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published in
Environmental Science and Technology
2004

DOI (link to publisher)
10.1021/es034648y

document version
Publisher's PDF, also known as Version of record

Link to publication in VU Research Portal

citation for published version (APA)

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In Vitro and in Vivo Antiestrogenic Effects of Polycyclic Musks in Zebrafish

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The polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,7,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB) are used as fragrance ingredients in perfumes, soaps, and household cleaning products. They are known to be ubiquitously present in the aquatic environment, and because of their lipophilic nature, they tend to bioaccumulate in aquatic biota. In surface waters, concentrations between 1 ng/L and 5 μg/L have been found, depending mainly on the proportion of sewage effluents in the water. In fish, under normal environmental conditions, concentrations in the microgram per kilogram fresh weight (fw) range are found. In a previous study we showed that AHTN and HHCB showed dose-dependent antagonistic effects in vivo using a transgenic zebrafish assay (1). In sewage water effluents concentrations of AHTN and HHCB were found up to 4 and 13 μg/L, respectively (3). Environmental concentrations of AHTN and HHCB were found up to 3 and 6.5 mg/kg of lipid (0.7 and 1.5 mg/kg fresh weight (fw)), respectively, with maximal concentrations which are 3 times higher (3). In other fish species lower concentrations were found, due to their lower fat content. Also in surface waters with a low or moderate input of sewage water, lower concentrations in the microgram per kilogram fw range were found.

Surprisingly, despite the fact that polycyclic musks are ubiquitously present in the aquatic environment and tend to bioaccumulate in aquatic biota, little is known of their ability to disrupt endocrine systems, such as estrogen homeostasis. Using cell lines stably transfected with an estrogen-responsive reporter construct and the human estrogen receptor α (ERα) or ERβ, we recently found that both polycyclic musks show antiestrogenic and weak estrogenic effects, depending on the cell line and ER subtype used (5). In the current study, we were interested to assess the (anti)estrogenic effects of these compounds in fish. Examples can be found of wild fish populations showing disturbances of normal endocrine functions, and occurrences of intersex and testis abnormalities (6–8). These effects have been associated with the exposure to natural, synthetic, and xenoestrogens in the aquatic habitats. Here, we assess the (anti)estrogenic effects of AHTN and HHCB on the zebrafish ERα, ERβ, and the recently cloned zfERγ. The experiments show no agonism of the musks on all three receptors, but antagonism on zfERγ. Furthermore, we investigated the antiestrogenic effects in vivo using a transgenic zebrafish assay (12), and we correlated these effects to the measured internal dose. Both AHTN and HHCB showed dose-dependent antagonistic effects at test concentrations of 0.1 and 1 μM, and internal doses which are roughly 600 times higher. As far as we know, in vivo antiestrogenic effects of environmental contaminants in fish have never been described before in the scientific literature.

Introduction

Two chemicals that are ubiquitously present in the aquatic environment are the polycyclic musks 6-acetyl-1,1,2,4,4,7-hexamethyltetraline (AHTN) and 1,2,4,6,7,8-hexahydro-4,6,7,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB). Polycyclic musks are used as fragrance compounds in laundry detergents, soaps, and cosmetics, with a worldwide production volume of about 6000 tons per year (1). They may reach the aquatic environment via wastewater treatment plants, and consequently, because of their lipophilic character, they tend to bioaccumulate in fish and other aquatic organisms. Log K_{ow} values of 5.7 and 5.9 have been found for AHTN and HHCB, respectively (1). Concentrations of HHCB vary from 1 ng/L in clean surface waters up to 5 μg/L in surface water with a high proportion of effluents of sewage treatment plants (2, 3). In sewage water effluents concentrations of AHTN and HHCB were found up to 4 and 13 μg/L, respectively (3). Environmental concentrations of AHTN and HHCB were found up to 3 and 6.5 mg/kg of lipid (0.7 and 1.5 mg/kg fresh weight (fw)), respectively, with maximal concentrations which are 3 times higher (3). In other fish species lower concentrations were found, due to their lower fat content. Also in surface waters with a low or moderate input of sewage water, lower concentrations in the microgram per kilogram fw range were found.

Materials and Methods

Chemicals and Reagents. 17β-Estradiol was purchased from Sigma (St. Louis, MO). HHCB and AHTN were kind gifts from International Flavours and Fragrances (IFF), Hilversum, The Netherlands, and PFW-Aroma Chemicals, Barneveld, The Netherlands, respectively. 4-Hydroxytamoxifen was a kind gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, U.K.).

Cell Culture. Human embryonal kidney 293 (HEK293) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM)
and Ham's F12 medium (DF; Life Technologies Inc., Gaithersburg, MD) supplemented with 7.5% fetal calf serum (FCS) (InterM, Linz, Austria). The cell line was cultured at 37 °C and 7.5% CO₂.

**Plasmids.** The human ERα expression plasmid pSGS-HEGO was kindly provided by Dr. P. Chambron (IGBMC, Strasbourg, France). The human ERβ expression plasmid pSGS-HERβ was kindly provided by Dr. J.-Å. Gustafsson (Karolinska Institute, Huddinge, Sweden). The estrogen-responsive reporter gene construct (3xERE-TATA-Luc), which contains three copies of a consensus estrogen response element (ERE) containing oligonucleotide and a TATA box, is inserted into the EcoRI site of the multiple cloning site of the pSGS expression vector (Stratagene) are described elsewhere (Zeinstra et al., manuscript in preparation).

**Transient Transfections.** HEK293 cells were plated in 800 μL of phenol red-free DF medium containing 30 mM selenite, 10 μg/mL transferrin, and 0.2% BSA, supplemented with 5% dextran charcoal stripped FCS, at 8 × 10⁴ cells per well of a 24-well plate (Costar). Cells were transfected using the calcium phosphate precipitation method (CaOx) 24 h after plating.

A total amount of 2.0 μg of DNA was transfected, consisting of 0.6 μg of luciferase reporter plasmid, 0.6 μg of PDM-LacZ internal control plasmid, 0.6 μg of pBluescript SK-, and 0.2 μg of ER expression plasmid. After an overnight incubation cells were given fresh media with or without test compounds (dissolved in ethanol, maximum 0.1% solvent).

After 24 h, the cells were lysed in 200 μL of lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT). A 50 μL portion of cell lysate was transferred to a black 96-well plate to which 50 μL of luciferase substrate (LucLite reporter gene assay kit, Packard Instruments, Meriden, CT) was added. Luciferase activity was measured in a Topcount liquid scintillation counter (Packard Instruments) for 0.1 min per well. To correct for variations in transfection efficiencies, β-galactosidase activity was measured (15).

**Transgenic Zebrafish Assay.** The development of the transgenic zebrafish assay is described elsewhere (12). Exposure studies were carried out with homoyzogous F4 juvenile fish of 4–5 weeks of age. Fish (n = 5–6) were exposed for 96 h in 200 mL of acclimated tap water (26–27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:1000 dilution. Fish were fed once daily with live brine shrimp (Artemia salinas). Half of the test medium was renewed daily. At the end of the exposure, fish were sacrificed in ice-water, transferred to Eppendorf vials, and immediately frozen at −80 °C. To assay luciferase activity, Eppendorf vials containing whole fish were transferred to ice, 500 μL of cold lysis solution (1% Triton X-100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA, and 1 mM DTT, pH 7) was added, and the fish were homogenized using a microcuble (Eppendorf). After centrifugation at 12000 rpm for 15 min at 4 °C, duplicate samples of 50 μL of supernatant were transferred to a black 96-well plate (Packard) to which 50 μL of luciferase substrate (LucLite reporter gene assay kit, Packard) was added. Luciferase activity was measured in a scintillation counter (Packard Topcount) for 0.1 min per well.

**Internal Exposure.** To assess the internal concentration of both musks in the zebrafish, six animals were exposed to 0.1 or 1 μM (i.e., 25.8 or 258 μg/L) musk for 1, 2, 3, or 4 days. Fish were exposed in 200 mL of acclimated tap water (26–27 °C) in beaker glasses. The compounds to be tested (dissolved in DMSO) were added to the water in a 1:10000 dilution. Fish were fed once daily with live brine shrimp (A. salinas). Each day, before half of the test medium was refreshed, water samples were taken. After exposure, fish were sacrificed, and cleaned up for GC–MS analysis. The same exposure experiment was done in the absence of zebrafish.

**Extraction and Cleanup of Fish Samples and Water Samples.** Fish sacrificed on ice were spiked with 1 μg of AHTN or HHCB as an internal standard, and ultrasonicated in a mixture of 10 mL of water and 4 mL of cyclohexane. Then 4 g of dry NaCl was added, and after centrifuging, the upper cyclohexane layer was transferred into a glass test tube. Again 4 mL of cyclohexane was added to the sample, and the procedure was repeated. After concentration under nitrogen to 1 mL, the organic layer was eluted on a silica column using a 6 μL mixture of cyclohexane and ethyl acetate (98:2 v/v). The eluate was concentrated under nitrogen and transferred into a vial for GC–MS analysis.

Water samples of 3 mL were extracted using 4 mL of cyclohexane. The organic layer was spiked with either 1 μg of AHTN or HHCB, concentrated under nitrogen, and transferred into a vial for GC–MS analysis.

**GC–MS Analysis.** AHTN and HHCB concentrations in all extracts were analyzed on a Carlo Erba 5300 GC (Milan, Italy) equipped with a split/splitless injector, a 30 m × 0.25 mm (0.25 μm film thickness) fused silica DB-5MS column (&W Scientific, Folsom, CA), and a QMD 1000 mass spectrometer (Carlo Erba Instruments, Milan, Italy). Analyses were carried out by splitless injection of 1 μL at 225 °C. The column temperature was maintained at 90 °C for 1 min, and raised by 30 °C/min to 150 °C followed by 4 °C/min to 210 °C. The mass spectrometer was operated using selected ion monitoring (SIM), for m/z 243. Unknown concentrations were quantified with the peak area ratio of the compound to be quantified and the internal standard, using a standard curve.

The recovery of this method is 100%.

**Data Analysis.** Luciferase activity per well was measured as light units. In the in vitro experiments, each concentration was analyzed in triplicate. The luciferase activity per well was divided by the concomitant β-galactosidase activity. In the transgenic zebrafish assay five to six fish per concentration were measured. From these values, the fold induction was calculated by dividing the mean value in exposed and nonexposed wells or fish. ERE-luc activity as a percentage of nonexposed wells or fish. ERE-luc activity as a percentage of estradiol induction is calculated by setting the fold induction of the nonexposed wells or fish. ERE-luc activity as a percentage of estradiol induction is calculated by setting the fold induction of the nonexposed wells or fish.

**Results**

**Human and Zebrafish ER Transactivation by Estradiol in a Transiently Transfected HEK293 Reporter Cell Line.** To compare the human and zebrafish ERs for E2-induced transcriptional activation, we used the HEK293 cell line. This cell line lacks significant endogenous levels of ER, can be easily transfected, and has been shown to be highly responsive to estrogens in transient transfections (16). Cells were transiently transfected with one of the tested ER expression plasmids and a reporter construct, consisting of three EREs upstream from a TATA box in front of luciferase cDNA.

Dose–response curves of E2 are shown in Figure 1. Transcriptional activity at hERα was about 1 order of magnitude lower than at hERβ.
magnitude higher than that of hERβ (Figure 1A), with EC50 values of 0.009 and 0.10 nM, respectively. When the zebrafish ERs were compared, ERα was the most responsive to E2, suggesting an important role in estrogen regulation (Figure 1B). Zebrafish ERβ and ERγ were about equally sensitive, but less sensitive than zfERα. We found EC50 values of 0.38, 0.73, and 0.09 nM for zfERα, zfERβ, and zfERγ, respectively. Comparison of human and zebrafish ERs revealed that E2-induced transcriptional activity of the human ERα was about 40 times higher than its zebrafish counterpart, while human ERβ was only about 7 times more responsive than zfERβ. Together with the small difference in sensitivity between zfERα and zfERβ, in contrast to the human ERs, these findings may suggest that zfERβ, compared to its ERα counterpart, is relatively more sensitive to estrogens than hERβ. These findings are consistent with another study (9). In addition, we used the dose–response curves to determine the submaximal E2 dose for each receptor subtype. At this concentration the response can be influenced most sensitively and can therefore be used in competition experiments, to assess antiestrogenicity.

Inhibition of E2-Induced Transactivation of Human and Zebrafish ER by AHTN and HHCB. To assess the antiestrogenic effects of AHTN and HHCB, we dosed the transiently transfected HEK293 cells with different concentrations of musk, and a submaximal concentration of estradiol. A submaximal dose of 0.01 nM E2 was used for hERα-mediated experiments, a dose of 0.1 nM was used for hERβ and zfERγ, and 1 nM E2 was used for zfERα and zfERγ. AHTN and HHCB alone showed a marginal transcriptional activation of the human ERα at the highest test concentration of 10 μM. This was also previously shown in stably transfected HEK293 cells (5). Neither of the musks could stimulate transcriptional activity of the zebrafish ERs and the human ERβ (data not shown). As a positive control for antiestrogenicity in this experiment, the well-known antiestrogen 4-hydroxytamoxifen (OHT) was used at a concentration of 0.01 μM. OHT strongly inhibited the E2-stimulated transcriptional activation of all tested estrogen receptors (Figure 2). A clear dose-dependent and significant suppression of E2...
induction by AHTN and HHCB was shown toward human ER\(\beta\) and zebrafish ER\(\alpha\) (Figure 2B,E). A weak antagonistic effect could be observed on human ER\(\alpha\) and zfER\(\beta\) only at the highest test concentration of 10\(\mu\)M (Figure 2A,D), whereas no effect was seen at zfER\(\alpha\) (Figure 2C). Co-administration of the musks with higher concentrations of E2 abolished the antagonistic effects of the musks, which suggests a competitive interaction at the level of the ER (data not shown).

**Inhibition of E2-Induced Transactivation by AHTN and HHCB in Transgenic Zebrafish.** Since both musks antagonized E2-induced transcription on the zebrafish ER\(\beta\) and ER\(\alpha\) in vitro, we wished to examine whether these antiestrogenic effects could also be observed in vivo. Therefore, we used a transgenic zebrafish assay, a rapid and specific in vivo assay for the detection of (xeno)estrogens (Figure 2B,E). A weak antagonistic effect could be observed on human ER\(\alpha\) and zfER\(\beta\) only at the highest test concentration of 10\(\mu\)M (Figure 2A,D), whereas no effect was seen at zfER\(\alpha\) (Figure 2C). Co-administration of the musks with higher concentrations of E2 abolished the antagonistic effects of the musks, which suggests a competitive interaction at the level of the ER (data not shown).

**TABLE 1. Concentrations of AHTN and HHCB Determined in Test Water during the Internal Exposure Experiment**

<table>
<thead>
<tr>
<th>compd</th>
<th>nominal abs. concn (mg/L)</th>
<th>presence of fish</th>
<th>absence of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t = 0 h</td>
<td>t = 24 h</td>
<td>t = 96 h</td>
</tr>
<tr>
<td>AHTN</td>
<td>25.8</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>HHCB</td>
<td>25.8</td>
<td>22</td>
<td>18</td>
</tr>
</tbody>
</table>

*See Materials and Methods for details. Concentrations are expressed as micrograms per liter.*

Discussion

In this study we were mainly interested in the in vitro and in vivo antioestrogenic effects of two polycyclic musks (AHTN and HHCB), which are ubiquitously present in the aquatic environment. For the in vitro experiments, we used the ER\(\alpha\) and ER\(\beta\) subtypes cloned from human and zebrafish, and also the novel zebrafish ER\(\alpha\) (9). Next to ER\(\alpha\) and ER\(\beta\), fish have a third ER subtype (ER\(\beta\/2\) or ER\(\gamma\)), which has been recently discovered in the Atlantic croaker (17), goldfish (18), and the zebrafish (10, 11, 19; see also Callard et al., unpublished results; GenBank Nos. AAK16740, AAK16741, and AAK16742). Comparison of the overall amino acid sequences of the three receptors indicates that each zfER protein was generated by a distinct gene (11). Alignment of
the ligand binding domains of the human and the zebrafish ERs reveals a homology of 55%. The percentage of identical amino acid residues of the human and the zebrafish ERβ is 57%, and that of ERβ and zfERβ is 61% (11). These alignment results taken together with phylogenetic analysis clearly indicated that zfERβ belongs to the ERβ subgroup and that both zfERβ and zfERβ belong to the ERβ subgroup (11).

The zebrafish ER subtypes, which have been cloned in our laboratory, have been shown to bind estradiol in a receptor-binding assay (Zeinstra et al., manuscript in preparation). This was partly to be expected, since all three zebrafish ERs contain the same specific amino acids for estradiol binding as are found in the human ERα, i.e., Glu353, Arg394, and His524 in hERα (20). In the present results we show that zebrafish ERβ is the most sensitive to estradiol, which was also shown in other studies (9, 11). Furthermore, the difference between E2-stimulated transactivation of human ERα and ERβ was not shown in the zebrafish situation, where ERα and ERβ are about equally potent and ERβ is the most responsive transactivator. Overall, zebrafish ERα and ERβ are less sensitive to E2 than their human counterparts, which also has been shown for rainbow trout ERs (21). This may explain the fact that both musk compounds do not show any agonistic effect on the three zebrafish ERs. On the human ERα, the transactivation potential of which is minimally a factor of 10 higher than those of the zebrafish ERs, a marginal agonism has been shown in stably transfected HEK293 cells (5). On the other hand, antiestrogenic effects of AHTN and HHCB on the human ERs have been recently shown (5). In the present study we also showed a dose-dependent antagonism on zfERβ already starting at 100 nM and which is almost as strong as that on hERα. Of the three zebrafish ERs, zfERβ showed the highest susceptibility for suppression of E2 induction by OHT and both musks.

In our in vivo transgenic zebrafish study, at test concentrations of 0.1 and 1 µM AHTN or HHCB, a significant repression of E2-induced transactivation was observed, especially at the EC50 E2 concentration. This repression is quite strong, namely, down to 20% of the E2 induction for both compounds at the highest test dose.

The nominal water concentrations used in this in vivo study (0.01, 0.1, and 1 µM musk, i.e., 2.58, 25.8, or 258 µg/L musk) were comparable to the nominal concentrations used in standard fish toxicity studies. In a 36-day early-life-stage test (OECD 210) with fathead minnow (Pimephales promelas), no observed effect concentrations (NOECs) were found of 35 µg of AHTN/L and 68 µg of HHCB/L. In a 21-day growth test (OECD 204) with bluegill sunfish (Lepomis macrochirus) NOECs were found of 89 µg of AHTN/L and 93 µg of HHCB/L (22). The actual concentrations at which we observe antiestrogenic effects are around or below the no-observed-effect levels from these studies. That is, no developmental disorders were or will be observed at the concentrations used in our transgenic zebrafish assay.

Furthermore, the nominal concentrations at which an antiestrogenic effect is observed are roughly 25 to more than 1000 times higher than the concentrations found in the environment. Under normal environmental conditions, in river and seawater, levels of musk in the nanogram per liter range are found (reviewed in refs 23 and 24). The higher the input of sewage water, the higher the concentration of musk that is found (3). Maximum levels in the low microgram per liter range have been found in water samples from lakes with a high input of sewage water. Higher concentrations have been found in sewage water effluents and sewage settlement ponds, with maximal levels in eel of 192 mg/kg fw (cited in ref 25). In eel under natural conditions mean levels of 43 µg/kg fw were found (cited in ref 3). In other fish species under natural conditions, comparable or lower concentrations are found, depending largely on the lipid content (reviewed in refs 23 and 24). The internal concentration that we have found at the lowest observed effect concentration for antiestrogenicity in vivo (i.e., 0.1 µM) is about 15.8 mg of AHTN/kg fw and 18.9 mg of HHCB/kg fw. These concentrations can be reached in fish from ponds of sewage treatment plants (24, 25) and might induce antiestrogenic effects in this particular situation. Under normal environmental conditions, the concentrations of the musks in receiving waters are too low to cause antiestrogenic effects.

Concentrations in the fish depend on the toxicokinetics and the biotransformation of the compound in the fish. The high antagonistic potency in vivo may be due to its high lipophilicity and thus to its strong bioaccumulation. From our GC–MS results it is clear that both musks are indeed bioaccumulating. In the zebrafish, concentrations have been found which are roughly a factor 600 higher than the nominal test dose. Internal concentrations stay roughly the same during the 96 h in vivo experiment, which means that the equilibrium has been reached within 1 day. Butte et al. (26) showed that for both polycyclic musks the steady state for uptake in zebrafish was reached in about 10 h. Concentrations in the fish also depend on the fate of the compound in the aquarium. When AHTN or HHCB is added to beaker glasses without fish, immediately after the first dosing, 15–30% has disappeared, and at the end of the 96 h experiment, 25–40% has disappeared. Compounds may sorb to the glass wall and detritus, or degradation of the compounds may take place. HHCB can be transformed into HHCB lactone by autoxidation, which can also occur as a biotransformation reaction (27). Nevertheless, a significant amount of musk has been accumulated in the fish, resulting in low actual water concentrations at the end of the exposure experiment, and also in the observed antiestrogenic effects.

The antiestrogenic effects seen in the in vivo transgenic zebrafish assay are probably for the greater part mediated by the zfERβ, which is the most responsive to estradiol in our in vitro assays. Also, the strongest antagonism by the musks was seen at this receptor subtype. However, it cannot be ruled out that the other two ER subtypes also play a role in the netto effect. Comparison between the in vitro system and the in vivo test system is possible because the measured end point (luciferase protein) is induced according to the same principle. In both assays the compound binds to the endogenous ER and activates it. Then the ER–ligand complex binds to EREs present on the luciferase target gene, which is introduced in the genome of both the cells and the fish. Finally the luciferase protein is induced. To our knowledge, this is the first time that environmental contaminants are shown to be antiestrogenic in an in vivo fish assay. In scientific literature, antiestrogenic effects have been observed in cultured fish hepatocytes by measuring the concentration or expression of the estrogen-regulated yolk protein vitellogenin (Vtg) (28, 29). Letcher et al. (29) showed the antiestrogenic effects of PCB metabolites in an in vitro carp hepatocyte assay by measuring Vtg, and made clear that these effects were ER-mediated, rather than aryl hydrocarbon receptor (AhR)-mediated. Monteverdi and Di Giulio (30) and Latonnelle et al. (28) observed antiestrogenic effects of tamoxifen in, respectively, channel cat fish and Siberian sturgeon hepatocyte cultures, by showing repressed Vtg synthesis. These effects were also postulated to be ER-regulated. However, these studies are in vitro studies, lacking important aspects of in vivo functioning, such as kinetics and biotransformation.

Several in vivo studies looked at the downregulation of Vtg synthesis by antiestrogenic AhR agonists. AhR agonism is assessed by measuring CYP1A induction. TCDD and nonortho PCBs function via an AhR-mediated mechanism of action, involving cross-talk between the ER and the AhR (31). In an in vivo study by Arukwe and colleagues (32), the...
antiestrogenic CYP1A1 inducer 3,3′,4,4′-tetrachlorobiphenyl (TCB) shows both potentiation and reduction of nonylphenol-
induced Vtg synthesis, depending on doses and temporal exposure sequence. Another in vivo study shows both potentiation and repression of E2-induced Vtg synthesis by the CYP1A1 inducer β-naphthoflavone, which has been shown to be antiestrogenic in vitro (33). These studies assessed antiestrogenicity which is both ER- and AhR-mediated. Only a few in vivo fish studies showed antagonism that was postulated to be solely ER-dependent. Tilapia showed decreased E2-induced Vtg levels after tamoxifen exposure for 12 days (34). Similar results were found in exposed female Tilapia by Lazier et al. (35). Recently, Panter et al. (36) exposed fathead minnows via the water. However, no dose-dependent antagonism was found when fish were exposed to ethino-
yestradiol and the antioestrogen ZM189,154. Using an AhR-
specific CALUX assay, we showed no in vitro (antagonistic effects of the musks on the human AhR (unpublished data).
Furthermore, AHTN is not a CYP1A1 inducer (37). We conclude that, in our in vivo assay, the observed antiestrogenic effects are not AhR-mediated, but solely ER-mediated. This makes the transgenic zebrafish assay a suitable tool for the rapid detection of both estrogenic and antiestrogenic effects of chemicals in an in vivo fish model.

Acknowledgments
This study was supported by BioDetection Systems B.V., Amsterdam, The Netherlands, and the Dutch Technology Foundation STW, Utrecht, The Netherlands.

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Received for review June 24, 2003. Revised manuscript received November 5, 2003. Accepted November 18, 2003.

ES03464B 1002 • ENVIRONMENTAL SCIENCE & TECHNOLOGY / VOL. 38, NO. 4, 2004