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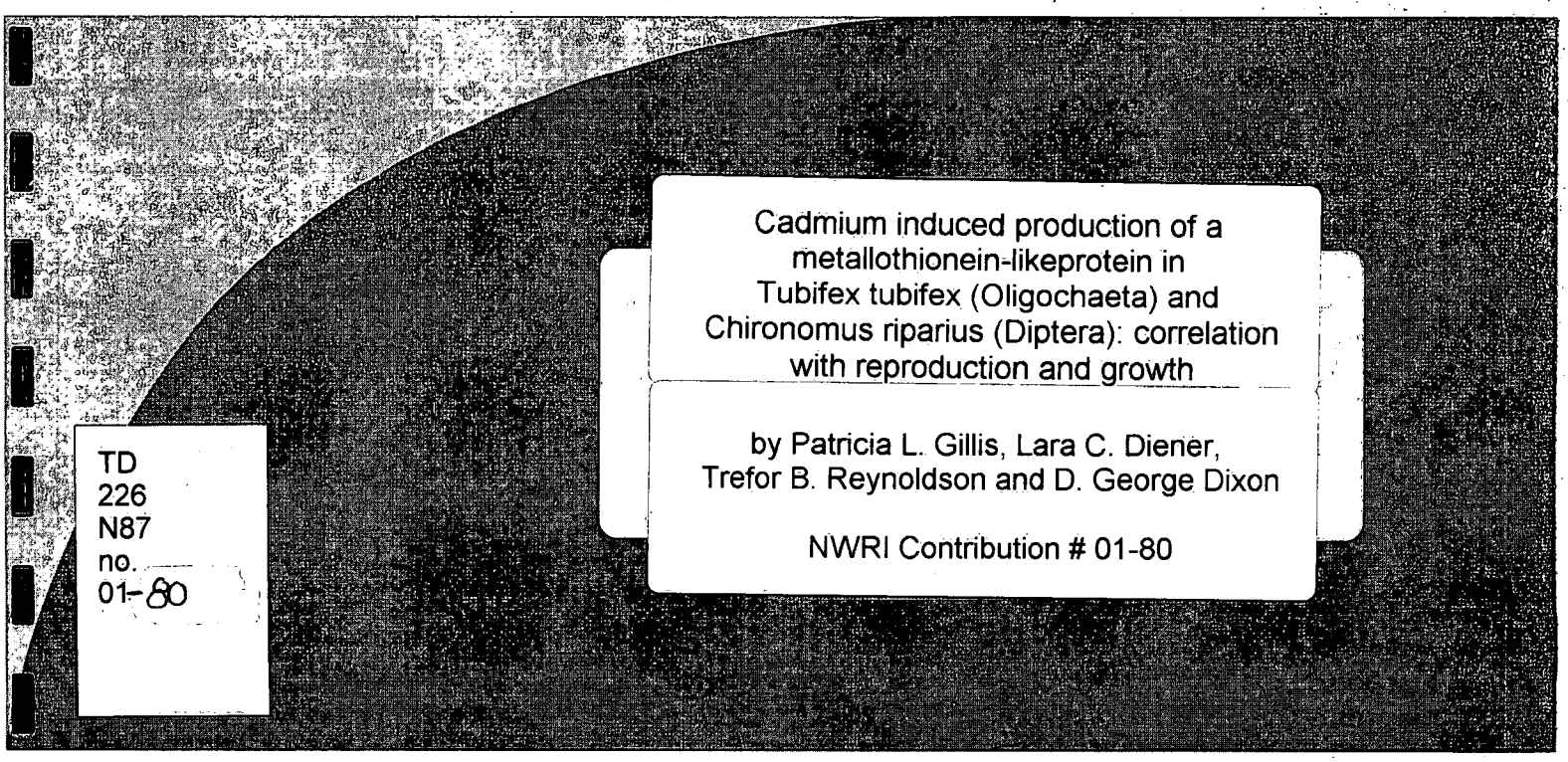
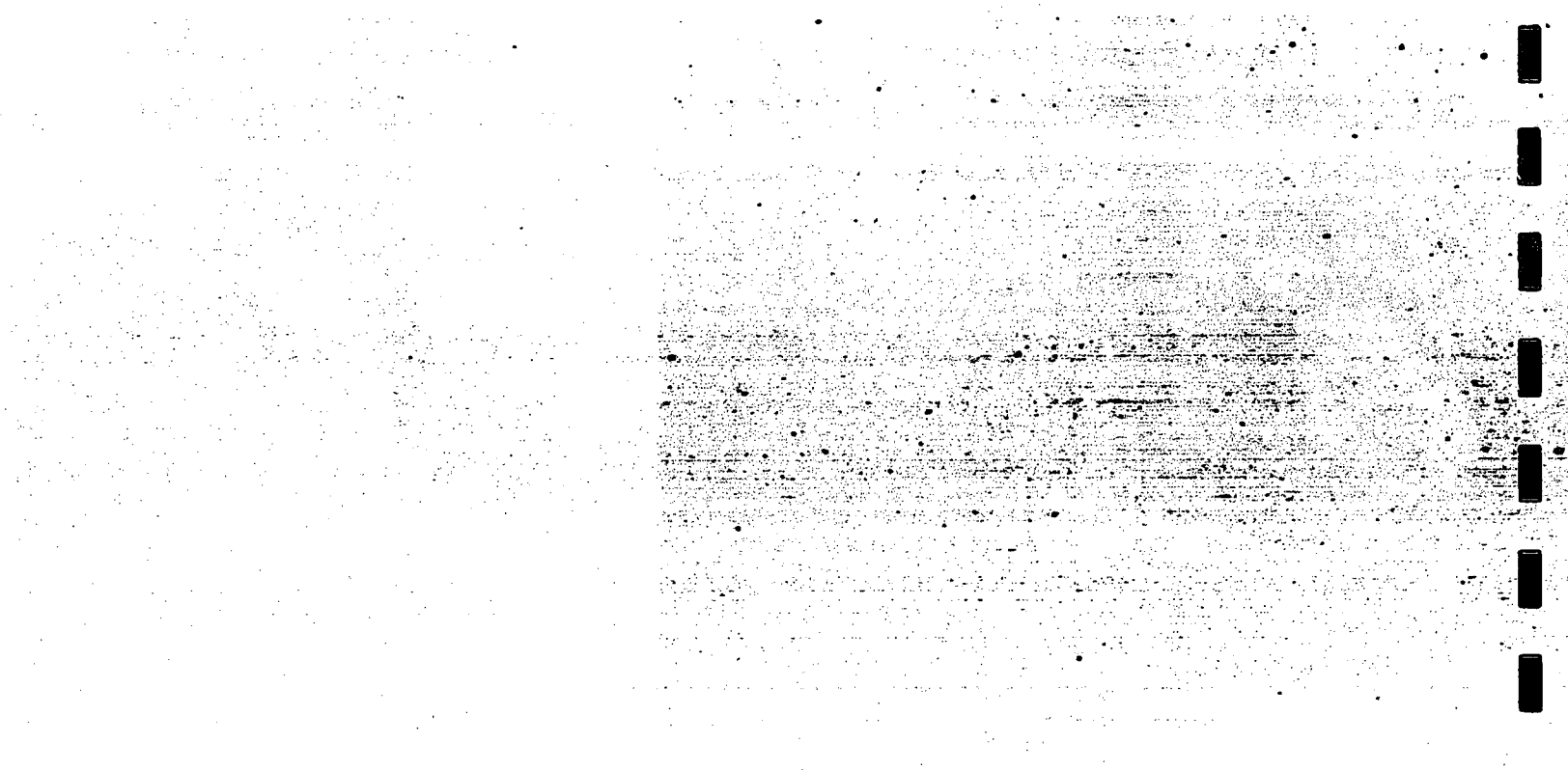


Cadmium induced production of a metallothionein-likeprotein in Tubifex tubifex (Oligochaeta) and Chironomus riparius (Diptera): correlation with reproduction and growth

by Patricia L. Gillis, Lara C. Diener, Trefor B. Reynoldson and D. George Dixon

NWRI Contribution # 01-80



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Cadmium induced production of a metallothionein-like protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): Correlation with reproduction and growth.

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Cadmium induced production of a metallothionein-like protein in *Tubifex tubifex* (Oligochaeta) and *Chironomus riparius* (Diptera): Correlation with reproduction and growth.

Management Perspective

This paper describes a method for measuring the metal binding protein, metallothionein in benthic invertebrates, in particular the worm, *Tubifex tubifex* and the insect larvae, *Chironomus riparius*. Aside from having a role in regulating cellular levels of essential metals, metallothionein has been shown to bind to nonessential metals to prevent possible damage to the cell. Previous studies have found that the metallothionein concentration in some organisms, increases after exposure to elevated levels of metals in the environment. Therefore metallothionein concentration has been proposed as tool for monitoring metal exposure and effects in aquatic organisms.

T. tubifex and *C. riparius* were exposed to field sediments artificially contaminated with the trace metal cadmium. The whole body endpoints of reproduction in *T. tubifex* and growth in *C. riparius* as well as two subcellular endpoints, metallothionein concentration and cadmium tissue concentration were measured following the exposure to cadmium contaminated sediments.

Both animals were negatively affected by the cadmium exposure and after an threshold concentration was reached, reproduction and growth were significantly reduced. *T. tubifex* and *C. riparius* were found to accumulate cadmium and produce metallothionein in a concentration-responsive manner. This suggests that metallothionein is produced in response to cadmium exposure in these two animals. Differences (increases) in the metallothionein concentration and cadmium tissue concentration were seen at lower sediment cadmium concentrations than were differences (reductions) in the whole body endpoints of reproduction and growth.

The findings of this study suggest that metallothionein and cadmium tissue concentrations are sensitive measurements that can be used as tools for biomonitoring, since they can provide an 'early warning' of metal contamination before negative effects are apparent at the whole organism level.

Sommaire à l'intention de la direction

Cet article décrit une méthode de mesure de la métallothionéine, la protéine liant les métaux chez les invertébrés benthiques, notamment chez le ver *Tubifex tubifex* et chez la larve de l'insecte *Chironomus riparius*. On a montré que la métallothionéine, en plus de jouer un rôle dans la régulation des teneurs cellulaires en métaux essentiels, lie aussi certains métaux non essentiels pour prévenir des dommage possibles aux cellules. Des études antérieures ont montré que les teneurs en métallothionéine de certains organismes augmentent après l'exposition à de fortes concentrations de métaux du milieu. On a donc proposé d'utiliser la concentration de métallothionéine comme outil pour la surveillance de l'exposition aux métaux et de leurs effets chez des organismes aquatiques.

On a exposé *T. tubifex* et *C. riparius* à des sédiments contaminés artificiellement sur place par des traces de cadmium. Après une période d'exposition à ces sédiments contaminés, on a mesuré, pour l'ensemble de l'organisme, les critères d'évaluation du taux de reproduction chez *T. tubifex* et du taux de croissance chez *C. riparius* , ainsi que deux critères d'évaluation infracellulaire, la concentration de métallothionéine et celle du cadmium tissulaire.

Chez ces deux organismes, on observait des impacts négatifs de l'exposition au cadmium et, une fois atteinte une concentration seuil, on notait une diminution significative des taux de reproduction et de croissance. On a noté que *T. tubifex* et *C. riparius* accumulaient du cadmium et produisaient de la métallothionéine selon un mécanisme dépendant de la concentration, ce qui semble indiquer que la production de la métallothionéine dépend de l'exposition au cadmium chez ces deux organismes. On observait des différences (augmentations) dans les concentrations de métallothionéine et de cadmium tissulaire aux faibles concentrations de cadmium dans les sédiments, ainsi que d'autres différences (réduction) pour les critères d'évaluation des taux de reproduction et de croissance (ensemble de l'organisme).

Les résultats de cette étude semblent indiquer que les concentrations de métallothionéine et de cadmium tissulaire sont des mesures sensibles utiles pour la biosurveillance, étant donné qu'elles peuvent servir d'outil d'« alerte précoce » de contamination par les métaux avant qu'on n'observe des effets négatifs pour l'ensemble de l'organisme.

ABSTRACT

Laboratory cultured *Chironomus riparius* and *Tubifex tubifex* were exposed to sediments artificially enriched with a range of cadmium concentrations. Both species accumulated Cd in a concentration-dependent manner. Metallothionein (MT) concentration, as measured by a mercury saturation assay, also increased with increasing Cd exposure. After reaching a threshold of Cd exposure, the whole body endpoints of reproductive output in *T. tubifex* and growth in *C. riparius* declined significantly. The threshold effect concentrations for *T. tubifex* and *C. riparius* were 2.68 and 0.134 μmol Cd/g dry sediment, respectively. Cadmium tissue residue and MT concentration were more sensitive indicators of exposure than the whole body endpoints. For *T. tubifex*, MT concentration and tissue residue were significantly elevated above control levels after exposure to the 0.67 μmol Cd/g dry sediment treatment. In *C. riparius*, MT concentration and tissue residue were both significantly elevated above control levels after exposure to $3.8x^{10\text{-}3}$ μmol Cd/g dry sediment exposures. These data suggest that MT concentration and tissue cadmium concentrations are sensitive sub-cellular endpoints, which can be used to predict effects at the individual or population level.

Key Words: Metallothionein, Biomarker, Oligochaete, Chironomidae, Cadmium toxicity

Production induite par le cadmium d'une protéine de type métallothionéine chez *Tubifex tubifex* (Oligochaeta) et *Chironomus riparius* (Diptera), et sa corrélation avec la reproduction et la croissance

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RÉSUMÉ

On a exposé des *Chironomus riparius* et des *Tubifex tubifex* de laboratoire à des sédiments artificiellement enrichis par une gamme de concentrations de cadmium. Ces deux espèces ont accumulé du Cd en fonction de la concentration. On observait également un accroissement en fonction de l'exposition au Cd de la concentration de métallothionéine (MT) mesurée par un essai de saturation au mercure. Une fois atteinte la concentration seuil d'exposition au Cd, on a observé une diminution notable des valeurs des critères d'évaluation servant à mesurer, pour l'ensemble de l'organisme, le taux de reproduction chez *T. tubifex* et le taux de croissance chez *C. riparius*. Les concentrations à effet de seuil pour *T. tubifex* et *C. riparius* étaient de 2,68 et de 0,134 µmol Cd/g de sédiments secs, respectivement. La concentration des résidus tissulaires de cadmium et celle de MT étaient des indicateurs de l'exposition plus sensibles que les critères d'évaluation pour l'ensemble de l'organisme. Dans le cas de *T. tubifex*, la concentration de MT et celle des résidus tissulaires étaient significativement plus élevées que celles mesurées chez des témoins après des expositions à des traitements pouvant atteindre 0,67 µmol Cd/g de sédiments secs. Chez *C. riparius*, la concentration de MT et celle des résidus tissulaires étaient significativement plus élevées que celles mesurées chez des témoins après une exposition à des teneurs de $3{,}8 \times 10^{-3}$ µmol Cd/g de sédiments secs. Ces données semblent indiquer que la concentration de MT et de cadmium tissulaire sont des critères d'évaluation infracellulaires sensibles, qu'on peut utiliser pour prévoir des effets au niveau des individus ou des populations.

INTRODUCTION

The use of biomarkers has been gaining recognition as a means of assessing the toxicity of a medium (water or sediment) to the biota. Biomarkers, biochemical or physiological indicators of either exposure to, or effects of, environmental contaminants at the suborganism or organism level [1] have a number of advantages over other methods of sediment toxicity analysis. Like more conventional endpoints in laboratory sediment bioassays, biomarkers provide measurements of toxicity and bioavailability, but can also be used in the field to determine the effect of toxicants in natural environments. Biomarkers can provide a link between exposure and ecologically relevant effects at a community or population level. Molecular and biochemical biomarkers respond quickly to changes in contaminant exposure, while a longer period may be required before a change is apparent at the population or community level [2,3]. This sensitivity permits sub-cellular indicators to be used as a first line of action in a monitoring program [4].

Metallothionein (MT), a cystine-rich, low molecular weight, metal binding protein has been used as a biomarker of metal exposure in a number of aquatic animals. In a 1990 review [3], MT or MT-like proteins had been reported in at least 80 species of fish and aquatic invertebrates, and many more have been reported since. The principal role of MT appears to be the homeostasis of Zn and Cu, but other functions are thought to include the detoxification, storage and regulation of heavy metals [5,3,6]. Metallothionein is also thought to sequester unbound metals thereby providing protection against toxicity from nonessential metals, such as Cd and Hg, with no known biological function [3]. It has been suggested that MT, because of its stress specificity, would be a useful biomarker for

exposure to certain metals including Cd, Cu, Zn, and Ag [7-9].

There has been a growing interest in using MT concentration as a biomarker in benthic invertebrates to assess the potential toxicity of sediment metals. There are reports of MT-like proteins in oligochaetes, chironomids and other closely related organisms, but to the best of our knowledge, no study has specifically characterized the MT protein in either of these groups. Suzuki et al. [10] measured inducible Cd binding proteins in the earthworm *Eisenia foetida*, which he suggested were MT because of their molecular characteristics. Bauer-Hilty et al. [11] isolated and partially characterized a Cd binding protein with metallothion-like characteristics in *Lumbriculus variegatus*. Morgan et al. [12] isolated metal binding proteins possessing characteristics typical of MT from two earthworms *Dendrodrilus rubins* and *Lumbricus rubellus*. Klerks and Bartholomew [13] found significantly higher levels of a MT-like protein in *Limnodrilus hoffmeisteri* collected from highly metal-contaminated sediment. Deeds and Klerks [9] also found strong evidence for the presence of a MT-like protein in *Limnodrilus udekenmianus*. Both studies [13,9] suggest that the metal binding protein in question is most likely MT because the molecular weight (15 000 KD) is consistent with the molecular weight of MT. After studying Cd accumulation in the chironomid *Chironomus thummi*, Seidman et al. [14] suggested that it has inducible metal binding proteins. Yamarura et al. [15] investigated Cd binding proteins in *Chironumus yoshimatsui* and suggested that the proteins are MT based on their molecular characteristics.

In order for MT to be valid as an indicator of metal stress and not just exposure, the levels of MT in an exposed animal should be related to the health or fitness of the

organism [2,8]. The purpose of this study was to investigate the potential of using MT as a biomarker of both metal exposure and effects. Metallothionein would be a useful biomarker of exposure if the tissue concentration reflected the degree of exposure to biologically available trace metals. Additionally, if the MT concentration was correlated to toxic effects at higher levels of biological organization (reproduction and growth) it would also be considered an indicator of effect.

Because of their close association with the sediment, infaunal organisms were used in this study. Since oligochaetes build burrows within sediment, and feed by ingesting sediment particles, they are constantly exposed to sediment contaminants through both feeding and bodily contact. Chironomid larvae also build burrows in the sediment and feed on organic detritus on the sediment surface. Oligochaetes and midge larvae have a restricted home range, thus making them true indicators of the sediment conditions from which they were collected. Both groups are known to be tolerant of many types of pollution and are frequently an abundant component of the benthic community of lakes [16,17]. These attributes are relevant to biomonitoring because an organism must be able to survive in contaminated areas in order to be used to assess such conditions. Tubifcids [13,18,19], and chironomids [14,20-23], have been found to accumulate trace metals and therefore have been proposed as potential biomonitors for metal exposure. The representative species *Tubifex tubifex* and *Chironomus riparius* were chosen for this work because they are known to be widespread in natural lake sediments and can be easily cultured in the laboratory.

In order to determine if these organisms produce MT in a concentration-responsive manner they were exposed to field sediment artificially contaminated with Cd using whole

sediment bioassays. The endpoints of the sediment bioassays included measurement of reproductive output in *T. tubifex* and growth in *C. riparius* as well as measurements of MT concentration and Cd tissue residue. Cadmium was used in this study because it is a highly toxic, nonessential metal that has been shown to induce the production of MT and MT-like proteins in other annelids and chironomids.

METHODS

Culturing *T. tubifex* and *C. riparius*

T. tubifex was cultured in the laboratory using uncontaminated, fine (70.3% silt, 29.1% clay; and 0.6% sand), organically rich (8.1% organic carbon), sediment collected from Long Point Marsh, Lake Erie. Prior to use, the sediment was passed through a 250 μm sieve to remove any indigenous animals [24]. *T. tubifex* cultures were initiated with 200 cocoons and held for a period of seven to eight weeks. The sexually mature worms and cocoons were separated from the sediment by sieving the contents of the culture aquarium through a 500 μm sieve. The mature worms were used in bioassays and the cocoons were used to initiate new cultures. Detailed *T. tubifex* culture methods are described by Reynoldson et al. [24].

C. riparius was also cultured in the laboratory using natural aquarium gravel as a substrate. Chironomid cultures were initiated with first instar larvae and fed crushed Nutrafin™ fish flakes *ad libitum* until pupation. The adults emerge after approximately three to four weeks and after mating, the females lay egg masses, which were either used in bioassays or to initiate new cultures. Detailed *C. riparius* culture methods are described in

Day et al. [25].

Both the *T. tubifex* and *C. riparius* cultures were aerated and held in climate controlled environmental chambers at 23±1°C. The worms were held in constant darkness and the chironomids were held under a 16 hour light, 8 hour dark photoperiod regime.

Cadmium exposures

T. tubifex and *C. riparius* were exposed to sediments artificially contaminated with a range of Cd concentrations in order to determine if MT is produced in a concentration-responsive manner. Environment Canada's protocols [26] for conducting whole sediment bioassays with *T. tubifex* and *C. riparius* were followed. In the *T. tubifex* bioassay, sexually mature worms were exposed to test sediments for a period of 28 days to assess effects on adult survival and reproduction. In the *C. riparius* bioassay, first instar larvae were exposed to test sediments for a period of 10 days to assess effects on growth and survival. The sediment bioassays were conducted using a reference sediment (site 303) collected near Long Point, Lake Erie (0.35% organic carbon; 60.9% silt, 33.5% clay; and 5.6% sand) which was artificially contaminated with Cd. The initial aim of the spiking was to find a range of Cd concentrations which resulted in observable effects on the whole body endpoints while still providing enough tissue to allow for measurement of MT and Cd tissue residue.

Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$) was used as the source of Cd. It was prepared as an aqueous stock solution and added on a per-dry-weight basis to the sediment to create a range of Cd exposures. Sediment and metal stock solution were mixed using a side-to-side shaker for a period of 90 minutes at 175 agitations per minute.

Test beakers for the *T. tubifex* exposures were prepared by mixing 80 mg of crushed Nutrafin™ flake fish food with 100 ml of spiked sediment, followed by 150 ml of dechlorinated tap water. Both the *T. tubifex* and the *C. riparius* bioassays used 250 ml glass beakers as test vessels. Test beakers for the *C. riparius* exposures received 50 ml of spiked sediment and 150 ml of dechlorinated tap water. Food was not initially added to the chironomid test beakers but 8 mg of crushed Nutrafin™ was added to each test beaker three times during the exposure (days 1, 4 and 8). The prepared test beakers were held at 4±1°C for a period of two weeks to allow for the metals to partition between the sediment, pore water and overlying water. The beakers were then aerated in the dark for one week in a 23± 1°C environmental chamber (earlier studies found that this was necessary to eliminate any excess ammonia before test initiation), after which the test animals are added. In the worm bioassay four sexually mature adult worms with well developed gonads were added to each beaker. Test beakers in the chironomid bioassay received fifteen, first instar, *C. riparius* larvae. All animals in one beaker were treated as a replicate. Five replicates per concentration were conducted in each of the bioassays. The sediment concentration ranges used for the worm and chironomid exposures were, respectively, 0 to 4.52 and 0 to 0.218 μ mol Cd/g dry sediment. The Cd exposures (including spiking) were repeated twice, independently, for each of the test animals.

Various water chemistry parameters including, temperature, conductivity, pH, as well as dissolved oxygen and ammonia concentration, were measured at the initiation and completion of each experiment. A 50 ml sample of sediment from each Cd treatment was kept for determination of actual sediment Cd concentration.

At the end of this exposure, the contents of the test beakers were sieved to separate the animals from the sediment. In the worm test, reproductive output was determined by the number of cocoons and young produced, as well as the percent of the cocoons that hatched. The whole-body endpoint for *C. riparius* was final wet weight, a surrogate for growth. Both *T. tubifex* and *C. riparius* were allowed to clear their gut contents before determination of wet weight. This was achieved by holding the test animals for 24 h in dechlorinated water. Faeces produced during this time were periodically removed with a pipette to prevent coprophagy. Following the gut clearing period, the animals were gently blotted with filter paper prior to obtaining a composite wet weight measurement for all surviving animals in each replicate. Finally, the samples were frozen and held at -80°C pending analysis for MT and Cd concentration; since a minimum of 13 mg wet weight was required for analysis, only samples over this weight were processed.

Metallothionein measurements

The Hg saturation assay of Dutton et al. [27] was adapted to measure MT in oligochaete and larval chironomid tissues. The Dutton et al [27] method is based on the principal that Hg has the strongest affinity for the binding sites of MT of all trace metals. In summary, an acidified solution of non-radioactive Hg was added to the homogenized worm tissue to replace any other metals bound to the MT molecule. Commercially available bovine haemoglobin (Sigma Chemical Company) was added in excess to bind any free Hg, thus removing it from solution. After centrifuging, the supernatant was retained for Hg analysis. Any Hg remaining in solution at this stage was assumed to be bound to MT. The

Hg was oxidized using potassium permanganate and direct heat and then measured using cold vapour Hg atomic absorption spectrometry. Finally, Hg concentrations were converted to tissue MT concentrations.

Metallothionein assay

Based on wet weight determined prior to freezing, the tissues were homogenized in a 20 times dilution using a 0.9% saline solution. Tissues and saline were homogenized in 1.5 ml microcentrifuge tubes using small plastic pestles. The homogenized tissue obtained from one replicate beaker was split into two parts, one for MT analysis and the other for tissue residue measurements. The tissue allotted for MT analysis was centrifuged at 14000 rpm for a period of five minutes. The supernatant was retained and the pellet discarded.

In a new microcentrifuge tube, 200 µl of the tissue supernatant and 200 µl of a 5% Hg reference (Fisher Scientific certified 1000 ppm) solution in 10% nitric acid were combined using a 'Vortex Mixer' for about 20 seconds. Following mixing, 400 µl of bovine hemoglobin solution (5% bovine hemoglobin in 0.9 % saline) was added, after which the sample was mixed using the 'Vortex Mixer' and centrifuged as above. The final supernatant was retained and held in a refrigerator pending Hg analysis.

Mercury measurements

A 300 µl aliquot of the supernatant from the MT assay was added to 200 µl of 5% potassium permanganate solution in a sealable 15 ml Pyrex™ test tube. The tube was gently swirled to mix the two liquids. The test tube and contents were then cautiously

heated over an open flame so as not to boil and/or expel the contents. To this mixture, 500 µl of hydroxylamine hydrochloride solution (25.3 g NH_2OH-HCL, 30 g of NaCl, made up to 500 ml with Milli-Q water) was added to clear the permanganate. The test tube was swirled gently until its contents became clear. The sample was then brought up to a final volume of 12 ml with 5% nitric acid. The test tube was sealed and finally mixed by repeated inversions.

Mercury in the sample was measured using cold vapor Hg atomic absorption spectrometry (Mercury Monitor LCD/Milton Roy). The resulting Hg concentrations were then converted to tissue MT concentration based on the molecular weight of Hg and the binding stoichiometry of MT as:

$$MT(nmol/g) = \frac{X\,D * 200.59}{7} \qquad (1)$$

where X is the concentration (µg/l) of Hg, D is the dilution factor accounting for all dilutions in the MT and Hg assays, 200.59 is the molecular weight of Hg (g/mol), and 7 is the number of binding sites per molecule of MT. Metallothionein is expressed as nmol per gram of wet tissue. It should be noted that as yet we have not yet attempted to confirm the identity of the metal binding protein that we are referring to as MT, so in the strictest sense it should be referred to as MT-like.

Cadmium measurements

Tissues were digested with acid prior to measurement of Cd concentration using atomic absorption spectrometry. Concentrated nitric acid (20 µl) was added to a 1.5 ml

Cryovial™ containing 100 µl of the homogenized tissue reserved for metal analysis. After six days, hydrogen peroxide (25 µl of 30% H_2O_2) was added and 24 h later, the samples were made up to 1 ml with 1% HNO_3. Cadmium concentrations were determined using a Varian SpectraAA 400 graphite furnace atomic absorption spectrophotometer with Zeeman background correction using ammonium phosphate modifier and platforms. Tissue Cd concentrations were converted to a per dry weight basis using a wet to dry weight correction factor. The correction factors were based on preliminary investigations that found mature, *T. tubifex* contain on average 89.1% water and 3rd instar *C. riparius* contain an average of 86.9% water. Bulk sediment was prepared for analysis according to the method of Agemian and Chau [28]. Sediment samples were freeze-dried, ground and homogenized then digested with 5% hydrochloric acid and analyzed using ICP-OES analysis (JY74 Optical Emission System).

Quality control

Commercially available rabbit liver MT (Sigma Chemical Company) prepared to a working concentration of 10 µg/l, was used to measure recovery of MT. Three replicates of MT reference material were prepared in the same manner as tissue samples and included in each analytical run. The mean recovery of MT was 73%. National Research Council lobster hepatopancreas (TORT-1) was used to determine of recovery of Cd (certified 26.3±2.1 µg Cd/g TORT). An amount of TORT equal in dry weight to the worm tissue in each sample (0.5445 µg) was digested as described for tissues samples. A minimum of three TORT digestions were included in each analytical run. The mean

recovery of Cd was 85%. The Cd and MT concentrations reported here were not corrected for recovery.

All glassware and pestles used in this method were acid washed (5% HCL) and rinsed thoroughly with Milli-Q water. Metals grade nitric acid was used for all steps requiring nitric acid. All solutions used in this method were made using Milli-Q water.

Statistical analysis

Initial analysis of variance (ANOVA), conducted to determine if data from the duplicate tests could be combined, revealed that the slope of the responses differed significantly between the two worm tests. Therefore the data could not be pooled and each of the experiments were analyzed separately. Comparisons of endpoints (reproductive output, wet weight, Cd tissue residue and MT concentration) between treatments (Cd concentrations) were made using ANOVA. In cases where data were not normally distributed, a log transformation was used. In cases where variances were unequal, a natural log transformation was used. Significant differences were determined using Dunnett's Test, which compared all experimental treatments to the control group ($p<0.001$). Regression analysis was conducted to determine if there was a significant relationship between Cd tissue concentration and MT concentration. Statistical analysis were conducted using the software package 'Sigma Stat' version 3.2.

RESULTS

T. tubifex survival and reproduction

Sediment Cd concentrations ranging from 0 to 4.52 μmol/g were found to be suitable for *T. tubifex* exposures (Table 1) in that the effects on the whole body endpoint (reproduction or growth) were significant at the highest concentration while still providing enough tissue for analysis. Worms exposed to the control sediment had 100% survival and produced a mean of 22 and 15 young in Test A and B respectively (Table 1). There was 100% survival at the highest concentration used (7.38 μmol Cd/g) but the worms experienced severe reproductive impairment, and did not meet the minimum tissue requirement. Reproductive output in *T. tubifex* was negatively correlated with increasing Cd exposure (Figure 1a). The most sensitive reproductive endpoint was the number of young produced per adult. In Test A, the first significant decline in the number of young produced occurred at 2.68 μmol Cd/g dry sediment whereas the number of cocoons produced per adult significantly declined in worms exposed to 3.67 μmol Cd/g dry sediment. In Test B, the first significant decline in cocoon production occurred at 1.85 μmol Cd/g dry sediment but 3.67 μ mol Cd was the concentration after which all higher concentrations produced a significant decline in cocoon production (Table 1). The percent of the cocoons that hatched did not change significantly with Cd concentration.

T. tubifex metallothionein concentration and Cd tissue residue

The worms from the control sediment had a mean MT concentration of 2.6 nmol/g wet tissue in Test A and 6.7 nmol/g tissue Test B. Metallothionein concentration in exposed worms was positively correlated with increasing Cd exposure (Figure 1b). Metallothionein

concentration was significantly elevated above the control level after exposure to 0.67 µmol Cd/g sediment in Test A and 0.77 µmol Cd/g in Test B (Table 1). Cadmium tissue residue in *T. tubifex* residue was also positively correlated with increasing Cd exposure, ranging from $3.60x^{10-3}$ µmol/g in the control to 40.51 µmol/g in the highest Cd concentration (Figure 1c). Cadmium tissue residue was significantly ($p<0.05$) elevated above the control level after exposure to 0.67 µmol Cd/g spiked sediment. Tissue residue measurements were only available from Test B. Regression analysis found a significant relationship between Cd concentration residue and MT concentration ($p<0.001$).

C. riparius survival and growth

Cadmium concentrations ranging from 0 to 0.218 µmol/g sediment were found to be suitable for *C. riparius* exposures. Increasing Cd concentration in the sediment had a negative effect on both survival and growth (Figure 2a). In the control sediment 87 to 88% of the larvae survived with a mean final wet weight of 2.71 mg in Test A and 2.60 mg in Test B (Table 2). At the highest useable concentrations (0.218 and 0.216 µmol/gm) the survival had declined to 72 and 73%, respectively and the mean final wet weight had significantly ($p<0.001$) declined to 0.89 mg in Test A and 0.42 mg in Test B (Table 2). Although some animals did survive above this concentration, they did not produce adequate tissue for analysis because of their limited growth. The first concentrations in which the final wet weight of *C. riparius* was significantly lower than the control was 0.134 and 0.150 µmol Cd/g sediment in Test A and B respectively.

C. riparius metallothionein concentration and Cd tissue residue

Metallothionein concentration in *C. riparius* showed a positive relationship with increasing Cd exposure (Figure 2b). The chironomid larvae from the control sediment had a mean MT concentration of 7.9 nmol/g in Test A and 6.6 nmol/g in Test B. The lowest sediment Cd concentrations at which MT concentrations were significantly ($p < 0.001$) elevated above those of the control were 3.8 and 9.1 nmol in Test A and B respectively (Table 2). Cadmium tissue residue in *C. riparius* also showed a positive relationship with increasing metal exposure and was significantly ($p < 0.001$) elevated above control levels at all Cd concentration used (Figure 2c). Mean Cd tissue concentration ranged from 0.03 and 0.04 µmol/g in control animals, to 1.97 and 2.10 µmol/g in those exposed to sediments with the highest Cd from which MT could be measured (Table 2). Tissue residue data was unavailable from the three highest concentrations because of reduced biomass. Regression analysis found a significant relationship between Cd tissue concentration and MT concentration ($p<0.001$).

DISCUSSION AND CONCLUSIONS

Cadmium toxicity

T. tubifex and *C. riparius* exposed to Cd through whole sediment bioassays exhibited both acute and chronic toxicological effects depending upon the exposure concentration. Increasing Cd exposure had negative effects on the reproductive output in *T. tubifex* as well as the growth and survival of *C. riparius*. The toxicity of Cd to tubificids including *T.*

tubifex has been well documented [29-32]. Reproduction was found to be a more sensitive indicator of toxicity than survival in *T. tubifex,* which is in agreement with other annelid studies [16,33,32]. The lower levels of Cd exposure did not have a significant effect on the reproductive output of *T. tubifex* until a critical level of Cd exposure was reached, a response also seen by Jenkins and Manson [33] in the polychaete, *Neanthes arenaceodenta.* The negative effects of Cd exposure on *C. riparius* larval growth and survival has also been well documented [17,34,22,35,32].

Chironomid larvae were found to be much more sensitive to Cd than the oligochaete. *C. riparius* exposed to 0.218 μmol Cd/g exhibited a significant ($p<0.001$) decrease in wet weight of over 67% and although not significant, the survival was also reduced (73% compared to 88% in controls). In comparison, the reproductive output of *T. tubifex* did not significantly decline until the sediment concentration reached 3.7 μmol Cd/g and there was still 100% survival at 4.5 μmol Cd/g. It should be noted that first instar chironomids larvae, which are know to be more sensitive than later instars [17] were used in this study, whereas the worms were mature. Therefore it may not be appropriate to make a direct comparison of the Cd sensitivity of the two animals used in this study because of the different life stages.

Cadmium accumulation

The data presented here show that both *T. tubifex* and *C. riparius* accumulate Cd in their tissues in a concentration-responsive manner. The accumulation of Cd by tubificids and chironmids has been previously reported [14,20,36,13,18,22,23,19]. Invertebrates such as such as chironomids and tubificids that burrow into and feed on sediment have been

shown to respond strongly to sediment Cd contamination [19].

Although the Cd concentrations used in this study are relatively high compared to most aquatic sediments, the levels of biaccumualtion are comparable with animals collected from metal contaminated environments. In this study, *T. tubifex* exposed to 2.68 µmol Cd/gm, the concentration at which reproduction was significantly reduced, accumulated 30.38 µmol Cd/g dry tissue. Klerks and Barthomew [13] collected *L. hofmeistri* with tissue residues of 9.5 µmol/g dry tissue from Foundry Cove on the Hudson River, NY. Krantzberg [18] reported the tissue residue of 'tubificids' collected from Hamilton Harbour, Lake Ontario ranged from 0.62 to 5.87 µmol Cd/g dry weight. This study found that chironomid larvae exposed to 134 nmol Cd/g sediment had mean tissue Cd concentration of 257 nmol/g dry tissue, which is comparable with the 185 nmol/g dry tissue reported by Warren et al. [19] in *Chironomus staegeri* collected from Lake St. Joseph, near Quebec City, PQ.

Metallothionein

The recovery of the MT standard (mean 73%) was lower than expected since Couillard et al. [37] reported recoveries over 90%. The method of Dutton et al. [28] was recently adapted for use with non-radioactive Hg and perhaps some fine tuning of the method is still needed to improve the recovery. Berger et al. [38] also found a decrease in sensitivity after adapting a saturation assay using radioactive Cd to one which used non-radioactive Cd. The detection limit in the Berger et al. [38] adapted assay was 10 µg/g compared to 0.1 µg/g in the original assay (actual percent recoveries not reported). There is a possibility that a similar reduction in recovery has occurred in this study because of the use

of non-radioactive Hg.

Metallothionein concentration increased in a concentration-responsive manner in both test species following exposure to Cd contaminated sediments. The significant relationship between Cd tissue residue and MT ($p<0.001$), suggests that the increase in MT concentration in the exposed animals is produced in response to the accumulation of Cd. Metallothionein is thought to be produced in response to increased intracellular levels of free metal in order to prevent or reverse potentially detrimental, non-specific binding of metals to other ligands [38,39].

The two sub-cellular endpoints, MT concentration and Cd tissue residue, were found to be more sensitive indicators of Cd exposure than either of the whole body endpoints. Metallothionein concentration and tissue residue were significantly elevated above the control levels at Cd concentrations well below those which resulted in a significant decline in reproductive output and growth. Significant decreases in whole body endpoints were not evident until after a two to five fold increase in MT concentration. This increased sensitivity of sub-cellular biomarkers was not unexpected since any stress response first occurs at the biochemical level, and then once regulatory mechanisms can no longer compensate, effects may be seen at the organism level [3,40].

If MT is to be used as a biomonitoring tool, a critical warning level of MT that corresponds with negative effects at the organism or population level could be set. For example, under the conditions of this study, *T. tubifex* with tissue MT concentration above 14 nmol/g would be expected to be experiencing significant reproductive impairment with implications for the health of the population. Similarly, *C. riparius* with MT levels over 20

nmol/g would be experiencing a significant reduction in larval growth. Correlating MT concentration to decreased growth in larval *C. riparius* could be used to predict the onset of negative effects at the population level since a substantial decrease in larval growth could delay or prevent emergence [41]. Reduced or retarded emergence has been related to a reduced number of swarming adults and hence to decreased mating success [17].

Metallothionein levels have often been labelled as an indicators of trace metal exposure rather than toxicological effect because most studies simply report levels of the protein without any indication of the effect of the exposure on the organism. Recently a few studies have investigated correlations between MT concentration and ecologically relevant endpoints. Deeds and Klerks [9] attempted to correlate MT levels with a sub-lethal indicator of stress in *L. udekenmianus* but concluded that respiration rate was a poor indicator of sub-lethal stress as it was not responsive at lower levels of Cd exposure. Couillard et al. [40] found that *Pyganodon grandis* (Unionidae) with elevated MT concentrations also showed symptoms of cellular toxicity and had a lower condition index. In this study we correlated MT concentration with the whole body effects of reduced reproduction and growth. Therefore we suggest that MT concentration can be used as a diagnostic tool of compromised individual health that can detect the effects of metal exposure before the onset of changes in population structure.

There are suggestions that MT concentration in some organisms can vary naturally with physiological condition of the animal and environmental conditions [42,4,43]. If MT is to be used as a biomarker of metal exposure and effects in chironomids and tubificids, the natural variation in MT concentration will need to be investigated. Also, the metal binding

protein that we have measured will need to be characterised to confirm its identity. Although at this stage MT measurements in *T. tubifex* and *C. riparius* are a long way from being used as a biomonitoring tools, we would suggest that based on laboratory experiments MT measurements show promise as a biomarker for trace metal exposure and effects in these organisms.

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Table 1. Mean (SD; n=5) of sediment Cd concentration, number of young and cocoons per adult, tissue Cd concentration and tissue MT concentration for exposure of *T. tubifex* to Cd in whole sediment bioassays.

Sediment Cd Concentration (μmol/g)	Test	Young Adult^{-1}	Cocoons Adult^{-1}	Percent Hatched	Tissue Cd Concentration (μmol/g)	Metallothionein Concentration (nmol/g)
ND	A	22.4 (3.5)	9.1 (3.2)	57	NA	2.6 (0.5)
ND	B	14.8 (2.4)	9.3 (2.3)	55	$3.60x^{10-3}$ ($4.4x^{10-3}$)	6.7 (1.3)
0.67	B	12.7 (2.4)	8.2 (2.6)	55	2.26 (0.25)[c]	9.6 (1.5)[d]
0.77	A	24.7 (2.1)	9.0 (3.6)	67	NA	5.4 (0.7)[d]
1.35	B	12.0 (2.8)	8.2 (4.1)	58	9.50 (2.61)	10.9 (2.4)
1.85	B	13.1 (1.0)	7.8 (2.3)*[b]	55	23.44 (4.65)	12.7 (1.3)
1.87	A	24.3 (2.5)	9.1 (5.4)	62	NA	6.4 (1.4)[c]
2.15	A	24.2 (4.5)	8.5 (3.0)	67	NA	11.7 (2.2)
2.68	B	10.6 (2.6)[a]	8.4 (5.0)	53	30.38 (4.60)	14.0 (2.4)
2.78	A	17.9 (3.9)[a]	7.5 (5.8)	63	NA	14.6 (3.1)
3.67	B	9.9 (2.0)	7.2 (1.8)[b]	52	32.18 (6.07)	15.5 (1.7)
3.67	A	12.2 (3.8)	6.9 (6.9)[b]	73	NA	17.9 (2.3)
4.31	A	8.2 (1.7)	6.5 (3.3)	69	NA	18.6 (5.1)
4.52	B	7.0 (2.0)	0.8 (0.6)	92	40.51 (3.68)	17.6 (3.5)

ND = Not detected, NA = Not available, [a]Lowest concentration at which number of young is significantly reduced compared to control. *[b]Lowest concentration at which number of cocoons is significantly reduced compared to control. [b]First concentration above which all concentrations result in a significant decrease in number of cocoons. [c]Lowest concentration at which tissue residue is significantly elevated above control. [d]Lowest concentration at which MT concentration is significantly elevated above control.

Table 2. Mean (SD; n=5) of sediment Cd concentration, survival, wet weight, tissue Cd concentration and tissue metallothionein concentration for exposure of *C. riparius* to Cd in whole sediment bioassays.

Sediment Cd Concentration (μmol/g)	Test	Survival (%)	Wet Weight (mg)	Tissue Cd Concentration (μmol/g)	Metallothionein Concentration (nmol/g)
ND	A	88	2.71 (0.33)	0.03 ($4.0x^{10-3}$)	7.9 (0.9)
ND	B	87	2.60 (0.36)	0.04 ($4.1x^{10-3}$)	6.6 (0.5)
$3.8x^{10-3}$	A	88	2.69 (0.06)	0.13 ($9.5x^{10-3}$)[b]	12.3 (2.6)[c]
$9.1x^{10-3}$	B	96	2.52 (0.22)	0.14 (0.02)[b]	9.2 (2.2)[c]
0.014	A	88	2.60 (0.11)	0.41 (0.06)	14.0 (1.3)
0.016	B	71	3.04 (0.21)	0.36 (0.06)	14.0 (1.9)
0.035	A	85	2.78 (0.32)	0.73 (0.05)	16.5 (1.4)
0.041	B	85	2.49 (0.21)	0.85 (0.16)	18.3 (5.7)
0.071	B	84	2.59 (0.19)	1.13 (0.16)	17.7 (1.2)
0.114	B	69	2.33 (0.47)	1.91 (0.15)	18.8 (1.0)
0.134	A	88	1.88 (0.35)[a]	1.97 (0.20)	20.1 (2.3)
0.151	B	85	1.49 (0.52)[a]	2.10 (0.21)	20.7 (3.6)
0.216	B	73	0.42 (0.27)	NA	21.4 (2.8)
0.218	A	72	0.89 (0.39)	NA	23.5 (6.1)
0.397	A	40	NA	NA	NA

ND=Not detected, NA=Not available, [a]Lowest concentration at which wet weight has significantly declined compared to the control, [b]Lowest concentration at which tissue residue is significantly elevated above control. [c]Lowest concentration at which MT concentration is significantly elevated above control.

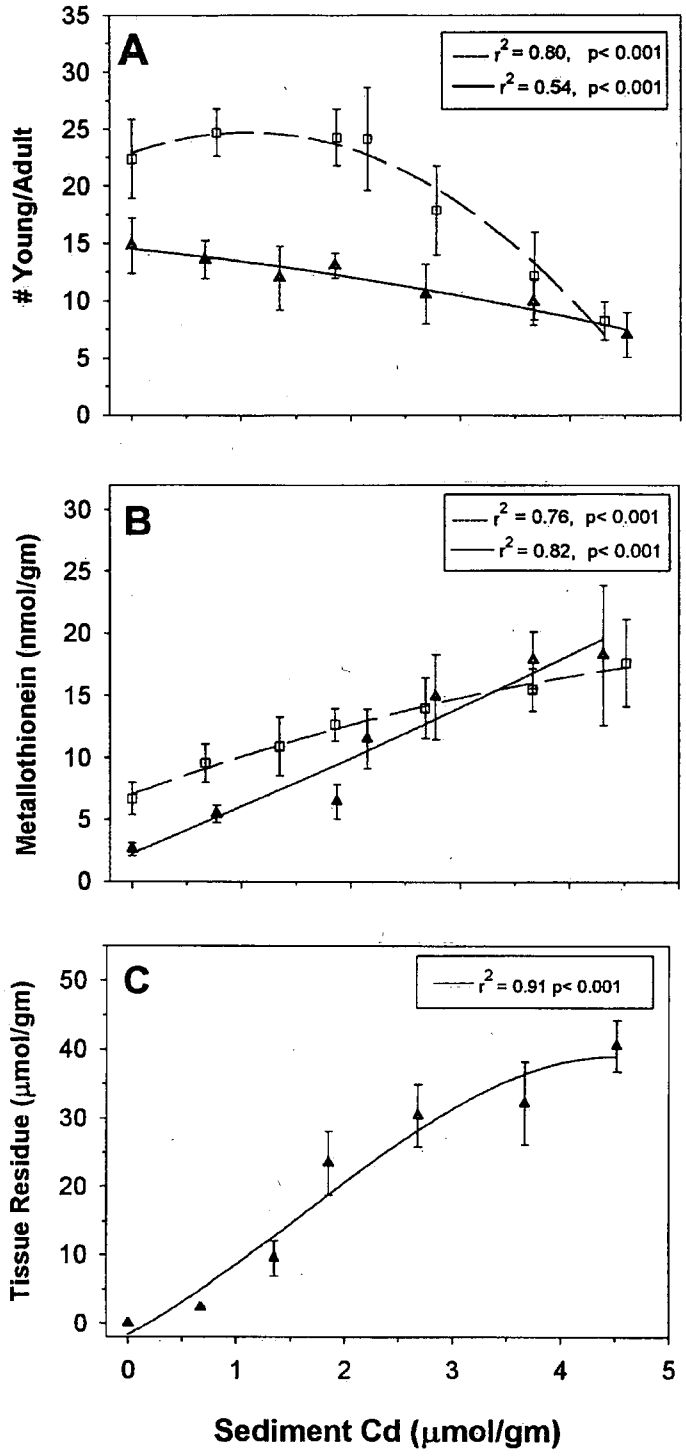

Figure 1. (A) Relationship of mean number of young produced per adult (A), mean metallothionein concentration (B) and mean tissue metal concentration (C), all to sediment Cd concentration, for exposure of *T. tubifex* to Cd-contaminated sediments for 28 d. Error bars represent standard deviations, n = 5. Dashed line and open squares represent Test A. Solid line and filled triangles represent Test B.

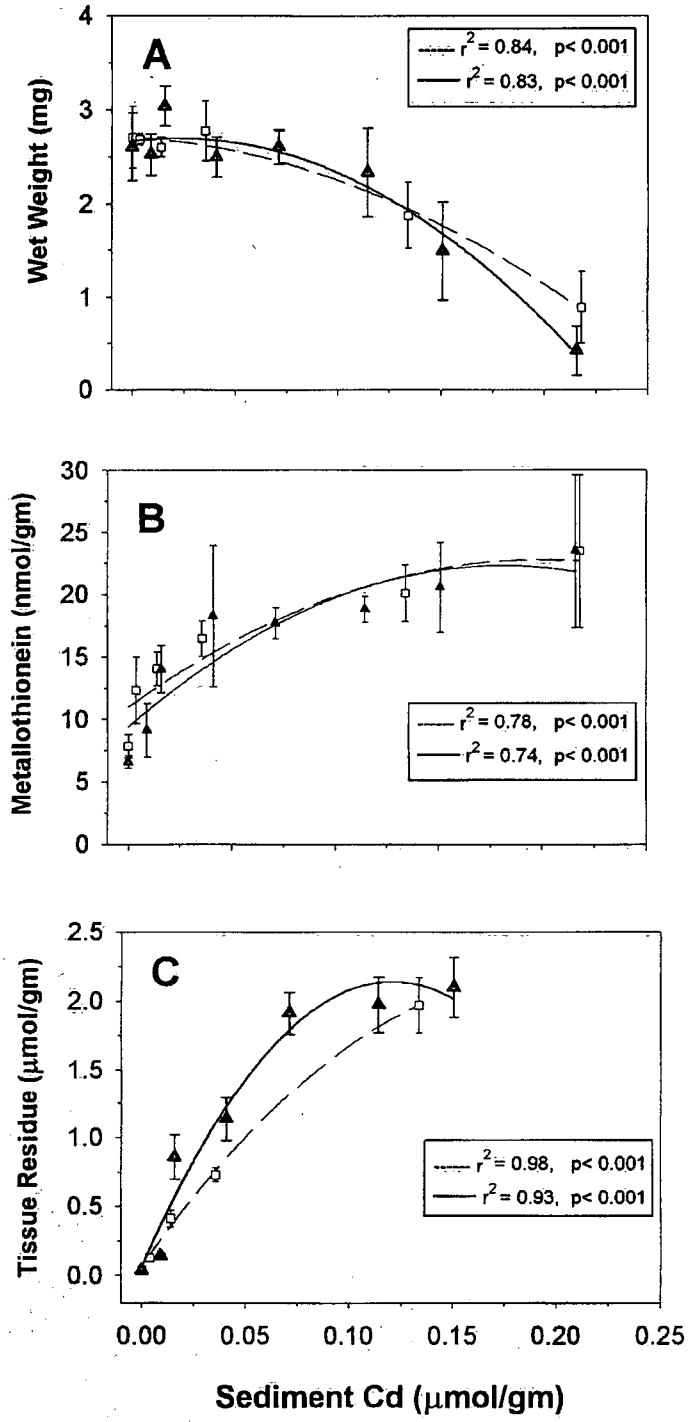

Figure 2. Relationship of mean wet weight (A), mean metallothionein concentration (B), and mean tissue Cd concentration (C), all to sediment Cd concentration, for exposure of *C. riparius* to Cd-contaminated sediments for 28d. Error bars represent standard deviations, n = 5. Dashed line and squares represent Test A, Solid line and triangles represent Test B.

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