Ecotoxicological Effect of Polycyclic Musks on *Caenorhabditis elegans*

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The polycyclic musks (PCMs), 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtha-lene (AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB), are widely used as fragrance compounds in laundry detergents, soaps and cosmetics. To assess the potential toxicological effects associated with AHTN and HHCB, *Caenorhabditis elegans* (*C. elegans*) was used as a model organism for eco-toxicity testing. We examined acute toxicity using 50% lethal concentrations (LC_{50}) after 24 hr PCM exposure and also examined changes in the test endpoints of growth and maturation such as body length, percentage of gravid worms and fecundity. The LC_{50} for *C. elegans* was found to be more than 255.2 mg/l for AHTN and 194.6 mg/l for HHCB. In growth tests, the lowest observed effect concentrations (LOEC) in *C. elegans* for AHTN and HHCB were 12.8 mg/l and 9.8 mg/l, respectively. In maturation tests, LOECs were estimated at 6.4 mg/l for AHTN and 9.8 mg/l for HHCB. In reproduction tests, while maximum LOECs of 19.5 mg/l were observed for HHCB, concentrations of more than 25.5 mg/l were obtained for AHTN.

Key words —— polycyclic musk, *Caenorhabditis elegans*, soil organism, 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtha-lene, 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran

INTRODUCTION

The polycyclic musks, 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphtha-lene (AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB), are used as fragrance compounds in laundry detergents, soaps and cosmetics.[1,2] Their combined global production has been estimated at approximately 6000 t per year.[1,2] Consequently, these chemicals have the potential to contaminate the aquatic environment via wastewater treatment plants. Furthermore, given their lipophilic characteristics, these compounds exhibit the ability to accumulate in fish and other aquatic organisms, and AHTN and HHCB have also been detected in human adipose tissue and breast milk.[3,4] There are also recent reports of HHCB and AHTN being detected in river runoff and sewage sludge.[5–10] In a previous study, we reported finding HHCB and AHTN in both atmospheric suspended particulate matter (APM) and in sedimentation particles at concentrations of 14.04 and 3.58 pg/m^3, and 80 and 37 ng/g, respectively.[9] The structure and some relevant characteristics of AHTN and HHCB are summarized in Table 1.

Caenorhabditis elegans (*C. elegans*) is a free-living, bacterivorous soil nematode with a transparent body that principally inhabits the liquid phase of soils. *C. elegans* 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 have body lengths of only 1 mm, they contain highly differentiated muscle tissue and well developed nervous, digestive and reproductive systems. *C. elegans* has a short life cycle spanning approximately three days at 20°C and can easily be grown on agar plates or in liquid media containing bacteria as food. In addition, the organism can be maintained under condi-

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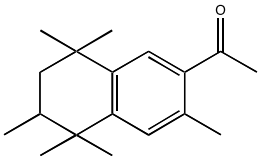
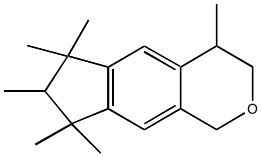
Table 1. Polycyclic Musks and Selected Properties[10)]

Compound	Structure formula	CAS No.	Molecular weight	Water solubility (mg/l)	log K_{ow}	Vapor pressure (Pa)
AHTN		1506-02-1	258.4	1.25	5.7	0.068
HHCB		1222-05-5	258.4	1.75	5.9	0.073

HHCB: 1,3,4,6,7,8-hexahydro-4,6,6,7,8-hexamethylcyclopenta-γ-2-benzopyran. AHTN: 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene.

tions of limited space and the entire genome has been sequenced. Given these characteristics, *C. elegans* is particularly well suited as an organism for bioassays involving both acute and chronic toxicity testing.[11,12)] Most studies on *C. elegans* have focused on the effects of metals or agricultural chemicals in the soil and water environments, but little is known about the toxic effects of environmental chemicals such as personal care products. The objective of this study was thus to evaluate the efficacy of several bioassay methods for estimating the eco-toxicological effects of polycyclic musks such as AHTN and HHCB on the post-embryonic development, growth, sexual maturation and fecundity of the soil organism, *C. elegans*.

MATERIALS AND METHODS

Chemicals —— Both, HHCB and AHTN were obtained from Promochem (Teddington, U.K.) and were dissolved in a solvent, dimethyl sulfoxide (DMSO), for the bioassays. Only reagent grade solvents were used in this study (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Strain and Cultivation —— The wild type N2 strain of the *C. elegans* nematode was used in this study. The N2 strain was grown in S-medium[13)] seeded with *Escherichia coli* strain DH5α in a sterile 1 l flask at 20°C and shaken continuously.

Bioassay Procedure —— Given the possible influence of age on chemical responsiveness, age synchronous populations were used for gravity (lethality test) and breech (growth, maturation and reproduction tests) bioassays.

Lethality Test —— Acute toxicity testing was performed according to the procedure of Donkin and Williams.[14)] Mixed-stage worm populations dominated by one-day-old larvae were grown on plates containing nematode growth medium (NGM) incubated at 20°C. The plates were washed gently with K-medium (32 mM KCl, 51 mM NaCl) to remove bacteria completely and age-synchronous populations (one-day-old larvae) were isolated using a glass centrifuge tube containing Sephadex G-25 after allowing for sufficient time for worms to settle by gravity. The supernatant was removed and worms were rinsed with K-medium to completely remove bacteria. Worms were then transferred to wells on a 24-well tissue culture plate (10 worms/well) containing 0.5 ml K-medium and varying nominal concentrations of AHTN and HHCB (AHTN: 4.0, 8.0, 16.0, 31.9, 63.8, 127.6, 255.2 mg/l; HHCB: 3.0, 6.1, 12.2, 24.3, 48.7, 97.3, 194.6 mg/l). All treatments were preformed in triplicate and DMSO was used as a control. Nematodes were deprived of food for the 24 hr duration of chemical exposure at 20°C. The number of dead and/or live worms was determined by lack of response to physical stimulation with a platinum wire under a dissecting microscope (Nikon, ECLIPSE, TS100, Japan). The median lethal concentration (LC_{50}) was calculated using the PROBIT method.

Growth and Maturation Tests —— Body length and percentage of gravid worms were used to assess growth and maturation endpoints. Eggs were collected by sodium hypochlorite treatment and placed on NGM agar plates without a food and incubated

overnight to obtain age-synchronous worms (one-day old). Plates containing the age-synchronous worms were then gently washed with S-basal buffer to remove any dead nematodes. Ten worms (0.22 ± 0.02 mm mean body length) were then dispensed into each well of a 24-well tissue culture plate 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 treatments were performed in triplicate and worms were administered food during the test. Two types of test plates were prepared: the first plate was used for assessing body length and the percentage of gravid worms, and the second type was used for conducting the reproductive toxicity test described below. Worms were exposed to PCMs for 55 ± 1 hr at 20°C and egg laying was not observed during this period. Plates were observed under a dissecting microscope (Nikon, ECLIPES, TE2000-U) to determine the number of gravid worms (eggs visible inside the body) and body length.

Reproduction Test —— In addition to the growth maturation test, worms were also exposed to chemicals during the reproduction test. After exposure, a randomly chosen selected worm was transferred from the second plate used for growth and maturation into a well containing a 0.5 ml test solution prepared using a new bioassay plate. Three replicate wells were used for each treatment and animals were exposed to chemicals under the same conditions as described previously. Brood size was counted everyday and new test solution added until egg production stopped.

Statistical Analysis —— All statistical analyses were performed using Stat View J 5.0 (SAS Institute Inc., Cary, North Carolina, U.S.A.) with all experimental data checked for assumptions of homogeneity of variance across treatments using a Bartlett test. Once assumptions had been satisfied, data were analyzed by one-way analysis of variance followed by Dunnett's multiple comparison tests.[15] When no homogeneity was observed in the data, a nonparametric Kruskal-Wallis test was used, followed by a Mann-Whitney *U* test with Bonferroni adjustment.[16] Differences were considered significant at $p < 0.05$.

RESULTS

Polycyclic Musk AHTN

We assessed the lethality, growth and maturation, and reproduction associated with AHTN exposure using *C. elegans*. A summary of the lowest observed effect concentrations (LOEC) for *C. elegans* is shown in Table 2. No significant effects were observed after AHTN exposure lethality testing until 255.2 mg/l AHTN (Table 2). Changes in body length, percentage of gravid worms and brood size after AHTN exposure are shown in Figs. 1–3. Exposure to AHTN at 12.8 and 25.5 mg/l had the effect of significantly decreasing mean body length. The percentage of gravid worms significantly decreased after AHTN exposure above 6.4 mg/l. Interestingly, no significant changes in brood size was observed.

Polycyclic Musk HHCB

We assessed the lethality, growth and maturation, and reproduction associated with HHCB exposure using *C. elegans*. A summary of the LOEC for *C. elegans* is shown in Table 2. No lethal toxicity was observed after HHCB exposure up to 194.6 mg/l (Table 2). However, changes in body length, percentage of gravid worms, and brood size after exposure to HHCB are shown in Figs. 1–3. A statistically significant effect of HHCB exposure on mean body and brood size was observed. Body length and the percentage 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.

DISCUSSION

To evaluate the impact of polycyclic musks on the post-embryonic development of *C. elegans*, we tested AHTN and HHCB using a sub-acute bioassay. The results are summarized Table 2 as LOEC data supplemented with No observed effect concentration (NOEC) data from other reports.

We previously demonstrated aquatic acute toxicity testing using PCMs in K-medium without bacteria and showed that LC_{50} after 24 hr (24 hr-LC_{50}) for benzo[*a*]pyrene, nonylphenol, benzophenone, bisphenol A, 17β-estradiol, aldicarb, styrene monomer, styrene dimmer, styrene trimmer, and ponasterone A, were 0.047–0.053, 6.7–7.7, 52.1–62.1, 301.1–349.5, > 1000, > 40, > 20, > 20, > 20, and > 10 mg/l, respectively.[11] In this study, the acute toxicities of AHTN and HHCB (LC_{50} in *C. elegans*) were found to be more than 255.2 and 194.6 mg/l, respectively. In trials using *Daphnia magna*,[17,18] 21-day LC_{50} concentrations of 0.34 mg/l for AHTN

Table 2. 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 sediment	0.02–1.10[b)]	0.22–0.92[b)]	Fromme *et al*., 2001
Concentration in dry sludge from sewage plants	34[b)]	63[b)]	Rimkus, 1999
Daphnia magna: Survival 21 day-LC_{50}	0.34[a)]	0.29[a)]	Wthrich, 1996a, b
Danio retio: Survival 21 day-LC_{50}	0.45[a)]	0.31[a)]	Tas *et al*., 1997
Pimephales promelas: Survival LOEC at 36 days	0.14[a)]	0.14[a)]	Balk and Ford, 1999b
C. elegans: Survival 24 hr-LC_{50}	> 255.2[a)]	> 194.6[a)]	Present study
C. elegans: Development LOEC at 60 hr	12.8[a)]	9.8[a)]	Present study
Pimephales promelas: Development LOEC at 36 days	0.14[a)]	0.14[a)]	Balk and Ford, 1999b
C. elegans: Maturation LOEC at 60 hr	6.4[a)]	9.8[a)]	Present study
Oncorhynchus mykiss: Reproduction 21-day EC_{50}	0.28[a)]	0.24[a)]	Tas *et al*., 1997
Eisenia fetida: Reproduction and food consumption LOEC at 8 weeks	250[b)]	105[b)]	Balk and Ford, 1999b
Eisenia fetida: Reproduction and food consumption NOEC at 8 weeks	105[b)]	45[b)]	Balk and Ford, 1999b
C. elegans: Reproduction LOEC at 3 days	> 25.5[a)]	19.5[a)]	Present study

a) mg/l, *b*) mg/kg.

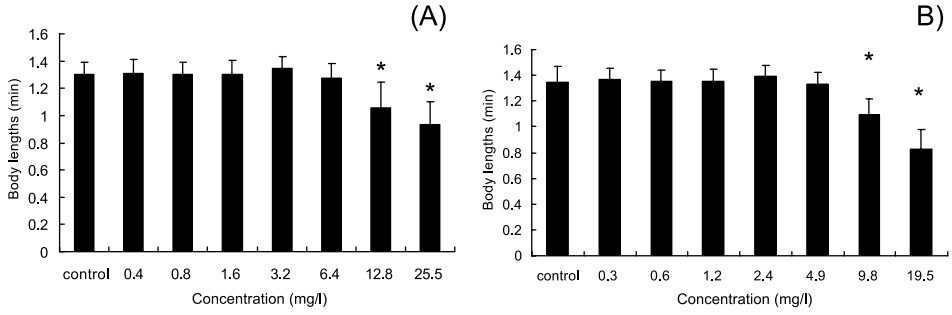


Fig. 1. Body Length of *C. elegans* after Exposure to AHTN (A) and HHCB (B) in the Growth and Maturation Tests

The dose indicates the nominal dose. Asterisk (*) denotes significant differences relative to the control group ($p < 0.05$). Error bars represent the standard deviation about the mean ($n = 9$).

and 0.29 mg/l for HHCB were observed. Similarly, 21-day LC_{50} levels in *Danio retio* were 0.45 mg/l for AHTN and 0.31 mg/l for HHCB. These findings illustrate that the acute toxicity of PCMs in *C. elegans* is therefore lower than it is in *Daphnia magna* and *Danio retio*.[19)]

The ease with which this test organism can be cultured, combined with the short duration required for conducting assays and the wealth of biological information that exists for this species has resulted in *C. elegans* being among the best characterized invertebrates. We therefore used PCMs, not only to test acute toxicity, but also to determine the endpoints related to growth, maturation and reproduction in *C. elegans*. These tests revealed that the LOECs for the effect of PCMs on the growth and reproduction of *C. elegans* were as follows: growth tests, 12.8 mg/l (AHTN) and 9.8 mg/l (HHCB); maturation tests, 6.4 mg/l (AHTN) and 9.8 mg/l (HHCB) (Table 2). In addition, the LOEC for HHCB

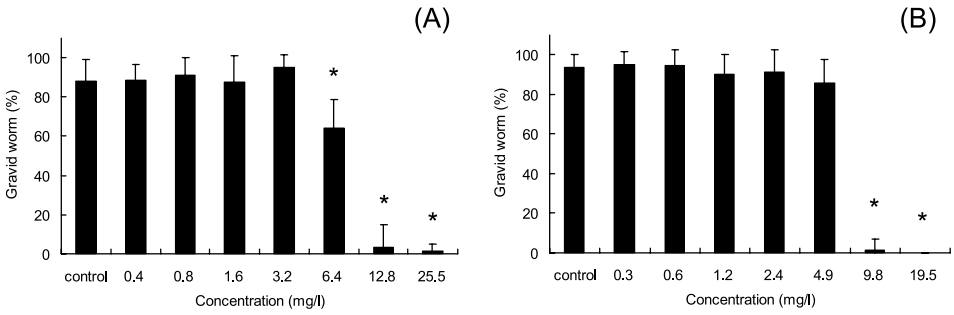


Fig. 2. The Percentage of *C. elegans* with Eggs after Exposure to AHTN (A) and HHCB (B) in the Growth and Maturation Tests

The dose indicates the nominal dose. Asterisk (*) denotes significant difference relative to the control group ($p < 0.05$). Error bars represent standard deviation about the mean ($n = 9$).

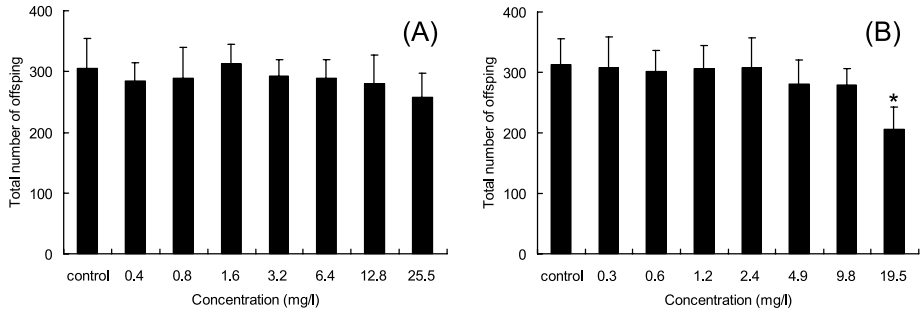


Fig. 3. Fecundity of *C. elegans* after Exposure to AHTN (A) and HHCB (B) in the Reproduction Test

The dose indicates the nominal dose. Asterisk (*) denotes significant difference relative to the control group ($p < 0.05$). Error bars represent the standard deviation about the mean ($n = 9$).

in reproduction tests was estimated at 19.5 mg/l, but for AHTN it was more than 25.5 mg/l. Conversely, the LOEC for 32 days post hatching (36 days overall) was 0.14 mg/l for AHTN and HHCB in *Pimephales promelas*.[20] The 21-day EC_{50} for reproduction in *Oncorhynchus mykiss* was reported to be 0.28 mg/l for AHTN and 0.24 mg/l for HHCB.[19] Compared to other environmental organisms, *C. elegans* was capable of withstanding relatively high PCM concentrations (Table 2). Since both AHTN and HHCB have been detected in sewage treatment plant water at maximum concentrations of 4.4 μg/l for AHTN and 6 μg/l for HHCB,[1] it would appear that the acute ecotoxicological effects associated with PCMs in the aquatic environment are lower than the concentrations tested in this study.

At present, the earthworm (*Eisenia fetida*, *E. fetida*) is a commonly used terrestrial invertebrate test organism for ecological risk assessment by the Organization for Economic Co-operation and Development (OECD) and Environmental Protection Agency (EPA).[21,22] However, in addition to requiring 41-days for acute toxicity testing, mortality and growth inhibition tests for adult earthworms exposed to AHTN and HHCB in artificial soil revealed that no mortality or growth inhibition occurred in adult earthworms exposed to AHTN and HHCB (AHTN: 250 mg/kg; HHCB: 250 mg/kg) after four weeks.[20] However, these authors also found that 100% mortality occurred after 14 days of exposure to 1000 mg/kg for both AHTN and HHCB, and that growth was not significantly inhibited at the highest concentrations of HHCB applied to earthworms (250 mg/kg). In addition, reproduction in earthworms was not significantly affected by AHTN or HHCB at concentrations of up to 105 and 45 mg/kg, respectively. Based on these findings, they proposed that the LOEC of AHTN and HHCB for reproduction tests in *E. fetida* was approximately 250 and 105 mg/kg, respectively. In *C. elegans*, we found that both AHTN and HHCB affect growth (AHTN: 6.2 mg/l; HHCB: 9.5 mg/l), maturation (AHTN: 12.5 mg/l; HHCB: 9.5 mg/l) and reproduction (HHCB: 19 mg/l). Although Peredney and Williams reported that the sensitivities of *C. elegans* and *E. fetida* in response to metals were similar,[23] *C.*

elegans appeared slightly more sensitive to PCM exposure in growth/maturation tests than *E. fetida*, despite the use of a different test system (Table 2).

In previous growth and maturation studies, test solutions were not replaced regularly and it is therefore not clear what the exact concentration of PCMs was at the time the experiments were conducted. Balk and Ford reported that AHTN and HHCB added to white rot fungus cultures disappeared within 6- and 3-days, respectively.[24] It is thought that the effects of AHTN and HHCB might impact growth and maturation for at least 1-day.

PCMs (AHTN, HHCB and their related compounds) have been proposed to have an adverse effect on the sediments of aquatic ecosystems.[1] Although the concentrations of PCMs in aquatic sediments in Berlin ranged between 0.02 to 1.10 mg/kg for AHTN and 0.22 to 0.92 mg/kg for HHCB (Table 2),[25] PCM levels have been found to vary widely in the environment and maximum concentrations for AHTN and HHCB of 34 and 63 mg/kg dry matter have been found in sludge from sewage plants.[1] In addition, it is likely that the concentration of PCMs in aquatic sediments may be correlated to the distance from point sources of pollution such as water treatment facilities. Taken together, these findings are of concern given their potentially deleterious effects on the survival of invertebrates.

C. elegans is thus a suitable test organism for ecotoxicological assessments of several environmental chemicals. We have established ecotoxicological tests that can be used to assess, not only acute toxicity, but also the endpoints associated with processes such as growth, maturation and reproduction. Within this context, the test system described here provides a comprehensive and systematic method for assessing the ecotoxicological impact of a variety of environmental chemicals. Consequently, future research on PCMs will focus on the ecotoxicogenomic attributes of *C. elegans* using DNA microarrays.

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