

## The Effect of Humic Acid on the Uptake of Mercury(II), Cadmium(II), and Zinc(II) by Chinook Salmon (*Oncorhynchus tshawytscha*) Eggs

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**Abstract.** The Chinook salmon (*Oncorhynchus tshawytscha*) is endangered or threatened in several of its ranges. The uptake of metals by Chinook salmon eggs and how humic acid (HA) affects the uptake is a subject of interest. Humic acid (0, 0.001, 0.01, and 0.05g/l) reduces the uptake of the metal ions Hg(II), Cd(II), and Zn(II), (1.0  $\mu$ M) by eggs. HA is more effective in reducing the uptake of Hg than that of Cd or Zn. At [HA] = 0.001 g/L Hg uptake is reduced by 44% compared to no HA, while Cd and Zn uptakes are slightly or not reduced. Once the metals are taken up by the eggs, Hg migrates more slowly from the chorion to the yolk than either Zn or Cd. In experiments in which the metal contents of the chorion and yolk were measured at up to 24 h and five days after uptake, the order of migration was Cd > Zn > Hg. This observation is important when discussing the effects of metals on biological processes in the yolk because when Hg is taken up by eggs, a smaller percentage reaches the yolk than does Cd and Zn.

The presence of metal pollutants in natural waters can have adverse effects on organisms by producing changes in the properties of the membrane interface between the organism and its aquatic environment, and affecting biochemical processes. For example, metal ion-induced changes in membrane properties can result in the loss of chemicals from the organism or affect the accumulation of soluble chemicals by the organism (Gottlieb and Swinehart 1988; Lee *et al.* 1983), including fish eggs (Swinehart 1994). A problem of continuing importance is “the beneficial or deleterious effects on biota caused by binding of metals . . . to humic substances” (Horth *et al.* 1988). It is clear that humic substances (HS) are a major metal complexing agent in natural waters (Weber 1988), but the effect HS may have on the distribution, speciation, and bioavailability of these metals is not well defined (Horth *et al.* 1988).

HS are the major organic fraction in soils (MacCarthy 1985), freshwater sediments (Ishiwatari 1985), and freshwater and saltwater (Thurman and Martinus 1985). HS components are defined according to their acid-base solubility. Humic is the

fraction that is insoluble in water, humic acid (HA) is insoluble at a pH of less than 2, and fulvic acid is soluble at all pH values (MacCarthy 1985). Humic substances derived from aquatic sources range in molecular weight (MW) from less than 700 to  $2 \times 10^6$  (Hiraide 1992). HA contains approximately 80% of its carbon in an aromatic form, a variety of metal complexing groups such as carboxylic acids, carbonyls and hydroxyls, and about 4.0 meq of carboxylate groups per gram (Thurman and Martinus 1985). Complexing groups per g of HA varies with MW, with there being 10 meq/g for MWs less than 5000 and about 4 meq/g for MWs up to  $2 \times 10^5$  (Rashid 1985).

The interaction between metal ions and HA is believed to be mainly with carboxylate and phenolic hydroxyl groups. The binding sites resemble the bidentate complexation of divalent metal ions by salicylic and phthalic acids (Livens 1991; Jackson *et al.* 1978 and references cited therein). The complexation sites may be slightly different for different metals and there is the possibility of complexation with N- and S-containing functional groups (Petersen 1991).

There is a limited amount of stability constant data for metal ion-HA complexes (Hiraide 1992). Rashid (1985) summarized such data from the works of Mantoura *et al.* (1978) and Jackson *et al.* (1978). Typical log of stability constants for metal ion-HA complexes of interest for this study are: Hg, 19.1; Zn, 5.1; and Cd, 4.6. Other workers (Campbell and Evans 1987; Chakravarti *et al.* 1984; Weber 1988) have summarized the field or determined a range of stability constants.

With respect to studies on the effects of HA on metal ion bioavailability and toxicity towards organisms, there are a number of reviews (Horth *et al.* 1988; Livens 1991; McCarthy 1989; Pagenkopf 1986; Rashid 1985). Although the binding of metals to HS potentially makes the metal unavailable for absorption through biological membranes (McCarthy 1989), there are cases in which dissolved HS increase, decrease, or do not affect the uptake of metal ions. For example, studies using the freshwater cladoceran, *Daphnia magna*, show the uptake of Cd and Zn to be unaffected (Winner 1984; Winner 1986; Winner and Gauss 1986) or decreased by HS (Poldoski 1979; Stackhouse and Benson 1989). For marine bivalve molluscs HS decrease Cd uptake for *Crassostrea virginica* (Huang 1982), but increase it for *Mytilus edulis* (George and Coombs 1977).

The objectives of this paper are to study the effects of HA, at environmentally relevant levels, on the uptake of the metal

ions Hg(II), Cd(II), and Zn(II) by Chinook salmon, *Oncorhynchus tshawytscha*, eggs and the time dependent distribution of these metals between chorion and yolk. The rationale for this study is that metals affect egg membrane properties and HA can affect the interaction between metals and eggs.

## Materials and Methods

### *Organisms and Associated Waters*

Salmon eggs (*Oncorhynchus tshawytscha*) were obtained from the California Department of Fish and Game, Nimbus Road Hatchery in Rancho Cordova, CA and the United States Department of Fish and Game, Coleman Hatchery at Battle Creek, Anderson, CA.

The eyed fish eggs were stored at 14–18°C in unchlorinated fresh water (UCW) which contained 0.50–0.85 mM Ca and 0.95–1.85 mM Mg (Environmental Health and Safety, University of California, Davis). Eggs that became opaque were discarded because they were no longer viable. Using graphite furnace atomic absorption spectroscopy no Zn, Cd, or Hg could be detected in this water, so the levels of these metals are at least a factor of 10 less than those used in this study. The pH of the water was in the range 7–8. To determine if HS were present, the fluorescence at 440 nm resulting from excitation at 360 nm was measured using a Perkin-Elmer Fluorescence spectrometer (Senesi *et al.* 1991). No fluorescence was detected. American River water (ARW) was collected at the Nimbus Road Hatchery. HS were detected in these waters using the fluorescence method. Calibration with humic acid yielded a HA concentration of 0.002 g/L, but since Aldrich HS is about 50% carbon, the value is probably closer to 0.001 g of carbon/L.

### *Radiochemicals and Chemicals*

The radioisotopes  $^{203}\text{Hg}$  and  $^{65}\text{Zn}$  (with carriers), and  $^{109}\text{Cd}$  (carrier free), in 0.01 M HCl solutions, were obtained from Amersham as the dichlorides. Humic acid was purchased from the Aldrich Chemical Co. as the sodium salt. All other chemicals were reagent grade.

### *Uptake Studies*

Thirty fish eggs, which weighed about 0.1 g/egg, were removed from their storage water, drained, and placed in 200 ml of water containing radiolabelled metal ion (1–4  $\mu\text{Ci}$ ), and metal ion and HA at the appropriate concentrations. Before eggs were introduced, the solution was equilibrated for a minimum of 6 h and there was no loss of activity during the equilibration. Duplicate aliquots were taken from the solution before the introduction of eggs for counting so that the actual moles of metal associated with the sample could be calculated. The solutions containing eggs were mechanically stirred and maintained at 14–18°C.

Two sets of three eggs were removed at various times, rinsed in water, allowed to drain, and each set of three eggs was placed in 12  $\times$  75-mm test tubes. An EDTA solution, 2.5 mM, sufficient to cover the eggs was added to all samples, but was only necessary in the case of Hg(II) samples to eliminate the conversion of Hg(II) to volatile methylmercury compounds by HS (Lee *et al.* 1985; Matthiessen 1996), which was observed in samples absent in EDTA. The tubes were sealed with rubber septum stoppers and counted for about 20 min with a Beckman Gamma 310 gamma counter. The amount of metal taken up was reported as moles/egg.

A study was carried out to determine if the uptake of metals by eggs was dependent on how long the eggs were stored under laboratory

conditions. Hg uptake experiments were performed daily on Nimbus Road Hatchery eggs that were stored for a period of nine days in UCW, ARW, and ARW with 0.05 g/L added HA. For this particular group of eggs the time period between delivery and hatching was nine days. Duplicate sets of three eggs were exposed to 1  $\mu\text{M}$  Hg containing radiotracer for 30 min, counted, and the moles of Hg taken up determined. The data show that for storage in UCW after transfer from Nimbus Hatchery ARW there was an elevated Hg uptake for two days before reduction to a constant value for the remaining seven days. Therefore, uptake experiments were carried out after two days of storage. There was no change in uptake versus storage time when the eggs were stored in ARW or ARW with 0.05 g/L HA, and the uptake was less than that in UCW due to the presence of HS in ARW and added HA.

The localization of metals in salmon eggs was determined in the following manner. After the eggs were counted as described in the uptake experiments, the eggs were removed from the counting tube, the chorion separated from the yolk, and each fraction counted. In addition, whole eggs from the uptake experiments were frozen at  $-10^\circ\text{C}$  for five days, the chorion and yolk were separated, and each fraction counted. When the eggs or their components were subjected to the complexing agent EDTA, the solution containing the complexing agent was also counted.

The percentage of total metal taken up by fish eggs ranged from 6.5% Hg by salmon eggs in HA free water to less than 1% for all other metals by salmon eggs with 0.05 g/L HA added. One gram of HA contains an average of 4 mmol of carboxylate groups (Rasid 1985; Thurman and Martinus 1985), which corresponds to a maximum divalent cationic binding capacity of approximately 2 mmol/g, assuming two carboxylate groups per divalent cation to maintain charge balance. Even though there are limitations in comparing various humic acid preparations (Malcolm and MacCarthy 1986), it is certain that when HA was present, the concentration of complexing groups was in excess of the metal concentrations.

## Results

### *Uptake of Metals as Affected by Humic Acid*

Figures 1–3 are typical least square fits to data for the uptake by Chinook salmon eggs of the metal ions Hg (Figure 1), Cd (Figure 2), and Zn (Figure 3) at 1.0  $\mu\text{M}$  as influenced by HA at 0.001, 0.01, and 0.05 g/L. The data show that the uptake of Hg is more strongly affected by the presence of HA than the other metal ions. Even at [HA] of 0.001 g/L the uptake of Hg is reduced by 44% at 30 min, compared to the uptake with no HA present. The uptakes of Cd and Zn are only slightly or are not reduced at 0.001 g/L HA, but at 0.01 g/L HA are reduced by 20% and 23%, respectively. Note, the data for Zn in Figure 3 is to 60 min, while those for Hg and Cd are to 30 min.

A question arises as to whether the long term uptake of Cd is reduced at HA levels of 0.001 g/L. The data in Figure 2 show that within experimental error at times up to 30 min 0.001 g/L HA does not affect Cd uptake, but uptake studies at longer times show a slight reduction of Cd uptake at 0.001 g/L HA,  $3.8 \pm 0.2$  nmoles/egg, compared to no HA,  $4.7 \pm 0.2$  nmoles/egg at 120 min. The effect of HA on Cd uptake is more similar to that of Zn than Hg.

Mercury uptake experiments were carried out daily over a period of nine days to determine if the uptake characteristics were dependent on storage time after transfer from ARW at the fish hatchery to UCW. As described in the Materials and

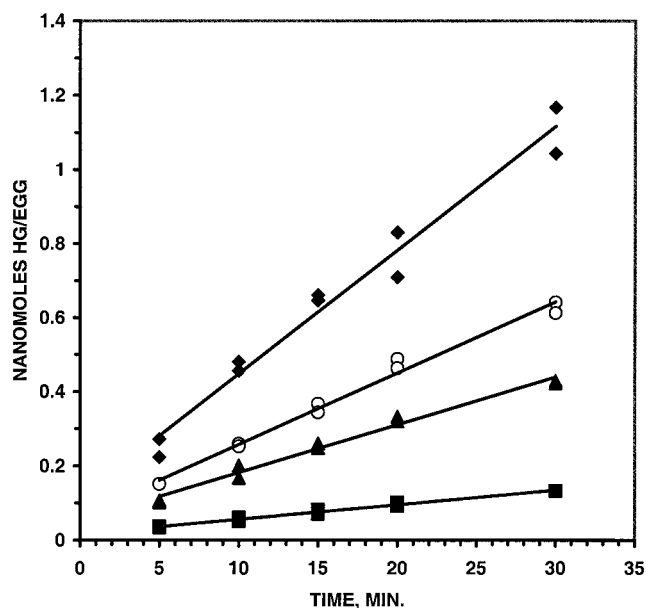


Fig. 1. Uptake of Hg(II) by salmon eggs as affected by HA.  $[\text{Hg(II)}]_{\text{total}} = 10^{-6}\text{M}$ ,  $[\text{HA}] = 0$ , ◆; 0.001, ○; 0.01, ▲; and 0.05 g/l, ■

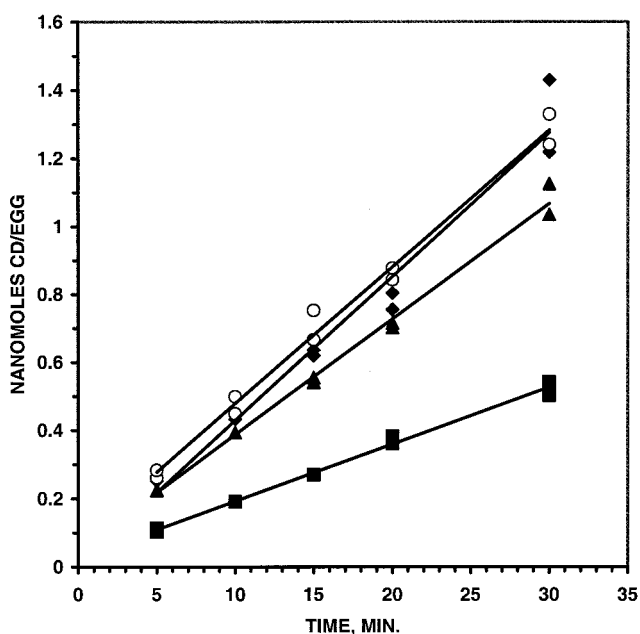


Fig. 2. Uptake of Cd(II) by salmon eggs as affected by HA.  $[\text{Cd(II)}]_{\text{total}} = 10^{-6}\text{M}$ ,  $[\text{HA}] = 0$ , ◆; 0.001, ○; 0.01, ▲; and 0.05 g/l, ■

Methods section, eggs were exposed for 30 min to  $1 \mu\text{M}$  Hg with radiotracer in UCW, ARW, and ARW with 0.05 g/L HA and the activity counted. The results show that Hg uptake by eggs in UCW required an adjustment period of two days, and for the remaining seven days the uptake was 62% of that for the first two days and remained constant. Mercury uptake in ARW and ARW with 0.05 g/L HA remained constant over the entire nine-day period. For UCW the uptake values are  $3.23 \pm 0.02$

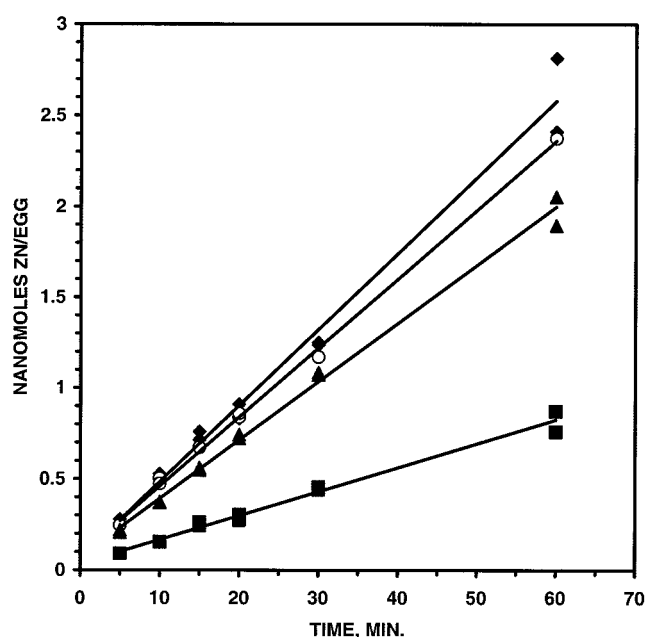


Fig. 3. Uptake of Zn(II) by salmon eggs as affected by HA.  $[\text{Zn(II)}]_{\text{total}} = 10^{-6}\text{M}$ ,  $[\text{HA}] = 0$ , ◆; 0.001, ○; 0.01, ▲; and 0.05 g/l, ■

and  $2.01 \pm 0.11$  picomoles ( $10^{-12}$  moles) of Hg/min, for days 1–2 and 3–9, respectively. For ARW and ARW with 0.05 g/L HA the values are  $0.93 \pm 0.16$  and  $0.29 \pm 0.06$  picomoles/min, respectively. The values are the arithmetic average and the error a standard deviation.

#### Sites of Metal Ions in Eggs

An investigation was carried out to determine the distribution of Hg, Cd, and Zn between the chorion and yolk of the egg at various times during the uptake process, and the affinity of the metals for the chorion as measured by EDTA extraction. Sets of three eggs were incubated with radiotracer for 30 min, the chorion separated from the yolk at various times after incubation, and the activity of both the chorion and yolk or the chorion and EDTA extract of the chorion were measured. Table 1 contains the data and the arithmetic means with the standard deviation.

After 1 and/or 4, and 24 h, the percentages of Hg, Cd, and Zn found in the chorion are  $98 \pm 2$ ,  $93 \pm 3$ , and  $90 \pm 3$ , respectively. Within experimental error, the amount of metal in the chorion did not decrease with time from 1 to 24 h.

In an experiment in which the eggs were stored in a frozen state at  $-10^\circ\text{C}$  for five days and both the chorion and yolk were measured, the percents of metal found in the chorion for Hg, Cd, and Zn are  $84 \pm 4$ ,  $59 \pm 4$ , and  $68 \pm 4$ , respectively. Thus, there is migration of Hg, Zn, and Cd from the chorion into the yolk between one and five days, and the order of migration is  $\text{Cd} > \text{Zn} > \text{Hg}$ .

In the five-day experiments the metal associated with the chorion was the sum of an EDTA extract of the chorion and the extracted chorion. The purpose of carrying out an EDTA extraction of the chorion was to determine how strongly Hg(II),

**Table 1.** Data for percent metal in the chorion-yolk at various times and amount EDTA-extractable from the chorion at five days

Time, type of measurement				
Metal	Time/Data Points			Mean $\pm$ standard deviation
1, 4, 24 h percent metal in chorion				
	<u>1 h</u>	<u>4 h</u>	<u>24 h</u>	
Hg	96	—	100	98 $\pm$ 2
Cd	97	88	93	93 $\pm$ 3
Zn	—	87	93	90 $\pm$ 3
5 days, percent metal in chorion				
	<u>5 days</u>			
Hg	79, 80, 87, 88			84 $\pm$ 4
Cd	62, 52, 63, 58, 61, 57			59 $\pm$ 4
Zn	68, 64, 75, 68, 65, 69			68 $\pm$ 4
5 days, percent of metal extracted from chorion by EDTA				
	<u>5 days</u>			
Hg	14, 12, 15, 13			14 $\pm$ 1
Cd	77, 48, 63, 65, 45, 56			59 $\pm$ 11
Zn	92, 95, 92, 94, 94, 94			94 $\pm$ 1

Cd(II), and Zn(II) were bound to the chorion, and to see if there was a correlation between the strength of metal-chorion binding, as expressed by an EDTA extraction, and the migration rate from chorion to yolk. The experiment shows the order of extraction by EDTA to be Zn > Cd > Hg, with the percentages of metal extracted from the chorion by EDTA being 94  $\pm$  1, 59  $\pm$  11, and 14  $\pm$  1, respectively.

## Discussion

The uptake of the metal ions Hg, Cd, and Zn at 1.0  $\mu$ M by Chinook salmon eggs as influenced by HA at concentrations of zero, 0.001, 0.01, and 0.05 g/L was studied. Humic acid is more effective in reducing the uptake of Hg than the other metal ions, and at a concentration of 0.001 g/L, probably near to the level of complexing functional groups found in natural waters, reduced the uptake of Hg by about 44%. The effects of HA on the uptake of Cd and Zn are similar, with HA being slightly more effective in reducing Zn than Cd uptake. The stability constants at pH = 8.0 for HS complexes with metal ions are Hg(II)  $\gg$  Zn(II) > Cd(II), where typical values of the log of the constants are 19.4, 5.1, and 4.6, respectively (Mantoura *et al.* 1978) These data indicate that the HA complexes of these metal ions are less susceptible to uptake by salmon eggs than complexes derived from the solvated metal ions, such as the aquated and hydroxide species.

Stackhouse and Benson (1989) studied the effects of HA at 0.0005, 0.005, and 0.050 g/L on the uptake of Cd(II), Cr(VI), and two forms of Cr(III) by the freshwater cladoceran, *Daphnia magna*, for up to 96 h. Their data show that a HA level of 0.005 g/L, but not 0.0005 g/L, reduces the uptake of Cd, the only metal in common between the two studies. Penttinen *et al.* (1998) studied the effect of dissolved organic material (DOM) in freshwater on the toxicity of Cd to *Daphnia magna* and found dissolved organic material (DOM) reduced the toxicity. In another study related to this discussion, Decho and Luoma

(1994) studied the effects of particle bound fulvic acid (FA) and HA on the uptake of adsorbed Cd(II) and Cr(III) by two suspension feeding, estuarine bivalves. They found that both FA and HA reduced the uptake of Cd. In agreement with these studies, the data from this study show the uptake of Cd by Chinook salmon eggs to be slightly reduced at 0.001 g/L HA and substantially reduced at 0.01 g/L.

Since Mg(II) and Ca(II) are constituents of both freshwater and saltwater, and complex HA with log of stability constants of 4.00 and 3.95, respectively, which approximate those of Cd-HA, 4.6, and Zn-HA, 5.1, complexes (Rashid 1985), it would be expected that changes in the concentrations of Mg and Ca would affect the uptake of Cd and Zn by organisms by competing with these metals for binding sites on HA. Penttinen *et al.* (1998) reported that 'hardness cations', especially Ca, compete with Cd for binding sites in DOM. This has a special bearing on the uptake of metal ions by organisms in seawater, where the average concentrations of Mg and Ca are 0.054 and 0.010 M, respectively; several orders of magnitude larger than their concentrations in freshwater. The observation of Pempkowiak (1991) that "aqueous solutions of HS exhibit decreasing complexing capacities with increasing salinity," where seawater was diluted with distilled water to change the salinity, could be a manifestation of the competition of Mg and Ca for sites in metal ion-HA complexes. However, the uptake of metals such as Cd also involves competition with other metals at the binding site on organism membranes or in transport through membranes. Penttinen *et al.* (1998) report that increasing concentrations of Ca reduced the uptake of Cd by *Daphnia magna* by competition for binding/transport to the organism. Calcium competition with other metals, such as Cd, has been used by Playle (1998) in modeling metal interactions at fish gills. In work related to Ca and Mg competition with Cd, Sunda and Huntsman (1988a, 1988b) examined the effects of Mn and Zn, two metals in the same size range as Mg and Cd, on the uptake of Cd by diatoms and found that both Mn and Zn reduce the uptake of Cd.

There are other variables that affect comparisons between studies. John *et al.* (1988) and Alberts and Giesy (1983) found that the conditional stability constant of the Cd-HA complex increased with increasing pH and decreasing concentration of humus. John *et al.* (1988) also reported that the HA fraction with molecular weights ranging from  $10^3$ – $10^4$  complexed the greatest amount of Cd(II). It has also been shown that the adsorption strength of Zn(II) with HS depends on the degree of humification (Matsuda and Uto 1970).

The correlation between the reduction in the uptake of metal ions and the stability constants for the metal ion-HA complexes results in the conclusion that the metal ion uncomplexed by HA is taken up by the eggs. However, differences in the uptake of metal ions will also occur due to differences in speciation, such as the formation of hydroxide (HO<sup>-</sup>) containing species and dimers. For example, the pKs for the loss of the first proton from the aquo species of the metal ion, M(H<sub>2</sub>O)<sub>n</sub><sup>2+</sup> are: Hg, 3.4; Zn, 9.0; and Cd, 10.1 (Stumm and Morgan 1981). Thus, at pH = 7, monomeric Hg(II) will be present as (H<sub>2</sub>O)<sub>n-1</sub>Hg(OH)<sup>+</sup>, while Zn and Cd will be present as Zn(H<sub>2</sub>O)<sub>n</sub><sup>2+</sup> and Cd(H<sub>2</sub>O)<sub>n</sub><sup>2+</sup>. Differences in charge can result in differences in the uptake of the metal ions at a particular pH, which are independent of the HA complexation process. In addition dimerization of metal ions occurs (Stumm and Morgan



1981), which may have dramatic effects on the uptake properties of the soluble form of the metals.

Using uptake information for Hg (Results, Uptake of Metals as Affected by Humic Acid) and the results of experiments on whether the uptake of metals by eggs was dependent on how long the eggs were stored under laboratory conditions (Materials and Methods, Uptake Studies), it is possible to calculate an amount of HA present in ARW. If the ratio of Hg uptake at 30 min with HA to that in UCW (data from Figure 1) is plotted versus added HA (0.56, 0.001 g/L; 0.40, 0.01 g/L; and 0.11, 0.05 g/L) and compared to the ratio of Hg uptake at 30 min in ARW and ARW with 0.05 g/l added HA to that in UCW, 0.46 and 0.14, respectively, the calculated values of HA are 0.004 g/L for ARW and 0.046 g/L for ARW with 0.05 g/L of added HA. Fluorescence measurements yield a value of 0.002 g/L HA for ARW (Materials and Methods, Organisms and Associated Waters). It is satisfying that such different methods yield similar results for the level of HA in ARW. It is likely that the method using data from Figure 1 and comparing them with egg uptake studies in ARW yields a more accurate result.

For the uptake of Hg, Cd, and Zn by Chinook salmon eggs, Hg migrates more slowly from the chorion to the yolk than either Zn or Cd, with the order of migration being Cd > Zn > Hg. This observation is important when discussing the effects of metals on biological processes in the yolk in that when Hg is taken up by eggs, less of it reaches the yolk than more labile metal ion such as Cd and Zn. Since Hg resists removal with EDTA, it is more strongly bound to complexing sites in the chorion than either Cd or Zn, with the order of extraction by EDTA being Zn > Cd > Hg. Guadagnolo *et al.* (2001) studied the accumulation of silver, Ag, by rainbow trout (*Oncorhynchus mykiss*) and found that up to 85% of Ag was in the chorion. In addition, they discussed the fact that the chorion contains proteins rich in sulfhydryl (-SH) groups, which bind Ag strongly. Sulfhydryl groups strongly complex Hg(II) and could participate in the conversion of ionic Hg(II) to methylmercury compounds, which may enter the yolk more rapidly than ionic Hg(II). The methylation of Hg(II) by fulvic acid has been studied by Lee *et al.* (1985), who found that 12 ng/L of methylmercury was produced in 44 h at pH 4.5 and 30°C, starting with [Hg(II)] = 20 mg/L (100 µM) and fulvic acid at 0.180 g/L organic carbon. Iron(III) or Fe(II), at about 100 µM, increased methylmercury production from 12 ng/L to 110 ng/L. Although the conditions are different from those in this work, HA and iron (in the egg) are certainly present and would result in increased methylmercury production.

## Conclusions

With respect to the uptake of the metal ions Hg, Cd, and Zn by Chinook salmon eggs, HA is more effective in reducing the uptake of Hg than the other metal ions. HA at a concentration of 0.001 g/L reduced the uptake of Hg by 44% while only slightly reducing the uptakes of the other metal ions.

For the uptake of Hg, Cd, and Zn by Chinook salmon eggs, Hg migrates more slowly from the chorion to the yolk than either Zn or Cd. This observation is important when discussing the effects of metals on biological processes in the yolk in that when Hg is taken up by eggs, less of it reaches the yolk than more labile metal ion such as Cd and Zn.

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