THE SEDIMENT-CONTACT TEST USING THE OSTRACOD *HETEROCYPRIS INCONGRUENS*: EFFECT OF FINE SEDIMENTS AND DETERMINATION OF TOXICITY THRESHOLDS.

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ABSTRACT

The toxicity test using freshwater ostracods of the species *Heterocypris incongruens* is a sub-chronic static test that exposes individuals to whole sediments over a period of 6 d, the endpoints being mortality and growth. We tested the hypothesis that endpoints of the sediment bioassay using *H. incongruens* are affected by the presence of fine sediment particles by testing control sediment supplied with the commercial test kit with increasing proportions of kaolin clay as a proxy for fines. While mortality is not affected, the results show that increasing the presence of clay reduces ostracod growth. Based on the variability in growth, a sublethal toxicity threshold of 35% is proposed to distinguish effects due to sediment properties from those due to toxicity. The relevance of this threshold was verified using data from toxicity tests of ambient sediment samples with low levels of contamination.

Keywords

Sediment toxicity

Ostracodtoxkit®

Clay content

Toxicity threshold

Grain size

1. Introduction

The toxicity test using freshwater ostracods of the species *Heterocypris incongruens* is a sub-chronic static test that exposes individuals to whole sediments over a period of 6 d, the endpoints being mortality and growth. This bioassay can be purchased as a kit (Ostracodtoxkit FTM, MicroBioTests Inc.) and it has a number of advantages over other conventional bioassays using benthic invertebrates in that it does not require stock culturing of organisms, is miniaturised into a microscale test that only requires limited bench space, sample volume and materials, and is user-friendly. Since its establishment in the early 2000s, it has been widely applied for sediment quality assessment either for toxicity screening or as part of a toxicity test battery (e.g. Chial-Belgis et al., 2003; Garcia-Lorenzo et al., 2009; Torokne and Toro, 2010; Mwanamoki et al., 2014) and for testing other complex matrices, including soils (e.g. Oleszczuk 2008; Plaza et al. 2010; Khanal et al., 2015). In 2012 a standard protocol for testing freshwater sediments with the ostracod *H. incongruens* was released (ISO 2012).

As for other analytical and testing methods, the assessment of ruggedness (robustness) for sediment bioassays should be part of the development phase (ASTM 1992; Dillon 1994). Because responses of benthic organisms may be influenced by a variety of sediment characteristics, test ruggedness addresses the sensitivity to sediment characteristics in addition to the sensitivity of the test method to deviations from normal test conditions and protocols (Dillon 1994). The experimental determination of ruggedness is meant to provide a matrix of conditions for which the sediment bioassay is or is not appropriate. Chial and Persoone (2002a; 2002b) studied the sensitivity of the ostracod test endpoints to deviations in the test protocol, including incubation conditions, and an interlaboratory comparison was carried out for its standardization (Janssen and Persoone 2010). The sensitivity of *H. incongruens* to unionized ammonia and pH changes has recently been determined (Watanabe et al. 2013). Test sensitivity to fine sediment (the term is used here as the load of silts and clays, which have diameters smaller than 0.0625 mm), an important factor in sediment toxicity assays (Dillon et al. 1994), has not yet been determined.

According to the ISO guideline 14371 (2012), the ostracod bioassay is based on the comparison of mortality and growth of freshly hatched ostracods in control and test sediments after a 6 d exposure. The controls are used to determine compliance with acceptability criteria (mortality $\leq 20\%$ and a mean ostracod length increase of at least 1.5-fold over the test duration) and to quantify growth inhibition as a % control response if the % mortality is low ($< 30\%$). A commercial (not toxic and not calcareous) river sand or marine sand, which is water-washed and sieved to eliminate dirt and debris and then air-dried, can be used as control sediment provided it is a medium-coarse sand, with a particle size < 2 mm (ISO 2012). However, the grain size of natural sediments can vary greatly, ranging from sand, through silty-sand, to sandy-silt, to clays. If fine sediments have an effect on test endpoints, the interpretation of results solely on the basis of comparison with responses in a medium-coarse control sediment may overestimate or underestimate toxicity.

One way of dealing with sediment matrix issues is by use of an uncontaminated reference sediment with similar properties to the test sediments. However, this approach may not be practicable in monitoring programs, either because the samples being tested vary in grain size composition, or due to difficulties finding appropriate uncontaminated reference sediments. An alternative to using reference sediments is to determine the natural variability of the test endpoints by testing natural sediments with a broad range of physico-chemical properties and low levels of contamination (Höss et al. 2010; Durand 2012). The main advantage of this method over the reference sediment approach is that it avoids additional testing.

In the present study, we tested the hypothesis that endpoints of the sediment bioassay using *H. incongruens* are affected by the presence of fine sediments. Tests were performed with control sediment supplied with the commercial test kit amended with increasing proportions of kaolin clay as a proxy for fine sediment load. The results were used to derive toxicity thresholds that distinguish the effect of grain size from that of toxicity, and the relevance of these thresholds was verified using data from toxicity tests of ambient sediment samples with low levels of contamination.

2. Materials and Methods

2.1. Test with ostracods

The sediment toxicity test using *H. incongruens* was performed following the corresponding ISO guideline 14371 with no modifications (ISO 2012). Tests with artificial sediments were performed using a commercial Ostracodtoxkit (MicroBioTests Inc.) which provides all materials and allows the test to be completed in accordance with the guideline. Tests with ambient sediment samples were carried out using commercially available cysts of *H. incongruens* (MicroBiotest Inc.), but they were fed *Pseudokirchneriella subcapitata* from a pre-culture in the exponential growth phase. The clean sand provided with the commercial kit was also used for all trials.

2.2. Test with artificial sediments

The medium-coarse sand (> 0.2 mm) provided with the commercial kit (this sediment is provided dried and has virtually no fine sediment and an organic matter content of < 1%) was mixed with a spatula with dry pure kaolin ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$; 0.1–4 μm ; Fluka, Sigma Aldrich) until complete homogenization to form the following mixtures (expressed as % clay): 0, 2.5, 5, 10, 15, 20, 40 and 80. Each sediment was tested in triplicate and two separate trials were performed.

2.3. Tests with natural sediments

Eight ambient sediment samples were tested in 6 replicates and three separate trials. These sediments are characterized by low levels of contamination and toxicity. Further information on the sediment sampling and characterization can be found elsewhere (Durand 2012; Burga-Perez 2012). The clay content in these sediments ranged between 0.04 and 19%, while the proportion of fine

sediment (including silt and clay) ranged between 6 and 91%. Organic matter content (measured as loss on ignition) ranged between 2 and 15%.

2.4. Data analysis

Statistical analyses were performed with GraphPad Prism®. For comparison of means a Kruskal-Wallis test was carried out because the requirements for a parametric test, normal distribution and equal variances, were not met. Mann-Whitney test was used for post-hoc comparisons.

Toxicity criteria were derived based on the minimal detectable difference and the maximal tolerable inhibition following Höss et al. (2010). Briefly, the variability of the test endpoints among replicates was expressed as the coefficient of variation (CV_i) and the minimal detectable difference (MDD), also referred to as minimum significant difference (Denton et al. 2003). The variability of endpoints when expressed in relative values (% effect with respect to controls) associated with differences in the sediment matrix was estimated using the coefficient of variation (CV_s) and the maximal tolerable inhibition (MTI). This approach was applied for all tests including those for which requirements for a parametric test were not formally met. Thus this paper presents indicative threshold values as they are calculated using parametric statistics as an approximate index of test precision and variability.

A CV_i for each test sediment was calculated as $CV_i = SD/Mean \times 100$ where Mean and SD are the mean and standard deviation of test endpoints (mortality and growth) from replicates of each test sediment sample. The mean, maximum and minimum CV_i were calculated separately for the set of controls (CV_{i_c}) and sediments (CV_{i_s}). Then the MDD, which is the smallest difference between the mean responses in a test sediment and a control that is statistically significant given the observed test variability and the number of replicates, was calculated as

$$\% MDD_s = 100t \sqrt{[(SD_c^2 / n_c) + (SD_s^2 / n_s)] / Mean_c} \quad (1)$$

where t is the tabulated value of the Student's t distribution ($\alpha = 0.05$, one-sided, $df = n_c + n_s - 2$), SD_c^2 , SD_s^2 and $Mean_c$ are, respectively, the variances or mean of the test endpoint for the control sediment (c) and the test sediment (s) and n_c and n_s are the number of replicates for the control sediment (c) and the test sediment (s), respectively. Finally, an average MDD is calculated over all single MDD_s as a measure of precision of each test endpoint.

To quantify the influence of fine particles on mortality and growth inhibition CV_s were calculated as $CV_s = SD_{RV} / Mean_{RV} \times 100$ where SD_{RV} and $Mean_{RV}$ are the mean and standard deviation of each endpoint expressed as % control response of all test sediments.

The MTI is the maximal inhibition compared to the control that is still within the natural variability of the endpoint under the given test conditions and is calculated as

$$\% \text{ MTI} = \text{Mean} (\% I_s) + \text{SD} (\% I_s) \quad (2)$$

where $\text{Mean} (\% I_s)$ and $\text{SD} (\% I_s)$ are, respectively, mean and standard deviation of percent inhibition of a toxicity endpoint in a test sediment (s) compared to the respective control sediment. Percent inhibition was defined as $\% I = 100 - \text{Mean}_s / \text{Mean}_c \times 100$ where Mean_s and Mean_c are the mean values of a certain endpoint in a test sediment (s) and the respective control sediment (c). A single MTI value was calculated as a measure of the variability of the endpoint associated with differences in sediment characteristics.

Appropriate toxicity criteria were calculated using the maximum value among the MDDs and MTI, rounded to the next multiple of five (Höss et al. 2010).

3. Results and discussion

3.1. Effect of % clay on ostracod growth and survival

Controls were within the validity criteria provided by ISO 14371 (ISO 2012): mortality was below 20% and mean ostracod length increased at least 1.5-fold. Survival in control sediment was $96 \pm 5\%$, while in control sediments amended with increasing concentrations of kaolin clay ostracod survival ranged between 95 and 100% (Table 1). Thus the presence of clay in the sediment samples had no effect on ostracod survival. On the other hand, growth decreased with increasing clay proportions. Ostracod growth in control sediment was $793 \pm 73 \mu\text{m}$. In sediments with the lowest proportion of clay tested (2.5%) growth was not significantly different from control sediment. However, when the proportion of clay was $\geq 5\%$, growth was significantly reduced (growth $< 700 \mu\text{m}$), and overall growth was significantly reduced to $\geq 20\%$ compared to controls. Maximum mean growth inhibition (38%), corresponding to an ostracod growth of $528 \pm 75 \mu\text{m}$, was observed in sediments amended with a clay content of 20%.

To our knowledge, our results provide the first evidence of the significant effect of fine sediments on ostracod growth measured according to the ISO protocol (ISO 2012). The effects of fine sediments in sediment toxicity are species- and endpoint-specific, although most commonly the processes behind such adverse effects remain unknown (Nipper et al., 1989; Suedel et al., 1994; Emery et al., 1997; Gratzner and Ahlf 2001). The composition of the kaolin clay used in our experiments ($\text{Al}_2\text{Si}_2\text{O}_5(\text{OH})_4$) excludes the possibility that growth inhibition was caused by high oxygen consumption due to organic matter content. The mixture of fine sediment particles together with the algal food might have affected test results. Ostracods may have ingested clay particles and the low nutritional value of kaolin clay compared with alternative food source (i.e. algae) could explain the observed growth inhibition. As for other test species in sediment toxicity assessment, some physical interaction cannot be excluded (Nipper et al., 1989; Emery et al., 1997; US EPA 2000).

Table 1: Survival and growth (mean $\pm$ SD) of ostracods at the end of the exposure (6d) to control sediment and sand/kaolin mixtures expressed as percentage of kaolin clay. The percentage of growth

inhibition is included in brackets). Significant difference ($p = 0.05$) between clay treatments and control sediment is marked with an asterisk

Sample	Survival (%)	Growth (μm)
Control sediment	96 ± 5	793 ± 73
Control/kaolin mixture		
2.5	100 ± 0	739 ± 15 (0)
5	100 ± 0	687 ± 40 (19)*
10	95 ± 7	644 ± 33 (24)*
15	97 ± 6	606 ± 14 (18)*
20	100 ± 0	528 ± 75 (38)*
40	100 ± 0	600 ± 18 (29)*
80	97 ± 6	604 ± 2 (29)*

3.2. Effect of fine sediment on ostracods exposed to natural sediments

Survival of ostracods in the tested natural sediments ranged from 67 to 100% and growth inhibition ranged from 10 to 37%. No significant relationship was found between mortality and growth inhibition, or between mortality and the presence of fine sediments (data not shown). Growth inhibition increased with increasing clay content (Fig. 1A, dots), as was observed in the tests with artificial sediments (Fig. 1A, squares; Table 1). However, growth inhibition was higher in natural sediments than in artificial sediments for the same proportion of clay (Figure 1A). The presence of clay in the tested natural sediments ranged between 0 and 20% whereas the presence of fine sediment (silt and clay) ranged between 0 and 90%. The extent of growth inhibition in natural sediments is most clearly explained when both silt and clay, but not clay alone, were considered as affecting ostracod growth (Figure 1B). This is in agreement with our hypothesis that clay could be used as a proxy for fine sediments.

Figure 1: Inhibition of ostracod growth plotted against the sediment clay content (A) and against the percentage of fine sediment including clay and silt (B) when exposed to artificial sediments (squares) and when exposed to natural sediments with low levels of contamination (dots)

3.3. Determination of toxicity thresholds for growth inhibition accounting for the presence of fine sediment

Ostracod growth and mortality showed low inherent variation among replicates independent of sediment characteristics (Table 2). The variability among replicates was similar for artificial and natural sediments, with slightly higher variability (CV_i) for the endpoint growth in natural sediments than in artificial sediments. The variability of ostracod responses associated with the variation of sediment clay content expressed as relative values with respect to the control sediment was $78.5 \pm 11.5\%$ for growth and $102.9 \pm 5.3\%$ for mortality, corresponding to CV_s of 14.6% and 5.2%, respectively. For natural sediments with low levels of contamination the variability of responses was similar to that observed for artificial sediments, with CV_s of 14.8% for the endpoint growth and 13.3% for mortality.

The relevant variation coefficients for the determination of toxicity criteria are the maximal MDD and MTI. When testing artificial sediments a maximal MDD of 7.7% and a MTI of 1.3% was calculated for the endpoint mortality whereas the maximal MDD was 10.8% and the MTI was 22.4%

for natural sediments. A toxicity criterion of 20% for mortality with respect to controls can be derived according to the results of testing natural sediments with low levels of contamination. For growth inhibition, maximal MDD and MTI were 9.6% and 34.3% for artificial sediments with increasing levels of clay and 22.0% and 35.7% when testing natural sediments. Based on our data, a toxicity criterion of 35% as a percentage control response was determined to account for effects of grain size on this endpoint.

3.4. Relevance of the determined toxicity criteria and implications for sediment toxicity assessment

Since its development, the bioassay using freshwater ostracods has been extensively used for sediment quality assessment either for toxicity screening or as part of a toxicity test battery. The toxicity criteria are, however, not explicitly mentioned in the literature or are fuzzy in that they are most commonly set at the acceptability criteria for the response in control sediments with no additional statistical tests applied. The acceptability criterion for mortality is 20% in control sediments according to intra- and inter-laboratory variability (Chial et al., 2002a; 2002b; Janssen and Persoone, 2010). According to our results this acceptability criterion is meant to account for species sensitivity to increasing fine sediment content and varying sediment composition. In order to account for other confounding factors on test results interpretation, information on the sensitivity of ostracod survival to unionized ammonia and pH is available (Watanabe et al. 2010).

Table 2: Coefficients of variation and other relevant coefficients calculated to derive effect thresholds.

CV_{IC} = coefficient of the test inherent variation of control sediment; Min = minimal value; Max = maximal value; CV_{IS} = coefficient of the test inherent variation of test sediment with varying proportions of clay; MDD = mean minimal detectable difference; CVs = coefficient of variation among test sediments with varying clay proportions; MTI = maximum tolerable inhibition

	Artificial sediments		Natural sediments	
	Growth inhibition	Mortality	Growth inhibition	Mortality
<i>Variability of test endpoints</i>				
% CV_{IC} (Min; Max)	4.4 (1.7; 7.1)	3.1 (0; 6.2)	5.7 (1.0; 13.0)	2.3 (0; 6.2)
% CV_{IS} (Min; Max)	3.0 (0.3; 5.8)	3.0 (0; 6.2)	7.9 (1.2; 17.5)	2.9 (0; 8.7)
% MDD (Min; Max)	3.9 (0.9; 9.6)	5.6 (4.1; 7.7)	10.9 (5.5; 22.0)	4.0 (0; 10.8)
<i>Variability of response in relative values (% of control)</i>				
Min	56.1	90.0	60.5	62.1
Max	101.2	107.2	97.1	100.0
Mean	78.5	102.9	73.8	89.7
Standard deviation	11.5	5.3	10.9	11.9
% CV_S	14.6	5.2	14.8	13.3
% MTI	34.3	1.3	35.7	22.4
Effect threshold (% control response)	35	5	35	20

Chronic and sub-lethal endpoints are potentially more sensitive to variations in environmental conditions. Indeed, toxicity thresholds have become more common with the implementation of sub-lethal tests. In the absence of a clear cut off value for the growth endpoint, such as the acceptability criterion of 20% for the endpoint mortality, the most common practice is to set the toxicity criterion at 20% also for growth inhibition. Effect thresholds determined for other standard test organisms range from 20% for macrophyte growth, 25% for nematode growth, or up to 50% for the endpoint

reproduction in the nematode test (Höss et al., 2010). The threshold for growth effects determined in the present study is set at 35%, based on the significant effect associated with the presence of fine sediment on ostracod growth, and this cut-off value is confirmed with data from tests with natural sediments. From the eight sediments with low levels of contamination only one exceeded this criterion, not corresponding to the sediment with the highest proportion of fines.

The ISO guideline specifies that “the test report shall include all biological, chemical, and physical information relative to the sediment under test” (ISO 2010), but at this time it is not common practice to report information of test sediment grain size together with ostracod test results. Yet the proportion of fine sediment at which significant effects start to be observed is environmentally relevant (INERIS 2010). Our study suggests that sediment characteristics that are known to affect test endpoints should be reported and test results interpreted accordingly, either with the aid of a reference sediment or using a toxicity criterion that takes into consideration potential confounding factors. Because fine-grained sediments high in organic matter are better able to adsorb pollutants than are coarser ones, further validation with data from natural sediments should ensure that the 35% threshold is a suitable criterion to account for natural variability of ostracod growth. This validation exercise should also overcome the limitation of using approximate estimates of test variability and precision in their calculation. Otherwise, the use of an appropriate reference sediment is recommended.

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