Arch Environ Contam Toxicol 53, 390–396 (2007)
DOI 10.1007/s00244-006-0190-4

Chub *(Leuciscus cephalus)* as a Bioindicator of Contamination of the Vltava River by Synthetic Musk Fragrances

K. Hájková · J. Pulkrabová · J. Hajšlová · T. Randák · V. Žlábek

Received: 4 September 2006 / Accepted: 10 March 2007

Abstract Synthetic musk fragrances, which are contained in almost all scented consumer products, enter aquatic environment mainly by way of wastewater paths. To monitor contamination of the Vltava River by these relatively persistent chemicals in the surroundings of Prague industrialized agglomeration, chub (*Leuciscus cephalus*) was employed as a bioindicator. Validated gas chromatography–mass spectrometry method was used for fish sample examination. Polycyclic musks, represented by 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(γ)-2-benzopyran (galaxolide) and 1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl)-ethanone (tonalide) were the most abundant representatives of this group; their levels in fillets were in the range of 1.7 to 105.9 μg/kg and 0.9 to 19.3 μg/kg wet weight, respectively. Nitro-musks, musk ketone, and musk xylene were also detected in most samples; nevertheless, their levels were lower, ≤ 2 μg/kg. Significant contamination of resident fish was recognised in the locality of Klecany, which is located 6 km downstream from a large municipal sewage-treatment plant (STP). High levels of musks found in the locality of Vraňany, 32 km downstream from Prague STP, were caused not only by this emission source but probably also by the effluents from the local plant that produces cleaning agents in Velvary.

K. Hájková · J. Pulkrabová · J. Hajšlová (✉)
Department of Food Chemistry and Analysis, Institute of Chemical Technology, Technická 3, 166 28, Prague 6, Czech Republic
e-mail: jana.hajslova@vscht.cz

T. Randák · V. Žlábek
České Budějovice, Research Institute of Fish Culture and Hydrobiology, University of South Bohemia, Zátiší 728/II, 389 01 Vodňany, Czech Republic

Thousands of organic pollutants have been produced and released into the environment during the last century. Since approximately the early 1960s, people have become aware of the potential adverse effects of these chemicals for humans as well as their risks for biota of aquatic and terrestrial ecosystems. The number of organochlorine and other ''classic'' persistent organic pollutants (POPs) has been investigated in various environmental compartments. Since the early 1990s, several ''new'' groups of relatively persistent xenobiotics have become a concern because of their widespread occurrence in aquatic environments. Synthetic musk fragrances are anthropogenic chemicals representing one of such groups.

Musk compounds (MCs) have been widely applied to scent various personal care products, such as perfumes, soaps, creams, detergents, etc. They have been synthesized to substitute precious and expensive natural musks, which have mainly been obtained in the past from male musk deer and some plants. The first generation of more affordable synthetic musk analogues included substituted trinitrobenzenes and dinitrobenzenes. This group of nitro-musks, mainly musk xylene (MX) and musk ketone (MK), has been used since the end of 19th century (Ricking et al. 2003; Rimkus 1999). In recent years, polycyclic musks have become the most important commercial synthetic musks because of concern about the environmental distribution and toxicologic effects of the nitro-musks and subsequent decrease in their use (Rimkus 2004).

Musks are semivolatile, relatively hydrophobic substances (Langedijk et al. 1999; Kuplet et al. 2004). The values of the octanol–water partition coefficient (K_{OW}) of polycyclic musks are comparable with those tabulated for some lipophilic environmental pollutants, *e.g.*, low-chlorinated PCBs or some organochlorine pesticides, such as hexachlorbenzene or DDT and its metabolites (Bester et al.

1998). The K_{OW} values of nitromusks are lower (an approximate difference between nitro-musks and polycyclic musks is approximately two orders of magnitude [Rimkus 1999]). Becasue of their relatively lipophilic nature, these musk fragrances are capable of bioconcentrating in various aquatic species (García-Jares et al. 2002; Gatermann et al. 2002a, 2002b; Wann et al. 2007). However, there is no evidence on their capacity for biomagnification. Contrary to halogenated POPs, the concentrations of chemicals in fish and other biota appear more likely to be a function of momentary exposure than the consequence of a lifetime of concentration from a contaminated aquatic environment (Dietrich 1999). On this account, fish is an excellent bioindicator of musk emission sources. These compounds find their way to freshwaters mainly by way of sewage-treatment plant (STP) wastewaters because both parent musks and their metabolites are poorly biodegradable (Kannan et al. 2005; Rimkus et al. 1995).

Because of environmental persistence, adverse effects on exposed biota can be assumed. Although the risk of acute toxicity is low, possible chronic effects must also be considered (García-Jares et al. 2002). For instance, endocrine modulation by nitro-musks in some biota has been reported. Unfortunately, present available data do not enable the completion of toxicologic risk assessment (Breitholtz et al. 2003). In any case, monitoring musks, in both aquatic and terrestrial environments, should be carried out to collect more information for this purpose.

In this article, the extent of aquatic ecosystem pollution by synthetic MC from STPs and other emission sources in the Prague area was assessed. Data obtained by analysis of chub (*Leuciscus cephalus*) collected at several localities alongside the Vltava River were used as the basis for evaluation.

Two groups of MCs were monitored within the study. The first included the following polycyclic MCs: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(γ)-2-benzopyran (galaxolide [HHCB]); 1-(5,6,7,8-tetrahydro-3,5,5,6,8,8-hexamethyl-2-naphthalenyl)-ethanone (tonalide [AHTN]); 6-acetyl-1,1,2,3,3,5-hexamethylindane (phantolide [AHMI]); 4-acetyl-6-*tert*-butyl-1,1-dimethylindane (celestolide [ADBI]); and 5-acetyl-3-isopropyl-1,1,2,6-tetramethylindane (traseolide [ATII]). The second group included nitro-MCs, such as 1-*tert*-butyl-3,5-dimethyl-2,4,6-trinitrobenzene (MX) and 1-*tert*-butyl-3,5-dimethyl-2,6-dinitro-4-acetylbenzene (MK).

Materials and Methods

Chemicals and Standards

Reference compounds of galaxolide, tonalide, phantolide, celestolide, and traseolide (purity 75%, 98%, 94.5%, 98%, and 98%, respectively) were purchased as solids and nitro-MCs as liquid standard solutions (LGC Promochem, Germany). The liquid internal standards ^{3}D tonalide and ^{15}D MX (both 10 μg/mL cyclohexane, 99% purity) were supplied by Dr. Ehrenstorfer (Germany). Stock standard solutions (1 mg/mL each of the polycyclic MCs) were prepared in isooctane. Working solutions of the mixtures of all analytes were prepared at various concentrations by appropriate dilution of the stock solutions in isooctane. Their concentrations were in the range of 0.5 to 1000 ng major musks (HHCB and AHTN), 0.5 to 100 ng ATII and MX, 1 to 100 ng MK, and 0.2 to 100 ng AHMI and ADBI/mL isooctane. Each working solution contained 40 ng/mL deuterated musk analogues.

All solvents were of analytic grade: ethyl acetate for pesticide residue analysis and dichloromethane for gas chromatography (Scharlau, Spain); and hexane and cyclohexane for gas chromatography as well as iso-octane for spectroscopy (Merck, Germany). Technical gases used for gas chromatography analyses were helium 6.0 and nitrogen 5.0 (Linde, Czech Republic). Anhydrous sodium sulphate (Penta, Czech Republic) used for dessication was heated to 600°C for 7 hours and stored in a dark vessel in dessicator before use. Styrene-divinylbenzene gel (Bio-Beads S-X3, 200 to 400 mesh) was purchased from Bio Rad (United States).

Sampling

Chub (*L. cephalus*) were caught by electrofishing in sampling sites located both upstream (Prague–Podolí) and downstream (Prague–Klecany, Vraňany) from the potential emission source represented by the Prague urban area on the Vltava River. The sampling locality of Klecany is located only 6 km downstream from the Prague Central STP, which has a capacity 135 million m^3 wastewater treated/y. This STP serves for 1.2 million inhabitants. The locality of Vraňany is situated 32 km downstream from the Prague STP. A fishpond near Vodňany town in South Bohemia was selected as an unpolluted area representing a control site of the study. The locations of the sampling sites are illustrated in Figure 1. Twelve fish samples from each locality were collected by the Hydrobiological Research Institute in Vodňany (Czech Republic) in June 2005. After the samples were weighed, body length measured, and fish sexed, skin-free muscle tissues were deep-frozen and transported in plastic bags to our laboratory.

Sample Preparation Procedure

The pooled samples of fish muscle were thoroughly homogenised, and 30 g was mixed with anhydrous sodium sulphate in a ratio of 1:4 (w/w). Pre-extracted cellulose

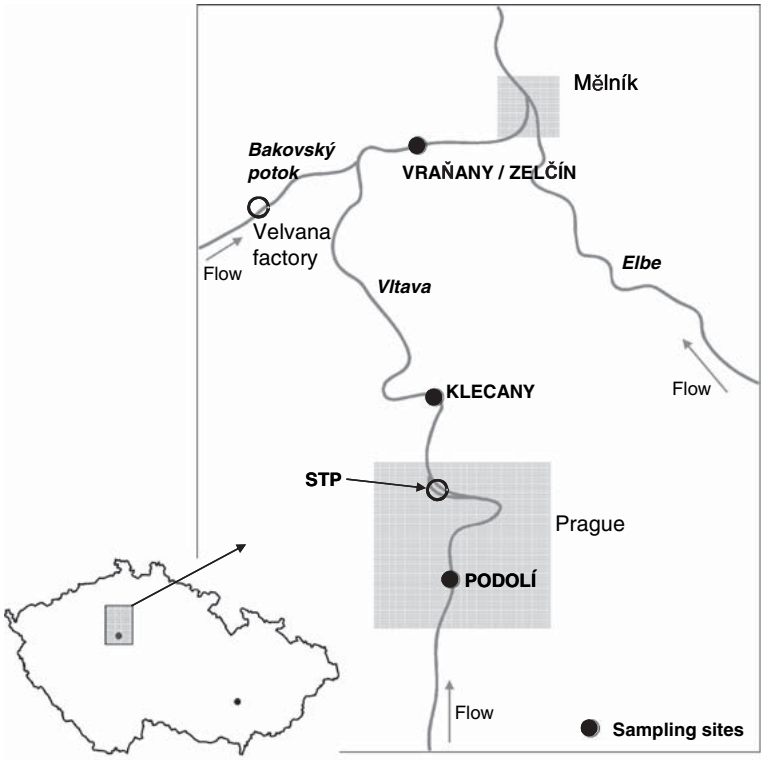

Fig. 1 Sampling sites investigated during the study. The fish were sampled in 2005 at several sampling sites located on the Vltava River

cartridges (Soxhlet extraction, 340 mL hexane and dichloromethane, 1:1 *v/v*, 2 hours) were filled with the prepared mixture of sample with sodium sulphate and plugged with glass wool. Sodium sulphate was added into the sample matrix, which was pit into an exsiccator for 12 hours. Soxhlet extraction of fish samples with 340 mL of a mixture of hexane and dichloromethane (1:1, *v/v*) was carried out and maintained for 7 hours with a frequency of 9 to 10 extraction cycles/h. The extract was rotary vacuum evaporated to the last drop, which was removed using a gentle nitrogen stream. Afterward, the lipid content in examined tissue was determined gravimetrically.

An automated gel permeation chromatography (GPC) system (Gilson 231XL, France), equipped with a stainless steel column (500 x 8 mm i.d.) packed with Bio-Beads S-X3 (200 to 400 mesh), was employed to separate target analytes from the coextracted lipids in crude extracts. A mixture of cyclohexane and ethyl acetate mixture (1:1 *v/v*) was used as mobile phase (mobile phase flow rate 0.6 mL/min). The residue left after removing the extraction solvent was dissolved in 10 mL GPC mobile phase, and an aliquot of 2 mL was transferred onto the column for the cleanup procedure. Eluate fraction of 14 to 30 mL, containing the major portion of target MCs, was collected and vacuum evaporated. A gentle nitrogen stream was applied to remove the last decrease of the residue solvent including isolated analytes. The sample residue was dissolved in 500 µL a syringe of internal standard mixture containing 40 ng/mL ^{3}D tonalide and ^{15}D MX in iso-octane and transferred into GC vials for instrumental analysis.

Identification and Quantification of Analytes

Identification and quantification of MCs was carried out using a gas chromatograph HP 6890 (Agilent Technologies, United States) coupled to a quadrupole (Q) mass selective detector ([MSD] HP MSD 5973) operated in electron ionization (EI) mode. A DB-5MS capillary column (60 m × 0.25 mm × 0.25 µm; J&W Scientific, United States) was used for separation. Oven temperature was programmed as follows: 60 °C (2 minutes), then 45 °C/min to 180 °C, 2 °C/min to 220 °C, and 60 °C/min to 280 °C (2 minutes). The mass spectrometer was operated in selected ion monitoring mode. A sample volume of 1 µL was injected into the gas chromatograph. Ions (*m/z*) used for identification (quantification ions are in italics) were *243*, 213, 258 (HHCB); *243*, 258, 201 (AHTN); *282*, 297, 283 (MX); *279*, 294, 280 (MK); *229*, 244, 173 (ADBI); *229*, 244, 187 (AHMI); and *215*, 173, 258 (ATII).

Internal standard calibration technique was used for quantification of MCs. The linearity range for the HHCB limit of quantification (LOQ) was defined as 10 times the background signal. Performance characteristics of the method are listed in Table 1.

Table 1 Performance characteristics of the method used for analysis of musk compounds in fish tissues

Compound	LOQ[a] (μg/kg wet weight)	LOQ[a] (μg/kg lipid weight)	Linearity range (ng/mL)	Recovery (%)	Repeatability (RSD, %)
HHCB	1.0	50	15–1000	99	1.9
AHTN	0.8	40	10–1000	92	2.2
AHMI	0.4	20	5–100	95	3.0
ADBI	0.4	20	5–100	90	2.1
ATII	0.4	20	5–100	94	2.7
MX	0.3	15	5–100	101	2.5
MK	0.3	15	5–100	96	1.8

[a] The value corresponds to the lowest calibration level for which the uncertainities were estimated. LOQ is defined as ten times the background signal

RSD = Relative standard deviation

Results and Discussion

Chub As a Bioindicator

In accordance with common practice employed in many monitoring studies concerned with the occurrence of organic micropollutants in freshwater (de Boer & Brinkman 1994), fish was used as a bioindicator of river pollution. The suitability of the chub (*L. cephalus*) that we employed for this purpose was demonstrated in our earlier monitoring studies (Hajšlová & Šetková 2004; Šetková 2004). Chub represents an omnivorous species and was available at all sampling sites. Normally, migration of fish alongside the river may occur, leading to some uncertainty regarding the reflection of particular locality pollution. In our case, this factor was almost eliminated because weirs surrounded selected sampling sites. It should be noted that according to similar results of measurements of other bioaccummulating pollutants, the levels of musk fragrances found in fish tissues indicate only the bioavailable fraction.

Levels of MCs in the Examined Sampling Sites

Overall, the most abundant musk fragrances found in our study were HHCB and AHTN, representing polycyclic musks. Both of them were present together in all analyzed fish samples (mixtures of these compounds are used for fragrance compositions). Their concentrations on a wet-weight basis ranged from 1.7 to 105.9 μg/kg (HHCB) and from 0.4 to 19.3 μg/kg (AHTN) (Table 2) and were well correlated with each other. The levels of HHCB and AHTN in fish from control locality were relatively low. Compared

Table 2 Mean concentration of musk compounds in chub from the river Vltava (2005)[a] and SD–in both μg/kg wet weight and lipid weight

Compound	Control site	Prague–Podolí	Prague–Klecany downstream STP	Vraňany
Lipid content (%)	2.3 (0.6)	1.9 (1.1)	1.7 (0.9)	2.4 (1.2)
Weight (g)	271 (153)	291 (61)	303 (144)	634 (222)
HHCB				
(ww)	3.1 (1.0)	13.5 (10.4)	32.1 (25.8)	44.0 (29.5)
(lw)	136 (31)	663 (326)	1900 (660)	1744 (405)
AHTN				
(ww)	1.7 (0.4)	2.6 (1.6)	8.4 (4.6)	5.9 (2.9)
(lw)	76 (13)	134 (59)	525 (164)	248 (36)
MX				
(ww)	<0.3	<0.3	1.7 (1.3)[a]	1.7 (1.2)
(lw)	<15	<15	92 (41)[a]	67 (20)
MK				
(ww)	<0.3	<0.3	0.6 (0.7)[a]	0.7 (0.4)[a]
(lw)	<15	<15	27 (21)[a]	30 (14)[a]

[a] These statistical data were calculated from all values using half of the LOQ if the compound could not be quantitatively determined

lw = lipid weight; ww = wet weight

SD = standard deviation

Table 3 Musk compounds in chub muscle (μg/kg lipid weight) from various years and localities (Hajšlová and Šetková 2004, Kněžourková 2004)

Analyte	Prague–Podolí		Prague–Klecany downstream STP					Zelčín–Vraňany	
	2003 (2 samples)	2005 (12 samples)	1998 (6 samples)	1999 (18 samples)	2000 (20 samples)	2003 (2 samples)	2005 (12 samples)	2000 (9 samples)	2005 (12 samples)
HHCB									
Mean	273	663	2693	1274	1555	3227	1900	855	1744
RSD (%)	35	49	25	23	24	30	35	22	23
AHTN									
Mean	81	134	2448	831	1283	754	525	802	248
RSD (%)	15	44	19	18	29	27	31	21	14
MX									
Mean	29	<15	494	160	169	138	92	146	67
RSD (%)	11	–	14	14	26	2	44	13	30
MK									
Mean	16	<15	213	121	127	65	26	87	30
RSD (%)	15	–	14	15	18	2	79	16	47

RSD = Relative standard deviation

with to fish from rivers, no relation between the levels of these two polycyclic musks was observed. This may be explained by a indirect contamination pathway of the pond. More so than STP wastewaters discharged into the pond, musk contamination probably occurred by way of atmospheric transport. None of the examined fish samples contained detectable amounts of minor polycyclic musks, such as AHMI, ADBI, and ATII. Nitro-musk fragrances were present in fish in considerably lower amounts than HHCB and AHTN; MX was found in 58% of the samples at levels ranging from <0.3 (LOQ) to 4.9 μg/kg wet weight; and MK was found in 40% of the examined samples at concentrations ranging from <0.3 (LOQ) to 2.4 μg/kg wet weight. The mean concentrations of the synthetic MCs in fish from localities involved in the current study followed the same order (HHCB > AHTN >> MX > MK), as reported in earlier Czech studies (Hajšlová & Šetková 2004; Šetková 2004). Also in studies conducted in Germany at the end of last century, HHCB—followed by AHTN—were the dominating musks in freshwater; however, MX levels were always higher than those of MK (Rimkus 1999; Gatermann et al. 1999). In Table 3, current data (2005) are compared with the available results obtained by examination of chub in monitoring studies carried out earlier. It is interesting to note that the ratio of two dominating musks, HHCB and AHTN, varied between the monitoring years in the range 1.1 to 4.9, which may indicate variability of fragrance sources used for source product scenting. Similar levels of total musks were reported in other studies in fish bioindicators caught in localities close to large urban industrialized areas, such as Prague. However, it should be emphasized that their levels are species dependent (Heberer 2002), and only using the same fish is fully relevant for the sake of comparison. This fact is not only related different accumulation parameters, however; it is also realted to different enzymatic transformation pathways (Gatermann et al. 2002a, 2002b).

Regarding the expression of generated data, most similar studies concerned with the occurrence of lipophilic persistent chemicals (mainly organochlorine contaminants) in biota have report their concentrations to be normalized to the lipid content. However, for the evaluation of fish total body burden, as well as for the purpose of a risk assessment for consumers related to dietary intake, information on concentration of contaminants in the edible portion of fish (expressed in wet weight) is more useful (Hajšlová & Šetková 2004). In any case, Fig. 2 well illustrates apparent discrepancies in the identification of the most polluted locality by means of bioindicator analysis. The ranking of sampling sites by pollution order depends on musk content expression: based on the lipid basis, Klecany was the most contaminated sampling site, whereas the highest levels of musks in wet fish tissue identified Vraňany as the locality with the most intensive pollution. In the latter case, the fish fat content from this sampling site was higher (see Table 2), which resulted in some ''dilution'' of accummulated contaminants in this receiving medium. Nevertheless, the total body burden of the employed bioindicator was higher in Vraňany compared with Klecany. Under common conditions, levels of musks and other compounds present in wastewaters succesively decreased with increasing distance from the STP. However, this was not the trend in our particular case, although no other municipal wastewater emission source exists between these two sampling sites. Therefore, we assume that the pollution of aquatic ecosystem in this small village, 26 km

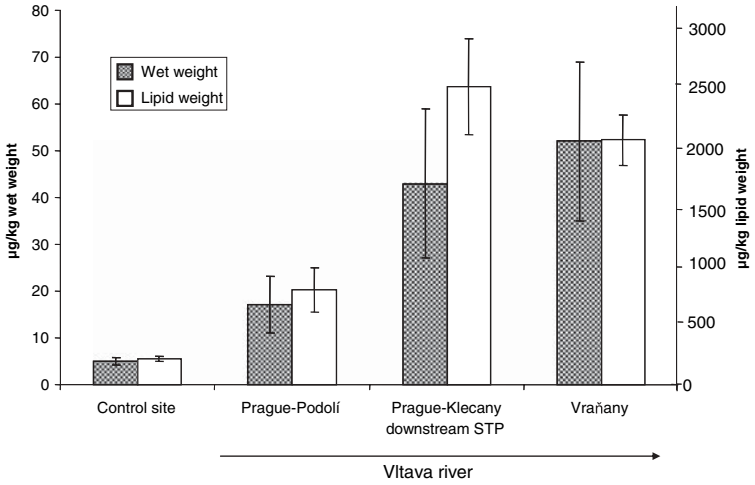

Fig. 2 Contamination of chub alongside the Vltava River by MCs. Sum of four main musks (HHCB, AHTN, MX, and MK) with standard deviations. The figure illustrates the apparent discrepancy in the identification of the most polluted locality by means of bioindicator analysis. The conclusion depends on musk content expression. On the lipid basis, Klecany was the most contaminated sampling site, whereas the highest levels of musks in wet fish tissue were in the locality of Vraňany

downstream from Klecany, was caused by effluents from a local producer of consumer cleaning agents Velvana nearby a stream called Bakovský potok (7 km to the Vltava River) (Fig. 1).

Although some decrease in levels of musk fragrances occurred in some surface waters in Europe, *e.g.*, significant decrease of nitro-musks and polycyclic compounds, was observed in trouts within the period from 1992 to 2003 (Duedahl-Olesen et al. 2005), no time-trend in concentrations of musks can be seen when comparing our data from 2005 with those obtained in the earlier studies conducted in 1998, 1999, 2000, and 2003 (mentioned in Table 3). One should be aware, however, that water flow in any particular sampling season plays an important role in influencing the concentration of musks in water; therefore, their content caused by bioconcentration in aquatic biota may vary largely.

In summary, pollution of the Vltava River was shown to occur alongside the river. The results suggest that the Prague STP is one of the most significant sources of musk fragrance contamination of the Vltava River ecosystem. Fish, such as chub (*L. cephalus*), was shown to be an excellent bioindicator of the proximity influents containing these contaminants.

Acknowledgments The development of analytical method for MCs was carried out within the projects COST 636 and MSM 6046137505 funded by Czech Ministry of Youth and Education. The study was supported by USB RIFCH No. MSM 6007665809 and Ministry of the Environment of the Czech Republic VaV/650/5/03.

References

Bester K, Hühnerfuss H, Lange W, Rimkus G, Theobald N (1998) Results of non target screening of lipophilic organic pollutants in the German bight II: Polycyclic musk fragrances. Wat Res 32:1857–1863

Boer de J, Brinkman UAT (1994) The use of fish as biomonitors for the determination of the aquatic environment by persistent organochlorine compounds. Trends Anal Chem 13:397–404

Breitholtz M, Wollenberger L, Dinan L (2003) Effects of four synthetic musks on the life cycle of the harpacticoid copepod *Nitocra spinipes*. Aquatic Toxicol 63:103–118

Dietrich DR (1999) Editorial. Toxicol Lett 111:1–4

Duedahl-Olesen L, Cederberg T, Pedersen KH, Hojgard A (2005) Synthetic musk fragrances in trout from Danish fish farms and human milk. Chemosphere 61:422–431

García-Jares C, Llompart M, Polo M, Salgado C, Macías S, Cela R (2002) Optimisation of a solid-phase microextraction method for synthetic musk compounds in water. J Chromatogr A 963:277–285

Gatermann R, Hellou J, Hühnerfuss H, Rimkus G, Zitko V (1999) Polycyclic and nitromusks in the environment: A comparison between Canadian and European aquatic biota samples. Chemosphere 38:3431–3441

Gatermann R, Biselli S, Hühnerfuss H, Rimkus GG, Hecker M, Karbe L (2002a) Synthetic musks in the environment. Part 1: Species-dependent bioaccumulation of polycyclic and nitro musk fragrances in freshwater fish and mussels. Arch Environ Contam Toxicol 42:437–446

Gatermann R, Biselli S, Hühnerfuss H, Rimkus GG, Franke S, Hecker M, et al. (2002b) Synthetic musks in the environment. Part 2: Enantioselective transformation of the polycyclic musk fragrances HHCB, AHTN, AHDI, and ATII in freshwater fish. Arch Environ Contam Toxicol 42:447–453

Hajšlová J, Šetková L (2004) Synthetic musk fragrances in the environment. In: Rimkus GG (ed) The handbook of environmental chemistry. Springer-Verlag, Germany, pp 151–188

Heberer T (2002) Occurrence, fate and assessment of polycyclic musk residues in the aquatic environment of urban areas-a review. Acta Hydrochim Hydrobiol 30:227–243

Kannan K, Reiner JL, Hun Yun S, Perrotta EE, Tao L, Johnson-Restrepo B, et al. (2005) Polycyclic musk compounds in higher trophic level aquatic organisms and humans from the United States. Chemosphere 61:693–700

Kněžourková K (2004) Sledování kontaminace vodního ekosystému organickými polutanty. Diploma thesis, Institute of Chemical Technology, Prague

Langedijk AC, Spinelli S, Anguille C, Hermans P, Nederlof J, Butenandt J, et al. (1999) Insight into odorant perception: The crystal structure and binding characteristics of antibody fragments directed against the musk odorant traseolide. J Mol Biol 292:855–869

Ricking M, Schwarzbauer J, Hellou J, Svenson A, Zitko V (2003) Polycyclic aromatic musk compounds in sewage treatment plant effluents of Canada and Sweden—First results. Marine Pollut Bull 46:410–417

Rimkus GG (1999) Polycyclic musk fragrances in the aquatic ecosystem. Toxicol Lett 111:37–56

Rimkus G (2004) The handbook of environmental chemistry. Volume 3, Part X: Synthetic musk fragrances in the environment. Springer-Verlag, Germany

Šetková L (2004) The fate of organic contaminants in aquatic ecosystem. Doctoral thesis, Institute of Chemical Technology, Prague

Wann Y, Wei Q, Hu J, Jin X, Zhang Z, Zhen H, et al. (2007) Levels, tissue distribution, and age-related accumulation of synthetic musk fragrances in Chinese sturgeon (*Acipenser sinensis*): Comparison to organochlorines. Environ Sci Technol 41:424–430