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Binding and movement of silver in the intestinal epithelium of a marine teleost fish, the European flounder (*Platichthys flesus*)☆

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Abstract

The intestine has been indicated as a site of waterborne silver toxicity in marine fish and chronic effects at the intestine have been observed at concentrations far below acutely toxic level. Thus, models of silver toxicity to marine fish need to consider the intestine as a biotic ligand. The present study characterises binding of silver to the intestine of the European flounder (*Platichthys flesus*). Everted intestinal sacks were prepared and submersed in a solution mimicking the intestinal fluid of the fish at the acclimation salinity (21‰). Silver was added as $^{110m}AgNO_3$ or $^{110m}AgNO_3$/$AgNO_3$ mixtures at concentrations ranging from 1.6 to 950 nM total silver. Appearance of ^{110m}Ag was analysed in mucosal scrapings, muscle layers, and in the plasma saline on the serosal side of the intestine. The latter represented uptake into blood and other extra-intestinal compartments. Mucosal scrapings consisted of both epithelial cells and mucus and, thus contained adsorbed as well as absorbed silver. Most of the silver in mucosal scrapings was bound to mucus. There was no difference in silver binding between the anterior, mid, and posterior regions of the intestine. Concentration-dependency of silver binding was sigmoidal and saturated above 100 nM total silver. The saturable appearance of ^{110m}Ag in the plasma saline suggest that silver passage across the intestine is transcellular and carrier mediated. Mucus likely influences uptake of silver by altering its speciation from that in the lumen and by serving as physical barrier for silver binding to the brushborder membrane. A biotic ligand model for marine fish to silver may have to consider these interactions.

Keywords: Fish; Marine; Metal; Intestine; Silver; Biotic ligand; Binding; Transport

1. Introduction

The gill has been implicated as the site for acute toxicity of several metals to freshwater fish (Wood, 2001). Unlike freshwater fish, marine teleost fish drink considerable amounts of water, which exposes the intestine to waterborne metal. One consequence of this is that the fish intestine becomes a potential target for toxicity of dissolved metals. In

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fact, it has been shown that impacts on the intestine contribute significantly to toxicity of dissolved silver in marine teleosts (Hogstrand et al., 1999; Grosell et al., 1999; Webb et al., 2001; Grosell and Wood, 2001). For the lemon sole (*Parophrys vetulus*), the intestinal contribution to overall acute silver toxicity was quantified to be 50–60% (Grosell and Wood, 2001). The physiological effects of dissolved silver to gastrointestinal function are a reduction of drinking and an inhibition of both solute-linked intestinal water uptake and HCO_3^- secretion (Hogstrand et al., 1999; Grosell et al., 1999). However, the biochemical mechanism(s) of the effects and the exact site(s) of interference have not been identified. The intestinal contribution to internal silver accumulation (as opposed to toxicity) was reported to be minor for the lemon sole (Grosell and Wood, 2001), but this may not be the case for other fish species where intestinal exposure to dissolved silver could result in accumulation through this route.

Prior to 1995, discharges of silver in the USA were regulated based on total recoverable silver in the water column. Since 1995, the US EPA has recommended that metals be regulated based on dissolved metal, as operationally defined by the portion of metal passing through a 0.45 μm filter (US EPA, 1995). In fresh water, a regulatory adjustment can be made for water hardness, but not for other factors (US EPA, 1986). The present US EPA water quality criterion for silver in seawater does not recognise any influence of water chemistry. It is now very clear that a number of environmental factors regulate acute silver toxicity to animals, the most important being the concentrations of reduced sulphur, dissolved organic matter, and chloride (Hogstrand and Wood, 1998; Wood et al., 1999, 2001; Bianchini et al., 2002). This has been realised by regulatory bodies in several countries and significant efforts are being made to devise new water quality criteria that can accommodate all factors that are important in modulating silver toxicity. One such approach is the biotic ligand model (BLM), which uses geochemical speciation models to calculate binding of Ag(I) to the sites of toxicity (the biotic ligand) on the animal. For freshwater fish, the gill is considered the biotic ligand of importance. The BLM is based on work by Pagenkopf (1983) who suggested that the gill will compete with other complexing agents in the water for metal ions present and that abundant ions such as Na^+ and Ca^{2+} would compete with transition metals for negatively charged sites on the gills. Janes and Playle (1995) put this approach into practice and used a geochemical speciation computer program to accurately calculate binding of Ag(I) to the gills of freshwater rainbow trout (*Oncorhynchus mykiss*). Several versions of the BLM for Ag(I) have been published and the main difference amongst these is the end-point modelled. Whereas Janes and Playle (1995) modelled binding of Ag(I) to the gill, McGeer et al. (2000) used stability constants of Ag(I) binding to the target molecule (Na^+/K^+-ATPase) and Paquin et al. (1999) and DiToro et al. (2001) used mortality as the end-point. The BLM approach is much more precise than hardness-based calculations in predicting acute effects of Ag^+ to rainbow trout, but current BLMs have limitations and are not universally applicable to all environments and species.

One such limitation is that BLMs designed to predict toxicity to freshwater fish cannot accurately predict toxicity of silver to fish in brackish and sea water. One reason for this is that Ag(I) barely exists in high chloride environments, and as yet the various binding constants, speciation equilibria, and toxicity for the many other forms of silver which predominate in saline waters have not yet been adequately characterised. A second very likely reason for this is that freshwater BLMs calculate binding of Ag(I) to sites in the gills while the intestine might be a more important target for silver toxicity in marine fish (Wood et al., 1999). Thus, a BLM to predict toxic effects of silver to marine fish needs to include the intestine as a site of toxicity, and to take into account the water chemistry and speciation of silver in intestinal fluids. Information on binding of metals to the intestine of marine fish is lacking. Therefore, in a first step to formulate a BLM applicable to marine fish, the aim of the present study was to characterise the binding of silver to the intestine of the European flounder (*Platichthys flesus*). For this purpose, we used an in vitro preparation consisting of everted sacs of intestinal segments. As it was found that silver was moving across the intestinal wall of this preparation, we also characterised the kinetics of transepithelial silver transport.

2. Materials and methods

2.1. Animals

European flounder *Platichthys flesus* (252–541 g) were collected locally by gill nets from the

coast off Funen, Denmark, and kept in flowing ambient sea water (21‰) at Kerteminde Marine Station, Kerteminde, Denmark. Water temperature was 13 °C and the fish were maintained without feeding in the holding tanks for at least a week prior to experimentation.

2.2. Experimental system

The general procedures for using European flounder intestinal sacs in ion transport experiments has been evaluated and described previously (Grosell and Jensen, 1999). Fish were euthanised with an overdose of anaesthetic (MS-222) and the intestine removed from immediately below the caecae to above the rectal sphincter. For the *time and location dependency* experiment, the intestine was cut into three equal segments, operationally denoted *anterior*, *mid*, and *posterior intestine*. For all other experiments, the anterior section constituting the proximal 1/3 of the intestine was used. The intestinal segment was everted and sealed off at the posterior end by a ligature. At the anterior end a 5-cm piece of flanged polyethylene (PE-50) tubing was inserted and tied to allow for administration and sampling of medium from the serosal side. The intestinal sac was then washed in *intestinal saline* ($[Na^+]=50$ mM, $[Cl^-]=107.5$ mM, $[SO_4^{2-}]=54$ mM, $[Mg^{2+}]=100$ mM, $[Ca^{2+}]=5.5$ mM, $[K^+]=4.5$ mM, $[HCO_3^-]=50$ mM; osmolarity = 280 mOsm l^{-1}), which was based on analyses of intestinal fluid in five flounders from the Kerteminde Marine Station holding tanks (Grosell et al., unpublished). Through the PE-50 tubing, the intestinal sac was filled with 3–5 ml of *plasma saline* (depending on experiment) consisting of $[Na^+]=149$ mM, $[K^+]=5$ mM, $[Mg^{2+}]=0.9$ mM, $[Ca^{2+}]=1.6$ mM, $[Cl^-]=142.3$ mM, $[SO_4^{2-}]=0.9$ mM, $[H_2PO_4^-]=3$ mM, $[HCO_3^-]=11.9$ mM and [Glucose] = 5.6 mM (osmolarity = 286 mOsm l^{-1}). Like the intestinal saline, the plasma saline was based on analyses of plasma from flounders kept at identical conditions as those used for the present study. Once filled, the intestinal sacs were closed by heat-sealing the PE tubing, checked for leakage, and submersed in a 500-ml polypropylene graduated cylinder filled with 500 ml of *transport medium*, which was *intestinal saline* with $^{110m}AgNO_3$ added, except for the *Effect of intestinal ion concentrations* and *cyanide* experiments. The transport medium was constantly gassed with a 99.5% O_2/0.5% CO_2 mixture ($PCO_2 \sim 3.75$ torr) from a Wösthoff (Bochum, Germany) gas mixing pump, resulting in a pH of 8.4. The temperature was 13 °C. Geochemical equilibrium calculations with MINEQL+ (ver. 4.01, Environmental Research Software, USA) predicted that 7% of the added silver was present as $AgCl_{aq}$, 78% as $AgCl_2^-$, 10% as $AgCl_3^{2-}$, 5% as $AgCl_4^{3-}$ and less than 0.04% as Ag^+. The calculated limit for cerargyrite ($AgCl_{(s)}$) precipitation in *intestinal saline* was 1.6 μM of total dissolved silver. The highest concentration of silver used in the present study was therefore limited to 0.95 μM (100 μg l^{-1}).

After the incubation period, the intestinal sac was rinsed for 30 s in 250 ml of ice-cold ^{110m}Ag-free intestinal saline to slow down any transport and remove unbound ^{110m}Ag. The PE tubing was cut open and the plasma saline extracted from the intestinal sac with a syringe. The plasma saline was then transferred to pre-weighed scintillation vials for measurements of final volume and ^{110m}Ag activity. The mucosa was blotted with paper towels to reduce mucus-bound ^{110m}Ag included in measurements of intestinal ^{110m}Ag binding. The intestinal sac was cut longitudinally, blotted dry on the serosal side, and the surface area traced on graph paper. The intestine was then transferred to a glass plate where mucosa and remaining mucus was scraped off underlying subepithelial tissue (operationally termed: *muscle layer*) with a glass slide. The *mucosal scraping* and the residual *muscle layer* were placed in separate pre-weighed scintillation vials. The vials were then reweighed and mass of each compartment recorded. Tissue and saline samples were analysed for ^{110m}Ag radioactivity on a gamma counter (1480 Wizard 3 Automatic Wallac, Turku, Finland) with appropriate standards, using energy windows as outlined by Hansen et al. (2002) The counting efficiency for ^{110m}Ag was 18.9 cpm Bq^{-1}.

Radiolabelled Ag(I) was purchased as $^{110m}AgNO_3$ from Risø National Laboratory (Roskilde, Denmark). The specific activity of ^{110m}Ag(I) was 22.1 TBq mol^{-1} at a radioisotopic concentration of 38.7 kBq μl^{-1}. With exception for the three highest Ag(I) concentrations in the '*concentration-dependency*' experiment, the radiolabelled Ag(I) was not diluted with stable isotope of Ag. This procedure enabled easy monitoring during experiments of actual total silver concentrations by counting of γ emission as the specific activity of the ^{110m}Ag isotope remained constant.

2.3. Experiment 1: Time and location dependency

Time-dependency of silver binding to 'mucosal scrapings' and 'muscle layer' was evaluated in three regions of the intestine. Everted sacs from anterior, mid, and posterior sections of the intestine (after stomach) were exposed to 11.4 ± 1.1 nM (mean $\pm$ S.E., $N=32$) of total silver in intestinal saline. Binding to epithelial scrapings and muscle layer was assessed after 0.5, 3, 6 and 12 h of exposure. In this first experiment, the mucosa was only briefly blotted on absorbent paper after exposure, resulting in most of the mucus being included in the mucosal scrapings.

2.4. Experiment 2: Concentration-dependency

Data from the time-dependency experiment suggested that there was no significant difference between the segments in terms of Ag(I) binding. In subsequent experiments only one of the sections, the anterior, was used. Measurements of ^{110m}Ag radioactivity in the plasma saline (inside of sacs) indicated that a significant amount of silver crossed the intestine. Therefore, the volume of the plasma saline after exposures was carefully measured by weighing and the total radioactivity in the plasma saline counted. These data were used to calculate unidirectional silver influx across the intestine. As results from Experiment 1 indicated that most of the ^{110m}Ag was bound to the mucus rather than the tissue, a more rigorous blotting protocol was adopted for Experiment 2 and all subsequent experiments. This entailed blotting of the mucosa with absorbent paper until no more water could be visibly removed. To establish concentration-dependency of silver binding and influx, everted sacs from the anterior intestinal segment were exposed to a range of different concentrations of silver (1.6–950 nM; 0.17–100 $\mu g\, l^{-1}$) for 6 h. After this incubation period, the concentration of silver on the serosal side was still less than 10% of that on the mucosal.

2.5. Experiment 3: Effect of intestinal ion concentrations

The European flounder can tolerate the full range of salinities from fresh water to sea water. The external salinity will influence the composition of the intestinal fluid (Shehadeh and Gordon, 1969) and this may in turn affect Ag(I) binding to and movement across the intestinal epithelium. Effect of intestinal salt concentrations on Ag(I) binding and influx was evaluated using everted sacs from the anterior segment. These were exposed to approximately 10 nM (11 $\mu g\, l^{-1}$) of Ag(I) in 10–107.5 mM of Cl^- for 6 h. The range of ion concentrations in the exposure medium was selected to reflect the range of salinities from 21‰ sea water to fresh water (Table 1). Formulation of the different media was based on measured osmolyte concentrations in anterior intestine of European flounder at 21‰ salinity and extrapolated to other salinities by using the data of Shehadeh and Gordon (1969) for rainbow trout. The concentrations of Mg^{2+}, Cl^- and SO_4^{2-} were altered without changing the concentrations of Na^+, Ca^{2+}, K^+ and HCO_3^-. This was achieved by supplying Mg^{2+} as a varied mixture of $MgSO_4$ and $MgCl_2$ salts in combination with constant amounts of KCl, $CaCl_2$ and $NaHCO_3$. The experiment was carried out with or without the addition of mannitol to compensate for measured changes in osmolality (Table 1). The measured average total silver concentration over the 6 h exposure period was 10.2 ± 1.0 nM (mean $\pm$ S.E., $N=36$) for the experiment without mannitol and 9.9 ± 1.3 nM (mean $\pm$ S.E., $N=24$) for the experiment with mannitol. In the medium representing intestinal fluid at 21‰ salinity, the modelled speciation of the total silver present was 6.7% $AgCl_{aq}$, 73.8%

Table 1
Composition of intestinal salines for investigation of effects of intestinal ion concentrations on binding and influx of silver

Constituents (mM)	Simulated salinity			
	21‰	9‰	3‰	FW
Na^+	50	50	50	50
Cl^-	107.5	55.5	44.5	15.5
Mg^{2+}	100	50	25	0
SO_4^{2-}	54	30	10.5	0
Ca^{2+}	5.5	5.5	5.5	5.5
K^+	4.5	4.5	4.5	4.5
HCO_3^-	50	50	50	50
Osmolality (mOsm l^{-1})[a]	280	200	140	120
Mannitol[b]	0	80	140	160

The four different media simulate composition of intestinal fluid when the fish resides in waters ranging from 21‰ sea water to fresh water (FW). See Section 2 for details.

[a] Osmolarities are for salines without mannitol added. Results of experiment are shown in Fig. 3.

[b] Mannitol (mM) added to adjust osmolarity of salines to 280 mOsm l^{-1}. Results of experiment are shown in Fig. 4.

$AgCl_2^-$, and 7.4% $AgCl_4$; speciation of total silver in the medium representing intestinal fluid of the fish in fresh water was 3.1% Ag^+, 46.4% $AgCl_{aq}$ and 49.9% $AgCl_2^-$ (MINEQL+ ver. 4.01, Environmental Research Software, USA).

2.6. Experiment 4: Effects of temperature and cyanide

Involvement of energy-dependent processes on silver binding and influx was investigated by changing incubation temperature and by pre-treatment with NaCN. Sacs of everted intestine (anterior segment) were placed in intestinal saline at either 15 °C (control), 0 °C, or at 15 °C with 1 mM of NaCN added. After 1 h of incubation under these conditions, control and 0 °C groups were transferred to 250-ml chambers at the same respective temperature as before and ^{110m}Ag was added. Intestinal sacs treated with NaCN were rinsed for 1 min in cyanide-free intestinal saline before transfer to the incubation chambers and exposed to ^{110m}Ag in absence of NaCN so as to avoid any direct cyanide *vs.* silver interactions. The preparations were exposed to ^{110m}Ag for 7 h at one of the three conditions. During this time period the mean exposure concentrations of total silver were 31 ± 1, 25 ± 0.3, and 40 ± 0.8 nM (mean $\pm$ S.E., $N=8$) for the 15 °C (control), 0 °C, and 15 °C + NaCN groups, respectively. The mean exposure concentrations of both experimental groups were significantly different from that of the control, mainly due to different rates of disappearance of silver from the intestinal saline. Differences in mucus production among treatment groups were believed to be involved as well as changes in binding to intestinal tissue and transepithelial silver movement.

2.7. Calculations

Binding of silver to the intestinal tissue was calculated as follows:

$$\text{Binding (mol cm}^{-2}) = B_{\text{cpm}}/(\text{SA} \times A)$$

where B_{cpm} is the number of counts per minute in intestinal scrapings or in the muscle layer, SA is the specific activity of total silver in the intestinal saline (cpm mol^{-1}), and A is the surface area of the intestinal sac. Binding to mucosal scrapings was also calculated on a per mass basis to reflect possible differences in folding between intestinal sections. As it was found that these results were qualitatively the same as those based on measured surface area, only the latter are presented.

Influx of silver from the intestinal saline to the plasma saline was calculated from the following:

$$\text{Influx (mol cm}^{-2}\,\text{h}^{-1}) = I_{\text{cpm}}/(\text{SA} \times A \times h)$$

where I_{cpm} is the total number of counts in the plasma saline and h is the duration of the experiment in hours. SA and A are as defined above for tissue binding.

Net flux of water across the intestinal wall was calculated according to the equation below:

$$\text{Net flux (ml cm}^{-2}\,\text{h}^{-1}) = (V_0 - V_t)/(A \times h)$$

where V_0 and V_t are the volumes of serosal fluid at the start (V_0) and the end (V_t) of the experiment (in ml), respectively. A and h are as defined above.

2.8. Statistical evaluation

All values are expressed as means $\pm$ S.E.M. Kruskal–Wallis analysis of variance (ANOVA) was used to assess differences in binding and influx of silver between multiple treatments and intestinal segments. Following Kruskal–Wallis ANOVA, the Mann–Whitney U-test was used to evaluate specific differences in silver binding or influx between control intestines at 15 °C, cyanide-treated intestines at 15 °C, and intestines at 0 °C. Statistical analysis was performed using STATISTICA computer package and $P<0.05$ was taken as the level of significance. Non-linear regression of data were carried out with SigmaPlot 7.0 graphing software. Data for concentration-dependency of silver binding and influx were fitted to the Hill equation to derive constants for binding and transport. In this equation, $B_{\max}$ is the maximum binding ($J_{\max}$ = maximum transport rate), $K_{0.5}$ is the affinity constant, and n is the Hill coefficient. A value of $n>1$ indicates cooperative binding.

3. Results and discussion

The average exposure concentration during the time and location dependency experiment was 11 nM (1.2 μg l^{-1}) and this resulted in a binding to the mucosal scrapings of maximally 200 fmol cm^{-2} (22 pg cm^{-2}). Binding of silver to mucosal scrapings of the anterior and mid segments of the intestine reached peak levels after 6

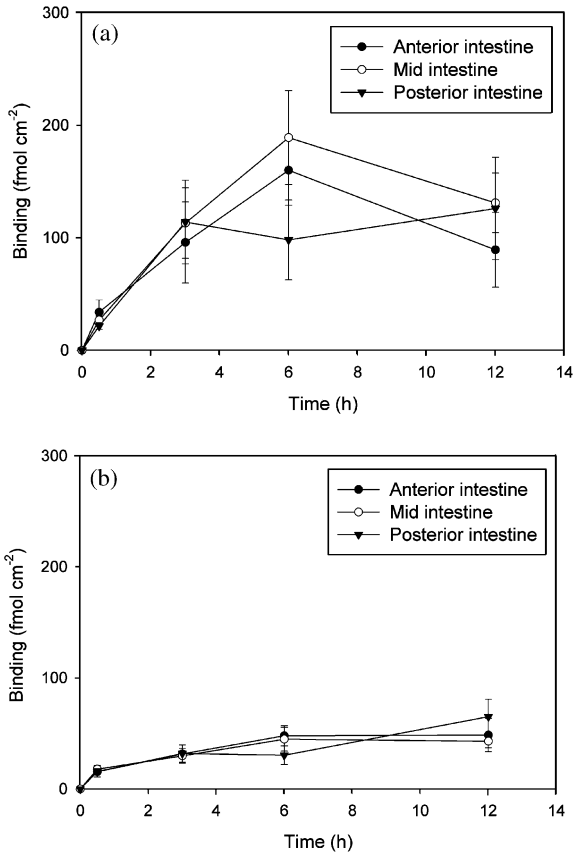

Fig. 1. Time dependency of silver binding to (a) mucosal scrapings and (b) muscle layer in everted intestinal sacs, prepared from the anterior, mid, and posterior sections of the intestine (from pylorus to rectum). The intestinal sacs were exposed to an average total silver concentration of 11.4 nM. At sampling, remaining liquid was removed by lightly blotting the mucosa with absorbent paper. Each data point represents the arithmetic mean of four fish and the error bars denote S.E.M. There were no statistically significant differences in Ag(I) binding between the three sections.

h of exposure and remained unchanged thereafter (Fig. 1a). The posterior portion appeared to reach equilibrium after 3 h, but there were no statistically significant differences in binding between the three segments at any time point. This apparent absence of localised uptake could be representative of the natural, in vivo situation, but it is also possible that it was a consequence of the in vitro condition, including lack of movement of intestinal fluid through the gut in the preparation of the present study. In this regard, Glover et al. (in press) found that the accumulation pattern of zinc in the intestine of the marine toadfish (*Opsanus beta*) was influenced by flow through the intestine. Pentreath (1977) fed ^{110m}Ag(I) labelled nereids to plaice (*Platichthys platessa*) and recovered most of the radioactivity (after intestinal evacuation) in the portion of the intestine directly posterior to the stomach (anterior segment in present study). In contrast, silver-exposed sea water acclimated rainbow trout (*Oncorhynchus mykiss*) and the lemon sole (*Parophrys vetulus*) seem to accumulate more silver in the posterior portion of the intestine than elsewhere along the gastrointestinal tract (Grosell and Wood, 2001; Playle and Nichols, personal communication). Bury et al. (2001), in the same batch of European flounder as studied here, found that iron uptake was also greatest in the posterior intestine. Although it should be kept in mind that some aspects of the silver accumulation patterns observed here (i.e. contribution to uptake by different sections; relative distribution between accumulation in mucosal scrapings, muscle layer, and plasma saline) could be influenced by the exposure system employed, the intestinal bag preparation offered a great deal of experimental control, in terms of definition of exposure medium, exposure time, and surface uptake area.

There was 3–7 times more ^{110m}Ag in mucosal scrapings than in the muscle layer (compare Fig. 1a, b, and Fig. 2a). This difference between mucosal scrapings and muscle layer in silver binding was partly attributed to mucus, because it could be reduced by further blotting of the mucosa (Fig. 2a). By using the values for B_{max} and $K_{0.5}$, derived with the more rigorous blotting protocol (Experiment 2), and comparing this to data generated for the anterior intestine at the 6 h point of Experiment 1, it can be calculated that more than 67% of the silver was trapped in removable mucus. Also, γ counting of the adsorbent paper used for blotting accounted for all of the removed ^{110m}Ag from the mucosal scrapings (data not shown).

Binding of silver to mucosal scrapings and muscle layer of the anterior segment were saturable processes (Fig. 2a). By log–log transforming (common logarithm) both axes it could be shown that dose-dependency was sigmoid, possibly indicating the presence of binding sites with higher affinity that saturated already at the lowest concentration of silver used. An alternative explanation would be cooperativity of silver binding. However, the derived Hill coefficient of 1.0 suggested that Ag(I) ions bound independently of each other with an affinity constant, $K_{0.5}$, of 350 nM for mucosal scrapings and 400 nM for muscle

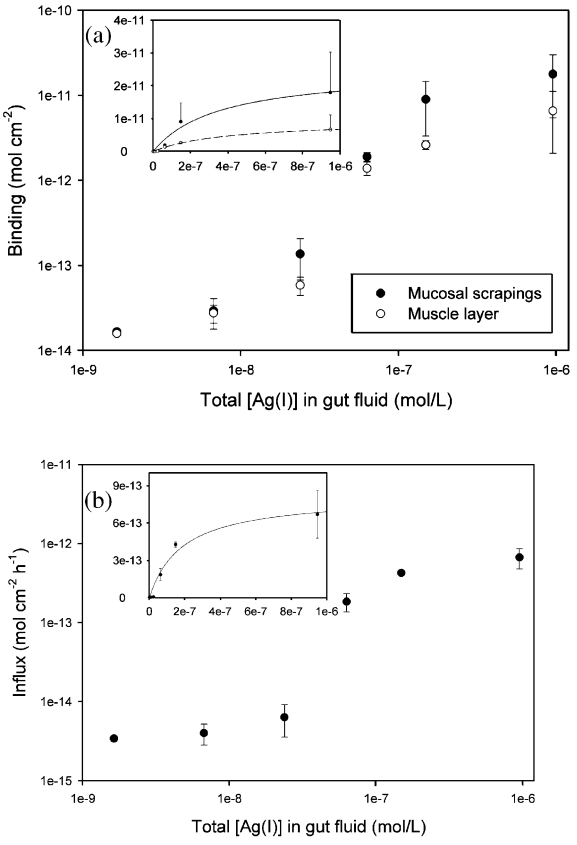

Fig. 2. Concentration-dependency of silver (a) binding to mucosal scrapings and muscle layer of intestinal sacs and (b) influx from intestinal saline to plasma saline. At sampling, the mucosa was blotted with absorbent paper until no further mucus could be removed. All data are for everted intestinal sacs prepared from the anterior section of the intestine and exposed for 6 h to silver. Each point represents the arithmetic mean of 2–4 fish and the error bars denote S.E.M. Data in the main graphs are shown on common log–log scales to accommodate the wide ranges. In the inserts, the same data are presented with linear scales to show the fitted hyperbolic regression curves. The constants describing the curves are displayed in Table 2.

layer (Table 2). Thus, the very similar log K values for mucosal scrapings and muscle layer were determined to be 6.5 and 6.4, respectively. In contrast, the density of binding sites, as indicated by B_{max}, was more than twice as high in the mucosal scrapings as in the muscle layer (Table 2).

Unidirectional influx of silver into the internal body fluid compartment, based on appearance of ^{110m}Ag(I) on the serosal side, also exhibited sigmoid concentration dependency if data were expressed on log–log scale. As for the binding, influx kinetics fitted best to a hyperbolic curve (i.e. Michaelis–Menten model). Affinity constant ($K_{0.5}$) for the uptake sites was determined to 180 nM, indicating a twofold higher affinity than for the 'binding sites' (Table 2). The maximal 'transport' rate was 0.82 pmol cm^{-2} h^{-1}, which means that the maximal amount of silver transferred across to the serosal side over the 6 h period (4.9 pmol cm^{-2}) was about half of the maximally bound silver to the muscle layer (9.4 pmol cm^{-2}). The fact that unidirectional silver influx was saturable indicates that intestinal silver transport is mediated by carrier proteins. It further suggests that intestinal silver uptake could be mechanistically similar to that of freshwater rainbow trout gill, in which silver enters apically through a Na^+-channel-like protein and is extruded basolaterally by a Ag^+-activated P-type ATPase (Bury and Wood, 1999; Bury et al., 1999).

Changing the intestinal fluid ionic concentrations to mimic changes in ambient salinity (Table 1) had no effect on silver binding (Fig. 3a), but resulted in a tendency for reduced silver influx with increased salinity (Fig. 3b). These results are consistent with data from whole animal experiments (Shaw et al., 1998; Webb and Wood, 2000) and from preliminary modeling (Hogstrand and Wood, 1998; Wood et al., 1999), suggesting that Ag(I) is more bioavailable at lower salinities than at higher. The tendency for reduced silver influx with increased salinity was not statistically significant in the present study (ANOVA, $P=0.06$) but it traced a significant decrease in net flux of water from mucosal to serosal side (Fig. 3b). In fact, there was a highly significant direct correlation

Table 2
Binding and influx constants for silver interactions with European flounder anterior intestine

Compartment	B_{max} (pmol cm^{-2}) or J_{max} (pmol cm^{-2} h^{-1})	$K_{0.5}$ (nM)
Mucosal scraping	24.9 ± 0.5	350 ± 150
Muscle layer	9.4 ± 0.8	400 ± 8
Influx	0.82 ± 0.10	180 ± 64

Data shown in Fig. 2a (binding) and b (influx) were fitted to the Hill equation which in each case returned a simple hyperbolic curve with a Hill coefficient of one, indicating no cooperativity of binding. Constants were derived from the fitted curves and are presented as mean ± S.E.M., $N=6$. B_{max} values pertain to 'mucosal scrapings' and 'muscle layer' whereas J_{max} is for 'influx' only. $r^2<0.9$ for all regressions (SigmaPlot 7.0).

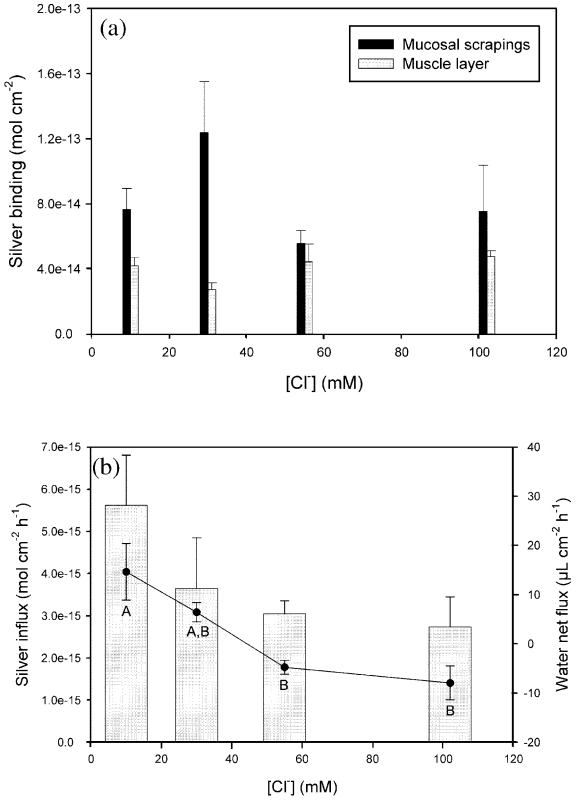

Fig. 3. Effect of co-varied ion concentrations and osmolality of intestinal saline on silver (a) binding to mucosal scrapings and muscle layer of intestinal sacs and (b) influx from intestinal saline to plasma saline. Also shown in (b) is the net water flux. A positive value indicates net movement of water from mucosal to serosal side. The intestinal salines represented intestinal fluid of fish at various salinities between 21‰ and FW (Table 1). All data are for everted intestinal sacs prepared from the anterior section of the intestine and exposed for 6 h to silver. Each point represents the arithmetic mean of four fish and the error bars denote S.E.M. The average total silver concentration during exposures was 10.2 nM. There were no statistically significant differences in silver binding or influx among the different salines. Net flux of water from mucosal to serosal side was significantly decreased with increasing salinities (ANOVA, $P<0.05$). Mean values that are labeled with different letters are significantly different (Tukey LSD test, $P<0.05$) from each other.

($r=0.72$, $P<0.0017$) between silver influx and water net flux. Furthermore, compensation for reduction in osmolality with mannitol abolished any trend in salinity-related silver influx, suggesting that it was caused by the osmotic gradient and not by changes in silver complexation or cation competition (Fig. 4b). Over the range of intestinal fluids representing brackish and coastal sea water (3–21‰), there were moderate changes in silver speciation of the intestinal saline (Fig. 5). Going from the 3 to 21‰ intestinal salines, the Ag^+ concentration went down by a factor three and the $AgCl_{aq}$ concentration decreased by a factor two. The concentration of $AgCl_3^-$ increased twofold in the same salinity range (3–21‰) while the $AgCl_2^-$ concentration changed very little. More pronounced changes were observed in the transition from fresh water to 3‰ brackish water. Most notably, there was 10 times less Ag^+, two times less $AgCl_{aq}$, and fourfold more $AgCl_3^{2-}$ in the 3‰ condition as compared with the saline representing intestinal fluid of flounders in fresh water.

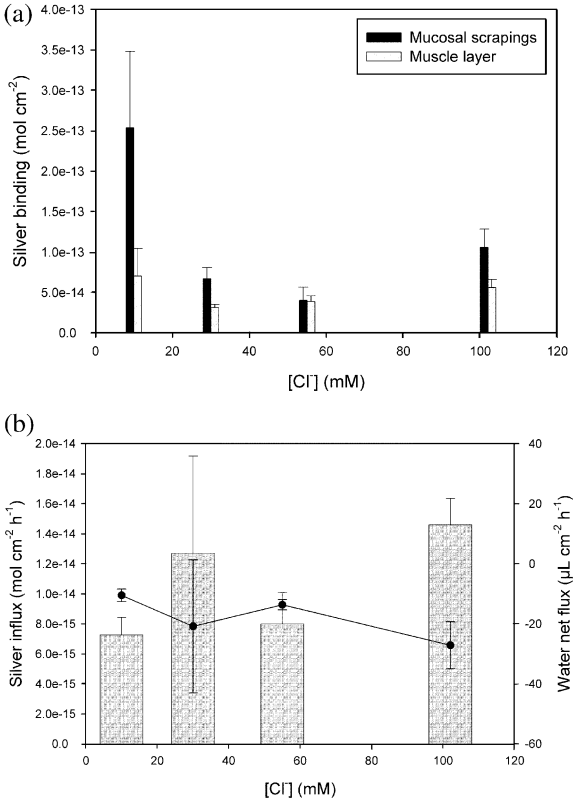

Fig. 4. Effect of varied ion concentrations of intestinal saline on silver (a) binding to mucosal scrapings and muscle layer of intestinal sacs and (b) influx from intestinal saline to plasma saline. The intestinal salines represented intestinal fluid of fish at various water salinities between 21‰ and FW (Table 1). Mannitol was used to adjust osmolality to that of intestinal saline representing 21‰ sea water. All data are for everted intestinal sacs prepared from the anterior section of the intestine and exposed for 6 h to silver. Each point represent the arithmetic mean of four fish and the error bars denote S.E.M. The average total silver concentration during exposures was 9.9 nM. There were no statistically significant differences in silver binding or influx between the different salines.

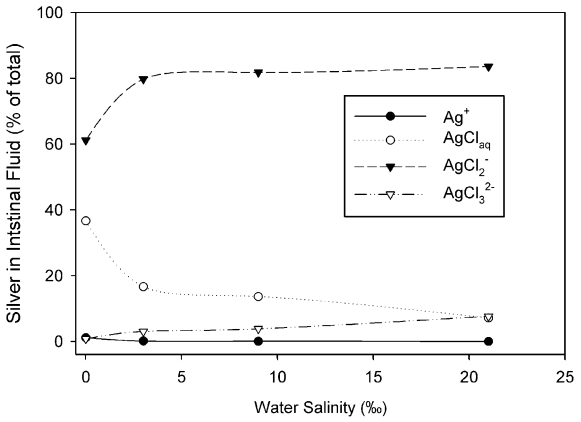

Fig. 5. Modeled speciation of silver in intestinal salines of Experiment 3. Results for this experiment are shown in Figs. 3 and 4. The intestinal salines were composed to mimic flounder intestinal fluid at the water salinities showed. Silver speciation was modeled using MINEQL+ (ver. 4.01; Environmental Research Software) and expressed at percentage of average measured total silver concentration during the experiment.

Geochemical equilibrium modelling using MINEQL+ (ver 4.01) and a BLM-type approach with Ag-intestine binding constants derived from the present study and other stability constants as in Wood et al. (1999), could not predict accumulation of silver in the intestine at any salt concentration (data not shown). Neither binding ($\log K =$ 6.4–6.5) nor kinetic affinity constants ($\log K_{0.5} =$ 6.7) were sufficiently high to account for the silver that in actual fact accumulated in intestinal tissue and plasma saline (number of binding sites (pmol l^{-1}) = B_{max} (pmol cm^{-2}) × Intestinal surface area (cm^{-2})/Volume of intestinal saline (l)). This was not particularly surprising since these conditional constants were derived in presence of complexing Cl^- and competing cations in the intestinal saline. What is evident, however, is that co-varying $[Cl^-]$ from 15 to 107 mM and $[Mg^{2+}]$ from 0 to 100 mM (Table 1, Fig. 4) had little or no effect on silver binding. Modelling changes in the log K for silver binding to the intestine, while keeping the total silver concentration in the intestinal saline at the measured 50% saturation (350 nM) for mucosal scrapings, suggested a theoretical 50% saturation of the binding sites at a log K of 10.0. Interestingly, this is exactly the measured log K for Ag(I) binding to the gill (Janes and Playle, 1995). This model predicted a tenfold increase in Ag(I) binding to the intestine when the $[Cl^-]$ concentration of the intestinal saline was decreased tenfold from 107.5 to 10 mM (Experiment 3). However, in reality changes in $[Cl^-]$ and $[Mg^{2+}]$ did not statistically affect Ag(I) binding, whether with or without compensation for osmotic gradient with mannitol (Fig. 3a and Fig. 4a). As the different ion compositions of intestinal salines in Experiment 3 were associated with dramatic differences in Ag(I) speciation, an effect on Ag(I) binding to the intestine was expected. Perhaps the initial interaction between Ag(I) and mucus has to be considered and that the epithelium itself never encounters $AgCl_n$ species, but rather Ag–mucus complexes.

In freshwater rainbow trout gill, Ag^+ uptake appears to be an active process, aided by a basolateral silver-activated ATPase activity (Bury et al., 1999). To investigate if intestinal silver binding and influx are similarly active, intestinal sacs were exposed to silver at either 0 °C, at 15 °C with cyanide pre-treatment (no cyanide was present in intestinal saline during exposure), or at 15 °C control condition. If uptake of silver was active, either treatment would have been expected to reduce binding and influx of silver. This did not occur. Decrease of incubation temperature had no effect on silver binding or influx and cyanide pre-

Table 3
Effect of decreased temperature and cyanide treatment on binding and influx of silver in intestinal sacs

Group	Ag(I) exposure (nM)	Binding (fmol cm^{-2})		Influx (fmol cm^{-2} h^{-1})
		Mucosal scrapings	Muscle layer	
Control (15 °C)	31 ± 1	114 ± 78	50.0 ± 8.6	3.67 ± 0.47
Low temp. (0 °C)	25 ± 0.3*	173 ± 11	40.6 ± 8.5	3.83 ± 0.53
Cyanide	40 ± 1*	1420 ± 746	827 ± 203*	8.08 ± 2.13*

Values are reported as mean ± S.E.M., $N = 4$. 'Ag exposure' is the mean ± S.E.M. of the measured time-averaged total silver concentration at each condition, based on values at start and end of the experiment. Significant differences from the 'Control' group at $P < 0.05$ are shown with an asterisk.

treatment markedly enhanced binding to muscle layer as well as influx (Table 3). These results were at first unexpected, but may be explained by a potential role for intestinal mucus in reducing uptake of silver. Production of mucus seems to be very important in moderating uptake of zinc by the intestine of fish (Glover and Hogstrand, 2002; Glover and Hogstrand, in press). Although not quantified, it was noted that cyanide as well as low temperature decreased the amount and consistency of mucus produced. Hence, the effect of either treatment may have been to remove some of the barrier for silver uptake. At the lowered temperature (0 °C), this effect may have been offset by a reduced activity of silver transport proteins, leaving binding and influx unchanged. Thus, it was not possible to evaluate if silver uptake is mediated by active transport, but the results provided useful insight into the possible role of intestinal mucus in decreasing silver surface-binding and uptake. In view of the indications that mucus influences silver uptake by altering its luminal speciation and by acting as a barrier, further work on silver–mucus interactions seems warranted.

The specific activity of the ^{110m}Ag and sensitivity of the γ counter limited the lowest exposure concentration of total silver to 1.6 nM (0.17 $\mu g\, l^{-1}$; concentration-dependency experiment). As a result of water uptake across the intestine in an intact marine fish, this concentration would probably correspond to approximately 0.6 nM of total silver in imbibed water, or approximately 30 times higher level than that encountered in contaminated sea waters (Schafer, 1995; Kramer et al., 2001). Thus, even with radiotracer it is difficult to design controlled environmentally realistic exposures of silver to marine organisms.

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