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The effects of kaolin clay on the amphipod *Eohaustorius estuarius:* Part Two

Prepared by

Brian Anderson, Department of Environmental Toxicology, University of California, Davis

Bryn Phillips, Department of Environmental Toxicology, University of California, Davis

Jennifer Voorhees, Department of Environmental Toxicology, University of California, Davis

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Final Report

Brian Anderson, Bryn Phillips, and Jennifer Voorhees

Department of Environmental Toxicology, University of California, Davis

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Abstract

A number of lines-of-evidence suggest that the amphipod *Eohaustorius estuarius* has variable tolerance to clay in sediments. In Phase 1 of the current RMP special study, laboratory dose-response experiments were conducted in 2014 with kaolin clay to evaluate whether clay effects varied with amphipod size. The results indicated that smaller amphipods were more tolerant of clay than larger individuals. Average survival was 81%, 79%, and 65% for small, medium and large amphipods, respectively, in concentrations $\geq$ 50% clay. As part of Phase 2 confirmation studies conducted in 2015-2016, the original kaolin dose-response experiments were repeated. Results of this experiment showed that average amphipod survival was 88%, 63%, and 41% for small, medium and large amphipods, respectively, in concentrations $\geq$ 50% clay. Standard 96-hour reference toxicant tests with cadmium chloride ($CdCl_2$) were conducted to determine whether there were size-specific differences in response to this metal reference toxicant. The $CdCl_2$ median lethal concentrations (LC50s) for small, medium and large amphipods were 6.78, 5.13, and 4.63 mg/L, respectively. Responses of all three size classes to cadmium were within historic confidence intervals for this reference metal, and were not significantly different from one another based on overlapping confidence intervals. Additional experiments with high clay reference site sediments from San Francisco Bay were conducted to confirm the size related response with field sediments. The results confirm that use of smaller amphipods in routine monitoring of high clay sediments will reduce the influence of this factor on test results.

Background

The 10-day whole sediment toxicity test protocol for the amphipod *Eohaustorius estuarius* is one of the principal tests recommended for toxicity monitoring in California and Canada (U.S. EPA, 1994). Several studies have shown this species is appropriate for this application, and this is the benchmark test used in regional monitoring programs in southern California and the San Francisco Estuary. Due to concerns about limitations of methods to determine causes of persistent moderate toxicity in field sediments and the relative influence of non-contaminant factors on amphipod survival, two recent workshops sponsored by the San Francisco Estuary Institute's Regional Monitoring Program (RMP) identified specific attributes of *E. estuarius* that require additional research. Among a list of non-contaminant factors considered, the relative impacts of grain size, particle shape, and test animal condition were identified as possibly important factors affecting amphipod survival.

As part of the initial evaluation of *E. estuarius* as a test species, Dewitt et al. (1989) assessed survival of *E. estuarius* in 42 uncontaminated field sediment samples from Puget Sound, Washington and Oregon. These authors reported that "*E. estuarius* showed little sensitivity to sediments of different grain sizes: mean survival was 92.4% in sediments with ≥80% silt-clay content and 96.7% for coarser sediments." Environment Canada published grain size recommendations for the 10-day test with *E. estuarius* (Environment Canada, 1998). Tay et al. (unpublished study described in Environment Canada, 1998) found mean survival was 74% in mixtures with 57% clay and 99% fines (silt and clay). Based on these experiments, they established tolerance limits of <90% coarse grained sediment, and <70% clay. The Environment Canada (1998) 10-day guideline states that "test materials with ≥ 70% clay must not be used in a 10-day sediment toxicity test with *E. estuarius*". UC Davis conducted similar experiments using mixtures of sand and field-collected reference sediment that was composed of silt and clay. The field reference material was sieved through a 75µm screen then mixed with sand to create sediments with 10 – 90% fines. *Eohaustorius estuarius* 10-day survival was ≥85% in sediments with ≤70% fines. Survival was 57% in sediment with 90% fines (Marine Pollution Studies Laboratory-Granite Canyon unpublished data). In addition to these studies, analyses of data from the RMP and elsewhere have shown that survival of *E. estuarius* in field sediments is negatively correlated with percent fine grained sediment, and with percent clay in sediment. Based on the preponderance of evidence, the effect of clay was prioritized for further study by participants of the two RMP workshops.

The toxicity workshops also identified the possible interaction of seasonal differences in amphipod health and their ability to tolerate fine-grained sediments as a high-priority topic for investigation. This is based on evidence suggesting sediment toxicity in San Francisco Bay is greater in winter, and the possibility that increased winter toxicity is related to variability of the health of field-collected amphipods. Seasonal changes in amphipod fitness related to nutrition, senescence, or reproductive activity have been suggested as the reason for such variations in sensitivity to San Francisco Bay sediments. The workshop participants also recommended measurement of amphipod lipid content as an indicator of animal condition. Measurement of amphipod lipid content may provide a valuable tool for interpreting the results of future sediment toxicity surveys, but information on the seasonal changes in this parameter and its association with changes in amphipod sensitivity to stressors is needed. Combining seasonal measurements of tissue lipid with studies of the sediment particle size effects on *E. estuarius* survival will provide the information needed to evaluate the usefulness of lipid measurements in toxicity testing.

A United State Geological Survey characterization of suspended sediments in the San Francisco Estuary found that water column suspended sediments contained three clay mineral types: illite, montmorillonite (=smectite), and chlorite + kaolinite (Knebel et al., 1977). Samples were collected in the spring, summer, fall and winter seasons and covered the northern and southern reaches of the estuary. The results demonstrated that waters in the northern reaches of the estuary were dominated by chlorite + kaolinite via inputs from the Sacramento-San Joaquin river systems. Kaolinite originates from the weathering of granite. Illite dominates the southern reach of the estuary where clay minerals are re-suspended from the estuary floor by tidal currents. Illite originates from the weathering of micas and feldspars. Samples of the estuary sediments (bedded, not suspended sediments) showed that sediments in the northern and southern reaches were dominated by chlorite + kaolinite clays, which compose a somewhat larger size fraction than illite clays (Knebel et al., 1977). Phase 1 experiments conducted in 2014 demonstrated there were size specific differences in response to kaolin clay.

Phase 1 Experiments (2014)

Reference sand was spiked with increasing concentrations of kaolin clay, the dominant clay found in San Francisco Estuary sediment (Knebel et al., 1977). Clay was received gratis from Ione Minerals. Ione Minerals kaolin is mined in Ione, California, in the Cosumnes River watershed. This is industrial kaolin, which has not been altered during or after the extraction process. Raw kaolin mixed with seawater results in a low overlying water pH, so prior to mixing with sand, the kaolin was repeatedly equilibrated with seawater to raise the pH. In this process, seawater was mixed with kaolin, the mixture was then rolled on a jar roller, and then allowed to equilibrate at 15°C for 24 hours. The overlying water pH was measured, and the water was then decanted and replaced. The process was repeated until the overlying water pH was equilibrated at approximately 7.5. Once equilibrated, the wet clay was then hand mixed with #60 reference sand (0.25 mm mesh size) at the following ratios: 0% (sand only), 10%, 30%, 50%, 70%, 90%, and 100% kaolin. Sediment from the amphipod collection site (home sediment from the beach at the Beaver Creek, OR collection site) was also tested as a control. Analyses of variance with post hoc Dunnett's tests were used to determine significant differences among amphipod responses in different concentrations of clay, and also among different size classes of amphipods ($\alpha = 0.05$).

Experiment 1

Amphipod survival in the sand-clay mixtures was determined using 10-day toxicity tests conducted with *E. estuarius* (U.S. EPA, 1994). The initial design for this project included a single range-finder test to establish the range of percent clay that inhibits amphipod survival, followed by two definitive experiments to confirm the dose-response relationship. This approach was revised based on the results of the range-finding experiment (Figure 1). Only the 50% clay concentration had a significantly different responses from the control ($p < 0.05$), but survival was reduced in all concentrations. There was no clear dose response observed with increasing clay concentration. Survival was also more variable in the clay treatments. The only plausible explanation for the variable results was that some characteristic of the amphipods caused variable response to the clay. The amphipods are collected from Beaver Creek Beach, Oregon by Northwestern Aquatic Sciences (NAS). The animals are collected by wet-sieving sand from the collection site through a 1-mm screen to exclude the smallest animals, and placing the remaining amphipods back in sand to acclimate to 20‰ seawater prior to shipment to the laboratory. The animals used in the first range-finding experiment therefore represented a mixture of size classes greater than 1 mm. Based on the hypothesis that the variable responses observed in the first experiment were size related, a second experiment was conducted to test the hypothesis that the variable responses to clay was related to the size of the amphipods.

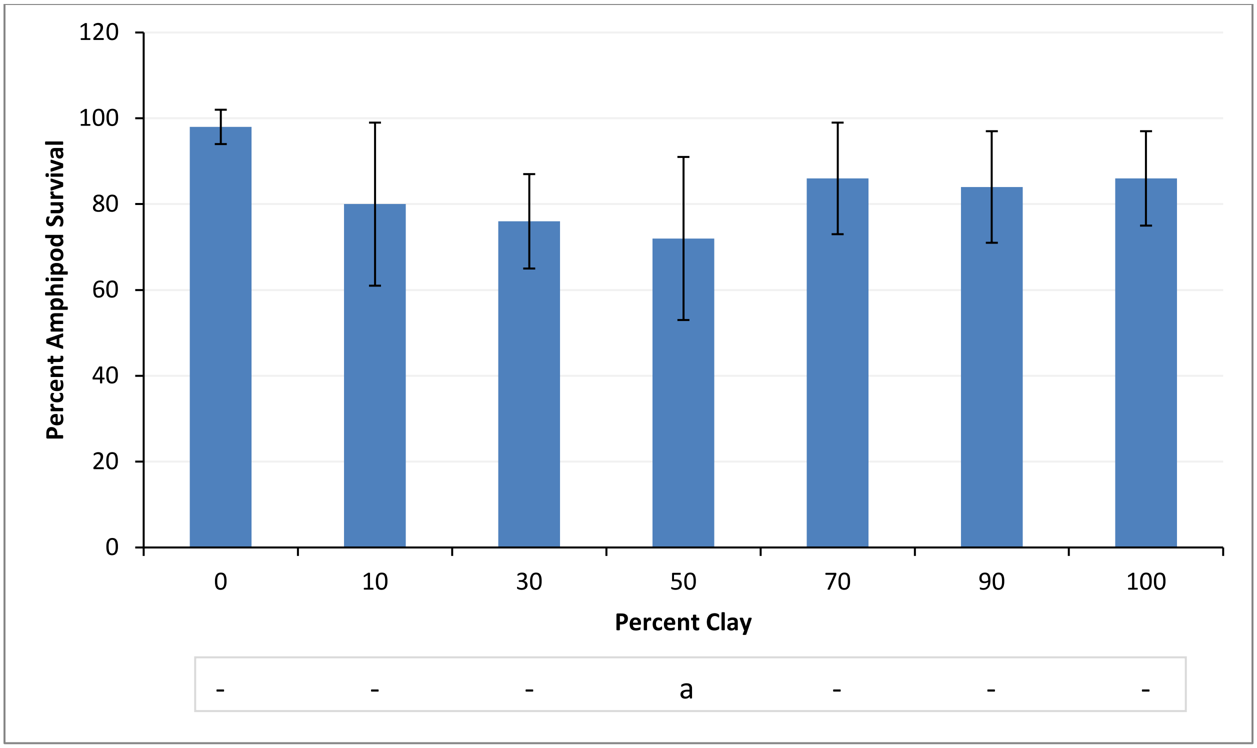


Figure 1. Percent amphipod survival in samples of sand with increasing concentrations of kaolin clay (Experiment 1). A mixture of amphipod sizes were exposed in this experiment. "a" indicates significant difference between response in a given clay concentration and response in the control ($\alpha < 0.05$).

Experiment 2

For this experiment, amphipods were pre-sorted visually by NAS into small, medium, and large size classes. Amphipods were collected from Beaver Creek Beach on July 3, 2014. The three size classes were exposed to sand or 100% kaolin for 10 days. A subset of each size class was measured to determine mean weight (n = 5 replicates of 5 animals from each size class; Table 1). The results of the 10-day exposures showed significantly lower survival in the large size class relative to the control, and although the response in the large size class was lower compared to the small size class, the difference was not statistically significant (Figure 2). Mean survival of small, medium, and large amphipods was 86%, 82% and 66%, respectively, in 100% kaolin clay. Survival was 98% in sand (note: a mixture of the three size classes was exposed to the sand control). These results suggested that some factor associated with amphipod size affected their tolerance to kaolin clay.

Table 1. Size distribution of amphipods exposed to 100% kaolin clay (corresponds to results presented in Figure 2). SD indicates standard deviation. Mean weight was determined from five replicates of 5 amphipods from each size class.

	Weight (mg)			
Size Class	Mean	SD	Minimum	Maximum
Small	1.128	0.108	0.980	1.260
Medium	1.544	0.092	1.420	1.660
Large	1.840	0.102	1.780	2.020

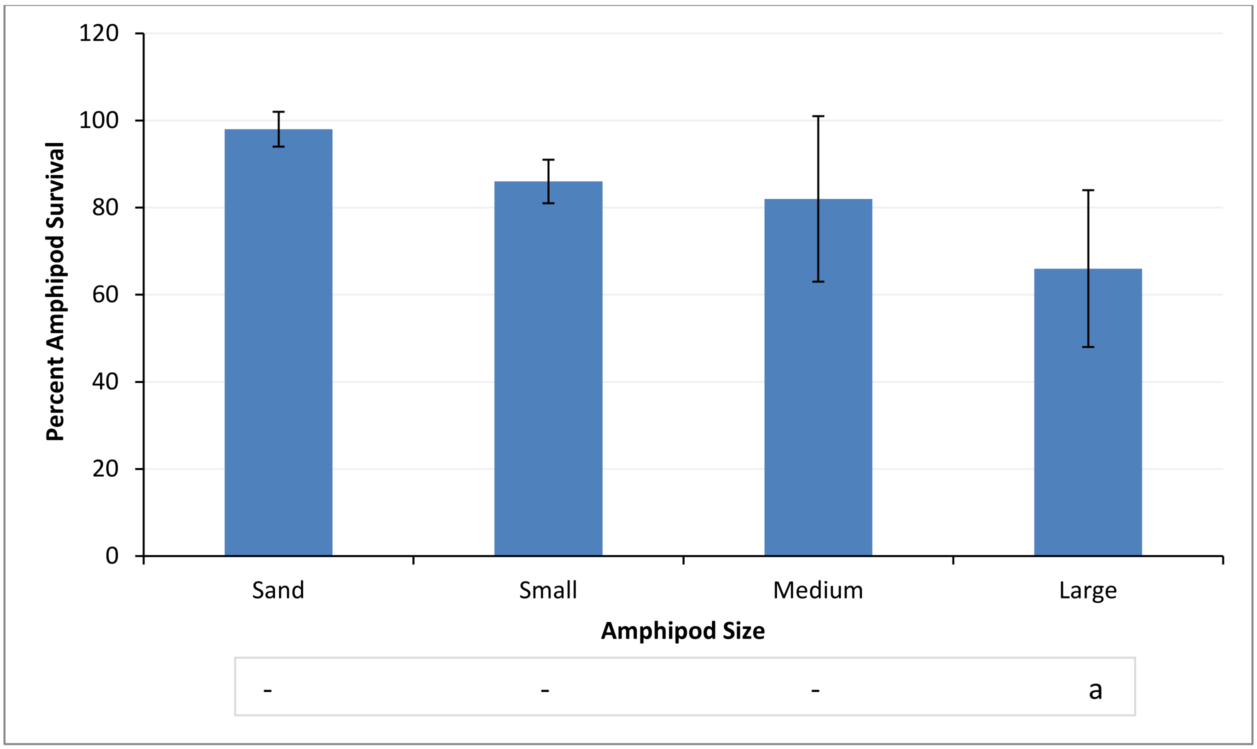


Figure 2. Percent survival of three size classes of amphipods in 100% kaolin clay, and a mixture of size classes in control sand (Experiment 2). "a" indicates significant difference between response of individual size class in a given clay concentration and response in sand ($\alpha < 0.05$).

Experiment 3

To further investigate this relationship, a third experiment was conducted where three size classes of amphipods were exposed to a full range of kaolin. Kaolin was mixed with sand to give the same kaolin percentages as in the first dose-response experiment (0, 10, 30, 50, 70, 90 and 100% kaolin). In this experiment, each size class was exposed to each treatment, including the sand control. Animals for this experiment were collected on August 5, 2014. Small, medium, and large amphipods were visually sorted by NAS, as before. NAS had difficulty finding larger amphipods for this experiment because the largest animals had largely disappeared from the collection site by August (Table 2). Because of this, only five amphipods were used in each replicate container for each kaolin treatment using the large amphipod size class. The amphipods were further visually sorted while loading them in the test replicates. Ten amphipods were used in each replicate container with the small and medium size classes. The animals were sorted by eye and the three size classes were less obvious in the third experiment than in the second experiment. This is reflected in the data.

The size classes used in Experiment 3 were not as distinct as those used in Experiment 2, and the weight ranges overlapped. Overall, the amphipods were smaller in the third experiment, and they weighed less. The weight of the large amphipods used in Experiment 3 was more comparable to the medium amphipods used in Experiment 2. The animals measured for weight presented in Table 2 were selected after each size class of animals was sorted into their respective replicate test containers. Because of the difficulty finding representative medium and large amphipods for the clay exposure, the animals used for the size measurements may not accurately represent the size of the animals used in the exposures. In retrospect, these data may have been more reflective of the actual sizes of animals used for the clay exposures of each size class if the animals measured for size had been haphazardly selected during the sorting process.

Table 2. Size distribution of amphipods exposed to clay ranging from 0 to 100% kaolin (corresponds to results presented in Figure 3). Mean weight was determined from five replicates of 5 amphipods from each size class for small and medium amphipods, and three replicates of 4 amphipods for large organisms. SD indicates standard deviation.

	Weight (mg)			
Size Class	Mean	SD	Minimum	Maximum
Small	0.940	0.245	0.720	1.340
Medium	0.932	0.239	0.620	1.220
Large	1.412	0.078	1.325	1.475

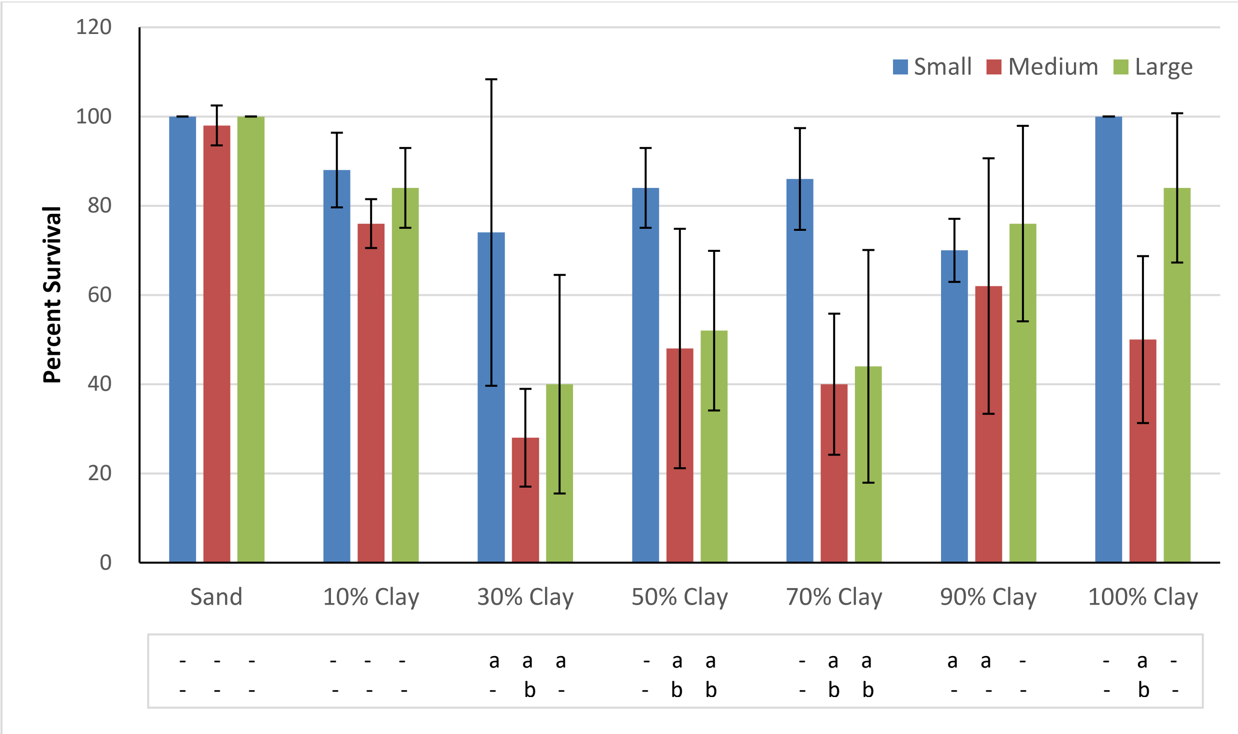


Figure 3. Survival of three size classes of *E. estuarius* in a range of kaolin clay concentrations in Experiment 3. "a" indicates significant differences between response of individual size class in a given clay concentration and response in sand ($\alpha < 0.05$). "b" indicates significant differences between response of medium or large size classes in a given clay concentration and response of small size class in the same concentration ($\alpha < 0.05$).

The average amphipod survival in Experiment 3 was greatest for the smallest size classes when all clay concentrations were combined (84%; Figure 3). The average survival for the medium and large size amphipods exposed to all clay concentrations was 51% and 63%, respectively. Survival in sand was >98% for all size classes. There was no clear dose-response relationship between amphipod size and percent kaolin clay for any of the size classes, but the responses of medium and large amphipods differed significantly from the control in more concentrations than the responses of the small amphipod size class. The survival of the medium and large amphipods in the individual clay concentrations was often significantly lower than the survival of the small amphipods. Results with the small and large amphipods showed an increase in survival at the two highest clay percentages (Figure 3). As was noted with the size measurements, this may have been because of the fact that fewer representative animals were available as each size class was loaded into the final replicate containers. Animals were loaded in sequence from the lowest percent clay to the highest. Therefore, the animals from the three size classes that were loaded into the final replicates used for the highest kaolin concentrations tested with each size class may have been larger than the animals loaded into the lower concentrations. It is also possible that flocculation of kaolin at the higher clay concentrations affected the results (see discussion below).

The grain size distribution in the kaolin-spiked sand treatments was initially analyzed using laser diffraction. Results of these analyses showed that the sand component was not detected by the instrument beyond 30% clay, presumably because the clay in the samples masked the detector in the instrument (data not shown; personal communication, Ivano Aiello, Moss Landing Marine Laboratories). The samples were re-analyzed using the ASTM hydrometer method (ASTM D422). Results of this analysis showed relatively close agreement between the nominal and measured percent clay in each treatment, up to 50% clay (Table 3). In the three higher clay concentrations the measured clay concentrations were approximately 10% lower than the nominal value, and this was accounted for by higher percentages of silt (particle size >4 µm and <63 µm) in these treatments. This likely resulted from flocculation of the clay in the higher clay treatments, and this occurred despite the fact that the samples are dispersed with sodium hexametaphosphate (personal communication, I. Aiello). This was confirmed by laser diffraction analysis of the 100% kaolin treatment. This analysis showed the mean clay size in the 100% kaolin was 2.37 µm, but that the sample was poorly sorted, with a main mode at 2.54 µm and a secondary mode at 5 µm. This analysis showed that only 67% of the sample was below the clay limit (4 µm) and that ~23% was very fine silt, presumably due to flocculation of the sample (despite the use of NaPO as a dispersant prior to analysis). These results suggest flocculation of the clay in the higher kaolin treatments could help explain the lack of a consistent dose-response using the three amphipod size classes.

Table 3. Results of Experiment 3 grain size analysis on kaolin-spiked sand using hydrometer method.

Treatment	Percent Sand	Percent Silt	Percent Clay
10% Kaolin	88.6	0	13.1
30% Kaolin	74.5	0	26.9
50% Kaolin	46.6	4.3	49.4
70% Kaolin	28.5	8.9	62.6
90% Kaolin	8.45	10.8	80.8
100% Kaolin	1.39	7.18	91.4

Experiment 4

The experimental design used in Experiment 3 was repeated in December 2014 when three distinct size classes were present at the Beaver Creek Beach amphipod collection site. Amphipods were again visually sorted into small, medium and large size classes by NAS. Animals were further sorted to assure uniform sizes within each size class during their loading into the test containers at Granite Canyon. A subset of amphipods were selected haphazardly during loading and these were later measured for dry weight, as described above. The three size distributions were more distinct than the previous experiments, and were all significantly different from one another (Table 4).

Table 4. Size distribution of amphipods exposed to clay ranging from 0 to 100% kaolin (corresponds to results presented in Figure 4). Mean weight was determined from five replicates of 5 amphipods from each size class. SD indicates standard deviation.

	Weight (mg)			
Size Class	Mean	SD	Minimum	Maximum
Small	1.004	0.065	0.900	1.080
Medium	1.700	0.189	1.520	1.920
Large	2.812	0.059	2.760	2.900

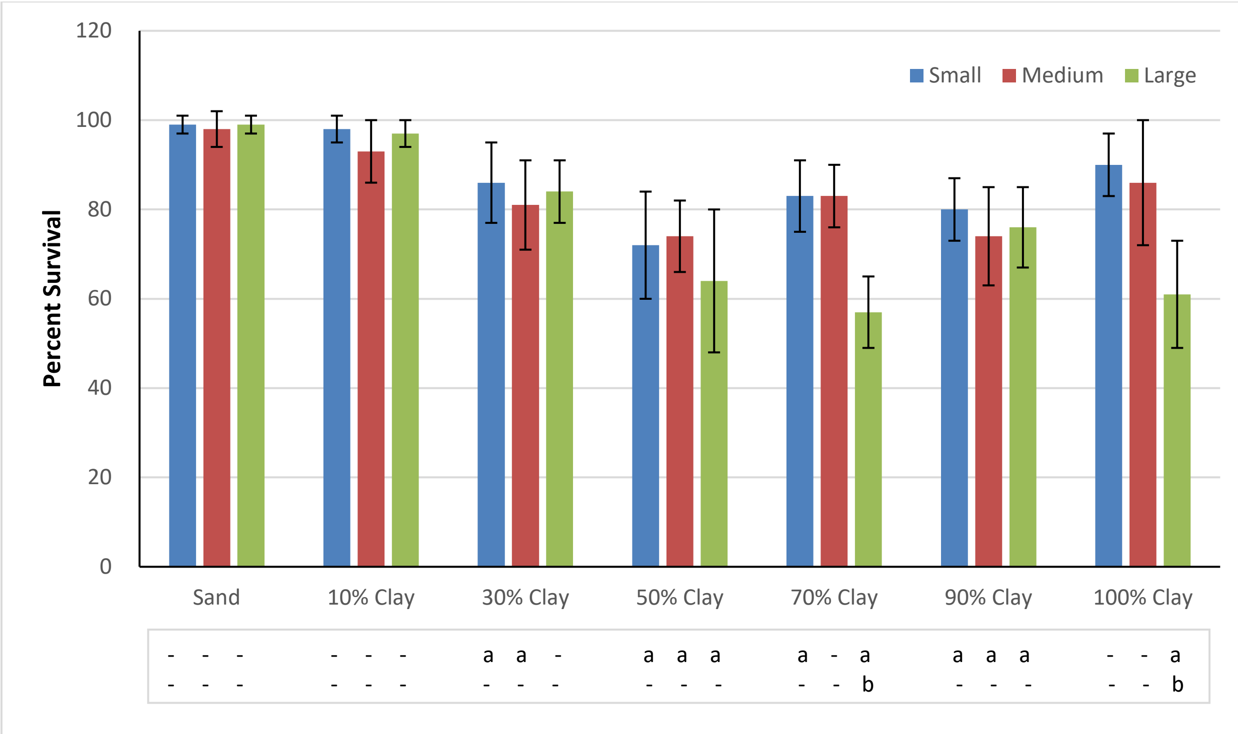


Figure 4. Survival of three size classes of *E. estuarius* in a range of kaolin clay concentrations in Experiment 4. "a" indicates significant differences between response of individual size class in a given clay concentration and response in sand ($\alpha < 0.05$). "b" indicates significant differences between response of medium or large size classes in a given clay concentration and response of small size class in the same concentration ($\alpha < 0.05$).

Survival in the pure sand control was ≥ 98% for all amphipod sizes after the 10 day exposure (Figure 4). As in the previous experiment, clay had the greatest effect on survival of the largest amphipods. Mean survival in all clay concentrations ≥ 10% clay was 84, 82, and 72% for small, medium and large amphipods, respectively. Survival was 81, 79, and 65% for small, medium and large amphipods, respectively in concentrations ≥ 50% clay. Significant effects on survival were observed in clay concentrations 30% and greater in all size classes. A dose-response relationship with lower survival in increasing clay concentrations was observed in the largest amphipods, and large amphipods had significantly lower survival in 70% and 100 % clay (Figure 4), but as was observed in Experiment 3, this relationship was not completely consistent. While there was a dose-dependent decline in survival of large amphipods in concentrations between 10% and 70% clay, survival increased to 76% in 90% clay, then dropped to 61% in 100% clay.

Grain size distributions in Experiment 4 were analyzed using the pipette method, which is an alternate sedimentation method that provides more accurate results (M. Galloway, Soil Control Laboratories, personal communication). These results showed that the measured clay concentrations were in close agreement with the nominal concentrations at clay concentrations between 0 and 50% kaolin (Table 5). As was observed in Experiment 3, the measured clay concentrations were from 7 to 13% lower than nominal in the three highest clay concentrations, and the percentage of particles in the silt size fraction increased in the three highest concentrations (note: pure clay is defined as the size fraction <4 µm; the silt fraction is defined as particles sizes >4 µm and <63 µm). The occurrence of increased flocculation in the higher clay treatments was confirmed with laser diffraction analysis, which showed only 65% of the particles were less than 4 µm in 100% kaolin. This suggests that flocculation also occurred in Experiment 4, and as in Experiment 3, this could help explain the lack of a consistent dose-response relationship at these highest clay concentrations.

Table 5. Results of Experiment 4 grain size analysis on kaolin-spiked sand using sedimentation pipette method.

Treatment	Percent Sand	Percent Silt	Percent Clay
Sand	99.64	0.15	0.21
10% Kaolin	88.04	1.62	10.34
30% Kaolin	72.59	3.28	24.13
50% Kaolin	48.73	5.91	45.36
70% Kaolin	29.33	6.87	63.80
90% Kaolin	12.19	9.09	78.72
100% Kaolin	1.48	11.24	87.28

Possible mechanisms of clay impact on *E. estuarius*

Regardless of the observed inconsistencies, the overall trend in these experiments suggests that *E. estuarius* are less tolerant of kaolin clay as they increase in size. It is not clear how clay impacts survival and hypotheses range from size-specific impacts on gill function to clogging of female marsupia. Smaller amphipods might have larger energy reserves and can therefore better withstand the energy demands required of functioning in high clay sediments. An additional possibility is related to the high density of setae that are characteristic of haustoriid amphipods.

In descriptions of several species of haustoriids Barnard and others (Barnard, 1962; Croker, 1967; Bousfield, 1970; Bosworth, 1976; Bousfield and Hoover, 1995) describe these amphipods as being highly setose (i.e., covered in coarse and fine setae), presumably as an adaptation to burrowing in sandy beach and subtidal habitats (see Figure 5). Microscopic examinations of amphipods from the three size classes used in the current experiments indicated that the number and density of setae increased with the size of the amphipods. To quantify this we selected a representative structure on the amphipods and developed several metrics to quantify the degree of setoseness. This structure was the third article of the second antenna which is near the head of the amphipod (Figure 6). The antennae of haustoriids are used for a number of functions including chemoreception, olfactory reception, and burrowing (Kaufman, 1994). This structure was selected because it is representative of other appendages, practical to quantify and easily identifiable under magnification. Setae were quantified on the posterior edge of this structure on five animals from each of the three size classes. The following metrics were measured using the digital micrometer feature on an Olympus IX71 inverted microscope: dorsal-ventral length of antenna 2 article 3, average number and length of primary setae, average length of secondary setae (Figures 6a and b; note - the secondary setae are the feathery plumes branching from the primary setae).

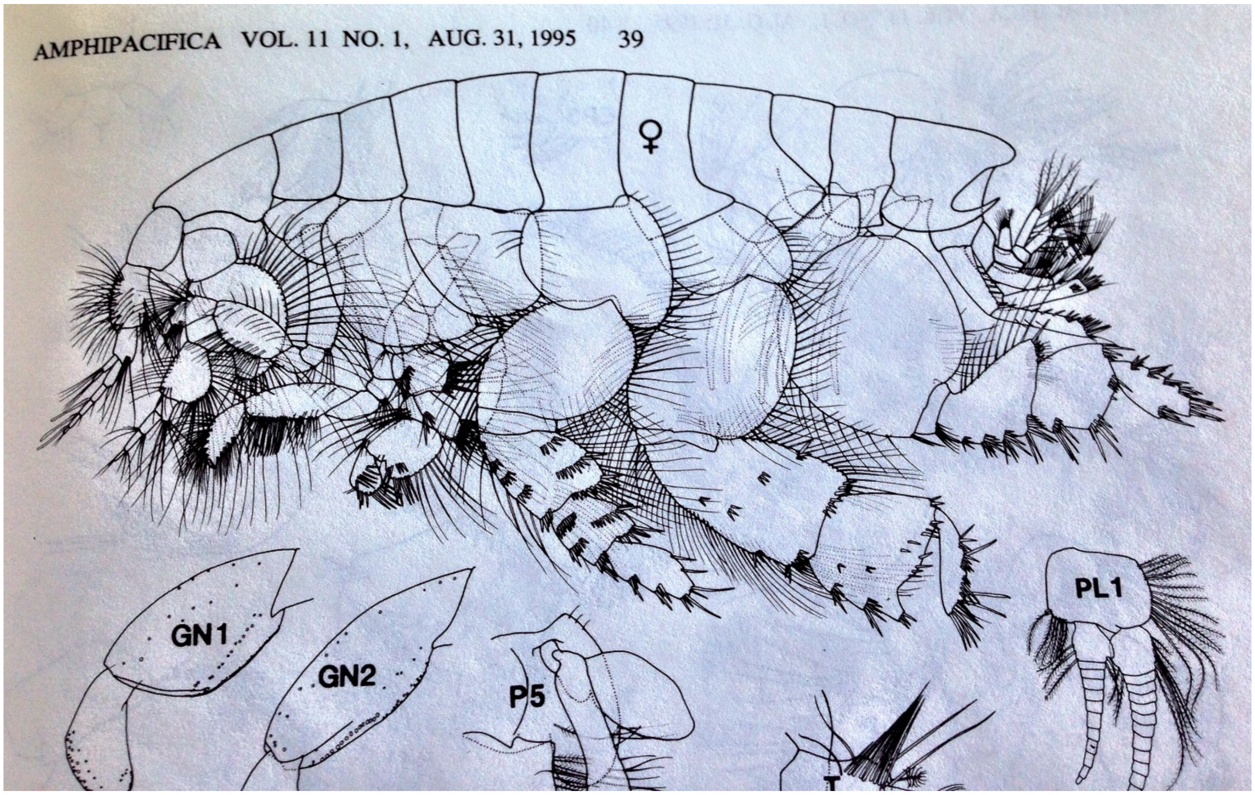
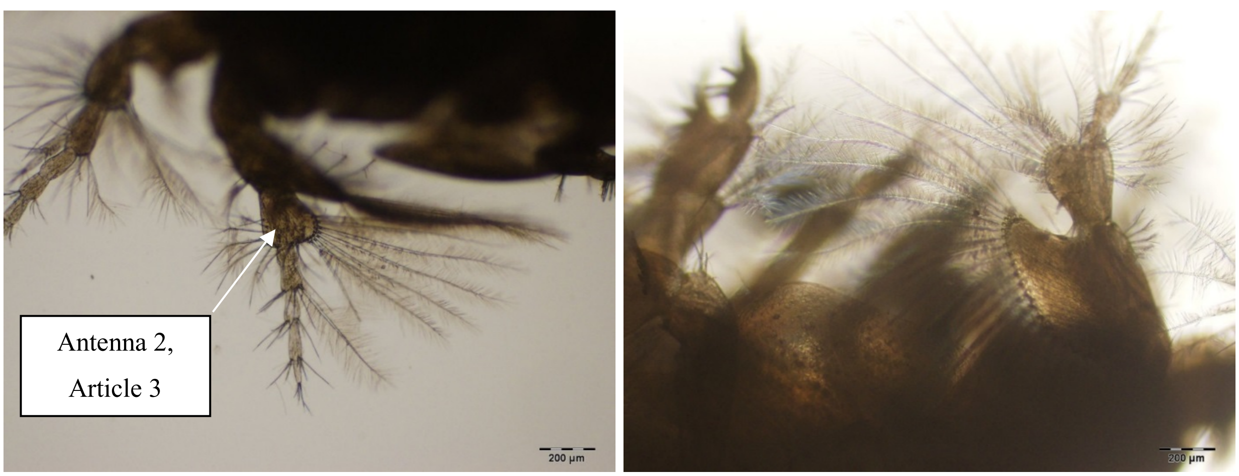


Figure 5. Drawing of *Eohaustorius sp*. by E. Bousfield, 1995.



(a: small amphipod) (b: large amphipod)

Figure 6a and b. Photomicrographs of 2nd antennae from small (a) and large (b) size classes of amphipods in Experiment 4.

All measurements confirmed that as *E. estuarius* grow, the size and degree of setoseness of the 2^{nd} antennae increases. The third articles of second antennae were significantly longer on the largest amphipods relative to small animals. In addition, large animals had longer primary and secondary setae (Table 6). Another way to examine these data is to normalize the appendage and seta lengths to the average mass of the organisms in each size class. When the metrics are divided by the average weights listed in Table 4, the proportional average decreases as the organism mass increases (Table 7). All metrics measured as a proportion of the weight were significantly smaller in the large amphipods.

The relationship between increasing amphipod size and increasing number and length of primary and secondary setae on the second antenna likely holds for the many appendages bearing setae on this species, and may explain why larger animals have a lower tolerance for increasing clay concentration. One hypothesis is that as *E. estuarius* grow, their setae increase in size and number and become more plumose with secondary setae, and the energy required to burrow through and function in high clay sediment increases. Smaller animals are less setose and therefore may expend less energy burrowing, and this is reflected in greater survival of smaller animals over a ten day exposure. Another hypothesis involves the proportional size of the setae. Because the appendage and setae length become proportionally shorter as the amphipod increases in size, it is possible that the functions of the setae become less efficient. In this case the greater size of primary and secondary setae may provide more surface area for clay to clog, and this clogging effect has a greater relative effect on the physiological functioning of larger amphipods. Setae are involved in a number of functions including burrowing, grooming and swimming, as well as food particle concentration and filtering. Setae are used to create respiratory currents and specialized setae are used in chemoreception and gustatory (i.e., food tasting) functions (Kaufman, 1994). It is possible that clay impacts a number of these activities and this negative effect is exacerbated as amphipods grow and become more setose. We note that these hypotheses are speculative and the direct impact of clay on amphipod physiology would require more direct experimentation.

Table 6. Average (standard deviation) size metrics of setae from amphipod antennae 2, article 3. See text for explanation of metrics.

Amphipod Size Class	Mean Antennae 2, Article 3 Length (µm)	Mean Primary Seta Length (µm)	Mean Secondary Seta Length (µm)	No. Primary Seta
Small	236 (21.6)	753 (56.5)	78.1 (8.7)	13.0 (0.84)
Medium	256 (15.4)	830 (52.4)	91.2 (11.0)	13.8 (0.89)
Large	297 (51.2)	923 (111)	98.3 (14.7)	15.2 (1.53)

Table 7. Proportional average (standard deviation) size metrics of setae from amphipod antennae 2, article 3. Average values listed in Table 6 were divided by average size class weights listed in Table 4.

Amphipod Size Class	Mean Antennae 2, Article 3 Length (µm)	Mean Primary Seta Length (µm)	Mean Secondary Seta Length (µm)
Small	235 (30.6)	750 (74.6)	77.7 (13.1)
Medium	151 (18.9)	488 (16.1)	53.7 (3.2)
Large	106 (10.0)	328 (14.0)	35.0 (4.1)

This effect may be compounded by seasonal life history characteristics. *E. estuarius* are late winter breeders and there are a higher percentage of larger and sexually mature animals in winter months (peaking in February and March; Bosworth 1976). The percentage of smallest animals in Oregon beach habitats peak in June, and no reproduction occurs in summer (Bosworth, 1976). Larger sexually active animals may also have lower energy reserves in winter. In addition, male *E. estuarius* die after breeding (Peter Slattery, Moss Landing Marine Laboratories; personal communication). It should be noted that our procedure avoids the largest amphipods when working with *E. estuarius,* primarily to avoid loading reproductive or senescent animals in test containers. Discussions with numerous testing laboratories indicate that this is part of the standard operating procedure with this species. However, this practice may include use of some larger animals, particularly in tests conducted in winter when the proportion of larger individuals increases. This could include ovigerous females. Clay could also interfere with gravid females by clogging the female marsupium (Peter Slattery, Moss Landing Marine Laboratories; personal communication).

The interaction of these factors may help explain the observation of seasonal differences in sediment toxicity in RMP samples. The number of toxic sites and the magnitude of toxicity is greater in the estuary in winter. Winter is when greater sediment contamination is measured in RMP sediments (Anderson et al., 2007), and is also coincident with greater percentage of larger animals at the amphipod collection site. Lower summer toxicity might be explained by lower sediment contamination combined with tests conducted with smaller amphipods. Previous analyses have not suggested that suspended clay in San Francisco Estuary waters vary by season (Knebel et al., 1977), but seasonal clay content in sediments has not been investigated. The degree to which these factors affect toxicity results in RMP sediments can be confirmed only through experimentation.

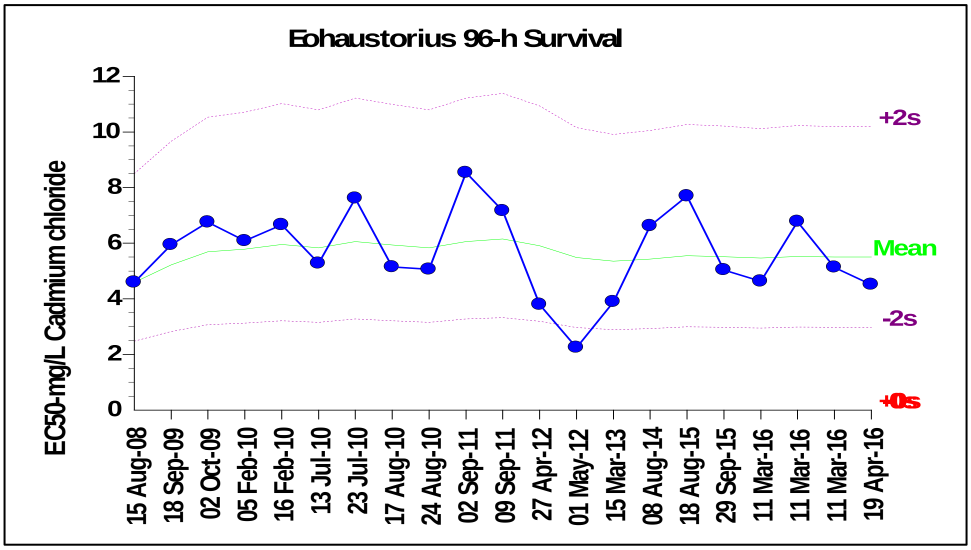
Phase 2 Experiments (2015- 2016)

Size Specific Responses to Cadmium

An additional series of experiments were designed to confirm the results suggesting that smaller amphipods tolerate high clay sediments better than larger amphipods. Prior to conducting experiments with field collected sediments, water only 96-hour cadmium chloride reference toxicant tests were conducted with small, medium and large amphipod size classes. Results of these experiments showed LC50s of 6.78, 5.13, and 4.63 mg/L, for small, medium, and large amphipods, respectively. These results indicated that larger amphipods were somewhat more sensitive to cadmium, though 95% confidence intervals overlapped for all LC50s (Table 8). When plotted within historical laboratory data for this metal, the results showed that all three LC50s fell within two standard deviations of the running mean LC50 for $CdCl_2$ toxicity to *E. estuarius* (Figures 7a and b). These results suggest minimal difference in sensitivity to cadmium among the three size classes.

Table 8. Results of 96-hour cadmium chloride reference toxicant tests with small, medium and large amphipod size classes. CI indicates confidence interval.

Size Class	Cadmium LC50 (mg/L)	95% CI
Small	6.78	5.15 - 8.92
Medium	5.13	3.90 - 6.70
Large	4.63	3.78 - 5.68

A.


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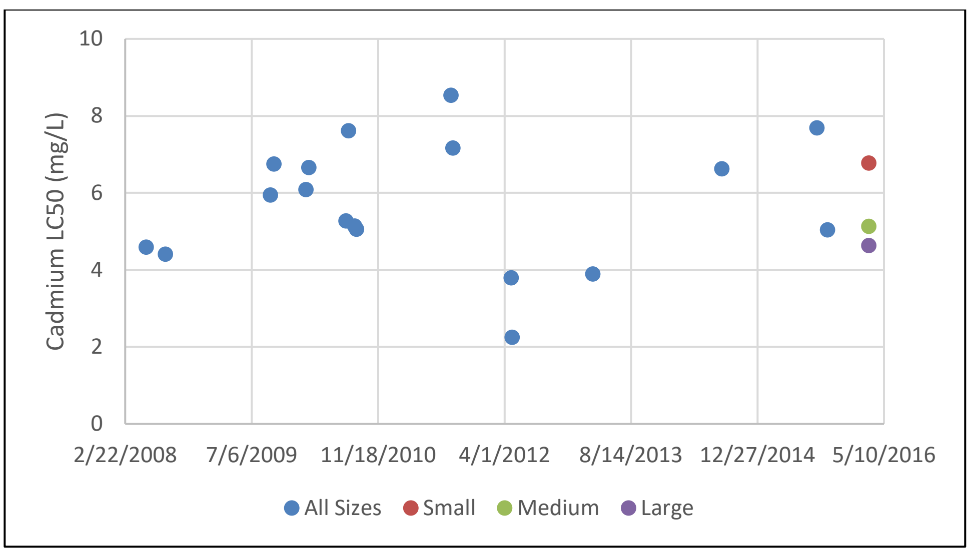

Figure 7a. Control chart plotting recent *E. estuarius* reference toxicant LC50 data for $CdCl_2$. The final three reference toxicant tests with small, medium, and large size classes are on far right. 7b. $CdCl_2$ LC50 data points used to construct the control chart with LC50s for the three size classes on the far right (red = small, orange = medium, blue = large).

Size Specific Responses to Kaolin

A dose response experiment was conducted with small, medium and large size classes exposed to sand-spiked kaolin. This experiment repeated experiments conducted in Phase 1 and was intended to confirm the previous results showing a size-specific response to clay (reported above). Methods for this experiment duplicated those described for Experiments 3 and 4 in Phase 1, where small, medium and large amphipods were exposed to a series of kaolin concentrations ranging from 0% (sand) to 100% kaolin. Measurements of representative animals from the three size classes showed that all three were distinct in terms of dry weight (Table 9), and concentrations of clay were similar to previous experiments (Table 10). Results of this experiment confirmed that smaller amphipods are more tolerant of kaolin (Figure 8). There was a clear dose-response relationship between increasing kaolin concentration and decreasing amphipod survival, particularly for the large amphipods. Average survival for small, medium and large amphipods exposed to kaolin concentrations $\geq$ 50% clay were 88%, 63%, and 41%, respectively.

Table 9. Size distribution of amphipods exposed to clay ranging from 0 to 100% kaolin (corresponds to results presented in Figure 8). Mean weight was determined from five replicates of 5 amphipods from each size class. SD indicates standard deviation.

	Weight (mg)			
Size Class	Mean	SD	Minimum	Maximum
Small	0.791	0.288	0.546	1.210
Medium	1.554	0.124	1.420	1.730
Large	1.966	0.123	1.840	2.110

Table 10. Results of Experiment 5 grain size analysis on kaolin-spiked sand using sedimentation pipette method (corresponds to results presented in Figure 8).

Treatment	Percent Sand	Percent Silt	Percent Clay
10% Kaolin	90.1	0.7	9.3
30% Kaolin	71.1	3.1	25.8
50% Kaolin	51.5	5.7	42.9
70% Kaolin	26.1	8.1	65.8
90% Kaolin	12.1	5.1	82.9
100% Kaolin	1	6.3	92.8

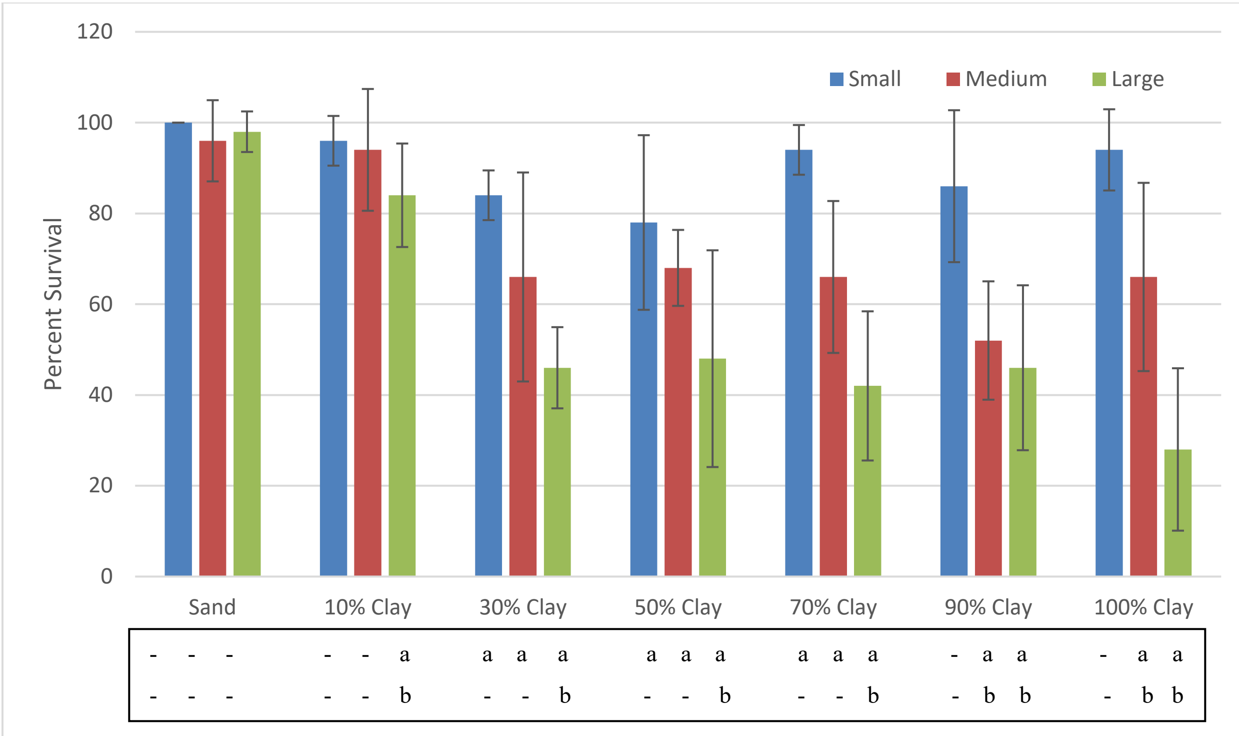

Figure 8. Survival of three size classes of *E. estuarius* in a range of kaolin clay concentrations in Experiment 5. "a" indicates significant differences between response of individual size class in a given clay concentration and response in sand ($\alpha < 0.05$). "b" indicates significant differences between response of medium or large size classes in a given clay concentration and response of small size class in the same concentration ($\alpha < 0.05$).

Size Specific Responses to Field Sediments

Experiments with field sediments were conducted to determine whether size specific differences also occurred in clay-rich sediments from the San Francisco Estuary. The RMP data base was screened to select stations that have historically been characterized with low contaminant concentrations and high clay content. The most recent RMP Status and Trends data were screened for low contaminant concentrations based on a mean sediment quality guideline quotient value (SQGQ), and moderate amphipod survival. Sites with percent clay concentrations greater than 60% were prioritized. Based on these parameters, sediments from six stations were collected in December 2015. These sites had historic mean SQGQ values of $\leq$ 0.15 (Fairey et al., 2001), and amphipod survival in samples from these sites was 73 to 88% in previous testing (Table 11). Toxicity tests were conducted on each sample using the mixture of $\geq$1mm size classes typically provided by Northwest Aquatic Sciences as part of routine test organism supply. Toxicity was higher than had been previously observed with survival ranging from 11 to 32% at these stations (Table 11). The percent clay at these sites were lower than previously observed, with clay ranging from 36 to 56%, and all but one sample had greater than 96% fine grained sediments. Based on these data, sediments with the highest clay content were selected for the final experiments to evaluate size-specific effects: Lower South Bay 073 (LSB073), San Pablo Bay 13 (SPB013), and San Pablo Bay 25 (SPB025). Although the toxicity of the current samples was much higher than that of the historic samples, chemical analysis of SQGQ analyte groups using the approach of Fairey et al. (2001) demonstrated that the current sediments had low contaminant concentrations, with quotient values of 0.16 for all three sediments (data not shown). Seven pyrethroid pesticides were also measured in these three sediments, and all pyrethroids were below detection limit with the exception of bifenthrin in LSB073. Bifenthrin was detected but not quantified, and had an estimated concentration of 0.3 ng/g, well below the bifenthrin LC50 for *E. estuarius* (7.9 ng/g (Anderson et al., 2008)).

Table 11. Amphipod survival in sediments from selected RMP stations in December 2015. Grain size characteristics of each sediment are also provided. Sediments selected for size-specific toxicity comparisons are indicated with *.

Station	Historical Data				Current Data					
	Sample Date	Mean % Surv.	% Clay	SQGQ	Mean % Surv.	SD	% Sand	% Silt	% Clay	% Fines
LSB017	8/3/04	88	69	0.15	23	11	1.5	52.0	46.5	98.5
LSB073*	8/24/05	73	69	0.11	30	9	3.0	41.1	55.9	97.0
Petaluma Riv.	NA	NA	NA	NA	21	11	2.2	52.4	45.4	97.8
SPB010	8/20/03	81	53	0.10	32	10	33.4	30.5	36.1	66.1
SPB013*	8/19/03	73	67	0.14	11	7	1.0	43.5	55.5	99.0
SPB025*	8/26/05	79	61	0.11	18	12	3.8	48.5	47.7	96.2
Control (Home)	NA	NA	NA	NA	100	0	NA	NA	NA	NA

Size Specific Responses to Field Sediments

Experiments to evaluate the responses of small, medium and large *E. estuarius* to these three sediments were conducted in April 2016. All sediments were diluted with reference sand to provide a range of sediment concentrations: 0 (sand), 25%, 50% and 100%, and three distinct amphipod size classes were exposed (Table 12). The undiluted samples had percent clay concentrations ranging from 48% to 56%, and diluting the samples brought the clay content down to approximately 8% to 16% (Table 13).

Table 12. Size distribution of amphipods exposed to 3 field sediments ranging from 0 to 100% field sediment (corresponds to results presented in Figures 9-11). Mean weight was determined from five replicates of 5 amphipods from each size class. SD indicates standard deviation.

	Weight (mg)			
Size Class	Mean	SD	Minimum	Maximum
Small	0.961	0.173	0.690	1.100
Medium	1.348	0.110	1.240	1.510
Large	1.900	0.178	1.720	2.190

Table 13. Grain size analysis of 3 field sediments used to assess size-specific responses of amphipods corresponding to results in Figures 9-11.

Treatment	Percent Sand	Percent Silt	Percent Clay
LSB073 100%	3.0	41.1	55.9
LSB073 50%	37.7	26.5	35.9
LSB073 25%	77.3	9.7	13.1
SPB013 100%	1.0	43.5	55.5
SPB013 50%	61.7	16.8	21.5
SPB013 25%	71.4	12.4	16.1
SPB025 100%	3.8	48.5	47.7
SPB025 50%	65.5	17.4	17.1
SPB025 25%	84.4	7.5	8.1

There was higher amphipod survival in all three sediment samples in April 2016 than were observed in the screening toxicity tests conducted in December 2015. Despite the reduced toxicity in these samples, the small size amphipods demonstrated significantly greater survival than the medium and large amphipods in the 50% and 100% sediment concentrations in the LSB073 and SPB013 sediments (Figures 9-10). Both the small and medium size classes had greater survival than the large amphipods in the two higher concentrations of the SPB025 sediment (Figure 11).

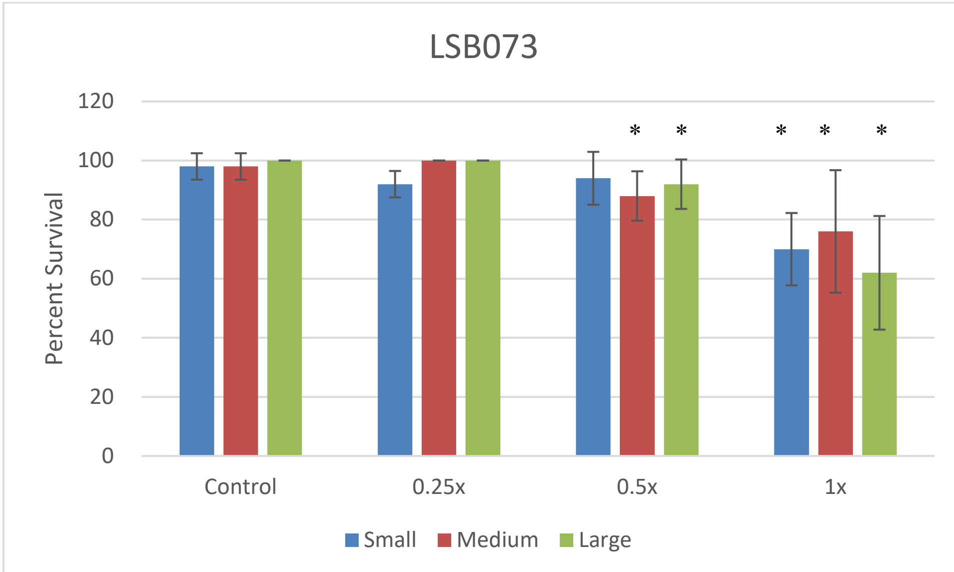

Figure 9. Survival of three size classes of *E. estuarius* to a dilution series of field sediment from station LSB073. Asterisk (*) indicates significant toxicity when compared to the control in the respective size class.

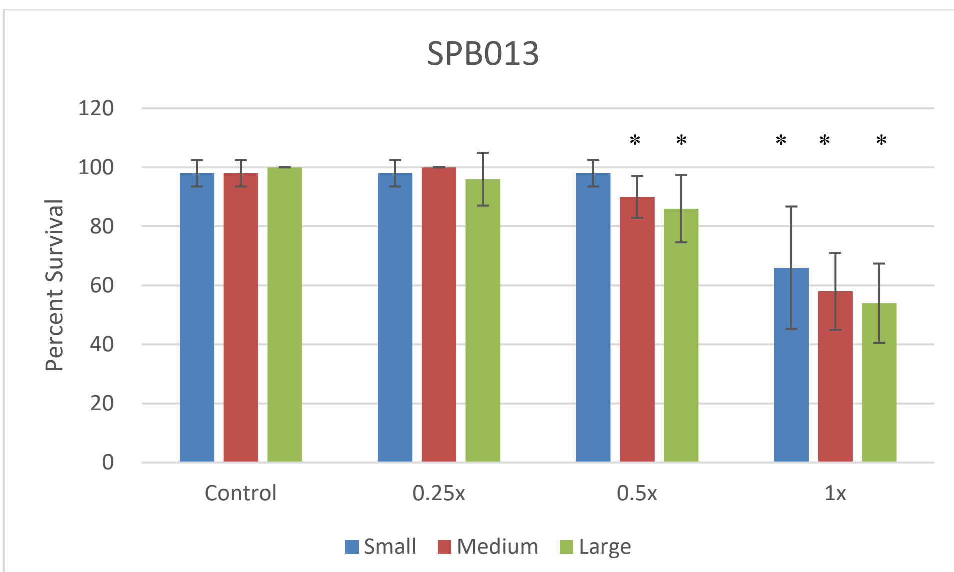

Figure 10. Survival of three size classes of *E. estuarius* to a dilution series of field sediment from station SPB013. Asterisk (*) indicates significant toxicity when compared to the control in the respective size class.

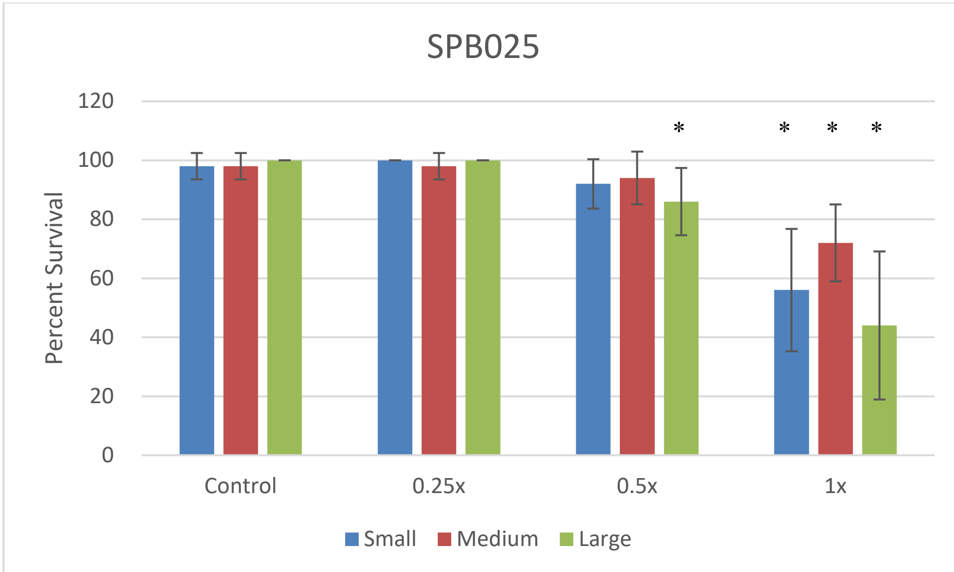

Figure 11. Survival of three size classes of *E. estuarius* to a dilution series of field sediment from station SPB025. Asterisk (*) indicates significant toxicity when compared to the control in the respective size class.

It is not clear why there was an apparent reduction of toxicity of these samples between the December and April testing. The samples were assumed to be relatively uncontaminated based on historical and current data, and any effects on amphipod survival were assumed to be due to effects of clay. One possible reason is that the lower amphipod survival in these samples in December was due to unmeasured contaminants, and these degraded between the December and April testing. These samples were analyzed for metals, PAHs, PCBs, and organochlorine pesticides following Fairey et al. (2001), and were also analyzed for seven pyrethroids. All measured concentrations were lower than known effect thresholds. A second and more plausible explanation is that the amphipods used in testing had variable sensitivities to the sediments between December and April. Amphipods used for the initial screening of the samples were collected in December 2015, and represented a mixture of size classes. Previous research suggests that the winter population of *E. estuarius* at the collection site have a greater proportion of larger individuals. The size ranges of the animals used in the December screening tests were not measured. It is also possible both factors interacted to influence the results.

The sediments were diluted with the sand to provide a dilution series of percent clay in the samples. The results showed better survival of all size classes in the lower dilutions. However, it is not possible to separate the effects of grain size (clay) from the possible influence of unmeasured contaminants on amphipod survival. Although the effects of contaminants are difficult to separate from those of grain size, smaller amphipods performed statistically better than the large size class.

Conclusions

The results of these experiments suggest that larger *E. estuarius* are less tolerant of clay than smaller individuals. Despite the lack of a clear dose-response relationship in some of the kaolin experiments, the survival in clay was greatest using the smallest size class of amphipods in all experiments where amphipods were tested by size, and a dose response relationship was observed in the final kaolin experiment conducted in Phase 2 of the project. All size classes showed lower survival relative to pure 100% sand, even when exposed to the lowest concentration of kaolin (10%).

Results of water-only reference toxicant tests with cadmium chloride showed some increase in response to this metal between small, medium and large size classes. Cadmium LC50s for all three size classes were within the historic range of responses to this metal. Size-specific exposures to sediments from three field stations also suggested that smaller amphipods tolerated sediments with moderate clay concentrations better than larger amphipods. Chemical analyses of the field samples demonstrated that contaminant concentrations in these samples were low relative to sediment quality guideline quotient values, and pyrethroid pesticide concentrations were below *E. estuarius* threshold values.

The specific mechanism by which clay inhibits amphipods was not conclusively demonstrated and may not be related to an increase in the degree of setoseness as amphipods increased in size. Seta metrics were quantified in the current experiments and the results showed that larger animals have larger primary and secondary setae. However, normalized to the mass of the amphipods, the relative size of the setae were proportionately smaller in the larger animals. It is possible therefore that the functioning of setae become less efficient in the largest animals as they became clogged with clay. In this scenario larger more plumose setae are more easily clogged with clay and this has a greater physiological cost for larger amphipods. However, because the impact was not linked to any physiological endpoint, the specific mechanism remains unclear. It is also possible the size-related clay inhibition relates to some other factor such as gill clogging, depletion of energy reserves, or inhibition of feeding (which is likely also related to functioning of setae).

These results suggest modifications could be made to the 10-day *E. estuarius* protocol for future testing of San Francisco estuary sediments. The most practical solution would be to conduct tests with the smallest amphipods available. This could be accomplished by requiring the supplier, Northwest Aquatic Sciences, to pre-sort amphipods prior to shipment and to supply only the smallest amphipods caught on the 1 mm sieve. In our experiments amphipods with an average weight of 0.97 mg dry wt. (average ranging from 0.79 – 1.13 mg dry wt.) had the best survival in clay. Testing of clay-rich sediments should use smaller size amphipods to reduce clay related mortality. We note that dry weight provides a more accurate measure of amphipod size because length is affected by the curvature of the amphipod body.

Based on the observation that even 10% kaolin tended to reduce amphipod survival relative to sand, a second modification might be required to allow for some mortality in clay rich sediments. Small amphipod survival averaged 84% in all clay concentrations in Experiments 3 and in 4, which was 16% lower than their survival in sand. Small amphipod survival averaged 88.6% in all clay concentrations in the kaolin dose-response experiment conducted in Phase 2, which was 11.4% lower than their survival in sand. The RMP criteria for significant toxicity using this protocol is based on a statistically significant difference from the control, as well as survival less than the minimum significant difference for this test ($\geq$18.8%). Depending on control survival and between-replicate variability, this usually results in samples with survival less than 72% being designated as "toxic". Use of smaller amphipods resulted in average survival of 84% to 88.6% to all clay concentrations, which would not be considered "toxic" based on these criteria. This ignores any potential interaction between anthropogenic contaminants that elicit a true toxic response, and clay effects which may be mechanical or related to organism health.

Additional seasonal measures of lipid content in field collected *E. estuarius* might help confirm the relationship between amphipods and energy reserves. This investigation could also determine whether lipid content varies with amphipod size.

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