Characterization of bioactive 3-trifluoromethyl-4-nitrophenol (TFM) lampricide formulation impurities.

Mark Hewitt
Department of Environmental Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada
Mark Servos
Aquatic Ecosystem Protection Branch, Environment Canada, Burlington ON, L7R 4A6, Canada
Glen Van Der Kraak
Department of Zoology, University of Guelph, Guelph ON, N1G 2W1, Canada
Keith Solomon
Department of Environmental Biology, University of Guelph, Guelph, ON, N1G 2W1, Canada
Kelly Munkittrick
Aquatic Ecosystem Conservation Branch, Environment Canada, Burlington ON, L7R 4A6, Canada

1. Introduction.

The application of lampricides containing 3-trifluoromethyl-4-nitrophenol (TFM) within the Great Lakes basin of North America has been the primary means of control of sea lamprey (*Petromyzon marinus*) since the late 1950s. Tributary streams are treated on a 3 to 4 year cycle, designed to reduce the numbers lamprey ammocoetes that reach the predatory adult phase of their life cycle. Treatment concentrations vary from 1.0 mg/L to 14 mg/L of TFM, and current usage approximates 50,000 kg/year. Induction of hepatic mixed function oxygenase (MFO) detoxification enzymes and altered levels of circulating sex steroids were recently associated with fish exposed to field formulations of TFM[1]. Induction of P450IA enzyme activity in fish has been associated with exposure to planar aromatic compounds such as PCBs[2], PCDD/PCDFs[3], and PAHs[4]. MFO induction was initially observed in white sucker *(Catostomus commersoni)* caged during a lampricide treatment and in static laboratory exposures using rainbow trout *(Oncorhynchus mykiss)*[1]. Attempts to isolate the responsible compound(s) showed that induction was associated with the field formulation and not TFM itself. Formulation concentrations of PCDD/DFs, and PAHs were below detection limits[1].

A full understanding of the significance of the contamination requires information on contaminant structure, levels in the formulation, environmental fate and a perspective on historical levels. The objective of this study has been to develop and apply a Toxicity Identification Evaluation (TIE) directed by biochemical endpoints to isolate the active chemical(s).

2. Methods.

The methods described are modifications of the Toxicity Identification and Evaluation (TIE) approach [5-7]. Fractionations were directed by rainbow trout hepatic MFO induction determined as ethoxyresorufin-*O*-deethylase (EROD) activity. Fish exposure procedures and MFO assays were optimized to reduce fish handling and analysis time and used consistently throughout all TIE

phases[8]. Solid phase extraction was employed to isolate bioactive formulation impurities from primary formulation ingredients which had been previously shown to not exhibit the ability to induce MFO activity[1]. Cartridge phase, packing size and elution solvents were optimized to isolate bioactivity[8]. All SPE fractionations were performed on a preparative scale using 150 µL formulation (1 formulation equivalent), or 4.6 mg/L TFM. Fish exposures were conducted directly with the fractions generated. Subsequent fractionations were performed using reverse phase preparative high pressure liquid chromatography (HPLC). Bioactive fractions were characterized by gas-chromatography-high-resolution-mass-spectrometry (GC-HRMS). Fractionations and exposures were conducted on the 1990-batch #2 field formulation manufactured by Hoescht Chemical (provided by Sea Lamprey Treatment Centre, Sault Ste. Marie, ON Canada). The formulation is 37% (w/v) TFM in a solution of isopropanol and aqueous sodium hydroxide.

3. Results.

SPE was employed to separate inducing formulation contaminants from TFM because of the large chromatographic interference and the acute toxicity associated with TFM; the isolation of bioactive formulation impurities was be facilitated after TFM removal. After optimizing the solvent/buffer systems and cartridge size, >99% of TFM was separated from inducing formulation impurities[9]. Bioassays verified that induction was recovered after SPE and HPLC fractionations and that toluene extractions would recover inducing compounds for GC-HRMS analysis. Induction was initially isolated in two fractions (19-3 and 19-5; Figure 2) and the presence of three diphenyl ethers (A, B, C) in these fractions was confirmed by synthesis (Figure 1).

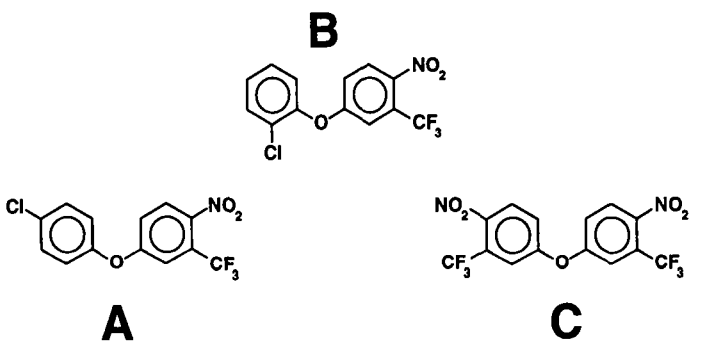

Figure 1. Diphenyl ether impurities confirmed in the TFM lampricide formulation.

However, no induction was observed after exposures to the pure compounds[9]. Induction was eventually isolated in a total of three final sub-fractions (30-3, 34-2, 31-3) depicted in Figure 2. A highly concentrated extract of the fraction with the highest induction potency, 34-2, showed trace levels of one unique constituent; its positive ion electron impact mass spectrum and assigned structure are presented in Figure 3. Additional evidence for this structure was gained after the molecular formulae and ratios of several fragment ions were corroborated by selected ion monitoring (SIM) analyses at 10,000 resolution. SIM analysis of 31-3 detected low levels of three apparent isomers[9].

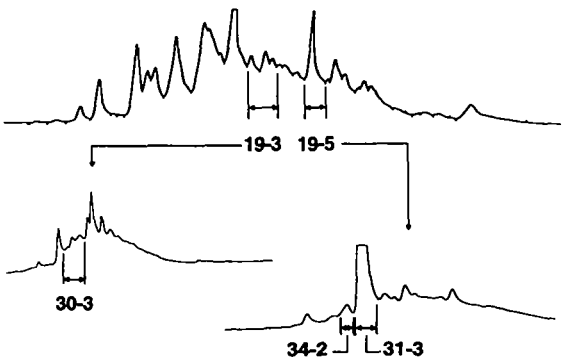

Figure 2. HPLC profiles of formulation impurities and fractions where MFO activity was isolated.

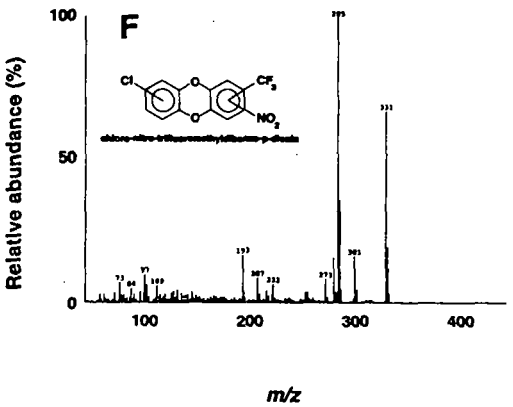

Figure 3. Mass spectrum and assigned structure to compound F, present in the fraction with the highest induction potency, 34-2.

Custom synthesis of the 2,3,7(or 8)-substituted isomer (F1) of this compound was undertaken and analysis of the product by GC-HRMS revealed this compound to be an isomer of the unknown (compound F) in TFM. MFO activities from waterborne trout exposures are presented in Figure 4.

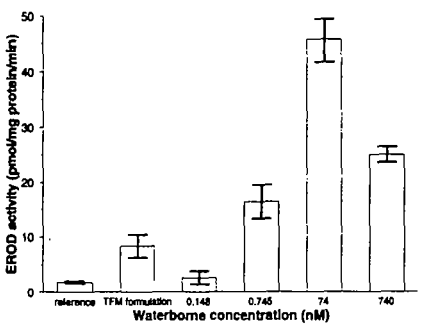

Figure 4. MFO activity determined for rainbow trout exposed waterborne to 2-trifluoromethyl-3-nitro-7(or 8)-chlorodibenzo-*p*-dioxin.

Given that an isomer of the unknown in the bioactive fraction causes induction under the identical, waterborne conditions employed throughout the TIE, custom synthesis of other isomers was undertaken to ascertain the substituent locations. The structures of the compounds synthesized to date are presented in Figure 5.

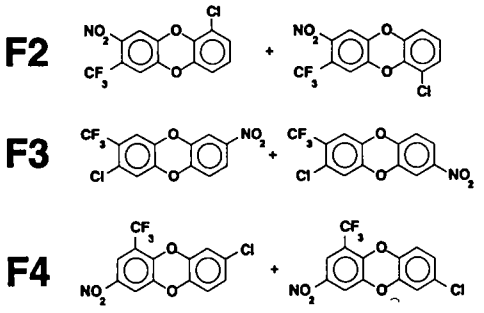

Figure 5. Structures of synthesized tri-substituted dioxins that can be eliminated from the isomer in fraction 34-2.

GC-HRMS analysis has shown that compound F2 is present in the TFM formulation but not in the bioactive fraction 34-2. Compounds F3 and F4 are also not present in the formulation and can be eliminated as possible structures. Once the location of the substituents is verified, a historical perspective and the environmental fate of these impurities can be addressed. Formulation concentrations for the diphenyl ethers in previous batches are presented in Figure 6.

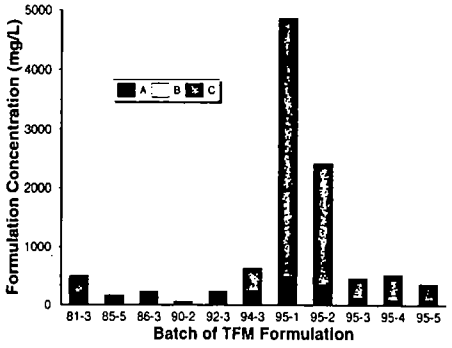

Figure 6. Formulation concentrations of confirmed diphenyl ether impurities from previous batches of TFM field formulations.

4. Conclusions.

TIE methodology directed by hepatic trout MFO induction was successfully developed and applied to the lampricide formulation containing TFM as the active ingredient. A minimum of two chemicals are responsible for induction in the formulation. Isomers of a chloro-nitro-trifluoromethyl-substituted dibenzo-p-dioxin have been identified in two final bioactive fractions. The 2-trifluoromethyl-3-nitro-7(and 8)-chloro- isomer causes *in vivo* induction in fish after waterborne exposures. Several possible isomers have been eliminated as candidates by synthesis.

Relatively high concentrations of other diphenyl ether impurities have been determined in previous batches.

5. References

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