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Kinetics of radiolabelled silver uptake and depuration in the gills of rainbow trout (*Oncorhynchus mykiss*) and European eel (*Anguilla anguilla*): the influence of silver speciation

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Abstract

We examined the influence of speciation on the kinetics of silver uptake and depuration in the gills of two freshwater fish, the rainbow trout (*Oncorhynchus mykiss*) which has high branchial Na^+ and Cl^- uptake rates and is relatively sensitive to silver, and the European eel (*Anguilla anguilla*, yellow stage) which has low ion uptake rates and is relatively resistant to silver. Fish previously acclimated to the appropriate chloride level were exposed to $^{110m}AgNO_3$ (1.3 μg l^{-1}, sublethal) for 24 h in synthetic softwater with either low (10 μM) or high (1200 μM) chloride concentration, and then followed over a subsequent 67-day post-exposure period in silver-free water of the same chloride content. The exposures were therefore mainly to the free ion, Ag^+ in the low chloride water versus mainly to the neutral aqueous complex, $AgCl_{aq}$ in the high chloride water. In trout, but not in eel, water chloride is known to protect against physiological disturbances and toxicity caused by Ag^+. In both fish species, at both chloride levels, silver uptake exhibited complex kinetics. Gill silver loading occurred slowly until 6 h, then rose greatly to a peak at 12 h, followed by significant net depuration thereafter during continued exposure. By 24 h, net gill loading was three- to fivefold greater from $AgCl_{aq}$ than from Ag^+ exposure in both species, and threefold greater in trout than in eel under both conditions, with trout holding a lower fraction of the whole body burden in their gills. During the post-exposure period, depuration of silver from the gills occurred rapidly in trout, but very slowly in eel, such that gill silver burdens were greater in eel throughout the 67-day period on both an absolute and relative basis (e.g. 35% of whole body burden in eel versus $<3\%$ in trout at day 8). The kinetics of depuration were described by two phase exponential models, with break points between the fast and slow phases at 1 and 15 days for trout and eel, respectively. We conclude that speciation affects not only uptake rates but also the kinetics of depuration. When silver is loaded from $AgCl_{aq}$ it is clearly more labile than from Ag^+ exposures, with 1.6–1.8-fold greater loss rates during the fast phases in both species. Differences in branchial silver uptake between eel and trout correlate well with differences in acute toxicity, but are not as large as differences in ion uptake rates. The complex time-dependent

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patterns of gill loading, and the higher loading from $AgCl_{aq}$ than from Ag^+, mean that gill total silver burden is not an appropriate endpoint for biotic ligand modelling. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Silver; Metal speciation; Rainbow trout; European eel; Gill; Fish; Metal uptake; Metal depuration; Pharmacokinetics; Biotic ligand model; BLM

1. Introduction

Research on the toxicity of silver to freshwater fish over the past few years has identified inhibition of branchial Na^+,K^+ATPase activity, and associated blockade of active Na^+ and Cl^- uptake at the gills, as the key mechanism of acute toxic action (reviewed by Hogstrand and Wood, 1998; Wood et al., 1999). At least in rainbow trout, only the free ionic form of silver (Ag^+) appears to be toxic (Galvez and Wood, 1997), whereas various complexed forms such as silver thiosulphate, silver chloride and silver-DOM (dissolved organic matter) appear to be relatively benign (LeBlanc et al., 1984; Hogstrand et al., 1996; Bury et al., 1999a; Karen et al., 1999). Much less is known about the actual uptake of silver into the gills and subsequently into the body, though one recent study on rainbow trout has indicated that Ag^+ enters gill ionocytes via the same route as Na^+, through an apical channel, driven by the electrical gradient created by an H^+ATPase (Bury and Wood, 1999). This study also found that gill silver levels increased, peaked, then decreased during continuous exposure, suggesting complex loading and clearance kinetics. Clearance from the gills appeared to be associated with accumulation by the rest of the body. A companion study (Bury et al., 1999b) indicated that the transport step across the basolateral membranes of the gills is carrier-mediated and ATP-dependent, suggesting active transport of silver. It is clear that silver can also accumulate in the gills and internal organs when present in the water as non-toxic silver thiosulphate or silver chloride complexes (Eisler, 1996; Wood et al., 1996b; Hogstrand et al., 1996; Hogstrand and Wood, 1998; Ratte, 1999). However nothing is known about the mechanism(s), kinetics of accumulation, efficiency relative to Ag^+, or even the form(s) in which silver is taken up from such complexes. Furthermore, nothing is known about the kinetics of depuration of silver from the gills once exposure to waterborne silver is over.

These issues are particularly topical because of recent interest in the construction of gill binding or 'biotic ligand models' for predicting metal toxicity to aquatic organisms in different water qualities (Renner, 1997; Bergman and Dorward-King, 1997; Playle, 1998). Several such models have been constructed for silver (Janes and Playle, 1995; Di Toro et al., 1998; Paquin et al., 1999), but the focus has been on only the short-term (2–3 h) binding of Ag^+ to rainbow trout gills. The influences of time (i.e. kinetics), the potential uptake of other forms of silver, and possible differences between species of fish have not been explicitly considered. There are several reasons to suspect that such factors may have a large influence. Firstly, at longer exposure times to several different forms of silver (including free Ag^+), McGeer and Wood (1998) and Bury et al. (1999c) could find no relationship between gill silver burden and Na^+,K^+ATPase inhibition. Secondly Grosell et al. (2000) reported large differences in toxic responsiveness to Ag^+ between the yellow stage European eel (resistant) and rainbow trout (sensitive), and that chloride complexation protected against Na^+, K^+ATPase inhibition and associated physiological disturbances in the trout, but not in the eel. However, using analysis by graphite furnace AAS at a single time point in each species (12 h exposure in trout, 38 h in eel), Grosell et al. (2000) were unable to detect any significant differences in gill silver burden either as a result of chloride complexation or between the two species.

Using the study of Grosell et al. (2000) as a point of departure and a more sensitive radiotracer technique (^{110m}Ag; cf. Garnier and Baudin, 1990; Garnier et al., 1990; Bury and Wood, 1999) for tracking silver accumulation, the present study

had several objectives. The first was to directly compare the gill silver burdens in the silver-sensitive trout versus the silver-tolerant eel at several different times from 3 to 24 h of continuous exposure to a sublethal level of ^{110m}Ag-labelled silver. The second was to compare in both species, at these same times, the influence on gill silver burden of presenting the same silver concentration either mainly as the free ion (Ag^+) or mainly as the neutral aqueous chloride complex ($AgCl_{aq}$). This was accomplished by exposing the fish in either low chloride or chloride-supplemented softwater to which they had been acclimated. The final objective was to compare the depuration kinetics of silver from the gills in trout versus eel, and in low chloride versus high chloride softwater, over a subsequent 67-day period during which silver was not present. In addition, because a large influence of water chloride on uptake and depuration was seen in the trout, the internal distribution of silver within various subcellular fractions of trout gills was also examined, using the differential centrifugation method of Julshamn et al. (1988).

2. Methods and materials

2.1. Experimental animals and acclimation

Rainbow trout (*Oncorhynchus mykiss*; 20–30 g) were obtained from Reersoe fish farm, Kalundborg, Denmark while European eels (*Anguilla anguilla*; 45–75 g, all yellow stage, non-migratory) were collected from Roskilde Fjord using fyke nets in autumn, 1997. Since the eels were collected from brackish water, they were first acclimated to flowing freshwater for 30 days ($Na^+ = 1$ mM, $Ca^{2+} = 2.6$ mM; $Cl^- = 1.6$ mM) for acclimation to laboratory conditions and experimental temperature (14 ± 0.5°C) at the RISOE laboratory. Trout, which had originated from freshwater of comparable composition, were held only 3–7 days in freshwater prior to acclimation to synthetic softwater (below). Eels were fed fresh mussels and trout were fed commercial pellets once a day.

For 16 days prior to the experimental period (December 1997–February 1998), approx. 200 individuals of each species were acclimated to flowing low chloride (10 μM) softwater and 200 to high chloride (1200 μM) softwater, in separate 150 l PVC tanks. This synthetic softwater (see Table 1 for composition) was created from tapwater by reverse osmosis for use as cooling water in the RISOE Nuclear Research Reactor. Chloride was added as KCl from a concentrated stock solution via a peristaltic pump. KCl was chosen as the chloride salt because K^+ does not interfere with Ag^+ uptake (Bury and Wood, 1999), does not alter Ag^+ toxicity (Galvez and Wood, 1997), and exerts no ill effects on salmonids at concentrations even five- to tenfold higher than used here (Wilkie et al., 1993). Measured chloride concentrations were always 9–12 μM in the low chloride water and 1050–1350 μM in the high chloride water.

2.2. Exposure to ^{110m}Ag

Feeding was suspended for 3 days prior to the experiments so as to minimize defecation during the exposure period. Exposure to ^{110m}Ag was per-

Table 1
Composition of the low chloride and high chloride softwater used in the ^{110m}Ag loading and depuration experiments[a]

	Low chloride water	High chloride water
Na^+ (μM)	50	50
Cl^- (μM)	10	1200
K^+ (μM)	10	1200
Ca^{2+} (μM)	10	10
Mg^{2+} (μM)	<1	<1
DOC (mg C l^{-1})	1.3	1.3
pH	7	7
Total Ag[b] (μg l^{-1})	1.3	1.3
Ag-DOC[b] (μg l^{-1})	0.5	0.5
Ag^{+}[b] (μg l^{-1})	0.78	0.23
AgClaq[b] (μg l^{-1})	0.02	0.51

[a] Silver preent only during the 24 h exposure to ^{110m}Ag; speciation according to MINEQL (see text details).

[b] Nominal values; measured values remained within ±15% of nominal values throughout.

formed in separate trials for the loading and depuration experiments, with the latter actually being performed first.

In the ^{110m}Ag exposures for the depuration experiments, trout and eels were exposed in separate tanks. For convenience, exposures for the two species were staggered by 24 h, to allow adequate time for sample processing ($\sim$6–9 h for 10 fish $\times$ 2 treatments) before the next sampling period occurred. For each exposure, 80 trout or 80 eels were placed in 200 l of their respective low chloride or high chloride softwater in temperature controlled (14°C), vigorously aerated glass aquaria, and allowed to settle for 1 h. Then ^{110m}Ag (as $AgNO_3$), custom-synthesized in the RISOE Nuclear Research Reactor, nominal specific activity = 470 gigaBecquerels (GBq per g Ag) was added to each 200 l volume, yielding a total silver concentration of about 1.3 μg l^{-1} in the exposure water. The walls of the tanks had been earlier pre-equilibrated at the same concentration and specific activity. Water samples were subsequently taken at 10, 20, 30, 40, 60 and 90 min, and at 2, 4, 8, 12 and 24 h for measurement of ^{110m}Ag radioactivity and total Ag concentration. As the radioisotopic addition was the only source of Ag, specific activity did not change appreciably, whereas net radioactivity declined by no more than 20% over the 24 h exposure due to uptake by the fish and adhesion to surfaces. All fish were exposed for 24 h, after which they were transferred to the depuration tanks and sampled at the appropriate times up to 67 days post-exposure.

In the ^{110m}Ag exposures for the loading experiments, eels and trout were exposed together in the same tanks. This approach was adopted based on our experience with the exposures for the depuration experiments, where small differences in water radioactivity, and therefore in absolute silver concentration ($\pm$15% relative to the nominal value of 1.3 μg l^{-1}) were noted between experiments as a result of differences in uptake and adhesion to the fish. In addition, exposures for each time point (3, 6, 12 and 24h) of loading were carried out separately and the number of fish per treatment at each sample time was reduced to six. Otherwise, the time needed to process the samples would have been greater than the time intervening between samples. In each exposure, six trout and six eels were placed in 100 l of the appropriate low chloride or high chloride softwater, allowed to settle for 1 h, and then the ^{110m}Ag was added to a total concentration of 1.3 μg l^{-1}, as for the depuration experiments, with water samples taken at the same time points. Sampling occurred immediately at the end of each exposure as outlined below.

2.3. Depuration

From each ^{110m}Ag exposure, the eighty fish of one species per chloride concentration were placed into one of the four identical glass depuration tanks (volume = 80 l each, 20 fish per tank), which received vigorous aeration and a one-pass flow-through (rate = 0.7 l min^{-1}) of the appropriate low chloride or high chloride softwater at 14 $\pm$ 0.5°C. No silver was present in the water. At each sampling time (3 h and 1, 2, 4, 8, 15, 32 and 67 days post-exposure), 10 fish were sacrificed from each treatment, except for trout at day 67 where $N = 4$ and 5 at low and high chloride respectively due to escapees. There were no mortalities. Feeding was resumed following the day 8 sample point.

In order to evaluate whether there were significant ^{110m}Ag losses to the water during the first few hours of depuration, fish of both species from the low chloride treatments only were placed in individual 0.6 l flux chambers (closed PVC tubes, with aeration and temperature control) for the first 1 to 3 h of the post-exposure period. Water samples were taken in duplicate at the start and end of these 2 h fluxes to detect ^{110m}Ag appearance in the water. Immediately at the end of these flux experiments, the fish were sacrificed and sampled for the 3 h post-exposure time point.

2.4. Sampling and calculations

At sampling, fish were rapidly anaesthetized with urethane, and then rinsed in isotope-free and anaesthetic-free water. A 0.5 ml blood sample was drawn by caudal puncture, and then the fish was euthanized by either a cephalic blow (trout) or cutting the spinal cord (eels). The gills were the

first tissue removed, and were scraped with a glass slide to displace the soft tissue from the filaments. This soft tissue was then manually homogenized to a fine slurry. An aliquot (~ 50–100 mg) was spread out evenly on a tared counting planchet, dried to a constant weight, and then counted for ^{110m}Ag radioactivity. All the other tissues were then dissected out, homogenized individually using a glass-Teflon® homogenizer (or a domestic food-processor in the case of the remaining carcass), and subsamples were plated onto tared planchets and dried to a constant weight in a similar manner. Thus, as homogenized subsamples of all tissues (plus plasma and bile) were prepared for radioactivity counting, and as the proportion of total body weight represented by each tissue or body fluid was known (C. Hogstrand, C.M. Wood, M. Grosell, and H. Hansen, unpublished results), then the total amount of radioactivity in the whole body and in each individual tissue could be calculated, as well as the relative contribution of each tissue to whole body radioactivity. In turn this could be converted to an absolute silver concentration, based on the known specific activity of the water during the exposure period. In order to compare data directly between different exposures where the mean measured silver concentration varied slightly from the nominal value of 1.3 μg l^{-1}, all specific activities used in calculations were normalized back to this nominal concentration.

In addition, at 3 h, and 2, 8 and 32 days of the post-exposure period, gills from five of the 10 trout in each sample group from both low chloride and high chloride softwater were subjected to a differential centrifugation procedure (Julshamn et al., 1988) to determine the subcellular distribution of ^{110m}Ag. We suspected that differences in the branchial uptake and depuration of ^{110m}Ag between and Ag^{+} and $AgCl_{aq}$ exposures might be related to differences in subcellular distribution. As the ^{110m}Ag radioactivity was measured in all fractions, then addition of the fractions produced a measurement of total gill radioactivity comparable to that in the initial homogenate and also comparable to those yielded by the simple homogenization technique used for the other five fish in the group. We initially attempted to investigate subcellular distribution of ^{110m}Ag in eel gills as well, but this portion of the experiment was aborted as it became apparent that eel gills could not be fractionated with the differential centrifugation method used, because their high mucous content caused the supernatant to gel.

2.5. Analytical techniques

^{110m}Ag emits both β- and γ-radioactivity. For measurement of ^{110m}Ag radioactivity in the majority of samples, water samples and tissue homogenates were dried onto planchets as described above, then counted for β-radioactivity to 99% statistical accuracy using two custom-built low background Geiger-type multi-sample counters (Bøtter-Jensen and Nielsen, 1989). This allowed 10 samples to be counted at once, an advantage in view of the more than 5000 samples processed in this entire study. For this type of counting, quench correction is not an issue, but uniform sample geometry is a concern, so care was taken to ensure the latter by making sure that the dried tissue homogenate was spread out uniformly on the planchet. For a few samples, radioactivity was great enough to exceed the analytical range of the β-counters; these samples were therefore recounted by traditional γ-counting, using appropriate standards to cross-calibrate the two detection systems. Additional details are provided by Hansen et al. (2001).

Water samples were taken to determine the exact concentration of total silver during the exposure period, and therefore also its specific activity. These samples were acidified with 1% trace-metal grade HNO_3 (Merck Chemicals) and analysed by graphite furnace atomic absorption (Varian AA-1275 with GTA-9 atomiser) as detailed by Grosell et al. (2000). Water chloride concentrations were checked periodically throughout the experiments using either a Radiometer CMT-10 chloridometer or the colorimetric assay of Zall et al. (1956), and water pH was measured using a Radiometer GK2401C electrode. Representative low and high chloride softwater samples were analysed for Na^{+}, K^{+}, Mg^{2+} and Ca^{2+} by atomic absorption (Varian AA-1275) and for dissolved organic carbon (DOC) by a Rosemount

Analytical DC-180 total organic carbon analyser. The speciation of silver in the exposure water was calculated using the MINEQL+ aquatic geochemistry program, version 3.01 (Schecher and McAvoy, 1992), with the addition of conditional equilibrium constants for Ag-DOC and H-DOC taken from Janes and Playle (1995).

For subcellular fractionation, trout gill samples (100–400 mg) were carefully weighed and individually homogenized in 2.00 ml of isotonic buffer, 35 mM Tris–HCl/0.2 M KCl/0.25 M sucrose, pH 7.4 (homogenization buffer), on ice, using a glass-Teflon® homogenizer. A 500-μl aliquot of each homogenate was withdrawn and saved for total gill tissue radioactivity counting. The rest of the homogenate was centrifuged at $370 \times g$, 4°C, for 5 min. Unusually high mucous content of the gill samples from the first two sampling times processed for subcellular fractionation (3 and 48 h post-exposure) made it difficult to separate the supernatant from the pellet after this low-speed centrifugation, because an interphase of gelled supernatant was formed. To deal with this problem, the upper liquid phase was withdrawn and 1.50 ml of homogenization buffer was added, followed by centrifugation at $1000 \times g$. The sediment produced ('nuclear' fraction = N) was saved while the supernatant was pooled with the supernatant from $370 \times g$ centrifugation. This combined supernatant was further centrifuged at $9200 \times g$, 4°C, for 5 min. The pellet ('mitochondria-lysosome' fraction = ML), was saved for subsequent analysis. Finally, microsomes were separated from the cytosol by centrifugation at $130\,000 \times g$, 4°C, for 60 min. The resulting pellet ('microsomal' fraction = MS) and the supernatant ('cytosolic' fraction = C) were saved. All subcellular fractions were transferred to individual planchets and counted for ^{110m}Ag radioactivity as described above. The influence of the added $1000 \times g$ centrifugation step on purity of the separated fractions was not tracked using marker enzymes. However, the weight of the subsequent mitochondrial fraction ($9200 \times g$ pellet) was not changed measurably and microscopic examination revealed no visible contamination by mitochondria in the 'nuclear fraction' ($1000 \times g$ pellet).

2.6. Statistical analyses

Data have been expressed as means ± 1 S.E (N) where N represents the number of different fish contributing to the mean. Differences between treatments, and/or between species at the same sample time were evaluated by Student's unpaired two-tailed t-test. The influence of time and chloride concentration on loading during the exposure period, and on depuration during the post-exposure period were evaluated by two-way ANOVA for each species. Gill subcellular fractionation data (trout only) were analyzed in the same manner. All percentage data were normalized by arcsin transformation prior to statistical tests. Rate constants (fractional loss day^{-1}) quantifying the depuration of ^{110m}Ag radioactivity from the gills during the post-exposure period were determined by fitting two phase exponential models (Causton, 1983) to the time-series data using individual data points for every fish; break-points between the two phases were chosen so as to maximize the r^2 value for the first phase. A significance level of $P \leq 0.05$ was employed throughout.

3. Results

3.1. Mortality and water chemistry

Exposure to approximately 1.3 μg l^{-1} of radioactively labelled silver as $AgNO_3$ for up to 24 h was clearly sublethal in both low chloride and high chloride softwater; no fish died during the exposures or during the 67 days thereafter. The low and high chloride exposure waters differed only in K^+ and Cl^- concentration (Table 1). Based on calculations with MINEQL+, approximately 38% of the total silver existed as Ag-DOC complexes in both exposure waters due to the very high binding affinity of DOC ($\log K = 9.0$, binding site density 35 nmol per mg C; Janes and Playle, 1995) at a measured concentration = 1.3 mg C l^{-1} (Table 1). However, based on the speciation calculations, the vast majority of the remainder existed as Ag^+ in the low chloride water, whereas the neutral dissolved aqueous $AgCl_{aq}$ complex predominated in the high chlo-

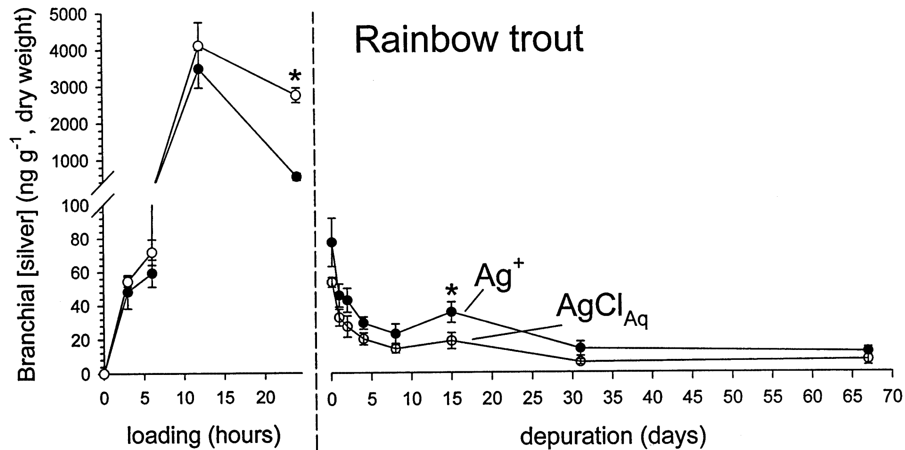

Fig. 1. Kinetics of uptake (over 24 h of exposure) and depuration (over 67 days post-exposure) of silver in the gills of rainbow trout exposed for 24 h to 1.3 μg l^{-1} silver in either low chloride water (silver mainly as Ag^+) or high chloride water (silver mainly as $AgCl_{aq}$). Means ± 1 S.E.M. ($N = 10$ at each sample point except day 67, where $N = 4$ for Ag^+ and $N = 5$ for $AgCl_{aq}$ treatments). Asterisks indicate significant differences ($P \leq 0.05$) between Ag^+ and $AgCl_{aq}$ treatments at the same sample time. The overall effects (by two-way ANOVA) of chloride and time were each significant ($P \leq 0.05$) during the 24h exposure period and during the 67-day post-exposure period.

ride water (Table 1). Thus relationships in figures have been labelled by this convention: Ag^+ for low chloride water; $AgCl_{aq}$ for high chloride water. However it should be noted that according to the speciation calculations, some Ag^+ was still present in the high chloride water, amounting to about 29% of that in low chloride water.

3.2. Gill loading kinetics

In both species and water qualities, silver loading onto the gills occurred slowly up to 6 h (Fig. 1 and Fig. 2). However, by 12 h there appeared to be a breakthrough phenomenon, such that gill silver loads in both trout and eel increased more than fiftyfold. By two-way ANOVA, the overall effect of time was significant for both species, but the overall effect of chloride was significant only for trout. For trout at 12 h, there was no significant difference between $AgCl_{aq}$ and Ag^+ exposures (Fig. 1), whereas for eel, net gill loading at this time was significantly greater from Ag^+ than from $AgCl_{aq}$ (Fig. 2). However, despite the continuation of the waterborne exposures, significant net depuration of the gill silver burden occurred in both species and water qualities between 12 and 24 h of exposure. For both species, the 'apparent extent' of the depuration in Ag^+ exposures (80–90% decreases) was much greater than in $AgCl_{aq}$ exposures (35–60%). However, it must be remembered that both loading and depuration are occurring simultaneously in this situation of continued external exposure, so differences in either or both factors could contribute to the observed patterns. Notably at 24 h, the gill burden from $AgCl_{aq}$ exposure was significantly higher than from Ag^+ exposure in both species, by threefold (eels; Fig. 2) to fivefold (trout; Fig. 1). For eels, this was a reversal of the situation at 12 h. Absolute gill burdens in rainbow trout at 24 h were threefold higher than in eels for both water qualities, a highly significant difference.

Despite the small absolute contribution of the gill tissue to body mass (eels = 0.55% of wet weight; trout = 0.42% of wet weight), the gills were the largest individual contributors (23–82%) to the whole body silver burden throughout the 24 h exposure period (Fig. 3). However, there were important differences between eels and trout in the partitioning of the silver burden between gills and the rest of the body. Throughout the exposure, a much smaller percent of the total was

retained in the gills of the trout. Considering that total body loading at this time averaged 33.0–51.2 ng g^{-1} in trout versus 17.5–23.1 ng g^{-1} in eels, this is a marked difference. In trout, the percentage of the total uptake in the gill tissue was greater during exposure to $AgCl_{aq}$ than to Ag^+ (Fig. 3). In eels, there was no significant effect of chloride on the partitioning of silver between gills and the rest of the body.

3.3. Gill depuration kinetics

By the time the first post-exposure sample was taken at 3 h, a remarkable further depuration of silver from the gills had occurred in the rainbow trout, gill silver burden falling from several thousand to less than 100 ng g^{-1} (Fig. 2). This trend was particularly marked in the trout which had been exposed to $AgCl_{aq}$ (> 90% decrease); branchial concentrations were now slightly below those of trout which had been exposed to Ag^+, though the difference was not significant. In eels, depuration over this first 3 h of post-exposure was much less, though again it was more marked in the eels (60% decrease) which had been exposed to $AgCl_{aq}$ than in those which had been exposed to Ag^+ (negligible change). Notably, gill silver burdens were now two- to threefold higher in eels than in trout, a highly significant difference, and a reversal of the situation 3 h earlier at the end of exposure.

Measurements of ^{110m}Ag flux to the water were made only on the fish which had been exposed to Ag^+, and which therefore had the slower depuration rates, and only from hours 1 to 3. ^{110m}Ag losses to the water over this period were undetectable in eel. For a few of the trout, there was a detectable elevation in water cpm (relative to a background of 5 cpm for a 5 ml sample) whereas for others there was not. However, we can calculate that even if all the cpm that were lost from the gills (0.42% of body weight or 0.1 g for a 25 g trout) between 24 h exposure and 3 h post-exposure had appeared in the 600 ml water volume of the flux chamber, water cpm would have increased by only about 10% above background, which is about the detection limit.

The kinetics of depuration of silver from the gills continued to be very different between trout and eel for much of the post-exposure period, with the gills releasing silver much more slowly in the latter. For example, at day 8, the gills still held about 35% of the whole body silver burden in eels, whereas this value was less than 3% in trout,

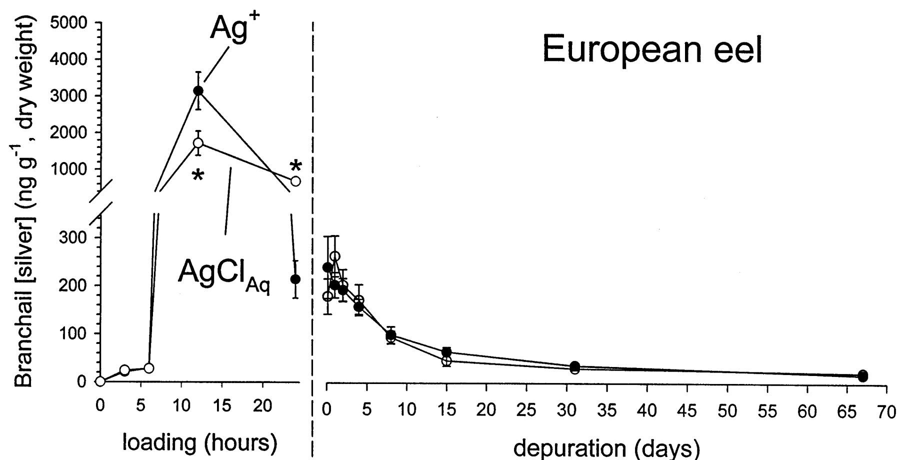

Fig. 2. Kinetics of uptake (over 24 h of exposure) and depuration (over 67 days post-exposure) of silver in the gills of European eel exposed for 24 h to 1.3 μg l^{-1} silver in either low chloride water (silver mainly as Ag^+) or high chloride water (silver mainly as $AgCl_{aq}$). Means ± 1 S.E.M. ($N = 10$ at each sample point). Asterisks indicate significant differences ($P \leq 0.05$) between Ag^+ and $AgCl_{aq}$ treatments at each sample time. The overall effects (by two-way ANOVA) of time were significant ($P \leq 0.05$) during the 24 h exposure period and during the 67-day post-exposure period, but the overall effects of chloride were not significant.

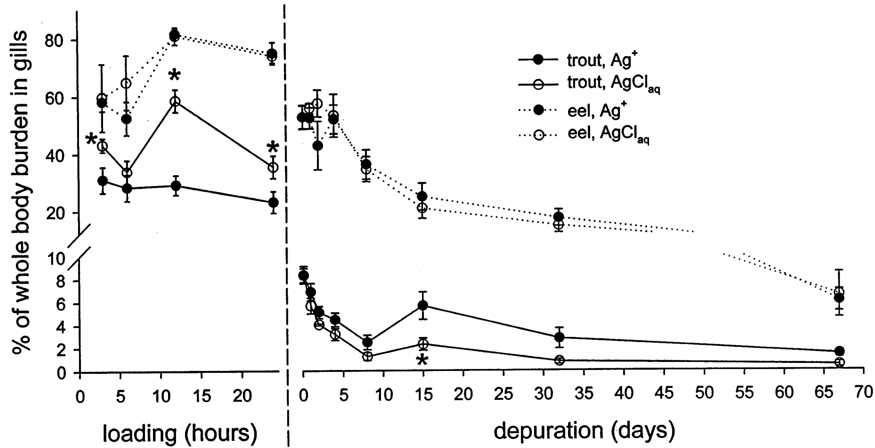

Fig. 3. Gill silver burden, as a percent of whole body burden, in rainbow trout (solid lines) and European eel (dotted lines) exposed for 24 h to 1.3 μg l^{-1} silver in either low chloride water (silver mainly as Ag^+) or high chloride water (silver mainly as $AgCl_{aq}$) and then allowed to depurate for 67 days in the same water. Means ± 1 S.E.M. ($N = 10$ at each sample point, except for trout at Day 67, where $N = 4$ for Ag^+ and $N = 5$ for $AgCl_{aq}$ treatments). Asterisks indicate significant differences ($P \leq 0.05$) between Ag^+ and $AgCl_{aq}$ treatments for rainbow trout at the same sample time. For eel, there were no significant differences between Ag^+ and $AgCl_{aq}$ treatments at the same sample time. However, at every sample time, eel values were significantly greater than trout values. For trout, the overall effects (by two-way ANOVA) of chloride and time were each significant ($P \leq 0.05$) during the 24 h exposure period and during the 67-day post-exposure period. For eel, the overall effects (by two-way ANOVA) of time were significant ($P \leq 0.05$) during the 24h exposure period and during the 67-day post-exposure period, but the overall effects of chloride were not significant.

regardless of water chloride concentration (Fig. 3). Right out to 67 days, gill silver burdens remained significantly above background in both species. For both species, the best fits to the data were obtained using two phase exponential models, but with very different breakpoints between the two phases — day 1 for trout, and day 15 for eels, at both high and low water chloride levels. Thus the rate constants (fractional loss day^{-1}) for the first phase were over an order of magnitude higher in trout than in eels, whereas the rate constants for the second phase were fairly comparable (Table 2). In both species, the rate constants of the first phase were significantly higher in fish which had been exposed to $AgCl_{aq}$ than in those which had been exposed to Ag^+. This difference persisted during the second phase in trout but not in eels. These results were in accord with analysis of the same data by two-way ANOVA, which demonstrated a significant effect of time in both species, but a significant effect of water chloride only in trout.

In contrast to the gills, the whole body burden of silver did not change at all in trout from 3 h to 67 days post-exposure period, averaging 12.8 ± 0.8 and 13.0 ± 1.0 ng g^{-1} dry weight in $AgCl_{aq}$-exposed and Ag^+-exposed fish respectively. However in eels, the whole body burden gradually decreased by about 50% over the 67 days from 3 h values of 8.1 ± 1.5 and 9.9 ± 2. 6 ng g^{-1} in the comparable treatments. The slow loss of silver from the gill tissues accounted for most of this decrease, with the gill silver burden dropping from about 50% of the total at the start of the post-exposure period (3 h) to about 7% by day 67 (Fig. 3). In trout, the decrease was from about 6% to 1% over the same time frame. Two way ANOVA demonstrated that the overall influence of chloride on % distribution was significant in trout but not in eels, whereas the overall influence of time was significant in both, comparable to the results of the earlier ANOVA for gill silver burdens expressed on an absolute basis.

3.4. Subcellular fractionation of trout gills

Despite the obviously faster depuration of silver from the gills of trout in the $AgCl_{aq}$ treatment,

Table 2
Rate constants indicating fractional loss per day of gill ^{110m}Ag burden in trout and eel in either low chloride or high chloride water during the post-exposure period[a]

		Phase 1			Phase 2		
		Rate constant	P	r^2 (%)	Rate constant	P	r^2 (%)
Trout	Low chloride	-1.867 ± 0.373	<0.0001	56.9	-0.022 ± 0.005	<0.0001	29.0
	High chloride	-3.354 ± 0.629[b]	<0.00005	61.3	-0.038 ± 0.007[b]	<0.00005	41.2
Eels	Low chloride	-0.099 ± 0.015[c]	<0.00005	41.3	-0.031 ± 0.005	<0.0005	55.5
	High chloride	-0.154 ± 0.016[b,c]	<0.00005	61.5	-0.019 ± 0.006[c]	<0.005	25.0

[a] Rate constants (mean $\pm$ 1 S.E.M.) were obtained by two phase exponential models with breakpoints at one day for trout, and 15 days for eels (see text for details). P and r^2 values for the relationships are given.
[b] $P<0.05$ relative to low chloride value for the same species.
[c] $P<0.05$ relative to comparable value for trout.

there were no significant differences in the fractional subcellular distribution of silver between the two treatments at any time during the post-exposure period, though there were significant changes over time in both groups revealed by two-way ANOVA (Fig. 4). The 'nuclear' fraction (see Methods and materials, Section 2.5 for definitions) held by far the largest relative amount of silver, and this increased from about 65% (at 3 h) to > 90% by day 67. The percentage of silver in the 'cytosolic' fraction decreased proportionately from about 20% to 5%. The relative contributions of the 'mitochondria-lysosome' and 'microsomal' fractions did not change significantly, remaining generally < 15% in total.

4. Discussion

4.1. Overview

The ^{110m}Ag-based measurements of the present study revealed complex differences in gill silver accumulation and depuration, both between species and between low chloride and high chloride water. Previous 'cold' measurements by graphite furnace AAS were unable to detect any of these differences, or indeed even the net accumulation of silver above control levels over a similar time frame, despite using considerably higher exposure concentrations (Grosell et al., 2000). This illustrates the power of the radioisotopic approach for silver metabolism, as earlier noted by Pentreath (1977), Garnier and Baudin (1990) and Garnier et al. (1990). However, it is worth noting that if the facilities of a large nuclear research reactor and a high capacity counting laboratory had not been

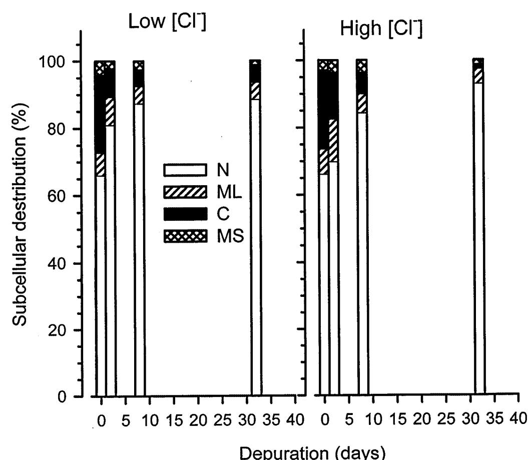

Fig. 4. Fractional subcellular distribution of silver in the gills of rainbow trout at 3 h, 2 days, 8 days, and 32 days of the post-exposure period in fish held in low chloride water (which had been exposed mainly to Ag^+) and fish held in high chloride water (which had been exposed mainly to $AgCl_{aq}$). Nominally, fractions are: N = nuclear; ML-mitochondria and lysosomes; C = cytosolic; MS = microsomal. Although absolute concentrations were greater throughout in the low chloride treatment, there was no significant difference ($P \leq 0.05$) at any time, or overall (by two-way ANOVA), in the fractional distributions between the two treatments. However, in both treatments, increases in the N fraction and decreases in the C fraction over time were significant.

available, the present study would have been difficult to perform because of safety, financial, and practical considerations associated with the very large amounts of radioisotope used.

Four important conclusions may be clearly drawn from this study. First, when freshwater fish are exposed to waterborne silver, loading of silver onto the gills does not occur linearly with time, and in fact net depuration starts to occur even while the exposure is maintained. Secondly, when the exposure is mainly in the form of $AgCl_{aq}$ (in high chloride water) silver loads into/onto the gills to a greater extent than if the exposure is mainly to Ag^+ (in low chloride water), though this is only clear for trout. Thirdly, silver is subsequently depurated from the gills more rapidly during the post-exposure period if the original exposure was mainly to $AgCl_{aq}$ and high chloride levels are maintained in the ambient water. Finally, the gills of the rainbow trout handle silver very differently from those of the European eel, loading to a much greater extent during exposure, and unloading much more rapidly during the post-exposure period, with a much greater influence of water chloride level on the attendant rates. The possible explanations for each of these conclusions, and their implications will be discussed.

4.2. Complex kinetics of silver loading onto the gills during the exposure period

The irregular pattern reported here (Figs. 1 and 2) with first a slow accumulation of silver through 6 h, then a 'breakthrough' to a peak at 12 h, with a significant net depuration by 24 h despite the continued exposure, is unusual relative to most other metals, but confirms that recently reported by Bury and Wood (1999) for rainbow trout exposed to mainly Ag^+ at a higher concentration (3.6–12.8 $\mu g\ l^{-1}$). In the latter study, the breakthrough was more rapid and did not occur at lower exposure concentrations, but water quality was different, and Bury and Wood (1999) used a different sampling method ('cold displacement') resulting in lower measured gill silver burdens. Studies with Cu, Cd and Zn have generally shown simple linear or hyperbolic uptake kinetics at the gills over time (e.g. Lauren and McDonald, 1986; Giles, 1988; Playle et al., 1993a,b; Hollis et al., 1997; Grosell et al., 1999; McCrae et al., 1999; Alsop et al., 1999), though it is possible that sampling regimes used in many of the cited investigations would have missed an intermediate peak. Indeed, one study on Cu has shown a similar pattern to the present, with an intermediate peak and depuration during continued exposure in rainbow trout gills (Grosell et al., 1997). For silver, the complex, irregular pattern seems to occur regardless of fish species or water chloride concentration, suggesting that it is a general characteristic of the response to this metal. The mechanism(s) behind this pattern are unclear, but must involve some sort of physiological regulation of the branchial entry or exit steps, or both. For entry, this could involve opening and then closing of apical Na^+ channels in gill ionocytes (Bury and Wood, 1999), whereas for exit this might involve changes in the active extrusion of silver by ATPase pumps on the basolateral membranes of the ionocytes (Bury et al., 1999a). Alternately or additionally, sloughing of mucus may be involved. For example, it is possible that silver accumulation in a large initial mucus production (early peak) causes a sloughing of that layer, perhaps by denaturation, followed by continued production and sloughing at a higher rate (later decline) as silver, especially Ag^+, contacts the epithelial cells. Such a pattern is thought to occur in trout chronically exposed to aluminum (McDonald et al., 1991).

Regardless of the mechanistic explanation, the findings are in accord with studies showing no relationship between gill total silver burden at a particular time and the extent of Na^+,K^+ATPase inhibition, which is the toxic mechanism (McGeer and Wood, 1998; Bury et al. 1999b; Grosell et al., 2000). These data reinforce the conclusion of Wood et al. (1999) that biotic ligand models for silver toxicity based simply on 'fast' gill silver burdens (e.g. Janes and Playle, 1995; Paquin et al., 1999) will be problematic, because only a very small fraction of a continually changing total gill burden may actually be causing the acute toxicity. A large fraction of the gill silver burden may be highly labile and 'non-toxic'. For this reason, a new biotic ligand model has recently been developed for acute silver toxicity, in which the mod-

elled endpoint is Na^+,K^+ATPase inhibition rather than gill silver burden (McGeer et al., 2000).

4.3. Branchial loading of silver from $AgCl_{aq}$ versus Ag^+ exposures

The extent of net branchial silver loading over the 24 h exposure was affected by speciation. The extent of net loading represents the difference between entry and depuration, the latter occurring via losses to either the rest of the body or the external environment. The fraction of total Ag bound to DOC was the same in the two exposures (Table 1), and Ag–DOC complexes are thought not to enter fish gills (Janes and Playle, 1995; Wood et al., 1999), so the difference in Ag^+ versus $AgCl_{aq}$ fractions in low chloride versus high chloride water was the probable explanation for the higher net branchial loading of silver by 24 h in the high chloride water. One cautionary note however is that there was still some Ag^+ present in the high chloride water (Table 1). At 12 h, net loading was either similar (trout; Fig. 1) or greater (eel; Fig. 2) when Ag^+ predominated in the exposure water, whereas by 24 h, the total gill silver loads had declined substantially in both species, such that the net remaining load was three- to fivefold higher when $AgCl_{aq}$ predominated.

Recent evidence suggests that Ag^+ enters branchial ionocytes via apical Na^+ channels driven by the electrical gradient created by an H^+ATPase (Lin and Randall, 1991; Bury and Wood, 1999). Therefore the pattern for Ag^+ might be explained by an initial rapid entry of the free ion through the Na^+ channels followed by a later closure of this channel due to a rise in intracellular Na^+ coincident with a slowly developing blockade of the basolateral Na^+ extrusion system, Na^+K^+ATPase (Wood et al., 1996a; Ferguson et al., 1997; Morgan et al., 1997). Continuing or accelerated removal of silver by the basolateral active transport system for silver (Bury and Wood, 1999) and/or mucus sloughing in the absence of further entry would cause the large decrease in gill silver load observed between 12 and 24 h. At least in trout, the time course of Na^+K^+ATPase blockade and accompanying Na^+ influx inhibition fit well with this scenario (Morgan et al., 1997; Webb and Wood, 1998). Furthermore, this scenario would be analogous to that seen with Cd^{2+}, where a rise in intracellular Ca^{2+} coincident with blockade of the basolateral Ca^{2+} extrusion system (high affinity Ca^{2+}ATPase) closes the apical Ca^{2+} channel through which Cd^{2+} originally enters, thereby greatly reducing Cd^{2+} entry over a very similar time course (Verbost et al., 1989).

Since a small amount of Ag^+ was also present during the '$AgCl_{aq}$ exposure' (Table 1), a similar explanation may apply to some extent here. However the higher gill silver loads at 24 h could be due to the large $AgCl_{aq}$ component. Nothing is known about the entry mechanism from $AgCl_{aq}$ exposures. However Wood et al. (1999) hypothesized that this neutral complex diffuses passively through the apical and basolateral membranes of the entire gill surface, not just through the 5–10% of the surface which is composed of ionocytes (Jurss and Bastrop, 1995; Perry, 1997). The $AgCl_{aq}$ complex has a higher K_{ow} than Ag^+ (0.09 vs. 0.03 according to Reinfelder and Chang, 1999), and therefore might be more diffusible through cell membranes. $AgCl_{aq}$ would therefore enter the gill at an overall rate comparable to the initial entry of Ag^+ through ionocyte Na^+ channels despite the lack of a selective uptake mechanism for the former. Furthermore, since silver readily penetrates the internal tissues of the body from $AgCl_{aq}$ exposures (Hogstrand et al., 1996; Hogstrand and Wood, 1998) without greatly inhibiting branchial Na^+K^+ATPase activity (McGeer and Wood, 1998; Bury et al., 1999c) or causing acute toxicity (Hogstrand et al., 1996; Galvez and Wood, 1997; Bury et al., 1999a), it seems likely that the basolateral exit step probably avoids the basolateral Na^+K^+ATPase or other active transport mechanism.

What is remarkable about the differences in uptake pattern and toxicity between Ag^+ and $AgCl_{aq}$ is that one might expect them to be rendered inconsequential by the internal environment of the branchial cells. The concentration of Cl^- in the whole cell water of gill cells is likely above 10 mM (Perry, 1997) relative to 1.2 mM in the high chloride water used here, and intracellular protein

and amino acid concentrations are high. One might expect all Ag^+ that enters to become $AgCl_{aq}$, or alternately that the very high affinity of various sulphydryl groups (log $K > 10$; Bell and Kramer, 1999) would strip Ag^+ off the weak ligand Cl^- (log $K = 3.3$). Clearly, physiological targetting (e.g. in the case of Ag^+, from the apical Na^+ channel to the basolateral Na^+K^+ATPase, perhaps by the intracellular tubule system or vesicular trafficking) must be much more important than simple geochemical equilibria might indicate. Again, these considerations dictate that biotic ligand models for silver toxicity must be based on the toxic mechanism, not on the gill silver burden (McGeer et al., 2000).

4.4. Influence of chloride on depuration of silver from the gills during the post-exposure period

When silver was loaded onto/into the gills from $AgCl_{aq}$ exposure, it was clearly much more labile during the post-exposure period than if loaded from Ag^+ exposure, a difference which was present in both species, but more marked in trout (Figs. 1–3 and Table 2). Silver was lost from the gills 1.6–1.8-fold faster during the first phase (0–1 day in trout, 0–15 days in eel) in the $AgCl_{aq}$ exposure group, and this difference persisted in the second phase (1–67 days) in trout only (Table 2). Thus gill silver burdens were consistently lower than in the Ag^+ exposure group on both an absolute basis (Fig. 1) and as a percentage of whole body burden (Fig. 3). These differences are likely related to the fact that silver does not bind to and inhibit branchial Na^+K^+ATPase to the same extent in high chloride water (McGeer and Wood, 1998; Grosell et al., 1999).

The gill silver burdens were different in the two treatments by the end of the loading period, yet the whole body loads were the same in the two treatments by 3 h post-exposure (Fig. 1 and Fig. 2). Therefore, it seems very probable that much of the large loss of silver from the gills during the first few hours of the post-exposure period was to the water, perhaps by simple diffusion of $AgCl_{aq}$ back out and/or mucous sloughing. The faster depuration in the high choride water might also be due to an increased availability of free Cl^- to strip away any Ag^+ moving out. Unfortunately, silver fluxes to the water were not quantified in the present study. Indeed, based on the sensitivity analysis presented in Results, it is likely that flux data would have been equivocal even if ^{110m}Ag appearance in the water had been measured right from the start (0 h) of post-exposure period in the $AgCl_{aq}$-exposed trout. This analysis underscores the fact that it is the ability of the organism to accumulate the ^{110m}Ag-labelled silver to manyfold environmental levels which made possible the present analyses based on radioisotope appearance/disappearance from tissues. Even with radioisotopic techniques, measurements of appearance/disappearance in the water are just not sensitive enough to detect the very small fluxes of silver which occur.

The distribution of ^{110m}Ag among subcellular gill fractions was dominated by the ‘nuclear (N) fraction’, which accounted for 65–95% of the total radioactivity in the tissue (Fig. 4). The ‘cytosolic (C) fraction’ never contained more than 30% of the gill ^{110m}Ag and cytosolic silver decreased exponentially with time. We initially speculated that the high level of ^{110m}Ag in the ‘nuclear fraction’ was due to Ag binding to external mucus, which indeed sedimented together with the ‘nuclear fraction’ however, the amount of mucus on the gills (as judged from gel formation during centrifugation) gradually declined during the experiment while the percentage of total gill ^{110m}Ag present in the ‘nuclear fraction’ actually increased over time. Thus, the ^{110m}Ag was probably not bound to external mucus, but to other unidentified components of the ‘nuclear fraction.’ The results further suggest that cytosolic silver was more rapidly eliminated from the gills than the silver in the ‘nuclear fraction.’ This finding is in accordance with the observed presence of both rapidly and slowly eliminating pools of ^{110m}Ag in the gills (Table 2). The subcellular distribution found in the present study is in stark contrast to a similar study (Galvez et al., 2001) in which rainbow trout were exposed to a ‘pulse’ of ^{110m}Ag, followed by continuous exposure to stable Ag. In that study, less than 10% of the radioactivity was present in the ‘nuclear fraction’ and as much as 70% was found in the ‘cytosolic fraction.’ This

difference in distribution may be explained by stable silver displacing ^{110m}Ag from the 'nuclear fraction' in the study with continuous exposure. The subcellular fractionation also sheds light on the mechanism of differential toxicity between Ag^{+} and $AgCl_{aq}$ (e.g. Hogstrand et al., 1996; McGeer and Wood, 1998; Bury et al., 1999a). In the present study, the subcellular distribution of ^{110m}Ag was practically identical in fish exposed in the two water qualities, which shows that the difference in toxicity is not because silver accumulated from Ag^{+} has a different intracellular fate from silver accumulated from $AgCl_{aq}$. Hence, these results support the current hypothesis that Ag^{+} is more toxic than $AgCl_{aq}$ because the route of passage across the gills is different (Wood et al., 1999).

4.5. Differences in branchial silver handling between trout and eel

On a qualitative basis, differences in the gill accumulation of silver during the 24 h exposure correlated with differences in the branchial turnover rates of Na^{+} between the European eel and the rainbow trout (Grosell et al., 2000). However, whereas Na^{+} influx at the gills was over twentyfold higher in trout than eel, branchial accumulation of silver was only threefold higher (Fig. 1 and Fig. 2), and differences in whole body uptake less than twofold higher after 24 h exposure even when the exposure was to the free ion Ag^{+}. Nevertheless, this threefold difference in gill accumulation does correlate well with the threefold difference in acute toxicity (i.e. threefold higher 96 h LC_{50} in eels) recorded by Grosell et al. (2000). Since absolute $Na^{+}K^{+}$ATPase activities are similar in the two species (Grosell et al., 2000), it seems likely that it is the apical entry steps which are limiting, at least for unidirectional Na^{+} influx rates. Corresponding large differences in gill permeability allow the eel to achieve ionic equilibrium at much lower ionic turnover rates than the trout (Bornancin et al., 1977; Perry et al., 1992; Goss and Perry, 1994). The fact that the difference in silver influx rates is much less than the difference in either Na^{+} influx or Na^{+} permeability suggests that other factors must come into play to dictate the rate(s) of silver movement.

Other important features of the ionoregulatory differences between the two species include a much lower density of mitochondrial rich-cells on the gills (Perry et al., 1992), and a virtual absence of Cl^{-} influx from the water in freshwater eels (Kirsch, 1972; Hyde and Perry, 1989). Nevertheless these 'negligible' whole body Cl^{-} uptake rates measured by Grosell et al. (2000) in eels in the same high chloride water (600 nmol kg^{-1} wet weight h^{-1}) were more than five hundredfold greater than the whole body silver uptake rates measured in the present study (<1 nmol kg^{-1} wet weight h^{-1} over 24 h). Thus even if silver enters exclusively as $AgCl_{aq}$, its impact on the fish's Cl^{-} budget will be insignificant. This comparison emphasizes the extremely low absolute rates of silver influx in both species. It also emphasizes the fact that simultaneous measurements of Cl^{-} uptake (using ^{36}Cl) will always be so much higher than those of ^{110m}Ag as to make it impossible to demonstrate $AgCl_{aq}$ uptake directly with such an approach.

The most striking difference in branchial silver handling was the much faster depuration of silver from the gills of trout during the post-exposure period. The very rapid removal over the first few hours in trout, even when the exposure had been to Ag^{+} (Fig. 1, Table 1), helps to explain why rainbow trout recover their Na^{+} uptake so quickly (within 2 h) after the end of a silver exposure (Morgan et al., 1997). Presumably, the inhibitory binding of silver to Na^{+} K^{+} ATPase (Ferguson et al., 1997) and the closure of the apical Na^{+} channels are both rapidly reversible once silver is removed. It would be interesting to test whether these effects are as quickly reversed in the eel, in light of the fact that 50% of the whole body load still remained in the gills of the eel during these first few hours, and 35% after as much as 8 days (Fig. 3). Trout gills probably lost silver to the water initially, but since whole body silver loads were stable from 3 h through 67 days, losses from the gills during this period probably redistributed to other body compartments. Interestingly, this did not appear to be the case in the eel, since the 50% decline observed in whole body silver burden of eels over the 67 days could be quantitatively accounted for by the gradual loss of this gill fraction (Fig. 2 and Fig. 3).

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