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Pathways of CH_3Hg and Hg Ingestion in Benthic Organisms: An Enriched Isotope Approach

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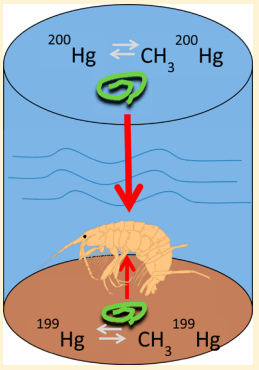
Supporting Information

ABSTRACT: Mercury is a widespread contaminant in marine food webs, and identifying uptake pathways of mercury species, CH_3Hg^+ and Hg^{2+}, into low trophic level organisms is important to understanding its entry into marine food webs. Enriched stable isotope tracers were used to study benthic vs. pelagic pathways of CH_3Hg^+ and Hg^{2+} uptake via food to the infaunal estuarine amphipod, *Leptocheirus plumulosus*. Algal cells differentially labeled with isotopically enriched CH_3Hg^+ or Hg^{2+} were added simultaneously to the sediment and water column of microcosms, and Hg species were monitored in amphipods and in sediment and water compartments. Methylation of Hg^{2+} occurred during the course of the experiment, enhancing the uptake of Hg^{2+} spikes. Trophic transfer of Hg from algae added to the water column was determined to be the major uptake route for amphipods, suggesting inputs of contaminated organic matter from the pelagic zone are important to mercury bioaccumulation even in organisms living in sediments.



INTRODUCTION

Methylmercury is efficiently accumulated through estuarine food webs, such that concentrations in fish are controlled by initial CH_3Hg^+ assimilation at the base of the food web.[1] Sources and pathways of CH_3Hg^+ and Hg^{2+} accumulation in lower trophic levels are therefore fundamental to understanding Hg cycling in estuaries and controlling Hg levels in top predator fish. Estuarine sediments are a sink for Hg^2 and also a dominant site for methylation of Hg^{2+} to the more bioavailable species, CH_3Hg^+.[3,4] Benthic organisms, which dwell at the sediment−water interface, are exposed to Hg from both sediments and the water column[2,5,6] and are also a food source to larger invertebrates and fish.[7] As such, they present an important link between Hg bioaccumulation in benthic and pelagic food webs, yet dominant sources and pathways of Hg uptake in benthic fauna are not well understood.[1,8,9] Benthic species are exposed to dissolved metals from porewater and overlying water[8−10] and also take up metals from dietary sources,[11,12] which can originate in both the sediment and water column. Identifying the uptake and trophic transfer between sediment and water food sources and primary consumers is important to linking water and sediment quality data to bioaccumulation.

An important benthic species along the east coast of North America is the estuarine amphipod, *Leptocheirus plumulosus*, which is abundant over a wide range of sediment types and salinity and temperature conditions.[13] *L. plumulosus* is also used as a test organism to assess both acute[14,15] and chronic[16,17] toxicity of estuarine sediments. *L. plumulosus* are facultative feeders; they live in U-shaped burrows through which they filter water and ingest suspended particles and also roam the surface of the sediment and deposit feed,[13] thus deriving their nutrition and exposure to contaminants from both the water column and sediments. Microcosm and modeling studies have shown dietary ingestion of organic matter, rather than uptake from the dissolved phase, to be the dominant process for CH_3Hg^+ and Hg^{2+} accumulation in *L. plumulosus*[6,18] Over a range of assimilation efficiencies (AE) and ingestion rates (IR), the relative contribution of food sources to body burden was modeled to be >80% for CH_3Hg^+ and 10−60% for Hg^{2+}, although accumulation was determined in water with no appreciable dissolved organic carbon (DOC),[18] and uptake of both species from natural water is inversely correlated to DOC concentrations.[6] Ingestion pathways are ignored in toxicity testing, which assumes metal exposure is predominantly from sediment porewater.[11,18] Biokinetic models of metal accumulation currently lack estimates of uptake from deposit vs suspension feeding.[18] Feeding behavior in *L. plumulosus* is a critical piece of information to understanding Hg accumulation and trophic transfer.

In this study, we used enriched isotopic CH_3Hg^+ and Hg^{2+} tracers in microcosm experiments to directly determine Hg uptake pathways in *L. plumulosus*. Two experiments were conducted to study feeding pathways of CH_3Hg^+ (experiment 1) and Hg^{2+} (experiment 2). This approach had several advantages: (1) Using multiple enriched isotopes of Hg allowed us to simultaneously decipher uptake from different pathways. (2) We could expose amphipods to CH_3Hg^+ and Hg^{2+} at environmentally relevant levels.[19] (3) Unlike radiometric tracer

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studies, this technique enabled Hg species transformation to be traced[20] throughout the experiment. Because AE of CH_3Hg^+ is much higher than that of Hg^{2+},[18,21] methylation of Hg^{2+} will increase availability to the biota.[22] Methylation occurs in anoxic zones in sediment[3] and has also been observed in algal cultures.[23−26] If Hg speciation is not monitored during incubation in uptake experiments, bioaccumulation of Hg species may be misinterpreted. Enriched stable isotopes of Hg species have been used as a powerful tool to study Hg cycling and uptake into freshwater foodwebs.[27−32] In this study, we applied the technique to identify trophic transfer of Hg species from live phytoplankton in water and sediment compartments in estuarine benthic infauna.

METHODS

Enriched Isotopic Tracers. All solutions were made up using ultrapure water (>18 MΩ cm^{-1}) produced by a PurelabPluswater purifier (US Filter, MA,USA). Enriched stable isotopes of Hg (as ^{199}HgO, ^{200}HgO, and ^{201}HgO) were purchased from Cambridge Isotope Laboratories (Andover, MA). Single isotope spikes of $^{199}Hg^{2+}$ and $^{200}Hg^{2+}$ were prepared by dissolution of HgO in HCl, followed by dilution to 400 mg/L in 5% HCl (Fisher Optima grade, Pittsburgh, PA, USA). Solutions of $CH_3{}^{200}HgCl$ and $CH_3{}^{201}HgCl$ were synthesized by conversion of ^{201}HgO to $^{201}HgCl_2$ followed by reaction with methylcobalamin.[33] Stock solutions were diluted to 50 mg/L of $CH_3{}^{200}Hg^+$ and 5 mg/L of $CH_3{}^{201}Hg^+$ in 0.5% acetic acid and 0.2% HCl (Fisher Optima). Isotopic abundances of natural Hg and the enriched isotope spikes are given in the Supporting Information.

***Isochrysis galbana* and *Leptocheirus plumulosus* Cultures.** The marine phytoplankton species, *I. galbana*, was used as the food source for the CH_3Hg^+ and Hg^{2+} exposures of *L. plumulosus*. Cells of *I. galbana* were grown in a 19 L carboy of 20 ppt artificial seawater (Instant Ocean prepared in ultrapure water) to a density of 1.3×10^6 cells per milliliter and then reduced by centrifugation to 800 mL. The concentrated culture was then divided into four 200 mL solutions, and each algal solution was spiked with one isotopically enriched Hg species. In experiment 1 (CH_3Hg^+ uptake), the suspensions were tagged with 2.5 μg $CH_3{}^{200}Hg^+$ or $CH_3{}^{201}Hg^+$ and in experiment 2 (Hg^{2+} uptake), with 34 μg of $^{199}Hg^{2+}$ or $^{200}Hg^{2+}$. The isotopically labeled cultures were allowed to equilibrate for 24 h and then spun down into pellets and the supernatant discarded. Algal cells were resuspended in 20 mL of artificial seawater. Aliquots (1 mL) of each cell suspension were removed and then centrifuged, and the resulting algal pellets were freeze-dried and weighed. The pellet and supernatant solutions (both $n = 4$) were analyzed for CH_3Hg^+ and THg, and the fraction of algal-bound Hg was determined (94 ± 1% for $CH_3{}^{200}Hg^+$, and 95 ± 2% for $CH_3{}^{201}Hg^+$; 98.1 ± 0.1% for $^{199}Hg^{2+}$ and $^{200}Hg^{2+}$). Concentrations of Hg spikes in the algal slurries are included in Tables 1 and 2.

Leptocheirus plumulosus was purchased from Aquatic Research Organisms (Hampton, NH) and cultured according to EPA method 1994.[14] Similarly sized (>1 mm) amphipods were retrieved from culturing tanks prior to the experiment and depurated for 4 h in salt water.

Microcosms. Microcosm experiments were conducted in 100 mL (5 cm diameter, 10 cm height) glass jars (Qorpak, Bridgeville, PA, USA). Sediment was collected from Webhannet Estuary in Wells, Maine. Sediment was sieved to <250 μm and homogenized. The organic carbon content was determined to be 3.2 ± 0.1% by LOI (dry sediment was heated at 550 °C for 4 h). For each experiment, 16 identical microcosms were assembled. In each microcosm, 1 mL of algal suspension containing enriched isotope CH_3Hg^+ (experiment 1) or Hg^{2+} (experiment 2) was mixed with ∼16 g of wet sediment, and the other enriched isotope-tagged algae was mixed with ∼60 mL of 20 ppt artificial seawater (Table 2; henceforth isotopically enriched Hg species are denoted by a subscript (Hg_{sed} or Hg_{water}) indicating the compartment to which the spiked algae was added). The sediment was added to a jar (∼1 cm depth), along with 5 amphipods, and overlying water was added. Microcosms were kept in an environmental chamber (20 °C, 10:14 (L/D) h photoperiod), covered loosely with plastic to minimize evaporation, and slowly agitated on an orbital shaker (100 rpm) to maintain aeration and suspension of algae. A water sample was taken from four microcosms immediately after assembly ($t = 0$); then four microcosms were dismantled for analysis after 12, 24, 36, and 48 h. This two-day time series was chosen to monitor relative accumulation of Hg from different sources in amphipods over time, and to minimize the effects of algal death and decay on bioavailability of CH_3Hg^+ and Hg^{2+} [34] associated with longer incubation periods. Changes in Hg concentration and speciation were monitored in whole and filtered water and sediment throughout the time series. A second set of microcosms was assembled under the same conditions but using algae suspensions not spiked with Hg species, to monitor dissolved oxygen (DO), pH, and oxidation reduction potential (Eh) throughout the experiment. Results are reported in the Supporting Information (SI).

To dismantle microcosms after each time point, overlying water was removed by pipet with minimal disturbance to the sediment. A 15 mL aliquot was filtered through a 0.45 μm poresize 25 mm syringe tip filter (Fisherbrand), and a second 15 mL sample was collected unfiltered. Both filtered and whole water samples were acidified to 0.5% HCl (Fisher Optima). Amphipods from each microcosm were removed from the sediment by Pasteur pipet and thoroughly rinsed in two sequential water baths and placed in a preweighed 15 mL glass vial (I-Chem). Sediments were removed from the microcosm jars and placed in 60 mL PFA vials (Sarstedt, Nümbrecht, Germany). Amphipod and sediment samples were freeze-dried; all concentrations are reported on a dry weight basis. All sample

Table 1. Concentrations (mean ± SD) of Isotopically Enriched CH_3Hg^+ in Algae, Sediment, and Water ($n = 4$/time point) in Experiment 1

compartment analyzed	time points	$CH_3{}^{201}Hg_{sed}{}^+$	$CH_3{}^{200}Hg_{water}{}^+$
concentration of Hg spike in algae (μg/L)	N/A	28.8 ± 2.5	42.0 ± 4.0
% Hg bound to algae	N/A	95.0 ± 1.7	93.6 ± 0.7
sediment CH_3Hg (ng/g)	12−48 h	1.3 ± 0.6	1.5 ± 0.8
whole water CH_3Hg (ng/L)	0 h	2.5 ± 1.0	562 ± 112
	12 h	1.1 ± 0.2	16.5 ± 1.9
	24 h	0.3 ± 0.1	2.7 ± 0.2
	36 h	0.5 ± 0.2	3.1 ± 1.1
	48 h	0.4 ± 0.1	2.7 ± 0.9
filtered water CH_3Hg (ng/L)	0 h	<MDL	39.7 ± 9.7
	12 h	0.3 ± 0.1	2.8 ± 1.9
	24 h	0.5 ± 0.2	3.0 ± 0.8
	36 h	0.3 ± 0.1	1.4 ± 0.4
	48 h	0.1 ± 0.02	0.8 ± 0.2

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Table 2. Concentrations (Mean ± SD) and % CH_3Hg^+ of Isotopically Enriched Hg^{2+} in Algae, Sediment, and Water (n = 4/time point) for Experiment 2

compartment analyzed	time points	$CH_3{}^{199}Hg_{sed}$ (%)	$^{199}Hg_{sed}{}^{2+}$	$CH_3{}^{200}Hg_{water}$ (%)	$^{200}Hg_{water}{}^{2+}$
Hg spike in algae (μg/L)	N/A	0.8 ± 0.1	954 ± 79	0.7 ± 0.1	834 ± 16
% Hg bound to algae	N/A		98.4 ± 0.3		99.0 ± 0.1
sediment (ng/g)	12−48 h	3.1 ± 1.8	97 ± 8	1.9 ± 1.2	71 ± 10
whole water (ng/L)	0 h	<MDL	21.8 ± 23.9	0.3 ± 0.1	2679 ± 2486
	12 h	4.2 ± 1.1	4.0 ± 1.3	1.0 ± 0.4	53.4 ± 22.0
	24 h	4.2 ± 4.6	5.5 ± 2.0	1.0 ± 0.7	27.5 ± 3.4
	36 h	6.6 ± 4.2	6.4 ± 3.1	2.6 ± 1.3	27.7 ± 13.6
	48 h	2.7 ± 1.6	21.7 ± 9.4	1.4 ± 0.9	69.4 ± 29.8
filtered water (ng/L)	0 h	<MDL	7.5 ± 10.5	4.5 ± 2.3	468 ± 648
	12 h	<MDL	2.2 ± 0.1	1.7 ± 0.1	19.3 ± 9.7
	24 h	3.5 ± 1.0	4.8 ± 2.2	2.2 ± 0.5	15.8 ± 2.3
	36 h	4.5 ± 1.1	3.6 ± 0.8	3.1 ± 0.7	12.3 ± 2.5
	48 h	3.0 ± 1.7	6.2 ± 0.8	4.5 ± 1.7	16.7 ± 3.2

containers were double bagged and stored refrigerated in the dark until analysis.

SAMPLE PREPARATION AND ANALYSES

CH_3Hg^+ Determination. Freeze-dried amphipod samples were weighed and extracted in glass vials. To each vial, 1.5 mL 4 M HNO_3 was weighed, and samples were heated overnight at 60 °C.[35] A 50 μL aliquot of each extract was transferred to a 40 mL amber glass vial with Teflon-lined septa (Brooks Rand Laboratories, Seattle, USA).[36] Samples (8 mL) of filtered and whole overlying water were weighed into 40 mL vials. Portions (0.5 g) of freeze-dried, homogenized sediment samples were leached with $KBr/H_2SO_4/CuSO_4$, extracted into CH_2Cl_2, then back extraction into 10 mL water;[37] then 2 mL of the extract was weighed into 40 mL glass vials.

Samples in amber glass vials were diluted to 40 mL with ultrapure water. Samples were buffered and derivatized, then analyzed using a MERX-M Methylmercury Analysis System (Brooks Rand Laboratories, Seattle, USA) coupled with a 7700x Agilent ICP-MS (Agilent Technologies, Santa Clara, USA); the details of this technique are given in Taylor et al.[36] Isotope deconvolution was used to separate Hg species derived from three sources: the ambient Hg in the sample (determined from the ^{202}Hg signal) and Hg inputs from two enriched Hg-tagged algal spikes added to the water and sediment compartments.[38] Details are given in the SI. External calibration was performed using six standards (0.025 to 25 ng/L), and a secondary source calibration check was analyzed every 10 samples. Method detection limits (MDL) were 0.05 ng/g (sediment), 5 ng/g (amphipods), and 0.13 ng/L water. Recoveries for CH_3Hg were 85 ± 13% (n = 2) in BCR-580 estuarine sediment (IRMM, Geel, Belgium) and 106 ± 10% (n = 3) in NIST 2976 mussel; spike recoveries in water were 99 ± 5% (n = 5).

Total Hg (THg) Determination. Quantification of total Hg from biological tissues was determined from 4 M HNO_3 extracts.[35] Aliquots (0.5 mL) of amphipod extracts were diluted 10 times by weight and analyzed directly by ICP-MS (Agilent 7700x), with a MDL of 20 ng/g. Portions of sediment samples (0.25 g) were weighed into 60 mL PFA tubes (Sarstedt) and 5 mL of 45% HNO_3: 5% HCl v/v was added to each tube. Samples were digested in an open vessel microwave (Mars Xpress, CEM Corps, Matthews, NC, USA) at 95 °C for 1 h. Digested samples were diluted to 50 mL with ultrapure (18 MΩ) water, and weighed, then analyzed by ICP-MS, with a MDL of 0.1 ng/g. Signal intensities were deconvoluted (SI) prior to conversion to concentration by external calibration. Recovery of standard reference materials NIST 2711a (n = 2) and NIST 2976 (n = 3) were 80 ± 1% and 115% ± 11%, respectively.

Filtered and whole overlying water and supernatant samples from algal suspensions were analyzed by cold vapor-ICP-MS, using an automated MERX−T purge and trap system (Brooks Rand) coupled with ICP-MS based on EPA Method 1631.[39] Samples (8 mL overlying water or 0.4 mL supernatant) were weighed into 40 mL clear glass vials with Teflon-lined septa (Brooks Rand) and diluted to 25 mL with 1% HCl. Samples were digested overnight in capped vials, by the addition of 0.1 mL freshly prepared BrCl (0.16 g KBr was dissolved in 15 mL HCl, then 0.38 g $KBrO_3$ added and stirred for 1 h). To each sample, 0.1 mL $SnCl_2$ and 0.1 mL hydroxylamine (Brooks Rand) were added, then vials were recapped for analysis. The MDL was 1.25 ng/L, and spike recoveries were 98−111% (n = 8).

Calculation of Bioaccumulation Factors (BAFs). Bioaccumulation factors (BAF) were calculated for each uptake pathway to normalize body burden to different exposure concentrations. Body burdens of isotopically enriched Hg from 36 and 48 h time points were divided by sediment concentrations in each microcosm; e.g., in experiment 1, BAF for $CH_3{}^{201}Hg_{sed}{}^+$ was calculated as the concentration of $CH_3{}^{201}Hg_{sed}{}^+$ (ng/g) in amphipods relative to its concentration in sediment.

Calculation of Ingestion Rates from Biokinetic Modeling. Metal accumulation from ingestion has been described by the model in eq 1[40,41]

$$C_{ss} = \frac{(AE \times IR \times C_f)}{(K_{ef} + g)} \quad (1)$$

where the body burden of a metal species taken up from food, C_{ss}, is calculated from the metal concentration in food (C_f), the assimilation efficiency of the metal species (AE, unitless), the ingestion rate, IR (mg food/g body wt/d), the metal efflux rate constant following uptake from food, K_{ef} (1/d), and the growth rate constant, g (1/d). The equation was rearranged to calculate IR. Reported values of AE (CH_3Hg^+ = 80%, Hg^{2+} = 6%), K_{ef} (CH_3Hg^+ = 0.052 d^{-1}, Hg^{2+}= 0.089 d^{-1}),[18] and g (0.07 d^{-1})[17,18] for *L. plumulosus* were applied, and concentrations of isotopic tracers in algae C_f and amphipods C_{ss} were used to distinguish ingestion from benthic and pelagic sources.

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Statistical Analyses. Statistical analyses were performed with the Stata 12.0 Statistical/Data Analysis package. Nonparametric analyses were chosen because distribution could not be ascertained due to small sample sizes. Significant trends in spike concentrations in sediment, water, and amphipods with time were assessed by Kruskal–Wallis rank tests; specific differences in concentration between time points were determined using the Mann–Whitney rank sum statistic. A statistical significance criterion was established as $p = 0.05$.

RESULTS

Experiment 1—CH_3Hg^+ Uptake. Using the two enriched isotopes, CH_3Hg^+ concentrations from spiked algae added simultaneously to the water column and sediment were tracked in the sediment, water column, and in amphipods over time (Table 1). For sediment, differences in concentrations of both enriched isotope Hg species were not significant between time points (Kruskal–Wallis: $d.f. = 3$, $p > 0.05$), so data for all time points were pooled for each experiment. Only 0.2% of the recovered $CH_3{}^{200}Hg_{water}{}^+$ was suspended in the water column after 12 h, whereas concentrations of $CH_3{}^{200}Hg_{water}{}^+$ and $CH_3{}^{201}Hg_{sed}{}^+$ in the homogenized sediment samples were similar.

Variation in CH_3Hg^+ tracer levels occurred in the water column between time points (Table 1). Levels of $CH_3{}^{200}Hg_{water}{}^+$ in the initial whole water sample ($t = 0$) were elevated and variable between microcosms. Concentrations of $CH_3{}^{200}Hg_{water}{}^+$ decreased from 0 to 24 h and then reached a steady state between 24 and 48 h (Mann–Whitney U, $p > 0.05$). In the filtered water fraction, $CH_3{}^{200}Hg_{water}{}^+$ was also elevated in the initial time point. Low levels of the $CH_3{}^{201}Hg_{sed}{}^+$ tracer were also present in the water compartment.

Enriched isotope CH_3Hg^+ tracers were present in amphipods after 12 h (Figure 1), and concentrations of both tracers in amphipods did not vary significantly across time series (Kruskal–Wallis, $d.f. = 3$, $p > 0.05$), although variance between replicates at each time point was large (16–46%). Uptake of $CH_3{}^{200}Hg_{water}{}^+$ was ~20 times higher in amphipods than $CH_3{}^{201}Hg_{sed}{}^+$.

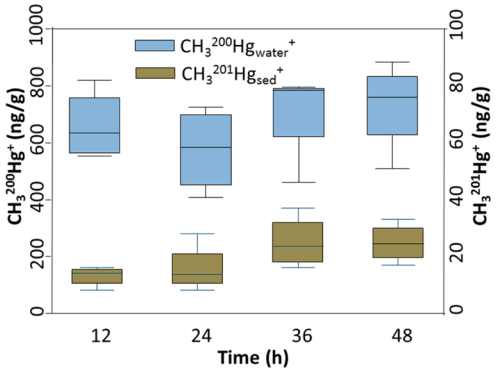

Figure 1. Body burdens of isotopically enriched CH_3Hg^+ tracers in *L. plumulosus* over time (experiment 1).

Experiment 2—Hg^{2+} Uptake. The two enriched isotopes of Hg^{2+} were tracked over time in microcosms (Table 2). As with experiment 1, both tracers were present in the bulk sediment, including 99.9% ^{200}Hg-tagged algae added to the water column. Concentrations of Hg^{2+} tracers in sediment did not vary with time (Kruskal–Wallis $d.f. = 3$, $p > 0.05$), whereas tracer concentrations in the whole water fraction were variable. Whole water samples were elevated in $^{200}Hg_{water}{}^{2+}$ at $t = 0$ (Mann–Whitney U, $z = 2.3$, $p = 0.02$) immediately following the addition of spiked algae to the surface water but reached an apparent steady state from 24 to 48 h ($p > 0.05$). Concentrations of $^{200}Hg_{water}{}^{2+}$ in the filtered water were also highly variable at $t = 0$ but did not change significantly from 0 to 48 h (Kruskal–Wallis, $d.f. = 3$, $p > 0.05$). Low levels of $^{199}Hg_{sed}{}^{2+}$ were present in whole and filtered water samples.

Methylation of the Hg^{2+} spikes was evident during incubation of the *I. galbana* suspensions (Table 2; 0.7–0.8% CH_3Hg^+). The $\%CH_3Hg^+$ of tracers in sediment increased slightly relative to the starting algal suspension (Mann–Whitney U, $\%CH_3{}^{199}Hg_{sed}{}^+$: $z = -2.5$, $p = 0.01$; $\%CH_3{}^{200}Hg_{water}{}^+$: $z = -2.0$, $p = 0.04$). The $\%CH_3Hg$ in the $^{199}Hg_{sed}{}^{2+}$ tracer, mixed with the sediment, was slightly higher than in the $^{200}Hg_{water}$ tracer initially added to the water column (Mann–Whitney U, $z = -2.2$ $p = 0.03$), although variability in $\%CH_3Hg^+$ in tracers among microcosms was high (RSD = 58–63%). In the whole and filtered water samples, $\%CH_3{}^{199}Hg_{sed}{}^+$ was higher than in the algal suspensions (Table 2) prior to addition to the sediment (Mann–Whitney U, $z = -2.5$, $p = 0.01$). In the overlying water, $\%CH_3{}^{199}Hg_{sed}{}^+$ was not significantly different than in the sediment ($p > 0.05$). There was no difference between $\%CH_3{}^{200}Hg_{water}{}^+$ in the water column and in the algal suspensions or sediments ($p > 0.05$). Neutral pH (8) and oxic conditions (DO > 6 mg/L) in the sediment and water column were maintained over the time course under these experimental conditions (SI).

Body burdens of isotopic tracers in amphipods, as THg, CH_3Hg^+, and $\%CH_3Hg$ are shown throughout the time series in Figure 2. Ambient THg in amphipods (from ^{202}Hg) was 15 ng/g with 39 ± 12% CH_3Hg (all time points; $n = 16$). At $t = 12$ h, body burdens of $^{200}Hg_{water}$ were higher than in later time

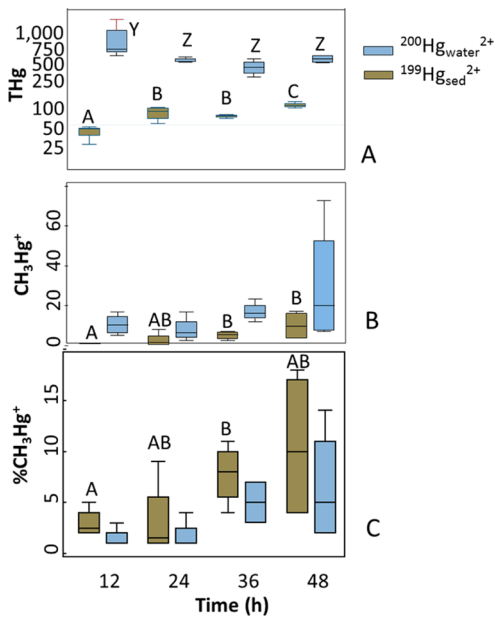

Figure 2. (A) Body burdens of isotopically enriched Hg^{2+} tracers, as THg concentrations. (B) CH_3Hg^+ concentrations and $\%CH_3Hg^+$ in *L. plumulosus* over time (experiment 2). Letters (A, B, C for $^{199}Hg_{sed}{}^{2+}$; or Y, Z for $^{200}Hg_{water}{}^{2+}$) signify statistical differences between time points by Mann–Whitney U rank sum ($p < 0.05$).

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points, even when one high value (1812 ng/g) was removed (Mann−Whitney U, $z = 2.3$, $p = 0.02$). Conversely, increases in $^{199}Hg_{sed}$ body burden were significant with time (Figure 2a).

Amphipods accumulated methylated forms of both tracers, comprising up to 18% of THg (Figure 2b and c). Uptake of $CH_3{}^{200}Hg_{water}{}^{+}$ was evident, but there was no relationship between accumulation and time (Kruskal−Wallis, *d.f.* = 3, $p > 0.05$), whereas $CH_3{}^{199}Hg_{sed}{}^{+}$ body burden was significantly related with time. The trend was also significant when normalized to total $^{199}Hg_{sed}$ (as $\%CH_3{}^{199}Hg_{sed}$).

Bioaccumulation Factors. Because 99.8% $CH_3{}^{200}Hg_{water}{}^{+}$ and 99.9% $^{200}Hg_{water}{}^{2+}$ added to the water column were recovered in the bulk sediment, BAFs for both enriched isotopes were calculated as body burdens normalized to sediment concentrations. The BAF for $CH_3{}^{200}Hg_{water}{}^{+}$ was 682 ± 442 (unitless), whereas for $CH_3{}^{201}Hg_{sed}{}^{+}$, the BAF was 30 ± 19, such that uptake of CH_3Hg^+ from organic material of pelagic origin was ∼23× higher than from algae mixed with the sediment. For experiment 2, BAFs for Hg^{2+} were 0.9 ± 0.2 for $^{199}Hg_{sed}{}^{2+}$ and 5.8 ± 2.0 for $^{200}Hg_{water}{}^{2+}$, and bioaccumulation from settling algae was only ∼6× higher than for sediment sources.

Ingestion Rates. For the CH_3Hg^+ experiment, IR was determined to be 65 mg/g/day from ingestion of algae added to the water column and 3.1 mg/g/day for ingestion of algae mixed in with the sediment. For the Hg^{2+} experiment, IR was 30 mg/g/day for algae originating in the water column and 5.2 mg/g/day for sediment feeding. The IR is a measure of the milligrams of food consumed (per body wt/day) and should not vary with metal species, although its calculation does depend on the accuracy of the applied constants (AE, K_{ef}, g) as well as the experimentally derived food concentration and body burden.

DISCUSSION

Microcosm studies using enriched stable isotopes showed trophic transfer from sedimenting algae was a major source of Hg to benthic-dwelling amphipods. This suggests that newly deposited phytoplankton is an important and potentially overlooked source of Hg to benthic infauna in ecosystem studies and toxicity testing.

Use of Enriched Stable Isotope Tracers in Microcosm Experiments. Enriched stable isotopes are a powerful tool for studying accumulation of Hg in aquatic food webs;[27−31] we applied this technique to a microcosm study to compare trophic transfer of Hg by two ingestion pathways. Microcosms were scaled down from recommended conditions for culturing *L. plumulosus*, to minimize the quantity of enriched isotopes needed, but maintaining recommended amphipod densities (<1.5 cm^{-2}), sediment depth (1 cm), and water conditions (20 ppt, 20 °C, DO >4.4 mg/L).[13]

Concentrations of CH_3Hg^+ and THg in sediments and whole surface waters in microcosms were within ranges found in New England[42] and estuaries worldwide,[7,43] although dissolved water concentrations were slightly higher. Body burdens of Hg from tracers were up to 6× higher than those found in New England estuaries (Chen, unpublished: 17−105 ng/g).

In estuaries, amphipods are found over a wide range of sediment characteristics and temperatures.[13,17] Sediment characteristics and food quality have been shown to affect AE and K_{ef} in amphipods,[11,44] as well as survival and reproduction,[17] but specific effects on amphipod feeding behavior have not been reported. Because amphipods feed selectively, behavior may change with habitat and food availability. Accumulation of Hg species from sediment mixed with algae was shown to be higher than from sediment alone,[6] suggesting the abundance of high quality food spiked with Hg in this experiment may lead to higher accumulation in amphipods than in a natural environment. Homogenized sediment and standardized conditions used in this experiment allow for direct comparison of metal accumulation from different feeding modes under optimal conditions.

Algae added to the water column was rapidly deposited to the sediment surface, such that >99.8% of $CH_3{}^{200}Hg^+$ and $^{200}Hg^{2+}$ were recovered from the bulk sediment within 12 h. Concentrations of Hg species added to the water column varied over time, due to settling of algae added to the water, and diffusion/suspension of tracers added to the sediment. Variability between samples was high at initial time points due to the capture of large suspended particles during sampling. While sediment concentrations were similar for both tracers added to the microcosm, spiked algae originating in the water column was deposited to the sediment surface, whereas the spikes originating in the sediment were thoroughly mixed in the bulk sediment.

Tracking speciation throughout the experiment proved important as methylation of Hg^{2+} spikes was observed. In radiometric methods, Hg species cannot be distinguished, and species transformation alters the bioavailability of Hg,[22] which could lead to inaccuracies in uptake modeling. Methylation of tracers was observed in spiked algal cultures (0.7−0.8%) and has previously been observed in cultures of periphyton.[23−26] Further methylation was also evident during sediment incubation (2−3% CH_3Hg^+), despite maintaining oxic conditions throughout the experiment. This may also occur during toxicity testing, where sediment incubation conditions are similar to those used here, altering the bioavailability of Hg to test organisms relative to natural environments.

Because the experiment focused on uptake, a short exposure duration was used to minimize artifacts from algal decay, water quality changes, and amphipod death/reproduction.[6] The body burden of CH_3Hg^+ reaches an apparent steady state after 12 h in this experiment, although variability at each time point was high, possibly due to feeding behavior, and to variable metal distribution in the algal food source. Lawrence and Mason reported an apparent steady state in amphipods sampled at three and six days, but calculated much longer exposures (∼50 day) are needed to achieve equilibrium.[6] The apparent steady state in body burden may be due to the long time intervals (12 h) used in this study, relative to reported gut passage times of 35−228 min for *L. plumulosus* fed *I. galbana*.[45] Longer exposure to Hg sources may lead to higher accumulation if a true steady state was not reached in this experiment, but body burdens represent relative accumulations from different feeding pathways. Because tracers were ingested simultaneously and prepared from the same algal source, AE and k_{ef} will be the same for both ingestion pathways.[11,44,45] Amphipods in this study were not depurated, although for CH_3Hg^+, this is likely to have little effect on the determined body burden due to its high AE[18] where rate of loss from excretion is negligible relative to growth dilution.[6]

Variability in body burden of Hg^{2+} tracers was high, likely due to spatial variability of tracers in both food sources. The body burden of $^{199}Hg_{sed}{}^{2+}$ increased significantly between time points, but $^{200}Hg_{water}{}^{2+}$ did not. Reasons for this apparent

difference in uptake rate from different pathways are unclear. The pattern may be explained by preferential uptake of $^{200}Hg_{water}^{2+}$ from settling algae during the first time interval (t = 0 to 12 h), then increased consumption of $^{199}Hg_{sed}^{2+}$ as amphipods stir up sediment while they burrow. An increase in $CH_3{}^{199}Hg_{sed}^{+}$ between time points was also evident. This may be explained by the higher AE of CH_3Hg^+ (80%) than Hg^{2+} (6%),[18] causing Hg^{2+} to be excreted while CH_3Hg^+ is mostly retained. This difference in assimilation should be consistent for both isotopic tracers, however, but was not significant for $CH_3{}^{200}Hg_{water}^{+}$.

Bioaccumulation Factors and Ingestion Rates. Uptake of Hg tracers initially added to the water column and deposited on the sediment surface was significantly higher than for tracers mixed in the sediment, suggesting ingestion from the sediment surface is the major feeding mode in amphipods. While BAFs are useful to normalize body burden to different exposure concentrations, they can be difficult to evaluate between different food sources and habitats. In this study, because the same algal food source was added to both compartments, BAFs were used to compare relative uptake from different sources. For both spikes, BAFs were determined using concentrations in amphipods relative to the sediment; this assumes that ingestion from the sediment surface or the bulk sediment were distinct uptake pathways and ignores accumulation from the water column. Conversely, if both sediment sources of CH_3Hg^+ were assumed to have the same BAF, then $BAF_{CH3201Hg+}$ (for bulk sediment) could be applied to the concentration of $CH_3{}^{200}Hg_{water}^{+}$ in the sediment compartment, to predict an amphipod body burden of 45 ng/g $CH_3{}^{200}Hg_{water}^{+}$. Determined body burdens in the experiment were ∼670 ng/g $CH_3{}^{200}Hg_{water}^{+}$. To accumulate another 625 ng/g of $CH_3{}^{200}Hg_{water}^{+}$ from the fraction of algae suspended in the water, a 5 mg amphipod would have to ingest 3.9 ng of $CH_3{}^{200}Hg_{water}^{+}$ (assuming AE = 80%). Whole water $CH_3{}^{200}Hg_{water}^{+}$ concentrations were 2.7 to 16.5 ng/L (12 to 48 h), meaning that only 0.15 to 1 ng of $CH_3{}^{200}Hg_{water}^{+}$ was available for uptake from the entire in 60 mL water compartments. Similar calculations suggest amphipods would need to acquire 40 ng of $^{200}Hg_{water}^{2+}$ from suspended algae to achieve determined body burdens, whereas only 1.5−4 ng of $^{200}Hg_{water}^{2+}$ were present in the microcosms.

Only a small portion of Hg spikes was present in the dissolved water fraction. Lawrence and Mason found an inverse effect of DOC-binding on Hg uptake and derived a laboratory-based relationship between DOC concentration (mg/L) and Water Bioaccumulation Factor (WBAF): Log $WBAF_{CH3Hg}^{+}$ = 1.74−0.173[DOC],[6] where $WBAF_{CH3Hg}^{+}$ is the concentration of CH_3Hg^+ in amphipods (ng/g wet wt) divided by the dissolved concentration of CH_3Hg^+ in the water column (ng/L). Using this equation, $WBAF_{CH3200Hg}^{+}$ was estimated to be 7.5 for a DOC concentration of 5 mg/L measured in this study. For a dissolved $CH_3{}^{200}Hg^+$ concentration of 2 ng/L (experiment 1; time points 12 to 48 h), amphipod concentrations are calculated to increase 100 ng/g (dry wt, assuming 15% dry/wet weight ratio),[6] which is less than 15% of the determined $CH_3{}^{200}Hg^+$body burden. $WBAF_{Hg2+}$ is reportedly 10 times lower than $WBAF_{CH3Hg}^{+}$, under the same conditions.[6] For a $WBAF_{Hg2+}$ of 0.75 and dissolved concentrations of $^{200}Hg^{2+}$ concentration of 16 ng/L (experiment 2; time points 12 to 48 h), amphipod concentrations are calculated to increase 80 ng/g (dry wt), which is also 15% of the determined body burden. Uptake from both dissolved and suspended algae sources was therefore considered minor in this study, and while newly deposited algae may be considered part of the sediment, this study suggests accumulation was much higher from this new surface layer than from the bulk sediment.

As expected, BAFs for CH_3Hg^+ were significantly higher than for Hg^{2+}. In phytoplankton, CH_3Hg^+ accumulates predominantly in the cytoplasm of algal cells, whereas Hg^{2+} adheres to the cell membrane;[21,34,46] the fraction of metal in the cytoplasm is attributed with higher AE during trophic transfer.[47] Comparing the bioaccumulation of the two Hg species, BAF_{CH3Hg}^{+} was relatively higher than BAF_{Hg}^{2+} for tracers originating in the water column than for sediment sources. There are several possible explanations for this relative difference. Uptake of CH_3Hg^+ into the cytoplasm of phytoplankton only occurs in live cells, whereas both Hg species are bound to the cell membrane in dead cells,[34] causing lower assimilation of CH_3Hg^+ during trophic transfer. Accumulation of CH_3Hg^+ in some invertebrates (copepods) was shown to decrease with decay of the marine algae, *Thalassiosira weissflogii*; however, CH_3Hg^+ assimilation in *L. plumulosis* was not affected, which is thought to be due to this species being well adapted to detrital feeding.[46] Release of trace metals from decaying algae decreases exponentially with time and varies between elements.[48] In this study, death and decay may have occurred more rapidly in the phytoplankton mixed in the sediment, which would explain this relative difference, but the microcosm incubation time was relatively short (48 h), and algal decay has been shown not to affect CH_3Hg^+ assimilation in amphipods.[46] Although amphipods were rinsed, residual particle binding may lead to adsorption of $^{199}Hg_{sed}^{2+}$ to the amphipod exoskeleton elevating apparent body burdens from the sediment. Given that Hg^{2+} is strongly particle reactive,[7] adsorption of $^{199}Hg_{sed}^{2+}$ to the exoskeleton may have been higher than for $CH_3{}^{201}Hg_{sed}^{+}$, causing the relatively higher BAF from sediment sources.

To advance biokinetic modeling studies, IR for both feeding pathways of *L. plumulosus* were calculated. Values of IR for surface feeding determined here are in reasonable agreement with values for suspension feeding determined from five different metal species using radiometric techniques (50 to 151 mg/g/day).[18] In this study and in Williams et al., IR was calculated using values of AE determined by radiometric techniques,[18] which may have led to overestimation of AE for Hg^{2+} if methylation of Hg^{2+} spikes occurred during incubation. This would lead to underestimation of IR, which was lower for Hg^{2+} than for CH_3Hg^+ in both studies. No other studies report IR from deposit feeding in amphipods, although Shlekat et al. reported a single measurement of 3 g/g/day, which is much higher than was observed here.[11] This may reflect variation in feeding behaviors and effects of food substrate on test organisms.

Implications of Amphipod Feeding Behavior to Hg Cycling and Toxicity Testing. Phytoplankton rapidly accumulate CH_3Hg^+,[34,49] but rapid algal growth has been shown to dilute Hg in algal food sources and therefore decrease uptake into higher trophic levels.[50] Deposition of phytoplankton blooms is a major source of organic carbon flux to sediment surfaces,[51] and this study suggests deposited algae are a source of Hg species to primary benthic infaunal consumers. In estuaries, increases in growth and reproduction of *L. plumulosis* have been correlated to *Chl a* production associated with sedimenting algae.[52] In a study of $\delta^{13}C$-labeled tracers, however, phytodeposition was found to be a less prominent

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food source than microphytobenthos production for benthic fauna, including amphipods. However, the study also demonstrated that pelagic organic carbon sources were consumed during pulses which mimicked deposition of spring phytoplankton blooms.[53]

Processes at the sediment surface are important to Hg bioavailability. Turbulence and resuspension of sediment can increase transfer of sediment CH_3Hg^+ into organisms,[54] and sedimenting algal blooms have also been shown to increase CH_3Hg^+ production in surface sediments.[55] Previously, bioavailability of Hg species in estuaries has been inversely correlated with sediment organic matter.[7,56] This study suggests Hg in suspended and freshly deposited algal cells are an important uptake source, and that newly deposited algal particles should be distinguished from bulk sediment organic carbon as compartments controlling Hg accumulation. In field collection, bulk sediments are typically collected from the top 4 to 15 cm. Field collection of amphipods is reported within the top 5−7 cm of the sediment,[13] but imaging of amphipod burrows in sediment incubations show the highest burrow volume within the top 1 cm of sediment.[57] Our findings suggest that amphipods feed primarily from the sediment surface, suggesting much smaller sampling depths (<1 cm) are appropriate for assessing Hg sources to benthic infauna.

L. plumulosus are routinely used in both acute and chronic toxicity tests for estuarine sediments.[14,16] In acute (10 d) tests, sediments are the only food source, whereas during 28 day chronic toxicity tests, *L. plumulosus* are fed by an external uncontaminated food source 2−3 times per week.[14] If in natural systems, algae originating in the water column are a preferred food source, these tests do not accurately assess exposure to contamination, as both tests overlook the influence of contamination originating in the water column, and acute toxicity tests ignore ingestion pathways completely. Bioaccumulation varies with feeding behavior, suggesting uptake pathways in test organisms need careful investigation to relate to sediment and water quality criteria.[12]

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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