

Toxicity of nitromusks in early lifestages of South African clawed frog (*Xenopus laevis*) and zebrafish (*Danio rerio*)

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Abstract

Musk xylene (MX), musk ketone (MK) and musk moskene (MM) are synthetic nitro-containing fragrances. Due to their inherent lipophilicity and environmental persistence, they are frequently detected in environmental samples and especially in aquatic ecosystems. Despite this, the current environmental toxicity database of nitromusks is limited. Although nitromusks have been shown to accumulate in aquatic organisms, little is known about their potential developmental effects in the respective aquatic species. To investigate the developmental toxicity of these compounds to amphibians and fish, early lifestages of xenopus (*Xenopus laevis*) and zebrafish (*Danio rerio*) were exposed to three nitromusks for 96 h to examine the developmental effects of these compounds in the two species. Nitromusk body concentration measurements were carried out in parallel for correlation with potential developmental effects. No increased mortality, malformation or growth inhibition was observed in either species following 96-h exposure to 400 µg/l MX, MK and MM. However, an approximately 20% reduced viability was observed in xenopus larvae when exposed to 400 µg/l MX, MK and MM for 11 days. Xenopus and zebrafish exposed to 10, 153, 871 and 1637 µg/l ¹⁴C-MX for 96 h resulted in whole-body concentrations of 0.7 ± 0.1 , 11.1 ± 1.1 , 38.7 ± 1.9 and 76.3 ± 18.3 µg/g, and 4.3 ± 0.6 , 73.3 ± 11.8 , 440.0 ± 72.7 and 664.0 ± 47.7 µg/g wet body weight, respectively. Exposure of xenopus larvae to 400 µg/l MX, MK and MM for 11 days, resulted in whole body concentrations (extrapolated from gas chromatographic determinations) of 4700 ± 5000 , 1300 ± 300 and 4600 ± 4800 µg/g wet weight for MX, MK and MM, respectively. The latter toxicity results, in conjunction with the fact that the concentrations used for the above experiments were between 400- and 10 000-fold higher than those detected in the environment, suggest that environmental concentrations of nitromusks are not hazardous for early lifestages of fish and amphibians. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Nitromusks; Uptake; Toxicity; Early lifestages; Fish; Amphibian

1. Introduction

Musk xylene (MX; 1-*tert*-butyl-3, 5-dimethyl-2, 4, 6-trinitrobenzene), musk ketone (MK; 4-acetyl-1-*tert*-butyl-3, 5-dimethyl-2, 6-dinitrobenzene) and musk moskene (MM; 4, 6-dinitro-1, 1, 3, 3, 5-pentamethylindane) are synthetic fragrances

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found in various products including detergents, cosmetics, perfumes, etc. (Sommer, 1993). Due to their lipophilicity (K_{ow} : 4.9 and 4.3 for MX and MK, respectively; Tas and van de Plassche, 1996) and planar nitro-containing aromatic structure, between 10 and 80% of nitromusks may either be removed during sewage treatment processes by direct adsorbing to the sludge fraction or transformed via nitro reduction to more polar amine metabolites (Rimkus et al., 1999) during the anaerobic processes in the sewage plant (Tas and van de Plassche, 1996; Rimkus et al., 1998). The remaining fraction of nitromusks, in addition to the more polar amine metabolites, is released via the sewage treatment plant into the environment. In consequence, nitromusks and their respective metabolites have been detected in the aquatic environment (ng- μ g/l) and in various aquatic organisms (μ g-mg/kg lipid; Hahn, 1993; Rimkus and Wolf, 1993; Gatermann et al., 1995; Tas and van de Plassche, 1996; Rimkus et al., 1999). However, the toxicological and/or developmental impact of these compounds on aquatic organisms in the environment is still not known. Only one toxicokinetic study regarding aquatic organisms has been previously published which indicated MX to accumulate rapidly in trout (Boleas et al., 1996). The LC_{50} s of MX and MK have been determined in various organisms (approximate range of 0.4–3.75 mg/l). These values are, however, well above the water solubility of the respective compounds (0.49 and 1.9 mg/l for MX and MK, respectively; Tas and van de Plassche, 1996) and also decisively exceed the concentrations detected in the environment (Ford, 1996; Tas and van de Plassche, 1996). In contrast, the NOECs (endpoints: mortality, swimming behavior and growth rate) of MX and MK determined in respective aquatic organisms are between 0.056 and 0.32 mg/l (Ford, 1996; Schramm et al., 1996; Tas and van de Plassche, 1996), lying therefore within the range of nitromusks water solubility. In view of this, no acute toxicity is therefore expected from the release of nitromusks into the environment. However, considering the fact that aquatic organisms are chronically exposed to nitromusk compounds in the receiving surface waters of sewage treatment plants, it was considered essen-

tial to study their effects on the development of aquatic organisms. Therefore, in this study, early life stages of xenopus (*Xenopus laevis*) and zebrafish (*Danio rerio*) were exposed to nitromusks to determine developmental endpoints, e.g. mortality, malformation, and normal growth (length).

2. Materials and methods

2.1. Chemicals

MX (CAS #: 81-15-2, MW: 297, water solubility: 0.49 mg/l), MK (CAS #: 81-14-1, MW: 294, water solubility: 1.9 mg/l) and MM (CAS #: 116-66-5, MW: 278, water solubility: 0.046 mg/l) were obtained from Givaudan-Roure (Clifton, NJ, USA). Quintozene was kindly provided by Dr Regina Suter (University Zürich, Switzerland), 14 C-musk xylene (18.2 mCi/mmol, purity > 96.7%) was a gift from Dr Lehman-McKeeman (Procter & Gamble, OH, USA). All other chemicals including Florisil® (mesh 60–100) were reagent grade and obtained from Sigma (St Louis, MO, USA).

2.2. Amphibian and fish embryos

2.2.1. Clawed frog (*X. laevis*)

Adult pairs were obtained from the in-house animal research facility (TFA) at the University of Konstanz. The adults were maintained in synthetic FETAX solution (ASTM, 1991) containing 625 mg NaCl, 96 mg NaHCO_3 , 30 mg KCl, 15 mg CaCl_2 , 60 mg $\text{CaSO}_4 \times 2\text{H}_2\text{O}$ and 75 mg MgSO_4 per liter of reagent grade water in a 20-l aquarium at $24 \pm 2^\circ\text{C}$ and with a 14-h/10-h, light/dark cycle. To obtain embryos, 250 and 150 IU of pregnant mare serum gonadotropin (PMSG) were injected into the dorsal lymph sac of female and male frogs, respectively, followed by an injection of 700 and 600 IU of human chorionic gonadotropin (HCG) to female and male adults, respectively, 48 h after the first injection. The resulting fertilized eggs were collected, dechorionated in 2% w/v cysteine solution (pH: 8.1), sorted for viability and staged according to the criteria of Nieuwkoop and Faber (1975). Embryos between stages 8 and 11 were selected for experiments.

2.2.2. Zebrafish (*D. rerio*)

Mature zebrafish were raised in-house and held in charcoal filtered, dechlorinated water in a 20-l aquarium at $28 \pm 1^\circ\text{C}$ with a 14-h/10-h, light/dark cycle. Males and females were isolated to prevent spawning until required. Eggs were collected, pooled, rinsed, and placed in embryo medium containing 800 mg NaCl, 350 mg NaHCO_3 , 40 mg KCl, 144 mg CaCl_2 , 246 mg $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 3.6 mg Na_2HPO_4 and 6 mg KH_2PO_4 per liter of reagent grade water at $24 \pm 2^\circ\text{C}$ until their development reached the stage of half epiboly (approx. 5 h; Westerfield, 1995). All experiments were carried out with embryos having reached, i.e. surpassed half-epiboly.

2.3. Test concentrations

Solutions (400 $\mu\text{g/l}$) of MX, MK and MM were made by dissolving the respective compounds in FETAX solution (see above) and mixing vigorously overnight. Samples of the solutions were taken (100 ml) periodically, added with quinotozene (extraction recovery control) and extracted with *n*-hexane (3×20 ml) in a separatory funnel. The organic phase was collected, reduced to a final volume of 10 ml using rotary evaporation and analyzed by gas chromatography (see below).

2.4. Toxicity of nitromusks in early lifestages of *xenopus*

2.4.1. Toxicity experiments

Musk testing solutions were prepared by dissolving 400 $\mu\text{g/l}$ musk xylene, musk ketone or musk moskene in FETAX solution (see above) with subsequent vigorous mixing overnight. Embryos were exposed for 96 h and 11 days. The 96-h exposure protocol followed the FETAX procedures (25 embryos per replicate in 10 ml testing media) as proposed by the ASTM guidelines (ASTM, 1991). The 11-day exposure followed the same protocol with the exception that a total of 1000 embryos per treatment were employed (50 embryos per petridish in 20 ml testing media) and that larvae were fed with algae (*Scenedesmus subspicatus*) starting on day 5 of development until termination of the exposure. For both exposures

(96 h and 11 days), media were renewed daily and the temperature was held at $24 \pm 2^\circ\text{C}$ with a 14-h/10-h, light/dark cycle. Upon termination of the exposure, mortality, malformation and growth inhibition was recorded.

Upon the termination of the 11-day experiment, larvae were collected on a nylon mesh in a glass funnel, washed with fresh FETAX solution, weighed and stored at -20°C for subsequent extraction and determination of nitromusk whole body concentrations (see below).

2.4.2. Ninety-six-hour ^{14}C -musk xylene uptake experiments

^{14}C -musk xylene stock solutions were prepared in absolute ethanol, subsequently diluted with FETAX solution giving the final concentrations of the testing media, i.e. 10, 153, 871 and 1637 $\mu\text{g/l}$. For each testing concentration, 20 embryos were exposed in 10 ml test solution in a 6-cm glass petridish. The exposure was static (without media renewal for 96 h) at $24 \pm 2^\circ\text{C}$ with a 14-h/10-h, light/dark cycle. Upon termination of the exposure, embryos were collected, washed with fresh FETAX solution and incubated with tissue solubilizer (BTS-450, Beckmann) in a glass scintillation vial at 40°C overnight. Following addition of the scintillation cocktail (Ready Safe, Beckmann) samples were submitted to liquid scintillation spectrometry.

2.4.3. Determination of larvae whole-body nitromusk concentrations following 11 days of exposure

Frozen embryos from the respective 11-day exposure experiments (see above) were thawed and subsequently submitted for nitromusk extraction. The extraction protocol involved adding anhydrous sodium sulfate (1:3 w/w) to the larvae in a porcelain mortar prior to homogenization with a porcelain pestle. Quinotozene, an extraction recovery control was added, the sample extracted for 8 h with 120 ml dichloromethane using a Soxhlet device and the resulting extract was rotary evaporated to near dryness and re-dissolved in dichloromethane. Half of this solution was used for gravimetric lipid determination while the remainder was used for nitro-musk quantification.

Florisil® columns were prepared by activation (650°C; 8 h, 110°C; 12 h) and subsequent deactivation with water in a dessicator overnight. The extract was passed through the Florisil® column using a solvent mixture of 1:4 dichloro-methane:petroleum ether at a flow rate of 1 drop/s. The resulting eluate was rotary evaporated to dryness, re-dissolved in *n*-hexane, and analyzed by gas chromatography.

Gas chromatography was performed using a Carlo-Erba GC 8000 series (Milan, Italy) equipped with a ⁶³Ni-electron capture detector (ECD). Samples were introduced into a splitless

injector with an AS800 Carlo-Erba autosampler. N₂ (150 kPa) and He (150 kPa) were used as the carrier and make-up gas, respectively. Analyses were separated in a 30 m, 0.25 mm i.d., 0.25 µm. f.t. DB-5 capillary column (J&W Scientific, CA, USA). Instrument settings: Detector temperature: 320°C; injector temperature: 250°C; injection method: 1 µl, 60 s; temperature program: 80°C for 1 min, 15°C/min linear ramp to 180°C, 5°C/min linear ramp to 300°C, 5 min isothermal at 300°C. The analytical detection limits for musk xylene, musk ketone, musk moskene and quintonzene were 1 pg at a peak height to background noise (S/N) ratio of 3:1. Background contamination levels were determined using control embryos. A typical GC chromatogram of xenopus tissue extracts is depicted in Fig. 1.

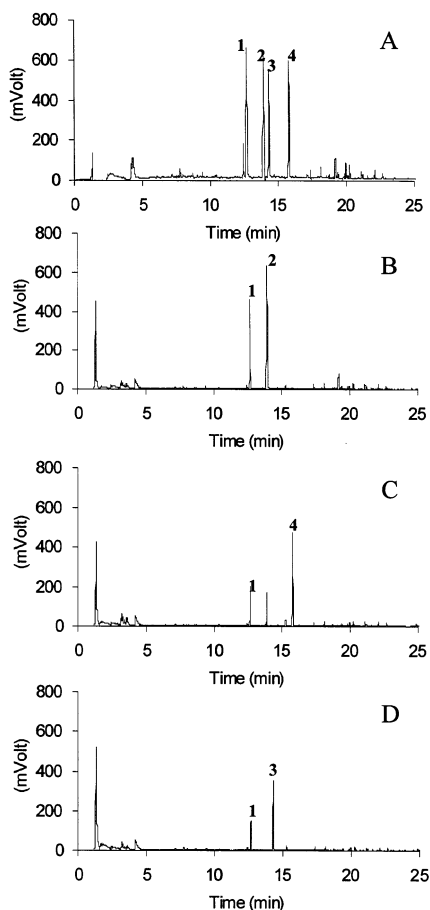


Fig. 1. Typical gas chromatograms of compounds obtained from standard (A) and xenopus (*X. laevis*) extracts following treatment with musk xylene (B), musk ketone (C) and musk moskene (D). Peak numbers refer to following compounds: 1, Quintozene; 2, MX; 3, MM; 4, MK.

2.5. Toxicity of nitromusks in early lifestages of zebrafish

2.5.1. Toxicity experiments

Nitromusk solutions were prepared by dissolving 400 µg/l musk xylene, musk ketone or musk moskene in embryo medium (see above). For each treatment, a total of 30 embryos were randomly distributed into two 6-cm glass petridishes (10 ml testing solution/petridish). Analogous to the tests with xenopus embryos, testing media were renewed daily and the temperature was held at $24 \pm 2^\circ\text{C}$ with a 14-h/10-h, light/dark cycle throughout the entire 96-h exposure period. Upon termination of the exposure, mortality, malformation and growth inhibition were determined.

2.5.2. Ninety-six-hour ¹⁴C-musk xylene uptake experiments

Experiments followed the same methods as described for xenopus exposed to ¹⁴C-MX. Embryo medium was used as testing medium and final nominal testing concentrations were 10, 153, 871 and 1637 µg/l.

2.6. Statistical analysis

Differences in survival, malformation and growth inhibition between nitromusk treatments and their respective controls for both xenopus and

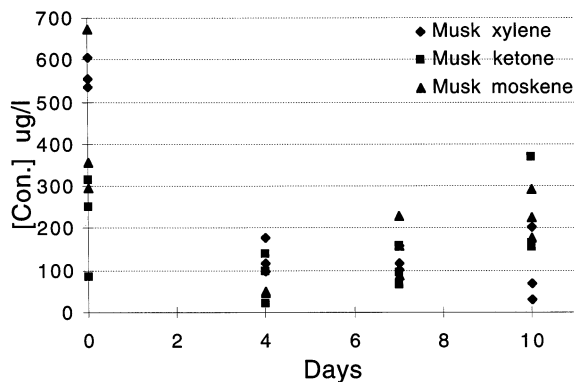


Fig. 2. Bio-available nitromusk concentrations during the 11-day xenopus experiment. The concentrations averaged 232 ± 223 ($n = 12$), 107 ± 82 ($n = 12$) and 221 ± 166 $\mu\text{g/l}$ ($n = 12$) for MX, MK and MM, respectively.

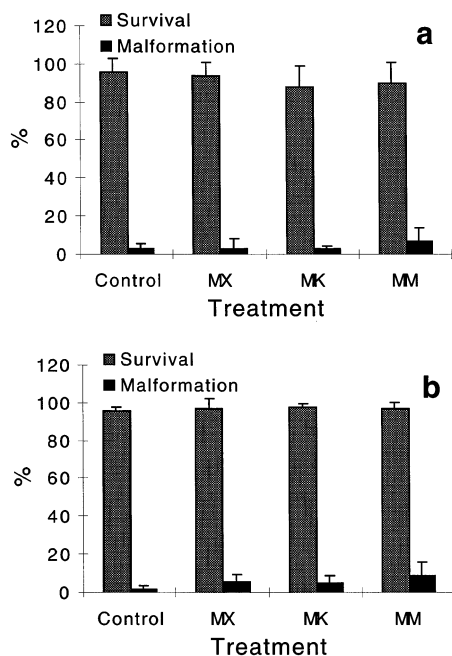


Fig. 3. Viability and malformation in xenopus (*X. laevis*) (2a) and zebrafish (*D. rerio*) (2b) embryos exposed to nominal concentrations of 400 $\mu\text{g/l}$ nitromusks for 4 days.

zebrafish were analyzed by ANOVA using the standardized mean of each group, followed by Tukey's multiple comparison test (Toxstat 3.3, University of Wyoming, 1991). Shapiro-Wilk's test and Bartlett's test were used to test the nor-

mality of the data distribution and the homogeneity of the variances, respectively.

3. Results

3.1. Nitromusk testing concentrations

Under the semi-static conditions chosen for experimentation, the nitromusk concentrations analyzed in the experimental media and therefore the bio-available concentrations averaged 232 ± 223 ($n = 12$), 107 ± 82 ($n = 12$), and 221 ± 166 $\mu\text{g/l}$ ($n = 12$) for MX, MK, and MM, accounting for 56, 27, and 55% of the nominal value of 400 $\mu\text{g/l}$, respectively (Fig. 2). The nitromusk extraction recovery, determined by using quintozene as internal standard, was $109 \pm 19\%$ ($n = 36$; Fig. 1).

3.2. Ninety-six-hour toxicity of nitromusks in early lifestages of xenopus and zebrafish

No mortality or malformation were observed in xenopus (Fig. 3(a)) and zebrafish embryos (Fig. 3(b)) exposed for 96 h to 400 $\mu\text{g/l}$ MX, MK or MM, respectively. Body length determination indicated that growth inhibition was not induced by the exposure regimen for either of the compounds tested (data not shown).

3.3. Toxicity of nitromusks in xenopus embryos following an 11-day semi-static exposure

In order to detect subtle changes in viability, incidences of malformation and growth inhibition, additional experiments with 1000 xenopus embryos per treatment (nitromusk compound) were carried out with a total duration of 11 days. In corroboration of the results obtained with the 96-h experiments, no increased malformation or growth inhibition was observed for any of the nitromusk compounds tested (data not shown). However, a statistically significant reduction of viability, i.e. 87 ± 5 , 85 ± 2 and $78 \pm 16\%$ of the control for MX, MK and MM, respectively, was observed (Fig. 4). Significantly increased mortality started as early as 96 h of exposure (survival was 99.6 ± 0.2 , 99.2 ± 0.4 and $99.6 \pm 0.3\%$ of the control for MX, MK and MM, respectively) and

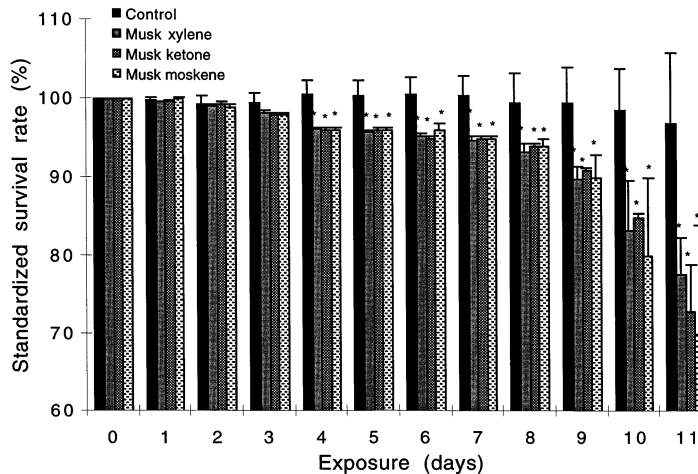


Fig. 4. Viability of xenopus (*X. laevis*) larvae exposed to nitromusks for 11 days. * Statistically different from control ($P < 0.05$).

became progressively more pronounced with continued exposure (Fig. 4).

3.4. Musk xylene whole body concentrations in xenopus and zebrafish following 96-h static exposure to ^{14}C -MX

Exposure of xenopus and zebrafish embryos for 96 h to 10, 153, 871 and 1637 $\mu\text{g/l}$ ^{14}C -MX resulted in xenopus whole-body concentrations of 0.7 ± 0.1 , 11.1 ± 1.1 , 38.7 ± 1.9 and 76.3 ± 18.3 $\mu\text{g/g}$ wet body weight, whereas 4.3 ± 0.6 , 73.3 ± 11.8 , 440 ± 72.7 and 664 ± 47.7 $\mu\text{g/g}$ wet body weight, respectively, were determined for zebrafish. Plotting of the whole body concentrations versus the exposure in an xy -diagram suggests a linear relationship between ambient exposure and body concentration (Fig. 5).

3.5. Nitromusks whole body concentrations in xenopus following 11 day semistatic exposure to MX, MK and MM

MX, MK, and MM whole body concentrations were determined in xenopus larvae following termination of the 11-day exposure. GC-ECD analysis was carried out following nitromusk extraction (Fig. 1) and the results expressed as per gram lipid (Table 1). The extraction recovery rate determined by adding quintozene to respective sample was

$112 \pm 20\%$ ($n = 15$), Fig. 1. Nitromusk concentrations per gram lipid were extrapolated to wet weight based on the average lipid concentrations determined from homogenates of 500 embryos from each experiment (3.1 ± 0.5 , 4.4 ± 0.7 and 2.8 ± 1.4 mg lipid/g wet weight for the MX, MK and MM treatments, respectively; Table 1).

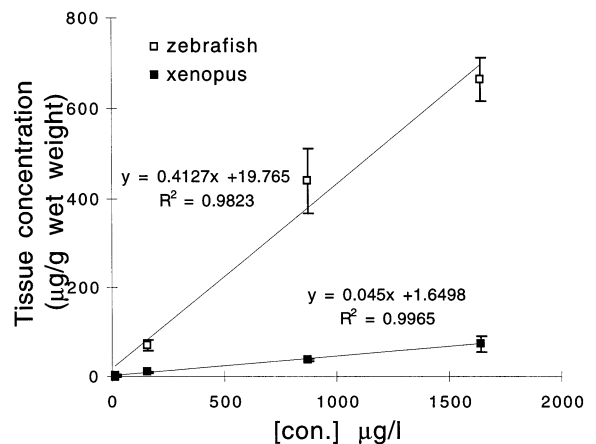


Fig. 5. Musk xylene whole body concentrations in xenopus (*X. laevis*) and zebrafish (*D. rerio*) exposed to ^{14}C -MX for 96 h. For the testing concentrations of 10, 153, 871 and 1637 $\mu\text{g/l}$, the respective tissue concentrations were 0.7 ± 0.1 , 11.1 ± 1.1 , 38.7 ± 1.9 and 76.3 ± 18.3 $\mu\text{g/g}$ wet weight for xenopus, and 4.3 ± 0.6 , 73.3 ± 11.8 , 440 ± 72.7 and 664 ± 47.7 $\mu\text{g/g}$ wet weight for zebrafish.

Table 1

Nitromusk whole-body concentrations in *X. laevis* larvae semi-statically exposed to 400 µg/l musk xylene, musk ketone and musk moskene for a period of 11 days^a

	Uptake			BCF	
	mg/kg lipid	µg/kg wet weight	mmol/kg wet weight	Uptake ^b	Uptake ^c
<i>Control</i>					
MX	3.3 ± 0.6	7.7 ± 1.4	–	–	–
MK	1.4 ± 1.4	2.1 ± 2.1	–	–	–
MM	4.9 ± 7.9	9.7 ± 15.6	–	–	–
<i>Treatment (400 µg/l)</i>					
MX	2012 ± 2112	4700 ± 5000	0.02 ± 0.02	5030	12
MK	857 ± 171	1300 ± 300	0.004 ± 0.001	2143	3
MM	2332 ± 2417	4600 ± 4800	0.02 ± 0.02	5830	12

^a Testing media were exchanged every 24 h.

^b Calculated from the uptake data, based on lipid content.

^c Calculated from the uptake data, based on wet body weight.

4. Discussion

The bio-available nitromusks concentrations prepared with FETAX solution have been determined to be approximately 30–50% of the nominal concentrations (Fig. 2). The moderate volatility of these compounds, adherence to glassware and possible partial water insolubilities of the concentrations employed could explain the high variability of soluble concentrations during the experiments. Indeed, discrepant water solubility data for MX, MK and MM have been reported by Tas and van de Plassche (1996) and Behechti et al. (1998). Furthermore, experiments with ¹⁴C-MX have indicated a compound loss of approximately 20–40% of the nominal concentration added that can be attributed to nitromusk adherence to the glassware.

Neither musk xylene, musk ketone nor musk moskene, when tested at the reported highest water soluble concentration (Tas and van de Plassche, 1996; Behechti et al., 1998), demonstrated any embryotoxic effect in xenopus or zebrafish embryos within the 96-h period of embryolarval development. Concurrent experiments with ¹⁴C-MX conclusively demonstrated that musk xylene was taken up by the embryos during this 96-h exposure period. The dose-whole-body concentration curves (Fig. 5) suggested that

the uptake and elimination processes of both xenopus and zebrafish embryos followed first-order kinetics. In addition, zebrafish have demonstrated an approximately ten-fold higher uptake than xenopus embryos through the testing concentration range. Whether it is due to the faster diffusion rate in zebrafish or the faster elimination rate in xenopus is not clear, as further investigation would be needed to provide a definitive answer. The dose-whole-body concentration curves indicated in neither of the species that saturation was reached, despite that musk xylene exposure concentrations exceeding the water-soluble concentration by a factor of 4 were employed. No mortality was observed with musk xylene even at 1673 µg ¹⁴C-MX/l tested in either xenopus or zebrafish embryos. Contrary to the findings above, the 11-day exposure with 1000 xenopus embryos per experiment resulted in a small but significant reduction in viability (< 20%). This may possibly be due to the use of higher number of embryos in these assays, leading to an increased statistical power for the detection of differences between the control and nitromusk treatment groups. The concurrently high whole-body concentrations determined via GC-ECD analysis (0.004–0.02 mmol/kg wet weight; Table 1), suggest that the observed mortality may not be due to a specific toxicity mechanism of the nitro-

musks tested but rather the result of generalized narcosis, as previously demonstrated for various other organic compounds in fish and amphibians (0.2–0.8 mmol/kg wet weight; McCarty et al., 1992). This hypothesis is also corroborated by studies of Adema and Langerwerf (1985), who reported musk xylene mediated mortality (LC₅₀ of 400 µg/l) in zebrafish exposed for 14 days using DMSO as the solvent for musk xylene.

As a linear relationship was suggested between ambient exposure and body concentration for xenopus exposed to ¹⁴C-MX for 96 h (no media change), an uptake of 19.6 µg/l and thus a BCF of 49 for a exposure of 400 µg/l ¹⁴C-MX could be extrapolated (Fig. 5). In contrast, a BCF of 12 (based on wet body weight) was calculated for xenopus exposed to 400 µg/l musk xylene for 11 days (media change daily) (Table 1). Xenopus exposed to a higher total dose of MX (media renewal daily for 11 days versus media non-renewal for 4 days), however, resulted in an approximately four-fold lower body concentration (BCF: 12 vs 49). As the exposure scenarios and mode of body burden determination were different in the 4- and 11-day experiments, it is highly questionable whether the four-fold difference in BCF is real. In view of the fact that the concentrations used for the above experiments were between 400- and 10 000-fold higher than the concentrations detected in the environment, it is not likely that nitromusks would pose a hazard to early-life stages of fish and amphibians. Indeed, the concentrations of nitromusks (MX and MK) detected in the environment (receiving waters of sewage treatment plants) rarely exceed 1 µg/l (Tas and van de Plassche, 1996). This conclusion is drawn despite the fact that zebrafish and xenopus are warm water species and thus have a much faster (ten-fold) embryonal development than indigenous cold water species.

However, nitromusk amine metabolites (Gatermann et al., 1998; Rimkus et al., 1999) have been recently detected in the aquatic biota at higher levels than the parent compounds toxicologically evaluated here. Furthermore, an EC₅₀ of 250 ng/l and 1070 µg/l has been determined in daphnia for 4-amino-MX and 2-amino-MX, respectively (Behechti et al., 1998). Thus, these metabolites are a

factor 22 400 and 5 more toxic in daphnia than their parent compound (Tas and van de Plassche, 1996). In view of this situation, further studies regarding the fate and biological effects of nitromusk amine metabolites in the aquatic environment appear warranted.

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