IMPORTANCE OF EQUILIBRATION TIME IN THE PARTITIONING AND TOXICITY OF ZINC IN SPIKED SEDIMENT BIOASSAYS

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Abstract—The influences of spiked Zn concentrations (1–40 μmol/l) and equilibration time (~95 d) on the partitioning of Zn between pore water (PW) and sediment were evaluated with estuarine sediments containing two levels (5 and 15 μmol/l) of acid volatile sulfides (AVS). Their influence on Zn bioavailability was also evaluated by a parallel, 10-d amphipod (Leptocheirulus plumulosus) mortality test at 5, 20, and 85 d of equilibration. During the equilibration, AVS increased (up to twofold) with spiked Zn concentration ([Zn]), whereas Zn—simultaneously extracted metals ([SEM]) Zn with AVS) remained relatively constant. Concentrations of Zn in PW decreased most rapidly during the initial 30 d and by 11- to 23-fold during the whole 95-d equilibration period. The apparent partitioning coefficient (Kapp, ratio of [Zn] in SEM to PW) increased by 10- to 20-fold with time and decreased with spiked [Zn] in sediments. The decrease of PW [Zn] could be explained by a combination of changes in AVS and redistribution of Zn into more insoluble phases as the sediment aged. Amphipod mortality decreased significantly with the equilibration time, consistent with decrease in dissolved [Zn]. The median lethal concentration (LC50) value (33 μM) in the second bioassay, conducted after 20 d of equilibration, was twofold the LC50 in the initial bioassay at 5 d of equilibration, probably because of the change of dissolved Zn speciation. Sediment bioassay protocols employing a short equilibration time and high spiked metal concentrations could accentuate partitioning of metals to the dissolved phase and shift the pathway for metal exposure toward the dissolved phase.

Keywords—Zinc Toxicity Equilibration time Sediment aging Acid volatile sulfides

INTRODUCTION

Aquatic sediments in industrialized areas can contain concentrations of metals that are orders of magnitude higher than background levels [1]. Because metal-contaminated sediments pose a threat to resident benthic organisms [2–4], a primary goal of managing contaminated sediments is to protect food webs from that threat. Sediment bioassays are commonly employed for evaluating the threshold concentrations at which specific metals have adverse effects on test organisms. Bioassays can also aid in understanding the processes that influence partitioning and bioavailability of metals in sediments. However, important uncertainties remain in extrapolating spiked-sediment bioassay results unambiguously across a range of conditions in nature [5]. In the present study, we illustrate that the metal concentrations and equilibration time used for bioassay can be critical in determining the partitioning of metals to different binding phases of sediment, subsequent acute toxicity to sediment-dwelling organisms, and thus, the reliability of extrapolations from sediment bioassays to nature.

A growing body of work has demonstrated that understanding uptake routes of metals to benthic organisms from contaminated sediments is critical to determining metal bioavailability from sediments [6–10]. Some contradictory results are observed among studies concerning major metal exposure routes (e.g., pore water [PW] vs dietary metals) [8,11–14]. Most equilibrium partitioning–based studies contend that PW metal is the dominant bioavailable pool in sediment [12,15,16]. Another body of work has employed laboratory microcosm studies, field transplantation experiments, or biokinetic models to demonstrate that dietary uptake from contaminated sediments and food particles is a major route of metal exposure for various marine invertebrates, especially in moderately contaminated conditions [6,7,17–19]. Differences in experimental protocols make it difficult to compare among these contradictory studies. However, one major comparable difference among studies, as well as between laboratory studies and field situations, is how the metal contaminants are introduced to sediments before bioassay.

Many laboratory studies employ a protocol that exposes sediments to a spike of dissolved, but surface-reactive, metal and then equilibrating the slurry for days before the bioassay. Metal contaminants in nature, however, are often introduced gradually and over a much longer period; therefore, these contaminants have long equilibration times with sediments. In a reanalysis of previous studies (e.g., [12,16,20,21]), Lee et al. [10] suggested that the apparent partition coefficient (ratio of sediment to PW metal concentration) of Cd, Ni, and Zn tends to decrease with spiked metal levels and increase with metal–sediment equilibration time. It is possible that high concentrations of dissolved metals introduced to sediment slurries can initially oversaturate the available binding sites on sediment surfaces until exchange reactions re-equilibrate partitioning [22]. Although numerous studies [10,23–26] have demonstrated effects of various geochemical parameters (e.g., acid volatile sulfide [AVS], pH, redox potential, organic content, salinity, etc.) on metal partitioning in sediments, the effects of metal–sediment equilibration time on metal partitioning remain poorly understood.

If the degree of metal contamination and metal–sediment equilibration time influences directly the partitioning of metals between PW and sediment, they could also influence the bioavailability and toxicity of metals in contaminated sediments.
In fact, Sae-Ma et al. [27] reported that bioaccumulation and mortality of midge (*Chironomus tentans*) exposed to Cd-spiked soils decreased with storage time (up to 120 d) of the experimental sediments. Although a few studies [27–29] have demonstrated reduction in bioavailability with metal–sediment equilibration time, this change in bioavailability has not been systematically linked to changes in metal geochemistry. Furthermore, the actual cause of reduction in bioavailability has rarely been explained.

In the present study, the influence of the equilibration time of Zn introduced to estuarine sediment particles on partitioning and toxicity was evaluated over 95 d. We tried to relate the temporal change of geochemical parameters, such as AVS, weak-acid extractable Zn, and pore-water Zn concentration (PW [Zn]), to that of mortality of test animals. For simultaneous sediment toxicity testing, estuarine amphipods (*Leptochirus plumulosus*) were exposed for 10 d to Zn-spiked sediments after 5, 20, or 85 d of equilibration. The relative concentrations of reactive sulfides called AVS (extracted by 1 N HCl and typically composed of amorphous iron sulfi des) and simultaneously extracted metals (SEM) with AVS in sediments [SEM − AVS] can control dissolved metal concentrations in PW [15]. So, a range of AVS and Zn-SEM in sediments was employed to investigate the dynamic interaction of these geochemical parameters with equilibration time.

**MATERIALS AND METHODS**

**Sediment manipulation**

The experimental sediment containing approximately 30 μmol/g of AVS was collected from a mud flat near Palo Alto (CA, USA) [30]. The collected sediment was screened through 1-mm nylon mesh at the site to remove macroinvertebrates. Mean particle size was 8.1  φ (phi), and mean sand, silt, and clay contents, as analyzed by the pipetting method [31], were 0.4, 43.8, and 55.8%, respectively. Loss on ignition for 5 h at 450°C was 7.7% ± 0.7% (mean ± standard deviation [SD], n = 6).

After being brought to the laboratory, a portion of the collected sediment was manipulated to achieve two nominal AVS concentrations (5 and 15 μmol/g) according to an established protocol [10]. Briefly, the sediment was mixed with an equivalent volume of deoxygenated saline water (20 practical salinity units [psu]) and divided into two batches. One batch of the sediment was kept in a closed container and purged with N₂ gas periodically. The remaining sediment was oxidized by bubbling continuously with air for one week. The AVS concentration after one week of aeration decreased to approximately 1 μmol/g. The oxidized and the remaining anoxic sediments (∼30 μmol/g) were mixed at appropriate ratios to achieve two nominal AVS levels (5 and 15 μmol/g).

Following the AVS manipulation, the experimental sediments (containing ∼1.2 μmol Zn/g) were mixed with an appropriate volume of Zn stock solution to achieve nominal Zn concentrations of 10, 20, 30, and 40 μmol/g for the low-AVS (5 μmol/g) series and 10, 20, 40, and 50 μmol/g for the high-AVS (15 μmol/g) series sediments. The Zn stock solution was prepared by dissolving ZnCl₂ in deoxygenated, deionized water. Additionally, the control sediments without Zn addition were included for each of two AVS series sediments. Following vigorous mixing with an electric mixer, the Zn-spiked and control sediments were kept in closed polyvinylchloride bags purged with N₂ gas and stored at 20 ± 1°C during the entire experimental period.

Aliquots of the stored sediments were removed from each treatment 4 d before the beginning of the bioassays (at t = 5, 20, and 85 d). The removed sediments were mixed well, and 300 ml were transferred to four replicate, 1-L glass beakers. Three beakers were used for bioassay replicates and one for chemical analysis. The transferred sediments were allowed to consolidate for 3 d in the beakers. The overlying water was then decanted, 700 ml of aerated seawater (20 psu) added into each beaker, and the sediments allowed to equilibrate for another day before bioassay.

**Bioassay procedure**

A 10-d amphipod sediment toxicity test [32,33] was used to evaluate the influence of equilibration time on the acute toxicity of spiked Zn in sediments. The estuarine amphipod *Leptochirus plumulosus* (approximately one month old) was obtained from laboratory culture and maintained in a salinity of 20 psu at 20°C. At the beginning of each bioassay (at t = 5, 20, and 85 d), 20 individual amphipods (length, 0.6–1.0 mm) were transferred to each of the four beakers. Each beaker contained previously consolidated sediments that were equilibrated with overlying water. The overlying water was continuously aerated, and test chambers containing beakers were illuminated for 24 h. Water quality (temperature, salinity, dissolved oxygen, pH, and total ammonia) was monitored at t = 0, 5, and 10 d of bioassay and met the recommended criteria for amphipod toxicity test in all cases [33]. Total ammonia concentration in the overlying water, sampled at 3 cm above the sediments, was always less than 100 μM, which is far below the acute toxicity concentration for this species [33]. Following 10-d exposure, live animals were collected from each of three experimental beakers by sieving sediments through 0.6-mm mesh. The remaining beaker was used for the later geochemical analysis.

Separate 96-h, water-only Cd and Zn toxicity tests were conducted using *L. plumulosus* in 20-psy海水 at 20°C. The Cd toxicity test was done as a positive control to compare sensitivity of the test animals used for each of three bioassays. Mean 96-h median lethal concentration (LC50) estimates (12 ± 0.2 μM) for the three concurrent Cd reference toxicant tests conducted in conjunction with the spiked-sediment bioassays were not significantly different, indicating similar sensitivity to metals among the batches of test organisms used. The LC50 values from the water-only Zn toxicity test would be compared later to the LC50 values for PW Zn from each of the three sediment bioassays.

Sediment samples for chemical analysis (AVS, SEM, and PW) were collected twice, at the beginning and end of each 10-d bioassay, from the beaker dedicated to chemical analysis. This sampling scheme resulted in six sediment-sampling events (at t = 5, 15, 20, 30, 85, and 95 d). The sediment in the beaker was homogenized with a plastic spatula after the overlying water was decanted. Following the homogenization, approximately 20 ml of sediment were taken using a plastic syringe and immediately analyzed for AVS and SEM with an established method (see below). Approximately 40 ml of sediment were transferred to a 50-ml centrifuge tube and spun for 30 min at 2,500 g, and then the supernatant was filtered with a 0.45-μm syringe filter. The filtrate was acidified immediately with Ultrex® (Baker, Phillipsburg, NJ, USA) HCl to a pH of 1 to 2 and used later for PW Zn analysis. Two replicate samples were taken from each treatment for AVS, SEM, and PW Zn analysis.
analytical quantification limit (mean ultrapure nitric acid (to mitigate chloride interference) before ICP emission spectroscopy and/or flame-atomic emission spectrometry. Pore-water samples were diluted 10-fold with 0.1 N HCl acid wash (1 N HCl), then soaked in N2-purged deionized water for one week. The sediment samples were handled under a glove bag filled with N2 gas. The AVS analysis was conducted by an N2 purge and trap method using an ion-specific sulfide electrode within a week of sample collection [34,35]. The detailed analytical procedures are described elsewhere [12]. Metal concentrations in SEM, metal extracts from total sediment digestion (HF-HClO4-HNO3), and PW samples were determined with inductively coupled argon plasma-atomic emission spectroscopy and/or flame-atomic emission spectroscopy. Pore-water samples were diluted 10-fold with 0.1 N ultrapure nitric acid (to mitigate chloride interference) before analysis. The analytical quantification limit (mean + 10 SD of procedure blank × dilution factor) for PW Zn was 1.5 μM.

Data analysis

The AVS, Zn-SEM, the molar difference between SEM with AVS and AVS in sediments, [SEM - AVS], and PW [Zn] data were analyzed by multiway analysis of variance using the Statistica® (StatSoft, Tulsa, OK, USA) package to test the effects of equilibration time and of AVS and SEM levels on the biogeochemical parameters. Dry weight-based concentrations were used for all sediment data. The [SEM - AVS] values were calculated by the difference between Zn-SEM and AVS [10,12]. The LC50 values for 96-h, water-only Cd and Zn tests and 10-d sediment tests were calculated using the trimmed Spearman-Karber method [36]. Limited statistical analyses were done for PW [Zn] data, because many data were under the detection limit. Statistical significance was set at $p = 0.05$ unless otherwise noted. Mortality data for the amphipod were analyzed by two-way analysis of variance to test the effects of Zn-SEM and equilibration time for both low- and high-AVS series treatments.

RESULTS

Geochemistry of sediment

The AVS in all sediments was significantly influenced by both spiked Zn concentration and equilibration time ($p < 0.001$) (Fig. 1). The AVS generally increased with equilibration time in the Zn-spiked sediments but remained relatively constant over time in the control sediments. The concentration of AVS changed most rapidly during the initial 30 d. The range of measured AVS at the beginning of equilibration ($t = 5 d$) was 3 to 7 μmol/g in the low-AVS series and 13 to 19 μmol/g in the high-AVS series, close to their nominal values (5 and 15 μmol/g). At the end of the experiment, AVS ranged from 3 to 16 μmol/g in the low-AVS series and from 13 to 30 μmol/g in the high-AVS series. The AVS also increased with Zn-SEM in both series (Fig. 1C).

The Zn-SEM in sediments was little influenced either by AVS or by equilibration time ($p > 0.05$). Mean Zn-SEM ($n = 12$) over the ranges of AVS and equilibration time was 1.2 ± 0.1 μmol/g for control and 9.8 ± 1.3, 15 ± 1, 24 ± 1, 30 ± 2, and 37 ± 2 μmol/g in sediments with nominal Zn level of 10, 20, 30, 40, and 50 μmol/g, respectively. Mean recoveries of Zn-SEM (ratio of SEM to total extractable Zn) were 57 ± 10% ($n = 12$) for control sediments and 87 ± 6% ($n = 48$) for all the Zn-spiked sediments.

Pore water [Zn] increased with spiked Zn concentrations, as expected. Pore water [Zn] decreased significantly with equilibration time, and its decrease was most apparent during the initial 30 d of equilibration (Fig. 1B). Pore water [Zn] decreased more in the high-AVS series (decrease of 23-fold) than in the low-AVS series (decrease of 11-fold) at the end of the 95-d equilibration period. This was least obvious in the sediments with Zn-SEM in both series (Fig. 1C).

Fig. 1. Variation of acid volatile sulfide (AVS) with sediment-Zn equilibration time in low-AVS (A) and high-AVS (B) series and with Zn simultaneously extracted metals (SEM) with AVS (C). Weak acid extractable Zn concentration [Zn-SEM] in low-AVS series increased from 1 (●), 10 (▲), 15 (■), 24 (▼), and 30 (□) μmol/g and those in high-AVS series from 1 (●), 10 (▲), 15 (■), 30 (□), and 37 (★) μmol/g. The AVS value at each sampling time represents the mean of two replicate measurements. The difference between two measurements was mostly less than 20%. Relationship between AVS and Zn-SEM in Zn-contaminated sediments in low- and high-AVS series was from the time-averaged values; error bars represents one standard deviation of AVS and Zn-SEM ($n = 6$).

Fig. 2. Variation of pore-water Zn concentrations with sediment equilibration time in low–acid volatile sulfide (AVS) (A) and high-AVS (B) series (see Fig. 1 for symbols). SEM = simultaneously extracted metals with AVS.
Fig. 3. Variation of simultaneously extracted metals (SEM) – acid volatile sulfide (AVS) with sediment equilibration time in low-AVS (A) and high-AVS (B) series. Relationship between pore-water Zn concentrations and [SEM – AVS] in low-AVS (C) and high-AVS (D) series at the different equilibration time is also shown.

Bioassays

The mortality of amphipods exposed to sediments for 10 d at day 5, 20, or 85 of Zn–sediment equilibration were compared to [SEM – AVS] (Fig. 5) or PW [Zn] (Fig. 6). The mortality of amphipods increased with [SEM – AVS] when [SEM – AVS] was greater than zero and increased with equilibration time for a given [SEM – AVS] (Fig. 4, C and D).

Bioassays

Fig. 4. Variation of apparent partitioning coefficient of Zn (Kpw) with sediment equilibration time (A and B) or with simultaneously extracted metals (SEM) – acid volatile sulfide (AVS) in low- and high-AVS series at the different equilibration time (C and D). Some Kpw in high-AVS series could not be calculated, because the pore-water Zn concentrations decreased below the detection limit. Decimal notation of exponential form is used for y axis scale.

Fig. 5. Mortalities of amphipod Leptocheirus plumulosus with respect to simultaneously extracted metals (SEM) – acid volatile sulfide (AVS) in low-AVS (A) and high-AVS (B) series. Amphipods were exposed to control and Zn-contaminated sediments for 10 d at the 5 (●), 20 (●), and 85 d (●) of equilibration. Error bars represent one standard deviation (n = 3).
Partitioning and toxicity of zinc in sediments

From the relationship in Figure 6, the 10-d LC50 of dissolved Zn in PW at 5 d was estimated as 17 μM (with a 95% confidence interval of 15–21 μM) and at 20 d as 33 μM (with a 95% confidence interval of 27–40 μM). These values were higher than the mean 96-h LC50 value of 14 μM Zn, independently determined with the water-only Zn toxicity test for the amphipod Leptocheirus plumulosus and pore-water Zn concentrations. Amphipods were exposed to control and Zn-contaminated sediments at the 5 (●), 20 (○), and 85 (□) d of equilibration. Vertical lines represent either 96-h median lethal concentration (LC50) of water-only Zn exposure or the limit of detection (LOD) for pore-water Zn.

Effect of equilibration time on metal partitioning

The 20-fold decrease of PW [Zn] during 85 d of equilibration could be explained mainly by the redistribution of Zn from labile/weak-binding phases to more insoluble phases, including AVS, and other binding phases, such as metal oxides and organic matter. Among these binding phases, AVS is recognized as a dominant binding phase in the anoxic sediments, and its effect on PW metals is relatively well studied [37].

The influence of AVS relative to SEM on PW metals has been well established for several divalent metals, including Zn (12,15,16,26). Typically, PW [Zn] is very low when [SEM - AVS] is less than zero and increases exponentially with [SEM - AVS] when [SEM - AVS] is greater than zero. In the present study, the decrease of [SEM - AVS] with time, which resulted in a reduction of PW [Zn], was largely controlled by the increase of AVS, because SEM was relatively constant over the equilibration period. The increase of AVS concentration ([AVS]) in Zn-contaminated sediments can be explained by sequestration of AVS (mostly in amorphous FeS) by Zn. If AVS could be rapidly converted from amorphous FeS to ZnS in the Zn-spiked sediments, the slower oxidation rate of ZnS compared to that of FeS could result in the buildup of AVS that was observed. For example, Simpson et al. [24,38] demonstrated that FeS was rapidly oxidized in aerated waters whereas ZnS was kinetically stable for oxidation. Similarly, other studies also observed an increase of AVS on addition of divalent metals in sediments [12,39].

An increase of AVS alone, however, does not entirely explain the observed reduction of PW [Zn] with equilibration time. For a given [SEM - AVS], PW [Zn] decreased with equilibration time when [SEM - AVS] was greater than 0 (Fig. 3). Furthermore, in anoxic sediment, PW [Zn] continuously decreased with time even when [SEM - AVS] was less than zero. These results collectively suggest that some other processes were responsible for further reduction of PW [Zn]. One process, other than AVS sequestration of PW [Zn], that could be responsible for PW [Zn] reduction is redistribution of Zn from labile binding sites to more refractory sites. In fact, the greater extraction efficiency of SEM in Zn-spiked sediments compared to unspiked control sediments in the present study suggests that Zn in spiked sediments is more labile than in the control sediments, which probably had a much longer metal–sediment equilibration time in nature. Confirming this observation, Griscom et al. [40] showed in the sequential extraction of metals in sediment aged up to 35 d that the labile fraction of Cd, Co, Se, and Zn decreased whereas the resistant fractions significantly increased with time. The labile fraction (i.e., low K_{pw}) includes the adsorbed and exchangeable phases of metals bound on the sediment surface; the resistant phases (i.e., high K_{pw}) include the hydrous iron or manganese oxides, organic matter, and mineralized fractions of sediments [41]. Other laboratory metal adsorption studies [41,42] demonstrated that slower partitioning processes (e.g., physical and microbial transformation) followed the initial rapid adsorption of metals that occurred within a few days.

The level of spiked metal is another important factor affecting PW metal concentrations. An order of magnitude decrease occurred in K_{pw} of Zn as Zn-SEM or [SEM - AVS] increased (Fig. 4). One possibility is association of Zn with binding sites of progressively lower stability as the [Zn-SEM] increases. Consistent with this, adsorption isotherms predict nonlinearity at high adsorbate (metal) concentrations because of the saturation of binding sites on the particle surface [22,43]. Other laboratory studies also showed decreases in K_{pw} of Cd, Ni, and Zn with the increase of [SEM - AVS] in laboratory-spiked sediments [10].

Bioavailability

Bioavailability (expressed as acute toxicity) of spiked Zn in sediments was also reduced significantly with Zn–sediment equilibration time. Other studies [39,44] have also evaluated the role of temporal variation of AVS and SEM for bioavailability and other biological responses, but to our knowledge, they have not established the potential reasons for the variation. In the present study, the reduction in toxicity was best explained by the concurrent reduction of dissolved Zn as represented by PW [Zn]. In contrast, [SEM - AVS] was a less accurate indicator of bioavailability/toxicity, because the dissolved Zn to [SEM - AVS] relationship was changed by the equilibration time. However, the reduction of dissolved Zn as represented by PW could not explain all the reduction of Zn bioavailability over time. The increase in LC50 values
of PW Zn in the sediment toxicity test by twofold during equilibration time suggests that the toxicity of dissolved Zn decreased over time. A plausible explanation for this observation could be that the Zn speciation of the dissolved phase had been changed over time. For example, a decrease in free Zn ion activity in PW, which could have occurred because of changes in concentrations and/or composition of dissolved Zn-binding ligands (e.g., dissolved organic matter), might be responsible for reduced toxicity of PW Zn. Another possible explanation could be related to the variation of dissolved Zn concentration in overlying water, which was not measured in the present study. It should be noted that the cause of mortality was assigned to dissolved Zn (either overlying water or PW Zn) rather than to PW Zn only. The dissolved [Zn] in the overlying water was not determined in the present study. However, it is reasonable to assume that overlying water [Zn] correlated and behaved similarly to PW [Zn], because [SEM – AVS] is a major co-factor controlling [Zn] in both overlying water and PW. Especially, the static renewal protocol employed in the present study could facilitate Zn to be equilibrated between PW and overlying water. Consistent with this idea, previous studies [19,21] have demonstrated that metal concentrations in overlying water strongly correlate with PW metal concentrations.

Many earlier studies (e.g., [16]) have suggested that acute mortality of organisms exposed to metal-contaminated sediments is controlled by exposure to dissolved metals. Our results generally support the utility of AVS normalization for predicting no acute toxicity of animals exposed to metal-contaminated sediments when [SEM – AVS] is less than zero. It is important to recognize, however, that the strong correlation between dissolved Zn and acute toxicity does not preclude chronic effects from other routes of uptake, including exposure to metals via diet, at lower Zn concentrations. In fact, a growing body of evidence [9,19,26,40,45] shows that bioaccumulation of metals in a variety of benthic invertebrates occurs when [SEM – AVS] is less than zero. Such results are little affected by the variation of [SEM – AVS]. Rather, bioaccumulation is related best to SEM concentrations and is best explained by ingestion of contaminated sediments [9,26,45]. One of the reasons for such results is that benthic invertebrates ingesting AVS-rich, pure-phase particles or anoxic sediments are able to assimilate metals with assimilation efficiencies similar to those for oxic particles [8,40,46]. A second reason, however, is that all such studies were conducted with less-than-extreme metal concentrations and realistic partitioning approaches.

The toxicological significance of complex, chronic exposure conditions are only beginning to be understood (e.g., [47]). However, whether the measure is toxicity or contaminant uptake, both short equilibration times and high spiked metal concentrations in sediments will accentuate partitioning of metals disproportionately to the dissolved phase and increase the probability of exposure and/or toxicity via dissolved metals. If metals are introduced gradually into contaminated sediment, equilibrated over long time scales, and/or have concentrations that are not extreme, then partition coefficients will be high, and chronic exposures are the concern. Results derived from spiked-sediment bioassays do not necessarily provide accurate/relevant estimates of the risk for sediments in nature.

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