Sublethal effects of oil-contaminated sediment to early life stages of the Eastern oyster, *Crassostrea virginica*

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Abstract

The explosion of the Deepwater Horizon (DWH) oil drilling rig resulted in the release of crude oil into the Gulf of Mexico. This event coincided with the spawning season of the Eastern oyster, *Crassostrea virginica*. Although oil bound to sediments constitutes an important source of polycyclic aromatic hydrocarbon (PAH) exposure to benthic organisms, toxicity of sediment-associated DWH oil has not been investigated in any bivalve species. Here, we evaluated the sublethal effects of acute exposure of gametes, embryos and veliger larvae of the Eastern oyster to different concentrations of unfiltered elutriates of sediment contaminated with DWH oil. Our results suggest that gametes, embryos and veliger larvae are harmed by exposure to unfiltered elutriates of contaminated sediment. Effective concentrations for fertilization inhibition were 40.6 µg tPAH50 L^{-1} and 173.2 µg tPAH50 L^{-1} for $EC20_{1h}$ and $EC50_{1h}$ values, respectively. Embryo exposure resulted in dose-dependent abnormalities (EC20 and EC50 values were 77.7 µg tPAH50 L^{-1} and 151 µg tPAH50 L^{-1}, respectively) and reduction in shell growth ($EC20_{24h}$ value of 1180 µg tPAH50 L^{-1}). Development and growth of veliger larvae were less sensitive to sediment-associated PAHs

compared to embryos. Fertilization success and abnormality of larvae exposed as embryos were the most sensitive endpoints for assessing the toxicity of oil-contaminated sediment. Bulk of measured polycyclic aromatic hydrocarbons were sediment-bound and caused toxic effects at lower tPAH50 concentrations than high energy water accommodated fractions (HEWAF) preparations from the same DWH oil. This study suggests risk assessments would benefit from further study of suspended contaminated sediment.

Summary:
Unfiltered sediment elutriates had negative effects on early life-stage oysters. Fertilization and embryogenesis were the most sensitive endpoints.

1. Introduction

The explosion of the Deepwater Horizon (DWH) oil drilling rig resulted in a deep (approximately 1500 m) subsurface release of an estimated 507 million liters of Louisiana crude oil into the Gulf of Mexico (GoM) from April 20th until July 15th 2010 (Operational Science Advisory Team, 2010; U.S. District Court, 2015). This led to the largest marine oil spill in United States history (National Commission, 2010). Although fractions of this oil were burned, skimmed from the surface, or chemically dispersed (Operational Science Advisory Team, 2010), some surface slicks were washed along shorelines of Louisiana, Mississippi, Alabama, and Florida (Michel et al., 2013; Nixon et al., 2016); and some oil fractions settled onto sediments of the northern GoM (Wang and Roberts, 2013). Oil remains in sediments for years or decades (Liu et al., 2012; Neff, 1979; Silliman et al., 2012; Turner et al., 2014) and oil accumulated in sediment particles exhibits slow weathering, which affects the chemical composition and toxicity of the oil (Brannon et al., 2006; Di Toro et al., 2007; Forth et al., 2017; Liu et al., 2012). Aromatic hydrocarbons, including polycyclic aromatic hydrocarbons (PAHs), are considered to be the most acutely toxic components of crude oil (Barron, 1999; Neff, 1985) and oil bound to sediments constitutes an important source of PAH exposure to benthic organisms (Albers, 2003; Geffard et al., 2007). The contamination of the water column by sediments occurs by diffusion and when sediments are re-suspended by natural factors (e.g., bioturbation, storms, wave, tide action) and by human activities (e.g., dredging activities) (Burgess et al., 1993; Chapman et al., 1998; Ciarelli et al., 1999, 2000; Geffard et al., 2007; Peterson et al., 1996).

Whole-sediment or unfiltered sediment elutriate (i.e., sediment supernatant) toxicity tests are commonly used to assess toxicity of oil-contaminated sediment (contaminated sediment) and bioassays have been developed to investigate the biological effects of contaminated sediments, using amphipods, copepods, echinoderms, bivalves, or fish (Brown-Peterson et al., 2014, 2015, 2017; Dubansky et al., 2013; Geffard et al., 2001, 2007; Ghirardini et al., 2005; Lotufo et al., 2016; Matthiessenn et al., 1998). While many studies have investigated impacts of DWH oil exposures on fish (Brewton et al., 2013; Brown-Peterson et al., 2014, 2015, 2017; Dubansky et al., 2013; Echols et al., 2015), mollusks (Carmichael et al., 2012; Finch et al., 2016; Langdon et al., 2016; Stefansson et al., 2016; Vignier et al., 2015, 2016, 2017; Volety et al., 2016), corals (Goodbody-Gringley et al., 2013), arthropod (Echols et al., 2015; Lotufo et al., 2016; McCall and Pennings, 2012), and zooplankton (Almeda et al., 2013), there is little information to date on the impact of oiled sediments from the DWH spill on marine species. Exposure to DWH-oiled sediment has been reported to alter normal embryogenesis and larval developments in fish, such as delayed hatching and reduced hatching success and growth in the Gulf killifish (*Fundulus grandis*, Dubansky et al., 2013) and developmental malformations in zebrafish (*Danio rerio*) embryos (Raimondo et al., 2014). Sediment-associated PAHs also reduce growth and survival in Southern flounder (*Paralichthys lethostigma*) juveniles (Brown-Peterson et al., 2015, 2017) and reduce offspring production, survival and growth of benthic amphipods (*Leptocheirus plumulosus*, Lotufo et al., 2016). Composition and toxicity of the oil deposited in sediments can be different than in the water column (Brannon et al., 2006; Di Toro et al., 2007; Forth et al., 2017; Liu et al., 2012). Though benthic organisms are easily exposed to PAHs in sediment, the effects of DWH-oiled sediment have not been investigated in any bivalve species.

The embryo- and larval-toxicity tests with oysters are among the most sensitive tests for evaluating sediment toxicity to bivalves (Geffard et al., 2002; His et al., 1999; McPherson and Chapman, 2000; Stefansson et al., 2016). The Eastern oyster (*Crassostrea virginica*) is distributed from Canada, along the East Coast of the USA, to the GoM (Galtsoff, 1964). It is the second most valuable bivalve fishery in the USA (NMFS, 2010), especially in the GoM, with total landings of this species in the northern GoM representing $74 million in value for 2012 (NMFS, 2012). In addition to its economic significance, the Eastern oyster is also ecologically important. It is a keystone species which has been the focus of conservation and restoration efforts because oyster populations have declined worldwide (Beck et al., 2011; Kirby, 2004), and so have the ecosystem services they provide, including improved coastal

water quality through filtration, and the creation of complex reefs that represent key habitat for numerous fish, invertebrate, and bird species (Beck et al., 2011; Coen et al., 2007; Newell, 2004).

In the northern part of the GoM, oyster spawning season occurs from mid-spring through late fall (Ingle, 1951), a period which coincided with the DWH-oil spill (April 20th until July 15th). Recent studies have demonstrated an acute toxicity of DWH-oil associated PAHs to gametes, embryos, and larvae of the Eastern oyster. Surface-collected and chemically dispersed DWH oil, and dispersant reduce fertilization success and normal development and survival of embryos and larvae in this species (Finch et al., 2016; Langdon et al., 2016; Vignier et al., 2015, 2016, 2017; Volety et al., 2016). Toxicity on early life stages (gamete, embryo, larva) can be evidenced within 1 hour of exposure at low concentration of PAHs (< 60 µg tPAH50 L^{-1}, Volety et al., 2016). The ecological and economical significance, and the sensitivity to pollutants of the Eastern oyster make it a suitable model species to investigate the effects of DWH oiled-sediment on early life stages of bivalve species.

In this study, we investigated i) the sublethal effects of unfiltered sediment elutriates contaminated with DWH oil on the fertilization success and early life stage development and growth in the Eastern oyster, and ii) determined the most sensitive life stages and endpoints for ecotoxicological assessment of contaminated sediment on the Easter oyster.

2. Materials and methods

2.1. Collection of sediment

Uncontaminated (control sediment) and contaminated sediment were collected for the *Deepwater Horizon* Natural Resource Damage Assessment (NRDA) (Krasnec et al., 2015). Contaminated sediment (LAAR38-B0123-SX401) was collected in 2011 from a site called Black Hole, LA (Lat. 29˚19’689’’N, Long. 89˚03’9’’W), an area that was classified during the NRDA as having “Heavier persistent” oiling (Nixon et al., 2016). Control sediment (LAAR42-C0208-SX403) was collected in 2012 from a reference site called Loomis II, LA (Lat. 29˚12’305’’N, Long. 89˚17’87’’W). Sediment samples were collected from the surficial layer (6-8”) with a shovel. Additional information regarding the methodology that field crews used to collect these sediments is provided in the National Oceanic and Atmospheric Administration (NOAA) DIVER data repository (DIVER, 2015; Krasnec et al. 2015). After collection, sediment samples were frozen and shipped under chain of custody to the

laboratory where they were stored at -20°C until needed for toxicity testing.

2.2. Sediment characteristics and analytical chemistry of elutriates

After field collection and before toxicity testing, a subsample of uncontaminated and contaminated sediment was sent to ALS Environmental (Kelso, WA, USA) for chemical analyses. ALS conducted analyses on PAHs, alkyl PAH homologues, and related hetero-compounds using gas chromatography with low-resolution mass spectrometry and using selective ion monitoring (GC/MS-SIM), based on U.S. Environmental Protection Agency (EPA) Method 8270D. These data were used to calculate the sum of 50 PAHs (tPAH50) (Forth et al., 2017). ALS also analyzed subsamples for other contaminants, including metals (6010C and 6020A) such as antimony (6020A), silver (6020A), and mercury (747IB); pesticides (808 IB); and polychlorinated biphenyls (PCBs; 8082A). Analyses describing the physical characteristics of the sediments [total organic carbon (TOC; ASTM D4129-05, 2013), particle size (PSEP PS), and total solids (TS-MET)] were also performed.

For all experiments, temperature, dissolved oxygen, salinity, ammonia and pH of elutriates were measured daily using a Pro ODO optic probe (YSI), a refractometer (Fisher Scientific), or a "Pinpoint" pH monitor (American Marine, Inc.). At the start and at the end of each exposure experiment, total ammonia was assessed using a Seal Analytical Auto Analyzer 3 and the G-171- 96 method. Water samples of each stock (100% stock) of unfiltered sediment elutriate, of the different concentrations of unfiltered sediment elutriates used for toxicity testing, and of control solutions were collected at exposure initiation. Water samples were not filtered and were stored at 4°C until they were shipped to ALS Environmental (Kelso, WA, USA) for chemical analysis. tPAH50, were quantified by gas chromatography with low-resolution mass spectrometry using selective ion monitoring (GC/MS-SIM).

2.3. Preparation of unfiltered sediment elutriates

Control sediment and contaminated sediment were thawed at 4°C for 48 hours before preparing unfiltered sediment elutriates according to modified protocol from Geffard et al. (2001). In a sterile glass beaker, UV-sterilized and 0.1 µm-filtered seawater, adjusted to 25°C and 22 PSU (filtered seawater, FSW), was added to control sediment or contaminated sediment in a ratio of 10:1 (i.e., 100 g of sediment mixed in 1000 mL of FSW) and mechanically stirred (300 rpm) for 6 hours using a stirring rod and a magnetic stirrer. After 12

hours of sediment settling, unfiltered contaminated sediment elutriates were prepared. Supernatant (100% stock) was siphoned off from the top of the beaker and then mixed with FSW in a dilution series to nominal concentrations of 100 (no dilution of the stock), 50, 25, 12.5, 6.25, 3.125, and 1.5625% of supernatant. The control sediment elutriate was prepared following the same methodology as the unfiltered sediment elutriates, except supernatant was not diluted (100% stock used). In addition to the control sediment elutriate, a FSW control (i.e., no sediment) was also tested. Solutions of sediment were neither filtered nor centrifuged.

2.4. Collection of oyster gametes

Oysters *Crassostrea virginica* were collected in September from natural populations in Estero Bay, Florida (Lat. 26˚19'50''N, Long. 81˚50'15''W). Average weight of oysters was 75 ± 20 g. They were kept under natural light conditions and ambient seawater salinity (20–30 PSU) for 2 weeks at 23˚C ± 1 using a flow-through system. Seawater was sand filtered (30-µm). Animals were fed with cultured fresh microalgae (*Chaetoceros muelleri*, *Tetraselmis chui*, and *Tisochrysis lutea)* at a daily ration of 3% of oyster dry body weight for conditioning (Utting and Millican, 1997). Ripeness of oysters was determined by microscopic observation of gonadal smears. Oocytes and spermatozoa were examined for motility (sperm), shape and absence of atresia (oocyte), and oysters showing immature gametes were discarded. For each ripe oyster, gametes were collected by stripping oyster gonad with a scalpel in 50 mL of FSW (Allen and Bushek, 1992). To remove gonadal and other tissue debris, sperm was sieved through 55-µm mesh and sperm from 3 males were pooled into 500-mL of FSW in a sterile beaker. Similarly, oocytes from 3 females were pooled into 2 L of FSW in a sterile beaker, after successive sieving through 150-µm and 55-µm mesh to remove gonadal tissue and debris, and collection on 20-µm mesh. Gamete concentration was determined by microscopic count using a Sedgewick-Rafter® counting cell (3 x 100 µL).

2.5. Sediment elutriate exposure of early life stages

2.5.1. Experimental design

Gametes (oocytes and spermatozoa), embryos, and larvae were exposed to the different concentrations of unfiltered sediment elutriates from sediment contaminated during the DWH oil spill, control sediment elutriate, or FSW control (i.e., no sediment).

Four replicates were set up for each condition. Exposures were conducted at 25 ± 1˚C and at a salinity of 21.5 ± 0.5 PSU. Elutriate solutions were not renewed, and no aeration was provided during the experiment, except for the veliger-larval exposure (48-hour exposure): gentle aeration (≈ 60 bubble min $^{-1}$) was delivered to maintain dissolved oxygen (DO) concentrations above 4 mg L^{-1}.

2.5.2. Gamete exposure

Sperm (2 x 10^6 spermatozoa mL^{-1}) and oocytes (20 oocytes mL^{-1}) from the pools were exposed separately for 30 minutes to unfiltered sediment elutriates, control sediment elutriate, or FSW. The exposure time was set for 30 minutes for gametes since broadcast spawning in the field allow fertilization to occur quickly after release of oocytes and spermatozoa in the surrounding seawater. After 30 minutes of exposure, oocytes were fertilized by adding 10 mL of exposed sperm from corresponding sperm-exposure replicate (same concentration of unfiltered sediment elutriate for oocytes and spermatozoa, 4 replicates/treatment).

To determine the fertilization success, a 10 mL aliquot was subsampled 1 hour after fertilization from each beaker. Samples were preserved with 300 µL of 10% buffered formalin until later determination of the fertilization success (number of embryos/initial number of oocytes). This was determined by counting embryos, characterized by first cell cleavage at 1-hour post fertilization, in at least 200 individuals per beaker.

Embryogenesis was assessed 24 hours after fertilization. A 10-mL aliquot was subsampled from each exposure beaker. Samples were preserved with 10% buffered formalin for later determination of the percentage of abnormal larval and shell measurements. A minimum of 100 randomly selected larvae per treatment were examined under a microscope to assess the percentage of abnormal larvae and shell length. About 24 hours after fertilization at 25°C, embryos develop to veliger-larvae. Abnormal larvae included: (1) segmented eggs, normal embryos, or malformed embryos that did not reach the veliger-larval stage; and (2) veliger-larvae with either a convex hinge, indented shell margins, incomplete shells, a protruded velum, or an extrusion of mantle as described in Vignier et al. (2015). Only live abnormal larvae were considered (Chapman, 1989). Shell lengths (the maximum distance between the anterior and the posterior margin measured parallel with the hinge axis) of 25 randomly selected 24-hour old live larvae from each beaker were measured using an Olympus IX73 inverted microscope equipped with an Olympus DP73 camera, and the CellSens Software.

2.5.3. Embryo exposure

Remaining unexposed oocytes from the pool of oocytes (2 L) were fertilized with 20 mL of the pool of unexposed sperm. The success of fertilization was confirmed forty-five minutes later by microscopic examination of the cell cleavage and the number of embryos was assessed (3 x 50 µL) as previously described in Section 2.5.2. One hour after fertilization, when the two- to four-cell stage was reached, embryos were transferred at a density of 15 mL^{-1} (3,000 individuals per beaker) into 200 mL of each concentration of unfiltered sediment elutriates, sediment elutriate control, and FSW control (4 replicates/treatment). After 24 hours of embryo exposure, an aliquot was subsampled from each beaker and fixed in 10% buffered formalin for later measurements of the percentage of abnormality and larval shell lengths as described in section 2.5.2. The exposure time was set for 24 hours to allow embryos to reach the next developmental stage of swimming veliger-larvae.

2.5.4. Veliger exposure

The remaining fertilized embryos that were not used for the embryo-toxicity assays described above were transferred to hatching tank at a final density of 40 embryos mL^{-1} in 50 L of FSW. At 24 hours post fertilization, veliger-larvae (developed from embryos) were collected on a 35 µm mesh and concentrated in 2 L FSW. Veliger-larvae were enumerated by microscopic count and distributed at a density of 11 larvae mL^{-1} (2,200 larvae/beaker) into 200 mL of the different unfiltered sediment elutriates, sediment elutriate control, or FSW control (4 replicates/treatment). Larvae were fed with cultured microalgae (*T. lutea*) at the start of the exposure at a concentration of 5 x 10^4 cells mL^{-1}. After 48 hours of exposure, 10-mL subsamples were collected from each beaker and 300 µL of 10% buffered formalin were added for later measurements of the percentage of abnormality and shell lengths as described in 2.5.2. The exposure time was set for 48 hours for veliger-larvae since preliminary range-finding experiments revealed that development and growth of veliger larvae are less sensitive to unfiltered sediment elutriate exposure than gamete and embryo stages.

2.6. Statistical analyses

Results are presented as mean ± SD. Log-logistic models with the *drc* package in R version 3.1.1 (2014) were used to fit dose-response curves (Ritz, 2010; Ritz and Streibig, 2005). A three-parameter log-logistic model was fitted for binomial response variables (fertilization, abnormality), while a 4-parameter log-logistic model was fitted for shell length. We estimated effective concentrations (ECx) from these fitted models for relevant quantiles. All results are reported with 95% confidence intervals (CIs) based on profile-likelihood using *bbmle* (Bolker and R Development Core Team, 2014).

3. Results

3.1. Sediment characteristics and analytical chemistry of elutriates

The chemical and physical characteristics of field-collected sediments are listed in Table 1. Temperature of sediment elutriates ranged from 24.2 to 26.4 °C throughout the exposure experiments. The pH and salinity averaged 7.9 ± 0.2 and 24 ± 4 PSU, respectively. Dissolved oxygen remained above 5.0 mg L^{-1}. Total ammonia concentrations remained at safe levels (< 1 mg L^{-1}) (Ferretti and Calesso, 2011; Losso et al., 2007).

The FSW used for the control contained very low levels of PAHs at background levels (tPAH50 = 0.08 µg L^{-1} ± 0.03). The composition of PAHs in the 100% stocks of unfiltered sediment elutriates were similar among the gamete, embryo and larval bioassays. Additionally, the composition of PAHs in the unfiltered sediment elutriate was very similar to the composition of PAHs in the field collected DWH contaminated sediment (Fig. 1, Supplementary Table 1), revealing that the PAHs in unfiltered sediment elutriates were likely from PAHs on suspended fine-grained particles rather that PAHs from water accommodated fractions (Supplementary Fig. 1). PAH concentrations were higher in higher concentrations of unfiltered contaminated elutriate (Supplementary Table 2).

3.2. Sub-lethal effects on fertilization

Fertilization successes of gametes exposed to FSW- and control sediment elutriate were 90 ± 3% and 87 ± 6%, respectively. At the highest dose of unfiltered sediment elutriate tested (152.8 µg tPAH50 L^{-1}) 54 ± 3% of the oocytes were unfertilized (Fig. 2). Fertilization decreased with increasing dose ($EC20_{1h}$ = 40.6 µg tPAH50 L^{-1} (95% CI =29.8, 54.1), $EC50_{1h}$ = 173.2 µg tPAH50 L^{-1} (95% CI =148.1, 209.6)).

3.3. Sub-lethal effects on embryogenesis and early larval development

Continuous 24-h exposure of gametes to control sediment elutriate induced a high percentage of abnormal larvae compared to FSW control. Percentage of larval abnormality was 60.4 ± 9.3% and 12.8 ± 3.0% in the sediment elutriate control and FSW control, respectively (Fig. 3A), therefore we do not report effects concentrations for gamete abnormality.

Embryo exposure resulted in dose-dependent abnormalities (Fig. 3B). High percentages of abnormal larvae were observed, with 100% of abnormalities at the highest dose of unfiltered sediment elutriate from contaminated with DWH oil (989.0 µg tPAH50 L^{-1}). Development of veliger larvae was less sensitive to sediment-associated PAHs compared to embryos. After 48 hours of exposure at the highest dose of PAHs, 46 ± 7% of the veliger larvae showed abnormalities (Fig. 3C).

EC20 and EC50 values of observed abnormality in larvae continuously exposed to unfiltered sediment elutriate from embryo or veliger-larva stages are presented in Table 2. Abnormality at the highest doses of unfiltered sediment elutriate tested in the veliger exposure was lower than 50% (Fig. 3C), so we do not report an EC50 value (Table 2).

3.4. Sub-lethal effects on larval size

The shell length of oysters exposed to FSW- and sediment elutriate controls were 65.3 ± 3 and 63.4 ± 3 µm, respectively, for 24 hour larvae developed from exposed gametes. The absence of data at the highest concentration of unfiltered sediment elutriate was due to high mortality. At 72 µg tPAH50 L^{-1} (i.e., the second highest concentration tested), mean shell length was 59.8 ± 2 µm (Fig. 4A).

In the 24h embryo exposures, shell lengths of larvae were 71.9 ± 3 and 71.4 ± 3 µm for FSW- and sediment elutriate controls, respectively, and 58.3 ± 4 µm at the highest concentration of unfiltered sediment elutriate tested. In the 48 hour exposures of veliger larvae, sediment-associated PAHs reduced larval shell length to 70.7 ± 1 µm compared to controls (75.5 ± 3 and 73.8 ± 3 µm for FSW- and sediment- control, respectively).

Unfiltered sediment elutriate exposure induced a dose-response decrease in shell length for larvae developed from exposed embryos, with an $EC20_{24h}$ value of 1180 µg tPAH50 L^{-1} (Fig. 4B). Limited decrease of shell length at the range of tPAH50 L^{-1}

concentrations in elutriates tested did not allow the calculation of EC20 and EC50 values in the gamete (Fig. 4A) and veliger-larval (Fig. 4C) exposures, or the calculation of EC50 in the embryo exposure (Fig. 4B).

4. Discussion

In the present study, we investigated the sublethal effects of unfiltered elutriates from sediment contaminated with DWH oil on fertilization success, embryogenesis, and larval development and growth of the Eastern oyster early life stages.

4.1. Sublethal effects of sediment-associated PAHs on early life stages

Fertilization success was reduced after exposure of gametes to unfiltered sediment elutriates. Vignier et al. (2015) reported higher values of effective levels of tPAH50 for inhibition of fertilization for exposure to HEWAF (high energy water accommodated fraction) prepared from surface DWH oil compared to unfiltered sediment elutriates (present study) in the same species. $EC20_{1h}$ and $EC50_{1h}$ values for fertilization inhibition were 1650 and 2250 µg tPAH50 L^{-1}, respectively, for exposure to HEWAF of DWH oil (Vignier et al., 2015); and 40.6 and 173.2 µg tPAH50 L^{-1}, respectively, for exposure to unfiltered sediment elutriates (present study). Reduced fertilization success is partly related to the negative effects of oil on marine bivalve spermatozoa (Renzoni, 1973, 1975). PAHs in DWH-oil induce cellular alterations in Eastern oyster spermatozoa, including changes in reactive oxygen species production and mitochondrial membrane potential (Vignier et al., 2017; Volety et al., 2016). These cellular characteristics play a crucial role in fertilizing ability of oyster spermatozoa (Boulais et al., 2017).

We found that sediment-derived PAHs induced abnormal development and reduced shell length in 24-h old larvae developed from exposed embryos. Similarly, unfiltered elutriates of PAH-contaminated sediment (freeze-dried sediment from Arès/Bidassoa region in France, mixing ratio of 4:1 for 8h at 500 rpm and settled for 8h) were reported to cause significant abnormalities in Pacific oyster (*Crassostrea gigas*) 24-h old larvae ($EC20_{24h}$ and $EC50_{24h}$ values were 5.3 and 22.4 g sediment L^{-1}) and sea urchin (*Paracentrotus lividus*) 48-h old larvae ($EC20_{48h}$ and $EC50_{48h}$ values were 16.2 and 42.3 g sediment L^{-1}) developed from exposed embryos (Geffard et al., 2001). Vignier et al. (2015) reported that HEWAFs of weathered DWH oils inhibit larval development in exposed embryos, though at higher

tPAH50 concentrations than unfiltered sediment elutriates in the present study. $EC20_{24h}$ and $EC50_{24h}$ values for abnormality induction in larvae development from exposed embryos were 218 and 342 µg tPAH50 L^{-1}, respectively, for exposure to HEWAF (water-accommodated fractions) of DWH oil (Vignier et al., 2015); and 77.7 and 151 µg tPAH50 L^{-1}, respectively, for unfiltered sediment elutriates (present study). Regarding shell length, the present study indicated that exposure to sediment-associated PAHs induced a dose-response decrease in shell length for larvae exposed as embryos, with an $EC20_{24h}$ value of 1180 µg tPAH50 L^{-1}. Shell length of larvae developed from exposed embryos was also reduced after a 24-h exposure to HEWAF of oil collected from the DWH incident in the Eastern oyster (Vignier et al., 2015; no calculation of effective concentrations). Similarly, crude oil adversely affected shell length of developing embryos at an $EC50_{14d}$ value of 1000 µg L^{-1} of oil in seawater in the surf clam, *Mulinia lateralis* (Renzoni, 1975). Increased developmental malformations (e.g., hatching success) with exposure to oil-contaminated sediment was also observed in embryos of fish, such as the fathead minnows *Pimephales promelas* (Colavecchia et al., 2004) and the zebrafish *Danio rerio* (Raimondo et al., 2014; Sogbanmu et al., 2016) (no calculation of effective concentrations). It was suggested that PAHs impair the mechanism of shell calcification of newly segmented embryos in the Eastern oyster and could interfere with protein synthesis, metabolism, and enzymatic activities (Vignier et al., 2015). Finally, PAH exposure causes high rates of abnormal larvae and DNA strand breakage in Pacific oyster embryos (Wessel et al., 2007). These mechanisms probably contribute to the observed abnormal development and the reduction of shell growth of Eastern oyster larvae exposed during the embryo stage to PAHs of unfiltered sediment elutriates.

Effects of PAHs on development of larvae exposed from the veliger stage have been less investigated compared to embryogenesis. Geffard et al. (2002) reported that Pacific oyster larval growth was sensitive to unfiltered elutriates of PAH-contaminated sediment after 5 days of exposure at 0.664 µg tPAH12 L^{-1} (12 polycyclic aromatic hydrocarbons analyzed), but not after 3 days of exposure. Similarly, shell length of larvae exposed from the veliger stage for 4 days was inhibited by HEWAF of DWH oil in the Eastern oyster at an EC20 value of 106 µg tPAH50 L^{-1} (Vignier et al., 2016), and water-soluble fractions of Southern Louisiana Crude oil affected larval growth (2-day old) of the quahog clam (Byrne and Calder, 1977). Effects of PAHs on veliger-larval development may be related to their bio-accumulation in larvae through feeding, which may induce the production of toxic metabolites during PAH metabolization, such as reactive radical cations (Colavecchia et al., 2004; Geffard

et al., 2002). Additionally, it was suggested that particulate oil could act on gills and velum, impairing the normal physiology of larvae (Vignier et al., 2016).

In the present study, the control sediment elutriate induced abnormalities in 24-h old larvae developed from exposed gametes. Chemical analyses did not reveal any contaminants (metals, pesticides, PCBs, and tPAH50; Supplementary Table 3) in the sediment elutriate control but there was a high percentage of fine particles (i.e., silt and clay, 82%) as elutriates were neither filtered nor centrifuged. Particles in the sediment elutriate control may have impeded early embryogenesis (i.e., embryo exposed during the first hour post fertilization, Fig. 3A) without impacting fertilization success and later embryogenesis (i.e., embryo exposed after the first hour post fertilization, Fig. 3B). Griffin et al. (2009) found a similar effect in the Pacific herring, *Clupea pallasi*, for which suspended sediment induced malformations in larvae developed from embryos exposed during the two first hours of embryogenesis, but did not reduce fertilization success of exposed gametes or induce larval malformations in older embryos (> 2-h old embryos). Our results suggest that exposure to fine particles during the first hour of embryogenesis may cause developmental abnormalities in the Eastern oyster. Mechanism for this effect of particles is not known but could be related to particles binding to embryo membranes, disturbing the first embryo cleavage (2 to 4-cell embryo at 1-hour post fertilization in oysters). However, because the sediment elutriate control was not necessarily a perfect representation of the physical characteristics of the contaminated sediment, separating the mechanical effects from the toxic effects of oil on this endpoint was not feasible. Further testing is warranted to determine whether particulate alone may have caused the high rates of abnormality in 24-h old larvae developed from exposed gametes.

4.2. Toxicity of sediment-associated PAHs compared to HEWAF-derived PAHs on early life stages

Material and methods used in the present study were the same as those used in Vignier et al. (2015), allowing the comparison of effective concentrations for inhibition of fertilization ability, embryogenesis, and larval development of the Eastern oyster between HEWAF and unfiltered sediment elutriate preparations from the same DWH oil. Unfiltered sediment elutriates caused toxic effects at lower tPAH50 concentrations than HEWAF preparations for fertilization success and embryogenesis of exposed embryos. $EC20_{24h}$ and $EC50_{24h}$ values for abnormality induction in larvae development from exposed embryos were 218 and 342 µg

tPAH50 L^{-1}, respectively, for exposure to HEWAF (water-accommodated fractions) of DWH oil (Vignier et al., 2015). The increase in toxicity in the contaminated sediment elutriate treatments cannot be explained by the presence of fine particles of sediment in the water column alone as the control sediment elutriate had the same fertilization success and larval abnormality as the seawater control, but rather due to the PAHs attached to the fine-grain particles suspended in the water column. Furthermore, fertilization success was higher than 87% and larval abnormality of exposed embryos was lower than 15% in control seawater and sediment elutriate control. However, we found almost two-fold higher percentages of 3 ring PAHs (dibenzothiophenes, DBT4) and 4 ring PAHs (naphthobenzothiophenes: NBT1, NBT2, and NBT3) in the composition of unfiltered sediment elutriates used in the present study compared to HEWAF of DWH oil used by Vignier et al. (2015). This is probably due do the low water-solubility (Djomo et al., 1996; Porte and Albaigés, 1993) and high affinity for organic carbon of these PAHs, resulting in their adsorption onto sediment particles, possibly limiting their degradation (Baumard et al., 1999; Dubansky et al., 2013; Raimondo et al., 2014; Turner et al., 2014). Higher toxicity of sediment-derived PAHs compared to HEWAF-associated PAHs may be related to the greater proportion of 3 and 4 ring-PAHs in oiled-sediment elutriates, as it was suggested that higher molecular weight PAHs are more toxic than lower molecular weight compounds (Achten et al., 2015). Toxicity of individual PAHs on oyster early life stages remain unknown and further research is needed to better elucidate the higher toxicity of sediment-derived PAHs compared to HEWAF-derived PAHs to Eastern oyster fertilization success and larval development from exposed embryos.

4.3. Choice of sensitive life stages for ecotoxicological assessment

The present study reveals that 1-h fertilization success and 24-h abnormality of larvae exposed as embryos endpoints are more sensitive than shell length (across all bioassays) and veliger larval abnormality endpoints with respect to sediment-derived PAHs in the Eastern oyster. Fertilization success was reduced at similar levels of tPAH50 than those affecting embryogenesis, and both of these endpoints showed strong dose-dependent response for the unfiltered elutriates of oil-contaminated sediment, indicating that they are both sensitive sublethal endpoints for assessing the acute toxicity of oiled sediment. Advantage for the 1-h fertilization success endpoint is that this assay is conducted over a shorter period of time (i.e., few hours) than the embryo abnormality assay. Endpoints using veliger larvae were less sensitive to sediment-derived PAHs compared to fertilization success and embryo

development. Although larval abnormality and shell length of exposed veliger larvae did not show strong dose-dependent response for concentrations of sediment-derived PAHs tested, it should be noted that larval abnormalities were more sensitive to sediment-derived PAHs than larval shell length.

5. Conclusion

In the present study, the effect of unfiltered elutriates from sediment contaminated with DWH oil on the early life stages of a marine bivalve species were investigated for the first time. The results of our study indicate that gametes, embryos and veliger larvae of the Eastern oyster can be adversely impacted by oil attached to suspended sediments, which indicates that sediments should also be evaluated and considered as a possible contaminant source for this exposure route. Fertilization success and 24-h abnormality of larvae exposed as embryos endpoints were the most sensitive endpoints for ecotoxicological assessment of oil-contaminated sediment to early life stages of this species.

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Figure captions

Fig. 1. PAH composition of unfiltered sediment elutriate stock and field-collected contaminated sediment, expressed in %. Avg: Mean composition of tPAH50 of unfiltered sediment elutriate stock (stock 100%) used for gamete, embryo, and veliger acute exposures. PAH abbreviations are provided in Supplementary Table 1.

Fig. 2. Dose response curve for fertilization success of gametes exposed continuously to unfiltered elutriates from sediment contaminated with DWH oil. Observed unfertilized oocytes (in %) were reported 1 hour after fertilization, for 4 replicates per treatment. Modeled for unfertilized oocytes for unfiltered sediment elutriates was fitted to tPAH50 exposure concentrations (µg L^{-1}). Gray filled circles represent sediment elutriate control; black filled circles represent seawater control (on the left) and unfiltered sediment elutriates. Dose-response curve was fitted using seawater control. Horizontal lines on curve represent 95% CI of EC20 and EC50.

Fig. 3. Dose response curve for larval abnormality developed from (A) gametes, (B) embryos and (C) veliger-larvae exposed continuously to unfiltered elutriates from sediment contaminated with DWH oil. Observed abnormalities (in %) were reported after 24 hours of exposure for gametes and embryos and 48 hours of exposure for veliger larvae; for 4 replicates per treatment. Modeled for abnormalities for unfiltered sediment elutriate were fitted to tPAH50 exposure concentrations (µg L^{-1}). Gray filled circles represent sediment elutriate control; black filled circles represent seawater control (on the left) and unfiltered sediment elutriates. Dose-response curves were fitted using seawater control. Horizontal lines on curves represent 95% CI of EC20 and EC50. (A) High percentage of abnormality in larvae developed from exposed gametes to sediment elutriate control did not allow determining EC20 and EC50 values. (C) Low percentage of abnormality at the highest doses of sediment elutriate tested in the veliger exposure did not allow the calculation of EC50 value.

Fig. 4. Dose response curve for shell length of larvae developed from (A) gametes, (B) embryos and (C) veliger-larvae continuously exposed continuously to unfiltered elutriates from sediment contaminated with DWH oil. Observed shell lengths (in µm) were reported after 24 hours of exposure for gametes and embryos, and 48 hours of exposure for veliger larvae; for 4 replicates per treatment. Modeled shell lengths for unfiltered sediment elutriate

were fitted to tPAH50 exposure concentrations (µg L^{-1}). Gray filled circles represent sediment elutriate control; black filled circles represent seawater control (on the left) and unfiltered sediment elutriates. Dose-response curves were fitted using seawater control. Limited decrease of shell length at the range of tPAH50 L^{-1} concentrations in sediment elutriate tested did not allow the calculation of EC20 and EC50 values in the (A) gamete and (C) veliger-larval exposures, and the calculation of EC50 in the (B) embryo exposure.

Tables

Table 1

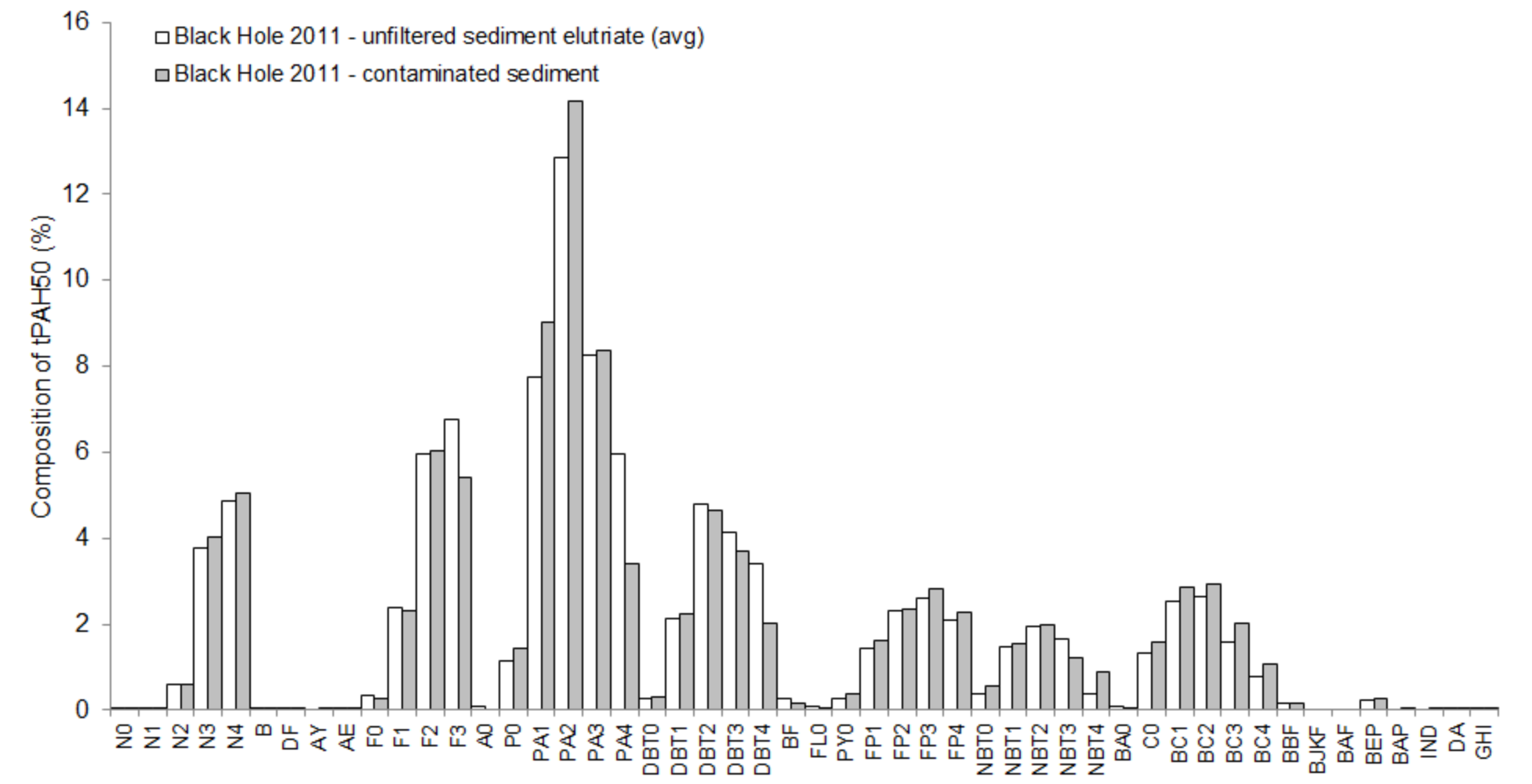
Chemical and physical characteristics of contaminated sediment (Black Hole 2011) and sediment control (Loomis II) used for toxicity testing. Sum of 50 PAHs (tPAH50) is expressed in mg kg^{-1}; Total Organic Carbon (TOC) and Total Solids (TS-MET) are expressed in %. Fines, corresponding to particle size (silt + clay), is expressed in %. Fines of contaminated sediment could not be assessed because of high oil content (N/A).

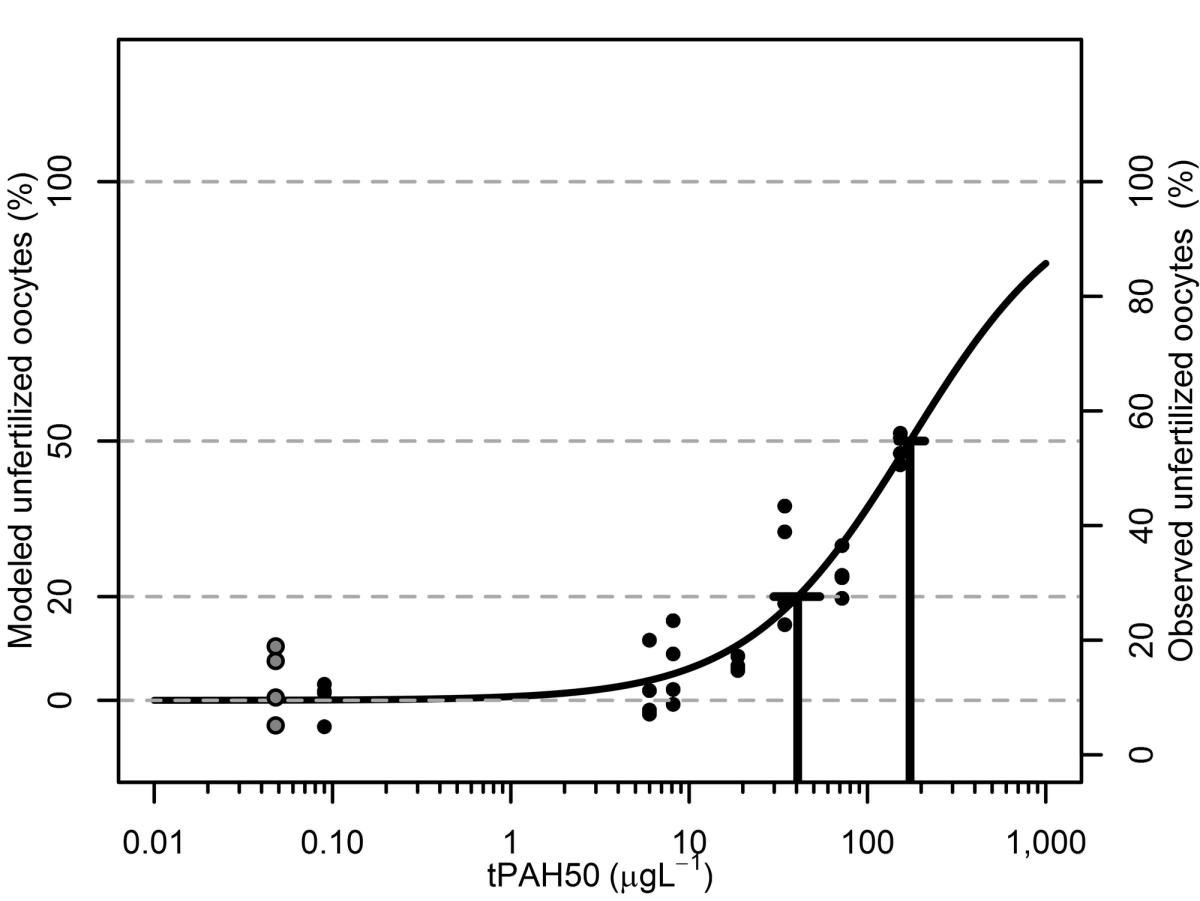
Sediment type	tPAH50 (mg kg^{-1})	TOC (%)	TS-MET (%)	Fines (%)
Contaminated sediment	3259	69.9	26.2	N/A
Sediment control	0	0.833	38.0	82.38

Table 2

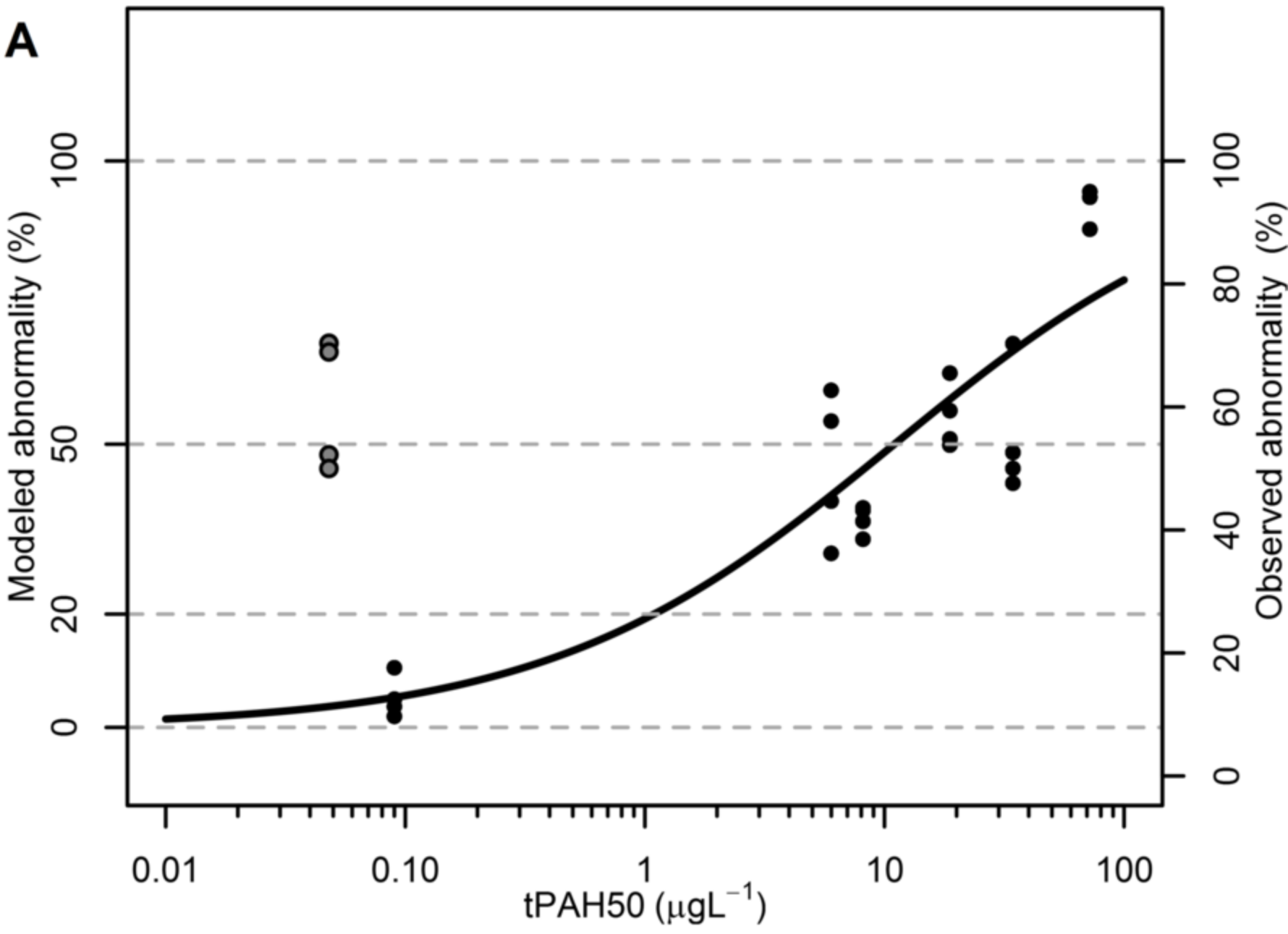
Concentration causing 20% and 50% inhibition (EC20/EC50) of observed abnormality in larvae continuously exposed to unfiltered elutriates of sediment contaminated with DWH oil for 24 hours from embryos, or for 48 hours from veliger. Data are expressed as measured concentrations of a sum of 50 PAHs (µg tPAH50 L^{-1}) for sediment ± 95% confidence intervals. NC: not calculated.

Initial stage	Embryo	Veliger larva
EC20	77.7 (62.8-96.9)	95.9 (42.3-174)
EC50	151 (134-172)	NC

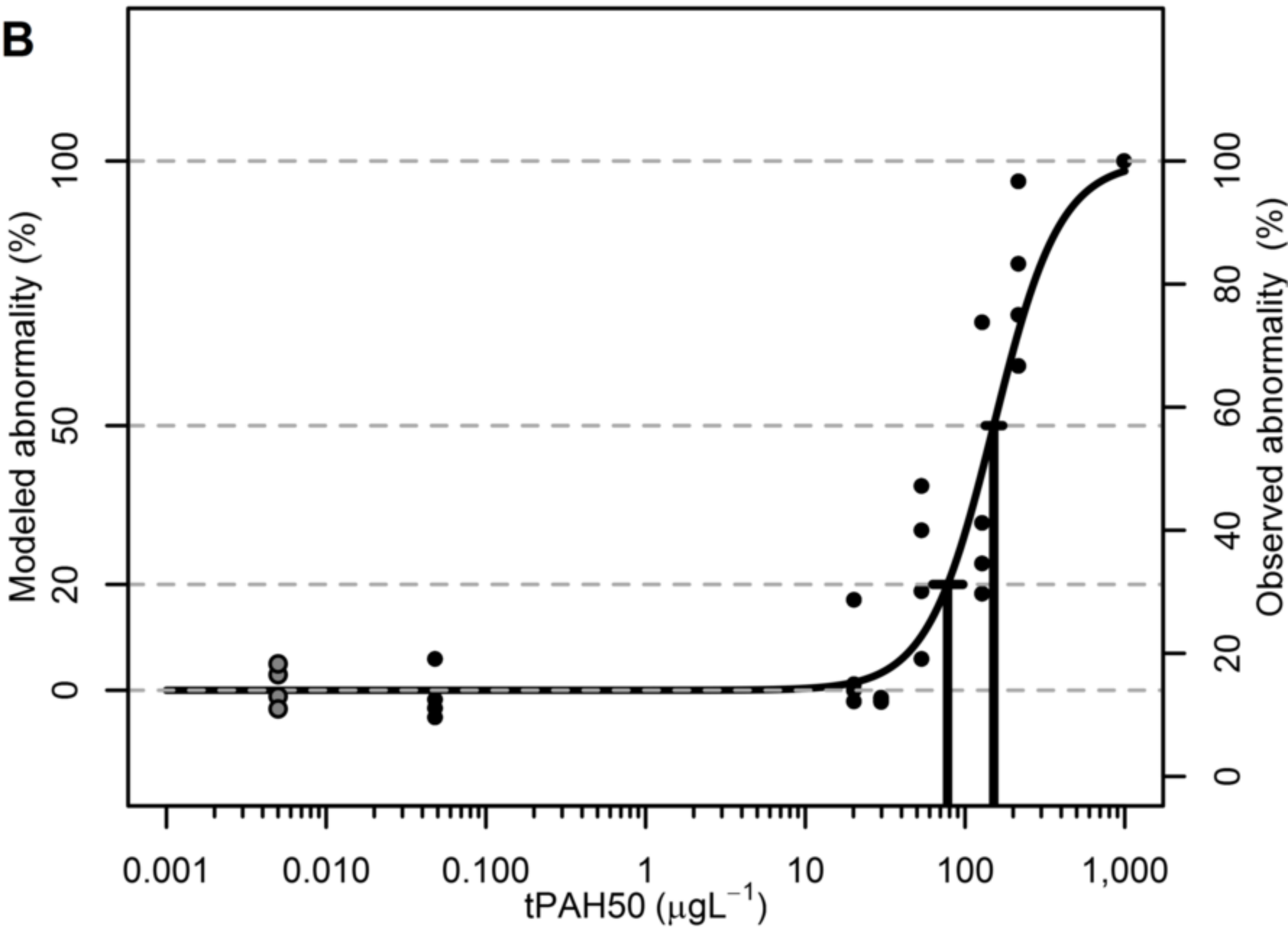

Black Hole 2011 - unfiltered sediment elutriate (avg)
Black Hole 2011 - contaminated sediment
Composition of tPAH50 (%)
0
2
4
6
8
10
12
14
16
N0
N1
N2
N3
N4
B
DF
AY
AE
F0
F1
F2
F3
A0
P0
PA1
PA2
PA3
PA4
DBT0
DBT1
DBT2
DBT3
DBT4
BF
FL0
PY0
FP1
FP2
FP3
FP4
NBT0
NBT1
NBT2
NBT3
NBT4
BA0
C0
BC1
BC2
BC3
BC4
BBF
BJKF
BAF
BEP
BAP
IND
DA
GHI


Modeled unfertilized oocytes (%)
100
50
20
0
Observed unfertilized oocytes (%)
100
80
60
40
20
0
0.01
0.10
1
10
100
1,000
tPAH50 (μgL^{-1})

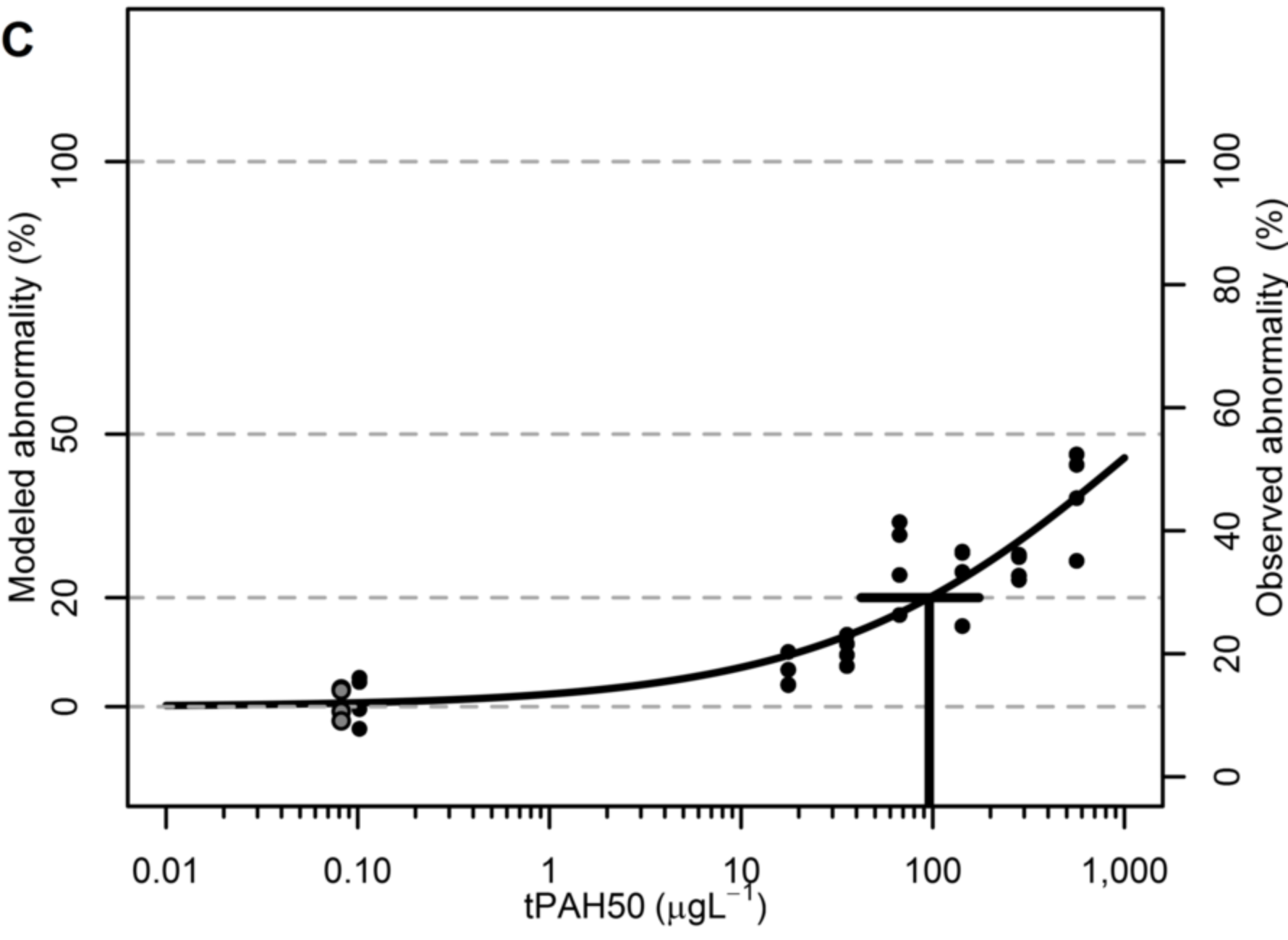
A

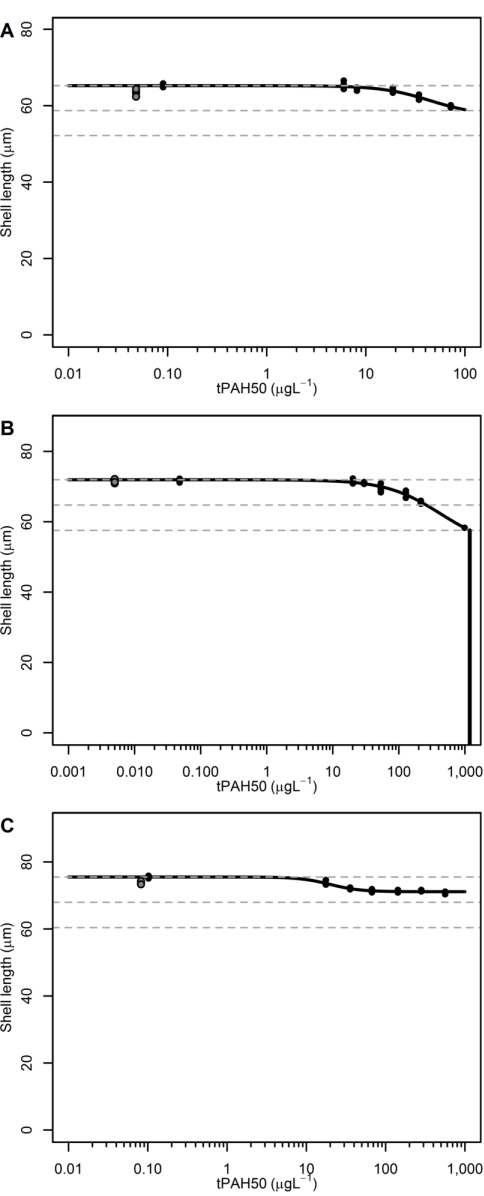

Modeled abnormality (%)
0
20
50
100
Observed abnormality (%)
0
20
40
60
80
100
0.01
0.10
1
10
100
tPAH50 (μgL^{-1})

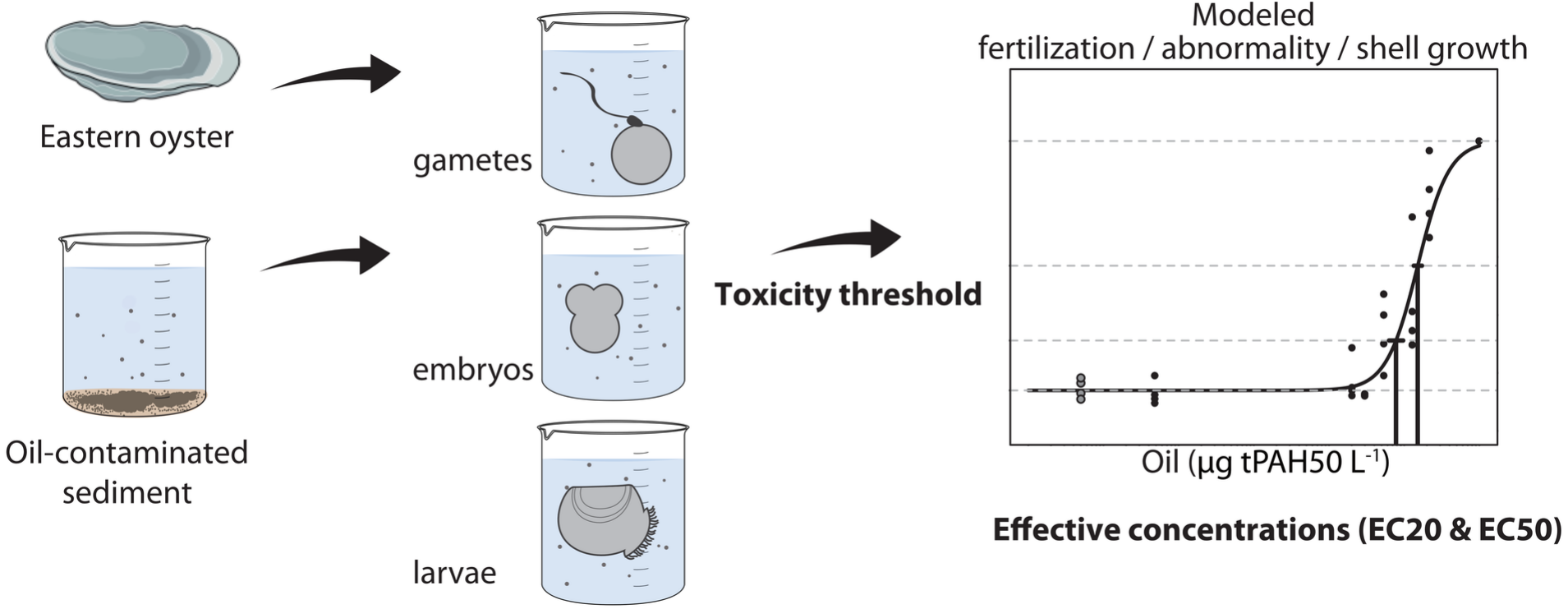
B


Modeled abnormality (%)
0
20
50
100
Observed abnormality (%)
0
20
40
60
80
100
0.001
0.010
0.100
1
10
100
1,000
tPAH50 (μgL^{-1})

C


Modeled abnormality (%)
0
20
50
100
Observed abnormality (%)
0
20
40
60
80
100
0.01
0.10
1
10
100
1,000
tPAH50 (μgL^{-1})


A
Shell length (µm)
0
20
40
60
80
0.01
0.10
1
10
100
tPAH50 (µgL^{-1})
B
Shell length (µm)
0
20
40
60
80
0.001
0.010
0.100
1
10
100
1,000
tPAH50 (µgL^{-1})
C
Shell length (µm)
0
20
40
60
80
0.01
0.10
1
10
100
1,000
tPAH50 (µgL^{-1})


Eastern oyster
Oil-contaminated sediment
gametes
embryos
larvae
Toxicity threshold
Modeled
fertilization / abnormality / shell growth
Oil (µg tPAH50 L-1)
Effective concentrations (EC20 & EC50)