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Toxic effects of di(2-ethylhexyl)phthalate on mortality, growth, reproduction and stress-related gene expression in the soil nematode *Caenorhabditis elegans*

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Abstract

In this study, di(2-ethylhexyl)phthalate (DEHP) toxicities to *Caenorhabditis elegans* were investigated using multiple toxic endpoints, such as mortality, growth, reproduction and stress-related gene expression, focusing on the identification of chemical-induced gene expression as a sensitive biomarker for DEHP monitoring. The possible use of *C. elegans* as a sentinel organism in the monitoring of soil ecosystem health was also tested by conducting the experiment on the exposure of nematode to field soil. Twenty-four-hour median lethal concentration (LC50) data suggest that DEHP has a relatively high potential of acute toxicity to *C. elegans*. Decreases in body length and egg number per worm observed after 24 h of DEHP exposure may induce long-term alteration in the growth and reproduction of the nematode population. Based on the result from the *C. elegans* genome array and indicated in the literatures, stress proteins, metallothionein, vitellogenin, xenobiotic metabolism enzymes, apoptosis-related proteins, and antioxidant enzyme genes were selected as stress-related genes and their expression in *C. elegans* by DEHP exposure was analyzed semi-quantitatively. Expression of heat shock protein (hsp)-16.1 and hsp-16.2 genes was decreased by DEHP exposure. Expression of cytochrome P450 (cyp) 35a2 and glutathione-*S*-transferease (gst)-4, phase I and phase II of xenobiotic metabolism enzymes, was increased by DEHP exposure in a concentration-dependent manner. An increase in stress-related gene expressions occurred concomitantly with the deterioration on the physiological level, which suggests an increase in expression of those genes may not be considered as a homeostatic response but as a toxicity that might have physiological consequences. The experiment with the soil from the landfill site suggests that the potential of the *C. elegans* biomarker identified in laboratory conditions should be calibrated and validated for its use *in situ*.
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Keywords: *Caenorhabditis elegans*; Di(2-ethylhexyl)phthalate; Stress-related gene expression; Ecotoxicity monitoring

1. Introduction

Di(2-ethylhexyl)phthalate (DEHP) is widely used in flexible polyvinyl chloride (PVC) as a plasticizer to soften plastic materials. Owing to its utility and cost effectiveness, DEHP has been used in a broad range of applications, such as wires and cables, floor tiles, garden hoses, containers, footwear and clothing (De Jonge et al., 2002; Chao and Cheng, 2007). Due to the widespread use of DEHP, it is ubiquitous in the environment and many environmental species are thus exposed to various levels of DEHP in their natural habitat. At present,

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hazard or risk assessments of DEHP conducted by international authorities are available (WHO, 1992; EPA, 1999; EU, 2001; ATDSR, 2002), some of which include an assessment of the ecological risk of DEHP to aquatic life. However, few studies have been performed on the ecotoxicity of soil organism.

Caenorhabditis elegans, a free-living nematode that lives mainly in the liquid phase of soils, is the first multicellular organism to have its genome completely sequenced. The genome showed an unexpectedly high level of conservation with the vertebrate genome, which makes *C. elegans* an ideal system for biological studies, such as those in genetics, molecular biology and development biology (Brenner, 1974; Bettinger et al., 2004; Leacock and Reinke, 2006; Schafer, 2006; Schroeder, 2006). *C. elegans* is also a good animal model for the study of ecotoxicology. Due to its abundance in soil ecosystems, its convenient handling in the laboratory, and its sensitivity to different kinds of stresses, *C. elegans* is frequently used in ecotoxicological studies utilizing various exposure media, including soil and water (Peredney and Williams, 2000; Willams et al., 2000; Boyd and Williams, 2003a). The range of *C. elegans* studies in ecotoxicology focuses on organism-level endpoints, such as mortality, behavior, growth or reproduction. However, using these classical test endpoints, it is difficult to find significant effects. Therefore, more specific and sensitive systems than classical ecotoxicological tests are needed.

In this study, to identify a suitable tool to develop a screening system for ecotoxicity monitoring, DEHP toxicities to *C. elegans* were investigated using multiple toxic endpoints, such as mortality, growth, reproduction and stress-related gene expression, focusing on the identification of chemical-induced gene expression as a sensitive biomarker for DEHP toxicity. *C. elegans* whole genome microarray was conducted for screening the differentially expressed gene list by DEHP exposure. Tested stress-related genes were selected according to the results of microarray data and literature. Alteration on stress-related gene expression by DEHP exposure was examined in a semi-quantitative manner. The response of green fluorescent protein (gfp) transgenic nematode, incorporated full-length heat shock protein (hsp)-16.2 and hsp-16.48 genes to DEHP exposure was also examined to test a possibility of transgenic worm as a biosensor of environmental monitoring. Additionally, *in situ* application of *C. elegans* toxicity indicator was investigated on the nematodes exposed to soil from landfill sites, using the same endpoints applied for the exposure of laboratory condition.

2. Materials and methods

2.1. Organisms

The wild-type *C. elegans* Bristol strain N2 was used in this study. *C. elegans* were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20 °C, using the standard method previously described by Brenner (1974). Young adults (3 days old) from an age-synchronized culture were used in all the experiments. Worms were incubated at 20 °C for 24 h without a food source, and were then subjected to the analysis.

2.2. Sample preparation

Four types of endpoints (mortality, growth, reproduction, and stress-related gene expression) were assessed for exposure to DEHP. Pure analytical-grade DEHP (Sigma–Aldrich Chemical, St. Louis, MO, USA) was used in the experiment and it was dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich Chemical). Nematodes were exposed to DEHP prepared in a K-medium (0.032 M KCl, 0.051 M NaCl) (Williams and Dusenbery, 1990). Three replicates for each concentration and a control were conducted for all the test types. The DEHP concentrations in a K-medium were nominal values.

Soil toxicity testing with *C. elegans* was performed as described in the American Society for Testing and Materials (ASTM, 2001). Briefly, for each sample, 2.33 g of soil as loaded in to a 35 mm × 10 mm petridish. The moisture was adjusted to 35% (dry weight) using K-medium, worms were added and were incubated at 20 °C for 24 h without a food source. The worms were then recovered with centrifugation/flotation using Ludox (Sigma–Aldrich). Three replicates were conducted for all the test types.

2.3. Lethal toxicity tests

Each test consisted of four concentrations and a control, in which 10 ± 1 of young *C. elegans* adults were transferred onto 24-well tissue culture plates containing 1 ml of the test solution for each of the five wells. The worms were exposed for 24 h at 20 °C. After the 24 h, the numbers of live and dead worms were determined through visual inspection and by probing the worms with a platinum wire under a dissecting microscope.

2.4. Measurement of growth and reproduction

Following the 24 h incubation with exposure to sublethal concentrations of DEHP, growth and reproduction were assessed. Growth was assessed by measuring the length of the worms that had been killed by the heat through microscopy, with a scaled lens in each sample. The average length of the unexposed control worm was in the range of 1.0–1.2 mm. Reproduction was assessed by counting the eggs of each worm through the microscopic inspection of the transparent *C. elegans* body in each sample. Although this procedure differs from

more commonly used reproduction tests of offspring counting from an age-synchronized single worm, this simple detection method seems appropriate for the rapid screening of the reproduction effect (Roh et al., 2006). The average number of eggs per worm in the unexposed controls was in the range of 10–25. One hundred worms were examined per treatment for growth and reproduction experiments.

2.5. RNA extraction

Following the 24 h incubation with exposure to sublethal concentrations of DEHP, nematodes were harvested for the preparation of RNA. Total RNA was prepared by phenol–chloroform extraction, according to the manufacturer's standard protocol. RNA concentrations were determined by the absorbance at 260 nm. The quality of total RNA was estimated based on the ratio of the optical densities from RNA samples measured at 260 and 280 nm.

2.6. Microarray analysis

Five micrograms of the total RNA extracted from nematodes exposed to DEHP and the control was used for reverse and *in vitro* transcription followed by application to a GeneChip® *C. elegans* Genome Array (Affymetrix, Santa Clara, CA, USA), which contains 22,500 probe sets against 22,150 unique *C. elegans* transcripts. The arrays were scanned with the GeneChip scanner 3000 (Affymetrix), controlled by GeneChip Operating Software (GCOS, Affymetrix).

2.7. Semi-quantitative reverse transcription-polymerase chain reaction

The two-step reverse transcription-polymerase chain reaction (RT-PCR) method was used with RT Premix (Bioneer Co., Seoul, Korea) and PCR Premix kits (Bioneer Co.), using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA). The primers were designed on the basis of the sequences retrieved from the *C. elegans* database (www.wormbase.org). Actin mRNA was used for expression-level normalization of the studied genes. The PCR products were separated through electrophoresis on 1.5% agarose gel (Promega, Madison, WI, USA) and were visualized with ethidium bromide (Bioneer Co.). All the tests were replicated at least three times, and the relative densities of each band were determined with the use of a Kodak EDAS 290 image analyzer (Kodak, Rochester, NY, USA), with a TFX-20.M UV transilluminator (Vilber Lourmat, Marne la Vallee, France).

2.8. Detection of green fluorescence protein transgenic *C. elegans*

The transgenic strains (hsp-16.2::gfp and hsp-16.48::gfp) of *C. elegans* were developed as previously described by Hong et al. (2004). Transgenic *C. elegans* were incubated for 24 h with 2 mg/l of DEHP, as well as, with soil from landfill sites, and the fluorescence signal was examined from 20 independent transgenic worms per treatment. Fluorescence was observed using a Leica DM IRB microscope (Leica, Wetzlar, Germany), and the image was taken using a Leica DC 300FX camera (Leica). Levamisole (Sigma–Aldrich Chemical) treatment (2 mM) was used to take pictures of the live worms.

2.9. Analysis of DEHP from soil of Korean landfill sites

Soil was collected from Korean landfill sites, namely Jeonju (J), Gwangju (G) and Mokpo (M). Reference soil (R) was sampled from the clean area. Soil samples were dried in the air at room temperature. DEHP was extracted using the Pressurized Solvent Extraction (SPE) method and was analyzed using 6890 Gas Chromatography–5973N Mass Spectroscopy systems (Agilent, Santa Clara, CA, USA).

2.10. Data analysis

Median lethal concentration (LC50) were derived through Probits analysis. The statistical differences between the control and treated worms were determined with the aid of the parametric *t*-test.

3. Results

Acute toxicity of DEHP on *C. elegans* was investigated using mortality as endpoint (Table 1). Twenty-four-hour LC50 of DEHP in *C. elegans* was estimated as 22.55 mg/l. Based on the results of the acute toxicity test, three concentrations corresponding to 1/1000, 1/100, and 1/10 of the 24 h LC50 were selected for laboratory sublethal exposure conditions, that is, 0.02, 0.2 and 2 mg/l.

The changes in the worms' body lengths and in the number of eggs per worm were investigated as a growth and a reproduction indicator, respectively (Fig. 1). The worms, which had been exposed to 0.02 and 0.2 mg/l of DEHP, showed a decrease in their body length, as well as, in the number of eggs per worm.

Suitability of the DNA microarray technique for the ecotoxicological approach was investigated in *C. elegans* exposed to 0.2 mg/l of DEHP. DEHP-induced genes expression was screened using *C. elegans* Genome Arrays. Major up- and down-regulated known genes by

Table 1
Estimation of 24 h LC10, LC50 and LC90 of DEHP in *C. elegans*

	24-h LC (mg/l)	Interval of confidence (95%)
LC10	3.650	$0.016 < LC10 < 11.33$
LC50	22.55	$4.200 < LC50 < 56.63$
LC90	139.4	$55.75 < LC90 < 5611$

LC means lethal concentration.

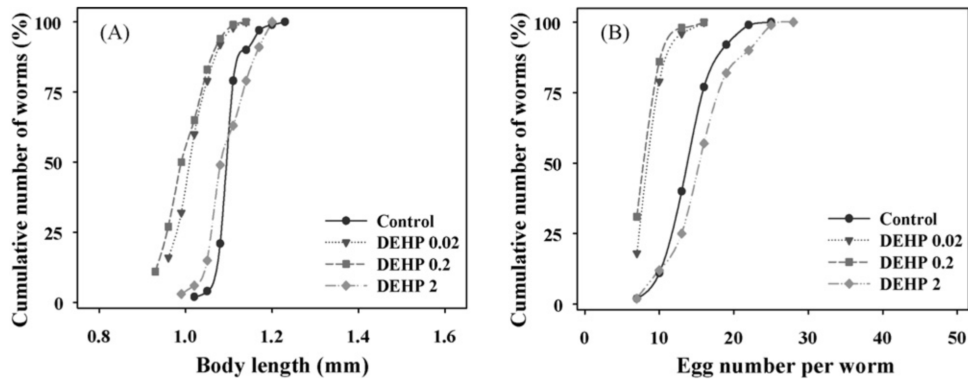


Fig. 1. Growth and reproduction indicators examined in the young adult of *Caenorhabditis elegans* exposed to DEHP for 24 h. Growth was assessed by measuring the length of the worms that had been killed by the heat through microscopy, with a scaled lens in each sample. Reproduction was assessed by counting the eggs of each worm through the microscopic inspection of the transparent *C. elegans* body in each sample.

DEHP exposure in *C. elegans* were shown in Table 2. For stress-related gene expression profiling analysis, based on the result from the microarray and what is found in the literature, we investigated alteration on the gene expression of heat shock proteins (hsp-16.1, hsp-16.2, hsp-16.48, hsp-70), metallothioneins (mt-1, mt-2), vitellogenins (vit-2, vit-6), xenobiotic metabolism enzymes (cyp35a2, gst-4), tumor suppressor and apoptosis proteins (cep-1, ape-1), and antioxidant enzymes (sod-1, ctl-2) in *C. elegans* by DEHP exposure.

The stress-related gene expression profile was investigated in the young *C. elegans* adults exposed to DEHP for 24 h (Fig. 2). Expression of hsp-16.1 and hsp-16.2 decreased at 0.02 and 0.2 mg/l of DEHP exposure. Expression of mt-2 increased at DEHP-treated *C. elegans*. However, due to high data variation, statistical significance was not observed. Expression of cyp35a2 and gst-4, phase I and phase II of xenobiotic metabolism enzymes, was increased by DEHP exposure in a concentration-dependent manner. Expression of genes related to vitellogenes, apoptosis, or antioxidant enzymes was not changed by DEHP exposure.

As shown in Fig. 3, the hsp-16.2 and hsp-16.48 gene expression levels were assayed using gfp-based

Table 2
Screening of DEHP induced gene expression using *C. elegans* whole genome microarray

Up-regulated genes			Down-regulated genes		
Wormbase no.	Sequence description	Flod change	Wormbase no.	Sequence description	Flod change
K02D7.3	Cuticular collagen	17	Y71D11A.5	Ion channel protein	0.03
C39E9.3	Collagen	6.4	W03G1.7	Sphingomyelin phosphodiesterase	0.03
R08F11.7	Peroxidase	6.2	F36D3.9	Cysteine protease	0.07
B0365.6	C-type lectin domain	5.8	C06A8.9	Glutamate receptor	0.07
CEC2033	Major sperm protein	5.0	F21F8.2	Protease	0.08
K08E7.9	Multidrug resistance protein	4.2	C58111	GTP-binding protein	0.08
C33A12.6	UDP-glucuronysltransferase	3.6	C52B9.9	AMP-binding protein	0.1
K09F5.2	vit-1	3.0	C37H5.1	Annexin	0.13
F09G2.3	Permease	2.9	C08H9.1	Serine carboxypeptidase	0.15
T11F9.3	Zinc metalloprotease	2.9	T07G12.1	Calmodulin	0.16
F34H10.1	Ubiquitin/ribosomal protein	2.7	K08C7.5	Flavin-containing monoxygenase	0.16
C03G6.15	Cytochrome P450	2.7	F53B7.2	G-protein coupled receptor	0.18
F28A12.4	Peptidase	2.6	F48E3.7	Acetylcholine receptor	0.18
F22D6.10	Collagen	2.6	Y46H3A.3	Heat shock protein	0.19
ZK1248.17	Major sperm protein	2.6	C42C1.2	Protein phosphatase	0.19
F08A8.3	Acyl-coenzyme A oxidase	2.5	E02H9.5	Glycosyl hydrolase	0.23
R07B1.3	Membrane glycoprotein	2.5	T27E4.8	Heat shock protein HSP16-1	0.28
W06D12.3	Fatty acid desaturase	2.4	T27E4.9	Heat shock protein	0.3
K08B4.3	Glucuronosyltransferase	2.4	K03A1.4	Calmodulin calcium-binding sites	0.31
F44G3.2	Arginine kinase	2.4	AU113943	Carbonic anhydrase	0.31

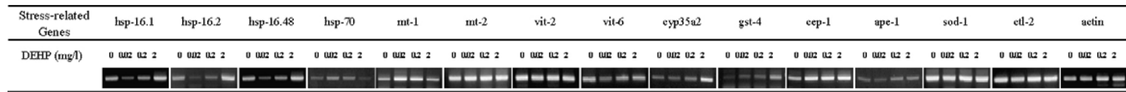


Fig. 2. Stress-related gene expression profiling in the young adult of *C. elegans* exposed to DEHP for 24 h. Stress related gene mRNA was amplified by RT-PCR method using the primers designed on the basis of the sequences retrieved from the *C. elegans* database (www.wormbase.org). Actin mRNA was used for expression-level normalization of the studied genes. The PCR products were separated through electrophoresis on 1.5% agarose gel and were visualized with ethidium bromide. All the tests were replicated at least three times.

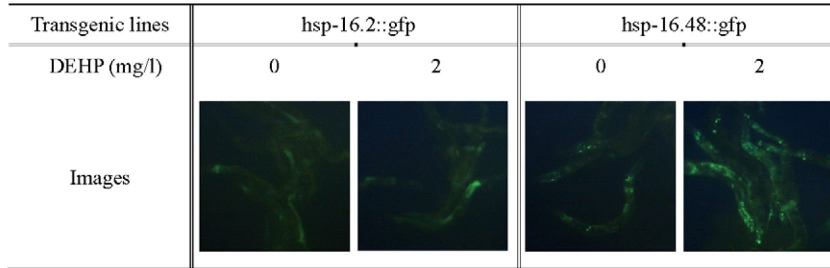
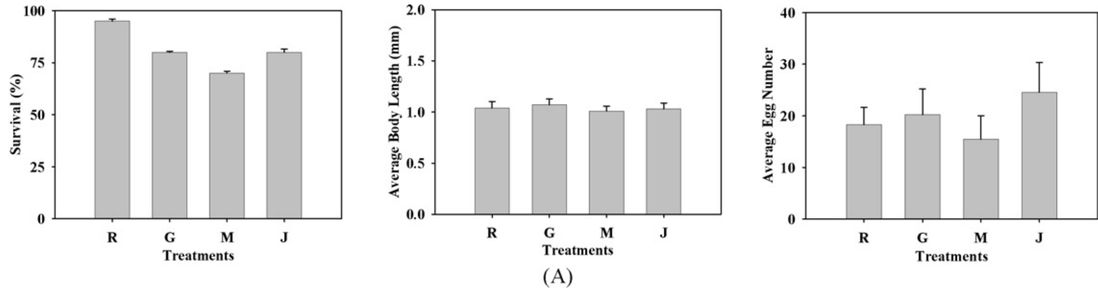
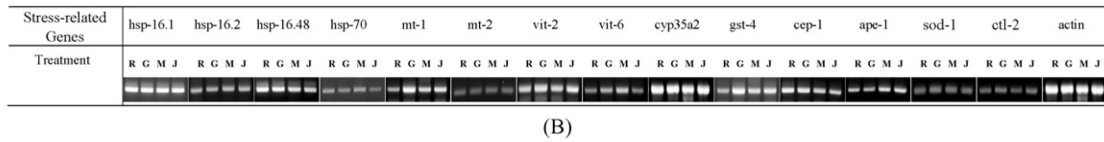


Fig. 3. Response of hsp-16.2::gfp and hsp-16.48::gfp transgenic *C. elegans* exposed to DEHP for 24 h. Transgenic *C. elegans* were incubated for 24 h with 2 mg/l of DEHP and the fluorescence signal was examined from 20 independent transgenic worms per treatment. Fluorescence was observed using a Leica DM IRB microscope, and the image was taken using a Leica DC 300FX camera.

reporter transgenic nematodes. Transgenic nematodes were exposed to 2 mg/l of DEHP and the fluorescence signals from both gfp transgenic lines increased after DEHP exposure. The response of hsp-16.48::gfp, however, was greater than that of hsp-16.2::gfp.

Concomitantly with bioassay using *C. elegans*, the analysis of DEHP was conducted on three Korean landfill sites (Table 3). The contamination levels of DEHP in soil from landfill sites were between 6 and 20 mg/kg soil. DEHP was not detected in the soil from the reference site.





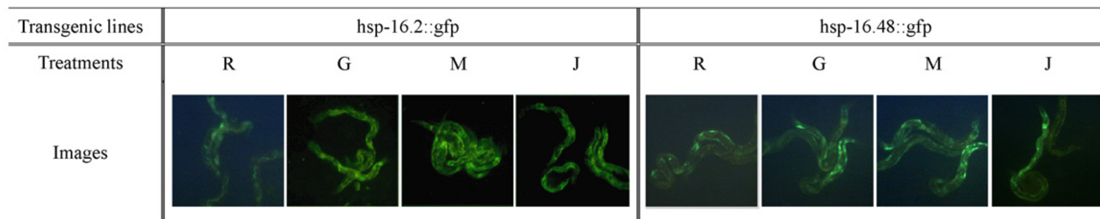


Fig. 4. Mortality, growth and reproduction indicators (A), stress-related gene expression profiling measured in the young adult of *C. elegans* exposed for 24 h to DEHP contaminated soil from Korean landfill sites (B). Response of hsp-16.2::gfp and hsp-16.48::gfp transgenic *C. elegans* exposed for 24 h to DEHP contaminated soil from Korean landfill sites (C). The soils were named as R, G, M, J (R: reference site soil, G: Gwangju landfill soil, M: Mokpo landfill soil, J: Jeonju landfill soil).

Table 3
Analysis of DEHP on soil from Korean landfill sites (number = 3; mean ± standard error of the mean)

	R[a]	G[b]	M[c]	J[d]
DEHP (mg/kg)	ND[e]	6.27 ± 0.65	20.05 ± 1.34	10.73 ± 1.37

[a] R: Reference soil site.
[b] G: Gwangju landfill site.
[c] M: Mokpo landfill site.
[d] J: Jeonju landfill sites.
[e] ND: non detected.

Fig. 4 shows the response of *C. elegans* on mortality, growth, reproduction, stress-related gene expression and the response of transgenic *C. elegans* exposed to soil from Korean landfill sites for 24 h. Increase in mortality occurred in the most severe extent (about 30%) in soil from the M landfill site, which showed the highest DEHP contaminated level among the three sites (20 mg/kg). Growth parameters did not change in the worm exposed to soil from the landfill site. The number of eggs per worm slightly increased in the nematode that had been exposed to soil from the J landfill site. Differently from that in Fig. 2, worms that had been exposed to soil from landfill sites did not show any statistically significant change in studied stress-related gene expression. The fluorescence signals from both gfp transgenic lines slightly increased in transgenic nematode exposed to soil from the landfill site.

4. Discussions

C. elegans is an attractive animal model for the study of the ecotoxicological relevance of chemical-induced molecular-level responses (Menzel et al., 2005; Reichert and Menzel, 2005). In this study, the utility of molecular parameters, such as stress-related gene expression, as biomarkers in *C. elegans* were investigated to identify specific and sensitive tools to develop a screening system for ecotoxicity monitoring. And the possible use of *C. elegans* as a sentinel organism in the monitoring of soil ecosystem health was also tested by conducting the experiment on the exposure of nematode to field soil.

Although DEHP is a widely used environmental compound, little study has been performed on its ecotoxicological properties. Twenty-four-hour LC50 data (Table 1) suggest that DEHP has a relatively higher potential of acute toxicity to *C. elegans* than previously studied metals have (Roh et al., 2006). Mortality is a reliable ecotoxicological endpoint. However, such a high level of exposure hardly occurred in the real environment. More sensitive indicators, physiological-level alterations, such as growth, reproduction, feeding, movement, or behavior, have been used as endpoints for chemical-induced toxicity testing in *C. elegans* (Dhawan et al., 2000; Anderson et al., 2001, 2004; Kohra et al., 2002; Tominaga et al., 2003). The effects of xenobiotics on the growth and reproduction of the test organisms are broadly accepted test parameters, and were found to be more sensitive indicators of toxicity than lethality, as also shown in this study (Fig. 1). The decreases in body length and egg number per worm observed after 24 h of DEHP exposure may induce alteration in the growth and reproduction of the nematode population in the long term. However, due to the low concentration of xenobiotics in the environment, it is hard to find a correlation between the occurrence of contaminants and a physiological effect of a test organism in the environment, even when using reliable ecotoxicological endpoints, such as, growth or reproduction, which emphasizes the need for understanding the sublethal effects at the biochemical and molecular levels where the toxicant-induced responses are initiated.

The effect of DEHP to the *C. elegans* whole genome gene expression was investigated. Using concentration corresponding to the 1/100 of LC50 value, which was 0.2 mg/l of DEHP, it was possible to show that a strong and differential gene induction or repression was detectable in response to DEHP exposure; they were cyp superfamily, hsp, vit, multidrug-resistance protein, etc. (Table 2). In case of some genes, such as C-type lectin, collagene, and major sperm protein genes, it is unclear what physiological meaning the over-expression has. Stress-related genes were selected from the array results and previously reported literature. Expression of selected stress-related genes to DEHP exposure was investigated at three different sublethal concentrations, using the semi-quantitative RT-PCR method. It is obvious that members of the cyp family, gst, hsp, and vit genes should be included in a selection of stress-related genes. Among the group of genes, which encode proteins belonging to different metabolic pathways, the metallothionein, apoptosis, and antioxidant enzyme genes were also included in the stress-related gene selection. As screened in the microarray result, the expression of hsp-16.1 and hsp-16.2 genes was decreased by DEHP exposure. DEHP exposure led to increases in the expression of some stress-related genes tested, including mt-2, cyp35a2 and gst-4. In particular, it has been reported that almost all cyp35 forms in *C. elegans* are moderately or strongly inducible by different xenobiotics in a cyp450 gene-expression screening experiment (Menzel et al., 2001, 2005). Increase in stress-related gene expressions occurred concomitantly with this deterioration on

the physiological level, which suggests that the increase in the expression of those genes may not be considered as a homeostatic response but as toxicity that might lead to physiological consequences (Figs. 1 and 2).

C. elegans also offers the advantage of transgenetic approaches, which may allow the development of a sensitive biosensor for environmental monitoring (Stringham and Candido, 1994; Jones et al., 1996; Chu et al., 2005). In our previous study (Roh et al., 2006), gfp transgenic nematode, incorporated full-length hsp-16.2 and hsp-16.48 genes were developed, since hsps are thought to play roles in various stress conditions. As shown in their response to the exposure to four metals (Roh et al., 2006), semi-quantitatively assayed using gfp-based reporter hsp-16.2 and hsp-16.48 transgenic nematodes were not very sensitive towards DEHP exposure. Even though, transgenic nematode seems to have a considerable potential as a biosensor for toxicity monitoring, to develop a sensitive biosensor using transgenic *C. elegans*, the responses of a broad range of stress-related gene promoters to various classes of chemicals should be screened and validated with environmentally relevant low-concentration samples.

Bioassay methods for soil toxicity monitoring have also been developed and frequently used in *C. elegans* (Peredney and Williams, 2000; Boyd and Williams, 2003b). As a soil-dwelling organism, *C. elegans* has a potential as a bioindicator for the detection of soil contamination because of its abundance in soil ecosystems and its sensitivity to different kinds of stresses, which was tested in this study using the same endpoints used for laboratory K-media exposure condition (Fig. 4). Increase in egg number per worm in the nematode exposed to soil from landfill sites suggests a mixture of contaminants, including DEHP, might have a stimulatory effect on the reproduction of the nematode. Expression of stress-related genes did not affect *C. elegans* exposed to soil from the landfill site, which were different from the response of nematodes exposed to DEHP prepared in K-media in laboratory conditions. The common feature of responses to DEHP of *C. elegans* between laboratory and field conditions was the increase in gst-4 gene expression. But, as gst is known to use a broad range of substrates, it is difficult to consider it as to DEHP-specific biomarker. Increase in fluorescence signal of hsp-16.2::gfp and hsp-16.48::gfp transgenic nematodes might be interpreted as a response of heat-shock protein upon exposure to mixture contaminants. The experiment with the soil from the landfill site suggests that the potential of the *C. elegans* biomarker identified in laboratory conditions should be calibrated and validated for their use *in situ*.

The data obtained from this study can comprise an important contribution to the knowledge of the toxicology of DEHP in *C. elegans*, about which little data are available. A link or correlation between a validated toxicity endpoint (e.g., growth and reproduction) and upstream-induced gene expression is interesting, particularly for ecotoxicological purposes. Direct experimental demonstrations of the wider relationships between molecular-/biochemical level effects and their subsequent consequences at higher levels of biological organization are needed in order to establish causal relationships. The characterization of the causal relationships between the molecular level responses and the effects at higher biological levels will help to define the sublethal hazards of chemicals in *C. elegans*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2007.05.008.

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