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# Estrogenic activity of bio-degradation products of C-heavy oil revealed by gene-expression profiling using an oligo-DNA microarray system

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ABSTRACT

Degradation of heavy oil by bacteria to decompose organic compounds such as aliphatic and aromatic hydrocarbons has been used in bioremediation. However, the biological and environmental effects of the degradation products including intermediates are still not clear. Here, we monitored the degradation of C-heavy oil by analyzing the products formed in cultures with oil-degrading bacteria (complex microbes or a single bacterial strain). Furthermore, proliferation assays using breast cancer MCF-7 cells and gene-expression profiling of MCF-7 cells using oligonucleotide-DNA microarrays were performed to evaluate the estrogenic activity of the degradation products. While the products did not show any significant cell-proliferative activity, the oil samples cultured for longer periods (2–3 months), whether cultured with mixed microbes or a single bacterial strain, showed gene-expression profiles similar to that of 17β-estradiol ($E_2$). These results suggest that oil-degradation products have estrogenic activity, and estrogen-like components could possibly be produced during the degradation process.



## 1. Introduction

Marine oil-spillage accidents cause serious damage to various marine organisms and also inflict heavy losses on human activities. To diminish the damage and economic losses, remediation techniques such as physical removal and dispersant spreading have been developed and used around the world. In addition, new techniques based on the stimulation of in situ microbial oil-degrading activity, so-called bioremediation, have received attention (Swannell et al., 1996). However, our knowledge of microbial communities and their behavior in oil-contaminated marine environments is still limited (Maruyama et al., 2003; Prince, 2005). Also, little is known about the safety of various intermediates in the degradation of oil by microbes enhanced by such active treatments in the sea.

To establish reliable oil-bioremediation techniques, the rapid, accurate and quantitative monitoring of oil-degradation processes is essential. To date, chemical fingerprinting approaches using high-resolution instruments, such as gas chromatography (GC), frame ionization detection (FID), and mass spectrometry (MS), have been applied to microbial oil-degradation experiments as well as the characterization of spilled oil (e.g., Wang et al., 2007). However, it is almost impossible to characterize all of the ingredients in oil products and monitor them one by one continuously. One of the most reliable methods with which to characterize all oil products is to use a chemical grouping or fractionation technique based on organic solvents such as a combination of thin-layer chromatography and frame ionization detection (TLC-FID). In contrast, nondestructive measurement techniques should be useful for rapid continuous monitoring of oil cleanup processes. In the present study, we applied these new measurement techniques to evaluate microbial oil-degradation.

Estrogenic endocrine-disrupting chemicals (EDCs) are ubiquitous in the environment because estrogenicity is mainly exhibited by chemicals with a simple phenolic core structure. Simple phenol-derivatives such as alkylphenols, bisphenols and parabens, and complex hydroxylated aromatic hydrocarbons such as natural estrogens and phytoestrogens are well-known estrogenic chemicals. On the other hand, some chemicals without phenolic hydroxyl groups, such as phthalate esters (Parveen et al., 2008) and perfluoroalkyl acids (Benninghoff et al., 2011), are also estrogenic. More complex events important for estrogenicity occur at the cellular level; variations of receptors for binding of estrogenic chemicals, cross-talk between receptors and subsequent signaling, and the modification of chemicals within cells such as in the case of

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methoxychlor (Inoue et al., 2002). Therefore, the estrogenicity of chemicals should be understood based on a comprehensive analysis with various detection and evaluation techniques. Numerous approaches have been suggested in European, US and OECD programs (reviewed in Bucher and Lucier, 1998; Menditto and Turrio-Baldassarri, 1999; Vos et al., 2000).

Several methods were used to examine gene-expression in response to compounds including estrogen. For example, quantitative RT-PCR was used to evaluate the transcriptional level of genes in thicklip grey mullets or in rainbow trout after exposure to oil (Bilbao et al., 2010; Gagné et al., 2012). Here, we used DNA microarray assay using estrogen-responsive genes to evaluate the estrogenicity of chemicals using MCF-7 cells, a human breast cancer cell line. We used this DNA microarray assay system to examine a variety of chemicals and mixtures of chemicals (Inoue et al., 2002; Ise et al., 2005; Dong et al., 2007; Parveen et al., 2008, 2009). The system is relatively simple but, as the results are evaluated statistically by the behavior of a number of genes, it gives information on how reliable the results are. Furthermore, as the assay results are based on combinations of different, sometimes independent, cellular events with markers in respective events, we can detect cellular events even when the cells do not show phenotypes, such as cell growth and apoptosis (Inoue et al., 2006). In this sense, the assay is more sensitive than animal tests or reporter gene assays, and the information about the response of human cells would be useful for assessing human exposure to chemicals.

## 2. Materials and methods

### 2.1. Cell culture

Human breast cancer MCF-7 cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% $CO_2$.

### 2.2. C-heavy oil-degradation and monitoring methods

The hydrocarbon degrader *Cycloclasticus* sp. strain HP-7 used in this study was isolated from a surface seawater sample collected at Heisei port in Tokyo Bay in August, 2003, using NSW medium (Higashihara et al., 1978) containing biphenyl (final 0.5%). For preparing complex microbes, a sample was collected from surface seawater at central Tokyo Bay in January, 2009, and used for culturing at 20 °C for about two weeks in a medium consisting of C-heavy oil (PetroChina, Beijing, China; final 0.5%), $NH_4NO_3$ (0.1%), $NaH_2PO_4$ (0.002%) and $FeC_6H_5O_7$ (0.002%). Then, a series of culture samples were prepared by 10-fold serial dilution using NSW medium (Higashihara et al., 1978) containing C-heavy oil (final 0.5%) and incubated at 20 °C for a month. Some of the most transparent samples showing almost the complete disappearance of C-heavy oil were sequentially incubated with the same medium containing C-heavy oil at 20 °C, and a sample showing persistent oil-degrading activity was selected and named as the microbial consortium TK09W.

Oil-degrading activity was examined using an image analyzer system, BioDoc-It/GDS-7900 (UVP, Upland, CA) equipped with LED lights and a monochrome CCD camera, after culturing the samples in 24-well polystyrene microplates (BD Falcon, Franklin Lakes, NJ). The images captured in the wells were quantified by Quantity One software (Bio-Rad Laboratories, Philadelphia, PA), and oil-degrading activity was calculated by normalization with that of the oil incubated without bacteria.

### 2.3. Component analysis of C-heavy oil

The chemical components in C-heavy oil used in this study were analyzed by a combination of thin-layer chromatography and frame ionization detection (TLC-FID) according to Goto et al. (1994) using an Iatroscan instrument (Mitsubishi Chemical, Tokyo, Japan) with minor modifications.

### 2.4. Cell-proliferation assay

Human breast cancer MCF-7 cells were cultured in phenol red-free RPMI 1640 medium containing 10% (v/v) dextran-coated charcoal-treated FBS (DCC-FBS) in a 10-cm culture dish for 2 days. The cells were replated in 96-well plates at a density of $3 \times 10^3$ and cultured in DCC-FBS medium for one more day. $E_2$ was dissolved in DMSO to create a 10 mM stock solution. The cells were then treated with 10 nM $E_2$ (diluted with RPMI 1640 medium; final 0.1% DMSO) or oil-degradation products (diluted with RPMI 1640 medium) at the indicated dilution rates for 3 days. Control cells were treated with 0.1% DMSO in the culture medium. The cell-proliferation assay was performed by the soluble tetrazolium/phenazine methosulfate method (Cory et al., 1991) with modifications (MTS/PES assay) using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, MI) according to the manufacturer's instructions.

### 2.5. DNA microarray assay

The oligonucleotide-DNA (oligo-DNA) microarray was manufactured by Invitrogen by mechanical spotting of oligo-DNA on a glass slide for 203 genes including a set of 172 estrogen-responsive genes as described previously (Terasaka et al., 2004). For the microarray assay, MCF-7 cells were cultured in phenol red-free RPMI 1640 medium containing 10% DCC-FBS for 3 days and treated with 10 nM of $E_2$, oil-degradation products (dilution rates of samples were all 100-fold), or vehicle (0.1%DMSO) for 3 days. RNA isolation, cDNA synthesis and hybridization were performed as described previously (Terasaka et al., 2006; Dong et al., 2011). The slides were then scanned using FLA-8000 (Fujifilm Corp., Tokyo, Japan). Image analysis and the data analysis were performed as described previously (Dong et al., 2011). The raw data of the assay is summarized in Supplementary Table 1.

## 3. Results

### 3.1. Evaluation of microbial oil-degrading activity using C-heavy oil

First, the C-heavy oil was analyzed by a combination of thin-layer chromatography and frame ionization detection technique (TLC-FID) to understand its initial composition. Its four major components were saturates (23%), aromatics (57%), resins (9%) and asphaltenes (11%) (Fig. 1A).

Using this C-heavy oil, oil-degrading activity of microbes was assessed by optical measurement followed by image analysis. Fig. 1B shows that oil-degradation was remarkably stimulated by the addition of microbes. After incubation with the microbial consortium TK09W, the brownish black C-heavy oil in microplates was reduced in 28 days (#1, Fig. 1C) and mostly disappeared in 101 days (#3, Fig. 1C). The activity was also found in the culture with a single microbial strain, HP-7, after incubation for 73 days, although it was not higher than that in TK09W (#4, Fig. 1C).

### 3.2. Effect of oil-degradation products on cell-proliferation

We examined the effect of the degradation products on the growth of MCF-7 cells (Fig. 2). The cells treated with 10 nM $E_2$ or the oil-degradation products at the indicated dilution (from $10^{-5}$ to $5 \times 10^{-1}$) were examined by MTS/PES assay based on Cory et al. (1991). The results showed that while cells treated with 10 nM $E_2$ exhibited enhanced cell growth, the oil-degradation products did not show any significant growth-promoting activity at any dilution (Fig. 2). Furthermore, anti-proliferative activity was observed at higher concentrations ($5 \times 10^{-1}$ for samples #2 and #3, Fig. 2).

### 3.3. Gene-expression profiling of oil-degradation products by oligo-DNA microarray assay

To evaluate the estrogenic activity of oil-degradation products, we used an oligo-DNA microarray containing 172 estrogen-responsive genes to examine gene-expression profiles in MCF-7 cells exposed to these products. Each of the profiles for oil-degradation products was compared with that of $E_2$ and a correlation coefficient ($R$-value) was calculated (Fig. 3). The oil cultured with the complex microbes (TK09W) for 2–3 months showed high $R$-values ($R = 0.82–0.88$) (Fig. 3C and E), while low $R$-values were observed for the control ($R = 0.21$) (Fig. 3I) and the sample incubated for 28 days ($R = 0.57$) (Fig. 3A). The oil cultured with a single microbial strain (HP-7) also showed a higher correlation ($R = 0.70$) (Fig. 3G). In addition, there was a high correlation between the samples treated with complex microbes and HP-7 for 73 days