

## Comparative bioaccumulation studies of colloiddally complexed and free-ionic heavy metals in juvenile brown shrimp *Penaeus aztecus* (Crustacea: Decapoda: Penaeidae)

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### Abstract

A large fraction of the trace metal pool in estuarine and coastal waters is complexed with colloidal-sized macromolecular organic matter. Bioaccumulation and bioavailability studies of trace metals have generally utilized free-ionic dissolved metal ions. Consequently, little is known about the uptake and depuration kinetics of the colloiddally bound species. Chemical characteristics such as the relative hydrophobicity of these metal–colloid complexes may alter their bioavailability by interfering with or enhancing transport across membrane lipid bilayers. We used radiotracers to compare the bioaccumulation and sites of accumulation of colloiddally complexed and free-ionic forms of Ag, Cd, Ba, Fe, Sn, Zn, Co, Hg, and Mn in juvenile brown shrimp *Penaeus aztecus*. In this paper, we present the results from 14-d uptake and bioaccumulation studies in which shrimp could take up radiotracers only from the water. Our results indicated that the colloiddally complexed forms were bioavailable to shrimp. Uptake kinetics appeared to be similar in the two treatments for most metals, although by the end of the uptake study, the whole-body activities of Ba, Sn, and Zn were significantly higher in shrimp exposed to free-ionic metals. While the hepatopancreas appeared to be the site of highest accumulation on a mass-specific basis for both treatments, the total activity level of radiotracer differed between the two treatments. In the colloiddally complexed treatment, the majority of total tracer activity was associated with the hepatopancreas while shrimp exposed to free-ionic metals exhibited the highest proportion of total activity in the abdomen. The mechanisms underlying these differences remain to be tested.

Heavy metals are some of the most toxic, persistent, and widespread contaminants in estuarine systems. Dissolved or suspended metals can degrade water quality and become available to plankton, nekton, and benthic filter and deposit feeders. Aquatic organisms can acquire trace elements from food, from suspended particles, or directly from the water (Luoma 1989; Luoma et al. 1992; Carvalho and Fowler 1993). Uptake of dissolved metals from solution through permeable surfaces into the bodies of marine invertebrates is generally considered to be a passive process (Williams 1981), while metals associated with macroscopic particles are taken up via active ingestion.

The solution speciation of trace metals is thought to be the primary determinant of their bioavailability to, and bioaccumulation in, aquatic organisms (Zamuda and Sunda 1982; Bruland et al. 1991; Luoma et al. 1992). Heavy metal bioaccumulation has also been shown to be a strong function of salinity (Phillips 1977; Part et al. 1985); pH (Hart and

Scaife 1977); size and nature of dissolved organic matter (DOM) (Laegreid et al. 1983); and presence and concentrations of other metals in the water (Jackim et al. 1977; Wright 1977).

The toxicity and potential for bioavailability of trace metals are frequently considered to be determined by the concentration of the free metal ion species. Many investigations of the bioaccumulation and toxicity of metals to marine organisms have employed a free-ion activity model to describe bioavailability (e.g., Sunda and Lewis 1978; Engel et al. 1981; Daly et al. 1990; Meador 1991; Campbell 1995). This model relates all observable metal effects to the concentration of free-ionic species and does not consider the concentrations of chelated species (Sunda and Lewis 1978). While the free-ionic model can explain a large fraction of the observed bioaccumulation results, recent data clearly show that organic ligands can mediate trace metal toxicity and bioaccumulation (Daly et al. 1990; Campbell 1995 and references therein).

Sequestration of free-ionic species by biogenic organic ligands is well documented as a mechanism to transport metal ions across cell membranes (Blust et al. 1986; Bruland et al. 1991). Chelating agents that form water-soluble species can reduce the uptake and toxicity of free-ionic heavy metal species. Conversely, complexing agents that form lipid-soluble uncharged species can increase bioavailability of metals to marine organisms (Connell 1990; Daly et al. 1990; Furness

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and Rainbow 1990; Phinney and Bruland 1994). Evidence is also emerging to support the importance of colloidal organic matter in mediating particle uptake and transport of trace metals in natural water (Honeyman and Santschi 1992; Santschi et al. 1997).

The charge structure and relative lipophilicity of organically complexed metal and organic contaminants may alter rates of transport across the lipid membranes of cells located on the gills, epithelium, and/or guts of marine organisms (Simkiss and Taylor 1989). Biogenically produced organic compounds are amphiphilic (i.e., they contain both hydrophilic and hydrophobic groups), and trace metals may be bound to functional groups associated with the hydrophobic "backbone" of the organic macromolecules. This makes it difficult to predict the bioavailability of organically complexed metals to marine organisms and their subsequent behavior once they have been taken up. The potentially hydrophobic character of metal-colloid complexes means that these compounds may behave differently within an organism than hydrophilic (i.e., charged) inorganic ion species or ions complexed with hydrophilic organic molecules.

This radiotracer study was undertaken to: (1) contrast bioaccumulation of free-ionic and colloiddally complexed trace metals in a common marine invertebrate—the brown shrimp *P. aztecus*, and (2) compare the subsequent fate of both forms of accumulated metals within shrimp tissues. In a subsequent paper, we will describe the depuration kinetics of selected metals.

## Materials and methods

**Experimental animals**—Juvenile *P. aztecus* (40–60-mm total length) were purchased on 5 May 1996 from a local bait dealer who had collected these animals in West Galveston Bay on the same day using an otter trawl. The dealer held his animals in 16-psu flowing seawater, and we selected healthy individuals and transported them to the laboratory in 17-psu 0.2- $\mu\text{m}$ -filtered seawater. Shrimp were held at 17 psu in a 40-liter glass aquarium containing 0.2- $\mu\text{m}$  filtered seawater and equipped with a power canister filter containing activated carbon and polyester filter floss. Shrimp generally had full digestive tracts when purchased and were not fed during the 27-h period prior to the onset of the uptake experiments.

**Radiotracers**—We prepared our working trace metal spikes from commercially sourced radiotracers ( $^{54}\text{Mn}$ ,  $^{59}\text{Fe}$ ,  $^{60}\text{Co}$ ,  $^{65}\text{Zn}$ ,  $^{109}\text{Cd}$ ,  $^{110\text{m}}\text{Ag}$ ,  $^{113}\text{Sn}$ ,  $^{133}\text{Ba}$ , and  $^{203}\text{Hg}$ ) that were either carrier free or had the highest available specific activities (Table 1). Carrier-free or high specific activity forms of radionuclides were employed to elevate the level of radioactivity in samples so that counting times and errors were minimized while animals were exposed to environmentally realistic (i.e., low) metal concentrations. The activity of all spikes was determined using a high-purity germanium well detector coupled to a Canberra S-100 multichannel analyzer and spectral analysis software (Genie PC). The gamma ray detector was calibrated with liquid standards of isotope spikes in geometries ranging from 1 to 10 ml. Typical resolution (full width, half maximum) was 2.24 keV at 1,332

Table 1. Radioisotope characteristics for the multi-ion uptake study.

Isotope	Supplier	Oxidation state	Specific activity (mCi mg <sup>-1</sup> )	Gamma detection energy (keV)
$^{54}\text{Mn}$	NEN	II	148.80	834.8
$^{59}\text{Fe}$	NEN	II	23.27	1,099.3
$^{60}\text{Co}$	Amersham	II	17.24	1,332.5
$^{65}\text{Zn}$	Amersham	II	405.00	1,115.0
$^{109}\text{Cd}$	NEN	II	3.11	88.0
$^{110\text{m}}\text{Ag}$	Amersham	I	0.95	657.7
$^{113}\text{Sn}^*$	Amersham	IV	4.19	391.1
$^{133}\text{Ba}$	NEN	II	3.16	355.9
$^{203}\text{Hg}$	Amersham	II	16.30	279.8

\* Hexachlorostannic acid.

keV. Gamma emissions are summarized in Table 1. Counting times were adjusted (minutes to hours) so that propagated counting errors were below 10% (1 sigma); however, some samples had such low activities that even with 12-h counts, 1-sigma errors were as high as 20%.

**Colloids**—Colloids were concentrated from 100 liters of 5-psu water (collected on 29 April 1996) from Dickinson Bayou, an inlet of Galveston Bay. Upon arrival in our laboratory, the water was prefiltered through a 5- $\mu\text{m}$  cartridge. Next, the colloids were concentrated by reducing the original volume to approximately 11 liters in an Amicon DC-10 cross-flow ultrafiltration system equipped with a spiral-wound 1-kDa cutoff cartridge. This concentrate was refrigerated at 4–5°C in a high-density polyethylene (HDPE) container until required. A 2-ml aliquot of the concentrate was diluted with 48 ml of 18-M $\Omega$  deionized water and analyzed with a Shimadzu model TOC-5050A total organic carbon (TOC) analyzer so that the colloidal organic carbon concentration of the concentrate could be determined. The mean  $\pm$  1 SD TOC concentration of the colloid concentrate was 78  $\pm$  1 mg TOC liter<sup>-1</sup>.

**Experimental treatments**—Shrimp were exposed to one of two treatments: (1) colloiddally complexed radiotracers, or (2) free-ionic radiotracers or an ultrafiltered seawater control without any radiotracers. All of the 17-psu seawater used in our experimental treatments was collected from a West Galveston Bay salt marsh and ultrafiltered under the same conditions as the water from Dickinson Bayou. The permeate from the ultrafilter was stored in HDPE containers until required.

Our studies were designed to evaluate uptake of colloiddally complexed and free-ionic species at environmentally realistic metal and organic carbon concentrations. We selected a TOC concentration of 10 mg C liter<sup>-1</sup>, a concentration that falls within the same order of magnitude as water measured from Galveston Bay (Benoit et al. 1994; Santschi et al. 1995; Guo and Santschi 1996; Wen et al. 1997a,b, 1999). The trace metal concentrations in our treatments (Table 2) were based on values reported for surface waters in Galveston Bay (Benoit et al. 1994; Stordal et al. 1996a; Wen

Table 2. Trace metal concentrations added to our experimental treatments as compared to published values from Galveston Bay and the North Atlantic and observed BE between metals and colloids after dialysis. The actual metal concentration added to our experimental treatments is a product of our targeted concentration and the BE.  $BE = 100 \times \text{postdialysis activity/predialysis activity}$ .

Trace metal	Estuarine concentration range ( $\mu\text{g liter}^{-1}$ )	Reference	Colloid binding efficiency (%)	Experimental concentration ( $\mu\text{g liter}^{-1}$ )
Mn	$1 \times 10^{-1}$ – $5.5 \times 10^1$	*	25	$5 \times 10^{-6}$
Fe	$1 \times 10^0$ – $1.5 \times 10^1$	*	82	$2 \times 10^{-4}$
Co	$2 \times 10^{-2}$ – $5 \times 10^{-1}$	§	0.5	$2 \times 10^{-7}$
Zn	$6 \times 10^{-1}$ – $4.5 \times 10^0$	*	10	$2 \times 10^{-6}$
Cd	$8 \times 10^{-3}$ – $1.9 \times 10^{-2}$	†	7	$2 \times 10^{-5}$
Ag	$1 \times 10^{-3}$ – $1 \times 10^{-2}$	*	74	$5 \times 10^{-3}$
Sn	$1 \times 10^{-3}$	§	27	$2 \times 10^{-5}$
Ba	$4.5 \times 10^{-2}$ – $1 \times 10^1$	§	14	$2 \times 10^{-3}$
Hg	$2.4 \times 10^{-4}$ – $2.7 \times 10^{-3}$	‡	22	$1 \times 10^{-3}$

\* Benoit et al. 1994.

† Wen et al. 1996.

‡ Stordal et al. 1996b.

§ Bruland 1983.

et al. 1997a,b, 1999) and the North Atlantic (Bruland 1983). In all cases, we utilized additional metal concentrations that were at or below the low end of reported values.

*Isolation of colloiddally complexed ions*—Aliquots of all the radiotracers, except  $^{203}\text{Hg}$ , were mixed with 1.2 liters of the concentrated colloid solution in an acid-cleaned Teflon beaker and mixed with a Teflon-coated magnetic stir bar for 24 h. The  $^{203}\text{Hg}$  spike arrived too late for addition with the other radioisotopes. It was added to the colloid–isotope mixture after 24 h of mixing had elapsed and was mixed for another 2 h.

Dialysis was employed to separate the colloiddally complexed ions from free-ionic forms that remained in solution. After mixing, the colloid–ion solution was distributed into a series of 30.5-cm-long cellulose dialysis bags (Spectra Por, 1-kDa cutoff), which were sealed with plastic dialysis bag closures and suspended in a 20-liter low-density polyethylene (LDPE) bucket containing 0.2- $\mu\text{m}$ -filtered 17-psu seawater that was stirred with a magnetic stir bar. At periodic intervals, 1-ml aliquots of the seawater were gamma counted. The relationship between seawater activity and time indicated when diffusion was approaching equilibrium, and at that time, the seawater was replaced and dialysis was continued. The dialysis procedure was terminated after approximately 24 h, when the rate of diffusion out of the bags had declined to near zero.

The colloid–ion complex was decanted into an acid-cleaned Teflon beaker, and the activity of a 1-ml aliquot was determined. The gamma activity determined how much of each isotope needed to be added to ultrafiltered seawater to establish a free-ion treatment solution with the same metal concentrations as the colloid–ion complex. It also provided an estimate of the binding efficiencies (BEs) of our colloid sample (Table 2). The BE was estimated as

$$BE = 100 \times \frac{\text{postdialysis decay} - \text{corrected activity}}{\text{predialysis decay} - \text{corrected activity}} \quad (1)$$

BE values were within the range obtained in other laboratory studies using radiotracers (Stordal et al. 1996b; Wen et al. 1997b). Based on the activities in the colloid–ion complex, the free-ion treatment solution was prepared by combining sufficient aliquots of each isotope with ultrafiltered 17-psu seawater in an HDPE bucket and mixing with a Teflon stir bar for 1 h. The high activities of our isotopes allowed us to dispense very small aliquots of each spike into a relatively large volume of seawater. As a consequence, changes in the pH of our treatment solutions were negligible. Figure 1 provides an overview of our experimental treatment procedures.

*Uptake experiment*—Uptake experiments were designed to expose shrimp to experimental treatments for 14 d, during which time animals were periodically sacrificed and counted for gamma activity. Our protocols were developed during preliminary studies and included periodic feeding and a mechanism for maintaining adequate oxygen concentrations. Animals were housed individually in 400-ml Teflon containers equipped with threaded lids. Each container in the colloid–ion treatment received 40 ml of the colloid–ion complex and 260 ml of oxygenated ultrafiltered 17-psu seawater, and each container in the free-ion treatment received 300 ml of ultrafiltered 17-psu seawater containing free-ionic metals. We randomly assigned 26 containers to each treatment, while another 11 containers were designated as controls and received 300 ml of ultrafiltered seawater. This level of replication was sufficient to allow two shrimp to be sacrificed from each treatment after 1, 4, 7, and 14 d of uptake and a single shrimp to be sacrificed from the control group at the same intervals. The remaining containers consisted of several spares to replace losses due to mortalities and to provide

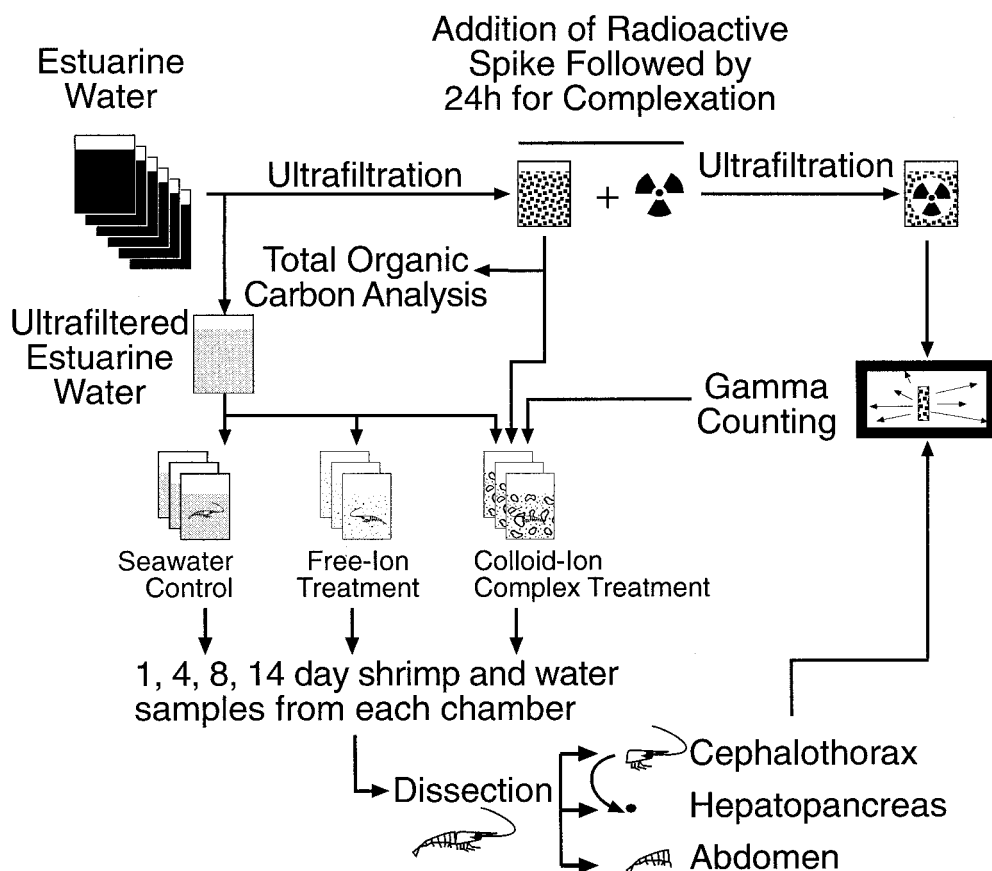


Fig. 1. Schematic diagram of the experimental protocols used to measure uptake of free-ionic and colloidally complexed radionuclides by juvenile brown shrimp.

additional duplicate samples for subsequent depuration studies on days 15–28.

Treatment and control solutions were added to containers on 6 May 1996, and one brown shrimp was allocated to each container in a random sequence. Lids were sealed, and pure oxygen was added to each container via a small resealable hole in the lid. This pure oxygen atmosphere maintained saturation oxygen concentrations for at least 24 h and was replenished daily. All containers were placed within a clean bench in a room with a 12:12 light:dark (LD) photoperiod, provided by overhead fluorescent illumination, and a room temperature of 22–23°C.

After 24 h, and at each subsequent sampling time, two a priori, randomly designated containers in each treatment and a single control were removed for sampling. A 1-ml aliquot of the treatment solution was withdrawn from each of the containers for gamma counting. Each shrimp was placed in a petri dish and rinsed with 0.2- $\mu\text{m}$ -filtered seawater to flush away external treatment solution. Stainless steel dissection instruments were used to separate the abdomen (exoskeleton, pleopods, telson, and uropods but not the digestive tract) from the cephalothorax (carapace, rostrum, pereopods, and all tissue within, except the hepatopancreas and posterior section of the digestive tract) and to extract the hepatopancreas with associated digestive tract from the cephalothorax (Fig. 1). All tissues except the hepatopancreas were blotted,

sealed in pretared counting vials, weighed, and gamma counted (Fig. 1). Counts were decay corrected to day zero of the experiment and expressed as Bq per fresh mass of tissue or volume of sample.

Preliminary studies indicated that shrimp could be held for several days without food; however, starvation-induced mortalities increased sharply after 1 week. The extended 14-d duration of our experiment mandated periodic feeding. Feeding could not be conducted within experimental chambers because radiotracers would be ingested along with food and because water quality rapidly declined after food was introduced. Shrimp were therefore removed from their treatments on days 2, 5, 7, and 12 of uptake for feeding, which took place in additional Teflon containers filled with ultrafiltered seawater. Each shrimp received one pellet (mean mass = 8.65 mg) of commercial shrimp food (Rangen) and was allowed to feed and excrete all visible material from its digestive tract before being returned to its experimental container. Experimental containers were reoxygenated and resealed.

Samples were collected on days 1, 4, 8, and 14 of the study. Animals that died during this period were replaced by shrimp that were randomly selected from a pool of spares that had been exposed to the same treatments. The depuration phase of the study commenced after 14 d of uptake. At that time, we transferred all remaining shrimp to clean, in-

dividual 400-ml Teflon containers holding 300 ml of ultrafiltered seawater with no additional trace metals. The results of that experiment will be presented in a separate paper.

Within each treatment, our mass balance calculations assumed that metal activity could reside within the shrimp or the treatment solution or could be adsorbed to container walls. Wall adsorption ( $C_{wall}$ ) was estimated indirectly from the decay-corrected concentration measurements of shrimp ( $C_s$ ), water activity, and their initial concentration ( $C_w^0$ )

$$C_{wall} = C_w^0 - C_s - (C_w \times 300 \text{ ml}) \quad (2)$$

**Bioaccumulation models**—We used a kinetic approach to model uptake (Farrington and Westfall 1986), which treats uptake of radiotracers from solution as a simple reversible reaction:



where  $C_w$  is the activity (Bq ml<sup>-1</sup>) of radiotracer in the treatment solution, and  $C_s$  is the corresponding activity (Bq ml<sup>-1</sup>) in the shrimp tissues, and  $k_1$  and  $k_2$  are uptake and clearance rate constants, respectively, with units of inverse time. The equation for metal uptake in shrimp is as follows:

$$\frac{dC_s}{dt} = k_1 C_w - k_2 C_s \quad (4)$$

For uptake from a solution with an initial activity of zero in the shrimp and an activity in the treatment solution that declines in response to uptake by shrimp, we can solve Eq. 4 to arrive at

$$C_{s(t)} = C_{\infty_w} \times K_{sw} (1 - e^{-(k_1+k_2)t}) \quad (5)$$

where  $K_{sw}$  is a partition coefficient that represents  $k_1/k_2$  at equilibrium ( $dC/dt=0$ ), and  $C_{\infty_w}$  is a constant water activity at equilibrium. Note that in this model, the activity of the treatment solution is allowed to decrease over time. If treatment solution activity were constant, then the model would have the form of eq. 2 from Hamelink et al. (1994).

The experimentally determined activity in the shrimp,  $C_{shrimp}$ , expressed in Bq g<sup>-1</sup>, is then as follows:

$$C_{shrimp} = \frac{C_s}{C_p} \quad (6)$$

At equilibrium, the partition coefficient ( $K_{sw}$ ) can be related to the concentration factor ( $CF$ ) by

$$CF = \frac{K_{sw}}{C_p} = \frac{C_{\infty_s}}{C_{\infty_w} \times C_p} \quad (7)$$

where  $CF$  has units of milliliters per gram,  $C_p$  indicates the concentration of shrimp mass in the water (grams per milliliter), and  $C_{\infty_w}$  and  $C_{\infty_s}$  are the activities associated with water and shrimp at equilibrium, respectively. During the uptake phase,  $CF$  can be expressed using

$$CF = \frac{K_{sw} C_{\infty_w} (1 - e^{-(k_1+k_2)time})}{C_w^0 - C_{\infty_w} (1 - e^{-(k_1+k_2)time})} \quad (8)$$

where  $C_w^0$  is the initial activity concentration in the water and  $C_w = C_w^0 - C_s$ .

## Results

**Mortality**—Mortality rates were low in both treatments and the control. By the end of the uptake and depuration study (21 d), cumulative mortality rates in each treatment and the control were almost identical at 26–27%. No gamma activity was noted in the control animals or their seawater media during counts that lasted up to 12 h, suggesting that our clean procedures were adequate to prevent contamination among treatments.

During daily inspections, we discovered that three shrimp molted during the study (two in the free-ionic treatment and one in a seawater control), and one of the free-ion animals undoubtedly consumed the molted exoskeleton and ingested radionuclides in the process. Animals that molted were excluded from the analysis and were replaced with spares.

**Mass balance**—Mass balance calculations revealed that wall adsorption was <5% in both treatments for Sn, Mn, or Fe. On day 14, loss to container walls of colloidal forms of Zn and Co increased from near zero to 25 and 50%, respectively. Wall loss in both Hg treatments increased from 10% on day 1 to 20% by day 14. There was little wall adsorption by the free-ionic forms of Cd and Ba; however, loss to walls in the colloidal treatments was 10–40% in Cd and 90% in Ba. Conversely, 40–80% of free-ionic Ag was associated with the container walls, while the colloidal form remained in solution.

The overall trends of treatment metal activities suggested that solution activity was declining exponentially as shrimp accumulated radiotracers and metals were adsorbed to container walls. Exponential curves,  $Activity_t = Activity_{t=0}(e^{-bt})$ , were fitted to the solution activity concentrations and used to estimate solution activities that were more representative than our point estimates of the conditions that prevailed in the containers over the course of the study. Concentration factors were then determined using the fitted solution activity curves and the measured tissue activities.

**Metal uptake**—Shrimp accumulated trace metals in all treatments. Uptake kinetics typically followed a  $1 - \exp(-kt)$  (Fig. 2, Eq. 5) model. Bioaccumulation appeared to level off by day 14 in the colloidal Cd, Ba, Ag, Mn, and Fe and in the ionic Cd, Hg, Ba, Sn, Mn, and Fe treatments. In the remaining treatments, activities were still increasing (Fig. 2). Free-ionic forms of Ba, Sn, Zn, and Co appeared to be accumulated at higher levels than the colloiddally complexed ions (Fig. 2), and by day 14, Ba, Sn, and Zn had accumulated to significantly higher levels in the free-ionic treatments (Table 3). Differences among treatments in the remaining metals during the bioaccumulation period were largely obscured by within-treatment variability (Fig. 2). Comparisons of whole-animal activities at the end of the uptake experiment (day 14) confirmed that most free-ionic metals were not accumulated at significantly higher levels than the comparable colloidal forms (one-tailed *t*-test,  $P > 0.05$  using equal or unequal variances as appropriate) (Table 3).

After 14 d of uptake, mean  $\pm$  SE CFs for whole animals ranged from  $77 \pm 2$  ml g<sup>-1</sup> in ionic Sn to  $54,076 \pm 49,812$  ml g<sup>-1</sup> in ionic Ag (Fig. 3). Concentration factor kinetics

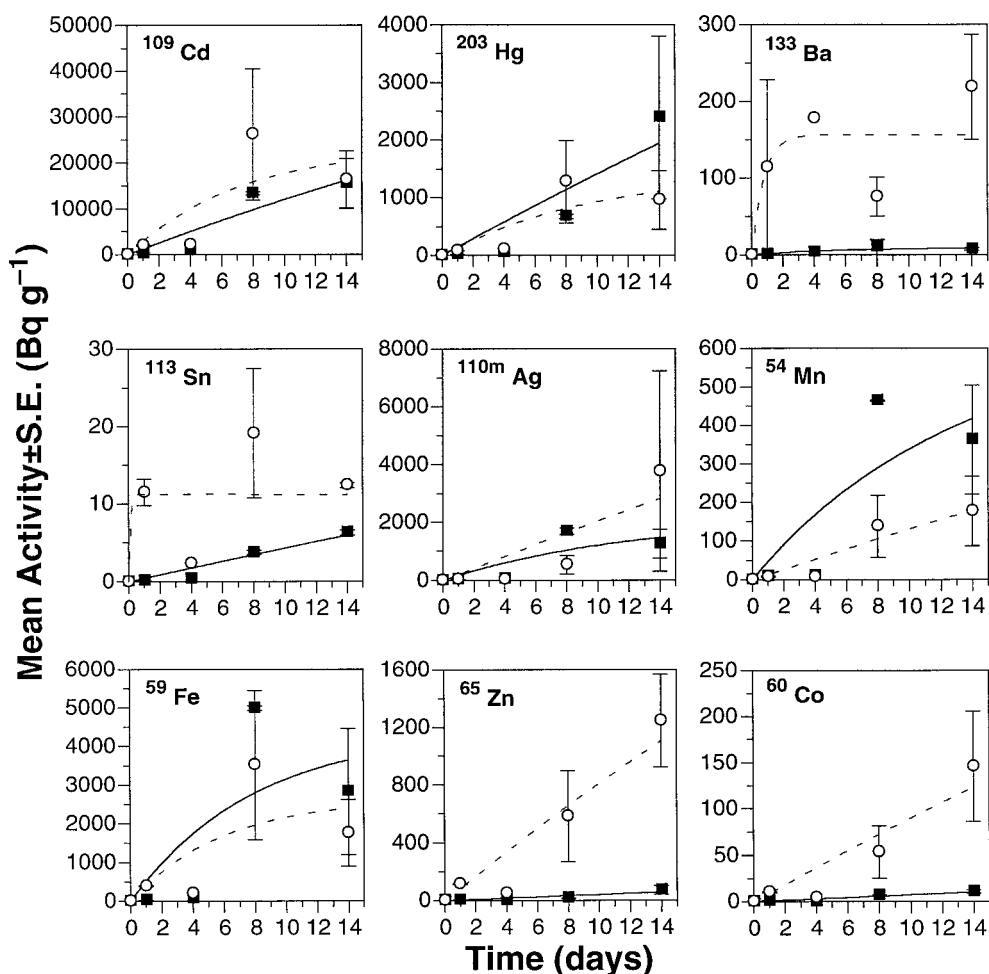


Fig. 2. Mean mass-specific activity in whole bodies of brown shrimp exposed to free-ionic (O, dashed line) and colloidally complexed (■, solid line) trace metals over 14 d.

Table 3. Mean  $\pm$  SE activity levels ( $\text{Bq g}^{-1}$ ) in whole shrimp after a 14-d exposure to treatment solutions. The probability indicates the significance level of a one-tailed  $t$ -test to evaluate the hypothesis that mean activities were greater in the free-ion treatment than in the colloidally complexed treatment. The magnitude of the difference between means was estimated by mean activity free-ion–mean activity colloid.

Isotope	Whole shrimp mean activity ( $\text{Bq g}^{-1}$ )		Probabilities
	Free-ion	Colloid	
$^{109}\text{Cd}$	$16,323 \pm 6,197$	$15,444 \pm 5,466$	$P \leq 0.423$
$^{203}\text{Hg}$	$958 \pm 511$	$2,394 \pm 1,414$	$P \leq 0.220$
$^{133}\text{Ba}$	$219 \pm 68$	$7 \pm 2$	$P \leq 0.001$
$^{113}\text{Sn}$	$20 \pm 8$	$6 \pm 0$	$P \leq 0.003$
$^{110\text{m}}\text{Ag}$	$3,764 \pm 3,467$	$1,248 \pm 489$	$P \leq 0.274$
$^{54}\text{Mn}$	$177 \pm 90$	$362 \pm 142$	$P \leq 0.192$
$^{59}\text{Fe}$	$1,760 \pm 855$	$2,825 \pm 1,631$	$P \leq 0.311$
$^{65}\text{Zn}$	$1,246 \pm 322$	$73 \pm 29$	$P \leq 0.034$
$^{60}\text{Co}$	$146 \pm 60$	$17 \pm 2$	$P \leq 0.133$

supported the apparent leveling off of bioaccumulation of colloidal Cd, Ba, Ag, and Fe (Fig. 3). Colloidal Hg, Zn, and Co also appeared to be approaching equilibrium, while colloidal Mn was still accumulating (Fig. 3). Ionic forms of Cd, Hg, Sn, and Fe had apparently reached equilibrium, and Mn, Zn, and Co were approaching equilibrium (Fig. 3). Only ionic Ag was still in the exponential phase of accumulation. The kinetics of Cd and Fe were similar in both treatments (Fig. 3; Table 4). Comparisons of rate constants ( $k_1 + k_2$ ) among the colloid and free-ionic treatments revealed differences ranging from 36% (Co) to 518% (Ag). Colloidally complexed Ag, Zn, and Co were concentrated at higher rates than their ionic counterparts, with the reverse pattern for Cd, Hg, Sn, Mn, and Fe (Fig. 3; Table 4). A rate constant for ionic Ba could not be calculated using our CF model. Statistical comparisons of differences in the mean CFs among the two treatments on day 14 were largely nonsignificant because of the small sample size and high within-treatment variability. The mean CF of the colloidally complexed form of Sn was significantly greater ( $P = 0.003$ ) than the CF in the ionic treatments.

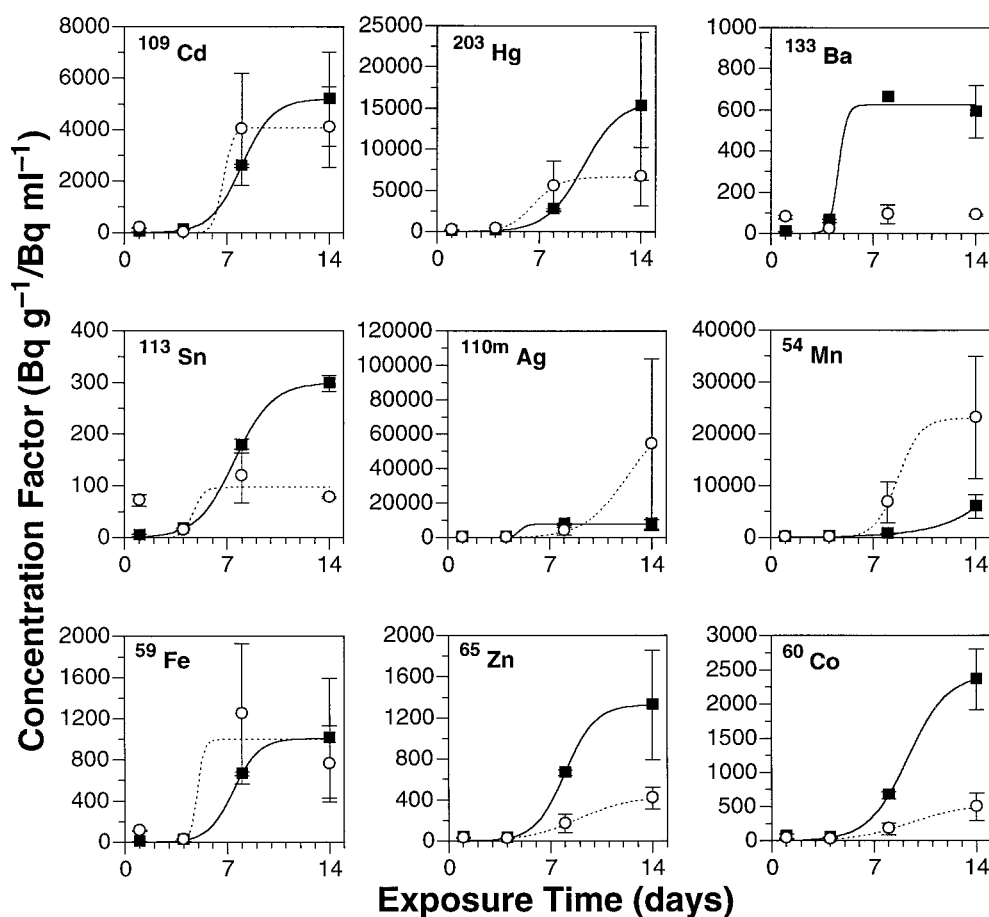


Fig. 3. Mean CF<sub>s</sub> ± 1 SE for whole shrimp exposed to trace metals in free-ionic (○, dashed line) and colloiddally complexed (■, solid line) forms. Models of the form  $CF = K_{sw} C_{\infty_w} (1 - e^{-(k_1+k_2)time}/C_w^0 - C_{\infty_w} (1 - e^{-(k_1+k_2)time})$  have been fitted to the mean CF data. The model could not be satisfactorily fitted to the free-ionic Ba data.

*Internal fate*—Colloidal complexation appeared to have a pronounced influence on the sites of metal accumulation. After the first day of uptake in both treatments, most radionuclides (Cd, Hg, Zn, Fe, Co, and Sn) were initially con-

Table 4. Values of rate constants ( $k_1 + k_2$ ) ( $d^{-1}$ ) and coefficients of determination ( $R^2$ ) estimated from least-squares fits of the model:

$$CF = \frac{K_{sw} C_{\infty_w} (1 - e^{-(k_1+k_2)time})}{C_w^0 - C_{\infty_w} (1 - e^{-(k_1+k_2)time})}$$

to the CF data ( $Bq\ g^{-1}/Bq\ ml^{-1}$ ) for whole shrimp.

Metal	Colloid		Free-ion	
	$k_1 + k_2$	$R^2$	$k_1 + k_2$	$R^2$
Cd	$9.44 \times 10^{-1}$	0.99	$2.97 \times 10^0$	0.99
Hg	$7.86 \times 10^{-1}$	0.99	$2.67 \times 10^0$	0.99
Ba	$3.48 \times 10^0$	0.99	—	—
Sn	$7.83 \times 10^{-1}$	0.99	$2.67 \times 10^0$	0.37
Ag	$4.10 \times 10^0$	0.99	$6.63 \times 10^{-1}$	0.99
Mn	$3.19 \times 10^{-1}$	0.99	$1.29 \times 10^0$	0.99
Fe	$1.13 \times 10^0$	0.99	$1.54 \times 10^0$	0.99
Zn	$9.61 \times 10^{-1}$	0.99	$5.45 \times 10^{-1}$	0.99
Co	$7.38 \times 10^{-1}$	0.99	$5.19 \times 10^{-1}$	0.99

centrated in the cephalothorax and abdomen, while Mn and Ag activities were approximately equally distributed in the three regions of the body (Fig. 4). From that time until the end of the uptake study, the metals that had been taken up in the colloiddally complexed form appeared to accumulate within the hepatopancreas, while activities in the abdomen and cephalothorax declined. This pattern was evident for Cd, Hg, Mn, Zn, Fe, Co, Ag, and Sn (Fig. 4). In contrast, metals from the free-ion treatments were generally accumulated in the abdomen, with the exception of Mn and Ag, which accumulated in the hepatopancreas (Fig. 4). The results for Ba were not plotted because of the high degree of loss to the chamber walls in the colloid treatment. The results for Ba from the colloiddally complexed treatment suggest accumulation in the cephalothorax, while the free-ion form accumulated in the abdomen.

### Discussion

Our results clearly show that colloiddally complexed trace metals can be bioaccumulated by shrimp at levels comparable to free-ionic forms. In typical estuarine waters, trace

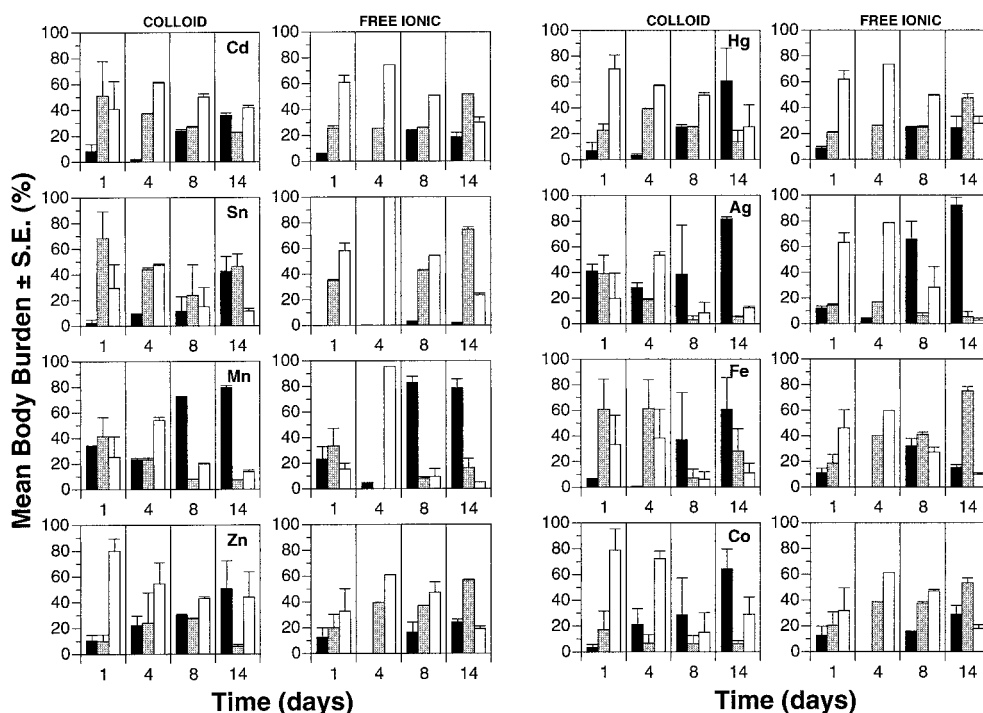


Fig. 4. Proportion of total body activity (mean  $\pm$  SE) within the hepatopancreas (■), abdomen (□), and cephalothorax (▨) during 14 d of uptake in *P. aztecus* exposed to colloiddally complexed and free-ionic metals.

metals can exist as ionic species as well as inorganic and organically complexed species. Studies by Wen et al. (1997b, 1999) in Galveston Bay show that a significant fraction of the naturally occurring metal pool is bound to colloiddally sized macromolecular organic matter (1 nm–0.4  $\mu$ m): 45  $\pm$  9% for Cd, 19  $\pm$  6% for Co, 91  $\pm$  5% for Zn, 79  $\pm$  11% for Fe, and 50  $\pm$  15% for Ag. Colloiddal metal concentrations correlated significantly with colloiddal organic carbon concentrations, suggesting that colloiddal metals resulted from metal–organic complexation.

The surface of the exoskeleton was undoubtedly covered by adsorbed metals, although our protocol included rinsing with clean seawater to reduce this superficial activity. Ionic species are probably adsorbed onto shrimp exoskeletons through chelation by functional groups on organic surfaces as seen with other divalent metals (Carvalho and Fowler 1993). One of our shrimp in the free-ion treatment molted shortly before the animal was to be sacrificed for sampling, and although this animal was not included in our analysis, it provided some data on the activities of the intact exoskeleton. Results indicated that it contained a substantial proportion (Ba 9%, Sn 14%, Cd 16%, Hg 18%, Ag 21%, Fe 48%, Mn 56%, Zn 70%, and Co 72%) of the total animal activity, although we do not know how much of this activity was due to surficial adsorption. Carvalho and Fowler (1993) found that after 21 d of uptake via solution, the exoskeleton of the caridean shrimp *Lysemata seticaudata* accounted for 27.5 and 36% of total activity of  $^{210}\text{Po}$  and  $^{210}\text{Pb}$ , respectively. Only three of our animals molted, and there was no evidence that any of the animals we sampled and used for final analysis had molted.

One uptake pathway into shrimp may have been via drinking of seawater, although we discount the importance of this route in our study. Carvalho and Fowler (1993) found that accumulation of  $^{210}\text{Pb}$  and  $^{210}\text{Po}$  by the *L. seticaudata* was linked to intake of water for osmotic regulation and that the hemolymph of penaeid shrimp was hypo-osmotic to seawater (35 psu). To compensate for water loss by osmosis, shrimp can drink seawater. This is followed by active excretion of salts and retention of water to balance levels of salt and water in tissues. At lower salinities, penaeid shrimp become iso-osmotic to the medium (McFarland and Lee 1963). The salinity we used for our bioaccumulation studies was typical of Gulf Coast estuaries. At this salinity (17 psu), the hemolymph of our shrimp should have been nearly iso-osmotic to the external medium. Further, typical drinking rates ( $\sim 1,700 \mu\text{l } 100 \text{ g}^{-1} \text{ h}^{-1}$ ) of a closely related species of penaeid shrimp *Penaeus duorarum* (Mantel and Farmer 1983) were not high enough to account for the high activities noted in our study. We estimate that our shrimp drank from 0.2 to 0.5 ml d $^{-1}$ , based on our range of shrimp masses and drinking rates cited by Mantel and Farmer (1983). Such rates are not high enough to account for the observed uptake rates. Assuming that our shrimp drank water at this rate and ignoring depuration and using the initial solution activities of each metal in our treatments, our most liberal estimate of uptake due to seawater drinking would account for <10% of the observed activity after 14 d of uptake.

Uptake of coagulated particles is another possible entry mechanism; however, active ingestion of even the largest coagulated particles would be unlikely since shrimp lack suitable appendages for manipulation of such small material.

Penaeid shrimp grasp food items with the chelae on their pereopods, which then pass it to their mouthparts for ingestion (Dall et al. 1990). The pereopod chelae are capable of grasping small particles such as sand grains (Hindley and Alexander 1978) and may be capable of handling detrital particles as small as 10–20  $\mu\text{m}$  (Moriarty et al. pers. comm. as cited in Hindley and Alexander 1978). Laboratory studies have demonstrated that shrimp can digest bacterial cells but can only manipulate the bacteria after they have been mixed with a binder and extruded into a large (several millimeters) pellet (Moriarty 1976). Condrey (1971) attempted to determine the nutritive value of benthic diatom cells to juvenile *P. aztecus* and *P. setiferus*. When he provided the juvenile shrimp with the opportunity to feed on single diatom cells, the individual cells were too small to be manipulated by the shrimp, and no ingestion occurred. The algal cultures had to be centrifuged and mixed with a binder to form 2–4-mm pellets before they could be successfully ingested by the shrimp. Given these results, it is clear that the maximum size that coagulated particles might reasonably be expected to attain (i.e., maximum of a few microns; Santschi et al. 1998) is still an order of magnitude below their lower size threshold for manipulation by juvenile penaeid shrimp.

The primary pathway for metals from solution into the internal tissues was likely across the gill epithelium during respiration. Crustacean gills act as a selective interface between the internal milieu and the external environment. In addition to their respiratory role, the gills are important for osmolyte, volume, and acid-base regulation. Absorption of the selected substances from the external environment and secretion of catabolic products occur through the gill filaments (Lucu 1990). In penaeid shrimp, the gill functions as the main location for gas transfer, nitrogenous waste excretion, acid-base regulation, and ion uptake to counter passive diffusive losses of ions. The gill epithelium is covered by negatively charged binding sites that contain phosphate, carboxyl, amino, and sulfate groups. Metals and other cations are attracted to these negative binding sites on the external surface of the gill epithelium (Janes and Playle 1995). Metals are then internalized via passive diffusion through hydrated pores and by carrier-mediated active transport mechanisms (Viarengo 1989). The presence of an impermeable exoskeleton reduces likelihood of metal uptake through other parts of the body surface except during molting.

It is possible that the gills were not the only uptake route. It is known that shrimp feed on their own fecal pellets while in captivity. We therefore cannot exclude fecal pellet ingestion as another route in our experiments, even though we were careful to remove fecal pellets daily from the water. Because we supervised shrimp for only about 12, rather than 24 h  $\text{d}^{-1}$ , such a mechanism would have been possible, though not very likely, because the shrimp were frequently fed and thus should not have been constantly hungry. If fecal pellet uptake were a significant uptake route, colloidal metals would have had to aggregate onto the suspended fecal pellet particles. In such a case, one would expect the free-ionic forms to adsorb to fecal pellets as well and thus, follow similar pathways in the shrimp. While we cannot unequivocally exclude ingestion as an uptake pathway, the metals appeared to follow different internal pathways in each treat-

ment, suggesting that some other factor is responsible for the observed patterns of accumulation.

Accumulation of radionuclides from the colloiddally complexed treatment raises questions about how the colloids influence the transfer of metals from solution to the shrimp. One hypothesis is that some portion of the complexed metals dissociated from the colloids in solution and subsequently entered the animals as free ions. Our experience from other experiments indicates that desorption rates from colloids are extremely low. This issue is discussed in more detail later in this section. The degree of dissociation should be related to the BE of the metal to the colloids. Where BEs were high, only a small portion of the bound metal should enter the ionic pool, and we would expect to see substantially lower activities within the shrimp in the colloid treatment. Where BEs were low, much of the bound metal might convert to the ionic form, leading to similar activities in both treatments. The metals with the highest BEs—Fe (82%), Ag (74%), Mn (25%), and Hg (22%)—displayed statistically comparable levels of uptake, with the exception of Sn (27%), while the three metals with low BEs—Ba (14%), Zn (10%), and Co (0.5%)—appeared to accumulate higher activities in the free-ionic treatments. A large proportion of the colloiddally complexed Ba was lost to container walls, which reduced the solution concentration and probably explains the low  $^{133}\text{Ba}$  tissue activities in that treatment. These results, combined with the intriguing evidence of different internal fates in the two treatments, do not support the dissociation hypothesis and suggest instead that the colloids were mediating uptake of the trace metals in some more complex manner.

Evidence suggests that metals cross cell membranes in a process that is essentially passive, although endocytosis may occur. It is thought that cell membranes possess aqueous channels that are lined with hydrophobic portions of protein and lipid molecules. The diameter of these channels could impede solute transport due to steric hindrances at the site of entrance. Simkiss (1983) noted that Type A metal cations permeate membranes by passing through polar pores in the lipid bilayer via passive diffusion or actively by conformational changes induced by energy supplied by metabolic activity. Type B metals such as Cd, Ag, and Hg can form complexes that cross membranes based on their lipid solubility. Campbell (1995) speculated that metal–ligand complexes might cross cell membranes accidentally in cases when membrane-bound permeases at the metal-binding site could not discriminate between the metal and the metal–ligand complex. Such a process might be possible for low-molecular-weight ligands such as amino acids; however, it is more difficult to conceptualize the much larger colloid–metal complexes in our study being carried by transport proteins or passing through aqueous membrane channels.

Organic ligands have an affinity for the gill epithelium, and studies with fish have demonstrated that dissolved organic carbon (DOC) will accumulate on the gill surface (Campbell 1995; Janes and Playle 1995; Hollis et al. 1996). Janes and Playle (1995) suggested that the DOC reduced the bioavailability of metals by lowering the free-ion concentration. The formation of ternary complexes L-M-X-cell (colloid-metal-metal sensitive membrane site) on the gill epithe-

lium discussed by Wilkinson et al. (1993) and Campbell (1995) may have occurred in our study. According to the free-ion activity model, bioaccumulation of metals will be a function of the free-metal ion concentration at the surface of the gill epithelium. In the colloid treatments, most metals were probably organically complexed, and the concentration of the colloid complex would determine bioavailability, provided that the metal dissociated from the colloid prior to passage across the membrane. Metal ion dissociation from organic complexes can be a slow process (Hering and Morel 1989), which is generally consistent with our observations of similar or reduced bioaccumulation of metals from the colloiddally complexed treatment, assuming that some process that enhances metal dissociation from the colloid is operating at the gill epithelium. Metals that could be easily dissociated from the colloids would be expected to enter the cells at rates equivalent to those in the free-ionic treatments, while metals that exhibited slower dissociation would be taken up at reduced rates. The BEs we observed after colloid complexation are probably not good predictors of the dissociation at the membrane surface, if this process is governed by biochemical factors at the cell surface. This leaves us with the problem of explaining why metals from the colloiddally complexed treatment were generally accumulated in the hepatopancreas, while the free-ionic metals tended to collect in the abdomen, if they both crossed the cell membrane as free ions.

Decapod crustaceans eliminate excess heavy metals via urine and feces, through molting (though the exoskeleton is frequently reingested), and through the gills (Langston and Spence 1995). Metals that cannot be rapidly eliminated are sequestered for subsequent elimination. Shrimp exposed to the colloiddally complexed metals demonstrated a highly consistent tendency to accumulate metals within the hepatopancreas. The crustacean hepatopancreas gland performs several functions: secreting digestive enzymes, serving as the main site of food adsorption, and storing metabolic reserves. This organ is also the site of vesicles containing metal-binding proteins (metallothioneins), which shrimp use to sequester excess heavy metals (Carvalho and Fowler 1993). We presently lack sufficient information to evaluate the mechanisms that underlie the hepatopancreatic accumulation of activity. Accumulation of radionuclides in the hepatopancreas may have been caused by an inability of shrimp in the colloid treatment to eliminate these metals as efficiently as the animals in the free-ionic treatment. Other interpretations are possible, and some direct physiological influence of the colloids on the shrimp cannot be excluded. As Campbell (1995) has pointed out, "natural DOM may act directly on aquatic organisms."

The success of the experiments hinged on how well the two treatments could maintain the colloid-bound and free-ionic states in the test solutions over time. We recognize that our colloid treatment contained some uncomplexed ionic metals. The dialysis procedure clearly indicated that diffusion of free ions out of the dialysis bags had slowed to a very low rate before we dispensed the contents of the bag into our colloid treatments. Subsequent dissociation of free-ionic metals was likely very low. Wen et al. (1997b) and Stordal et al. (1996b) documented that over a 5–10-d period,

colloiddally bound trace metals did not release or desorb any trace metals. On the contrary, a significant fraction coagulated into particles  $>0.45 \mu\text{m}$ . Similar evidence of very low dissociation rates for colloiddally complexed  $^{59}\text{Fe}$  with or without bacteria present was demonstrated by Barbeau et al. (1996). The free-ionic treatment undoubtedly gained organic ligands contributed by microbial action and shrimp excretory products, though we were careful that feeding and defecation occurred outside the experimental vessel. However, such a problem would have affected all published results where free metal ions were initially added and was thus unavoidable. Subsequent investigations would benefit from electrochemical measurements of the free-ion pool.

Our colloids were collected at 5 psu, while our experiments were run at 17 psu. This raises a potential concern that the salinity changes may have altered the stability of the colloids. We have not observed large changes in water-column trace metal partitioning between colloiddal and dissolved forms that could have been attributed to salinity changes (Wen et al. 1996, 1997b, 1999). Kilduff and Weber (1992) and Logan and Jiang (1990) demonstrated that ionic strength changes would mainly affect lower molecular weight forms with molecular weights in the neighborhood of the molecular weight cutoff of the cartridge, i.e., in our case, 1 kDa.

Our low level of replication was a consequence of logistical and space constraints. However, we are aware that it imposed limits on the power of our statistical tests to detect differences between our treatments. Both the activity and CF data indicated that for most metals, uptake had reached, or was approaching, equilibrium with depuration after 14 d of exposure. It should be noted that the small sample size and limited number of sampling intervals in this study also made it difficult to assess whether equilibrium had actually been attained. Substantial variability in uptake rates and levels of accumulation was probably a consequence of differences in the physiological state (e.g., molt stage) and health of individual animals. We recognize that a nondestructive sampling protocol such as gamma counting of live animals would allow kinetics to be followed in the same individuals over time. Unfortunately, the penaeid shrimp we used did not survive our attempts to develop such a protocol, and the genus *Penaeus* does not appear to be sufficiently hardy or behaviorally placid for live counting. A physiologically similar and comparably sized decapod, the daggerblade grass shrimp *Palaemonetes pugio*, has been the subject of new uptake and depuration experiments that were completed just before the submission of this manuscript. Using this species, we were able to maintain healthy shrimp for over 60 d while subjecting individual shrimp to 30 min of gamma counting every 3 d. The results demonstrate that, while there is substantial variability among replicate animals, individual uptake and depuration kinetics follow smooth, predictable models.

Decapod crustaceans exposed to colloiddally complexed heavy metals at environmentally representative concentrations are capable of accumulating radionuclides to levels comparable to those observed in solutions where the bulk of the metal pool was ionic. The uptake and depuration rates and pathways appeared to be different for our two treat-

ments. Consequently, we conclude that shrimp were able to discriminate between colloidal and ionic species; however, we are less certain about the exact uptake route of colloiddally bound metals. Experiments that do not allow any fecal pellets or other sources of ingestible material in the water are needed to more rigorously evaluate uptake pathways. (Note added in proof: Recent short-term experiments with daggerblade grass shrimp (*Palaemonetes pugio*) that were actively prevented from feeding on their fecal pellets demonstrated conclusively that both colloiddally-bound metals and ionic forms are taken up.)

Penaeid shrimp and other estuarine-dependent organisms are exposed to heavy metals in a milieu that includes colloidal organic material, and much of the metal pool in such systems is complexed with organic ligands. Studies of this type provide a basis for determining how organisms respond to metal-colloid complexes and how bioavailability models such as the free-ion activity model may be modified to account for complexed metals.

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