

Trophic transfer of trace metals from protozoa to mesozooplankton

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Abstract

Radiotracer techniques were used to quantify the assimilation and subsequent efflux of silver, cadmium, iron, mercury, thallium, and zinc by mesozooplankton fed ciliates, heterotrophic dinoflagellates, or heterotrophic flagellates, and the results were compared with published values measured for phytoplankton prey. The subcellular distribution of the metals within the prey cells was also determined and related to their bioavailability. Marine copepods assimilated 59–82% of Ag, 83–92% of Cd, 32–66% of Fe, 14–49% of Hg, and 71–78% of Zn ingested with protozoan prey. Higher Ag, Cd, Fe, and Hg assimilation efficiencies were observed for at least one of the species of protozoa than reported in the literature for phytoplankton. Significant differences in assimilation were not observed for Zn or for Tl fed to freshwater *Ceriodaphnia* in either protozoa or phytoplankton. The higher assimilation efficiencies of protozoan metals, when observed, were matched by higher fractions of metals in the cytoplasm of protozoa. A biokinetic model used to calculate steady-state metal concentrations in copepods indicates that copepods may contain 119% more Ag and 44% more Zn when feeding on ciliates instead of diatoms, possibly resulting in sublethal toxic effects at Ag and Zn concentrations reported for the Hudson River estuary. Further, higher assimilation and efflux rates of protozoan Fe may enhance remineralization of this limiting nutrient by mesozooplankton in high nutrient, low chlorophyll regions.

Aquatic animals can accumulate metals both directly from their aqueous environment and from the prey they ingest, but metals accumulated through these two routes can have different physiological effects and geochemical fates. Food is often the dominant uptake pathway for metals in crustacean zooplankton (Munger and Hare 1997; Wang and Fisher 1998), and zooplankton can be more sensitive to metals accumulated through this pathway (Hook and Fisher 2001*a,b*). In addition to having greater physiological effects, metals that are ingested and assimilated by zooplankton can build up in food chains or be biologically recycled; these metals generally display longer residence times in surface waters than unassimilated metals, which get packaged into fecal pellets and sink out of surface waters (Fowler and Knauer 1986; Fisher et al. 1991). Metals bound to the exoskeleton may also be exported with molts (Fowler 1977).

Most studies that have examined the assimilation of metals by zooplankton grazers have focused on phytoplankton prey (e.g., Sick and Baptist 1979; Fisher et al. 1991; Wang et al. 1996), but heterotrophic protists can also serve as an important food source for mesozooplankton. As the dominant grazers of heterotrophic and autotrophic picoplankton in many aquatic systems (Sherr and Sherr 1994), protozoa are an important trophic link between the microbial loop and the metazoan food web (Stoecker and Capuzzo 1990). Mi-

crozooplankton consume a significant portion of bacterial and phytoplankton production in oligotrophic regions (Landry et al. 1995) as well as in more eutrophic coastal (Caron et al. 1991) and freshwater ecosystems (Sherr et al. 1991). Protozoa can also graze on larger prey, with heterotrophic dinoflagellates having been observed to ingest centric diatoms in some coastal ecosystems (Neuer and Cowles 1994; Buck and Newton 1995). Protozoa, in turn, can be an important food source for copepods (White and Roman 1992; Ohman and Runge 1994; Rollwagen Bollens and Penry 2003), particularly during summer months when the dominant cells in the phytoplankton community may be too small to be efficiently grazed by mesozooplankton (Gifford and Dagg 1988).

It has also been proposed that protozoa may constitute a higher quality food for copepods than certain phytoplankton. Studies have shown that egg production and hatching success are higher in protozoa-fed copepods than in copepods fed algae (e.g., Stoecker and Egloff 1987; Nejstgaard et al. 2001). This may be because protozoa produce necessary fatty acids and sterols lacking in algal prey (Klein Breteler et al. 1999) or simply have a more balanced nutritional composition (Kleppel 1993). These findings may explain why copepods have been observed to preferentially consume protozoa over phytoplankton prey (Stoecker and Capuzzo 1990; Verity and Paffenhof 1996).

Despite the importance of heterotrophic protozoa as a food source for mesozooplankton, very few studies have yet examined the trophic transfer of metals between these two groups. Metals ingested in protozoa prey might be more bioavailable to copepod grazers than those ingested in algae. It has been well established that the efficiency with which copepods assimilate ingested metals is related to the distribution of the metal within the phytoplankton cell (Reinfelder and Fisher 1991). Herbivorous grazers assimilate the metals contained within the cytoplasm of prey cells and egest metals bound to cell walls and membranes. Through the process

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of phagocytosis, protozoa ingest algal and bacterial cells, concentrating prey cells and their internally and externally bound metals within endocytotic vacuoles, where they are chemically digested. Once the endocytotic vacuole is separated from the cell membrane, the pH is rapidly lowered to ~ 3 as water is expelled from the contracting vacuole (Fok et al. 1982). Iron colloids are dissolved during protozoan phagocytosis (Barbeau and Moffett 2000), and it is likely that the acidic endocytotic vacuole is an appropriate environment for the dissolution of membrane-bound metals as well. Thus, actively feeding phagotrophic protozoa may contain a higher fraction of dissolved metals than phytoplankton, which do not ingest metal-containing particles and must transport externally bound metals to achieve desired internal concentrations. Such differences in subcellular partitioning of metals within prey could cause differences in metal bioavailability for invertebrate zooplankton predators.

In order to better understand the toxicological and geochemical implications of protozoan grazing by copepods, it is necessary to quantify differences in metal bioavailability in protozoa and phytoplankton prey. Elements ingested by zooplankton may be either assimilated across the gut lining of the animal and later excreted or not assimilated and packaged into fecal pellets that are egested. Assimilated elements, therefore, are more likely to be retained in surface waters while unassimilated elements may be vertically exported in sinking fecal pellets (Fisher et al. 1991; Fisher and Reinfelder 1995). Further, only assimilated elements can exert a biological response. We employed well-developed radiotracer techniques to measure the assimilation and subsequent efflux of a suite of trace metals by invertebrate zooplankton grazing on heterotrophic protozoa under controlled laboratory conditions. Iron and zinc are both essential to many biological functions, whereas silver, mercury, and thallium have no known biological use and can be toxic at elevated concentrations. Cadmium may substitute for Zn in certain enzymes and is a pollutant metal of concern in some contaminated waters. Additionally, Fe and Tl can undergo redox transformation that may affect their bioavailability.

Materials and methods

Culture maintenance—The heterotrophic ciliate *Uronema* sp. (clone BBcil, isolated by D. Caron) and the heterotrophic dinoflagellate *Oxyrrhis marina* (CCMP 604) were maintained in 0.2- μm -filtered surface seawater (SHSW) collected 8 km off Southampton, New York. The ciliates were fed a natural assemblage of bacteria enriched with yeast extract, and the dinoflagellates grazed on the autotrophic chlorophyte *Dunaliella tertiolecta* (CCMP 1320). The diatom *Stephanodiscus hantzschii* (UTCC 267) and the cyanobacterium *Synechococcus leopoliensis* (UTEX 625) were maintained axenically in WCL-1 medium (Guillard 1975). The mixotrophic chrysophyte *Ochromonas danica* (UTEX 1298) was also maintained in WCL-1 medium but fed an aliquot of *Synechococcus* twice a week prior to the trophic transfer experiments. The heterotrophic bacterium *Vibrio natriegens* (ATCC 14048) was maintained in Difco Nutrient Broth amended with 1.5% NaCl.

Calanoid copepods were collected from Stony Brook harbor with a 64- μm plankton net several days prior to the marine experiments. The dominant species were *Acartia tonsa*, *A. hudsonica*, and *Temora longicornis*, depending on the time of the year. Reinfelder and Fisher (1991) found no significant difference in the assimilation of selenium by these three species fed radiolabeled phytoplankton. Further, these authors observed a remarkably close relationship between metal assimilation efficiency and cytoplasmic distribution in algal food in experiments with the different copepod species, providing indirect evidence that food digestion and assimilation processes in these three species are similar. Monospecific assemblages of the three copepod species were used interchangeably in this study; the species used for each experiment is noted in Table 1. Adult copepods were hand picked and transferred to filtered SHSW and allowed to completely depurate their guts immediately prior to the feeding experiments. The freshwater cladoceran *Ceriodaphnia dubia* was purchased from Aquatic BioSystems and maintained in WCL-1 media on *Selenastrum* sp. prior to the feeding experiments.

Ciliate-copepod experiments—Five separate experiments were performed to assess the assimilation of Ag, Cd, Fe, Hg, and Zn by calanoid copepods fed *Uronema*. In the first experiment, Cd and Zn assimilation efficiencies were measured simultaneously, while subsequent experiments tested Ag, Fe, and Hg assimilation separately (Hg was tested twice, using two different copepod feeding times). The protocol was the same for each experiment and is outlined in Fig. 1. A batch culture of *V. natriegens* was washed and resuspended in sterile, filtered SHSW and radiolabeled with either $^{110\text{m}}\text{Ag}$, ^{55}Fe , ^{203}Hg , or ^{109}Cd and ^{65}Zn for 24 h. The radioisotope additions are summarized in Table 1; each flask also received equimolar NaOH to offset the acid matrices of the radioisotopes. A small inoculum of ciliates was then added to the bacteria, and the ciliates were allowed to grow on the radiolabeled bacteria for 75–100 h. Ciliate growth was monitored through daily cell counts. The *Uronema* cells underwent 4–6 divisions, ensuring that all cells were uniformly labeled. After radiolabeling, late log-phase ciliates were harvested by gravity filtration onto 5- μm polycarbonate membranes (the filters were never allowed to dry out) and resuspended in SHSW. The resuspension was then filtered through 10- μm polycarbonate membranes to remove clumped cells. Samples were taken for cell counts (preserved with 4% Lugols), and the resuspended ciliates were then split into 250-ml glass screw-top flasks. For the experiments with ^{203}Hg , all solutions were kept in ground-glass stoppered flasks and all manipulations were performed in a fume hood. Initial cell concentrations in the feeding flasks were between 2,000 and 7,300 cells ml^{-1} (1–3.9 mg C L^{-1} ; Table 1), comparable with prey densities in earlier phytoplankton feeding studies (Wang et al. 1996; Wang and Fisher 1998; Xu and Wang 2001).

For the Ag, Cd, Hg, and Zn experiments, copepods were added (0.5–0.75 individuals ml^{-1}) to the radiolabeled suspensions held in triplicate flasks and allowed to consume the ciliates, while an additional flask received resuspended ciliates but no copepods. After 10 h (4 h for the second Hg experiment), the copepods were removed from the feeding

Table 1. Radioisotope additions, prey concentrations, and zooplankton feeding times used for the trophic transfer experiments. Prey densities were converted to C densities by calculating the volume of the prey cells from cell dimensions and using the C: vol relationships of Menden-Deuter and Lessard (2000) and Ohman and Snyder (1991).

Uronema						Oxyrrhis					
Radioisotopes		Prey concentrations		Zooplankton feeding		Radioisotopes		Prey concentrations		Zooplankton feeding	
(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)	(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)	(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)
Ag	18.6	185	2.7	5,050	2.7	<i>Temora</i>	10	27.0	177	<i>A. hudsonica</i>	4
Cd	14.5	308	1.1	2,120	1.1	<i>Temora</i>	10	21.0	348	<i>A. hudsonica</i>	4
Fe	5.1	733	2.6	4,960	2.6	<i>Temora</i>	4	5.1	733	<i>Temora</i>	4
Hg	12.3	183	3.9	7,300	3.9	<i>Temora</i>	10	11.2	83	<i>A. tonsa</i>	16
Hg	12.3	67	3.5	6,720	3.5	<i>A. hudsonica</i>	4	1.1	222	<i>A. hudsonica</i>	4
Zn	0.3	111	1.1	2,120	1.1	<i>Temora</i>	10	1.1	222	<i>A. hudsonica</i>	4
Stephanodiscus						Ochromonas					
Radioisotopes		Prey concentrations		Zooplankton feeding		Radioisotopes		Prey concentrations		Zooplankton feeding	
(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)	(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)	(nmol L ⁻¹) (kBq L ⁻¹)	(Cells ml ⁻¹) (mg C L ⁻¹)	Species	Time (hours)
Tl	4.2	148	1.2	70,000	1.2	<i>C. dubia</i>	1.5	4.2	148	<i>C. dubia</i>	1.5

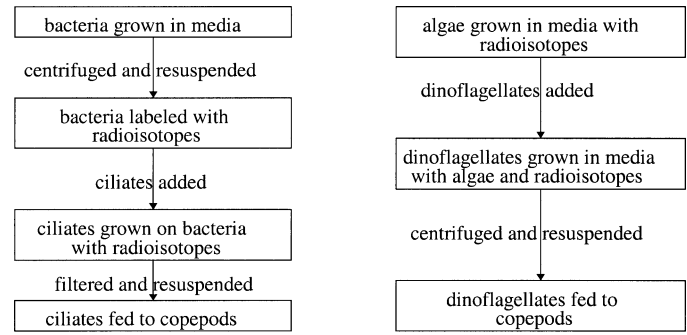


Fig. 1. Schematic of protocols used to grow radiolabeled protozoa for pulse feeding to copepods.

suspension with a 100- μ m filter cup, resuspended in 15 ml SHSW, and the accumulated radioactivity assessed immediately. The fecal pellets produced during the feeding period were collected in a 40- μ m filter cup, rinsed, and the radioactivity measured. After assessing their radioactivity, the copepods were gently transferred to SHSW with a mixture of cultured phytoplankton (*Isochrysis galbana*, *Rhodomonas lens*, *Prorocentrum minimum*, *Thalassiosira pseudonana*) and allowed to depurate for 48 h. At various times during the depuration (4, 8, 24, 36, 48 h), the copepods were gently removed from the feeding suspension and the remaining gamma radioactivity assessed nondestructively. At these times, the copepods were carefully observed to ensure that they appeared active and healthy, and additional unlabeled food was added.

At the end of the copepod feeding period, the ciliates in the control flask containing no copepods were gently filtered with a 0.2- μ m polycarbonate membrane and copepods added to the flask for a period equal to the duration of the original pulse feeding. The accumulation of radioactivity by these copepods was used to assess the contribution of metals that desorbed or was otherwise released into the water from the ciliates during feeding and were subsequently accumulated from the dissolved phase by the feeding copepods.

Because ⁵⁵Fe emits β particles, it was necessary to dissolve the radiolabeled copepods prior to radioassaying samples labeled with this isotope. In order to account for this difference, radiolabeled ciliates were added to 17 replicate 125-ml glass flasks and copepods were added to 15 of these (leaving two control flasks). At the end of a 4-h feeding period, the copepods in four of the flasks were collected in 100- μ m filter cups, rinsed thoroughly with SHSW, and collected on 10- μ m polycarbonate membranes before being transferred to 20-ml scintillation tubes. The copepods were dissolved with Solvable (Packard BioScience) for 2 h at 60°C prior to the addition of Ultima Gold XR scintillation cocktail. The fecal pellets from these four feeding flasks were collected onto a 40- μ m filter and assayed for radioactivity. The copepods from the other feeding flasks were immediately transferred into unlabeled water containing phytoplankton and allowed to depurate. At the subsequent depuration time points, the copepods in triplicate flasks were collected as above and radioassayed.

Dinoflagellate-copepod experiments—Similar protocols were used to measure the copepods' assimilation of Ag, Cd, Fe, Hg, and Zn from dinoflagellate prey, although the methods used to radiolabel and resuspend the prey cells were slightly different (Fig. 1). The dinoflagellates were fed cultures of *Dunaliella* that were radiolabeled in SHSW amended with f/2 nutrients (Guillard and Ryther 1962), excluding Cu, Zn, and ethylenediaminetetraacetic acid (EDTA), and allowed to undergo several divisions before an aliquot of *Oxyrrhis* was added. The dinoflagellates were allowed to grow to the end of the log phase, at which point most of the *Dunaliella* prey had been consumed. The dinoflagellates were then centrifuged at $40 \times g$ for 45 min at 4°C and resuspended in sterile, filtered SHSW. The choice of prey for the protozoa (heterotrophic bacteria for the ciliates and chlorophytes for the dinoflagellates) was matched to the prey upon which each was maintained.

Ochromonas-Ceriodaphnia experiments—The freshwater ^{204}Tl trophic transfer experiments followed similar protocols, with some modifications. Like ^{55}Fe , ^{204}Tl primarily emits β particles, so the same radiolabeled zooplankton could not be repeatedly assayed during their depuration, and 16 replicate flasks of *Ceriodaphnia* were labeled so that duplicate flasks could be filtered and counted at each depuration time point. Because no measurements of Tl assimilation from phytoplankton prey are available in the literature for comparison, an additional experiment was performed to measure the assimilation of Tl from diatom prey. For this experiment, the diatom *Stephanodiscus* was radiolabeled with ^{204}Tl in WCL-1 without Cu, Zn, or EDTA for 4 d (Table 1). The cells were then filtered onto a 1- μm polycarbonate membrane and resuspended into WCL-1 that was split into sixteen 125-ml flasks. *Ceriodaphnia* were added to 14 of these (1 individual ml^{-1}) and allowed to feed on the radiolabeled diatoms for 1.5 h. At the end of this period, the cladocerans were transferred to depuration flasks, as before, and unlabeled cladocerans added to the filtrate of the two control uptake flasks. At each depuration time point, the *Ceriodaphnia* from duplicate flasks were collected in a 250- μm filter cup, rinsed with WCL-1, and collected on a 14- μm polycarbonate membrane. The *Ceriodaphnia* were dissolved with Solvable prior to the addition of scintillation cocktail.

For the *Ochromonas* feeding experiment, *Synechococcus* were radiolabeled with ^{204}Tl in WCL-1 without Cu, Zn, or EDTA for 3 d. An inoculum of *Ochromonas* was added and allowed to graze, dividing several times in 3 d. The radiolabeled flagellates were centrifuged ($1,500 \times g$ for 15 min) and resuspended in WCL-1 two times and then fed to *Ceriodaphnia* as above.

Depuration of metals from the mesozooplankton was plotted as the percent of ingested metal (calculated as the sum of radioactivity in the zooplankton at the end of feeding plus any radioactivity in the fecal pellets produced during feeding) retained in the zooplankton during the 48 h following the pulse feeding. Assimilation efficiencies were calculated by fitting an exponential decay function ($y = be^{mx}$) to the depuration curve between 4 and 48 h. The assimilation efficiency was estimated by the y -intercept of the curve (b), while the efflux rate constant, k_e , is described by the coef-

ficient m (Wang and Fisher 1998; Xu and Wang 2001). For comparison, assimilation efficiencies were also calculated as the percent of ingested metal (metal in copepods + metal in fecal pellets at end of feeding) retained by the copepods after 4 h of depuration (Hutchins et al. 1995; Mason et al. 1996; Wang et al. 1996).

Fractionation experiments—Separate experiments were conducted to measure the distribution of Ag, Cd, Fe, Hg, and Zn within the protozoan prey. The experiments were similar to those performed with phytoplankton (Fisher et al. 1983; Reinfelder and Fisher 1991) but were adapted to account for the heterotrophic nature of the copepod prey. *Uronema* and *Oxyrrhis* were radiolabeled as described above. At the end of the protozoan growth/radiolabeling period, cells were filtered or centrifuged out of their radiolabeling suspension, resuspended in SHSW, and left to equilibrate for 1 h (as in the trophic transfer experiments). The cells were then filtered or centrifuged again and resuspended in Milli-Q water, lysing the cells (verified microscopically). The lysed cell suspensions were mixed well, split into triplicate tubes, sampled for radioactivity, and then centrifuged at $10,000 \times g$ for 15 min, which separates membranes (including large intracellular membranes and membrane-bound organelles) from cytoplasm (Sheeler 1981; Reinfelder and Fisher 1991). Following centrifugation, the supernatant was aspirated and the pellet sampled for radioactivity.

Analytical procedures—Gamma emissions were measured with a large-well NaI(Tl) gamma detector for $^{110\text{m}}\text{Ag}$ (885 keV), ^{109}Cd (88 keV), ^{203}Hg (279 keV), and ^{65}Zn (1,115 keV). The β emissions of ^{55}Fe and ^{204}Tl were measured with a Packard Tri-Carb 2100TR liquid scintillation analyzer. Counting times were adjusted to enable propagated counting errors <5%. Radioactive counts of ^{203}Hg were corrected for decay prior to data analysis. Cell counts were performed with a Coulter Multisizer II particle counter or microscopically using a hemacytometer or Palmer cell. Cell density of bacterial cultures was estimated from absorbance at 480 nm (Ohman and Snyder 1991) using the equation $\text{cells ml}^{-1} = (6 \times 10^9)\text{Abs} - (2 \times 10^8)$.

Results

Following the radiolabeling incubations and resuspension in unlabeled water, the ciliates and dinoflagellates were examined microscopically and were observed to be intact and swimming. The copepods actively ingested the radiolabeled protozoa during the pulse feedings, with each copepod consuming between 4,000 and 11,700 cells (equivalent to 0.12–0.69 $\mu\text{g C copepod}^{-1} \text{ h}^{-1}$; data not shown) during the various experiments. In addition to the metal measured in the copepods at the end of the feeding periods, a variable fraction of ingested metal was present in their fecal pellets produced during the feeding period. This fraction was generally smaller for the shorter feeding experiments: <22% of the metal was found in fecal pellets, with the exception of the 16-h *Oxyrrhis*-Hg feeding (57% of the metal in the fecal pellets). Although it was not possible to quantify the excretion of ingested metal into the dissolved phase during the short

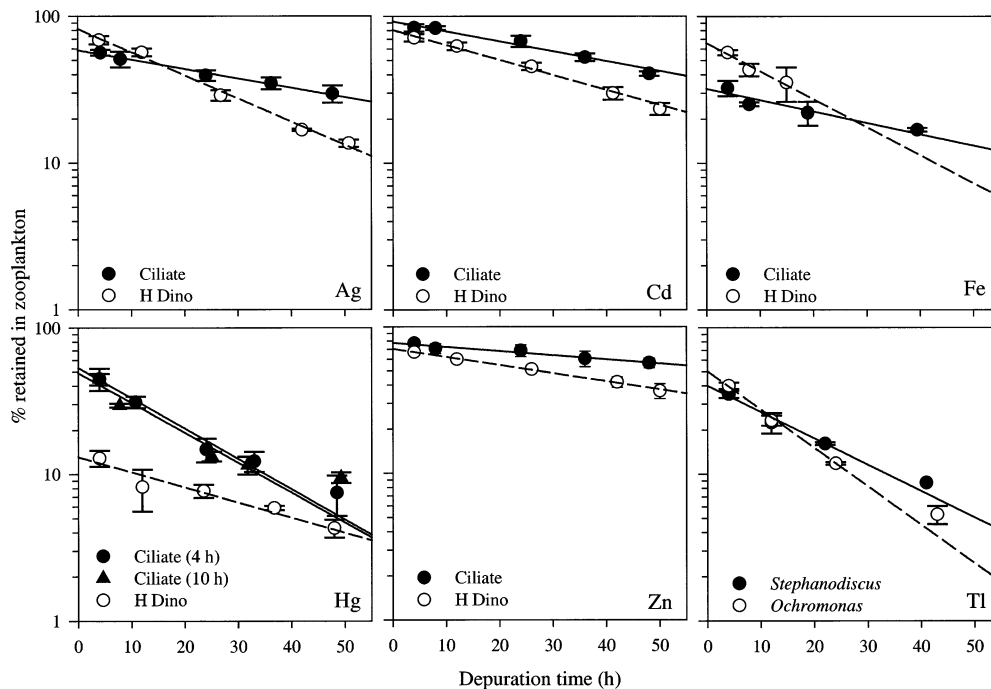


Fig. 2. Depuration of trace elements from mesozooplankton following feeding on radiolabeled ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H Dino; *Oxyrrhis marina*), diatoms (*Stephanodiscus*), or heterotrophic flagellates (*Ochromonas danica*). Data points are means of two to four replicates \pm range/2 (for $n = 2$) or standard deviation [SD] (for $n = 3-4$).

pulse feedings, metal that desorbed from the prey cells and was subsequently sorbed by the copepods was generally <5% of the total metal accumulated during the feedings, indicating that trophic transfer of metal was clearly the dominant source of metal for the copepods in these experiments.

During the depuration period, the metals were generally lost from the copepods in a two-phase pattern (Fig. 2). For each metal, there was a rapid initial loss (<4 h) corresponding to the egestion of metal remaining in the guts of the animals at the end of feeding; fecal pellets were observed in the feeding flasks, indicating that copepods produced fecal pellets when grazing on the protozoa in these experiments. This was followed by a more gradual loss phase corresponding with the excretion of assimilated metals during normal metabolic turnover. The copepods were monitored during depuration and the animals remained actively swimming. The radioactivity in the copepods during the depuration was normalized to the total radiolabel ingested during the feeding period, calculated as the sum of the activity in the copepods and their fecal pellets at the end of the radioactive feeding period.

The assimilation efficiencies as calculated using both the entire depuration curve and only the 4-h time point are compared in Table 2. In general, the calculations yield very similar results, and significant differences (t -test, $P < 0.05$) were observed only in the cases of Ag- and Fe-labeled *Oxyrrhis*. In both cases, the assimilation efficiency calculated using only the 4-h time point is lower than the y-intercept assimilation efficiency because it does not account for the assimilated metal lost by excretion during the first 4 h of depuration. The Hg-assimilation efficiencies measured in two

separate *Uronema* feeding experiments characterized by different pulse feeding times (4 h, 10 h) were not significantly different (t -test, $P > 0.05$) (Table 2), suggesting that feeding time does not influence the assimilation efficiency calculation (as long as fecal pellet activity is measured). Copepods assimilated Cd and Hg more efficiently from *Uronema* than from *Oxyrrhis*, but no significant difference was noted for Zn (t -test, $P > 0.05$). Conversely, copepods assimilated Ag and Fe more efficiently from *Oxyrrhis* than from *Uronema*. *Ceriodaphnia* appeared to assimilate more Tl from the heterotrophic flagellates than from diatom prey, although the difference was not statistically significant (t -test, $P > 0.05$).

The calculated efflux rate constants (k_e) are compared between the different feeding treatments and metals in Table 3. Again we observed no significant difference in the results between the 4-h and 10-h Hg feeding experiments. There are marked differences in metal retention by copepods fed the two prey items, however. Copepods feeding on *Oxyrrhis* excreted Ag, Cd, Fe, and Zn twice as fast, on average, as did copepods feeding on *Uronema*, while Hg was lost 1.5 times more rapidly from copepods fed ciliates (t -test, $P < 0.05$). *Ceriodaphnia* excreted Tl obtained from *Ochromonas* significantly faster than Tl obtained from the diatom *Stephanodiscus* (t -test, $P < 0.05$).

The partitioning of Ag, Cd, Fe, and Zn in the ciliates and dinoflagellates fed to the copepods is shown in Fig. 3. For the ciliates, the percentage of metal contained within the cytoplasmic fraction (i.e., the cytoplasm, endoplasmic reticulum, ribosomes, or Golgi complex [Sheeler 1981]) ranged from 36% for Fe to 92% for Zn. This fraction was higher and less variable in the dinoflagellates, ranging between 58%

Table 2. Metal assimilation efficiencies (%) for copepods fed ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H. Dino; *Oxyrrhis marina*), heterotrophic flagellates (H. flag; *Ochromonas danica*), or diatoms.

Food type	Ag	Cd	Fe	Hg	Zn	Food type	Tl
Ciliate y-intercept	58.6±2.6	92.3±2.8	32.3±2.0	49.2±3.2*	77.7±4.7	H. flag y-intercept	50.4±2.4
4-h depuration	56.5±5.2	83.5±3.4	32.5±3.9	53.3±6.0†	77.2±5.2	4-h depuration	40.2±2.1
H. Dino y-intercept	82.0±5.5	82.8±4.8	66.4±5.6	44.6±8.6*	70.7±2.1		
4-h depuration	68.9±7.6	73.5±6.0	56.8±2.2	44.9±5.5†	67.1±4.4		
Diatom	8–19 ^{2,7}	30–37 ^{1,2,7}	7–25 ^{3,4,8}	15–21 ^{1,5}	47–64 ^{1,2,7}	Diatom y-intercept	40.6±3.1
		85–95 ⁶			65–80 ⁶	4-h depuration	35.1±2.1

¹ Fisher et al. (1991).

² Reinfelder and Fisher (1991).

³ Hutchins and Bruland (1994).

⁴ Hutchins et al. (1995).

⁵ Mason et al. (1996).

⁶ Wang et al. (1996).

⁷ Wang and Fisher (1998).

⁸ Schmidt et al. (1999).

* Ten-hour pulse feeding.

† Four-hour pulse feeding.

and 70% for Ag, Cd, and Zn; iron fractionation was not measured in the dinoflagellates.

Discussion

Assimilation of metals from protozoa and phytoplankton—The metal-assimilation efficiencies measured for copepods consuming protozoan food are compared with published assimilation efficiencies for diatom food in Table 2 and Fig. 4. Given the similarity of assimilation efficiencies calculated using the y-intercept and the 4-h depuration time point, we consider here only the assimilation efficiencies calculated from the y-intercept for simplicity. Copepods assimilated 3–5 times more Ag from heterotrophic prey than from autotrophic prey. Cadmium in protozoa also appears to be 2–3 times more bioavailable to copepod predators, given algal-based assimilation efficiencies of 30–37% reported by several different studies (Fisher et al. 1991; Reinfelder and Fisher 1991; Wang and Fisher 1998). Our results are similar to the Cd-assimilation efficiency measured for copepods feeding on natural seston and within the range of assimilation efficiencies observed for some phytoplankton prey by Wang et al. (1996), although they could not explain the discrepancy between their results and other published values.

The protozoan Zn assimilation efficiencies are at the high end of the wide range of phytoplankton assimilation efficiencies that have been observed, but they do not show the notable departure seen for Ag and Cd. Similarly, only a small difference in Tl assimilation was observed for daphnids fed either heterotrophic flagellates or diatoms.

For Fe and Hg, more metal was assimilated from one of the two protozoa than from phytoplankton. Iron contained in the heterotrophic dinoflagellates was assimilated twice as efficiently as Fe in the ciliate and at least threefold more efficiently than Fe in diatoms. The opposite trend appears for Hg, which was threefold more available in ciliates than in the heterotrophic dinoflagellates or phytoplankton. Interestingly, the assimilation of Hg from ciliates is similar to the assimilation of methylmercury from diatom prey. Iron assimilation by copepods has been shown to be weakly related to the Fe content of the prey (Hutchins et al. 1995; Schmidt et al. 1999), but it is unlikely that metal:C ratios of the cells were markedly different because the Fe and Hg concentrations used to label the protozoa were very close. The protozoa were also grown on different prey (heterotrophic bacteria vs. algae), and this may have affected the bioavailability of the metals contained in the protozoa. It is difficult to separate the effects of different protozoa and dif-

Table 3. Efflux rate constants (k_e , d⁻¹) for copepods fed ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H. dino; *Oxyrrhis marina*), heterotrophic flagellates (H. flag; *Ochromonas danica*), or diatoms.

Food type	Ag	Cd	Fe	Hg	Zn	Food type	Tl
Ciliate	0.355±0.037	0.374±0.016	0.445±0.104	1.128±0.057† 1.143±0.008‡	0.158±0.032	H. flag	1.446±0.117
H. dino	0.874±0.030	0.560±0.042	1.093±0.262	0.728±0.227	0.310±0.043		
Diatom	0.395–0.424 ¹	0.41–0.685 ^{1,2}	0.085–0.186 ³	—	0.248–0.59 ^{1,2}	Diatom	1.031±0.159

¹ Wang and Fisher (1998).

² Wang et al. (1996).

³ Schmidt et al. (1999).

† Ten-hour pulse feeding.

‡ Four-hour pulse feeding.

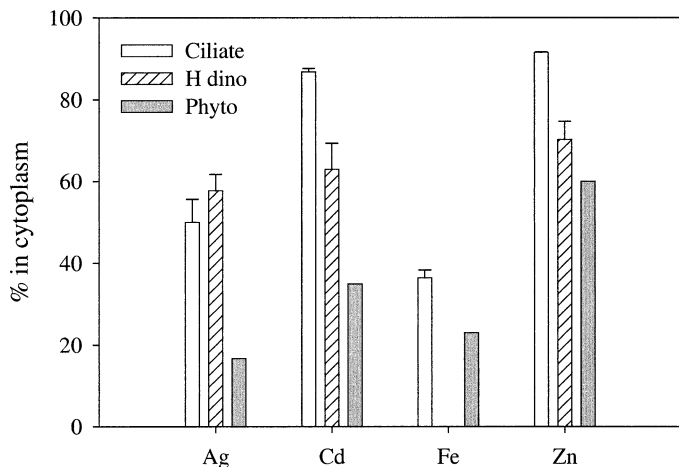


Fig. 3. Subcellular fractionation of metals in ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H dino; *Oxyrrhis marina*), and diatom cells (Phyto). The bars are means \pm SD ($n = 3$) for the protozoa and single data points for the phytoplankton (data taken from Reinfelder and Fisher [1991] and Hutchins et al. [1995]).

ferent protozoa prey, however; nor was that the objective of this study. Rather, the goal was simply to compare metal bioavailability in protozoa and phytoplankton.

The differences in metal bioavailability noted between phytoplankton and at least one of these protozoa are in contrast with observations of only minor differences in the assimilation of metals from a diverse group of phytoplankton species, including the autotrophic dinoflagellate *P. minimum* (Wang et al. 1996). Further, in no case was the assimilation of ingested metal in copepods lower from protozoa food than from phytoplankton food. Thus, it appears that Ag, Cd, Fe, and Hg are more bioavailable to mesozooplankton from some types of protozoa than from phytoplankton. To our knowledge, the only other study to quantify the transfer of metals from protozoa to mesozooplankton was performed by Hutchins and Bruland (1994), who measured the accumulation of Fe, Mn, and Zn by mesozooplankton grazing on radiolabeled diatoms or flagellated protozoa. Although most of the metal originally associated with the prey was regenerated into the dissolved phase, less recycling was observed for protozoan prey than diatom prey, suggesting that metals in these cells were more efficiently retained in the grazers. Calculated assimilation efficiencies, though, were higher only for Fe in one experiment (18% for flagellates compared with 7% for diatoms) and were both lower than those measured in this study.

Past studies have employed a range of techniques for calculating zooplankton assimilation efficiencies. The studies with which we have compared our results fed copepods for between 30 min (Wang and Fisher 1998) and 18 h (Fisher et al. 1991) and took several different approaches to calculating the assimilation efficiency, including using the y -intercept of the curve describing loss of metal from the slowly exchanging pool and considering only the radioactivity in copepods at the end of egestion (usually taken as 4–6 h). Our results indicate that these methodological differences will not significantly affect the calculated assimilation effi-

ciencies. Further, we obtained nearly identical Hg-assimilation efficiencies in separate experiments using 4-h and 10-h pulse feeding periods, and the calculated assimilation efficiencies were very close whether using the y -intercept of the fitted depuration curve or the 4-h time point. Others have also found these assimilation efficiency calculations to yield similar results (Stewart and Fisher 2003). Therefore, we believe that the trends noted between protozoa and phytoplankton prey are not attributable to differences in methods of calculating assimilation efficiencies. Further, the similar assimilation of Hg in ciliates fed to *T. longicornis* and *A. hudsonica* confirms that these species assimilate ingested metals in a similar manner, as noted previously (Reinfelder and Fisher 1991). While it is also possible that the age and sex of the copepods may influence metal assimilation, this was not investigated during this study.

The food concentrations used in our study (1,000–3,900 $\mu\text{g C L}^{-1}$) fall at the upper range of those used for published phytoplankton experiments, and the calculated ingestion rates (0.12–0.69 $\mu\text{g C copepod}^{-1} \text{ h}^{-1}$) overlap those measured for natural populations of *Acartia* feeding on phytoplankton and protozoa in San Francisco Bay (Rollwagen Bollens and Penry 2003). Studies on the relationship between food concentration and phytoplankton metal assimilation present conflicting results: Wang et al. (1996) found no relationship between food concentration (varied between 16 and 800 $\mu\text{g C L}^{-1}$) and assimilation while Xu and Wang (2001) found that assimilation decreased at higher food concentrations (varied between 54 and 3,500 $\mu\text{g C L}^{-1}$). Therefore, the assimilation efficiencies measured for protozoa here may, in fact, underestimate values that would be obtained using lower food levels.

Assimilation efficiencies were calculated in relation to total ingested metal, taken as the sum of the radioactivity in the copepods and their fecal pellets at the end of the feeding period. Assimilated metal that was excreted during the feeding period is not included in this calculation, and therefore total ingested metal could be underestimated, leading to an overestimation of assimilation efficiency. The magnitude of this potential error was estimated by calculating the percentage of assimilated metal that would be excreted during the various feeding periods given the measured excretion rates. For Ag, Cd, Fe, and Zn, less than 20% of metal present in the copepods at the beginning of the feeding period would be excreted during feeding. This is clearly an upper limit of this potential error because metal was not ingested instantly but was consumed gradually over the course of the feeding period. In the case of Hg, which showed fast efflux rates, 46–49% of metal present in the copepods at the beginning of feeding might have been excreted during the long feeding but only 18% in the short feeding experiment. Because the 4-h and 10-h ciliate feedings produced nearly identical Hg-assimilation efficiencies, we conclude that this is probably a minor effect.

Subcellular distribution of metals in prey—The higher bioavailability of metals in some protozoa may be largely explained by the different distribution of these metals within the cells: Ag, Cd, Fe, and Zn were partitioned more in the cytoplasmic fraction of the protozoa studied here than in

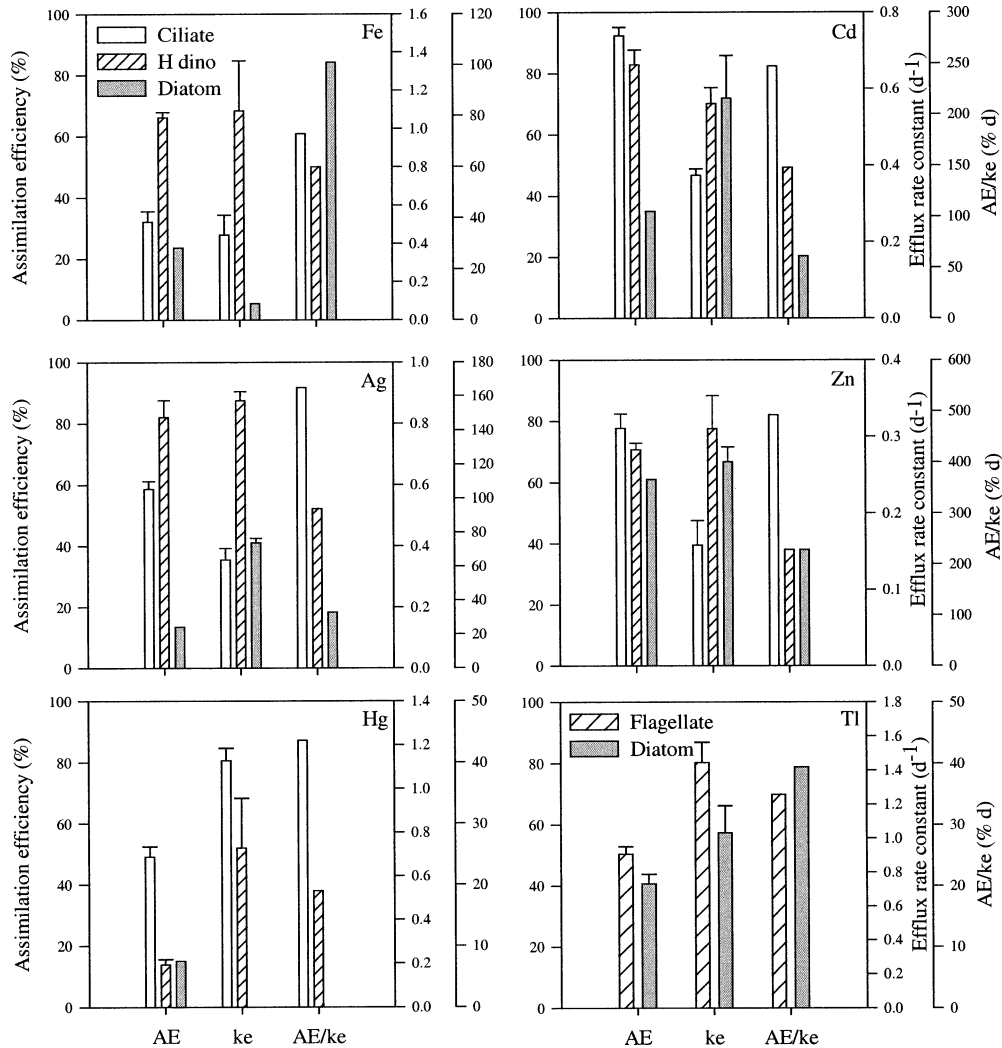


Fig. 4. Assimilation efficiency (AE, %), efflux rate constant (k_e , d^{-1}), and the ratio of these two parameters (AE/k_e , % d) for copepods fed ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H dino; *Oxyrrhis marina*), or phytoplankton radiolabeled with Ag, Cd, Fe, Hg, Tl, or Zn. For AE and k_e , the bars are means \pm SD ($n = 3$); for AE/k_e , the ratios are based on mean values.

phytoplankton cells (Fig. 3). A close relationship between metal partitioning in phytoplankton prey and assimilation within mesozooplankton has been observed with these and other metals (Reinfelder and Fisher 1991; Hutchins et al. 1995; Stewart and Fisher 2003). Although the correlation between these two parameters is not quite as close when protozoa are used as food, the higher protozoan assimilation efficiencies are matched by a higher percentage of metals within the protozoan cells (Fig. 5). In fact, for Ag, Cd, and Fe in *Uronema* and Zn in *Oxyrrhis*, assimilation efficiency and cytoplasmic partitioning are essentially equal. Different subcellular partitioning of metals in autotrophic and heterotrophic protists may result from phagotrophic processes occurring with the protozoa. While phytoplankton rely on active transport of adsorbed metals across the cell membrane to accumulate trace metals from the aqueous environment (Williams 1981), phagotrophs actively engulf prey cells

within digestive vacuoles, concentrating the metals inside the protozoa cells.

Metals ingested by protozoa may be converted into a bioavailable liquid fraction within digestive vacuoles. Harris and Ramelow (1990) found that Cd, Cu, and Zn rapidly desorbed from algal biomass at pH 3, similar to that found in protozoan digestive vacuoles (Fok et al. 1982); and Frey and Small (1979) observed the release of bioavailable Fe from phytoplankton cell homogenate following in vitro replication of the protozoan digestion pH cycle. Barbeau et al. (1996) detected a similar result following the ingestion of bacteria by protozoa. Furthermore, photosynthetic pigments are degraded within protozoan food vacuoles (Strom et al. 1998), including the vacuoles of *O. marina* (Barlow et al. 1988). While the chemical nature of the degraded pigments is not known, metals associated with these pigments may be converted to a form that is more likely to cross the copepod gut

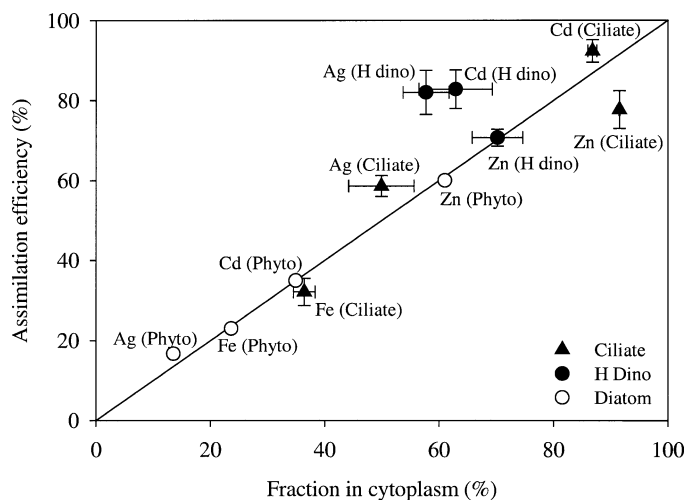


Fig. 5. Assimilation efficiency (%) of ingested elements in copepods fed ciliates (*Uronema* sp.), heterotrophic dinoflagellates (H dino; *Oxyrrhis marina*), or diatoms (Phyto) as a function of the cytoplasmic fraction (%) of those elements in the prey cells. Also plotted is a 1:1 line.

lining, thereby leading to higher assimilation of the protozoan metals than phytoplankton metals.

Accumulation of metals by copepods—The accumulation of metals by copepods from food is controlled both by the assimilation efficiency and the rate at which assimilated metals are lost. A comparison of metal efflux rate constants (k_e) for copepods fed *Uronema* or *Oxyrrhis* reveals that Ag, Cd, Fe, and Zn are lost more rapidly following ingestion of the latter while the reverse is true for Hg (Table 3; Fig. 4). Efflux rates for Ag, Cd, and Zn ingested with diatoms are comparable with one of the two protozoa, but phytoplankton Fe and Tl are excreted more slowly. Thus, although copepod efflux rates do not always show a consistent relationship to prey type, higher assimilation was accompanied by faster loss for four of the six metals tested (Ag, Fe, Hg, and Tl). This suggests that metal assimilated from protozoan prey may partition into a different physiological compartment with a faster turnover rate than metals assimilated from phytoplankton. Zinc (and possibly Cd, which can act as an analog for Zn in some cases) may be actively regulated by copepods, as in other marine invertebrates (Wang and Fisher 1996), and therefore behave differently.

In order to assess the impact of the different uptake and loss rates on metal accumulation, we have used a biokinetic model to calculate the steady-state metal concentrations in marine copepods feeding on either phytoplankton or one of the two protozoa used in this study. This model, which has been field tested for evaluating metal (including Ag, Cd, and Zn) accumulation in marine copepods (Fisher et al. 2000), considers metal accumulation from both the dissolved phase and food, as well as the efflux of metal from the organism following uptake from both pathways, to calculate the resulting metal concentration within the organism at steady state (e.g., Reinfelder et al. 1998; Wang and Fisher 1998). The model is given as

$$C_{ss} = \frac{C_w \times k_u}{k_{ew} + g} + \frac{C_f \times IR \times AE}{k_{ef} + g} \quad (1)$$

where C_{ss} is the concentration of a given metal in the organism at steady state ($\mu\text{g g}^{-1}$), C_w is the dissolved metal concentration (g L^{-1}), k_u is the uptake rate constant from water ($\text{L g}^{-1} \text{d}^{-1}$), k_{ew} and k_{ef} are the efflux rate constants following uptake from water and food (d^{-1}), respectively, C_f is the metal concentration in the protistan prey ($\mu\text{g g}^{-1}$), IR is the ingestion rate ($\text{g g}^{-1} \text{d}^{-1}$), AE is the assimilation efficiency (%) of the ingested metal, and g is the growth rate constant (d^{-1}). Dissolved metal concentrations from Sañudo-Wilhelmy and Gill (1999) and model coefficients from Wang and Fisher (1998) were used to calculate the steady-state Ag, Cd, and Zn concentrations in copepods feeding on either phytoplankton, ciliates, or heterotrophic dinoflagellates in a coastal system with elevated metal concentrations such as the Hudson River estuary. The model parameters and resulting concentrations for each metal in copepods are given in Table 4. Note that we used the metal efflux rate constants for phytoplankton-fed copepods measured following the pulse feeding (Wang and Fisher 1998: Table 1), as these are more comparable with our data.

In order to calculate C_{ss} , it is necessary to know the metal concentration in the prey, C_f , be it phytoplankton or protozoa. Unfortunately, there are currently no published studies that compare the metal concentrations of co-occurring phytoplankton and protozoa. In fact, there are no published measurements of trace elements in natural populations of heterotrophic protozoa at all, and the data on metal concentrations in natural assemblages of phytoplankton are complicated by the inclusion of co-occurring abiotic particles and heterotrophic cells in the same size range. This dearth of data stems from the difficulty in separating autotrophic and heterotrophic protists of the same size with standard size-fractionation techniques. Bulk element analysis techniques that rely on filtration to separate and concentrate protists, therefore, cannot distinguish between these groups, which are generally grouped together as plankton (e.g., Martin and Knauer 1973). New techniques that enable cell-specific element analysis, such as synchrotron x-ray fluorescence microscopy (SXRF) (Twining et al. 2003), should enable comparisons between the elemental composition of autotrophs and heterotrophs, but as yet there is a sparse data set available for metal concentrations in field-collected protists. Therefore, we have assumed that protozoa concentrate Ag, Cd, and Zn to a similar extent as phytoplankton and applied the volume concentration factors ($(\text{mol metal } (\mu\text{m}^3 \text{ cell})^{-1}) \div (\text{mol metal } (\mu\text{m}^3 \text{ ambient water})^{-1})$) measured for phytoplankton to protozoa. This is the simplest assumption to make given the lack of species-specific data, and this calculation most clearly highlights the role of the varying assimilation efficiencies and efflux rate constants in altering the resulting mesozooplankton metal concentrations.

If protozoa and phytoplankton metal concentrations are equal, the Ag concentrations in copepods eating protozoa are predicted to be 1.5–2 times higher than in animals eating phytoplankton, and Zn levels should be 44% higher in copepods consuming ciliates than in copepods grazing either phytoplankton or heterotrophic dinoflagellates (Table 4). In

Table 4. Biokinetic model parameters and resulting steady-state metal concentrations for copepods consuming either diatoms, ciliates (*Uronema* sp.), or heterotrophic dinoflagellates (H. dino; *Oxyrrhis marina*). Dissolved metal concentrations (C_w) are from Sañudo-Wilhelmy and Gill (1999) for the Hudson River Estuary, the ingestion rate (IR) was taken from Lonsdale et al. (1996) as calculated by Wang and Fisher (1998), the uptake (k_u) and efflux (k_{ew}) rate constants for dissolved metals, as well as the growth rate (g) and the diatom assimilation efficiencies (AE) are from Wang and Fisher (1998). The concentrations of metals in prey (C_f) were calculated from C_w using volume concentration factors (VCF) for phytoplankton from Fisher and Wentz (1993) (Ag: 6×10^4 , Zn: 1×10^4) and Fisher and Reinfelder (1995) (Cd: 1×10^3).

	C_w ($\mu\text{g L}^{-1}$)	k_u ($\text{L g}^{-1} \text{d}^{-1}$)	k_{ew} (d^{-1})	C_f ($\mu\text{g g}^{-1}$)	IR ($\text{g g}^{-1} \text{d}^{-1}$)	AE (%)	k_{ef} (d^{-1})	g (d^{-1})	C_{ss} ($\mu\text{g g}^{-1}$)	% metal from food
Ag										
Diatom	0.01	10.42	0.173	1.75	0.42	13.5	0.388	0.09	0.64	33
Ciliate	0.01	10.42	0.173	1.75	0.42	58.6	0.355	0.09	1.39	69
H. dino	0.01	10.42	0.173	1.75	0.42	82.0	0.874	0.09	1.06	59
Cd										
Diatom	0.11	0.694	0.108	0.30	0.42	34.9	0.519	0.09	0.47	16
Ciliate	0.11	0.694	0.108	0.30	0.42	92.3	0.374	0.09	0.65	39
H. dino	0.11	0.694	0.108	0.30	0.42	82.8	0.560	0.09	0.56	29
Zn										
Diatom	6.5	3.29	0.108	175	0.42	61.0	0.265	0.09	235	54
Ciliate	6.5	3.29	0.108	175	0.42	77.7	0.158	0.09	339	68
H. dino	6.5	3.29	0.108	175	0.42	70.7	0.310	0.09	238	55

the case of Cd, the higher assimilation of protozoa metal is largely balanced by the faster loss of the assimilated metal, resulting in metal concentrations only 20–40% higher. Of course, the absolute metal concentrations in copepods will ultimately depend on dissolved ambient metal concentrations, which will affect the prey metal concentration and the solute metal contribution to the copepods. Because the dissolved metal concentrations shown in Table 4 for a contaminated estuary far exceed surface-water concentrations in open ocean waters (Donat and Bruland 1995), copepods in uncontaminated waters would also have proportionately lower metal body burdens.

The higher metal body burdens calculated for copepods feeding on protozoa may have toxicological implications. Silver body burdens calculated for copepods eating phytoplankton are 37% higher than the measured effect concentration ($0.46 \mu\text{g g}^{-1}$) (Hook and Fisher 2001b), but Ag body burdens in copepods grazing on protozoa are two- to three-fold higher than sublethal concentrations. In the case of Cd, body burdens calculated here are below those found to cause sublethal toxicity in copepods (Hook and Fisher 2001a), but calculated Zn concentrations for all three prey types are above those found to exert sublethal toxicity in copepods (Hook and Fisher 2002), although within the range of concentrations measured for natural zooplankton populations (Fisher et al. 2000). This apparent anomaly can be explained by considering the rates of Zn uptake relative to the rates of production of detoxifying compounds (Hook and Fisher 2002; Rainbow 2002). The higher assimilation of these protozoan metals also increases the importance of food as a source of Ag and Cd to the copepods. For example, while animals ingesting phytoplankton will only accumulate 33% of their total Ag body burden from food, copepods feeding on protozoa will accumulate an average of 64% from prey. Given the higher sensitivity of invertebrate zooplankton to metals accumulated from food (Hook and Fisher 2001a,b),

copepods living in the Hudson River estuary may experience a heightened risk of sublethal toxicity (i.e., reduced egg production, hatching rate, ovary development, and egg protein content)—particularly from Ag—in the summer, when protozoa may be an important food source for copepods (Lonsdale et al. 1996).

The biokinetic model discussed above relies on a suite of measurements that may vary by location or season (such as ingestion rate and metal concentrations in the water and food) or that may not be available for all metals (such as dissolved uptake and efflux rate constants for Fe and Hg). Another way to compare the fates of metals ingested by copepods is to calculate the ratio of assimilation efficiency to efflux rate constant (AE/k_e) for phytoplankton and protozoan food; this ratio is analogous to the trophic transfer potential discussed by Reinfelder et al. (1998). For Ag, Cd, and Zn, the AE/k_e ratio is higher for one or both of the protozoa than for phytoplankton, while the opposite is true for Fe (Fig. 4). Thus, for Ag, Cd, and Zn, trophic transfer would be more efficient in food chains sustained by a protozoan diet. However, biomagnification of these metals along the food chain, even involving copepods feeding on protozoa, is unlikely given the high efflux rates of these metals (Reinfelder et al. 1998).

In the case of Fe, the higher Fe assimilation efficiencies combined with the lower Fe AE/k_e ratios for protozoa prey suggest that Fe may be recycled more efficiently by mesozooplankton consuming protozoa than animals consuming phytoplankton. Hutchins and Bruland (1994) also noted that more Fe may be recycled more efficiently through a protozoa–mesozooplankton food chain. Although the experiments in the present study were not conducted at the low Fe concentrations typical of oceanic waters, Schmidt et al. (1999) found that copepods assimilated more Fe from Fe-limited diatoms and excreted the assimilated metal at a faster rate. If such an effect held for protozoa prey, it would further

enhance remineralization of protozoan Fe. This may have biogeochemical consequences in high-nutrient, low-chlorophyll regions where recycled Fe is thought to sustain much of the primary production (Landry et al. 1997).

This study provides the first direct measurements of trophic transfer of Ag, Cd, Fe, Hg, Tl, and Zn from heterotrophic protozoa to mesozooplankton. The results suggest that metals contained within some protozoa are more efficiently assimilated by copepods than metals in phytoplankton. Phagotrophic protists display remarkable taxonomic diversity, however, and significant differences in assimilation efficiency and efflux rates were observed even between the two species used here. Therefore, future studies should examine metal trophic transfer with a wider range of organisms. The higher bioavailability of some metals was correlated with a higher fraction of metals in the cytoplasmic, or liquid, fraction of the protozoa. This matches the relationship observed previously with phytoplankton and suggests that copepods use a similar digestive strategy for both types of prey. These differences, as well as significant differences in the efflux rates of the assimilated metals, have implications for both the bioaccumulation of toxic metals and remineralization of nutrient metals in systems where protozoa comprise a major fraction of mesozooplankton prey.

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