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Resolving false-negatives with nonpolar organics amendment

RESOLVING THE FALSE-NEGATIVE ISSUES OF THE NONPOLAR ORGANIC
AMENDMENT IN WHO 0000-0002-6750-4487LE-SEDIMENT TOXICITY
IDENTIFICATION EVALUATIONS¹

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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.

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

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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 0000-0003-4504-5125 (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 noncontaminated [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 $<44 \mu\text{m}$, 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): Glynn's 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- μ m 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) 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 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 $\mu\text{g/g}$ organic carbon, respectively. The low and high nominal concentrations of permethrin were 75 and 150 $\mu\text{g/g}$ 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 (Mettler 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)

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

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 <ZAQ;1>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 discussed above <ZAO;2>. 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; Burgess et al. 2008), 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 Glynn's 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 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 chemicals, survival, growth, and emergence varied with concentrations and the activated carbon ratio used (excluding growth for permethrin) <ZAQ;3>. 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 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 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 ($\geq 90\%$) 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 sediment bioassays (Beckingham and Ghosh 2011; Besser et al. 2011; Boyle et al. 2016; Burgess et al. 2008) <ZAQ;4>. 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.

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

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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.

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).

<<ENOTE>>AQ1: Please confirm replacement of "-ECD"

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<<ENOTE>>AQ8: Mehler et al. 2017b was deleted because it was the same title, journal, volume, page range. All citations are now to “2017” in text. OK? Or was 2017b supposed to be a different source?

<<ENOTE>>AQ9: The 2 Nybom refs (2016 and 2012) have not been cited anywhere in the article. Please add citations where appropriate or, if not needed, delete from ref list. (See footnote to Table 4, which is missing a citation.)

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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					
	Site	AC	Site:AC	Permethrin			Carbaryl		
				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.

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

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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

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

^a Different letters indicate significant differences ($p < 0.05$) among the treatments for a specific endpoint for a specific concentration level.

^bThe 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.

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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[<i>g,h,i</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 Burgess et al. (2008). The equilibrium sediment benchmarks for naphthalene, phenanthrene, pyrene, and benzo[*g,h,i*]perylene are as reported in <ZAQ;10>. 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.

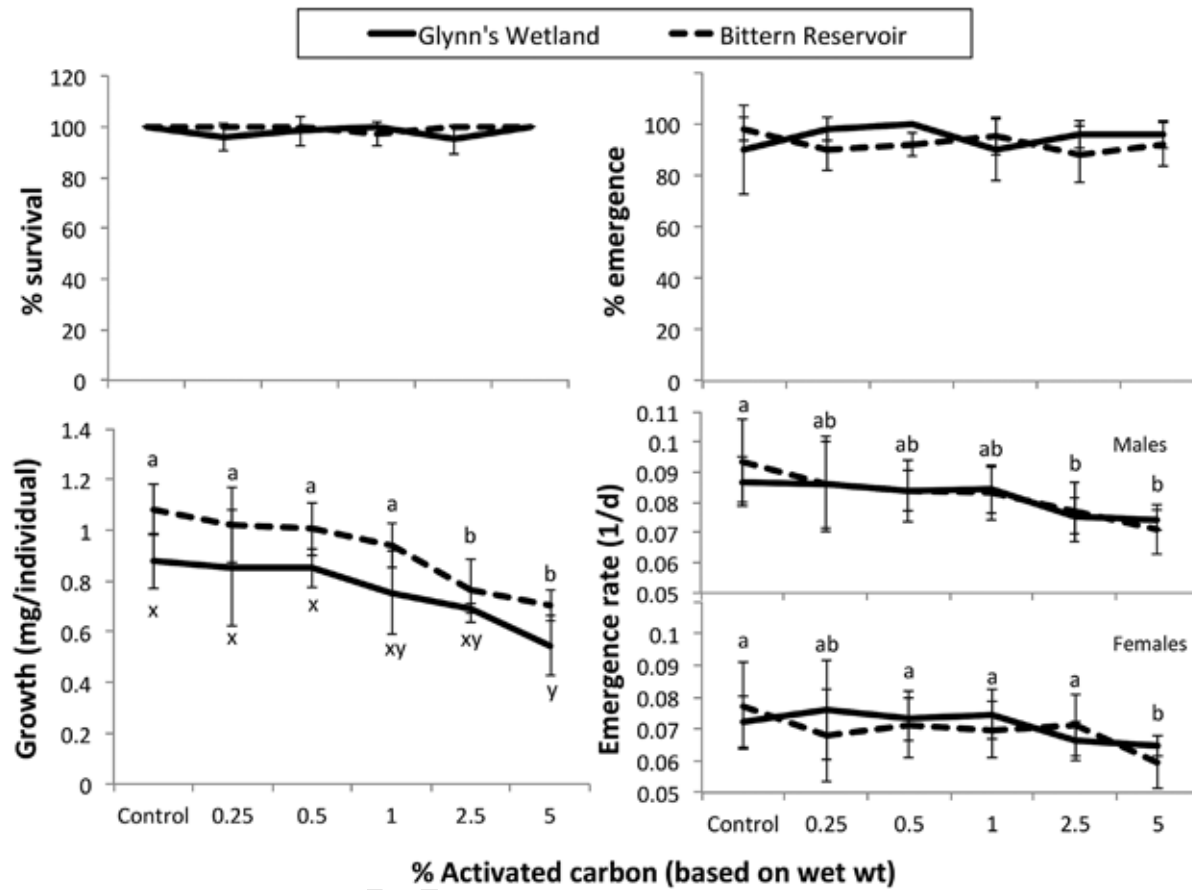
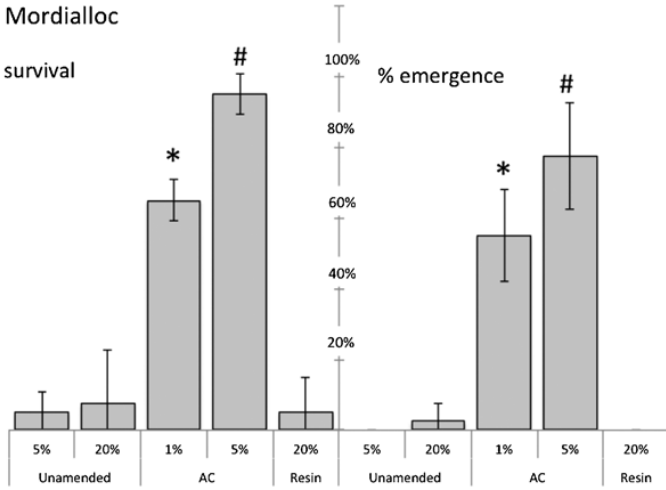


Figure 1

A) Mordialloc

% survival

% emergence



B) National Business Park

% survival

% emergence

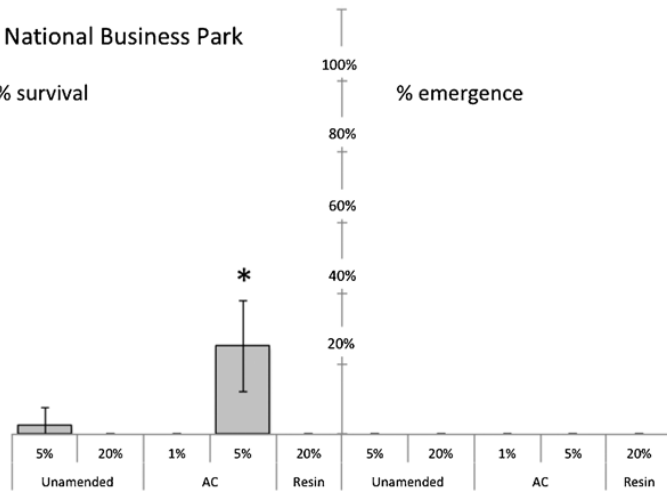


Figure 2