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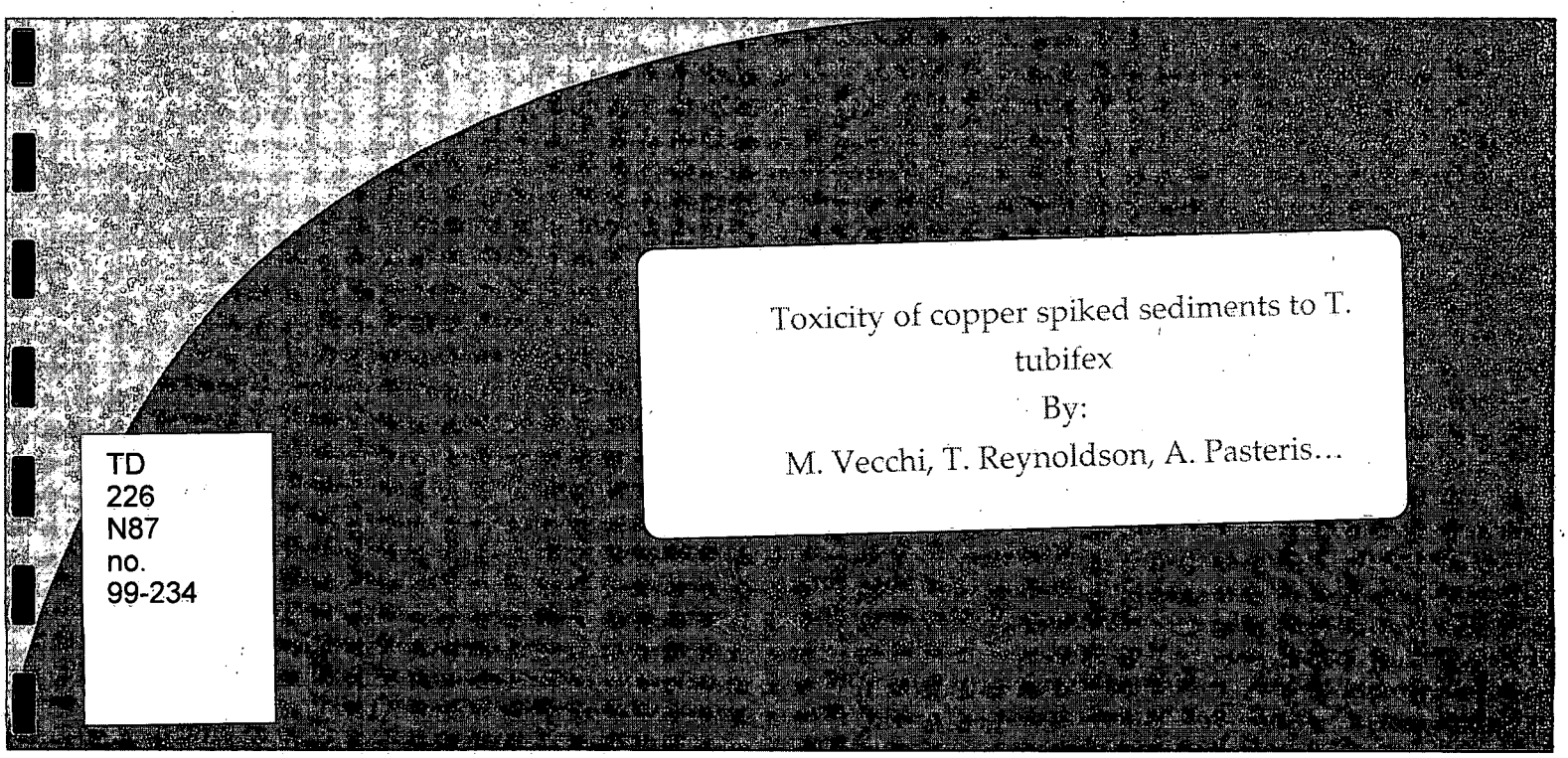

Toxicity of copper spiked sediments to T. tubifex

By:

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MANAGEMENT PERSPECTIVE

Title	Toxicity of copper spiked sediments to *T. tubifex*:
Authors	M. Vecchi, **T.B. Reynoldson**, A. Pasteris and G. Bonomi
NWRI Publication No:	99-234
Citation:	Environmental Toxicology and Chemistry
EC Priority/Issue:	Methods and protocols for aquatic effects testing are an important component of several departmental programmes and initiatives (e.g., EEM, AETE and the GL2000 RAP's). NWRI has been developing new methods for sediment toxicity testing as well as criteria setting for these tests. As a part of this process comparative testing with other laboratories is an important part of the verification and calibration of the methods.
Current status:	A reproductive bioassay with the oligochaete worm *Tubifex tubifex* was developed by NWRI. A major criticism of laboratory tests is their lack of ecological realism. The validity of this 28 day test as a surrogate for population level effects is being investigated in a collaborative study with the University of Bologna (Italy). This paper describes the first results of that collaboration; a comparison of the NWRI (adult reproduction) test with an early life stage test and demonstrates the greater sensitivity of the former.
Next steps	Future collaboration will describe the sufficiency of the adult reproduction test as a surrogate for higher level biological responses.

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TOXICITY OF COPPER-SPIKED SEDIMENTS TO *TUBIFEX TUBIFEX* (OLIGOCHAETA, TUBIFICIDAE): COMPARISON OF THE 28-DAY REPRODUCTIVE BIOASSAY WITH AN EARLY-LIFE-STAGE BIOASSAY

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Abstract—Two sediment bioassay methods using *Tubifex tubifex* (Müller, 1774) as the test species were compared. The first was an adult reproduction test, the second an early-life-stage survival test. The duration of both bioassays is 28 d and the amount of work required was similar; they may be useful alternatives to each other in different circumstances (e.g., the early life stage bioassay could be carried out with smaller volumes of sediment). The two bioassays were performed simultaneously on copper-spiked sediments. Sediments from two freshwater and two terrestrial sites were used; five separate, nonsimultaneous experiments were performed, one for each sediment or soil and a further experiment with soil with a food supplement. In the adult bioassay, there were large differences in the production of cocoons, eggs, and young among the control treatments of the five experiments. There were also major differences in the NOEC (no-observed-effect concentration) and LOEC (lowest-observed-effect concentration) for copper between the tested substrates. The early life stage bioassay appears to be less sensitive to copper toxicity than the adult reproductive bioassay since NOECs and LOECs are higher for early survival than for the most sensitive endpoints of the adult bioassay in three experiments out of five.

Keywords—Toxicity tests *Tubifex tubifex* Sediment Copper Spiking

INTRODUCTION

The environmental relevance of contaminated sediments is widely recognized, and the role of sediment as both a sink and a secondary source of toxic chemicals has been described by many authors [1–4]. It is also widely accepted that laboratory bioassays are an important component in the assessment of sediment contamination and in setting sediment quality criteria [5,6].

Nevertheless, sediment toxicity testing is still a developing subject and there are only a few standardized bioassays that have been specifically designed for determining sediment toxicity [7–9]. Many of those currently used are adaptations of water-column assays using sediment elutriates or sediment pore water with nonbenthic species. These methods have the advantage of being rapid, simple, inexpensive, and using known standard test species and are thus useful as screening tests. However, their primary disadvantage is a lack of ecological realism. Recently, several protocols for bioassays that test whole sediments using true sediment-dwelling species have been proposed [6–12].

Several characteristics of tubificid oligochaetes make them potentially useful test organisms for sediment bioassays. They are widely distributed, frequently dominating the macrobenthic community in freshwater habitats (e.g., the profundal of lakes). Furthermore, they dwell in the sediment, burrowing and ingesting large volumes of sediment for feeding and are thus exposed to contaminants both through interstitial water and through contact with sediment particles. They also play a major role in bioturbation and in decomposition of organic matter.

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Laboratory tests can focus on different levels of biological organization, from the cellular to the population scale and finally to the whole community. As described by Calow [13], each of these organization levels has advantages and disadvantages and, in simplistic terms, as one increases the level of complexity, ecological relevance increases and interpretability and diagnostic capability decline. A series of studies are ongoing to investigate the utility and relative sensitivity at these different levels of organization in the oligochaete worm *Tubifex tubifex*. In this paper, we have focused on endpoints at the level of the individual organism with links between the individual and population levels.

The long life cycles of tubificid oligochaetes have been considered as reasons discouraging their use in standard bioassays [14–16]. However, to overcome these problems, Reynoldson et al. [17] proposed a test starting with sexually mature *T. tubifex* that measures survival and reproduction over 28 d. The test is reasonably rapid, simple, and inexpensive; in particular, very little maintenance work is required during the test, yet the test is aimed at giving information on chronic toxicity and could be considered a rapid surrogate for more traditionally designed long-term experiments such as those conducted by Milbrink [14], Wiederholm et al. [15], and Casellato [16].

An alternative to the method of Reynoldson et al. [17] is to use an early life stage bioassay. Early life stage bioassays are the standard approach to rapidly assessing chronic toxicity. The presumption in early stage bioassays is that juveniles are the most sensitive stage in the life cycle of an individual and, as a consequence, little additional information is gained by extending observations over a longer time. However, as it is usually impossible to test reproduction using early life stage bioassays, the most frequently used endpoints are survival and growth.

The aim of the work reported here was to conduct a comparison between the adult reproduction bioassay proposed by Reynoldson et al. [17] and a 28-d early life stage bioassay, starting with cocoons and measuring hatching and early survival and requiring a similar amount of work. This was done by simultaneously performing the two bioassays on copper-spiked sediments, thus assessing their relative sensitivity to an important and widely studied contaminant.

MATERIALS AND METHODS

Stock cultures

In a previous study, Reynoldson et al. [18] demonstrated that there were few differences in reproductive performance and sensitivity to contaminants in cultures of *Tubifex tubifex* (Müller, 1774) established from populations derived from Canada and Spain. Accordingly, laboratory stock cultures of *T. tubifex* have been established using worms collected from Lake Suviana, a reservoir located on the mountains of Appennino Tosco-Emiliano in northeastern Italy.

Worms were reared in groups of about 30 individuals in circular glass containers (diameter, 11 cm; height, 6 cm), half filled with sand and then filled with aerated tap water. Frozen lettuce was put under the sand as food. Every week, each container was checked, the sand washed, the residual food removed, and fresh lettuce added; cocoons were also removed and either discarded or placed in separate containers to start new stock cultures. Cultures were kept in the dark at 23 ± 1°C and discarded when 6 months old.

Collection, handling, and spiking of sediments and soils

Sediments from two freshwater and two terrestrial sites were used in these experiments. Aquatic sediments were collected with an Ekman grab from Lake Suviana at a depth of 50 m and from Lake Maggiore in northwestern Italy at a depth of 360 m. In the laboratory, sediments were wet sieved through 250-μm mesh, using aerated tap water, to remove indigenous macrofauna and coarse particles. The resulting slurry was allowed to settle for 72 h, and then the overlying water was removed and discarded. Sediment was stored in the dark at 4°C and spiked within 7 weeks of collection.

Terrestrial sediments were collected with a spade from two plots, one at Sala Bolognese near the city of Bologna and one at Cà Bosco near the city of Ravenna; both are located in northeastern Italy in the River Po alluvial plane and have not been chemically treated for at least 10 years. A superficial layer of 1 to 2 cm was scraped and discarded and then the soil was collected to a depth of 15 cm. In the laboratory, sediments were dried at room temperature, manually ground using a mortar, and dry sieved through 500-μm mesh to remove coarser particles; 500-μm mesh was used instead of 250-μm mesh since it was not necessary to remove potentially confounding indigenous macrofauna. The sieved soil was mixed 1:1 by volume with aerated tap water. The resulting slurry was allowed to settle for 72 h, and then the overlying water was removed and discarded. After this treatment, the soils had the appearance and consistency of wet sediment.

Five separate, nonsimultaneous experiments were performed, one for each sediment or soil and a further experiment with the Cà Bosco soil with the addition of a food supplement.

The 10-g/L copper stock solution used to spike the sediments was prepared dissolving reagent grade $CuSO_4 \cdot 5H_2O$ in double distilled water (for the Sala experiment, a 40-g/L solution was used). Prior to spiking each batch of sediment, four 50-ml samples of wet sediment were weighed, oven dried at 80°C for 12 h, and then reweighed to estimate the dry weight/wet weight ratio. Spiking was performed by mixing the required weight of wet sediments (approximately 1.5 L) with 2 L of commercial mineral water and the required volume of Cu stock solution in a 5-L glass jar. The contents of each jar were stirred manually for 5 min using a stainless steel spoon. Jars were then placed in a refrigerator at 4°C for 14 d. Every 3 d, stirring was repeated.

Table 1. Measured concentrations in copper-spiked sediments used in comparison of two *Tubifex tubifex* bioassays

Nominal concn. (mg/kg)	Measured concentration (mg/kg)				
	Lake Suviana	Sala Bolognese	Lake Maggiore	Cà Bosco, unfed	Cà Bosco, fed
0	48.31	36.51	53.10	18.95	19.00
12.5	59.78	—	—	—	—
25	70.06	67.25	—	—	—
50	98.76	—	91.77	69.06	62.64
100	147.1	166.7	127.3	108.8	101.4
200	236.1	—	231.7	211.7	191.8
400	427.3	550.0	385.8	403.7	370.9
800	802.1	—	521.0	775.1	807.4
1,600	—	2,092	—	—	—
6,400	—	8,042	—	—	—

Commercial mineral water was used to provide a standard water source, as a reliable source of good quality natural water with constant characteristics was unavailable. Reconstituted water is known to be often unsuitable for rearing freshwater organisms. The chemical composition of the mineral water (pH, 7.77; conductivity, 357 μS/cm; hardness, 200 mg/L) was within the range reported by Reynoldson et al. [17,18] in previous toxicity testing with *T. tubifex* in laboratories in Canada and Spain. The target nominal concentration range used in the first experiment (Lake Suviana sediment) was 0 (control) to 800 mg/kg. For the second experiment (Sala Bolognese soil), the concentrations were 0 (control) to 6,400 mg/kg. For the other three experiments, the concentrations were 0 (control) to 800 mg/kg. The measured concentrations are shown in Table 1.

Sediment analysis

Percentage of sand and fines (silt + clay) was determined as dry weight (80°C) after wet sieving through a 63-μm mesh. The fine fraction was not further characterized. As all the analyses were performed on the sediments after they had been prepared for the bioassays, the size range for the sand fraction was 63 to 250 μm for aquatic sediments and 63 to 500 μm for terrestrial soils. Carbonate corrected TOC concentrations in the samples were measured using a Leco (St. Joseph, MI, USA) CR12 carbon analysis system [19]. Phosphorus concentrations were determined using a spectrophotometric technique that measures molybdenum blue formed from the reaction of ammonium molybdate with orthophosphates extracted from the sediments by treatment with a sulphuric and nitric acid solution [19]. Total nitrogen in the sediment was measured by using a sulphuric acid catalyst solution to extract and convert nitrogen to ammonium sulphate into a sulphuric acid solution and back-titration with sodium hydroxide [19]. Concentrations of trace metals in the sediments were determined with inductively coupled plasma-atomic emission spectroscopy (ICP-AES) on a multichannel Jarrell-Ash (Franklin, MA, USA)

Atom Comp® 1100 after a two-step nitric acid–hydrochloric acid digestion [20]. To define general sediment mineralogical characteristics, concentrations of major oxides in the sediments were determined by calculation after measurements of aluminium, calcium, iron, magnesium, silicon, and titanium. Sample preparation for the oxide determination consisted of combining the sediment with a lithium meta-/tetraborate mixture, followed by fusion at 950°C and subsequent transfer into 10% nitric acid before detection and quantification of the analytes on a sediment dry-weight basis.

Bioassays

The protocol for the early life stage bioassay was developed to be comparable with the adult reproduction bioassay. The latter was performed according to the protocols described by ASTM [7] and Reynoldson et al. [17] with minor modifications.

Day minus 5. All the cocoons were removed from the stock cultures and discarded. This ensured that the cocoons found in the stock culture 5 d later and used in the early life stage bioassay were at most 5 d old.

Day minus 1. Each 250-ml bioassay beaker received 100 ml of spiked (or control) sediment and 100 ml of overlying water from a jar. Each beaker of the second experiment with Cà Bosco soil also received 80 mg of powdered Tetramin® fish food (TetraWerke, Melle, Germany) suspended in approximately 1 ml of water. The beakers were then placed in the dark in the test incubator at 23 ± 1°C to settle; the position of each beaker was chosen at random and maintained over the experimental period. Eleven replicated beakers were prepared for each nominal concentration, including the unspiked control; five were used in the adult bioassay, five in the early life stage bioassay, and one, a "blank" containing no animals, supplied the sample for the analytical determination of copper concentration in the spiked sediments. After the beakers had been filled, the excess control sediment was set aside for particle size and chemical characterization.

Day 0. The overlying water in each beaker was gently aerated for 1 h. Then temperature, pH, and dissolved oxygen were measured from the overlying water of each beaker. At each concentration, a sample for the analytical determination of the copper concentration was taken from the blank beaker. For the adult reproduction bioassay, sexually mature specimens were transferred from the stock culture to small Petri dishes, four per dish. When sufficient animals had been collected, each group of four was added to a bioassay beaker chosen at random; beakers were then returned to the incubators. For the early life stage bioassay, cocoons were picked from the stock cultures and the number of eggs in each of them was determined by observation with a dissecting microscope. Cocoons were used for the bioassays only when the number of eggs was visible through the wall; this resulted in cocoons with more than five eggs being discarded. Groups of three to four cocoons, containing a total of 12 to 13 eggs, were formed, distributing as evenly as possible cocoons containing the same number of eggs among groups. For the second experiment with Cà Bosco soil, groups of five cocoons, corresponding to 20 eggs, were used. Each group was added to a bioassay beaker chosen at random; using a pipette, each cocoon was placed in a hole drilled in the mud, which was then filled. Beakers were then returned to the incubators. All the beakers were kept in the incubators for 28 d; the overlying water was continuously and gently aerated. The beakers were examined every 2 or 3 d for loss of water due to evaporation and double distilled water was added if required.

Day 14. Temperature, pH, and dissolved oxygen were measured from the overlying water of each beaker. Beakers were otherwise left undisturbed until day 28.

Day 28. Beakers were removed from the test incubator and temperature, pH, and dissolved oxygen were measured. The content of each beaker was individually sieved through 500-μm and 250-μm mesh, and the surviving adults were counted immediately. The residues from the two sieves were washed separately into two vials, preserved in 70% alcohol, and examined later. Samples were enumerated with a dissecting microscope; cocoons were dissected to count eggs and embryos if their number was not visible through the wall.

Endpoints. The raw endpoints measured in the adult reproduction bioassay were surviving adults, laid cocoons (empty and full), embryos present inside the cocoons, and hatched young (large or small, if retained, respectively, by the 500-μm mesh or by the 250-μm mesh). Total offspring was calculated as the sum of young and embryos. Embryos were counted, although this endpoint was not used by ASTM [7] and Reynoldson et al. [17] to verify if and how much this would increase the sensitivity of the bioassay.

The raw endpoints measured in the early life stage bioassay were large young and small young. Survival rate was calculated for each replicate beaker as the ratio between the number of worms present at day 28 and the number of eggs placed in the beaker at day 0.

Data analysis

One-way ANOVA was used to test the overall significance of the effect of copper concentration on the endpoints. A treatment was excluded from the analysis when there was a 100% response in all five replicates, as these treatments have null variance and a statistical test is not needed to detect difference from the control; furthermore, their inclusion in the ANOVA computation would violate the assumption of homogeneity of variance and strongly bias the estimate of within-group variance.

When ANOVA detected significant differences ($\alpha \leq 0.05$), the Scheffé's post hoc test at 5% significance level was used to identify the NOEC (no-observed-effect concentration), which is the highest concentration not significantly different from the control, and the LOEC (lowest-observed-effect concentration), which is the lowest concentration that is significantly different from the control. This test was chosen among other available post hoc tests because of its demonstrated robustness, i.e., its low sensitivity to violations of the assumptions of normality and homogeneity of variances [21].

Data on total young, eggs plus embryos, and total offspring from the adult bioassay were transformed by means of square root before the statistical analysis to attain homogeneity of variances and independence of variance from the mean. No transformation was necessary for total cocoons. Data on survival from the early life stage bioassay were processed with the arcsine transformation before analysis, based on recommendations for application of ANOVA to proportions and percentages [22].

RESULTS

Sediment characteristics

There are a few major differences between the four sediments used for copper spiking in this study. The results of

Table 2. Summary of major elements and total metals for four test substrates

	Site				
	Lake Suviana	Sala Bolognese	Lake Maggiore	Cà Bosco, unfed	Cà Bosco, fed
Silt + clay (<63 μm) (%)	96.3	69.7	65.2	71.5	62.5
Fe_2O_3 (%)	6.49	5.08	6.2	3.64	3.47
CaO (%)	2.00	6.41	1.76	12.54	12.86
TOC (%)	3.22	1.41	1.56	1.05	1.03
Total N (mg/kg)	3,220	1,290	1,260	899	906
Total P (mg/kg)	744	674	2,090	1,270	1,360
Cr (mg/kg)	59	62	54	33	34
Ni (mg/kg)	80	57	70	38	37
Cu (mg/kg)	51	36	58	21	20
Zn (mg/kg)	150	78	202	59	56
As (mg/kg)	19	17	62	30	20
Cd (mg/kg)	<1	<1	1	<1	<1
Pb (mg/kg)	18	15	43	14	14

analyses for particle size, major elements, nutrients, and metals are shown in Table 2.

The sediment from Suviana has a much higher silt content than the other three sediments and, as a consequence, the total organic carbon (TOC) concentrations are two to three times greater than for the other sediments. Nickel levels are highest in the sediment from this site, and zinc is also elevated. The sediments from Lake Maggiore have notably high levels of total phosphorus as well as the highest levels of zinc, arsenic, and lead. Sediment from Cà Bosco have lower levels of iron, and calcium is elevated. Background metals levels are lowest at this site, which has the lowest concentrations of arsenic, chromium, copper, nickel, and zinc.

Lake Suviana sediment

The number of cocoons and young produced in the unspiked (control) treatment for the adult bioassay is low compared with figures reported by others [18,23] as well as with the results from the other sediments tested in this study (Table 3). Furthermore, there were no large young present after 28 d.

There is no significant effect of sediment copper in the concentration range used here for any of the endpoints and therefore NOECs and LOECs have not been established (Table 4).

Sala Bolognese soil

The numbers of cocoons and young produced in the control treatment for the adult bioassay are much higher than in the Suviana experiment and are close to values reported by previous authors (Table 3). In this sediment, large young were present at day 28, although the ratio of large young/total young (0.09) is rather low.

There is an obvious effect of sediment copper concentration on all the endpoints that is not merely due to the wider concentration range, as there is a marked response even at 166.7 mg/kg. The NOEC is 67.25 mg/kg and the LOEC is 166.7 mg/kg for all the endpoints (Table 3). However, these estimates for both NOEC and LOEC may be refined by using a narrower concentration range.

Table 3. Comparison of adult bioassay test endpoints from different studies

	Cocoons/adult		Young/adult	
Sediment source	Average	SD	Average	SD
Previous studies				
Spain (4 sites), fed[a]	8.6	1.5	12.9	15.6
Great Lakes (41 sites), unfed[b]	8.9	1.3	17.4	7.2
Great Lake (166 sites), fed[c]	9.8	1.3	28.8	8.4
This study				
Lake Suviana, unfed	2.7	0.7	3.5	2.2
Sala Bolognese, unfed	7.4	1.8	12.7	3.9
Lake Maggiore, unfed	5.6	0.8	2.5	1.5
Cà Bosco, unfed	3.3	0.9	7.7	0.7
Cà Bosco, fed	8.3	0.9	20.7	7.1

[a] [18]
[b] Reynoldson and Day (unpublished data, NWRI)
[c] [23]

Lake Maggiore sediment

The background copper concentration at 58 mg/kg (Table 2) is the highest of all four test sediments and is in the heavily polluted range based on U.S. EPA quality criteria [11]. The highest spiked concentration achievable (521.0 mg/kg) was substantially less than the target nominal concentration (800 mg/kg), suggesting that the binding capacity of the sediment was saturated.

The number of cocoons produced in the unspiked sediment (control) was rather low. The number of young is lowest of all the observed values, and there were no large young present after 28 d.

Copper concentration had a significant effect on all the endpoints. From the adult bioassay, LOECs are 385.8 mg/kg for total cocoons and total offspring and 521.0 mg/kg for eggs plus embryos (Table 4). Despite the significance of the overall ANOVA, the post hoc test could not detect significant differences between the control and any treatment for total young; consequently, the LOEC and NOEC were not determined for this endpoint. In the early life stage bioassay, the LOEC was 521.0 mg/kg for survival.

Cà Bosco, unfed

The background copper concentration is low, 21 mg/kg, in this sediment (Table 2). The number of cocoons produced in the control beakers is substantially less than those reported from sites in both the Great Lakes and Spain (Table 3). Of the four test sediments, only that from Suviana is lower. However, the number of young produced is higher, although there were no large young present at the end of the 28-d period.

There was a clear response to increasing copper concentration, the animals responding at the lowest concentrations used. The LOEC is 69.06 mg/kg for all the endpoints of the

Table 4. The LOEC for copper (mg/kg) in different sediments and for different *Tubifex tubifex* bioassay endpoints

Endpoint	LOEC (mg/kg)				
	Lake Suviana	Sala Bolognese	Lake Maggiore	Cà Bosco, unfed	Cà Bosco, fed
Adult bioassay					
Total cocoons	>802.1[a]	166.7	385.8	69.06	101.4
Total young	>802.1[a]	166.7	—[b]	69.06	101.4
Eggs plus embryos	>802.1[a]	166.7	521.0	69.06	191.8
Total offspring	>802.1[a]	166.7	385.8	69.06	101.4
Early-life-stage bioassay					
Survival	>802.1[a]	166.7	521.0	108.8	191.8

[a] No response within exposed range.
[b] Response observed but variability too great to identify a significant difference ($\alpha \leq 0.05$) from control.

adult bioassay (Table 4); since this was the lowest concentration to which the animals were exposed, the NOEC cannot be established. There was a large difference between survival at 0 and 69.1 mg/kg in the early life stage bioassay, although, due to high intratreatment variability, the difference was not significant and the NOEC and LOEC are, respectively, 69.06 and 108.8 mg/kg for this endpoint (Table 4).

Cà Bosco, fed

For this experiment, a second batch of soil was collected from the same location as in the previous experiment. The measured concentrations for background copper and other constituents were similar (Table 2). This experiment was run in the same way as the previous experiments except that food was added as described in the methods section.

The number of cocoons and particularly young produced in the control beakers were considerably higher than in the unfed experiment (Table 3) and were within the range reported for Great Lakes and Spanish reference sites.

While there is a clear dose–response relationship between copper concentration and all the test endpoints, the effect of feeding appears to reduce the sensitivity of *T. tubifex* to copper concentration. This has the result that both the NOEC and LOEC are higher. A distinct and, in some cases, steep concentration–response curve was observed for all the endpoints. The LOEC and NOEC are estimated, respectively, at 62.64 and 101.4 mg/kg for total cocoons, total young, and total offspring and at 101.4 and 191.8 for eggs plus embryos and for survival in the early life stage bioassay (Table 4).

DISCUSSION

Differences in the sensitivity among the endpoints of the adult bioassay were detected in only two of the experiments (Lake Maggiore and soil from Cà Bosco, fed). In both cases, total cocoons and total offspring were the most sensitive, having the lowest NOEC and LOEC. While total young was as sensitive as total cocoons and total offspring in the Cà Bosco fed experiment, it was not possible to estimate NOEC and LOEC for this endpoint in the Lake Maggiore experiment. Eggs plus embryos displays the highest NOEC and LOEC in both experiments.

Counting the individuals remaining inside the cocoons is required to measure both eggs plus embryos and total offspring. Our results showed that, while this increases the amount of work required to perform the adult bioassay, it does not improve the sensitivity to copper toxicity. Total cocoons, which is probably the most quickly measured endpoint, appears as sensitive as total offspring and more sensitive than eggs plus embryos. Therefore, we would not recommend counting embryonic stages in the routine protocol of the adult *Tubifex* bioassay.

The suggested higher sensitivity of total cocoons and total offspring seems to be due to the lower intratreatment variability of these endpoints. To illustrate this point, the coefficient of variation for the five replicates of each treatment, including controls, were calculated. These coefficients were then averaged for each experiment. These average coefficients of variation are reported on Table 5. The average coefficients of variation for total cocoons and total offspring tend to be lower than for other endpoints in every experiment and, consequently, the means pooled over the five experiment are the lowest.

The early-life-stage bioassay appears to be less sensitive to copper toxicity than the adult bioassay. The NOEC and LOEC are higher for survival than for the most sensitive endpoints of the adult bioassay in the Lake Maggiore experiment and in both the Cà Bosco experiments; such a difference was not detected in the Sala experiment, but this could be due to the wider range between concentrations.

The early-life-stage bioassay also seems to have an inferior discriminatory power. All the LOECs estimated for survival correspond to a 100% response and the effect of the toxicant

Table 5. Average coefficients of variation for *Tubifex tubifex* bioassay endpoints

	Lake Suviana	Sala Bolognese	Lake Maggiore	Cà Bosco, unfed	Cà Bosco, fed	Pooled
Adult bioassay						
Total cocoons	44	61	37	66	26	46
Total young	65	60	57	42	62	59
Eggs plus embryos	92	73	68	83	31	74
Total offspring	59	60	50	46	37	53
Early-life-stage bioassay						
Survival	48	75	100	103	41	68

is all or nothing; as a result, sediments can only be classified as either toxic or nontoxic.

The effect of copper concentration on the endpoints of the adult bioassay are more continuous, and responses lower than 100% can be recognized as significant. Therefore, the bioassay can discriminate different degrees of toxicity. Also, the power of the adult bioassay is enhanced by the several endpoints that can respond differentially.

Even though the adult bioassay has greater sensitivity and discriminatory power, toxicity testing using cocoons may be useful. While in this study the early life stage bioassay was performed using the same 250-ml beakers and the same amount of sediment as the adult bioassay, a protocol could be developed requiring a smaller volume of sediment; this is not feasible for the adult bioassay. This could be useful when acquiring large amounts of sediment for the test is difficult.

It was notable that there were large differences in the production of cocoons, eggs, and young among the control treatments of the five experiments. Since the experiments were nonsimultaneous, it is not possible to eliminate differences in the experimental conditions or in the individuals used for the bioassays. Nevertheless, it seems likely that the capacity of the different sediments to support the growth and reproduction of *Tubifex* is responsible for a large part of the observed variability, as several repeated tests with the Cà Bosco soil and the addition of food (unpublished data) displayed a much smaller interexperiment variability. However, it was not possible to find any significant correlation between the endpoints of the bioassay and the characteristics of the sediment reported in Table 2. In particular, total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP) prove completely inadequate as measures of food availability. Not only are these variables uncorrelated with the endpoints, the addition of a food supplement to the Cà Bosco soil, which increases dramatically the production of cocoons and young, affects TOC, TN, and TP only slightly.

The addition of food to the Cà Bosco soil has one more effect on the test endpoints, i.e., the shift of LOECs and NOECs to higher values. It is not possible to exclude that this is caused by a decreased copper bioavailability. Indeed, while the measured pH of overlying water was hardly influenced and the amount of organic matter added is insufficient to significantly change TOC of sediment, addition of food could have increased dissolved organic carbon of overlying and pore water. However, it seems more likely that the better condition of the animals, due to the increased food availability, improved their capacity to withstand the toxic stress, decreasing their sensitivity.

Effects of food availability on sensitivity to toxicants is well studied, at least for water-column tests [24–26]; both decreased and increased sensitivity have been observed. Addition of food may improve physiological conditions and accelerate growth and other biological processes but also may influence partition of chemicals and their bioavailability; moreover, feeding is a possible route of exposure. Thus, the final effect is difficult to predict in advance. This seems particularly true in whole sediment bioassays because of the complex environment and because the test species can use the sediment itself as a food source.

Taking food availability into account is also important when assessing the toxicity of field-collected sediments relative to a reference sediment. In this case, food limitation may be a cause of false positives. It has been shown that incorporating a standard feeding regime in the assay protocols greatly decreases the importance of this possible source of error [27].

Standardized feeding, following the same method described here, is routinely used in the *Tubifex* adult reproductive bioassay when applied to field sediments. However, since the aim of this work was to compare the performance of the two protocols in an array of conditions, sediments of different characteristics (and different values as food sources) and one sediment with addition of food were tested. It seems noteworthy that, while LOECs change with the addition of food to Cà Bosco soil for both the bioassays, the difference between their sensitivities is retained.

There were major differences in the NOEC and LOEC for copper in the four tested substrates across the test endpoints. The NOECs ranged from 62.64 to >802.1 mg/kg and the LOECs from 69.06 to >802.1 mg/kg. This variability illustrates the fact that the concentration at which toxicity within a species occurs is primarily related to the availability of the contaminant and is most likely due to the geochemical properties of the sediment matrix and not to differences in the biological processes used to estimate toxicity. Of the test endpoints used in these experiments, total cocoon production showed the least intrareplicate variability and was most sensitive to copper effects and may best illustrate the effects of geochemistry on availability and toxicity. A comparison between geochemical characteristics (Table 2) and LOEC for this endpoint (Table 4) suggests a relationship between decreasing toxicity and the proportion of silt plus clay and TOC in the sediment as well as the concentrations of iron and calcium.

Both particle size and the proportion of organic material (TOC) present are known to influence the availability of metals to organisms [3], and the low bioavailability and thus toxicity in the sediment from Suviana is most likely related to their relatively higher proportion in this sediment. Iron is known to bind free metal ions, and it has the lowest concentration in the sediment from Cà Bosco, where copper was most available. Finally, the role of calcium is of interest, and the increase in calcium in these sediments seems to relate to availability; calcium may compete for the same binding sites as copper and thus affect availability. The relationships were tested by Kendall's nonparametric correlation coefficient. As estimated LOEC depends on the concentrations actually tested, a coefficient that uses only the information on rank seems more reliable; moreover, the actual LOEC value for Lake Suviana was not available. Only correlation of LOEC with TOC and Fe_2O_3 are confirmed as significant ($\alpha \leq 0.05$); however, the power of the test is obviously low, as only five observation are available.

In summary, our results, though relative to copper only, confirm the importance of reproduction-based endpoints and suggest that, for *T. tubifex*, the adult reproduction test represents a more effective approach than the traditional early life stage survival bioassays.

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