# Synthetic musks fragrances in the aquatic environment: *in vitro* toxicological studies of their biotransformation and potential negative effects

S. Focardi, C. Della Torre, M. Monti, T. Biagini & I. Corsi
*Department of Environmental Sciences "G. Sarfatti", University of Siena, Italy*

## Abstract

The aim of the present study was to investigate the interaction of musk xylene (MX) and Tonalide (AHTN) with CYP1A by looking at gene transcription (*cyp1a*) and EROD activity in Poeciliopsis lucida hepatoma cell line (PLHC-1). MX and AHTN were studied individually and combined with classical inducer of CYP1A as B(a)P and PCB126. After 24h of exposure a different cytotoxicity has been observed with an $LC_{50}$ of 35.76μM for AHTN and $LC_{50}$ 123.6μM for MX. After 6h of exposure to MX, a dose-dependent reduction of *cyp1a* was observed respect to controls. At 24h, the same pattern was observed but with slight induction at the lowest concentration (2μM) and a dose-dependent reduction at the higher concentrations. Co-exposure to MX with B(a)P did not alter *cyp1a* transcription levels compared to the inducer alone. After 6h AHTN determined a slight induction of *cyp1a* transcription reaching maximum induction of 2.3 folds respect to controls at 2μM. No modulation of *cyp1a* transcription was observed after 24h. Co-exposure to AHTN with B(a)P and PCB126 at 6h determined a 55% reduction of *cyp1a* transcription respect to inducers alone which recovered at 24h. At 24h, MX caused a dose-dependent decrease of EROD activity. No modulation of EROD activity was detectable at 6h and 24h of exposure to AHTN. Co-exposure with both MX and AHTN did not alter EROD activity induced by B(a)P and PCB126. Results suggest different toxicological properties of MX and AHTN toward CYP1A in PLHC-1. MX reduced *cyp1a* basal transcription but did not alter *cyp1a* induction by B(a)P and PCB126. This suggests that MX cellular pathway is not mediated by AhR. On

WIT Transactions on Biomedicine and Health, Vol 15, © 2011 WIT Press
www.witpress.com, ISSN 1743-3525 (on-line)
doi:10.2495/EHR11171

the contrary AHTN did not alter significantly *cyp1a* basal levels but decreased *cyp1a* induction by B(a)P and PCB126. A potential role of AHTN as competitive antagonist of AhR could thus be hypothesized.
*Keywords: synthetic musks, PLHC-1, CYP1A.*

## 1 Introduction

In recent years, emerging organic contaminants known as pharmaceutical and personal-care products (PPCPs) have increasingly been released into the environment. PPCPs include many substances with a broad spectrum of uses, including musk fragrances [1]. They are divided into two categories, nitro- and polycyclic musks and are commonly added to detergents, perfumes, soaps and cosmetics. Nitromusks, as musk xylene (1-*tert*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene, MX) are synthetic di- and tri-nitrobenzene derivatives, while polycyclic musks such as Tonalide (7-acetyl-1,1,3,4,4,6-hexamethyl-tetrahydroxaphtalene, AHTN) are indane and tetraline derivatives highly substituted by methyl groups.

Considered widespread environmental contaminants, MX and AHTN have been detected in the atmosphere, water, sludge and sediments. Levels in surface waters are in the ng-µg/L range for both musks [1–5] so that exposure of aquatic biota is mainly related to waste water treatment plants.

The lipophilic nature of both compounds (log Kow MX = 4.90; log Kow AHTN = 5.70) suggests high bioaccumulation potential for aquatic biota, leading to levels in the µg-mg/kg lipid basis range [4, 6].

With regards to their toxicity to aquatic species, MX and AHTN inhibits multixenobiotic resistance in mussel (*Mytilus californianus)* with $IC_{50}$ of 0.97 µM for MX and $IC_{50}$ of 2.05 µM for AHTN [7, 8]. MX has been reported to be embryotoxic in zebrafish (*Danio rerio*) [9] and AHTN induce alteration of hearth rate at 33µM [9]. Some studies describe the potential estrogenic/anti-estrogenic effects of MX and its metabolites as well as AHTN. MX metabolites 2-amino-MX and 4-amino-MX seem to bind oestrogen receptors (ER) competitively *in vitro* in rainbow trout (*Onchorynkus mykiss*) and in the South African clawed frog (*Xenopus laevis*) [10]. In zebrafish, antiestrogenic effects have been reported for AHTN mainly mediated by ERγ [11]. An induction of ERα and vitellogenin expression has been reported in males of *Oryzias latipes* at 500µg/L of AHTN [12]. Despite acute and chronic effects have been observed at doses exceeding environmental concentrations, the persistence in the aquatic environment and bioaccumulation by biota, suggest a potential concern for aquatic biota due to their presence in the aquatic environment.

The toxicological pathways of synthetic musks, in terms for instance of detoxification, are poorly understood in mammalians and barely documented in aquatic organisms. Only a species-specific metabolization has been suggested for aquatic biota [4].

In this view, studies focusing on toxicological mechanisms of action of both compounds in fish species are needed, with particular regard to the interaction with major detoxification systems, such as cytochrome P450 responsible for

metabolism/detoxification of both endogenous and exogenous compounds. CYP1A in particular is a major enzyme subfamily involved in phase I of the detoxification response to various xenobiotics, well characterised in fish species [13].

The involvement of CYP450 in musks metabolism is supposed to be responsible for the species-specific accumulation of these compounds. In mammalian species, MX affects various CYPs, such as CYP1A1, CYP1A2, CYP2B and CYP3A. Rats exposed to high doses of MX (0.1 mM) show strong induction of CYP1A2, presumably by post-transcriptional induction [14]. Other studies in mice and rats showed that MX (up to 200 mg/kg/day) caused phenobarbital-like pretranslational induction of CYP2B mRNA and protein levels. Significant inhibition of the related pentoxyresorufin-O-deethylase (PROD) activity, probably related to MX amino metabolites, and no effects on CYP1A1 have also been reported [15, 16]. Long-term exposure of adult Long Evans rats to low doses of MX (0.7-0.8 mg/kg/day) showed increased CYP1A and CYP2B proteins and related resorufin activities [17], similar to those observed at higher doses of acute exposure. On the contrary AHTN resulted to be hepatotoxic, without altering CYPs activities [18].

The only study on fish indicated that among musks, MX was the strongest inhibitor of CYP1A activity *in vitro* ($IC_{50}$ 37 ± 7 μM) while AHTN determined a 30% inhibition [19]. Hence a different mechanism of interaction of MX and AHTN with CYP1A is likely.

The aim of the present study is to investigate the interaction of MX and AHTN with CYP1A by looking at gene transcription (*cyp1a*) and EROD activity in *Poeciliopsis lucida* hepatoma cell line (PLHC-1). PLHC-1 are commonly used in ecotoxicology to study toxicological pathways of chemicals, including PPCPs [20]. With regard to the biotransformation pathway, they are suitable by virtue of their high capacity to metabolize xenobiotics and they also express the gene for the aryl receptor AhR [21]. MX and AHTN were studied in short term exposure individually and combined with classical inducer of CYP1A as Benzo(a)pyrene (B(a)P) and 3,3'-4,4',5-pentachlorobiphenyl (PCB126).

## 2 Materials and methods

### 2.1 Cell culture

PLHC-1 cells (ATCC; LGC Promochem, Teddington UK) were kindly provided by Dr Tvrtko Smital of Ruder Boskovich Institute, Zagreb, Croatia. They were grown in 75 $cm^2$ plastic flasks (PBI International) at 30°C in 20 ml Dulbecco's Modified Eagles Medium (Sigma-Aldrich) supplemented with 5% FBS (Invitrogen). Cells were subcultured every 4 days by detaching with 1 ml per flask of 1:4 trypsin-EDTA solution (Gibco).

### 2.2 Neutral red assay

Cell viability was measured by neutral red assay [22]. Cells were grown in 200 μl/well of medium for 24 h. One hundred micro litres of medium was then

removed and replaced with the same volume of medium containing serial dilutions (range 0-250 µM) of MX and AHTN (LGC standards) or 0.1-2 µM B(a)P and 1nM PCB 126 (Sigma-Aldrich) dissolved in DMSO (maximum concentration 0.1%). Unexposed cells and DMSO-exposed cells were used as controls. The plates were incubated for another 24 h. The medium was then removed and the plates washed with 200 µl PBS, adding 100 µl neutral red medium, containing 40 µg/ml Neutral Red dissolved in DMEM. Cells were incubated for 2 h under culture conditions. The medium was then removed and the cells washed with 150 µl PBS. Then 100 µl neutral red destain solution (50% ethanol, 49% deionized water, 1% glacial acetic acid) was added and the plate shaken for 10 min. The optical density (OD) of neutral red extract was measured by spectrophotometer at 540 nm. Each experiment was performed at least three times. The IC50 for MX and AHTN were calculated by fitting the OD of a typical experiment to a classical sigmoidal dose-response model using GraphPad Prism5 software.

### 2.3 RNA extraction and Q-PCR

In order to evaluate CYP1A modulation at gene transcription by MX, AHTN, and the two known inducers B(a)P and PCB 126, singly and combined, cells were grown in 4 ml/well of medium for 24 h. Two millilitres of medium was then removed and replaced with 2 ml medium containing MX (2, 4 and 20 µM final concentrations ) and AHTN (1 nM, 2 µM f.c.) singularly dissolved in DMSO 0.1%, B(a)P 2 µM f.c. and B(a)P 2 µM combined with MX 20 µM and 2 µM AHTN. PCB126 1 nM f.c. and PCB126 1 nM combined with 1 nM and 2 µM AHTN. The plates were incubated for another 6 and 24 h. The medium was then discarded and the plates washed twice with 1.2 ml PBS. Cells dissolved in PBS were recovered with a cell scraper and centrifuged 10 minutes at 1200 x g. Cell pellets were stored at -80°C.

Total RNA was isolated using Qiagen RNeasy MiniKit (Qiagen) according to the manufacturer's protocol. RNA was treated with DNase from RNase-Free DNase set (Qiagen) to avoid any traces of genomic DNA. RNA concentrations were measured using a Shimadzu spectrophotometer at 260 nM. 250 ng total RNA was transcribed to cDNA using i-script cDNA Reverse Transcription Kit (Biorad). Real-time PCR was used to evaluate *cyp1a* gene expression. Specific primers were designed using IDTDNA www.idtdna.com. 18S rRNA was used as housekeeping gene based on high stability in this cell line. Primer sequences for PLHC-1 *r18S* were kindly provided by Jovica Loncar from Ruder Boskovic Institute, Zagreb, Croatia.

The following primers were used:

*cyp1a* Fw: 5'-GCATTTGGCGTGCTCGAAGAAA-3',
Rev: 5'-TTGCAGATGTGCTCCTCCAACA-3';
*r18S* Fw: 5'-CCTTTAACGAGGATCCATTGGA-3',
Rev: 5'-CGAGCTTTTTAACTGCAGCAACT-3'.

Each amplification reaction contained 12.5 µl SYBR green mix, 1 µl cDNA and 0.75 µl forward and reverse primers 10 µM, in 25 µl total volume. The

cycling parameters were: 3 min denaturing at 95°C, 40 cycles at 95°C for 15 s, annealing at 55°C for 45 s, elongation at 72°C for 1 min. All primer pairs gave a single peak of dissociation in all reactions, and no amplification occurred in reactions without template. PCR efficiencies for each primer were determined from a standard curve using dilutions of pooled cDNA ($r^2 > 0.97$ for all primers). Data was analysed by the ΔΔCt method [23].

### 2.4 EROD activity

EROD activity was determined by microplate assay [24]. Cells were grown in 200 µl/well of medium for 24 h. One hundred micro litres of medium was then removed and replaced with 100 µl of medium containing serial dilutions (0-20 µM) of MX, AHTN, B(a)P and PCB 126 and in combination, dissolved in DMSO (maximum concentration 0.1%). Unexposed cells and DMSO-exposed cells were used as controls. The plates were incubated for another 6 or 24 h and then washed with 200 µl PBS, adding 100 µl 2 µM 7-ethoxyresorufin (7-ER) dissolved in phosphate buffer pH 8.0 to each well. The kinetics of resorufin production were monitored for 10 min using a Victor3 microplate reader (Wallak) at $\lambda_{Excitation}$ = 530 nm and $\lambda_{Emission}$ = 590 nm. Protein content was quantified by a photometric assay [25] using bovine serum albumin as standard (0-0.5 mg/L $r^2$= 0.9728). Results of EROD assay shown in Tables 1 and 2 are expressed as mean ± SD of at least three independent experiments.

## 3 Results

### 3.1 Citotoxicity

After 24 h of exposure, a different cytotoxicity has been observed for the two compounds with an $LC_{50}$ of 35.76 µM for AHTN and $LC_{50}$ 123.6 µM for MX. No cytotoxic effect was encountered at the concentration of 2 µM of B(a)P and 1nM PCB126 (data not shown).

### 3.2 Interaction with CYP1A

After 6 h of exposure to MX, a dose-dependent reduction in *cyp1a* gene transcription was observed with respect to control. At 24 h, the same pattern was observed but with slight induction at the lowest concentration (2 µM) and still a dose-dependent reduction at the higher concentrations of 4 µM and 20 µM (Fig. 1). Co-exposure to MX with B(a)P did not alter *cyp1a* transcription levels compared to cells exposed to B(a)P alone (Fig. 1).

On the contrary after 6 h AHTN determined a slight induction of *cyp1a* transcription reaching maximum induction of 2.3 folds respect to controls at 2 µM. No modulation of *cyp1a* transcription was observed after 24 h (Fig. 2). Co-exposure to AHTN with PCB126 and B(a)P at 6 h determined a 55% reduction of *cyp1a* transcription respect to the inducers alone which seemed to recover at 24 h. No differences on *cyp1a* transcription were evident at 24h between single exposure to inducers and combined to AHTN (Fig. 2).

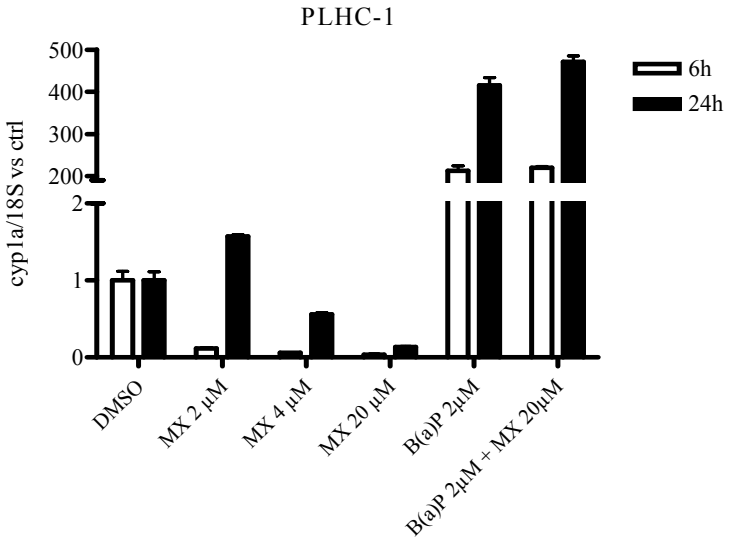


Figure 1: Transcription levels of *cyp1a*/*18S* of the cell line PLHC-1, exposed for 6 and 24 h to MX, B(a)P and B(a)P plus MX with respect to control (DMSO). Results are mean ± SD (N=3).

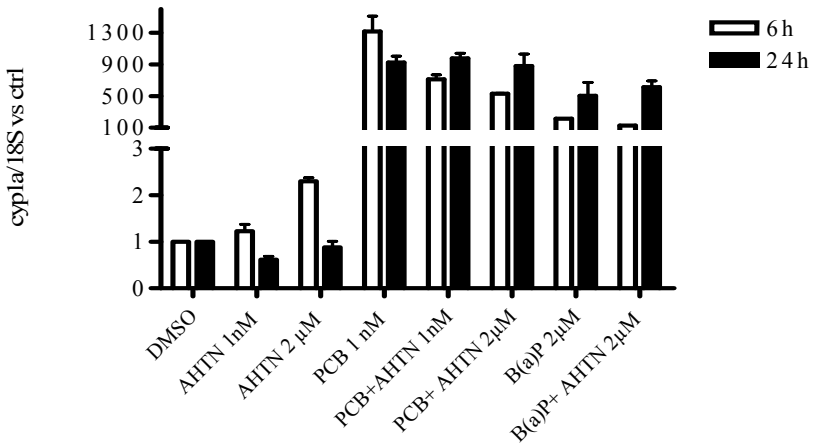


Figure 2: Transcription levels of *cyp1a*/*18S* of the cell line PLHC-1, exposed for 6 and 24 h to AHTN, B(a)P and PCB126 singly and combined with respect to control (DMSO). Results are mean ± SD (N=3).

PLHC-1 cells exposed to MX for 6 h all showed increasing EROD activity, with maximum induction at the highest concentration of MX (20 µM). On the

contrary, at 24 h, MX caused a dose-dependent decrease. Only a slight increase (about 2 folds vs ctrl) of EROD activity was detectable at 6 h and 24 h of exposure to AHTN (Tab. 1). Co-exposure with both MX and AHTN did not alter EROD activity induced by PCB126 and B(a)P (Tab. 2).

Table 1: Modulation of EROD activity by tested compounds at 6h and 24h. Indicated are maximal fold increase in activity ± s.d. and the maximal concentration where the highest activity has been found.

| | **6h** | | **24h** | |
|---|---|---|---|---|
| | Max folds induction | conc Max | Max folds induction | conc Max |
| MX 6h | 5.5 ± 0.53 | 20 μM | 0.24 ± 0.1 | 20 μM |
| AHTN | 2.32 ± 0.79 | 2 μM | 2.18 ± 0.01 | 4 μM |
| B(a)P | 80.35 ± 37.75 | 0.2 μM | 79.15 ± 1.10 | 0.2 μM |
| PCB | 40.53 ± 3.55 | 1 nM | 149.79 ± 16.34 | 1 nM |

Table 2: Induction of EROD activity by tested compounds in co-exposure at 24h. Indicated are fold increase in activity ± s.d.

| | EROD induction vs ctrl |
|---|---|
| PCB126 1 nM + AHTN 2 μM | 160.83 ± 8.87 |
| B(a)P 0.2 μM + AHTN 2 μM | 67.11 ± 5.31 |
| PCB126 1 nM + MX 2 μM | 127.43 ± 2.96 |
| B(a)P 0.2 μM + MX 2 μM | 88.85 ± 10.07 |

## 4 Discussion

The different extent of MX and AHTN citotoxicity observed in the present study has been already reported in liver rainbow trout cell lines (RTL-W1), in which a 30 folds lower $LC_{50}$ was measured for AHTN respect to MX [26]. These findings underline the need for more caution in the use of AHTN in household and personal care products. In Europe the use of AHTN is higher than nitromusk as MX with 358 tonnes per year for AHTN against 67 for MX (OSPAR data for 2000) [27].

Concerning CYP1A, results suggest that the MX and AHTN act differently in the PLHC-1 model.

MX seemed to affect CYP1A at transcriptional and enzymatic level with different profiles of *cyp1a* transcription after 6 h and 24 h of exposure. At 6 h, MX significantly reduced basal levels of transcription of the *cyp1a* gene in a dose-dependent manner. Partial recovery of the reduction but not dose-dependent occurred within 24 h. Positive feed-back involving amino metabolites, which act in the opposite manner to the parent compound, seems likely and reflects observations in mammals *in vivo* [15]. Although the cellular transformation pathways of MX are unknown, after 6 and 24 h of exposure our results suggest

that MX and its metabolites undergo different CYP1A biotransformation. Regarding the co-exposure experiment, MX reduced also *cyp1a* basal transcription but did not alter *cyp1a* induction by B(a)P. Other authors reported similar behaviour of rainbow trout hepatocyte cultures exposed in vitro to 17β-estradiol (E2) and 17α-ethinylestradiol (EE2), respectively [28,29]. In both studies, E2 and EE2 inhibited basal expression of the *cyp1a* gene and no interaction with induced levels of *cyp1a* by βNF (co-exposure) was observed. The effects on *cyp1a* expression found in the present study indicate that more research is needed into the behaviour of MX and its metabolites towards ER.

In mammals, it has been postulated that the MX cellular pathway is not mediated by AhR. A peculiar profile of drug-metabolizing enzyme induction by MX, not mediated by AhR, was suggested [14]. According to these authors, MX determined specific induction of CYP1A2 and phase II enzymes by a post-transcriptional induction mechanism. This hypothesis is in line with previous observations [30]. Under co-exposure conditions with 2-aminoanthracene (2-AA) or aflatoxin $B_1$ ($AFB_1$), MX caused an increase in genotoxicity of these compounds, whereas co-exposure with B(a)P did not cause any increase in toxicity. Thus the present results sustain the hypothesis of a distinct signalling pathway for MX not mediated by AhR.

On the contrary AHTN did not alter significantly *cyp1a* basal levels but decreased *cyp1a* induction by B(a)P and PCB126. A potential role of AHTN as competitive antagonist of AhR could thus be hypothesized. AHTN seems to interact with CYP1A similarly to other pollutants as PBDE (85) and PCB (105, 128) [31, 32].

The involvement of metabolites which act in the opposite manner to the parent compound, seems likely for both musks, due to the different profile of *cyp1a* transcription.

With regard to the effects of MX on EROD activity, a reduction was generally observed after 6 and 24 h of exposure, as observed for gene transcription, except for the increase at the highest concentrations (10-20 μM). A similar inhibitory effect was reported in the microsomal fraction of carp *in vitro,* where MX reduced EROD activity by 71 ± 5% [19]. Besides clear evidence of inhibition of EROD activity by MX *in vitro*, the present results show temporal permanence of the inhibitory effect over 24 h. The large discrepancy between 6 and 24 h exposure to the higher concentrations suggests compensatory positive feedback in the first 6 h of exposure, seemingly cancelled out over 24 h. A possible explanation could be the observed significant reduction in *cyp1a* transcription.

With regard to AHTN, no interaction with EROD activity is observed both at 6 h and at 24 h, both in single and in co-exposure. Consequently, the alteration at the transcriptional level by AHTN does not seem to interfere with the catalytic function of CYP1A.

This observation is in agreement with what has been reported in carp [19], where a higher ability of nitromusk to interact with CYP1A compared to polycyclic musk has been observed. AHTN in fact determines only a slight inhibition of EROD activity compared to other musks while it has been reported

as the strongest inhibitor of CYP3A activity (BFCOD) [19]. Moreover in rat microsomes AHTN seems not altering EROD activity [18], thus confirming the hypothesis that it is not metabolized by CYP1A, but possibly by other enzymes as CYP3A.

Based on the overall results, it can be hypothesized that other enzymes could be responsible for rapid metabolism of MX and AHTN in PLHC-1.

First useful data have been thus obtained from the present study in order to understand toxicological pathways of MX and AHTN in relation to the CYP1A system in fish. Overall results of this study suggest a different mechanism of interaction with CYP1A of a nitro musk as MX and a polycyclic musk as AHTN. Therefore, both compounds seem to affect CYP1A detoxification of toxic compounds known inducers of the system as PAHs and PCBs. A reduction in the detoxification of toxic molecules could affect negatively fish cells with unexpected detrimental effects for aquatic organisms and the ecosystem.

## 5 Conclusions

The present study clarifies the interaction of MX and AHTN with the CYP1A system in fish both in single and co-exposure with classical CYP1A inducers.

Regarding MX, data suggests that it interferes with the biotransformation system but not involving AhR mediated pathway as confirmed by co-exposure with B(a)P. AHTN seems not to be metabolized by CYP1A but at the same time able to reduce the metabolization of classical toxic inducers as B(a)P and the dioxin-like PCB 126. Exposure to musks could thus result in reduced detoxification capacity of CYP1A and may also interfere with important physiological processes in the organism. Therefore, under conditions of chronic exposure unexpected adverse effects could occur, especially in areas chemically impacted by mixture of toxic pollutants. The use of co-exposure experiments confirmed a great investigative tool to clarify the interaction and behaviour of emerging contaminants in respect of important cellular defence systems.

## Acknowledgements

The study was financed by the Italian Ministry for Universities and Research (MIUR) based on PRIN project 2007J23NNX_001 2007.

The authors are grateful to Roko Zaja and Jovica Loncar from the Ruder Boskovic Institute for setting up the PLHC-1 analysis and to Tvrtko Smital for great advice and support.

## References

[1] Daughton, C.G., & Ternes T.A., Pharmaceuticals and personal care products in the environment: agent of subtle change? *Environmental Health Perspectives*, **107**, pp. 907–938, 1999.

[2] Rimkus, G., Polycyclic fragrances in the aquatic environment. *Toxicology Letters*, **111**, pp. 37-56, 1999.
[3] Gaterman, R., Hühnerfuss, H., Rimkus, G., Attar, A., & Kettrup A., Occurrence of musk xylene and musk ketone metabolites in the aquatic environment. *Chemosphere*, **36**, pp. 2535–2547, 1998.
[4] Gaterman R., Biselli S., Hühnerfuss H., Rimkus G., Hecker M., Karbe L., 2002. Synthetic musks in the environment. Part 1: Species dependent bioaccumulation of polycyclic and nitro musk fragrances in freshwater fish and mussels. *Archives of Environmental Contamination and Toxicology*, **42**, pp. 437–446, 2002.
[5] Sumner, N.R., Guitart, C., Fuentes, G., & Readman, J.W., Inputs and distributions of synthetic musk fragrances in an estuarine and coastal environment; a case study. *Environmental Pollution*, **158**, pp. 215-22, 2010.
[6] Rudel, H., Bohmer W., & Schroter-Kermani, C., Retrospective monitoring of synthetic musk compounds in aquatic biota from German rivers and coastal area. *Journal of Environmental Monitoring*, **8**, pp. 812-823, 2006.
[7] Luckenbach, T., Corsi, I., & Epel, D., Fatal attraction: synthetic musk fragrances compromise multixenobiotic defense systems in mussels. *Marine Environmental Research* 58, pp. 215-9 2004.
[8] Luckenbach, T., & Epel, D., Nitromusk and polycyclic musk compounds as long-term inhibitors of cellular xenobiotic defence systems mediated through multidrug transporters. *Environmental Health Perspectives* **113**, pp. 17 – 24, 2005.
[9] Carlsson, G., & Norrgren L., Synthetic musk toxicity to early life stages of zebrafish (*Danio rerio*). *Archives of Environmental Contamination and Toxicology*, **46**, pp. 102–105, 2004.
[10] Chou, Y., & Dietrich, D.R., Interactions of nitromusk parent compounds and their amino-metabolites with the estrogen receptors of rainbow trout (*Oncorhynchus mykiss*) and the South African clawed frog (*Xenopus laevis*). *Toxicology Letters*, **111**, pp. 27–36, 1999.
[11] Schreurs, R.H.M.M., Legler, J., Artola-Garicano, E., Sinnige, T.L., Lanser, H., & Van Der Burg B., In vitro and in vivo antiestrogenic effects of polycyclic musks in zebrafish. Environmental Science and Technology **38**, pp. 997-1002, 2004.
[12] Yamauchi, R., Ishibashi, H., Hirano, M., Mori, T., Kim, J-W., & Arizono, K., Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (Oryzias latipes). *Aquatic Toxicology* **90**, pp. 261-268, 2008.
[13] Goksøyr, A., & Förlin, L., The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicology* 22, pp. 287–312, 1992.
[14] Iwata, N., Minegishi, K., Suzuki, K., Ohno, Y., Igarashi, T., Satoh, T., & Takahashi, A., An unusual profile of musk xylene-induced drug-metabolizing enzymes in rat liver. *Biochemical Pharmacology*, **45**, pp. 1659-1665, 1993.

[15] Lehman-McKeeman, L.D., Caudill, D., Vassallo, J.D., Pearce, R.E., Madan, A., & Parkinson, A., Effects of musk xylene and musk ketone on rat hepatic cytochrome P450 enzymes. *Toxicology Letters*, **111**, pp. 105–115, 1999.
[16] Lehman-McKeeman, L.D., Johnson, D.R., & Caudill, D., Induction and inhibition of mouse cytochrome P-450 2B enzymes by musk-xylene. *Toxicology and Applied Pharmacology*, **142**, pp. 169-177, 1997.
[17] Suter-Eichenberger, R., Boelsterli, U.A., Conscience-Egli, M., Lichtensteiger, W., & Schlumpf, M., CYP 450 enzyme induction by chronic oral musk xylene in adult and developing rats. *Toxicology Letters*, **115**, pp.73–87, 2000.
[18] Steinberg, P., Fischer, T., Arand, M., Park, E., Elmadfa, I., Rimkus, G., Brunn, H., Dienes, & H-P., Acute hepatotoxicity of the polycyclic musk 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthaline (AHTN). *Toxicology Letters*, **111**, pp. 151-160, 1999.
[19] Schnell, S., Martin-Skilton, R., Fernandes, D., & Porte, C., The interference of nitro- and polycyclic musks with endogenous and xenobiotic metabolizing enzymes in carp: an in vitro study. *Environmental Science and Technology*, **43**, pp. 9458 – 9464, 2009.
[20] Fent, K., Fish cell lines as versatile tools in ecotoxicology: assessment of cytotoxicity, cytochrome P4501A induction potential and estrogenic activity of chemicals and environmental samples. Toxicology in Vitro, **15**, pp. 477 – 488, 2001.
[21] Hahn, M.E., The aryl hydrocarbon receptor. A comparative perspective. *Comparative Biochemistry and Physiology C*. **121**, pp. 23 – 54, 1998.
[22] Repetto, G., del Peso, A., & Zurita, J.L., Neutral red uptake assay for the estimation of cell viability/cytotoxicity. Nature Protocols **3**, pp. 1125-1132, 2008.
[23] Pfaffl, M., A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acid Research*, **29**, pp. 2002-2007, 2001.
[24] Traven, L., Žaja, R., Lončar, J., Smital, T., & Mićović, V., CYP1A induction potential and the concentration of priority pollutants in marine sediment samples – In vitro evaluation using the PLHC-1 fish hepatoma cell line. *Toxicology in Vitro*, **22**, pp. 1648 – 1656, 2008.
[25] Bradford, M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, pp. 248-254, 1976.
[26] Schnell, S., Bols, N.C., Barata, C., & Porte, C., Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. *Aquatic Toxicology,* **93**, pp. 244-252, 2009.
[27] OSPAR Commission. *Ospar Background Document on Musk Xylene and Other Musks*, Hazardous Substances Series; OSPAR Commission: London, pp. 45, 2004.

[28] Navas, J.M., & Segner, H., Estrogen-mediated suppression of cytochrome P4501A (CYP1A) expression in rainbow trout hepatocytes: role of estrogen receptor. *Chemico – Biological Interactions*, **138**, pp. 285–298, 2001.
[29] Grans, J., Wassmur, B., & Celander, M.C., One-way cross-talk between arylhydrocarbon receptor (AhR) and estrogen receptor (ER) signalling in primary cultures of rainbow trout hepatocytes. *Aquatic Toxicology*, **100**, pp. 263-270, 2010.
[30] Mersch-Sundermann, V., Emig, M., & Reinhardt, A., Nitro musks are cogenotoxicants by inducing toxifying enzymes in the rat. *Mutation Research*, **356**, pp. 237–245, 1996.
[31] Chen, G., Konstantinov, A.D., Joyce, E.M., Bols, N.C., and Bunce, N.J., Synthesis of polybrominated diphenyl ethers and their capacity to induce CYP1A by the Ah receptor mediated pathway. *Environmental Science and Technology*, **35**, pp. 3749-3756, 2001.
[32] Hestermann, E.V., Stegeman, J.J., & Hahn, M.E., Relative contributions of affinity and intrinsic efficacy to aryl hidrocarbon receptor ligand potency. *Toxicology and Applied Pharmacology,* **168**, pp. 160-172, 2000.