Individual and Combined Effects of Petroleum Hydrocarbons Phenanthrene and Dibenzothiophene on Reproductive Behavior in the Amphipod *Hyalella azteca*

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Individual and Combined Effects of Petroleum Hydrocarbons Phenanthrene and Dibenzothiophene on Reproductive Behavior in the Amphipod *Hyalella azteca*
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Dedication

I dedicate this work to my late grandmother, Shubhada Sadashiv Bhagat, without whose dreams and aspirations this work would not have been possible. She boosted my confidence throughout the application process and during my initial transition phase at the University of Louisiana at Lafayette. She supported and cherished my dreams and decisions till her last breath.

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Introduction

The present thesis addresses the individual and combined effects of petroleum hydrocarbons—phenanthrene and dibenzothiophene—on reproductive behavior in the amphipod *Hyalella azteca* (Saussure). The reproductive behavior is studied using two variables: time until initiation of mate-guarding (TIMG) and the proportion of time spent in amplexus (PTA). It is hypothesized that firstly, that there is an inverse relationship between hydrocarbon concentration and the time spent on reproductive behaviors; secondly, that an equimolar mixture of hydrocarbons exerts additive toxicity on amphipods with respect to their reproductive behavior.

Water pollution has been widely studied in order to assess the effects of both organic and inorganic pollutants on aquatic organisms and the pollutants' fate in water bodies. Organic contaminants include insecticides, pesticides, herbicides, petroleum hydrocarbons, polychlorinated biphenyls, polychlorinated dibenzodioxins, polychlorinated dibenzofurans, and ethinylestradiol. Inorganic contaminants include metals like cadmium, copper, mercury, or even nutrients such as nitrates and phosphates. Once these pollutants enter a water body, they may be dissolved or remain as suspended particulate matter or settle to the bottom and become adsorbed onto the sediment. This study focuses on the effects of a specific group of organic pollutants, petroleum hydrocarbons.

Oil spills have received a lot of attention and have been drivers of toxicological studies on aquatic systems. Several studies have documented the spills' adverse effects on aquatic ecology. These studies looked at different organisms at various oil-spill events in order to assess the ecological impacts of these spills. A study focusing on the December 1999 "Erika" oil spill along the French Brittany coast assessed the extent of DNA damage in the mussel *Mytilus edulis* (Bocquené et al., 2004), and showed that the damage was positively correlated with the oil contamination. Another study looked at the effects of the November 2002 "Prestige" oil spill along the Spanish coast (Peteiro et al., 2008). This study determined the effects on the mussel *Mytilus galloprovincialis* and did not detect any effects on growth and lipid metabolism. The recent "Macondo" crude oil spill from the Deepwater Horizon (DWH) explosion is becoming the most widely studied spill. The DWH spill had negative effects on several invertebrates. A study showed elevated stress response in corals, including *Paragorgia regalis* and *Acanthogorgia aspera* (White et al., 2012). The DWH oil, along with the dispersant "Corexit" negatively affected the survival and reproduction in the rotifer *Brachionus plicatilis* (Rico-Martínez et al., 2013), the nematode *Caenorhabditis elegans* (Zhang et al., 2013), and copepods including *Acartia tonsa* and *Temora turbinate* (Almeda et al., 2014).

Apart from these studies on actual oil-spill events, several studies have focused on the effects resulting from exposure to oil and individual oil components. A study on the copepod *Schizopera knabeni* (Lotufo, 1998) showed that phenanthrene and diesel fuel have similar toxic effects with respect to mortality. Furthermore, copepods had a lower feeding rate and offspring production in the presence of phenanthrene and they avoided sites contaminated with phenanthrene when burrowing. It has also been shown that the hydrocarbon fluoranthene caused mortality in the amphipod *Leptocheirus plumulosus* (Schaffner and Dickhut, 1998). Elevated mortality was seen in the grass shrimp *Palaemonetes pugio* when exposed to phenanthrene (Unger et al., 2007) or to naphthalene, dibenzothiophene or fluorene (Unger et al., 2008).

Most studies on the effects of petroleum hydrocarbons have focused on the effects brought about by exposure to individual petroleum hydrocarbons, yet real-world exposures generally involve a mixture of petroleum hydrocarbons. The exposure to multiple chemicals can result in these chemicals interacting with respect to toxicity. When multiple contaminants are present simultaneously, they can act independently or have various types of interactions. Contaminants acting synergistically will cause the combined effect of the contaminants to be larger than what is expected from each individual component in the mixture. If contaminants interact in an antagonistic manner, their combined effect will be smaller than what is expected from each individual component in the mixture. Finally, when contaminants do not interact, their effects are independent or additive, wherein their combined effect is equal to the sum of the effects of the individual components. For polycyclic aromatic hydrocarbons (PAHs), multiple types of interactions have been reported. PAHs generally had additive effects on amphipod mortality (Engraff et al., 2011; Landrum et al., 2003; Swartz et al., 1997) whereas they had synergistic effects in bacterial communities (Verrhiest et al., 2002). Cadmium and phenanthrene acted independently (i.e., effects were synergistic) with respect to amphipod mortality (Gust, 2006), whereas they acted antagonistically with respect to oligochaete mortality (Gust and Fleeger, 2006).

Studies focusing on organic contaminants generally used acute-toxicity tests. There is therefore a need to assess the impacts of PAHs, when present together at sublethal concentrations, in order to obtain a realistic understanding of oil-spill impacts on aquatic ecosystems. Few studies have shed light on how interactions between different PAHs affect reproductive behavior, which is essential for the fitness of organisms. As a substantial portion of spilled oil tends to end up at the sediment–water interface, investigating such effects becomes especially important for the epibenthic organisms that live at this interface. Amphipods are good representatives of this group of organisms and are exposed to contaminants at the sediment–water interface. They are very sensitive to environmental contaminants, have short life span and exhibit precopulatory mate-guarding. Studies using amphipods as test organisms have looked at mortality following exposure to hydrocarbons (Engraff et al., 2011; Kreitinger et al., 2007; Landrum et al., 2003; Lotufo and Landrum, 2002), insecticides (Deanovic et al., 2013), and pesticides (Ingersoll et al., 2005; Negro et al., 2013). This thesis addresses the effects of the petroleum hydrocarbons phenanthrene and dibenzothiophene (for which chemical structures are shown below in Fig.1 and Fig. 2), both individually and together, on mate-guarding behavior in the amphipod *Hyalella azteca*.

Fig. 1. Chemical structure of phenanthrene

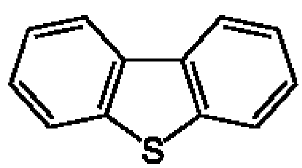

Fig. 2. Chemical structure of dibenzothiophene

Amphipods, like many other crustaceans, exhibit precopulatory mate-guarding behavior (Wen, 1993) which can be used as an end-point to assess the sub-lethal impacts of toxicants. Amphipods are widely used by US-EPA to assess the effects of environmental contaminants, and have many characteristics that make them ideal test organisms. In *Hyalella azteca* (Saussure), mate-guarding is initiated in the 8th instar stage of their life cycle and males hold the females with the help of gnathopods, situated on the males' ventral side (Geisler, 1944). *Hyalella azteca* males preferentially select larger females when initiating mate guarding (Hatcher and Dunn, 1997; Hume et al., 2002; Wen, 1993). This mate-guarding has both—advantages and disadvantages. A study with the amphipod *Gammarus pulex* showed that females involved in mate-guarding had a shorter time interval between molting cycles and thus reached reproductive stage earlier, making precopulatory mate-guarding behavior an advantageous mating strategy for females (Galipaud et al., 2011). That same study also showed that males involved in mate-guarding have a higher fitness than males that were unpaired. With respect to disadvantages, mate-guarding in *Hyalella azteca* has been shown to increase the predation risk to the amphipods, as it effectively increases the size of the organisms and makes them more easily spotted by predators such as fishes (Cothran, 2004). However, it is advantageous when the predators are dragonfly larvae because the latter prefer single unpaired amphipods over paired amphipods as their prey (Cothran, 2004). Because mate-guarding behavior affects the fitness of amphipods, it is important to determine whether exposure to environmental contaminants affects this behavior. Amphipods serve as prey items to invertebrates like nemerteans and arthropods, vertebrates like fish, birds, and a few mammals. If mate-guarding is affected by petroleum hydrocarbons the impact on fitness may eventually affect amphipod populations and potentially the overall local ecosystem.

The precopulatory mate-guarding behavior assay was reported to be a "cheap, reliable and effective indicator of toxicological impact" in the amphipods *H. azteca* (Blockwell,

1998) and *G. pulex* (Watts, 2001). In *Hyalella azteca,* amplexing pairs separated due to exposure to the insecticide lindane (Blockwell, 1998), and amplexus in *Gammarus pulex* was disrupted by exposure to a pyrethroid insecticide (Heckmann et al., 2005). A study of the effects of ethinylestradiol and bisphenol A on precopulatory behavior in the amphipod *Gammarus pulex,* showed that there were no effects at relatively low concentrations of these estrogen derivatives (Watts, 2001). However, the time taken to re-establish pairs among separated males and females was elevated at higher concentrations of these estrogens derivatives. These studies demonstrated that these behavioral bioassays can be used to evaluate toxicity and that they provide an alternative to studies with traditional endpoints of mortality, reduced growth and reduced fecundity. Because the occurrence of molting affects mate-guarding behavior, studies on precopulatory behavior often include an assessment of molting (Cornet et al., 2012; Jormalainen et al., 1994). In the present study, the effects of individual and combined PAHs on amplexing behavior were addressed, taking into account the molting and amplexus status during the hydrocarbon exposure. This study hypothesizes that with higher hydrocarbon concentration, the initiation of mate-guarding is delayed and the proportion of time spent in amplexus is reduced.

Materials and Methods

Experimental organism

The freshwater amphipod *Hyalella azteca* was selected as the experimental organism. This crustacean is found throughout North America in lakes, ponds and streams. The amphipods were collected from the roots of water hyacinths *Eichhornia crassipes* in Lake Martin, near Lafayette, Louisiana. These amphipods were maintained in the laboratory in two HDPE containers (size: 73.7 cm × 45.7 cm × 15.2 cm, source: Rubbermaid, USA) with aerated dechlorinated tap water at room temperature (around 23°C) and a 14:10 h light:dark cycle. The amphipods were fed with YCT (yeast-cerophyl-trout chow) every two days and cotton gauze was added to the storage containers to provide substrate for the amphipods.

Experimental design

The amphipods were exposed to various concentrations of the petroleum hydrocarbons phenanthrene (Phen), dibenzothiophene (DBT), or the mixture of Phen–DBT at equimolar concentrations (see "Toxicants" section below). The experiments were conducted using dechlorinated water that was aerated overnight prior to use. Reconstituted freshwater (moderately soft), tap water, non-aerated dechlorinated water, and aerated dechlorinated water were used to select the optimum conditions for the experiment. It was found that dechlorinated water that was aerated overnight was optimal because it did not affect the water-solubility of hydrocarbons, the extraction process using dichloromethane solvent or the survival of the amphipods. Glass beakers of 250 mL capacity were used for the experiment. Amphipod pairs consisting of two similarly-sized individuals were selected by choosing pairs that were engaged in amplexing behavior at the beginning of the experiment. Such pairs were selected in order to minimize the variability associated with preferential mating, female choice and female molting stage. If an amphipod pair separated before adding the hydrocarbons, then it was replaced with a new amplexing pair. Initially, 200 mL aerated dechlorinated water was added in a beaker, then a pair of amphipods, and lastly the toxicant was added. The toxicants were added at five different concentrations (0, 1, 2, 4, and 8 µM). 12 replicates were used, each containing a single pair of amphipods, for each concentration in a treatment. After exposing the amphipod pair to the toxicant for 24 h, at 23.5°C, with a 14:10 h light:dark cycle, the exposure was stopped by transferring the pair to another beaker with clean water. Just prior to this transfer, two factors—the amplexing state of the amphipod pair (i.e. whether or not the pair was engaged in amplexing behavior) and the presence of molt shed during the exposure were noted. Amphipod pairs were transferred into a transparent plastic (HDPE) cup containing 50 mL dechlorinated water that had been aerated overnight (explained diagrammatically in Fig. 3). I decided to transfer the amphipods to clean beakers rather than just replacing the water in exposure beakers in order to get rid of most of the hydrocarbons adsorbed on the surface of glass beakers. After five min, the male and the female were placed in two different HDPE cups containing 50 mL aerated dechlorinated water. This second transfer further diluted any remaining PAHs and also helped to insure that all the amphipods were separated at the start of the behavior observations. After five min, the female and male were introduced together into a different HDPE cup with 50 mL aerated dechlorinated water. A tripod-mounted video camera ("Sony" digital video camera recorder; Model: DCR-DVD301) was used to record amplexing behavior of amphipods. The amplexing behavior was recorded for ten min. The video was later evaluated to quantify time until initiation of mate-guarding and the proportion of time spent in amplexus, as measures of mate-guarding (see the “Behavioral analyses” section below).

In this study, the term “amplexus” refers to the behavior of an amphipod pair where the male was exactly behind, and in contact with the female. A pair is not considered to be in amplexus when the amphipod pair faced each other, were parallel to each other, or if there appeared to be some distance between the two individuals. The term “mate-guarding” refers to the behavior of an amphipod pair where the male was exactly behind, and in contact with, the female and they stayed together in contact for a minimum of 6 s. A pair is not considered to be mate-guarding when the amphipod pair faced each other, were parallel to each other, or if there appeared to be some distance between the two individuals.

Toxicants

Phenanthrene (Phen)

This is one of the most commonly used hydrocarbons for toxicity testing. It is a US-EPA priority pollutant (Kafilzadeh et al., 2011). Phenanthrene is a polycyclic aromatic hydrocarbon containing three benzene rings (Fig. 1) and has a mass of 178.23 g/mol. Phenanthrene was dissolved in acetone to make a stock solution of 40 g/L. Initially different solvents (including dimethyl sulfoxide, dichloromethane, hexane, and ethanol) were tested to choose the optimal solvent. Acetone was the optimum solvent for this application because hydrocarbons were completely soluble in acetone and there was no precipitation of hydrocarbons once the stock solution was added to water; also acetone did not affect the amphipods’ amplexing behavior and did not interfere with hydrocarbon extractions or the chromatography column in the Gas Chromatography-Flame Ionization Detector (GC-FID) instrument. Concentrations of 1, 2, 4, and 8 μM, and both a solvent (acetone) control and a control with no solvent (no acetone) and no toxicant (no hydrocarbons) were used in this study. The actual concentrations were measured both at the beginning and at the end of the experiment (see the "Chemical analyses" section below).

Dibenzothiophene (DBT)

This is a heterocyclic aromatic hydrocarbon that is found in the heavier fractions of petroleum (Nyman et al., 2007) and is less commonly studied than priority PAH pollutants, in ecotoxicology. It is a compound with two benzene rings and a thiophene ring (Fig. 2) and has a mass of 184.26 g/mol. The same procedures were used for DBT (including solvent, concentrations, extraction and quantification methods) as were used for Phen (see the "Chemical analyses" section below).

Mixture of Phen and DBT (Phen–DBT mixture)

As stated earlier, petroleum hydrocarbons do not occur in nature as individual hydrocarbons but occur as a mixture, so testing the interactive effects of these hydrocarbon mixtures is essential. Hence, a mixture of Phen and DBT was used. The effect of Phen and DBT was studied using Phen at 0, 0.5, 1, 2 and 4 µM and DBT at 0, 0.5, 1, 2 and 4 µM in mixtures with equimolar concentrations of each. Analytical methods used were similar to those described for the individual hydrocarbons and these two hydrocarbons were quantified using GC-FID (see the "Chemical analyses" section below).

Behavioral analyses

Time until initiation of mate-guarding (TIMG)

TIMG in this study is defined as the period of time, that elapsed between the time point that the male was introduced into the cup containing the female to which he had been "paired" before (during the preceding 24-h exposure), and the point that the male and female initiated amplexus. TIMG was quantified from video recordings captured during the 10-min observation period, using the video camera recorder ("Sony" digital video camera recorder; Model: DCR-DVD301), the same stop-watch used for observations and noting the time (in seconds) where mate-guarding was initiated. A pair is defined to be mate-guarding when the male was exactly behind, and in contact with, the female and they stayed together for a minimum of 6 s. The two amphipods were not considered to mate-guarding if they faced each other, were parallel to each other, or if there appeared to be some distance between the two individuals.

Proportion of time in amplexus (PTA)

PTA in this study is defined as the period of time spent in amplexus during the 10-min observation period that started when the male was introduced in the cup that already contained the female. This behavior was assessed using video recordings and amplexus was defined as the proportion of time out of 10-min spent in amplexus. A pair is defined to be in amplexus when the male was exactly behind, and in contact with the female. The two amphipods were not considered to be in amplexus if they faced each other, were parallel to each other, or if there appeared to be some distance between the two individuals.

Although the PTA defined in such a way is not fully independent of TIMG (PTA and TIMG are inversely related), the two variables can vary independently to a degree because brief periods of amplexus are counted towards PTA but not towards TIMG (which required prolonged amplexus lasting longer than 6 s).

Chemical analyses

At the beginning of the experiment, 200 mL beakers with aerated dechlorinated tap water, containing 8 µM Phen, 8 µM DBT, or 8 µM Phen–DBT mixture (at 4 µM each) were used for quantifying initial hydrocarbon levels. These beakers did not contain amphipods. After the 24-h exposures and following the transfer of the amphipods out of the exposure beakers, the water contents of these beakers were used to quantify the final hydrocarbon levels. The Phen, DBT, and the Phen–DBT mixture were extracted from the 200 mL water samples using four sequences of liquid–liquid extraction (LLE). Four portions of 60 mL, water-immiscible organic solvent—dichloromethane (CH_2Cl_2) were used for extraction of the organic hydrocarbons. Anhydrous sodium sulfate (Na_2SO_4) was then added to absorb any moisture that might be present in the extracted organic layer containing the hydrocarbons. The dichloromethane was evaporated using a rotary evaporator at 22°C at a speed of 3.5 rotations per min. The hydrocarbons left behind in the evaporator flask were then dissolved in hexane. Additional portions of hexane were used to rinse the flask to recover all traces of the examined hydrocarbons. The volume was then concentrated to 1 mL by purging nitrogen gas. The extracts were refrigerated under nitrogen till further analysis. The Phen and DBT concentrations were then quantified using gas chromatography with a flame ionization detector (GC-FID). An aliquot of 8 µL of the organic extract was injected into an Agilent technologies 7820 A GC System, under the following conditions: The injector and detector temperatures were set at 250 and 300°C, respectively; helium was used as the carrier gas; samples were injected in the splitless mode; the oven temperature was programmed to increase from an initial temperature of 60°C to 300°C at a rate of 5°C/min and was then maintained at final temperature of 290°C for 25 min. The peak areas of hydrocarbons, computed by a software (Agilent company software) were obtained on a printed chromatogram. The concentrations corresponding to these areas were obtained using standard calibration curves. The standard calibration curves were obtained by using different concentrations in the range of 0.1 μL to 8 μL of Phen standard (Restek; 1 μg Phen per 1 μL methanol) and in the range of 0.5 μL to 8 μL laboratory-prepared DBT standard (1 μg DBT per 1 μL acetone).

Data analyses

In order to analyze the data for molt status at $t = 24$ h, amplexus status at $t = 24$ h, TIMG and PTA, the results for regular control and solvent control were combined. This was done because there was no significant difference between these two control groups for any of these variables. Using this single control group increased the sample size and thus also the statistical power. All the data analyses were performed using JMP Software (Version 10, SAS Institute Inc, 2012). The average measured hydrocarbon concentrations for each treatment, at the beginning of the experiment ($t = 0$ h) and at the end of the experiment ($t = 24$ h) were compared using Student's *t*-test.

The proportion of amphipod pairs that shed at least one molt (most of those had a single molt in the beaker) after 24 h was calculated. An arcsine-square-root transformation was performed on these data (because the data were in the form of proportions and that transformation typically yields a normal distribution for such data) prior to further analyses. In order to see if the PAH exposure affected the molt status at the end of the exposure, a regression analysis was conducted for each chemical treatment. Molting incidence was also included as a covariate in the analyses of TIMG and PTA (see below).

The amplexus status at 24 h was calculated as the proportion of the pairs in a treatment that were in amplexus at the end of the 24-h exposure. Data were again arcsine-square-root transformed. In order to see if the PAH exposure affected the amplexus status at the end of the exposure, a regression analysis was conducted for each chemical treatment. Amplexus status at 24 h was also included as a covariate in the analyses of TIMG and PTA (see below).

TIMG was quantified using video recordings of amphipods during the 10-min observation period following the 24-h contaminant exposure (see earlier). For amphipod pairs that did not amplex during the observation period, their TIMG was censored at the 10-min period. The data were censored because the behavioral observation period was finite resulting in an indefinite ("at least 10-min") time taken to initiate amplexus for those amphipods that did not initiate amplexus during this observation period. The survival analysis proportional hazards method (which evaluates the time to an event in the presence of a covariate) was used to assess the effects of molt status at 24 h, amplexus status at 24 h, and hydrocarbon concentration on TIMG. This was done for each of the three chemical treatments (Phen, DBT, Phen–DBT).

To analyze PTA, the proportion of time during the 10-min observation period that amphipod pairs spent mate-guarding was calculated. An arcsine-square-root transformation was performed prior to further analyses. In order to determine whether molt status at 24 h affected PTA, a *t*-test was used to see whether PTA differed between those pairs with and those without a molt at 24 h. Similarly, a *t*-test was conducted to determine whether PTA differed between pairs in amplexus at 24 h and those not in amplexus at that time. Regression analyses were conducted (one for each treatment) of PTA against PAH concentration in order to assess whether exposure to the hydrocarbons affected PTA during the 10-min observation period. Although TIMG and PTA are generally inversely related, they both are quantified and analyzed independently with respect to hydrocarbon treatment and the hydrocarbon concentration. As referred to earlier, these variables are not mutually exclusive but can capture different aspects of the effects of contaminants on amplexing behavior. The TIMG and PTA variables are neither analyzed together nor are analyzed in relation to one another.

The interaction between Phen and DBT with respect to their effect based on amplexus status at the end of the 24-h exposure and TIMG was quantified. The arcsine-square-root transformed proportion of amphipod pairs in amplexus at the end of the 24-h exposure was plotted against the concentration for each of the two individual PAHs and the Phen–DBT mixture. The slope of regression line (observed effect for Phen–DBT mixture) was then statistically compared to the slope of the line predicted if the two PAH effects were additive (exerting toxicity independent of each other). The slopes of two regression lines were compared using “Tests of significance in regression” (Sokal and Rohlf, 1981). The predicted relationship was based on the average arcsine-square-root transformed proportion from exposures to Phen and DBT individually. The observed relationship of the Phen–DBT interaction was compared to the predicted Phen–DBT interaction by assessing whether the regression coefficients differed statistically. If the slopes of regression lines obtained for observed Phen–DBT and predicted Phen–DBT effects differed from each other, then the Phen and DBT interacted either synergistically or antagonistically. If the slopes of observed

Phen–DBT and predicted Phen–DBT regression lines did not differ significantly, then the Phen and DBT had no interactive effects and their toxicity was additive.

The interaction between Phen and DBT with respect to their effect based on TIMG was quantified on the basis of 25% quartile values (time until 25% of the amphipod pairs had initiated mate-guarding) at each concentration. These values were obtained as output of the non-parametric survival analysis. While mean or median values are commonly used, a valid mean could not be determined because the censored data (for those pairs that had not initiated mate-guarding at the 10-min observation period) caused the mean to be biased. Moreover, less than 50% of pairs initiated mate-guarding in some treatments; therefore, medians could not be determined. The time until 25% of the pairs had initiated mate-guarding was plotted against the concentration for each of the two individual PAHs and the Phen–DBT mixture. The slope of the latter line (observed effect for Phen–DBT mixture) was then statistically compared to the slope of the line predicted if the two PAH effects were additive (exerting toxicity independent of each other). The slopes of the two regression lines were compared using “Tests of significance in regression” (Sokal and Rohlf, 1981). The predicted relationship was based on the average TIMG values from exposures to Phen and DBT individually. The observed relationship of the Phen–DBT interaction was compared to the predicted Phen–DBT interaction by assessing whether the regression coefficients differed statistically. If the slopes of regression lines obtained for observed Phen–DBT and predicted Phen–DBT interaction differed from each other, then the effects of Phen and DBT were not additive and showed an interaction that was either synergistic or antagonistic.

To assess the overall interactive effects of hydrocarbon concentrations on PTA, the EC_{50} values (the concentration at which the proportion of time spent in amplexing was reduced by 50% relative to the control) were calculated. These EC_{50} values were then used to obtain the sum of toxic contributions (S) value. This S value indicates the type of interaction of a binary mixture of chemicals seen as an overall effect (Bailey et al., 1997; Pape-Lindstrom and Lydy, 1997; Swartz et al., 1997).

This value was obtained using the following formula:

$$S\ (PTA) = \frac{EC_{50}\ \text{for Phen in presence of DBT}}{EC_{50}\ \text{for Phen individually}} + \frac{EC_{50}\ \text{for DBT in presence of Phen}}{EC_{50}\ \text{for DBT individually}}$$

If–S = 1, the interactive effects are additive; if S > 1, the interactive effects are antagonistic; if S < 1, the interactive effects are synergistic.

Results

Chemical analyses

The actual concentrations of the hydrocarbons were quantified only for the 8 µM for individual hydrocarbons and 4 µM each in the mixture treatments, since concentrations at the end of the 24-h exposure period were below detection limits for the lower treatment levels. For the beakers with the nominal concentration of 8 µM Phen the measured concentration at $t = 0$ h was 4.82 µM and this declined (in beakers with amphipods) at $t = 24$ h to 0.74 µM (Table 1). This decline was statistically significant ($t = -11.24$, $n = 7$, $p = 0.0005$). For the beakers with the nominal DBT concentration of 8 µM; the measured concentration for $t = 0$ h averaged 5.37 µM, and this declined to 1.86 µM at $t = 24$ h (Table 1). This decline was statistically significant as well ($t = -8.80$, $n = 6$, $p = 0.007$). For the Phen–DBT mixture with nominal concentrations of 4 µM for each chemical, the Phen declined from 2.95 µM at $t = 0$ h to 0.29 µM at $t = 24$ h, whereas the DBT concentration declined 2.37 µM at $t = 0$ h to 0.31 µM at $t = 24$ h (Table 1). This decline in concentrations for the Phen–DBT mixture is illustrated using chromatograms for the water samples obtained at $t = 0$ h (Fig. 4) and at $t = 24$ h (Fig. 5). The decline was statistically significant for Phen ($t = -8.38$, $n = 5$, $p = 0.008$) but not for DBT ($t = -2.88$, $n = 5$, $p = 0.10$).

Effects on molt status at the end of the 24-h exposure

There were no statistically significant effects of the hydrocarbon concentration on the molting status of amphipods after 24 h. This lack of statistical significant effects was observed for Phen (Fig. 6, $F_{1,3} = 3.92$, $t = 1.98$, $n = 60$, $p = 0.14$), for DBT (Fig. 7, $F_{1,3} = 0.03$, $t = 0.18$, $n = 57$, $p = 0.87$), as well as for Phen–DBT mixture (Fig. 8, $F_{1,3} = 1.14$, $t = 1.07$, $n = 52$, $p = 0.36$).

Effects on amplexus status at the end of the 24-h exposure

The incidence of amplexus at the end of the 24-h exposure period showed a statistically significant inverse relationship with the Phen concentration (Fig. 9, $F_{1,3} = 49.31$, $t = -7.02$, $n = 60$, $p = 0.006$). The same trend was observed for Phen–DBT mixture (Fig. 10, $F_{1,3} = 477.59$, $t = -21.85$, $n = 52$, $p = 0.0002$). However, the relationship was not significant for DBT (Fig. 11, $F_{1,3} = 6.56$, $t = -2.56$, $n = 57$, $p = 0.08$).

Effects on time until initiation of mate-guarding

Phenanthrene

The overall proportional hazards model that included molt status after 24-h exposure, amplexus after 24-h exposure, and Phen concentration was highly significant ($\chi^2 = 14.17$, $df = 3$, $n = 60$, $p = 0.003$; data not shown). This analysis showed that TIMG was significantly affected by the molt status ($\chi^2 = 5.25$, $df = 1$, $n = 60$, $p = 0.02$) and amplexus status ($\chi^2 = 6.01$, $df = 1$, $n = 60$, $p = 0.01$), but not by the Phen concentration ($\chi^2 = 0.36$, $df = 1$, $n = 60$, $p = 0.55$). Having a molt at 24 h increased TIMG and being in amplexus at 24 h decreased TIMG. In line with the results from the proportional hazards model, the non-parametric survival analyses detected a significant effect of molt status on TIMG (Fig. 12, $\chi^2 = 6.11$, $df = 1$, $n = 60$, $p = 0.01$) and a significant effect of amplexus status on TIMG (Fig. 13, $\chi^2 = 11.51$, $df = 1$, $n = 60$, $p = 0.0007$), but no significant effect of the Phen concentration on TIMG (Fig. 14, $\chi^2 = 3.65$, $df = 4$, $n = 60$, $p = 0.46$).

Dibenzothiophene

The overall proportional hazards model that included molt status, amplexus status, and DBT concentration was highly significant ($\chi^2 = 12.33$, $df = 3$, $n = 57$, $p = 0.006$; data not shown). This analysis also showed that TIMG was significantly affected by the molt status at

24 h (χ^2 = 4.14, *df* = 1, *n* = 57, *p* = 0.04) and amplexus status at 24 h (χ^2 = 9.72, *df* = 1, *n* = 57, *p* = 0.002), but not by the DBT concentration (χ^2 = 0.03, *df* = 1, *n* = 57, *p* = 0.87). Presence of molt at 24 h decreased TIMG and being in amplexus at 24 h decreased TIMG. No significant effect of molt status was detected on TIMG using non-parametric survival analysis (Fig. 15, χ^2 = 1.48, *df* = 1, *n = 57*, *p* = 0.22*)*. However, a significant effect of amplexus status at 24 h on TIMG was seen (Fig. 16, χ^2 = 9.91, *df* = 1, *n = 57*, *p* = 0.002), showing that being engaged in amplexus at 24 h was associated with a reduced TIMG during the subsequent behavioral assay. No significant effect of the DBT concentration on TIMG was found (Fig. 17, χ^2 = 2.31, *df* = 4, *n* = 57, *p* = 0.68) in the non-parametric survival analysis.

Mixture of phenanthrene and dibenzothiophene

The overall proportional hazards model (with molt status, amplexus status, and Phen–DBT concentration) was not statistically significant (χ^2 =4.98, *df* = 3, *n* = 52, *p* = 0.17; data not shown). The non-parametric survival analyses did not detect a significant effect of molt status on TIMG (Fig. 18, χ^2 = 0.007, *df* = 1, *n* = 52, *p* = 0.93) or a significant effect of the Phen–DBT concentration on TIMG (Fig. 20, χ^2 = 4.59, *df* = 4, *n* = 52, *p* = 0.33). The analysis did however did detect a significant effect of amplexus status at 24 h on TIMG (Fig. 19, χ^2 = 5.12, *df* = 1, *n* = 52, *p* = 0.02), indicating that amphipod pairs engaged in amplexus at 24 h had a reduced TIMG during the subsequent observation period.

Effects on proportion of time spent in amplexus

Phenanthrene

While there was a tendency for PTA to be lower for those pairs of amphipods in which at least one of the individuals had molted than for pairs that had not shed a molt during the 24–h exposure period, this difference was not statistically significant (Fig. 21, $F_{1,58}$ = 3.73, t = 1.93, n = 60, p = 0.06). The PTA was clearly and significantly higher in those pairs that were in amplexus at the end of the 24-h exposure period than in those that were not engaged in amplexus at that time (Fig. 22, $F_{1,58}$ = 15.53, t = –3.94, n = 60, p = 0.0002). The Phen concentration had no statistically significant effect on PTA (Fig. 23, $F_{1,3}$ = 2.03, t = –1.42, n = 5, p = 0.25).

Dibenzothiophene

While there was a tendency for PTA to be higher for those amphipod pairs that had shed at least one molt during the exposure period compared to those pairs in which none of the amphipods molted, this difference was not quite statistically significant (Fig. 24, $F_{1,55}$ = *3.66*, t = –1.91, n = 57, p = 0.06). The PTA was significantly higher in those pairs that were in amplexus at the end of 24-h exposure period, compared to the PTA of pairs not in amplexus at that time (Fig. 25, $F_{1,55}$ = *14.39*, t = –3.79, n = 57, p = 0.0004). The DBT concentration had no statistically significant effect on PTA (Fig. 26, $F_{1,3}$ = 1.77, t = –1.33, n = 5, p = 0.28).

Mixture of phenanthrene and dibenzothiophene

The PTA was not significantly affected by the molting status of amphipods after the 24-h exposure period (Fig. 27, $F_{1,50}$ = 0.43, t = –0.65, n = 52, p = 0.52). However, the PTA was significantly higher in pairs that were in amplexus at the end of the 24-h exposure period than in those that were not in amplexus at that time point (Fig. 28, $F_{1,50}$ = *14.42*, t = *–3.80*, n = *52*, p = 0.0004). The concentration of the Phen–DBT mixture had no statistically significant effect on PTA (Fig. 29, $F_{1,3}$ = 5.04, t = –2.25, n = 5, p = 0.11).

Interactive effects

Amplexus status at the end of the 24-h exposure period.

For the relationship between the amplexus status at the end of the 24-h exposure and the Phen–DBT concentrations, a comparison between the observed relationship and the relationship predicted on the basis of the effects of the two components being additive showed that the slopes of the two regression lines did not differ significantly (Fig. 30, $\beta_{observed} = -0.10$, $\beta_{predicted} = -0.08$, $t = -1.02$, $n = 10$, $p = 0.35$).

Effects on TIMG

A comparison between the observed relationship of TIMG and Phen–DBT concentrations and the relationship predicted on the basis of the effects of the two components being additive, showed that the difference between the two regression lines was somewhat a function of the exposure concentration. At concentrations below 4.23 µM, the combined effect of Phen and DBT was less than predicted on the basis of the individual effects (i.e. their interaction was antagonistic) while, at concentrations of Phen–DBT above 4.23 µM, the interactive effect on TIMG was synergistic in nature. However, the difference in slopes between the observed and predicted relationship for the dependence of TIMG on the concentration of the mixture, was not statistically significant (Fig. 31, $\beta_{observed} = 42.98$, $\beta_{predicted} = 7.68$, $t = 1.08$, $n = 10$, $p = 0.32$).

Effects on PTA

The interaction between Phen and DBT with respect to their inhibition of PTA was assessed on the basis of their EC_{50} values (concentrations at which PTA was reduced by 50%). The EC_{50} was 13.74 µM for Phen by itself, 16.35 µM for dibenzothiophene by itself, and 9.44 µM for the equimolar mixture of the chemicals (each of the chemicals present at 4.72 µM). The resulting value for the interaction coefficient S was 0.63. This value is less than 1, indicating that the interaction between Phen and DBT with respect to PTA was synergistic in nature. The toxic effects of this study's equimolar mixture of Phen and DBT on PTA were therefore more potent than the toxic effects predicted from the toxicity of Phen and DBT by themselves.

Discussion

Chemical analyses

The results of this study showed a substantial reduction in the hydrocarbon concentration from $t = 0$ h to $t = 24$. The decrease in concentrations of polycyclic aromatic hydrocarbons or their low concentrations may have been the responsible for lack of significant effects of concentration on both the variables—TIMG and PTA. Such a substantial decrease has been demonstrated by other studies (most of which also used higher concentrations than were used here). Sediment concentrations of naphthalene also showed substantial decline after 72 h of exposure, and even at the reduced concentrations of 5 μg/g sediment and 50 μg/g, sediment significantly affected the reproductive behavior in the amphipod *Corophium volutator* (Krang, 2007). . In the present study, the decline in the measured hydrocarbon concentrations may be due to loss by evaporation. This loss may be high due to the relatively high surface area of water in the beakers used. The decrease in concentrations can also be attributed to adsorption of hydrocarbons on the surface of the amphipods, and to ingestion or degradation of the hydrocarbons. Earlier studies have shown that amphipod *Diporeia* spp. have a negligible ability to biotransform PAHs (Lotufo and Landrum, 2002). In the current study, the GC-FID chromatograms did not have any peaks for obvious breakdown products like naphthalene (Fig. 4 and Fig. 5). This indicates that the decrease in concentration was not due to degradation, but is probably due to adsorption or ingestion by amphipods. Further research in phenanthrene and dibenzothiophene toxicokinetics at sub-lethal levels of these hydrocarbons can provide a better understanding of their toxicity and toxicity interaction with respect to the reproductive behavior in *Hyalella azteca*.

Effects on molt status at the end of the 24-h exposure

It has been shown that the molting cycle is affected by exposure to petroleum hydrocarbons. For example, pyrene delayed molting in the males of grass shrimp *Palaemonetes pugio* (Oberdörster et al., 2000), and these authors suggested this relationship existed because the P_{450} system which plays an important role in detoxification processes is also responsible for metabolism of 20-hydroxyecdysone which regulates molting (Oberdörster et al., 2000). It is also known that molting state and amplexing decisions in the amphipod in *G. pulex* are correlated; hence, controlling for molting becomes necessary while studying their reproductive behavior (Cornet et al., 2012). In the present study, no statistically significant relationship was seen between molting and the concentration of either Phen, DBT, or Phen–DBT. The lack of statistically significant effects of Phen and DBT (individually or in mixture) on molting indicates that these two hydrocarbons did not elicit an ecdysosteroid response or that the 24-h exposure was too short to do so. Consequently, any changes observed in this study on mate-guarding behavior are thus likely to be due to the hydrocarbon exposure itself rather than an indirect effect from changes in molting.

Effects on amplexus status at the end of the 24-h exposure

The amplexus status was recorded at the end of the 24-h exposure period, as it was expected that this status may affect mate-guarding during the subsequent observation period. For Phen and Phen–DBT treatments, the exposure to hydrocarbons resulted in fewer pairs of amphipods being engaged in amplexus at the end of the 24-h exposure period. These results are in accordance with the results from a previous study in *H. curvispina* , where higher concentrations of endosulfan (pesticide) resulted in fewer amphipod pairs remaining in amplexus (Negro et al., 2013). Initiation of amplexus is an energetically costly affair in the amphipod *Gammarus pulex*, and these amphipods tend to remain in amplexus till molting occurs (Plaistow et al., 2003). The reduction in incidence of amplexus at the end of the 24-h exposure period was not statistically significant for the DBT treatment. Since the effect for DBT by itself was not statistically significant, but the effect of Phen–DBT treatment was, more research is needed to understand if the latter effect was due to the presence of Phen alone.

Reproductive behavior in amphipods has also been affected by the presence of predators and parasites that tend to lower the incidence of mate-guarding. In the presence of predatory sunfish, *Gerris remigis* (Insecta) significantly decreased their mating activity and reproductive investment (Sih et al., 1990) whereas parasitic infection decreased mate-guarding in the amphipod *G. pulex* (Ward, 1986). These results show that under environmental conditions causing stress, reproductive behavior is altered. The polycyclic aromatic hydrocarbons have been shown to cause a stress response in other amphipods (Engraff et al., 2011; Krang, 2007; Landrum et al., 2003). Contaminants adversely affected mate-guarding in amphipods, with studies reporting this effect for naphthalene exposure in the amphipod *Corophium volutator* (Krang, 2007) and pyrethroid exposure in the amphipod *G. pulex* (Heckmann et al., 2005). The results of the present study for Phen and the Phen–DBT mixture are in agreement with these previous studies. It is unclear what mechanisms were responsible for this effect. It can be hypothesized that reproductive behavior is altered by a change in energy allocation or by toxicant-induced damage. Such damage may involve the sensory system, because it was shown for the amphipod *Corophium volutator* that exposure to the PAH naphthalene impairs the males' ability to recognize females (Krang, 2007). Because the present study had only one male and one female, whether recognition was affected could not be assessed. Further investigations that specifically focus on energy allocation or recognition may provide a clearer picture of the mechanisms underlying the effects of Phen and DBT on amphipods' reproductive behavior.

Effects on time until initiation of mate-guarding

The present study detected overall significant effects using proportional hazards models that included the hydrocarbon concentrations as well as molting status and amplexus status at the end of the 24-h exposures, for the individual Phen and DBT treatments, but not for the Phen–DBT mixture. There was also a significant effect of molting status on TIMG using proportional hazards model for individual hydrocarbons Phen and DBT, but not for the Phen–DBT mixture. The mechanism causing this effect is still unclear. The effect of amplexus state at the end of 24 h was significant in the proportional hazards model for Phen, DBT, as well as the Phen–DBT mixture. The nonparametric survival analyses showed that TIMG was significantly affected by molting status for the Phen treatment only, whereas hydrocarbon concentrations had no effect on TIMG for either of the three chemical treatments. The TIMG during the observation period was significantly affected by amplexus status at the end of the exposure period for each of the three chemical treatments. As described earlier, the incidence of amplexus at the end of the 24-h exposure period was significantly reduced at higher hydrocarbon levels. Thus, the hydrocarbon exposures indirectly delayed the time taken to initate amplexing behavior. These results support our hypothesis that at higher hydrocarbon concentrations, the time until initiation of mate-guarding is increased. This effect may be due to damage to sensory system involved in recognition of mates in *Hyalella azteca.* A previous study showed that PAHs like naphthalene can affect the pheromone recognition in the amphipod *Corophium volutator* and in turn affect the amphipods' reproductive behavior and fitness (Krang, 2007). Another potential reason for the relationship with amplexus status at the end of the exposure is that pairs not engaged in amplexing behavior were more affected by hydrocarbons since they had higher surface area for the hydrocarbon absorption. The mechanisms delaying the TIMG are unclear. Further research on the metabolic costs involved in the reproductive behavior in presence of Phen and DBT might shed light on delayed initiation of mate-guarding.

Effects on proportion of time spent in amplexus

Amphipod pairs that had shed at least one molt at the end of the 24-h Phen exposure had significantly reduced PTA. This reduction in PTA was observed only for Phen and not for DBT or the Phen–DBT mixture. Further research is needed to explain this difference. While the two chemicals have a fairly similar structure and may thus have the same mode of action, modes of action for these chemicals in the mixture are yet to be reported. Amphipod pairs engaged in amplexus after 24-h exposure to Phen, DBT, and Phen–DBT had significantly higher PTA during the subsequent mate-guarding assay in clean water. As described earlier, the incidence of amplexus at the end of the 24-h exposure was significantly reduced at Phen, DBT and Phen–DBT concentrations. Consequently, the proportion of time spent in amplexing behavior during the observation period was reduced indirectly by the hydrocarbon exposures—again by affecting the amplexus status at the end of the preceding exposure period. These results support the hypothesis that at higher hydrocarbon concentrations, the proportion of time spent in amplexus by amphipods is reduced.

One potential reason for the higher PTA at lower hydrocarbon concentrations is that pairs that were still engaged in mate-guarding at the end of the 24-h exposure period could have recognized each other faster once both were paired up again for the observation period. As mentioned earlier, the study on the amphipod *C. volutator* showed that naphthalene affected the pheromone recognition and thus the ability to recognize the mates earlier (Krang, 2007). Also, in amphipods, a male can evaluate a female's quality (Cornet et al., 2012; Lemaître et al., 2009). Being engaged in mate-guarding (with the same mate earlier) could result in males making a quicker decision to choose the female, thereby initiating mate-guarding earlier, and thus remaining in amplexus for a longer time duration. Increased metabolic demand required for reproductive behavior in amphipods has shown to increase the sensitivity to contaminants (Negro et al., 2013). A potential reason for observation in this study is that animals which spent more energy on reproductive behavior had less energy to invest in metabolic and detoxification activities. The present study did not evaluate the energetic demand for both mate-guarding and detoxification processes; hence, more research on energetic expenditure of amphipods in presence of contaminants is needed.

Interactive Effects

While other studies have shown the effects of different PAHs on amphipods, the present study is the first one to do so for precopulatory mate-guarding and for the mixture of phenanthrene and dibenzothiophene. Studying effects on this behavior is important because at environmentally relevant concentrations, the toxicants may not be lethal but they can still affect the organisms at sub-lethal concentrations. This study's findings showed that interactive effects tended to differ for the different variables measured and, for those interaction assessments that provided such detail, for the different exposure concentrations. The effects of Phen and DBT on the amplexus status at the end of the 24-h exposure were additive. For the effect on TIMG during the post-exposure observation period, the interaction was generally additive in nature, because the observed and predicted slopes were not significantly different from each other. There was a tendency though for an antagonistic effect at concentrations below 4.23 µM and a synergistic effect at higher concentrations. Such a dependency of the interaction type on the exposure concentration has been reported previously (Moreau et al., 1999). The effect on PTA (again during the post-exposure period) where the methodology provides only an overall assessment of the interaction (i.e., no distinction by concentration), the two chemicals were acting synergistically. Studies on PAH interactions with respect to amphipod mortality (Engraff et al., 2011; Landrum et al., 2003) have generally reported such effects to be additive. This additive interaction is expected where mixture components have similar modes of action. There is insufficient information on the modes of action of Phen and DBT in amphipods to evaluate the similarity in mode of action.

This study's observation of a mixed additive and synergistic interaction for the effect of Phen and DBT on mate-guarding indicates that these chemicals may differ in their mode of action when it comes to their effect on mate-guarding behavior. This study has shown that amphipod mate-guarding is affected by exposure to the two hydrocarbons—phenanthrene and dibenzothiophene, and that the two chemicals interact with respect to this effect in a complex way. This illustrates the challenges that are faced when trying to predict the impacts of oil spills on aquatic communities, where large numbers of chemical constituents (with even larger number of potential interactions) are involved, with larger numbers of aquatic species at risk, and where each species may be affected in effects as subtle as a change in reproductive behavior.

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FIGURES

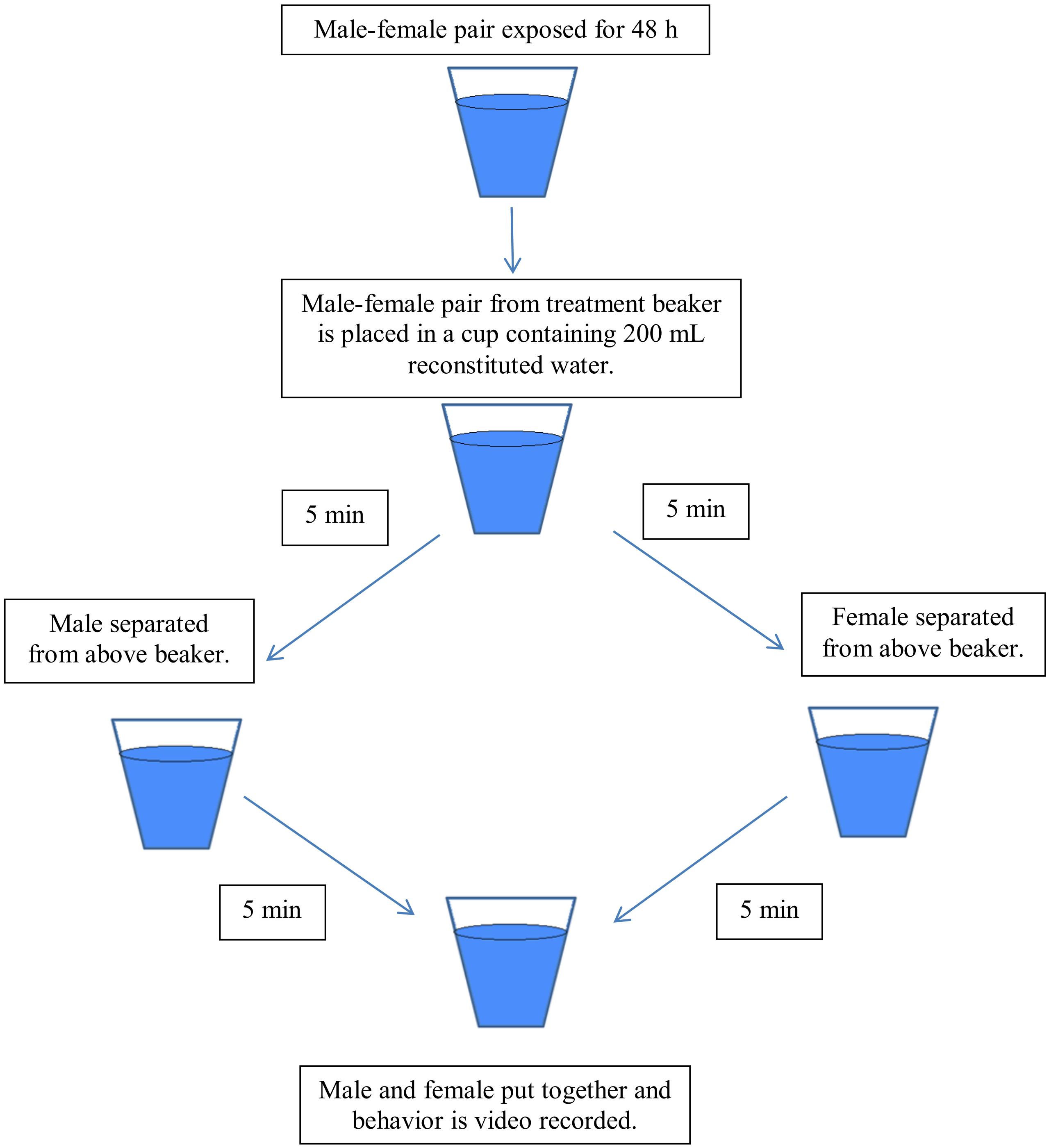
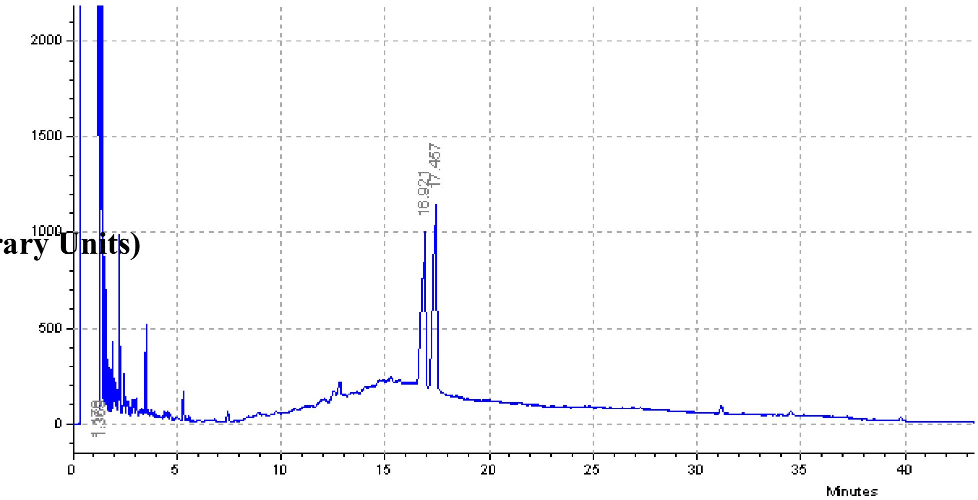


Fig. 3. Protocol for quantifying the mate-guarding behavior measures time until initiation of mate-guarding (TIMG) and proportion of time spent in amplexus (PTA).

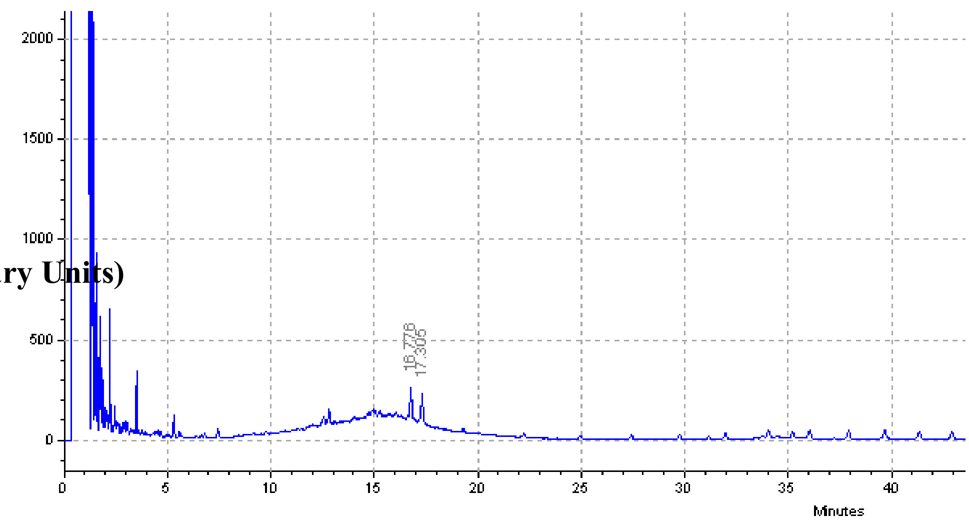
Peak height (Arbitrary Units)



Minutes

Fig. 4. A chromatogram of 8 µM phenanthrene and dibenzothiophene, at 4 µM each, at the beginning of the experiment ($t = 0$ h), with the peak of phenanthrene is at 16.921 min and the one of dibenzothiophene at 17.457 min.

Peak height (Arbitrary Units)



Minutes

Fig. 5. A chromatogram of 8 µM phenanthrene and dibenzothiophene, at 4 µM each, at the end of the experiment ($t = 24$ h), with the peak of phenanthrene at 16.776 min and the one of dibenzothiophene at 17.305 min.

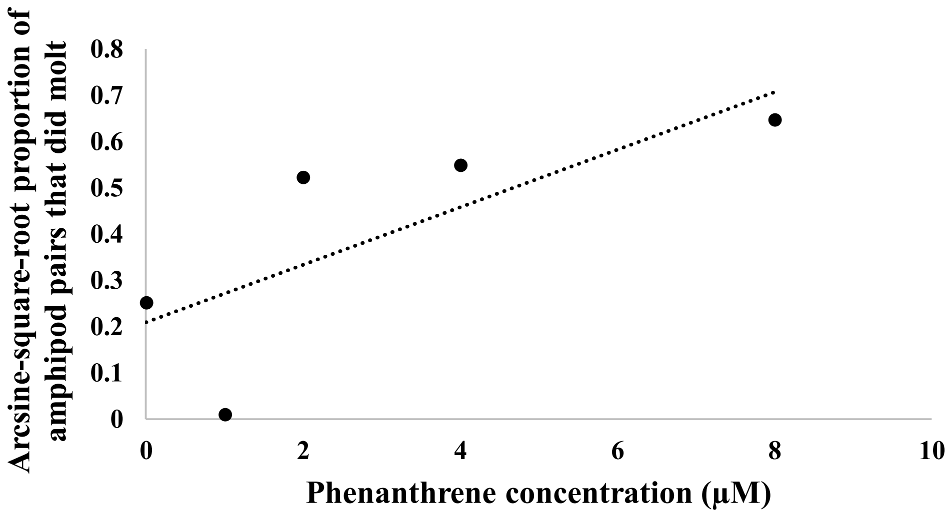


Fig. 6. Proportion of amphipod pairs in which at least one individual had molted during the 24-h exposure as a function of the phenanthrene concentration. The dotted line represents the regression line.

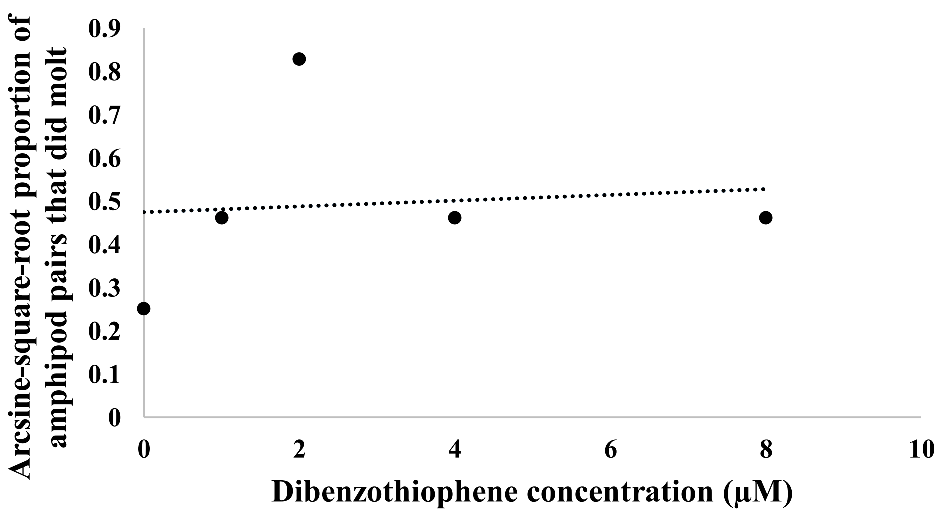


Fig. 7. Proportion of amphipod pairs in which at least one of them had molted during the 24-h exposure as a function of the dibenzothiophene concentration. The dotted line represents the regression line.

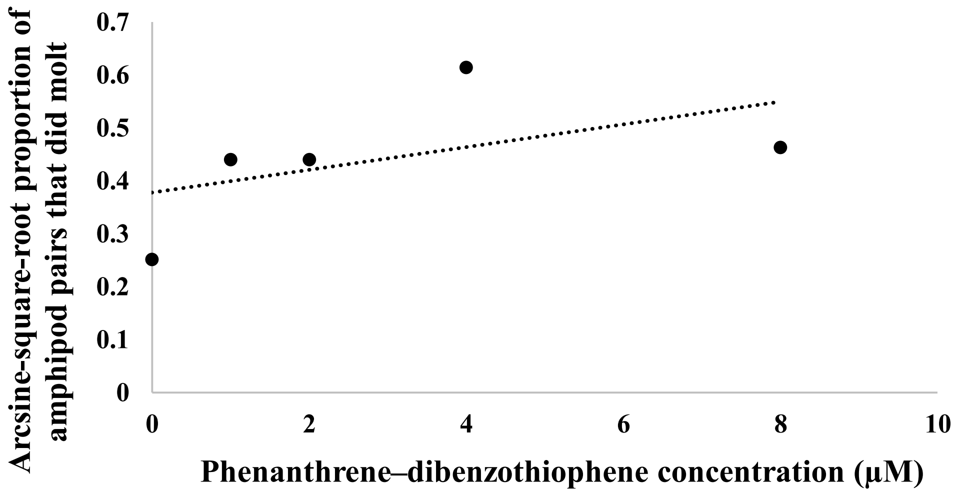


Fig. 8. Proportion of amphipod pairs in which at least one individual had molted during the 24-h exposure as a function of the phenanthrene and dibenzothiophene concentrations. The dotted line represents the regression line.

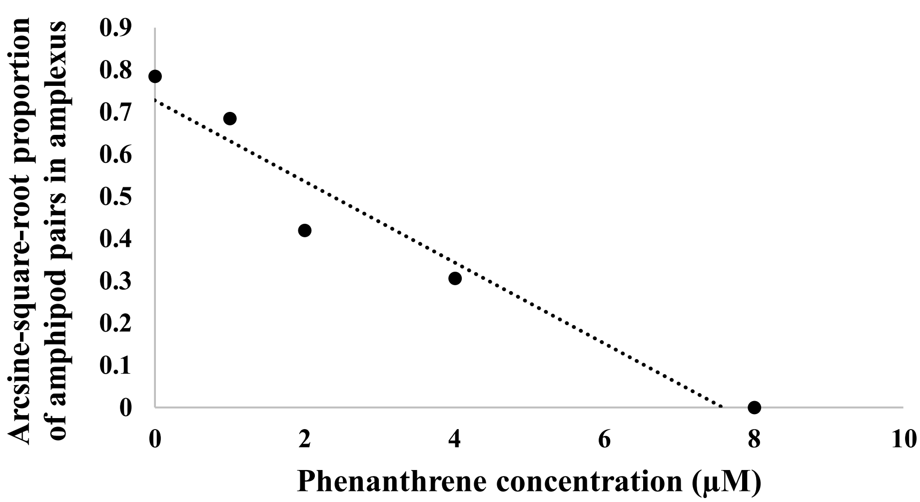


Fig. 9. Proportion of amphipod pairs that were engaged in amplexus at the end of the 24-h exposure period as a function of the phenanthrene concentration. The dotted line represents the regression line.

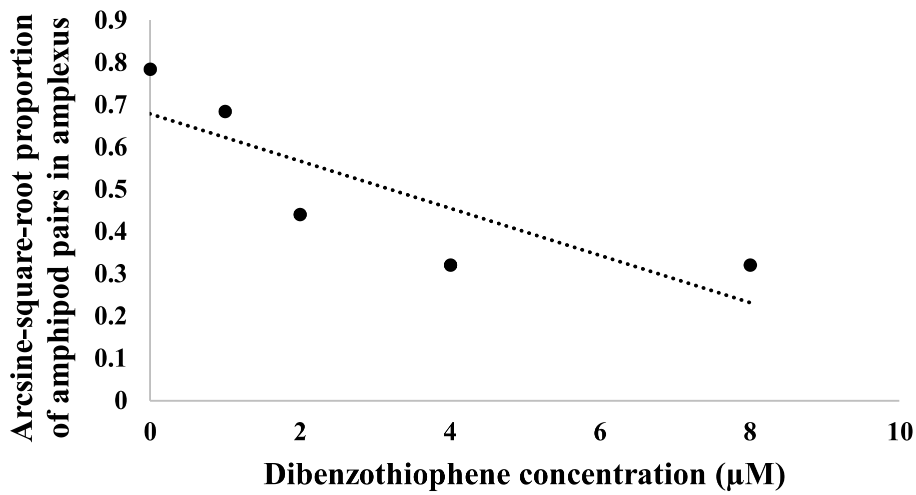


Fig. 10. Proportion of amphipod pairs that were engaged in amplexus at the end of the 24-h exposure period as a function of dibenzothiophene concentration. The dotted line represents the regression line.

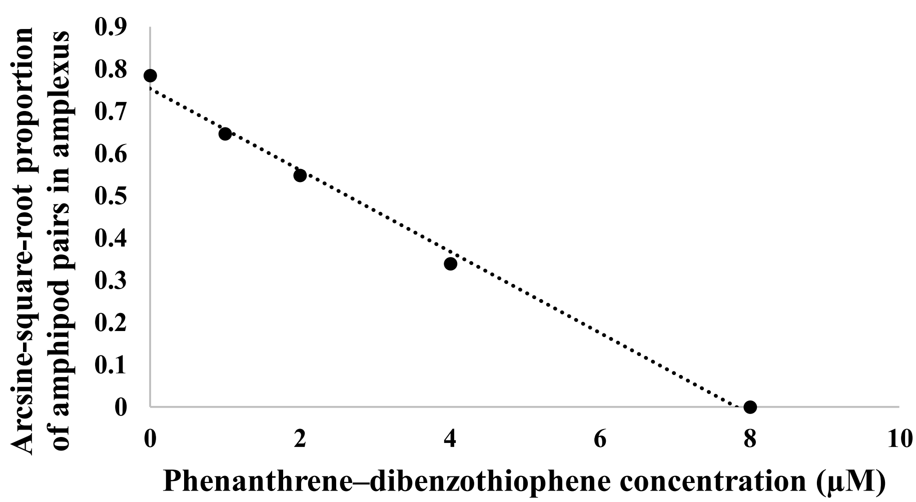


Fig. 11. Proportion of amphipod pairs that were engaged in amplexus at the end of the 24-h exposure period as a function of phenanthrene and dibenzothiophene concentrations. The dotted line represents the regression line.

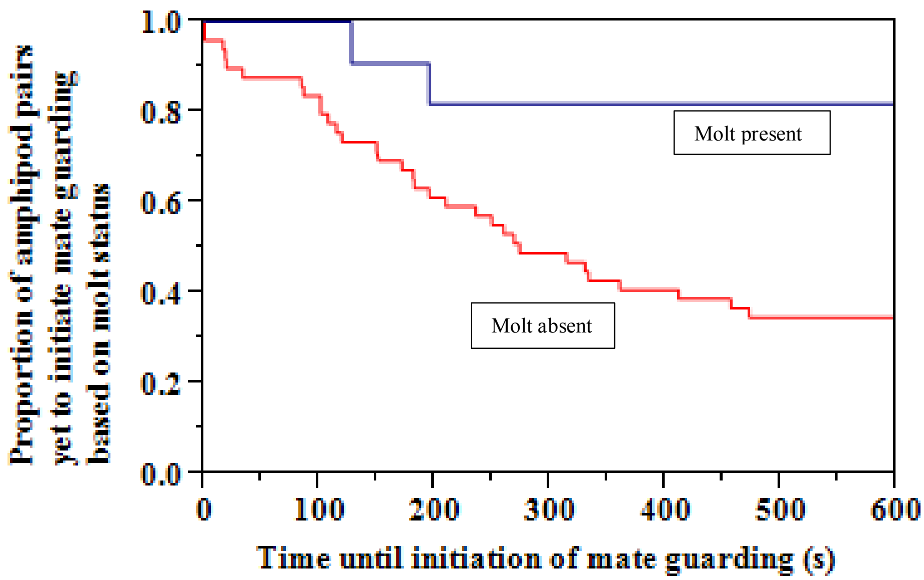


Fig. 12. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for pairs that did not molt (‘Molt absent’) and those that did molt (‘Molt present’) during 24-h exposure to phenanthrene.

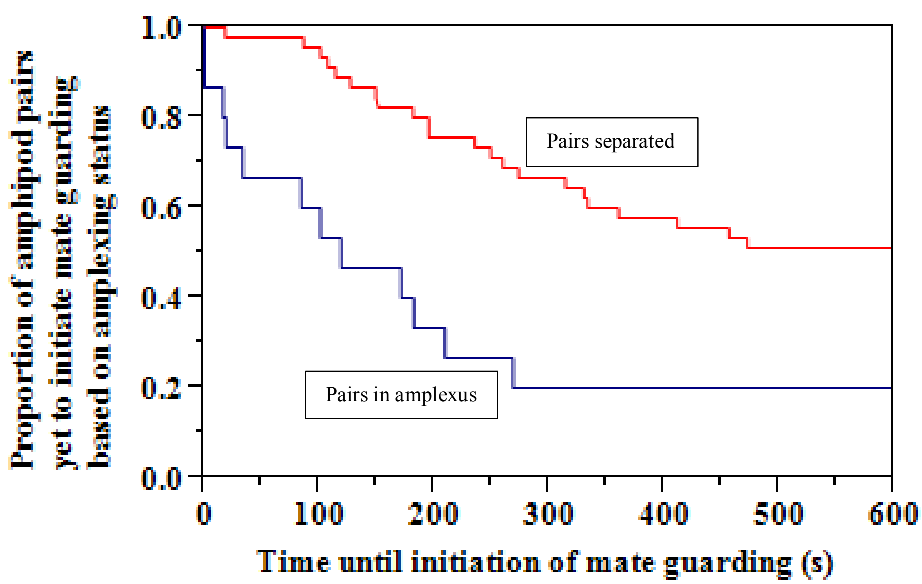


Fig. 13. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for pairs still in amplexus (‘Pairs in amplexus’) and those no longer in amplexus (‘Pairs separated’) at the end of the 24-h exposure to phenanthrene.

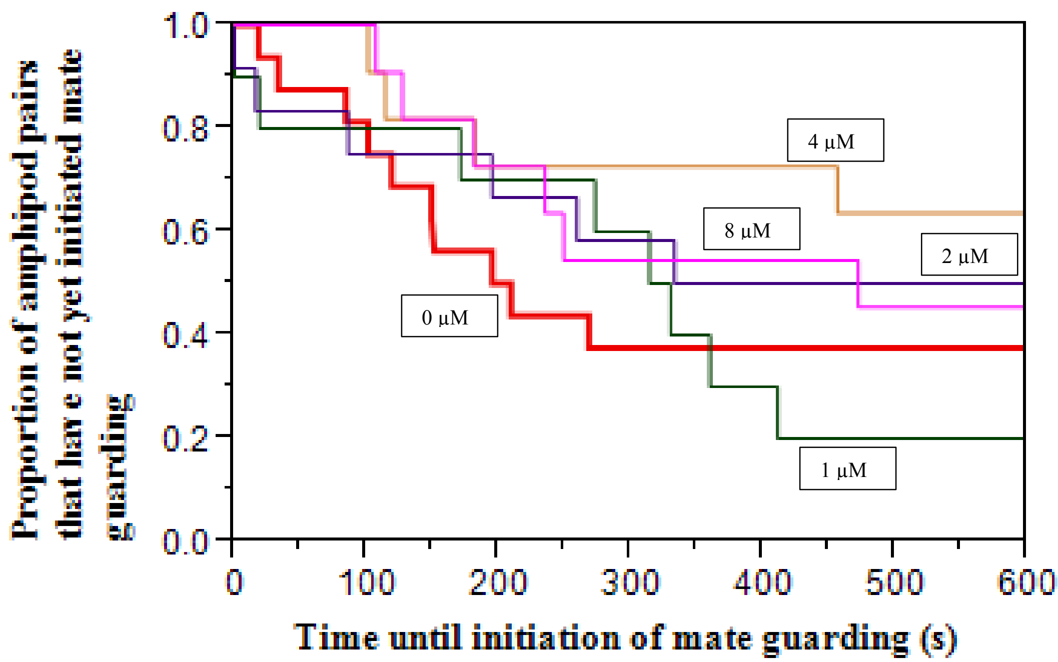


Fig. 14. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for amphipods previously exposed to different concentrations of phenanthrene.

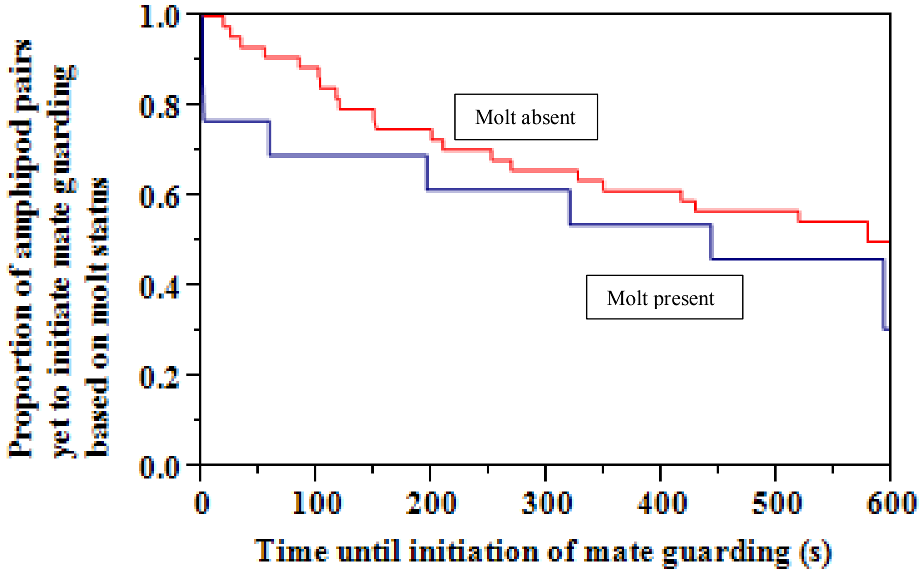


Fig. 15. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for pairs that did not molt ('Molt absent') and those that did molt ('Molt present') during the 24-h exposure to dibenzothiophene.

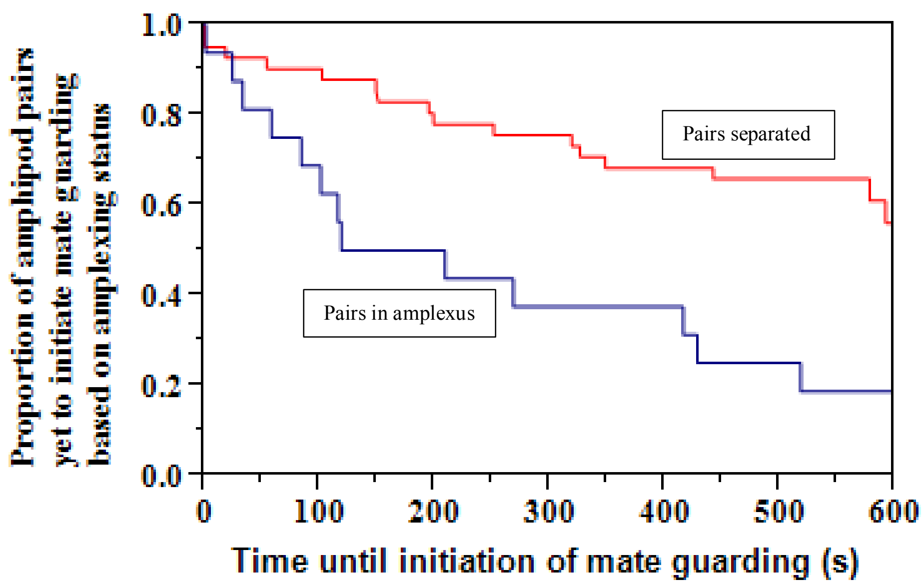


Fig. 16. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for pairs still in amplexus ('Pairs in amplexus') and those no longer in amplexus ('Pairs separated') at the end of the 24-h exposure to dibenzothiophene.

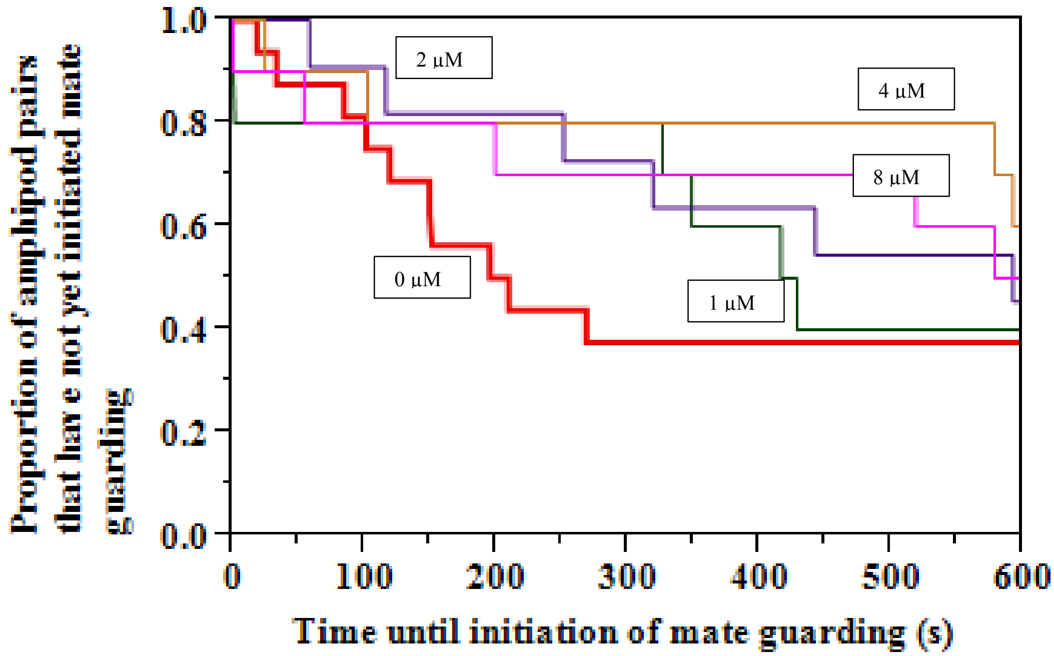


Fig. 17. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for amphipods previously exposed to different concentrations of dibenzothiophene.

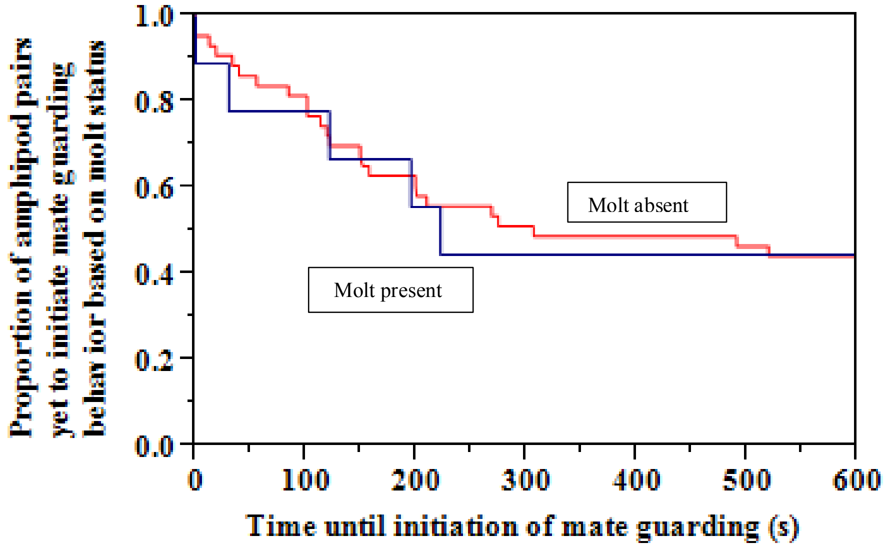


Fig. 18. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for pairs that did not molt ('Molt absent') and those that did molt ('Molt present') during the preceding 24-h exposure to phenanthrene and dibenzothiophene.

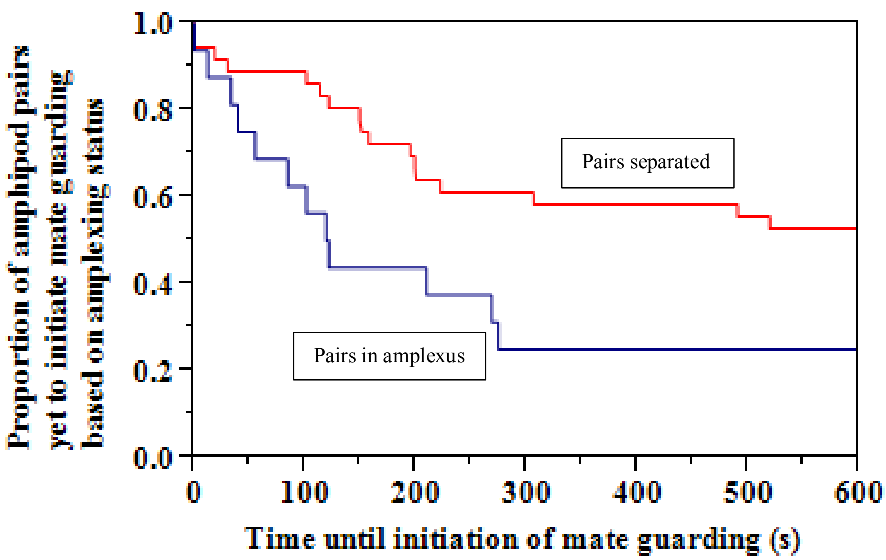


Fig. 19. Survival plot indicating proportion of amphipod pairs yet to initiate amplexing behavior during the 600-s observation period for pairs still in amplexus ('Pairs in plexus') and those no longer in amplexus ('Pairs separated') at the end of the 24-h exposure to phenanthrene and dibenzothiophene.

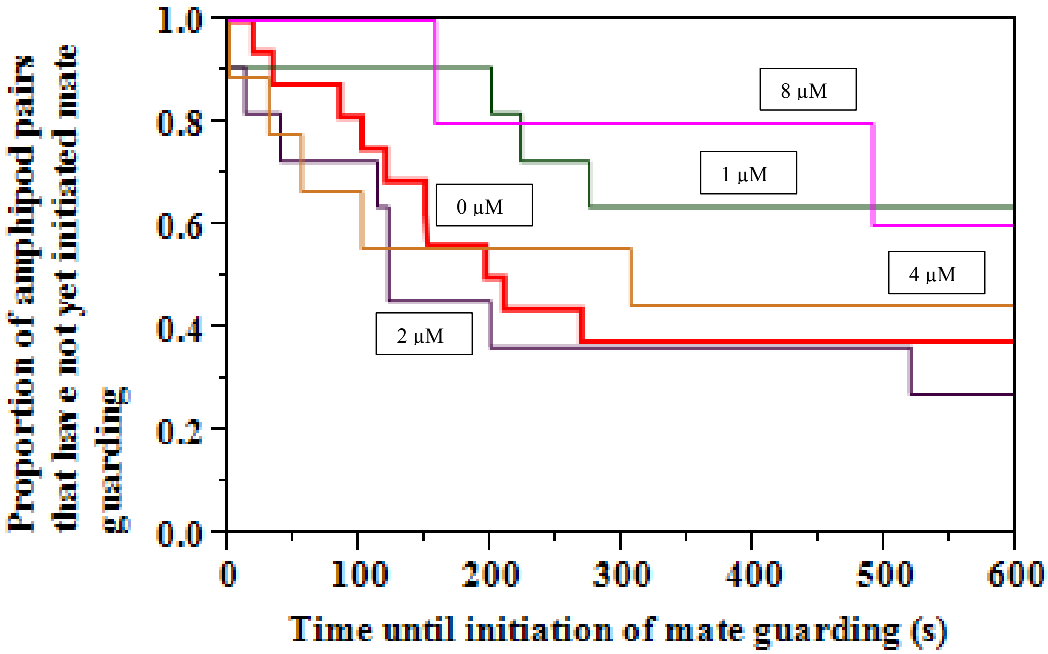


Fig. 20. Survival plot indicating proportion of amphipod pairs yet to initiate mate guarding during the 600-s observation period for amphipods exposed to different concentrations of phenanthrene and dibenzothiophene.

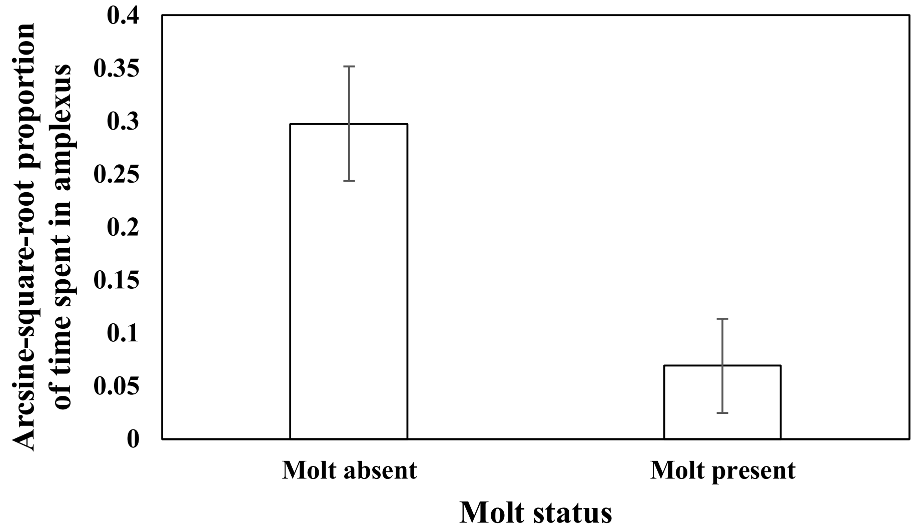


Fig. 21. Proportion of time spent in amplexus (during the 10-min observation period) for pairs of amphipods in which neither individual had molted ("Molt absent") or for which at least one individual had molted ("Molt present") during the preceding 24-h exposure to phenanthrene. Error bars indicate standard error.

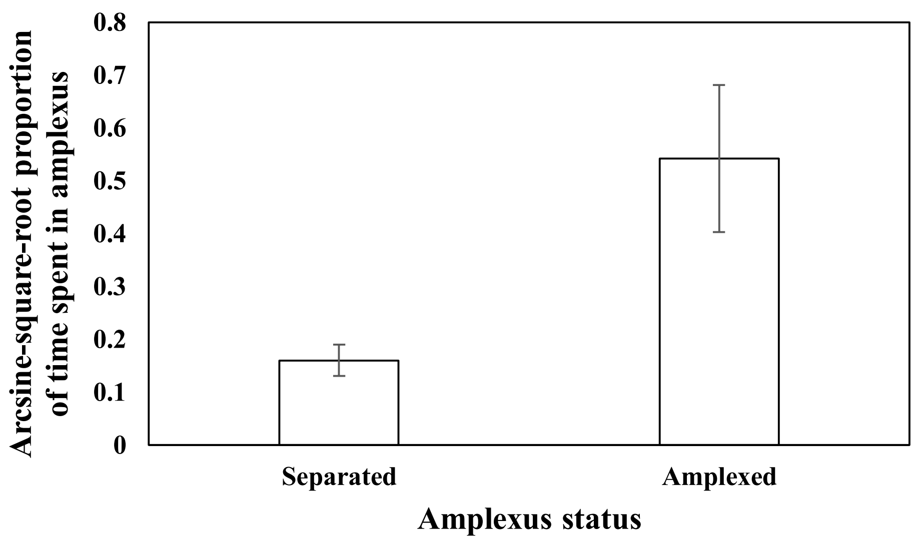


Fig. 22. Proportion of time spent in amplexus (during the 10-min observation period) by amphipod pairs that were not engaged in amplexus ("Separated") or pairs that were engaged in amplexus ("Amplexed") at the end of the preceding 24-h exposure to phenanthrene. Error bars indicate standard error.

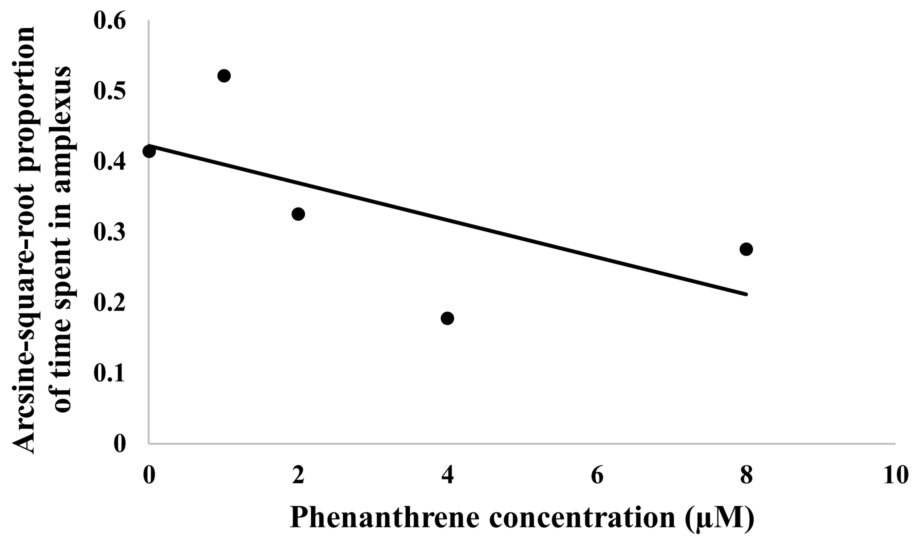


Fig. 23. Proportion of time spent in amplexus (during the 10-min observation period) as a function of the phenanthrene concentration to which the amphipods had been exposed in the preceding 24 h period.

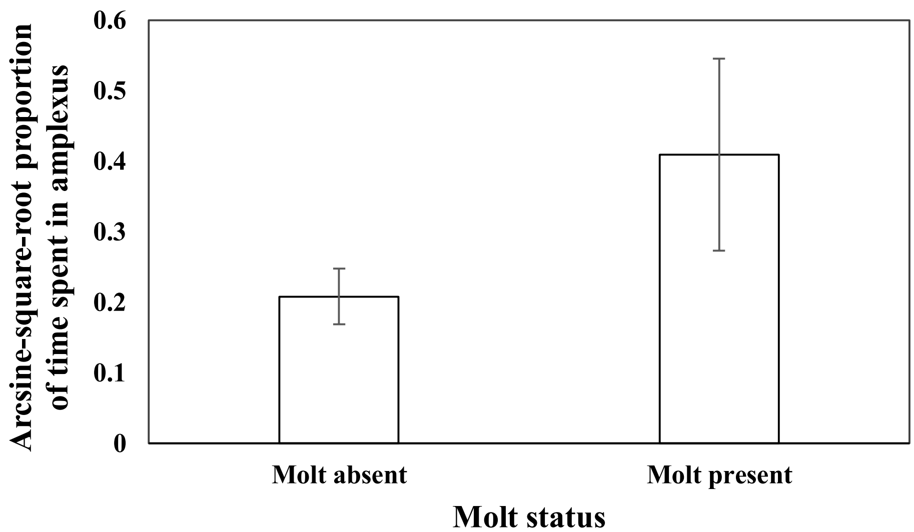


Fig. 24. Proportion of time spent in amplexus (during the 10-min observation period) for pairs of amphipods in which neither individual had molted ("Molt absent") or for which at least one individual had molted ("Molt present") during the preceding 24-h exposure to dibenzothiophene. Error bars indicate standard error.

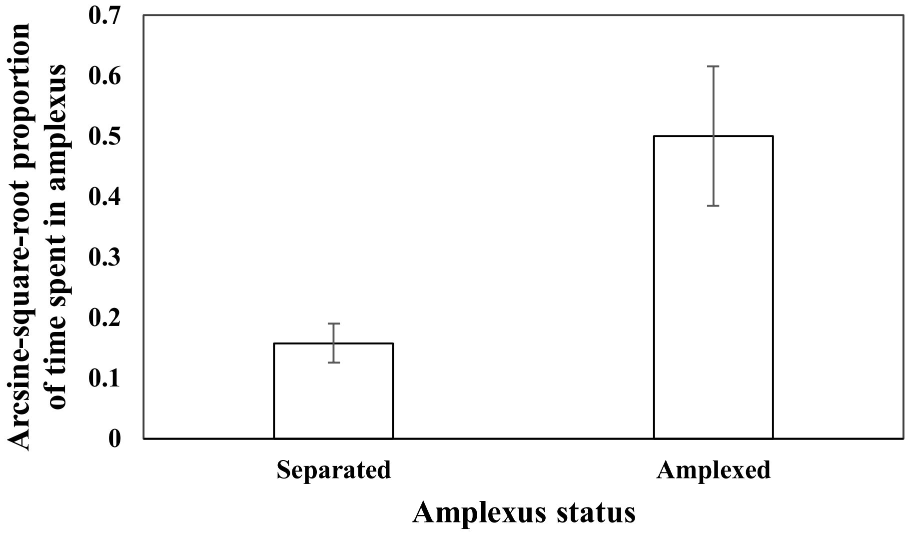


Fig. 25. Proportion of time spent in amplexus (during the 10-min observation period) by amphipod pairs that were not engaged in amplexus ("Separated") or pairs that were engaged in amplexus ("Amplexed") during the preceding 24-h exposure period. Error bars indicate standard error.

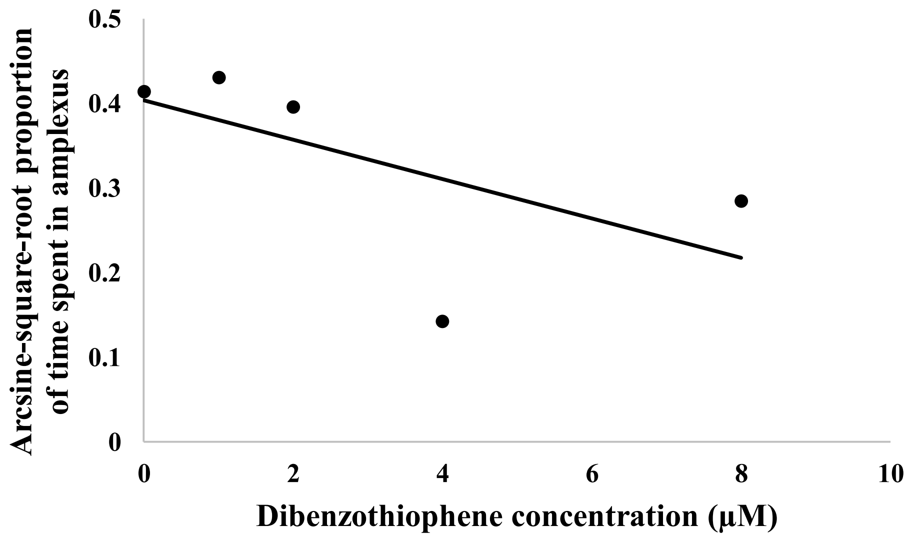


Fig. 26. Proportion of time spent in amplexus (during the 10-min observation period) as a function of the dibenzothiophene concentration to which the amphipods had been exposed in the preceding 24 h period.

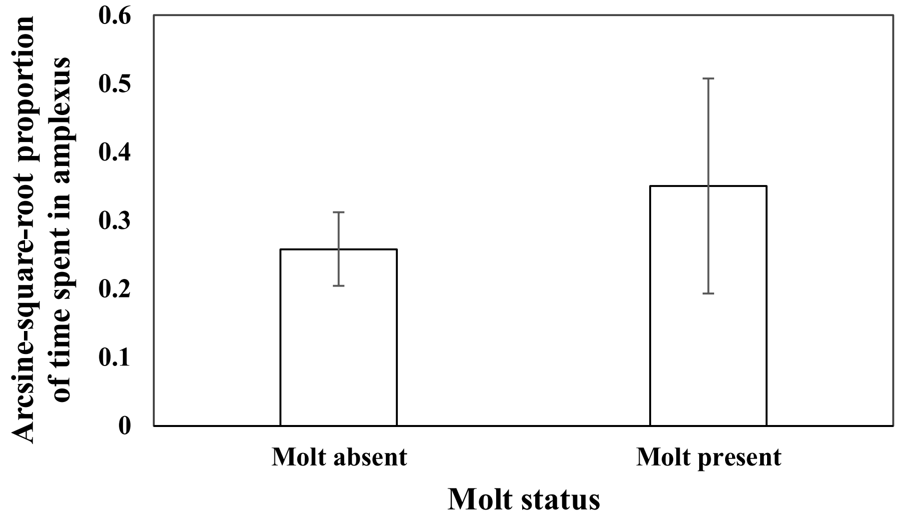


Fig. 27. Proportion of time spent in amplexus (during the 10-min observation period) for pairs of amphipods in which neither individual had molted ("Molt absent") or for which at least one of them had molted ("Molt present") during the preceding 24-h exposure to phenanthrene and dibenzothiophene. Error bars indicate standard error.

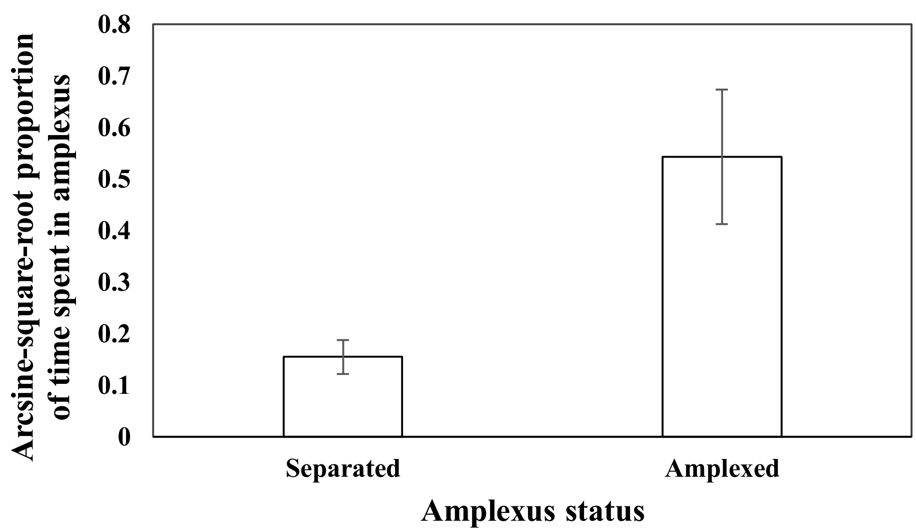


Fig. 28. Proportion of time spent in amplexus (during the 10-min observation period) by amphipod pairs that were not engaged in amplexus ("Separated") or pairs that were engaged in amplexus ("Amplexed") at the end of the preceding 24-h exposure to phenanthrene and dibenzothiophene. Error bars indicate standard error.

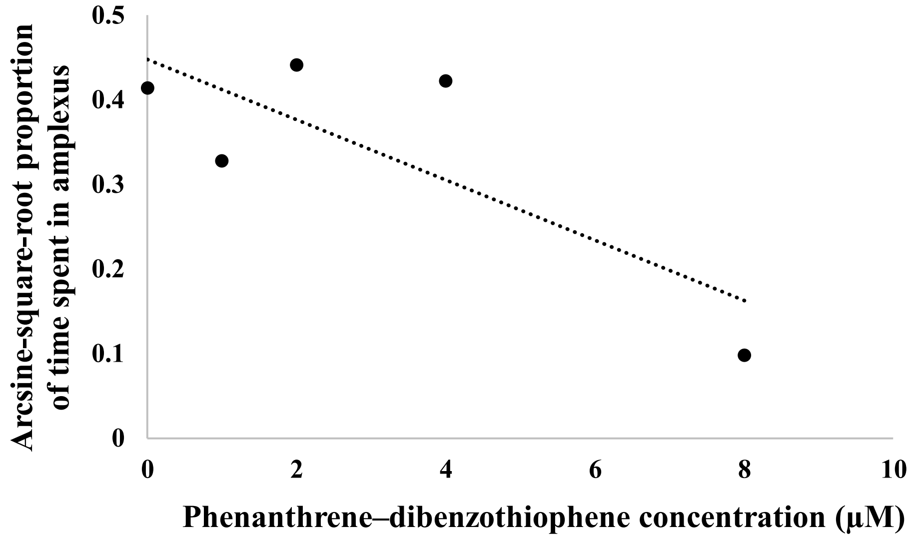


Fig. 29. Proportion of time spent in amplexus (during the 10-min observation period) as a function of the phenanthrene and dibenzothiophene concentration to which the amphipods had been exposed in the preceding 24 h period.

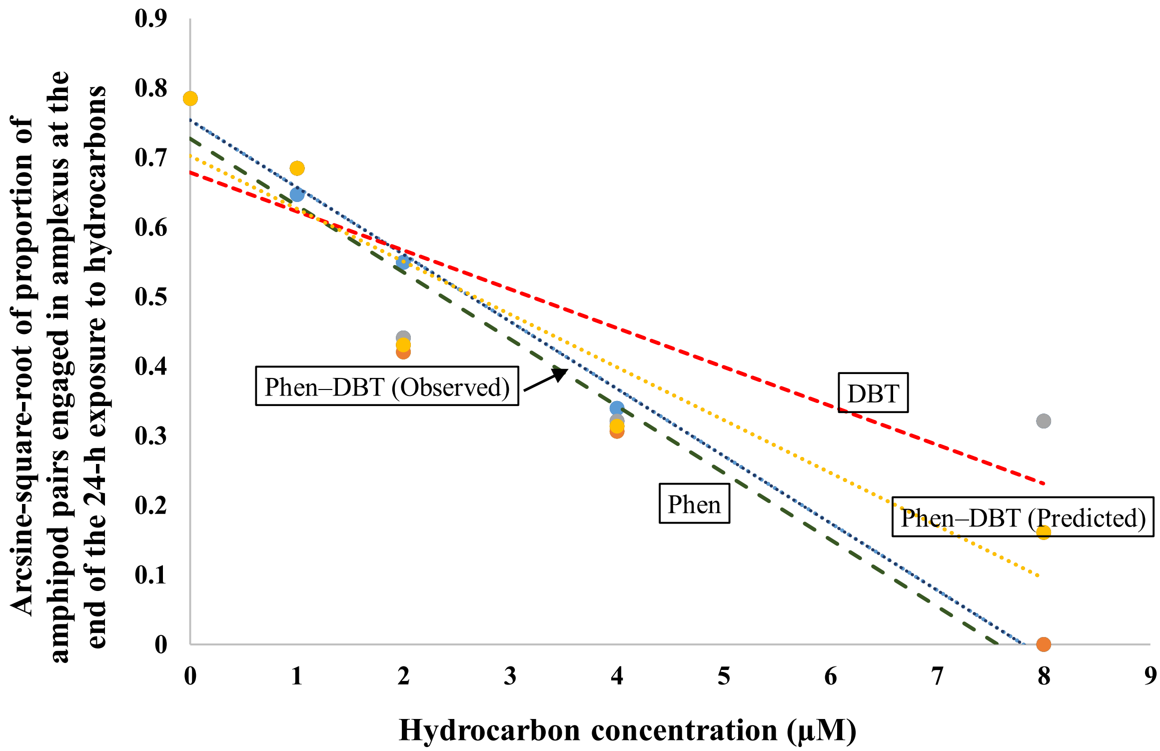


Fig. 30. Proportion of amphipod pairs that were engaged in amplexus at the end of the 24-h exposure to hydrocarbons phenanthrene "Phen", dibenzothiophene "DBT", and phenanthrene and dibenzothiophene "Phen–DBT observed". The "Phen–DBT predicted" are predicted values based on additive effects between phenanthrene and dibenzothiophene in the mixture. The lines represent regression lines (green, dash–phenanthrene; red, dash–dibenzothiophene; blue, dotted–phenanthrene and dibenzothiophene observed; light yellow, and dotted– phenanthrene and dibenzothiophene predicted).

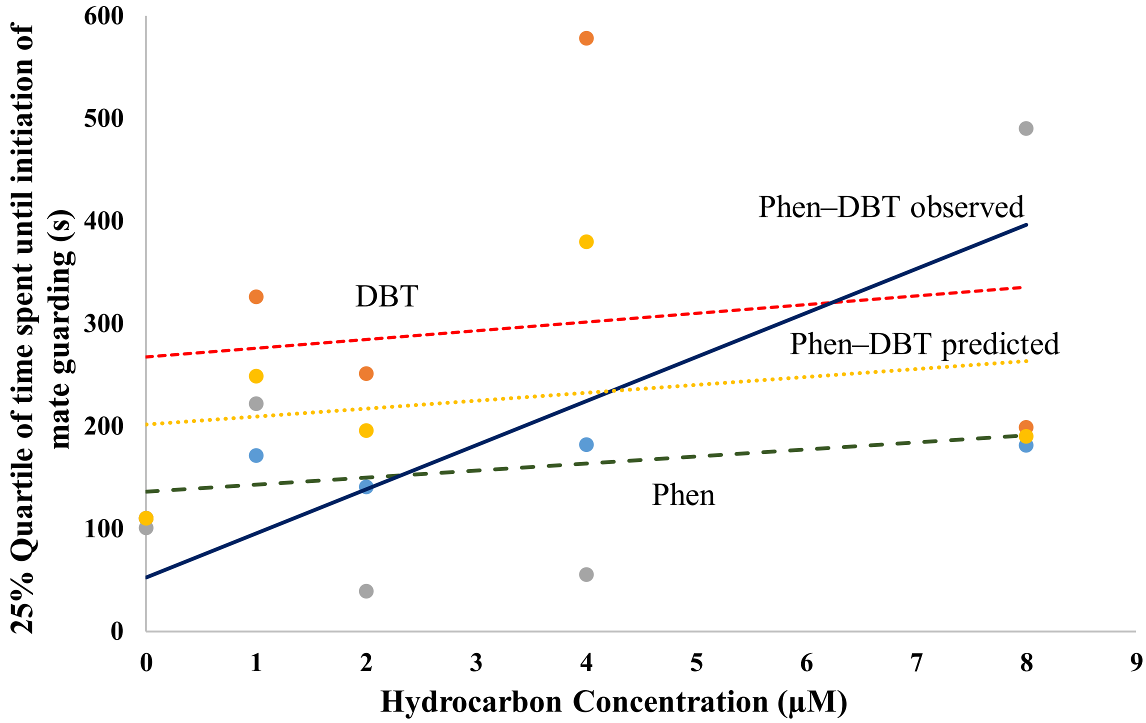


Fig. 31. Time till 25% of pairs had initiated mate-guarding (during the 10-min observation period) as a function of exposure concentration of phenanthrene "Phen", dibenzothiophene "DBT", and phenanthrene and dibenzothiophene "Phen–DBT observed" to which these amphipods were exposed in the preceding 24-h period. The "Phen–DBT predicted" are predicted values based on additive effects between phenanthrene and dibenzothiophene in the mixture. The lines represent regression lines (green, dash–phenanthrene; red, dash–dibenzothiophene; blue, solid–phenanthrene and dibenzothiophene (observed); and yellow, dotted– phenanthrene and dibenzothiophene (predicted).

Table

Table 1. Mean concentration ± SD of polycyclic aromatic hydrocarbon in water samples taken at $t = 0$ h (prior to introduction of amphipods) and at $t = 24$ h (in beakers that had amphipods).

Toxicant		Mean conc. ± SD
		(μM)
Phen	Nominal	8
	Measured at $t = 0$	4.82 ± 0.68
	Measured at $t = 24$ h	0.74 ± 0.22
DBT	Nominal	8
	Measured at $t = 0$	5.37 ± 0.66
	Measured at $t = 24$ h	1.86 ± 0.22
Phen in mixture	Nominal	4
	Measured at $t = 0$	2.95 ± 0.52
	Measured at $t = 24$ h	0.29 ± 0.13
DBT in mixture	Nominal	4
	Measured at $t = 0$	2.37 ± 1.22
	Measured at $t = 24$ h	0.31 ± 0.15

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Pages in Thesis: 69; Words in Abstract: 299.

ABSTRACT

Predicting impact of oil spills on aquatic life requires a better understanding of effects on aquatic organisms, both for single hydrocarbons and for their interactions. In this study, the individual and combined effects of petroleum hydrocarbons phenanthrene (Phen) and dibenzothiophene (DBT) were assessed on the reproductive behavior of the freshwater amphipod *Hyalella azteca*. Following a 24-h exposure to single PAHs, or an equimolar mixture of Phen–DBT, mate-guarding behavior was assessed at the end of the exposure and during a subsequent 10 min behavioral observation period with the animals in clean water. The endpoints of the study during the behavior observation period were—time taken to initiate mate-guarding (TIMG), and proportion of time spent in amplexus (PTA). The study demonstrated that the exposure to Phen and DBT reduced the incidence of mate-guarding during the actual exposure period, but not during the observation period. However, whether or not pairs were involved in mate-guarding at the end of the exposure period did affect both TIMG and PTA during the observation period. Thus, the effects of Phen and DBT on amplexus status at the end of the exposure period indirectly affected TIMG and PTA during the observation period. The interaction between Phen and DBT with respect to their effects on mate-guarding varied among the mate-guarding measures. For the amplexus status at the end of the exposure period and for the effect on TIMG, the interaction did not deviate statistically from an additive effect. For PTA, the overall interaction was a synergistic one.

This study's findings point out that assessments of hydrocarbon toxicity need to take into account that subtle reproductive behaviors (that may play an important role in population persistence) may be negatively affected. The results also show that the general assumption of additive effects among different PAHs may be an oversimplification.

BIOGRAPHICAL SKETCH

Kruuttika Milind Satbhai was born in Pune, Maharashtra, India, on 22 July, 1986, to Milind D. Satbhai and Shubhashree M. Satbhai. She completed her undergraduate degree in Microbiology in June 2007, and a Masters' degree in Biodiversity in December 2010 from the University of Pune. She was accepted into graduate school at the University of Louisiana at Lafayette in fall 2011. Kruuttika has presented her research at SC-SETAC Conference three times. She earned a Master of Science in Biology at the University of Louisiana at Lafayette in fall 2014.