo-osmotic environment and, the inhibition of this enzyme leads to a net loss of Na^+. In the present study, Na^+,K^+-ATPase activity and Na^+ influx were inhibited in both rainbow trout and European eel (Figs. 5 and 1) indicating that the key target for silver toxicity in both species is the branchial Na^+,K^+-ATPase activity. At least in freshwater rainbow trout, branchial Cl^- influx seems to be coupled to the active transport of Na^+ by the Na^+,K^+-ATPase (Wood, 1991; Jurss and Bastrop, 1995; Perry, 1997). With this in mind, it is not surprising the Cl^- influx, like the Na^+ influx, in rainbow trout was inhibited by silver exposure (Fig. 2) and this is in agreement with findings reported by Morgan et al. (1997) and Webb and Wood (1998). In the European eel, however, Cl^- influx was not influenced by silver exposure (Fig. 2). Unlike the rainbow trout where Na^+ and Cl^- influx rates were comparable, the Cl^- influx rate in the European eel was only a few percent of the corresponding Na^+ influx rate. The almost non-detectable Cl^- influx rate was the reason for the choice of a longer flux measurement and exposure period for the eel (38 h) than for the rainbow trout (12 h). It is well established that Cl^- influx rates in eels are very low. In the European eel it has been reported to be 1.2 μmol kg^{-1} h^{-1} (Kirsch, 1972) and 3.6 μmol kg^{-1} h^{-1} (Bornancin et al., 1977), and 2.0 μmol kg^{-1} h^{-1} (Goss and Perry, 1994) in the closely related American eel (*Anguilla rostrata*). In the present investigation we found that Cl^- influx rates were even lower than the above-mentioned values even at the elevated water Cl^- concentration (Fig. 2). The mechanisms for maintaining Cl^- homeostasis in freshwater eels is unknown, but could include a significant dietary contribution of Cl^- uptake and/or an extremely low selective Cl^- permeability resulting in very low Cl^- loss. At least in the study of Kirsch (1972), conducted on silver stage eels (sexually mature), the dietary contribution to Cl^- homeostasis can be disregarded since silver stage eels have a degenerated gastro-intestinal tract and do not eat. Cl^- uptake in the eels of the present experiment was dependent on ambient Cl^- concentration; at 1.2 mM Cl^-, the influx rate was 0.5 μmol kg^{-1} h^{-1} but only 0.05 μmol kg^{-1} h^{-1} at 0.06 mM Cl^- (Fig. 2). The latter is, to our knowledge, the lowest Cl^- uptake rate ever reported in fish.

4.3. Why is the European eel more silver tolerant than the rainbow trout

The difference in tolerance to silver between the rainbow trout and European eel likely reflects differences in ionoregulation. Na^+ influx in rainbow trout was slightly more sensitive to silver exposure than in the European eel (Fig. 1) and

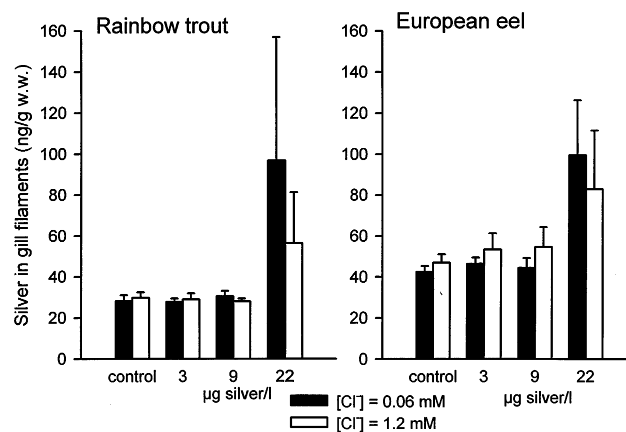

Fig. 6. Silver concentration in gill filaments (ng g^{-1}, wet weight) from rainbow trout and European eel exposed to a range of silver concentrations at two different ambient Cl^- concentrations for a total of 12 and 38 h, respectively. Mean ± S.E.M. ($n = 8$). There were no significant differences from control values ($P < 0.05$).

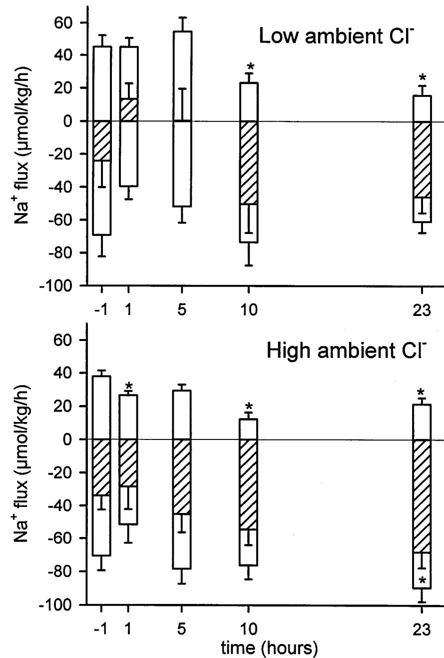

Fig. 7. Unidirectional Na^+ efflux and influx rates (open bars) and net Na^+ flux rates (hatched bars) in European eel at low ambient [Cl^-] (top) and high ambient [Cl^-] (bottom) prior to and during exposure to 3.9 ± 0.3 µg silver l^{-1}. Mean ± S.E.M. ($n = 10$). Positive values represent Na^+ transport into the fish and negative values represent Na^+ loss from the fish. * indicates statistically significant difference from initial control ($P < 0.05$).

this may be part of the explanation. However, a particularly marked difference between the osmoregulation of freshwater rainbow trout and European eel is the turnover rate of whole body Na^+. In the control fish, the turnover of the whole body Na^+ pool can be calculated from the Na^+ influx rates and whole body Na^+ concentration. In the European eel, this turnover rate is 1.1% per day (Na^+ influx, 19 µmol kg^{-1} h^{-1}; whole body Na^+, 42.00 ± 3.31 mmol kg^{-1} (mean ± S.E.M., $n = 20$)) but in the rainbow trout, the corresponding turnover rate is as high as 19% per day (Na^+ influx, 450 µmol kg^{-1} h^{-1}; whole body Na^+, 57 mmol kg^{-1}; Laurén and McDonald (1987)). This is probably one of the most important reasons for the difference in tolerance between the two species.

This means that the internal Na^+ pool will be depleted a lot faster in rainbow trout than in European eel. The mortality of rainbow trout exposed to silver (as silver nitrate) or low pH occurs when the plasma Na^+ concentration is reduced by around 30% (McDonald et al., 1980; Wood et al., 1996a; Webb and Wood, 1998). The rainbow trout exposed to ionic silver in this study experienced an almost total inhibition of Na^+ influx (Fig. 1) and a complete inhibition of branchial Na^+,K^+-ATPase activity (Fig. 5). Assuming that the entire whole body Na^+ pool is readily exchangeable, the plasma Na^+ pool would be depleted by 30% and mortality would occur after around 38 h (19% Na^+ turnover per 24 h) of silver exposure in the rainbow trout. This calculated 'time to mortality' at total Na^+ influx inhibition in the European eel would be 655 h (1.1% Na^+ turnover per 24 h) a point which is addressed subsequently. In the rainbow trout exposed to the highest silver concentration as ionic silver, the plasma Na^+ concentration was reduced by 27% (Fig. 3). These fish were close to death, showing severe hyperventilation and impaired vertical orientation. This is in close agreement with the above mentioned findings of mortality occurring when the plasma Na^+ pool is depleted by 30%. These rainbow trout were only exposed to silver for 12 h—about one third of the 'calculated time to mortality'. This discrepancy probably arises from the assumption that the entire whole body Na^+ pool is readily exchangeable, which is not the case.

Despite the longer silver exposure period, European eels still maintained some Na^+,K^+-ATPase activity even after exposure to the highest silver concentration as ionic silver. Under these conditions, but after a shorter exposure period, the Na^+,K^+-ATPase activity was totally inhibited in rainbow trout (Fig. 5). This apparent difference in enzyme sensitivity to silver exposure could be part of the explanation for the observed difference in tolerance. Interestingly, the enzyme activity of the control European eel, with a very low Na^+ uptake, was as high as the corresponding enzyme activity of the rainbow trout with a much higher Na^+ uptake. This could be an artifact arising from the assay procedure but may suggest that the apical entry step, rather than the extrusion of

Na^+ across the basolateral membrane of the branchial epithelial cells, is the rate-limiting step in Na^+ uptake.

The higher tolerance of the European eel is not due to a compensatory reduction in Na^+ efflux during silver exposure. Silver exposure inhibited unidirectional Na^+ influx in eels but the Na^+ efflux was not reduced correspondingly, resulting in an increased net Na^+ loss during silver exposure (Fig. 7). The European eel can reduce Na^+ efflux in response to transfer from hard to soft freshwater apparently by an increased synthesis of wax-alcohols (Hansen et al., 1999). This decrease occurs immediately after a substantial reduction in Na^+ influx due to a reduction in ambient Na^+ concentration. In the present study, a decreased Na^+ uptake caused by silver exposure was not followed by increased Na^+ retention. This is in close agreement with reported findings for rainbow trout (Morgan et al., 1997; Webb and Wood, 1998). The explanation for the lack of down-regulation could be that the experimental fish were adapted to softwater and presumably had maximally reduced Na^+ efflux rates prior to silver exposure.

Another important reason for the different tolerance may be that branchial Cl^- influx in the rainbow trout is critical to Cl^- homeostasis, and is very sensitive to silver exposure whereas European eel does not depend on branchial Cl^- influx for the Cl^- homeostasis. Furthermore, the extremely low branchial influx of Cl^- in the eel is not influenced by exposure to silver. The plasma Cl^- concentration in the rainbow trout was reduced by as much as 35% after exposure to the highest silver concentration (as ionic silver). In agreement with the Cl^- influx data, the plasma Cl^- concentration of the European eel was not influenced by silver exposure. The chain of events leading to mortality associated with acute silver toxicity in rainbow trout has been summarized by Hogstrand and Wood (1998). In brief, the net loss of Na^+ and Cl^- sets up an osmotic imbalance between plasma and tissues, causing a net shift of water from the extracellular to the intracellular compartment leading to severe hemoconcentration, which eventually leads to death by circulatory failure. In contrast to the rainbow trout, which faces a net loss of both Na^+ and Cl^- during silver exposure, the eel apparently only faces a net loss of Na^+ and the osmotic imbalance arising from silver exposure must thus be less severe. The implications of this for charge balance and acid–base regulation in the blood are unclear at present, but may indicate that the eel suffers a more severe acidosis than the trout. Even given a total inhibition of branchial Na^+ influx at the highest silver concentration applied in the bioassay (100% mortality at 96 h) for European eel, Na^+ depletion alone cannot explain mortality (see 'calculated time to mortality' above). Additional toxic mechanisms, such as acidosis, thus seems likely in the European eel and should be investigated.

4.4. The effect of ambient chloride concentration on silver toxicity

Elevated ambient Cl^- clearly protected against the physiological disturbances induced by silver in rainbow trout (Figs. 1–5). However, in European eel there was no clear protective effect of water Cl^- on any of the measured physiological parameters. The observed protective effects of Cl^- against physiological disturbances in trout agree with other reports on this species (McGeer and Wood, 1998; Bury et al., 1999a) and also agree well with the results of classical toxicity studies (Galvez and Wood, 1997; Bury et al., 1999b). As outlined below, silver toxicity was reduced in proportion to the removal of ionic Ag^+ by complexation into silver chloride complexes.

However, several classical toxicity studies have now shown that there is little or no protective effect of water Cl^- against silver toxicity in the fathead minnow (*Pimephales promelas*) (Brooke et al., 1996; Erickson et al., 1998; Karen et al., 1999; Bury et al., 1999b). In the present study on European eel, the possible protective effects of Cl^- against silver have been assessed only on physiological disturbances, and not on classical toxicity (i.e. 96 h LC50 values), whereas for the fathead minnow, the reverse is true. Thus while no absolute conclusion can be drawn, the available information suggests that the protective effect of Cl^-

does not occur in two of the three species examined to date. Clearly more work is needed to evaluate the suggestion of Wood et al. (1999) that water Cl^- concentration should be taken into account when generating water quality criteria for silver. Other factors such as dissolved organic carbon may be more important in ameliorating silver toxicity to the majority of freshwater fish species.

4.5. How does Cl^- protect against silver toxicity in rainbow trout?

The protective effect is believed to be associated with the geochemical speciation of silver. At low Cl^- concentrations, silver is present mainly as ionic silver, whereas AgCl complexes become dominant at higher Cl^- concentrations. Ionic silver has been shown to be a more potent inducer of toxic effects, in rainbow trout, than other silver complexes (Section 1). This concurs with work on the toxicity of other metals (Borgman, 1983; Luoma, 1983; Brezonik et al., 1991) where the free metal ion has been found to be the most toxic form. One additional possible explanation could be that membrane characteristics of the branchial epithelium may be different at different water Cl^- concentrations, rendering the epithelium more or less vulnerable to silver-induced disturbances.

The target of silver toxicity—the Na^+,K^+-ATPase-enzyme is located at the basolateral membrane of the branchial epithelial cells. This means that silver has to enter the cell across the apical membrane and move through the cytosol before it can act on the Na^+,K^+-ATPase. Intracellular Cl^- concentrations in branchial epithelial cells are likely to be higher than 10 mM (Perry, 1997) so regardless of the speciation of silver in the water, the dominating form of silver in the cytosol would be AgCl complexes (disregarding the potential complexation by amino acids, polypeptides, and proteins in the cytosol). In the present study, exposure to ionic Ag^+ did not yield greater accumulation of silver than did AgCl complexes in either European eel or rainbow trout gills (Fig. 6). The same lack of effect of water Cl^- on gill silver accumulation in trout has also been reported by McGeer and Wood, (1998) and Bury et al. (1999a). Speciation-specific gill loads of silver can thus not explain the protective effect of water Cl^-. Silver speciation could, however, indirectly influence silver toxicity by differences in site-specific accumulation. Accumulation of ionic Ag^+ probably occurs via uptake through Na^+ channels in the apical membrane (Bury and Wood, 1999) whereas the uncharged AgCl complex could accumulate by diffusion through the entire apical surface of the gill epithelium (mainly pavement cells). This would lead to a site-specific accumulation of silver presented as ionic silver in the cells responsible for Na^+ uptake, and a more diffuse accumulation of silver when presented as AgCl complexes that could explain the effect of speciation on silver toxicity. At least two questions still deserve attention. Is there site-specific accumulation of ionic Ag^+ and AgCl complexes in rainbow trout? And, if so, why is this not important for fathead minnow or European eel?

5. Conclusions

The mechanism of silver toxicity in European eel and rainbow trout seems at least partly similar. In both species, the branchial Na^+,K^+-ATPase enzyme was inhibited by silver exposure leading to reduced Na^+ influx and consequently a net loss of Na^+. The difference in tolerance to silver exposure between the two species correlates with differences in whole body Na^+ turnover rates and differences in chloride homeostatic mechanisms. In rainbow trout, both Na^+ and Cl^- influx were impaired by silver exposure. In contrast, European eel showed only impaired Na^+ influx. The well documented ameliorating effect of water chloride on silver-induced physiological disturbances in rainbow trout does not apply to the European eel.

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References

Borgman, U., 1983. Metal speciation and toxicity of free metal ions to aquatic biota. In: Nriagu, J.O. (Ed.), Aquatic Toxicology. Wiley, New York, pp. 47–72.

Brezonik, P.L., King, S.O., Mach, C.E., 1991. The influence of water chemistry on trace metal bioavailability and toxicity to aquatic organisms. In: Newman, M.C., McIntosh, A.W. (Eds.), Metal Ecotoxicology: Concepts and Applications. Lewis, Chelsea, MI, pp. 1–31.

Bornancin, M., De Renzis, G., Maetz, J., 1977. Branchial Cl transport anion-stimulated ATPase and acid–base balance in *Anguilla anguilla* adapted to freshwater: effects of hyperoxia. J. Comp. Physiol. 117, 313–322.

Brooke, L., Erickson, R., Kahl, M., 1996. Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. In: Proceedings of the Second International Conference on Transport, Fate and Effects of Silver in the Environment, Madison, WI, USA, September 11–14, 1994, pp. 119–122.

Bury, N.R., McGeer, J.C., Wood, C.M., 1999a. Effects of altering freshwater chemistry on the physiological responses of rainbow trout to silver exposure. Environ. Toxicol. Chem. 18, 49–55.

Bury, N.R., Galvez, F., Wood, C.M., 1999b. Effects of chloride, calcium and dissolved organic carbon on silver toxicity: comparison between rainbow trout and fathead minnows. Environ. Toxicol. Chem. 18, 56–62.

Bury, N.R., Wood, C.M., 1999. The mechanism of branchial apical silver uptake by rainbow trout is via the proton-coupled Na^+-channel. Am. J. Physiol. (in press).

Bøtter-Jensen, L., Nielsen, S.P., 1989. Beta multicounter systems for low-level measurements of environmental samples. In: Proceedings of the XV Regional Congress of IRPA, pp. 459–464.

Davies, P.H., Goettl, J.P., Sinley, J.R., 1978. Toxicity of silver to rainbow trout (*Salmo gairdneri*). Water. Res. 12, 113–117.

Erickson, R.J., Brooke, L.T., Kahl, M.D., Venter, F.V., Harting, S.L., Markee, T.P., Spehar, R.L., 1998. Effects of laboratory test conditions on the toxicity of silver to aquatic organisms. Environ. Toxicol. Chem 17, 572–578.

Galvez, F., Wood, C.M., 1997. The relative importance of water hardness and chloride levels in modifying the acute toxicity of silver to rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 16, 2363–2368.

Goss, G.G., Perry, S.F., 1994. Different mechanisms of acid–base regulation in rainbow trout (*Oncorhynchus mykiss*) and American eel (*Anguilla rostrata*) during $NaHCO_3$ infusion. Physiol. Zool. 67, 381–406.

Grosell, M.H., Hansen, H.J.M., Rosenkilde, P., 1998. Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. Comp. Biochem. Physiol. C. 120, 295–305.

Hansen, H.J.M., Grosell, M., Rosenkilde, P., 1999. Gill lipid metabolism and unidirectional Na^+ flux in the European eel (*Anguilla anguilla*) after transfer to dilute media: The formation of wax alcohols as a primary response. Aquaculture (in press).

Hogstrand, C., Galvez, F., Wood, C.M., 1996. Toxicity, silver accumulation and metallothionein induction in freshwater rainbow trout during exposure to different silver salts. Envion. Toxicol. Chem. 15, 1102–1108.

Hogstrand, C., Wood, C.M., 1998. Toward a better understanding of the bioavailability, physiology, and toxicity of silver in fish: implications for water quality criteria. Environ. Toxicol. Chem. 17, 547–561.

Holliday, C.W., 1985. Salinity-induced changes in the gill Na/K-ATPase activity in the mud fiddler crab, *Uca pugnax*. J. Exp. Zool. 233, 199–208.

Janes, N., Playle, R.C., 1995. Modelling silver binding to gills of rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 14, 1847–1858.

Jurss, K., Bastrop, R., 1995. The function of mitochondria-rich cells (chloride cells) in teleost gills. Rev. Fish Biol. Fisher. 5, 235–255.

Karen, D., Ownby, D.R., Forsythe, B.L., Bills, T.P., LaPoint, T.W., Cobb, G.B., Klaine, S.J., 1999. Influence of water quality on silver toxicity to rainbow trout (*Oncorhynchus mykiss*) fathead minnows (*Pimephales promelas*) and the water flea (*Daphnia magna*). Environ. Toxicol. Chem. 18, 63–70.

Kirsch, R., 1972. The kinetics of peripheral exchanges of water and electrolytes in the silver eel (*Anguilla anguilla* L.) in fresh water and seawater. J. Exp. Biol. 57, 489–512.

Laurén, D.J., McDonald, G.D., 1986. Influence of water hardness, pH and alkalinity on the mechanisms of copper toxicity to rainbow trout, *Salmo gairdneri*. Can. J. Fish. Aquat. Sci. 43, 1488–1496.

Laurén, D.J., McDonald, G.D., 1987. Acclimation to copper by rainbow trout, *Salmo gairdneri*: physiology. Can. J. Fish. Aquat. Sci. 44, 99–104.

LeBlanc, G.A., Mastone, J.D., Paradice, A.P., Wilson, B.F., Lockhart, H.B., Robillard, K.A., 1984. The influence of speciation on the toxicity of silver to fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 3, 37–46.

Luoma, S.N., 1983. Bioavailability of trace metals to aquatic organisms—a review. Sci. Total. Environ. 28, 1–22.

Maetz, J., 1956. Les échanges de sodium chez les poissons *Carassius auratus* L. Action d'un inhibiteur de l'anhydrase carbonique. J. Physiol. (Paris) 48, 1085–1099.

McDonald, D.G., Hobe, H., Wood, C.M., 1980. The influence of calcium on the physiological responses of the rainbow trout *Salmo gairdneri*, to low environmental pH. J. Exp. Biol. 88, 109–131.

McDonald, D.G., Reader, J.P., Dalziel, T.R.K., 1989. The combined effects of pH and trace metals on fish ionoregulation. In: Morris, R., Taylor, E.W., Brown, D.J.A., Brown, J.A. (Eds.), Acid Toxicity and Aquatic Animals, Society for Experimental Biology, Seminar Series 34, pp. 221–242.

McGeer, J.C., Wood, C.M., 1998. Protective effects of water Cl^- on physiological responses to waterborne silver in rainbow trout. Can. J. Fish. Aquat. Sci. 55, 2447–2454.

Morgan, I.J., Henry, R.P., Wood, C.M., 1997. The mechanisms of acute silver nitrate toxicity in freshwater rainbow trout (*Oncorhynchus mykiss*) is inhibition of gill Na^+ and Cl^- transport. Aquat. Toxicol. 38, 145–163.

Nebeker, A.V., McAuliffe, C.K., Mshar, R., Stevens, D.G., 1983. Toxicity of silver to steelhead and rainbow trout, fathead minnows and *Daphnia magna*. Environ. Toxicol. Chem. 2, 95–104.

Perry, S.F., 1997. The chloride cell: structure and function in the gills of freshwater fishes. Ann. Rev. Physiol. 59, 325–347.

Schecher, W.D., McAvoy, D.C., 1992. MINEQL+: a software environment for chemical equilibrium modelling. Environ. Urban Sys. 16, 65–76.

Schou, J.C., 1973. The relationship of the $(Na^+ + K^+)$-activated enzyme system to transport of sodium and potassium across the cell membrane. Bioenergetics 4, 1–30.

Webb, N.A., Wood, C.M., 1998. Physiological analysis of the stress response associated with acute silver nitrate exposure in freshwater rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 17, 579–588.

Wilkie, M.P., Wright, P.A., Iwama, G.K., Wood, C.M., 1993. The physiological responses of the Lahontan cutthroad trout (*Oncorhynchus clarki henshawi*), a resident of a highly alkaline lake (pH 9.4), to challenge at pH 10. J. Exp. Biol. 175, 173–194.

Wood, C.M., 1991. Branchial ion and acid–base transfer in freshwater teleost fish: environmental hyperoxia as a probe. Physiol. Zool. 64, 68–102.

Wood, C.M., Hogstrand, C., Galvez, F, Munger, R.S., 1996a. The physiology of waterborne silver exposure in freshwater rainbow trout (*Oncorhynchus mykiss*) I: the effects of ionic Ag^+. Aquat. Toxicol. 35, 93–109.

Wood, C.M., Hogstrand, C., Galvez, F., Munger, R.S., 1996b. The physiology of waterborne silver exposure in freshwater rainbow trout (*Oncorhynchus mykiss*) II: the effects of silver thiosulphate. Aquat. Toxicol. 35, 111–125.

Wood, C.M., Playle, R.C., Hogstrand, C., 1999. Physiology and modelling of mechanisms of silver uptake and toxicity in fish. Environ. Toxicol. Chem. 18, 71–83.

Zall, D.M., Fisher, D., Garner, M.D., 1956. Photometric determination of chlorides in water. Anal. Chem. 28, 1665–1678.