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Relationships between Free Cadmium Ion Activity in Seawater, Cadmium Accumulation and Subcellular Distribution, and Growth in Polychaetes

by Kenneth D. Jenkins*† and Brenda M. Sanders*‡

We have examined the relationships between Cd ion activity $\{Cd^{2+}\}$, in seawater, Cd accumulation and subcellular distribution and growth in the polychaete *Neanthes arenaceodentata*. Organisms were exposed for 3 weeks to a range of $\{Cd^{2+}\}$ in a Cd-chelate buffer system. Cadmium accumulation and growth were monitored weekly for each exposure group and subcellular Cd distributions were determined at the end of the 3-week period. We found Cd associated with all of the subcellular fractions except the very low molecular weight ligands. Total Cd accumulation was greatest at day 7 and decreased over time in all but the highest $\{Cd^{2+}\}$ where it remained constant. For each point in time, however, there was a linear relationship between total Cd and $\{Cd^{2+}\}$ in seawater. Linear relationships were also observed between $\{Cd^{2+}\}$ and Cd loading in each subcellular ligand pool. Specific growth rates varied with both $\{Cd^{2+}\}$ and time in a nonlinear manner.

Introduction

The bioavailability and toxicity of trace metals such as Cd, Cu, and Zn are related to the activity of the free metal ion rather than the total metal concentration (*1–5*). For Cd it is the $CdCl_2$ complex that predominates in seawater (*3*). Therefore, salinity is the overriding factor which can alter free Cd ion activity $\{Cd^{2+}\}$, and hence, bioavailability and toxicity in marine systems. Yet the relationships between $\{Cd^{2+}\}$ in seawater and Cd bioaccumulation, metabolism, and toxicity are not well understood. We do know that the major mechanism underlying these relationships involves interactions between Cd and both membrane-bound and soluble ligands (*6*).

In this study we have examined the relationships between $\{Cd^{2+}\}$ in sea water, Cd bioaccumulation and subcellular distribution, and growth in a polychaetous annelid. These organisms were exposed to a range of several orders of magnitude of $\{Cd^{2+}\}$. Cadmium ion activity was maintained with a Cd-chelate buffer system which included ^{109}Cd as a tracer. Organisms were exposed for 3 weeks, and both Cd accumulation and growth were determined weekly.

There was a linear relationship between $\{Cd^{2+}\}$ and Cd accumulation at each point in time. Surprisingly, Cd accumulation was greatest after 1 week and declined over the second and third weeks for all but the highest $\{Cd^{2+}\}$ where it remained constant. Further, physiological stress, as evidenced by hormesis and growth inhibition, was observed within the range of $\{Cd^{2+}\}$ used in this experiment.

At the end of the 3-week period, the subcellular distribution of Cd was determined for organisms from each exposure regime. We found Cd associated with all subcellular fractions and the cytosol. Cytosolic Cd was associated with high molecular weight (HMW) and metallothionein (MT) ligand pools but not the very low molecular weight (VLMW) ligands. As we observed for total Cd accumulation, cadmium in the subcellular fractions and cytosolic pools was also linear relative to $\{Cd^{2+}\}$. Throughout the entire range of exposures there were no shifts in Cd accumulation or its relative distribution among the subcellular fractions.

Materials and Methods

Polychaetous annelids (*Neanthes arenaceodentata*) were obtained from a laboratory population which has been under continuous culture since 1964 (*7*). These organisms were randomly divided into six groups, each of which was exposed to a different free cadmium ion activity $\{Cd^{2+}\}$. As a control, a seventh group was included which was exposed to the same buffer system with no added Cd. There were five sets of 10 organisms each within each group. Individual organisms were maintained in separate acid-cleaned plastic petri dishes.

Metal-chelate buffer systems made up in Millipore-fil-

*Molecular Ecology Institute, California State University, Long Beach, CA 90840.
†Department of Biology, California State University, Long Beach, CA 90840.
‡Duke University Marine Laboratory, Beaufort, NC 28516.

tered (0.45 μm) seawater (35°/oo) were used to control $\{Cd^{2+}\}$ as well as the activities of Cu, Zn, Mn, Co, Fe, and Mo (*3*). Each of the buffer solutions contained 10^{-5} M EDTA, 4×10^{-5} M NaOH, 5.2×10^{-7} M Cu, 1.33×10^{-6} M Zn, 9.0×10^{-8} M Mn, 6.8×10^{-8} M Co, 10^{-7} M Fe, and 3×10^{-8} M Mo. The calculated metal ion activities for these metals were $\{Cu^{2+}\} = 10^{-12}$ M, $\{Zn^{2+}\} = 10^{-9.3}$ M, $\{Mn^{2+}\} = 10^{-8}$ M, $\{Co^{2+}\} = 10^{-6}$ M. The amount of Cd added to the buffers ranged from 4.5×10^{-9} M to 4.5×10^{-5} M to achieve $\{Cd^{2+}\}$ which ranged from 10^{-12} M to 10^{-8} M. To facilitate measurement of Cd uptake and subcellular distribution, the buffers were spiked with radiolabeled ^{109}Cd (0.01 μCi/mL). The addition of trace concentrations of ^{109}Cd did not significantly alter the total Cd concentration or the $\{Cd^{2+}\}$.

Organisms were removed at weekly intervals, blotted to remove excess water, weighed, and counted for ^{109}Cd activity. They were then placed in clean culture dishes containing fresh metal buffered seawater at the appropriate $\{Cd^{2+}\}$. After 3 weeks of exposure the organisms were weighed and frozen at –80°C until subsequent analysis.

All organisms from each exposure regime were pooled to determine subcellular Cd distributions. Each sample was suspended in homogenization buffer (0.05 M Tris-HCl, pH 7.4), and homogenized with a glass homogenizer and Teflon pestle. The homogenate was then fractionated by differential centrifugation (*8*). The cellular debris (200*g* pellet), nuclear-mitochondrial fraction (10,000*g* pellet), microsomal fraction (100,000*g* pellet) and cytosol (100,000*g* supernatant) were collected and their ^{109}Cd activity determined. The cytosol was brought to uniformity with a vortex mixer, divided into 1 mL aliquots, and stored at –80°C for subsequent fractionation by high performance liquid chromatography (HPLC). Individual aliquots of the cytosol were rapidly thawed, passed through a 0.2 μm nylon filter, and 800 μL was immediately fractionated on an HPLC steric exclusion column (SEC) (*4*); 1-mL fractions were collected, and ^{109}Cd activities were determined on a Beckman 4000 gamma counter which has a counting efficiency of 25%. Cadmium concentrations were calculated from the specific activities of each metal buffer system and assume no isotope effect.

Data on Cd accumulation, subcellular distribution and growth were analyzed by correlation analysis, linear regression analysis, and analysis of variance (*9*). Our confidence limits were set at 0.05.

Results

The total accumulation of ^{109}Cd was determined weekly over the 3-week exposure period by measuring the ^{109}Cd activity in intact worms. After 7 days, there was a direct relationship between Cd accumulation and $\{Cd^{2+}\}$ in seawater ($r^2 = 0.993$, slope = 1.004; Fig. 1). Similar relationships between Cd accumulation and $\{Cd^{2+}\}$ in seawater were also observed following exposures of 14 days ($r^2 = 0.990$; slope = 1.028; Fig. 1) and 21 days ($r^2 = 0.985$; slope = 1.086; Fig. 1). Cadmium accumulation

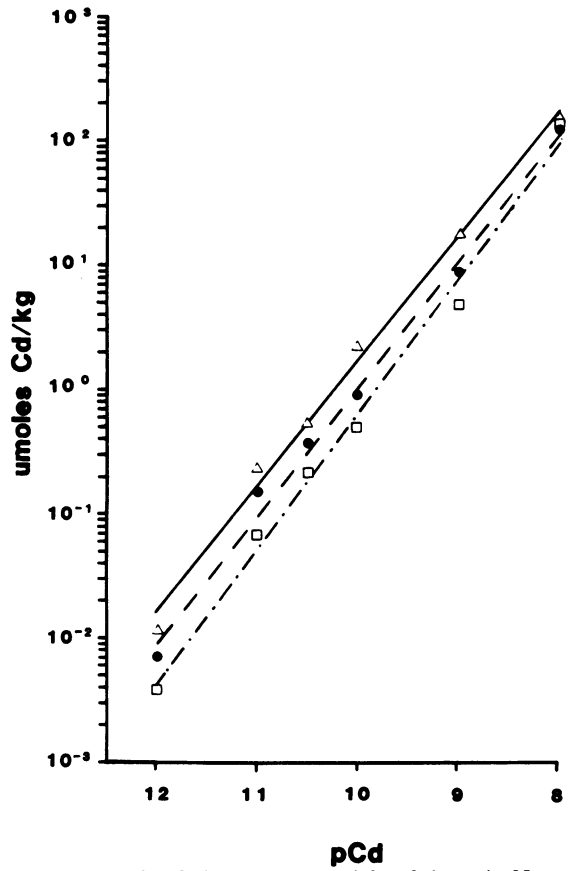

FIGURE 1. Total cadmium per wet weight of tissue in *N. arenaceodentata* exposed to a range of $\{Cd^{2+}\}$ (pCD = $-\log$ of $\{Cd^{2+}\}$) for (△) 7, (●) 14, and (□) 21 days. Model curves were fitted by standard linear regression.

decreased significantly from day 7 through day 21 (F = 17.2, 27.3, 37.9, 51.2, 110.1, with d.f. = 2,14 for 10^{-12}M to 10^{-9}M, respectively) at all but the highest $\{Cd^{2+}\}$ of 10^{-8} M where it did not change with time.

After 3 weeks of exposure, ^{109}Cd was present in all polychaetes exposed to Cd and was associated with the cellular debris, the nuclear-mitochondrial and microsomal fractions, and the cytosol (Fig. 2). The cytosol usually contained the highest Cd concentrations and was followed in order by the cellular debris, the nuclear-mitochondrial fraction, and the microsomal fraction. As with the accumulation of total Cd, cadmium accumulated linearly in these fractions with increased $\{Cd^{2+}\}$ (r^2 = 0.96, 0.988, 0.992, 0.974; slope = 1.02, 1.06, 1.11, 1.05; for cytosol, debris, nuclear-mitochondrial fraction, and microsomal fraction, respectively).

Within the cytosol, ^{109}Cd accumulated in two ligand pools (Fig. 3). It was most prominent in the metallothionein-like ligand pool (MT; 8–10 kD) and was also associated with the high molecular weight pool (HMW; > 20 kD). The very low molecular weight ligands (VLMW; < 5 kD) did not accumulate significant ^{109}Cd activity in the course of this experiment. The cadmium associated with both MT and HMW ligands also increased linearly with increased $\{Cd^{2+}\}$ in seawater (Fig. 4; r^2 = 0.954, 0.976; slope = 1.01, 1.2; for MT and HMW, respectively).

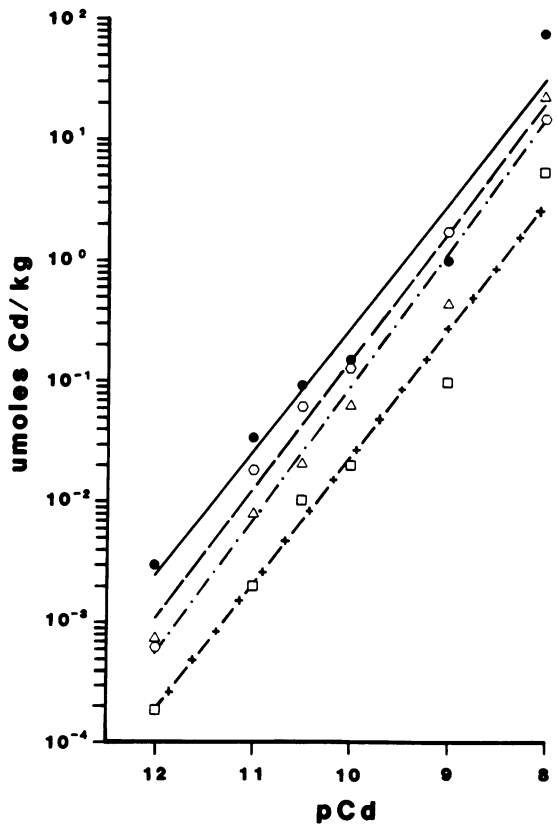

FIGURE 2. Subcellular distributions of Cd in polychaetes from Fig. 1. Accumulation is shown in (●) the cytosol, (△) nuclear-mitochondrial fraction, (□) microsomal fraction, and (○) cellular debris after 21 days. Model curves for Cd accumulation in each fraction were fitted by standard linear regression.

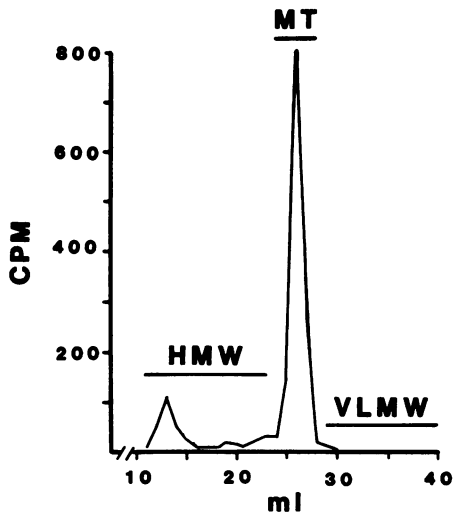

FIGURE 3. Cytosolic distribution ^{109}Cd expressed as counts per minute (CPM) in polychaetes exposed to $10^{-10.5}$ M $\{Cd^{2+}\}$ for 21 days. HMW, MT, and VLMW represent high molecular weight, metallothionein, and very low molecular weight pools, respectively. The bars represent fractions which were combined for each pool.

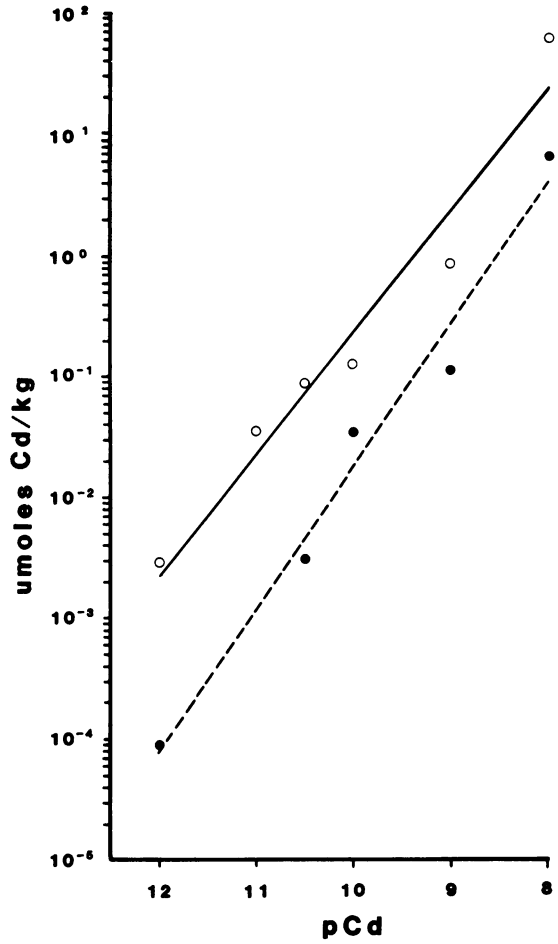

FIGURE 4. Cadmium in polychaetes from Fig. 1 (○) in the MT pool and (●) in the HMW pool after 21 days of exposure. Model curves for each pool were fitted by standard linear regression.

The relationship between $\{Cd^{2+}\}$ and growth was nonlinear and time-dependent (Fig. 5). The specific growth $\bar{R}$ defined as the change in weight divided by weight at day 0, did not differ significantly between exposure regimes on days 7 and 14. On day 21, however, $\bar{R}$ increased with increased $\{Cd^{2+}\}$ in seawater up to 10^{-9} M, and was reduced at 10^{-8} M (F = 4.1; d.f. = 6,34).

Over the course of this experiment, specific growth did not change significantly for organisms exposed to buffer without added Cd and to the lowest $\{Cd^{2+}\}$ (10^{-12} M). For those organisms exposed to all other activities, however, wet weight changed with time; it increased with time at exposures of 10^{-11}, $10^{-10.5}$, 10^{-9} (F = 9.6; 18.6; 24.2 for d.f. = 2,14). At 10^{-10} and 10^{-8} M of $\{Cd^{2+}\}$, $\bar{R}$ increased at day 14 and decreased at day 21 (F = 19.3; 6.9; for d.f. = 2,14).

Discussion

Cadmium Accumulation

In this study, there was a direct, one-to-one relationship between Cd accumulation and exposures to $\{Cd^{2+}\}$ of over four orders of magnitude. A similar relationship

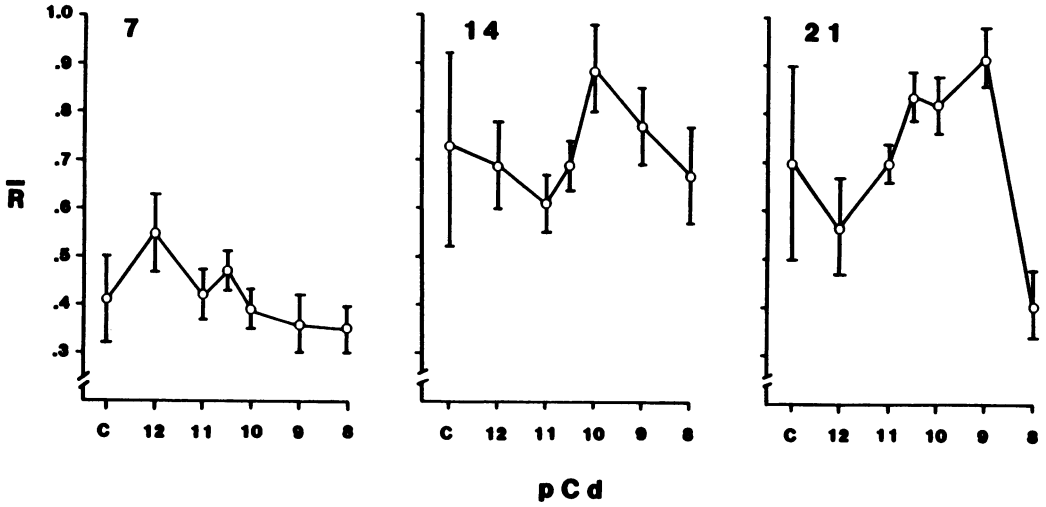

FIGURE 5. Specific growth $\bar{R}$ for polychaetes from Fig. 1 for days 7, 14, and 21. Specific growth is equal to the change in wet weight from day 0 divided by the wet weight at day 0 (C = control organisms exposed to the buffer system with no added Cd).

is observed in juvenile and adult oysters (*10*). Relationships between free metal ion activity and bioaccumulation are found for Cu, Zn, and Mn in other organisms (*1,11–14*). Depending upon the metal and the organisms, both linear and sigmoidal relationships have been described.

Cross and Sunda (*15*) used thermodynamic considerations to explain the dependence of the bioaccumulation of these metals on their free ion activity: The free metal ion activity is a measure of the free energy of the metal and, as such, reflects the potential for interactions between the metal and available ligands. Many metals, including Cd (*16*), require a protein to mediate transport across cell membranes. Metal uptake then will depend upon the interactions between the metal and the transport proteins and the free metal ion activity reflects the potential for these interactions.

We observed no increase in total Cd accumulation between day 7 and 21, even though the polychaetes were continuously exposed. In fact, except for the highest exposure regime, where Cd accumulation did not change with time, total Cd in the polychaetes decreased from day 7 to 21. These observations indicate that although at any point in time total accumulation is dependent upon $\{Cd^{2+}\}$, over a time span of several weeks this species has some capacity to regulate rates of Cd uptake and excretion to reduce Cd accumulation.

Subcellular Distribution

Within the cell much of the Cd was found in the cytosol, where it ranged from 30 to 66% of the total tissue burden of Cd (Fig. 2). Cadmium accumulates in the cytosol of mammals, lower vertebrates and invertebrates where it is usually associated with the low molecular weight metal-binding protein metallothionein (*17*). In this experiment most of the cytosolic Cd (77 to 100%) was associated with a metal-binding pool that has characteristics similar to metallothioneins (Figs. 3 and 4) (*17*). This pool: had an apparent molecular weight of 9,000 daltons; comigrated with fish and mouse metallothioneins on SEC-HPLC; increased with Cd exposure; also bound Cu and Zn; was resolved as two to three isoforms on DEAE–Sephadex; and had a high 250/280 nm ratio (unpublished data). Cadmium-binding proteins with similar characteristics have been isolated from an oligochaetous annelid (*18*). In addition, a low molecular weight Cu-binding protein has been found in the polychaete *Eudistylia vancouveri* (*19*).

We found cytosolic Cd was also associated with heterogeneous high molecular weight ligands (HMW; Fig. 4). A number of marine invertebrates accumulate Cd in this pool (*20,21*). This accumulation is thought to be a consequence of nonspecific binding of Cd to soluble macromolecules and may represent an important mechanism of metal toxicity.

The Cd in the nuclear-mitochondrial fraction may be enclosed within membrane-bound vesicles such as the Cd containing granules found in the mitochondrial–lysosomal pellets of mussels and other marine invertebrates (*22,23*). These vesicles are thought to be tertiary lysosomes and could function in metal homeostasis and detoxication (*24*). Cadmium also accumulates in the nuclear fraction of mussel and mammalian cells where it may cause DNA lesions (*25,26*). Since the procedures used in this study do not allow us to distinguish between the nuclear, mitochondrial, and lysosomal fractions the specific distribution of Cd between these organelles and their possible involvement in Cd metabolism is not clear. Because of the prominence of Cd in these fractions, we are currently carrying out a more extensive examination of their role in Cd metabolism.

In previous studies we examined the relationships between $\{Cd^{2+}\}$ in seawater and cytosolic Cu accumulation and distribution in crab larvae (*4,27*). In those studies, cytosolic Cu was independent of $\{Cu^{2+}\}$ at lower exposures and increased significantly at $\{Cu^{2+}\}$ beyond those measured in the estuary where the crabs had been col-

lected. This increased Cu accumulation at higher Cu exposures reflected increases in Cu associated with both MT and VLMW ligands. Copper associated with HMW ligands, however, remained constant over the entire range of $\{Cu^{2+}\}$. From these data we concluded that crab larvae could regulate both the accumulation of Cu and the distribution of Cu within the cell. This control over Cu uptake, efflux, and metabolism would allow the larvae to maintain some independence from external $\{Cu^{2+}\}$.

Since total Cd accumulation and accumulation in each of the subcellular fractions can be described as linear functions of $\{Cd^{2+}\}$ in seawater, these polychaetes demonstrated no independence from $\{Cd^{2+}\}$ in their environment. Differences in Cd concentrations among the subcellular fractions can be explained solely by differences in the number and affinity of Cd-binding sites within each fraction. Also, there was no redistribution of Cd between the subcellular ligand pools, even at $\{Cd^{2+}\}$ indicative of stressful conditions. We conclude that this species cannot regulate Cd distribution between subcellular fractions under these experimental conditions. Redistribution within the fractions, of course, cannot be precluded from these data. We do not know if this qualitative difference between larval crabs and polychaetes in their ability to regulate subcellular metal distribution is the result of differences between the metabolisms of essential and nonessential metals or is species specific. Experiments are underway to clarify this issue.

Growth

We monitored growth over the course of this experiment in order to understand the ecological significance of Cd accumulation and subcellular distribution. The major differences in specific growth occurred at day 21 where $\bar{R}$ increased in worms exposed from 10^{-12} to 10^{-9} M $\{Cd^{2+}\}$ and then dropped off significantly at 10^{-8} M (Fig. 5). Reductions in growth are indicative of stress and occur in marine organisms exposed to high concentrations of Cd and other toxicants (*27–30*). At lower concentrations of these toxicants, growth increases (*29–31*). These responses of growth enhancement at low toxicant concentrations and inhibition at higher concentrations are similar regardless of the physical or chemical characteristics of the toxicant (*30*). The apparent enhancement of a physiological process by low concentrations of a contaminant is termed hormesis. It is considered an integral component of the etiology of stress (*31*) and has been attributed to transient overcorrections of internal regulatory mechanisms in response to inhibitory challenges (*28*). The presence of a hormetic response followed by growth inhibition indicates that after three weeks of exposure to $\{Cd^{2+}\}$ greater than $10^{-10.5}$ M, these organisms were experiencing physiological stress.

In summary, these data reveal a linear relationship between Cd accumulation in this polychaete species and $\{Cd^{2+}\}$ in seawater, indicating that Cd accumulation is simply a function of bioavailability. The relative distribution of Cd between the subcellular fractions was constant at all $\{Cd^{2+}\}$ and Cd accumulation within each fraction could also be related in a linear manner to external $\{Cd^{2+}\}$. No shifts in the rate of Cd accumulation within each fraction or relative Cd distribution between the fractions were observed, even in the higher $\{Cd^{2+}\}$ where the growth data suggest that these organisms were stressed. However, since the rate of accumulation decreases at all but the highest $\{Cd^{2+}\}$ from day 7 to 21, this species appears to have some capacity to adjust their Cd uptake and efflux to reduce total accumulation over a time span of several weeks.

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