

Eco-toxicological effect of polycyclic musks for *C. elegans*

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Abstract

Laundry detergents, soaps and cosmetics often include fragrance compounds such as 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydro naphthalene (AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzopyran (HHCB), that are polycyclic musks (PCMs). We assessed the potential toxicological effects associated with AHTN and HHCB using *Caenorhabditis elegans* (*C. elegans*) as a model soil organism. We examined changes in the test endpoints of growth and maturation such as body length, ratio (%) of gravid worms and fecundity. The 50% lethal concentrations (LC₅₀) in *C. elegans* were >255.2 mg/l (AHTN) and >194.6 mg/l (HHCB), respectively. Growth tests showed that the lowest observed effective concentrations (LOEC) of AHTN and HHCB in *C. elegans* were 12.8 and 9.8 mg/l, respectively. The estimated LOEC values in maturation tests were 6.4 mg/l for AHTN and 9.8 mg/l for HHCB. The estimated LOEC for HHCB in reproduction tests was 19.5 mg/l, but this could not be determined for AHTN. We also investigated the suitability of the DNA microarray as an eco-toxicogenomic approach to determine the physiological function of PCMs in *C. elegans*. Both AHTN (25.5 mg/l) and HHCB (19.5 mg/l) were examined mainly as cytochrome P450 (CYP) gene expression using a customized chip for *C. elegans*. Among 79 CYP genes, 26 and 17 were up-regulated upon exposure to AHTN and HHCB, respectively.

Keywords: DNA microarray, AHTN, HHCB, *C. elegans*

Introduction

The polycyclic musks (PCMs), 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta- γ -2-benzopyran (HHCB), are used as fragrances in laundry detergents, soaps and cosmetics and their estimated combined global production is 6,000 tons per year (Rimkus, 1999). Consequently, these compounds have the potential for aquatic contamination via wastewater treatment plants. Furthermore, the lipophilic characteristics of these compounds mean that they can accumulate in fish and other aquatic organisms, and AHTN and HHCB have also been detected in human adipose tissue and breast milk (Rimks and Wolf, 1996). Furthermore, HHCB and AHTN have recently been detected in river runoff and sewage sludge (Boxall *et al.*, 2004; McArdell *et al.*, 2003). We have found HHCB and AHTN both in atmospheric suspended particulate matter (APM) and in sedimentation particles at concentrations of 14.04 and 3.58 pg/m³,

and at 80 and 37 ng/g, respectively (Mori *et al.*, 2004).

Caenorhabditis elegans (*C. elegans*) is a free-living, bacterivorous soil nematode with a transparent body that principally inhabits the liquid phase of soils. It occurs naturally as either a self-fertile hermaphrodite capable of producing >300 self-progeny, or as males that can cross-fertilize hermaphrodites. While adult hermaphrodites are composed of only 959 somatic cells and are only 1 mm long, they contain highly differentiated muscle tissue and well-developed nervous, digestive and reproductive systems. The life cycle of *C. elegans* is approximately 3 days at 20°C and the organism can easily be grown on agar plates or in liquid media containing bacteria as food. In addition, it can be maintained under conditions of limited space and the entire genome has been sequenced. Given these characteristics, *C. elegans* is particularly suited to bioassays of both acute and chronic toxicity testing (Ura *et al.*, 2002; Graves *et al.*, 2005). Most

studies of *C. elegans* have focused on the effects of metals or agricultural chemicals in soil and aquatic environments, but little is understood about the toxic effects of environmental chemicals such as personal care products including PCMs AHTN or HHCB. The present study evaluated the eco-toxicological effects of PCMs on the post-embryonic development, growth, sexual maturation and fecundity of *C. elegans* to determine the eco-toxicological effect of PCMs. We also applied an eco-toxicogenomic approach to determine the physiological function of PCMs using a DNA microarray based on the cytochrome P450 (CYP) genes.

Materials and methods

Chemicals

We obtained HHCB and AHTN from Promochem (Teddington, UK) and stored them in dimethyl sulfoxide (DMSO). All solvents were reagent grade in this study (Wako Pure Chemical Industries Ltd., Osaka, Japan).

Strain and cultivation

The wild type N2 strain of the *C. elegans* nematode was cultivated in S-medium (Sulston and Williams, 1974) seeded with *Escherichia coli* strain DH5 α in sterile 1-l flasks at 20°C and shaken continuously.

Bioassay procedure

Worm populations were age-synchronized for gravity (lethality test) and brench (growth, maturation and reproduction) bioassays to negate the possible influence of age on chemical responsiveness.

Lethality test

Acute toxicity was tested as described (Donkin and Williams, 1995). Mixed-stage worm populations dominated by one-day-old larvae were grown on plates containing nematode growth medium (NGM) at 20°C. The plates were washed gently with K-medium (32 mM KCl, 51 mM NaCl) to remove bacteria and then age-synchronized populations (one-day-old larvae) were isolated in glass centrifuge tubes containing Sephadex G-25 after allowing sufficient time for the worms to settle by gravity. The supernatant was removed, the worms were rinsed with K-medium to completely remove bacteria and then transferred to 24-well tissue culture plates (10 worms/well) containing 0.5 ml K-medium and various nominal concentrations of AHTN (4.0, 8.0, 16.0, 31.9, 63.8, 127.6, 255.2 mg/l) and HHCB (3.0, 6.1, 12.2, 24.3, 48.7, 97.3, 194.6 mg/l). All experiments proceeded in triplicate and the control was DMSO. The nematodes were deprived of food during 24 h of chemical exposure at 20°C. The number of dead and/or live worms was determined as the absence of a response to physical stimulation

with a platinum wire under a dissecting microscope (ECLIPSE TS100; Nikon, Tokyo, Japan). The median lethal concentration (LC₅₀) was calculated using the PROBIT method.

Growth and maturation tests

Growth and maturation endpoints were determined by assessing the body length and ratio (%) of gravid worms. Eggs were collected in sodium hypochlorite, placed on NGM agar plates without food and incubated overnight to obtain worms synchronized at one day of age. Plates containing these worms were then gently washed with S-basal buffer to remove any dead nematodes. Ten worms (0.22 \pm 0.02 mm mean body length) were dispensed into 24-well tissue culture plates containing 0.5 ml of S-medium and varying nominal concentrations of xenobiotics per well (AHTN, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8, 25.5 mg/l; HHCB, 0.3, 0.6, 1.2, 2.4, 4.9, 9.8, 19.5 mg/l). All experiments proceeded in triplicate and the worms were administered with food during the test. Body length and the ratio (%) of gravid worms were assessed on one type of plate and reproductive toxicity was tested on another. Worms were exposed to PCMs for 55 \pm 1 h at 20°C and no eggs were laid during this period. The number of gravid worms (eggs visible inside the body) and body length was determined in plates under a dissecting microscope (ECLIPSE TE2000-U; Nikon).

Reproduction test

The worms were also exposed to chemicals during the reproduction test. After exposure, randomly selected worms were transferred from the plates used for growth and maturation into wells containing 0.5 ml of test solution in new bioassay plates. The worms were exposed to the agents in triplicate wells under the conditions described above. Brood size was determined daily and new test solution was added until egg production ceased.

RNA extraction

Following 24 h of exposure to AHTN (25.5 mg/l) and HHCB (19.5 mg/l), we prepared RNA from harvested *C. elegans*. Frozen nematode pellets were ground into a fine powder using a liquid nitrogen-chilled mortar and pestle before homogenization in Trizol as a described (Liao and Freedman, 1998). Poly(A)⁺ RNA was purified using an Oligotex mRNA kit (Takara Bio Inc., Shiga, Japan).

DNA microarray chip

We constructed custom DNA microarray chips on glass slides spotted mainly with 79 cytochrome P450s (CYPs) and reference genes (ACT: 4, *ama-1*: 1) (Arizono, 2007).

Microarray analysis

Control and experimental cDNA samples were labeled with Cy3-dUTP (Amersham) and Cy5-dUTP (Amersham), respectively and hybridized to the customized *C. elegans* genome microarray (focusing on approximately 300 metabolism-related genes) in a chamber placed in a water bath for 16 at 42°C. Dye was swapped several times. Data were extracted from the microarrays using Scan Array and Quant Array. The normalized Cy5/Cy3 ratios were >2.0 and <0.5 for up- and down-regulation, respectively.

Statistical analysis

All data were statistically analyzed using Stat View J 5.0 (SAS Institute Inc., Cary, NC, USA) with all experimental data checked for assumptions of homogeneity of variance across manipulations using the Bartlett test. Once assumptions had been satisfied, data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (Dunnett, 1995). When homogeneity was not evident in the data, we applied the nonparametric Kruskal-Wallis test, followed by the Mann-Whitney *U* test with Bonferroni adjustment (Glantz, 1992). Differences were considered significant at $p < 0.05$.

Results

Eco-toxicological effects of polycyclic musk AHTN

We assessed the lethality, growth and maturation, and reproduction of *C. elegans* exposed to AHTN. Table 1 summarizes the LOEC for *C. elegans* as well as changes in body length, ratio (%) of gravid worms and brood size after AHTN exposure. Lethality testing showed that AHTN up to 255.2 mg/l did not elicit significant effects in *C. elegans* although 12.8 mg/l significantly decreased mean body length. The ratio of gravid worms significantly decreased after

exposure to >6.4 mg/l of AHTN. Interestingly, brood size was not significantly altered.

Eco-toxicological effects of polycyclic musk HHCB

We assessed the lethality, growth and maturation, and reproduction of *C. elegans* exposed to HHCB. Table 1 summarizes the LOEC for *C. elegans* as well as changes in body length, ratio (%) of gravid worms and brood size after HHCB exposure. Lethality testing showed that HHCB did not elicit significant effects in *C. elegans* up to 194.6 mg/l when the effect on mean body and brood size became statistically significant. Body length and the ratio (%) of gravid worms both decreased at doses of 9.8 and 19.5 mg/l, respectively. The effect of HHCB on brood size at a concentration of 19.5 mg/l was not estimated.

DNA microarray analysis

We applied DNA microarray analysis as an ecotoxicogenomic approach, using a custom chip mainly comprising 79 CYP genes for *C. elegans*. The growth, maturation, and reproduction were significantly decreased in *C. elegans* exposed to 25.5 mg/l AHTN or 19.5 mg/l HHCB. Thus, these concentrations were selected as the PCM doses for the microarray analysis. Among 79 CYP genes, 26 and 17 of them including CYP14As, CYP34As and CYP35As were up-regulated by exposure to either AHTN or HHCB (Table 2). In contrast, only AHTN exposure affected CYP33B1, 33C5, 33E2, 33E3, 13A11, 34A6, 35B1, 35D1, and 42A1 (Table 2).

Discussion

The ease of culture, short assay duration and wealth of available biological information has resulted in *C. elegans* being among the best characterized of the invertebrates. To investigate the eco-

Table 1. The summary of eco-toxicological effects of AHTN and HHCB on the lethal toxicity and post-embryonic development.

	AHTN	HHCB	References
Concentration in water	0.0044 ^{a)}	0.006 ^{a)}	Rimkus, 1999
Concentration in dry sludge from sewage plants	34 ^{b)}	63 ^{b)}	Rimkus, 1999
<i>Danio retio</i> : Survival 21 day – LC ₅₀	0.45 ^{a)}	0.31 ^{a)}	Tas <i>et al.</i> , 1997
<i>Pimephales promelas</i> : Survival LOEC at 36 days	0.14 ^{a)}	0.14 ^{a)}	Balk and Ford, 1999
<i>Pimephales promelas</i> : Development LOEC at 36 days	0.14 ^{a)}	0.14 ^{a)}	Balk and Ford, 1999
<i>Oncorhynchus mykiss</i> : Reproduction 21-day EC ₅₀	0.28 ^{a)}	0.24 ^{a)}	Tas <i>et al.</i> , 1997
<i>Eisenia fetida</i> : Reproduction and food consumption LOEC at 8 weeks	250 ^{b)}	105 ^{b)}	Balk and Ford, 1999
<i>Eisenia fetida</i> : Reproduction and food consumption NOEC at 8 weeks	105 ^{b)}	45 ^{b)}	Balk and Ford, 1999
<i>C. elegans</i> : Survival 24 hr - LC50	>255.2 ^{a)}	>194.6 ^{a)}	Present study
<i>C. elegans</i> : Development LOEC at 60 hr	12.8 ^{a)}	9.8 ^{a)}	Present study
<i>C. elegans</i> : Maturation LOEC at 60 hr	6.4 ^{a)}	9.8 ^{a)}	Present study
<i>C. elegans</i> : Reproduction LOEC at 3 days	>25.5 ^{a)}	19.5 ^{a)}	Present study

a) mg/l, b) mg/kg

Table 2. The *C. elegans* CYP genes induced by treatment with AHTN and HHCB.

Gene name	AHTN	HHCB	Gene name	AHTN	HHCB
	25.5 mg/l	19.5 mg/l		25.5 mg/l	19.5 mg/l
	Ratio	Ratio		Ratio	Ratio
<i>CYP13A11</i>	2.06	<i>1.06</i>	<i>CYP34A6</i>	2.16	<i>0.54</i>
<i>CYP13B2</i>	1.97	2.38	<i>CYP34A8</i>	2.31	2.55
<i>CYP14A1</i>	2.43	2.34	<i>CYP34A9</i>	4.06	3.11
<i>CYP14A2</i>	2.81	5.07	<i>CYP34A10</i>	7.78	13.04
<i>CYP14A3</i>	5.52	9.99	<i>CYP35A1</i>	5.92	6.80
<i>CYP14A4</i>	2.03	2.26	<i>CYP35A2</i>	6.00	6.21
<i>CYP14A5</i>	3.03	2.50	<i>CYP35A3</i>	3.28	4.01
<i>CYP33B1</i>	2.16	<i>1.52</i>	<i>CYP35A4</i>	3.92	4.17
<i>CYP33C5</i>	2.22	<i>0.96</i>	<i>CYP35A5</i>	3.45	3.80
<i>CYP33E2</i>	2.14	<i>0.70</i>	<i>CYP35B1</i>	2.61	<i>0.79</i>
<i>CYP33E3</i>	2.97	<i>1.40</i>	<i>CYP35C1</i>	2.73	4.97
<i>CYP34A1</i>	2.01	<i>1.41</i>	<i>CYP35D1</i>	2.46	<i>0.50</i>
<i>CYP34A4</i>	3.18	2.12	<i>CYP42A1</i>	2.38	<i>1.54</i>
<i>CYP34A5</i>	2.34	2.31			

The italic values in the table were found by not regulation the tested dose range.

toxicological effect of PCMs, we evaluated post-embryonic development, growth, sexual maturation and fecundity in *C. elegans* exposed to AHTN and HHCB. To further analyze the potential effects of PCMs on the physiological function of invertebrates, we investigated the molecular mechanisms of these compounds using a customized DNA microarray chip based on the CYPs genes.

The LOECs for the effect of AHTN and HHCB on the growth and maturation of *C. elegans* were 12.8 and 9.8 mg/l, respectively and 6.4 and 9.8 mg/l, respectively. In addition, the estimated LOEC for HHCB and AHTN in reproduction tests was 19.5 and >25.5 mg/l, respectively. In contrast, the LOECs for 32 days post-hatching (36 days overall) are 0.14 mg/l for AHTN and HHCB in *Pimephales promelas* (Balk and Ford, 1999). The 21-day EC₅₀ values of AHTN and HHCB for *Oncorhynchus mykiss* reproduction are 0.28 and 0.24 mg/l, respectively (Tas *et al.*, 1997). Compared with these organisms, *C. elegans* could tolerate relatively high PCM concentrations (Table 1). Since both AHTN and HHCB have been detected in sewage treatment plant water at maximal concentrations of 4.4 and 6 µg/l, respectively (Rimks, 1999), the acute eco-toxicological effects associated with PCMs in the aquatic environment are apparently lower than the concentrations tested in the present study.

Our DNA microarray analysis revealed that AHTN or HHCB exposure up-regulated 16 CYPs in *C. elegans* including CYP14As, 34As and 35As, indicating that these CYPs are involved in the potential eco-toxicogenomic effects of PCMs on *C. elegans*. This is the first report to describe CYP gene expression elicited by AHTN and HHCB in *C. elegans*.

Based on the phylogenetic analysis of *C. elegans* CYP genes reported by Gotoh *et al.*, the CYP genes up-regulated by AHTN and HHCB were comparable

with the findings of several reports. The *C. elegans* CYP genes up-regulated by AHTN and by HHCB were mainly positioned in the rat CYP2A2 clade. We previously showed that the CYP genes up-regulated by exposure to nonylphenol are also positioned in this clade (Arizono, 2007), whereas few up-regulated genes were positioned in the rat CYP3A1 clade. These results suggest that *C. elegans* CYPs as well as rat CYP2A2 are influenced by exposure to specific PCMs. The analysis of CYP gene expression in *C. elegans* is important for studying the potential ecotoxicological effects of environmental chemicals including PCMs in this model organism.

References

- Arizono, K. (2007) *C. elegans* microarray as bioassay for ecological impact by environmental pollutants. *Cell Technology*, **26**, 1408-1411. (in Japanese).
- Balk, F. and Ford R.A. (1999) Environmental risk assessment for the polycyclic musks, AHTN and HHCB. II. Effect assessment and risk characterisation. *Toxicol. Lett.*, **111**, 81-94.
- Boxall, A.B.A., Fogg, L.A., Blackwell, P.A., Kay, P., Pemberton, E.J. and Croxford, A. (2004) Veterinary medicines in the environment. *Rev. Environ. Contam. Toxicol.*, **180**, 1-91.
- Donkin, S.G. and Williams, P.L. (1995) Influence of developmental stage, salts and food presence on various and endpoints using *Caenorhabditis elegans* for aquatic toxicity testing. *Environ. Toxicol. Chem.*, **14**, 2139-2147.
- Dunnett, C.W. (1955) Multiple comparison procedure for comparing several treatments with a control. *J. Am. Stat. Assoc.*, **50**, 1096-1121.
- Glantz, S.A. (1992) Primer of Biostatistics, 3rd ed McGraw-Hill, New York, NY, USA.
- Gotoh, O. (1998) Divergent structures of *Caenorhabditis elegans* cytochrome P450 genes suggest the frequent loss and gain of in trons during the evolution of nematodes. *Mol. Biol. Evol.*, **15**, 1447-1459.
- Graves, A.L., Boyd, W.A. and Williams, P.L. (2005) Using transgenic *Caenorhabditis elegans* in soil toxicity testing. *Arch. Environ. Contam. Toxicol.*, **48**, 490-494.

- Liao, V. H. and J. H. Freedman. (1998) Cadmium-regulated genes from the nematode *Caenorhabditis elegans*. Identification and cloning of new cadmium-responsive genes by differential display. *J. Biol. Chem.*, **273**, 31962-31970.
- McArdell, C.S., Molner, E., Suter, M. J-F. and Giger, W. (2003) Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. *Environ. Sci. Technol.*, **37**, 5479-5486.
- Mori, T., Arizono, K. and Takemasa, T. (2004) The (anti) estrogen activities of atmospheric suspended particulate matter estimated by yeast system. *Proc. International Joint Conference Risk Assessment and Management with SRA Japan & China / KOSET / SETAC Asia / Pacific*, pp.120.
- Rimkus, G.G. (1999) Polycyclic musk fragrances in the aquatic environment. *Toxicol. Lett.*, **111**, 37-56.
- Rimkus, G.G. and Wolf, M. (1996) Polycyclic musk fragrances in human adipose tissue and human milk. *Chemosphere*, **33**, 2033-2043.
- Sulston, J. E. and Brenner, S. (1974) The DNA of *Caenorhabditis elegans*. *Genetics*, **77**, 95-104.
- Tas, J.W., Balk, F., Ford, R.A. and van de Plassche, E.J. (1997) Environmental risk assessment of musk ketone and musk xylene in The Netherlands in accordance with the EU-TGD. *Chemosphere*, **35**, 2973-3002.
- Ura, K., Kai, T., Sakata, S., Iguchi, T. and Arizono, K. (2002) Aquatic acute toxicity testing using the nematode *Caenorhabditis elegans*. *J. Health Sci.*, **48**, 583-586.

