UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JAN 20 1995

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Response to the 2,4-D Reregistration Standard: Fish Metabolism Study (MRID # 43378801, CBRS # 14481, Barcode No. D208093)

FROM: R. B. Perfetti, Ph.D., Chemist
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THRU: William J. Hazel, Ph.D., Section Head
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TO: Esther Saito, Chief
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Attached is a review of a fish metabolism study submitted in response to the 2,4-D Reregistration Standard. This review was completed by Dynamac Corporation under supervision of CBRS, HED. It has undergone secondary review in the branch and has been revised to reflect Agency policies.

The submitted fish metabolism study is adequate. Following dosing of bluegill sunfish with [^{14}C]2,4-D at 10.6 ppm in the water for four consecutive days, the level of radioactive residues in fillet samples was 0.406 ppm. Approximately 95% of the total radioactive residues (TRR) was extractable and 90% of the TRR was identified/characterized by HPLC. The principal residue was 2,4-D, accounting for 80% of the TRR (0.325 ppm); 2,4-DCP accounted for 8% of the TRR (0.032 ppm). The identities of the metabolites were confirmed by GC/MS. The registrant also tentatively identified radioactive residues in fish viscera. In viscera, 100% of the TRR was extractable. 2,4-D and 2,4-DCP each accounted for approximately 30% of the TRR and a mixture of chlorophenoxyacetic acid (CPAA) and chlorophenol (CP) accounted for 40% of the TRR. The 2,4-D metabolic pathway in fish is proposed by the registrant to proceed via formation of 2,4-DCP

and conjugates of 2,4-D and 2,4-DCP. The residue to be regulated in fish and shellfish is 2,4-D.

This metabolism study also indicates that magnitude of the residue studies on fish and shellfish are needed. No acceptable studies have been submitted to date. The experiments should reflect exposure of fish and shellfish to water containing levels of 2,4-D at the maximum treatment concentration for any aquatic use.

If you need additional input please advise.

Attachment 1: Review of 2,4-D Fish Metabolism Study.

cc (With Attachment 1): RBP, 2,4-D Reregistration Standard File, 2,4-D Subject File and Dynamac.

cc (Without Attachments): RF.

DYNAMAC
CORPORATION
Environmental Services

Final Report

2,4-D
Shaughnessy No. 030001
Case No. 0073
(CBRS No. 14481, DP Barcode D208093)

Registrant's Response to Residue Chemistry Data Requirements

November 21, 1994

Contract No. 68-D4-0010

Submitted to:
U.S. Environmental Protection Agency
Arlington, VA 22202

Submitted by:
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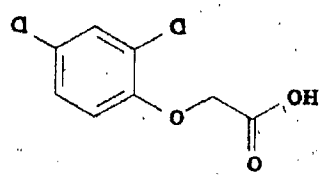
2,4-D



Shaughnessy No. 030001; Case 0073

(CBRS No. 14481; DP Barcode D208093)

REGISTRANT'S RESPONSE TO RESIDUE CHEMISTRY DATA REQUIREMENTS

BACKGROUND

The 2,4-D Guidance Document dated 9/88 required a metabolism study in which fish are exposed to water containing [^{14}C]2,4-D. Complete quantification and characterization of ^{14}C-residues in edible tissue (flesh and skin) were required. In response, the Industry Task Force II on 2,4-D Research Data submitted a study (1994; MRID 43378801) pertaining to the metabolism of [^{14}C]2,4-D in fish. These data are reviewed here for their adequacy in fulfilling residue chemistry data requirements. The Conclusions and Recommendations stated herein pertain only to data requirements for nature of the residue in fish and shellfish [Guideline 171-4(f)].

The qualitative nature of the residue is adequately understood in wheat but not in apples or potatoes. The Agency has concluded that given the present established uses on apples and potatoes, it may be impossible to get enough radioactivity into these two commodities for identification and for validation of analytical methods. Nevertheless, because of the widespread use of 2,4-D, the Agency still requires that plant metabolism studies with at least three dissimilar crops be conducted.

The qualitative nature of the residue in ruminants and poultry is adequately understood. The HED Metabolism Committee has determined that the residue to be regulated in animal products is 2,4-D *per se*.

Tolerances for residues of 2,4-D (2,4-dichlorophenoxyacetic acid) in/on plant, processed food/feed, and fish commodities are expressed in terms of 2,4-D *per se* [40 CFR §180.142 (a through f, i and j), §185.1450 (a), and §186.1450]. Tolerances in animal commodities are currently established in terms of residues of 2,4-D and/or its metabolite 2,4-dichlorophenol (2,4-DCP) [*40 CFR §180.142 (h)*]. Adequate methods are available for data collection and tolerance enforcement. Three GC methods with microcoulometric detection (MCD) and one GC method with electron capture detection (ECD) are listed in Pesticide Analytical Method (PAM) Vol, II as Methods A, B, C, and D.

There are no Codex MRLs in effect for residues of 2,4-D in water, fish, or shellfish. Therefore, no compatibility questions exist with respect to Codex MRLs.

CONCLUSIONS AND RECOMMENDATIONS

1. The submitted fish metabolism study is adequate. Following dosing of bluegill sunfish with [^{14}C]2,4-D at 10.6 ppm in the water for four consecutive days, the level of radioactive residues in fillet samples was 0.406 ppm. Approximately 95% of the total radioactive residues (TRR) was extractable and 90% of the TRR was identified/characterized by HPLC. The principal residue was 2,4-D, accounting for 80% of the TRR (0.325 ppm); 2,4-DCP accounted for 8% of the TRR (0.032 ppm). The identities of the metabolites were confirmed by GC/MS. The registrant also tentatively identified radioactive residues in fish viscera. In viscera, 100% of the TRR was extractable. 2,4-D and 2,4-DCP each accounted for approximately 30% of the TRR and a mixture of chlorophenoxyacetic acid (CPAA) and chlorophenol (CP) accounted for 40% of the TRR. The 2,4-D metabolic pathway in fish is proposed by the registrant to proceed via formation of 2,4-DCP and conjugates of 2,4-D and 2,4-DCP. The residue to be regulated in fish and shellfish is 2,4-D.

2. This metabolism study also indicates that magnitude of the residue studies on fish and shellfish are needed. No acceptable studies have been submitted to date. The experiments should reflect water containing levels of 2,4-D which utilize the maximum treatment concentration for any aquatic use.

DETAILED CONSIDERATIONS

Nature and Magnitude of the Residue in and in Fish and Shellfish

According to a REFs search conducted on 11/3/94, there are four products containing 2,4-D as an active ingredient that are registered for applications to aquatic areas and drainage systems. Tolerances of 1 ppm have been established for residues of 2,4-D in fish and shellfish.

Industry Task Force II submitted data (1994; MRID 43378801) depicting the metabolism of uniformly phenyl-labeled [^{14}C]2,4-D in bluegill sunfish. The fish were exposed to 10.6 ppm of [^{14}C]2,4-D in the water under static conditions for 4 consecutive days. The test substance had a radiochemical purity >99% and a specific activity of 2.37 mCi/mmol (2.38×10^4 dpm/μg). The study was conducted in four treated aquaria and one control aquarium. The five aquaria containing 70 fish each were immersed in a water bath (22 C); temperature, oxygen, and pH were monitored. A small aquarium treated with [^{14}C]2,4-D, but not holding any fish, was immersed in the same water bath as the test aquaria. This small aquarium was used to monitor the effect of the environment on 2,4-D. During the dosing period, the fish were fed *ad libitum*.

One whole fish and water samples from each aquarium were collected daily. After sampling, the whole fish were frozen with dry ice, homogenized, pooled, and analyzed for TRR. Fish collected at the completion of dosing (Day-4) were dissected into fillets and viscera. The fillets consisted of muscle, skin, and skeleton; the viscera consisted of fins, head, and internal organs. Samples were stored frozen (ca. -20 C) until analysis. The registrant stated that analyses were performed within 6 months of sample collection. The in-life and analytical phases of the study were conducted by ABC Laboratories, Inc., Columbia, MO.

Total radioactive residues (TRR)

Triplicate aliquots of homogenized whole fish, fillet, and viscera samples were combusted and radioassayed by liquid scintillation spectroscopy (LSS). Water and liquid samples were radioassayed in triplicate directly by LSS. The limit of quantitation was 0.004 ppm (calculated by the reviewer). Sample calculations were submitted. The TRR, expressed as 2,4-D equivalents, are presented in Table 1.

Table 1. Total radioactive residues (TRR) in samples from fish dosed with [^{14}C]2,4-D at 10.6 ppm for four consecutive days.

Matrix		TRR (ppm)
Whole fish	Day-1 [a]	0.410
	Day-2	0.511
	Day-3	0.600
Viscera		1.91
Fillet		0.406

[a] On each day, one fish from each of the four treated aquaria was collected and pooled for whole fish ^{14}C-residue determinations.

Extraction and hydrolysis of residues

Residues in the fillet sample were extracted with acidic ACN (0.1 M HCl, 4:1, v/v). A subsample of the initial acidic ACN fraction was analyzed by HPLC and the remaining extract was partitioned with ACN-saturated hexane. The hexane fraction was not analyzed further. Residues in the acidic ACN fraction were concentrated, acid hydrolyzed (2 N HCl; reflux for 8 hours, cool overnight), and then partitioned with diethyl ether (Et_2O). Residues in the Et_2O fraction were concentrated, dissolved in ACN, and analyzed by HPLC and GC/MS. The aqueous fraction was not analyzed further.

Residues in the solid fraction (PES) were sequentially hydrolyzed with acid and base. Briefly, residues were acid hydrolyzed (2 N HCl with ACN as co-solvent, reflux for 6 hours, cool overnight) and filtered, resulting in an acid filtrate and a solid fraction. The solid

fraction was then base hydrolyzed (0.5 N NaOH, reflux for 6 hours, cool overnight), and the residues were filtered, resulting in a base filtrate and a solid fraction. Residues in the acid and base filtrates were each partitioned with H_2O-saturated ethyl acetate (EtOAc) resulting in aqueous and EtOAc fractions. The acid and base EtOAc fractions were combined and the residues concentrated, dissolved in ACN, and analyzed by HPLC. The aqueous fractions and remaining solids were not analyzed further.

A subsample of the PES was sequentially hydrolyzed with acid (2 N HCl with ACN as co-solvent, reflux for 16 hours) and base (2 N NaOH with ACN as co-solvent, reflux for 16 hours). The acid and base hydrolysates were partitioned as described above. The EtOAc fraction resulting from partitioning the acid filtrate was analyzed by HPLC. The EtOAc fraction resulting from partitioning the base filtrate, the aqueous fractions, and the remaining solids were not analyzed further.

The distribution and characterization of ^{14}C-activity in solvent extracts of the fish fillet samples are summarized in Table 2. Similar information was provided from refluxing the PES fillet sample in acid and base for 6 or 16 hours. Only the information provided for the 6-hour procedure is presented in this document.

Residues in the viscera were extracted with ACN and partitioned with hexane as described above. Only solvent soluble residues were characterized/identified by HPLC. The registrant explained that because viscera are not edible, attempts were not made to confirm identities of the residues.

To demonstrate extractability and stability of 2,4-D through the extraction procedure, control fillet and viscera samples were fortified with [^{14}C]2,4-D, extracted with ACN, and partitioned with hexane in a similar manner to the treated samples.

Characterization of Residues

Reverse phase HPLC analyses were performed on a system equipped with a UV absorbance detector at 280 nm and a radiodetector, and using a H_2O (1% acetic acid, v/v):acetonitrile (ACN; 1% acetic acid, v:v) solvent system. The R_ts of individual reference standards and of a reference standard mixture consisting of 2,4-D, [^{14}C]2,4-D, 2,4-DCP, and 2,4-DCA were determined and compared to the treated samples. Identifications of 2,4-D and 1,4-DCP were confirmed by GC/MS in the EI mode. Representative HPLC and GC/MS chromatograms were submitted.

A summary of identified and characterized ^{14}C-residues in fillet samples is presented in Table 3 and the molecular structures and chemical names of 2,4-D and its metabolites in fish are presented in Figure 1.

HPLC analyses of the water from the aquaria indicated that 2,4-D was stable in the test environment. The extraction of ^{14}C-fortified samples demonstrated that 2,4-D was stable during the extraction and characterization procedures.

Table 2. Distribution and characterization of TRR in fillet samples from fish dosed with [^{14}C]2,4-D at 10.6 ppm for four consecutive days.

Fraction	% TRR	ppm	Characterization/Identification
Fillet (0.406 ppm)			
Acidic ACN	83.7	0.340	Concentrated.
ACN	80.8	0.328	Acid hydrolyzed and solvent partitioned.
Et_2O	81.5	0.331	HPLC detected 2,4-D (73.6% TRR; 0.299 ppm), 2,4-DCP (7.9% TRR, 0.032 ppm) and one polar unknown accounting for 1.5% of the TRR (0.006 ppm). GC/MS analyses confirmed the identities of 2,4-D and 2,4-DCP.
Aqueous	0.0	<LOQ	Not analyzed further.
Hexane	1.2	0.005	Not analyzed further.
PES	10.1	0.041	Acid hydrolyzed (refluxed 6 hours) and solvent partitioned.
EtOAc	5.2	0.021	Combined with EtOAc below.
Aqueous	0.0	<LOQ	Not analyzed further.
Solids	NR *	NR	Base hydrolyzed (refluxed 6 hours) and solvent partitioned.
EtOAc	2.5	0.01	Combined with acid EtOAc fraction above. Combined fraction (7.1% TRR; 0.029 ppm) was analyzed by HPLC. HPLC detected 2,4-D (6.4% TRR; 0.026 ppm) and a polar unknown (0.7% TRR; 0.003 ppm)
Aqueous	0.0	<LOQ	Not analyzed further.
Solids	0.0	<LOQ	Not analyzed further.

* NR=Not reported.

Table 3. Summary of characterization/identification of ^{14}C-residues in fillet samples from fish dosed with [^{14}C]2,4-D at 10.6 ppm for four consecutive days.

Metabolite/Component	%TRR	ppm
2,4-D	80.0	0.325
2,4-DCP	7.9	0.032
Total identified	**87.9**	**0.357**
Polar Unknowns	2.2	0.009
Solids	0.0	<LOQ

In summary, the metabolism of 2,4-D in fish is adequately understood. The TRR was 0.406 ppm in bluegill sunfish fillets exposed to 10.6 ppm of [^{14}C]2,4-D under static conditions for 4 consecutive days. Approximately 95% of the TRR was extractable and 90% of the TRR was identified/characterized by HPLC. The principal residue was 2,4-D, accounting for 80% of the TRR (0.325 ppm); 2,4-DCP accounted for 8% of the TRR (0.032 ppm). The identities of the metabolites were confirmed by GC/MS. The registrant also tentatively identified radioactive residues in fish viscera. In viscera, 100% of the TRR was extractable. 2,4-D and 2,4-DCP each accounted for approximately 30% of the TRR and a mixture of CPAA and CP accounted for 40% of the TRR. The registrant suggested that the additional metabolites detected in the viscera were due to microbial degradation. Literature was cited to support this hypothesis. The 2,4-D metabolic pathway in fish is proposed by the registrant to proceed via formation of 2,4-DCP and conjugates of 2,4-D and 2,4-DCP. The residue to be regulated in fish and shellfish is 2,4-D.

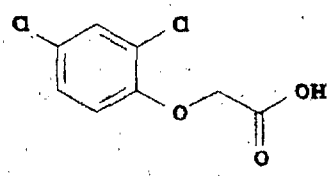
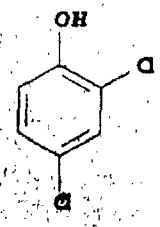
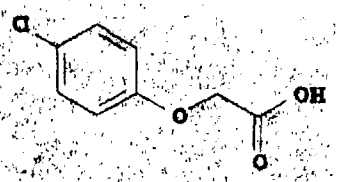
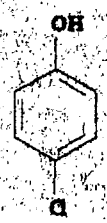
This metabolism study also indicates that magnitude of the residue studies on fish and shellfish are needed. No acceptable studies have been submitted to date. The experiments should reflect exposure of fish and shellfish to water containing levels of 2,4-D at the maximum treatment concentration for any aquatic use.

MASTER RECORD IDENTIFICATION NUMBERS

The citation for the MRID document referred to in this review is presented below:

43378801 Premkumar, N.D. and Stewart, S. (1994). Uniformly ^{14}C-Ring Labeled 2,4-Dichlorophenoxyacetic Acid: A Metabolism Study in Bluegill Sunfish. ABC Study No. 41116. Unpublished study conducted by ABC Laboratories, Inc. 128 pp.

Figure 1. Chemical names and structures of 2,4-D and its metabolites.*

Chemical/Common Name	*Structure*
2,4-Dichlorophenoxyacetic acid 2,4-D	
2,4-Dichlorophenol 2,4-DCP	
4-Chlorophenoxyacetic acid 4-CPAA	
4-Chlorophenol 4-CP	

* Reference standards also used in the fish study included 2,4-dichloroanisole (2,4-DCA), 2,4-dichlorophenoxyacetic acid methyl ester (2,4-D methyl ester) and 2,5-dichloro-4-hydroxyphenoxyacetic acid, potassium salt (4-OH-DCAA).