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Characterization of endocrine disruption potentials of coastal sediments of Taean, Korea employing H295R and MVLN assays–Reconnaissance at 5 years after *Hebei Spirit* oil spill

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ABSTRACT

Endocrine disrupting potentials were assessed for sediment samples collected near *Hebei Spirit* oil spill (HSOS) site, between December 2007 and January 2012. For comparison, major crude oil (CO) of HSOS, or its weathered form were assessed. Both raw extracts (REs) and their fractionated samples were tested using H295R and MVLN*luc* bioassays. In H295R cells, REs of crude and weathered oil (WO), and nine of 14 sediments significantly increased E2 levels, which were correlated with the concentrations of PAHs. Steroidogenic disruption potentials of the sediments generally decreased over time. Among silica fractions of all REs, aromatic hydrocarbons (F2) and polar compounds (F3) caused greater E2 levels. While, in MVLN cell bioassay, only three of 14 sediment REs showed estrogen receptor binding potencies, and no temporal trend was observed. In conclusion, oil spill can cause endocrine disruption in the affected ecosystem through steroidogenic alteration for years, and such potencies attenuate over time.

1. Introduction

The *Hebei Spirit* oil spill (HSOS) that occurred near the west coast of Korean peninsula on December 7, 2007, is the nation's biggest oil spill accident. The accident led to the spillage of approximately 10,900 tons of crude oil into the sea near the Taean coastline (Yim et al., 2012). Even though extensive cleanup activities were implemented immediately following the spill, continuous monitoring of the area's sediments has shown that the sediments were still affected by oil spill and the stranded oil residues have served as reservoirs for oil contamination. Sorbed oil residues can be slowly released to and would contaminate the neighboring environment over time. Crude oil consists of numerous hydrocarbon compounds including polycyclic aromatic hydrocarbons (PAHs), volatile organic molecules, and many other compounds. Many of these compounds are toxic (Page et al., 2002; Peterson et al., 2003), and therefore, oil spill can cause adverse effects on the affected ecosystem including benthic organisms overtime (Peterson et al., 2003; Vrabie et al., 2012).

Once spilled into environment, oil undergoes various physicochemical and biological degradation or transformation processes, including evaporation, dissolution, photolysis, and microbial degradation, that would significantly change the composition of oil (Kim et al., 2010). Generally, more volatile, soluble, and/or lighter compounds would easily dissipate, and relatively high molecular weight hydrocarbons, such as alkylated PAHs, may persistent and remain on site. This compositional change of oil by weathering affects toxicological characteristics of oil spill as well (Hong et al., 2012). For example, the aryl-hydrocarbon receptor (AhR)-binding potencies of oil residues in sediments tend to decrease through weathering as time passes by (Hong et al., 2012; Hong et al., 2016).

PAHs, including alkylated PAHs, are common constituents of oil, and are one of the major contributors to the toxicity of oil residues (Adams et al., 2014; Lee et al., 2011). PAHs may cause endocrine disrupting effects through several mechanisms of action. For example,

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11H-benzo[*b*]fluorene and retene exert antagonistic effects on androgen and progesterone receptors of zebrafish embryo, or inhibited activities of steroidogenic enzymes such as cyp17 and cyp19 in ovary tissue of flounder (*Platichthys flesus* L.) (Monteiro et al., 2000). In addition, benzo[*a*]pyrene was reported to alter plasma 17β-estradiol (E2) and testosterone (T) levels of rainbow trout leading to decreased estrogenicity (Kennedy and Smyth, 2015). Using a human adrenocortical carcinoma cell line (H295R), we reported that major PAHs and their alkylated analogues could disturb steroidogenic pathways and disrupt sex steroid hormone balance (Lee et al., 2017).

After HSOS, many studies have been conducted and reported toxic effects of the oil spill with an emphasis on PAHs. However, most of these studies are focused on AhR-mediated dioxin-like effects and to lesser extent on genotoxicity (Incardona et al., 2005; Lee et al., 2011; Hong et al., 2015, 2016; Jeong et al., 2015; Kennedy and Smyth, 2015). Only a few studies looked at endocrine disrupting potentials of oil residues, but accumulating literature shows possible endocrine disruption by oil spills. Early studies have reported that oils and oil spill-affected waters could impair reproduction of yellow perch and fathead minnows (He et al., 2012; van den Heuvel et al., 2012). S. Kim et al. reported that water-accommodated fractions of crude oil could disrupt thyroid function of larval zebrafish (S. Kim et al., 2016). Wang and others confirmed that naphthenic acid derived from crude petroleum could negatively impact development and endocrine function of zebrafish (Wang et al., 2015).

Previously, we reported endocrine disrupting potentials from the raw extracts (REs) of the sediments collected from shoreline affected by HSOS, even two years after the oil spill (Ji et al., 2011). However, the study did not provide any clue regarding responsible constituents of the sediments that might explain the observed hormonal disruption. In the present study, first we looked at endocrine disruption potentials of the sediments that were collected during the first 5 years after the oil spill, using H295R and MVLN cell bioassays. H295R cell is a human adrenocortical carcinoma cell that has frequently been used to evaluate effects of chemicals on steroidogenesis by measuring transcription of several key steroidogenic genes and associated changes in sex hormone levels (Hecker et al., 2006). MVLN cells are human breast cancer cell line that is stably transfected with a vitellogenin-A2 promoter/luciferase reporter construct (Harris et al., 2005). MVLN assay has been widely used for measuring receptor binding transactivation by xenoestrogens. In addition, major fractions of given samples that are responsible for the endocrine disruption potentials were identified by serial fractionation of the sediment samples. Such effect-directed analysis (EDA) is a powerful tool to narrow down major toxicants that occur in complex mixtures in environment samples, including sediments, soil and effluents (Regueiro et al., 2013; Vrabie et al., 2009). The results of this study will increase our understanding on the persistence of oil spill related damages, and on major contributors to the oil related ecotoxicological impact.

2. Materials and methods

2.1. Sediment collection and oil samples

A total of 14 sediments were collected along the coastline of Taean between December 2007 and January 2012. These samples include sediments collected from Sinduri dune (five samples collected at December 2007, December 2010, and January 2012, SDD1-SDD5), Sinduri mudflat (five samples collected at January 2008, December 2010, and January 2012, SDM1-SDM5), and Sogeunri mudflat (four samples collected at December 2010 and January 2012, SGM1-SGM4). Sampling locations are shown in Fig. 1. Sampling sites were carefully chosen based on visual sign of oil contamination (Ji et al., 2011; Kim et al., 2013). Samples were kept at 4 °C during transfer to the laboratory, and stored at − 20 °C until further processing. In addition, crude oil (CO) and weathered oil (WO) samples were employed to investigate whether the toxicity observed from the sediment samples are related to oil residues (Table S1).

2.2. Sample preparation and chemical analysis

To obtain raw extracts (REs) of the sediment samples, approximately 10 g (wet-weight) of the sediment samples were mixed with anhydrous sodium sulfate, and Soxhlet-extracted with 300 mL of high-purity dichloromethane (DCM). Extracts were initially concentrated by rotary evaporator and then free sulfur was removed by treatment with acid-activated copper. The extracts were subsequently concentrated to 5 mL by a gentle stream of nitrogen. The final raw extract (RE) of 0.4 mL (2 g sediments/mL) was obtained by replacing DCM solvent with dimethyl sulfoxide (DMSO). For REs of CO and WO samples, methods used in Ji et al. (2011) were followed. Briefly, 0.1 g of Iranian heavy crude oil (CO) and the artificially weathered oil (WO) were extracted with 300 mL of DCM. Concentrations of PAH and alkyl-PAH was measured from both CO and WO using an Agilent 7890 gas chromatograph (GC) coupled to a model 5975C mass-selective detector (MSD, Agilent Technologies, Avondale, PA, USA). The analytical data were reported elsewhere (Hong et al., 2012) and are also summarized in Table S1.

To identify major chemicals that are responsible for the toxicity in H295R and MVLN cells, REs of CO and WO, and all sediment REs were further fractionated by silica gel. Briefly, 1 mL of the sediment RE, or 0.1 g CO or WO, was passed through 10 g of activated silica gel in a packed glass column for fractionation (Hong et al., 2015). The first fraction (F1) containing saturated hydrocarbons was eluted with 40 mL of hexane. The second fraction (F2) that contains PAHs and alkylated PAHs was collected by elution with 50 mL of 20% DCM (v/v) in hexane. The third fraction (F3) containing resins and polar compounds was eluted in 50 mL of 60% DCM (v/v) in acetone. All eluents were concentrated using a rotary evaporator, dried with a gentle stream of nitrogen to 1 mL, and replaced with 1 mL DMSO for use in the cell bioassays. For F2 of CO and WO, further separation was made into six sub-fractions (F2.1–F2.6) using a normal-phase HPLC column (Hong et al., 2015), for more toxicity assessment.

2.3. H295R cell bioassay

The human adrenal cells (H295R) were used to determine the effects on hormone synthesis and transcription of related genes following Liu et al. (2012). H295R were obtained from the American Type Culture Collection (Manassas, VA, USA, ATCC CRL 2128) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F12) (Sigma D-2906; Sigma-Aldrich,) supplemented with 1% ITS + Premix (BD Biosciences, San Jose, CA, USA), 2.5% Nu-Serum (BD Biosciences), and 1.2 g/L Na_2CO_3 (Sigma–Aldrich). Briefly, H295R cells were seeded at a density of 3×10^5 cells/mL and allowed to attach and stabilize for 24 h. To minimize the influence of cytotoxicity, non-cytotoxic doses were determined by WST-1 bioassay (Roche Applied Science, Mannheim, Germany). Then the cells were exposed to a series of dilution of REs or their fractions in three replicates for 48 h to evaluate effects on hormone synthesis or gene expression. The dose ranges determined for REs of CO and WO, their silica fractions (F1, F2, F3), or HPLC sub-fractions (F2.1–F2.6) were 30, 6, and 1.2 μg/mL, and sediment REs and their silica fractions were 2000, 400, and 80 μg/mL. After exposure, culture medium and remaining cells were used for hormone measurement and quantification of gene transcription, respectively. Hormones were measured by competitive enzyme-linked immune-sorbent assay (ELISA) using commercial kits (Cayman chemical; testosterone [Cat # 582701], 17β-estradiol [Cat # 582251]). Only the samples that showed significant changes in sex hormone levels were further assessed for gene transcriptional changes. Transcription of *StAR*, *CYP11A*, *CYP17*, and *CYP19* genes was measured by RT-PCR (ABI 7300, Applied Biosystems, Waltham, MA, USA).

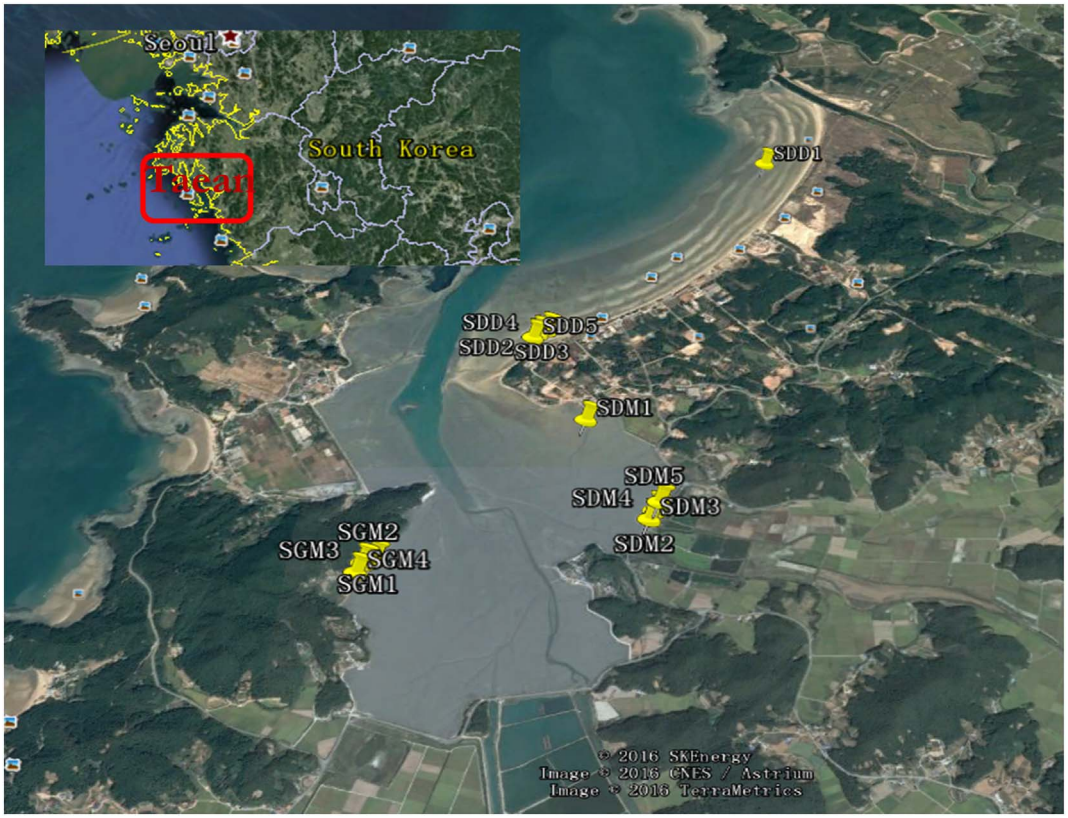

Fig. 1. Location of 14 sampling sites in Sinduri dune (SDD1-SDD5), Sinduri mudflat (SDM1–SDM5), and Sogeunri mudflat (SGM1–SGM4) along the coast of Taean, Korea (Satellite photo provided by Google Earth version 7.1.5. 2015. Image providers are shown in the bottom of map).

Detailed methods and primer sequences can be found elsewhere (Liu et al., 2012). After exposure, total RNA was isolated from the cells and the cDNA was synthesized with 100 ng/μL of RNA using a commercial kit using commercial kits (Agilent Technologies, Ontario, CA, USA BioRad, Hercules, CA, USA). Then, quantitative real-time PCR was performed following the manufacturer's instructions. A final reaction volume of 20 μL was made up with 10 μL TaqMan gene expression master mix, 5 μL nuclease-free distilled water, and 4 μL cDNA template. Thermal cycle was 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

2.4. MVLN cell bioassay

MVLN*luc* cells, i.e., MCF-7 human breast carcinoma cells stably transfected with luciferase reporter gene under the control of estrogen response elements (EREs), were cultured and tested following the protocols described previously (Liu et al., 2012). Briefly, cells were cultured with hormone-free DMEM/F12 nutrient mixture, 1 mM sodium pyruvate, and 1 mg/L insulin (Sigma-Aldrich) supplemented with 10% dextran-charcoal stripped fetal bovine serum (Hyclone, Logan, UT, USA). Cells for bioassay were plated into the 60 interior wells of the 96-well plates at a density of approximately 1.25×10^5 cells per well. After 24 h, the cells in the well plate were dosed with 2.5 μL of the appropriate standards (17β-estradiol, E2), sample extracts including silica fractions, and HPLC sub-fractions, and the solvent control (0.1% DMSO). All the tests were conducted in triplicates. After 72 h of exposure, luciferase activities were measured using Steady-Glo-Luciferase Assay System (Promega Corp., Madison, WI, USA), with a microplate reader (Tecan, Infinite 200, Mannedorf, Switzerland). The maximum response of E2 was set to 100% and the relative light unit (RLU) for each sample well was calculated as a percentage of the maximum induction of luciferase activity (% E2 max). MVLN cell bioassay-derived estrogen equivalents (E2-EQs) were determined from the dose-response curves of the test samples. To avoid the uncertainty caused by deviation of the concentration-response curve of a given sample from parallelism to the standard curve obtained by E2, E2-EQs were calculated for a range of responses from EC20 to EC80.

2.5. Statistical analyses

Normality and homogeneity of the data were determined using Shapiro-Wilk's and Levene's test, respectively. Dunnett's one-way analysis of variance (ANOVA) test or Kruskal-Wallis test were employed to assess differences among treatments and control, depending on the distribution of dataset. For testing dose-response relationship and correlations, linear regression analysis and Spearman correlation test were performed, respectively. The p values less than 0.05 were considered statistically significant. For statistical analysis, SPSS 16.0 for Windows® (SPSS, Chicago, IL, USA) was used.

3. Results

3.1. Effects on steroidogenesis in H295R cell bioassay

The H295R cells treated with REs of CO or WO showed significantly increased 17β-estradiol (E2) levels or increased E2/T ratio (Fig. 2). Among silica gel fractions of CO, increased estrogenicity, e.g., greater production of E2, or increased E2/T ratio, was observed from F2 (aromatics) and F3 (resins and polar compounds), whereas F1 (saturates) did not show any significant response. Similar trend was observed from WO, e.g., increased estrogenicity by F2 and F3 fractions. Interestingly, in WO, F1 also induced greater E2/T ratio. Fractions of CO induced 2.09–2.61 fold increase in E2 levels. Exposure to fractions of WO resulted in 1.82–3.20 fold increase in E2 levels (Fig. 2, Table S1). To examine the major compounds that are responsible for the increased E2 level, F2 fractions of both CO and WO were further separated to six

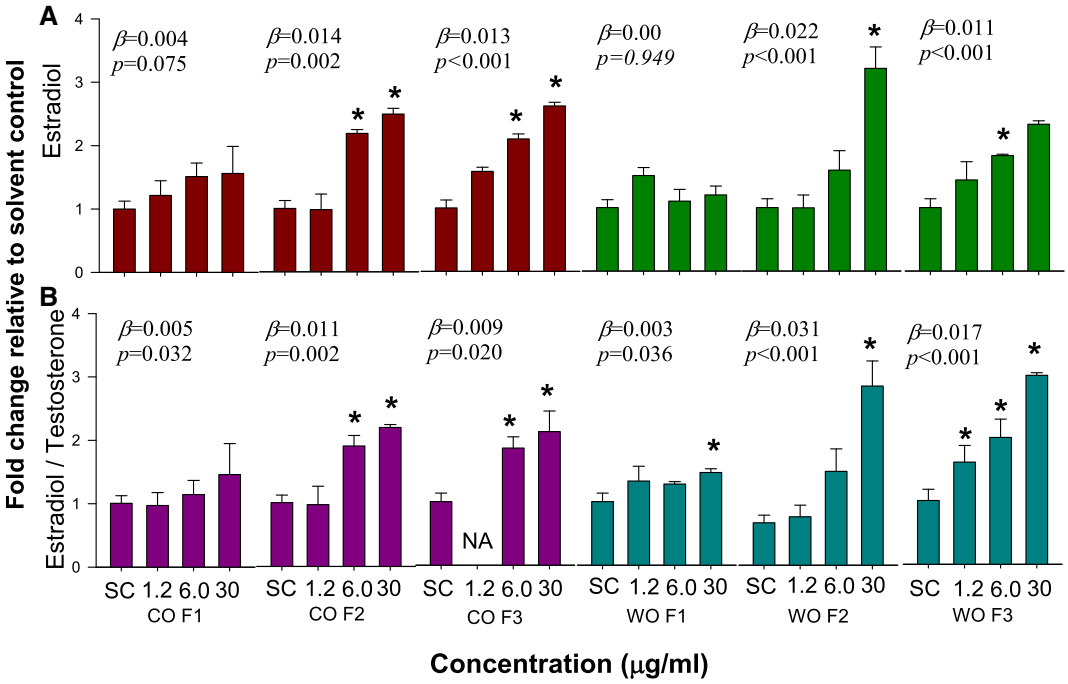

Fig. 2. Effects of oil silica fractions on 17β-estradiol (E2) and E2/testosterone (T) ratio in H295R cell bioassay. CO: crude oil, WO: weathered oil, NA: not available, error bar: Mean ± SD, asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO). The β and *p* values were determined based on linear regression analysis.

sub-fractions (F2.1 to F2.6) based on the number of aromatic rings. Among the sub-fractions, F2.2 of CO and F2.5 of WO showed greater levels of E2 in H295R cells (Fig. 3).

Significant increases of E2 levels in H295R cells were observed in nine of 14 sediment REs (Fig. 4). Those samples with high E2 levels include four Sinduri dune samples (SDD1, SDD2, SDD4 and SDD5), three Sinduri mudflat samples (SDM1, SDM2 and SDM5), and two Sogeunri mudflat samples (SGM2 and SGM3). Results of Spearman correlation analysis showed that increased E2 levels after exposure to extracts of the sediment samples were correlated with the concentrations of PAHs ($r = 0.684$, $p = 0.012$) but not those of alkyl-PAHs ($r = 0.182$, $p = 0.535$) of the sediment samples (Jeong et al., 2015, Table S2). Significantly higher E2/T ratio was also observed in the samples from Sinduri dune or mudflat collected in 2007, 2008, and 2010.

Potency of steroidogenic alteration in H295R cells by sediment REs tends to decrease over time. Extracts of SDD1 which was collected in December 2007 (SDD1) showed greatest production of E2 (~5 fold) and highest E2/T ratio (~9 fold). In contrast, those measured from the samples collected later in January 2008 or December 2010 showed lessor E2 production, and those of the most recent samples collected in January 2012 showed even lessor production of E2 (Fig. 4, Table S1). For most sediment REs, fractions of F2 and/or F3 were determined to be significant contributors to increased E2 production in the cell, with some exceptions (Fig. 5). For SDD2, F2 significantly decreased E2 productions, and for SDD5, SDM3, SDM4, and SGM4, F1 also significantly increased production of E2 in H295R cells.

SDD1, SDD5, SDM2, and SDM2 REs showed significant up-regulation of the *CYP19* gene in H295R cells. In addition, SDM5 RE up-regulated *STAR* in adrenal cells (Table 1). Significant up-regulation of *CYP19* gene was also observed following exposure to RE, F2, or F3 of both CO and WO, while regulation of *CYP11a*, *CYP7*, and *STAR* were not influenced.

3.2. MVLN cell bioassay

In the MVLNluc cell bioassay, WO exhibited greater estrogenic potency (19.5% E2 max) than did CO (8% E2 max) (Fig. 6). Estrogenic potency was observed mostly in F2 of CO, and F1 of WO. Among the six sub-fractions of both CO and WO F2, estrogenic potencies were observed in F2.2 of WO, and F2.3, and F2.4 of CO, which contained 3 to 5 ring aromatics and/or aromatics of similar molar mass.

Estrogenic responses mediated by ER transactivation were observed only for three REs among 14 sediments. SDD1, SDM5 and SGM2 elicited responses that were 31%, 85.9%, and 84.2% E2 max in the MVLN bioassay, respectively (Fig. 7). Estimated concentrations of bioassay-derived E2-EQs ranged between 0.5 and 711.8 pg/E2-EQ/g dry mass (Table S1). The measured E2-EQs were associated with neither total concentrations of 16 PAHs nor alkyl-PAHs based on Spearman correlation analysis ($r = 0.048–0.116$, $p = 0.693–0.871$) (Table S2). Among the three fractions, F2 and F3 generally showed greater estrogenic potency in MVLN cells (Fig. 8), with an exception of F1 of SGM3 which caused 21.7% E2 max.

4. Discussion

Results of H295R and MVLN assays indicated that endocrine disrupting potentials persist in sediments of the Taean coastline even 5 years after HSOS. While sediments were collected only from locations where visible signs of contamination remain, observations show that endocrine disruption potentials of the oil spill could persist and cause long-lasting ecotoxicological or public health concerns. Recently, it was reported that oxidative stress markers measured among the people who live closer to the HSOS site and participated longer as remediation workers were greater than the others, even 6 years after HSOS, implying that the public health consequences of the oil spill could last for several years (J.A. Kim et al., 2016). In coastal ecosystems, oil spill could cause long-lasting effects on predator-prey dynamics as well, which was reported for the Prestige oil spill (Moreno et al., 2013; J.A. Kim et al., 2016).

Observations of endocrine disruption by oil spill-affected sediment have suggested that adverse effects on steroidogenesis rather than ER-binding potencies are important (Figures 4, 5, 7, 8). This observation is consistent with previous reports that showed increased E2 synthesis by H295R cells (Ji et al., 2011), but negligible induction of ER dependent

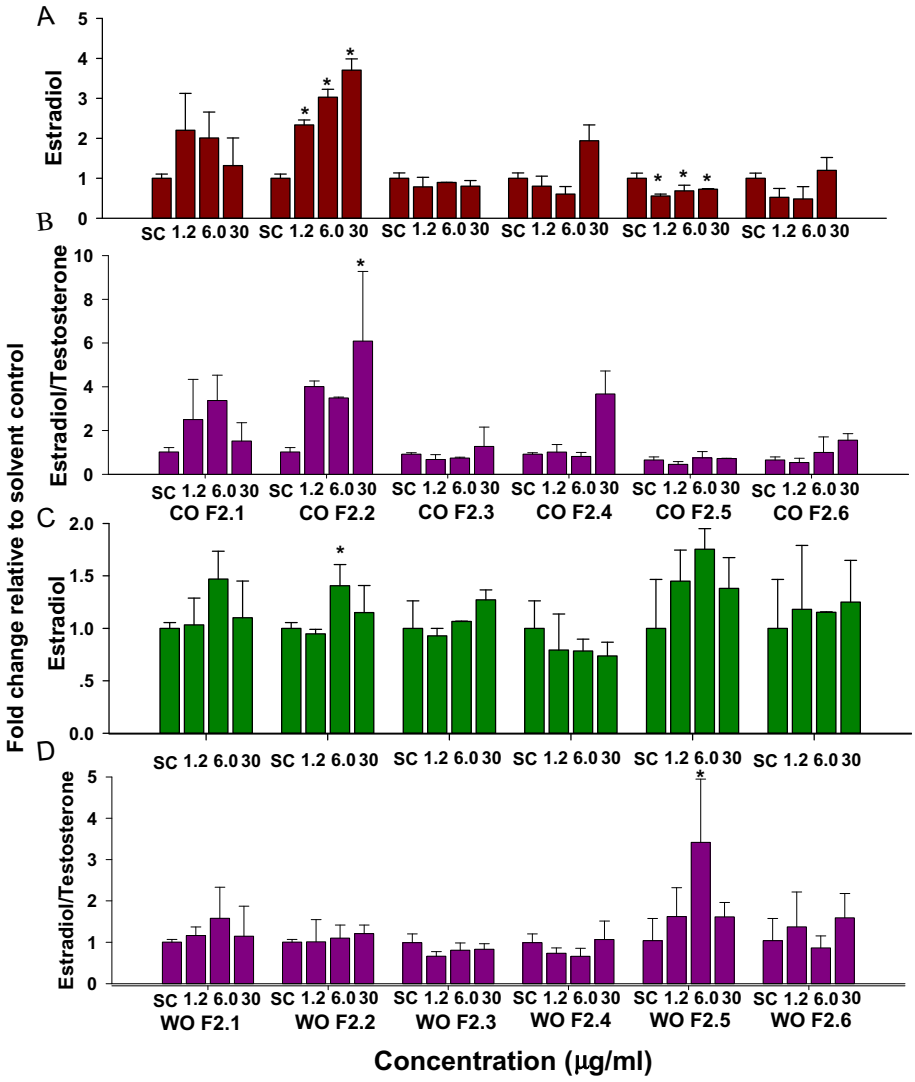

Fig. 3. Effects of HPLC sub-fractions of the F2 of CO on (A) E2, (B) E2/T ratio, or effects of sub-fractions of the F2 of WO on (C) E2 and (D) E2/T ratio in H295R cell bioassay. CO: crude oil, WO: weathered oil, error bar: Mean ± SD, asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO).

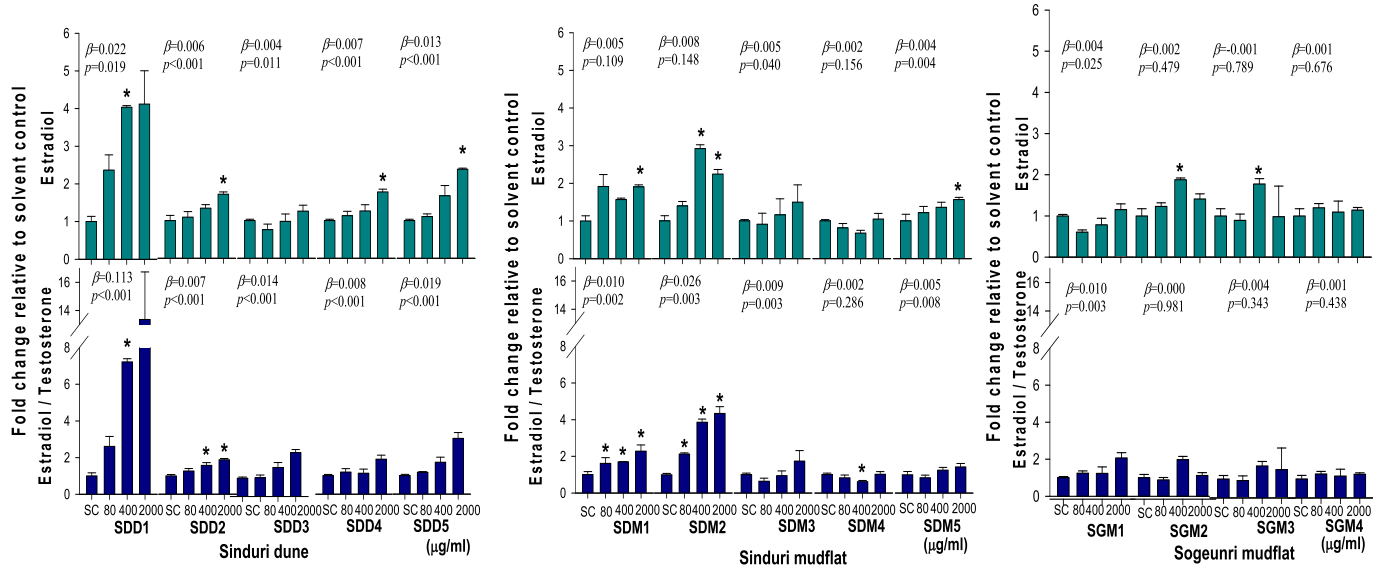

Fig. 4. Effects of sediment raw extracts on E2 and E2/T ratio in H295R cell bioassay. Error bar: Mean ± SD, asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO). The β and *p* values were determined based on linear regression analysis.

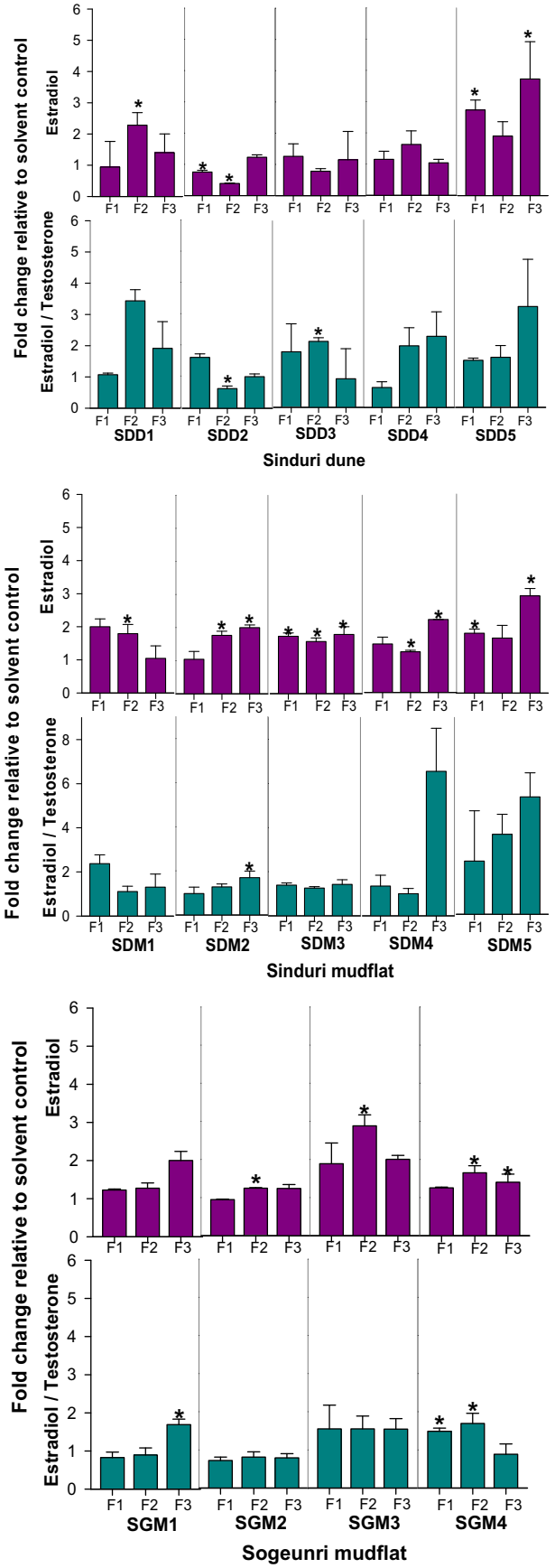

Fig. 5. Effects of 20% sediment silica fractions on E2 and E2/T ratio in H295R cell bioassay. Error bar: Mean ± SD. Asterisk indicates significant difference from solvent control (SC, treated with 0.1% DMSO).

Table 1
Expression of *StAR*, *CYP11a*, *CYP17*, and *CYP19* genes in H295R cells following exposure to raw extracts (REs) of crude oil (CO), weathered oil (WO), or sediment samples collected from Taean area, Korea.

Sample	*StAR*	*CYP11A*	*CYP17*	*CYP19*
CO	0.93 ± 0.10	1.13 ± 0.35	1.13 ± 0.19	**2.20 ± 0.16***
WO	1.45 ± 0.26	1.5 ± 0.18	1.26 ± 0.16	**3.41 ± 0.22***
CO-F2	2.36 ± 0.47	**2.81 ± 0.08***	2.10 ± 0.21	**2.38 ± 0.24***
CO-F3	0.98 ± 0.08	1.15 ± 0.19	1.15 ± 0.19	**2.70 ± 0.12***
WO-F2	0.98 ± 0.07	1.38 ± 0.44	1.38 ± 0.44	**2.62 ± 0.40***
WO-F3	0.93 ± 0.09	1.26 ± 0.22	1.26 ± 0.22	**2.63 ± 0.18***
SDD1	1.07 ± 0.09	1.17 ± 0.23	1.17 ± 0.22	**4.90 ± 0.57***
SDD2	0.87 ± 0.18	0.94 ± 0.26	0.92 ± 0.11	1.90 ± 0.54
SDD4	1.59 ± 0.47	0.85 ± 0.03	0.65 ± 0.02	2.26 ± 0.62
SDD5	0.91 ± 0.08	0.95 ± 0.11	0.66 ± 0.04	**2.78 ± 0.20***
SDM1	1.14 ± 0.67	1.11 ± 0.14	1.11 ± 0.13	**1.84 ± 0.01***
SDM2	1.20 ± 0.35	1.18 ± 0.34	1.18 ± 0.34	**3.87 ± 0.12***
SDM5	**2.76 ± 0.29***	0.91 ± 0.05	0.62 ± 0.11	1.52 ± 0.06
SGM2	1.39 ± 0.07	0.96 ± 0.20	0.67 ± 0.88	1.12 ± 0.10
SGM3	1.57 ± 0.16	1.28 ± 0.06	0.92 ± 0.03	1.42 ± 0.03

* Statistically significant compared to that of control ($p < 0.05$)' in the footnote of Table 1.

responses by exposure to extracts of costal sediments of HSOS (C. Kim et al., 2016).

Increased synthesis of E2 or the E2/T ratio could be explained by significant up-regulation of *CYP19* by extracts of either sediment or oil. Aromatase or CYP19 enzyme facilitates conversion of androstenedione into estrone and T to E2 (OECD, 2011). Up-regulation of *CYP19* in H295R cells by exposure to extracts of HSOS sediment has been reported (Ji et al., 2011). No significant changes in transcription of *StAR*, *CYP11A*, or *CYP17* were observed in the present study (Table 1), which suggests that these genes may not play roles in alteration of steroidogeneis by the oil spill residues. Significant regulatory changes of *CYP19*, *CYP11B2*, and *3βHSD2* by extracts of sediments collected near HSOS or oil, suggested that these three genes might be more responsive than *StAR*, *CYP11A*, or *CYP17* to oil residue (Ji et al., 2011). In the present study, among the fractions, not only F2 but also F3 up-regulated transcription of *CYP19*. Major constituents of F3 that might explain up-regulation of *CYP19* are not clear, and warrant further investigation.

Increased synthesis of E2 by H295R cells was correlated with concentrations of PAHs but not alkylated PAHs. The greatest production of E2 was observed after exposure to extracts of SDD1, which also contained the greatest concentration of total PAHs among sediments studied (Table S1). Concentrations of E2 observed after exposure to extracts of samples of Sogeunri mudflat were consistent with PAHs concentrations. These observations support the hypothesis that PAHs play an important role in synthesis of E2 in the body. More potent endocrine disrupting effects of F2 comparing to the other fractions of both crude and weathered oils also support greater effects of aromatic compounds on steroidogenesis. It was reported that major PAHs in crude oil, including naphthalene, fluorine, dibenzothiophene, phenanthrene and thrysene, and their alkylated analogues, could stimulate synthesis of steroid hormones (Lee et al., 2017).

Fractions of extracts caused greater endocrine disrupting potency compared to their mixture (Table S1), as shown in samples such as SDD5 or SDM5 for H295R cell bioassay, and SDD5, SDM3, or SGM1 in the MVLN assay. One explanation for these observations is competition or interaction between the constituents of different fractions (Hong et al., 2016; Vrabie et al., 2009). One cannot rule out a possibility that F1 might antagonize and/or mask potency of F2 or F3 (Koh et al., 2006; C. Kim et al., 2016), that F2 and F3 antagonize one another (Vrabie et al., 2012). Similar observations were reported previously (C. Kim et al., 2016), suggesting that EDA using fractionated samples was often not representative of toxic potencies of whole samples. Future experiments aimed at elucidating and understanding interactions between constituents of sediment extracts could help interpretation and

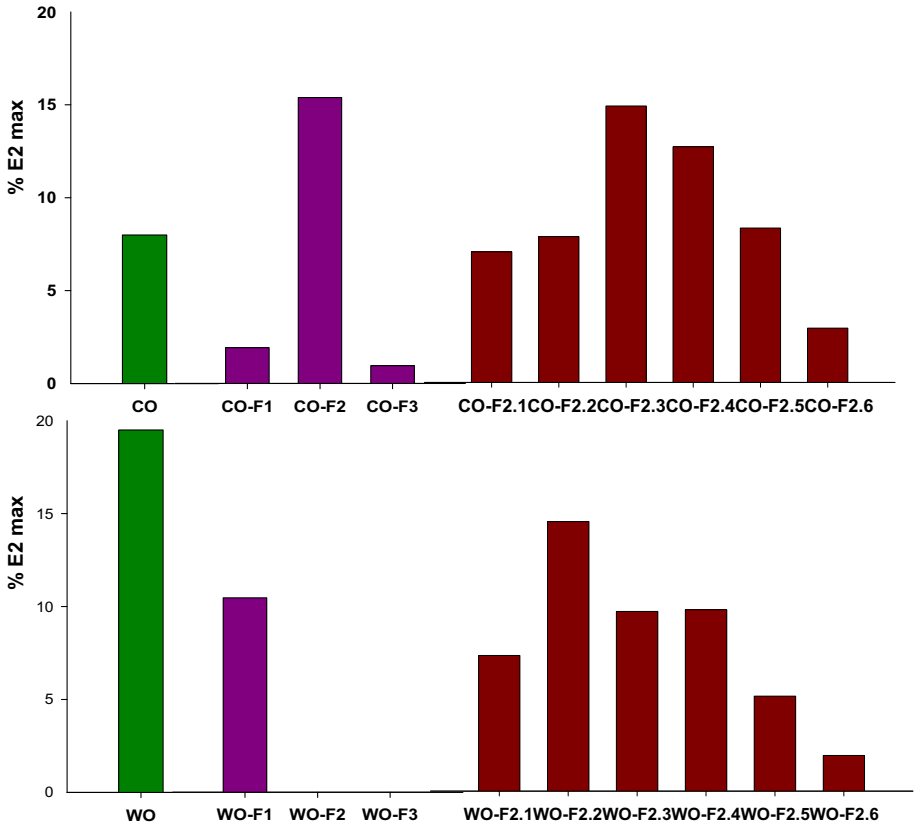

Fig. 6. ER-binding affinity of crude oil (CO) and weathered oil (WO), silica fractions (F1, F2, F3) and F2 HPLC sub-fractions in MVLN cell bioassay. Response magnitude presented as percentage of the maximum response observed for E2 (% E2 max).

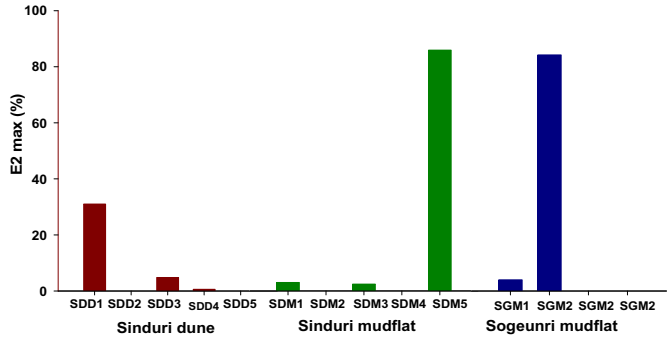

Fig. 7. ER-binding affinity of sediment raw extracts in MVLN cell bioassay. Response magnitude presented as percentage of the maximum response observed for E2 (% E2 max).

understanding endocrine disruption by oil mixtures.

Sediment quality in terms of endocrine disruption appears to recover over time, as indicated by observations of less or steroidogenic alteration in more recently collected sediments. Several reports have suggested that sediment quality in the Taean area has improved over time (Yu et al., 2013; Kim et al., 2010; Ji et al., 2011; Hong et al., 2012). Weathering of spilled oil over time might explain the decrease of endocrine disrupting potencies of oil spill-affected sediment. By calculating ratios of concentrations of alkylated PAHs, Hong et al. (2015, 2016) reported that SDM1 and SGM4 were more weathered and contained greater proportions of C3 and C4 alkyl-PAHs, and that SDD1, SDM2, and SGM1 which contained less concentrations of alkyl-PAHs were less weathered (Hong et al., 2015). Greatest production of E2 by H295R cells was observed after exposure to the extract of SDD which is slightly weathered sediment, followed by extracts of moderately weathered sediments such as SDD2–SDD5. Among samples from Sinduri mudflat, greatest production of E2 was caused by exposure to extracts of the slightly weathered sediment SDM2, followed by moderately or more weathered sediments. These observations indicate that the weathering process could change chemical composition of sample, and hence influence steroidogenic alteration potentials caused by extracts of oil contaminated sediments. Components of oil spill residue that are responsible for alteration of steroidogenesis appear to be changed by weathering. For example, while in crude oil, F2.2 containing two to three aromatic rings significantly increased production of E2, by weathered oil, F2.5 containing four to five aromatic rings was most potent of altering steroidogeneis.

Similar to oil fractions, with some exceptions F2 and/or F3 of sediments showed greater alteration of steroidogenesis. In contrast, F1 of SDM3, SDM5 and SDD5 significantly increased E2, but F1 of SDD2 significantly decreased production of E2, which suggests that other types of contaminants might be present in coastal sediments possibly sources other than the oil spill and should be considered in interpreting oil spill related consequences in the ecosystem.

5. Conclusions

Sediments collected near the HSOS at the Taean, west coast of Korean peninsula, showed endocrine disruption potentials on sex steroidogenesis but not due to transactivation of the estrogen receptor. However, endocrine disruption by affected sediment on sex steroidogenesis have decreased over time. Such decrease in steroidogenic alteration coincided with the decrease of concentrations of PAHs. Further fractionation of extracts of endocrine active sediments using silica gel revealed that generally constituents present in F2 and F3 fractions were responsible for steroidogenic alteration of adrenal cells. However, the sum of potencies of fractions was not equal to that caused by a given RE, which suggests either loss of potencies by fractionation or interaction among the constituents of different fractions within an RE. Result of this study showed that contamination by PAHs from the spilled oil could cause endocrine disruption through altering steroidogenic pathway, and such potencies of the sediment extracts attenuate

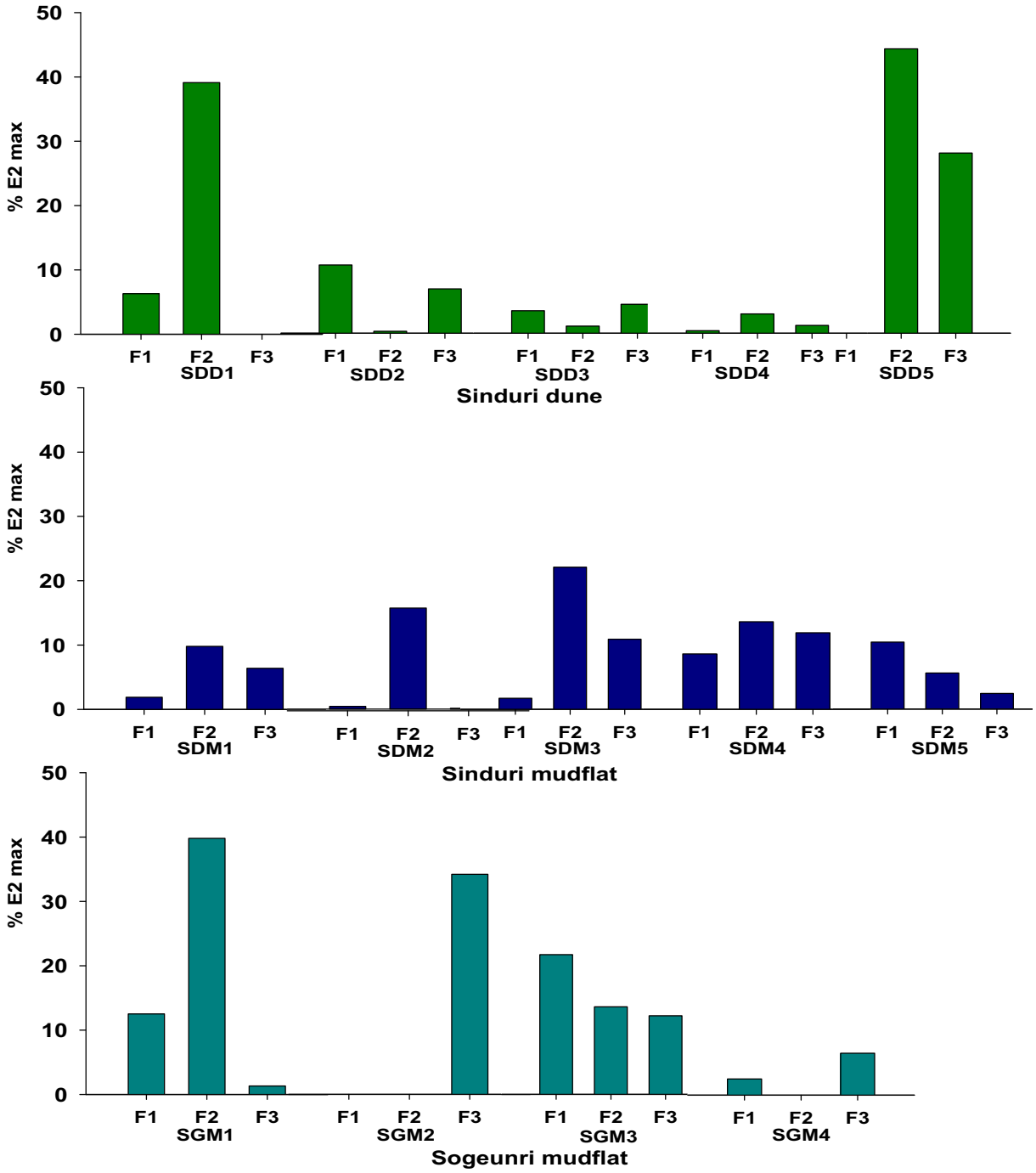

Fig. 8. ER-binding affinity of sediment silica fractions in MVLN cell bioassay. Response magnitude presented as percentage of the maximum response observed for E2 (% E2 max).

over time.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2017.11.055.

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Characterization of endocrine disruption potentials of coastal sediments of Taean, Korea employing H295R and MVLN assays–Reconnaissance at 5 years after *Hebei Spirit* Oil Spill

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Contents of Supporting Information

Table S1. Concentrations of 16 priority PAHs and major alkyl PAHs, and their relative fold changes in H295R bioassay. S2

Table S2. Relationships between E2 induction potency or estrogenic activity of RE or F2, and PAHs or alkyl PAH concentrations (n=16) based on Spearman correlation analyses and bivariate regression analyses …. . . . . ….S3

Table S1. Concentrations of 16 priority PAHs and major alkyl PAHs, and their relative fold changes in H295R bioassay.

Sample Site	Sampling Year	Sample ID	Chemical assay (ug/g dry weight)		H295R bioassay			MVLN bioassay		
					E2 Relative fold change			E2-EQ (pg/g dry weight)		
			∑16 PAHs	∑Alkyl PAHs	RE	F2	F3	RE	F2	F3
Oil		Crude oil	$3.4X10^3$	$3.7X10^3$	2.34	2.49	2.61	2066	3151.2	1382.3
		Weathered			3.29	3.60	2.31	136135	-	-
Sinduri dune	2007	SDD1	3.35	66.44	4.04	2.28	1.40	0.54	32.15	1.64
	2010	SDD2	0.08	9.36	1.70	0.39	1.23	0.59	3.82	3.00
	2012(1)	SDD3	0.28	40.53	1.25	0.78	1.15	1.7E-06	0.03	2.11
	2012(2)	SDD4	0.26	33.88	1.76	1.79	1.04	7.9.E-05	0.06	0.17
	2012(3)	SDD5	1.01	124.76	2.36	2.74	3.73	3.7.E-07	0.97	0.54
Sinduri mudflat	2008	SDM1	0.09	4.60	1.91	1.79	1.21	0.34	10.21	13.02
	2010	SDM2	1.30	165.75	2.92	1.76	2.00	0.01	2.33	2.06
	2012(1)	SDM3	2.49	268.55	1.50	1.57	1.79	1.26	23.49	10.05
	2012(2)	SDM4	1.00	84.76	1.05	1.23	2.21	0.10	0.09	0.10
	2012(3)	SDM5	0.58	63.26	1.57	1.66	2.94	711.78	23.53	107.52
Sogeunri mudflat	2010	SGM1	0.52	40.95	1.15	1.27	2.00	1.7.E-04	77.35	65.28
	2012(1)	SGM2	1.66	138.10	1.88	1.24	1.24	568.50	0.01	0.09
	2012(2)	SGM3	0.43	16.63	1.77	2.88	2.00	0.02	0.01	0.00
	2012(3)	SGM4	0.26	1.3	1.20	1.65	1.40	0.38	0.01	0.03

RE= raw extract, E2-EQ =E2 equivalency in MVLN cell bioassay. --: not available； Measured PAHs concentrations were based onJeong et al. (Jeong et al., 2015).

Table S2. Relationships between E2 induction potency or estrogenic activity of RE or F2, and PAHs or alkyl PAH concentrations (n=16) based on Spearman correlation analyses and bivariate regression analyses

Independent variables	E2 induction r (*p* value)		Estrogenic activity	
	RE	F2	RE	F2
∑16 PAHs[a]	0.648（0.012*）	0.267（0.356）	0.048（0.871）	0.450（0.104）
∑Alkyl-PAHs[b]	0.182（0.535）	0.087（0.767）	0.116（0.693）	0.294（0.391）

a: ∑16 PAHs; b:∑Alkyl-PAHs; *:statistically significant;

Reference

Jeong H.J., Lee H.J., Hong S., Khim J.S., Shim W.J., Kim G.B., DNA damage caused by organic extracts of contaminated sediment, crude, and weathered oil and their fractions recovered up to 5 years after the 2007 Hebei Spirit oil spill off Korea. *Mar. Pollut. Bull*. **95**, 2015, 452–457.