Original Article

16-00757

Environmental Toxicology

W.T. Mehler et al.

Development of whole-sediment TIE techniques in Australia

DEVELOPMENT OF WHOLE-SEDIMENT TOXICITY IDENTIFICATION AND EVALUATION (TIE) TECHNIQUES FOR TWO AUSTRALIAN FRESHWATER SPECIES: *CHIRONOMUS TEPPERI* AND *AUSTROCHILTONIA SUBTENUIS*[1]

W. TYLER MEHLER,* MICHAEL J. KEOUGH, and VINCENT PETTIGROVE

School of Biosciences, The University of Melbourne, Parkville, Victoria, Australia

(Submitted 27 October 2016; Returned for Revision 9 December 2016; Accepted 5 March 2017)

[1] This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi:10.1002/etc.3787

Abstract

Most of the public literature and available guidance documents on the conduct of freshwater whole-sediment toxicity identification and evaluations (TIEs) detail the use of test organisms and amending agents that are readily available in North America. These commonly used test organisms and the supported amending agents, however, are not available and largely inappropriate (i.e., not native species) for conducting whole-sediment TIEs outside of North America. The overall objective of the present study was to build foundational methods for performing freshwater whole-sediment TIEs in Australia. We examined the capability of 3 amending agents: ANZ38 Zeolite (for ammonia; Castle Mountain Zeolites), Oxpure 325B-9 Activated Carbon (for nonpolar organics; Oxbow Activated Carbon), and Lewatit MonoPlus TP 207 (for cationic metals; Lanxess Deutschland) on 2 Australian native freshwater species: the midge *Chironomus tepperi* and the amphipod *Austrochiltonia subtenuis.* To evaluate the effectiveness of each amendment, bioassays were conducted with spiked sediments of ammonia, permethrin (as part of a commercial formulation), and copper using acute median lethal concentrations (LC50s) for both species and growth median effect concentration (EC50) of midges as the endpoints of interest.

Keywords: Toxicity identification and evaluations (TIEs), Australia, Sediment toxicity, Freshwater toxicology

* Address correspondence to wmehler@student.unimelb.edu.au

Published online 7 March 2017 in Wiley Online Library (www.wileyonlinelibrary.com).

DOI: 10.1002/etc.3787

INTRODUCTION

Toxicity identification and evaluations (TIEs) are useful tools in characterizing the responsible contaminant in a toxic medium, especially in scenarios of complex mixtures. In general, TIEs use direct manipulations of a contaminated medium to either increase or decrease toxicity of a certain contaminant or contaminant class when evaluated through the use of a bioassay. These changes in toxicity elucidate which contaminant class is contributing to the toxicity of the medium. In 2007, the United States Environmental Protection Agency (USEPA) issued guidance documents on appropriate techniques to conduct TIEs on whole sediment [1]. The USEPA guidance documents provided a variety of ways to characterize toxicity of various classes of contaminants in sediments. Not surprisingly, most of the materials (amending agents) and test species used in the bioassays are oriented around the United States. Amending agents including Clinoptilolite (ammonia amendment; Aquatic Eco-Systems), Carbon-G coconut charcoal (organics amendment; Calgon Carbon), and SIR-300 (metals amendment; ResinTech) are not readily available in Australia. Similarly, most of the research in the USEPA guidance documents, as well as in the published literature, has been conducted with standard test species that are native to the United States, such as *Chironomus dilutus* and *Hyalella azteca* [1]. These test organisms are not native to Australia and importing them for risk assessments would be inappropriate because it would pose a risk of accidental introduction of nonnative species.

Because much of the current sediment TIE research is based on nonnative species and largely unavailable amending agents in Australia, the use of sediment TIEs in Australia has been quite limited. To date, most published TIE studies in Australia have focused on overlying water, effluents, or porewater [2–4]. Kellar et al. [5] used a

sediment TIE as part of a weight-of-evidence approach in the assessment of sediments in Upper Dandenong Creek (an urban waterway near Melbourne) with variable success. Kellar et al. [5] performed the TIE using amendments from the United States and assessed survival, growth (via length), and emergence of *Chironomus tepperi*. Although the test was able to characterize toxicity in selected sites, some of the amended sediments in the TIE bioassays showed high variability (using the emergence endpoint) and in some cases provided inconclusive results on the source of toxicity (reference sites appeared to be impacted by nonpolar organics and metals although none were found via chemistry analysis). Because this was one of the first studies to use this species (*C. tepperi*) and the first to use sediment TIE techniques in Australia, the associated variability is understandable. Regardless, sediment TIE methods in the study by Kellar et al. [5] were able to isolate the cause of contamination for some sites in the study; however, more refinements to the methods may have been able to further strengthen these results.

One of the major benefits claimed for the TIE technique is that it is an effective and cost-efficient tool for determining the cause of toxicity [1]. Unfortunately, that is not currently the case for the use of sediment TIEs in Australia because the baseline procedures have yet to be developed in this country. The objective of the present study was to make the use of whole-sediment TIE techniques for use in Australia more practical and effective. The specific goals of the present study were to: 1) identify TIE amending agents that are both readily available in Australia and could successfully characterize toxicity of ammonia, nonpolar organics, and cationic metals in whole sediments; and 2) develop TIE methods using 2 native freshwater species (*C. tepperi* and

Austrochiltonia subtenuis) that could be used to assess the acute (via mortality) or sublethal risk (via growth of *C. tepperi*) of impacted freshwater sediments.

MATERIALS AND METHODS

Chemicals

A single contaminant for each of 3 contaminant classes (nonpolar organics, ammonia, and cationic metals) was evaluated as part of the present study: permethrin, ammonia, and copper. The formulated and commercially available pesticide Brunnings Ant, Spider & Cockroach Killer, which contained permethrin as the active ingredient (concentration of 100 g/L; 25:75), was used as the test nonpolar organic. Using this formulation produced a contaminated sediment that was more environmentally realistic than using neat chemicals (because other constituents are present in insecticide formulations) and avoided the needed for carrier solvents. Reagent grade copper ($CuCl_2 \bullet 2H_2O$; APS Ajax Finechem) and reagent grade ammonium (NH_4Cl; Chem-Supply Pty) represented the metals and ammonia classes of compounds, respectively.

TIE amendments and preparation

Zeolite has been successfully used to preferentially bind ammonia [6–9], and it was expected that the zeolite produced in Australia would be no different. The zeolite amendment ANZ38 is mined, produced, and manufactured in Australia (Castle Mountain Zeolites). The ANZ38 zeolite product comes as a fine (<76 µm) powder from the manufacturer and required little manipulation. Zeolite was rinsed with deionized water and allowed to rest overnight before being decanted before use, resulting in a thick paste that could be added to test sediments. An amending ratio of 20% (via wet wt) was used in TIE bioassays, based on published literature with similar zeolite products [1,7].

Powdered coconut charcoal or a carbonaceous resin (e.g., the Ambersorb Resins), which are suggested by the USEPA [1], could not be easily acquired in Australia. Although not manufactured in Australia, Oxpure 325B-9 (Oxbow Activated Carbon) was readily available through Filchem Australia Pty. Oxpure 325B-9 is a powdered activated carbon with a virgin bituminous coal base, with a diameter of <44 µm. Similar to zeolite, this product required very little manipulation. The activated carbon was saturated and stored in deionized water and allowed to rest overnight. Before use, the material was decanted and rinsed again with deionized water and then decanted a final time. The resulting product had a wet/dry ratio of approximately 22%. An amending ratio of 1% (via wet wt) was used in TIE bioassays. This ratio was slightly lower than reports in previous published literature with similar activated carbon products (~2% [1,7,10]), but was still able to reduce acute toxicity and showed limited effects to growth in preliminary studies and thus was used in the present study.

Similar to the activated carbon, Lewatit MonoPlus TP 207 (Lanxess Deutschland) is not manufactured in Australia, but is readily available in Australia through Filchem Australia Pty. This metals-amending agent is a weakly acidic, macroporous cation exchange agent (with chelating iminodiacetic**<ZAQ;1>** groups) that preferentially binds cationic metals. The product as purchased comes in a sodium ionic form that cannot be used in bioassays (because it causes adverse effects to the test organism). Therefore before use, the resin needed to be changed to the calcium form. To do this, approximately 700 g of resin were stored in 1 L of 2 M $CaCl_2 \bullet 2H_20$ (for at least 24 h) at 4 °C. Before use, the resin was repeatedly rinsed with deionized water until the conductivity of the decanted overlying water was below 250 µS/cm. Based on preliminary testing, improper

conversion to the calcium form (resulting in high pH values and/or a void of a true sediment–water interface) or inadequate rinsing of the final product (resulting in high salinity in test waters) proved toxic to the test organisms. An amending ratio of 20% (via wet wt) was used in TIE bioassays, based on published literature with similar metals' amendments [1,11].

Organisms

Two different freshwater test organisms were used in sediment bioassays: *C. tepperi* (midge) and *A. subtenuis* (amphipod). Both species are broadly similar to the Northern Hemisphere species *C. dilutus* and *H. azteca* in appearance but are most likely confined to Australia in regard to their distribution. Although international toxicity testing standards or government-based guidelines have yet to be developed for these native-Australian species, their use for such purposes continues to grow [12–15].

Cultures of the freshwater midge were originally acquired from temporary ponds in the Yanco Agricultural Institute (New South Wales, Australia). Cultures of this organism were maintained in ethanol-sterilized tissue paper using modified Martin's solution [16,17]. For bioassay work, adult flies were collected from the cultures and allowed to breed. Egg masses from adults were collected and resulting larvae were used in testing after 7 d (resulting in 5–7-d-old second instars).

Cultures of the freshwater amphipod *A. subtenuis* were originally obtained from Deep Creek (Victoria, Australia) and then from Devilbend Reservoir (Tuerong, Victoria, Australia). The cultures were maintained in aquaria with mesh gauze (as a substrate) using artificial water based on on-site conditions. Cultures were sieved using 2 sieves, 250 and 400 µm, with individuals collected on the smaller sieve being used. Organisms

were held for an additional 24 h before use to avoid using organisms that may have been injured during the sieving process.

Sediment preparation

Two sediments were collected for midge and amphipod bioassays. Sediments for midge testing were collected at Glynns Wetland, North Warrandyte (Victoria, Australia). Sediment used in amphipod testing was collected from one of the sites of origin, Bittern Reservoir (Tuerong, Victoria, Australia), because a stronger control response (i.e., less mortality) variability was noted with this sediment in comparison with Glynns Wetland. Both of these sites have been used and/or evaluated in the past and have been shown to be free of toxicity and have limited contamination [18,19]. Collected sediment was sieved through a 500- and 63-µm nylon mesh net for *C. tepperi* and *A. subtenuis*, respectively. The smaller mesh net was used for amphipods because of the small size of the test organism being used in the bioassay (using a finer particle-size sediment made the termination of the bioassay much simpler). Sediments were stored in 20-L buckets at 4 °C in the dark until use [20].

All 3 spiked sediments were prepared differently and held for differing amounts of time because of spiking procedures and chemical volatility. A high-concentration or "super-spike" sediment was prepared for both copper and permethrin (in formulation) bioassays [21]. Super-spike sediments for permethrin and copper were aged for at least 14 d before being diluted with clean sediment to ascertain the desired testing concentrations. These diluted test sediments (for copper and permethrin) were then aged for at least another 10 d before the initiation of bioassays. Desired ammonia test concentrations in sediments were prepared individually and aged for 7 d before the

beginning of bioassays. During the aging process, all sediments were manually mixed then rolled on a Stovall low profile roller. The relevant amending agent or sand (to account for any dilution effect) was added to sediments 3 d before the addition of test organisms.

Copper and ammonia were both spiked using formulations of the chemical ($CuCl_2 \bullet 2H_2O$ and NH_4Cl, respectively) that led to low pH in sediments [7]. The pH of both of these test sediments was adjusted using 10 M NaOH. Sediments were adjusted to a pH of approximately 7. Super-spike copper test sediments were also purged with nitrogen during the aging period to produce more environmentally realistic sediments [21].

Bioassays

For each contaminant class, 3 types of treatments were evaluated: amendment only, contaminant only, and the combination of the amendment and contaminant (as well as controls—containing neither the contaminant nor the amendment). Six concentrations were spiked for testing with *C. tepperi* and both growth and survival were evaluated as part of this bioassay, and only 5 concentrations were used for testing with *A. subtenuis* because only survival was measured. Six replicates with 15 individuals per replicate were used for *C. tepperi*, whereas 5 replicates and 10 individuals per replicate were used for *A. subtenuis*.

Bioassays were conducted in 350-mL beakers and the total weight of the sediment and the artificial volume of water used per replicate was 60 g wet weight and 250 mL, respectively. Test water for bioassays was prepared in accordance with culture waters' preparation as mentioned. All toxicity testing was conducted using a standard 16:8-h light:dark photoperiod and a temperature of 21 ± 1 °C following previously used

protocols [5]. Water-quality parameters including dissolved oxygen, pH, conductivity, and temperature were measured daily in midge testing and every other day for amphipod testing.

Bioassays evaluating metals and nonpolar organics with *C. tepperi* and *A. subtenuis* were conducted over a 5- [5] and 10-d test period (to be comparable with USEPA protocols for *H. azteca*) [22], respectively. For copper and permethrin bioassays, water changes occurred twice per day using a static renewal system (150–200 mL per change), with *C. tepperi* being fed every other day (10 mg TetraMin) and *A. subtenuis* being fed daily (1 mL of yeast, cereal leaves, and TetraMin and 0.5 mg TetraMin). In ammonia bioassays, approximately 30 mL were collected from each replicate per day to assess water quality; replicates were then replenished with fresh water of that same volume. Excluding this small replenishment, the ammonia bioassays were a 5-d static test (to avoid dilution of ammonia by replacement of overlying water) [9,23] for both species; in addition, neither organism was fed during the bioassay (to avoid any issues with dissolved oxygen). Mortality of both species was assessed in all TIE bioassays, with growth also being assessed for *C. tepperi* in all TIE bioassays. The dry weight of midges was used to assess growth [24]; organisms were dried at 90 °C to a constant temperature (Memmert drying oven) and weighed using a Kern ABS/ABJ analytical balance (reproducibility ± 0.1 mg; Kern & Sohn).

Chemical analysis

Before the addition of sand or amendment, approximately 50-g aliquots of each treatment were collected to serve as an initial concentration for each treatment. These initial concentrations were used in calculating acute median lethal concentrations (i.e.,

LC50s) and median sublethal effect concentrations using growth (i.e., EC50 values). Post amendment concentrations were only evaluated to better understand the level of concentration reduction that occurred with the addition of the amendment for that respective chemical class. Initial (day –3)**<ZAQ;2>** and post amendment concentrations (day 1) were analyzed in porewater for ammonia and whole sediment for permethrin and copper. In the case of copper, the resin was removed from the post amendment sediment by sieving through a 250-µm sieve before analysis. Porewater was extracted from sediment by centrifuging a 50-g aliquot (Avanti J-E Centrifuge; Beckman Coulter) at 2900 *g* at 4 °C for 45 min. Commercial laboratories accredited to ISO 17 025 and ISO 9001 carried out chemical analyses of all chemicals. Total ammonia (mg N/L) in porewater was analyzed using APHA 4500-NH3 H [25], which uses a Buchi steam distillation coupled with a titrimetric finish. For quantifying metals' concentration, moisture content of sediments containing copper was first determined using a gravimetric procedure (dry at ~105 °C, over a 12-h period). Afterward, 1 g of air-dried sediments (<50% moisture content) was refluxed and digested with both nitric (4 mL, 50% HNO3) and hydrochloric (10 mL, 20% HCl) acids for 2 h or until the volume was sufficiently reduced [26]. After digestion the solution was cooled and hydrogen peroxide (30% H_2O_2) was added. Solutions were heated and cooled again with the solution being diluted to a volume of 50 mL using deionized water and allowed to settle before extraction for analysis. Concentrations of metals in sediments were analyzed using inductively coupled plasma-atomic emission spectrometry (Method 200.7 [26]). For analysis of permethrin, sediments were extracted using acetone and hexane and analyzed using capillary injection followed by high-performance gas chromatography coupled with determination

by tandem mass spectrometry [27]. Permethrin was determined with a limit of reporting of 0.01 mg/kg. Total organic carbon (TOC) was analyzed using high-temperature combustion. The sample was air-dried, pulverized, and subjected to an acid reaction to remove inorganic carbonates, then combusted in a LECO furnace in the presence of strong oxidants and catalysts. The evolved (organic) carbon (as CO_2) was measured using an infrared detector. Five to 20 mg of treated sample were placed into the boat sampling module furnace at 800 °C, where all the carbonaceous matter was oxidized to CO_2, which was quantified by the infrared detector in the Dohrmann Chromatograph-190 TOC analyzer. Measured concentrations are reported throughout the present study.

Data analysis

Statistical analysis was conducted using R [28], utilizing the dose–response curve package [29,30]. Akaike's information criteria were used to select the most appropriate dose–response curve model function, including logistic, log-logistic, log-normal logistic, and Weibull (either 2- , 3- , or 4-parameter), of both an unaltered model as well as a reduced model in which dose–response curves of both the unamended and amended sediment (using the initial sediment concentrations) were set to have common upper and lower limits. The chosen unaltered model was compared statistically with the reduced model using analysis of variance (ANOVA). If no significant differences existed between the curves, the reduced model was chosen and used to derive the LC50 and/or EC50 values. In addition, as described by the USEPA [1], if when using an ANOVA any significant differences were noted between the treatments, a Dunnett's multiple comparison test was employed to compare the individual treatments with their respective baseline control sediment. For those treatments in which significant differences were

noted, further analysis was accomplished using Fisher's**<ZAQ;3>** least significant difference test to compare the unaltered sediment with the same sediment that was amended.

RESULTS

Water quality

Temperature (21 ± 1 °C) and dissolved oxygen (>60%) in overlying water was consistent in all TIE bioassays conducted. The pH in the overlying water for ammonia and copper TIEs was 7.0 ± 0.23 and 7.9 ± 0.09 for midges and amphipods, respectively. Not surprisingly, conductivity in the overlying water increased with increasing concentration for both ammonia (ranging from 962–5970 µS/cm and 1216–2289 µS/cm in *C. tepperi* and *A. subtenuis*, respectively) and copper (ranging from 292–893 µS/cm and 1371–1471 µS/cm in *C. tepperi* and *A. subtenuis*, respectively). Interestingly, the pH and conductivity of the overlying water in the ammonia TIE bioassays were slightly decreased with the addition of the amendment when compared with the unamended sediment (conductivity decreases of up to 10%; pH decreases of up to 0.28 units). The pH and conductivity of the overlying water in the copper TIE bioassays increased with the addition of the amendment (conductivity increases of up to 5%; pH increases of up to 0.66 units). These changes were more pronounced in testing with *C. tepperi* when compared with *A. subtenuis*, most likely caused by the high concentrations used in midge testing and the higher conductivity test water used in amphipod testing. These trends were not seen in nonpolar organics' TIE bioassays (conductivity: *C. tepperi* 198 ± 9.94 µS/cm; *A. subtenuis* 1528 ± 214 µS/cm and pH: *C. tepperi* 6.2 ± 0.15; *A. subtenuis* 7.86 ± 0.19).

TIE amendments alone

The addition of all 3 TIE amendments to control sediment showed little-to-no acute toxic effect to *C. tepperi* and *A. subtenuis* because survival was above or near 80% for both controls and amended controls (Figure 1). These control levels meet the specified control criteria for *C. tepperi* [31]. Control thresholds for *A. subtenuis* do not currently exist but are expected to be similar for those of the amphipod *H. azteca*, which are also 80% [22]. In the metals' TIE bioassays, average individual weights were similar for controls and amended controls for *C. tepperi* with weights of 0.75 ± 0.17 mg dry weight/organism and 0.82 ± 0.18 mg dry weight/organism, respectively. Growth, however, was significantly reduced by the addition of the activated charcoal amendment in TIEs for nonpolar organics (Oxpure 325B-9 Activated Carbon) because the individual weights of *C. tepperi* ranged from 1.0 ± 0.07 mg dry weight/organism without the amendment and 0.81 ± 0.17 mg dry weight/organism when the amendment was present. In ammonia TIE bioassays, average growth of *C. tepperi* was not reduced by the addition of the amendment; however, it was lower overall when compared with the other TIE bioassays for both treatments because controls in both unamended and amended sediments had individual weights of 0.36 ± 0.05 mg dry weight/organism and 0.45 ± 0.06 mg dry weight/organism, respectively.

TIE amendment capabilities: Effects-based response

The LC50s for both species and the EC50s (via weight) for midges, in both unamended and amended test sediments (using the initial sediment concentration), for all TIE bioassays as well as the estimated ratio of concentration effect doses (i.e., the amended EC50/unamended EC50) are shown in Table 1. The LC50 values for ammonia

using *C. tepperi* when amended with ANZ38 Zeolite were 1.54 times higher than those in unamended sediments. The amphipod *A. subtenuis* had a much larger ratio of concentration effects doses because the LC50 values in amended sediments were 3.92-fold higher than the LC50s for unamended sediments. The use of growth as an endpoint for *C. tepperi* in the proposed ammonia TIE methods was not satisfactory and will be discussed in further detail (see *Further refining the TIE methodology* and *Test species use* sections). The LC50 and EC50 (growth) values for copper using *C. tepperi* when amended with the Lewatit MonoPlus TP 207 were 1.70 and 3.15 times higher than unamended sediments. *Austrochiltonia subtenuis* showed an even greater difference because LC50 values of amended sediments were 5.22 times higher than LC50 values of unamended sediments. The LC50 and EC50 (growth) values for permethrin (in formulation) using *C. tepperi* when amended with the activated carbon were 4.52 and 3.02 times higher than for unamended sediments. Similar to the ammonia and copper results, *A. subtenuis* again showed a higher ratio of concentration effect dose because LC50 values for the amended sediments were 4.48 times higher than for unamended sediments.

TIE amendment capabilities: Concentration-based response

The ammonia amendment (ANZ38 Zeolite) significantly reduced total ammonia concentrations in the porewater of both *C. tepperi* and *A. subtenuis* TIE ammonia studies. The addition of the amendment reduced porewater total ammonia concentrations in spiked samples with *C. tepperi* and *A. subtenuis* by 41 to 80% and 74 to 86%, respectively (Figure 2). In addition, at the conclusion of the amphipod test, ammonia in the overlying water was also evaluated. In all 5 of the test treatments, reductions of total

ammonia in the overlying water when compared with the unamended sediment ranged from 64 to 99%. In fact, with the 3 lowest test concentrations amended with zeolite (21.7, 44.8, and 127 mg N/L), total ammonia concentrations in the overlying water were below the detection limit, which was not the case for the unamended sediment (overlying water concentrations of 2.1, 5.4, and 10.9 mg N/L, respectively). It should also be noted (as reported earlier<**ZAQ;4**>) that the conductivity and pH in test waters in ammonia TIE bioassays were slightly lower when zeolite was present, further suggesting that zeolite was removing the ammonia/ammonium ion.

The metals' amendment (Lewatit MonoPlus TP 207) reduced copper concentrations in sediment (which was sieved to remove the resin and adsorbed copper) for nearly all test concentrations for both *C. tepperi* and *A. subtenuis* with a reduction ranging from 1.2 to 32% and 6.8 to 38%, respectively—excluding 2 treatments in which slight increases were observed (356.5 mg/kg using *C. tepperi*, 2.7% increase and 916 mg/kg using *A. subtenuis*, 10.3% increase). Greater increases were noted in test sediments with higher test concentrations. The cause for seemingly no reduction (in the 2 sediments above<**ZAQ;5**>) as well as the lower than expected decreases overall, although surprising, can be best explained by a past study that evaluated the removal capabilities of ResinTech SIR-300 (the traditionally used metals' amendment in North America) over time. Burgess et al. [11] evaluated metal chemical concentrations in the overlying water during whole-sediment TIE bioassays and for many metals (including cadmium, lead, zinc, and nickel) an observed reduction of the metal concentration was nearly instantaneous at the start of the bioassay in the presence of the amendment. Copper behaved differently however because differences in the overlying water

concentrations were not noted until more than 24 h after the bioassay was initiated. Because we evaluated the amendment effects on chemical concentrations in the porewater before the bioassay even started (i.e., day –1), the observed reductions are most likely underestimated. To confirm, we evaluated the porewater concentrations of a single copper concentration (nominal concentration 250 mg/kg) at 4 time points (day –1, day 0, day 1, and day 5) in a separate bioassay. Results from this small analysis (Figure 3) showed that reductions of copper in the porewater were not noticeably lower until day 1 of the bioassay. The reduction in porewater copper concentrations in the present small pilot study at day 1 (83% reduction), using Lewatit MonoPlus TP 207, were similar to reports by Burgess et al. [11] that showed a copper porewater concentration reduction of 72% at 24 h. Similar to ammonia, the overlying water-quality data also provide further evidence that copper was being actively removed by the addition of the resin amendment <**ZAQ;6**>as increases in conductivity and pH were noted. The increases in these 2 parameters strengthen the notion of an overall chemical reduction because the copper would cause displacement of the calcium ion on the metal amendment bead causing increases in the conductivity and pH.

Unfortunately, quantifying the capacity of the nonpolar organics' amendment (activated carbon) to remove the nonpolar organics was more difficult than for the other 2 chemical classes. The activated carbon amendment itself could not be removed from the sediment (because it could be with the resin in the metals' TIE) and it was unknown to what extent the chemical extraction process would remove the permethrin from the activated carbon (concentrations for initial, unamended, and amended for samples<**ZAQ;7**> in the midge TIE bioassay showed a coefficient of variation of less

than 40%, suggesting that the extraction process may have at least partially removed permethrin from the activated carbon). An additional bulk sediment sample was prepared using permethrin at a concentration constituent with the midge LC50 (nominal concentration 150 µg/g organic carbon<**ZAQ;8**>) to evaluate whether the activated carbon was able to reduce the porewater concentrations. Although only a single amended and unamended sample were evaluated (as a result of the volume of sediment required for analysis), the analytical results confirmed that the activated carbon was reducing the concentration of this nonpolar organic (unamended 10 µg/L; amended with activated carbon 2.9 µg/L). Additional work using more porewater samples, passive sampling techniques (such as Tenax and/or solid-phase microextraction), or evaluating different nonpolar organics may provide more information to better understand the reduction capacity of the carbon.

False-positives/False-negatives

The possibility of false-positives has been well discussed [1,7,10,11] and, although unlikely, they are possible as amending materials such as those used in the present study, and may slightly reduce constituents other than the target contaminant class. For example, zeolite has an affinity for some cationic metals and polar organic toxicants, although its affinity for ammonia is much greater [1]. If the zeolite slightly improves the endpoint of interest in the TIE bioassay, so does the metal chelating resin; a weight-of-evidence analysis should be used to identify the source of toxicity. Thus conducting the TIE procedures for all contaminant classes is imperative and if the results are still inconclusive, additional TIE techniques and/or analytical chemistry may be warranted (i.e., addition of *Ulva lactuca* for further ammonia clarification).

False-negatives are also possible and were evident in the present study (8 of 28 toxic concentrations—those concentrations in which growth or survival were significantly different from control; see Figure 1). Three types of false-negatives were observed in the present study and can be classified as "too toxic," "not toxic enough," and "secondary effects caused by the amendment." All 3 of these false-negatives are discussed in the USEPA whole-sediment TIE guidance documents [1] and thus observing them in the present study was not surprising. In short, too toxic false-negatives occur when the sample has a high enough concentration that the amending material is overwhelmed and unable to reduce the toxicity in a significant manner (shown in 3 of the toxic concentrations in the present study; see 13 600 mg/kg copper using midge survival in Figure 1, as an example). Samples that are not toxic enough are those in which the amending material has reduced toxicity but cannot do so in a significant manner caused by the low degree of effect of the contaminant itself (found in 2 of the toxic concentrations; see 3315 mg N/L of porewater ammonia using midge survival in Figure 1, as an example). In all samples that were deemed not toxic enough, the amended sample was not significantly different from control, suggesting that although it was able to remove the toxicity it was just not able to do so significantly. The last false-negative that occurred was a result of the secondary effects caused by the amendment itself. This only occurred in the growth endpoint for *C. tepperi* in the nonpolar organics' TIE bioassay with the use of activated carbon (observed in 2 of the toxic concentrations; see 46.6 µg/g OC of permethrin [in formulation] using midge survival in Figure 1, as an example). This finding, however, is nothing new because the secondary effects of activated carbon and/or charcoal are well documented [1,10,32–34]. The use of 1% activated carbon in

control sediments had no acute effects or significant reduction in growth in preliminary testing; nevertheless, as shown in the results listed, it did significantly decrease growth in *C. tepperi* by 19% in control sediments. This statistically significant reduction, although seemingly small, could lead to false-negatives; thus it is important to have amendment controls in TIE bioassays to understand the potential impact of the amendment itself. The possibility of using an even lower percentage of carbon is possible; however, by lowering the amount of carbon used, the ability to remove the contaminant may become an issue and also lead to false-negatives (i.e., too toxic). Further work in this area, perhaps using different endpoints such as emergence, could resolve the secondary effect issues of activated carbon and should be investigated in the future.

Further refining the TIE methodology

The TIE techniques as implemented proved successful but various limitations to the methods still exist—the aforementioned issue caused by the secondary effects of the activated carbon and issues evaluating growth for ammonia were also problematic. The proposed ammonia TIE method (i.e., 5-d static testing; no feeding during testing) appeared to limit the growth of *C. tepperi*. Substantial differences in the weight of control organisms of the ammonia static bioassays (0.36 ± 0.05 mg per organism) were observed when compared with control organisms from either the metals' (0.75 ± 0.17 mg dry wt/organism mg per organism) or nonpolar organic TIE bioassays (1.0 ± 0.07 mg per organism) that utilized water renewals and feeding. The difference between the metals' and nonpolar organic TIE is somewhat surprising because the same static renewal and feeding schedule were used. But the disparity may be accounted for by use of a different clutch of organisms and a different batch of control sediment because testing was not run

simultaneously. Although water quality met the necessary parameters for testing in ammonia bioassays, overlying water was more turbid than usual and this, coupled with no feeding (which was done to avoid lowering dissolved oxygen even further), most likely led to the reduced growth. To resolve these issues, water changes and feeding could be conducted because they have been shown to be necessary in some sediment bioassays [35]. However, if this approach were taken, the required concentration of a water-soluble chemical, such as ammonia, would need to be much higher to observe effects. Although the growth-TIE method for *C. tepperi* was unsuccessful, what was elucidated from the ammonia TIE bioassay was the overall lack of sensitivity of this organism to ammonia and that the use of this organism may not be suitable for assessing ammonia risk. If water changes were to take place, this lack of sensitivity would become even more apparent. The lack of sensitivity of this organism to ammonia makes this species rather impractical for assessing risk of ammonia in freshwater.

Test species use

Differences in life cycles and test methods make it difficult to compare the work in the present study with published literature values for the more traditionally used Northern Hemisphere species of midge (*C. dilutus* and *Chironomus riparius*) and amphipod (*H. azteca*); however, both Australian species appear to have relatively similar sensitivities to the northern species. For instance, available copper 10-d LC50 values for *C. dilutus* and *C. riparius* ranged between 487.3 and 2296 mg/kg dry weight (*C. dilutus* [36,37], *C. riparius* [38]), with 10-d EC50 values (using dry wt) at approximately 210.3 mg/kg (*C. riparius* [38]). *Chironomus tepperi*, even with the shorter 5-d test duration, had relatively similar sensitivities with copper LC50 and EC50 values of 5748 and 252.2

mg/kg, respectively. The copper 10-d LC50 values in the public literature for *H. azteca* ranged from 262 to 1078 mg/kg [36,37], which is slightly lower than 10-d LC50 values calculated for *A. subtenuis* (2064 mg/kg); but again these disparities may in part be caused by differences in test procedures (i.e., differences in test water and control sediment characteristics of the bioassay).

Perhaps more importantly, however, are comparing these 2 Australian species with one another and with what is environmentally relevant in freshwater ways of Australia. For all 3 contaminant classes, survival of *C. tepperi* was the least sensitive endpoint and showed survival sensitivities that are most likely not environmentally relevant (as based on concentrations reported in the greater Melbourne area [5,18,20,39,40]). The use of growth for *C. tepperi*, however, was a much more sensitive endpoint, more environmentally relevant, and is still an easy and time-efficient addition to the bioassay procedure. To our knowledge, the present study is one of the first studies to use an Australian freshwater amphipod species for sediment toxicity testing purposes. Although culturing and baseline bioassays' procedures for the studied amphipod species *A. subtenuis* are still in their infancy (as evident in the observed higher variability than for *C. tepperi*), the strong TIE response (as evident in the high estimated ratio of concentration effects dose in Table 1) and higher sensitivity of this species (when compared with *C. tepperi*), as well as occupying a unique role in benthic ecosystems similar to *H. azteca* (i.e., epibenthic shredder), make it a strong candidate for future use in bioassays, especially freshwater sediment TIEs.

CONCLUSIONS

The results presented in the present study build on the current TIE-technique literature and provide Australia with the necessary foundation to conduct sediment-based TIEs using native species. Although limitations to the method still exist, by using the TIE technique as part of a weight-of-evidence approach many of the issues are of less concern. Perhaps, not surprisingly, Australia is not unique in lacking foundational whole-sediment procedures because other countries outside of North America would be in a similar situation. The following work<**ZAQ;9**> not only shows how countries can develop country-specific TIE procedures but also the importance of identifying and understanding the relative sensitivities of 2 easily cultured native test species and how to ensure that these organisms effectively work in TIE-based procedures. It is hoped that these baseline methods will not only ensure more successful implementation of TIEs in future risk assessments as well as increase the overall use of this technique being employed in Australia, but also that similar methods (acquiring local-sourced amendments, determining functionality of amendments, and ascertaining suitability of local test species) can be employed for the use of TIEs in other countries as well.

Acknowledgment—We thank S. Ware and the rest of the Filchem team for providing assistance as well as generously donating samples of Oxpure 325B-9 Activated Carbon and Lewatit MonoPlus TP 207. In addition, we express similar gratitude to G. Heath and the team members at Castle Mountain Zeolites for donation as well as advice when working with ANZ38 Zeolite. The present study was partially funded by the Holsworth Wildlife Research Endowment<**ZAQ;10**>.

Data Availability—Data, associated metadata, and calculation tools are available from the corresponding author (wmehler@student.unimelb.edu.au).

References

1. US Environmental Protection Agency. 2007. Sediment toxicity identification evaluation (TIE) phases I, II, and III guidance document. EPA/600/R-07/080. Washington, DC.

2. Golding C, Krassoi R, Baker E. 2006. The development and application of a marine toxicity identification evaluation (TIE) protocol for use with an Australian bivalve. *Ecotoxicology* 12:37–44.

3. Hogan AC, Stauber JL, Pablo F, Adams MS, Lim RP. 2005. The development of marine toxicity identification evaluation (TIE) procedures using the unicellular alga *Nitzschia closterium*. *Arch Environ Contam Toxicol* 48:433–443.

4. Strom D, Ralph PJ, Stauber JL. 2009. Development of a toxicity identification evaluation protocol using chlorophyll-a fluorescence in a marine microalga. *Arch Environ Contam Toxicol* 56:30–38.

5. Kellar CR, Hassell KL, Long SM, Myers JH, Golding L, Rose G, Kumar A, Hoffmann AA, Pettigrove V. 2014. Ecological evidence links adverse biological effects to pesticide and metal contamination in an urban Australian watershed. *J Appl Ecol* 51:426–439.

6. Burgess RM, Pelletier MC, Ho KT, Serbst JR, Ryba SA, Kuhn A, Perron MM, Raczelowski P, Cantwell MG. 2003. Removal of ammonia toxicity in marine sediment TIEs: A comparison of *Ulva lactuca*, zeolite and aeration methods. *Mar Pollut Bull* 46:607–618.

7. Besser JM, Ingersoll CG, Leonard EN, Mount DR. 1998. Effect of zeolite on toxicity of ammonia in freshwater sediments: Implications for toxicity identification evaluation procedures. *Environ Toxicol Chem* 17:2310–2317.

8. Mehler WT, Maul JD, You J, Lydy MJ. 2010. Identifying the causes of sediment-associated contamination in the Illinois River (USA) using a whole-sediment toxicity identification evaluation. *Environ Toxicol Chem* 29:158–167.

9. Mehler WT, You J, Maul JD, Lydy MJ. 2010. Comparative analysis of whole sediment and porewater toxicity identification evaluation techniques for ammonia and non-polar organic contaminants. *Chemosphere* 78:814–821.

10. Ho KT, Burgess RM, Pelletier MC, Serbst JR, Cook H, Cantwell MG, Ryba SA, Perron MM, Lebo J, Huckins J, Petty J. 2004. Use of powdered coconut charcoal as a toxicity identification and evaluation manipulation for organic toxicants in marine sediments. *Environ Toxicol Chem* 23:2124–2131.

11. Burgess RM, Cantwell MG, Pelletier MC, Ho KT, Serbst JR, Cook HF, Kuhn A. 2000. Development of a toxicity identification evaluation procedure for characterizing metal toxicity in marine sediments. *Environ Toxicol Chem* 19:982–991.

12. Phyu YL. 2005. The toxicity and bioavailability of atrazine and molinate to *Chironomus tepperi* larvae in laboratory and river water in the presence and absence of sediment. *Chemosphere* 58:1231–1239.

13. Kefford BJ, Salter J, Clay C, Dunlop J, Nugegoda D. 2007. Freshwater invertebrates' response to gradients of salinity and turbidity: Using as a rapid sub-lethal test. *Australas J Ecotoxicol* 13:131–142.

14. Ahsanullah M, Florence T. 1984. Toxicity of copper to the marine amphipod *Allorchestes compressa* in the presence of water- and lipid-soluble ligands. *Mar Biol* 84:41–45.

15. Vu HT, Keough MJ, Long SM, Pettigrove VJ. 2016. Effects of two commonly used

fungicides on the amphipod *Austrochiltonia subtenuis*. *Environ Toxicol Chem* 36:720–726.

16. Martin J, Kuvangkadilok C, Peart DH, Lee BTO. 1980. Multiple sex determining regions in a group of related *Chironomus* species (Diptera: Chironomidae). *Heredity* (*Edinb*) 44:367–382.

17. Jeppe KJ, Carew ME, Long SM, Lee SF, Pettigrove V, Hoffmann AA. 2014. Genes involved in cysteine metabolism of *Chironomus tepperi* are regulated differently by copper and by cadmium. *Comp Biochem Physiol C Toxicol Pharmacol* 162:1–6.

18. Pettigrove V, Hoffmann A. 2005. A field-based microcosm method to assess the effects of polluted urban stream sediments on aquatic macroinvertebrates. *Environ Toxicol Chem* 24:170–180.

19. Hale R, Marshall S, Jeppe K, Pettigrove V. 2014. Separating the effects of water physicochemistry and sediment contamination on *Chironomus tepperi* (Skuse) survival, growth and development: A boosted regression tree approach. *Aquat Toxicol* 152:66–73.

20. Marshall S, Pettigrove V, Carew M, Hoffmann A. 2010. Isolating the impact of sediment toxicity in urban streams. *Environ Pollut* 158:1716–1725.

21. Besser JM, Brumbaugh WG, Kemble NE, Ivey CD, Kunz JL, Ingersoll CG, Rudel D. 2011. Toxicity of nickel-spiked freshwater sediments to benthic invertebrates—Spiking methodology, species sensitivity, and nickel bioavailability. US Department of the Interior, Washington, DC.

22. US Environmental Protection Agency. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates. EPA/600/R-94/024. Washington, DC.

23. Besser JM, Ingersoll CG, Leonard EN, Mount DR. 1998. Effect of zeolite on toxicity of ammonia in freshwater sediments: Implications for toxicity identification evaluation procedures. *Environ Toxicol Chem* 17:2310–2317.

24. Schuler LJ, Landrum PF, Lydy MJ. 2007. Response spectrum of pentachlorobenzene and fluoranthene for *Chironomus tentans* and *Hyalella azteca*. *Environ Toxicol Chem* 26:1248–1257.

25. American Public Health Association. 1995. *Standard Methods for the Examination of Water and Wastewater*, 19th ed. Washington, DC.

26. US Environmental Protection Agency. 1994. Determination of metals and trace elements in water and wastes by inductively coupled plasma-atomic emission spectrometry. Method 200.7. Revision 4. Washington, DC.

27. US Environmental Protection Agency. 1986. Test methods for evaluating solid waste: Physical/chemical methods. SW-846. US Document 955-001-00000. Washington, DC.

28. R Development Core Team. 2009. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

29. Ritz C, Streibig J. 2005. Bioassay analysis using R. *J Stat Softw* 12:1–22.

30. Ritz C, Cedergreen N, Jensen JE, Streibig JC. 2006. Relative potency in nonsimilar dose–response curves. *Weed Sci* 54:407–412.

31. Simpson SL, Batley GE. 2016. *Sediment Quality Assessment: A Practical Guide*, 2nd ed. CSIRO, Clayton South, Victoria, Australia.**<ZAQ;11>**

32. Kupryianchyk D, Reichman EP, Rakowska MI, Peeters ET, Grotenhuis JT, Koelmans AA. 2011. Ecotoxicological effects of activated carbon amendments on

macroinvertebrates in nonpolluted and polluted sediments. *Environ Sci Technol* 45:8567–8574.

33. Nybom I, Abel S, Waissi G, Väänänen K, Mäenpää K, Leppänen MT, Kukkonen JVK, Akkanen J. 2016. Effects of activated carbon on PCB bioaccumulation and biological responses of *Chironomus riparius* in full life cycle test. *Environ Sci Technol*, in press. DOI: 10.1021/acs.est.6b00991.

34. Janssen EML, Beckingham BA. 2013. Biological responses to activated carbon amendments in sediment remediation. *Environ Sci Technol* 47:7595–7607.

35. Ankley GT, Benoit DA, Hoke RA, Leonard EN, West CW, Phipps GL, Mattson VR, Anderson LA. 1993. Development and evaluation of test methods for benthic invertebrates and sediments: Effects of flow rate and feeding on water quality and exposure conditions. *Arch Environ Contam Toxicol* 25:627–635.

36. Cairns MA, Nebeker AV, Gakstatter JH, Griffis WL. 1984. Toxicity of copper-spiked sediments to freshwater invertebrates. *Environ Toxicol Chem* 3:435–445.

37. Suedel BC, Deaver E, Rodgers JH. 1996. Experimental factors that may affect toxicity of aqueous and sediment-bound copper to freshwater organisms. *Arch Environ Contam Toxicol* 30:40–46.

38. de Haas EM, Léon Paumen M, Koelmans AA, Kraak MHS. 2004. Combined effects of copper and food on the midge *Chironomus riparius* in whole-sediment bioassays. *Environ Pollut* 127:99–107.

39. O'Brien ML, Pettigrove V, Carew ME, Hoffmann AA. 2010. Combining rapid bioassessment and field-based microcosms for identifying impacts in an urban river. *Environ Toxicol Chem* 29:1773–1780.

40. Carew ME, Pettigrove V, Cox RL, Hoffmann AA. 2007. The response of *Chironomidae* to sediment pollution and other environmental characteristics in urban wetlands. *Freshw Biol* 52:2444–2462.

Figure 1. Results show the acute toxic effects for *Chironomus tepperi* (survival and growth) and *Austrochiltonia subtenuis* (survival) in the presence of ammonia (mg N/L), copper (mg/kg), and permethrin (µg/g organic **<ZAQ;12>**carbon). The single asterisk indicates that survival or growth of unamended sediment was significantly decreased when compared with the control ($p < 0.05$). The # sign used as a unit of weight identifies a significant increase of survival or growth of the species when the amended was added. NA = not available.

Figure 2. Ammonia concentrations (mg N/L) of amended (with zeolite) and unamended samples in bioassays with both *Chironomus tepperi* and *Austrochiltonia subtenuis* when compared with the initial concentration of the sediment.

Figure 3. Comparing the amended (with Lewatit MonoPlus TP 207; Lanxess Deutschland) and unamended porewater concentrations (for a single copper concentration, nominal concentration 250 mg/kg) over time (in days).

<<ENOTE>>AQ1: Edit OK? Changed from “iminodiacetate”

<<ENOTE>>AQ2: Correct edit? Day –3 or is order for (day 3) and (day 1)?

<<ENOTE>>AQ3: Fisher's least significant difference test?

<<ENOTE>>AQ4: If “earlier” refers to a section other than *TIE amendment capabilities: Concentration-based response* please add the section heading.

<<ENOTE>>AQ5: Please clarify which sediments not using “above”

<<ENOTE>>AQ6: Here, does as mean at the same time? If not, please change as to because.

<<ENOTE>>AQ7: amended samples?

<<ENOTE>>AQ8: Is organic carbon the correct expansion of OC?

<<ENOTE>>AQ9: Do mean work, research that will follow the present study?

<<ENOTE>>AQ10: Please provide location in Australia.

<<ENOTE>>AQ11: Reference 31: Please check completed reference

<<ENOTE>>AQ12: [0]Is organic carbon the correct expansion of OC?

<<ENOTE>>AQ13: Is organic carbon the correct expansion of oc?

Table 1. Effect concentrations (for *Chironomus tepperi*[a] and *Austrochiltonia subtenuis*[b] as well as the estimated ratio of effects dose between the unamended and amended sediment[c] for 3 contaminant classes[d,e]

Contaminant	Endpoint	EC50 (± SE)		Estimated ratio of concentration effects dose (± SE)
		Unamended	Amended	
Ammonia (mg N/L)	*C. tepperi*—survival	3849 (75.96)	5909 (144.1)	1.54 (0.0469)*
	C. tepperi—growth	NA	NA	NA
	A. subtenuis—survival	141.4 (16.47)	554.9 (35.05)	3.92 (0.529)*
Copper (mg/kg)	*C. tepperi*—survival	5748 (128.2)	9776 (189.2)	1.70 (0.0497)*
	C. tepperi—growth	252.2 (72.1)	795.2 (67.0)	3.15 (0.838)*
	A. subtenuis—survival	2064 (353.7)	10 794 (2252)	5.23 (1.42)*
Permethrin[f]	*C. tepperi*—survival	411.9 (57.53)	1861 (396.2)	4.52 (1.07)*

(µg/g organic carbon)<**ZAQ;13**>	*C. tepperi*—growth[g]	139.7 (14.17)	422.64 (142.0)	3.02 (1.06)*
	A. subtenuis—survival	205.55 (8.157)	920.54 (34.78)	4.48 (0.229)*

[a] Using survival and growth.

[bc] Using the initial (i.e., before amendment) sediment concentration.

[d] Ammonia, cationic metals, and nonpolar organics.

U[e] Standard error and estimated ratio of effects dose were calculated using R utilizing dose–response curve methods [29,30]. Estimated ratio of effect doses is equal to amended EC50/unamended EC50.

s[f] The values for permethrin are measured concentrations in sediment; however, it should be noted that other constituents (namely hydrocarbons) may have been present in the

sediment because a commercial formulation (Brunnings Ant, Spider & Cockroach Killer) containing permethrin was used.

i[g] Curves could not be confined to have the same lower and upper limits because of significant differences in control response with the addition of the carbon amendment. In addition, control concentrations were below the detection limit; thus one-half of the detection limit (DL = 0.1 mg/kg) was used as the control value for computing EC50 values.

n*The difference in EC50 values between amended and unamended is significant at $p < 0.05$.

gEC50 = median effect concentration; SE = standard error; NA = not available.

survival.