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Chapter 9

Ecotoxicology of Musks

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Due to the fact that both nitro and polycyclic musks and their metabolites are found in the aquatic environment and appear to accumulate in some of the species, the past and most recent research has focused mainly on possible ecotoxicological effects of musks in aquatic rather than terrestrial species. The compilation of the newest available data for aquatic interactions demonstrates in general that neither parent compounds nor the metabolites of nitro and polycyclic musks pose any significant hazard for the aquatic ecosystem. The observation that amphibians appear more susceptible to endocrine modulating effects of xenobiotics than other species mandates that the interactions of the nitro musk metabolites with the *Xenopus laevis* estrogen receptor, as presented in this review, are investigated in more detail. Such an investigation appears warranted despite the fact that all observed adverse interactions of nitro and polycyclic musks occur at concentrations several orders of magnitude higher than those detected in the environment.

Introduction

The yearly global production of nitro and polycyclic musk fragrances has been estimated to be approximately 2000 (for the year 1988) and 5600 tons, respectively (*1-3*). The use of musks as fragrances and fragrance fixatives in a

wide array of personal care products (e.g., washing detergents, detergents in general, perfumes, lotions, soaps and shampoos, cosmetics, etc.) stipulates that most of these compounds will appear in municipal sewage treatment plants (STP). The removal of nitromusks (NMs) and polycyclic musks (PCMs) during municipal sewage treatment processes has been estimated at approximately 60-80% and 40-60%, respectively. The higher retention of NMs in the STP are explained by the presence of the aromatic ring and thus higher affinity for particles, a rather low water solubility, and a moderately high lipophilicity [K_{ow}: 4.9 and 4.3 for musk xylene (MX) and musk ketone (MK), respectively]. In contrast, PCMs have a high water solubility, despite their inherently high lipophilicity (K_{ow}: 5.7 and 5.9 for AHTN and HHCB, respectively; see next section for abbreviations) and biological stability (*4*). In view of the lipophilicity of NMs and PCMs and their broad form of application, it is not surprising to find these compounds as contaminants in the aquatic environment. Indeed, the concentrations detected in environmental samples range from ng/L to µg/L in effluent and surface waters and from µg/kg lipid to mg/kg lipid in aquatic organisms (*3, 5-8*). Furthermore, most recent analyses point to NM and PCM metabolites as being of greater environmental concern, due to greater metabolic stability and environmental persistence and thus higher concentrations present in biological samples, e.g., in fish flesh, than the respective parent compounds (*1, 2, 9-11*).

All available analytical data, while showing the capability of musk fragrances to bioconcentrate in various aquatic species, do not demonstrate any capacity of these compounds for biomagnification in the aquatic ecosystem. The capacity for "bioconcentration/bioaccumulation" must be differentiated in that for musk compounds this appears more likely to be a function of momentary exposure of the species in question, rather than that of a lifetime up-concentration from a chronically contaminated environment. Indeed, age class analyses of fish taken from the Elbe river demonstrated no significant differences in tissue levels of NMs and PCMs from younger and older fish of the same species (*12*). The concentrations of musk fragrances in the aquatic environment, including species, e.g., fish, are highly related to the distance from the STP (*11*). In consequence and contrary to the situation with PCBs, the potential for toxicological effects resulting from musk exposure stems largely from the actual concentrations the species are exposed to via the ambient water *in situ* (*13*). In view of this the following paragraphs represent primarily a compilation of data for the acute, subacute, and "potential" for subchronic-chronic toxicity of musk fragrances in "target" species (algae, daphnia, fish, and amphibians) and not with imaginable but highly unlikely indirect effects in other species.

Acute and Subacute Toxicity

The acute toxicity and potential environmental effects of NMs and PCMs were summarized in several publications either using the EU-Technical Guidance Documents as a basis for environmental risk assessment (*4, 14-15*), test procedures in conformity with OECD guideline 201 and 202 for testing of chemicals (*16-17*), or test procedures identical or analogous to ASTM guideline E 1439-91 (*18*). The latter publications include studies with algae (*P. subcapitata*), *Daphnia magna*, bluegill sunfish (*L. macrochirus*), rainbow trout (*O. mykiss*), zebrafish (*D. rerio*), fathead minnow (*P. promelas*), and the South African clawed frog (*X. laevis*). The most prominent results are compiled in Table I.

The main focus of the latter studies was on musk xylene (MX), musk ketone (MK) and the three polycyclic musks AHTN (7-acetyl-1,1,3,4,4,6-hexamethyltetraline), HHCB (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(g)-2-benzopyran) and ADBI (4-acetyl-1,1-dimethyl-6-*tert*-butylindane). Additional data can be found for the three amino-metabolites of MX and MK (*18*) as well as for musk moskene, tibetene, and ambrette (*17*). Toxicity of either NMs and PCMs was observed at rather high concentrations of these respective compounds (Table I), i.e., in many cases at or exceeding the inherent water solubilities (Table II).

The mechanism(s) involved in the acute toxicity of the NMs and PCMs is presently unknown. However, a generalized narcosis, as previously demonstrated for various other organic compounds in fish and amphibians (*19*), may be suggested in view of the high concentrations necessary to induce acute mortality (*17, 18, 20*) and the erratic behavior noted with daphnia (*17*). The latter findings are contrasted by the report of Behechti et al., (*16*) who found acute toxicity of low concentrations of the amino-metabolites of MX, especially of the 4-amino-MX in *D. magna* (EC_{50} = 250 ng/L; 95% CI 230-280 ng/L). Whether the findings of Behechti et al. are generally applicable to other aquatic organisms is presently unknown. It is, however, a fact that these toxic concentrations lie approximately 1-2 orders of magnitude above those found in STP effluents and 3 orders of magnitude above those found in surface waters (*1, 2*).

In contrast, more specific effects are noted when embryos of *X. laevis* and *D. rerio* are exposed to PCMs but not NMs (*18, 20*). Both *D. rerio* and *X. laevis* embryos present with a significant increase in malformations (Fig. 1 and 2a). Surprisingly, while all three PCMs (ADBI, AHTN, HHCB) induced malformations in zebrafish embryos, malformations are observed only in ADBI treated *X. laevis* embryos (Fig. 2a.). While ventro-dorsal curvature of the tail was the most prominent and characteristic malformation for PCM exposure in both species, the concentrations necessary to induce malformations in *D. rerio* were approx. one order of magnitude lower than those necessary to produce the same effects in the amphibian embryos. Of the PCMs tested, AHTN demonstrated the greatest degree of teratogenicity, with the steepest dose-

Table I: Compilation of acute and subacute toxicity values obtained with nitro and polycyclic musks in various species

Species	*Endpoint*	*MX [mg/L]*	*MK [mg/L]*	*MM [mg/L]*	*AHTN [mg/L]*	*HHCB [mg/L]*	*ADBI [mg/L]*	*Ref.*
Algae	$EC_{50\ growth}$	NE[a]	0.244	-	>0.797	>0.854	-	4, 14-15
	$EC_{50\ biomass}$	NE[a]	0.118	-	0.468	0.723	-	
Daphnia magna	24hr EC_{50}	NE[a]	NE[a]	NE[a]	-	-	-	4, 14-15, 17
	21d IC_{50}	0.680	0.338-0.675	NE[a]	0.341	0.293	-	
	21d EC_{50} rep.	-	0.169-0.338	NE[a]	0.244	0.282	-	
O. mykiss	96hr LC_{50}	>1000	-	-	-	-	-	4, 14
	21d LC_{50}	-	>0.50	-	-	-	-	
Lepomis macrochirus	96hr LC_{50}	1.20	-	-	-	-	-	4, 14-15
	21d LC_{50}	-	-	-	0.314	0.452		
Danio rerio	14d LC_{50}- adult fish	0.4	-	-	-	-	-	14, 18-19
	96hr LC_{50}- embryo	>0.4	>0.4	>0.4	>0.67	>0.67	>1.0	
	96hr EC_{50}- embryo-hatching	>0.4	>0.4	>0.4	>0.67	>0.67	>1.0	
	96hr EC_{50}- embryo-teratog.	>0.4	>0.4	>0.4	0.18	0.39	0.69	
	96hr EC_{50}- embryo-growth	>0.4	>0.4	>0.4	>1.0	>1.0	>1.0	
P. promelas	32d LC_{50}- embryo-adult	-	-	-	0.100	>0.140	-	15
Xenopus laevis	96hr LC_{50}- embryo	>0.4	>0.4	>0.4	> 2.0	>2.0	>4.0	18-19
	96hr EC_{50}- embryo-teratogen	>0.4	>0.4	>0.4	>4.0	>4.0	>4.0	
	96hr EC_{50}- embryo-growth	>0.4	>0.4	>0.4	>1.0	>2.0	>4.0	

[a]: NE, No effect found at concentrations exceeding compound solubility in H_20 (Table II)

Table II: Calculated water solubilities of nitro and polycyclic musks

	MK [mg/L]	*MX [mg/L]*	*MM [mg/L]*	*AHTN [mg/L]*	*HHCB [mg/L]*
H_2O sol.	1.9[a]; 0.46[b]	0.49[a]; 0.15[b]	0.046[b]	1.25[c]	1.75[c]

[a]: (*14*); [b]: (*17*); [c]: (*11*)

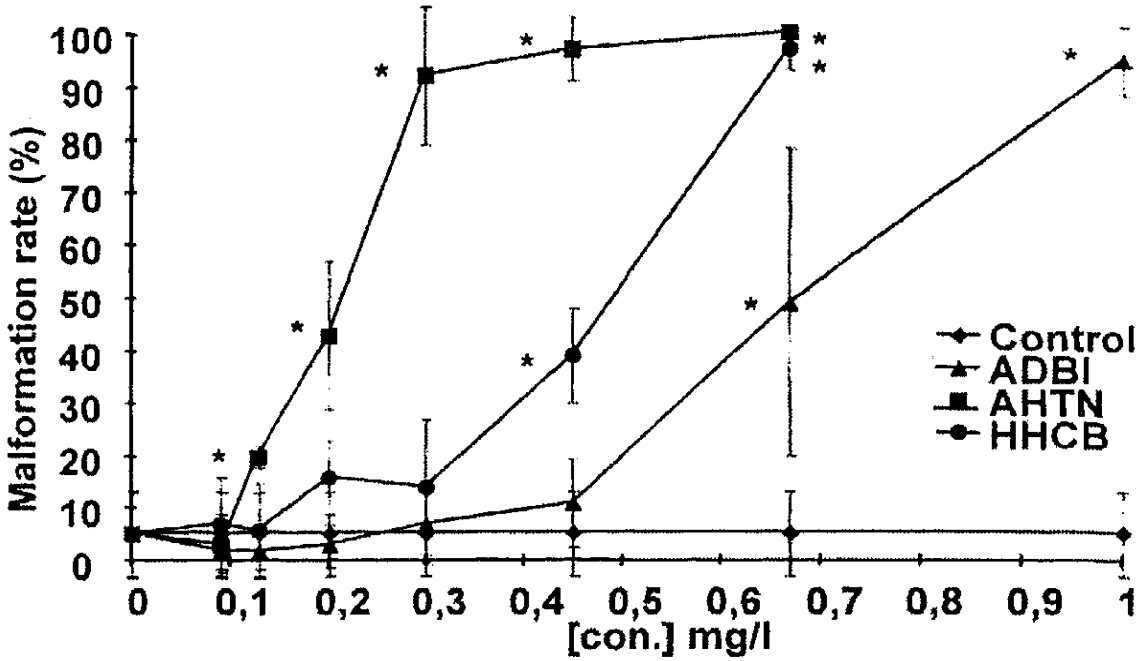

*Figure 1. Malformation in early-life-stage Danio rerio following 96 hours of exposure to polycyclic musks (n=3). (ANOVA and Dunnett's T test. * p<0.05).(Reproduced with permission from (20))*

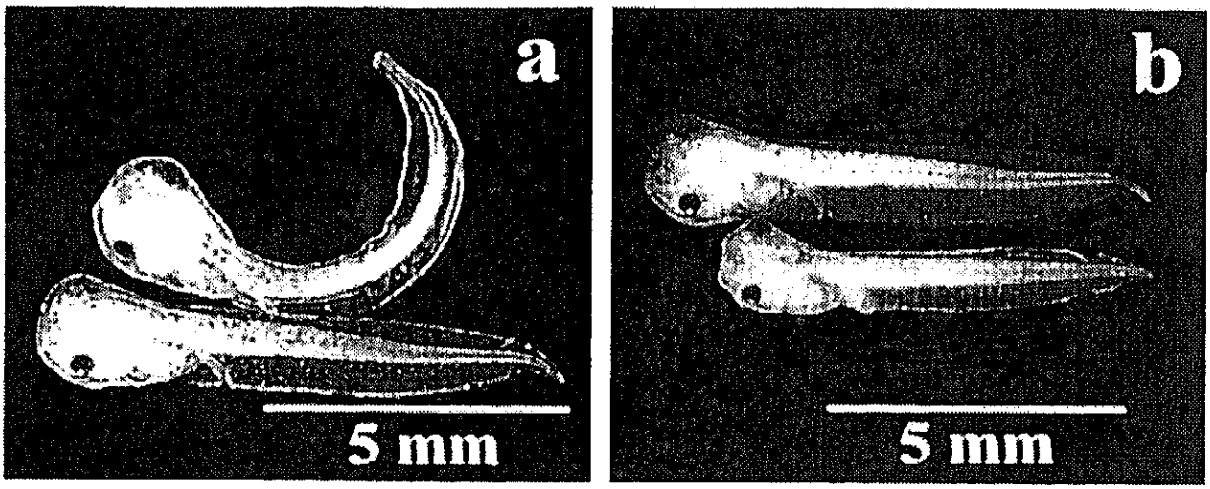

Figure 2. *Malformation and growth inhibition in early-life-stage xenopus (Xenopus laevis) exposed to polycyclic musks for 96 hours. a) ADBI treatment (4 mg/L) (top) and control (bottom): occurrence of ventro-dorsal curvature. b) Control (top) and AHTN treatment (2 mg/L) (bottom): stunted growth).(Reproduced with permission from (20))*

response curve (Fig. 1), while ADBI was teratogenic at high concentrations only. AHTN-induced malformations appear to be specific for cyprinid embryos, as tail-loss was noted in *P. promelas* embryos exposed to 0.067 or 0.14 mg/L AHTN, while no malformations were observed in *X. laevis* embryos exposed to AHTN or HHCB (*20*) or in *P. promelas* exposed to HHCB (*15*). Of the three PCMs tested in a semi-static embryotoxicity test with *X. laevis*, AHTN

and HHCB demonstrated a significant and dose-dependent effect on growth at concentrations below those which were acutely toxic to the embryos (Fig. 2b, Table I). No effects on growth were observed in zebrafish embryos, as the doses necessary to induce a significant growth inhibition exceeded those inducing acute toxicity (Table I). Similar effects were noted in *P. promelas* exposed to 0.140 mg/L HHCB but not for AHTN (*15*).

The comparison of the NM and PCM concentrations found in environmental samples (*1-3, 9-10, 21-22*) with those concentrations inducing acute and subacute toxicity in various aquatic species, as discussed above, strongly suggests that NMs and PCMs do not pose an acute risk for the aquatic ecosystem. This conclusion is also supported by the instrumentalized risk assessment processes for NMs and PCMs using the EU-Technical Guidance Documents (*4, 14-15*), which predict no effects of these musk fragrances in the aquatic environment.

Subchronic-Chronic Toxicity

At present, only limited data are available for assessing the risk to the aquatic environment, i.e., the populations of aquatic species exposed sub-chronically or chronically to low concentrations of parent compounds and metabolites of NMs and PCMs. In general, there are three potential adverse interactions of xenobiotics with the health and sustainability of a population that are of primary importance: (i) an extremely high incidence of pathological changes, e.g., tumors (*23*) resulting from genotoxic or a tumor promoting activity; (ii) suppression of the immune system and thus higher susceptibility of the population to pathogens (*24*); and (iii) endocrine modulation affecting the reproductive success of the population.

Neither the parent compounds nor the metabolites of NMs and PCMs have been demonstrated to possess carcinogenic activity, with the exception of a species-specific promotion of liver tumors at high concentrations of MX observed in mice (*25*). This process was shown to be not of genotoxic (*26-27*), but rather of an epigenetic nature, i.e., driven by the induction of microsomal enzymes, particularly those of the CYP2B family (28), and the pattern of induction was consistent with that observed for phenobarbital, the classical CYP2B inducer and mouse liver carcinogen (*29-30*).

No information is as yet available regarding the potential interaction of NMs and PCMs on immune parameters of aquatic species. However, the present expectation is that no immune-suppressive activity is to be expected in aquatic species as no evidence was found suggesting immune suppressive activity of these compounds in mammalian species exposed subchronically or chronically to high concentrations of these compounds (*25, 31-32*).

Although the present database on potential endocrine modulating activity of NMs and PCMs is still rather scant, the compilation of mammalian data and data from *in vitro* assays with cells and tissue homogenates from aquatic species

suffices for a primary assessment, at least of the potential (anti)estrogenic activity of these compounds. Neither subchronic or chronic administration of NMs, PCMs or mixtures of NMs and PCMs (*25, 31-32*) suggests any form of (anti)estrogenic activity in rodent species. The basis for this assessment was organ weight and histopathological examination of the uterus, seminal vesicles, mammary gland, testes, ovaries, and vaginas. These findings are corroborated by a study of Seinen et al. (*33*) who exposed juvenile mice to high dietary levels of AHTN and HHCB and found no evidence for an increase in uterine weight. On the other hand, the same scientists reported a very weak estrogenic activity of both compounds using ERα- and ERβ-dependent gene transcription assays with human embryonal kidney 293 cells. The reported estrogenic activity was approximately six to eight orders of magnitude lower than the endogenous ligand estradiol (E_2). The latter findings demonstrated that only extremely high concentrations of AHTN and HHCB have measurable estrogenic potency and that the current levels of wildlife and human exposure to these compounds are too low to induce any estrogenic effects in the exposed species. The interaction of the PCMs with the hepatic estrogen receptor(s) of rainbow trout, carp, or the amphibian *X. laevis* was also shown in an *in vitro* competitive binding assay (Fig. 3). In comparison to the endogenous ligand E_2, approximately four orders of magnitude higher concentrations of AHTN were necessary to elicit the same degree of ligand competition (IC_{50}) in the *X. laevis* receptor binding assay. Very weak binding of AHTN and HHCB were found in the rainbow trout receptor binding assay (*34*), corroborating the findings by Seinen et al. (*33*). Neither AHTN nor HHCB, but ADBI bound to the carp estrogen receptor (*34*), corroborating earlier findings by Smeets at al. (*35*), who investigated AHTN and HHCB induced synthesis of vitellogenin in carp hepatocytes *in vitro*. Neither of the two compounds was capable of inducing vitellogenin in this system suggesting that these compounds do not interact with the fish estrogen receptor(s) to the degree or with the high concentrations necessary for estrogen dependent gene transcription. Although metabolites of AHTN and HHCB, as found in environmental samples (*3, 9-10*), were not analyzed for (anti)estrogenic activity, it can safely be assumed that these metabolites were also formed during incubation of the primary carp hepatocytes used as the screening method for estrogenic activity. If indeed these metabolites had any form of estrogenic activity the lack of vitellogenin induction in the carp hepatocyte system suggests that the metabolites were not formed in adequate concentrations to have an estrogenic effect. Overall it can be concluded that the current environmental PCM levels are too low to induce estrogenic effects in aquatic species.

In contrast to the PCMs neither of the two nitro musk parent compounds (MX and MK) had any competitive binding activity to either the rainbow trout or the Xenopus estrogen receptor(s). However, amino-metabolites of MX and MK, formed during the sewage treatment process, were able to bind to the estrogen receptors of rainbow trout (Fig. 4) and *X. laevis* (Fig. 5). The concentrations of the 2-amino-MX metabolite necessary to displace 50% of the

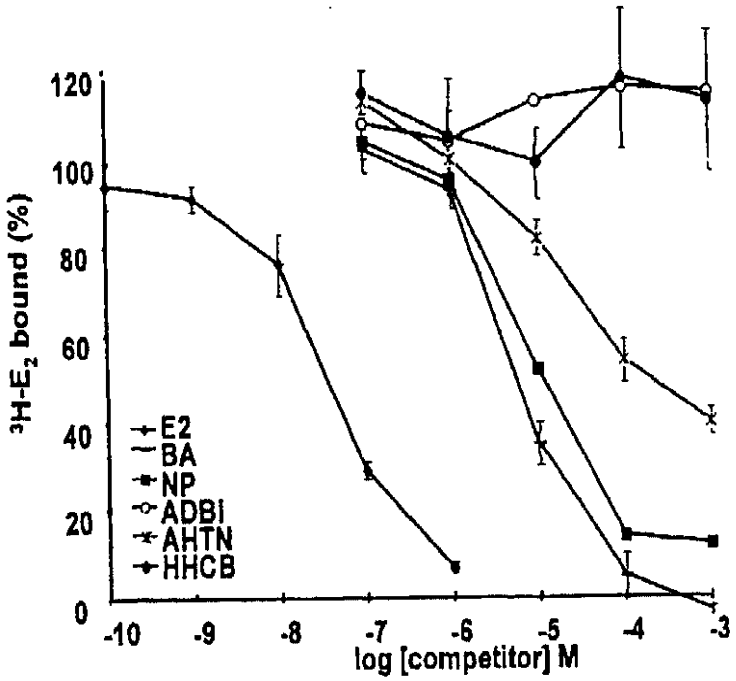

Figure 3. Competitive binding of estradiol (E_2), bisphenol A (BA), nonylphenol (NP), and polycyclic musks to Xenopus ER. The incubation concentrations were 10^{-10}-10^{-6} M for E_2, 10^{-7}-10^{-3} M for BA, NP, and PCMs. IC_{50} values (n=3) were 24.0±0.5 nM (E_2), 3.7±0.1 μM (BA), 24.0±0.6 μM (NP) and 257±6 μM (AHTN). [Reproduced with permission from (34)]

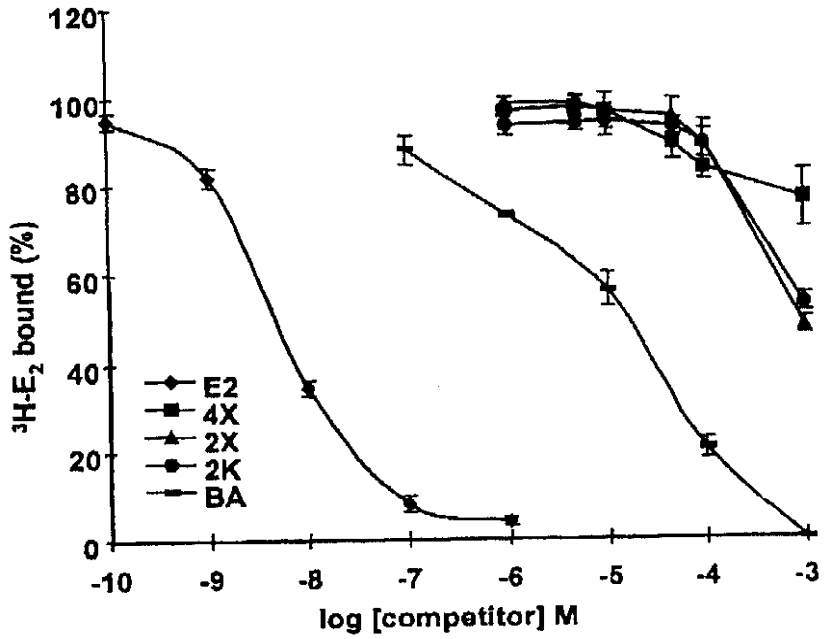

Figure 4. Competitive binding of estradiol (E_2), bisphenol-A (BA), 4-NH_2-MX (4X), 2-NH_2-MX (2X) and 2-NH_2-MK (2K) to the rainbow trout ER. The incubation concentrations were 10^{-10}-10^{-7} M for E_2, 10^{-7}-10^{-3} M for BA, and 10^{-6}-10^{-3} M for amino metabolites. IC_{50}s were 5.3 ± 1.2 nM for E_2, 8.8 ± 1.8 μM for BA and 1.2 ± 1.1 mM for 2X. [Reproduced with permission from (38). Copyright 1999 Elsevier Science Ireland Ltd.]

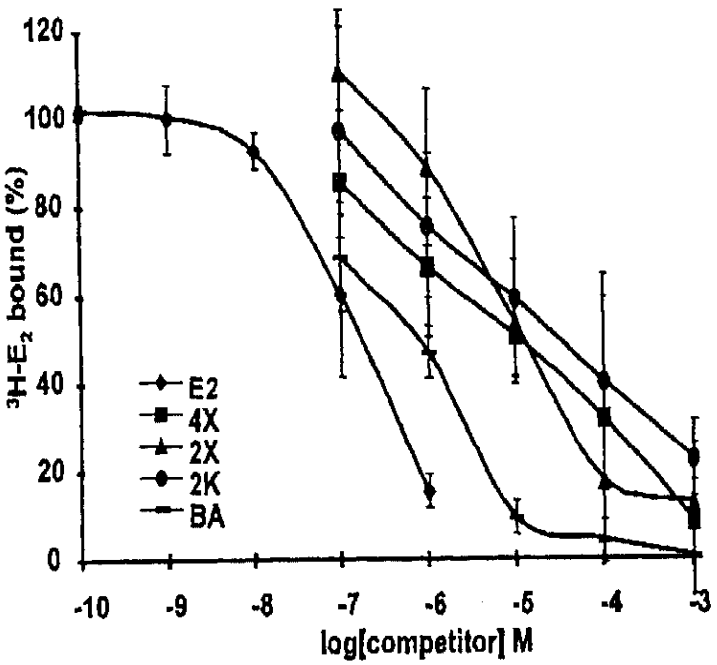

Figure 5. Competitive binding of estradiol (E_2), bisphenol-A (BA), 4-NH_2-MX (4X), 2-NH_2-MX (2X), and 2-NH_2-MK (2K) to the Xenopus ER. The incubation concentrations were 10^{-10}-10^{-7} M for E_2, 10^{-7}-10^{-3} M for BA, 4X, 2X, and (2K). IC_{50}s were 187 ± 76 nM for E2, 441 ± 247 nM for BA , 30.8 ± 28.5 µM for 4X, 12.9 ± 10.3 µM for 2X and 70.1 ± 88.3 µM for 2K. [Reproduced with permission from (38). Copyright 1999 Elsevier Science Ireland Ltd.]

endogenous ligand at the rainbow trout estrogen receptor(s) was approximately six orders of magnitude greater than that of the endogenous ligand (E_2) itself, again demonstrating that unrealistically high concentrations of these metabolites were needed to elicit any estrogenic activity in rainbow trout (Fig. 4).

Surprisingly the binding curves derived from the *X. laevis* estrogen receptor binding assay, demonstrated that all three known amino-metabolites of MX and MK were able to compete with the endogenous ligand. The concentrations necessary for competition were only 2-3 orders of magnitude higher than those of E_2 (Fig. 5). Furthermore, the concentrations of 2-amino-MX necessary for E_2 competition at the *X. laevis* estrogen receptor(s) were nearly 3 three orders of magnitude lower than those needed for competing at the rainbow trout estrogen receptor(s). The latter suggests that there are some species-specific susceptibilities with regard to potential estrogenic activities of nitro musk metabolites. Indeed, the findings in the *X. laevis* system (Fig. 5) are unique in that these *in vitro* findings were indicative for the endocrine modulating effects observed for bisphenol A (BA) *in vivo* (*36*). Chronic exposure of *X. laevis* embryos to low concentrations of BA induced a feminization of male embryos (*37*). Although the above *in vitro* systems may be indicative that some of the NM metabolites and PCMs may have the potential for endocrine modulation in aquatic species, the mere interaction of a xenobiotic with the estrogen receptor(s) of a given aquatic species does not imply that this interaction will

also lead to all of the specific associated downstream events. Indeed, an investigation of the estrogenic activity of complex STP effluents using several *in vitro* assay systems demonstrated that while an interaction with the rainbow trout ER(s) was observed, no simultaneous inductions of ER and vitellogenin mRNA in primary rainbow trout hepatocyte cultures were detectable (*39-40*).

The concurrent chemical analysis of these STP effluents revealed the presence of ethoxylates and plant steroids in ng-µg/L quantities, thus strongly suggesting that, with the exception mentioned above, high concentrations of these estrogenic xenobiotics are necessary to elicit a demonstrable endocrine modulating effect at the individual or population level.

Conclusions

Although the present data base for ecotoxicological effects of NMs and PCMs and of their respective metabolites is still too small for a concluding risk assessment, there is little evidence that would suggest that these compounds, despite their overt presence in environmental samples, generally would have an adverse impact on the aquatic ecosystem. The concentrations of musk fragrances in the aquatic environment are highly related to the distance to the STP (*11*). Indeed, as indicated also via the comparison between the tissue levels of various ages of fish exposed to NMs and PCMs, no biomagnification within the same species (age classes) or various trophic levels appears to occur (*12*). In consequence and contrary to the situation with PCBs, the potential for toxicological effects resulting from musk exposure stems largely from the actual concentrations the species are exposed to via the ambient water *in situ* (*13*) and this risk appears to be negligible when using the presently available database for risk estimation. However, as pointed out above, amphibians appear to be more susceptible to endocrine modulating compounds than most of the species investigated so far (*37*). In light of this, the interaction of the MX and MK metabolites with the estrogen receptor of *X. laevis* (*38*) must be taken more seriously and should encourage others to investigate the mechanisms of this interaction, the potential effects, and risks associated with these amino metabolites for amphibians in more detail.

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