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Slow avoidance response to contaminated sediments elicits sublethal toxicity to benthic invertebrates

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Abstract
Advanced analytical techniques have identified the heterogeneity of sediments in aquatic environments which may impact the exposure of benthic organisms to contaminants. Acute and chronic toxicity associated with short, intermittent exposure to four field-collected contaminated sediments were assessed for the epi-benthic amphipod Melita plumulosa and the harpacticoid copepod Nitocra spinipes. Increasing the duration of exposure caused a decrease in survival of M. plumulosa and N. spinipes during 10-d bioassays. Increasing the frequency of exposure to a total exposure time >96-h resulted in a significant toxicity to M. plumulosa. Reproduction decreased for both species from exposure to contaminated sediment. For M. plumulosa, reproductive effects occurred for shorter exposures than the time taken to sense and avoid contaminant exposure. Thus, while avoidance behaviors may prevent acute lethality, slow responses may not prevent sublethal effects. Exposure of M. plumulosa to contaminated sediment appeared to cause a physiological change in females which reduced fecundity. This study indicates that sediment toxicity methods which utilize static continuous exposures may overestimate the toxicity that would occur at a field location. However, by preventing organisms from avoiding unfavorable sediments, these methods provide a precautionary assessment of possible effects, which is usually the aim of most assessments frameworks.

Keywords
slow, toxicity, sublethal, elicits, invertebrates, sediments, benthic, contaminated, response, avoidance, CMMB

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Slow avoidance response to contaminated sediments elicits sub-lethal toxicity to benthic invertebrates

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ABSTRACT: Advanced analytical techniques have identified the heterogeneity of sediments in aquatic environments which may impact the exposure of benthic organisms to contaminants. Acute and chronic toxicity associated with short, intermittent exposure to four field-collected contaminated sediments were assessed for the epi-benthic amphipod *Melita plumulosa* and the harpacticoid copepod *Nitocra spinipes*. Increasing the duration of exposure caused a decrease in survival of *M plumulosa* and *N. spinipes* during 10-d bioassays. Increasing the frequency of exposure to a total exposure time >96-h resulted in a significant toxicity to *M. plumulosa*. Reproduction decreased for both species from exposure to contaminated sediment. For *M. plumulosa*, reproductive effects occurred for shorter exposures than the time taken to sense and avoid contaminant exposure. Thus, while avoidance behaviours may prevent acute lethality, slow responses may not prevent sub-lethal effects. Exposure of *M. plumulosa* to contaminated sediment appeared to cause a physiological change in females which reduced fecundity. This study indicates that sediment toxicity methods which utilise static continuous exposures may over estimate the toxicity that would occur at a field location. However, by preventing organisms from avoiding unfavourable sediments, these methods provide a precautionary assessment of possible effects, which is usually the aim of most assessments frameworks.

Keywords: pulse, sediment toxicity, chronic, reproduction, fecundity

INTRODUCTION

A major portion of the dissolved contaminants that enter aquatic systems bind with dissolved organic matter, inorganic colloids or suspended particulates and eventually deposit in bottom sediments.[1] The distribution of contaminant concentrations in surface sediments is often heterogeneous.[2, 3] Variations in sediment contaminant concentrations may occur over large geographic regions (i.e. across large bays and estuaries),[4, 5] or within much smaller and localised patches (<1 cm), often referred to as micro-niches.[6] In addition to total concentrations, the speciation of contaminants may be even more heterogeneous, with bioturbation resulting in mixing of redox layers that were created in a stratified manner and deposition of organic matter (faeces/defunct remains).[6]

The heterogeneity of contaminated sedimentary environments influences the contaminant exposure for mobile benthic organisms. As invertebrates move throughout the environment (for foraging, mate searching, migration etc.), they may move in and out of sediments with high and low levels of contamination, thus resulting in intermittent exposures to contaminants. However, it is well recognised that many benthic organisms can detect and actively avoid areas of contamination.[7-9] Avoidance behaviour exhibited by mobile aquatic organisms is considered a significant factor determining the extent of exposure of an organism[8, 10, 11] and therefore the magnitude of the hazard and the overall risk the sediment poses to ecosystem health. Thus a benthic organism's exposure to contaminants may vary temporally and spatially due to the erratic nature of contaminant inputs.

Pulses of dissolved contaminants (both metal and organic) have the potential to cause toxic effects to aquatic organisms despite having short intermittent exposure times,[12, 13] however, if the mode of action of the toxicant is reversible, recovery of the organisms between exposures to contaminant pulses may be possible.[14, 15] While there has been significant research into the effect of dissolved contaminant pulses on aquatic life, little has been done to determine the impact of sediment heterogeneity on organism exposure to sediment-bound contaminants. Results of past studies have demonstrated the ability of benthic organisms to avoid contaminated sediment which suggests that exposure to contaminated sediment in the environment may not be continuous.[16, 17] Instead, it is more likely that organisms will come into contact with contaminated sediment as short intermittent

exposures similar to aquatic pulse exposures. Ward et al.[17] demonstrated that the rate of avoidance of contaminated sediments by an amphipod, harpacticoid copepod and a snail differed significantly. These species started avoiding contaminated sediments within 1 to 6 h, but sometimes as long as 48 h was taken for a significant avoidance response to be observed. It was speculated that slow avoidance behaviour could result in toxicity to sensitive benthic invertebrates.

Bioassays used for sediment quality assessment typically rely on static continuous exposure of a test organism to a contaminant or contaminated sediment. Static bioassay methods which force a continuous exposure throughout the duration of the experiment (often 10 days) will not suitably represent the nature of exposure that mobile benthic organisms have to the same contaminants in field locations. To further our understanding of the influence of exposure duration and frequency on toxicity caused by exposure to contaminated sediment, the acute and chronic toxicity associated with short, 'pulsed' exposures to contaminated sediment was assessed. This study investigated the potential toxic effects to two benthic invertebrates, an epibenthic amphipod (*Melita plumulosa*) and a harpacticoid copepod (*Nitocra spinipes*), associated with short intermittent exposures to contaminated sediment. The effect of exposure duration and frequency on acute and chronic toxicity endpoints was assessed following exposure to four field-contaminated sediments.

MATERIALS AND METHODS

General Chemistry. New plasticware was used for all chemical analyses. All chemicals were analytical reagent grade or equivalent analytical purity. Measurements of pH, salinity, temperature and dissolved oxygen were made in accordance with the instrument manufacturers' instructions. Analyses of sediments included particle size (by wet sieving and gravimetry), organic carbon (high temperature TOC analyser), and particulate metals (2:1 concentrated HCl:HNO_3, heated).[18, 19] Overlying water samples were rapidly filtered through acid-washed 0.45 µm membrane filters (Minisart, Sartorius) immediately following collection and acidified to 2% HNO_3 (v/v) with concentrated HNO_3 (Tracepure, Merck). Acid-volatile sulfide (AVS) and simultaneously extracted

metals (SEM) were analysed according to Simpson.[20] Dissolved metal concentrations in water samples and digested sediments were determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Varian 730-ES, Varian Australia) calibrated with matrix-matched standards.[21] Analyses of filter and digest blanks, replicates for 20% of samples, analyte sample-spikes and the certified reference material (PACS-2, National Research Council Canada, Ottawa, ON, Canada) were made as part of the quality assurance, and recoveries were within 85-110% of expected values. The limits of reporting for the various methods were less than one tenth of the lowest reported values. All sediment related concentrations are reported on a dry mass basis.

Test media. Clean seawater was collected from Port Hacking, Sydney, Australia, membrane filtered (0.45 μm), and acclimated to the room temperature of 21±1°C. Where necessary, the salinity of the filtered seawater was adjusted to the test salinity of 30 PSU using deionised water (18 MΩ/cm; Milli-Q Academic Water System).

Relatively clean silty sediments were collected as described previously from the Bonnet Bay estuary, Port Hacking, Sydney, Australia shown to have low or negligible concentrations of metal and organic contaminants.[22] The sediment was stored at 4°C for no longer than 1 month before use. Contaminated sediments were collected from estuarine field sites of unspecified locations, stored at 4°C in the dark, and toxicity testing undertaken within 8 weeks.[5, 22] Sediments from these locations had been used in earlier studies of sediment avoidance behaviour for these species, and in that study it was demonstrated that the differences in sediment properties (e.g. particle size, organic carbon (OC), AVS) would not result in avoidance behaviour.[17] Analyses of physicochemical properties (pH, OC, particle size, AVS) and metal contaminants were made on all sediments collected.[23] Previous analyses of sediments from these sites have consistently found that they contain negligible concentrations of common organochlorine or organophosphate pesticides (0.005-0.05 mg/kg), polychlorinated biphenyl (PCB) aroclors (<0.01-0.1), (<250 mg/kg) or polycyclic aromatic hydrocarbons (PAHs), BETX (<0.25 mg/kg benzene, toluene, ethyl benzene, xylene), and total petroleum hydrocarbons (<1 mg/kg).[5, 22]

Test species. Both species used in this study were epibenthic invertebrates found in intertidal estuarine environments of south eastern Australia. *Melita plumulosa* (Zeidler) is commonly found in estuarine tidal mudflats ranging from silty to sandy sediments in freshwater, estuarine and marine environments throughout south-eastern Australia.[24] Adult specimens of *M. plumulosa* typically range from 8-10 mm in length. The harpacticoid copepod species *Nitocra spinipes* (Boeck) is known to adapt to a wide range of environmental conditions (including salinity and temperature) and as such has a world-wide distribution.[25] Mature copepods of this species are approximately 400 µm long. *M. plumulosa* and *N. spinipes* were obtained from laboratory cultures, maintained as described previously.[22, 25]

Toxicity test procedures. Glass beakers and acrylic beaker-lids used for toxicity tests were cleaned in a dishwasher (Gallay Scientific Pty Ltd) programmed for a phosphate-free detergent wash (Clean A, Gallay Scientific Pty Ltd), a dilute acid wash (1% HNO_3), followed by thorough rinsing with Milli-Q water. New plasticware was used for each copepod test performed.

The acute 10-day lethality tests with *M. plumulosa* were conducted as described previously.[18, 19] In brief, tests were performed at 21 ± 1˚C in a constant environmental chamber (Labec Refrigerated Cycling Incubator) on a 12-h light/ 12-h dark cycle (light intensity = 3.5 µmol photons/s/m^2). Dissolved oxygen (>85%), pH (7.5-8.2), salinity (30 ± 1 PSU) and temperature (21 ± 1°C) were monitored and maintained. The method was modified slightly to include three replicate 250 mL beakers containing 30 g of test sediment, 200 mL seawater and 15 adult *M. plumulosa* per treatment. This amount of sediment created a depth of 1-2 cm within the beaker and has been previously demonstrated to provide plenty of substrate and nutrition (>3% OC) for this shallow-burrowing species during 10-day acute lethality and sub-lethal tests.[19, 22, 26] Every two to three days, 80% of the overlying water was replaced with clean seawater. In all tests, water samples were collected before and after each water change, and at the end of tests for dissolved metal analysis. Although the source of the metals in the overlying waters was from the pore waters, previous studies have found that the overlying water concentrations provide a suitable level of information for interpreting the dissolved metal exposure for this epibenthic species. No food was added throughout the duration of the tests.[19]

At the termination of the tests, live organisms were sieved from the sediments and identified by movement and the remaining sediment was fixed with neutral phosphate buffered formalin and stained with Rose Bengal solution. After 72 h, any surviving amphipods (now stained) that were missed initially were added to the count. Tests were considered acceptable if the physico-chemical parameters in beakers remained within the limits of pH 7.7-8.2, at 20-22 °C, dissolved oxygen >80% saturation, and salinity 28-32 PSU throughout the test, and if survival of amphipods was on average ≥80% in the controls.

Sublethal bioassays with pulsed contaminant exposure. Sublethal toxicity tests that assess reproductive output of *M. plumulosa* and *N. spinipes* were modified from previously published methods for the purpose of achieving pulsed exposures to contaminated sediments.[27, 28] The pulsed exposure durations were chosen based on avoidance times determined previously.[17] Visual presentation of the various pulses exposures are shown in Figure S1 of the Supporting Information. Each pulsed exposure period was separated by a period of at least 48 h where the test organisms were in contact with uncontaminated control sediment.

Female *M. plumulosa* were isolated at least 7 days prior to commencing chronic assays and placed into trays containing uncontaminated sediment. This allowed any pre-existing embryos to develop and ensure that non-gravid females were used at the commencement of the test. An excess of males and females were placed into the sediment exposure chambers that had been specifically designed and constructed to allow for the quick and easy transfer of the amphipods, minimising disturbance to the organisms while allowing them to be in contact with the test sediment.

The chambers were made from a polycarbonate container (diameter = 7 cm; height = 10 cm) with the base removed and a large hole (diameter = 6 cm) cut into the lid (Figure S2, Supplementary Information). A 250 μm mesh was secured over the base and lid of the container so that when pressed down onto the surface of the test sediment, the surface layer of the sediment could push through the mesh and overlying water could exchange with water outside of the chambers. The test vessels and sediments were placed into glass tanks containing filtered seawater, an aquarium pump and an air stone for the duration of the tests (see Supporting Information for additional details). The chambers

were carefully rinsed with clean seawater to remove all sediment particles when being transferred between sediments. Controls were handled in the same way as the treatments. Test chambers were set up with sediment and filtered seawater and equilibrated for 24 h before the initiation of the experiment. The overlying water was replaced immediately before the test organisms were added.

Separate exposures to contaminated sediments were undertaken for male and female amphipods to provide information of the effect of gender. Following a 24- or 48-h exposure period, 7 male and 5 female amphipods were randomly selected and transferred to beakers containing uncontaminated sediment and the test continued under the same conditions as used for the acute toxicity tests. The sublethal assays were terminated after 10-days. Juveniles present were sieved from the sediment and counted. Females were inspected under a light microscope to count the number of eggs/embryos being carried in the brood pouch. In the case that amphipods were still amplexed when the test was terminated, the pair were placed in a beaker containing clean seawater and returned to the incubator for a further 48-h before the number of embryos were counted on the female. The test endpoint was 'total offspring' which included the juveniles and the embryos produced from the mating of the amphipods exposed to the test sediments.

For the copepod, *N. spinipes*, gravid females were selected from the cultures and placed into a series of 10 mL polycarbonate tubes (maximum of 20 females for tube) containing test sediment. The females were exposed to the test sediment for the desired period and then gently removed from the tubes (using a pasture pipette) and pooled in a Petri dish containing clean seawater. Five females were randomly selected from the pool and placed into each of a series of 10 mL polycarbonate tubes (5 replicates per treatment) containing uncontaminated sediment for 10 days. A small amount of food (150 µl vial from a stock of 1×10^4 cells/ml of both *Isochrysis* sp. and *Tetraselmis* sp. and 0.3 mg Sera Micron fish food sieved to <63 µm) was added to each vial at the beginning of the exposure and when copepods were transferred to vials with uncontaminated sediment. In addition, a small volume of food was added to the vials following water changes every 2 to 3 days during the assay. This species is iteroparous, meaning that females are capable of producing multiple broods of offspring from reserves of sperm stored after mating giving this species the ability to produce multiple broods

187 in a short period of time.[29] This characteristic makes *N. spinipes* a suitable species to assess sediment
188 toxicity using a reproductive output as a chronic endpoint.[25] The test endpoint was 'total offspring'
189 which included all nauplii and copepodites.

190 **Amphipod gender-exposure tests.** Trays containing uncontaminated sediment and either
191 solely male or female *M. plumulosa* were set up in the laboratory at least 7 days prior to commencing
192 these tests. This was considered sufficient time for fertilised or gravid females to drop young and
193 become non-gravid. Individuals of these males and non-gravid females were randomly selected and
194 separately exposed to contaminated sediment for 48-h. By separating and exposing male and female
195 *M. plumulosa* to contaminated sediment, the mating of exposed and unexposed organisms over a 10-d
196 period could be controlled. Following the 48-h exposure period, 7 males and 5 females were placed
197 into a 250 ml beaker containing uncontaminated sediment for 10 days. Four reproduction scenarios
198 were investigated as follows: a) unexposed males × unexposed females, b) exposed males ×
199 unexposed females, c) unexposed males × exposed females, and d) exposed males × exposed females.
200 Microscopy was used to determine the number of offspring produced per female during the last 10
201 days of the bioassay.

202 **Data Analyses.** Descriptive statistics were generated using the Microsoft Excel (2007) data
203 analysis tool pack. Survival and reproductive output of test organisms in bioassays are reported as a
204 percentage relative to controls. All statistical analyses were performed using Toxcalc for Microsoft
205 Excel (TidePool Scientific Software). Survival and reproductive output data, expressed as percent
206 control, was tested for normality (using the Shapiro-Wilk's test) and homogeneity of variance
207 (Bartlett's test) prior to analysis.

208 The *t*-tests were performed to assess a significant reduction in survival and reproduction of
209 amphipods and copepods exposed to contaminated sediments compared to controls. Dunnet's test was
210 then used if assumptions of normality and homogeneity of variances were met. Where nonparametric
211 analysis was required, Steel's test was used. For toxicity tests on single sediments, *t*-tests were used to
212 determine significant differences in the response of the amphipods and copepods exposed to test

sediment compared to that in the control sediment. Significance in all statistical tests was set at the $p < 0.05$.

RESULTS AND DISCUSSION

The properties of the contaminated sediments are shown in Table 1. The AVS concentrations were <5 μmol/g in all sediments and were consistent with them being predominantly oxic/sub-oxic in nature. While the metal concentrations in these sediments were considered very high, both test species demonstrated sensitivity to metal contaminants, with sublethal effects occurring when dilute acid-extractable metal concentrations exceed lower guideline values.[22]

Dissolved metal concentrations in the overlying water of *M. plumulosa* bioassays remained well below reported 10-d and 96-h LC50 values for both adult and juvenile amphipod survival.[30] Concentrations of dissolved Cu, As, Cd and Pb measured in the overlying water did not exceed 40 μg/L in any bioassay, and dissolved zinc reached a maximum of 280 μg/L. Because of the small volume of seawater used in the copepod bioassays, it was not possible to subsample the overlying water during the assays to analyse dissolved metal concentrations. It is assumed that dissolved metal concentrations were similar to those measured in the amphipod tests and this species has been found to have a similar sensitivity to metals as the amphipod.[22, 28]

10-day lethality from continuous or pulse exposures to contaminated sediment.

For 10-day lethality tests with continuous exposures, all contaminated sediments were highly toxic to *M. plumulosa*, with survival of 12±5 %, 24±6 %, 32±2 % and 59±11 % (relative to controls) for Sediments 1, 2, 3 and 4, respectively (Table 1). In our previous research, it was demonstrated that in some cases *M. plumulosa* has the ability to avoid contaminated sediment within 6-h of the initial exposure and all toxic sediments were avoided within 48 h.[17] Therefore, a 48-h exposure period was used to represent a 'worst case' scenario for exposure to contaminated sediments. In all experiments, the survival in control treatments was >95%. Control amphipods were transferred from clean

Table 1

Table 1. Properties of control and contaminated sediments used in sediment pulse experiments

Parameters [a]	Test sediment				
	Control	**1**	**2**	**3**	**4**
Silt, %	98	76	15	79	27
AVS, µmol/g	4.5	<5	<5	<5	<5
TOC, %	4.7	3.9	5.9	6.8	3.0
As, µg/g	4	3430	7	61	ND
Cd, µg/g	0	83	0	19	1.1
Cu, µg/g	13	1100	1070	108	71
Pb, µg/g	40	14400	60	830	470
Zn, µg/g	210	14500	260	2630	1230
Static 10-d amphipod survival	100±2	12±5	24±6	32±2	59±11
48-h exposure amphipod survival	100±3	100±5	93±2	96±2	ND

[a] Silt = percent of particle <63 µm. AVS = acid-volatile sulfide. TOC = total organic carbon. Concentrations of total petroleum hydrocarbons were <250 mg/kg and polycyclic aromatic hydrocarbons (PAHs) were <1 mg/kg in all sediments. All metal concentrations are 1-M HCl extractable metals. Amphipod survival expressed as % Control ± SE. ND = not determined.

238 sediment to clean sediment to mimic the handling stress that may occur when creating the pulsed
239 exposures.

240 When exposed to Sediments 1, 2 and 3 for 48-h during the 10-day assay (remaining time in
241 clean sediments), the survival was 100±5 %, 93±2 % and 96±2 %, respectively, and not significantly
242 different from controls. Thus, as *M. plumulosa* would avoid these contaminated sediments within
243 48 h, acute lethality would not be expected to occur if cleaner sediments are located suitably close to
244 where this species can move. It can also be concluded that latency effects did not result from the 48-h
245 exposure, as surviving amphipods were still alive after eight days in the clean sediment.

246 To investigate the effect of increased exposure frequency and duration on toxicity, the
247 survival of *M. plumulosa* was determined for various exposure scenarios (Figure 1; see Figure S3 of
248 the Supporting Information for a graphical representation of exposure scenarios). This series of
249 experiments used Sediment 1 as it resulted in the greatest toxic effect in the continuous 10-d exposure
250 (Table 1). Significantly lower survival (75±8 %) was observed following two 48-h exposure periods
251 (total exposure time of 96 h) with a period of 48-h of no-exposure between exposure pulses (Figure
252 1). For three 48-h exposure pulses (total exposure time of 144 h during the 10-day test) much greater
253 mortality occurred (14±5 % survival) and was comparable to the toxicity observed for continuous
254 exposure to Sediment 1.

255 To consider the influence of exposure duration on toxicity to *M. plumulosa*, the exposure
256 period was reduced to 24-h. Survival of *M. plumulosa* exposed to Sediment 1 for three 24-h exposure
257 pulses did not decrease significantly in comparison to controls (96±5 % survival; total exposure time
258 of 72-h, with each exposure pulse separated by a 72-h period of exposure to uncontaminated
259 sediment). This confirmed that the duration of exposure is a significant factor affecting the toxic
260 response of organisms that come into contact with contaminated sediment.

261 In the case of *N. spinipes*, significant toxicity occurred following a 48-h exposure to
262 Sediment 1 with survival reduced to 28±2 % (relative to controls). When the exposure time was
263 reduced to a 24-h duration, survival remained significantly lower than the control, however remained

higher than the 48-h treatment (24-h survival >60% relative to controls). This is a clear indication of the significance of exposure duration in the expression of acute toxicity caused through the exposure to contaminated sediment. However, this species was demonstrated to have a fast avoidance response time, being able to avoid contaminated sediment within 1-6 h of exposure.[17] Again, this would imply that acute toxicity could be avoided if clean uncontaminated habitat quickly be found.

The lethality tests conducted in the present study suggest that acute lethality from exposure to contaminated sediment may not occur in mobile benthic invertebrates if there is a possibility to escape the contaminant and inhabit uncontaminated sediment. The lethality observed in the contaminated sediments was clearly dependent on the extent of exposure an organism had to the contaminated sediment (Figure 1). The extent of the exposure varies considerably between the traditional continuous exposure commonly used for acute and chronic toxicity methods, which may not reflect the true nature of exposure for mobile benthic invertebrates.[22, 31, 32] Under most scenarios, where non-toxic sediments exist within a suitable distance, *M. plumulosa* and *N. spinipes* would likely avoid contaminated sediments and lethality to adults could potentially be avoided. However, the research has also confirmed that the exposure duration is a major factor contributing to the toxic effects elicited by sediment contamination and that the species may not adequately recover during periods in clean sediment between multiple exposures to contaminated sediment.

Sublethal toxicity from pulsed exposure to contaminated sediment. For the amphipod, *M. plumulosa*, and copepod, *N. spinipes,* effects to reproduction are known to occur in sediments that do not effect survival of adults.[22, 28, 33] Both of these organisms have shown the ability to avoid contaminated sediment within as little as 6 hours of exposure.[17] Based on the results presented above, the effective avoidance of contaminated sediment by *M. plumulosa* and *N. spinipes* may alleviate acute toxicity, however it is speculated that sublethal effects may occur as a result of short exposures to contaminants.

Sublethal effects were assessed when *M. plumulosa* was exposed to contaminated Sediments 1 and 4 which caused 88% and 41% lethality during 10-day continuous exposure, respectively (Table 1, Figure 2). A 48-h exposure pulse to Sediment 4 resulted in a 46% decrease in reproductive output

291 from 6.7±0.8 to 3.6±1.2 juveniles per female in control and contaminated treatments, respectively.
292 The 48-h exposure to Sediment 1 resulted in the reproductive output decreasing from 6.7±0.8 to
293 2.4±0.6 juveniles per female in control and contaminated treatments (a decrease of 65%),
294 respectively. Increasing the time of exposure to Sediment 1 increased the magnitude of the toxicity
295 exhibited by *M. plumulosa*, with reproductive output being 85±15%, 66±3% and 34±8% (of control)
296 for 8-, 16- and 48- h exposures, respectively (Figure 1 – shaded area represents time period where
297 avoidance of contaminated sediment by *M. plumulosa* is likely to occur[17]). Despite not being acutely
298 toxic, sublethal effects to *M. plumulosa* were significant following the 16- and 48- h exposures
299 ($p<0.05$).

300 For the benthic copepod *N. spinipes*, exposure to Sediment 1 and 2 for 24- and 48-h pulses,
301 respectively, significantly lowered reproductive output after 10 days (Figure 3). Significant lethality
302 occurred when gravid copepod females were exposed to Sediment 1 for 48-h (27 ± 2% survival, mean
303 ± SE, n = 4) and the exposure time was reduced to 24 h for subsequent tests. Following a single 24-h
304 exposure to Sediment 1, the reproductive output (Figure 3) was 16% (reduced from 10±0.8 to 2±0.4
305 juveniles per female in control and Sediment 1 exposed treatments, respectively). Nauplii and
306 copepodite numbers declined by 82% and 95%, respectively (Figure 3). Following a single 48-h
307 exposure to Sediment 2, the reproductive output was 63% and nauplii and copepodite numbers
308 declined by 31% and 49%, respectively (Figure 3).

309 **Gender of *M. plumulosa* influencing reproduction.** For *M. plumulosa*, effects on
310 reproductive output were observed following a single 16-h pulse exposure to contaminated field
311 sediment. While the identification of this sub-lethal effect is ecologically important in implicating
312 significant ecotoxicological consequences at the population level, the gender responsible for limiting
313 the reproductive output of the population has not been identified.

314 The influence of the amphipod gender being exposed to contaminated sediment prior to
315 mating was investigated. It was found that fecundity was significantly lower in treatments where
316 females had been exposed to Sediment 1 for 48-h prior to mating. When unexposed males were mated
317 with exposed females, reproductive output over 10-d was 34% of controls. In the scenario where

318 exposed males were mated with exposed females, fecundity was 63% of controls (Figure 4).
319 Conversely, when exposed males were mated with unexposed females there was a slight increase in
320 offspring production (of 12%) although this was not significantly different from control treatments.
321 These findings are consistent with field population studies which found that female *M. plumulosa*
322 collected from polluted sites along the east-coast of Australia were less fecund than those obtained
323 from uncontaminated sites.[34] It is important to note that there is no statistically significant difference
324 ($p>0.05$) in the reproductive output observed between scenarios A and B or between C and D (Figure
325 4).

326 Interestingly, reproductive output was slightly increased in scenarios where male amphipods
327 were exposed to contaminated sediment compared to the corresponding scenario with similar females.
328 McCurdy et al.[35] found that parasitised male *C. volutator* were more likely to mate immediately after
329 being introduced to a receptive female when compared to unparasitised males. The slight increase in
330 offspring production where only males were exposed may be the result of similar factors that increase
331 the male drive to reproduce. McCurdy et al.[35] also observed that pairings of parasitised males resulted
332 in higher initial brood sizes. In a similar study, it was reported that females showing a greater degree
333 of infection by parasites are less likely to invest in reproduction.[36] It has also been found that mating
334 in the isopod *Lirceus fontinalis* is ultimately controlled by the female of this species.[37] As for
335 amphipods, pair-formation is controlled by the female as they must position themselves correctly to
336 allow amplexing to occur.[38] In light of this, it is concluded that contaminant exposure in female *M.*
337 *plumulosa* results in a physiological or behavioural change that is responsible for reducing its
338 reproductive output. This could be due to the resorption/abortion of eggs and/or embryos,[36] or non-
339 cooperative behaviour which limits the ability of male *M. plumulosa* to amplex with receptive
340 females.[38] These results are also reflected in field studies of this species, with adult female *M.*
341 *plumulosa* living in contaminated habitats found to be generally smaller and less fecund.[34, 39]

342 **Implications.** Lethality associated with contaminated sediment resulting from 10-d static
343 toxicity test methods was alleviated when the organism exposure was limited to short and intermittent
344 exposures. This result suggests that the toxic effect resulting from contaminant exposure is
345 determined by the frequency and duration of exposure to contaminants. *M. plumulosa* and *N. spinipes*
346 are known to avoid contaminated sediment within 6 to 24-h and 1 to 6-h, respectively.[17] Our results
347 indicate that acute toxicity may not occur for these species if suitable uncontaminated habitat can be
348 found within 48-h of exposure to contaminated sediment.

349 The present study also confirmed that while latent effects of short pulses may not be observed
350 in terms of lethal effects, sublethal effects to reproduction may occur as a result of exposure before *M.*
351 *plumulosa* or *N. spinipes* sense and avoid contaminated sediment. It is also speculated that exposure
352 of *M. plumulosa* to contaminated sediment causes a physiological change in females which reduces
353 fecundity. This has significant implications for the design of sediment bioassays and the use of acute
354 toxicity tests for sediment quality assessment purposes which may over estimate the toxicity
355 associated with sediment in a field location. Specifically, these results imply that traditional standard
356 toxicity tests which employ static continuous exposure methods do not provide an assessment of the
357 impact contaminated sediments have on aquatic ecosystems at the population level.

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362 and suggestions which have improved the quality of this manuscript.

363 **Supporting Information.** Supporting Information includes further information on exposure
364 scenarios, the experimental method and a summary of results. This information is available free of
365 charge via the internet at http://pubs.acs.org/

366

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Figure 1

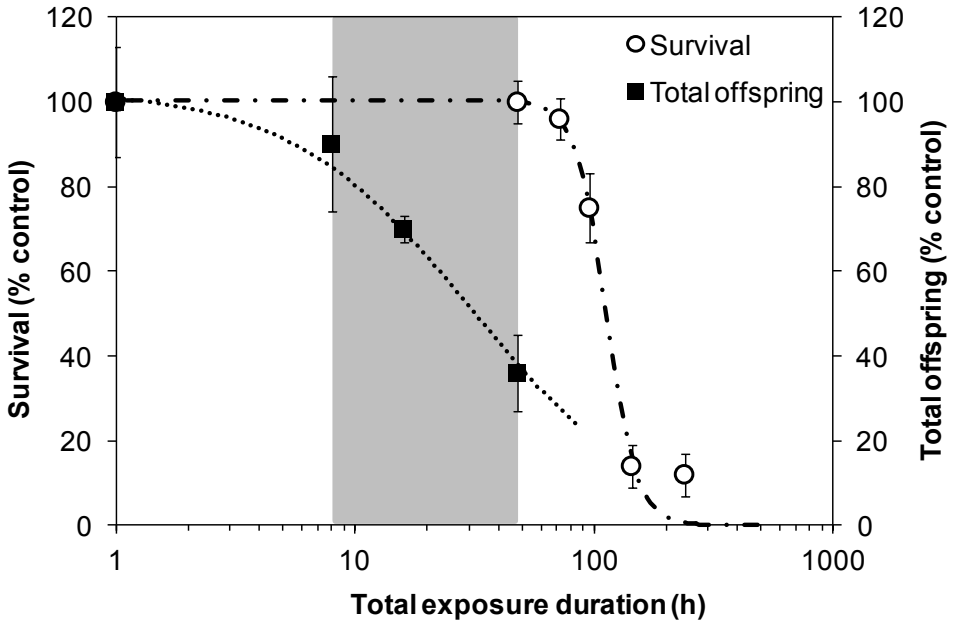

Figure 1. The effect of increasing periods of exposure to contaminated Sediment 1 on survival and reproduction of *M. plumulosa*. The shaded region represents duration of exposure required to elicit contaminant avoidance (from Ward et al.[17]). The error bars represent standard error (n=4).

FIGURE 2

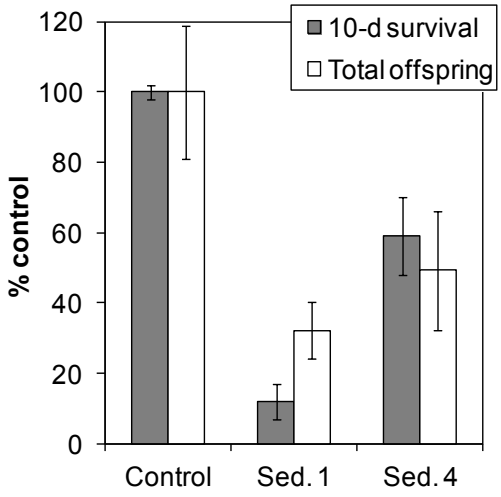

Figure 2. The effect of short (48-h) exposure to contaminated sediment on the survival and reproductive output of *M. plumulosa* exposed to uncontaminated sediment (control) and field-collected contaminated sediments (Sediments 1 and 4; mean ±SE, n=4).

FIGURE 3

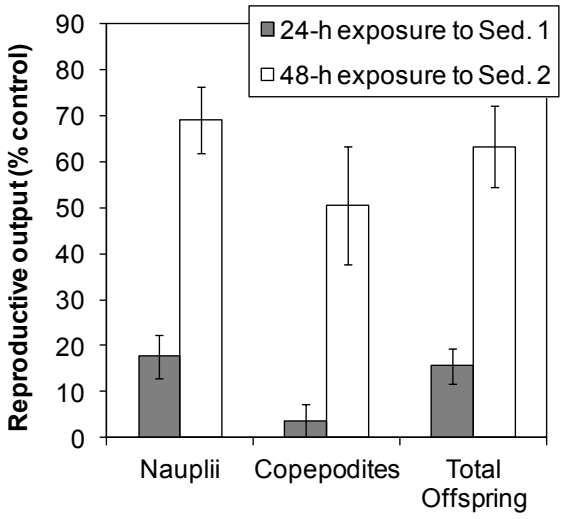

Figure 3. The effect of short exposure to contaminated sediment on the reproductive output of *N. spinipes* (mean±SE) exposed to Sediment 1 for 24-h (n=4) and Sediment 2 for 48-h (n=5). Total offspring was counted after a 10-d period in uncontaminated sediment following prior exposure to contaminated sediment.

FIGURE 4

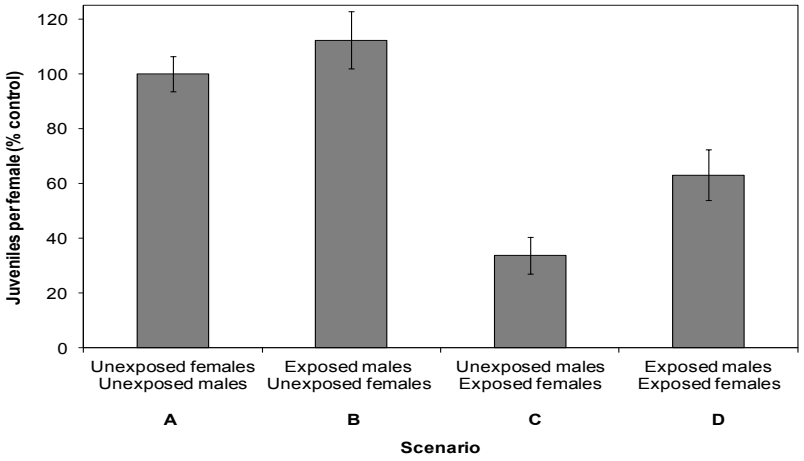

Figure 4. Reproductive success observed for four gender-exposure scenarios generated by exposing male and female amphipods to contaminated Sediment 1 (mean ±SE, n=4). A reduction in reproductive success was found to occur in treatments where females had been exposed to contaminated sediment prior to mating.

Supplementary material for manuscript: "Slow avoidance response to contaminated sediments elicits sub-lethal toxicity to benthic invertebrates"

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Pages: 5

Summary of Supporting Information

Figure S1: A visual representation of pulse exposure scenarios used in bioassays with *Melita plumulosa* and *Nitocra spinipes*.

Additional details of the experimental method used to create the pulsed exposure to contaminated sediment is included on Page S3.

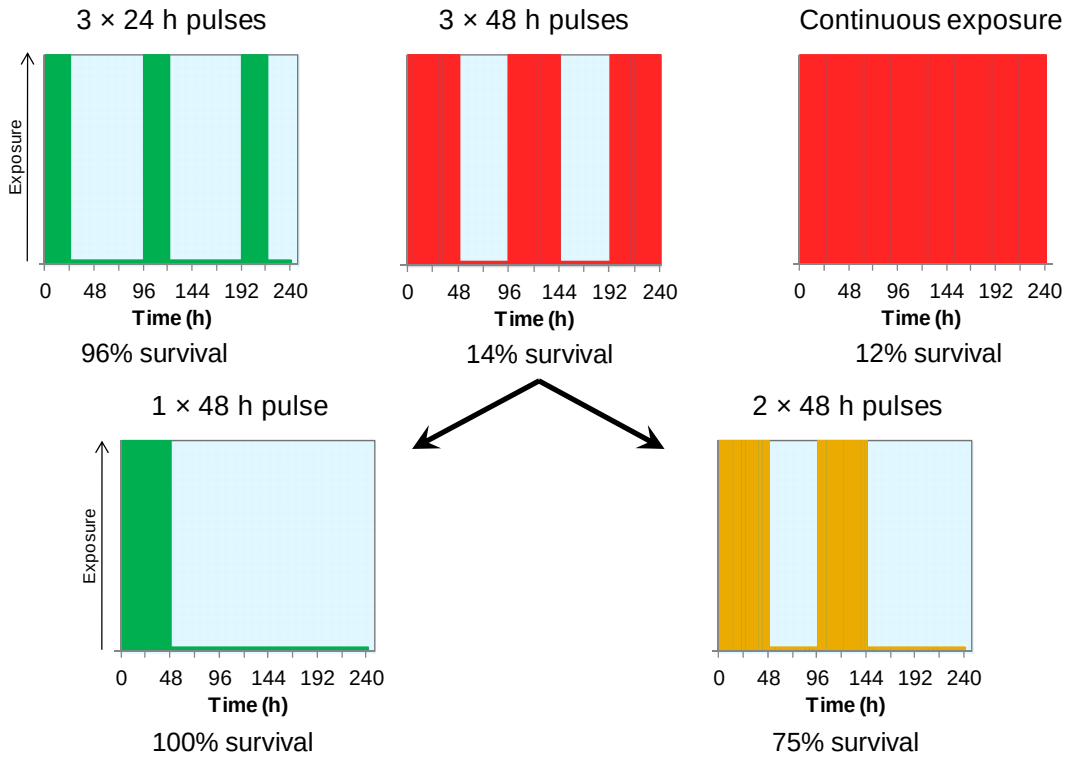
Figure S2: A photograph of the setup used for in this study.

Figure S3: Acute lethality observed for *Melita plumulosa* following pulsed exposure to Sediment 1.

Table S1: A summary of acute and chronic toxicity observed for *M. plumulosa* and *N. spinipes* during pulsed exposure bioassays conducted in this study.

Pulse scenarios

a)



b)

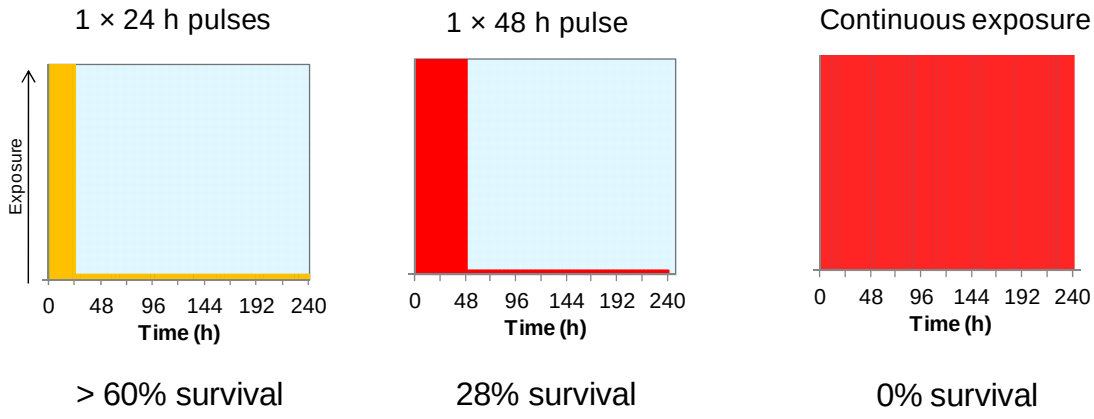


Figure S1. Visual representation of exposure scenarios used for a) amphipod and b) copepod bioassays. Acute toxicity (survival) is also shown - green, yellow and red represents non-toxic, moderatley toxic and toxic, respectively.

Further Method Details

Test vessels for *M. plumulosa* exposure experiments were constructed from 250 mL polycarbonate containers with a screw lid (diameter = 7 cm; height = 10 cm). The base of the container was removed and a hole was cut into the top of the lid (approximately 6 cm diameter). Nylon mesh (250 µm mesh size) was stuck to the lid and the base of the container with aquarium safe silicone (Selleys®). The silicone was allowed to cure for 4 days (as per manufacturer recommendations) and containers were rinsed thoroughly with deionised water before use. Toxicity of all materials used in the construction of the vessels was checked prior to use in bioassays. Sediment was placed in a disk with a diameter slightly larger than the base of the 250 mL polycarbonate container. The test vessels were pressed down onto the surface of the sediment discs so that the test organisms inside the vessels were in contact with the sediment surface through the mesh. Test vessels were housed in glass tanks measuring approximately 40 × 40 × 40 cm^3 filled with approximately 30 L of filtered seawater. An aquarium pump and air stone was added to the tanks to provide aeration and circulation of the water around the vessels, facilitating the transfer of water into and out of the vessels during tests. Only one type of sediment was contained within the tanks at a time and tanks were thoroughly washed with 10% HNO_3 and deionised water prior to reuse. This method allowed for the quick and easy transfer of organisms between sediments to create the 'pulsed' exposure scenarios whilst minimising stress caused from handling *M. plumulosa.*

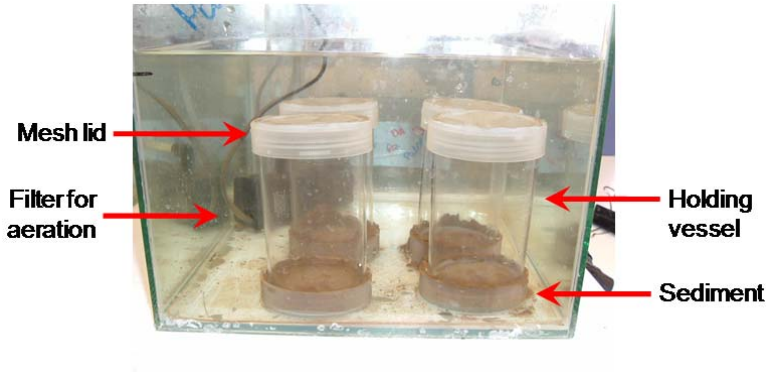


Figure S2. Experimental setup showing the placement of the holding vessels on contaminated sediment and the aquaria in which the vessels were housed.

Acute lethality resulting from the intermittent exposure of *M. plumulosa* to contaminated sediment.

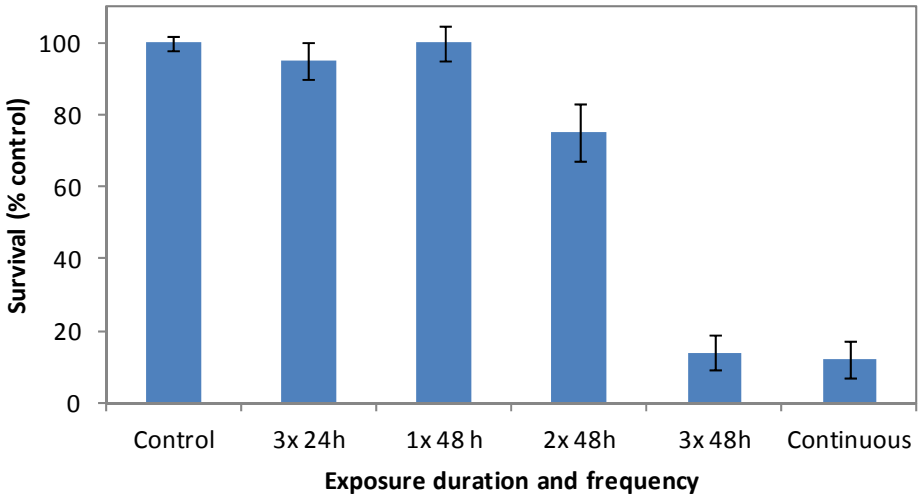


Figure S3. The effect of exposure duration and frequency on the acute toxicity of *M. plumulosa* exposed to Sediment 1.

Summary of Results

Table S1: A summary of acute and chronic toxicity (relative to controls ± standard error) observed for a) *M. plumulosa* and b) *N. spinipes* following pulsed exposure to contaminated sediment.

a)

Sample	Endpoint	Duration of Exposure	Result	Organism
Sediment 1	Acute Lethality	48-h	100±5%	*M. plumulosa*
	Acute Lethality	72-h (3×24-h exposures)	96±5%	*M. plumulosa*
	Acute Lethality	96-h (2×48-h exposure)	75±8%	*M. plumulosa*
	Acute Lethality	144-h (3×48-h exposures)	14±5%	*M. plumulosa*
	Acute Lethality	240-h	12±5%	*M. plumulosa*
	Reproductive Toxicity	8-h	85±15%	*M. plumulosa*
	Reproductive Toxicity	16-h	66±3%	*M. plumulosa*
	Reproductive Toxicity	48-h	34±8%	*M. plumulosa*
	Reproductive Toxicity	48-h (pre-exposure of females)	34±7%	*M. plumulosa*
	Reproductive Toxicity	48-h (pre-exposure of males)	112±10%	*M. plumulosa*
Sediment 2	Acute Lethality	48-h	93±2%	*M. plumulosa*
	Acute Lethality	240-h	24±6%	*M. plumulosa*
Sediment 3	Acute Lethality	48-h	96±2%	*M. plumulosa*
	Acute Lethality	240-h	32±2%	*M. plumulosa*
Sediment 4	Acute Lethality	240-h	59±11%	*M. plumulosa*
	Reproductive Toxicity	48-h	54±18%	*M. plumulosa*

b)

Sample	Endpoint	Duration of Exposure	Result	Organism
Sediment 1	Acute Lethality	24-h	> 60%	*N. spinipes*
	Acute Lethality	48-h	28±2%	*N. spinipes*
	Reproductive Toxicity	24-h	16±4%	*N. spinipes*
Sediment 2	Reproductive Toxicity	48-h	63±9%	*N. spinipes*