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Reproductive toxicity of metals in calanoid copepods

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Abstract This study investigated the effect of exposure route on metal accumulation, tissue distribution, and toxicity in the marine copepods *Acartia hudsonica* and *A. tonsa*. Sublethal toxicity was measured as decreases in egg production, hatching rate, ovarian development and protein (yolk) content of the egg. When algal food, exposed to Hg at 1 nM or Cd at 5 nM resulting in cells containing 34 and 64 nmol metal g^{-1} dry weight, respectively, was ingested over a 4-h period by copepods, the total copepod body burden increased nine-fold for Hg and two-fold for Cd over background concentrations, and egg production decreased by 50%. Sublethal concentrations of metals were >2 orders of magnitude lower than LC_{50} concentrations. Hatching rate, ovarian development and egg protein content all decreased following trophic exposure to metals, implying that the process of yolk accumulation (vitellogenesis) was affected. Exposure to dissolved Cd had no effect, but dissolved Hg at concentrations as low as 0.25 nM did affect egg production. Different toxic effects following different exposure routes were related to different metal distributions in the copepods: exposure to dissolved metal resulted in metal deposition in the exoskeleton, whereas exposure to dietary metal resulted in metal deposition in internal tissues. These findings indicate that enrichment of metal concentrations in internal tissues, which occurs primarily after exposure to dietary metal, affects vitellogenesis. The reproduction rate decreases by about 75% at metal concentrations only moderately higher than levels in coastal waters. Toxicity tests involving aquatic animals need to consider effects following uptake by different pathways, including the trophic transfer of metals.

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Introduction

Water quality criteria for potentially toxic substances are established in part through the application of toxicity tests, which typically measure the mortality of a test species following a period of exposure to a dissolved contaminant. Standardized test protocols generally focus on a few organisms and measure lethal toxicity, but do not consider uptake of contaminants from food, do not account for contaminant bioavailability, and usually overlook sublethal toxic effects (Luoma 1995; Wood et al. 1997). While these tests can provide useful information in evaluating the potential toxicity of a contaminant, they may not accurately predict toxicity as it occurs in natural systems.

Although contaminants are only toxic if they are incorporated into an organism's tissues, the contaminant concentrations in tissue (the body burden) are seldom measured in the course of toxicity tests. Instead, most studies express toxic effects as a function of the ambient contaminant concentration rather than a function of the contaminant concentration in tissue. There are many chemical and biological factors that influence metal uptake, and thereby affect toxicity, complicating the relationship between ambient metal concentrations and toxic responses. For instance, the bioavailability of metals typically decreases with increases in DOC, salinity and hardness (Campbell 1995; Sunda and Huntsman 1998). In particular, dissolved organic carbon (DOC)–metal interactions can be complex and are influenced by the composition of the DOC (Phinney and Bruland 1994; Sunda and Huntsman 1998; Roditi et al. 2000). Biological factors, such as age, size and sex can also modify uptake and elimination of metals (Langston and Spence 1995; Wang and Fisher 1997).

Relatively few studies have evaluated the toxic effects of contaminants, particularly metals, on marine zooplankton, despite the fact that these organisms are dominant grazers as well as important food sources in marine food webs. Further, diverse zooplankters can

greatly influence the biological recycling of metals within surface waters and their flux out of surface waters. For example, elements that are assimilated by copepods can be passed to higher trophic levels, whereas those that are unassimilated and packaged in fecal material sink rapidly out of surface waters within days (Fowler and Knauer 1986; Fisher and Reinfelder 1995).

Copepods accumulate metals by assimilating them from their food or by absorbing them from water (Reinfelder et al. 1998; Wang and Fisher 1998); the relative importance of a given uptake pathway varies considerably among metals. The uptake pathway of a metal can determines its internal distribution (Hare et al. 1991; Wang and Fisher 1998), hence evaluating the toxicity of an element that is principally taken up from food (e.g., Se in copepods: Fisher and Reinfelder 1991; Wang and Fisher 1998) by using a toxicity test in which the animals are only exposed to the dissolved form would be inappropriate. Very few studies (Sunda et al. 1987; Hook and Fisher 2001) have examined the toxic effects of metals in marine zooplankton taken up from food.

We therefore conducted a series of experiments to examine the role of exposure route and internal metal distribution on metal toxicity to marine copepods. We focused on sublethal toxicity in assessing metal effects on the copepods. Recently we reported that egg production and viability in marine and freshwater crustacean zooplankton are the most sensitive parameters following dietary exposure to Ag (Hook and Fisher 2001). In this study we have chosen to focus on reproductive impairment caused by Cd and Hg. We compared the toxicity of these metals to Ag following exposure through both food and water, and related toxic effects to internal metal concentration.

Materials and methods

Calanoid copepods (*Acartia tonsa* and *A. hudsonica*) were collected from uncontaminated Stony Brook harbor at high tide using a 64-μm plankton net. Adults were sorted from the total sample and kept at 15–25°C, depending on season. Animals were maintained in the laboratory for at least 24 h before experiments began. Prior to experiments, copepods were maintained on a mixed phytoplankton diet consisting of the diatom *Thalassiosira pseudonana,* the cryptophyte *Rhodomonas salinas,* the prymnesiophyte *Isochrysis galbana,* and the dinoflagellate *Prorocentrum minimum*. No significant differences were noted in how copepods accumulated or responded to metals in different seasons or at different temperatures.

For experimental treatments in which copepods were exposed to dissolved metals, experimental water was prepared by adding Cd to 0.2 μm filtered surface seawater collected 8 km off Southampton, Long Island, N.Y., to produce concentrations of 0, 1, 2, 5, or 10 nM Cd (from a stock solution in 0.1 N Optima HCl) or Hg to produce concentrations of 0, 0.25, 0.5, 1 or 2 nM Hg (in 1 N Optima HCl). For each treatment, there were five replicate flasks containing 200 ml and 20 adult copepods. Following additions of metal, the pH was adjusted to 8.0 by adding dilute NaOH. Dissolved solutions were prepared at least 12 h prior to experiments to allow metals sufficient time to react with ligands present in the seawater (Ma et al. 1999). Copepods were exposed to these solutions for 12 h without food to ensure that metal exposure was from the dissolved phase only. Following exposure, animals were transferred to filtered seawater, fed the mixture of algal cells noted above, and toxic effects were assessed (see below).

To expose copepods to metals via food, *T. pseudonana* cells were grown in f/2 media (Guillard and Ryther 1962), but containing no added Cu, Zn, or EDTA, prepared with sterile-filtered Southampton seawater. Concentrations of Cd and Hg were the same as those used for solute exposure tests with the copepods. Cells were grown for six to ten generations (3–5 days) to ensure a uniform metal concentration per cell for each treatment. Cells were harvested onto 1-μm Nuclepore polycarbonate membranes and resuspended in 0.2 μm filtered seawater to yield a cell density of 10^4 cells ml^{-1} (or 22 μg dry weight l^{-1}). The copepods were fed metal-contaminated cells (metal concentrations in cells given in Table 1) for 4 h, during which metal exposure was only from ingested food, and were then transferred to 0.2 μm filtered seawater and fed a mixture of uncontaminated algal cells as above.

Toxicity was monitored for a week following exposure. Lethal effects were determined by exposing three replicates of 20 copepods to metal solutions for 48 h continuously, following standard protocols (American Society for Testing and Materials 1995). Periodically during exposure, animals were inspected using a dissecting microscope, and surviving animals were returned to solution. Exposure periods were 48 h only to avoid starvation-based mortality. To determine sublethal toxicity, replicate samples were gravity-filtered onto two Nitex meshes, 210 μm to catch adults, 60 μm to catch eggs. The number of surviving animals and the eggs produced were counted using a dissecting microscope. Those eggs that were produced were put into one of three replicate flasks with filtered seawater and *I. galbana* and monitored for 4 days to determine hatching rate. Thus, hatching rate and egg production were measured independently of each other. Following hatching, nauplii were counted. To determine the percentage of females with devel-

Table 1 Tissue concentrations of Hg and Cd (nmol g^{-1} dry weight) in diatom food and copepods. Metal concentrations in the copepods are given following uptake from food and from the dissolved phase. For comparison, data for Ag are also given (Hook and Fisher 2001). Tissue metal concentrations were determined using parameters given in Table 2 following a kinetic model (Wang and Fisher 1998). Background Cd and Ag concentrations (0 added metal) are from Fisher et al. (2000) and background Hg concentration from Fowler (1977). The body burden at which toxicity was first observed is given in bold. No sublethal toxic response was observed following exposure to dissolved Ag and Cd

Exposure concentration (nM)	Hg					Cd					Ag				
	0	0.25	0.5	1	2	0	1	2	5	10	0	0.5	1	2	5
Food															
Concentration in diatoms		8.5	16.9	33.8	67.7		12.8	25.6	64	128		19	39	77	193
Copepod body burden	0.3	0.9	1.5	**2.7**	5.0	22.1	26.1	30.0	**42.0**	61.8	1.3	2.7	**4.3**	7.1	15.9
Internal body burden added		0.6	1.2	**2.3**	4.7		3.1	6.1	**15.3**	30.6		1.2	**2.7**	5.1	13.0
Dissolved															
Copepod body burden	0.3	**26.7**	41.5	71.0	130	22.1	23.1	24.0	26.8	31.5	1.3	2.4	3.7	6.1	13.1
Internal body burden added		**1.9**	2.9	5.0	9.1		0.9	1.2	2.5	4.2		0.4	0.8	1.7	4.2

oped ovaries, adults were fixed with formalin 2–3 days after exposure to metals and their reproductive state characterized (Mauchline 1998; Niehoff 1998;). If a darkened ovary was observed, the animal was considered developed.

Eggs produced were also analyzed for total protein content. To prepare protein samples for analysis, 500 eggs from each treatment were collected, filtered onto a GF-F glass fiber filter, and homogenized using a Biospec Products mini-bead beater with 500 µl of 10 mM phosphate-buffered saline solution (Sigma). The homogenized sample was centrifuged at 14,000 *g* and the supernatant, which contained the yolk protein (Lee and Walker 1995) but none of the impurities associated with phytoplankton debris and fecal material, was collected and analyzed for total protein content using BCA analysis (Smith et al. 1985).

To relate metal toxicity to tissue body burdens of metals, parallel experiments using the gamma-emitting radioisotopes ^{109}Cd and ^{203}Hg were conducted. These experiments followed the same exposure protocols as described above and used the same total metal concentrations. Total metal concentrations were adjusted by adding appropriate amounts of stable metal to water with radioactive metal. When zooplankton were exposed via food, uniformly radiolabeled *T. pseudonana* cells were harvested (Wang and Fisher 1998) when cell densities were at 1.3×10^6 ml^{-1} (= 29.1 µg ml^{-1}) and fed to copepods as in the toxicity experiments described above. Radioactivity in the copepods was measured at the end of feeding, and at regular time intervals afterwards to determine the assimilation efficiency of metals, as described in Fisher et al. (1991) and Wang and Fisher (1999a). When copepods were exposed to dissolved metals, solutions were made by adding ^{203}Hg or ^{109}Cd to 0.2 µm filtered seawater, as described previously. Amounts of added radioactivity were adjusted to ensure sufficient counts in all samples. For ^{203}Hg, 29.6 kBq l^{-1} were added to the algal suspensions to radiolabel the diatoms, and 20.7 kBq l^{-1} were added to the suspensions of copepods exposed to dissolved Hg. For ^{109}Cd, 88.8 kBq l^{-1} were added to the algal suspensions to radiolabel the diatoms, and 148 kBq l^{-1} were added to the suspensions of copepods exposed to dissolved Cd. Radioactivity in the copepods was measured at the end of uptake and periodically after different periods of depuration to determine metal absorption (Wang and Fisher 1998). As shown in previous studies (Wang et al. 1996), it was confirmed that metals desorbed from algal prey cells into the dissolved phase were a negligible source of metal to the copepods during the 4-h feeding period. The radioactivity of all samples was measured using a Pharmacia-Wallac LKB Compugamma counter equipped with a NaI(Tl) well detector. The gamma emissions of ^{109}Cd were determined at 88 keV and of ^{203}Hg at 279 keV. Counting times were adjusted to yield propagated counting errors of < 5%.

Metal body burdens were determined using the uptake and assimilation parameters obtained from these radiotracer experiments and a kinetic model (Wang and Fisher 1998). Uptake parameters used to determine body burdens are given in Table 2. The increase in copepod metal concentration following exposure from food was determined to be: (metal concentrationper diatom cell)× (the number of cells eaten) × (the assimilation efficiency)/the dry weight of the copepod. The feeding rate of each copepod was taken to be 2 µg dry weight cells per 4-h feeding period, an intermediate feeding rate for these copepod species (Mauchline 1998). Following uptake from water, the increase in metal concentration in copepods was: (metal absorbed per copepod)/(the dry weight of the copepod . These experimental increases were added to the background metal concentrations in zooplankton, using data from field-collected zooplankton from relatively uncontaminated waters in the Ligurian Sea [Cd, 22 nmol g^{-1} dry weight in copepods, Fisher et al. (2000); Hg, 0.3 nmol g^{-1} dry weight in euphausiids, Fowler (1977)].

To determine how the metals were deposited in the copepods following exposure, copepod tissues were separated using a chemical fractionation technique (Munger and Hare 1997; Wang and Fisher 1998). Zooplankton were exposed to radioisotopes, via either food or water, as previously described. Metals were assumed to be in one of three fractions: either exchangeably bound (in which case they are released with an EDTA rinse), bound to the exoskeleton, or bound to internal tissues. After 12 h exposure, animals were collected on a 210-µm mesh, rinsed with filtered seawater, then with a solution of 200 µM EDTA in filtered seawater, after which they were rinsed onto a 10-µm polycarbonate membrane and dissolved in 2 ml of 0.2 N NaOH heated to 60°C for 12 h. Preliminary experiments indicated that this dissolution time was sufficient to dissolve all internal tissues but not the exoskeleton. Following dissolution, the zooplankton debris was filtered onto a 10-µm polycarbonate filter and rinsed with 1 ml of 0.2 N NaOH. Exoskeletons were then separated from dissolved internal tissue using a 10-µm filter.

Table 2 Parameters used in modeling uptake and distribution of Cd and Hg following exposure through food (the diatom *Thalassiosira pseudonana*) and from the dissolved phase in *Acartia* spp. For comparison, data for Ag are also given (Hook and Fisher 2001). Concentration factors are on a dry weight basis. Uptake and distribution parameters are based on values obtained from means of three replicates, 50 copepods per replicate. *n.d.* Not determined

	Hg	Cd	Ag
Food			
Concentration factor in *T. pseudonana*	1.4×10^5	1.8×10^4	2.3×10^5
Assimilation efficiency	14%	62%	15%
Distributed internally	99%	77%	89%
Dissolved			
Concentration factor in copepod	1.3×10^4	1.0×10^3	3.0×10^3
Absorption efficiency	59%	n.d.	30%
Distributed internally	7%	40%	35%

Results

Following exposure to Hg and Cd via food, egg production decreased in *Acartia* sp. by at least 50% (Fig. 1). This decrease occurred when food was exposed to 1 nM Hg and 5 nM Cd (Table 3), yielding metal concentrations in the algal food of 28 nmol Hg g^{-1} and 60 nmol Cd g^{-1}, respectively (Table 1). Previously published data on the response of copepods to Ag (Hook and Fisher 2001) are also given in Fig. 1 and Table 1 for comparison. When animals were exposed to similar concentrations of dissolved metals, egg production was not affected by Cd at any tested concentration but decreased following exposure to ≥0.25 nM Hg (Fig. 1). Concentrations ≥300 nM Hg or 1,000 nM Cd caused mortality of *Acartia* (Table 4). Thus, sublethal reproductive effects occurred at metal concentrations 2–3 orders of magnitude below concentrations that caused lethality (Table 4).

Exposure route also caused differences in metal distribution (Table 5). When metals were accumulated by copepods from ingested food, they were primarily distributed in internal tissues, whereas metals taken up from the dissolved phase were primarily in the exoskeleton. For Hg, > 90% of the total burden was deposited in internal tissues following exposure through food and nearly 100% was associated with the exoskeleton following solute exposure. For Ag, 89% was internal following exposure from food and 54% was in the

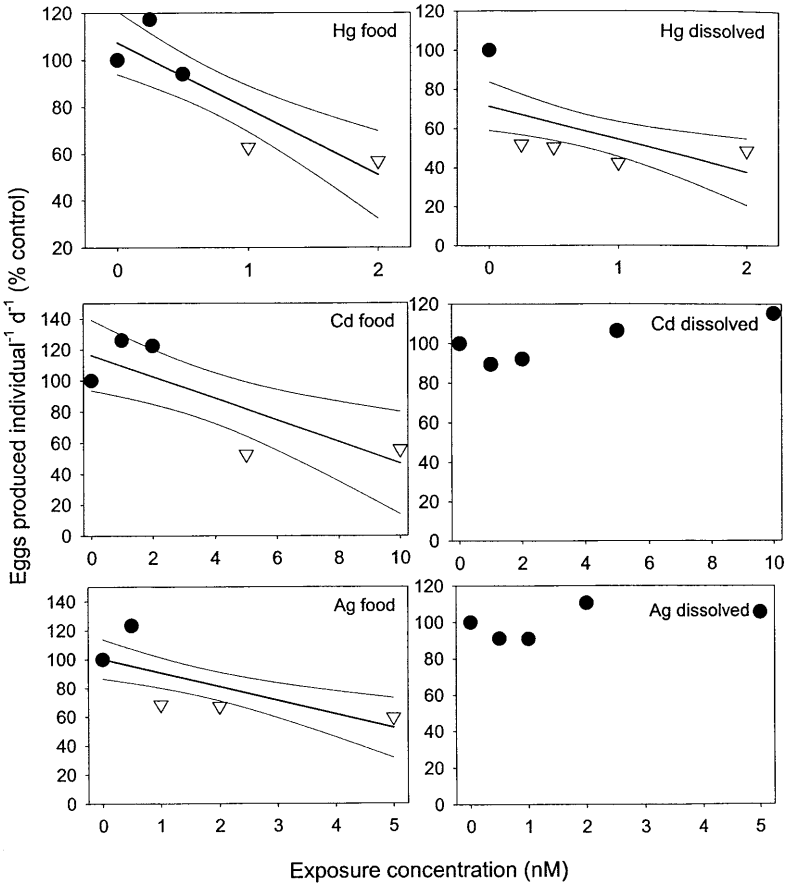

Fig. 1 Egg production individual^{-1} day^{-1} as % control in the copepods *Acartia tonsa* and *A. hudsonica* following exposure to dietary or dissolved Ag, Hg, and Cd. The *x-axis* denotes the dissolved metal concentration (nM) to which either the algal food (*left-hand panels*) or copepods (*right-hand panels*) were directly exposed. Points shown are means of five replicates, 20 adult copepods per replicate. Egg production was significantly lower than controls (Student's *t*-test, $P < 0.05$) when food was exposed to 1 nM Hg or 5 nM Cd (denoted by *open triangles*). Egg production was significantly lower than in controls at 0.25 nM dissolved Hg. *Lines* and *95%* confidence intervals are drawn to depict trends and use least squares linear regression by a Levenberg-Marquart iterative search procedure (Sigma Plot 4.0). Previously published (Hook and Fisher 2001) Ag data are also plotted for the purpose of comparison with Hg and Cd. All *lines* shown are significant ($P < 0.05$). Equations for the regression lines (drawn through all data points) are: Hg (food), $y = -28.1x + 107.3$, $r^2 = 0.453$; Hg (dissolved), $y = -16.8x + 71.5$, $r^2 = 0.260$; Cd (food), $y = -7.0x + 116.4$, $r^2 = 0.299$; Ag (food), $y = -9.5x + 100.1$, $r^2 = 0.238$

exoskeleton following dissolved-phase exposure. For Cd, 77% was internal when taken up from food and 46% was in the exoskeleton when taken up from water. Additionally, 11% of the Ag and 13% of the Cd accumulated following solute exposure were removable with EDTA rinsing (Table 5).

When toxicity results were expressed as a function of metal body burden, effects of food-borne metals on egg production were evident for Hg and Cd at ≥2.7 and ≥42.0 nmol g^{-1} dry weight tissue, respectively, for whole animals and ≥2.3 and ≥15.3 nmol g^{-1} dry weight tissue, respectively, in internal tissues (Table 1). Hg accumulated from the dissolved phase depressed egg production at tissue concentrations ≥26.7 nmol g^{-1} dry weight tissue for whole animals and ≥1.9 nmol g^{-1} dry weight in internal tissue, but body burdens following exposure to dissolved Cd had no significant toxic effect on egg production (Table 1). For comparison, results of Ag toxicity experiments indicate that, like Cd, only the dietary metal exerts a toxic effect on egg production, at concentrations ≥4.3 nmol g^{-1} dry weight tissue in whole animals and ≥2.7 nmol g^{-1} dry weight in internal tissues (Table 1).

After copepods were exposed to metals, the toxic effects on the hatching rate of produced eggs showed a similar pattern to that of egg production. Following exposure to Hg and Cd via food, hatching rate decreased by ≥50% when the parent's food was exposed to 1 nM Hg or 5 nM Cd (Fig. 2, Table 3). As with egg production, dissolved Hg exposure also affected hatching rate (Fig. 2, Table 3). Egg hatching rate was diminished following parental exposure to dissolved Hg concentrations > 0.25 nM Hg, whereas dissolved Cd had no detectable effect in the nanomolar range. Previously published results for Ag (Hook and Fisher 2001) are plotted in Fig. 2 for comparison.

Ovarian development also declined following exposure to dissolved Hg, and to Hg and Cd via food (Fig. 3, Table 3). The concentrations of metal required to reduce ovarian development were similar to those necessary to depress egg production: 0.25 nM dissolved Hg, 1 nM Hg via food, and 5 nM Cd via food (Fig. 3). Figure 3 also

Table 3 Response of copepods (% of controls) exposed to dietary or dissolved metals. Exposure concentrations are metal concentrations to which algal food or copepods were directly exposed. Egg production data for all metals and hatching rate data for Hg and Cd are means ±1 SD; all other data were evaluated with analysis of frequencies (Sokal and Rohlf 1976) ($n = 28$ per treatment for Ag egg hatching, $n = 50$ per treatment for ovarian development)

			Exposure Concentration (nM)				
			0	0.25	0.5	1	2
Hg	Egg production	Food	100 ± 5	117 ± 20	94 ± 8	63 ± 7*	56 ± 7*
		Dissolved	100 ± 10	52 ± 7*	51 ± 4*	42 ± 2*	49 ± 5*
	Hatching rate	Food	100 ± 9	89 ± 18	92 ± 12	70 ± 17*	54 ± 4*
		Dissolved	100 ± 15	57 ± 5*	56 ± 9*	58 ± 7*	53 ± 4*
	Ovarian development	Food	100	85	81	71**	53**
		Dissolved	100	75	54**	37**	31**
			0	1	2	5	10
Cd	Egg production	Food	100 ± 17	126 ± 16	122 ± 34	53 ± 3*	56 ± 7*
		Dissolved	100 ± 14	89 ± 10	92 ± 23	106 ± 22	115 ± 20
	Hatching rate	Food	100 ± 18	106 ± 26	80 ± 17	53 ± 23*	53 ± 14*
		Dissolved	100 ± 18	85 ± 8	86 ± 11	82 ± 13	85 ± 14
	Ovarian development	Food	100	106	76	66**	30**
			0	0.5	1	2	5
Ag	Egg production	Food	100 ± 12	123 ± 16	69 ± 12*	67 ± 16*	59 ± 9*
		Dissolved	100 ± 17	91 ± 16	91 ± 28	110 ± 32	106 ± 23
	Hatching rate	Food	100	120	83	31**	50**
		Dissolved	100	98	110	79	96
	Ovarian development	Food	100	79	58**	n.d.	35**

*$P < 0.05$ (significant difference from control using Student's *t*-test), **$P < 0.01$ (significant difference using goodness-of-fit frequency analysis)

contains previously published data obtained for Ag plotted for the purpose of comparison (Hook and Fisher 2001).

The protein content of the eggs produced by adults exposed to Hg, but not Cd, declined (Table 6). When the food was exposed to 1 nM Hg, the protein content of eggs decreased by approximately 50%. Exposure to Hg concentrations as low as 0.25 nM dissolved Hg also depressed egg protein concentrations. There were insufficient eggs produced by copepods exposed to 2 nM dissolved Hg to measure protein accurately.

Discussion

These findings demonstrate that exposure to metals via food causes reduced reproductive output in copepods when the algal food is exposed to metals at the nanomolar level. Since both hatching rate and egg production decrease by 50%, the total reproductive rate of the copepods decreases by about 75%. We found these reductions (and no other effects) in both copepod species regardless of the season in which the animal was collected, indicating that stress was not a significant contributor towards the observed toxic effects. The effects on egg production and hatching are consistent with previous work on Ag toxicity in *Acartia* (Hook and Fisher 2001) in which exposure to 1 nM Ag via food caused a 50% reduction in egg production and hatching rate, whereas exposure to dissolved Ag had no discernible effects. These results also agree with another study which examined the influence of metal accumulation via food on the reproductive rates of copepods (Sunda et al. 1987) in which copepods exposed to Zn displayed a decreased reproductive rate at nanomolar concentrations. Overall, these impacts would be missed by studies which only examine lethal toxicity or studies measuring effects of dissolved metals. Lethal toxicity occurred at concentrations 200–400 times higher than those which caused sublethal effects.

Table 4 Lethal (LC_{50}) and sublethal (PC_{50}) concentrations of Hg, Cd, and Ag for *Acartia* spp. LC_{50} is the concentration at which half the population died when exposed to the metal in solution for 96 h. PC_{50} is the concentration to which the phytoplankton food was exposed that resulted in a 50% reduction in egg production by the copepods after 4 h feeding on the food. Lethal experiments represent means of three replicates of 20 individuals. Sublethal values represent means of five replicates of 20 individuals

	PC_{50} (nM)	LC_{50} (nM)	LC_{50}/PC_{50}
Hg	1	300	300
Cd	5	1,000	200
Ag	1	400	400

Table 5 Distribution of metals in *Acartia* spp. (% of total body burden) following uptake from food or water. Samples represent means of three replicates, 50 individuals per replicate

	Hg	Cd	Ag
Food			
Exoskeleton	0	17	11
Internal tissues	100	77	89
Easily exchanged	0	5	0
Dissolved			
Exoskeleton	93	46	54
Internal tissues	7	40	35
Easily exchanged	0	14	11

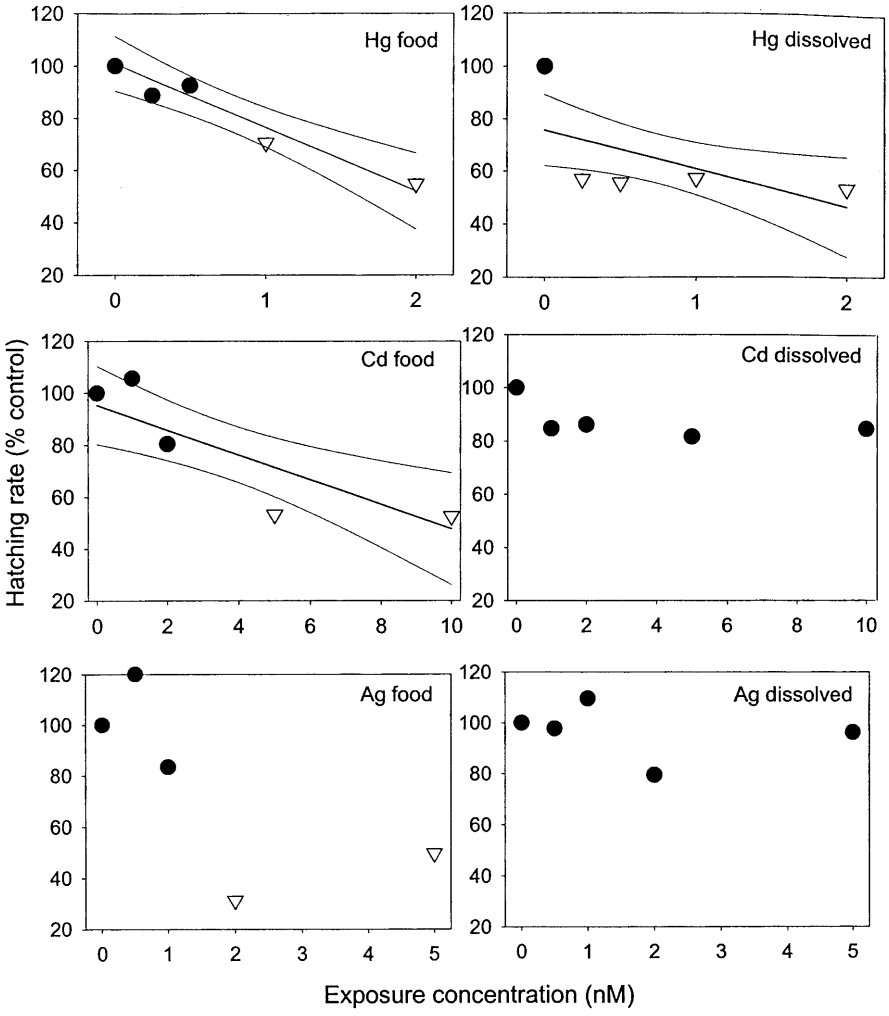

Fig. 2 Egg hatching frequency as % control in the copepods *Acartia tonsa* and *A. hudsonica* following exposure to dietary or dissolved Ag, Hg, and Cd. The *x-axis* denotes the dissolved metal concentration (nM) to which either the algal food (*left-hand panels*) or copepods (*right-hand panels*) were directly exposed. Points shown are means of five replicates, 20 adult copepods per replicate. Hatching was significantly lower than in controls (Student's *t*-test, $P < 0.05$) when food was exposed to 1 nM Hg or 5 nM Cd (denoted by *open triangles*) or when copepods were exposed to dissolved Hg > 0.25 nM. Lines and *95%* confidence intervals are drawn to depict trends and use least-squares linear regression by a Levenberg-Marquart iterative search procedure (Sigma Plot 4.0). Previously published (Hook and Fisher 2001) Ag data are also plotted for the purpose of comparison with Hg and Cd. These data were analyzed using goodness-of-fit frequency analysis (Sokal and Rohlf 1976). All *lines* shown are significant ($P < 0.05$). Equations for the regression lines (drawn through all data points) are Hg (food), $y = -24.4x + 100.5$, $r^2 = 0.659$; Hg (dissolved), $y = 14.5x + 75.6$, $r^2 = 0.288$; Cd (food) $y = -4.7x + 95.4$, $r^2 = 0.466$

In this study, toxicity occurred at concentrations that approach those found in natural waters. For example, Hg was toxic at 1 nM; it has been detected at concentrations as high as 340 pM in Narragansett Bay (Vandal and Fitzgerald 1995), although oceanic surface water concentrations are typically about 1–3 pM, mostly as elemental Hg^0 (Mason et al. 1998). Cd, which was toxic at 5 nM, has been detected at 1.5 nM in San Francisco Bay (Flegal et al. 1991), but again oceanic surface water concentrations are generally about 3 orders of magnitude lower (Bruland 1983). Ag is also sublethally toxic at environmentally realistic concentrations: it is toxic at 1 nM (Hook and Fisher 2001) and has been detected at 250 pM in estuarine waters (Smith and Flegal 1993; Flegal et al. 1996), and again at much lower concentrations (≤ 0.7 pM) in oceanic surface waters (Flegal et al. 1995). Because deeper ocean waters have substantially higher metal concentrations for Hg, Cd, and Ag, upwelling regions may display higher surface water concentrations (especially for Cd) than in open ocean regions (Bruland 1983). Concentrations that are sublethally toxic were well below lethally toxic levels, which are in the micromolar range (Table 4). Algal food became toxic when concentrations of Hg were 34 nmol g^{-1}, a level that is about 170 times particulate concentrations of Hg in oceanic waters (Mason et al. 1998) or about 70 times particulate concentrations in estuarine waters (Riedel et al. 2000). Cd concentrations in food were toxic at concentrations ≥ 64 nmol g^{-1}, or about 130 times phytoplankton concentrations of Cd in coastal waters (Fisher et al. 2000). Concentrations of Ag in algal food become significantly

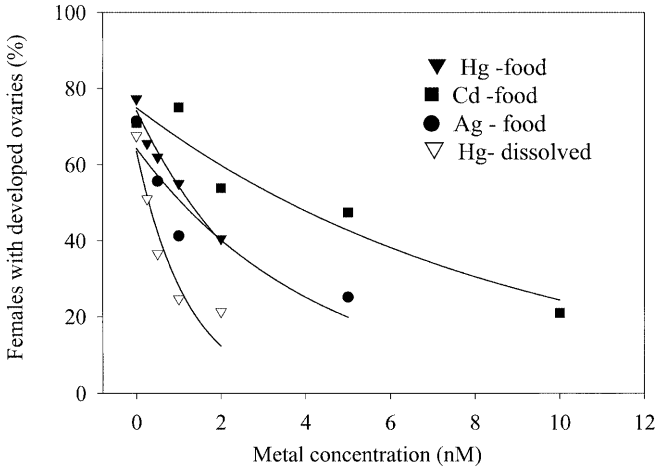

Fig. 3 Ovarian development (% of females with developed ovaries) in the copepods *Acartia tonsa* and *A. hudsonica* following exposure to Ag, Hg and Cd. The *x-axis* denotes the dissolved metal concentration (nM) to which either algal food (*closed symbols*) or copepods (*open symbols*) were directly exposed. Ovarian development was significantly lower than in controls (chi square, $P < 0.01$) when food was exposed to 1 nM Hg, 1 nM Ag, or 5 nM Cd. Ovarian development was significantly lower than in controls (chi square, $P < 0.01$) when animals were exposed to dissolved Hg≥0.25 nM. All *lines* denote a statistically significant toxic response

toxic to egg production at a concentration of 39 nmol g^{-1}, or about 100 times typical coastal concentrations in suspended particles (Fisher et al. 2000). Generally, these findings imply that copepods could be exposed to harmful metal concentrations in urbanized estuaries.

No attempts were made to compare the relative toxicity of metals following long-term exposure with short-term exposures such as used in this study. Experiments involving long-term metal exposures and focusing on physiological acclimation or adaptation to metals should be performed. While acclimation/adaptation to elevated metal concentrations may occur following long-term chronic exposures, we believe that the most direct comparisons of laboratory toxicity data to those of natural copepod populations may be to express toxicity as a function of metal body burdens, recognizing, however, that metals sorbed to the exoskeleton from the dissolved phase (but still contributing to the overall body burden in the copepods) would not exert toxic effects at concentrations likely to be encountered in natural waters.

Calculations of body burdens of metals in the copepods were based on parameters such as metal enrichment in algal food, assimilation efficiency of ingested metal, and distribution of metal in the copepod tissues. Overall, values for these parameters were comparable with findings from other studies. The concentration factors of Hg (1.4×10^5), Cd (1.8×10^4), and Ag (2.3×10^5) in the diatom food (*T. pseudonana*) were within a factor of 2.5 of mean values reported for this species (Hg: 2.5×10^5; Cd: 8.4×10^3; Ag: 9.2×10^4) (Fisher and Reinfelder 1995). The assimilation efficiency of ingested Hg (14%) from this diatom food was comparable to that of other studies with this metal: 15% from *Thalassiosira weissflogii* food in three copepod species, including *A. tonsa* (Mason et al. 1996) and 21% from *I. galbana* food in the copepod *Anomalocera patersoni* (Fisher et al. 1991). The assimilation efficiency of ingested Cd (62%) was within the very large range of assimilation efficiencies (30% to >90%) in diverse copepod species recorded for this metal from different algal diets (Reinfelder and Fisher 1991; Wang et al. 1996; Wang and Fisher 1998). The fractionation of Cd and Ag found in the internal tissues of the copepods following dietary exposure was comparable to Wang and Fisher's (1998) findings with the copepod *Temora longicornis* but generally somewhat lower than Wang and Fisher's findings following solute exposure; Wang and Fisher (1998) did not include Hg in their study. We are not aware of studies which examined the tissue distribution of metals in field-collected copepods, but in a study of the euphausiid *Meganyctiphanes norvegica* in the Mediterranean, only 4% of the total body burden of Hg, 22% of Cd, and 31% of Ag were bound to the exoskeleton (Fowler 1977), indicating that dietary uptake probably is the dominant accumulation pathway in this organism, although long-term equilibrium in metal distributions between internal tissues and exoskeleton may occur following solute uptake.

When expressed as a function of the metal concentration in tissues needed to generate a toxic effect following exposure via food, egg production decreased by 50% when the copepod's tissue metal concentration increased nine-fold for Hg and two-fold for Cd over typical back-

Table 6 Protein content of eggs produced by copepods exposed to metals (expressed as % of controls). Treatment concentrations are those to which either the diatom *T. pseudonana* was exposed (for food), or those to which the copepods themselves were exposed (dissolved)

Hg (food)		Hg (dissolved)		Cd (food)		Ag (food)	
Treatment	Protein content	Treatment	Protein content	Treatment	Protein content	Treatment	Protein content
0 nM	100	0 nM	100	0 nM	100	0 nM	100
0.25 nM	86	0.25 nM	28*	1 nM	101	0.5 nM	n.d.
0.5 nM	95	0.5 nM	25*	2 nM	73	1 nM	78
1 nM	48*	1 nM	49*	5 nM	110	2 nM	56*
2 nM	50*	2 nM	n.d.	10 nM	103	5 nM	56*

*$P < 0.05$ (ANOVA, statistically significant difference from controls, Sokal and Rohlf 1976)

ground concentrations in copepods inhabiting relatively clean coastal waters (Fisher et al. 2000). Cd was not sufficiently accumulated from water to generate a toxic response. The decrease in egg production following exposure to dissolved Hg occurred only when tissue concentrations of Hg increased 50-fold, a much higher body burden than that needed to induce toxicity following exposure via food. Egg production significantly declines when Ag body burdens increase 3.3-fold following dietary exposure, but comparable body burdens obtained via dissolved exposure have no effect (Hook and Fisher 2001).

The relative increase in a metal's concentration in internal tissues seems to be the best predictor of a toxic effect. Exposure to metals via food always resulted in a toxic response given a sufficient internal concentration. Since most of the metal taken up from the dissolved phase is distributed in the exoskeleton, that exoskeletal sorbed metal is less likely to interfere with metabolic processes. A small portion of the metal associated with the copepods was released with the EDTA rinse, regardless of the uptake pathway, indicating that metals bound to the exoskeleton were tightly bound and probably not easily mobilized to result in higher internal metal concentrations. Of the three metals examined from the dissolved phase, only Hg exposure resulted in decreased egg production, probably because the relative increase in internal tissues in the copepods was greatest following Hg exposure. Thus, the increase in internal Hg concentration following exposure to dissolved Hg at 0.25 nM (1.9 nmol g^{-1} dry wt) was simular to that following ingestion of diatoms exposed to 1 nM Hg (2.3 nmol g^{-1}dry wt) (Table 1). None of the exposures to dissolved Cd resulted in increases in internal Cd concentrations approaching that (15.3 nmol g^{-1} dry wt) which depressed egg production following dietary exposure (Table 1). Still, the fractionation technique used in our study is imprecise, and only separates tissue types, not individual organs. One consequence is that based on the range of internal tissue concentrations shown in Table 1, it would be predicted that Ag should interfere with egg production at 5 nM, as this treatment resulted in an internal tissue concentration (4.2 nmol g^{-1}) that was higher than that (2.7 nmol g^{-1}) produced by animals fed algae containing 39 nmol g^{-1} which did display depressed egg production. Since no toxic effect of the dissolved Ag was observed, it is likely that Ag accumulated from food was deposited in different internal organs than Ag accumulated from water.

There are metabolic pathways that could lead to remobilization of metals from the exoskeleton into internal tissues in copepods and other crustaceans. During ecdysis (or molting), Ca salts and metal salts are resorbed (Brusca and Brusca 1990). Crabs normally use metallothioneins to regulate metal levels, which vary in the hemolymph during molting as a result of the breakdown and synthesis of hemocyanin (Engel and Brouwer 1991). Excess metal is then excreted with the feces and urine (Engel and Brouwer 1993). Although metal concentrations in the hemolymph are regulated by metallothionein and glutathione complexes (Engel and Brouwer 1993), it is theoretically possible that in copepods exposed to high concentrations of dissolved metals, these regulatory mechanisms could be overwhelmed, and the resorbed metal could exert a toxic effect. This possibility was not addressed in our study, and variability in individual egg production following ecdysis would have been missed since the copepods did not molt synchronously. However, many metals are not resorbed during molting in crustacean zooplankton and stay bound to the exoskeleton after it is released (Fowler 1977; Fisher et al. 1983). We think it likely that Hg, Cd, and Ag are also not resorbed in copepods during molting.

The mechanism underlying metal toxicity to egg production in copepods appears to be altered vitellogenesis. Normally, during oogenesis, the ovary becomes enlarged and dark as oocytes accumulate lipovitellin (the dominant yolk protein in crustaceans). We have observed that the ovary remained undeveloped and that the eggs contained less protein following exposure to Hg and Cd via food, or to Hg via water. For Cd, Hg, and Ag (Hook and Fisher 2001), the response pattern is the same: the ovaries of the adults are less developed following exposure to metals via food, fewer eggs are produced, the eggs that are produced hatch less frequently and contain less yolk protein. These results imply that metals interfere with the process of yolk accumulation (vitellogenesis) during oogenesis, consistent with an earlier study with Cd in crabs (Lee and Noone 1995). Since vitellogenesis is a common process among oviparous animals, the decline in egg production observed in copepods may occur in other taxa as well. In San Francisco Bay, for instance, the percentage of *Macoma balthica* (clam) individuals with mature gametes is negatively correlated with Ag and Cu body burdens (Hornberger et al. 2000).

Unlike Hg and Ag, exposure to Cd via food did not cause a reduction in egg protein content, even though egg production, hatching rate and ovarian development all showed significant impairment. We have no clear explanation as to why Cd's behavior was unlike that of Ag and Hg, but perhaps the animal detoxified and excreted the Cd dose following the 4-h feeding period before a sufficient quantity of eggs could be collected for protein analysis. We hypothesize that Cd would have a shorter lasting effect than Hg or Ag because Cd's affinity for S (and thus protein) is much lower (Martell and Smith 1977). To better simulate conditions in contaminated environments, future studies should examine egg development following long-term, chronic exposure to metals, especially metals that have weaker affinities for protein like Cd, Pb, and Zn.

The importance of uptake pathway in toxicity seen here suggests that toxicity tests need to consider varying uptake pathways. The inclusion of trophic accumulation in toxicity test design is especially important given that food can often be the main route of contaminant uptake (Fisher and Reinfelder 1995; Wang and Fisher 1999a, b). Expressing toxic effects as a function of contaminant body burden is preferable to reporting effects as a

function of ambient concentrations. The body burden reflects the bioavailable fraction and only this fraction of the ambient metal can elicit a toxic effect.

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