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New Artificial Sediment for *Chironomus riparius* Toxicity Testing

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Ecotoxicological testing of sediments began in the late 1970s as a consequence of by-products from dredging operations (Burton 1991). Thus, sediment contamination has become an emphasis on toxicological studies. Although the effects of substratum type and presence on aquatic organisms have been demonstrated, some essential problems remain unresolved (Swartz and Lee 1979).

Laboratory testing using natural sediments cannot be easily reproduced due to site-specificity and variable composition of test substrata when taken at different times (Walsh *et al.* 1992, Ankley *et al.* 1994). Furthermore, no natural sediment is totally uncontaminated and has the same physical features as the chemically corrupted sediment (Watzin *et al.* 1994). Moreover, natural sediments may exhibit toxicity due to naturally occurring properties (Burton 1991). Sediment variability, which results in the variable partitioning of chemicals between sediment and water, suggests the importance of having a standard sediment bioassay using a reference sediment (Hill *et al.* 1993, Naylor and Rodrigues 1995). Thus, it is desirable for toxicity measurements of natural sediments to be made relative to an internationally available, standardized control.

In the present work, a contaminant-free synthetic sediment of well-defined composition was developed. Natural sediments are composed mainly of an organic fraction, clay, and sand. As with other artificial sediments, the clay fraction is represented by kaolinite as it is an aluminosilicate mineral common in clay fractions of soil, but with a small sorption capacity relative to other clay minerals (Homenauth and McBride 1994). Sand in different forms is ubiquitous in sediments and soil (Churchman *et al.* 1993). The main variation in the proposed formulated sediment is in the composition of the organic fraction, which is composed of humic substances and laboratory grade alpha-cellulose. Thus, the composition of this organic fraction is probably less variable than other organic components used in previous works (e.g., moss-peat or cow manure) (Walsh *et al.* 1992). A common problem of standardized sediment compositions, and their subsequent use in toxicity testing, is the inherent variability of this composition.

The 3,4-DCA is present in many natural aquatic systems and in wastewaters of dye industries, and is produced by hydrolytic reactions and microbial metabolism

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Table 1. Details of substrates (subst.), experimental treatments and replicates (repl.) used in the three experiments (exp.). Number of replicates refers to individual test chambers used.

Exp.	Subst.	Treatments	Replicates	Larvae	Duration
i	Sand	Tetramin	10	yes	All 10 days
	Sand	Tetramin:cellulose	10	yes	
	Sand	(50:50)			
		cellulose	10	yes	
ii	Sand	0.0 mg L^{-1} 3,4-DCA	10	yes	All 10 days
	LOM	0.0 mg L^{-1} 3,4-DCA	10	yes	
	LOM	1.25 mg L^{-1} 3,4-DCA	10	yes	
	LOM	2.5 mg L^{-1} 3,4-DCA	10	yes	
	LOM	5.0 mg L^{-1} 3,4-DCA	10	yes	
	LOM	10.0 mg L^{-1} 3,4-DCA	10	yes	
	LOM	20.0 mg L^{-1} 3,4-DCA	10		
iii	HTM	All 5, 10 and	3 (each conc.)		1 day
	HTM	15 mg/l 3,4-DCA	3 (each conc.)		2 days
	HTM		3 (each conc.)		3 days
	HTM		3 (each conc.)		4 days
	LTM		3 (each conc.)		1 day
	LTM		3 (each conc.)		2 days
	LTM		3 (each conc.)		3 days
	LTM		3 (each conc.)		4 days

several pesticides (Kuiper and Hanstveit 1984, Tatsumi *et al.* 1992). This toxicant is a reference compound used in several inter-laboratory toxicity ring tests (Diamantino *et al.* 1997).

This work had three main objectives: (i) to test the suitability of alpha-cellulose as an inedible fraction for sediment testing with *C. riparius* larvae, (ii) to evaluate the performance of a simple low organic matter sediment with alpha-cellulose in a test of chronic toxicity of 3,4-DCA to *C. riparius* larvae, and (iii) to investigate the ability of a more complex high (10% d.w.) and low (1% d.w.) organic matter content sediment formulation, incorporating humic acid complexes, to adsorb the same toxicant.

MATERIALS AND METHODS

Three experiments were performed: (i) a test where alpha-cellulose was included as a feeding treatment to *C. riparius* larvae, (ii) a chronic toxicity test of 3,4-DCA to *C. riparius* larvae using alpha-cellulose as the organic fraction of the sediment, and (iii) an experiment where the sorption of the same toxicant to a more complex high (10% d.w.) and low (1% d.w.) organic matter content sediment formulation incorporating humic acid complexes, was assessed. In all experiments were 90 mL capacity glass jars were used as test chambers and all tests were conducted at a temperature of 20.0°C, with a 16 hr light and 8 hr dark photoperiod.

Table 2. Relative composition (% of dry weight, d.w.) of high-treated (HTM) and low-treated (LTM), and untreated (LOM) organic matter content artificial sediments.

Component	LOM (% d.w.)	LTM (% d.w.)	HTM (% d.w.)
Untreated kaolin	25.0	19.06	0.00
Treated kaolin	0.0	5.94	40.00
Untreated cellulose	1.0	0.00	3.32
Treated cellulose	0.0	0.99	6.66
Humic acids	0.0	0.01	0.02
Sand	74.0	74.00	50.00

In the first experiment, 20.00±0.05 g of acid washed, organic-matter free (ignited for 8 hr at 550°C) calcinated sea sand (0.1-0.4 mm particle size range; supplied by Merck Co.) and 40 mL of aerated ASTM hardwater medium (ASTM 1980) were added to each test chamber. This mixture was allowed to settle for 24 hr. The various feeding treatments (Table 1) consisted of 1.0 mg/larva/day, as a single 10.0±0.l mg dose on Day 0, just prior to the addition of one *C. riparius* 2nd instar larvae per replicate chamber. At the end of the experiment, larvae and pupae were weighed (fresh weight) to the nearest 0.001 mg. Mortality, pupation, and emergence data were analyzed for significance using the Fisher exact test.

In the second experiment, a simple artificial sediment low in organic matter (LOM) was prepared with three ingredients: alpha-cellulose (from Sigma Co.), kaolin with a 0.1-0.4 µn particle size (from Sigma Co.), and sand (see first experiment; Table 2). This sediment was produced by mechanically mixing the dried components for 1 hr, without pre-treatment. A small adjustment with $CaCO_3$ was required to produce a circumneutral sediment pH. To each test chamber, 20.00±0.05 g of LOM sediment was added and 40 mL of solution was then introduced. The sediment:solution mixture was allowed three days to equilibrate prior to the start of the test. Two controls were also established: LOM sediment with pure ASTM medium, and sand plus ASTM. Tetramin (from TetraWerck) was the sole source of food added; the ration was again set at 10.0± 0.1 mg for the 10 days of the experiment, added when the introduction of one *C. riparius* 2nd instar larvae per replicate chamber (Day 0). Gentle aeration was applied to all treatments. A stock solution of 30 mgL^{-1} 3,4-DCA was prepared by dissolving 3,4-DCA crystals (from Aldrich Co.) in ASTM hardwater medium. This solution was shaken for 24 hr in an orbital incubator at 180 r.p.m., in darkness. The dissolution method used was previously validated with good agreement between nominal and actual 3,4-DCA concentrations (Ribeiro *et al.* 1995). In order to obtain the different 3,4-DCA concentrations for the test (Table l), this stock solution was serially diluted with ASTM hardwater medium. On Day 0 and Day 10, 3,4-DCA actual concentrations were analyzed in the overlying water of three replicates (Table 1). For all 3,4-DCA analyses, water overlying sediments was removed from test chambers by pipette, centrifuged, and

Table 3. First experiment: mortality, pupation, emergence and fresh weight ($\bar{x}$ ± SD in mg, and sample size inside brackets) of *Chironomus riparius* after 10 days under different feeding treatments.

	Feeding treatments		
Parameter	Tetramin	Tetramin+cellulose	Cellulose
Organisms	10	10	10
Mortality	40 %	20 %	70 %
Pupation	0%	70 %	0 %
Emergence	0 %	10%	0 %
Fresh weight	4.51±1.143 (6)	4.55±1.132 (7)	0.40±0.120 (3)

concentrated in Bond Elut columns. The 3,4-DCA was then extracted with acetonitrile and analyzed by HPLC at a wavelength of 242 nm (Merck-Hitachi L-6200A pump, L-4260 UV-Vis detector, RP 18 column, 5 µn, 250 x 4 mm, with 1.0 mL min^{-1} flow rate, eluent: nanopure water:methanol 25:75). Averages of actual 3,4-DCA concentrations in the overlying water during the test were computed assuming an exponential and constant decay between Day 0 and Day 10. Since no larvae survived after the fifth day in the two highest concentrations, the mean of actual 3,4-DCA concentrations in the two most toxic solutions were computed only for the first live days of the test. At the end of the experiment, larvae and pupae were freeze-dried and weighed to the nearest 0.001 mg. Mortality, pupation, and emergence data on toxicant solutions were analyzed for significance using the Fisher exact test. Dry weights were compared with l-way ANOVAs.

In the third experiment, the kaolinite clay was mixed with alpha-cellulose. This mixture was then saturated with $CaCl_2$ by stirring in a solution of 1M $CaCl_2$ for 48 hr, at 80°C. This procedure bound calcium ions to the kaolin and cellulose in order to increase the amount of humic acid that binds to the kaolin and cellulose. The kaolin/alpha-cellulose mixture was then centrifuged and washed with nanopure water until complete removal of chlorine occurred, determined by the $AgNO_3$ test (APHA 1989). Following drying (55°C), the kaolin/alpha-cellulose mixture was crushed to powder and stored. When required, this dry powder was resuspended in nanopure water. Humic acids (sodium salt, from Aldrich Co.) were dissolved in KOH 0.lN. This solution was mixed in a stirring device together with the suspension of kaolin/alpha-cellulose. Treated and non-treated alpha-cellulose/kaolin mixtures were then mixed together and the sediment was completed with the sand fraction. After neutralization with 1N H_2SO_4, both sediments were dried at 55°C. The low and high content sediments (1% and 10% cellulose fraction are designated, respectively, LTM and HTM; Table 1).

The conditions for the second experiment were repeated here, but with 5.00±0.05 g of either LTM or HTM sediment added to each test chamber and 50 mL of 5, 10, or 15 mg L^{-1} 3,4-DCA solution. At the commencement of the experiments, and at subsequent time intervals (1, 2, 3, and 4 days), 3,4-DCA levels were tested in the overlying water of three replicates for each sediment type (Table 1). Comparisons

Table 4. Second experiment: 3,4-DCA concentrations in overlying water (in mgL^{-1}), mortality (Mort.), pupation (Pup.), emergence (Emerg.), and dry weight ($\bar{x}$ ± SD in mg, and sample size inside brackets) of *Chironomus riparius* after 10 days of toxicant exposure.

3,4-DCA concentrations						
Initial	Day 0	Mean	Mort.	Pup.	Emerg.	Dry weight
Control	0.00	0.00	10%	100%	33 %	0.93±0.167 (6)
1.25	0.57	0.31	20 %	12.5 %	12.5 %	1.06±0.132 (7)
2.5	0.85	0.45	10 %	44%	0%	0.90±0.121 (7)
5.0	1.80	0.96	30%	0%	0%	1.05±0.159 (7)
10.0	4.13	3.02	100%	0%	0%	--- (0)
20.0	9.69	7.10	100%	0%	0%	--- (0)

of actual toxicant concentrations between the two sediment formulations (LTM and HTM), at 0, 24, 48, 72, and 96 hr, were made with 1 -way ANOVAs.

RESULTS AND DISCUSSION

In the first experiment, mortality was observed in all three treatments, being significantly higher (Fisher exact test: $p<0.05$) where no Tetramin was given to midge larvae (Table 3). In this treatment, surviving larvae were similar in appearance to start of the test. This result indicated that cellulose is an inedible organic fraction to this species, being ideal as an organic fraction in formulated sediments to be used with *C. riparius.* Using an edible organic fraction in sediment formulations for toxicity testing could permit the alteration of the sediment composition during the test period. Since cellulose is an inedible fraction, then the better larval performance in the cellulose+Tetramin treatment can not be due to the value of cellulose as a food source. Although the fresh weight of surviving pupae with this treatment was similar to the weight of surviving larvae when only Tetramin was supplied (t-test: $p>0.5$), the larvae development was much slower: pupation+emergence was significantly lower (Fisher exact test: $p<0.05$). One plausible explanation to the better performance when cellulose was present is that cellulose fibers could have been used for larval tube building, which is of vital importance to *C. riparius* (Naylor and Rodrigues 1995).

At the start of the second experiment (Day 0), i.e. three days after the preparation of the sediment:solution mixtures, 3,4-DCA concentrations in the water column were much lower than initial values (Table 4). On average, the remaining 3,4-DCA concentrations at Day 0 was 41.0 % of initial values, ranging from 33.8 and 48.4 %. Since significant losses of 3,4-DCA, under laboratory conditions, by processes such as photodegradation or adsorption to glass were not expected (Ribeiro *et al.* 1995), the 3,4-DCA loss in the water column indicated that a strong sorption to the sediment occurred meanwhile. *C. riparius* larvae in the two highest exposure concentrations did not survive beyond the fifth day of the experimental

Table 5. Third experiment: 3,4-DCA concentrations ($\bar{x} \pm SD$ in mg L^{-1}), during the four days of the experiment.

Sediment type - - time of exposure	Nominal concentration		
	5 mg L^{-1}	10 mg L^{-1}	15 mg L^{-1}
LTM - 0 hrs	5.50	11.18	13.62
LTM - 24 hrs	0.60 ± 0.044	8.80 ± 0.24	11.67 ± 0.31
LTM - 48 hrs	0.39 ± 0.004	7.95 ± 0.30	11.20 ± 0.12
LTM - 72 hrs	0.36 ± 0.013	6.83 ± 0.59	10.02 ± 0.32
LTM - 96 hrs	0.28 ± 0.069	5.83 ± 0.64	8.63 ± 0.17
HTM - 0 hrs	5.50	11.18	13.62
HTM - 24 hrs	0.37 ± 0.100	7.16 ± 0.51	10.80 ± 0.44
HTM - 48 hrs	0.31 ± 0.026	6.29 ± 0.23	9.60 ± 0.34
HTM - 72 hrs	0.28 ± 0.002	5.57 ± 0.30	8.56 ± 0.33
HTM - 96 hrs	0.18 ± 0.021	3.74 ± 0.49	7.95 ± 0.17

period. In all other toxicant concentrations, mortality values were not significantly different from the control (Fisher exact test: $p < 0.05$). A significant reduction in development rate was found in all toxicant concentrations: the proportion of pupae was significantly lower than in the control (Fisher exact test: $p < 0.05$), indicating that this endpoint is very sensitive to 3,4-DCA. Dry weights of surviving organisms did not differ among toxicant concentrations (l-way ANOVA: d.f. = 3, 26; $F = 2.26$; $p = 0.11$). Therefore, below lethal concentrations *C. riparius* larvae exposed to 3,4-DCA attain a similar weight, but are primarily affected in development rate.

In the third experiment, both formulations of the more complex LTM and HTM artificial sediments had a short sedimentation time, and low turbidity was observed in the water column of the test chambers. Significant reductions of toxicant in solution were registered in all 3,4-DCA concentrations (ANOVAs: $p < 0.05$), especially in the 5 mg L^{-1} 3,4-DCA concentration. The HTM sediment formulation presented a significantly higher sorption capacity than the LTM formulation, at all 3,4-DCA concentrations, except at the 5 mg L^{-1} concentration after 96 hr (ANOVA: d.f. = 1,4; $F = 6.24$; $p = 0.07$).

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