III. MATERIAL AND METHODS

A. Reference Standards

The following analytical standards, along with pertinent documentation, were received in good condition from Valent U.S.A Corporation. The certificates of analysis of the analytical standards can be found in Appendix 1.

Compound	Date received	Lot No.	Physical Description	% Purity	Expiration Date
Flumioxazin	6/12/2007	AS1663i	white powder	99.2	4/10/2008
APF	6/12/2007	AS1981c	yellowish brown powder	98.1	5/21/2009
482-HA	6/12/2007	AS1997b	white powder	98.4	4/26/2009

Upon receipt, the reference substances were stored in a freezer maintained at approximately -20°C. Stock solutions of Flumioxazin and APF were prepared in acetonitrile. A stock solution of 482-HA was prepared in acetone. Fortification solutions and analytical calibration standards were prepared from the stock solution as described:

Compound	Solvent	Concentration of Fortification Solution
Flumioxazin	Acetone	1.01 µg/mL
	Acetone	29.4 µg/mL
APF	Acetone	0.999 µg/mL
	Acetone	29.1 µg/mL
482-HA	Acetone	1.01 µg/mL
	Acetone	30.6 µg/mL

Nominal Concentration of Analytical Calibration Standard		
Flumioxazin and APF in Methanol	APF and 482-HA in Acetone	482-HA in Acetone
0.5 ng/mL	0.5 ng/mL	0.5 ng/mL
2.5 ng/mL	2.5 ng/mL	2.5 ng/mL
5.0 ng/mL	5.0 ng/mL	5.0 ng/mL
10 ng/mL	10 ng/mL	10 ng/mL
20 ng/mL	20 ng/mL	20 ng/mL
40 ng/mL	40 ng/mL	40 ng/mL

Spiking solutions were stored in a freezer (~20° C). Calibration

standards of Flumioxazin and APF were stored in a refrigerator (~5° C). The other two sets of calibration standards (APF and 482-HA) were stored in a freezer (~20° C).

B. Sample Receipt and Tracking

The dates pond water and sediment samples from the sites were received are summarized below:

Date Received	Site	Events
August 14, 2007	Iowa	S1-S5
August 21, 2007	Iowa	S6 and S7
August 28, 2007	Iowa	S8
August 30, 2007	Florida	S1-S8
September 11, 2007	Iowa	S9
September 20, 2007	Florida	S9
October 10, 2007	Iowa	S10
November 6, 2007	Iowa	S11
November 13, 2007	Florida	S10 and S11

All samples were received frozen and delivered by Federal Express. Upon receipt, samples were immediately inventoried and placed into freezers. All samples were stored frozen (approximately -20°C) at GPL except when removed for subsampling to extract and analyze. A sample history table including the summary of the date sampled, date received, date extracted and number of days from sampling to extraction for each field-generated sample is presented in Table 39.

C. Method of Analysis

1.0 Scope of Method Validation

GPL analytical method GPL-MTH-064 entitled "Determination of Flumioxazin and its APF and 482-HA Metabolites in Basic Pond Water and Pond Sediment," was validated at GPL prior to the analysis of the study samples. The method validations, consisting of a low and high reagent spike, a control sample, three low level fortification and three high level fortification samples, were conducted to establish the performance of the methodology and optimize the method for the analysis of Flumioxazin, APF and 482-HA in pond water and pond sediment samples.

2.0 Method Summary for the Collection and Analysis of Pond Water

Two samples of pond water were sent to GPL for each individual sample. One sample was acidified upon collection with 10-mL of 100 mM pH 5 buffer to stabilize the pH for analysis of Flumioxazin. Samples that were acidified were indicated with an "A" as a suffix to the sample number, e.g., 185538A. The pond water, without any pH 5 buffer, was analyzed for residues of the APF and 482-HA metabolites.

For the analysis of pond water, an aliquot of the sample was transferred to a chromatography vial and analyzed by HPLC/MS/MS. Samples having higher residue levels were diluted to an appropriate final volume, using Optima water so that the response fell within the calibration range of the standards. Calibration standards and samples were injected at 10 µL.

3.0 Method Summary for Collection and Analysis of Pond Sediment

Residues of Flumioxazin and its APF metabolite were extracted from a 20 gram pond sediment sample with 80 mL of Acetone/ 2.5% Formic acid in Water (90:10 v/v). The sample was shaken for 15 minutes on a platform shaker and centrifuged for 4 minutes at 3000 rpm. The supernate was vacuum filtered through a Whatman GF/A filter paper into a 250 mL volumetric cylinder. The pond sediment was extracted a second time with 80 mL of Acetonitrile/ 2.5% Formic Acid in Water (80:20 v/v) and the extracts combined. The volume was adjusted to 200 mL using Optima grade water and mixed thoroughly. An aliquot of the extract was diluted 1:1 with Optima grade water and samples were analyzed using HPLC/MS/MS for residues of Flumioxazin and its APF metabolite. Samples having higher residue levels were diluted to an appropriate final volume, using Optima grade water so that the response fell within the calibration range of the standards. All calibration standards were injected at 10 µL and all sample extracts were injected at 20 µL.

Residues of the 482-HA metabolite were extracted from a 20 gram pond sediment sample with 80 mL of Acetone/ 2.5% Ammonium Hydroxide in Water (90:10 v/v). The sample was shaken for 15 minutes on a platform shaker and centrifuged

for 4 minutes at 3000 rpm. The supernate was vacuum filtered through a Whatman GF/A filter paper into a 250 mL volumetric cylinder. The pond sediment was extracted a second time with 80 mL of Acetonitrile/ 2.5% Ammonium Hydroxide in Water (80:20 v/v) and the extracts combined. The volume was adjusted to 200 mL using Optima grade water and mixed thoroughly. An aliquot of the extract was diluted 1:1 with Optima grade water and samples were analyzed using HPLC/MS/MS for residues of the 482-HA metabolite. Samples having higher residue levels were diluted to an appropriate final volume, using Optima grade water so that the response fell within the calibration range of the standards. All calibration standards were injected at 10 µL and all sample extracts were injected at 20 µL.

4.0 Instrument Parameters – HPLC/MS/MS

For most of the early samples, two HPLC/MS/MS instruments were used simultaneously to minimize delay in sample analysis. For analysis of water samples, one instrument was used for the analysis of Flumioxazin and APF and the second instrument for the analysis of the 482-HA metabolite. For analysis of sediment samples, one instrument was used for the analysis of Flumioxazin and the second instrument for the analysis of APF and the 482-HA metabolite.

Instruments:	Sciex API 4000 HPLC/MS/MS with two Shimadzu LC-10AD VP HPLC Pumps, Shimadzu SCL-10A VP Controller and Perkin Elmer Series 200 Autosampler
	Sciex API 4000 HPLC/MS/MS with two Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller and SIL-20AC Autosampler
HPLC Column:	Phenomenex Luna 3µ C18(2) 100Å (50 mm x 3.0 mm), Catalog #00B-4251-Y0
Guard Column:	Security Guard Column
Data System:	Analyst Chromatography Data System version 1.4

Mobile Phase (Gradient Program):

Time	A%	B%
0.0	20	80
4.0	90	10
4.1	20	80
8.0	20	80
where A = 0.2% Formic Acid in Acetonitrile and B = 0.2% Formic Acid in Water		

Flow Rate: 0.5 mL/minute

Injection Volume: 10 or 20 µl

Retention Time: Flumioxazin ~4.45 minutes
APF ~2.60 minutes
482-HA ~3.21 minutes

Mass Spectrometer Parameters: (operated in LC/MS/MS mode)

Interface: Turbo-Ion Spray (ESI)
Polarity: Positive
Scan Type: MRM Monitoring with Low and Unit resolution

Ions Monitored:
(Flumioxazin) m/z 355.2 (Q1)
m/z 327.2 (Q3)
(APF) m/z 221.1 (Q1)
m/z 163.1 (Q3)
(482-HA) m/z 373.2 (Q1)
m/z 221.0 (Q3)

LC/MS/MS conditions varied slightly from run to run when instrument and column maintenance were performed. The instrument parameters were optimized for analyte sensitivity and resolution prior to each chromatographic run, and the exact parameters were documented with each data set.

5.0 Analytical Study Samples

Study sample analysis commenced on August 14, 2007, and was completed November 20, 2007. A total of 390 pond water samples and 111 pond sediment samples were analyzed. Samples were analyzed in sets that consisted of at least one field UTC sample, two laboratory fortification samples, and four to eleven treated samples. Samples needing further dilutions were reanalyzed with the control and one laboratory

fortification sample unless the entire set was diluted and reanalyzed. Prior to extraction, a unique laboratory sample code designation was assigned by GPL to each sample extracted. The laboratory code consisted of the last three digits of the GPL study number, the sample set, and a sample number (e.g. 274SET01-1).

Laboratory fortifications were prepared by using Hamilton glass syringes, Wiretrol® micropipettes or glass pipettes and directly fortifying the samples. The pond water samples were fortified at approximately 1 ppb and 100 ppb for each compound. The fortification levels for pond sediment samples were approximately 10 ppb and 1000 ppb for each compound.

6.0 Storage Stability

The stability of Flumioxazin and its APF and 482-HA metabolite residues in pond water and pond sediment was investigated by spiking untreated samples of pond water and pond sediment from the Iowa test site with the individual compounds, and periodically analyzing the stored samples. The table below summarizes the spiking programs:

Compound	Pond Water Spike Level	Pond Sediment Spike Level
Flumioxazin	10.1 ppb	103 ppb
APF	9.99 ppb	102 ppb
482-HA	10.1 ppb	107 ppb

Thirty-six 10 mL aliquots of control pond water were transferred into disposable test tubes (Fisher Cat. No. 14-959-35A). Twelve of the test tubes were acidified with 1 mL 100 mM pH 5 buffer solution and fortified with 100 µL of a 1.01 µg/mL solution of Flumioxazin. Twelve of the test tubes were fortified with 100 µL of a 0.999 µg/mL solution of APF. Twelve of the test tubes were fortified with 100 µL of a 1.01 µg/mL solution of 482-HA.

Thirty-six 20 ± 0.05 g samples of control pond sediment were weighted in 250 mL Nalagene® bottles (Fisher Cat. No. 2105-0008) and fortified accordingly. Twelve of the samples were fortified with 0.07 mL of a 29.4 µg/mL solution of Flumioxazin. Twelve of the samples were fortified with 0.07 mL of a 29.1 µg/mL solution of APF. Twelve of the samples were fortified with 0.07 mL of a 30.6 µg/mL solution of 482-HA.

The spiked samples were stored in a freezer (approximately -20° C) along with the study samples. Samples were analyzed at 0, 7, 14, 21 and 48 days of storage. For each storage stability interval after the initial analysis, two freshly fortified samples were also prepared for analysis with the duplicate stored samples.

7.0 Quantitation Procedures

Analyst Chromatography Data System (Analyst 1.4) was used to acquire, integrate and calculate the concentration of Flumioxazin and its metabolites as ng/mL using the linear regression function. The HPLC/MS/MS responses (peak areas) were determined for a series of calibration standards. The concentration of the standards and their corresponding responses were complied. The concentration was designated as the independent variable and plotted on the x-axis. Peak response was designated as the dependent variable and plotted on the y-axis. From this, Analyst calculated a standard calibration curve. The slope, y-intercept and correlation coefficient of the standard curve run with each analytical set were calculated. The correlation coefficients were all greater than 0.998. Calibration standards were injected every two to five sample injections, as well as at the beginning and ending of the injection sequence. Six different standard concentrations were injected within each analytical set. The concentrations (ng/mL) of Flumioxazin and its metabolites detected in sample extracts were interpolated from the standard calibration curve. The concentration as ppb (ng/g) found in the samples, was then calculated by Microsoft Excel using the following equation:

For Pond Water:

$$\textit{Flumioxazin/ metabolites in ppb} = \frac{\textit{(ng/mL from curve) x (Final Vol. in mL)}}{\textit{(sample volume in mL)}}$$

For Pond Sediment:

$$\textit{Flumioxazin/ metabolites in ppb} = \frac{\textit{(ng/mL from curve) x (Final Vol. in mL)}}{\textit{(sample amount in grams) x (Injection Volume Factor)}}$$

Both samples and standards were analyzed under the same

HPLC/MS/MS conditions and within the same analytical sequence.

Recovery of the analyte from fortified samples was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{(Measured Concentration, ppb)} \times 100}{\text{(Theoretical Concentration, (ppb)}}$$

An example calculation for a Flumioxazin laboratory fortification on pond sediment in set 274SET43, sample 274SET43-2 Laboratory Spike at 10.1 ppb, is as follows:

standard curve equation: y = 1.04e+003 (x) + 9.95
where x = Flumioxazin concentration in ng/mL and
y = peak response = 1069.5
Flumioxazin concentration from the curve = 1.01 ng/mL

$$\text{ppb} = \frac{\text{(1.01ng/mL Flumioxazin) (400 mL)}}{\text{(19.99 grams) (2)}} = 10.1 \text{ ppb}$$

$$\% \text{ Recovery} = \frac{10.1 \text{ ppb}}{10.1 \text{ ppb}} \times 100 = 100\%$$

The field samples were not corrected for any residues detected in corresponding field control samples. However, all control samples were <LOQ. Field samples were not corrected for average laboratory fortification recoveries.

8.0 Statistics

Statistical evaluations performed on the residue data include mean and standard deviation calculations of fortified samples analyzed. Linear regression analysis was applied to calibration curves to determine the slope and intercept values.