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Use of biological detection methods to assess dioxin-like compounds in sediments of Bohai Bay, China

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ABSTRACT

Bohai Bay, in the western region of northeastern China's Bohai Sea, receives water from large rivers containing various pollutants including dioxin-like compounds (DLCs). This study used the established zebrafish (*Danio rerio*) model, its known developmental toxicity endpoints and sensitive molecular analyses to evaluate sediments near and around an industrial effluent site in Bohai Bay. The primary objective was to assess the efficacy of rapid biological detection methods as an addition to chemical analyses. Embryos were exposed to various concentrations of sediment extracts as well as a (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD)) positive control. Exposure to sediment extract the discharge site (P1) resulted in the most- and highest change in embryos and larvae, suggesting that DLC contaminated sediment probably did not occur much beyond it. P1 extract resulted in concentration dependent increases in mortality and pericardial edema. The highest concentration caused up-regulation of P-450 (CYP)-1A1(*CYP1A)* mRNA expression at 72 h post fertilization (hpf), an increase in its expression in gill arches as observed by whole mount in situ hybridization, and an increased signal in the *Tg*(*cyp1a: mCherry*) transgenic line. The pattern and magnitude of response was very similar to that of TCDD and supported the presence of DLCs in these sediment samples. Follow-up chemical analysaes confirmed this presence and identified H7CDF, O8CDF and O8CDD as the main components in P1 extract. This study validates the use of biological assays as a rapid, sensitive, and cost-effective method to evaluate DLCs and their effects in sediment samples. Additionally, it provides support for the conclusion that DLCs have limited remobilization capacity in marine sediments.

1. Introduction

The Bohai Sea in northeastern China receives water from more than 40 rivers (Zhang et al., 2006). Its coastal areas have seen rapid economic growth over the past several decades and these have led to environmental impacts from agricultural runoff, city sewage, and various industrial pollutants including heavy metals and organic chemicals (Gao et al., 2016; Li et al., 2015; Peng, 2015; Zhang et al., 2009, 2006). As a result, the region has been inundated with persistent organic pollutants

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(POPs) and heavy metals becoming one of the most polluted sea areas (Meng et al., 2017). Bohai Bay, on western region of the Bohai Sea, receives water from multiple large rivers (Wang and Wang, 2007). Many of the pollutants originate inland and enter watersheds via runoff and atmospheric deposition, with particularly high concentrations appearing at the mouths of the Yellow- and Haihe Rivers that discharge into this Bay (Bao et al., 2012; Liu et al., 2003; Meng et al., 2017).

Concerns over human and ecological risks have led to monitoring programs for pollutants worldwide (Zhang et al., 2006). Chemical approaches have been used to quantify levels of individual components within abiotic media (i.e., water and sediment) via techniques such as high resolution gas chromatography and high resolution mass spectrophotometry (GC/HRMS) (Kashimoto et al., 1987; Wu et al., 2016). Such analytical approaches provide direct evidence of pollutant presence as well as specific and selective compounds (Jin et al., 2017). However, they are costly and often fail to provide critical insights into bioavailability and organismal responses.

Among these pollutants, dioxin-like compounds (DLCs) have been tightly associated with suspended particles that eventually enter sediments (Soubaneh et al., 2015), serving as a depository for DLCs and a other contaminants that adversely affect benthic organisms and suspension feeders (Ren et al., 2017; Soubaneh et al., 2014). DLCs have received increasing attention due to their persistence in the environment, bioaccumulation and biomagnification characteristics, toxic alterations in aquatic organisms, risks to aquatic ecosystems, and potential health risks to humans through trophic transfer (i.e., via the consumptions of DLC-contaminated seafood) (Eisler, 1986; Jiang et al., 2005; Zhang et al., 2011).

DLCs have structural similarities and modes of action in organisms and include various groups of organic pollutants such as polychlorinated dibenzo-p-dioxins and -furans (PCDD/Fs) and polycyclic aromatic hydrocarbons (PAHs) (Brown et al., 2018; Verhaert et al., 2013, 2017). These similarities have led to the use of toxic equivalents (TEQ), a term operationally defined by the sum of the products of the concentration of each compound multiplied by its toxic equivalency factor (TEF) (Van den Berg et al., 2006). While levels of DLCs are generally low (0.5–17.7 ng/g or ng/mL) in environmental media (i.e., water and sediments), they proven to be potent toxic contaminants with potential to cause toxicity in aquatic organisms following long-term exposure (Eljarrat et al., 2005; Macikova et al., 2014; van den Dungen et al., 2016). To date, toxicology research has focused on endocrine disruptions caused by DLCs as well as adverse effects mediated through the aryl hydrocarbon receptor (AhR2) pathway (Dong et al., 2002; Fang et al., 2016; Prasch et al., 2003). The latter can alter the expression of target genes, resulting in disruptions of biochemical processes, cell differentiation, and growth (Kim et al., 2012). Various bioanalytical detection methods (BDMs) have been developed for the screening and diagnosis of DLCs based on their binding potential to the AhR2 and induction of cytochrome P-450 (CYP)-1A1 (Brown et al., 2015). The limitation inherent with these assays has spurred efforts to develop new BDMs that utilize the latest advancements while remaining simple, sensitive, rapid, and cost-effective.

A main objective of this study was to test BDMs using embryo and larval zebrafish (*Danio rerio*), a well-known laboratory model fish, with environmentally relevant DLCs as a way to integrate information on organismal responses at different levels of biological organization. To do this, sediments were collected from an industrial effluent discharging site near the Bohai Bay and shown to contain DLCs in porewater. Established developmental toxicity endpoints and molecular responses were used to evaluate biological effects of DLCs in a rapid, cost-effective manner and one that demonstrated the presence of effects of sediment contaminants Chemical analysis of DLCs in sediment was then performed to validate the findings of these biological assays.

2. Materials and methods

2.1. Chemicals and reagents

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (98% purity, Andover, MA). Anti-β-actin antibody, mouse anti fish CYP1A antibody, and goat anti-mouse immunologlobulin were purchased from Cayman Chemical Company (Ann Arbor, MI). Chemicals or reagents listed in the methods below were of the highest commercially available purity.

2.2. Zebrafish culture

Adult zebrafish were maintained at 28 °C in a re-circulating aquatic habitat system under a 14 h:10 h (light:dark) photoperiod. Spawning groups of 12 males and 6 females were placed in breeding chambers, their embryos collected and maintained in Danio medium (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, and 0.17 mM KCl) under the conditions as adults. Adult care and reproductive techniques were noninvasive and approved by the Duke University Institutional Animal Care and Use Committee.

2.3. Sample collection and preparation

Surface sediments were collected in 2017 from the Bohai Bay (Shandong, China) according a previously published protocol (Wu et al., 2016). All collection and processing equipment were thoroughly cleaned between sampling sites. Sampling sites were near the exit of the Zhanli River within the Yellow River Delta (YRD) (Fig. S1). Sampling site 1(P1) was near the wastewater discharge site of a petroleum refinery factory, which has been in operation since 2012. Sampling site 2 (P2) and site 3 (P3) were approximately 1 and 2 km downstream of P1, respectively (Fig. S1). At each site, sediment samples were collected with a stainless-steel Ekman grab sampler (Dynamic Aqua-Supply, Ltd. Surrey, British Columbia, Canada) and homogenized with a stainless-steel spoon for 10 min. Approximately 20 g of un-sieved wet material was then placed in a labeled glass container. A field blank was also prepared using MilliQ water (MilliQ Integral, Millipore, Germany) following the same procedures without sediment. Samples were then transferred on ice to the laboratory and stored at −20 °C until analysis.

From one jar per sampling site, approximately 10 g of sediment was transferred to a clean glass beaker and treated with 100 mL ethyl alcohol (HPLC grade, Fisher Chemical Co., Beijing, China) by dipping overnight. Next, samples were filtered through Whatman filter paper No. 42 and then filtrates were dried by evaporation at room temperature. Finally, extracts from each sample site were dissolved in 1 mL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) for embryo exposures as described in Section 2.4.

Results from zebrafish exposures revealed P1 samples to show the most effects. Therefore, only these sediments underwent chemical analysis. P1 sediment samples were freeze-dried (FreeZone 4.5 L −50 °C Benchtop Freeze Dryer, Labconco Corp. Kansas City, MO), ground, and put through a 0.2 mm mesh sieve. Approximately 1 g of dried sediment was transferred into a 15 mL centrifuge tube (Corning, Inc., Corning, NY). To each tube a mixture of mass-labeled surrogates was added including

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$^{13}C_4$-perfluorooctane sulfonate (PFOS), ethyl-perfluorooctane sulfonamido acetic acid (EtFOSAA-d_{5+}), $^{13}C_4$-bis (1H, 1H, 2H, 2H) perfluorooctyl phosphate ($^{13}C_4$–8:2 diPAP) (all three from Wellington Laboratories Inc., Guelph, Ontario, Canada) and Tris (2-ethylhexyl)-d51 phosphate (TEHP-d51) (Hayashi Pure Chemical Ind. Ltd., Osaka, Japan) (Zhang et al., 2018). After equilibration at room temperature for 2 h, 5 mL methanol was added to each tube. The mixtures were shaken on an orbital shaker for 10 min and centrifuged at 2710*g* for 10 min. The supernatants were transferred to a new centrifuge tube. The extraction procedure was repeated twice, and all extracts were combined and concentrated to 1 mL under nitrogen flow. An Envi-Carb cartridge (1 mg, 1 mL; Supelco, Bellefonte, PA) was selected for the removal of interferences in the subsequent clean-up procedure. The columns were pre-conditioned with 1 mL methanol (Sigma-Aldrich) containing 0.5% NH_4OH and 1 mL of a mixture of methanol and dichloromethane (v/v, 1/1; Sigma-Aldrich). The concentrated extract was introduced into the cartridge and the cartridge was eluted sequentially with 1 mL of methanol containing 0.5% NH_4OH and 1 mL of the mixture of methanol and dichloromethane (v/v, 1:1). The drainage and eluate were combined and dried under a gentle stream of nitrogen. The dried samples were re-dissolved with 200 μL of methanol prior to UPLC-MS/MS analysis (Waters, Milford, MA).

2.4. Acute exposure to sediment extract in zebrafish embryos

OECD, Test No. 236 guidelines were used to assess effects on survival and pericardial alterations of zebrafish following acute exposure to sediment extracts (OECD, 2013). Embryos at 4 h post fertilization (hpf) were selected and placed into 10 mL wells of a six-well tissue culture plate each containing 5 mL of Danio media, with 10 embryos per well and 3 wells per treatment. Exposure solutions were prepared in a 2-step process: 1) 0.1, 0.2, and 1 g of dried sediment extract from each sampling site were individually dissolved in 1 mL of DMSO to create sediment equivalent stock solutions and then 2) 5 μL of stock solution were added to a well containing embryos in 5 mL Danio medium resulting in final exposure concentrations of 01, 0.2, or 1 mg/mL of sediment extract. The final concentration of DMSO in the exposure medium of each well was 0.1%. A negative, solvent control (SC) and a positive control containing 3.1 nM (1ppb) TCDD were run with each test. Embryos were scored daily for mortality, pericardial edema, and other developmental malformations until they reached 72 hpf. The pericardial edema area was quantified using ImageJ 1.52a (Rasband, 1997–2018).

2.5. Total RNA isolation and quantitative real-time polymerase chain reaction

The above exposure design was repeated with P1 sediment extracts to obtain samples for analysis of gene expression. At 48 h, 72 h and 96 h of exposure, 20 larvae from each treatment were sampled and pooled. Total RNA from these larvae was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. Purity of the RNA samples was analyzed by the absorbance ratio of 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA) (ratio range from 1.85 to 2.06 for all samples). cDNA was obtained via reverse transcription from purified RNA samples using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems Inc., Foster City, CA) in accordance with the protocols of the manufacturer. The cDNA samples were stored at −20 °C until use.

Real time PCR (qRT-PCR) was used to quantify the expressional levels of *CYP1A* and *18S* transcripts. The primers were designed using PrimerQuest (Integrated DNA Technologies, San Diego, CA). For *CYP1A*, forward primer was 5′-CGCTGAAGTCTTCCTGTTCC −3′ and reverse primer was 5′-TTTTGGATGCATGATGAGGA −3′. For *18S* forward primer was 5′-CCTGCGGCTTAATTTGACTC-3′ and reverse primer was 5′-GACAAATCGCTCCACCAACT-3′. *CYP1A* and *18S* cDNAs were amplified separately in triplicate using a Step One Plus Sequence Detection System (Applied Biosystems) in a 96-well PCR plate. qRT-PCR amplification was carried out for 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s *18S* ribosomal RNA was used to normalize target gene expression. In order to compensate for intrinsic variability in the amount of RNA between embryos, threshold cycle (Ct) was normalized with *18S* RNA of the same sample according to a previous method (Wang et al., 2018). The relative transcriptional level of *CYP1A* to SC was analyzed using the $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001).

2.6. AHR2-MO knockdown exposure to P1 sediment extract

A morpholino proven to block the translation of zebrafish aryl hydrocarbon receptor 2 (AHR2-MO) was purchased from Gene Tools, LLC (Philomath, OR) (Teraoka et al., 2003). The morpholinos were diluted to 100 μM working stocks in 30% Danieau medium. Approximately 3 nL of 100 μM morpholino solution was injected into the yolk of embryos at the 1-cell or 2-cell stage with a fine glass needle connected to an automatic injector (IM-300, Narshige). Fifteen surviving embryos (5 for each of 3 replicates) were used to test effects of sediment extract on developing zebrafish hearts. Based on preliminary results (data not shown), only sediments from P1 were tested for phenotypic differences between the wild type and the AhR2 morpholino knockdown mutant. The exposure lasted for 72 h.

2.7. 2.7 Whole mount in situ hybridization

Embryos at 4 hpf were exposed to P1 sediment extract as described in Section 2.4 until they reached 36 hpf. This stage was selected because embryos had developed blood vessels and had normally high expression of melanin. Upon reaching 36 hpf, zebrafish embryos were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) in phosphate saline solution (PBS, pH 7.4) overnight, dechorionated using watchmaker's forceps, and then stored at −20 °C. Whole mount in situ hybridization (WISH) was carried out according to our previously published method (Hollert et al., 2018). Briefly, embryos were hybridized with an antisense probe of zebrafish *CYP1A*, in which the cDNA (483 base pairs) was originally cloned by degenerate-PCR. Following hybridization overnight at 64 °C, embryos were washed with 2× saline sodium citrate (SSC 300 mM NaCl, 30 mM sodium citrate, pH 7.0) and 0.2× SSC twice for 30 min, respectively. Then embryos were blocked with 2% blocking reagent (Roche, Mannheim, Germany), followed by incubation overnight with 3000× diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) at 4 °C. The color reaction was carried out by incubation with BM-purple substrate (Roche).

2.8. Western blot analysis

Embryos were exposed to 0.1, 0.2 and 1 mg/mL of P1 sediment extract from 4 hpf until 72 hpf, with 10 individuals in each of 3 replicates. All larvae in a replicate were pooled and frozen in liquid nitrogen. Next, samples were ground and then dissolved in a lysis solution containing 500 μL RIPA solution (Sigma-Aldrich) with 1 mM phenylmethyl sulfonyl fluorine (PMSF; Sigma-Aldrich). Samples were allowed to lyse for 30 min on ice

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after which they were centrifuged at 10,000–14,000*g* for 5 min at 4 °C. The supernatant was aliquoted and stored at −20 °C until further analysis. Total protein levels in the supernatants were set to 3 mg/mL by dilution of the lysates. The proteins were separated by gel electrophoresis and electro-transferred to PVDF membranes (Thermo Fisher Scientific). The PVDF membranes were incubated in a solution containing anti-β-actin antibody and mouse anti-fish CYP1A antibody (Cayman Chemical Company). The membranes were placed in a 2% PBST solution containing the biotin-labeled goat anti-mouse secondary antibody (1:200) for 1 h at room temperature, followed by 1 h incubation with A-B solution from the VECTASTATIN Elite kit (Vector Laboratories, Burlingame, CA). The signals were detected with a ECL system (Sigma-Aldrich).

2.9. Transgenic zebrafish line Tg(cyp1a:mCherry) exposure to sediment extract

The transgenic zebrafish line *Tg*(*cyp1a: mCherry*) with CYP1A promoter driving *mCherry* expression was has recently been shown to be a sensitive, efficient and convenient tool for monitoring PAHs and TCDD contamination in the environment (Luo et al., 2018; Xie et al., 2018). Therefore, it was chosen for testing sediment extracts. Embryos were exposed to sediment extract as described in Section 2.4 and evaluated according to methods described by Xie et al. (2018). At 72 hpf, larvae were anesthetized with MS-222 (Aladdin, China), examined and imaged using a Leica M125C fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Leica DMC5400 digital camera. Fluorescence intensity was quantified from images using the RiFRET plugin (Roszik et al., 2009) for ImageJ.

2.10. Statistical analyses

Data are expressed as mean ± SEM unless otherwise stated. GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA) was used for statistical analyses. Differences between means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of sediment extracts on zebrafish embryo development

The effects of sediment extracts from three sampling locations (P1, P2, and P3) and TCDD on mortality of zebrafish embryos and size of pericardial cavities in survivors are shown in Fig. 1. In general, exposure to 0.1 and 0.2 mg/mL of the sediment extracts from the three sampling locations did not affect the mortality of the zebrafish embryos (Fig. 1A). However, 1 mg/mL of P1 sediment extract resulted in 26.6% mortality, significantly more than control ($p < 0.05$). This mortality rate was close to that of TCDD (28.7%). The same concentration of P2 and P3 sediment extracts did not result in significant differences in mortality. The effect of sediment extracts and TCDD on the pericardial cavities of zebrafish embryos showed a similar pattern to that of mortality. An approximately 370% increase in the pericardial cavity area was observed in the 1 mg/mL P1 treatment, indicating presence of pericardial edema (Fig. 1B). This value was also similar to that of fish from the TCDD treatment (Fig. 1B).

No obvious differences were observed in the larvae from the solvent control (SC) and those with AhR2 gene knockdown (Fig. 2A-B). Pericardial edema was observed in larvae from the 0.2 mg/mL P1 treatment (Fig. 2C) and became significantly larger with an increase in concentration to 1 mg/mL (Fig. 2E,I) as well as with TCDD (Fig. 2G,I). Interestingly, AhR2 gene knockdown larvae did not develop pericardial edema (Fig. 2B,D,F,H).

3.2. Expression of CYP1A mRNA in zebrafish embryos exposed to P1 sediment extract

WISH showed the expression of *CYP1A* in the zebrafish at 36 hpf (Fig. 3). By contrast, *CYP1A* mRNA expression was not detected in control embryos (Fig. 3A). Expression increased with the increasing concentrations of P1 sediment extract (Fig. 3B-D). At 0.1 and 0.2 mg/mL, the expression of *CYP1A* mRNA was seen as punctate reactions on the skin of fish (Fig. 3 B,C). At 1 mg/mL, signal was greater in the gill arches, where it was seen in developing vasculature and on the skin (Fig. 3D). The fish from the TCDD treatment showed a similar albeit slightly less extensive pattern when compared to that seen in fish from the 1 mg/mL treatment (Fig. 3E).

RT-qPCR analysis showed *CYP1A* expression in all P1 and TCDD treatments at each time point and were significantly higher compared to controls. At 48 hpf, this expression was up-regulated by approximately 50 times in the 0.1 and 0.2 mg/mL treatments and approximately 100 times in 1 mg/mL and TCDD treatments. At 72 hpf, expression remained comparable that at 48 hpf in the 1 mg/mL and TCDD treatments, while it decreased approximately 40% in the 0.1 and 0.2 mg/mL treatments (Fig. 4). By 96 hpf, expression in the 1 mg/mL and TCDD treatments dropped over 50% relative to the earlier time points, nearing levels of the other two treatment groups (Fig. 4).

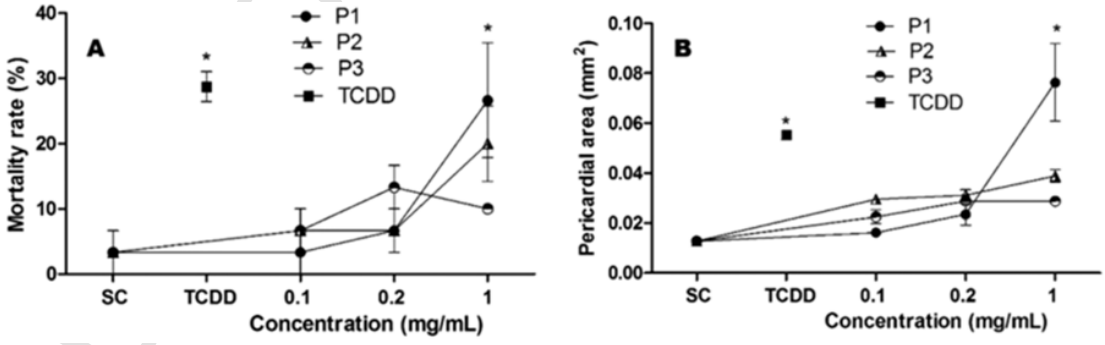

Fig. 1. Effects of sediment extracts from Bohai Bay on mortality and pericardial cavities of zebrafish embryos. Embryos were exposed from 4 h past fertilization (hpf) to 72 hpf extracts collected from three locations (P1, P2, and P3), a solvent control (SC), and a positive control containing TCDD. Areas of pericardial cavities were measured in ImageJ. Asterisks (*) indicated significant differences ($p < 0.05$) between the treatment groups and the SC group.

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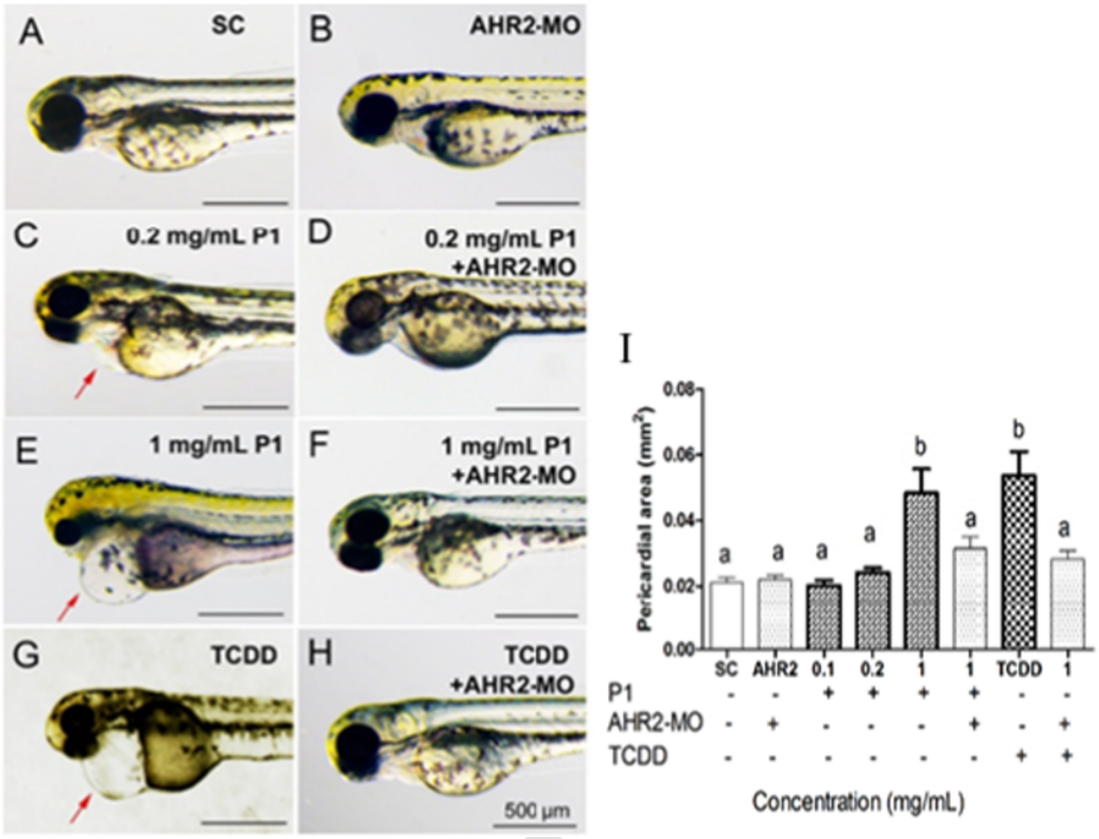

Fig. 2. Representative images of pericardial cavity changes in zebrafish larvae exposed to P1 sediment extracts from Bohai Bay, a solvent control (SC), or a positive TCDD control. Column 1 (A, C, E,G) shows control and exposed larvae without AHR2-morpholino (MO) knockdown injection. Column 1 (B, D, F, H) shows control and exposed larvae with AHR2-MO knockdown. Red arrows indicate pericardial edema. Histogram (I) shows pericardial area quantified in ImageJ from each treatment (3 replicates each with 5 individuals). Different letters indicate significant differences compared to SC ($p < 0.05$). Scale bars are 500 μm.

3.3. CYPIA protein levels in P1 exposed zebrafish

At 72 hpf of exposure to sediment P1 extract, expression of CYP1A protein showed a concentration-dependent increase, with 1 mg/mL significantly higher than SC ($p < 0.001$, Fig. 5). Densitometric analysis showed that the expression of *CYP1A* was increased by 30, 178, and 305 fold in the 0.1, 0.2 and 1 mg/mL treatments, respectively, compared to that of the SC treatment. In the TCDD treatment, expression of *CYP1A* was 363 times higher than that of SC (Fig. 5).

3.4. Response of Tg(cyp1a:mCherry) transgenic zebrafish to sediment extract exposure

Expression of *mCherry* was not detectable in the SC treatment fish (Fig. 6A-B). After 6 h of exposure, larvae exposed to 1 mg/mL P1 sediment extract and those exposed to TCDD h showed high increases in fluorescence intensity (Fig. 6C-F,K). Comparatively, fish exposed to P2 or P3 sediment extracts had lower fluorescence intensity (Fig. 6G-J,K). Quantification of the fluorescence intensity supported these observations. All treatment groups had statistically higher fluorescence intensity values compared to SC, with a site distance from source dependent decrease (P1 > P2 > P3) and TCDD falling between values of P1 and P2 (Fig. 6K).

3.5. Chemical analysis of sediments from P1

Based on the results of the biochemical responses in the zebrafish, the chemical analyses were only carried out in the sediments collected from P1 location from the Bohai Bay. Of the 16 DLCs, T4CDF, P5CDD and T4CDD were not detected (Table 1). The levels of the remaining compounds ranged from 4.9 pg/g dry wt (dw) for O8CDD to 0.2 pg/g dw for H6CDD. The total level of DLCs was calculated to be 23.35 pg/g dw, while the TEQ was estimated to be approximately 1.1 pg/g (Table 1). After conversion to μg/L, these two values were 4–5 orders of magnitude lower than that of the positive control (3.1 nM ~ 1 μg/L).

4. Discussion

4.1. Biological assays to examine effects of sediment extracts on zebrafish

Exposure to sediment extracts from the P1 site led to a concentration-dependent increase in mortality of zebrafish embryos. The absence of significant mortality in sediment extracts collected from P2 and P3 sites were indicative of lower levels of toxic chemicals in sediments further removed from effluent discharging sites. These results suggested DLC contaminated sediment particles probably did not occur beyond the area immediately around the effluent discharge site. Similarly, a chemical analysis of dioxins in sediments from a Houston Ship Channel waste pit showed contaminated particles were not remobilized

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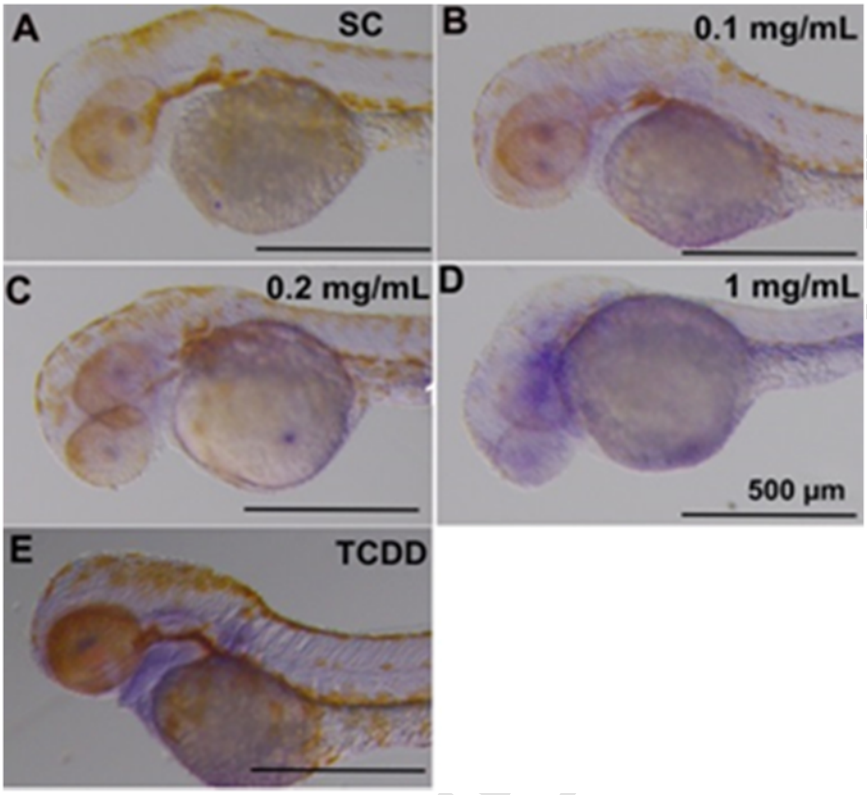

Fig. 3. Detection of *CYP1A* mRNA by whole-mount in situ hybridization (WISH) in 36 hpf zebrafish exposed to either 0.1, 0.2, or 1 mg/mL of P1 sediment extract, a solvent control (SC), or a positive TCDD control. Blue color indicated *CYP1A* mRNA expression. Scale bars are 500 μm.

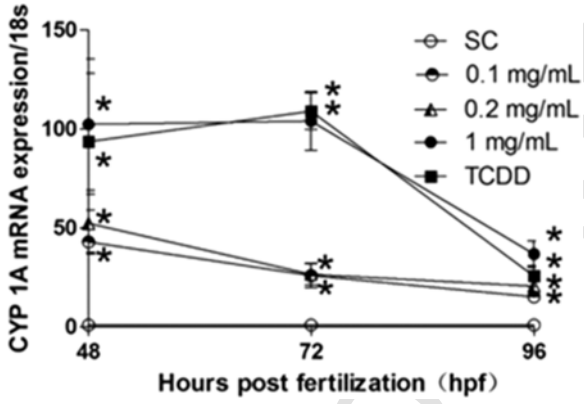

Fig. 4. Detection of CYP1A mRNA by qRT-PCR at 48, 72 and 96 hpf zebrafish exposed to 0.1, 0.2, or 1 mg/mL P1 sediment extract from Bohai Bay, a solvent control (SC), or a positive TCDD control. Data are expressed as mean ± SEM (3 replicates each with 10 embryos). Asterisks (*) indicate significant differences from SC ($p < 0.05$).

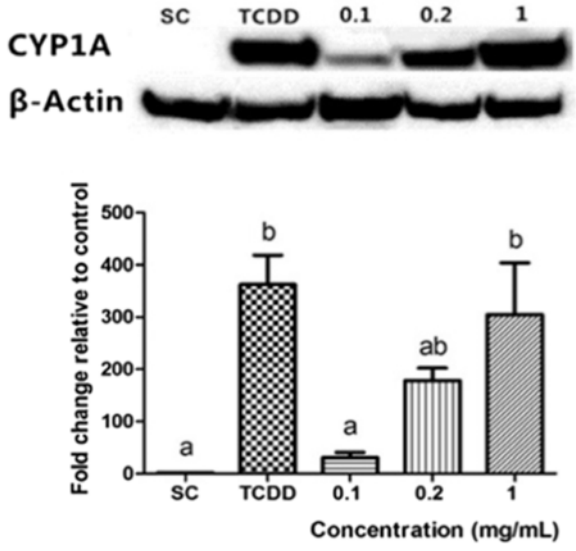

Fig. 5. Detection of CYP 1 A protein levels in 72 hpf larvae that had been exposed to either 0.1, 0.2, or 1 mg/mL of P1 sediment extract, a solvent control (SC), or a positive TCDD control. Image of Western blot (top) show CYP1A levels compared to β-actin. Histogram (bottom) shows CYP1A levels quantified by densitometric analysis (3 replicates each with 5 individuals). Different letters indicate significant differences compared to SC ($p < 0.05$).

beyond the pit (Louchouarn et al., 2018). We also observed highest concentration of P1 sediment elicited the same mortality as 3.1 nM of TCDD, even though it was ≥ 4 magnitudes lower in total DLCs and TEQ. In general, chemical analysis derived TEQs correlate with- but are often less than those derived from biological analyses in their evaluation of DLCs in sediments (Eichbaum et al., 2016; Hong et al., 2014). Eichbaum et al. (2016) evaluated biological equivalent quotients (BEQs) with TEQs by comparing results exposures of sediment extracts of DLCs in juvenile common roach (*Rutilus rutilus*) with those from 7-ethoxyresorufin-O-deethylase (EROD) assays of mammalian (H4IIE)- and fish (RTL-W1) liver cell lines. Those authors reported that BEQs and TEQs reflected sediment-specific uptake patterns independent of initial sediment contamination levels. They also suggested that higher the BEQs they observed might be the result of synergism and/or additive effects of non-target compounds. We had also considered the complexity of the sediment

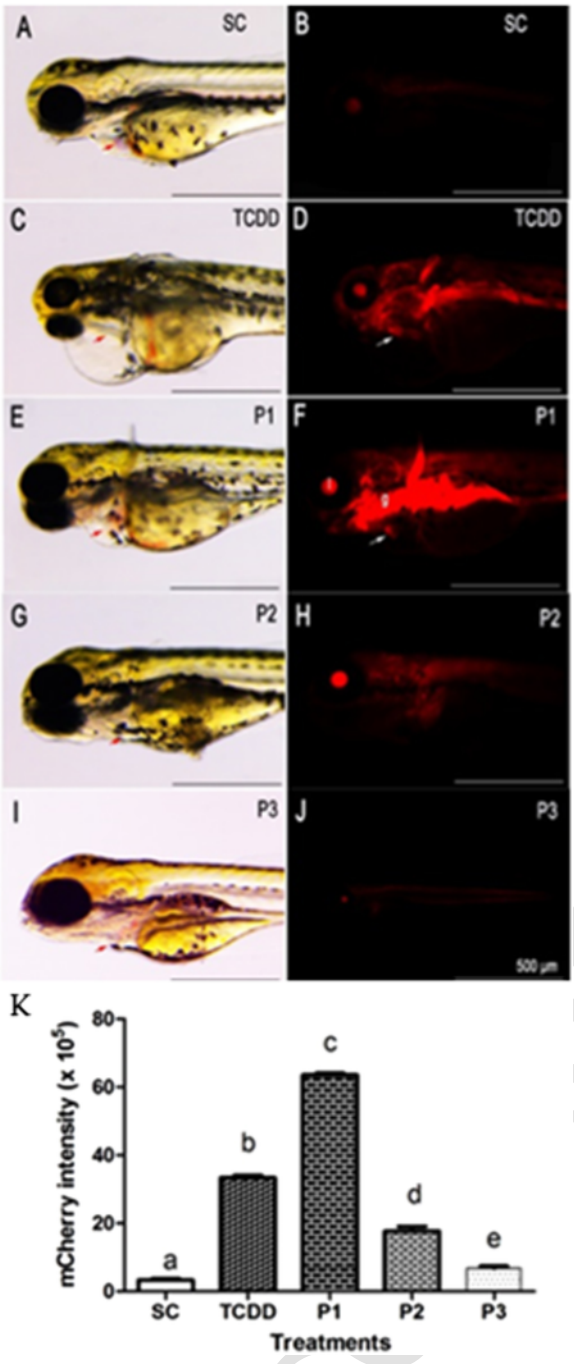

Fig. 6. Detection of CYP1A levels in transgenic zebrafish, *Tg(cyp1a:mCherry)*, larvae exposed to exposed to either 1 mg/mL P1, P2, or P3 sediment extract, a solvent control (SC), or a positive TCDD control for 6 h. Left panels are light microscopic images (A,C, E, G, I) and right panels are fluorescent microscopic images (B, D, F, H, J). Arrows pointed to the position of the heart in left panels. Expression of CYP1A in fluorescent images is indicated by red color. Histogram (K) shows results of quantification of fluorescence intensity. Different letters indicate significant differences compared to SC ($p < 0.05$). Scale bars are 500 μm.

samples used in the present study. It is likely the samples had other compounds such as PAHs or heavy metals. Synergism between PAHs and DLCs should not be excluded as they could explain some of the larger than expected effects of P1 extracts. A representative mixture of polychlorinated biphenyls (PCBs) with TCDD also resulted in exacerbated effects in mice (Shan et al., 2014). In addition, incomplete quantification of DLCs and matrix effects may explain differences we observed between derived TEQs of chemical and biological analyses (Hong et al., 2014).

Table 1
Levels (ng/g dry weight) of dioxin-like compounds in the sediments collected from sampling locations P1, P2, and P3 near Bohai Bay, China.

Compound Name		TEF	P1	P2	P3
T4CDD	2378	1	0	0.00	0.00
P5CDD	12378	1	0	0.00	0.00
H6CDD	123478	0.1	0.2	0.00	0.00
H6CDD	123678	0.1	0.55	0.00	0.00
H6CDD	123789	0.1	1.2	0.00	0.10
H7CDD	1234678	0.01	1.4	0.44	0.22
O8CDD	12346789	0.0003	4.9	0.50	1.51
T4CDF	2378	0.1	0	0.00	0.00
P5CDF	12378	0.03	1	0.34	0.00
P5CDF	23478	0.3	1.4	0.19	0.14
H6CDF	123478	0.1	1.3	0.26	0.00
H6CDF	123678	0.1	1.35	0.20	0.42
H6CDF	123789	0.1	0.45	0.18	0.00
H6CDF	234678	0.1	1	0.16	0.34
H7CDF	1234678	0.01	3.5	0.20	0.10
H7CDF	1234789	0.01	0.8	0.00	0.00
O8CDF	12346789	0.0003	4.3	0.50	0.21
Total levels (pg/g)			23.35	2.97	3.04
Total TEQ (pg TEQ/g)			1.1	0.15	0.13

Note: TEQ refers to toxic equivalent.

The developmental toxicity of TCDD in zebrafish has been studied extensively (Dong et al., 2002; Henry et al., 1997). It causes a variety of adverse effects in zebrafish including reduction of blood flow, pericardial edema, reduced heart rate, yolk sac edema, impaired swim bladder inflation, and eventual mortality (Dong et al., 2002). The heart is a main target for TCDD and DLCs (Antkiewicz et al., 2005), with pericardial edema being an early sign of the cardiotoxicity. Because of this, it is considered to be a sensitive biomarker to exposure to DLCs (Dong et al., 2010; Teraoka et al., 2014). DLCs, along with other ligands binding with the AhR2, can induce the expressions of *CYP1A* and cause subsequent cardiotoxicity including pericardial edema in zebrafish and other fish species (Clark et al., 2010; Dong et al., 2010; Lanham et al., 2014). When we exposed zebrafish sediment P1 extract at 1 mg/mL, we observed a 5 fold increase in pericardial cavity area, indicative of severe edema. Importantly, pericardial edema did not occur with knockdown of the AhR2 gene by antisense morpholino (Antkiewicz et al., 2006; Prasch et al., 2003). These results supported our contention that P1 sediments contained DLCs. It is important to note that pericardial edema is also an early sign of cardiac toxicity in fish larvae exposed to PAHs (Clark et al., 2013; Incardona et al., 2004) and some emerging contaminants (Zhang et al., 2017).

TCDD and DLCs are known to cause developmental toxicity in fish via the mediation of AhR2 (Zodrow and Tanguay, 2003). AhR2 and AhR2 nuclear translocator protein (ARNT1) isomers produce TCDD toxicity in zebrafish (Antkiewicz et al., 2006), and the AhR2/ARNT heterodimer regulates transcription of many downstream genes including P450 1 A (*CYP1A*) (Antkiewicz et al., 2006). Induction of *CYP1A* expression has been used as a biomarker of the activation of AhR2 pathway by DLCs (Zodrow et al., 2004). In our study, the level of *CYP1A* in larvae at 72 hpf from the 1 mg/mL P1 treatment group was comparable to that of the 3.1 nM TCDD treatment. These results indicated that *CYP1A* was responsive to TCDD exposure and confirmed the presence of DLCs in sediment extracts. WISH analysis localized *CYP1A* mRNA expression to the gill arches of larvae at 36 hpf, consistent with earlier studies (Andreasen et al., 2002a). Our RT-qPCR analysis showed concentration dependent increase in

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CYP1A mRNA in 72 hpf larvae exposed to P1 sediment extact, followed by a sharp decrease iby 96 hpf. The magnitude and temporal pattern of *CYP1A* mRNA in 1 mg/mL P1 sediment extract followed closely to that of TCDD, reflecting a similar mode of action. This temporal pattern was also reported when zebrafish were exposed to TCDD, but did not occur in *zfahr2* morpholinos (Prasch et al., 2003). These results suggest that there is a window of opportunity for DLC and adds additional evidence of their presence in our sediments Lastly, use of the transgenic zebrafish line *Tg*(*cyp1a*:*mCherry*)showed an interesting result. This line has been shown to be sensitive to TCDD and PAHs (≥ 4-rings) (Xie et al., 2018), and we observed a concentration-dependent response to P1 extract, with a higher intensity of *mCherry* in the 1 mg/mL treatment than the TCDD treatment.

Taken together, our results demonstrated that biological assays based on the AhR2 pathway were reliable and sensitive for the assessment of DLCs in sediments. CYP1A expressions at the transcriptional and protein levels were sensitive to exposure to TCDD and DLCs, but developmental stage at which exposure is initiated requires careful attention (Andreasen et al., 2002b). Developmental endpoints combined with molecular analyses can be used to assess both the presence of DLCs in sediments as well as their effects on early life stages of fish.

4.2. Chemical analysis of DLCs in sediments from P1 sediment

A chemical analysis of DLCs was conducted for P1 sediment to confirm presence of DLCs and validate the findings of our biological assays. Among the 14 DLCs detected in the P1 sediment, H7CDF, O8CDF and O8CDD were the most abundant species, with concentrations greater than 3 pg/g dry wt. The total TEQ value of approximately 1.1 was in the range of reported TEQ for coastal sediments from sites receiving municipal and industrial wastewaters in Spain (Eljarrat et al., 2005), sediments from the Masan Bay, Korea (Khim et al., 2001), and sediments from the UK estuaries (Hurst et al., 2004). This value was also close to those of the sediments collected from Bays surrounding the Bohai Sea (Hong et al., 2012).

The occurrence of these DLCs partially explained the toxicity we observed with P1 sediment extract. Differences in observed toxicity, considering TEQ, likely arise from other contaminants in effluents from this petroleum factory, whether they are antagonistic or synergistic modes of action. Additional study is necessary to parse out mixture effects as well as and including site specific phenomena.

5. Conclusion

In summary, the present study used the zebrafish laboratory model with established morphological endpoints for developmental toxicity (e.g., pericardial edema) along with analyses of CYP1A at the transcriptional and protein levels and use of a transgenic for the evaluation of DLCs in sediments. Results of these tests aligned with the positive control, TCDD, provide evidence of the presence of DLCs in sediments of Bohai Bay. Follow-up chemical analysis confirmed the DLCs in specific sediments. Our battery of biological assays proved to be rapid, sensitive, and cost-effective ways to evaluate chemical contaminants and their effects in a heavily polluted environment.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.01.116.

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