ESTABLISHING, IMPROVING, AND ADAPTING THE WHOLE-SEDIMENT TOXICITY IDENTIFICATION EVALUATION (TIE) FOR USE IN AUSTRALIA

Submitted by

Wesley Tyler Mehler

ORCID#: 0000-0002-6631-3697

Submitted in fulfillment of the degree of Doctor in Philosophy

February 2018
School of Biosciences
Faculty of Science
The University of Melbourne

ABSTRACT

Sediment risk assessments have commonly employed what is known as the 'conventional' method to address risk and determine causality of toxic sediments. The 'conventional' method compares effects (determined through bioassays) to exposure data (via analytical evaluations of the contaminated sediment). However, this approach has numerous limitations that make the use of this method in many circumstances unreliable (including biasing classification towards priority pollutants and a lack of understanding of issues such as bioavailability and mixtures). In response to these limitations, researchers developed the 'toxicity-based' method, which uses the response of organisms to identify causal links. Whole-sediment toxicity identification evaluations (TIEs) are one such, using physical and/or chemical manipulations of the sediment to enhance or decrease the toxicity of a given chemical or chemical class. If the manipulation affects the toxicity of the media this confirms that the toxicant, which was being manipulated, is causing the given effects. This tool is still in its relative infancy, as guidance only became available in 2007 in the United States. To date, this tool has yet to be effectively developed or implemented in Australia. This dissertation provides the foundation for future whole-sediment TIE work in Australia. Additionally, this research expands on past work to make the technique more effective, and adapts it for various types of sediment contamination, such as mining sites. This research complements Northern Hemisphere whole-sediment TIE work, while providing additional techniques and modifications that will assist in making the use of the whole-sediment TIE method more user-friendly, cost-effective, and practical.

DECLARATION

This is to certify that:

i.   The thesis comprises only my original work towards the PhD

ii.   Due acknowledgement has been made in the text to all other material used

iii.   The thesis is less than 100,000 words in length, excluding tables, maps, bibliographies, and appendices

W. Tyler Mehler

February 2017

PREFACE

This thesis comprises four scientific papers (Chapters 2 – 4).

Chapter 2

W. Tyler Mehler, Jing You, Michael J. Keough, Michael J. Lydy, and Vincent Pettigrove. 2018. Improvements and cost-effective measures to the automated intermittent water renewal system for toxicity testing with sediments. *Ecotoxicology and Environmental Safety* 151: 62-67.

The majority of research in this chapter is my own work. Other co-authors provided scientific advice, training in laboratory and data analysis techniques, and review of the manuscript before submission.

Chapter 3

W. Tyler Mehler, Michael J. Keough, and Vincent Pettigrove. 2017. Development of whole-sediment toxicity identification evaluation (TIE) techniques for two Australian freshwater species: *Chironomus tepperi* and *Austrochiltonia subtenius. Environmental Toxicology and Chemistry* 36: 2476-2484.

The majority of research in this chapter is my own work. Other co-authors provided scientific advice and review of the manuscript before submission.

Chapter 4

W. Tyler Mehler, Michael J. Keough, and Vincent Pettigrove. 2018. Resolving the false-negative issues of the non-polar organic amendment in whole-sediment toxicity identification evaluations (TIEs). *Environmental Toxicology and Chemistry* (issue and page number not yet issued).

The majority of research in this chapter is my own work. Other co-authors provided scientific advice and review the manuscript before submission.

Chapter 5

W. Tyler Mehler, Bryant Gagliardi, Michael J. Keough, and Vincent Pettigrove (in preparation). The role of acidic pH in freshwater tasmanian mining sediment toxicity.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

There is growing concern regarding the environmental health of both freshwater and marine ecosystems worldwide. Although much of the concern for the deterioration of these systems is derived from human-health implications, the importance of these systems to other necessary environmental functions (such as nutrient cycling, climate regulation, and production of oxygen) cannot be overstated [1]. One of the potential causes of this increase in degradation of aquatic systems (in which there are many) is the increasing level of aquatic pollution [2]. As aquatic systems become subjected to increased levels of contamination, one of the most susceptible habitats in these systems is the benthic zone, the environment at, in, and or near the bottom of the water body. Detritus and other decaying matter of these aquatic systems typically settle into these benthic zones. The settling matter, collectively referred to as sediments, is generally rich in organic matter. This property of sediments coupled with the high hydrophobicity of many chemicals results in these benthic habitats (and more specifically sediments) tending to become "environmental sinks" for aquatic pollution [3]. Unfortunately, organisms in the benthic habitat (i.e. benthos) are then at risk to being exposed to increased levels of contamination. The benthos plays a major role in aquatic systems in regulating the flow of energy and materials in aquatic systems, so understanding the implications of aquatic pollution to these organisms is of the utmost importance [4–8] and methods to better understand that risk are the focus of this dissertation.

1.1. Risk Assessment Methodologies

Aquatic risk assessment frameworks can employ a variety of approaches to characterize risk of aquatic contaminants [9,10]. Risk from aquatic contaminants is the likelihood (i.e. exposure) of a contamiant causing an adverse effect ot an individual, population, or community. Many of the traditional approaches or "tools" generally provide insight into the characterization of effects or exposure, with relatively few tools doing both [11]. Perhaps one of the commonest approaches is the "conventional" approach in which a 'toxic' medium – determined via bioassay(s) – is analyzed for priority or suspected pollutants. The concentrations of each of the pollutants is then compared to available published literature values for toxicity or water quality criteria [9,12,13]. Although commonly used, the "conventional" based approach has major limitations. This approach tends to rely on professional judgment to understand how chemicals interact with one another (i.e. multiple stressors) and how they interact with the environment (i.e. bioavailability), these relationships are difficult to interpret as most guidelines are based on single toxicant bioassays in standard

laboratory settings. Therefore, accurately characterizing risk at these sites using the "conventional" approach is not only difficult and costly, but also considerably flawed [12]. To resolve many of the drawbacks to the approach, the toxicity-based approach was developed, which uses the response of the organism to determine the presence of a toxicant or toxicant class.

Table 1: Ecological Risk Assessment (ERA) Techniques*

Evauation Characteristic	Chemistry Evaluation	Toxicity Bioassays	Field Surveys	Microcosms	*In situ* Assays	TIE Bioassays
Evaluates Exposure	+	-	-	-	-	+
Evaluates Effects	-	+	+	+	+	+
Environmentally Relevant	+/-	-	+	+	+	-
Causality	-	+/-	-	+/-	+/-	+
Bioavaiability	-	+	-	+	+	+
Controlled Variability	+	+	-	-	-	+
Widely Used	+	+	+	-	-	-
Standardized	+	+	+	-	-	+
Effects Assessment Level	Not Applicable**	Single Species	Community	Community	Single Species	Single Species

TIE- Toxicity Identification Evaluation
* Adapted from Burton *et al.* 2005 [11]. Bioaccumulation is not included in the above table although it is an endpoint that can be used as part of an ERA. Data for bioaccumulation is considered an exposure endpoint. Using this data to evaluate exposure and effects is difficult due to a lack of published literature, however this data can be used for food web modeling which is a unique feature of this endpoint.
**Chemistry data itself does not quantify effects per se; data must be compared to available published literature to determine effects.

1.2. Toxicity Identification Evaluations (TIEs)

One toxicity-based technique is the toxicity identification evaluation (TIE), which combines toxicity testing and characterization of contamination into a single bioassay. TIEs are able to provides evidence as to which contaminant(s) are causing the effects in the testing media through experimental means, and as such eliminate a large degree of the professional judgment that is needed in the "conventional" based approach. The TIE technique uses physical and/or chemical manipulations to enhance or decrease the toxicity of a given chemical or chemical class in a medium (whether that be using effluent, pore water, or sediment as the media) [14]. If the manipulation affects the toxicity of the media this provides evidence that the toxicant, which was being manipulated, is causing the given effects. For instance, a toxic medium could be divided into two

samples. One of these samples is not manipulated and is tested to show the baseline effect, while the other sample is manipulated in a manner that will reduce the effects of non-polar organics (e.g. such as being subjected to activated carbon). If the manipulation causes no changes in toxicity of the sample it can be inferred that non-polar organics are not the cause of the toxicity in that sample, but if the manipulation alters the toxicity of the sample, non-polar organics are a source of toxicity in that sample [15–17].

These TIE approaches offer many advantages over the "conventional" based approach, especially in a contaminated media with a suite of different contaminants. A variety of chemicals, pharmaceuticals, metals, herbicides, pesticides, ammonia, oxidants, etc. can be found in aquatic systems, and our understanding how these chemicals interact with one another is extremely limited. This lack of understanding, becomes even more problematic as the probability of observing complex mixtures when addressing risk is one that continues to rise worldwide [18]. Addressing mixtures using the "conventional" approach is extremely difficult as traditionally, evaluations of chemical toxicity have only been on a single constituent (via spiked laboratory bioassays). Thus, determining the risk that each contaminant may play could easily be under- or over-estimated. As the TIE bioassay works with the whole sample to draw inferences, rather than the chemicals within the sample, it provides more systematic evidence of toxicity. As this approach continues to advance, not only will it provide researchers an accurate tool to define causality, but could address many of the questions that currently exist regarding mixture toxicity.

1.3. Expanding and improving the whole-sediment TIE

Aquatic risk assessments have used a variety of test media as part of bioassays to evaluate causality of stressors in aquatic ecosystems including: overlying water, effluent, pore water and sediments. Recent guidelines have been developed for the use of TIEs with sediments, which some have argued may provide a more environmentally relevant media for assessment purposes over that of overlying water, pore water, or effluent [14,19, 20]. Not only are sediments more environmental relevant, but aspects such as bioavailability alteration, sampling issues, volume requirements (for pore water and effluents), and other other artifacts are commonly present in non-sediment bioassays. Toxicity identification evaluation techniques with sediments have been developed for various classes of contaminants, such as ammonia [21,22], non-polar organics [15], and cationic metals [23], with additional techniques being developed for individual groups of contaminants such as pyrethroids [24–27]. As sediment TIEs are still a fairly new concept, the integration of these techniques into common aquatic risk assessment applications has been quite

limited, most likely due to cost, resource availability and lack of standardization. With that being said, the use of these techniques as part of a weight of evidence approach has been promising in linking exposure to effects in many watersheds of the United States [28–31]. This PhD thesis will aim to further improve the sediment TIE process for more accurate and cost-effective evaluations of sediment risk by addressing some of the limitations as discussed below.

1.3.1. Managing laboratory and resource utilization for TIE purposes

One of the difficulties with employing sediment-bioassays in many laboratories (and specifically for TIEs) is the large amount of time and effort that is needed to conduct water changes to control confounding factors that are associated with the water quality of bioassays. In many laboratories, sediment bioassays are performed in incubators with parameters such as dissolved oxygen, pH, conductivity, ammonia, light intensity, and temperature monitored regularly. Studies typically employ static (no water changes) or static renewal (infrequent changes throughout the assay – typically once every day or two days) due to the amount of time required to do so manually [1]. Automated systems have been developed for bioassay use in the past, which allow for more frequent water changes that are also more precise and less disruptive, but also save time and money [32–34]. Expanding on these previous plans and ensuring that they can be used with Australian test species is the first step in ensuring that TIE bioassays can be conducted more effectively in both a practical as well as an economic sense in Australia.

1.3.2. Lack of country specific standardized sediment TIE processes

In Australia specifically, the use of TIE techniques has been limited, with a majority of this work revolving around using these techniques in water, such as effluent or marine waters [35–39], with few applications in freshwater. Many of the principles and basic concepts of sediment TIEs are appropriate regardless of location, but the test species and materials used to derive these concepts may not be appropriate (for example the use of non-native species) or available to use in an area outside the United States. Most Australian guidelines discuss the use of these techniques in a general sense but do not require or provide information into how to conduct such research [9,13,40]. The two major hurdles in Australia for building foundational freshwater sediment TIE methods are: (1) identifying native test organisms that are appropriate for use in TIE testing (2) identifying suitable and easily accessible manipulation or amending materials (known as "TIE amendments"). By building these foundational freshwater TIE methods, Australian researchers will have a means to identify causes of aquatic degradation as part of freshwater aquatic risk

assessments, and the results would also add to the growing base of literature for using Australian native freshwater species in sediment bioassays in general.

1.3.3. Secondary TIE amendment effects and lack of TIE method refinement

Outside of developing TIE methods for Australia specifically, it is also important to understand the limitations for TIEs on a global scale, and one possible limitation is that the TIE amendments may themselves have effects. Although the secondary effects of activated carbon (AC) to many different species have been well studied [17,41,42], especially regarding remediation activities, the implications of these secondary effects on whole-sediment TIEs are less well known. These secondary effects may mask toxicity reductions caused by the carbon amendment and thus produce a 'false-negative', suggesting that non-polar organics are not an issue. To remedy this issue, most whole-sediment TIEs employ an amendment control to better understand the implication of possible secondary effects caused by the use of AC. Although this amendment control assists in rectifying secondary effect issues, it does not completely remedy the issue as potential for false-negatives and false-postives still exist.

1.3.4. Adapting whole-sediment TIEs

Traditional whole-sediment TIE techniques are currently employed in complex sites wherein the nature of toxicity is unknown, or there is a mixture of contaminants, typically ammonia, non-polar organics, and cationic metals. However, various other contaminants exist in the environment and in many scenarios evaluating all three contaminant classes is not necessary or pertinent. For instance, two of these contaminant classes, ammonia and non-polar organics, are generally not perceived to cause aquatic degradation at mining sites. As such, traditional sediment TIEs have only been used in a limited capacity to assess risk in these areas. To our knowledge, whole-sediment TIE techniques have not been adapted to aquatic waterways impacted by mining activities even though detrimental impacts could be caused by a complex mixture of chemicals including: cationic (metals) and anionic (sulfate) constituents, as well as acidity. Adapting TIE methods for assessing risk associated with mining contaminants would better clarify the relative contributions of these contaminants in mining areas and help develop better pollution reduction and remediation strategies. The TIE approach stands to improve our understanding of these issues, especially for those constituents whose contribution to mining contamination may be underappreciated (such as sulfate and acidity).

1.4. Thesis aims and overview

This thesis aimed to further enhance and improve the sediment TIE process by addressing the limitations identified above. In doing so, there were four aims:

- Improve the time and cost efficiency of whole-sediment TIEs,
- Develop whole-sediment TIE procedures for use specifically in Australia,
- Further refine existing whole-sediment TIE procedures to better improve the characterization of contaminants, and
- Adapt whole-sediment TIEs beyond their traditional contaminant class use

This thesis includes four experimental chapters and a discussion chapter, as outlined below.

Chapter 1: Introduction

Aim: Literature review of current knowledge on the state of whole-sediment TIEs and identify gaps in the literature.

Chapter 2: Improvements and cost-effective measures to the automated intermittent water renewal system for toxicity testing with sediments.

Aim: The first aim was to establish an automated system for conducting whole-sediment TIEs, which would eliminate much of the time associated with manual water changes while improving the water quality parameters. After the systems were built, bioassays were conducted in the system using *Chironomus tepperi* (midge) to ensure that the system provided suitable water change capacity when compared to manual static and static-renewal methods by evaluating mortality, growth, development rate, and emergence.

Chapter 3: Development of whole-sediment toxicity identification and evaluation (TIE) techniques for two Australian freshwater species: the larvae of the non-biting midge *Chironomus tepperi* and the crustacean *Austrochiltonia subtenuis.*

Aim: In this chapter, foundational methods for performing whole-sediment TIEs in Australia were developed. TIE methods were developed for three classes of contaminants including ammonia, non-

polar organics, and cationic metals. This method development involved identifying suitable and readily available amendments as well as evaluating these amendments on sediments spiked with the three contaminant classes and assessment with two native to Australia freshwater species *C. tepperi* and *A. subtenuis*.

Chapter 4: Improving the sensitivity for non-polar organics characterization in whole-sediment toxicity identification evaluation (TIE) bioassays with *Chironomus* while resolving the secondary effects of activated carbon.

Aim: A common issue with whole-sediment TIEs are the secondary effects associated with the TIE amendment for non-polar organic characterization, activated carbon (AC). This chapter aimed to reduce the likelihood of false-negatives of the *C. tepperi* TIE bioassay, while adding additional evidence to assess the adverse effects of sediment-bound contaminants to aquatic benthos. To do this, the present study (1) evaluated the use of AC in two different 'control' sediments to determine the possibility of secondary effects of AC, (2) evaluated the possible use of multiple amendment ratios to better understand the secondary effects of AC while also providing further evidence to characterize sediment toxicity, and (3) trialed the use of additional chronic endpoints (emergence and mean development rate). These objectives were evaluated using both spiked sediments (using two commercial insecticide formulations) as well as contaminated field sediments from Victoria, Australia.

Chapter 5: The role of acid sulfate sediments on metal toxicity in Tasmanian mining sediments

Aim: In this chapter, sediment toxicity in western Tasmania mining areas was investigated using modified whole-sediment TIE techniques developed specifically for mining. Rather than using traditional amendments (such as activated carbon for non-polar organics or zeolite for ammonia), cationic resins and an anion resin (for sulfate/acidity) were employed to better understand the toxic nature of mining sediments and provide evidences to the roles that both metals and acidity (via sulfate reduction, a mining contaminant whose role in sediment toxicity is poorly understood) play in these types of sites.

Chapter 6: Conclusions

Aim: Provide an overview of the main findings from this dissertation and recommend further research.

1.5. References

1. Rand G. 1995. *Fundamentals of aquatic toxicology: effects, environmental fate and risk assessment.*

2. Schwarzenbach RP, Egli T, Hofstetter TB, von Gunten U, Wehrli B. 2010. Global Water Pollution and Human Health. *Annu. Rev. Environ. Resour.* 35:109–136.

3. Chapman PM, Mann GS. 1999. Sediment Quality Values (SQVs) and Ecological Risk Assessment (ERA). *Mar. Pollut. Bull.* 38:339–344.

4. Carlisle DM, Clements WH. 2005. Leaf litter breakdown, microbial respiration and shredder production in metal-polluted streams. *Freshw. Biol.* 50:380–390.

5. Burton GA, Johnston EL. 2010. Assessing contaminated sediments in the context of multiple stressors. *Environ. Toxicol. Chem.* 29:2625–43.

6. Holomuzki JR, Feminella JW, Power ME. 2010. Biotic interactions in freshwater benthic habitats. *J. North Am. Benthol. Soc.* 29:220–244.

7. Lamberti G a., Chaloner DT, Hershey AE. 2010. Linkages among aquatic ecosystems. *J. North Am. Benthol. Soc.* 29:245–263.

8. Townsend CR, Uhlmann SS, Matthaei CD. 2008. Individual and combined responses of stream ecosystems to multiple stressors.:1810–1819. doi:10.1111/j.1365-2664.2007.0.

9. EPA Victoria. 2004. *Guideline for Environmental Management: Risk-Based Assessment of Ecosystem Protection in Ambient Waters. Publ. 961.*

10. United States Environmental Protection Agency. 1998. Guidelines for Ecological Risk Assessment. *EPA/630/R-95/002F.* 63:26846–26924.

11. Burton GA, Greenberg MS, Rowland CD, Irvine CA, Lavoie DR, Brooker JA, Moore L, Raymer DFN, McWilliam RA. 2005. In situ exposures using caged organisms: a multi-compartment approach to detect aquatic toxicity and bioaccumulation. *Environ. Pollut.* 134:133–44.

12. United States Environmental Protection Agency. 1991. *Methods for Aquatic Toxicity Identification Evaluations; Phase I Toxicity Characterization Procedures. EPA/600/6-91/003.* Washington, DC.

13. Australia and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand. 2000. *Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Volume 1, The Guidelines.* 1.

14. United States Environmental Protection Agency. 2007. *Sediment toxicity identification evaluation (TIE) Phases I, II, and III Guidance Document. EPA/600/R-07/080.* Washington, DC.

15. 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.

16. McKay G, Bino M, Altamemi A. 1985. The adsorption of various pollutants from aqueous solutions on to activated carbon. *Water Res.* 19:491–495.

17. Kupryianchyk D, Reichman E, Rakowska M, Peeters E, Grotenhuis J, Koelmans A. 2011. Ecotoxicological effects of activated carbon amendments on macroinvertebrates in nonpolluted and polluted sediments. *Environ. Sci. Technol.* 45:8567–74.

18. Schwarzenbach R, Escher B, Fenner K, Hofstetter T, Johnson C, von Gunten U, Wehrli B. 2006.

The Challenge of Micropollutants. *Sci. Technol.* 313:1072–1077.

19. 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.

20. Chapman PM, Wang F, Germano JD, Batley G. 2002. Pore water testing and analysis: The good, the bad, and the ugly. *Mar. Pollut. Bull.* 44:359–366.

21. 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.

22. 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.

23. 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.

24. Amweg EL, Weston DP. 2007. Whole-sediment toxicity identification evaluation tools for pyrethroid insecticides: I. Piperonyl butoxide addition. *Environ. Toxicol. Chem.* 26:2389–2396.

25. Weston DP, Jackson CJ. 2009. Use of engineered enzymes to identify organophosphate and pyrethroid-related toxicity in toxicity identification evaluations. *Environ. Sci. Technol.* 43:5514–20.

26. Weston DP, Lydy MJ. 2010. Focused toxicity identification evaluations to rapidly identify the cause of toxicity in environmental samples. *Chemosphere*. 78:368–74.

27. Harwood AD, You J, Lydy MJ. 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. *Environ. Toxicol. Chem.* 28:1051–1058.

28. Greenstein DJ, Bay SM, Young DL, Asato S, Maruya K a, Lao W. 2014. The use of sediment toxicity identification evaluation methods to evaluate clean up targets in an urban estuary. *Integr. Environ. Assess. Manag.* 10:260–8.

29. 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.* 29:158–67.

30. Phillips BM, Anderson BS, Hunt JW, Clark SL, Voorhees JP, Tjeerdema RS, Casteline J, Stewart M. 2009. Evaluation of phase II toxicity identification evaluation methods for freshwater whole sediment and interstitial water. *Chemosphere*. 74:648–53.

31. Watanabe H, Nakajima F, Kasuga I, Furumai H. 2013. Application of whole sediment toxicity identification evaluation procedures to road dust using a benthic ostracod Heterocypris incongruens. *Ecotoxicol. Environ. Saf.* 89:245–251.

32. Benoit D a., Phipps GL, Ankley GT. 1993. A sediment testing intermittent renewal system for the automated renewal of overlying water in toxicity tests with contaminated sediments. *Water Res.* 27:1403–1412.

33. Leppanen CJ, Maier KJ. 1998. An inexpensive and efficient modular water-renewal system for bulk sediment toxicity testing. *Environ. Toxicol. Chem.* 17:969–971.

34. Rand GM, Wheat J V, Carriger JF, Lee TA. 2003. An automated overlying water-renewal

system for sediment toxicity studies. *Environ. Pollut.* 122:169–175.

35. Leusch F, Chapman H. 2011. *The role of toxicity testing in identifying toxic substances : A framework for identification of suspected toxic compounds in water.*

36. 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.

37. 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.

38. Kumar A, Kookana R. 2006. *Impact of winery wastewater on ecosystem health–an introductory assessment. Final Report, CSIRO Adelaide.*

39. 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.

40. Simpson BSL, Batley GE, Chariton AA, Stauber JL, King CK, Chapman JC, Hyne R V, Gale SA, Roach AC, Maher WA, Simpson SL. 2005. *Handbook for Sediment Quality Assessment Quality Assessment.*

41. Jonker MTO, Suijkerbuijk MPW, Schmitt H, Sinnige TL. 2009. Ecotoxicological effects of activated carbon addition to sediments. *Environ. Sci. Technol.* 43:5959–5966.

42. Nybom I, Abel S, Mäenpää K, Akkanen J. 2016. Effects of activated carbon amended sediment on biological responses in Chironomus riparius multi-generation testing. *J. Hazard. Mater.* 318:388–395.

CHAPTER 2: IMPROVEMENTS AND COST-EFFECTIVE MEASURES TO THE AUTOMATED INTERMITTENT WATER RENEWAL SYSTEM FOR TOXICITY TESTING WITH SEDIMENTS.

Improvements and cost-effective measures to the automated intermittent water renewal system for toxicity testing with sediments

W. Tyler Mehler[a], Jing You[b,*], Michael J. Keough[a], Michael J. Lydy[c], Vincent Pettigrove[a]

[a] *Department of Biosciences, Centre for Aquatic Pollution Identification and Management (CAPIM), The University of Melbourne, Royal Parade, Parkville 3010, Australia*
[b] *School of Environment, Guangzhou Key Laboratory of Environmental Exposure and Health, and Guangdong Key Laboratory of Environmental Pollution and Health, Jinan University, Guangzhou 510632, China*
[c] *Center for Fisheries, Aquaculture and Aquatic Sciences and Department of Zoology, Southern Illinois University, Carbondale, IL 62901, USA*

ABSTRACT

The push to make bioassays more sensitive has meant an increased duration of testing to look at more chronic endpoints. To conduct these longer bioassays through the use of traditional bioassay methods can be difficult, as many traditional bioassays have employed manual water changes, which take considerable time and effort. To that end, static-renewal systems were designed to provide researchers a technique to ease the manual water change burden. One of the most well-known static-renewal designs, the static intermittent renewal system (STIR) was produced by the United States Environmental Protection Agency in 1993. This system is still being used in laboratories across the globe today. However, these initial designs have become rather dated as new technologies and methods have been developed that make these systems easier to build and operate. The following information details changes to the initial design and a proof of concept experiment with the benthic invertebrate, *Chironomus tepperi*, to validate the modifications to the original system.

1. Introduction

Sediment bioassays are increasingly being used to assess ecological effects as part of aquatic risk assessments. Standardized techniques to address sediment toxicity have been adopted by the Organization for Economic Co-operation and Development, OECD (2004), the United States Environmental Protection Agency (2000), and American Society for Testing and Materials International (ASTM, 1997) among others. The need for water changes in bioassays (due to water quality issues) has become more critical as more sensitive and longer bioassay procedures, such as reproductive and other chronic evaluations, have been developed (ASTM, 1997; Ankley et al., 1993). Manual water changes, as one might suspect, are quite time-intensive and, if not carefully done, disruptive to the test organisms and test sediments in the bioassay. With the technical difficulties of performing manual water changes and the increasing size of bioassays (such as for toxicity identification evaluation bioassays), the need for automated water renewal procedures was obvious and automated procedures were developed. Even with these developments, many laboratories still manually perform water changes for bioassays. While the drawbacks to manual water changes revolve around the amount of time required to perform a change and the potential for re-suspension of sediments, the major drawback to the automated water change hinges mainly on the initial cost and the technical expertise involved to build such a system.

Perhaps one of the most well known automated systems is the stationary and portable Sediment Testing Intermittent Renewal (STIR) system (Benoit et al., 1993). This system was designed to be economical and practical, while still being an effective and less time-consuming approach to conducting water changes in sediment bioassays. To date, the designs of the STIR as prepared in 1993 still meet those expectations. However, much of the information (e.g. cost, equipment choices, etc.), as detailed in that publication, has become dated, and modifications for easier construction with the use of new technologies have become possible, and they allow for construction that requires little to no building experience.

Similar to the initial publication describing the STIR system, the objective of this project is to provide researchers with enough detail to construct an automated system for sediment testing of their own. The information presented below is based on the construction of multiple automated sediment systems in laboratories in the United States, China, and Australia. With the construction of each system, modifications and improvements were made to make the system more user-friendly, while still reducing cost and space requirements. In some circumstances, proposed changes that we have utilized were suggested in other static
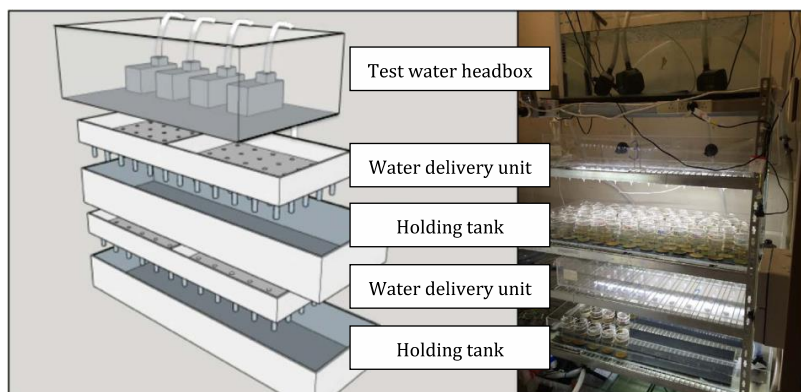
renewal test designs (Rand et al., 2003; Zumwalt et al., 1994; Leppanen and Maier, 1998). The details provided here have further simplified the construction, making the system easier to use, increasing throughput while saving space, and/or making it more cost-effective to build in comparison to the STIR system.

2. Materials and methods

The original specifications for the STIR had five major components, but the modified system described has four: test water headbox, water delivery unit, holding tank, and exposure test chambers. The current setup does not require a water tank as specified in the original design as the holding tank itself doubles as a water bath. An optional filtration water discharge system will be also discussed. The proposed design does differ dramatically from the original schematics of the STIR design (as shown in Fig. 1). As space and cost are limitations for most laboratories, a description of how to build this system is provided with the understanding that modifications will be made based on an individual laboratory needs and resources.

2.1. Housing the system

The initial STIR system design was a table top design or was oriented more horizontally (Benoit et al., 1993); the proposed modified system described herein is a tiered system requiring ~ 2 m of vertical space, and ~ 1 m in width, and a depth of 0.5 m, which increases throughput and decreases space requirements (as it utilizes vertical space). Also, the new modified design can handle more replicates, as the initial design could conduct an automated change for 96 replicates for 12 different sediments, while the most recently created system (housed at the University of Melbourne) can house 120 replicates. This design neither limits the number of sediments that could be run simultaneously (thus, 120 different sediments could be evaluated if so desired) – the reason for this difference will be discussed in the delivery unit section. As this system is tiered and will be holding a considerable amount of water, suitable heavy-duty shelving that can handle large weights is required. This shelving houses the test water headbox, two water delivery units and two holding tanks as well as the exposure chambers. The weight of this system when used at full capacity (containing water and exposure beakers) can exceed 40 kg for an individual level of the shelf.

2.2. Test water headbox

The test water headbox as specified in the original STIR design was fabricated from welded stainless steel, with additional weldings being required for couplers that would connect the headbox to the rest of the STIR system (Benoit et al., 1982). Similarly, designs by Rand et al. (2003)

require the construction of a headbox that utilizes various glass compartments that are constructed using plate glass. In our automated system this headbox has been simplified dramatically. Large aquaria, positioned at the top of the unit, can be used as the test water headbox. If space is not a limitation, tubs or trashcans can be placed adjacent to the system as a substitute for holding the test water. The modified system proposed here uses submersible pumps and reinforced PVC plumbing hose to connect the test water headbox to the water delivery unit, eliminating the need for modifications to the test water headbox. This is different than most other designs which require the use of solenoids.

As for the choice of pumps, the main consideration should be the discharge rate. A fast discharge rate ensures that the water delivery unit will fill quickly and result in a uniform delivery of test waters. In the University of Melbourne system (four test water headbox units/30 replicates per unit), 230–240 V pumps with a Q_{max} of 2400 L/h were used. It should be noted that if the test water headbox is placed at the top of STIR system (as shown in Fig. 1), water would continually discharge even when the submersible pumps shut off (i.e. siphoning). Thus, the PVC hosing will need to be fitted with a PVC "T" fitting, which will discharge water back into the test headbox during water renewals. Once the pump shuts off, this fitting will allow air into the hose, which will stop the siphoning action.

The starting and stopping of the submersible pumps are controlled by electronic timers. The initial STIR design used solenoid valves (as have many past designs) that were to be wired into 24-h timers. This portion of the construction can now be avoided with the development of specialized timers. Since the publication of the initial STIR design, timers with the ability to complete cycles in the duration of seconds as well as having extensive programming capabilities have become readily available (for example MistKing Seconds Timer (Jungle Hobbies Ltd, Ontario, Canada). These specialized timers have an electrical socket so that pumps can be plugged directly into the timer. Calibration of the units (and hence the timers) will be discussed in greater detail below.

2.3. Water delivery unit

The water delivery unit in the initial STIR system pumps water into a holding tank containing up to eight exposure test beakers. This tank would fill slowly and replace water in the exposure test beakers through a water renewal hole in the exposure test beakers. The modified design uses a different technique than the initial STIR design that allows each exposure test beaker to be filled separately (similar to (Zumwalt et al., 1994; Leppanen and Maier, 1998)). In turn, this allows for beakers to be randomly distributed throughout the holding tank and for various levels of replication.

The PVC plumbing hoses that are attached to the pumps in the test water headbox are connected to the water delivery unit by a PVC fitting, which is positioned at the back of the water delivery unit. The water
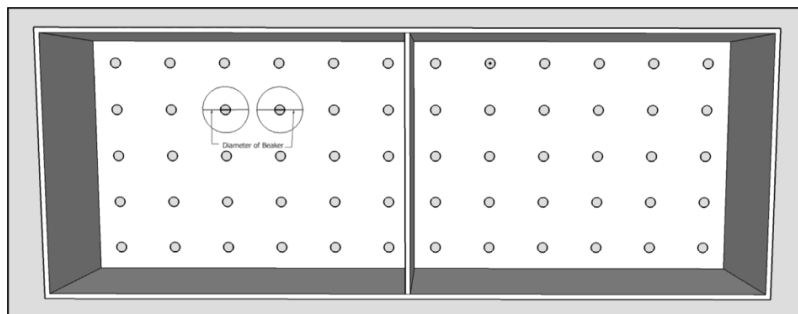
Fig. 2. Water-delivery unit design as built for the modified static-renewal system.

delivery unit itself is made entirely of poly (methyl methacrylate) – or more commonly known as acrylic glass (or by its trade name as Perspex™ or Plexigas™). The water delivery unit is essentially a modified open box (as shown in Fig. 1). Although the specifications for length and width of the water delivery unit are shelving dependent, the unit must be deep enough to contain the necessary water volume that is supplied to the test chambers. For the system as shown in Fig. 2 the depth is 14 cm. Similarly, the thickness of the glass for both the water delivery unit and the holding tank is important as thin acrylic glass has the potential to break or warp with high volumes of water (thicknesses of greater than 0.5 cm have been used successfully). Each water delivery unit is separated into two units (with each unit being filled by a separate pump). Each unit is able to yield 25–30 experimental replicates depending on design. The objective of separating the units into smaller blocks is so that the system can still be employed in smaller experiments, while using the necessary amount of water required for testing and also filling the chamber quickly to get more precise water volumes amongst replicates. The use of a poly (methyl methacrylate) solvent cement should be used when constructing the water delivery unit (as well as the holding tank) over silicon. These types of cement melts the glass together for a stronger and neater seal and should be used for the construction of both the water delivery unit as well as the holding tank.

The base of the water delivery unit has holes cut at a little more than a test chamber width apart (as shown in Fig. 2), which will house syringes (the use of syringes as part of a static renewal system has also been discussed in other designs (Rand et al., 2003; Zumwalt et al., 1994)). As the water delivery unit fills, these syringes will discharge the test water into the exposure test beakers, which sit below the unit in the holding tank. Various sizes of syringes (without the needle) have been used successfully in the past (10–50 mL) although the use of smaller volume syringes is encouraged as it allows for the delivery unit to fill

more quickly (providing a more uniform water distribution), less disturbance to the sediments in the beaker, as well as saving vertical space. Syringe holes should be cut to the correct diameter to house the syringes to avoid leaks; in many cases a 'lip' on the syringes can be beneficial as it can provide additional area for a better seal.

Each separate system is calibrated before use and the volume and water change frequency is dependent on the research requirements. In general, we have typically employed a frequency of two water changes per day aiming for 100–150 mL per change. The system should be calibrated before use and in our experience each 30 replicate unit is able to produce volumes consistently that have less then a 10% relative standard deviation among all replicates. Water changes generally take between 14 and 17 s using the pumps that were mentioned earlier. Syringes can be adjusted vertically if the designed hole is cut snugly as suggested, to further aid in calibration. Intial setup of the design should not only determine the pump frequency and duration, but also the syringe to chamber distance that allows for the fresh water to circulate with the old, while still not resuspend the sediment in the chamber.

2.4. Holding tank

Similar to the water delivery unit, the holding tank has been made from acrylic glass and the specifications for length and width are again shelving dependent. The depth of the tank should be greater than that of the exposure test beakers (as the beakers will be discharging water into the holding tank). The only additional assembly required with the holding tank is the waste overflow PVC fitting. The fitting needs to be positioned at such a height that the water in the bath is lower than that of the release hole in the test beakers (as shown in Fig. 3). The location of the fitting can be either on the side of the box (as shown in the depiction) or on the bottom the tank. As mentioned earlier the holding

Fig. 3. Excess valve (A) and various test beaker designs (mesh screen (B) silicone, (C) held with rubber bands, and (D) hosing fitted with screen.

Fig. 4. Water discharge filtration system as built for the modified static renewal system.

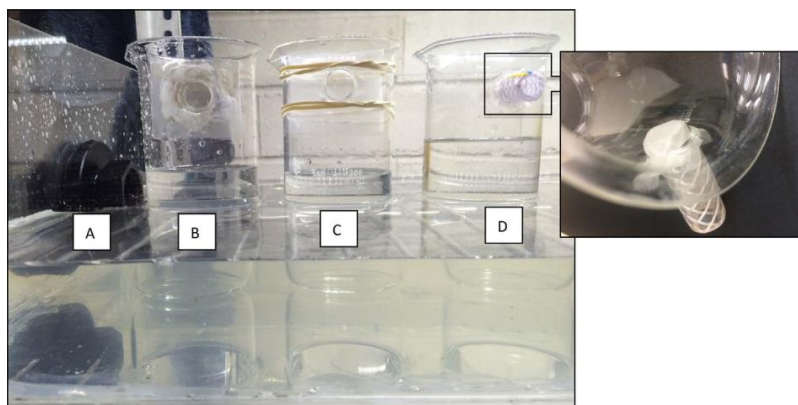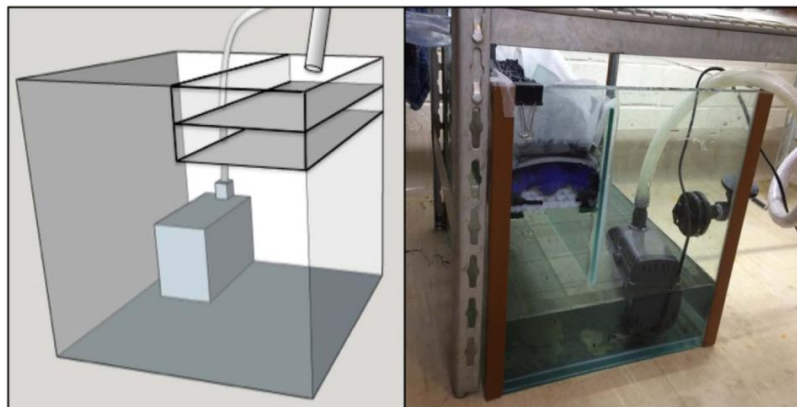tank also serves as a water bath. In many circumstances temperature controls are not required if the system is placed into a temperature-controlled room. If this is not possible, chiller units and heating units have also been successfully used to ensure that water temperature is maintained to the desired level.

2.5. Water discharge filtration system

The excess water from the test beakers will be discharged into the holding tank, which will dispose of this water through the excess flow valve. The flow valve is affixed with hosing which takes this water into the filtration water discharge system (see depiction in Fig. 4). This system has been put into place to ensure that any chemicals in the water are captured before the water is discharged as waste. The discharge system is fixed with a top compartment that can be filled with bioballs, activated carbon, or other agents to clean the test waters (depending on possible contaminants). After passing through the top compartment, the settled waters can be pumped out and disposed of as required.

2.6. Exposure test beakers

The size of beakers to be used for bioassays is laboratory specific as cost, availability, and types of bioassays being performed must all be taken into consideration. It is important to note that the size of the beakers will also play a large role into the size of the flow-through system – so the size of the beakers should be considered early on in the construction process. Previous designs have used standard beakers fixed with mesh over the top lip or have had a small notch cut at the top (Rand et al., 2003; Zumwalt et al., 1994; Leppanen and Maier, 1998). This technique works well with many organisms and provides an inexpensive means for exposure vessels; however, in the past we have had issues with overflowing and loss of test species especially when working with pelagic and/or small test organisms. For this reason, we use a similar construction method of exposure vessels as Benoit et al. (1993). The initial STIR design uses 300 mL beakers that have a hole drilled near the top of the vessel to release excess water. The hole should be positioned approximately 3–5 cm below the lip of the beaker (as shown in Fig. 3). In the construction proposed here, various types (customized beakers, mason jars) and sizes (200–600 mL) have been successfully used. The holes can be cut with a drill press using a diamond cutting bit with care being taken while cutting. Similar to the initial STIR design, the exposure test beaker hole should be covered with fine mesh screen (typically around 200–250 µm) to avoid the release of test organisms with the test water. This screen can be attached to beaker through the use of silicon or other non-toxic adhesives as mentioned in the initial design specifications (Fig. 3). However, as mentioned in the initial

design, much care needs to be taken to avoid creating areas where organisms can become lodged. An additional concern with the attachment of the screen to the beakers via silicon was the cleaning of the test beakers – as in many laboratories (including the University of Melbourne) cleaning of test beakers is conducted using an acid-cleaning step, which over time destroyed the silicon and would cause breaks in the seal. To remedy the above issues, two different solutions have been devised and should be used dependent on the test organism being used. As shown in Fig. 3, besides the use of silicon, the mesh can be held on the beakers through the use of rubber bands, this has been shown to be effective for test organisms that reside in the sediment and are unlikely to be in the water column. The other, and more preferred option, is to use the mesh in combination with flexible tubing that fits within the diameter of the hole snugly. This is simple in practice – the mesh is placed over the hole from the outside and the tubing can be pushed through the hole to make a tight seal (as shown in Fig. 3). This option ensures that test organisms will not escape or be lodged in the crevices of the mesh, while still allowing for the test vessels to be easily cleaned. It is important to note that the mesh size should not to be too small as water tension and/or build up may block the vessel from releasing its water.

3. Results

The following discussion details the benefits and drawbacks of both water change approaches (manual water changes and via the static renewal system) in conjuction with a "proof of concept" experiment.

3.1. Proof of concept

As a means to evaluate the suitability of the static renewal system that has been developed, a control experiment (using 5-d growth and survival as well as emergence as endpoints) with a commonly used freshwater species in Australia (*Chironomus tepperi*) was employed to understand the difference among the various methods currently used (manual water changes with aeration, aeration only, manual water changes only, no manual water changes with no aeration) with this static renewal system. The methods for the experiment can be found in the Supplemental material. Water quality was acceptable for all of the methods tested with the exception of the method that used no manual water changes with no aeration (due to low dissolved oxygen < 50%). Interestingly, each method showed various water quality trends (pH (Fig. S1), conductivity (Fig. S2), and dissolved oxygen (Fig. S3)), which can be explained by the method employed; further details on those trends can be found in the Supplemental material. Control survival (Fig. S4) and growth (Fig. S5) using all five methods for the five day acute
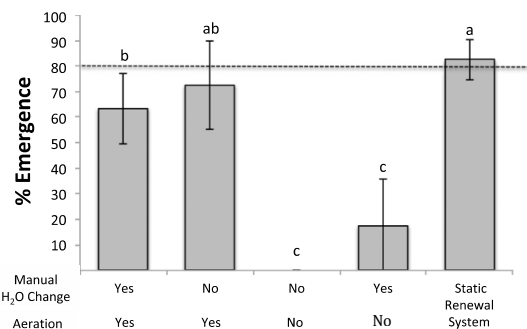
Fig. 5. Emergence (%) of *C. tepperi* for each control method tested after a 5-d bioassay period. Error bars are the standard deviation for each mean. The emergence threshold (± 80%) as required per the *C. tepperi* guidelines (Simpson and Batley, 2016) is depicted by the dashed line. Different letters indicate significant differences among the control methods tested ($p < 0.05$; single-way ANOVA).

portion of the experiment were comparable and not significant from one another (> 80% survival and growth ranged from 0.70 to 0.78 mg/individual) and survival met recently developed guidelines for *C. tepperi* (Simpson and Batley, 2016). However, it should be mentioned that significant differences were found between the treatments as the static renewal system had the highest survival (98% survival) and was significantly different then the manual water change with aeration method which just met the guideline threshold for control mortality (80%). These differences were further distinguished in the emergence results (Fig. 5), as only the static renewal system met the newly developed *C. tepperi* toxicity guidelines of > 80% (Simpson and Batley, 2016). This result was surprising, as the manual water changes with aeration method has been used in our laboratory successfully in the past using emergence as an endpoint (Boyle et al., 2016). The reason for the differences between the past studies that used aeration and manual water changes and those used here could be due to a smaller beaker (350 mL vs. 600 mL, respectively) with a smaller diameter being used (6 cm vs. 8.5 cm, respectively), possibly resulting in unwarranted disturbance to the organism. Issues with the sediment and/or fitness of the organism might have be an issue in the present study as emergence using the static renewal system (82.5%) was also just over the guideline threshold. Regardless, the results suggests that the newly-developed static renewal system can be employed with a great deal of confidence and may be more favorable then traditional methods. It also further shows that regardless of the method employed, control experiments should be conducted to ensure that the chosen method will work for the test organism of interest.

3.2. Time and cost savings

Manual water changes are quite time-intensive; in our experience testing using *Chironomus tepperi* (a benthic species) a water change takes 1–2 min per replicate per change. Most of the time required in the manual water change procedure is an attempt to avoid disturbing the sediment when adding fresh water. Additional time is required when conducting manual water changes for epi-benthic and pelagic species as these species reside in the water column itself and thus more care must be taken to avoid losing and/or stressing these organisms. The automated system described here can change water for approximately 120 replicates in less than 30 s and requires little to no researcher assistance during the change. Standard manual techniques conduct water changes every other day (for *C. tepperi*) or even less often for other test species (*Austrochiltonia subtenuis* (amphipod): one water change per week). To conduct a manual water change for 120 replicates, a single person would need to allocate at a minimum of 3–4 h to do the work. This automated technique not only is quicker, but also allows for additional

water changes to be conducted per day. In contrast, the only responsibility of the researcher with an automated system is initial calibration, ensuring that the test water headbox is filled throughout the bioassay, and that the beakers are lined up under the syringes. In our experience, the system can easily be built within a few days and if constructed by the researcher under $1000 AUD (Perspex/plexiglass: ~ $600, pumps: ~ $100, and timers, hosing, syringes and pvc material: ~ $300). Even if one does not have the necessary resources to build a system, an outside entity can still produce a relatively inexpensive system. For instance, two entire systems (240 replicates) were built by a consultant for the University of Melbourne for less than $3000 AUD. If a laboratory is frequently running bioassays the amount of time and money saved will be obvious within the first few bioassays run.

3.3. Comparison with other static renewal systems

The merits of using a static renewal system over manual water changes are abundantly apparent. Comparing the strengths and limitations of specific designs to one another, however, becomes much more difficult. First and foremost, the system discussed herein can only be used for sediment exposures, while other systems (such as the mini-diluter system (Benoit et al., 1982)) can be used for both sediment and water exposures. The systems that can do both exposure types generally have many more moving parts to ensure that accurate dilutions can be made, but also so that the system can easily be taken apart for cleaning purposes (as the entirety of the system would need cleaned between bioassays). As such, these systems are more costly and generally harder to build. Additionally, as the use of sediments to assess risk is now being recognized by many in the field as a more environmentally relevant media for assessment purposes over that of overlying water, pore water, or effluent (Mehler et al., 2010; United States Environmental Protection Agency, 2007; Chapman et al., 2002), many laboratories may not need a diluter system and instead would focus solely on sediment toxicology (especially those facilities doing considerable biomonitoring work). In these circumstances, the described system, offers considerable advantages over older designs, not because these designs are flawed, but rather that this design utilizes the strengths of each of these past designs (Benoit et al., 1993; Rand et al., 2003; Zumwalt et al., 1994) while taking advantage of more reason technologies. It should be noted, however, that a variety of renewal systems exists (mainly for water only exposures) many of which were designed specifically for a type or class of organism (i.e. fish embryos (Lammer et al., 2009), pelagic microscopic organisms (Smith and Hargreaves, 1983; Lauth et al., 1996; Novak et al., 1982), and even larval or juvenile fish (Brenniman et al., 1976; Diamond et al., 1995) systems). As many systems exists, researchers should first determine the needs of the laboratory as well as budget constrains and then consult the literature for designs before embarking on building a system of their own.

4. Conclusions

The use of static-renewal systems provides researchers added flexibility, saves times and money, and produces consistent results for bioassays. The initial design produced in 1993 (Benoit et al., 1993) produced a system that met these specifications. With the following modifications, we feel that even less resources and/or money and less constructional "know-how" is required to build a static-renewal system. It is our opinion that if a laboratory is conducting sediment bioassays at regular intervals (i.e. at least once a month) that the construction of a static-renewal system, such as the one discussed here, is well worth the investment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.12.051

References

Ankley, G.T., Benoit, D.A., Hoke, R.A., Leonard, E.N., West, C.W., Phipps, G.L., Mattson, V.R., Anderson, L.A., 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.

ASTM, 1997. Standard test methods for measuring the toxicity of sediment-associated contaminants with freshwater invertebrates. ASTM Annu. B Stand. 1138–1220. http://dx.doi.org/10.1520/E1706-05E01.tion.

Benoit, D.A., Mattson, V.R., Olson, D.L., 1982. A continuous-flow mini-diluter system for toxicity testing. Water Res. 16, 457–464.

Benoit, D.A., Phipps, G.L., Ankley, G.T., 1993. A sediment testing intermittent renewal system for the automated renewal of overlying water in toxicity tests with contaminated sediments. Water Res. 27, 1403–1412.

Boyle, R.L., Hoak, M.N., Pettigrove, V.J., Hoffmann, A.A., Long, S.M., 2016. Comparing the impacts of sediment-bound bifenthrin on aquatic macroinvertebrates in laboratory bioassays and field microcosms. Ecotoxicol. Environ. Saf. 133, 489–500.

Brenniman, G., Hartung, R., Weber, W.J., 1976. A continuous flow bioassay method to evaluate the effects of outboard motor exhausts and selected aromatic toxicants on fish. Water Res. 10, 165–169.

Chapman, P.M., Wang, F., Germano, J.D., Batley, G., 2002. Pore water testing and analysis: the good, the bad, and the ugly. Mar. Pollut. Bull. 44, 359–366.

Diamond, S., Oris, J., Guttman, S., 1995. A system for conducting flow-through toxicity tests with larval fish. Environ. Toxicol. Chem. 14, 1389–1391.

Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish embryo toxicity test (FET) with the zebrafish (Danio rerio) a potential alternative for the fish acute toxicity test? Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 149, 196–209.

Lauth, J.R., Dyer, S.D., Belanger, S.E., Cherry, D.S., 1996. A novel flow-through method for toxicity assessments using Ceriodaphnia dubia. Environ. Toxicol. Water Qual. 11, 335–343.

Leppanen, C.J., Maier, K.J., 1998. An inexpensive and efficient modular water-renewal system for bulk sediment toxicity testing. Environ. Toxicol. Chem. 17, 969–971.

Mehler, W.T., You, J., Maul, J.D., Lydy, M.J., 2010. Comparative analysis of whole sediment and porewater toxicity identification evaluation techniques for ammonia and non-polar organic contaminants. Chemosphere 78, 814–821.

Novak, A.J., Berry, D.F., Walters, B.S., Passino, D.R.M., 1982. New continuous-flow bioassay technique using small crustaceans. Bull. Environ. Contam. Toxicol. 29, 253–260.

OECD, 2004. Test No. 218: Sediment-Water Chironomid Toxicity Using Spiked Water, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publ.:1–21. ⟨http://doi.org/10.1787/9789264070264-en⟩.

Rand, G.M., Wheat, J.V., Carriger, J.F., Lee, T.A., 2003. An automated overlying water-renewal system for sediment toxicity studies. Environ. Pollut. 122, 169–175.

Simpson, S.L., Batley, G.E., 2016. Sediment Quality Assessment: A Practical Guide. CSIRO Publishing, Clayton, Victoria, Australia.

Smith, R.L., Hargreaves, B.R., 1983. A simple toxicity apparatus for continuous flow with small volumes: demonstration with mysids and naphthalene. Bull. Environ. Contam. Toxicol. 30, 406–412.

United States 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.

United States Environmental Protection Agency, 2007. Sediment Toxicity Identification Evaluation (TIE) Phases I, II, and III Guidance Document. EPA/600/R-07/080. Washington, DC.

Zumwalt, D.C., Dwyer, F.J., Ingersoll, C.G., 1994. A water-renewal system that accurately delivers small volumes of water to exposure chambers. Environ. Toxicol. Chem. 13, 1311–1314.

26

Supplemental Information

Methods

Control sediment was collected at a site near the city of Melbourne (Victoria, Australia), Bittern Reservoir (Tuerong). This site has been used in the past and have been shown to be free of toxicity and have limited contamination [1,2]. Collected sediment was sieved through a 500 μm net. Sediments were stored in 20 L buckets at 4 °C in the dark until use [3]. Cultures of the freshwater midge, *Chironomus tepperi*, were originally acquired from temporary ponds in Yanco Agricultural Institute (New South Wales, Australia). Cultures for this species were maintained in ethanol-sterilized tissue paper using modified a Martin's solution [4,5]. For conducting 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 days (resulting in 5 - 7 days old organisms, second instar).

The control experiment was conducted in 350 mL beakers. A total weight of 60 g wet wt. sediment was used with approximately 250 mL of artificial water. Five control methods were evaluated including the static renewal system (150 - 200 mL per change, twice per day) and control treatments that had aeration and manual water changes (every other day; ~70% water renewal), aeration only, manual water change only, and neither aeration and manual water change. Test water for bioassays was prepared in the same manner as culture water noted above. All toxicity testing was conducted using a standard photoperiod of 16:8 light:dark and a temperature of 21 ± 1°C. Bioassays evaluated survival and growth (via dry wt.) in the first five days and emergence (testing was terminated at 30 days). Eight replicates were screened for acute toxicity evaluating survival and growth. The dry weight of midges was used to assess growth [6]; organisms were dried at 90 °C to a constant temperature (Memmert drying oven, Schwabach, Germany) and weighed using a Kern ABS/ABJ Analytical Balance (reproducibility ± 0.1 mg; Kern & Sohn, Balingen, Germany). Another eight replicates were covered using nylon stockings to avoid losing emerged adults and were subsequently evaluated for emergence. Emergence was evaluated daily and emerged adults were collected using an aspirator. For collected adults, the date and sex was recorded at time of emergence. Water

quality parameters (in three replicates chosen at random) for all control methods were evaluated daily during the acute portion of the experiment (first five days) and then subsequently every other day until the test terminated. Water quality parameters evaluated included: dissolved oxygen, pH, conductivity and temperature. Statistical analysis was conducted using R [7]. Survival, growth, and emergence were analyzed using a single factor ANOVA. If any significant differences were noted between the treatments, a Tukey's post-hoc comparison test was employed to further understand which treatments were different from one another.

Water Quality Trends

The water quality parameter trends observed in the each control method are shown below. As the room used to conduct the bioassay is temperature controlled, temperature was consistent regardless of the method used (21 ± 1°C). The pH (Figure S1) was considerably consistent throughout the 30-d bioassays using the static renewal system, especially after day 5. Additionally, it appears that by not changing the water throughout the test the pH will continue to increase over the bioassay when no aeration is used and decrease when aeration is used. Similarly, the static renewal system showed the most consistent conductivity (Figure S2) throughout the 30-d bioassays. Not surprisingly, conductivity continues to increase when water changes were not performed (in aeration only and no aeration/manual water change methods) most likely due to evaporation. Dissolved oxygen for all control methods generally had a "U" shaped response this coincides with the growth and eventual emergence of *C. tepperi* (i.e. oxygen demand increases as the organisms growth and decreases as organisms emerge). Aeration methods (aeration only and aeration and manual water change methods) had the highest saturation levels with oxygen generally being at or above 80% as expected. The static renewal system had dissolved oxygen levels that were comparable to the manual water change method if not a little lower (at or above 70% saturation). The lower dissolved oxygen levels noted (which is still within the acceptable range of >60%; [8]) in the static renewal system occurred between days 10-20, which coincides with the growth of *C. tepperi* as the majority of *C. tepperi* emergence occurred between days 18-23. It should be noted that the static renewal system could be adjusted to conduct more water changes

if higher dissolved oxygen levels were desired – although this does mean that water would need to be added more regularly.

Figure A1. The pH trends of each control method employed over the 30-d bioassay. Trends lines were fitted using locally weighted scatterplot smoothing (LOESS) in R. Each circle is a single replicate.
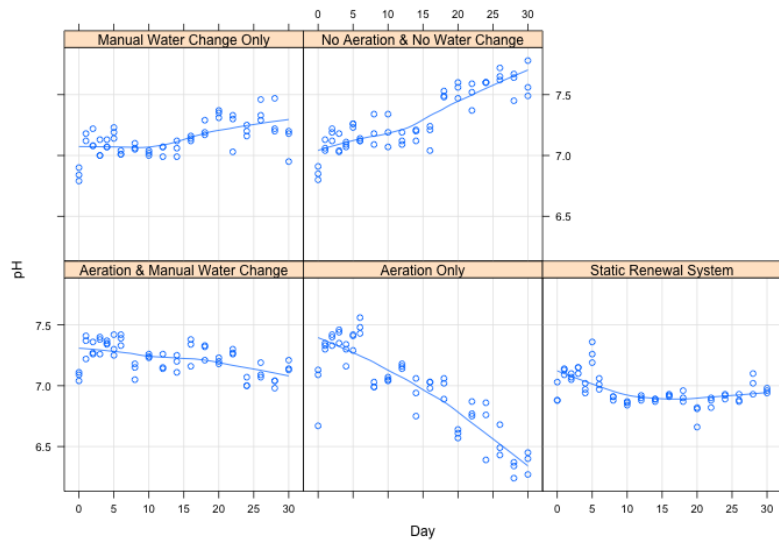
Figure A2. The conductivity (µS/cm) trends of each control method employed over the 30-d bioassay. Trends lines were fitted using locally weighted scatterplot smoothing (LOESS) in R. Each circle is a single replicate.
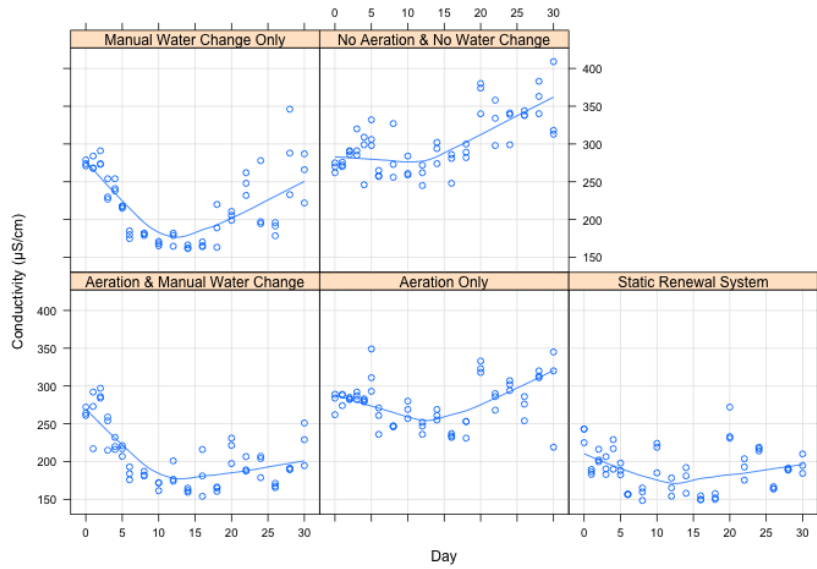
Figure A3. The dissolved oxygen (% saturation) trends of each control method employed over the 30-d bioassay. Trends lines were fitted using locally weighted scatterplot smoothing (LOESS) in R. Each circle is a single replicate.
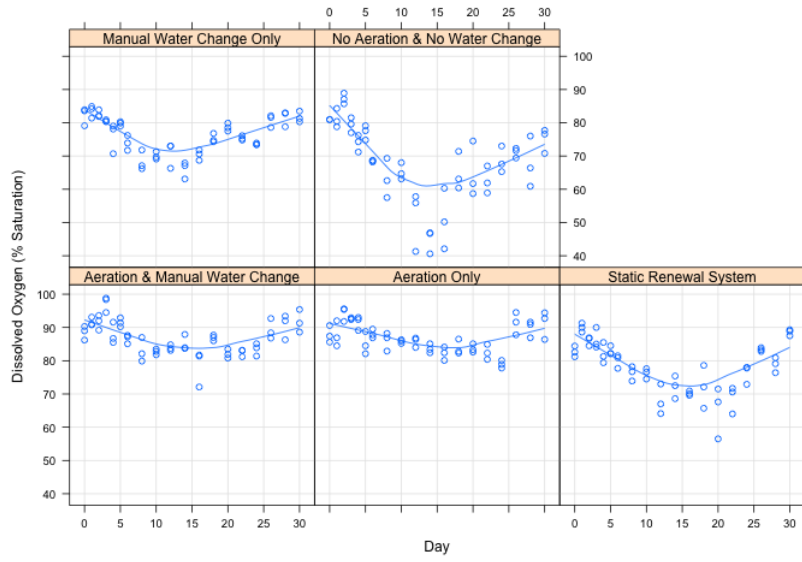
Figure A4. Survival (%) of each control method tested after a 5-d bioassay period. Error bars are the standard deviation for each mean. All control methods tested met the survival threshold ($\pm$ 80%) as required per the *C. tepperi* guidelines [8]. Different letters indicate significant differences ($p < 0.05$; single factor ANOVA) between the control methods tested.
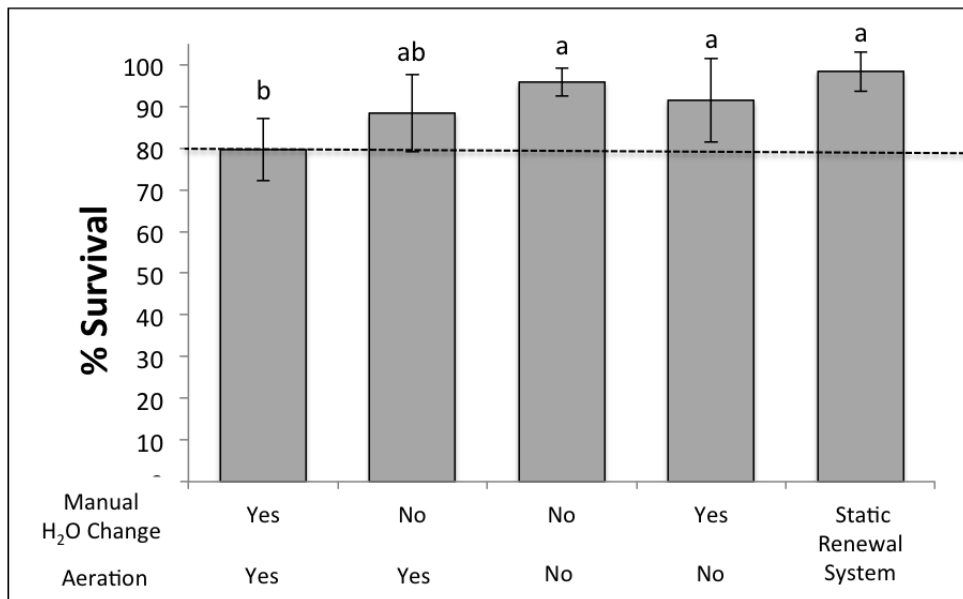
Figure A5. Growth (mg/individual) of each control method tested after a 5-d bioassay period. Error bars are the standard deviation for each mean. Currently no growth threshold exists for *C. tepperi*. No significant differences ($p < 0.05$; single factor ANOVA) were noted between the control methods tested.
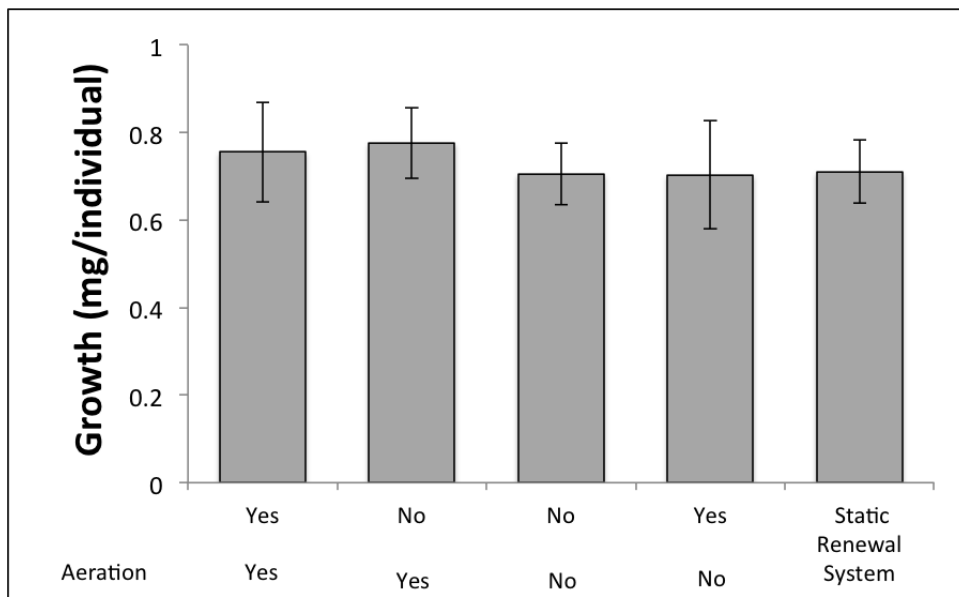
Citations:

1. 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.

2. 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.

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

4. 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.

5. 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.

6. Schuler LJ, Landrum PF, Lydy MJ. 2007. Response spectrum of pentachlorobenzene and fluoranthene for *Chironomus tentans* and *Hyalella azteca*. *Environ. Toxicol. Chem.* 26:1248–57.

7. Knezevic SZ, Streibig JC, Ritz C. 2007. Utilizing R Software Package for Dose-Response Studies: The Concept and Data Analysis. *Weed Technol.* 21:840–848.

8. Simpson SL, Batley GE. 2016. *Sediment quality assessment: A practical guide*. CSIRO Publishing, Clayton, Victoria, Australia.

CHAPTER 3: DEVELOPMENT OF WHOLE-SEDIMENT TOXICITY IDENTIFICATION EVALUATION (TIE) TECHNIQUES FOR TWO AUSTRALIAN FRESHWATER SPECIES: CHIRONOMUS TEPPERI AND AUSTROCHILTONIA SUBTENIUS.

W. Tyler Mehler, Michael J. Keough, and Vincent Pettigrove. 2017. Development of whole-sediment toxicity identification evaluation (TIE) techniques for two Australian freshwater species: *Chironomus tepperi* and *Austrochiltonia subtenius. Environmental Toxicology and Chemistry* 36: 2476-2484.

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

W. Tyler Mehler,* Michael J. Keough, and Vincent Pettigrove
School of Biosciences, The University of Melbourne, Parkville, Victoria, Australia

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. *Environ Toxicol Chem* 2017;36:2476–2484. © 2017 SETAC

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 non-native 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 ($<76\,\mu m$) 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 $<44\,\mu m$. 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 ($\sim$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 iminodiaceticacid 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 $CaCl_2 \bullet 2H_2O$ (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 $250\,\mu S/cm$. 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- to 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 $21 \pm 1\,°C$ 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) 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 least significant difference (LSD) test to compare the unaltered sediment with the same sediment that was amended.

RESULTS

Water quality

Temperature ($21 \pm 1\,°C$) 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
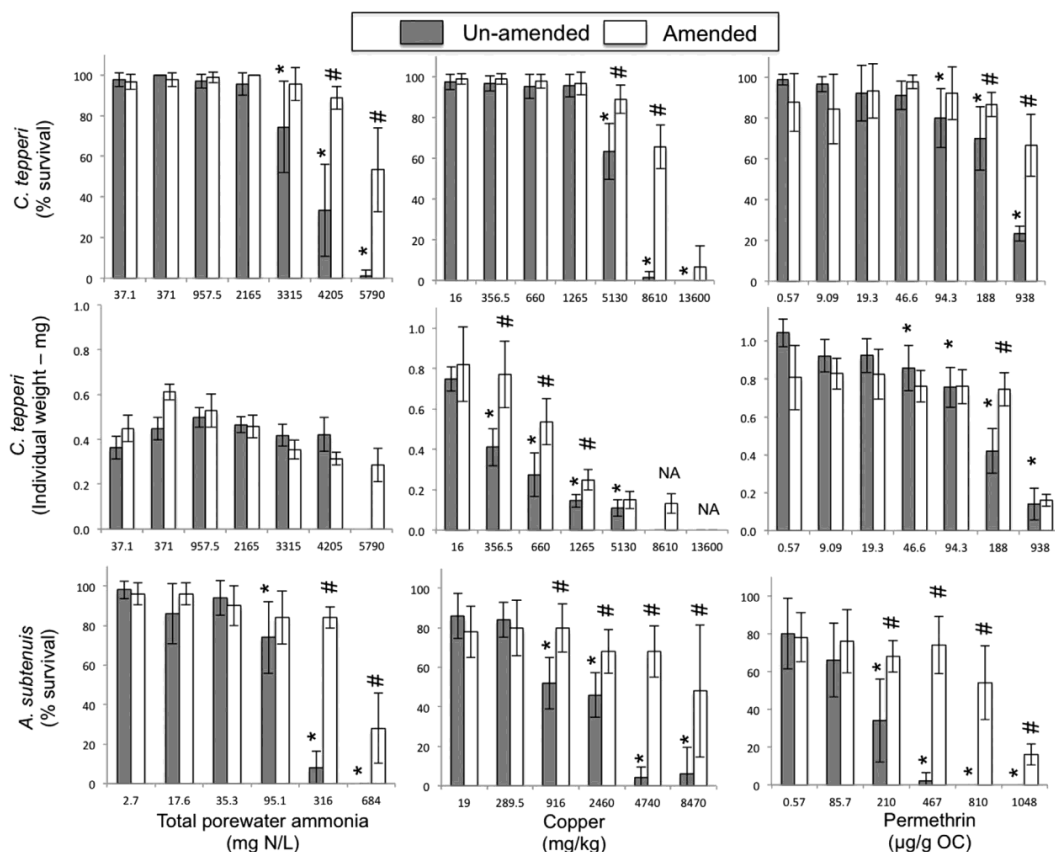
Figure 1. Results show the acute toxic effects for *Chironomus tepperi* (survival and growth) and *Austrochiltonia subtenuis* (survival) in the presence of ammonia (mg N/L), copper (mg/kg), and permethrin (μg/g organic carbon). The asterisk (*) indicates that survival or growth of unamended sediment was significantly decreased when compared with the control ($p < 0.05$). The pound sign (#) identifies a significant increase of survival or growth of the species when the amended was added. NA = not available.

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 in *Water Quality* section) 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

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 ($\pm$ SE) | | Estimated ratio of concentration effects dose ($\pm$ 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] (μg/g organic carbon)	*C. tepperi*——survival	411.9 (57.53)	1861 (396.2)	4.52 (1.07)*
	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.
[b]Using survival.
[c]Using the initial (i.e., before amendment) sediment concentration.
[d]Ammonia, cationic metals, and nonpolar organics.
[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.
[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.
[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.
*The difference in EC50 values between amended and unamended is significant at $p < 0.05$.
EC50 = median effect concentration; SE = standard error; NA = not available.
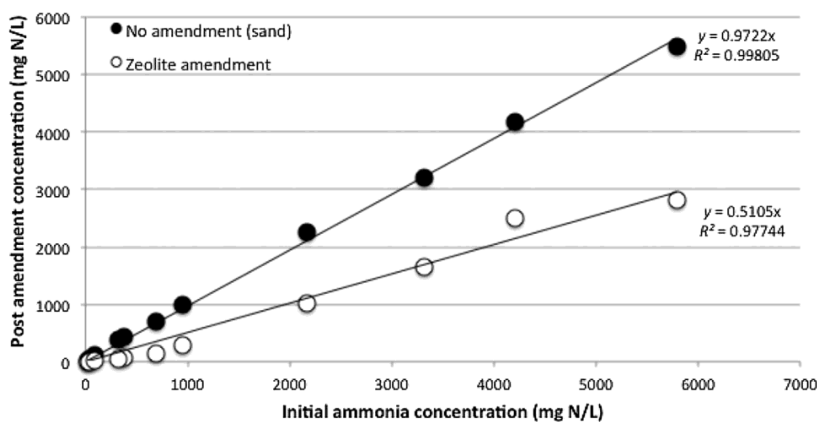
Figure 2. Ammonia concentrations (mg N/L) of amended (with zeolite) and unamended samples in bioassays with both *Chironomus tepperi* and *Austrochiltonia subtenuis* when compared with the initial concentration of the sediment.

2 sediments that showed no reduction) 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 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 samples 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) 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
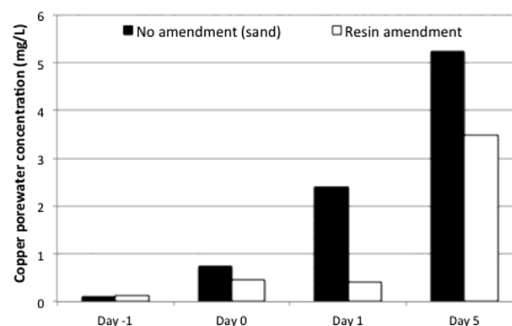
Figure 3. Comparing the amended (with Lewatit MonoPlus TP 207; Lanxess Deutschland) and unamended porewater concentrations (for a single copper concentration, nominal concentration 250 mg/kg) over time (in days).

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 bodies 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 present study 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.

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* 158:716–1725.
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.

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.

CHAPTER 4: RESOLVING THE FALSE-NEGATIVE ISSUES OF THE NON-POLAR ORGANIC AMENDMENT IN WHOLE-SEDIMENT TOXCITY IDENTIFICATION EVALUATIONS (TIES).

Remediation And Restoration

Resolving the False-negative Issues of the Nonpolar Organic Amendment in Whole-sediment Toxicity Identification Evaluations

W. Tyler Mehler,* Michael J. Keough, and Vincent Pettigrove

Department of Biosciences, Centre for Aquatic Pollution Identification and Management (CAPIM), The University of Melbourne, Royal Parade, Parkville, Australia

Abstract: Three common false-negative scenarios have been encountered with amendment addition in whole-sediment toxicity identification evaluations (TIEs): dilution of toxicity by amendment addition (i.e., not toxic enough), not enough amendment present to reduce toxicity (i.e., too toxic), and the amendment itself elicits a toxic response (i.e., secondary amendment effect). One such amendment in which all 3 types of false-negatives have been observed is with the nonpolar organic amendment (activated carbon or powdered coconut charcoal). The objective of the present study was to reduce the likelihood of encountering false-negatives with this amendment and to increase the value of the whole-sediment TIE bioassay. To do this, the present study evaluated the effects of various activated carbon additions to survival, growth, emergence, and mean development rate of *Chironomus tepperi*. Using this information, an alternative method for this amendment was developed which utilized a combination of multiple amendment addition ratios based on wet weight (1%, lower likelihood of the secondary amendment effect; 5%, higher reduction of contaminant) and nonconventional endpoints (emergence, mean development rate). This alternative method was then validated in the laboratory (using spiked sediments) and with contaminated field sediments. Using these multiple activated carbon ratios in combination with additional endpoints (namely, emergence) reduced the likelihood of all 3 types of false-negatives and provided a more sensitive evaluation of risk. *Environ Toxicol Chem* 2017;9999:1–12. © 2017 SETAC

Keywords: Whole-sediment toxicity identification evaluation; Activated carbon; *Chironomus tepperi*; False-negative; Secondary effect

INTRODUCTION

The release of whole-sediment toxicity identification evaluation (TIE) guidelines by the US Environmental Protection Agency (USEPA) in 2007 stimulated a worldwide increase in the use of whole-sediment TIEs to evaluate risk of aquatic contamination (US Environmental Protection Agency 2007). This increase in use is not surprising because whole-sediment TIEs can identify contaminant classes that may be contributing to toxicity in a manner that is meant to be efficient and cost-effective. Although many practices exist, most whole-sediment TIEs use the addition of TIE amendments to sediment to reduce the toxicity of the target class. The ideal amendment should be added at a low enough level that it does not noticeably dilute overall toxicity, while still having the ability to significantly remove toxicity of the target contaminant class. In addition, the amendment should not itself cause lethal or sublethal effects to the test organism. If the addition of the amendment violates one of these assumptions, it can lead to one of 3 types of false-negatives, which have been outlined in previous whole-sediment TIE work: dilution of toxicity by amendment addition (i.e., not toxic enough), overwhelming the amendment (i.e., too toxic), and a secondary amendment effect (wherein the amendment itself elicits a toxic response; US Environmental Protection Agency 2007; Mehler et al. 2017).

In past studies, amendments used in nonpolar organic characterization, namely activated carbon and powdered coconut charcoal, have encountered all 3 types of false-negative results (US Environmental Protection Agency 2007; Mehler et al. 2017). The USEPA guidance (US Environmental Protection Agency 2007) suggests conducting preliminary evaluations with the carbon and test organism of choice to determine the levels, typically on a wet weight basis, that should be used to avoid these issues (typically 2% for fine and 5% for medium charcoal in freshwater sediments). Even in doing so, this preliminary evaluation as part of a

whole-sediment TIE still has its limitations, and the possibility of false-negatives still exists.

The need for reducing false-negatives when working with nonpolar organics amendments becomes even more apparent when considering the likelihood of a nonpolar organic contributing to the risk in contaminated sediments. Ho and Burgess (2013) reviewed 30 marine and freshwater sediment TIEs over a 20-yr time span (1993–2013) and found that approximately 90% of studies identified a nonpolar organic as a source of toxicity in sediments, with 70% of evaluations characterizing nonpolar organics as the sole source of toxicity. Because nonpolar organics are one of the key classes causing toxicity, understanding the limitations and working to improve the use of activated carbon or powdered coconut charcoal are critical.

The objective of the present study was to better understand the types of false-negatives caused by the addition of activated carbon and how this may affect TIE studies and to determine if an alternative means, namely through a combination of endpoint choice and differing the activated carbon amendment ratio used, could be applied to limit those potential interferences. To meet this objective, we explored the secondary effects of activated carbon alone (in 2 non-contaminated [i.e., control] sediments) on not only survival and growth of *Chironomus tepperi* but also 2 more nonconventional endpoints, emergence and mean development rate. Using this information, a combination of multiple activated carbon amendment ratios and additional endpoints was evaluated using nonpolar organic–spiked sediments as well as contaminated field sediments to determine if this additional information yielded more effective results than current practices with this amendment.

MATERIALS AND METHODS

Chemicals, sand, and activated carbon

Two formulated pesticides were used to represent nonpolar organics: Bunnings Ant, Spider and Cockroach Killer, which contains permethrin as the active ingredient (concentration 100 g/L, 25 cis:75 trans; Brunnings Garden Products), and Richgro Carbaryl Caterpillar, Grasshopper, and Millipede Insecticide, which contains carbaryl as the active ingredient (concentration 100 g/L; Richgro Garden Products). Using commercially formulated products helped create contaminated sediment that was more environmentally realistic (because these products contain other filler agents that would also be found in nature) while also avoiding the need for carrier solvents.

To be cost-effective, TIEs typically used locally sourced products. Sand, to account for any type of dilution effect in TIE testing, was purchased from Chem-Supply and had a size of 300 to 350 μm. Oxpure 325B-9 (Oxbow Activated Carbon) is an activated carbon and was acquired through FilChem Australia. Oxpure 325B-9 is a virgin bituminous coal base with a diameter of <44 μm, a total ash content of 13%, and an apparent density of 0.575 g/cc. Although powdered coconut charcoal is a more common nonpolar organics amendment (Ho et al. 2004; US

Environmental Protection Agency 2007; Mehler et al. 2010), previous whole-sediment TIE work in Australia has shown the effectiveness of this activated carbon as a nonpolar organics amendment as well (Mehler et al. 2017). Before use, the activated carbon was wetted and stored in deionized water. The mixture was allowed to rest at least overnight before use. The activated carbon mixture was decanted, rinsed again with deionized water, and then decanted a final time before use. The resulting product had a wet to dry ratio of $33.8 \pm 8.2\%$.

Organism

In all bioassays, the freshwater midge *C. tepperi* was used. Cultures were originally acquired from temporary ponds in Yanco Agricultural Institute. Cultures for this species were maintained in ethanol-sterilized tissue paper using a modified Martin's solution (Martin et al. 1980; Jeppe et al. 2014). For conducting bioassay work, adult flies were collected from the cultures and allowed to breed. Egg masses from adults collected and resulting larvae were used in testing after 7 d (resulting in 5- to 7-d-old organisms, second instar).

Sediment collection and bioassays

Two control sediments were collected at sites near the city of Melbourne (VIC, Australia): Glynns Wetland (–37.740261, 145.1963920) and Bittern Reservoir (–38.302235, 145.117613). Both sites have been used and/or evaluated in the past and have been shown to be free of toxicity and to have limited contamination (Pettigrove and Hoffmann 2005; Hale et al. 2014; Mehler et al. 2017). Three contaminated storm water retarding basins in Victoria were also collected: National Business Park storm water retarding basin (–37.649604, 144.946685), Chandler storm water retarding basin (–38.00015578, 145.17971), and Mordialloc storm water retarding basin (–38.011928, 145.104851). All collected sediment was sieved through a 500-μm net. Sediments were then stored in 20-L buckets at 4 °C in the dark until use (Marshall et al. 2010).

All bioassays were conducted in 350-mL beakers in the same manner as previous TIE work in Australia (Mehler et al. 2017). In each replicate, a total of 60 g wet weight sediment was used with approximately 250 mL of artificial water. Test water for bioassays was prepared in the same manner as culture water noted above. All toxicity testing was conducted using a standard photoperiod of 16:8-h light: dark and a temperature of 21 ± 1 °C. Bioassays evaluated survival and growth (via dry wt) of *C. tepperi* in the first 5 d and emergence and mean development rate until 30 d. Water quality parameters including dissolved oxygen, pH, conductivity, and temperature were measured every 2 d in the first 5 d of the bioassay and then weekly during the remaining portion of the study. For all bioassays, water changes occurred twice per day using a static renewal system (150–200 mL per change) (Mehler et al. 2018) and replicates were fed every other day (10 mg TetraMin). Three different sediment bioassay experiments were conducted: secondary effects of activated carbon bioassays (activated carbon alone), nonpolar organic spiked

sediment bioassays, and contaminated field sediment bioassays; specific details unique to each bioassay are detailed in the following sections.

Secondary effects of activated carbon bioassays. Five amendment addition ratios (nominal addition ratios [wet wt activated carbon/wet wt sediment]: 0.25, 0.5, 1.0, 2.5, and 5%) as a well as a control were used to understand the impacts of activated carbon on survival, growth, emergence, and mean development rate of *C. tepperi* in 2 control sediments (Glynn's Wetland and Bittern Reservoir). A total of 10 replicates were used per amendment addition ratio (5 replicates were terminated at day 5 for survival and growth, and the remaining 5 were used for emergence and mean development rate endpoints). Activated carbon was added 3 d before the initiation of testing as performed in previous TIE work, and this time frame for amendment addition was utilized in all subsequent bioassays (Mehler et al. 2017). The results of this test were then used to determine appropriate activated carbon amendment ratio thresholds to use in the nonpolar organic spiked sediment bioassays and the contaminated field sediment bioassays that are discussed in the following sections.

Nonpolar organic–spiked sediment bioassays. Three treatments were used for each pesticide formulation (carbaryl and permethrin) that was studied including a control, a low concentration (expected to cause a growth effect but low toxicity), and a high concentration (expected to cause both significant mortality and pronounced growth effects). The low and high nominal concentrations of carbaryl were 170 and 340 µg/g organic carbon, respectively. The low and high nominal concentrations of permethrin were 75 and 150 µg/g organic carbon, respectively. Additionally, 3 activated carbon amendment ratios were evaluated (no addition, a 1% addition, and a 5% addition based on wet wt) for each treatment. These 3 amendment addition ratios were chosen based on the secondary effects of activated carbon outlined in the preceding sections. The outcome was a 3×3 factorial design (i.e., 3 amendment addition ratios and 3 concentrations) using 8 replicates (4 for survival and growth and 4 for emergence and mean development rate) per treatment. All spiked sediments for this portion of the study were prepared using a single high concentration, or a "superspike," sediment that was diluted with Bittern Reservoir sediment to ascertain the desired testing concentrations (Besser et al. 2011). Superspike sediments were aged for at least 7 d before dilution; diluted sediments were then aged for at least an additional 7 d. During the aging process, all sediments were manually mixed by hand as well as rolled on a low-profile roller (Stovall Life Sciences). The unamended treatment had 5% sand added per wet weight to ensure that no dilution effects were occurring by simply adding the activated carbon (the 5% activated carbon amendment addition was chosen over the 1% because it provided a more conservative means of evaluating the potential dilution of toxicity).

Contaminated field sediment bioassays. The general procedure of the whole-sediment field TIE bioassays with contaminated field sediments was the same as discussed in the nonpolar organic–spiked sediment bioassays, with 2 additional treatments included to evaluate the risk of cationic metals. One treatment had a 20% addition of a cation exchange resin, Lewatit Monoplus TP 207 (Lanxess; as sourced from FilChem Australia). The other treatment was a 20% addition of sand to the field sediment to ensure that the addition alone did not dilute toxicity (similar to the 5% sand addition for activated carbon discussed earlier). Preparation details for the chelating resin can be found in previous TIE work (Mehler et al. 2017). The risk of metals toxicity in Victoria (Marshall et al. 2010; Kellar et al. 2014) warranted the addition of these treatments. On the other hand, ammonia risk was not characterized as part of the present study because past work in these areas has suggested that ammonia is generally not a major source of toxicity (Morris and Keough 2002; O'Brien et al. 2010) and *C. tepperi* has been shown to be insensitive to ammonia (Mehler et al. 2017).

Endpoints

In the subset of replicates that was screened for survival and growth, surviving organisms were weighed (via dry wt) to assess growth of the test organism (Schuler et al. 2007). 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). The remaining replicates were covered using nylon stockings to avoid losing emerged adults and subsequently evaluated for emergence and mean development rate. Emergence was evaluated daily, and emerged adults were collected using an aspirator. For collected adults, the date and sex were recorded at the time of emergence. The mean development rate, or the reciprocal of the mean time span between the introduction of *C. tepperi* and the emergence of individuals, was calculated using (Goedkoop et al. 2010)

$$\text{MRD} = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

where MDR is the mean development rate, m represents the maximum number of inspection intervals, i is the index of the inspection intervals, f_i represents the number of emerged individuals in a given time interval, n_e is the total number of emerged individuals at the end of the experiment, and x_i is the development rate of midges emerged in a given interval (or i), calculated as

$$x_i = \frac{1}{\text{day}_i - \frac{l_i}{2}}$$

where day_i is the inspection day and l_i is the duration in days of the inspection interval (i.e., 1). Using this scheme, larger values correspond to faster emergence rates.

Chemical analysis

In the spiked sediments, before the addition of sand or amendment (day −3), an approximately 50-g aliquot of the high-concentration treatment was collected for evaluation. Spiked sediments with formulations of permethrin and carbaryl were evaluated using commercial laboratories accredited to ISO 17025 and ISO 9001. For analysis of permethrin and carbaryl, a single sediment sample (10 g) was treated with sodium sulfate to dry, then each was extracted using a mixture of acetone and hexane (US Environmental Protection Agency 1986). Permethrin and carbaryl were analyzed using capillary injection followed by high-performance gas chromatography (GC) coupled with determination by tandem mass spectrometry (MS/MS; Agilent 7000C) for permethrin and GC–electron capture detector for carbaryl. The limit of reporting for both chemicals was 0.01 mg/kg. Analytical-grade standards (Accustandard) were used for determination of accuracy and precision as part of standard quality assurance and quality control protocols. The recoveries of the spiked sediments compared with nominal concentrations for permethrin and carbaryl were 104 and 54%, respectively. The lower recoveries of carbaryl were unexpected but could be caused by a variety of issues (especially because a commercial formulation was used). Because the objective was to understand the ramifications of using multiple activated carbon amendment ratios when different levels of effects are observed (rather than understanding the concentrations needed to cause toxicity), we feel that these lower recoveries are still acceptable and, as such, the nominal concentrations of each chemical are used throughout the present study.

Unamended field site sediments were analyzed for a suite of different nonpolar organics (15 polycyclic aromatic hydrocarbons [PAHs], 23 organochlorine pesticides, 3 carbamates, 32 organophosphate pesticides, and 9 synthetic pyrethroid pesticides) and 10 metals using the same commercial laboratories as spiked sediment samples. Nonpolar organics were analyzed using the same procedure as for spiked sediment, with the exception that the PAHs were analyzed using single-quadrupole GC-MS, whereas all other compounds were measure using GC-MS/MS. For metals analysis, air-dried sediment (1 g) was refluxed and digested with 4 mL of 50% nitric and 10 mL of 20% hydrochloric acids for approximately 2 h. The heavy metals solution was cooled, hydrogen peroxide (30%) was added, and then it was heated and cooled again. Concentrations of metals in sediments were analyzed using inductively coupled plasma–atomic emission spectrometry (Method 200.7 [US Environmental Protection Agency 1994]). The reporting limit for the insecticides evaluated was 0.1 mg/kg, and the detection limit for the PAHs and metals was 0.5 mg/kg. Total organic carbon was analyzed in both spiked and field site sediments using high temperature combustion (800 °C) and subjected to an acid reaction followed by infrared detection (Dohrmann Chromatograph-190 total organic carbon analyzer). Chemical concentrations in field sediments were compared with available 50% lethal concentration (LC50) values for nonpolar organics (Maund et al. 2002; Maul et al. 2008; Harwood et al.

2009), equilibrium sediment benchmarks for PAHs and dieldrin (Hansen et al. 2003; US Environmental Protection Agency 2003), and probable effect concentrations for metals (MacDonald et al. 2000).

Statistical analysis

Statistical analysis was conducted using R with the drc package (Ritz and Streibig 2005; R Development Core Team 2009). The secondary effects of activated carbon and the spiked sediments bioassays of the study were first subjected to a 2-factor analysis of variance (ANOVA). This was to determine if the type of sediment would impact the observed secondary effects of activated carbon and whether activated carbon behaved differently with varying concentrations of the spiked chemical. In all experiments, survival, growth, emergence, and mean development rate data were then separately analyzed using a single-factor ANOVA. If any significant differences were noted between the treatments, Tukey's post hoc comparison test was employed to further understand which treatments were different from one another. Mean development rate was analyzed separately for males and females because studies have shown that male *C. tepperi* typically emerge earlier than females (Stevens 1998).

RESULTS

Secondary effects of activated carbon

No significant differences were noted between the sites or activated carbon addition treatment levels for survival or emergence in the secondary effects of activated carbon bioassays (Table 1), and the endpoints were within acceptable control levels (Simpson and Batley 2016). However, differences between the sites and the activated carbon addition treatment levels in these bioassays were noted for growth (Table 1). In addition, mean development rate (i.e., time to emergence) of *C. tepperi* was significantly different between the activated carbon ratio treatment levels, but no differences were evident between the sites (Table 1). Differences for growth and mean development rate when compared at the activated carbon addition treatment level were observed at the higher levels of activated carbon use (typically at and above 2.5%; Figure 1). Although differences were noted between the sites for growth (because organisms in Bittern Reservoir sediment grew larger than those in Glynns Wetland sediment), no interaction effect was observed with activated carbon ratios. No interaction effects were noted for any of the other endpoints tested as well (Table 1). These results suggest that activated carbon behaved similarly among the 2 control sediments. These results also provide information as to which activated carbon amendment addition ratio would have a low likelihood of causing secondary effects on growth (1%) and a ratio that would be suspected to have a higher reduction of contaminant with no effect on survival and emergence (5%). Using these 2 amendment ratios coupled with the addition of nonconventional endpoints would provide not only evidence to support or refute false-negatives occurring in whole-sediment

TABLE 1: Two-factor analysis of variance for the secondary effects of the activated carbon alone bioassay and the nonpolar organic spiked sediments bioassay

	Activated carbon alone bioassays[a]			Spiked sediment bioassays[b]					
				Permethrin			Carbaryl		
	Site	AC	Site:AC	Conc	AC	Conc:AC	Conc	AC	Conc:AC
Survival	0.358	0.525	0.149	<0.005[c]	<0.005[c]	0.001[c]	<0.005[c]	<0.005[c]	<0.005[c]
Growth	<0.005[c]	<0.005[c]	0.877	<0.005[c]	0.081	0.092	<0.005[c]	<0.005[c]	<0.005[c]
Emergence	0.159	0.862	0.060	<0.005[c]	<0.005[c]	<0.005[c]	<0.005[c]	<0.005[c]	<0.005[c]
Development rate									
Male	0.804	0.001[c]	0.907	NA	NA	NA	0.056	0.086	0.492
Female	0.260	<0.005[c]	0.087	NA	NA	NA	0.067	0.826	0.076

[a]Model predictors for activated carbon alone bioassays include site (Glynn's Wetland and Bittern Reservoir) and activated carbon (added 0, 0.25, 0.5, 1, 2.5, and 5%).
[b]Model predictors for spiked sediment bioassays included concentration (permethrin, 75 and 150 μg/g organic carbon; carbaryl: 170 and 340 μg/g organic carbon) and activated carbon (added 0, 1, and 5%).
[c]Indicates model predictors that explained significant variation of the response endpoint ($p < 0.05$).
AC = activated carbon; Conc = concentration; NA = not available. Because of a lack of emergence in the "no amendment" treatment, statistical analysis could not be completed.
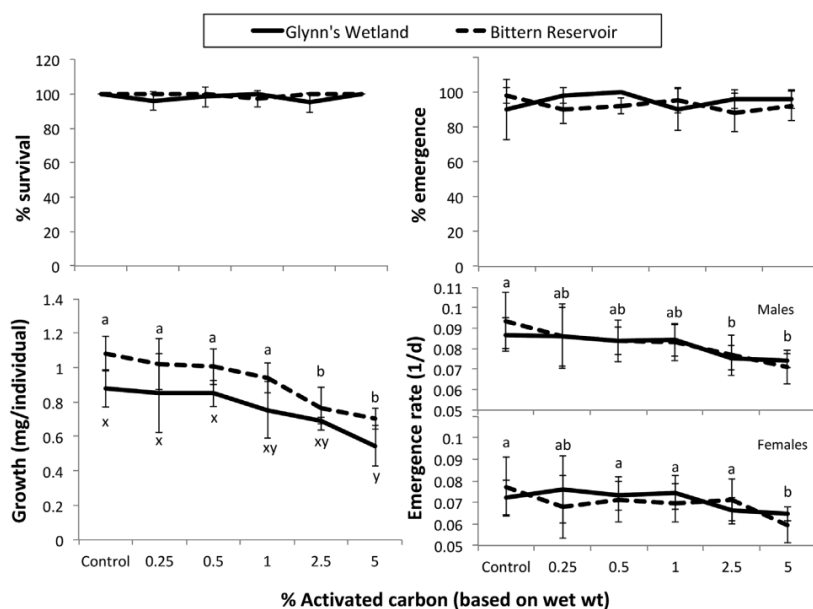
FIGURE 1: Secondary effects of activated carbon alone in control sediment. Five different activated carbon amendment ratios (wet wt/wet wt) in 2 control sediments (Glynn's Wetland and Bittern Reservoir) were evaluated. Using *Chironomus tepperi*, 4 endpoints were evaluated: survival, growth, emergence, and mean development rate. Different letters indicate differences between the treatments for that control sediment only. Significant differences in emergence rate were only noted in the Bittern Reservoir sediment.

TIEs but also additional, and more sensitive, evidence to assess the contamination risk at these sites. It should be noted that these trends for 1 and 5% activated carbon alone were also evident in the spiked sediment and field-based bioassays.

Nonpolar organic–spiked bioassays

A low and a high concentration containing either permethrin (Table 2) or carbaryl (Table 3) were used in the present study to investigate the potential of using 2 activated carbon amendment ratios (1 and 5%) using survival, growth, emergence, and mean development rate with *C. tepperi*. For both chemicals, the survival, growth, and emergence varied with concentrations and the activated carbon ratio used (excluding growth for permethrin). Similarly, these effects (again excluding permethrin growth) showed an interaction between concentration and activated carbon level used (Table 1). The 5% activated carbon ratio addition resulted in higher survival of *C. tepperi* when compared with the 1% activated carbon ratio addition for both the low and high concentrations of permethrin in spiked sediments (Table 2). In contrast, the reduction of acute toxicity in the low and high carbaryl-spiked sediments was no different because mortality was reduced to control levels after the addition of both 1 and

TABLE 2: Spiked sediment bioassay with permethrin (75 and 150 µg/g organic carbon) evaluating activated carbon amendment addition ratios (wet wt/wet wt: 1 and 5%) of activated carbon using *Chironomus tepperi*

| | Permethrin[a] | | | | | | | | |
| | Control | | | 75 µg/g OC | | | 150 µg/g OC | | |
Endpoints	0	1%	5%	0	1%	5%	0	1%	5%
% Survival	85	98	93	65	80	95	30*	69	93
	(13)	(5)	(10)	(13)A	(14)AB	(6)B	(8)X	(14)Y	(5)Z
Growth (mg/individual)	0.76	0.70	0.72	0.45*	0.69	0.58	0.16*	0.3*	0.48*
	(0.08)	(0.11)	(0.14)	(0.08)A	(0.05)B	(0.16)AB	(0.05)X	(0.18)XY	(0.15)Y
% Emergence	95	98	88	55*	78	88	3.0*	40*	85
	(6)	(5)	(5)	(10)A	(17)AB	(10)B	(5)X	(14)Y	(13)Z
Mean development rate (1/d)									
Male	0.081	0.075	0.070	0.080	0.087	0.082	—	0.079	0.078
	(0.012)	(0.006)	(0.013)	(0.01)	(0.001)	(0.008)		(0.011)	(0.005)
Female	0.072	0.070	0.066	0.075	0.079	0.070	—	0.076	0.068
	(0.005)	(0.01)	(0.012)	(0.007)	(0.002)	(0.004)		(0.002)	(0.003)

[a]The permethrin used was part of a formulation: Bunnings Ant, Spider and Cockroach Killer.
*Significant differences ($p < 0.05$) between that treatment and the control with no amendment ("0"). Different letters indicate the significant differences ($p < 0.05$) among the treatments for a specific endpoint for a specific concentration level.
OC = organic carbon.

TABLE 3: Spiked sediment bioassays with carbaryl (170 and 340 µg/g organic carbon) evaluating 2 activated carbon amendment addition ratios (wet wt/wet wt: 1 and 5%) of activated carbon using *Chironomus tepperi*[a]

| | Carbaryl[b] | | | | | | | | |
| | Control | | | 170 µg/g OC | | | 340 µg/g OC | | |
Endpoint	0	1%	5%	0	1%	5%	0	1%	5%
% Survival	98	100	100	52.5*	100	100	42.5*	100	97.5
	(5)	(0)	(0)	(12.6)A	(0)B	(0)B	(17.1)X	(0)Y	(5)Y
Growth (mg/individual)	0.95	0.85	0.72	0.47*	1.01	0.69*	0.26*	0.85	0.70*
	(0.19)	(0.11)	(0.09)	(0.18)A	(0.10)B	(0.05)A	(0.08)X	(0.13)Y	(0.08)Y
% Emergence	85	90	85	40*	85	85	5.0*	90	85
	(10)	(8.2)	(12.9)	(25)A	(13)B	(13)B	(5.8)X	(11.5)Y	(12.9)Y
Mean development rate (1/d)									
Male	0.077	0.071	0.064	0.078	0.081	0.071	—	0.073	0.061
	(0.011)	(0.008)	(0.004)	(0.01)	(0.012)	(0.005)		(0.012)	(0.014)
Female	0.072	0.063	0.062	0.067	0.066	0.065	—	0.076	0.059
	(0.004)	(0.005)	(0.006)	(0.008)	(0.006)	(0.007)		(0.014)	(0.006)

[a]Different letters indicate significant differences ($p < 0.05$) among the treatments for a specific endpoint for a specific concentration level.
[b]The carbaryl used was part of a formulation: Richgro Carbaryl Caterpillar, Grasshopper, and Millipede Insecticide.
*Significant differences ($p < 0.05$) between that treatment and the control with no amendment ("0").
OC = organic carbon.

5% activated carbon (Table 3). Growth, however, showed the opposite trend to survival because larger increases in growth were observed with addition of 1 rather than 5% activated carbon for both chemicals (with the exception of the high concentration of permethrin). This result was not surprising because it was expected (based on the secondary effects of activated carbon bioassays) that the 5% addition of activated carbon alone would cause a secondary decreased growth effect and would only be able to increase growth back to the 5% activated carbon ratio control blank level (which was the case). Emergence of *C. tepperi* was significantly reduced at both the low and high concentrations for both spiked sediments with no activated carbon addition when compared with emergence in control organisms. Similar to survival, the 5% addition of activated carbon removed toxicity of permethrin to near control levels (>80%). Although the 1% activated carbon addition did show a significant increase in emergence when compared with unamended sediment, it did not decrease toxicity to control levels (Table 1). Both the 1 and 5% activated carbon additions increased emergence to near control-acceptable levels (>80%) in carbaryl-spiked sediment bioassays (Table 3). Excluding the secondary effect of the addition of activated carbon alone, no differences were noted between the mean development rates (for both males and females) for either activated carbon addition for both chemicals when compared with control levels (Tables 2 and 3). Evaluation of mean development rates was not possible in the high permethrin and carbaryl concentrations because of low emergence. The lack of change in mean development rate in spiked sediments regardless of the activated carbon addition used was unexpected because it was believed that the 5% addition would have caused a significant decrease in development, but that was not the case.

Contaminated field sediment bioassays

Organisms in all controls (amended and unamended) in the 3 field sediment bioassays exhibited high survival (>90%) and emergence (>80%). As expected from earlier work, growth and the mean development rate (for both males and

females) were significantly reduced with the addition of the 5% activated carbon amendment when compared with the remaining control treatments. Sediments collected from National Business Park and Mordialloc storm water retarding basins showed significantly lower *C. tepperi* survival and emergence when compared with the control (Figure 2). Growth
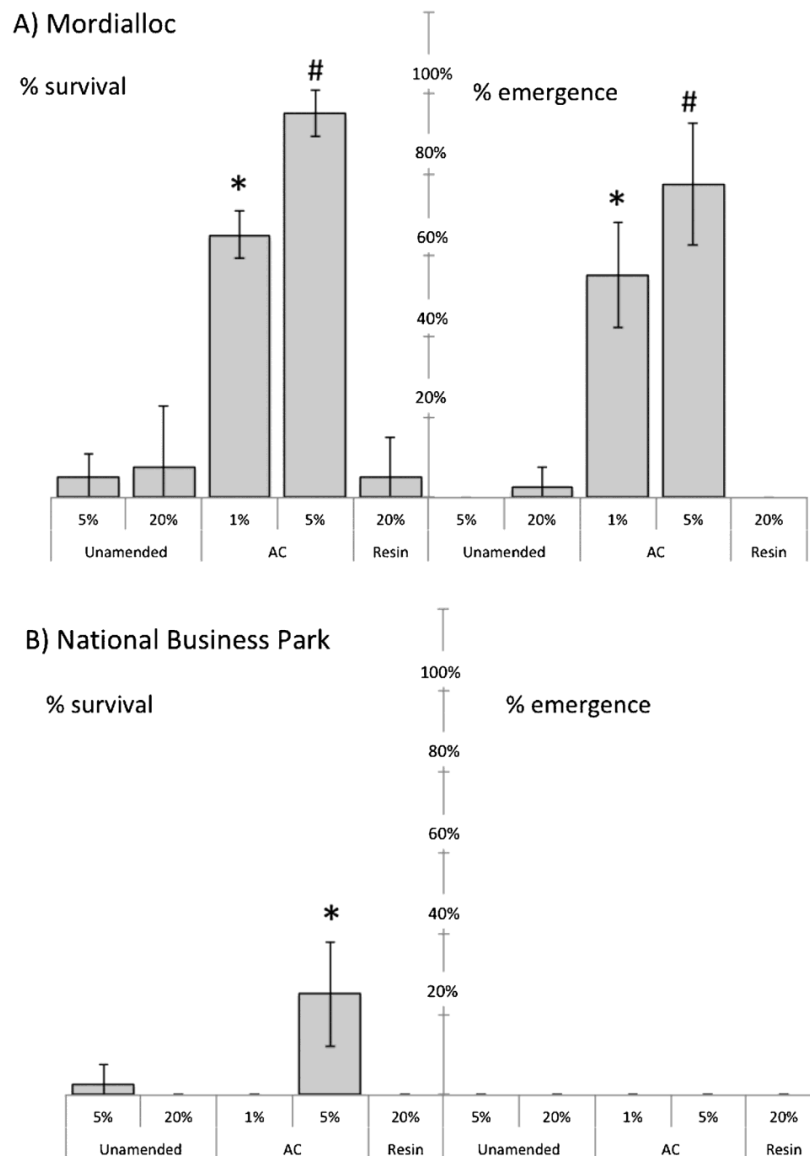
FIGURE 2: Survival and emergence results using toxicity identification evaluation (TIE) bioassays with *Chironomus tepperi* for 2 field site sediments from Victoria, Australia. The TIE bioassays utilized 2 amendment ratios (based on wet wt) of activated carbon and the cationic metals resin and compared those results with unamended sediment. Unamended sediments were amended with sand (at 5 and 20%) to account for any type of dilution effect that might have occurred. Control results (including those with amendment additions) were within acceptable limits (data not shown; Simpson and Batley 2016). * = Treatments that were significantly increased ($p < 0.05$) from the addition of the activated carbon amendment but yet also significantly lower than control. # = Treatments that were significantly increased ($p < 0.05$) from the addition of the activated carbon amendment and were not significantly different from control ($p > 0.05$; suggesting complete removal of toxicity for that endpoint.)

and mean development rate could not be evaluated in both of these sites because of low survival in these 2 test sediments. In sediment TIE bioassays from both sites, the cationic resin did not reduce toxicity, suggesting that heavy metals were not responsible for the noted toxicity. This result coincides with the analytical chemistry results for metals because the concentrations of metals in these sediments were below probable effect concentrations (Table 4) for nearly all metals evaluated (excluding zinc for both sites and mercury for National Business Park), which most likely overestimated risk in this scenario.

Both the 1 and 5% activated carbon additions significantly increased survival and emergence in the Mordialloc storm water retarding basin sediment, but only the 5% addition rate was able to reduce toxicity back to control levels for both survival and emergence. The National Business Park storm water retarding basin sediment was highly toxic (<5% survival in 5-d acute test and 0% emergence in the chronic 30-d test) and was only characterized for nonpolar organic toxicity using the 5% activated carbon addition. The analytical chemistry results support these characterizations of causality because concentrations of chlorpyrifos and cyhalothrin were slightly above the LC50 for *C. dilutus* for the Mordialloc storm water retarding basin and permethrin concentrations were 7 times greater than the LC50 for *C. dilutus* for the National Business Park storm water retarding basin (Table 4).

The Chandler storm water retarding basin unamended sediments (both the 5 and 20% sand dilutions) did not exhibit overt toxicity. Survival (>90%), growth (5% sand, 1.0 ± 0.18 mg/individual; 20% sand, 1.2 ± 0.25 mg/individual), and emergence (≥90%) in this unamended sediment were not significantly different from control. Interestingly, the mean development rates for *C. tepperi* for the unamended Chandler site, with the 20% sand dilution, were significantly higher (males, 0.106 ± 0.005; females, 0.100 ± 0.013) when compared with control organisms (males, 0.086 ± 0.011; females, 0.075 ± 0.004); and a similar, although not significant, trend was noted with the 5% sand dilution (unamended Chandler males, 0.100 ± 0.011; females, 0.098 ± 0.014; control males, 0.088 ± 0.006; females, 0.077 ± 0.010). Regardless, neither the activated carbon (1 or 5%) nor the resin changed the mean development rate in the Chandler field sediment when compared with the unamended sediment. In addition, the chemical concentrations for this site sediment suggested that most chemical concentrations were too low to cause adverse effects (Table 4). As such, the causes for the changes in mean development rate in this field sediment are unknown and require further investigation.

DISCUSSION

Species and chemical differences for activated carbon

The secondary effects of high levels of activated carbon and powdered coconut charcoal are well documented in the literature for many species, including those commonly used in

TABLE 4: Nonpolar organics and metal concentrations that were detected in at least one of the 3 storm water retarding basins[a]

	Toxicity threshold	Mordialloc	National Business Park	Chandler
Total organic carbon (%)	—	3.4%	7.5%	5.7%
Nonpolar organics (µg/g organic carbon)				
Chlorpyrifos	6.68	6.76[b]	6.53	BRL
Bifenthrin	6.2	3.24	2.67	BRL
Cyhalothrin	2.8	2.94[b]	BRL	BRL
Cypermethrin	13	4.41	6.27	BRL
Permethrin	24.5	BRL	186.7[b]	BRL
Dieldrin	12	0.71	BRL	BRL
Naphthalene	385	BRL	17.33	BRL
Phenanthrene	596	BRL	10.80	BRL
Pyrene	697	BRL	8.00	BRL
Benzo[*g,h,i*]perylene	1095	BRL	8.27	BRL
Metals (mg/kg)				
Antimony	NA	0.92	6.7	1.6
Arsenic	33	23	7.5	21
Cadmium	4.98	0.66	1.3	0.77
Chromium	111	42	74	56
Copper	149	71	310	91
Lead	128	51	95	79
Mercury	1.06	BRL	1.2[b]	BRL
Nickel	48.6	25	62	42
Silver	NA	0.61	6	BRL
Zinc	459	1030[b]	2220[b]	1150[b]

[a]The 50% lethal concentration (LC50) for chlorpyrifos for *Chironomus dilutus* is as reported by Harwood et al. (2009). The LC50 values for bifenthrin, cyhalothrin, and permethrin for *C. dilutus* are as reported by Maul et al. (2008). The LC50 for cypermethrin for *C. dilutus* is as reported by Maund et al. (2002). The equilibrium sediment benchmark for dieldrin is as reported by US Environmental Protection Agency (2003). The equilibrium sediment benchmarks for naphthalene, phenanthrene, pyrene, and benzo[*g,h,i*]perylene are as reported in Hansen et al (2003). The probable effect concentrations for the metals are as reported in MacDonald et al. (2000). Nonpolar organic chemicals that were below the reporting limit in all 3 field sites are not shown (which includes 31 organophosphates, 5 pyrethroids, 3 carbamates, 11 polycyclic aromatic hydrocarbons, and 22 organochlorines).
[b]Indicates a chemical was above the toxicity threshold.
BRL = below reporting limit; NA = not available.

sediment bioassays (Jonker et al. 2009; Mehler et al. 2017; Nybom et al. 2012, 2016; US Environmental Protection Agency 2007). These studies have also shown that a combination of factors including dose, particle size of the activated carbon, organism health, and organic carbon in the sediment may influence the toxicity of this amendment (US Environmental Protection Agency 2007; Lillicrap et al. 2015; Mehler et al. 2017). In addition, studies have shown that the ability to tolerate these additions varies by species (Kupryianchyk et al. 2012; Janssen and Beckingham 2013). These variations in toxicity make using a single activated carbon amendment ratio for multiple species problematic. For this reason, the whole-sediment TIE guidance suggests conducting pretesting to determine a threshold that does not elicit a toxic response for the chosen test species but that is high enough to reduce the bioavailability of nonpolar organics. Even in so doing, the chosen ratios used are usually "very close to concentrations that cause blank toxicity to these organisms" and, even when pretesting of the amendment has occurred, blank toxicity can still be possible (US Environmental Protection Agency 2007; Mehler et al. 2017). These same sentiments were recently echoed in whole-sediment TIE work in Australia, with this exact activated carbon amendment; preliminary testing suggested that the level of activated carbon used (1%) was not toxic, but during whole-sediment TIE the same amendment ratio elicited a toxic growth response (Mehler et al. 2017). Although the use of control blanks (amendment alone) as part of the TIE evaluation will help elucidate these potential false-negatives, it does not rectify the problem and assumes that all sediments behave in a similar manner to the control blank, which may not be the case. Current whole-sediment TIE guidance bases amendment addition on wet weight of the sediment, and this was also the case in the present study, which could be problematic because this means that additions might not be the same for all sediments (because not all sediments would have the same wet to dry ratio). This issue becomes even more complex considering that density and particle size differences would most likely also exist between the sand and activated carbon used in the TIE bioassays. Interestingly, the 2 control sediments (Glynn's Wetland and Bittern Reservoir) evaluated in the present study showed a similar response to activated carbon. However, the differences in growth rate between the 2 control sediments further confirm the variability that can exist between sites and can make choosing an appropriate activated carbon amendment ratio difficult (Figure 1).

The other aspect that could potentially affect the characterization of nonpolar organics using activated carbon is the nature of the contaminants themselves. The nonpolar organic–spiked sediment bioassays showed that the effectiveness of TIE methods can also be chemical-specific. Effects on emergence were similar at both high concentrations for both chemicals (carbaryl and permethrin, 5 and 3% emergence, respectively), but the effectiveness of toxicity reduction with the same addition ratio of amendment was starkly different. In the carbaryl bioassays both the 1 and 5% activated carbon amendment ratios returned toxicity to control levels, whereas in the permethrin bioassays only the 5% activated carbon amendment ratio was able to do this. This could be in part attributable to the

differences in the hydrophobicity and structure of each chemical. Permethrin has a high K_{OC} value (16 400–550 000 [Imgrund 2003]) and will bind more tightly to the sediment; thus, it may require a higher activated carbon addition ratio or a longer acclimation time with the activated carbon to ensure a more complete reduction of toxicity when compared with compounds with lower K_{OC} values, such as carbaryl (K_{OC}, 100–600 [Xu 1995]). Also, the chemical properties of each pesticide (i.e., structure) may play a role in the noted differences because the carbaryl structure is quite planar (when compared with permethrin), and this too would affect the binding of the chemical to the activated carbon. Coupling these differences with the notion that permethrin is generally considerably more toxic to invertebrates than carbaryl and that a similar amount of permethrin may be able to be bound by the activated carbon (in comparison with the carbaryl), the residual chemical would still have a higher likelihood of causing toxic effects (Xu 1995; Imgrund 2003; Parsons and Surgeoner 2008). These notions coincide with work using activated carbon for remediation purposes, which has suggested that a thorough understanding of the effectiveness of the activated carbon chosen and the potential ramifications to biota (among other things) should be considered prior to remediation application (Jonker et al. 2009; Beckingham and Ghosh 2011; Janssen and Beckingham 2013; Patmont et al. 2015). Collectively, these results suggest that a single activated carbon treatment level may not be appropriate for all test organisms or even all nonpolar organics.

Reducing false-negatives and defining causality

The main objective in whole-sediment TIEs is to determine causality in complex matrixes that have multiple contaminants present. The proposed method alterations of the present study effectively reduce the possibility of all 3 types of false-negatives (not toxic enough, too toxic, and secondary effects caused by the amendment) in whole-sediment TIE bioassays caused by the addition of activated carbon. If the sediment is not toxic enough or, in other words, if growth and/or survival was significantly different from the control, but only slightly, it is possible that the addition of the amendment would not be able to reduce the toxicity in a significant manner; thus, characterization would not be possible. In these events, under current guidance, testing may need to be repeated with additional replicates to determine causality. Although not observed in the present study, adding another and more sensitive endpoint, such as emergence (which has been shown to be more sensitive than survival or growth for many chemicals [Du et al. 2013, 2014]), would allow the amendment to reduce more toxicity (and possibly significantly) and enhance the likelihood of accurate characterization of contamination. One false-negative type that was reduced in the present study was a sediment sample being too toxic. The 5% activated carbon addition rate was able to reduce the acute toxicity in the National Business Park sediment, whereas the 1% activated carbon addition was not able to show a significant reduction. Whole-sediment TIE methods using only the 1% activated carbon addition would have either resulted in the mischaracterization of toxicity for this sediment or required

retesting using a dilution series to determine causality. With the 5% activated carbon addition this did not occur, and the addition was not completely overwhelmed (as was most likely the case for the 1% addition) because such causality could be determined without retesting. The last false-negative is the possibility of a secondary effect of the amendment itself. In the spiked bioassays and field contaminated sediment the 1% activated carbon amendment ratio did not elicit a toxic effect to any test endpoint (which was the goal of this amendment ratio). However, as noted earlier, the amount of amendment chosen is usually close to the amount that would cause toxicity. If a secondary effect of the amendment were noted (especially if using only the growth endpoint), testing may need to be repeated, especially if the sediment also elicits the "not toxic enough" false-negative. Rather than retesting, the emergence results would most likely provide the necessary information for accurate characterization. The complexity of these types of false-negatives illustrates why having additional endpoints and amendment ratios could simplify interpretation. Outside of rectifying false-negatives, the proposed method alterations (adding chronic endpoints and expanding the treatment additions of activated carbon) also provide a thorough evaluation of causality and a more sensitive evaluation of risk.

Additional advantages

The combination of additional amendment ratios and endpoints not only provided a more conclusive evaluation of causality but also, in some scenarios, elucidated the degree of contamination. The low–permethrin concentration sediment can be used to illustrate this point. In this example, low mortality was observed (not significantly different from control), so the growth results would then generally be consulted to define risk as part of a whole-sediment TIE. The addition of 1% activated carbon increased growth significantly, and thus the use of 1% activated carbon alone with standard whole-sediment TIE practices was successful. Although the 1% activated carbon addition alone was successful (as expected), by adding the 5% addition a significant increase in survival was also observed, providing further evidence to support the causality assessment. In addition, emergence as an endpoint coupled with the 2 activated carbon addition ratios lends further evidence to support causality caused by nonpolar organics and provides additional proof to show the extent of risk to aquatic life. The 5% activated carbon addition also suggested that a nonpolar organic might be the sole source of toxicity because it was able to increase survival, growth, and emergence to near control levels, something that was not evident by simply using the 1% activated carbon addition. The degree of contamination is an attribute that in many cases might not be evaluable using one amendment ratio alone (because low amendment ratios may be overwhelmed). This better understanding of causality was noted not only in the low–permethrin concentration bioassay but also in the Mordialloc storm water retarding basin field sediment (because toxicity was completely removed with the 5% activated carbon amendment ratio but not the 1%).

This combination of additional amendment ratios and additional endpoints not only provides a better and more thorough investigation of causality and risk than current guidance but also importantly comes at not much additional cost or time (~2–3 wk with this species). The current guidance outlines that pretesting needs to be completed prior to any TIE work, which is consistent with our findings as well. Pretesting would be similar to current guidance to evaluate multiple activated carbon amendment ratios, with the only additional cost being associated with the additional replicates required for the added endpoints (emergence and mean development rate). The other factor associated with time and cost discussed in the guidance is the number of replications and possible dilution experiments. Because of the size of bioassays, the number of replications in current whole-sediment TIE guidance is usually 3 to 4, which is a "workable compromise between statistical power and practicality" (US Environmental Protection Agency 2007). Guidance states that additional replicates may be warranted in circumstances with low toxicity (i.e., not toxic enough). Similarly, the guidance states that in circumstances in which complete toxicity has occurred subsequent dilution studies may be needed to define causality (i.e., too toxic). As previously noted, the additional activated carbon amendment ratios and endpoints could alleviate the need for additional testing because the more sensitive endpoints (i.e., emergence) should be usable to assess causality for the "not toxic enough" scenario, and the higher amendment ratio (5%) should be usable to assess the "too toxic" scenario. In these circumstances, the additional activated carbon amendment ratios and endpoints could save time and money in comparison with current guidance.

Further improving the whole-sediment TIE procedure

Adding endpoints (namely emergence) add a measure that, as previously noted, is more sensitive than either survival or growth for many chemicals (Du et al. 2013, 2014). "Chronic" endpoints (such as emergence and mean development rate) have been utilized in TIEs for primarily effluents (US Environmental Protection Agency 1992, 1993), with little use in whole-sediment TIEs to date. Although the use of these endpoints provides a more sensitive evaluation of risk, in many cases the use of *Chironomus* may still underestimate risk because this species is considered very tolerant (Carew et al. 2011; Mehler et al. 2017). However, the proposed alterations could also be used with more sensitive species. For example, amphipods (i.e., *Hyalella azteca*) have also shown reduced growth and in some cases mortality with the addition of the nonpolar organic amendment (US Environmental Protection Agency 2007). Adding endpoints (perhaps ability to molt and/or reproduction) and activated carbon treatments could further enhance the accuracy and influence of whole-sediment TIEs for this species as well. Rather than changing test species, more sensitive endpoints could be used with *Chironomus* as well. Initially, the hope was that mean development rate could be one of these endpoints; however, the results from the Chandler retarding basin sediment suggest that this might

not be a suitable endpoint. The increased mean development rate of the sediment when compared with control could have been attributable to a combination of factors, such as organic carbon differences, nutrient loadings, or other sediment factors (Ristola et al. 1999). Studies have also shown increases (Boyle et al. 2016) and decreases (Hatakeyama and Yasuno 1981; Goedkoop et al. 2010) in mean development rate attributable to the presence of contamination; hence, characterizing risk using this endpoint alone is difficult and should be done with caution. Further work to understand the significance of an alteration in mean development rate and its implications in understanding risk is needed before this could be used as a suitable endpoint in whole-sediment TIE testing. Additional evaluations using biomarkers (such as lipid content), metabolomics, or other behavioral/physiology endpoints may make the use of *Chironomus* more environmentally relevant. For instance, wing length has been used successfully in multiple studies to characterize fitness of *Chironomus* when exposed to contaminants (Frouz et al. 2002; Goedkoop et al. 2010; Boyle et al. 2016). Further work to better understand the overall sensitivity of these additional endpoints to contaminants as well as their sensitivity to activated carbon would enhance the accuracy of whole-sediment TIEs.

CONCLUSIONS

The present results suggest that the use of multiple activated carbon addition rates (in this case, 1 and 5%) as well as the use of additional endpoints (in this case, emergence) could resolve many of the false-negative issues of activated carbon in whole-sediment TIE testing with *C. tepperi*. The proposed method reduces the likelihood of all 3 types of false-negatives (not toxic enough, too toxic, and secondary effects of amendments), while providing a more accurate characterization of causality and a more sensitive and robust evaluation of risk. Although the species used, *C. tepperi*, may be rather tolerant, the proposed method was shown to be successful using both spiked and field sediments from Victoria, Australia, and appears to be a promising method for characterizing toxicity in sediments impacted by nonpolar organics.

REFERENCES

Beckingham B, Ghosh U. 2011. Field-scale reduction of PCB bioavailability with activated carbon amendment to river sediments. *Environ Sci Technol* 45:10567–10574.

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. Scientific Investigations Report 2011–5225. US Geological Survey, Reston, VA.

Boyle RL, Hoak MN, Pettigrove VJ, Hoffmann AA, Long SM. 2016. Comparing the impacts of sediment-bound bifenthrin on aquatic macroinvertebrates in laboratory bioassays and field microcosms. *Ecotox Environ Safe* 133:489–500.

Carew ME, Miller AD, Hoffmann A. 2011. Phylogenetic signals and ecotoxicological responses: Potential implications for aquatic biomonitoring. *Ecotoxicology* 20:595–606.

Du J, Li Y, Huang ZC, You J. 2014. Chronic toxicity thresholds for sediment-associated benzo[a]pyrene in the midge (*Chironomus dilutus*). *Arch Environ Con Tox* 66:370–378.

Du J, Pang J, You J. 2013. Bioavailability-based chronic toxicity measurements of permethrin to *Chironomus dilutus*. *Environ Toxicol Chem* 32:1403–1411.

Frouz J, Ali A, Lobinske RJ. 2002. Influence of temperature on developmental rate, wing length, and larval head capsule size of pestiferous midge *Chironomus crassicaudatus* (Diptera: Chironomidae). *J Econ Entomol* 95:699–705.

Goedkoop W, Spann N, Åkerblom N. 2010. Sublethal and sex-specific cypermethrin effects in toxicity tests with the midge *Chironomus riparius* Meigen. *Ecotoxicology* 19:1201–1208.

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.

Hansen DJ, DiToro DM, McGrath JA, Swartz RC, Mount DR, Spehar RL, Burgess RM, Ozretich RJ, Bell HE, Linton TK. 2003. Procedures for the derivation of equilibrium partitioning sediment benchmarks (ESBs) for the protection of benthic organisms: PAH mixtures. EPA/600/R-02/013. US Environmental Protection Agency, Washington, DC.

Harwood AD, You J, Lydy MJ. 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: Toxicokinetic confirmation. *Environ Toxicol Chem* 28:1051–1058.

Hatakeyama S, Yasuno M. 1981. A method for assessing chronic effects of toxic substances on the midge, *Paratanytarsus parthenogeneticus*—Effects of copper. *Arch Environ Con Tox* 10:705–713.

Ho KT, Burgess RM. 2013. What's causing toxicity in sediments? Results of 20 years of toxicity identification and evaluations. *Environ Toxicol Chem* 32:2424–2432.

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.

Imgrund H. 2003. Environmental fate of permethrin. California Department of Pesticide Regulation, Sacramento, CA.

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

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 Phys C* 162:1–6.

Jonker MTO, Suijkerbuijk MPW, Schmitt H, Sinnige TL. 2009. Ecotoxicological effects of activated carbon addition to sediments. *Environ Sci Technol* 43:5959–5966.

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.

Kupryianchyk D, Peeters ET, Rakowska MI, Reichman EP, Grotenhuis JT, Koelmans AA. 2012. Long-term recovery of benthic communities in sediments amended with activated carbon. *Environ Sci Technol* 46:10735–10742.

Lillicrap A, Schaanning M, Macken A. 2015. Assessment of the direct effects of biogenic and petrogenic activated carbon on benthic organisms. *Environ Sci Technol* 49:3705–3710.

MacDonald DD, Ingersoll CG, Berger TA. 2000. Development and evaluation of consensus-based sediment quality guidelines for freshwater ecosystems. *Arch Environ Con Tox* 39:20–31.

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

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

Maul JD, Brennan AA, Harwood AD, Lydy MJ. 2008. Effect of sediment-associated pyrethroids, fipronil, and metabolites on *Chironomus tentans* growth rate, body mass, condition index, immobilization, and survival. *Environ Toxicol Chem* 27:2582–2590.

Maund SJ, Hamer MJ, Lane MCG, Farrelly E, Rapley JH, Goggin UM, Gentle WE. 2002. Partitioning, bioavailability, and toxicity of the pyrethroid insecticide cypermethrin in sediments. *Environ Toxicol Chem* 21:9–15.

Mehler WT, Keough MJ, Pettigrove V. 2017. Development of whole-sediment toxicity identification evaluation (TIE) techniques for two Australian freshwater species: *Chironomus tepperi* and *Austrochiltonia subtenuis*. *Environ Toxicol Chem* 36:2476–2484.

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* 29:158–67.

Mehler WT, You J, Keough MJ, Lydy MJ, Pettigrove V. 2018. Improvements and cost-effective measures to the automated intermittent water renewal system for toxicity testing with sediments. *Ecotoxicol Environ Saf* 151:62–67.

Morris L, Keough MJ. 2002. Organic pollution and its effects: A short-term transplant experiment to assess the ability of biological endpoints to detect change in a soft sediment environment. *Mar Ecol-Prog Ser* 225:109–121.

Nybom I, Abel S, Mäenpää K, Akkanen J. 2016. Effects of activated carbon amended sediment on biological responses in *Chironomus riparius* multi-generation testing. *J Hazard Mater* 318:388–395.

Nybom I, Werner D, Leppänen MT, Siavalas G, Christanis K, Karapanagioti HK, Kukkonen JVK, Akkanen J. 2012. Responses of *Lumbriculus variegatus* to activated carbon amendments in uncontaminated sediments. *Environ Sci Technol* 46:12895–12903.

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.

Parsons JT, Surgeoner GA. 2008. Effect of exposure time on the acute toxicities of permethrin, fenitrothion, carbaryl and carbofuran to mosquito larvae. *Environ Toxicol Chem* 10:1219–1227.

Patmont CR, Ghosh U, LaRosa P, Menzie CA, Luthy RG, Greenberg MS, Cornelissen G, Eek E, Collins J, Hull J, Hjartland T, Glaza E, Bleiler J, Quadrini J. 2015. In situ sediment treatment using activated carbon: A demonstrated sediment cleanup technology. *Integr Environ Assess Manag* 11:195–207.

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

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

Ristola T, Pellinen J, Ruokolainen M, Kostamo A, Kukkonen JVK. 1999. Effect of sediment type, feeding level, and larval density on growth and development of a midge (*Chironomus riparius*). *Environ Toxicol Chem* 18:756–764.

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

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.

Simpson SL, Batley GE. 2016. *Sediment Quality Assessment: A Practical Guide*, 2nd ed. CSIRO, Clayton, VIC, Australia.

Stevens MM. 1998. Development and survival of *Chironomus tepperi* Skuse (Diptera:Chironomidae) at a range of constant temperatures. *Aquatic Insects* 20:181–188.

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

US Environmental Protection Agency. 1992. EPA toxicity identification evaluation: Characterization of chronically toxic effluents, Phase I evaluation. EPA/600/6-91/005F. Duluth, MN.

US Environmental Protection Agency. 1993. Methods for aquatic toxicity identification evaluations: Phase II toxicity identification procedures for samples exhibiting acute and chronic toxicity. EPA/600/R-92/080. Duluth, MN.

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

US Environmental Protection Agency. 2003. Procedures for the derivation of equilibrium partitioning sediment benchmarks (ESBs) for the protection of benthic organisms: Dieldrin. EPA/600/R-02/010. US Environmental Protection Agency, Washington, DC.

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

Xu S. 1995. Environmental fate of carbaryl. California Department of Pesticide Regulation, Sacramento, CA.

CHAPTER 5: THE ROLE OF ACIDIC pH IN FRESHWATER TASMANIAN MINING SEDIMENT TOXICITY

ABSTRACT

Mining-impacted aquatic systems could be at risk from various pollutants, including metals, sulfate, and acidic pH. The present study first evaluated toxicity of mining site sediments from western Tasmania using a conventional contaminant-based approach (i.e. comparing chemical concentrations (in this case metals, sulfate, and acidic pH) to published literature (such as sediment quality guidelines), but this provided only a limited understanding of causality (as all three contaminants appeared to be causing toxicity). In similarly complex sediments, toxicity identification evaluation (TIE) techniques have been employed to provide a better understanding of causality, so a subset of these site sediments was evaluated using a modified whole-sediment TIE technique wherein a cationic resin (to characterize metals toxicity) and an anionic resin (to characterize sulfate and acidic pH toxicity) were utilized. Anionic resins reduced toxicity completely in TIE bioassays while also reducing the target contaminants (sulfate and acidic pH). However, since metals bioavailability (and toxicity) is highly dependent on pH, metal toxicity could not be discounted. Unexpectedly, the cationic resin also removed toxicity completely, but this was believed to be due to a reduction of the acidic nature of the sediment rather than removing metals (as the resin did not reduce metals concentrations directly). These results are problematic because the causal role of each of these constituents could not be differentiated using current cationic TIE approaches. Although the modified TIE was unsuccessful in pinpointing the most important pollutant, the present study shows the importance of acidic pH in these site sediments as well as the potential, with further refinement, of TIE procedures in mining sediments.

Keywords: mining impact assessment, toxicity identification evaluation (TIE), metals, acidity, sulfate

5.1. INTRODUCTION

Australia has the some of the world's largest deposits of lead, iron ore, rutile, zircon, nickel, uranium and zinc (where profitable extraction is possible; [1]). Western Tasmania is one such area where mining has been abundant and profitable [2,3]. In 2014 – 2015,

approximately 59% of Tasmania's export income was associated with mining production [3]. Although many mines have closed in the area due to plummeting metal prices and increasing production costs, mining is still one of the central economic staples of this Australian state. Mining activities are not only economically important for this area, but also ecologically important as discharges from current mines and seepage from active and closed mines pose risks to aquatic local ecosystems.

Mining activities in western Tasmania over the last 100 years have resulted in a large amount of sedimentation and releases of slag, mining tail waste, and acid mine drainage (AMD). Acid mine drainage is a problem as it contains high concentrations of metals and the oxidation of pyrite results in high concentrations of sulfate (which can cause osmoregulatory stress in organisms [4]) and sulfuric acid [5]. The combination of these contaminants has led to the severe deterioration of many local aquatic ecosystems, and this toxicity is complex to interpret [6–8]. Research to understand the risk of aquatic contaminants in freshwater mining systems of Tasmania has been quite limited to date [6, 7], with a majority of research in Tasmania focusing on the presence of metals in effluents or overlying water. Although the risks of metals contamination have been studied in the area, the risk of other contaminants caused by mining, including sulfuric acid (resulting in acidic pH) and sulfate, have generally been overlooked.

Additionally, research in these Tasmanian systems (which has been mainly marine and estuarine focused) has investigated risk (typically of metals) to aquatic biota using the conventional contaminant-based approach [10,11]. The conventional approach evaluates risk by comparing chemical concentrations of the chosen test media to published laboratory studies and compares these relationships to observed effects of a chosen test species. The limitations of this approach have been well discussed and include: (1) a lack of available threshold or effects data, (2) inability to quantify all potential contaminants (3) a lack of understanding of possible mixture effects, and (4) the effects of bioavailability and other physical factors (including issues such as acidity) on toxicity are difficult to interpolate [12]. In response, researchers have developed an alternative approach, the toxicity identification evaluation (or TIE).

Whole-sediment TIEs bioassays use manipulations of the sediment to change toxicity of a certain contaminant class to "define causality" (i.e. determine whether it contributes to sediment toxicity). Whole-sediment TIE techniques are currently employed in complex site sediments wherein the cause of toxicity is unknown or where there is a

mixture of contaminants. To date, however, TIEs have rarely been used to assess mine-impacted aquatic systems [13,14], and have instead been applied predominantly in urban, industrial and agricultural environments [15–17]. Contamination issues from mines are as complex as in these other environments, but chemically quite distinct (acidic pH, sulfate, and metal contamination; [5]). Development of TIE methods specific to mine-impacted systems therefore stands to improve our understanding of aquatic impacts of this major international land use.

The present study first evaluated sediment toxicity in mining areas of western Tasmania using the conventional contaminant-based approach. These investigations highlighted the current limitations with this approach and suggested that TIE techniques were warranted. The present study also attempted to develop a whole-sediment TIE technique specifically for mining-impacted sediments. Rather than using traditional amendments in the whole-sediment TIE (such as activated carbon for non-polar organics or zeolite for ammonia [18]), we used amendments specifically for mining associated contaminants. These were a cationic resin and an anionic resin, which would elucidate the roles that metals and sulfate/acidity, respectively, play in sediment of mining impacted systems.

5.2. MATERIALS AND METHODS

5.2.1. Organisms

In conventional bioassays (i.e. non-TIE bioassays), two freshwater organisms were used as test species, the midge *Chironomus tepperi* and the amphipod *Austrochiltonia subtenuis.* These species have been used in initial whole-sediment TIE work in Australia [19] and are starting to be used more broadly in ecotoxicology work in Australia [20–22]. Midge cultures were originally acquired from temporary ponds in Yanco Agricultural Institute (New South Wales, Australia). Cultures for this species were maintained in ethanol-sterilized tissue paper using modified Martin's solution [23,24]. For conducting experiments, adult midges were collected from the cultures and allowed to breed. Egg masses from adults were collected and resulting larvae were used in testing after 7 days (resulting in 5 - 7 day old larvae, second instar).

Cultures of the freshwater amphipod were originally obtained from two locations in Victoria, Deep Creek (Bulla Rd, Victoria, Australia) and Devilbend Reservoir (Hodgins Rd,

Tuerong, Victoria, Australia). The cultures were maintained in aquaria with mesh gauze (as a substrate) using artificial water based on on-site conditions (see **Table S1** for artificial water recipes for both species). Cultures were sieved using two sieves, 212 and 297 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.

5.2.2. Site sampling and sediment preparation

Sediments from twelve sites were chosen for analysis in Tasmania based on accessibility and proximity to mine locations (**Figure 1**). Of the twelve sites, three sites were also chosen as controls in the area, based on their relative remoteness from mines and relatively low contaminant levels (sites: C2, C3, and C4). All sites were chosen from one of four mining areas including: Waratah (W1, C2, C3), Queenstown (Q1-Q5), Zeehan (Z1-Z2), and Que River (QR1, C4). Different mines were present at each location with different metals being mined at each: this includes the Mt. Bischoff Bluestone Mines in Waratah (mainly tin mining), the Mount Lyell CMT mine in Queenstown (primarily copper, silver, and gold mining), the Ivy Resources Helleyer Tailings, the Bass Metals Fossey Zone, the Bass Metals Que River along the Que River (mainly silver, zinc, gold, lead, and copper mining), and in Zeehan the Bluestone Mines Renison Bell (mining tin and copper) as well as Stellar Resources Mine (mining primarily tin and copper). Many of these mines are still in use (such as those in Queenstown), but some have also been abandoned (such as those in Zeehan). An additional control (C1) from the Melbourne area (Bittern Reservoir (Tuerong, VIC)) was also used, and although the geochemistry and sediment characteristic of this control would differ from Tasmanian sediments, it was also evaluated at it has been previously shown not to elicit sub-lethal toxic responses and has limited contamination [19].

This product incorporates data that is copyright owned by the Crown in Right of Tasmania. The data has been used in the product with the permission of the Crown in Right of Tasmania. The Crown in Right of Tasmania and its employee and agents: (a) give no warranty regarding the data's accuracy completeness, currency, or suitability for any particular purpose; and (b) do not accept liability, howsoever arising, including but not limited to negligence, for any loss resulting from the use of or reliance upon the data

Figure 1. Location of field sites in Tasmania (sites C2 – C4; W1; QR1; Q1-Q5; and Z1-Z2).

Sediment collection occurred in June of 2017. In the field, sediment was sieved through a 500 µm net. Sediments were transported back to The University of Melbourne in 20 L buckets and stored at 4 °C in the dark until use [25]. Before sediment was used, sediments were decanted and thoroughly mixed using an impact drill with a paddle mix rotary bit.

5.2.3. Conventional bioassays

To evaluate the toxicity of sediments, 'conventional' whole-sediment bioassays were conducted with sediments from the twelve Tasmanian sites. Bioassays were conducted for all 12 site sediments in 350 mL beakers with approximately 60 g wet wt. sediment and 250 mL of artificial water [19]. Artificial waters were prepared in the same manner as culture water for the respective species. Toxicity testing was conducted using a standard photoperiod of 16:8 light: dark and a temperature of 21 ± 1 °C. Water quality parameters (dissolved oxygen, pH, conductivity, and temperature) were measured every two days for both midge and amphipod bioassays. For all bioassays, water changes occurred twice per day using a static renewal system (150 - 200 mL per change). Replicates containing midges were fed every other day using a suspended solution of tetramin (10 mg tetramin per replicate), while replicates containing the amphipods were fed tetramin and yeast-cerophyll-trout chow mixture (YCT; 0.9 mg per replicate every other day).

Bioassays with *A. subtenuis* were conducted over 10 d and survival recorded using five replicates per treatment and ten organisms per replicate. Bioassays with midges evaluated four endpoints, including survival, growth, emergence, and mean development rate, with eight replicates used per treatment and ten organisms per replicate. Four midge replicates were terminated at 5 d to assess acute toxicity endpoints (*i.e.* survival and growth) and the remaining four were used to evaluate emergence and mean development rate. Surviving midges from each replicate of the acute portion of the study were dried at 90 °C to a constant temperature (using a Memmert drying oven for ~48 h) and weighed using a Kern ABS/ABJ Analytical Balance (reproducibility ± 0.1 mg; Kern & Sohn) to assess growth [26]. The remaining four replicates were covered using nylon stockings to avoid losing emerged adults and were subsequently evaluated for emergence and mean development rate over 30 d. Emergence was evaluated daily and the date and sex recorded at time of emergence. The mean development rate (MDR) calculations are discussed in greater detail in the supplemental section. Using this approach, larger values correspond to

faster emergence rates. Males and females are analyzed separately as males emerge earlier than females [27].

5.2.4. TIE amendments and preparation

We developed a TIE method aimed at differentiating sulfate/acidic pH and metal toxicity in sediments. We sourced amendments that we expected would attenuate the toxicity of these respective contaminant types, with minimal impacts on the toxicity of non-target contaminants. We therefore sourced the commercial resins Lewatit MonoPlus TP 207 and Lewatit A365 (Lanxess Deutschland GmbH). Lewatit MonoPlus TP 207 is a weakly acidic, macroporous cation exchange agent with chelating iminodiacetate groups, which preferentially binds cationic metals (and is hereby referred to as the 'cationic resin [28]'). This product has been previously used in whole-sediment TIE work in Australia [19]. Lewatit A365 (which will be referred to as the 'anionic resin') is a weakly basic anion exchange resin based on a crosslinked polyacrylate, which is able to remove large anions like sulfate but also acids such as sulfuric acid among others [29]. To be cost-effective and practical for future Australasian studies, both TIE amendments used were locally sourced and readily available in Australia via FilChem Australia Pty Ltd.

Before use, both resins were altered from their original form (resins are shipped as sodium (TP207) or free base (A365)), as this form cannot be used for TIE purposes directly. To accomplish a base change, approximately 700 g of resin was stored in 1 L of 2 M $CaCl_2$ $2H_20$ (for at least 24 h) at 4 °C. This transitions the resin to a calcium and chloride form for the metals and anionic resin, respectively. Prior to use, the resins were removed from the solution and rinsed repeatedly with deionized water until the conductivity of the decanted overlying water was below 250 μS/cm.

5.2.5. Whole-sediment TIE bioassays

To evaluate our whole-sediment TIE method for mining impacted sites, three acutely toxic sites were evaluated (W1, Q1, and Q5). These were evaluated using survival and growth as endpoints using the midge, *C. tepperi*. These three sites represented those which showed high toxicity of those sampled as based on screening bioassays, while also having differing levels and types of contaminants present. Unfortunately, additional bioassays with the other sites or with *A. subtenuis* could not be conducted due to limited availability of sediment.

Water quality, feeding, and water change frequency was run in the same manner in these tests as in conventional bioassays. For each site sediment, three types of treatments were evaluated: 'no amendment', 'anionic amended', and 'cationic amended'. The relevant amending agent (Lewatit MonoPlus TP 207 for 'cationic amended' and Lewatit A365 for 'anionic amended') or sand for the 'no amendment' (to account for any dilution effect) was added to sediments 3 d prior to the addition of test organisms. An amending ratio of 20% (via wet wt.) was used for both resins, based on previous work and published literature with similar metals amendments [18,19,30]. During this 3 d holding period, sediments were manually mixed then rolled on a Stovall low profile roller for at least two hours per day. A single replicate was added during this test to be used for analytical chemistry of the eluted resin. This replicate had no organisms added and no water changes performed to try to control contaminant loss via water changes and uptake by the organism. Resins were sieved from the replicate after the 5-d testing regime and thoroughly rinsed using RO water before being placed into a 50 mL falcon tube for resin elution.

5.2.6. Sediment Chemical Analysis

Commercial laboratories accredited to ISO 17025 and ISO 9001 carried out chemical analyses of total organic carbon, sulfate, and a suite of metals (21 metals) for all site sediments (**Table 1**). For quantifying concentrations in sediment of sulfate and metals, moisture content was first determined using a gravimetric procedure (dry at ~105 °C over a 12 h period). Afterwards, 1 g of air-dried sediment was refluxed and digested with 4 mL of 50% nitric and 10 mL of 20% hydrochloric acids for approximately two hours (for metals) and with 30 mL of HCl for sulfate analysis [31]. The metals solution was cooled and hydrogen peroxide (30%) was added and then heated and cooled again. Both solutions were then diluted to a volume of 50 mL using de-ionised water and allowed to settle prior to extraction for analysis. Concentrations of metals (Method 200.7; [31]) and sulfate in sediments were analyzed using Inductively Coupled Plasma – Atomic Emission Spectrometry. For quantifying concentrations in eluted resins, metals and anionic resins were weighed and then eluted with 20 mL of 7.5% HCL and 5% NaOH solution (by wt.), respectively. Resins were rolled with the eluting solution for roughly 10 hours before the solution was removed and analyzed using the same procedures as solutions from sediment samples. Total organic carbon (TOC) was analyzed by infrared detection (Dohrmann Chromatograph-190 TOC analyzer) after combustion at 800 °C and an acid reaction.

The reporting limit for sulfate was 1 mg/L, while metals reporting limits ranged from 0.001 to 0.05 mg/L (**Table S2**). As standard practice, method blanks, laboratory controls, matrix spikes and laboratory duplicates were run with each analysis and no outliers were observed. Laboratory spike recoveries for sediments were 87-111% and 68-136% for sulfate and metals, respectively. Laboratory spike recoveries of elution samples were 97-111% and 70-130% for sulfate and metals, respectively.

| | Control | | | | Mining Impacted | | | | | | | | |
| | | | | | Waratah | Que River | Queenstown | | | | | Zeehan | |
	C1	C2	C3	C4	W1	QR1	Q1	Q2	Q3	Q4	Q5	Z1	Z2
Total Organic Carbon (%)	1.2	6.62	8.41	6.77	4.97	9.02	1.96	0.69	5.16	1.3	0.3	7.63	7.35
Aluminium	18000	33200	51300	23200	4760	23100	2630	4680	9290	3210	2460	8990	9090
Barium	60	40	100	110	30	140	100	280	320	150	120	40	30
Beryllium	<1	<1	2	<1	2	<1	<1	<1	<1	<1	<1	2	2
Boron	<50	<50	<50	<50	<50	<50	90	<50	<50	100	100	<50	<50
Cobalt	4	24	34	67	<2	27	5	138	57	8	14	11	14
Iron	24800	105000	112000	120000	34500	47700	340000	274000	189000	340000	419000	256000	232000
Manganese	144	317	298	322	12	365	60	149	212	83	237	1400	813
Selenium	<5	<5	<5	<5	<5	6	<5	<5	<5	<5	<5	<5	<5
Sulfate (SO4 2-)	350	1560	1600	1960	4650	2860	105000	52000	9850	95600	147000	7520	3070
Tin	<5	<5	<5	<5	12	<5	<5	6	<5	<5	<5	21	25
Vanadium	49	105	164	90	64	40	26	21	39	30	46	16	40
Antimony	<5	7	<5	<5	9	8	13	11	6	13	15	50	114
Arsenic	<5	12	6	<5	133	163	16	48	32	21	31	525	436
Cadmium	<1	<1	<1	<10	<1	12	<10	<10	<10	<10	<10	<10	<10
Chromium	27	114	227	82	46	48	13	13	18	12	10	32	39
Copper	54	17	24	47	27	455	223	1500	786	403	715	196	116
Lead	15	80	22	23	60	1530	30	107	140	68	79	4930	7860
Mercury	<0.1	<0.1	<0.1	0.1	<0.1	0.3	<0.1	<0.1	0.2	0.1	<0.1	0.3	0.6
Nickel	8	28	94	117	6	50	3	20	19	4	3	26	41
Silver	<2	<2	<2	<2	<2	5	<2	<2	<2	<2	<2	15	21
Zinc	21	80	152	608	41	2620	19	215	195	47	56	2000	2160

Table 1. Total recovery metals in sediment (mg/kg) with field sites from Tasmania. Constituents below the double line in the table had available interim sediment quality guidelines - high (ISQG – High) values, whereas those above the double line in the table were unavailable. Shaded values are those sites where the concentration for the metal at that site exceeded the ISQG – high values [32].

ISQG-high values: Sb: 25 mg/kg; As: 70 mg/kg Cd: 10 mg/kg; Cr: 370 mg/kg; Cu: 270 mg/kg; Pb: 220 mg/kg; Hg: 1 mg/kg; Ni: 52 mg/kg; Ag: 3.7 mg/kg; and Zn: 410 mg/kg.

5.2.7. Statistical analysis

Statistical analyses were conducted using R [33,34]. Binomial data, survival (with both *C. tepperi* and *A. subtenuis*) and emergence (with *C. tepperi*), were first arcsine square-root transformed before statistical analysis. In the conventional bioassays, endpoints were analyzed separately using a single factor ANOVA after being tested for normality and homogeneity of variances. As statistical differences existed, a Tukey's post-hoc comparison test was done to compare the sites to one another. Similarly, in the TIE bioassays, the unamended treatments (controls and site sediments) were evaluated using a single factor ANOVA. If significant differences were noted for an individual site, the unamended and amended sediments were further evaluated using a Tukey's post-hoc comparison test to determine if the amendment significantly altered toxicity ($p < 0.05$).

5.3. RESULTS

5.3.1. Conventional bioassay – water quality

Dissolved oxygen (DO >70%) and temperature (21 ± 1°C) were consistent among all sites in the conventional bioassay with *C. tepperi*. Differences, however, were noted between the sites when comparing pH and conductivity. In the midge bioassays, Tasmanian controls (C2 - C4) had average pH (~6.3) and conductivity values (~177 µS/cm) that although lower were similar to the Victorian control (C1; pH: ~6.8 and conductivity: ~204 µS/cm). Most of the remaining impacted sites showed consistently lower pH values and higher conductivity, suggesting influence from acid mine drainage (**Table 2**). For instance, sites sampled in the Queen River at Queenstown showed pH levels as low as 3.5 during midge bioassays.

In the amphipod bioassay, DO and temperature readings were consistent with the midge bioassay. However, stark differences for pH and conductivity between sites were not noted with the amphipod bioassays. In these bioassays, the pH in all sites evaluated was between 7.1 and 7.5 and similarly, conductivity was rather consistent amongst all sites ranging from 1264 – 1387 µS/cm (Table 2). It is believed that the noted acidic pH values observed in midge bioassays were not observed in this bioassay due to the artificial water used (Table S1). This amphipod requires artificial water with a much higher salt content (when compared to midges), which most likely generated a buffering capacity that reduced the acidic pH effects that we encountered in the midge bioassay.

Table 2. Toxicity data and water quality data (pH and conductivity) for *C. tepperi* (survival, growth, emergence, and mean development rate (MDR)) and *A. subtenuis* (survival) conventional bioassays. Values in parentheses represent the standard deviation of the mean. Differing letters in a single row indicate significant differences amongst the site sediments for that endpoint. Shaded cells indicate those endpoints for a given site sediment that were significantly different from all three Tasmania control sediments. Growth and MDR could not be calculated for sites Q1, Q4, and Q5 due to low numbers of surviving midges.

	Control				Mining Impacted								
					Waratah	Que River	Queenstown					Zeehan	
	C1	C2	C3	C4	W1	QR1	Q1	Q2	Q3	Q4	Q5	Z1	Z2
Midge Bioassays (± standard deviation)													
pH	6.8 (0.3)	6.2 (0.5)	6.4 (0.4)	6.2 (0.6)	4.6 (0.6)	6.3 (0.5)	3.8 (0.3)	4.3 (0.6)	5.3 (0.8)	3.7 (0.2)	3.5 (0.2)	6.1 (0.6)	6.1 (0.6)
Conductivity (µS/cm)	204 (45.5)	175 (26.3)	185 (26.8)	171 (11.7)	198 (25.4)	181 (17.6)	267 (53.3)	218 (21.6)	186 (22)	295 (53.7)	395 (160)	174 (15.4)	177 (21.4)
Survival (%)	100 (0) *a*	92.5 (9.57) *a*	100 (0) *a*	95 (5.77) *a*	55 (12.91) *bc*	100 (0) *a*	12.5 (5) *de*	87.5 (12.58) *ab*	97.5 (5) *a*	25 (10) *cd*	0 (0) *e*	87.5 (25) *a*	85 (12.91) *a*
Growth (mg/individual)	1.34 (0.09) *a*	0.78 (0.1) *abc*	0.84 (0.09) *a*	0.81 (0.07) *ab*	0.41 (0.1) *bc*	0.6 (0.11) *bcd*	NA	0.39 (0.04) *d*	0.58 (0.11) *cd*	NA	NA	0.6 (0.09) *bcd*	0.68 (0.13) *abc*
Emergence (%)	93.3 (11.5) *a*	85 (19.1) *a*	80 (21.6) *a*	87.5 (9.6) *a*	0 (0) *b*	82.5 (12.6) *a*	0 (0) *b*	0 (0) *b*	65 (5) *a*	0 (0) *b*	0 (0) *b*	82.5 (17.1) *a*	62.5 (18.9) *a*
MDR — Males	0.094 (0.001) *a*	0.084 (0.006) *a*	0.085 (0.005) *a*	0.08 (0.005) *a*	NA	0.075 (0.006) *a*	NA	NA	0.074 (0.017) *a*	NA	NA	0.083 (0.007) *a*	0.057 (0.002) *b*
MDR — Females	0.089 (0.017)	0.067 (0.008)	0.084 (0.023)	0.073 (0.004)	NA	0.065 (0.005)	NA	NA	0.064 (0.006)	NA	NA	0.069 (0.011)	0.054 (0.006) *b*
Amphipod Bioassays (± standard deviation)													
pH	7.5 (0.3) *a*	7.3 (0.4) *a*	7.4 (0.4) *a*	7.4 (0.3) *a*	7.2 (0.4) *bc*	7.3 (0.5) *ab*	7.1 (0.5) *bc*	7.5 (0.4) *bc*	7.3 (0.3) *ab*	7.1 (0.7) *cd*	7.2 (0.5) *d*	7.5 (0.4) *ab*	7.4 (0.4) *ab*
Conductivity (mS/cm)	1.36 (0.14) *a*	1.29 (0.13) *a*	1.30 (0.13) *a*	1.29 (0.12) *a*	1.30 (0.13) *a*	1.32 (0.12) *a*	1.32 (0.11) *a*	1.36 (0.15) *a*	1.32 (0.13) *a*	1.33 (0.10) *a*	1.39 (0.13) *a*	1.32 (0.13) *a*	1.31 (0.15) *a*
Survival %	81 (13.42) *a*	82 (10.95) *ab*	85 (12.91) *a*	86 (13.42) *ab*	26 (11.4) *bc*	66 (20.74) *ab*	32 (27.75) *bc*	32 (21.68) *bc*	60 (18.71) *ab*	14 (16.73) *cd*	0 (0) *d*	72 (13.04) *ab*	62 (16.43) *b*

5.3.2. Conventional bioassay – toxicity

All Tasmanian sediments, including the three potential Tasmanian controls (sites: C2, C3, and C4), caused a lower growth in *C. tepperi* when compared to the Victorian control site (Table 2). Although the three controls (C2 - C4) had lower growth than the Victorian control (C1) they were still identified as controls as metals concentrations were relatively low and survival of both midges and amphipods were above 80%. Effects on growth in the midge could have been occurring due to moderately different contaminant levels, differing nutrient levels (i.e. nutrient levels are more likely to be lower in Tasmanian control sediments), or differences in sediment characteristics (e.g. particle size, etc.) and geochemistry between Tasmania and Victoria, and thus comparing Tasmanian sediments to Victoria sediments would be misleading.

Of the 9 mining sites, six (W1, Q1, Q2, Q4, Q5, and Z2) showed toxicity in at least one test endpoint for both test species. Site Z2 was unique in that only significant effects were noted for mean development rate of male midges and no other endpoint. The remaining five sites showed much more pronounced toxicological effects. In three of these sites, growth in *C. tepperi* could not be assessed due to low survival. Not surprisingly, emergence was even more sensitive in these sites as no organisms emerged from these site sediments (and hence why MDR could not be evaluated). In general, sites that exhibited toxicity exhibited it in both species. However, interestingly, midges and amphipods showed differing survival responses across sites, as in some circumstances midge survival was more sensitive (e.g. site: Q1) and in other sites amphipod survival was more sensitive (e.g. site W1 and Q2). Overall, sites closer to mining areas (Figure 1) showed greater impacts in both species (such as sites Q4 and Q5) when compared to sites further downstream (such as Q1-Q3).

5.3.3. Chemical analysis of metals and sulfate

In general, control sediments had low concentrations of metals (with the exception of a nickel and zinc in a few control sites), while mining site sediments showed moderate to very high levels of metal contamination (Table 1). Concentrations of copper were highest in the Queen River sites (Q1-Q5), which was not surprising as these sites are below a copper mine. Other metals varied significantly between the sites, with the highest concentrations being noted in Zeehan as well as the Que River sediments. Concentrations of sulfate ranged dramatically between the sites ranging from 1,560 – 1,960 and 2,860-147,000 mg/kg in

Tasmanian control sediments (C2-C4) and Tasmanian field sites, respectively (Table 1). As expected, sediments with low pH (via midge bioassay water quality data; Table 2) had corresponding higher sulfate concentrations. Interestingly, this relationship also held true for TOC as well, as sediment with low TOC had higher sulfate concentrations.

5.3.4. Whole-sediment TIE bioassays

Water quality data from the whole-sediment TIE are shown in **Table S3**. The three unamended acutely toxic sediments (W1, Q1, and Q5) showed similar water quality trends as in previous conventional bioassay results (i.e. DO: > 70%, 21 ± 1°C, pH 3.4 – 5, and conductivity: 192 - 435 μS/cm). Both the anionic and cationic resins increased pH levels to control levels in all three sites (pH range of 6.3 – 6.9). Conductivity generally increased in bioassays where the cationic resin was present and decreased where the anionic resin was present (Table S3), a trend that has been noted in previous work [19].

Toxicity data from the whole-sediment TIEs are shown in **Figure 2**. Unamended site sediments exhibited similar toxicity profiles as in the conventional bioassays. The anionic resin caused a significant decrease in growth in the Victorian control (C1), but this same effect was not observed in the Tasmanian control (C3). Surprisingly, nearly a 100% removal of toxicity (survival (for Q1 and Q5) and growth (Q1, Q5, and W1)) with both the cationic and anionic resins was noted for all three sites.

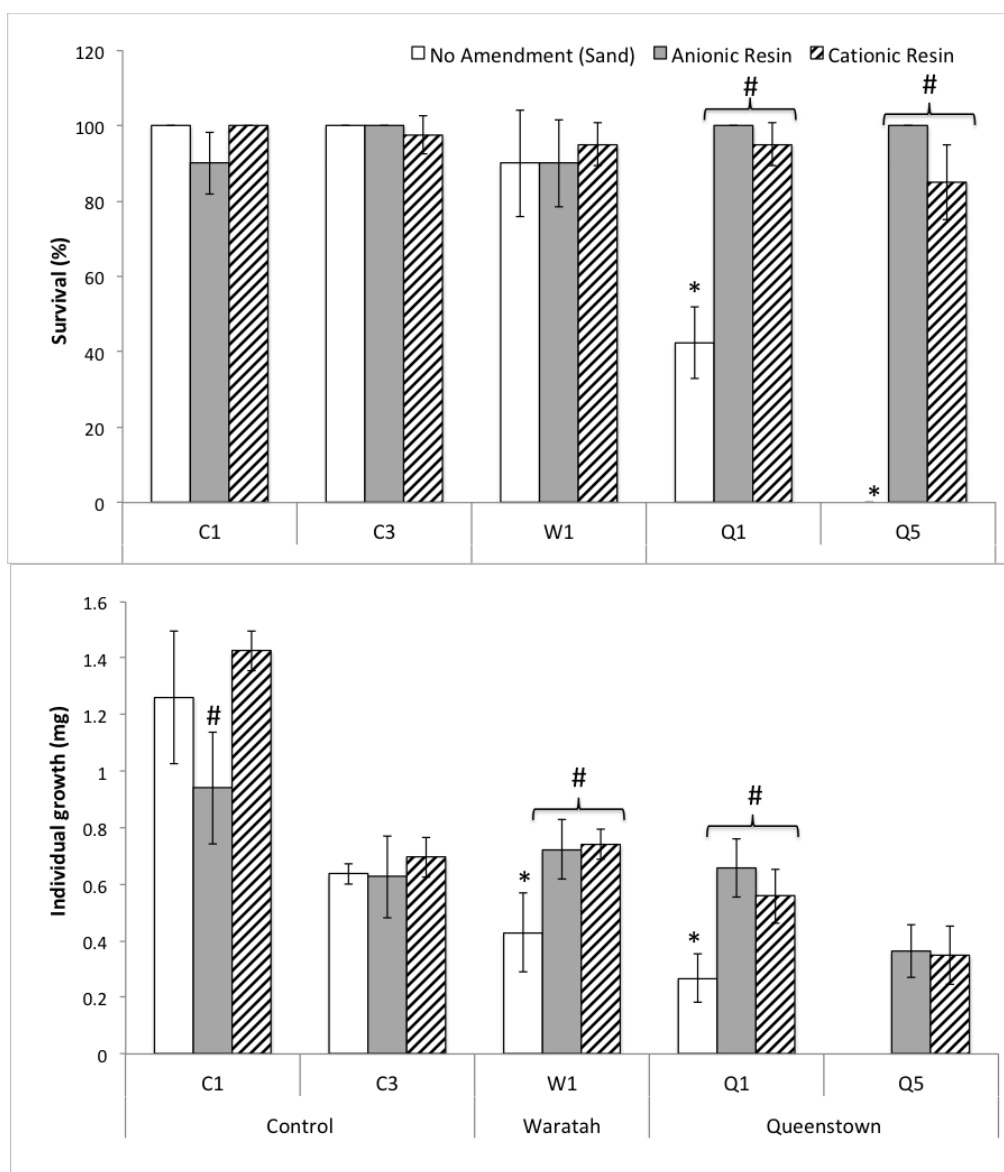Figure 2. Whole-sediment TIE results using no amendment (sand addition), anionic resin amendment, and cationic resin amendment. Asterisks (*) indicate where an unamended site treatment was significantly different from the Tasmania control (C3). Hashtag (#) indicates where toxicity was significantly altered in an unamended treatment by the addition of an amendment. Growth in site Q5 could not be assessed due to complete mortality.

5.3.5. Resin elution chemistry

The chemical analysis of the eluted resins showed that the anionic resin worked as expected removing sulfate and the acidic pH (as noted earlier with the pH increases) and only slightly reduced metal concentrations (**Table 3**). The resin reduced sulfate concentrations in these three site sediments by 24 – 55%. The cationic resin was only able to reduce total metal concentrations in sediments from these three sites by 0.3% - 7%. In fact, site W3 had similar reductions of total metal concentrations using both resins, showing the ineffectiveness of this resin. It should be noted, however, that the cationic resin also did not reduce sulfate concentrations (reduction of <1%).

	W1		Q1		Q5	
Resin Type	Anionic	Cationic	Anionic	Cationic	Anionic	Cationic
Aluminium	4.58	16.61	6.16	11.85	1.87	74.80
Antimony	0.07	0.01	0.01	0.01	BDL	BDL
Arsenic	0.36	0.10	0.03	0.04	BDL	0.05
Beryllium	BDL	0.01	BDL	BDL	BDL	BDL
Barium	0.03	0.18	0.16	1.24	0.03	1.00
Cadmium	BDL	0.01	BDL	0.00	BDL	0.00
Chromium	0.06	0.05	0.04	0.03	0.01	0.03
Cobalt	BDL	0.22	BDL	0.38	0.01	2.00
Copper	0.02	0.31	0.24	10.93	0.02	14.33
Lead	0.10	1.32	0.03	1.17	0.01	0.91
Manganese	0.01	1.59	0.43	17.54	0.17	102.08
Nickel	0.03	0.84	0.04	0.23	0.02	0.39
Selenium	BDL	BDL	BDL	BDL	BDL	BDL
Silver	BDL	0.01	BDL	0.03	BDL	BDL
Tin	0.03	0.02	0.06	0.08	0.02	0.13
Vanadium	0.23	1.19	BDL	BDL	BDL	0.12
Zinc	0.44	3.36	0.18	1.02	0.08	3.95
Boron	BDL	BDL	BDL	BDL	BDL	BDL
Iron	30.66	2776.72	221.20	3173.67	22.77	1236.78
ΣMetals	36.63	2802.57	228.58	3218.22	25.02	1436.57
Sulfate (as SO_4^{2-})	1115.80	6.81	57895.50	205.81	36416.24	1325.12

Table 3. Concentrations of sulfate and metals from eluted resins as converted back to sediment concentrations (mg/kg) for three toxic sites in Western Tasmania (sites: W1, Q1, and Q5). BDL – below detection limit.

5.4. DISCUSSION

5.4.1. Limitations of the conventional contaminant-based approach

As mentioned in the introduction, one of the major limitations with the conventional contaminant-based approach is an overall lack of threshold guidelines. Approximately half of the metals evaluated in the present study did not have associated ISQG threshold values and were not evaluated for risk any further. Additionally, other potential mining issues, such as acidic pH or sulfate, have not been focused on as intently as metals and as such are generally data poor. Although literature values exist for the classification of acidic pH and sulfate as potential toxicants, it is difficult to extrapolate. For instance, acidic pH has been studied with the midge *C. riparius* and, in soft water, survival can be impacted at pHs as low as 5.8 (30-d test: 52.3% survival at a pH of 5.8 and 11% at a pH of 4.5) [35]. In the present study, soft water was not used and the test duration was much shorter (5-d). However, as much lower pHs (as low as 3.5) was observed in these bioassays, one could speculate that the acidic pHs of these sediments could be causing direct toxicity. Similarly, for sulfate, water only bioassays (at a pH of 7.9) with midges (*Chironomus dilutus*) and amphipods (*Hyalella azteca*) are currently only available. No sediment data are available. This study suggests a relative high tolerance for midges as LC50 values for sulfate were 14,134 mg/L (48-h), while *H. azteca* was substantially more sensitive with LC50 values (96-h) of 502 mg/L [4]. Although comparing sediment concentrations to water concentrations are ill advised, concentrations of sulfate in sediments from Tasmania exceeded these concentrations dramatically, with concentrations of up to 146 g SO^-_4/kg (Table 2), suggesting that sulfate could also be directly causing toxicity. Another important point, is that although a large suite of metals was evaluated in this study, other metals and even other major cations/anions (such as calcium, sodium, chloride, nitrates) could have also been at high or even low enough (in the case of nutrient requirements) levels to cause effects at these sites, but due to cost restrictions in risk assessments not all possible sources of contamination can be evaluated [5].

Another issue with the conventional contaminant-based approach is that even when published literature is available for a given contaminant, various other environmental parameters (i.e. TOC, pH, conductivity, acid volatile sulfides (AVS), mixtures, etc.) can highly influence bioavailability and affect toxicity. For instance, metals concentrations in most of the Tasmania sites sampled (10 of 12 – which includes the three controls), exceeded at least one available interim sediment quality guideline threshold-high (ISQG – high) value [32] (Table 1). The only sites to not exceed a guideline value were sites C2 and Q1, and these

sites still exceeded at least one of the more conservative ISQG-low threshold values [32]. Each site would therefore be expected to induce toxicity. However, a large number of sites (including the controls) in fact did not exhibit toxicity. Similarly, the Zeehan site (Z2) and the Que River (QR1) showed the greatest number of interim sediment quality guideline threshold-high exceedances (5 and 6 exceedances, respectively) and the highest metal concentrations, but yet had relatively low toxicity. These results are perhaps even more surprising when the degree of exceedance is considered. For example, site Z2 had lead values that were approximately 220 fold higher than the ISQG – high threshold, but again only showed weak signs of toxicity (Table 2).

Comparing metals and sulfate concentrations as well as acidic pH values to available literature suggests that all three contaminants could be contributing to toxicity. However, once effects information from these sites (i.e. conventional bioassays) is also considered, it becomes evident that defining causality for many of these chemicals (namely metals) would not be reliable. Application of the conventional contaminant-based approach in the present study, for example, may have led to the erroneous inference that well-studied metals (such as Zn, Cu, or Pb) were the only toxicants, given the relative paucity of available data on possible alternative toxicants (e.g. sulfate and/or acidic pH toxicity). As such, the TIE approach was used in hopes to better resolve toxicity from these respective contaminant groups, without the need for an extensive literature of data for each.

5.4.2. Defining causality through the TIE

The anionic resin directly reduced toxicity of *C. tepperi* in whole-sediment TIEs back to control levels in these three mining sediments (Figure 2). Additionally, the resin removed both the acidic pH (via increases in pH; Table S3) as well as sulfate (Table 3), but not the metals present (Table 3). These results, under standard TIE procedures, would suggest that these contaminants (sulfate or acidic pH) are therefore causing toxicity in these sites. Unfortunately, these sediments are much more complex and this amendment alone cannot elucidate direct effects of these constituents, as cationic metals bioavailability decreases dramatically with increases in pH [36]. Thus, as the anionic resin increases pH it would indirectly reduce metal concentrations.

As such, it is imperative that the cationic resin behave as devised, by only reducing metal concentrations and not affecting sulfate or acidic pH. Unfortunately this was not the case, as the cationic resin, rather than reducing metals concentrations (Table 3), instead

reduced the effects of acidic pH (as apparent in increases in pH overlying water (Table S3)). It should be noted, however, that sulfate concentrations were also not reduced by the cationic exchange resin. The reasons for this anomalous result are discussed in more detail in a following section. These confounding results make interpreting causality in these site sediments difficult and ultimately mean that characterizing the role of direct toxicity by acidic pH is not possible. Additionally, as metals toxicity is driven by pH, it is hard to characterize how much toxicity this class of contaminants contributed as well. The dynamics of the acidic environment and metals toxicity are especially complex in mining sediments and further work to differentiate toxicity between the two using TIE procedures is still needed.

Although the TIE was not able to distinguish whether toxicity is caused directly by acidic pH or whether the acidic pH 'drives' an increase in bioavailability of metals, what it does show is the overall importance of acidic pHs in these site sediments. Of the 12 Tasmanian sites sampled, five showed toxic effects with both species and these same five were the only ones to exhibit low pHs (i.e. below 5). Site Q3 further distinguishes the importance of acidic pH. This site was not in the Queen River itself, but was in an upstream tributary that may occasionally receive sediments from the river during high water events. As such, concentrations of copper and other metals (Table 1) were similar to other actual Queen River sites, but yet as it was not below the mine directly it did not exhibit the same acidic pH issues as the other sites (pH: 5.3). Interestingly, although this site contained high concentrations of metals, toxicity was not observed for this site, most likely due to the higher pHs observed in the bioassay. Although the whole-sediment TIE was unable to truly isolate causality, it was still able to provide risk assessors much needed information as to what is 'driving' causality, which is the acidic pH.

5.4.3. Method and species choice importance

Previous studies have suggested that amphipods are generally more sensitive than midges to most metals [43, 44]. In the present study, however, survival responses between the two species were quite similar (Table 1). The similarities in sensitivities between these two species are again most likely a product of acidic pH. As mentioned in the results, acidic pH was much less variable (ranging from 7.1 to 7.5) in amphipod bioassays than in midge bioassays. As such, we believe that acidic pH is not influencing toxicity in this bioassay to the same extent as in midge bioassays.

These respective midge/amphipod bioassay findings are important though, as they illustrate that not only does test organism choice affect the assessment of risk, but also could affect the determination of causality in TIE testing. The bias posed from using either whole-sediment or pore water as a TIE testing media has been discussed previously [39], but this study shows that even aspects at a finer scale, including test species use and bioassay methods (e.g. salt content of laboratory artificial water used), could have major implications in defining TIE causality. For instance, if only amphipods were used in conventional and subsequent TIE bioassays, the acidic pH itself would not have been considered as a possible toxicant or even an issue in regards to metals toxicity, even though low pHs have been reported in the field [8,9]. Outside of differing sensitivities, the other factor that should be considered when conducting this type of bioassay work (especially in remote areas of Tasmania) is the suitability of the environment (sans contaminant issues) and whether the chosen test species would actually inhabit these areas. Perhaps even more importantly is to ensure that the species reside in this area at all, as although *C. tepperi* are more commonly found in Victorian waters, they have only been observed in Tasmanian aquatic systems in a very limited capacity [40]. The biological needs of *C. tepperi* could be quite different then local Tasmanian *Chironomus* species and could explain the lower growth rates exhibited in controls. These factors combined illustrate that the choice of bioassay method and test species should be carefully considered when evaluating sediment risks and this is especially true for mining areas of Tasmania.

5.4.4. Moving TIEs forward for mining risk assessments

Before this TIE technique can be truly implemented as part of a mining risk assessment, issues with the cationic resin must be first addressed. In the current study, a weak base cation exchange resin was utilized with a reported operating pH range of 1.5 - 9 [28], but when used in acidic sediments it was unable to effectively reduce metal concentrations and instead increased pH of the overlying water. Reports have suggested that weak base cation exchange resins at lower pHs do have higher affinity for H+ ions over many metals cations and thus may resulted in increasing pH values [41]. Most previous whole-sediment TIE work has used similar cation exchange resin [15,17–19,30] that report similar operating ranges (such as ResinTech SIR 300 [42]), and have worked successfully in the past as pH was not an issue in these systems. Further work with other resins, such as

strong acid resins, may suggest better alternative cationic exchange resins for TIE mining procedures.

Additionally, incorporating bioavailability aspects in the TIE procedure might provide additional information that could be used to determine causality. For instance, studies in Macquarie Harbor (an estuarine system which is downstream of our sites (Q1 - Q5) in the Queen River) have also reported high concentrations of copper, but unlike its upstream counterparts, does not possess the same acidic pH issues (pH: 7.3 – 7.9 [10]). In this system, studies have compared chemical concentrations in water to biological effects in algae and noted a lack of toxicity although total metals concentrations exceeded threshold values effects for this organism [10]. Following up on this work, a study by Eriksen et al. [11] reported that the cause for this lower than expected toxicity is most likely a lack of bioavailability of these metals, as they showed that 99.9% of freely available copper was bound to ligands (composed of humic compounds) and could be colloidal due to the high concentrations of iron and manganese. As iron concentrations (and manganese most likely) were also extremely high in the freshwater sediments of the present study, it is also possible that these metals are largely unavailable in these freshwater systems as well. Evaluating the bioavailable nature of metals (via acid volatile sulfides, extractable metals, pore water concentrations, etc.) in these freshwater site sediments could provide additional information as to the role and risk that these contaminants have, but also the capabilities of TIE resins to remove ligands and other complexed metals.

5.4.5. Tasmania mining risk assessment and mitigation

Perhaps, the more important finding is that environmental impacts of mining in Tasmania (for even those mines that are closed) are apparent. Mount Lyell (Q1 - Q5 sites) is one of the largest mining operations in Australia and has produced over 100 million tonnes of waste since it initiated operations [10]. This is alarming, considering that this is only one mine and another nine large mines are/were in operation in Tasmania (as of 2014) with many of those located in the same study area (Figure 1). Although risks have and are being investigated in these areas, most aquatic research has focused on the impacts in Macquarie Harbor and other estuarine/marine systems [6,10,11] rather than where these mines typically discharge into, the freshwater environments of western Tasmania.

Additionally, these past studies have primarily focused on the toxicity of metals; the results presented here suggest that focusing on the acidic pH of these stream sediments

should also be a main priority. Our results suggest that minimization of sediment in effluents, and/or treatment/removal of the acidic nature of these sediments, can improve environmental outcomes in receiving waters. While this result may not be that surprising, it does provide additional information regarding the severity and complexity of sediments from these areas to risk assessors. Further assessment of western Tasmanian freshwater sediments is warranted to better understand the role of metals, sulfate, and acidic pH (as well as the joint toxicity of these constituents) and the potential these constituents have on local aquatic biota. Improving the TIE procedure and further development of toxicity-based approaches (rather than solely conventional contaminant-based approaches) would provide additional evidence to assist in future prevention, mitigation, and recovery assessment of these types of sites.

5.5. ACKNOWLEDGMENTS

Supplemental Information. Additional method details (mean development rate) as well as water quality data for the whole-sediment TIE can be found in the supplemental information.

5.6. REFERENCES

1. Australian Trade Commission. 2016. *Mining Equipment, Technology and Services.* Sydney, NSW, Australia.

2. Solomon M. 1981. An introduction to the geology and metallic ore deposits of Tasmania. *Econ. Geol.* 76:194–208.

3. Department of State Growth. 2016. *The mining and mineral processing industry in Tasmania.*

4. Soucek DJ, Kennedy AJ. 2005. Effects of hardness, chloride, and acclimation on the acute toxicity of sulfate to freshwater invertebrates. *Environ. Toxicol. Chem.* 24:1204–1210.

5. Banks D, Younger PL, Arnesen RT, Iversen ER, Banks SB. 1997. Mine-water chemistry: The good, the bad and the ugly. *Environ. Geol.* 32:157–174.

6. Saunders KM, Harrison JJ, Butler EC V, Hodgson DA, McMinn A. 2013. Recent environmental change and trace metal pollution in World Heritage Bathurst Harbour, southwest Tasmania, Australia. *J. Paleolimnol.* 50:471–485.

7. Harle KJ, Britton K, Heijnis H, Zawadzki A, Jenkinson A V. 2002. Mud, mines and rainforest: A short history of human impact in western Tasmania, using pollen, trace metals and lead-210. *Aust. J. Bot.* 50:481–497.

8. Featherstone AM, O'Grady B V. 1997. Removal of dissolved copper and iron at the freshwater-saltwater interface of an acid mine stream. *Mar. Pollut. Bull.* 34:332–337.

9. Gault AG, Cooke DR, Townsend AT, Charnock JM, Polya DA. 2005. Mechanisms of arsenic attenuation in acid mine drainage from Mount Bischoff, western Tasmania. *Sci. Total Environ.* 345:219–228.

10. Stauber JL, Benning RJ, Hales LT, Eriksen R, Nowak B. 2000. Copper bioavailability and amelioration of toxicity in Macquarie Harbour, Tasmania, Australia. *Mar. Freshw. Res.* 51:1–10.

11. Eriksen RS, Mackey DJ, Van Dam R, Nowak B. 2001. Copper speciation and toxicity in Macquarie Harbour, Tasmania: An investigation using a copper ion selective electrode. *Mar. Chem.* 74:99–113.

12. United States Environmental Protection Agency. 1991. *Methods for Aquatic Toxicity Identification Evaluations; Phase I Toxicity Characterization Procedures. EPA/600/6-91/003.* Washington, DC.

13. Lee S, Ha H, Hong J, Kang G, Hong C. 2017. Toxicity identification and evaluation for the effluent from a nonmetallic mineral mining facility in Korea using *D. magna*. *Toxicol. Ind. Health*. 33:681–686.

14. Elphick, JR Bailey HMF. 2001. Toxicity identification evaluation of effluent from a mine. *Proc. 25th Annu. Br. Columbia Mine Reclam. Symp.*:80–91.

15. 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.

16. Phillips BM, Anderson BS, Hunt JW, Clark SL, Voorhees JP, Tjeerdema RS, Casteline J,

Stewart M. 2009. Evaluation of phase II toxicity identification evaluation methods for freshwater whole sediment and interstitial water. *Chemosphere*. 74:648–53.

17. 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.

18. United States Environmental Protection Agency. 2007. *Sediment toxicity identification evaluation (TIE) Phases I, II, and III Guidance Document. EPA/600/R-07/080.* Washington, DC.

19. Mehler WT, Keough MJ, Pettigrove V. 2017. Development of whole-sediment toxicity identification and evaluation (TIE) techniques for two Australian freshwater species: *Chironomus tepperi* and *Austrochiltonia subtenuis*. *Environ. Toxicol. Chem.* 36:2476–2484.

20. 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.

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

22. 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.

23. 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.

24. 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.

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

26. Schuler LJ, Landrum PF, Lydy MJ. 2007. Response spectrum of pentachlorobenzene and fluoranthene for *Chironomus tentans* and *Hyalella azteca. Environ. Toxicol. Chem.* 26:1248–57.

27. Stevens MM. 1998. Development and survival of *Chironomus tepperi* Skuse (Diptera : Chironomidae) at a range of constant temperatures. *Aquat. Insects Int. J. Freshw. Entomol.* 20:37–41.

28. Lanxess. 2011. *Product Information Lewatit Monoplus TP 207*.

29. Lanxess. 2012. *Product Information Lewatit A365*.

30. 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.

31. United States 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.

32. Australia and New Zealand Environment and Conservation Council, Agriculture and Resource Management Council of Australia and New Zealand. 2000. *Australian and New Zealand Guidelines for Fresh and Marine Water Quality. Volume 1, The Guidelines*. 1.

33. R Development Core Team. 2009. R: a language and environment for statistical computing.

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

35. Palawski DU, Hunn JB, Chester DN, Wiedmeyer RH, Hunn JB, Chester DN, Interactive RHW. 1989. Interactive effects of acidity and aluminum exposure on the life cycle of the midge *Chironomus riparius* (Diptera). *J. Freswater Ecol.* 5:155–162.

36. Smith KS, Balistrieri LS, Todd AS. 2015. Using biotic ligand models to predict metal toxicity in mineralized systems. *Appl. Geochemistry*. 57:55–72.

37. Besser JM, Brumbaugh WG, Ingersoll CG. 2014. Characterizing toxicity of metal-contaminated sediments from mining areas. *Appl. Geochemistry*.:1–12.

38. Liber K, Goodfellow W, den Besten P, Clements W, Galloway T, Gerhardt A, Green A, Simpson S. 2007. In situ-based effects measures: considerations for improving methods and approaches. *Integr. Environ. Assess. Manag.* 3:246–258.

39. 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.

40. Martin J, Lee BTO, Connor E. 1978. Apparent Incipient Speciation in Midge *Chironomus-Oppositus Walker* (Diptera-Chironomidae). *Aust. J. Zool.* 26:323–329.

41. Crittenden JC, Trussell RR, Hand DW. 2012. *Water treatment principles and design*. John Wiley & Sons.

42. ResinTech Inc. *ResinTech SIR 300 Product Data Sheet*. West Berlin, NJ.

5.7. SUPPLEMENTAL INFORMATION

Mean development rate calculation

　　　　The mean development rate (MDR) or the reciprocal of the mean time span has been used in past studies [1] to assesses the rate between the introduction and the emergence of individuals. The mean development rate was calculated using the following equation:

$$MDR = \sum_{i=1}^{m} \frac{f_i x_i}{n_e}$$

with i representing the index of the inspection intervals, m being the total number of inspection intervals, f_i the number of emerged individuals in a given time interval, x_i the development rate of midges emerged in a given interval (or i), n_e being the sum of emerged individuals at the end of the experiment and x_i, the development rate of midges emerged in a given interval (or i), calculated as:

$$x_i = \frac{1}{day_i - \frac{l_i}{2}}$$

with day_i being a single inspection day, and l_i being the duration of the inspection interval (in this study it is 1 day).

Citations:

Goedkoop W, Spann N, Åkerblom N. 2010. Sublethal and sex-specific cypermethrin effects in toxicity tests with the midge Chironomus riparius Meigen. *Ecotoxicology*. 19:1201–1208.

Table S1. Artificial water recipes for amphipod (*Austrochiltonia subtenuis*) and midge (*Chironomus tepperi*) bioassays.

Grams per 100L	*A. subtenuis*	*C. tepperi*
$MgCl_2$ $6H_2O$	42.3	2.2
$CaCl_2$ $2H_2O$	10.8	1.2
$NaHCO_3$	23.2	1.2
NaBr	0.11	--
KCl	0.78	--
$MgSO_4$	4.68	1.2
NaCl	33.06	12
KH_2PO_4	--	1.2
Iron Chelate	--	0.24

Table S2. Corresponding Limit of Reporting (LOR; mg/kg) for each of the metals evaluated as well as sulfate.

Contaminant	LOR (mg/kg)
Beryllium	1
Cadmium	1
Barium	10
Chromium	2
Cobalt	2
Nickel	2
Silver	2
Antimony	5
Arsenic	5
Copper	5
Lead	5
Manganese	5
Selenium	5
Tin	5
Vanadium	5
Zinc	5
Aluminum	50
Boron	50
Sulfate	100

Table S3. Average conductivity (µS/cm) and pH values and the standard deviation for the TIE bioassay with the midge (5 d).

	Resin Type	pH	Conductivity (µS/cm)
C1	Sand	6.6 (0.2)	213.2 (40.7)
	Anionic	6.8 (0.3)	175.5 (7.8)
	Cationic	6.7 (0.2)	202.7 (27.7)
C3	Sand	6.7 (0.1)	160.3 (7.8)
	Anionic	6.8 (0.4)	152.9 (8.6)
	Cationic	6.9 (0.2)	185.4 (6.8)
W1	Sand	5 (0.7)	192 (31.9)
	Anionic	6.5 (0.4)	154 (11.7)
	Cationic	6.5 (0.3)	230.9 (40.3)
Q1	Sand	3.8 (0.4)	308.8 (147.1)
	Anionic	6.3 (0.6)	177.9 (22.1)
	Cationic	6.4 (0.3)	312.5 (60.4)
Q5	Sand	3.4 (0.1)	434.5 (160.2)
	Anionic	6.7 (0.3)	211.3 (17.4)
	Cationic	6.3 (0.5)	339.3 (89.9)

CHAPTER 6: CONCLUSIONS

With increasing contamination world-wide the development of methods for addressing causality in complex sites is needed, and TIEs are one method to address risk at these sites. As whole-sediment TIEs are essentially just more complex sediment bioassays, basic bioassay aspects, such as species choice and method conditions, should be strongly considered before testing and the importance of these aspects was apparent in this dissertation as well. Additionally, the TIE methods in this dissertation were laboratory and field validated and this dissertation shows the importance of doing both. The importance of those aspects as well as the future of TIEs and further research to advance these techniques moving forward is discussed below.

6.1. Implications of species choice

Standardized guidelines were recently developed for bioassays with native species from Australia; one of those that was developed was for the freshwater chironomid, *Chironomus tepperi* [1]. Interestingly, those methods were slightly different from those used in the present study, as the initiation of this dissertation preceded the standardized guidelines. Perhaps the major difference between the bioassays used herein and the guidance document is that the guidance document suggests using first instar midges, while in this dissertation we chose to use second instar in a similar manner to previous research conducted in our lab [2–4]. Further work is needed to determine if instar choice affects sensitivity, especially as the toxicity results from Chapters 3 – 4 show the high tolerance of this instar stage of this species when exposed to a variety of contaminants, especially using survival. Chapters 4-5 showed that growth and emergence of *C. tepperi* were both more sensitive than survival, which was not surprising, as it would be assumed that survival would be one of the least sensitive endpoints. Perhaps just as important as sensitivity is the ease of the use of the endpoint. The growth endpoint is conducted in the same time frame as survival and does not require a significant amount of additional time or resources. While emergence does take additional time (as it took up to 30 d), this endpoint was not only more sensitive than survival, it also provided mean development rate and sex ratios that could be used to better understand effects on development/fecundity (and population effects) and compounds that may affect sex determination (such as endocrine disruptors). The variety of endpoints for this species provides risk assessors with a better

understanding of risk, but as illustrated in this dissertation, more work is still needed to identify more sensitive endpoints with this species.

The other species that was evaluated in this PhD was *Austrochiltonia subtenuis*, an amphipod species. This species in Chapter 3, showed a higher sensitivity to three different contaminants including ammonia, copper (a metal), and permethrin (a non-polar organic pesticide) when compared to *C. tepperi* (when using survival as an endpoint for both). These results are not surprising considering that past work has suggested that amphipod species (such as *Hyalella azteca,* a native North American amphipod species) are more sensitive than many *Chironomus sp.* to a variety of contaminant including many metals and non-polar organics [5–8]. These results suggest that the use of this amphipod in risk assessments moving forward would be advantageous as it is easy to use, utilizes a unique niche (epi-benthic), and appears to be a relatively sensitive species (although additional work with other species is still needed to further confirm such sensitivity). The sensitivity of this species could be further increased by evaluations of other sub-lethal endpoints, similar to those utilized in the chironomid assays (growth, molting time, etc.), in fact it is only just recently that these additional endpoints evaluations have started to be developed place for this species [9]. However, before additional endpoints can be fully utilized with this species further work to understand the ecology and biology of this species as well as further refinements to the methodology is needed. In this PhD (Chapters 3 and 5) and also other recent studies [9,10] with this amphipod species, a high degree of variation (such as high control mortality: ~20% mortality) was observed with a variety of test endpoints. The lack of refinement with the current bioassay method was believed to be the cause of this higher than expected variation in both acute and sub-lethal endpoints. The need for methodology refinements for bioassays with this species became even more obvious when working with contaminated field sediments from Tasmania (Chapter 5) and is discussed in more detail in the section entitled "*Method choice implications*" below.

Although laboratory work with both of these species has helped to better understand the strengths and limitations for each, the bioassays with contaminated field sediment from retarding basins of Melbourne (Chapter 4) and mining sediments from Tasmania (Chapter 5) showed the strengths of using multiple species and multiple endpoints. Using multiple endpoints (in both Chapters 4 and 5) and species (Chapter 5) provided a stronger weight of evidence to better understand the levels of risk associated with each site, which allowed for not only a more thorough assessment, but also a

prioritization scheme to understand the sites at most risk. The use of multiple endpoints and species used for Tasmanian site sediments (Chapter 5) also provided much needed information for subsequent TIE analysis, as it allowed the use of less sensitive techniques (*i.e.* survival and growth) that were not only effective (as most sites exhibited high toxicity) in addressing risk, but also that could be done in a more timely and cost-effective manner when compared to more sensitive species or endpoints (*i.e.* emergence or amphipods). Using multiple species and endpoints maybe cost or time prohibitive in many risk assessments, but the strengths and additional value of using a combination of endpoints and species should not be overlooked and should be strongly considered in all sediment risk assessments.

6.2. Method choice implications

Although surprising, freshwater sediment bioassays are still in their infancy in Australia. In 2005, *The Handbook for Sediment Quality*, as produced by CSIRO Land & Water, stated that "There are few whole-sediment tests available for freshwater sediments using local species, largely because the demand has been not as great as for marine whole-sediment tests." [11]. This statement should not be misinterpreted to suggest that freshwater systems in Australia are not at threat to chemical contamination as many studies have suggested otherwise [4,12–14]. Rather, with limited resources the focus has been curtailed to the marine environments where the need has been far greater. Although recent standardized guidelines have just become available for a few native Australian freshwater species, including *C. tepperi* and *Physa acuta* [1], further resources are still needed for refining these the aforementioned species, and especially for species that have yet to have standardized guidelines developed, such as the amphipod *Austrochiltonia subtenuis*. The shortcomings of current methods with *C. tepperi* and *A. subtenuis* were highlighted in many of the sediment bioassays of this dissertation, and two examples are discussed below.

After considerable research, the US EPA developed growth thresholds (for quality control purposes) for the commonly used North American chironomid bioassay species (*Chironomus dilutus*: 0.6 mg dried or 0.48 mg ash free dry mass; United States Environmental Protection Agency 2000), but unfortunately these thresholds are not available for *C. tepperi*. Bioassay test acceptability thresholds for survival and emergence (≥80%) were already available (and were met throughout this dissertation) and based on the work presented dry weights of *C. tepperi* after 5-d using second instar varied from 0.7 –

1.4 mg per individual. Although it should be noted that differences between control sediments from Melbourne (Glynn's Wetland and Bittern Reservoir) and those from Tasmania were also observed, which makes determining a threshold difficult. Outside of determining a growth threshold, these differences show that the choice of control sediment when collecting field sediments should always be considered in sediment bioassays, to reduce the possibility of biasing results.

Another example of a current shortcoming with current sediment bioassay techniques arose with the artificial water used for *A. subtenuis*. Initial work with *A. subtenuis* used artificial water wherein the recipe was based on water chemistry from the collection location of this species. Concentrations of major ions in this water recipe, although high, did not appear to affect laboratory contaminant-spiked bioassays when comparing the results to other amphipod data (Chapter 3), although the actual implications of using using this water source are unknown. However, as discussed in Chapter 5, amphipods and midges unexpectedly showed similar sensitivities when exposed to Tasmanian sediments, which was most likely caused by differences in artificial water (i.e. higher concentrations of major ions reduced the effects of acidity in Tasmania sediment when compared to midge artificial water). Outside of the direct effects of acidity, this buffering capacity may also reduce the secondary effects of lower pHs, which affect contaminants such as metals and ammonia [15,16]. It is possible that this organism may not be able to survive in low conductivity streams, and that using this species to address risk of all sediments would lead to misleading results. Before this species can be used further for risk assessment work (especially for mining sediment risk assessments), further work to understand the ecology and water constituent requirements for this species are needed to better understand if the artificial water can be further modified to produce consistent and non-biased results.

6.3. Importance of field validation

Field validation is critical for establishing methods to prove that the developed techniques work. In this dissertation, field validation was conducted using contaminated retarding basin sediments from Melbourne (Chapter 4) and contaminated mining sediments from Tasmania (Chapter 5). Field validation confirmed whether the methods worked sufficiently, and provided additional insight into the strengths and limitations of the method, which cannot be provided by simple laboratory evaluations. Perhaps just as

important as validating the method, field validations also provided an evaluation of risk in these areas. Many of the aforementioned contaminated site sediments were toxic to midges and based on the sensitivity (as discussed above) of species, this is concerning, and shows the need for further work in these areas. As the research (especially in the case of Chapter 4 -- Melbourne retarding basin sediments) was focused on method development, the results provide only a snapshot of risk of contaminants to aquatic ecosystems. Chapter 5 evaluated sediment toxicity of mining sediments with multiple species and endpoints as well as evaluated sediment chemistry, but even still this is only a limited evaluation of risk. Further investigations evaluating spatial and temporal trends and also community and population effects in the field are warranted to truly understand and prioritize the risk of contaminants to downstream environments in these mining areas. Overall, the use of field validation provided this dissertation with a means to combine a pure and an applied research aspect that benefited both research areas.

6.4. TIE techniques moving forward and recommendations for future studies

Not only does this dissertation focus on bringing whole-sediment TIEs to Australia, it also works to improve and enhance whole-sediment TIE use globally. For instance, Chapter 4 addressed one of the current limitations of whole-sediment TIEs, which is the secondary effects of the TIE amendment, activated charcoal (which would be applicable for powdered coconut charcoal as well– although not used in this dissertation). This work was needed as Ho and Burgess (2013) showed that of 30 sediment TIEs conducted (in both marine and freshwater over a 20 year time span (1993-2013)) that approximately 90% of these studies identified a non-polar organic as a source of toxicity in sediments, with 70% of evaluations characterizing non-polar organics as the sole source of toxicity. As this contaminant is responsible (or at least partially responsible) for a majority of risk in whole-sediment TIEs, work to enhance its effectiveness was warranted, and with the findings present here future TIE work using this amendment has less chance for errors. Chapter 5 did more than just try to improve whole-sediment TIEs, it attempted to adapt these methods to be used for sediments impacted by mining. Although the attempts were unsuccessful, it did provide valuable information as to the importance of acidic pH in these sites. Overall, the results from this Tasmania work are encouraging for future work with mining sediment TIEs and also provided much needed freshwater chemistry and toxicity data for this geographical area. As such, two of the four data chapters of this dissertation

focused on improving or adapting TIE techniques. The other two chapters took on a different focus and that was making TIEs more accessible and cost-effective.

In the sediment TIE review mentioned above, the analysis showed that whole-sediment TIE, especially freshwater ones are still not common place in risk assessment (as of 67 sediments studied using TIE procedures only 15 were evaluated using freshwater whole-sediment TIEs [17]). The real-world applications of TIEs are apparent as it provides systemic evidence as to the contaminant(s) that are causing toxicity. As the complexity of sediment-bound contamination continues to increase this additional evidence is imperative for risk assessors to accurately understand risk at aquatic sites. As such, improving TIEs to make not only make them more accurate and effective, but also more practical (i.e. inexpensive and time efficient) should be a priority.

Chapter 2 detailed the building of an automated water change system for sediment bioassays to make performing bioassays (especially large bioassays such as whole-sediment TIEs) easier, inexpensive, and less time consuming. This system can dramatically ease the burden of the large bioassays that are typically required with whole-sediment TIE procedures. As TIEs advance, work to make even better systems, utilizing new technologies, that can meet multiple user needs (such as including water only exposures, etc.) should be considered, but for purposes of most sediment toxicology laboratories – the system as described in Chapter 1 will more than meet their needs. Similarly, Chapter 3 also attempted to make TIE methods more user-friendly and less expensive. This was accomplished by adapting similar conventional North American whole-sediment TIE guidelines but also by providing baseline information for how to acquire and use TIE amendments that could be sourced locally (again saving in costs, as shipping more commonly used products such as ResinTech SIR 300, is cost-prohibitive). As whole-sediment TIEs continue to become more reliable and more user-friendly/cost-effective, the frequency of their use will continue to increase and so will the science behind these novel techniques.

The whole-sediment TIE method is still a rather novel technique (as methods only came out by the US EPA in 2007), especially in Australia, and is a method that is evolving to be quicker, more cost-effective, and use a battery of different endpoints, species, and test methods. Perhaps even more exciting is to see how the techniques continue to evolve scientifically. A majority of TIE studies have investigated toxicity using 'contaminant class' as the tier to be evaluated (i.e. metals, non-polar organics, etc.). Studies have shown, however, that in some circumstances TIEs can be more specific. For instance, similar mode

of action chemicals can be distinguished using TIE practices, such as manipulating temperature or adding piperonyl butoxide for pyrethroids, to characterize within a chemical class [18,19]. This has further progressed and now enzymes are being commercially manufactured that can serve the same purpose [20]. This is especially exciting for the mining TIEs (as discussed in Chapter 5) as recent studies have started evaluating and producing imprinted resins, which can reduce the toxicity of a single metal (such as copper;[21]). Not only are TIE procedures expanding regarding contaminant evalutaion but *in situ* procedures are also being developed that allow for field-based evidence of causality that could be used in conjunction with sediment TIEs for more accurate characterizations of risk to aquatic benthos [22,23]. Outside of changing TIEs, methods are also now being devised to be run in tandem with TIE procedures, which would further improve the outcomes of risk assessments. One of those techniques is effect direct analysis (EDA), which is used for organic toxicants and utilizes analytical techniques to fractionate test samples for both chemical analyses and biological tests. In doing so, organic contaminants eliciting toxicity can be identified in the toxic fractions. This technique is considerably useful in complex urban sites as it does not depend on a specified target list (traditionally evaluated non-polar organics) but can screen a variety of contaminants of unknown identity under the guidance of the bioassay. In turn, this provides not only much needed information regarding causality when caused by non-polar organics, but also provides a means to discover toxicants that are not commonly monitored and potentially regulated. Although not a focus of this PhD, the usefulness of this tool and how it can complement TIEs was evident with collaborations with scientists from Jinan University [24]. Overall the future of whole-sediment TIEs is bright, and with further research evaluating small aspects (such as native species sensitivity) to advancing new methods to be used with TIEs (such as EDA analysis) can only increase whole-sediment TIE use and functionality not only in Australia, but world-wide.

6.5. References

1. Simpson SL, Batley GE. 2016. *Sediment quality assessment: A practical guide*. CSIRO Publishing, Clayton, Victoria, Australia.

2. Townsend KR, Pettigrove VJ, Hoffmann AA. 2012. Food limitation in Chironomus tepperi: Effects on survival, sex ratios and development across two generations. *Ecotoxicol. Environ. Saf.* 84:1–8.

3. Boyle RL, Hoak MN, Pettigrove VJ, Hoffmann AA, Long SM. 2016. Comparing the impacts of sediment-bound bifenthrin on aquatic macroinvertebrates in laboratory bioassays and field microcosms. *Ecotoxicol. Environ. Saf.* 133:489–500.

4. 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.

5. Liber K, Doig LE, White-Sobey SL. 2011. Toxicity of uranium, molybdenum, nickel, and arsenic to Hyalella azteca and Chironomus dilutus in water-only and spiked-sediment toxicity tests. *Ecotoxicol. Environ. Saf.* 74:1171–1179.

6. Harwood AD, Landrum PF, Lydy MJ. 2013. Bioavailability-based toxicity endpoints of bifenthrin for Hyalella azteca and Chironomus dilutus. *Chemosphere*. 90:1117–1122.

7. Ding Y, Weston DP, You J, Rothert AK, Lydy MJ. 2011. Toxicity of sediment-associated pesticides to Chironomus dilutus and Hyalella azteca. *Arch. Environ. Contam. Toxicol.* 61:83–92.

8. Milani D, Reynoldson T, Borgmann U, Kolasa J. 2003. The relative sensitivity of four benthic invertebrates in spiked-sediment exposures and application to contaminated field sediment. *Environ. Toxicol. Chem.* 22:845–854.

9. 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.

10. Jeppe KJ, Kellar CR, Marshall S, Colombo V, Sinclair GM, Pettigrove V. 2017. Bifenthrin Causes Toxicity in Urban Stormwater Wetlands: Field and Laboratory Assessment Using *Austrochiltonia* (Amphipoda). *Environ. Sci. Technol.*:acs.est.7b01472. doi:10.1021/acs.est.7b01472.

11. Simpson BSL, Batley GE, Chariton AA, Stauber JL, King CK, Chapman JC, Hyne R V, Gale SA, Roach AC, Maher WA, Simpson SL. 2005. *Handbook for Sediment Quality Assessment Quality Assessment*.

12. 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.

13. 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.

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

15. Schubauer-Berigan MK, Monson PD, West CW, Ankley GT. 1995. Influence of pH on the Toxicity of Ammonia to Chironomus tentans and Lumbriculus variegatus. *Environ. Toxicol. Chem.* 14:713–717.

16. Smith KS, Balistrieri LS, Todd AS. 2015. Using biotic ligand models to predict metal toxicity in mineralized systems. *Appl. Geochemistry*. 57:55–72.

17. Ho KT, Burgess RM. 2013. What's causing toxicity in sediments? Results of 20 years of toxicity identification and evaluations. *Environ. Toxicol. Chem.* 32:2424–2432.

18.   Amweg EL, Weston DP. 2007. Whole-sediment toxicity identification evaluation tools for pyrethroid insecticides: I. Piperonyl butoxide addition. *Environ. Toxicol. Chem.* 26:2389–2396.

19.   Harwood AD, You J, Lydy MJ. 2009. Temperature as a toxicity identification evaluation tool for pyrethroid insecticides: toxicokinetic confirmation. *Environ. Toxicol. Chem.* 28:1051–1058.

20.   Weston DP, Jackson CJ. 2009. Use of engineered enzymes to identify organophosphate and pyrethroid-related toxicity in toxicity identification evaluations. *Environ. Sci. Technol.* 43:5514–20.

21.   Shamsipur M, Fasihi J, Khanchi A, Hassani R, Alizadeh K, Shamsipur H. 2007. A stoichiometric imprinted chelating resin for selective recognition of copper(II) ions in aqueous media. *Anal. Chim. Acta*. 599:294–301.

22.   Burton GA, Nordstrom JE. 2004. An in situ toxicity identification evaluation method Part I: Laboratory validation. *Environ. Toxicol. Chem.* 23:2844–2850.

23.   Burton GA, Nordstrom JF. 2004. An in situ toxicity identification evaluation method Part II: Field validation. *Environ. Toxicol. Chem.* 23:2851–5.

24.   Qi H, Li H, Wei Y, Mehler WT, Zeng EY, You J. 2017. Effect-Directed Analysis of Toxicants in Sediment with Combined Passive Dosing and in Vivo Toxicity Testing. *Environ. Sci. Technol.* 51:6414–6421.