

DIOXIN 2008

Prof. Giesy, research team presented four papers to Dioxin 2008: 28th International Symposium on Halogenated Persistent Organic Pollutants (POPs), which was held. August 17-22, in Birmingham, UK.

“Biomagnification Factors of PCDD/Dfs for Great Blue Heron and Belted Kingfisher Residing in the Tittabawassee River Flood Plain, MI, USA.” With R.M. Seston, M.J. Zwiernik, T.B. Fredricks, D.L. Tazelaar, J.N. Moore, S.J. Coefield, P.W. Bradley, M. S. Shotwell, and Denise P. Kay.

“Characterization of Mixed Function Monooxygenase Genes CYP1A1 and CYP1A2 in Mink (*Mustela Vison*) Exposed to Polychlorinated Dibenzofurans (PCDFs).” With X. Zhang, J.N. Moore, J.L. Newsted, M.J. Zwiernik, M. Hecker, P.D. Jones and S.J. Bursian

“Effects of Polychlorinated Dibenzofurans on Mink.” With D.P. Kay M. Zwiernik, S.J. Bursian, K. Beckett, L. Aylward, R. Budinsky, Melissa Shotwell, J.N. Moore, and J.L. Newsted.

“Hepatic P450 Enzyme Activity, Tissue Morphology and Histology of Mink (*Mustela Vison*) Exposed to Polychlorinated Dibenzofurans (PCDFs).” With J.N. Moore, J.L. Newsted, M. Hecker, M.J. Zwiernik, S.D. Fitzgerald, D. P. Kay, X. Zhang, Eric B. Higley, L.L. Aylward, K.J. Beckett, R.A. Budinsky, S. J. Bursian.

BIOMAGNIFICATION FACTORS OF PCDD/DFS FOR GREAT BLUE HERON AND BELTED KINGFISHER RESIDING IN THE TITTABAWASSEE RIVER FLOODPLAIN, MI, USA



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INTRODUCTION

The great blue heron (*Ardea herodias*) (GBH) and belted kingfisher (*Ceryle alcyon*) (KF) were selected as species of interest in an ecological risk assessment being performed on the Tittabawassee River, Michigan, USA. The trophic status of both the GBH and KF, along with their strong site fidelity and territoriality, make them ideal species to investigate bioaccumulative contaminants. The study area, which includes 38 km of river from the upstream boundary at the city of Midland, MI to the confluence of the Tittabawassee and Shiawassee Rivers, has previously been shown to contain elevated concentrations of polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzo-p-dioxins (PCDDs) in soils, sediments, and biota in comparison to reference areas upstream of Midland^{1,2}. The site-specific mixture of dioxin-like compounds in the study area is predominately furan congeners, specifically 2,3,7,8 TCDF (TCDF) and 2,3,4,7,8 PeCDF (4-PeCDF)^{1,2}. To understand the movement of this site-specific mixture of furans and dioxins up the food chain, comparisons of the biomagnification factors (BMFs) of different congeners were made within a species and also between species. Differences in congener-specific BMFs may suggest a difference in the half-lives between various congeners.

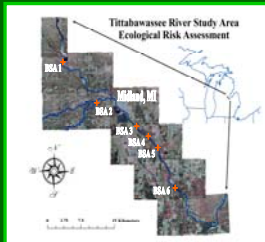


Figure 1. Target area consists of 38 km of river, stretching from the city of Midland, MI to the confluence of the Tittabawassee and Shiawassee Rivers. Reference areas include the Tittabawassee River upstream of Midland, along with the Chippewa and Pine Rivers.

METHODS AND MATERIALS

- Prey items (forage fish, crayfish, and frogs) were collected from six biological sampling areas (BSAs) (Figure 1).
- GBH nestling tissues (liver, adipose, and blood plasma) were collected from rookeries within the study area and analyzed separately.
- KF nestlings were collected from nest burrows located within the study area. Nestlings were homogenized whole minus stomach contents, feathers, legs, and beak.
- Site-specific BMFs were calculated by dividing the lipid-normalized concentrations of select PCDF/D congeners in receptor tissues by those in the diet (Table 1).
- Analyses of the seventeen 2,3,7,8 substituted PCDF/D congener concentrations were conducted atASUREQuality Limited (Lower Hutt, New Zealand) using EPA method 8290.
- All TEQ values based on avian World Health Organization TCDD equivalent factors³.

RESULTS

- Site-specific dietary composition for the KF was determined to be 82% fish, 13% crayfish, and 5% frog through the identification of prey remains. Percentages based on prey item occurrence.
- Literature-derived dietary composition for GBH was 96% fish, 2% crayfish, 2% frog^{4,5}.
- Mean total TEQs in GBH and KF diet was 2.1x10² ng/kg (ww) and 2.0x10² ng/kg (ww), respectively (Table 1).
- Mean total TEQs in GBH and KF tissues ranged from 1.4x10³ to 1.2x10² ng/kg (ww) (Table 1).

Table 1. Sum WHO_{AVIAN} TEQs ng/kg (ww)(1/2DL) from PCDF/Ds in diet, GBH, and KF collected from the Tittabawassee River study area. Biomagnification factors of 4-PeCDF, TCDF, and TCDD in GBH and KF.

Species	Tissue	Mean TEQ (range)	% Lipid	Biomagnification Factors			Ratio
				4-PeCDF	TCDF	TCDD	
GBH Diet		210	3.8				
	Plasma (n=13)	1.4 (0.52 – 2.8)	0.47	0.27	0.042	0.75	6.6
	Liver (n=9)	4.1 (1.7 – 7.7)	3.6	0.075	0.0084	0.21	9.0
KF Diet	Adipose (n=9)	120 (64 – 320)	72	0.081	0.018	0.34	4.4
	Whole body (n=9)	200	3.5				
		91 (33 – 180)	4.5	1.8	0.18	1.1	10

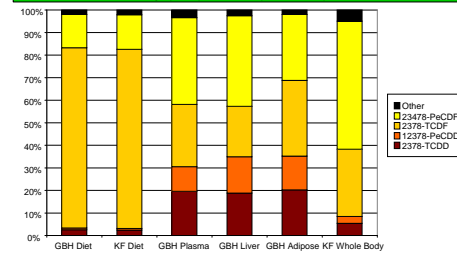


Figure 2. Percent contribution of the predominant PCDF/D congeners to the sum TEQ, based on WHO_{AVIAN} TEQ (1/2 DL). Each receptor tissue profile represents an average of all individuals analyzed.

- TCDF accounted for 80% and 79% of the total TEQs in the diet of the GBH and KF, respectively, while only contributing 28%, 22%, 34%, and 30% to total TEQs in GBH plasma, GBH liver, GBH adipose, and KF nestlings, respectively (Figure 2).
- 4-PeCDF is the predominant congener in most receptor tissues, accounting for 38%, 40%, and 57% of total TEQs in GBH plasma, GBH liver, and KF nestlings, respectively. An exception is GBH adipose; 4-PeCDF accounts for only 29% of total TEQs. In both diets, 4-PeCDF accounts for only 15% of total TEQs (Figure 2).

DISCUSSION

- Although GBH and KF have relatively equal trophic status, the KF had greater BMFs for the three most important congeners. This disparity could be due to:
 - Misunderstanding of dietary compositions of the species
 - Low variation of BMFs based on different dietary compositions suggests this is not driving the lesser BMFs in GBH (Table 2).
 - Off-site foraging
 - GBH have a larger foraging area and can travel much further from the nest⁶ than KF⁷ and thus have a greater potential to forage off-site in "clean areas". An underestimation of off-site foraging would result in overestimating dietary exposure and lesser BMFs.
 - GBH off-site foraging would likely increase inter-nest variability, however tissue concentrations and congener profiles were remarkably similar among nests.
 - Different metabolism and elimination of the congeners by the two species
 - Cytochrome P450 liver enzyme activity, as measured by EROD and MROD (ethoxyresorufin O-deethylase and methoxyresorufin O-deethylase), does not explain the BMF differences between species as activity was lesser for GBH (data not presented)⁸.
- Relative metabolism and/or elimination of TCDF and 4-PeCDF was similar between species despite the difference in the magnitude of BMFs (Table 1).
 - Similar ratio of 4-PeCDF:TCDF BMFs (5.1) observed in whole body herring gulls based on a diet of alewife from Lake Ontario⁹.
 - In mink fed a mixture of TCDF and 4-PeCDF, the half-life of TCDF was only 3.8 hours compared to 7.4 days for 4-PeCDF. It was also found that TCDF experienced increased metabolism and depuration when co-administered with other aryl hydrocarbon receptor (AHR)-mediated compounds¹⁰.
- There was a consistent shift in relative proportions of TCDF and 4-PeCDF in diet and tissues (Figure 2).
 - Similar trend seen in mink collected from the study area (Diet: 31% TCDF, 37% 4-PeCDF; Liver: <1% TCDF, 56% 4-PeCDF)².

Table 2. 2,3,4,7,8-PeCDF biomagnification factors for GBH tissues using different dietary compositions.

Dietary Composition	BMF of 4-PeCDF		
	GBH Plasma	GBH Liver	GBH Adipose
70% fish, 20% crayfish, 10% frog	0.19	0.053	0.057
82% fish, 13% crayfish, 5% frog	0.22	0.061	0.066
96% fish, 2% crayfish, 2% frog	0.27	0.075	0.081
100% fish	0.29	0.081	0.087



CONCLUSIONS

- TCDF is the predominant congener present in dietary items, while 4-PeCDF is the predominant congener in most receptor tissues.
- 4-PeCDF:TCDF BMF ratio remains similar between examined piscivorous avian species, suggesting similar metabolism and/or elimination of the two congeners by each species.
- Differences in calculated BMFs between GBH and KF are not likely due to differences in dietary composition or metabolism
- One plausible explanation for difference in calculated BMFs between GBH and KF is an overestimation of GBH dietary exposure through an assumption of 100% site use
- Based on the 4-PeCDF:TCDF BMF ratio observed in these and other similarly exposed species, 4-PeCDF has a much greater potential to biomagnify than TCDF.

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CHARACTERIZATION OF MIXED FUNCTION MONOOXYGENASE GENES CYP1A1 AND CYP1A2 IN MINK (*MUSTELA VISON*) EXPOSED TO POLYCHLORINATED DIBENZOFURANS (PCDFs)



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INTRODUCTION

- Mink have been proposed as a model sentinel or surrogate species for assessing the exposure and effects of environmental persistent organic chemicals such as polychlorinated dibenzo-p-dioxins (PCDDs), -dibenzofurans (PCDFs) and -biphenyls (PCBs) that are known to act through the aryl hydrocarbon receptor (AhR)
- Cytochrome P450s are up-regulated by AhR agonist and have been proposed as indicators or biomarkers for the exposure of these compounds in ecological risk assessments.
- While two CYP enzymes that include 7ethoxy O-deethylase (EROD) and 7-methoxy O-demethylase (MROD) have been measured in mink, gene expression (CYP1A1 and CYP1A2, respectively) associated with these enzymes has not yet been characterized in mink.

Objectives

- Clone and sequence CYP1A1 and CYP1A2 genes in mink
- Determine the relationship among CYP1A endpoints (gene expression, protein levels and enzyme activities) in mink liver
- Explore the relationships between CYP1A endpoints and other toxicological endpoints as well as adipose/liver TCDF and PeCDF concentrations.

MATERIALS AND METHODS

Experimental Design

- Fifty first-year mink were randomly assigned to a control group (n=8) or seven treatment groups (n=6) while 3 additional mink were used as Day 0 controls.
- Targeted low, mid, and high spiked feed concentrations for PeCDF and TCDF were 110, 390, 1600 ng/kg and 500, 2000, 9700 ng/kg ww, respectively. Mixture concentrations were 490 and 2200 ng/kg ww for PeCDF and TCDF, respectively.
- Mink were sampled on Days 90 and 180 of the study where livers were removed, weighed and subsamples were taken for chemical, biochemical and molecular analysis.

Biochemical and Molecular Methods

- Total RNA was extracted with Agilent Total RNA Isolation Kit
- Homologous genes of CYP1A1, CYP1A2, and beta actin were identified in other mammalian species used to design primers based on conserved regions. Gene-specific primers designed on partial cDNA sequences, and used in 5'- and 3'-RACE PCR reactions. PCR products were sequenced and used to design primers for real-time PCR reactions.
- Liver microsomes were prepared and enzyme activities were measured according to standard laboratory protocols. Western blots with anti-dog CYP1A antibody were used to identify CYP1A proteins.

Chemical Methods

- Feed and mink tissues were analyzed by USEPA methods 8290 and 1668, respectively.
- All concentrations were converted to 2,3,7,8-TCDD equivalents (TEQs) using toxicity equivalency factors (TEFs) of 0.3 for PeCDF and 0.1 for TCDF (van den Berg et al. 2006).

RESULTS and DISCUSSION

Liver Concentrations

- TCDF and PeCDF liver concentrations increased with dose (Table 1)
- TCDF liver concentrations ranged from 30% greater than daily dose to 65% less than daily dose across treatments.
- PeCDF liver concentrations ranged from 67 to 179-fold greater than daily dose across treatments.
- While slight differences in TCDF and PeCDF hepatic concentrations were observed between Day 90 and 180, these differences were not statistically significant.
- Regression analysis of adipose and liver TEQ concentrations showed that the slope for PeCDF was less than that of TCDF (p<0.05) indicating that PeCDF was either accumulated to a greater extent in the liver or was not metabolized and excreted as was observed with TCDF.

RESULTS AND DISCUSSION (Cont.)

EROD and MROD Activities

- In mink exposed singly to TCDF or PeCDF, hepatic EROD and MROD activities increased in a dose-dependent manner (Table 2).
- While differences in enzyme activities between Day 90 and 180 were observed in mink from the different treatments, these differences were not statistically significant.
- For the mixture, EROD and MROD activities were significantly greater than control activities but were similar to activities measured in mink exposed to 3.8 and 2.2 ng TEQ/kg bw/d TCDF and PeCDF, respectively

CYP1A Gene Characterization

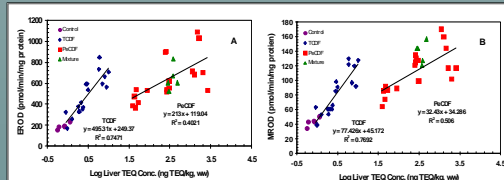
- CYP1A1 cDNA consists of 2607 bp with a 1554 bp ORF encoding 512 amino acid residues with a predicted molecular mass of 58.5 kDa.
- CYP1A2 cDNA consists of an ORF of 1539 bp encoding for 512 amino acid residues and has a predicted molecular mass of 57.9 kDa.
- Mink CYP1As are most closely related to sea otters with 95% and 93% overall amino acid identity for CYP1A1 and CYP1A2, respectively. For non-aquatic mammals, dog CYP1A genes are most closely related to mink with 89% and 81% overall amino acid identities for CYP1A1 and CYP1A2, respectively.
- Phylogenetic analysis showed that mink CYP1A1 and CYP1A2 belong to carnivores *Caniformia* CYP1A1 and CYP1A2 clades, respectively.

CYP1A Gene Expression and Protein Concentrations

- Exposure to either TCDF or PeCDF resulted in a dose-dependent increase in CYP1A1 mRNA levels (Table 2).
- Dose-dependent increases in CYP1A1 mRNA induced by PeCDF were greater than those observed with TCDF.
- Response profiles of CYP1A2 gene expression was similar to CYP1A1 for mink exposed to either TCDF or PeCDF, singly.
- For CYP1A protein concentrations at Day 90, the response profiles in mink exposed to either TCDF or PeCDF were similar to those observed for CYP1A1 and CYP1A2 gene expression, however at Day 180 several differences were observed, especially for PeCDF.
- Correlation analysis indicated that expression levels of CYP1A1, CYP1A2, P4501A proteins, EROD and MROD activities were all significantly (p<0.05) and positively related to each other.

Liver TEQ Concentration and CYP1A relationships

- CYP1A gene expression, protein levels, and enzymatic activities were significantly and positively correlated with dietary dose and liver TEQs when all mink were included in the analysis. These trends were also observed when TCDF and PeCDF data were analyzed separately.
- Regression slopes for liver TCDF and PeCDF TEQ concentrations to EROD (p=0.002) statistically differed (Figure 1). This trend was also observed for MROD activities.
- Slopes determined from the regression of CYP1A1 gene expression with either TCDF or PeCDF did not statistically differ (p=0.450). This observation was also seen with CYP1A2 gene expression slope comparisons (p=0.415) (Figure 2).
- Slopes associated with the regression of P4501A protein to TCDF and PeCDF liver TEQ concentrations (Figure 3) were not statistically different (p=0.262) (Figure 3).



Acknowledgements

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Table 1. Mean dietary and tissue concentrations of TCDF and PeCDF measured in the mink exposed through the diet. ^a

Treatment	Final Diet (ng TEQ/kg feed)	ADD (ng TEQ/kg bw/d)	Adipose (ng TEQ/kg ww)	Liver (ng TEQ/kg ww)
Control	<LOD ^b	<LOD ^b	2.7 ± 1.7	0.69 ± 0.39
TCDF	9.9	0.98	75 ± 9.1	5.2 ± 1.8
	40	3.8	300 ± 21	270 ± 25
	190	20	530 ± 100	1600 ± 530
PeCDF	6.6	0.62	9.9 ± 3.8	1.5 ± 0.56
	24	2.2	22 ± 5.4	2.6 ± 0.49
	96	9.5	62 ± 9.5	7.7 ± 1.6
Mix ^c	74	6.9	210 ± 22	360 ± 7.9

^a TEQ concentrations from 90 and 180 d were combined and given as mean and standard deviations. ADD is the average daily dose calculated from feed consumption and body weight data collected during the study.

^b Limit of detection was LOD = 0.1 ng TEQ/kg ww

^c Concentration in the mixture is given as total TEQ for both TCDF and PeCDF measured in the feed or tissue.

Table 2. Average daily dose and liver cytochrome P4501A gene, protein and enzyme responses in mink exposed to TCDF or PeCDF singly or in mixture via feed. ^a

Treatment	ADD (ng TEQ/kg bw/d)	EROD (pmol/min/mg)	MROD (pmol/min/mg)	CYP1A1 mRNA	CYP1A2 mRNA	P4501A Protein
Control	<0.01	177 ± 33	41 ± 5.9	0.85 ± 0.17	0.75 ± 0.29	0.87 ± 0.17
TCDF	0.02	452 ± 74 ^b	82 ± 11 ^b	1.36 ± 0.18	0.84 ± 0.26	1.30 ± 0.25
	2.2	672 ± 179 ^b	123 ± 13 ^b	2.52 ± 1.0 ^b	1.81 ± 1.18	2.21 ± 0.95 ^b
	9.5	811 ± 219 ^b	142 ± 27 ^b	4.21 ± 1.12 ^b	2.43 ± 0.67 ^b	4.07 ± 0.97 ^b
TCDF	0.98	311 ± 76	55 ± 9.0	0.90 ± 0.16	0.77 ± 0.38	1.02 ± 0.25
	3.8	473 ± 114 ^b	77 ± 17 ^b	1.13 ± 0.10 ^b	0.84 ± 0.17	1.18 ± 0.28
	20	579 ± 238 ^b	115 ± 16 ^b	1.93 ± 0.64 ^b	1.56 ± 0.56 ^b	2.22 ± 0.49 ^b
Mixture	6.9	646 ± 117 ^b	139 ± 14 ^b	2.83 ± 1.71	1.78 ± 1.26 ^b	2.71 ± 0.75 ^b

^a Day 90 and 180 data combined and given as mean and standard deviation. CYP1A1 and P4501A protein responses are fold-change from control values. ^b denotes significant differences from controls (p<0.05)

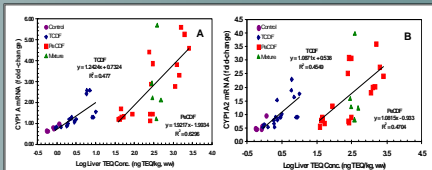


Figure 2. Relationship between liver TEQ concentrations and relative CYP1A gene expression. A) CYP1A1 and B) CYP1A2. Fold changes relative to control mRNA levels.

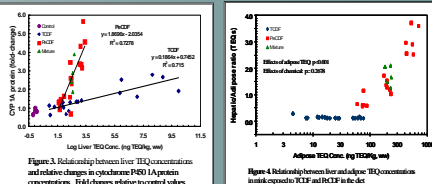


Figure 3. Relationship between liver TEQ concentrations and relative changes in cytochrome P4501A protein concentration. Fold changes relative to control values.

Conclusions

- The basic mechanisms of CYP1A induction via the AhR mediated pathway are conserved in mink.
- Predicted protein sequences of CYP1A1 and CYP1A2 indicate that mink have preserved several conserved traits with other mammalian species and are most closely related to marine mammals.
- TCDF and PeCDF behaved as full AhR agonists and displayed high-intrinsic induction of CYP1A
- Positive correlations between liver TEQ concentrations and the expression of CYP1A mRNAs, proteins, EROD and MROD activities show that tissue concentrations were the best predictors of AhR pathway activation.
- Plots liver/adipose TEQ concentrations to adipose TEQs (Figure 4) along with CYP1A responses indicate that PeCDF may have been sequestered in the liver unlike that observed for TCDF.



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EFFECTS OF POLYCHLORINATED DIBENZOFURANS ON MINK

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INTRODUCTION

Mink are often predicted to have the greatest potential for adverse effects in multi-species risk calculations for sites with a substantial aquatic habitat where polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and other dioxin-like compounds are the contaminants of concern (COC)¹. This is because mink:

- ◆ are apical carnivores
- ◆ consume a great amount of food relative to their body mass
- ◆ are among the mammals that are more sensitive to aryl hydrocarbon receptor (AhR)-mediated effects

Thus, remedial criteria are often derived for mink in situations where risks are predicted to occur due to AhR-active compounds². Hence it is important that exposure concentrations at which adverse effects are predicted to occur be as accurate as possible to appropriately protect wildlife from adverse effects due to chemical exposure but also to protect from habitat destruction due to remediation based on misunderstanding of critical effect concentrations.

Considerable toxicological information is available on the effects of PCBs and PCDDs on mink, but limited toxicological information is available for PCDFs. This report compares the toxic effects reported for laboratory and field studies on mink with both mixed and single dioxin-like congener exposures and demonstrates that exposure concentrations at which adverse effects occur cannot be determined reliably for complex mixtures in which PCDFs dominate the total calculated TEQ values, thereby suggesting that the values of the mammalian-specific TEFs suggested by the WHO may overestimate the toxic potency of PCDFs to mink.

Table 1. Estimated average first-order elimination rate constants, based on data from both 90- and 180-d time points, for 2,3,7,8-TCDF and 4-PeCDF by dose group. N=6 except where noted.

Daily dose TEQ (ng kg ⁻¹ d ⁻¹)	First order rate constant, d ⁻¹ Mean (S.D.)	Estimated half-life, d Mean
2,3,7,8-TCDF		
0.98	1.6 (0.6)	0.43
3.8	2.6 (0.7)	0.27
20	4.1 (0.6)	0.17
Mixture: 4.1 TCDF and 2.8 PeCDF (n=5)	4.3 (0.7)	0.16
2,3,4,7,8-PeCDF		
0.62	0.086 (0.012)	8.1
2.2	0.095 (0.008)	7.3
9.5	0.087 (0.019)	8.0
Mixture: 2.8 PeCDF and 4.1 TCDF (n=5)	0.094 (0.008)	7.4

DISCUSSION

In both the Tittabawassee River Field Study and the Laboratory Chronic Exposure to 2,3,7,8-TCDF, concentrations of TEQ_{2006-WHO-mammal} to which the mink were exposed exceeded those at which adverse effects, based on studies with PCDD or PCB congeners, would have been expected. Yet in both instances where PCDF congeners were the sole or predominant source of the TEQ_{2006-WHO-mammal}, predicted adverse effects were not observed. The apparent discrepancy between predicted and observed relative potency for 2,3,7,8-TCDF and mixtures containing 2,3,7,8-TCDF as compared to TCDD- and PCB 126-containing mixtures may be in part due to dissimilar metabolic transformation and elimination. Uncertainties associated with the relative potencies of individual components which would differentially affect mixtures of varying composition can be demonstrated by comparing the data collected from the 2,3,7,8-TCDF study reported herein to a parallel study, conducted at the same facility (MSU Experimental Fur Farm) and using the same methodologies, of 3,3',4,4',5-pentachlorobiphenyl (PCB 126)⁵ (Table 2).

Table 2. Reproductive outcomes resulting from mink dietary exposure to PCB 126 and 2,3,7,8-TCDF.

Chemical studied	Concentration in diet	Reproductive outcome
PCB 126 ⁵	240 TEQ/kg diet	Complete reproductive failure
2,3,7,8-TCDF ⁴	240 TEQ/kg diet	Whelping rate not different from control (80%)

The most comprehensive comparison of mixture and congener toxicological potency can be made by comparing all of the available dose response relationships between concentrations of TEQ and occurrence of squamous epithelial cell proliferation or jaw lesions. Jaw lesions are a sensitive response of mink to 2,3,7,8-TCDD, PCB 126, and mixtures of dioxin-like compounds.

The environmental mixtures that resulted in jaw lesions had great proportions of non-ortho PCBs, specifically, PCB 126 (Table 3). There was no clear relationship between the presence or frequency of jaw lesions and the total concentration of TEQ_{2006-WHO-mammal} contributed by PCDD or PCDF, 2,3,7,8-TCDF or mono-ortho PCBs. This does not mean that there is not a dose response for these compounds but rather the data set is limiting.

CONCLUSION

The results of these studies suggest that the values of the mammalian-specific TEFs suggested by the WHO overestimate the toxic potency of PCDFs to mink. Therefore, hazard cannot be accurately predicted by making comparisons to TRVs derived from exposure studies conducted with PCBs or PCDDs in situations where mink are exposed to TEQ mixtures dominated by PCDFs.

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Table 3. Effect levels for mink dietary exposure to dioxin-like compounds in ng TEQ_{2006-WHO-mammal}/kg diet, ww.

Study	Sum TEQs	2,3,7,8 TCDF TEQs		% Jaw lesions	Dioxin TEQs	Furan TEQs	Non-ortho PCB TEQs
		Sum TEQs	2,3,7,8 TCDF TEQs				
Housatonic River fish lab study ⁸	50	41	0.3	100% (6/6)	0.9	1.9	44
PCB 126 lab study ⁵	24	24	0	80% (12/15)	0	0	24
Saginaw River fish lab study ⁹	57	19	2.1	75% (6/8)	20	14	20
Saginaw River fish lab study ⁹	36	11	0.9	57% (4/7)	14	8.8	12
Housatonic River fish lab study ⁸	12	9.8	0.1	33% (2/6)	0.3	0.5	10
Saginaw River fish lab study ⁹	22	7.2	0.7	0% (0/8)	11	3.0	7.2
Housatonic River fish lab study ⁸	6.8	5.4	0.1	17% (1/6)	0.3	0.3	5.8
Housatonic River fish lab study ⁹	4.3	3.2	0.1	0% (0/6)	0.3	0.3	3.4
Tittabawassee River wild mink ³	31	2.4	8.8	0% (0/22)	4.4	22	2.5
2,3,7,8-TCDF lab study ⁴	240	0	240	0% (0/8)	0	240	0

RESULTS

Tittabawassee River Field Study³

A mink hazard assessment based on concentrations of furans, dioxins, and PCBs in site-specific dietary items from the Tittabawassee River, and toxicity reference values (TRVs) derived from mixtures of other Ah-R active compounds resulted in values of hazard quotients (HQ) that were greater than 1.0, which suggested potential adverse effects for mink³. However, there were no statistically significant differences in any of the measured parameters between mink exposed to a median estimated dietary dose of 31 ng TEQ_{2006-WHO-mammal}/kg ww, and mink from an upstream reference area where they had a median dietary exposure of 0.68 ng TEQ_{2006-WHO-mammal}/kg ww. Surveys of the conditions of individual mink, and the mink population, including track surveys, trapping, age distributions and sex ratios indicated that the mink population was not being adversely impacted. 75% of the 31 ng TEQ_{2006-WHO-mammal}/kg, ww in the mink diet were due to PCDFs with a majority of that originating from TCDF (31%) and 4-PeCDF (37%)³.

Laboratory chronic exposure to 2,3,7,8-TCDF⁴

Similarly, chronic exposure of mink to TCDF concentrations as great as 2400 ng TCDF/kg ww feed (240 ng TEQ_{2006-WHO-mammal}/kg ww feed) exhibited transient decreases in body masses of kits relative to the controls as the only statistically significant effect observed.

Laboratory toxicokinetic evaluation of 2,3,7,8-TCDF and 4-PeCDF¹

The laboratory study of the toxicokinetics of 2,3,7,8-TCDF and 4-PeCDF in mink demonstrated that 2,3,7,8-TCDF is quickly metabolized relative to 4-PeCDF¹ (Table 1).

“The apparent discrepancy between predicted and observed relative potency for 2,3,7,8-TCDF and mixtures containing 2,3,7,8-TCDF as compared to TCDD- and PCB 126-containing mixtures may be in part due to dissimilar metabolic transformation and elimination.”

“It is important that exposure concentrations at which adverse effects are predicted to occur be as accurate as possible to appropriately protect wildlife from adverse effects due to chemical exposure but also to protect from habitat destruction due to remediation based on misunderstanding of critical effect concentrations.”

METHODS AND MATERIALS

Three primary studies discussed herein include a three-year field study of mink chronically exposed to a mixture of PCDFs and PCDDs under natural conditions, a laboratory chronic exposure study in which mink were exposed to 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF) through diet, and a laboratory evaluation of the toxicokinetics of 2,3,7,8-TCDF and 2,3,4,7,8-pentachlorodibenzofuran (4-PeCDF).

Tittabawassee River Field Study³

Forty-eight wild mink, 22 from the study area and 26 from reference areas, were collected throughout the Tittabawassee River, Midland, Michigan, USA drainage basin during the winters of 2003-2005.

- ◆ Concentrations of dioxin, furan, and dioxin-like PCB congeners were measured in the dietary items and livers of mink
- ◆ Estimates of the daily dose were created from site-specific dietary composition and measured dietary item residue concentrations
- ◆ Necropsies included gross and histological examinations
- ◆ Jaws were examined histologically for the presence of squamous epithelial cell proliferation as described in Beckett et al.⁸

Laboratory chronic exposure to 2,3,7,8-TCDF⁴

This laboratory study was designed to determine the toxic effects threshold for mink exposed to 2,3,7,8-TCDF through the diet.

- ◆ Thirty 10-m old adult female mink (F0) were fed diets containing 0.0 (Control), 240, or 2400 ng 2,3,7,8-TCDF/kg feed on a wet-weight (ww) basis (0, 26, and 240 ng TEQ_{2006-WHO-mammal}/kg, respectively)⁵
- ◆ Dietary exposure was started 3 wk prior to the initiation of breeding
- ◆ Adults and kits were examined for sub-lethal effects including kit growth, organ masses, and tissue histology
- ◆ Necropsies included gross and histological examinations
- ◆ Jaws were examined histologically for the presence of squamous epithelial cell proliferation

Laboratory toxicokinetic evaluation of 2,3,7,8-TCDF and 4-PeCDF¹

A controlled laboratory feeding study was performed to determine the toxicokinetics of 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF using mink as a mammalian model.

- ◆ Mink were exposed to three concentrations each of the congeners and to a binary mixture of the two congeners through the diet (Table 1)
- ◆ Three animals from each of the dose groups were sampled on day 90 and 180
- ◆ 2,3,7,8-TCDF and 4-PeCDF residues were measured in liver, adipose, and scat
- ◆ Necropsies included gross and histological examinations
- ◆ Jaws were examined histologically for the presence of squamous epithelial cell proliferation
- ◆ CYP1A1 and CYP1A2 enzyme activities were measured

HEPATIC P450 ENZYME ACTIVITY, TISSUE MORPHOLOGY AND HISTOLOGY OF MINK (*Mustela vison*) EXPOSED TO POLYCHLORINATED DIBENZOFURANS (PCDFs)

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Abstract
Dose- and time-dependent effects of environmentally relevant concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin equivalents (TEQ) of 2,3,7,8-tetrachlorodibenzofuran (TCDF), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or a mixture of these two congeners on hepatic P450 enzyme activity and tissue morphology, including jaw histology, of adult ranch mink were determined under controlled conditions. Three doses each of TCDF (0.98, 3.8, or 20 ng TEQ_{TCDF}/kg bw/d) or PeCDF (0.62, 2.2, or 9.5 ng TEQ_{PeCDF}/kg bw/d) or one dose of a mixture of TCDF and PeCDF (4.1 ng TEQ_{TCDF}/kg bw/d and 2.8 ng TEQ_{PeCDF}/kg bw/d, respectively) were administered in feed to adult female ranch mink for 180 d. Based on estimates of TCDF and PeCDF concentrations in dietary components, these doses bracketed those received by wild mink inhabiting the Tittabawassee River, Michigan, USA where soil and sediment contain elevated concentrations of these two furan congeners. Activities of the cytochrome P450 IA enzymes, ethoxresorufin O-deethylase (EROD) and methoxyresorufin O-deethylase (MROD), were significantly greater in livers of mink exposed to TCDF, PeCDF and a mixture of the two congeners. However, there were no significant histological or morphological effects observed. Under the conditions of this study, the adaptive response of EROD/MROD induction occurred at doses that were less than those required to cause histological or morphological changes.



Discussion
PCDF Concentrations in Liver
2,3,4,7,8-Pentachlorodibenzofuran accumulated in the liver of the mink to a much greater extent than TCDF when administered as a single congener or in combination with TCDF (Table 1). The hepatic sequestration of PeCDF relative to PCDDs and other PCDFs including TCDF is consistent with what has been reported in other studies with mammals (Brewster and Birnbaum 1987, 1988; Van den Berg et al. 1989; Devito et al. 1997). The reason that PeCDF accumulates in the liver of the rodent is that it avidly binds to hepatic CYP1A2 protein (Dilbert et al. 1999). Presumably, PeCDF is sequestered in the liver of the mink by the same mechanism (Zwiernik et al. 2008b). The lesser concentrations of TCDF in the liver of the mink suggest an efficient elimination and/or metabolism of the congener.

Histology
In this study, TCDF and PeCDF, administered singly or in combination, at environmentally relevant doses for 180 d did not result in changes in gross morphological or histological endpoints that have been reported in other mink exposure studies utilizing dioxin or dioxin-like compounds (Hochstein et al. 1988, 1998; Rander et al. 2000a, 2000b, 2001). In the present study, only one animal fed 9.5 ng TEQ_{PeCDF}/kg bw/d had a single cyst of squamous epithelial cells at 180 d. The concentration of PeCDF in the liver of that mink was 1.3 ng TEQ_{PeCDF}/kg ww. There are two possible explanations for the scarcity of the jaw lesions in the present study. One possibility is that the age at which exposure was initiated was too late and/or the duration of exposure was not sufficient. A second possibility is related to the specific PCB/PCDD/PCDF congeners contributing to the TEQs. In studies of ranch mink utilizing individual congeners, TEQs were provided by either TCDF or PCB 126 (Rander et al. 2000a, 2000b, 2001). In those studies of mink fed diets containing contaminated fish, the majority of TEQs were contributed by congeners other than furans. For example, in a study that assessed the effects of feeding diets containing fish from the Housatonic River, PCB 126 and TCDF contributed 61% of the total TEQs while TCDF and PeCDF contributed 4% (Bursian et al. 2006a, b). In a similar study utilizing fish from the Saginaw River, PCB 126 and TCDF contributed 39% of the total while TCDF and PeCDF accounted for 25% of the total. It is possible that TCDF and PeCDF are less effective than PCB 126 and TCDF in inducing proliferation of mandibular and maxillary squamous epithelia. Furthermore, it has been determined that the effects of PCDFs can not be accurately predicted from the use of TEQ-based TRVs developed from studies of PCDDs and PCBs (Blankenship et al. 2007). This suggests that there are differences in the sensitivity of mink to PCBs and PCDFs that are not appropriately reflected by the currently utilized TEQ approach (Van den Berg et al. 2006).

Introduction
The mink (*Mustela vison*) is utilized as a sentinel species for ecological risk assessments at sites where contaminants of concern are chemicals that can bind to the aromatic hydrocarbon receptor (AHR) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and structurally similar compounds (Giesy et al. 1994; Tillitt et al. 1996; Blankenship et al. 2007). The mink is considered to be among the more sensitive mammals to TCDD and related compounds (Aulerich et al. 1977, 1985, 1987, 1988; Bleavins et al. 1980; Hochstein et al. 1988, 1998, 2001; Heaton et al. 1995; Tillitt et al. 1996; Restum et al. 1998; Beckett et al. 2005; Bursian et al. 2006a, 2006b, 2006c). Effects that occur in mink exposed to TCDD and related compounds include reproductive and developmental effects such as fewer kits whelped and mortality of kits, lesser body mass or body mass gain, and maxillary and mandibular lesions in juveniles (Mitsko et al. 2004; Beckett et al. 2005; Blankenship et al. 2007). In addition to their sensitivity to AHR-active compounds, mink have a relatively great potential for exposure to persistent, bioaccumulative chemicals due to their position at the top of aquatic food chain and relatively great consumption of fish and other prey (Basu et al. 2007). Recently, there has been concern about elevated concentrations of polychlorinated dibenzofurans (PCDFs), as well as detectable concentrations of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated biphenyls (PCBs) in floodplain soil and sediment from the Tittabawassee River, which flows into the Saginaw River and Saginaw Bay, Michigan, USA, as part of the Lake Huron watershed (Hilcherova et al. 2003). A preliminary risk assessment, using previously-established toxicity reference values (TRVs) derived primarily from studies of the effects of TCDD and other AHR-active compounds on mink and concentrations of TCDD equivalents (TEQs) in dietary components and tissues of mink inhabiting the Tittabawassee River, indicated that mink might be at risk of being adversely affected (GES 2003). Results of subsequent extensive monitoring of individual mink and the mink populations in the area indicated that the health of individuals was comparable or superior to those collected in reference areas and that the population was robust, despite accumulating relatively great concentrations of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in their livers (Zwiernik et al. 2008a).

Results
PCDF Concentrations in Liver
Concentrations of TCDF and PeCDF in the livers of mink receiving daily doses of TCDF, PeCDF or a mixture of the two congeners for up to 180 d did not differ at 90 and 180 d, thus a single hepatic concentration for each treatment group is presented (Table 1)

Gross Morphology and Histology
There were no treatment-related changes in gross morphology and histology. No external lesions or abnormalities that were attributable to treatment were observed and the nutritional status of all mink, except for one individual was classified as "good" to "very good". Jaw lesions classified as mild were observed at the termination of the study in two mink from the 9.5 ng TEQ_{PeCDF}/kg bw/d treatment group. One of these mink exhibited a single cyst consisting of squamous epithelial cells (Figure 2). However, the presence and severity of this lesion was not dose-dependent, and therefore, was considered incidental.

Enzyme Induction
The basal EROD activities measured in this study fell within a range of control activities that have been reported in other studies with mink (Smits et al. 1995; Shipp et al. 1998; Brunström et al. 2001; Käkälä et al. 2001; Martin et al. 2007). Values in this study were those reported by Smits et al. (1995), Käkälä et al. (2001) and Martin et al. (2007), but were less than those values reported by Brunström et al. (2001). However, given the inconsistencies between all of these studies relative to experimental design, age and sex of animals, as well as potential contaminants associated with their feed, a direct comparison between these studies is not possible. Given that the basal EROD activities in our study are similar to those enzyme activities measured in other studies, it can be assumed that the cytochrome P4501A1 system was functioning properly.

Following this preliminary risk assessment, a 180-day mink study was conducted to determine rates of induction of environmentally relevant doses of TCDF, PeCDF or a combination of the two congeners in liver tissue (Zwiernik et al. 2008b). In addition, the induction of hepatic ethoxresorufin O-deethylase (EROD) and methoxyresorufin O-deethylase (MROD) was examined as a predictive biomarker for morphological and histological changes. The effects of TCDF and PeCDF on hepatic EROD and MROD activities and selected morphological and histological parameters in mink are presented here.

EROD and MROD Activities
Mink administered daily doses of TCDF had significantly greater EROD and MROD activities in the liver compared to controls. Because there were no significant treatment by time interactions, enzyme activities at 90 and 180 d of exposure were combined into a single value. Exposure to TCDF resulted in significantly greater activity of both EROD and MROD in mink at doses of 3.8 and 20 ng TEQ_{TCDF}/kg bw/d (Figure 3). Additionally, EROD and MROD activities in animals dosed with 20 ng TEQ_{TCDF}/kg bw/d were significantly greater than activities in the 3.8 ng TEQ_{TCDF}/kg bw/d group. Both EROD (Figure 4A) and MROD (Figure 4B) activities were positively related to concentrations of TCDF expressed as TEQ in the liver.

There were no significant differences in enzyme activity in mink receiving daily doses of TCDF and/or PeCDF between 90 and 180 d. This indicates that maximum induction of CYP1A1s in mink as a function of time in response to the exposure with TCDF and PeCDF occurs earlier than the first sampling time point at 90 d.



Figure 1. Adult female mink were housed individually in wire mesh breeder cages (61 cm L x 76 cm W x 46 cm H) with wooden nesting boxes (22 x 22 x 22 cm) in a group housing facility at the Michigan State University (MSU) Experimental Fur Farm (East Lansing, MI, USA).

EROD and MROD activities in livers of mink dosed with a combination of TCDF (4.1 ng TEQ_{TCDF}/kg bw/d) and PeCDF (2.8 ng TEQ_{PeCDF}/kg bw/d) were significantly greater than control activities (Figure 7). EROD activity in the livers of mink fed the mixture of TCDF and PeCDF were similar to the activities in mink dosed with 3.8 ng TEQ_{TCDF}/kg bw/d and in mink dosed with 2.2 ng TEQ_{PeCDF}/kg bw/d. MROD activity in livers of mink fed the mixture was significantly greater than enzyme activity in mink in the 3.8 ng TEQ_{TCDF}/kg bw/d dose group, but did not differ from activity in mink in the 2.2 ng TEQ_{PeCDF}/kg bw/d PeCDF dose group (Figure 7B).

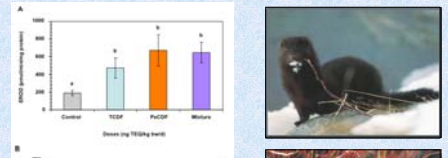
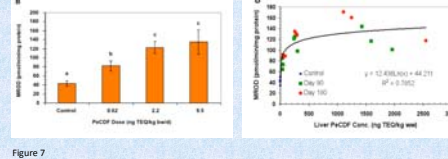
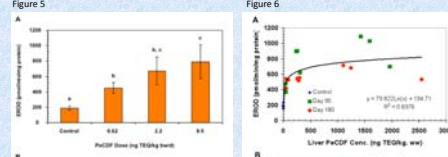
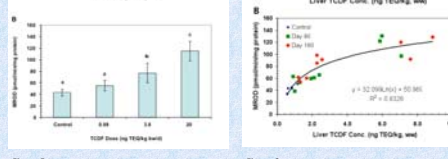
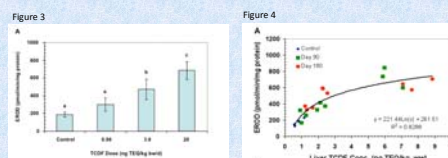
While activity and tissue concentrations increased slightly after 90 d for the TCDF treatments, maximum exposure could not be determined. Similarly in a female mink sub-chronic feeding study, TCDF and PeCDF administered singly for 13 weeks increased all enzyme activities reported in a dose-dependent manner, however, no clear maximum was attained for the TCDF (De Vito et al. 1997).

Materials and Methods

- Adult female mink were housed individually in wire mesh breeder cages (61 cm L x 76 cm W x 46 cm H) with wooden nesting boxes (22 x 22 x 22 cm) in a group housing facility at the Michigan State University (MSU) Experimental Fur Farm (East Lansing, MI, USA).
- A total of 51 female mink were distributed among eight treatments with six individuals in each of seven furan-dosed groups (three TCDF groups, three PeCDF groups and one TCDF plus PeCDF group) and eight female mink in the control group.
- Doses were expressed as TEQs (Table 1) calculated by use of toxic equivalency factors (TEFs) reported by Van den Berg et al. (2006).
- Each morning for 180 d, 25 g of feed containing the furan congener(s) was given to each animal. After this feed was consumed, an additional 100 g of uncontaminated feed was given to each animal.
- Individual body masses (g) were measured at the beginning of the study (January 31, 2006) and every 30 d thereafter.
- Three animals from the control group were euthanized by asphyxiation with carbon dioxide at initiation of the exposure (0 d) and three animals from each of the eight treatment groups were euthanized at 90 and 180 d of exposure for subsequent necropsy.
- Body mass (g) and length (cm) including and excluding the tail were recorded for each female mink.
- Mink were examined externally and internally for overall condition, nutritional status and the presence of gross abnormalities.
- Livers were removed and weighed. Sub-samples of liver were frozen in liquid nitrogen for subsequent measurement of EROD and MROD activities. Approximately 2.0 g of liver tissue was placed in a 10% formalin-saline solution (10% formalin in 0.9% sodium chloride) for histological examination.
- In addition, the spleen, kidney, thymus, mesenteric lymph node, and brain were removed and preserved for subsequent histological examination. The head was placed in formalin-saline solution for subsequent histological examination of mandibular and maxillary squamous epithelial cell proliferation as described by Beckett et al. (2005).
- The MSU Institutional Animal Care and Use Committee approved this study (AUF 12/05 – 165 – 00).
- EROD/MROD assays were optimized and conducted in 96-well plates (Corning Costar Corp., Corning, NY, USA) where both microsomal cytochrome P450 activity and protein concentration were measured simultaneously using a Fluorescent Assay microplate fluorimeter (Thermo Fisher Scientific Inc., Waltham, MA, USA).
- EROD and MROD activities were determined from the linear range of the time-curves for each well and the results were expressed as pmol substrate converted per min per mg protein (pmol/min/mg).
- To ensure that co-contaminants were not a factor in the study, the concentrations of 17 individual 2,3,7,8-substituted PCDF and PCDD congeners and 12 individual PCB congeners were measured in the dietary items and mink livers by use of United States Environmental Protection Agency (USEPA) Methods 8290 and 1668, respectively (USEPA 1994). Concentrations of TEQ were calculated as the sum of the products of the concentrations of congeners multiplied by their respective TEF (Van den Berg et al. 2006).
- All statistical analyses were performed with SAS (SAS, Ver. 8.1; Cary, NC, USA). Because of the nature of the parameters, several statistical models were used for data analyses. The study was designed for the application of both fixed effects models (test for differences among exposure groups) and regression analysis (correlation of liver PeCDF and TCDF concentrations and EROD and MROD enzyme activities).



Figure 2. A single cyst of squamous epithelial cells within the mandibular tissue at 4x (A) and 20x (B).



EROD/MROD INDUCTION ARE NOT PREDICTIVE OF CHANGES AT THE GROSS MORPHOLOGICAL OR HISTOLOGICAL LEVEL

The results of this study indicated that exposure to TCDF and PeCDF at concentrations equal to or greater than those found in the environment induce EROD and MROD enzyme activities in the livers of mink. However, TCDF and PeCDF doses resulting in enzyme induction did not cause any changes in gross morphological or histological parameters associated with exposure of mink to TCDD-like compounds. Because doses used in this study were approximately eight times greater than those reported in a parallel field study, it is unlikely that these field concentrations pose a risk to adult mink populations. However, long-term exposures, characteristic of multi-generation studies, are needed before final conclusions can be drawn as to the potential risk that these compounds may pose to wild mink. In addition, the results of this study indicate that use of the TEQ approach with use of the TEFs assigned to TCDF and PeCDF (Van den Berg et al. 2006) may overestimate their toxic potential in mink based on the lack of adverse effects in the present study in comparison to effects caused by PCB 126 and TCDD at equivalent or lesser TEQ concentrations. Finally, it was determined that while EROD and MROD activity can be used as sensitive biomarkers of exposure to PeCDF and TCDF in adult female mink, it cannot be determined at this time that EROD and MROD activity can be used to predict morphological or histological changes in wild mink.

Conclusions
The results of this study indicated that exposure to TCDF and PeCDF at concentrations equal to or greater than those found in the environment induce EROD and MROD enzyme activities in the livers of mink. However, TCDF and PeCDF doses resulting in enzyme induction did not cause any changes in gross morphological or histological parameters associated with exposure of mink to TCDD-like compounds. Because doses used in this study were approximately eight times greater than those reported in a parallel field study, it is unlikely that these field concentrations pose a risk to adult mink populations. However, long-term exposures, characteristic of multi-generation studies, are needed before final conclusions can be drawn as to the potential risk that these compounds may pose to wild mink. In addition, the results of this study indicate that use of the TEQ approach with use of the TEFs assigned to TCDF and PeCDF (Van den Berg et al. 2006) may overestimate their toxic potential in mink based on the lack of adverse effects in the present study in comparison to effects caused by PCB 126 and TCDD at equivalent or lesser TEQ concentrations. Finally, it was determined that while EROD and MROD activity can be used as sensitive biomarkers of exposure to PeCDF and TCDF in adult female mink, it cannot be determined at this time that EROD and MROD activity can be used to predict morphological or histological changes in wild mink.

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Table 1. Daily dose and concentrations of 2,3,7,8-tetrachlorodibenzofuran (TCDF) and/or 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in the liver of mink (*Mustela vison*)

Treatment	Daily dose (ng TEQ/kg bw/d)	Liver concentration (ng TEQ/kg ww)	
		0 d	90 & 180 d
Control	TCDF	<LOD ^a	0.79 ± 0.24
	PeCDF	<LOD ^a	0.61 ± 0.46
			NA
TCDF	0.98	NA	1.2 ± 0.27
	3.8	NA	2.3 ± 0.22
	20	NA	7.1 ± 1.1
PeCDF	0.62	NA	53 ± 18
	2.2	NA	270 ± 25
	9.5	NA	1600 ± 530
Mixture	TCDF	4.1	1.4 ± 0.24
	PeCDF	2.8	360 ± 80
			NA

^aEach treatment group had six mink while the control group had eight mink. Control animals were sampled at 0, 90 and 180 d; three treated animals per dose group were sampled at 90 and 180 d. All concentrations were converted to 2,3,7,8-TCDD equivalents.
Liver concentrations are presented as mean ± SD.
^bLOD = 0.1 ng TEQ/kg ww.
^cLOD = 0.01 ng TEQ/kg ww.
^dNA = no data was calculated, one mink was euthanized because of kidney failure that was not treatment related.
NA indicates that samples were not collected.

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