SUMMARY

Analytical methods were developed and validated for the detection and quantitation of 2,4-D Acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; and 2,4-DCA in soil (sandy loam and silt loam) and sediment (loamy sand and clay loam); and 4-CP and 4-CPA in sediment (loamy sand and clay loam).

The method limit of quantitation (LOQ) of 2,4-D Acid in soil and sediment was 0.0019 ppm (mg/kg). The limit of detection (LOD) was defined as approximately 1/3 LOQ or 0.00057 ppm (mg/kg). The method limit of quantitation (LOQ) of 2,4-D 2-EHE; 2,4-D DMAS; and 2,4-DCP; in soil and sediment; and 4-CP and 4-CPA in sediment was 0.010 ppm (mg/kg). The limit of detection (LOD) was defined as approximately 1/3 LOQ or 0.003 ppm (mg/kg). This method was validated at 0.0019, 0.019, and 0.19 ppm for 2,4-D Acid; and 0.010, 0.10, and 1.0 ppm for 2,4-D 2-EHE; 2,4-D DMAS; and 2,4-DCP in soil and sediment; and 4-CP and 4-CPA in sediment using an LC-MS/MS system in negative ion mode.

The method limit of quantitation (LOQ) of 2,4-DCA in soil and sediment was 0.010 ppm (mg/kg). The limit of detection (LOD) was defined as approximately 1/3 LOQ or 0.003 ppm (mg/kg). This method was validated at 0.010, 0.10, and 1.0 ppm in soil and sediment via GC-MSD.

The methods were validated on silt loam (Brierlow) and sandy loam (Hanford) soils, and loamy sand (Golden Lake) and clay loam (Goose River) sediments supplied by Dow AgroSciences, Indianapolis, Indiana.

Residues of 2,4-D Acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; and 2,4-DCA were extracted from soil matrices; and 2,4-D acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCA, 4-CP and 4-CPA were extracted sequentially from sediment via vortexing, sonication, and centrifugation using 5% acetic acid in methanol; 50:50, 5% acetic acid in methanol:5% acetic acid in water; and 5% acetic acid in water. The extracts were decanted, combined, and brought to a known volume with water. The sample preparation scheme for each analyte continues as follows:

- **FRACTION A** (2,4-DCA): An aliquot of extract was partitioned with isooctane, the volume reduced under nitrogen blow down, diluted with 0.2% peanut oil in isooctane, and submitted for GC-MSD analysis.
- **FRACTION B** (2,4-D 2-EHE and 2,4-D DMAS): An aliquot of extract was hydrolyzed with 1N NaOH (to 2,4-D Acid), acidified with 1:1, HCl:water, cleaned up via an HLB Solid Phase Extraction (SPE) column, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.
- **FRACTION C** (2,4-D Acid, 2,4-DCP, 4-CP, and 4-CPA): An aliquot of extract was diluted with 0.1N HCl (aq), cleaned up via an HLB SPE column, the volume reduced under nitrogen blow down, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.

The confirmatory analyses for the GC-MSD and LC-MS/MS methods were based on detection of secondary parent-to-daughter ion transitions monitored during the validation.

1 INTRODUCTION

1.1 Scope

These methods are applicable for the quantitative determination of residues of 2,4-D Acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; and 2,4-DCA in soil (sandy loam and silt loam) and sediment (loamy sand and clay loam); and 4-CP and 4-CPA in sediment (loamy sand and clay loam). The soil/sediment methods were validated over the concentration range of 0.0019 to 0.19 ppm with a limit of detection of 0.00057 ppm for 2,4-D Acid, and 0.010 to 1.0 ppm with a limit of detection of 0.003 ppm for all other analytes. Common and chemical names, molecular formulas, and the nominal masses for the analytes are given in Table 1.

This study was conducted to fulfill data requirements outlined in the U. S. EPA Residue Chemistry Test Guidelines, OPPTS 860.1000 (1) and OCSPP 850.6100 (2).

1.2 Method Principle

Residues of analytes 2,4-D acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; and 2,4-DCA were extracted from soil matrices; and analytes 2,4-D acid; 2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 2,4-DCA; 4-CP and 4-CPA were extracted sequentially from sediment via vortexing, sonication, and centrifugation using 5% acetic acid in methanol; 50:50, 5% acetic acid in methanol:5% acetic acid in water; and 5% acetic acid in water. The extracts were decanted, combined, and brought to a known volume with water. The sample preparation scheme for each analyte continues as follows:

- **FRACTION A** (2,4-DCA): An aliquot of extract was partitioned with isooctane, the volume reduced under nitrogen blow down, diluted with 0.2% peanut oil in isooctane, and submitted for GC-MSD analysis.
- **FRACTION B** (2,4-D 2-EHE and 2,4-D DMAS): An aliquot of extract was hydrolyzed with 1N NaOH (to 2,4-D acid), acidified with 1:1, HCl:water, cleaned up via an HLB Solid Phase Extraction (SPE) column, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.
- **FRACTION C** (2,4-D acid, 2,4-DCP, 4-CP, and 4-CPA): An aliquot of extract was diluted with 0.1N HCl (aq), cleaned up via an HLB SPE column, the volume reduced under nitrogen blow down, diluted with 0.1% formic acid (aq), and submitted for LC-MS/MS analysis.

1.3 Reference Standards

Standard	Lot Number	Purity	Expiration Date
2,4-D Acid	MORRIS/1710	99.5% 99.1%	19 October 2016 04 October 2018
2,4-D 2-EHE	YB1-100780-094	99.3%	20 October 2018
2,4-D DMAS	V43-037861-8	99.3%	21 October 2017
2,4-DCP	OCR 696-132-1	100%	07 October 2023
2,4-DCA	S67935	100%	16 October 2018
4-CP	MKBJ7452V	100%	07 October 2017
4-CPA	089F002125	100%	13 October 2023

The above standards were obtained from the Sponsor Monitor. The certificates of analysis were provided by Dow AgroSciences, Indianapolis, Indiana, and are located in Appendix C.

1.4 Characterization of Control Matrices

Brierlow and Hanford soils, and Golden Lake and Goose River sediments were supplied by Dow AgroSciences, Indianapolis, Indiana; details of the GLP characterization results are filed with Dow AgroSciences and included in the raw data.

1.5 Equipment, Glassware, and Materials

Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by appropriate tests. Common laboratory glassware and supplies are assumed to be readily available. Unless specified otherwise, Class A volumetric glassware is used to prepare analytical standards, fortification solutions, and calibration standards.

1.5.1 Laboratory Equipment

Gas chromatograph, Agilent 7890 GC with 5975C inert XL EI/CI MSD and Gerstel multi-purpose sampler

Gas chromatography data system, ChemStation G1701EA E.02.02

Liquid chromatograph, Shimadzu System Controller CBM-20A, Shimadzu Degasser DGU-20A3R, Shimadzu Pump LC30AD, Shimadzu Column Heater CTO-20AC, Shimadzu Autosampler SIL-30ACMP

Mass spectrometer, Applied Biosystems/Sciex API 6500 Q-Trap, Applied Biosystems

Mass spectrometer data system, Model Analyst 1.6.2, Applied Biosystems

Column, analytical, (GC-MSD) HP-5MS 30 x 0.25 mm, 0.25 µm

Column, analytical, (LC-MS/MS) Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm

1.5.2 Prepared Solutions

0.1% Formic Acid (aq)
Add 2 L of water to a 2-L bottle and add 2.0 mL of formic acid. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

20:80, ACN:0.1% Formic Acid (aq)
Add 800 mL of 0.1% formic acid (*aq*) to a 1-L storage container. Add 200 mL ACN to the conainer. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

1:1 HCl:Water
Add 500 mL of water to a 1-L storage container. Slowly add 500 mL of concentrated HCl to the container. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

0.1N HCl
Add 16.53 mL of concentrated HCl to a 2-L graduated mixing cylinder containing ~1400 mL of water. Bring to volume with water. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

1N Sodium Hydroxide
Weigh 40 g of sodium hydroxide and add to a 1-L graduated cylinder containing ~800 mL of water. Allow pellets to dissolve and bring to a final volume of 1000 mL with water. Transfer the solution to a 1-L plastic bottle and hand shake well to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

Sodium Hydroxide (1M)
Weigh 60.08 g of sodium hydroxide and add to a 2-L graduated cylinder containing ~1000 mL of water. Allow pellets to dissolve and bring to a final volume of 1500 mL with water. Transfer the solution to a 4-L amber glass bottle and hand shake well to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

0.2% Peanut Oil in Isooctane
Add 500 mL of isooctane to a 500-mL glass jar. Add 1 mL of peanut oil to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

0.1% Peanut Oil in Isooctane
Add 100 mL of 0.2% peanut oil in isooctane to a 500-mL glass jar. Add 100 mL of isooctane to the jar. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5% Acetic Acid in Methanol (First Extraction Solution)
Add approximately 400 mL of methanol to a 1-L mixing cylinder. Add 50 mL of acetic acid to the 1-L mixing cylinder containing the methanol. Bring to a 1-L volume with methanol. Cap and shake to mix. Transfer the solution to an empty 1-L bottle. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

50:50, 5% Acetic Acid in Methanol:5% Acetic Acid in Water (Second Extraction Solution)
Add ~400 mL of water to one 1-L mixing cylinder and ~400 mL of methanol to another 1-L mixing cylinder. Add 50 mL of acetic acid to each of the 1-L mixing cylinders, containing water and the other containing methanol. Bring each to a 1-L volume, the one containing methanol with methanol and the one containing water with water. Cap and shake to mix. Combine the solutions from both 1-L mixing cylinders in an empty 2-L bottle. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

5% Acetic Acid in Water (Third Extraction Solution)
Add approximately 400 mL of water to a 1-L mixing cylinder. Add 50 mL of acetic acid to the 1-L mixing cylinder. Bring to a 1-L volume with water. Cap and shake to mix. Transfer the solution to an empty 1-L bottle. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

1.0M Ammonium Acetate (aq)
Measure approximately 50 mL of water into a 100-mL mixing cylinder. Measure and add 7.71 g of ammonium acetate to the same mixing cylinder, and bring to volume with water. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

5mM Ammonium Acetate (aq)
Pipette approximately 20 mL of 1.0M ammonium acetate into a 4-L bottle of water. Cap and shake to mix. The solution may be stored at room temperature for up to 3 months. Adjust volumes accordingly for different quantities.

1:1:1, Acetonitrile:Methanol:Water
Add approximately 4 L each of acetonitrile, methanol, and water to a carboy. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

1:1:2, Acetonitrile:Methanol:Water
Add approximately 4 L each of acetonitrile, and methanol, and 8 L of water to a carboy. Cap and shake to mix. The solution may be stored at room temperature for up to 12 months. Adjust volumes accordingly for different quantities.

2 EXPERIMENTAL

2.1 Instrumental Conditions

2.1.1 Typical Gas Chromatography Operating Conditions

Instrumentation:	Agilent 7890 Gas Chromatograph ChemStation G1701EA E.02.02 data system
Detector:	5975C inert XL EI/CI MSD with triple-axis detection
Injector:	Gerstel Multi-Purpose Sampler
Column:	HP-5MS 30 x 0.25 mm, 0.25 µm
Oven Temperature:	Hold at 80 °C for 3 min, then 80 to 150 °C at 10 °C/min, then 150 to 310 °C at 40 °C/min, then hold 5 min
Injector Temperature:	275 °C
Detector Temperature:	EI Source: 230 °C Quad: 150 °C Thermal Aux: 280 °C
Carrier Gas:	Helium
Carrier Gas Flow Rate:	0.8 mL/min
Head Pressure:	30 psi 1 min, 6.84 psi initial
Inlet Liner:	Gooseneck splitless liner packed with CarboFrit, 4 mm i.d.
Injector Purge Delay:	1.5 min
Septum Purge:	3.0 mL/min
Injection Volume:	2.0 µL
Wash #1:	Acetone
Wash #2:	Isooctane

Analyte:	Quantitation Ion	Qualifier Ion 1	Qualifier Ion 2	Dwell Time (msec)
2,4-DCA	178	161	163	100

2.1.2 Typical Liquid Chromatography Operating Conditions

2,4-D Acid; 2,4-DCP; and 4-CPA

Instrumentation: AB SCIEX Q-Trap API 6500 LC-MS/MS System
AB SCIEX Analyst 1.6.2 data system

Column: Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm

Column Temperature: 40 °C

Injection Volume: 25 µL

Injection Wash Program: Autosampler loop and needle washed with:
1:1:1 ACN:MeOH:H_2O, followed by
1:1:2 ACN:MeOH:H_2O

Run Time: 9.00 minutes

Mobile Phase: A: 5mM ammonium acetate (aq)
B: Methanol

Flow Rate: 1.0 mL/min, no split

Gradient:

Time, min	A, %	B, %
6.00	10	90
7.50	10	90
7.60	70	30
9.00	Stop	

4-CP

Instrumentation: AB SCIEX Q-Trap API 6500 LC-MS/MS System
AB SCIEX Analyst 1.6.2 data system

Column: Phenomenex Synergi Hydro-RP 75 x 4.6 mm, 4µm

Column Temperature: 40 °C

Injection Volume: 35 µL

Injection Wash Program: Autosampler loop and needle washed with:
1:1:1 ACN:MeOH:H_2O, followed by

1:1:2 ACN:MeOH:H_2O

Run Time: 6.00 minutes

Mobile Phase: A: 5mM ammonium acetate (aq)
B: Methanol

Flow Rate: 1.0 mL/min, no split

Gradient:

Time, min	A, %	B, %
3.00	10	90
4.50	10	90
4.60	60	40
6.00	Stop	

2.2 Typical Mass Spectrometry Operating Conditions

2,4-D Acid; 2,4-DCP; and 4-CPA

Interface: APCI
Polarity: Negative (-)
Scan Type: MRM
Resolution: Q1 – unit, Q3 – unit
Curtain Gas (CUR): Nitrogen @ setting of "20"
Collision Gas (CAD): Nitrogen @ setting of "High"
Temperature (TEM): 500°C
Ion Source Gas 1 (GS1): 30
Ion Source Gas 2 (GS2): 0

Entrance Potential (EP): -10 volts
Cell Exit Potential (CXP) -10 volts

Analytes:	Precursor Ion Q1	Product Ion Q3	Dwell Time (msec)	Declustering Potential (DP)	Collision Energy (CE)
2,4-D Acid					
(quantitation)	219	161	100	-15 V	-21 V
(confirmation)	221	163	100	-15 V	-21 V
2,4-DCP					
(quantitation)	161	125	200	-70 V	-23 V
(confirmation)	163	127	200	-70 V	-23 V
4-CPA					
(quantitation)	185	127	100	-25 V	-19 V
(confirmation)	187	129	100	-25 V	-19 V

Note: 2,4-D 2-EHE and; 2,4-D DMAS are detected as 2,4-D.

4-CP

Interface:	Electrospray
Polarity:	Negative (-)
Scan Type:	MRM
Resolution:	Q1 – unit, Q3 – unit
Curtain Gas (CUR):	Nitrogen @ setting of "15"
Collision Gas (CAD):	Nitrogen @ setting of "High"
Temperature (TEM):	600°C
Ion Source Gas 1 (GS1):	70
Ion Source Gas 2 (GS2):	60
IonSpray Voltage (IS):	-4500 volts
Entrance Potential (EP):	-10 volts
Cell Exit Potential (CXP)	-10 volts

Analytes:	Precursor Ion Q1	Product Ion Q3	Dwell Time (msec)	Declustering Potential (DP)	Collision Energy (CE)
4-CP					
(quantitation)	127	91	100	-80 V	-22 V
(confirmation)	127	35	100	-80 V	-36 V

2.3 Preparation of Standard Solutions

Approximately 10 mg (corrected for purity) of each analytical standard was weighed into a 10-mL volumetric flask. The solutions were brought to volume with acetonitrile to make stock standard solutions of approximately 1000-µg/mL. 2,4-D DMAS was weighed out as 2,4-D acid equivalent using a conversion factor of 221.04 g/mol (molecular mass of 2,4-D) / 266.12 g/mol (molecular mass of 2,4-D DMAS). Stock standards were stored at ~2-8 °C (refrigerator) in 4-dram amber glass bottles.

2.4 Preparation of Fortification Solutions

The following concentrations of fortification standard solutions were prepared using individual stock standard solutions and stored capped at ~2-8 °C.

2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; and 4-CPA

100 µg/mL: (19 µg/mL for 2,4-D Acid)	Pipette 1.00 mL (0.190 mL of 2,4-D Acid) of each (2,4-DCP; 4-CP; and 4-CPA) of the 1000-µg/mL stock standards and 0.980 mL of the 2,4-DCA 1020-µg/mL stock standard into a 4-dram amber bottle, then add 5.83 mL of HPLC acetone. Cap and vortex mix.

10 µg/mL: (1.9 µg/mL for 2,4-D Acid)	Pipette 1.00 mL of the 100-µg/mL (19.0-µg/mL for 2,4-D Acid) mixed standard solution into a 4-dram amber bottle and bring to a final volume of 10 mL with HPLC acetone. Cap and vortex mix.
1.0 µg/mL: (0.19 µg/mL for 2,4-D Acid)	Pipette 1.00 mL of the 10-µg/mL (1.90-µg/mL for 2,4-D Acid) mixed standard solution into a 4-dram amber bottle and bring to a final volume of 10 mL with f HPLC acetone. Cap and vortex mix.
0.30 µg/mL: (0.057 µg/mL for 2,4-D Acid)	Pipette 0.300 mL of the 10-µg/mL (1.90-µg/mL for 2,4-D Acid) mixed standard solution into a 4-dram amber bottle and bring to a final volume of 10 mL with HPLC acetone. Cap and vortex mix.

2,4-D 2-EHE

100 µg/mL:	Pipette 1.00 mL of the 1000-µg/mL 2,4-D 2-EHE stock standard into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
10 µg/mL:	Pipette 1.00 mL of the 100-µg/mL 2,4-D 2-EHE standard solution into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
1.0 µg/mL:	Pipette 1.00 mL of the 10-µg/mL 2,4-D 2-EHE standard solution into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
0.30 µg/mL:	Pipette 0.300 mL of the 10-µg/mL 2,4-D 2-EHE standard solution into a 4-dram amber bottle containing 9.7 mL of HPLC acetone. Cap and vortex mix.

2,4-D DMAS

100 µg/mL:	Pipette 1.00 mL of the 1000-µg/mL 2,4-D DMAS stock standard into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
10 µg/mL:	Pipette 1.00 mL of the 100-µg/mL 2,4-D DMAS standard solution into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
1.0 µg/mL:	Pipette 1.00 mL of the 10-µg/mL 2,4-D DMAS standard solution into a 4-dram amber bottle containing 9.0 mL of HPLC acetone. Cap and vortex mix.
0.30 µg/mL:	Pipette 0.300 mL of the 10-µg/mL 2,4-D DMAS standard solution into a 4-dram amber bottle containing 9.7 mL of HPLC acetone. Cap and vortex mix.

2.5 GC Calibration Standards (2,4-DCA, Fraction A Analysis)

Calibration standards were injected interspersed with samples throughout each analytical run. Standards were prepared by using calibrated pipettes to measure 10 mL of 0.1% peanut oil in isooctane into 4-dram amber glass bottles, removing from the standard bottle the indicated aliquot volumes, and using the same calibrated pipette to add the corresponding amount of standard solution. Calibration solutions were stored refrigerated at ~2-8 °C.

Intermediate Standard Solutions:

10 µg/mL: Add 0.100 mL of 1000-µg/mL stock standard to a 4-dram amber glass bottle containing 9.900 mL of 0.1% peanut oil in isooctane. Mix well.

1.0 µg/mL: Add 1.00 mL of 10-µg/mL standard solution to a 4-dram amber glass bottle containing 9.00 mL of 0.1% peanut oil in isooctane. Mix well.

Calibration Standard Solutions:

0.100 µg/mL: Add 1.00 mL of 1.00-µg/mL standard solution to a 4-dram amber glass bottle containing 9.00 mL of 0.1% peanut oil in isooctane. Mix well.

0.0500 µg/mL: Add 0.500 mL of 1.00-µg/mL standard solution to a 4-dram amber glass bottle containing 9.500 mL of 0.1% peanut oil in isooctane. Mix well.

0.0250 µg/mL: Add 0.250 mL of 1.00-µg/mL standard solution to a 4-dram amber glass bottle containing 9.750 mL of 0.1% peanut oil in isooctane. Mix well.

0.0100 µg/mL: Add 0.100 mL of 1.00-µg/mL standard solution to a 4-dram amber glass bottle containing 9.900 mL of 0.1% peanut oil in isooctane. Mix well.

0.00500 µg/mL: Add 0.500 mL of 0.100-µg/mL standard solution to a 4-dram amber glass bottle containing 9.500 mL of 0.1% peanut oil in isooctane. Mix well.

0.00300 µg/mL: Add 0.300 mL of 0.100-µg/mL standard solution to a 4-dram amber glass bottle containing 9.700 mL of 0.1% peanut oil in isooctane. Mix well.

2.6 HPLC Calibration Standards (2,4-D Acid; 2,4-DCP; 4-CP; and 4-CPA, Fractions B and C Analysis)

Calibration standards were injected interspersed with samples throughout each analytical run. Standards were prepared by using calibrated pipettes to measure 10 mL of 20:80 ACN:0.1% formic acid (*aq*) into 4-dram amber glass bottles, removing from the standard bottle the indicated aliquot volumes, and using the same calibrated pipette to add the corresponding amount of standard solution. Calibration solutions were stored refrigerated at ~2-8 °C.

Intermediate Standard Solutions:

100 ng/mL: (19 ng/mL for 2,4-D Acid)	Add 0.100 mL of 10-µg/mL (1.9-µg/mL for 2,4-D Acid) stock standard to a 4-dram amber glass bottle containing 9.900 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.
10 ng/mL: (1.9 ng/mL for 2,4-D Acid)	Add 1.00 mL of 100-ng/mL (19-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 9.00 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.

Calibration Standard Solutions:

6.0 ng/mL: (1.14 ng/mL for 2,4-D Acid)	Add 0.600 mL of 100-ng/mL (19-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 9.400 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.
2.5 ng/mL: (0.475 ng/mL for 2,4-D Acid)	Add 0.250 mL of 100-ng/mL (19-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 9.750 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.
1.0 ng/mL: (0.19 ng/mL for 2,4-D Acid)	Add 1.00 mL of 10-ng/mL (1.9-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 9.00 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.
0.40 ng/mL: (0.076 ng/mL for 2,4-D Acid)	Add 0.400 mL of 10-ng/mL (1.9-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 9.600 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.
0.12 ng/mL: (0.0228 ng/mL for 2,4-D Acid)	Add 1.20 mL of 1.0-ng/mL (0.19-ng/mL for 2,4-D Acid) standard solution to a 4-dram amber glass bottle containing 8.80 mL of 20:80 ACN:0.1% formic acid (*aq*). Mix well.

2.7 Sample Origin, Numbering, Preparation and Storage

Untreated control soil and sediment samples were supplied by the Sponsor Monitor. Chain of Custody documentation is included in the raw data.

Sample preparation was performed by EAG Laboratories. Soil and sediment samples were ground using a Hammermill. Dry ice was passed through to cool it before sample processing. Each frozen sample was then ground in the presence of enough dry ice to keep the sample frozen.

After grinding, the samples were placed in pre-labeled containers and the dry ice was allowed to sublime in a freezer over several days. The sample grinding equipment was cleaned after each sample was processed.

The control soil and sediment samples were processed and stored frozen prior to analysis.

2.8 Sample Analysis

Each 10.0-g sample was weighed into a 25 x 150 mm glass culture tube. Recovery samples were made by adding appropriate aliquots of appropriate standard solution to obtain concentrations of 0.01 (0.0019), 0.10 (0.019) or 1.0 (0.19) ppm for each analyte as detailed in the table below. (The percent recovery found was calculated for each analyte.)

Matrix	Sample Type	Fortifying Compound	Fortification Level[a] (ppm)	Fortification Solution		No. of Samples
				Concentration[a] (µg/mL)	Amount Added (mL)	
Soil	Reagent Blank	N/A	0.0	N/A	N/A	1
	Control	none	0.0	N/A	N/A	2
	Fortified control	2,4-D Acid; 2,4-DCP; 2,4-DCA	LOD: 0.003 (0.00057)	0.30 (0.057)	0.100	1
		2,4-D 2-EHE	LOD: 0.003	0.30	0.100	1
		2,4-D DMAS	LOD: 0.003	0.30	0.100	1
		2,4-D Acid; 2,4-DCP; 2,4-DCA	LOQ: 0.01 (0.0019)	1.0 (0.19)	0.100	5
		2,4-D 2-EHE	LOQ: 0.01	1.0	0.100	5
		2,4-D DMAS	LOQ: 0.01	1.0	0.100	5
		2,4-D Acid; 2,4-DCP; 2,4-DCA	10×LOQ: 0.10 (0.019)	10 (1.9)	0.100	5
		2,4-D 2-EHE	10×LOQ: 0.10	10	0.100	5
		2,4-D DMAS	10×LOQ: 0.10	10	0.100	5
		2,4-D Acid; 2,4-DCP; 2,4-DCA	100×LOQ: 1.0 (0.19)	100 (19)	0.100	5
		2,4-D 2-EHE	100×LOQ: 1.0	100	0.100	5
		2,4-D DMAS	100×LOQ: 1.0	100	0.100	5

Matrix	Sample Type	Fortifying Compound	Fortification Level[a] (ppm)	Fortification Solution		No. of Samples
				Concentration[a] (µg/mL)	Amount Added (mL)	
Sediment	Reagent Blank	N/A	0.0	N/A	N/A	1
	Control	none	0.0	N/A	N/A	2
	Fortified control	2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	LOD: 0.003 (0.00057)	0.30 (0.057)	0.100	1
		2,4-D 2-EHE	LOD: 0.003	0.30	0.100	1
		2,4-D DMAS	LOD: 0.003	0.30	0.100	1
		2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	LOQ: 0.01 (0.0019)	1.0 (0.19)	0.100	5
		2,4-D 2-EHE	LOQ: 0.01	1.0	0.100	5
		2,4-D DMAS	LOQ: 0.01	1.0	0.100	5
		2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	10×LOQ: 0.10 (0.019)	10 (1.9)	0.100	5
		2,4-D 2-EHE	10×LOQ: 0.10	10	0.100	5
		2,4-D DMAS	10×LOQ: 0.10	10	0.100	5
		2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; 4-CPA	100×LOQ: 1.0 (0.19)	100 (19)	0.100	5
		2,4-D 2-EHE	100×LOQ: 1.0	100	0.100	5
		2,4-D DMAS	100×LOQ: 1.0	100	0.100	5

[a] Values in parentheses are for 2,4-D Acid only.

To each sample, 20 mL of 5% acetic acid in methanol were added before vortexing for ~30 seconds, sonicating for ~20 minutes, and centrifuging for ~10 minutes at ~2000 rpm. The supernatant from this extraction was decanted into a 100-mL graduated mixing cylinder, and the sample was re-extracted with 20 mL of 50:50, 5% acetic acid in methanol:5% acetic acid in water, by vortexing for ~30 seconds, sonicating for ~20 minutes, and centrifuging for ~10 minutes at ~2000 rpm. The supernatant was decanted into the same 100-mL graduated mixing cylinder, and the sample was re-extracted with 20 mL of 5% acetic acid in water by vortexing for ~30 seconds, sonicating for ~20 minutes, and centrifuging for ~10 minutes at ~2000 rpm. The supernatant was decanted into the same 100-mL graduated mixing cylinder, and brought to a final volume of 100 mL with water.

2.8.1 Fraction A (2,4-DCA)

A 20-mL aliquot of extract was transferred to a 25 x 150 mm glass culture tube and 5 mL of isooctane was added to the sample, which was capped and shaken by hand for ~30 seconds, then shaken in a horizontal position at high speed on a platform shaker for ~5 minutes and centrifuged at ~1000 rpm for ~2 minutes. The isooctane (top) layer was transferred to a 15-mL graduated polypropylene centrifuge tube, 5 mL of isooctane was added to the sample, which was capped and shaken by hand for ~30 seconds, then shaken in a horizontal position at high speed on a platform shaker for ~5 minutes and centrifuged at ~1000 rpm for ~2 minutes, before

combining the isooctane layer in the polypropylene centrifuge tube. The volume of the isooctane was reduced to ≤1 mL on an N-evap set to approximately 40 °C (not allowing to go to dryness) and returned to 1-mL volume with isooctane, then diluted to 2 mL with 0.2% peanut oil in isooctane. The sample was further diluted with 0.1% peanut oil in isooctane (if necessary) to bring the sample concentration within the standard curve range before vialing in a 2-mL clear glass injection vial containing a 500-µL glass insert and submitting for GC-MSD analysis.

2.8.2 Fraction B (2,4-D 2-EHE and 2,4-DMAS)

A 2-mL aliquot of extract was transferred to a 25 x 150 mm glass culture tube and 10 mL of 1N NaOH was added to the sample, which was capped and vortexed to mix, then incubated for ~30 minutes in a water bath set at ~40 °C. The sample was cooled to room temperature and 2 mL of 1:1 HCl:water was added before vortexing to mix. If needed to achieve pH <2, additional 1:1 HCl:water was added.

HLB SPE Cleanup

For each sample, an HLB SPE column was placed onto a vacuum manifold and, using gravity, the SPE column was conditioned with one column volume (~3 mL) of methanol followed by one column volume (~3 mL) of water (not allowing the column to go dryness). The sample was loaded onto the SPE column using a transfer pipette, passing the sample through the column by gravity at a flow rate of 0.5-1 mL/min (discarding the eluent). The sample tube was washed with 2 mL of water, and using the same transfer pipette, the water was passed through the SPE column as before (discarding the eluent). The sample tube was washed with 2 mL of acetonitrile, and using the same transfer pipette, the acetonitrile was passed through the SPE column as before, collecting the eluent in a 15-mL graduated polypropylene centrifuge tube. Strong vacuum was applied to recover any remaining eluent, which was diluted to 10 mL with 0.1% formic acid (aq). The sample was further diluted with 20:80, ACN:0.1% formic acid (if necessary) to bring the concentration within the standard curve range, then vialed in a 2-mL clear glass injection vial containing a 500-µL glass insert and submitted for LC-MS/MS analysis.

2.8.3 Fraction C (2,4-D Acid; 2,4-DCP; 4-CP; and 4-CPA)

A 2-mL aliquot of extract was transferred to a 16 x 125 mm glass culture tube and 12 mL of 0.1N HCl (aq) was added to the sample, which was capped and vortexed to mix.

HLB SPE Cleanup

For each sample, an HLB SPE column was placed onto a vacuum manifold and, using gravity, the SPE column was conditioned with one column volume (~3 mL) of methanol followed by one column volume (~3 mL) of water (not allowing the column to go dryness). The sample was loaded onto the SPE column using a transfer pipette, passing the sample through the column by gravity at a flow rate of 0.5-1 mL/min (discarding the eluent). The sample tube was washed with 2 mL of water, and using the same transfer pipette, the water was passed through the SPE column as before (discarding the eluent). The sample tube was washed with 2 mL of acetonitrile, and using the same transfer pipette, the acetonitrile was passed through the SPE column as before, collecting the eluent in a 15-mL graduated polypropylene centrifuge tube. Strong vacuum was applied to recover any remaining eluent. The volume of the sample was reduced to ≤1 mL on an N-evap set to approximately 40 °C (not allowing to go to dryness), then

returned to a 1-mL volume with acetonitrile and diluted to 5 mL with 0.1% formic acid (aq). The sample was further diluted with 20:80, ACN:0.1% formic acid (if necessary) to bring the concentration within the standard curve range, then vialed in a 2-mL clear glass injection vial containing a 500-µL glass insert and submitted for LC-MS/MS analysis.

2.9 Calculations

Calculations for instrumental analysis were conducted using AB Sciex Analyst, version 1.6.2 (validated software application) for LC-MS/MS analysis or Excel 2010 for GC-MSD analysis to create a standard curve based on linear regression. The regression functions were used to calculate a best-fit line (from a set of standard concentrations in ng/mL or µg/mL versus peak area response) and to determine concentrations of the analytes found during sample analysis from the calculated best-fit line. For each analytical batch, calibration standards were injected over the linear range of the instrument (typically 0.00300 to 0.100 µg/mL for GC-MSD analysis and 0.12 to 6.0 ng/mL [0.0228 to 1.14 ng/mL for 2,4-D acid] for LC-MS/MS analysis). All standards injected and their corresponding peak responses were entered into the program to create the standard curve. Weighting (1/x) was used.

The equation used for the least squares fit is:

$$Y = \text{slope} \times X + \text{intercept}$$

Y = detector response (peak area) for each analyte

X = analyte concentration in the sample in ng/mL or µg/mL

$$X = \frac{Y - \text{intercept}}{\text{slope}} = \text{ng/mL or } \mu\text{g/mL}$$

The standard (calibration) curve generated for each analytical set was used for the quantitation of 2,4-D Acid; 2,4-D 2-EHE and 2,4-D DMAS, detected as 2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; or 4-CPA in the samples from the set. The correlation coefficient (r) for each calibration curve was greater than 0.990 (r^2 equal to or greater than 0.98). The sample results in ng/mL from Analyst and peak areas (µg/mL results were calculated in Excel) from ChemStation were subsequently transferred to Microsoft Excel® for sample concentrations calculated with full precision. There may be slight differences in the calculated results due to rounding.

For the determination of 2,4-DCA in soil and sediment (in terms of ppm), the following equation was used:

GC-MSD:

$$sample\ ppm = \frac{\mu g/mL\ Found \times Final\ Volume\ (mL) \times Extract\ Volume\ (mL) \times DF}{Sample\ Wt\ (g) \times Aliquot\ Volume\ (mL)}$$

For the determination of 2,4-D acid; 2,4-D 2-EHE and 2,4-D DMAS, detected as 2,4-D Acid; and 2,4-DCP in soil and sediment; and 4-CP and 4-CPA in sediment (in terms of ppm), the following equation was used:

LC-MS/MS:

$$sample\ ppm = \frac{ng/mL\ Found \times Final\ Volume\ (mL) \times Extract\ Volume\ (mL) \times DF}{Sample\ Wt\ (g) \times Aliquot\ Volume\ (mL) \times Conversion\ Factor}$$

Note: For 2,4-D 2-EHE, multiply the sample ppm by the molecular mass conversion factor [333.25 g/mol (2,4-D EHE) / 221.04 g/mol (2,4-D) = 1.50765]

where:

µg/mL Found	=	analyte concentration found in the GC-MSD sample
ng/mL Found	=	analyte concentration found in the LC-MS/MS sample
Final Volume (mL)	=	total volume of the LC-MS/MS or GC-MSD sample (2 mL for Fraction A; 10 mL for Fraction B; 5 mL for Fraction C)
Extract Volume (mL)	=	volume of total extract (100 mL)
Sample Wt (g)	=	amount of sample weighed for analysis (10 g)
Aliquot Volume (mL)	=	volume of extract taken for cleanup and analysis (20 mL for Fraction A; 2 mL for Fraction B; 2 mL for Fraction C)
DF	=	Dilution Factor
Conversion Factor	=	1000 (ppb to ppm)

Procedural recovery data from fortified samples are calculated via the following equation:

$$\text{Percentage Recovery} = \frac{\text{ppm found - ppm found in control}}{\text{ppm added}} \times 100$$

GC-MSD Example: 2,4-DCA recovery of soil sample 83640-108A, Fortified Control @ 0.01 ppm, Set #005. See Appendix D.

The concentration determined from the standard curve is = 0.00836 µg/mL.

The residue of 2,4-DCA in the final solution is calculated as follows:

$$2{,}4-DCA\ (ppm) = \frac{0.00836\ \mu g/mL \times 2\ mL \times 100\ mL \times 1}{10.00\ g \times 20\ mL} = 0.00836\ ppm$$

$$Percent\ Recovery = \frac{0.00836\ ppm\ found - 0.00\ ppm}{0.01\ ppm} \times 100 = 84\%\ 2{,}4-DCA$$

LC-MS/MS Example: 2,4-D Acid recovery of soil sample 83640-108-1-C, Fortified Control @ 0.0019 ppm, Set #005-1. See Appendix D.

The concentration determined from the standard curve is = 0.06322 ng/mL.

The residue of 2,4-D Acid in the final solution is calculated as follows:

$$2{,}4-D\ Acid\ (ppm) = \frac{0.06322\ ng/mL \times 5\ mL \times 100\ mL \times 1}{10.00\ g \times 2\ mL \times 1000} = 0.00158\ ppm$$

$$Percent\ Recovery = \frac{0.00158\ ppm\ found - 0.00\ ppm}{0.0019\ ppm} \times 100 = 83\%\ 2{,}4-D\ Acid$$

2.10 Confirmation of Residue Identity

The methods are specific for the determination of 2,4-D acid; 2,4-D 2-EHE and 2,4-D DMAS, detected as 2,4-D Acid; 2,4-DCP; 2,4-DCA; 4-CP; and 4-CPA by virtue of the chromatographic separation and selective detection systems used. To demonstrate further confirmation, at least one additional ion transition was monitored.

2.11 Statistical Treatment of Data

Statistical evaluations including percent recoveries, mean percent recoveries, and standard deviations were made using Microsoft Excel®.

Table 1 Identity and Structures of 2,4-D Acid;2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 4-CP; and 4-CPA

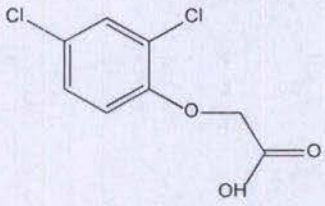
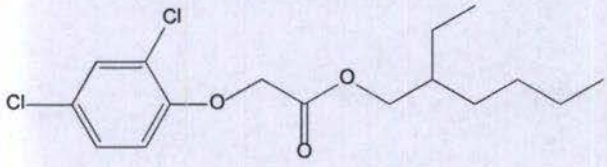
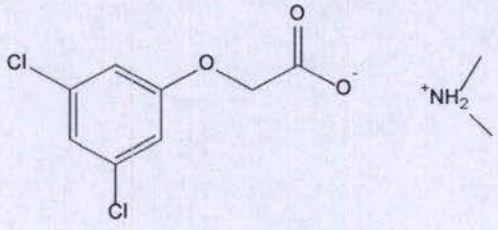
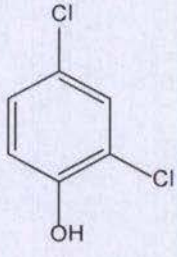
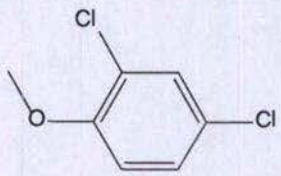
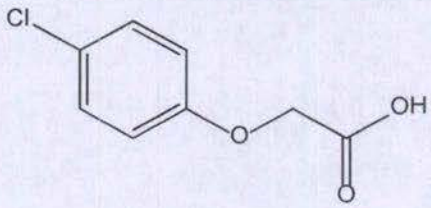
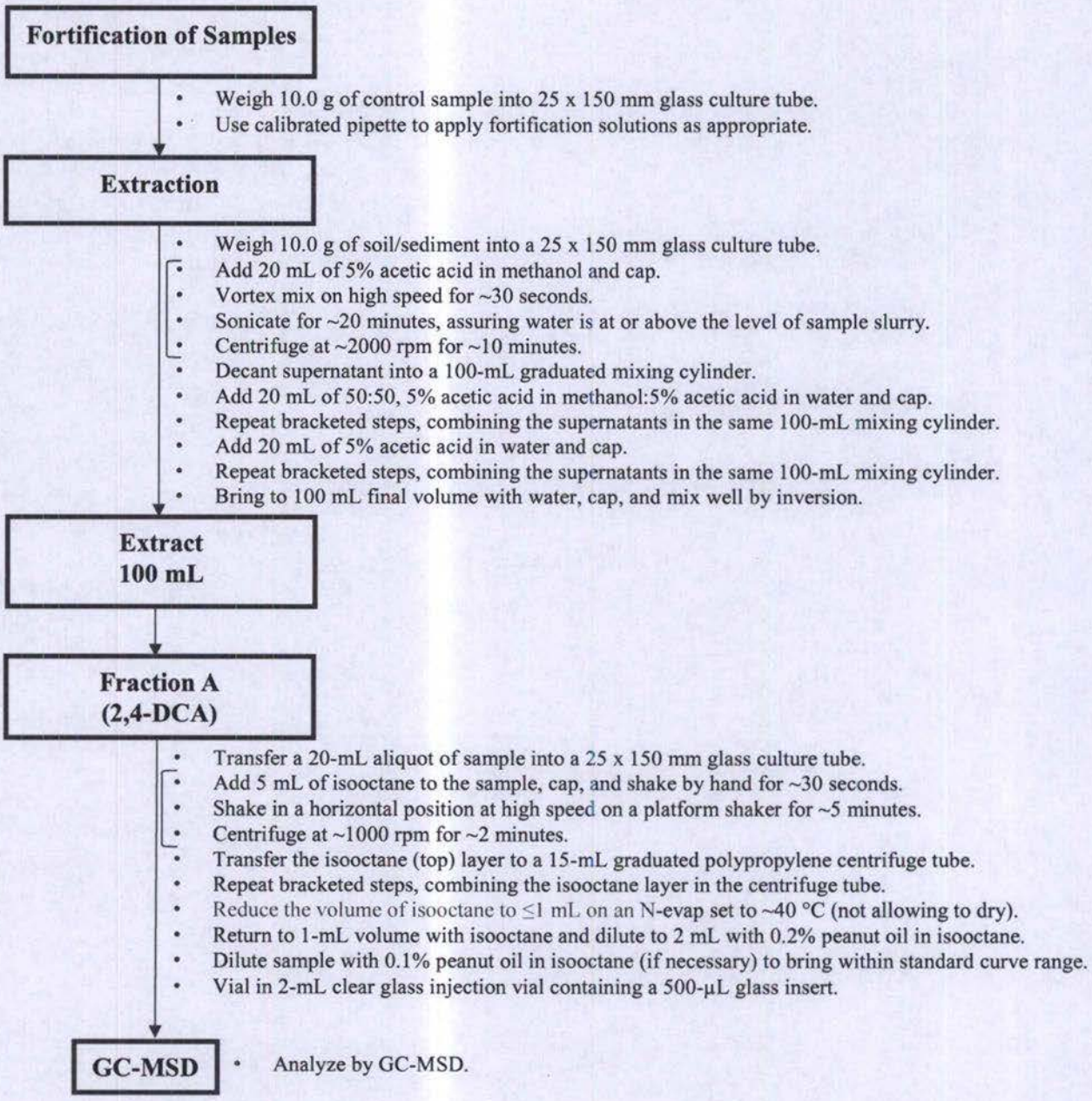
Common Name of Compound	Structure and Chemical Name
2,4-D Acid Molecular Formula: $C_8H_6Cl_2O_3$ Formula Weight: 221.04 g/mol CAS Number 94-75-7	 2,4-dichlorophenoxy acetic acid
2,4-D 2-EHE Molecular Formula: $C_{16}H_{22}Cl_2O_3$ Formula Weight: 333.25 g/mol CAS Number 1928-43-4	 2,4-dichlorophenoxyacetic acid 2-ethylhexyl ester
2,4-D DMAS Molecular Formula: $C_8H_5Cl_2O_3 \cdot C_2H_8N$ Formula Weight: 266.12 g/mol CAS Number 2008-39-1	 (2,4-dichlorophenoxy)acetic acid dimethylamine salt
2,4-DCP Molecular Formula: $C_6H_4Cl_2O$ Formula Weight: 163.0 g/mol CAS Number 120-83-2	 2,4-dichlorophenol

Table 1 Identity and Structures of 2,4-D Acid;2,4-D 2-EHE; 2,4-D DMAS; 2,4-DCP; 4-CP; and (continued)

Common Name of Compound	Structure and Chemical Name
2,4-DCA Molecular Formula: $C_7H_6Cl_2O$ Formula Weight: 177.03 g/mol CAS Number 553-82-2	 2,4-dichloroanisole
4-CP Molecular Formula: C_6H_5ClO Formula Weight: 128.56 g/mol CAS Number 106-48-9	 4-chlorophenol
4-CPA Molecular Formula: $C_8H_7ClO_3$ Formula Weight: 186.59 g/mol CAS Number 122-88-3	 4-chlorophenoxyacetic acid

APPENDIX A: SOIL ANALYSIS FLOWCHART



APPENDIX A : SOIL ANALYSIS FLOWCHART (CONTINUED)