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Baseline

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

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ABSTRACT

We examined the degree of DNA damage caused by three fractions (F1, aliphatic hydrocarbons; F2, aromatic hydrocarbons; and F3, polar compounds) of the organic extract of sediments taken from Taean, Korea, following the *Hebei Spirit* oil spill. DNA damage was measured using the comet assay with blood cells of the striped beakfish (*Oplegnathus fasciatus*). DNA damage was also examined for fractions of crude oil (Iranian Heavy Crude Oil, IHC), weathered oil and six subfractions (F2.1–F2.6). The greatest DNA damage was found from the Sinduri dune region and DNA damage decreased to 40% weathered oil in F2 fraction compared with crude oil. The DNA damage of the sum of fractions was found higher than the organic extracts of sediments, suggesting antagonistic interactions between the genotoxic compounds. This study confirmed the persistence of potential genotoxicity in sediments of the severely affected regions as long as 5 years after the oil spill.

The *Hebei Spirit* oil spill at Taean, Chungnam Province, Korea on December 7, 2007 was reportedly the nation's biggest marine accident, with an oil flow volume more than twice that from the 1995 *Sea Prince* accident off Korea (Taean Coast Guard, 2008), and larger than the *Prestige* incident off the Spanish coast in 2002, the *Tasman Spirit* in the Arabian Sea in 2003, and the *Solar No. 1* in 2006 off the Philippines. The oil spilled by the *Hebei Spirit* accident caused a serious level of pollution not only to the Taean area, but also to neighboring coastal areas, especially mud flats and beaches (Seo et al., 2011). Crude oil comprises mostly hydrocarbon compounds, particularly diverse volatile organic molecules such as benzene, toluene, ethylbenzene, xylene (collectively known as BTEX), and polycyclic aromatic hydrocarbons (PAHs) (Lemiere et al., 2005). PAHs diffuse into the sea after oil spills, and are easily absorbed and then deposited into sedimentary layers (Taylor and Jones, 2001). This is because of their low solubility and volatility and high hydrophobicity, resulting in bioaccumulation through food chains. In addition, in the case of the 1989 *Exxon Valdez* oil spill off Alaska, and the 2002 *Prestige* oil spill off Spain, oil pollution in sediments lasted for a long time because of the persistence of PAHs (Bence et al., 1996; Irvine et al., 2006; Morales-Caselles et al., 2008). Accordingly, such oil spills have had long-term and serious impacts on marine ecosystems, not only on the coast affected, but also in the open sea (Jackson et al., 1989; Houghton et al., 1991; Varela et al., 2006).

PAHs show genotoxicity when tested in bivalves (White, 2002), neurotoxicity in mammals (Saunders et al., 2002), and immunotoxicity in experiments using human DNA chips (Iwano et al., 2010). In addition, after the long-term exposure of marine organisms to oil, PAHs caused population declines arising from impaired viability and reproduction (Peterson et al., 2003). PAH accumulation interrupts the transcription of mRNA by transforming the DNA structure of organisms, and lowers enzyme activities and metabolic functions (Kurelec, 1993). Accordingly, DNA damage has been widely used as a biomarker to assess genotoxicity caused by environmental pollution. Various bioassays have been developed to investigate genotoxicity in organisms. Among them, the DNA single-cell gel electrophoresis or 'comet' assay has been used frequently, because it is a fast and easy technique to assess single-stranded DNA breakage with excellent sensitivity (Seo et al., 2006; Woo et al., 2006).

In marine environments, the spilled oil is subjected to various physicochemical, biological weathering and degradation processes,

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including evaporation, dissolution, photolysis, and microbiological degradation (Yim et al., 2007; Kim et al., 2010). Depending on the level of weathering and degradation, the composition of crude oil compounds and its toxicity change with time (NRC, 2003). Accordingly, for oil-spill accident areas, it is necessary to carry out environmental monitoring research on toxicity over time. After the *Hebei Spirit* oil spill, there has been active research on PAHs in various materials including seawater and pore-water (Kim et al., 2010), and the atmosphere (Lee et al., 2008). The assessment of DNA damage using organic extracts from oil-polluted sediments has been performed (Lee et al., 2011, Seo et al., 2011), whereas research on the toxicity of organic extract from the weathered sediments, crude oil, and weathered oil has been very limited (Hong et al., 2012). In particular, there has been little research on DNA damage in this context.

Here, we examined how changes in the components of crude oil consequent to weathering affected oil toxicity and investigated which fractions of crude and weathered oil induced DNA damage. We used the DNA comet assay with blood cells of the striped beakperch (*Oplegnathus fasciatus*). Further, we aimed to determine the spatiotemporal distribution of pollution caused by PAHs by ascertaining whether there remained any potential genotoxicity of sediments at the Sinduri dune, and the Sinduri and Sogeunri mud flat since the years 2007, 2008, 2010, and 2012, documented as heavily oil-contaminated sites in previous studies (Ji et al., 2011; Jung et al., 2012; Kim et al., 2013; Yim et al., 2007).

Fractionation of crude and weathered oil was carried out using the method of Hecker and Hollert (2009) to determine which fraction showed genotoxicity. One milliliter of the organic extract of sediment or 0.1 g of crude oil (Iranian Heavy Crude, IHC) or weathered oil (28.8% weathered IHC) were passed through 10 g of activated silica gel (70–230 mesh, Merck, Darmstadt, Germany) in a packed glass column for fractionation (Khim et al., 1999). The first fraction (F1) containing saturates was collected by elution with 40 mL hexane (Burdick and Jackson). The aromatic fraction (F2) was collected by elution with 50 mL of 20% (v/v) dimethyl chloride (DCM) in hexane. The third fraction (F3) contained resins and polar compounds collected by elution with 50 mL of 60% (v/v) DCM in acetone (Burdick and Jackson). Conditions of solvent elution for separation were optimized and confirmed using gas chromatography and mass selective detection (GC/MSD). All eluents were concentrated with a rotary evaporator, dried with a gentle stream of nitrogen to 1 mL, replaced by 1 mL dimethyl sulfoxide (DMSO) for use in the DNA comet assay, and further fractionated using a rotary evaporator and nitrogen concentrator. In addition, to evaluate the relative toxicity contribution of PAHs, the F2 fraction was further fractionated into six subfractions (F2.1–F2.6) based on the number of aromatic rings by use of a normal-phase high-performance liquid chromatography (HPLC) column (Nucleosil 100-5 NO_2, 250 mm long, 5 μm particle size, 4.6 mm internal diameter; Macherey–Nagel, Düren, Germany) (Brack and Schirmer, 2003). Separation and fraction collection were performed in an Agilent 1260 HPLC System (Agilent Technologies, Palo Alto, CA, USA) with ultraviolet (UV) detection at 254 nm wavelength. The hexane: DCM ratio (95:5, v/v) as the mobile phase was delivered isocratically at a flow rate of 0.7 mL min^{-1}

Sediments were collected from heavily contaminated areas of the Taean coast over a 5-year period after the accident in December 2007: within ~1 month (December 2007 and January 2008); after ~3 years (December 2010); and after ~4 years (January 2012). A total of 14 surface sediment samples were collected from the Sinduri dune, the Sinduri coastal mud flat, and the Sogeunri mud flat (Fig. 1). Ten grams of wet sediment were mixed with anhydrous sodium sulfate (Sigma–Aldrich, St. Louis, MO, USA), and extracted with 300 mL DCM (Burdick and Jackson, Muskegon, MI, USA) in a Soxhlet extractor for 24 h. Elemental sulfur was removed using activated copper (Merck, Darmstadt, Germany) and the extracts were concentrated to 5 mL under a gentle stream of nitrogen. The extracts were replaced with DMSO for the comet assays, and we used the rest of the extracts for additional fractionation and chemical analyses. The isotopically-labeled surrogate standards were not added in the extraction and fractionation procedures, because the chemicals would contribute to the DNA damage. For QA/QC purpose, the matrix spike test was performed for five sediment samples in a separate experiment using 16 EPA PAHs, and recoveries were generally acceptable, ranging from 57 (for naphthalene) to 104% (for fluoranthene) (on average = 85%).

The weathered oil was prepared for these studies using an evaporation simulation technique (Fieldhouse et al., 2010) by Environment Canada.

Striped beakperch fish (*O. fasciatus*) were provided by the Gyeongnam-do Fisheries Resource Research Institute, and then maintained at a constant temperature of 20 °C in filtered seawater at the culture facility of the Marine Organism Education Center of the College of Marine Science at Gyeongsang National University. Whole blood samples (10–100 μL) were collected by tail vein puncture into heparinized syringes and transferred to Hank's balanced salt solution (HBSS). After placing 50 μL of the suspended blood sample, 940 μL of HBSS, and 10 μL of sediment/crude oil/weathered oil extracts into 24-well plates (Falcon Plastics, Los Angeles, CA, USA), and then adjusting the final volume to 1 mL, plates were incubated in the dark at room temperature (25 °C) for 1 h.

The procedure for the comet DNA fragmentation assay followed that of Singh et al. (1988), with a few modifications. Prior to starting any experiment, agarose-coated glass microscope slides were made by inserting them into Coplin jars containing 1% normal melting point agarose diluted in TAE buffer solution (0.04 M Tris-acetate and 1 mM ethylenediaminetetraacetic acid, EDTA), wiping the rear side with a tissue and then air drying them. The blood cells were placed in a microcentrifuge separation tube and spun at 5,000 rpm for 5 min. The supernatant was discarded and the precipitate was resuspended using 50 μL of 0.65% low-melting agarose (LMA) diluted in Kenny's salt solution (0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$), placed onto the prepared slide and covered with a cover slip. After gel solidification for 3 min, 25 μL LMA was again added to the slide. Following the second solidification, the cover slip was removed. The slides were soaked in a Coplin jar containing lysis buffer solution(2.5 N NaCl, 0.1 M EDTA, 0.01 M Tris–HCl, 10% DMSO, 1% Triton X-100) and kept at 4 °C for more than 2 h. The slides were then placed in a Coplin jar containing DNA denaturing buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C for 15 min. Then, slides were transferred into electrophoresis chambers filled with DNA denaturing buffer and electrophoresis was carried out for 25 min at 25 V and 300 mA. Slides were washed three times in a jar containing 0.4 M Tris–HCl solution (pH 7.5) for 2 min, followed by transfer to ethanol for 5 min, and then dried at room temperature. Preparations were stained with 20 μL ethidium bromide solution (20 μg/mL). DNA strand breaks in cells reflected as extracellular 'comet tails' were determined using a Nikon Eclipse E200 inverted fluorescent microscope (×20 magnification). Cell images were captured using a high-sensitivity charge-coupled device camera connected to a computer. A computerized image analysis system (Komet version 5, Kinetic Imaging Ltd, Liverpool, UK) was used to determine DNA damage as the 'DNA tail moment' (amount of DNA in each tail × the tail length). For each experimental condition, 50 cells from each slide were examined randomly. All treatments were repeated three times. Finally, DNA fold induction (the DNA tail

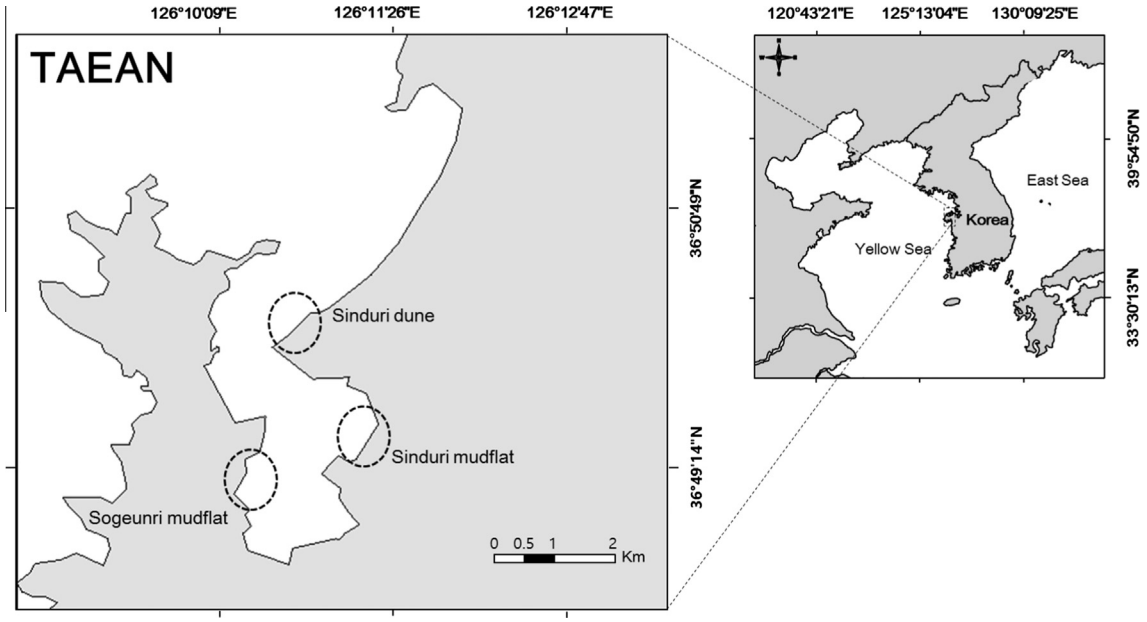

Fig. 1. Location of sampling sites.

moment in a treatment divided by the DNA tail moment in a control) was used for comparing DNA damage levels between treatments.

Blood cells of the striped beakperch were exposed to the organic extracts of the sediments, and DNA damage levels were examined using the comet assay (Fig. 2). Table 1 shows the concentrations of PAHs in the sediment extracts. The total concentrations of 16 PAHs in the sediment extracts ranged from 0.08to 3.35 μg/g dry weight (dw). The highest level of PAHs was observed in the Sinduri mud flat with a mean concentration of 1.09 μg/g dw, followed by the Sinduri dune (1.00 μg/g dw), and the Sogeunri mud flat (0.72 μg/g dw). In contrast, the DNA damage level was found to be highest in cells exposed to the Sinduri dune extract (6.85-fold induction), followed by the Sinduri mud flat (4.51), and the Sogeunri mud flat (3.56). There was no temporal trend in sediment PAH concentrations or DNA damage for any site (Table 1). However, the comet assay showed much higher DNA damage from all samples of the Taean area from 2007 until 2012 than in the controls (>1-fold induction), suggesting that there was still potential genotoxicity in the sediment of heavily oil-contaminated sites over 5 years after the oil spill occurred.

Organic extracts of sediments from heavily polluted areas were fractionated into three fractions (F1, F2, and F3) and blood cells were exposed to them for comet assays (Fig. 3). The fold induction of DNA damage ranged from 0.77 to 9.22. All samples showed higher DNA damage than in the controls (>1-fold induction) except for the Sinduri mud flat 2 (#9). Organic extracts from Sinduri dune sediments (#3–5) collected in 2012 produced higher DNA damage than the other two sites. On the contrary, the total DNA damage found for three fractions (F1–F3) of the sediment was in the following descending order: Sogeunri mud flat (4.19), Sinduri mud flat (3.96), and Sinduri dune (3.36). In addition, the total DNA damage caused by the three fractions was greater than that caused by the unfractionated organic extracts of sediments, suggesting that there might be antagonistic effects between genotoxic compounds in the unfractionated extracts. Similarly, Hong et al. (2015) reported antagonistic genotoxic effects in the same crude oil and sediment extraction samples.

The oil was fractionated into F1 (saturated hydrocarbons), F2 (aromatics), and F3 (resins and polar compounds) fractions using silica gel chromatography according to solvent polarity, and DNA damage responses were compared after blood cells had been exposed to the three fractions. We also examined the degree of DNA damage induced by chemical composition changes in IHC oil consequent to weathering (Fig. 4).

DNA damage levels caused by the fractions of crude and weathered oil were 3.08–10.31, and 2.92–8.31, respectively. Of these fractions, the IHC oil produced the highest DNA damage (10.31) in its F2 fraction, while the weathered IHC oil showed the highest damage (8.31) in its F1 fraction. The DNA damage caused by theF2 fraction of weathered IHC oil decreased to 40% of the level caused by IHC oil, suggesting that DNA damage might be influenced by differences in oil composition. Crude oil such as IHC is subjected to physicochemical and biological weathering and degradation after it enters the environment (Prince et al., 2003; Hong et al., 2012). A weathering experiment (Kim et al., 2012) showed that evaporation of IHC oil—the major type spilled in this accident—removed alkane and low molecular weight PAHs. This was consistent with previous studies on crude oil (Short and Heintz, 1997; Cao et al., 2013; Wang et al., 2005), showing changes in the composition of PAHs caused by weathering. Accordingly, this change in the composition of low molecular weight alkane and aromatic compounds after weathering might have caused the difference in DNA damage levels in F2 fractions between IHC and weathered IHC oil. Nevertheless, the F2 fraction of weathered IHC oil still produced a high level of DNA damage compared with its F3 fraction. Moreover, all fractions of IHC and weathered IHC oil showed DNA damage three to 10 times higher than the controls (>1-fold induction), suggesting the existence of genotoxic substances in all fractions. Therefore, further research is warranted for identifying and measuring the concentrations of genotoxic substances in all three fractions.

The weathered IHC oil produced a decrease in DNA damage to 40% of that produced by the F2 fraction of IHC oil (Fig. 4). Therefore, we examined the degree of DNA damage by subfractionation of the F2 fractions of IHC and weathered IHC oil using HPLC (Fig. 5). All six F2 subfractions induced DNA damage in the range 1.15–4.11, of which the F2.1 and F.2.5 subfractions showed higher DNA damage in weathered IHC than in IHC oil. In addition, the total fold induction value of subfractions was lower than the

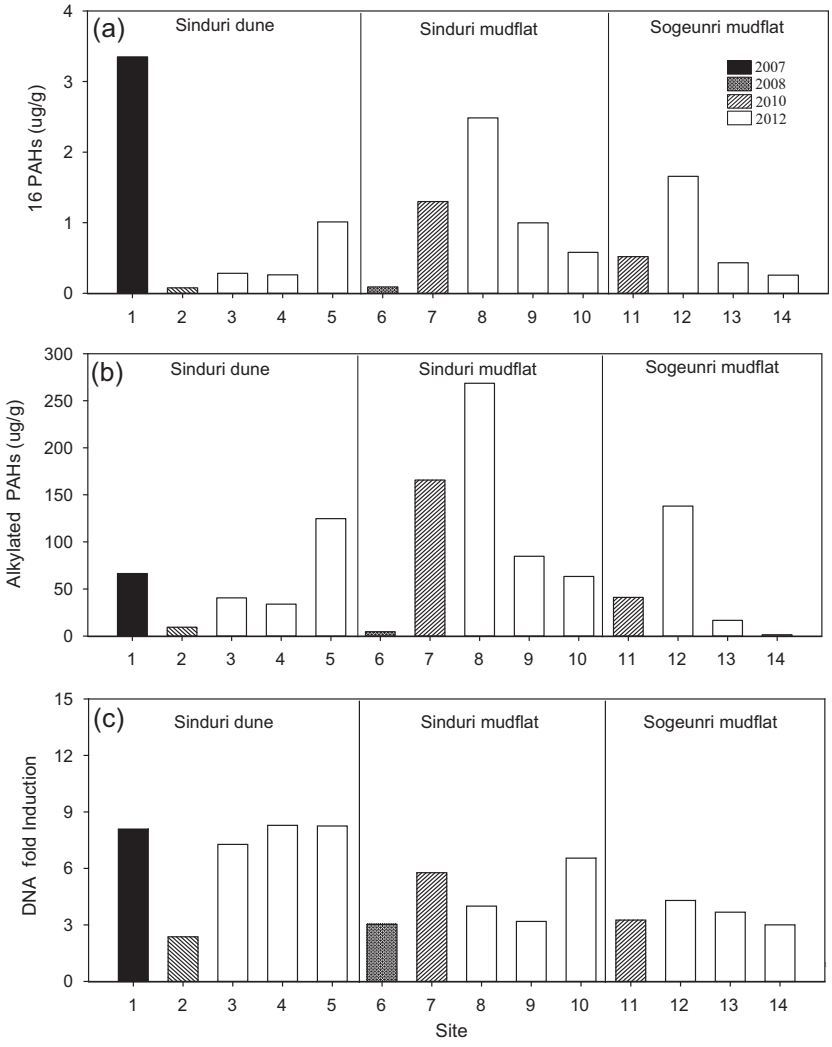

Fig. 2. Σ_{16} PAHs (a) and alkylated PAHs (b) in sediments, and relative DNA damage levels (c) induced by organic extracts of the sediments.

Table 1
Σ_{16} PAHs, concentrations of alkylated PAHs, and relative DNA damage.

Sample No.	Site	Sediment type	Year	Month	Σ_{16} PAHs	Alkyl-PAHs	Relative DNA damage	
					μg/g dw		Organic extract[a]	F2 fraction[a]
1	Sinduri dune	Sand	2007	12	3.35	66.44	8.09	1.73
2	Sinduri dune 3	Sand	2010	12	0.08	9.36	2.37	5.16
3	Sinduri dune 1	Sand	2012	1	0.28	40.53	7.27	2.98
4	Sinduri dune 2	Sand	2012	1	0.26	33.88	8.29	2.23
5	Sinduri dune 3	Sand	2012	1	1.01	124.76	8.25	2.23
6	Sinduri mud flat	Mud	2008	1	0.09	4.60	3.03	5.38
7	Sinduri mud flat 1	Mud	2010	12	1.30	165.75	5.77	5.50
8	Sinduri mud flat 1	Mud + gravel	2012	1	2.49	268.55	4.00	3.68
9	Sinduri mud flat 2	Mud + gravel	2012	1	1.00	84.76	3.18	2.08
10	Sinduri mud flat 3	Mud + gravel	2012	1	0.58	63.26	6.55	3.47
11	Sogeunri mud flat 1	Mud	2010	12	0.52	40.95	3.26	6.14
12	Sogeunri mud flat 1	Mud	2012	1	1.66	138.10	4.30	3.01
13	Sogeunri mud flat 2	Mud	2012	1	0.43	16.63	3.68	2.43
14	Sogeunri mud flat 3	Mud	2012	1	0.26	1.30	3.00	5.98

[a] Change in DNA comet 'tail' moment compared with controls, measured as the length of the tail × DNA content.

Faction 2, these also showed antagonistic effects between genotoxic components in subfractions, as shown in sediments (Fig. 3). The sharp decrease of DNA damage in F2 fraction shown in Fig. 4 might result from the decrease in DNA damage caused by F2.2 and F2.4 in Fig. 5.

By 2012, 5 years after the *Hebei Spirit* oil spill, DNA damage was induced from all organic extracts of those polluted coastal sediments at Taean, Korea. We could confirm potential genotoxic effects from the sediments of the severely affected regions. In addition, this study confirmed DNA damage caused

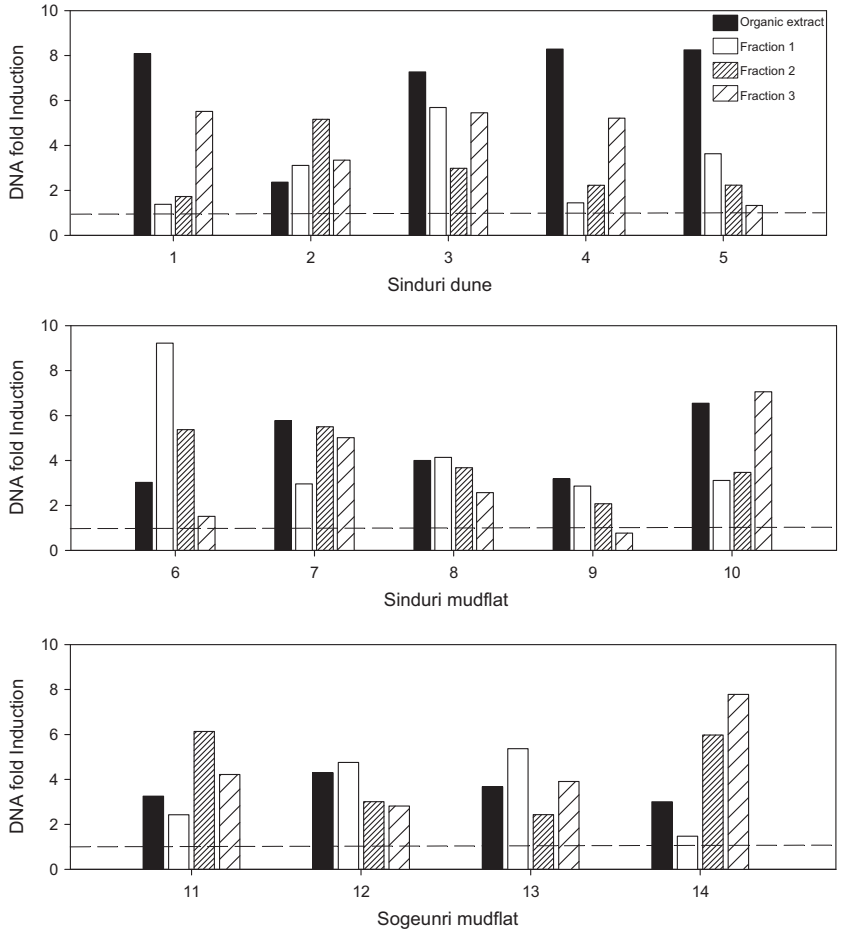

Fig. 3. Relative DNA damage caused by the F1, F2, and F3 fractions of organic extracts of sediment from the Taean area.

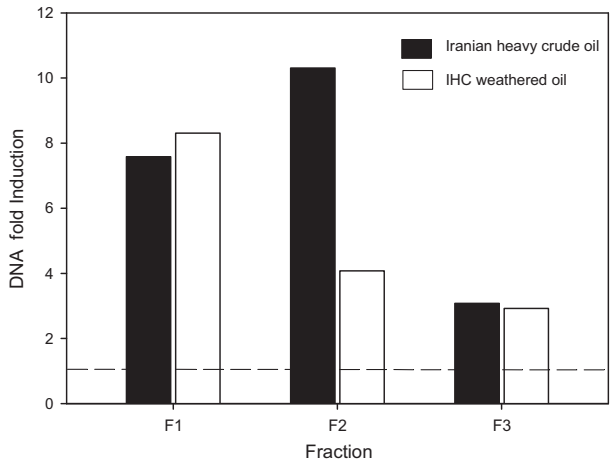

Fig. 4. Relative DNA damage levels induced by exposure to fractions 1–3 of Iranian Heavy Crude oil (IHC) and IHC weathered oil.

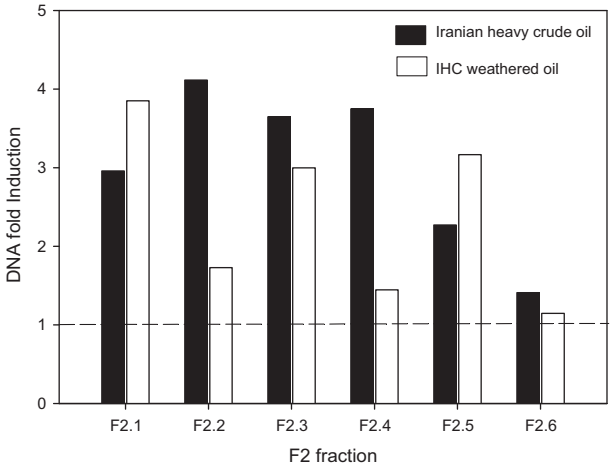

Fig. 5. Relative DNA damage induced by exposure to the F2 subfractions of Iranian Heavy Crude oil (IHC) and IHC weathered oil.

by organic extracts, their fractions (F1–3) and by subfractions (F2.1–2.6) of the F2 fractions of IHC and weathered IHC oil. A decrease in DNA damage caused by the F2 fraction after weathering of IHC oil was also confirmed by F2 subfractionation (F2.1–6) of the weathered oil. Both the sediments and two oil types showed antagonistic effects between genotoxic compounds in all three fractions. Further research is needed to identify and quantify the genotoxic substances in all three fractions, and in the F2 subfractions of IHC and weathered IHC oil samples.

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