Final Report

Study Title	PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)
Study Guideline	OPPTS Biological Effects Test Guidelines 850.1400 Fish Early-Life Stage Toxicity Test
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Study Number	2901/001
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Report issued	August 2008
Page Number	1 of 64

All procedures to be carried out on live animals as part of this study will be subject to the provisions of United Kingdom National Law, in particular the Animals (Scientific Procedures) Act 1986, to ensure that minimum suffering and distress is caused to animals on study.

STUDY DIRECTOR AUTHENTICATION AND GLP COMPLIANCE STATEMENT

PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)

The study was performed in accordance with the agreed protocol and with Covance Laboratories Limited Standard Operating Procedures, unless otherwise stated, and the study objectives were achieved. The data generated are scientifically acceptable and valid. This report provides a true and accurate record of the results obtained.

The study was conducted in compliance with the:

United Kingdom Statutory Instrument 1999 No. 3106, The Good Laboratory Practice Regulations 1999 as amended by the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004.

OECD Principles on Good Laboratory Practice (revised in 1997, issued January 1998) ENV/MC/CHEM(98)17.

Studies performed in compliance with the GLP regulation and standards referenced above will be fully acceptable to the US EPA and Japanese regulatory authorities.

7 August 2008
Date

QUALITY ASSURANCE STATEMENT

PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)

This study has been reviewed by the Quality Assurance Unit of Covance Laboratories Ltd. and the report accurately reflects the raw data. The following inspections were conducted and findings reported to the study director (SD) and associated management.

Critical procedures, which are performed routinely in an operational area, may be audited as part of a "process" inspection programme. This can be in addition to phases scheduled on an individual study basis. Selected process inspections conducted and considered applicable to this study are included below.

In addition to the inspection programmes detailed below, a facility inspection programme is also operated. Details of this programme, which covers all areas of the facility annually (at a minimum), are set out in standard operating procedures.

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
22 Nov 2007	22 Nov 2007	Protocol Review	22 Nov 2007
18 Dec 2007	18 Dec 2007	Protocol Amendment Review	18 Dec 2007
11 Jan 2008	11 Jan 2008	Protocol Amendment Review	11 Jan 2008
17 Jun 2008	17 Jun 2008	Protocol Amendment Review	17 Jun 2008
17 Jun 2008	17 Jun 2008	Protocol Amendment Review	17 Jun 2008
19 Jul 2008	20 Jul 2008	Draft Report and Data Review	21 Jul 2008
07Aug 2008	07 Aug 2008	Final Report Review	07 Aug 2008

Process

Inspection Dates			Date Reported to SD and SD Management
From	To	Phase	
02 Apr 2008	02 Apr 2008	Animal Arrival	02 Apr 2008
08 Apr 2008	08 Apr 2008	Dose Preparation	08 Apr 2008
15 Apr 2008	15 Apr 2008	Dose Administration	15 Apr 2008
25 Apr 2008	25 Apr 2008	Unannounced	25 Apr 2008
12 May 2008	12 May 2008	Test Article Receipt	12 May 2008

7.8.08
Date

REVIEWING AND RESPONSIBLE SCIENTISTS' STATEMENTS

PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)

I, the undersigned, hereby declare that I have reviewed this report in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

05.08.08
Date

I, the undersigned, hereby declare that I have reviewed the analytical procedure in conjunction with the Study Director and that the interpretation and presentation of the data in the report are consistent with the results obtained.

5 August 2008
Date

RESPONSIBLE PERSONNEL

PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)

The following personnel were responsible for key elements of the study:

STUDY MANAGEMENT	**NAME**
Study Director (from 20 May 2008 to present)	
Study Director (from 1 November 2008 to 20 May 2008)	
Scientific Reviewer	
Study Co-ordinator	

OPERATIONAL SUPERVISION

Biology
Chemistry
Named Veterinary Surgeon
Named Animal Care Welfare Officer

QUALITY ASSURANCE

Director, Quality Systems

ARCHIVE STATEMENT

PFH Ammonium Salt: Fish, Early Life Stage Toxicity Test to *Oncorhynchus mykiss* (Rainbow Trout)

All primary data (including electronic data), or authenticated copies thereof, specimens and the final report will be retained using appropriate storage media in the Covance Laboratories Limited archives for one year after issue of the final report. At this time the Sponsor will be contacted to determine whether the data should be returned, retained or destroyed on their behalf. Sponsors will be notified of the financial implications of each of these options at that time.

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SUMMARY

Introduction
The objective of the study was to establish the effect of PFH Ammonium Salt on the growth and development of embryos and larvae of the freshwater fish species *Oncorhynchus mykiss* (Rainbow trout) in a Fish Early-Life Stage (ELS) Toxicity Test. The study was performed in accordance with the OPPTS Biological Effect Test Guideline No. 850.1400, Fish Early-Life Stage Toxicity Test (1996).

Toxicity was determined in terms of the active moiety as the salt does not exist in aquatic environments. The test was conducted at nominal concentrations of 0.095, 0.304, 0.972, 3.11 and 9.96 mg/L active moiety, equivalent to 0.2, 0.641, 2.05, 6.56 and 21.0 mg/L in terms of PFH ammonium salt with a purity of 50% and conversion factor of 1.054. These concentrations were based on the results of an acute toxicity test. Both solvent and dilution water controls were included in the test design, with duplicate test vessels at all exposure concentrations including the controls.

The test was conducted with a flow through test design.

Concentrated stock solutions and test media were analysed during the ELS test. Measured concentrations of PFH active moiety in the concentrated stock solutions ranged between 98 and 100% of the nominal concentrations, test media concentrations ranged between 94 and 108% of nominal corresponding to geometric mean measured concentrations of 0.103, 0.310, 0.916, 3.14 and 10.1 mg/L.

Hatching success in the control group was 74%, satisfying the validation criterion for hatching success (>66%). The NOEC and LOEC for hatching success were determined as 9.96 and >9.96 mg/L, respectively.

Larval survival until Day 28 post-hatch in the control group was 93% thereby exceeding and satisfying the validity criteria for post-hatch survival (70%). Post-hatch survival across all remaining treatments ranged between 96 and 100%. In terms of nominal concentrations, the NOEC and LOEC for post-hatch larval survival until Day 28 were both considered to be equal to or greater than 9.96 mg/L.

For fish total lengths, the NOEC and LOEC determined on Day 28 post-hatch were 9.96 and >9.96 mg/L PFH active moiety, respectively.

For fish dry weights, the NOEC and LOEC determined on Day 28 post-hatch were 9.96 and >9.96 mg/L PFH active moiety, respectively.

All validity criteria were satisfied during the test, therefore the test was considered to be valid.

INTRODUCTION

The objective of the study was to establish the effect of PFH Ammonium Salt on growth and development of embryos and larvae of the freshwater fish species, *Oncorhynchus mykiss* (Rainbow trout), in a Fish Early-Life Stage Toxicity Test (ELS).

The study was conducted in accordance with the requirements of OPPTS Biological Effects Test Guideline 850.1400, Fish Early-Life Stage Toxicity Test.

PFH ammonium salt is an ammonium salt of perfluorohexanoic acid, that dissociates (*ca* 100%) to the active moiety on contact with water. The conversion factor between the salt and active moiety is 1.054, therefore 105 mg/L PFH ammonium salt is equivalent to 100 mg of the active moiety. Toxicity in this ELS test was determined in terms of the active moiety as the salt does not exist in aquatic environments.

The study was initiated (protocol issued) on 1 November 2007. Study completion is defined as the date of signing of the final report by the Study Director.

The experimental work (in-life and chemical analysis) was conducted between 18 February 2008 and 19 May 2008. The study was conducted in the Department of Environmental Sciences, Covance Laboratories Ltd., Harrogate, North Yorkshire, United Kingdom.

MATERIALS AND METHODS

Protocol Adherence

The study was conducted in accordance with the agreed definitive protocol and three protocol amendments. Minor deviations that do not affect the integrity of the study are presented in Appendix 4.

Test Substance

Test substance name	PFH Ammonium Salt
Sponsor batch number	Lot 7001
Description	Clear liquid
Purity	50% solution
Storage	Refrigerated (1-10°C) in the dark
Expiry date	3 June 2009
Date received	4 November 2007

Test substance details were supplied by the Sponsor.

Test Organism

The Rainbow trout (*Oncorhynchus mykiss*) eggs and milt used in this study were supplied by a recognised external supplier. Supplier details are maintained in the study raw data.

At the start of the test, approximately 40 fertilised eggs were added randomly to each test vessel, divided equally between two incubation chambers per vessel. The fertilised eggs were less than 24 hours old on addition to the test system.

Egg addition was achieved by careful addition of the required number of eggs directly to the incubation chambers as soon as possible after receipt to avoid handling stress. The test started after the final batch of eggs had been added to the last incubation chamber.

Preparation of Test Media

The dilution water used for conducting the definitive test was dechlorinated mains water that had been passed through particulate and activated charcoal filters and treated with ozone (O_3) to remove residual algal cells and fungal spores for improved water quality. The typical constituents of dilution water are presented in Appendix 1.

The definitive test was conducted at nominal concentrations of 0.095, 0.304, 0.972, 3.11 and 9.96 mg/L PFH active moiety (equivalent to 0.2, 0.641, 2.05, 6.56 and 21.0 mg/L in terms of PFH ammonium salt with a purity of 50% and conversion factor of 1.054)**.**

Concentrated aqueous stocks were prepared by direct addition of *ca* 48, 154, 492, 1574 and 5040 mg and made to a final volume of 15 L with treated mains water at each treatment level.

All media were stirred for at least 24 hours to aid dissolution of the test substance and to ensure that the test was conducted using the active moiety. This assumption was based on the known kinetics of the test substance. Complete dissociation to the active moiety is considered to occur between 12 and 24 hours following addition to water. The concentrated aqueous stock vessels were covered with black plastic to comply with the storage conditions of the test substance.

Following stirring, each concentrated stock solution was pumped using peristaltic pumps into duplicate test vessels (1.0 mL/min) via a glass mixing vessel where it was mixed with diluent water to provide the final test concentrations at each treatment level, including a control (water only).

The flow rate to each test vessel was measured daily. Dilution water was set at a nominal flow rate of 15 mL/min to achieve a total volume replacement rate (VRR) of (7.7) per 24 hour period.

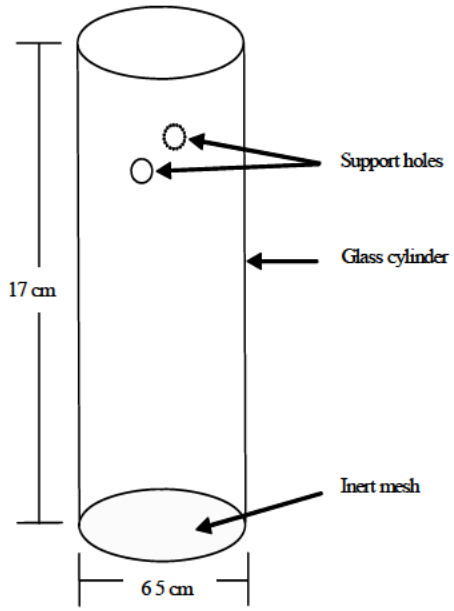
Appearance of Test Media
The appearance, colour and behaviour of the test substance in the test media was recorded.

Test Procedures
The test tanks were nominal 4 L constructed glass aquariums with an operating volume of approximately 3 L. Two replicate tanks were used at each test concentration, including the dilution water control treatment. The sides of all of the test vessels were covered with black plastic to reduce disturbance to embryos and fry and to comply with the storage conditions of the test substance.

Egg incubation chambers used in the test were 17 cm long glass cylinders (6.5 cm internal diameter) with a 0.5 – 1.0 mm non-reactive mesh attached to one end (bottom) using silicon adhesive (Diagram 1).

Opposing holes were drilled into the glass cylinder approximately 6.5 cm from the top of the cylinders (at the opposite end to the mesh) and a stainless steel rod was inserted to enable each chamber to be suspended across the width of each replicate test vessel. Each replicate test tank contained one incubation chamber.

Diagram 1 – Schematic representation of an egg chamber



To ensure adequate movement of water within the test vessels and maintain dissolved oxygen concentrations in the egg incubation chambers, two aeration tubes were added to each replicate vessel. The first was maintained in the test medium surrounding the incubation chamber and the second aerator was maintained within the incubation chamber, positioned with the tube end at approximately 1 cm from the mesh on the end of the cylinder. The agitation/turbulence caused by the aerators on the water column within the chambers ensured that a dynamic equilibrium of concentration was achieved between the environment within the chamber and surrounding test medium. The turbulence created slight movement of the embryos and maintained good levels of aeration within the chamber.

Egg Fertilization, Addition and Observations

Approximately 6000 *Oncorhynchus mykiss* eggs were supplied, these were washed with a 0.9 g/L sodium chloride solution. Milt was added to the eggs and gently mixed. The eggs were then washed with treated mains water and transferred to an egg holding tray with continuous renewal of water at a rate of 500 mL/minute.

A total of *ca* 40 eggs were transferred using a spoon into each incubation chamber. The eggs were chosen randomly from the holding vessel and added under reduced light conditions.

During the pre-hatch period, where possible, non-viable or necrotic eggs were removed, avoiding disturbance of adjacent viable eggs.

The post-hatch phase started once all of the viable eggs were considered to have hatched in the control groups. Egg hatch at each treatment was then assessed relative to control group performance. Once all viable eggs had hatched across all treatments and fish were actively feeding and mobile, the chambers were removed.

At the start of the post-hatch phase (>90% hatching in the control, equal to 28 days after fertilisation) an estimate of hatching success was determined.

On Day 28 of the post-hatch phase, hatching success was further confirmed by definitive counts of all surviving fish. These counts, for each vessel, were then corrected for larvae lost during the post-hatch phase. Discrepancies between the corrected hatched larvae counts and the number of eggs added to each chamber were considered to be due to losses during the pre-hatch phase.

On Day 28, the total numbers of surviving larvae were counted and individual total fish lengths and dry weights of all remaining fish were determined.

The percentage post-hatch survival was determined by expressing the number of surviving larvae on Day 28 post-hatch as a percentage of the hatched larvae at Day 0 (post-hatch).

Test Medium Renewal Procedure
The test was conducted with continuous renewal of the test media (flow through).

Individual concentrated stock solutions were prepared for each treatment level. Following preparation each bulk test medium was pumped, using a peristaltic pump, at a rate of 1 mL/minute into a mixing vessel. Dilution water (treated mains water) was pumped at a rate of 15 mL/minute into each mixing vessel. The test medium and dilution water were mixed using a magnetic stirrer and then continually added to the appropriate test vessels.

Feeding
The developing larvae were fed using Nutrafry trout feed. Between days 10 and 13 a 40 g/L solution of ground Nutrafry 01 in treated mains water was added drop-wise to the test vessels. From day 14 onwards the fish were fed with Nutrafry 00 *ad libitum*, in excess, three times per day.

Surplus feed and faecal material were siphoned from the test vessel on (at least) daily intervals during the test.

Water Quality and Environmental Conditions
Water temperatures (°C) were measured in freshly prepared and old test media on each test media renewal occasion and were monitored continuously in two vessels (V1 (control) and V12 (nominal 21 mg/L)) using minimum / maximum digital thermometers. Temperature ranges in vessels V1 and V12 were 11.6 to 12.6°C and 11.7 to 12.9°C, respectively, over the duration of the test.

The test was conducted in a light-controlled facility, with a 16-hour photoperiod.

The pH and dissolved oxygen concentration, expressed as a percentage of the air saturation value (% ASV) and as mg/L O_2, were measured in freshly prepared and old test media at each renewal. In addition, the total hardness (mg/L $CaCO_3$) and residual chlorine were measured in fresh control media at the start of the test and at each renewal.

Statistical Analysis
The statistical analysis results are presented in Appendix 3.

Day 28 total lengths and dry weights were analysed using analysis of variance, fitting fixed effects for group and vessel within group. The test concentrations were compared with the water control using Dunnett's test. Linear contrasts were performed to compare the two vessels within each group. All tests were interpreted with two-sided risk.

The number of hatched larvae and hatched larvae surviving at Day 28 post-hatch were analysed using a Fisher's exact test for pairwise comparisons of test concentrations with the control. The tests were interpreted for decreasing incidence with increasing concentration.

The Lowest Observed Effect Concentration (LOEC) is defined as the lowest test concentration, which produces a statistically significant adverse effect ($P<0.05$) when compared with the control(s). All test concentrations above the LOEC were required to show an effect that is statistically different from the control.

The No Observed Effect Concentration (NOEC) is defined as the highest test concentration, immediately below the LOEC, which does not produce a statistically significant adverse effect ($P<0.05$) when compared with control(s).

Chemical Analysis of PFH Ammonium Salt in Test Media
The Analytical Procedure CLE (E) 2901-001-01V, used to confirm the concentration PFH Ammonium Salt (as the active moiety) in samples of test media during this test is presented in Appendix 2.

Concentrations of PFH Ammonium Salt in fish dilution water were determined LC-MS/MS.

Samples of fresh and expired media were taken at least weekly for analytical determination.

Duplicate samples (20 mL) of test media from each of the test and control vessels were taken for analysis on days 0, 1, 8, 14, 22 and 28 during the pre-hatch period, and on days 0, 7, 11, 14, 17, 21, 27 and 28 during the post-hatch period.

Samples of freshly prepared concentrated aqueous stock solutions were taken for analysis at -24 hours, Day 0, 4, 8, 12, 16, 20, 24 and 28 pre-hatch and on Day 3, 7, 11, 14, 15, 19, 23 and 27 during the post-hatch period.

Samples of expired concentrated aqueous stock solutions were taken for analysis on Day 4, 8, 12, 16, 20, 24 and 28 pre-hatch and on Day 3, 7, 11, 19, 23 and 27 during the post-hatch period.

Throughout the results, some numerical data may have been rounded for presentation purposes. Therefore, manual recalculation of the data may result in slightly different values to those shown.

Validity Criteria
The criteria for test validity were as follows:

- the dissolved oxygen concentration must be maintained above 60% of the air saturation value,
- the water temperature must not differ by more than 1.5°C between test chambers or successive days at any time during the test,
- hatching success in the control treatments must be > 66%,
- post-hatch survival of fish larvae in the control treatments must be > 70%.

RESULTS

Test Organism

Eggs were fertilised and added to test vessels within 30 minutes of arrival at the laboratory.

Chemical Analysis of PFH Ammonium Salt in Test Media

The results of the chemical analysis for PFH Ammonium Salt (as the active moiety) in samples of aqueous stock and test media taken during the test are presented in Table 1 and 2

Example chromatograms for fresh and expired aqueous stock and test media prepared are presented in Figure 4 to Figure 18.

The overall geometric mean measured concentrations of PFH active moiety in samples of test media were 0.103, 0.310, 0.916, 3.14 and 10.1 mg/L. These concentrations corresponded to between 94 and 108% of the nominal concentrations.

The limit of detection for PFH Ammonium Salt (as the active moiety) was 0.012 µg/mL.

Appearance of Stock and Test Media

On preparation the stock and test media appeared as colourless solutions.

Hatching Success

The results of hatching success and fish larval survival are presented in Table 3.

Hatching success in the control group was 74%. As this exceeded 66%, the validity criterion for hatching success was satisfied.

First egg hatch in treatment and control vessels occurred in the 24-hour period between the Day 25 and Day 26 pre-hatch observation timepoints. This indicated no difference in time to first hatch across all treatments when compared to the control group.

NOEC and LOEC were determined as nominal concentrations of 9.96 and >9.96 mg/L as PFH active moiety.

Survival

Post-hatch larval survival results are presented in Table 3.

Larval survival until Day 28 post-hatch in the control group was 93%. As this exceeded 70%, the validity criterion for larval survival was satisfied.

Post-hatch larval survival across all remaining treatments ranged between 96 and 100%.

In terms of measured concentrations, the NOEC and LOEC for post-hatch larval survival until Day 28 were both considered to be equal to or greater than 10.1 mg/L (highest mean measured concentration).

Fish Total Lengths and Dry Weights
Fish total lengths and dry weights are presented in Table 5 and Table 6.

The NOEC and LOEC for both total fish length and fish weight were determined on Day 28 post-hatch and were considered to be 9.96 and >9.96 mg/L respectively.

Abnormalities
There were no dose related abnormalities recorded during the test.

Water Quality and Environmental Conditions
Results of water quality determinations during the fish early-life stage test are shown in Table 7 to Table 11.

All water quality parameters were within the specified ranges stated in the Guidelines.

CONCLUSIONS

The toxicity of PFH Ammonium Salt to fish embryos and hatched fish larvae was determined.

Hatching Success
Hatching success in the control group was 74%, satisfying the validity criterion for hatching success (>66%). The NOEC and LOEC were determined as 9.96 and >9.96 mg/L, respectively.

Survival
Larval survival until Day 28 post-hatch in the control group exceeded 70% (93%), satisfying the validity criterion for post-hatch survival.

In terms of nominal concentrations, the NOEC and LOEC for post-hatch survival until Day 28 were 9.96 and >9.96 mg/L, respectively.

Fish Total Lengths and Dry Weights
For both fish total lengths and dry weights, the NOEC and LOEC determined on Day 28 post-hatch were 9.96 and >9.96 mg/L, respectively.

Validity Criteria
All validity criteria were satisfied during the test, therefore the test was considered to be valid.

REFERENCES

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TABLES

Table 1
PFH Ammonium Salt (expressed as mg/L of the active moiety) in concentrated stock media during the fish early-life stage test with *Oncorhyncus mykiss*

Pre-hatch

Nominal concentration (mg/L)	Nominal PFHA concentration (mg/L)	-1 day (New)	0 day (New)	8 day (Old)	8 day (New)	16 day (Old)	16 day (New)	28 day (Old)	28 day (New)	Pre-hatch mean measured concentration (mg/L)
3.2	1.52	1.65	1.77	1.72	1.75	1.99	1.79	1.64	1.16	1.67
10.3	4.89	7.11	5.27	4.83	4.76	11.5	5.02	4.48	4.29	5.59
32.8	15.6	17.1	17.4	16.0	16.5	16.6	16.8	15.1	15.6	16.4
105	49.8	53.4	55.9	47.0	50.3	44.7	51.1	47.5	48.6	49.7
336	159	178	189	153	155	183	168	171	172	171

Post-hatch

Nominal concentration (mg/L)	Nominal PFHA concentration (mg/L)	3 day (Old)	3 day (New)	7 day (Old)	7 day (New)	14 day (New)	23 day (Old)	23 day (New)	27 day (Old)	27day (New)	Post-hatch mean measured concentration (mg/L)	$Mean measured concentration (mg/L)	Mean as % of nominal
3.2	1.52	1.23	1.15	1.18	1.30	1.51	1.56	1.46	1.51	1.48	1.49	1.53	98
10.3	4.89	4.85	4.68	4.96	3.98	4.66	5.10	4.93	5.13	4.94	4.78	4.98	98
32.8	15.6	16.9	15.3	16.4	17.1	14.8	16.8	16.4	17.1	17.0	15.7	15.9	101
105	49.8	52.0	47.1	53.4	52.3	42.2	53.3	53.1	53.4	53.2	49.6	49.6	100
336	159	174	146	166	168	157	167	171	174	172	157	160	98

$ Overall geometric mean measured concentration for the complete duration of the test.

Table 2
PFH Ammonium Salt (expressed as mg/L of the active moiety) in test media during the fish early-life stage test with *Oncorhyncus mykiss*

Nominal TA concentration (mg/L)	Nominal PFHA concentration (mg/L)	Pre-hatch						Pre-hatch mean measured concentration (mg/L)
		0 day	1 day	8 day	14 day	22 day	28 day	
Control	0	- 0	- 0	- 0	0.0139 -	- 0	0 0.0301	-
0.20	0.0949	0.0886 0.105	0.106 0.129	0.106 0.0975	0.120 0.114	0.107 0.118	0.125 0.109	0.104
0.641	0.304	0.285 0.286	0.36 0.319	0.287 0.279	0.344 0.347	0.336 0.303	0.343 0.333	0.312
2.05	0.972	0.835 0.876	0.979 1.02	1.04 0.974	1.01 0.963	0.923 0.967	0.937 1.01	0.929
6.56	3.11	3.04 3.42	3.31 3.42	3.04 3.21	3.71 3.89	3.18 3.11	3.61 3.45	3.19
21.0	9.96	9.97 8.86	10.8 12.6	10.2 10.3	12.5 10.6	10.2 9.83	16.4 11.6	10.3

Nominal TA concentration (mg/L)	Nominal PFHA concentration (mg/L)	Post-hatch								Post-hatch mean measured concentration (mg/L)	$Mean measured concentration (mg/L)	Mean as % of nominal
		0 day	7 day	11 day	14 day	17 day	21 day	27 day	28 day			
Control	0	0 0	0 0	0 0	0 0	- -	0 0	0 0	0 0	-	-	-
0.20	0.0949	0.101 0.123	0.112 0.0967	0.101 0.0922	0.119 0.111	0.0760 0.0904	0.0993 0.0920	0.0973 0.0971	0.0995 0.101	0.100	0.103	108
0.641	0.304	0.345 0.460	0.308 0.288	0.300 0.284	0.331 0.314	0.327 0.319	0.240 0.249	0.296 0.286	0.302 0.307	0.306	0.310	102
2.05	0.972	0.928 0.991	0.848 0.892	0.678 0.798	0.840 0.856	0.860 0.902	0.722 1.15	0.963 0.94	1.03 0.992	0.892	0.916	94
6.56	3.11	3.00 2.69	2.96 2.95	2.84 2.64	2.68 2.85	3.11 3.08	2.93 2.90	3.53 3.45	3.62 3.62	3.04	3.14	101
21.0	9.96	11.8 10.6	8.54 9.49	8.20 9.05	8.51 8.55	9.13 9.70	9.23 9.39	10.1 10.0	11.1 10.9	9.59	10.1	101

- Not detected above limit of detection (0.012 µg/mL)

Table 3
Hatching success and fish larval survival during the fish early-life stage test

Nominal concentration (mg/L)	Vessel number	Number of eggs added at start of test		Number of hatched larvae		Percentage of hatched larvae		Number of larvae surviving at 28 days post-hatch		Percentage of larvae surviving at 28 days post-hatch	
		Individual vessels	Pooled vessels	Individual vessels	Pooled vessels	Individual vessels	Pooled vessels	Individual vessels	Pooled vessels	Individual vessels	Pooled vessels
Control	V1	40	80	28	59	70	74	27	55	96	93
	V2	40		31		78		28		90	
0.20	V3	40	80	31	64	78	81	29	61	94	96
	V4	40		33		83		32		97	
0.641	V5	40	80	28	54	70	68	28	54	100	100
	V6	40		26		65		26		100	
2.05	V7	40	80	23	50	58	63	22	48	96	96
	V8	40		27		68		26		96	
6.56	V9	40	80	27	52	68	66	27	52	100	100
	V10	40		25		63		25		100	
21.0	V11	40	80	22	52	55	65	22	51	100	99
	V12	40		30		75		29		97	

Table 4
Post-hatch observed larval fish mortality during the fish early-life stage test

Nominal concentration (mg/L)	Vessel number	Day Number														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	V1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
0.20	V3	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
	V4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.641	V5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.05	V7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6.56	V9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.0	V11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V12	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0

Table 4 (continued)
Post-Hatch Observed Larval Fish Mortality during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day Number														
		15	16	17	18	19	20	21	22	23	24	25	26	27	28	Total dead larvae
Control	V1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
	V2	0	0	0	0	0	2	0	0	0	0	0	0	0	0	3
0.20	V3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
	V4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
0.641	V5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2.05	V7	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
	V8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
6.56	V9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21.0	V11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	V12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1

Table 5
Total Length of Fish (cm) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)											
	Control		0.20		0.641		2.05		6.56		21.0	
	Vessel number											
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
1	3.6	3.5	3.5	3.4	3.3	3.3	3.5	3.5	3.5	3.6	3.6	3.3
2	3.6	3.6	3.5	3.2	3.7	3.5	3.4	3.1	3.3	3.5	3.6	3.4
3	3.8	3.1	3.6	2.7	3.6	3.2	3.6	3.4	3.5	3.4	3.5	3.3
4	3.0	3.5	3.7	3.5	3.6	3.1	3.5	3.5	3.3	3.4	3.6	3.4
5	3.6	3.6	3.5	3.5	3.0	3.5	3.5	3.1	3.6	3.4	3.5	3.5
6	3.2	3.3	3.4	3.2	3.0	3.3	3.6	3.3	3.5	3.2	3.4	3.4
7	3.5	3.6	3.6	3.3	3.7	3.7	3.5	3.3	3.4	2.9	3.6	3.3
8	3.4	3.5	3.5	3.6	3.5	2.7	3.5	3.3	3.5	3.6	3.6	3.4
9	3.4	3.6	3.2	3.7	3.3	3.2	3.4	3.1	3.4	3.6	3.6	3.6
10	3.4	3.6	3.6	3.6	3.5	3.3	3.6	3.0	3.5	3.5	3.2	3.3
11	3.6	3.5	3.5	3.5	3.5	3.4	3.4	3.5	3.6	3.6	3.2	3.4
12	3.5	3.4	3.3	3.5	3.7	3.3	3.4	3.5	3.5	3.4	3.5	3.2
13	3.7	3.2	3.6	3.5	3.6	3.3	3.2	3.4	3.5	3.3	3.4	3.6
14	3.6	3.4	3.5	3.5	3.5	3.3	3.6	3.4	3.5	3.5	3.4	3.2
15	3.4	3.5	3.4	3.4	3.4	3.1	3.5	2.7	3.5	3.5	3.4	3.5
16	3.6	3.5	3.6	3.6	3.3	3.3	3.4	3.5	3.6	3.4	3.3	3.4
17	3.5	3.4	3.2	3.5	3.3	3.6	3.3	3.3	3.5	3.1	3.5	3.4
18	3.5	3.4	3.4	3.2	3.4	3.5	3.3	3.5	3.5	3.3	3.4	3.5
19	3.5	3.4	3.3	3.6	3.2	3.4	3.3	3.6	3.5	3.5	3.5	3.4
20	3.6	3.6	3.3	3.3	3.3	3.0	3.0	3.5	3.3	3.0	3.2	3.4
21	3.4	3.6	3.6	3.5	3.3	3.4	3.4	3.3	3.6	3.5	3.3	3.6
22	3.4	3.5	3.3	3.4	3.5	3.5	3.3	3.5	3.4	3.5	3.1	3.5
23	3.2	3.3	3.4	3.3	3.3	3.2	-	3.4	3.5	3.6	-	3.4
24	3.4	3.5	3.6	3.3	3.4	3.5	-	3.5	3.5	3.4	-	3.1
25	2.6	3.1	3.5	3.4	3.3	3.2	-	3.1	3.3	3.5	-	3.3
26	3.4	3.2	3.6	3.4	3.3	3.3	-	3.2	3.5	-	-	3.5
27	2.6	3.6	3.5	3.4	3.6	-	-	-	3.4	-	-	3.3
28	-	3.1	3.5	3.6	3.3	-	-	-	-	-	-	3.4
29	-	-	3.4	3.4	-	-	-	-	-	-	-	2.5
30	-	-	-	3.2	-	-	-	-	-	-	-	-

- Not applicable

Table 5 (continued)
Total Length of Fish (cm) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)											
	Control		0.20		0.641		2.05		6.56		21.0	
	Vessel number											
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12
31	-	-	-	3.6	-	-	-	-	-	-	-	-
32	-	-	-	3.1	-	-	-	-	-	-	-	-
-	Not applicable											
Individual mean	3.4	3.4	3.5	3.4	3.4	3.3	3.4	3.3	3.5	3.4	3.4	3.4
Pooled mean	3.4		3.5		3.4		3.4		3.5		3.4	
Individual minimum	2.6	3.1	3.2	2.7	3.0	2.5	3.0	2.7	3.3	2.9	3.1	2.5
Pooled minimum	2.6		2.7		2.7		2.7		2.9		2.5	
Individual maximum	3.8	3.6	3.7	3.7	3.7	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Pooled maximum	3.8		3.7		3.7		3.6		3.6		3.6	
Individual standard deviation	0.284	0.166	0.129	0.196	0.186	0.204	0.147	0.209	0.091	0.187	0.155	0.204
Pooled standard deviation	0.230		0.170		0.199		0.187		0.147		0.186	

Table 6
Dry Weights of Fish (g) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)									
	Control		0.20		0.641		2.05		6.56	
	Vessel number									
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
1	0.0682	0.0579	0.0571	0.0528	0.0470	0.0515	0.0705	0.0693	0.0672	0.0657
2	0.0694	0.0713	0.0709	0.0440	0.0657	0.0632	0.0650	0.0494	0.0521	0.0622
3	0.0535	0.0440	0.0632	0.0275	0.0625	0.0360	0.0756	0.0535	0.0599	0.0616
4	0.0328	0.0585	0.0683	0.0544	0.0585	0.0355	0.0601	0.0639	0.0483	0.0563
5	0.0640	0.0580	0.0583	0.0612	0.0365	0.0647	0.0626	0.0512	0.0660	0.0637
6	0.0389	0.0556	0.0602	0.0476	0.0343	0.0481	0.0794	0.0590	0.0570	0.0421
7	0.0697	0.0725	0.0794	0.0494	0.0705	0.0654	0.0524	0.0591	0.0569	0.0351
8	0.0628	0.0613	0.0633	0.0824	0.0600	0.0281	0.0571	0.0580	0.0630	0.0652
9	0.0534	0.0688	0.0415	0.0785	0.0539	0.0526	0.0488	0.0454	0.0582	0.0659
10	0.0459	0.0688	0.0675	0.0752	0.0511	0.0429	0.0603	0.0383	0.0543	0.0596
11	0.0707	0.0652	0.0633	0.0465	0.0592	0.0583	0.0576	0.0624	0.0605	0.0653
12	0.0578	0.0510	0.0503	0.0515	0.0789	0.0534	0.0535	0.0563	0.0672	0.0546
13	0.0717	0.0416	0.0755	0.0513	0.0660	0.0503	0.0482	0.0518	0.0610	0.0489
14	0.0624	0.0505	0.0613	0.0744	0.0690	0.0536	0.0624	0.0529	0.0536	0.0603
15	0.0564	0.0674	0.0475	0.0471	0.0612	0.0415	0.0586	0.0230	0.0592	0.0661
16	0.0697	0.0636	0.0688	0.0592	0.0538	0.0597	0.0623	0.0713	0.0566	0.0573
17	0.0612	0.0495	0.0532	0.0652	0.0500	0.0742	0.0593	0.0531	0.0584	0.0375
18	0.0644	0.0571	0.0624	0.0475	0.0631	0.0677	0.0553	0.0616	0.0520	0.0445
19	0.0539	0.0534	0.0550	0.0724	0.0435	0.0636	0.0546	0.0730	0.0598	0.0551
20	0.0644	0.0751	0.0476	0.0518	0.0554	0.0371	0.0524	0.0565	0.0441	0.0373
21	0.0455	0.0629	0.0543	0.0661	0.0605	0.0522	0.0568	0.0508	0.0674	0.0680
22	0.0554	0.0582	0.0548	0.0535	0.0740	0.0646	0.0507	0.0644	0.0526	0.0625
23	0.0407	0.0485	0.0618	0.0484	0.0591	0.0547	-	0.0563	0.0631	0.0779
24	0.0701	0.0609	0.0740	0.0569	0.0654	0.0577	-	0.0600	0.0617	0.0657
25	0.0185	0.0360	0.0603	0.0518	0.0569	0.0558	-	0.0424	0.0497	0.0634
26	0.0585	0.0498	0.0756	0.0541	0.0532	0.0519	-	0.0478	0.0603	-
27	0.0249	0.0577	0.0564	0.0449	0.0700	-	-	-	0.0515	-
28	-	0.0308	0.0595	0.0581	0.0563	-	-	-	-	-
29	-	-	0.0730	0.0676	-	-	-	-	-	-
30	-	-	-	0.0598	-	-	-	-	-	-

- Not applicable

Table 6 (continued)
Dry Weights of Fish (g) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)									
	Control		0.20		0.641		2.05		6.56	
	Vessel number									
	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10
31	-	-	-	0.0601	-	-	-	-	-	-
32	-	-	-	0.0027	-	-	-	-	-	-
-	Not applicable									
Individual mean	0.0557	0.0570	0.0615	0.0551	0.0584	0.0532	0.0593	0.0550	0.0578	0.0577
Pooled mean	0.0564		0.0583		0.0558		0.0571		0.0578	
Individual minimum	0.0185	0.0308	0.0415	0.0027	0.0343	0.0281	0.0482	0.0230	0.0441	0.0351
Pooled minimum	0.0185		0.0027		0.0281		0.0230		0.0351	
Individual maximum	0.0717	0.0751	0.0794	0.0824	0.0789	0.0742	0.0794	0.0730	0.0674	0.0779
Pooled maximum	0.0751		0.0824		0.0789		0.0794		0.0779	
Individual standard deviation	0.0144	0.0108	0.0093	0.0150	0.0103	0.0112	0.0080	0.0106	0.0060	0.0110
Pooled standard deviation	0.0126		0.0129		0.0110		0.0097		0.0087	

Table 6 (continued)
Dry Weights of Fish (g) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)	
	21.0	
	Vessel number	
	V11	V12
1	0.0659	0.0505
2	0.0754	0.0654
3	0.0651	0.0625
4	0.0666	0.0645
5	0.0670	0.0588
6	0.0585	0.0623
7	0.0698	0.0571
8	0.0737	0.0572
9	0.0676	0.0650
10	0.0445	0.0589
11	0.0485	0.0563
12	0.0588	0.0371
13	0.0666	0.0654
14	0.0573	0.0464
15	0.0600	0.0616
16	0.0498	0.0515
17	0.0674	0.0603
18	0.0669	0.0644
19	0.0595	0.0523
20	0.0443	0.0608
21	0.0529	0.0718
22	0.0463	0.0688
23	-	0.0596
24	-	0.0420
25	-	0.0603
26	-	0.0427
27	-	0.0377
28	-	0.0794
29	-	0.0345
30	-	-

- Not applicable

Table 6 (continued)
Dry Weights of Fish (g) at the End of the Fish, Early-life Stage Test - 28 Days Post-Hatch

Fish Number	Nominal concentration (mg/L)	
	21.0	
	Vessel number	
	V11	V12
31	-	-
32	-	-
-	Not applicable	
Individual mean	0.0606	0.0571
Pooled mean	0.0588	
Individual minimum	0.0443	0.0345
Pooled minimum	0.0345	
Individual maximum	0.0754	0.0794
Pooled maximum	0.0794	
Individual standard deviation	0.0094	0.0107
Pooled standard deviation	0.0102	

Table 7
Temperature (°C) Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number pre-hatch				Day number post-hatch											
		21 (new)	23 (new)	25 (new)	28 (new)	1 (new)	3 (new)	6 (new)	8 (new)	10 (new)	13 (new)	15 (new)	17 (new)	21 (new)	23 (new)	26 (new)	28 (new)
Control	V1	12.1	12.1	12.1	12.0	12.0	11.8	11.7	11.8	11.9	11.8	12.2	12.4	11.8	12.0	12.0	11.9
	V2	12.2	12.1	12.2	12.0	12.0	12.0	11.7	11.7	12.0	11.7	12.3	12.4	11.8	12.0	12.0	11.9
0.20	V3	12.2	12.2	12.2	11.9	12.1	11.8	11.8	11.8	12.0	11.8	12.3	12.3	11.7	11.8	11.9	11.9
	V4	12.2	12.2	12.2	12.1	12.1	11.8	11.8	11.8	11.9	11.9	12.3	12.3	11.6	11.8	11.9	11.9
0.641	V5	12.2	12.1	12.2	12.1	12.1	12.0	11.8	11.7	11.8	11.8	12.2	12.3	11.6	11.8	11.9	11.8
	V6	12.3	12.1	12.2	12.1	12.0	11.9	11.8	11.7	11.9	11.8	12.2	12.2	11.6	11.9	11.8	11.8
2.05	V7	12.2	12.0	12.3	12.1	12.0	12.0	11.9	11.8	12.0	11.7	12.3	12.2	11.6	11.9	11.8	11.8
	V8	12.2	12.0	12.1	12.2	12.0	12.0	12.0	11.8	12.0	11.7	12.3	12.1	11.8	11.9	11.7	11.9
6.56	V9	12.2	12.2	12.1	12.1	12.0	11.8	11.8	11.7	11.8	11.8	12.3	12.1	11.8	12.0	11.9	12.0
	V10	12.2	12.2	12.2	12.2	12.1	11.8	12.0	11.7	12.0	11.8	12.3	12.2	12.0	12.1	12.0	12.1
21.0	V11	12.3	12.2	12.3	12.2	12.1	11.8	12.0	11.8	11.9	11.7	12.4	12.3	12.0	12.1	12.0	12.1
	V12	12.4	12.3	12.3	12.2	12.2	11.8	12.0	11.8	11.9	11.8	12.4	12.4	12.1	12.2	12.1	12.2

Table 8
pH Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number pre-hatch														
		0 (new)	1 (new)	2 (new)	3 (new)	6 (new)	7 (new)	9 (new)	11 (new)	14 (new)	16 (new)	18 (new)	21 (new)	23 (new)	25 (new)	28 (new)
Control	V1	7.2	7.3	-	7.3	7.2	7.3	7.6	7.6	7.4	7.5	7.4	7.3	7.2	7.1	7.3
	V2	7.2	7.3	-	7.3	7.2	7.3	7.6	7.6	7.4	7.5	7.4	7.3	7.2	7.1	7.3
0.20	V3	7.2	-	-	7.4	7.2	7.4	7.6	7.6	7.4	7.6	7.4	7.3	7.2	7.1	7.3
	V4	7.2	-	-	7.3	7.3	7.4	7.6	7.7	7.5	7.5	7.5	7.3	7.2	7.2	7.2
0.641	V5	7.3	-	-	7.3	7.3	7.4	7.7	7.7	7.5	7.5	7.5	7.3	7.2	7.2	7.2
	V6	7.3	7.3	-	7.3	7.3	7.4	7.7	7.7	7.5	7.5	7.5	7.3	7.2	7.2	7.2
2.05	V7	7.3	7.3	-	7.3	7.3	7.4	7.7	7.7	7.6	7.5	7.5	7.3	7.2	7.2	7.2
	V8	7.3	-	-	7.3	7.3	7.4	7.7	7.7	7.5	7.4	7.5	7.3	7.2	7.2	7.2
6.56	V9	7.3	-	-	7.3	7.3	7.4	7.7	7.7	7.6	7.4	7.5	7.2	7.2	7.2	7.2
	V10	7.3	-	-	7.3	7.3	7.4	7.7	7.8	7.6	7.4	7.5	7.2	7.2	7.2	7.2
21.0	V11	7.3	7.3	-	7.3	7.3	7.4	7.7	7.8	7.6	7.4	7.5	7.2	7.2	7.2	7.2
	V12	7.3	7.3	-	7.3	7.3	7.4	7.7	7.8	7.6	7.4	7.5	7.2	7.2	7.2	7.2

Table 8 (continued)
pH Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number post-hatch											
		1 (new)	3 (new)	6 (new)	8 (new)	10 (new)	13 (new)	15 (new)	17 (new)	21 (new)	23 (new)	26 (new)	28 (new)
Control	V1	7.3	7.2	7.2	7.2	7.1	7.1	7.2	7.2	7.6	7.5	7.4	7.1
	V2	7.3	7.2	7.2	7.3	7.1	7.1	7.2	7.2	7.6	7.5	7.4	7.1
0.20	V3	7.3	7.2	7.1	7.3	7.0	7.0	7.2	7.2	7.6	7.5	7.4	7.1
	V4	7.3	7.2	7.2	7.2	7.0	7.0	7.2	7.2	7.5	7.5	7.4	7.1
0.641	V5	7.3	7.2	7.1	7.2	7.0	7.0	7.1	7.2	7.5	7.5	7.4	7.1
	V6	7.3	7.2	7.1	7.2	7.0	7.0	7.1	7.2	7.5	7.4	7.3	7.1
2.05	V7	7.4	7.2	7.1	7.2	6.9	7.0	7.1	7.2	7.5	7.4	7.3	7.1
	V8	7.4	7.2	7.1	7.3	6.9	7.0	7.1	7.2	7.4	7.4	7.3	7.0
6.56	V9	7.4	7.2	7.1	7.2	7.0	7.0	7.1	7.2	7.4	7.4	7.3	7.0
	V10	7.4	7.2	7.1	7.2	6.9	7.0	7.1	7.2	7.4	7.4	7.3	7.0
21.0	V11	7.4	7.2	7.1	7.2	6.9	7.0	7.1	7.2	7.4	7.4	7.3	7.0
	V12	7.3	7.2	7.1	7.3	6.9	7.0	7.1	7.2	7.4	7.4	7.3	7.0

Table 9
Dissolved Oxygen (% ASV) Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number pre-hatch														
		0 (new)	1 (new)	2 (new)	3 (new)	6 (new)	7 (new)	9 (new)	11 (new)	14 (new)	16 (new)	18 (new)	21 (new)	23 (new)	25 (new)	28 (new)
Control	V1	98	93	-	94	95	96	95	91	92	95	96	98	92	94	95
	V2	99	94	-	93	94	95	95	91	92	94	97	95	93	93	95
0.20	V3	97	-	-	92	93	94	96	92	92	96	96	96	94	92	96
	V4	98	-	-	93	93	96	97	93	94	96	96	96	94	92	96
0.641	V5	98	-	-	94	93	94	96	91	91	97	93	95	94	94	94
	V6	99	95	-	95	94	95	96	91	90	95	94	96	93	95	96
2.05	V7	101	93	-	95	95	95	97	95	90	96	94	95	94	92	95
	V8	100	-	-	94	95	96	97	94	92	96	95	96	95	92	95
6.56	V9	99	-	-	94	94	95	95	93	92	96	95	97	94	93	96
	V10	98	-	-	95	94	92	96	93	95	96	96	96	92	93	97
21.0	V11	97	92	-	93	93	95	96	91	94	94	96	96	93	94	93
	V12	97	93	-	95	95	94	96	91	93	95	95	96	94	94	98

Table 9 (continued)
Dissolved Oxygen (% ASV) Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number post-hatch											
		1 (new)	3 (new)	6 (new)	8 (new)	10 (new)	13 (new)	15 (new)	17 (new)	21 (new)	23 (new)	26 (new)	28 (new)
Control	V1	89	103	105	100	95	102	93	83	90	85	81	88
	V2	90	103	98	89	96	103	93	85	90	86	80	89
0.20	V3	90	104	92	94	96	102	91	85	92	87	83	89
	V4	88	105	104	95	96	105	94	83	89	86	79	90
0.641	V5	90	104	105	96	97	102	94	84	91	88	81	88
	V6	91	104	104	95	97	104	92	84	92	88	81	88
2.05	V7	89	103	104	96	97	104	92	86	89	89	77	90
	V8	89	104	105	95	97	105	93	81	88	86	82	90
6.56	V9	90	105	104	96	96	105	92	82	89	87	80	88
	V10	91	104	98	95	96	102	95	83	93	87	84	87
21.0	V11	90	105	98	96	97	103	90	83	92	88	83	89
	V12	90	103	96	96	97	104	94	85	90	88	80	89

Table 10
Dissolved Oxygen (mg/L) Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number pre-hatch														
		0 (new)	1 (new)	2 (new)	3 (new)	6 (new)	7 (new)	9 (new)	11 (new)	14 (new)	16 (new)	18 (new)	21 (new)	23 (new)	25 (new)	28 (new)
Control	V1	10.90	10.38	-	10.11	10.21	10.41	10.21	9.72	9.85	10.41	10.31	10.01	9.31	10.00	10.51
	V2	10.95	10.42	-	10.05	10.18	10.32	10.23	9.73	9.83	10.38	10.38	9.72	9.50	9.91	10.49
0.20	V3	10.81	-	-	9.93	10.07	10.22	10.29	9.80	9.83	10.52	10.32	9.70	9.53	9.82	10.59
	V4	10.91	-	-	10.04	10.06	10.37	10.38	9.92	10.00	10.50	10.30	9.75	9.55	9.82	10.61
0.641	V5	10.90	-	-	10.13	10.05	10.28	10.30	9.78	9.73	10.61	10.01	9.69	9.54	10.01	10.45
	V6	10.94	10.55	-	10.20	10.16	10.32	10.31	9.76	9.62	10.40	10.08	9.72	9.50	10.13	10.58
2.05	V7	11.10	10.32	-	10.20	10.19	10.38	10.36	10.09	9.70	10.48	10.11	9.70	9.56	9.81	10.50
	V8	11.00	-	-	10.15	10.22	10.32	10.37	10.05	9.72	10.52	10.20	9.76	9.68	9.80	10.50
6.56	V9	10.95	-	-	10.11	10.18	10.30	10.23	9.90	9.80	10.52	10.21	9.90	9.52	9.89	10.58
	V10	10.90	-	-	10.19	10.16	10.31	10.30	9.90	10.08	10.54	10.28	9.79	9.30	9.87	10.66
21.0	V11	10.82	10.27	-	10.05	10.06	10.42	10.30	9.70	10.00	10.36	10.25	9.76	9.45	10.03	10.21
	V12	10.81	10.32	-	10.20	10.20	10.40	10.29	9.72	9.91	10.45	10.20	9.75	9.52	10.04	10.77

Table 10 (continued)
Dissolved Oxygen (mg/L) Measurements during the Fish, Early Life Stage Test

Nominal concentration (mg/L)	Vessel number	Day number post-hatch											
		1 (new)	3 (new)	6 (new)	8 (new)	10 (new)	13 (new)	15 (new)	17 (new)	21 (new)	23 (new)	26 (new)	28 (new)
Control	V1	9.61	10.74	11.28	10.45	9.34	11.12	9.72	8.72	9.31	9.00	9.02	9.51
	V2	9.70	10.73	10.58	9.34	9.66	11.17	9.70	8.91	9.32	9.09	8.92	9.62
0.20	V3	9.71	11.03	9.99	9.97	9.67	11.09	9.49	8.92	9.50	9.12	9.26	9.61
	V4	9.52	11.23	11.30	10.23	9.92	11.49	9.81	8.76	9.23	9.05	8.86	9.72
0.641	V5	9.73	11.17	11.48	10.34	10.06	11.20	9.82	8.81	9.41	9.20	9.04	9.51
	V6	9.80	11.20	11.39	10.29	10.12	11.38	9.65	8.81	9.56	9.22	9.05	9.51
2.05	V7	9.65	11.01	11.41	10.40	10.10	11.36	9.61	8.99	9.17	9.32	8.79	9.72
	V8	9.60	11.21	11.57	10.37	10.08	11.48	9.73	8.52	9.12	9.04	9.12	9.71
6.56	V9	9.70	11.39	11.44	10.42	9.88	11.55	9.58	8.60	9.17	9.12	8.92	9.49
	V10	9.82	11.22	10.73	10.39	9.90	11.28	9.90	8.70	9.60	9.14	9.36	9.42
21.0	V11	9.75	11.35	10.71	10.45	10.30	11.30	9.39	8.71	9.54	9.25	9.27	9.53
	V12	9.74	10.88	10.51	10.52	10.34	11.31	9.81	8.92	9.36	9.21	8.94	9.54

Table 11
Water Hardness and Residual Chlorine Measurements in Dilution Water during the Fish, Early Life Stage Test

Day number	Water hardness (mg/L as $CaCO_3$)	Residual chlorine (mg/L)
0 pre-hatch (new)	61	0.01
3 pre-hatch (new)	75	0.00
7 pre-hatch (new)	65	0.02
14 pre-hatch (new)	68	0.01
18 pre-hatch (new)	68	0.01
21 pre-hatch (new)	61	0.00
28 pre-hatch (new)	50	0.02
6 post-hatch (new)	55	0.08
13 post-hatch (new)	57	0.08
21 post-hatch (new)	60	0.02
26 post-hatch (new)	59	0.00
28 post-hatch (new)	60	0.01

FIGURES

Figure 1
Chromatogram of a 0.02 µg/mL Standard Solution of Perfluorohexanoic acid in hplc mobile phase (Internal Standard of Perfluoroheptanoic acid at 0.01 µg/mL)

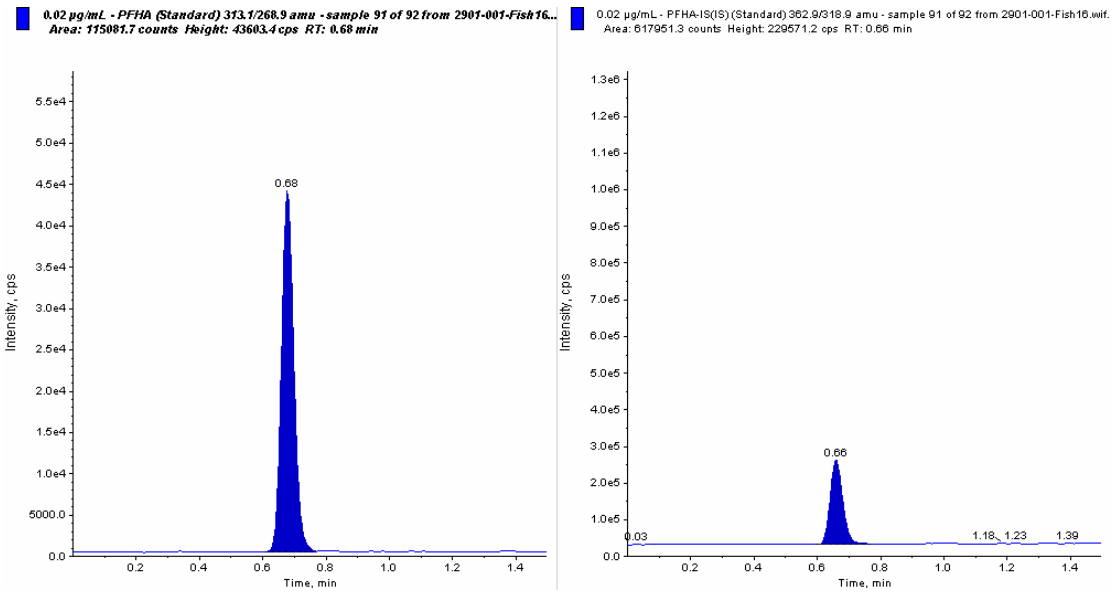


Figure 2
Chromatogram of a 0.0002 µg/mL Standard Solution of Perfluorohexanoic acid in HPLC mobile phase (Internal Standard of Perfluoroheptanoic acid at 0.01 µg/mL)

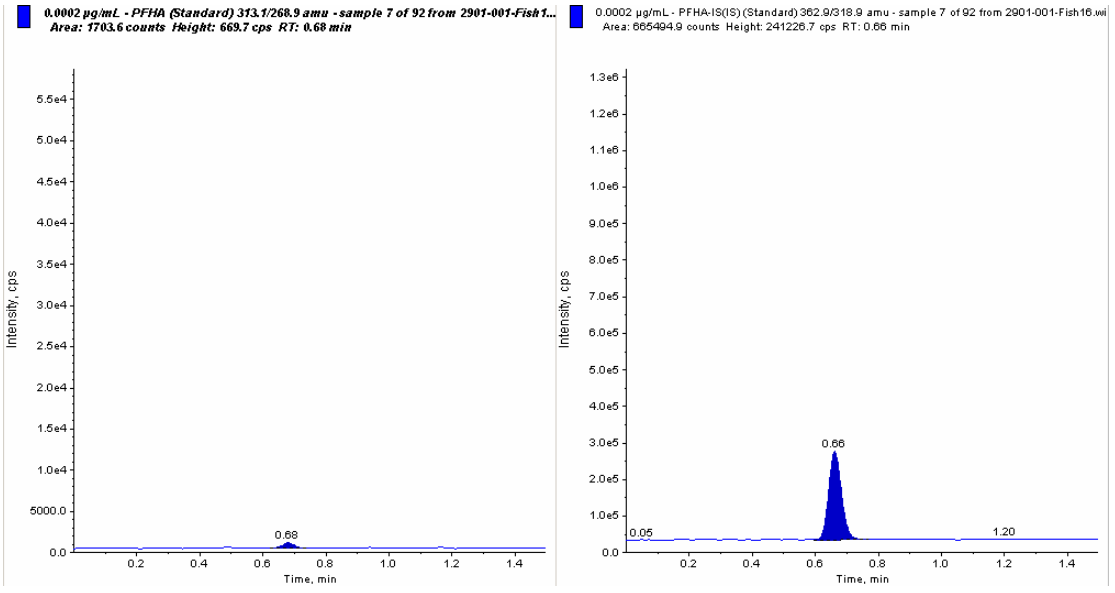


Figure 3
Chromatogram of a Control Test Water Sample at Day 0.)
(Dilution factor = 10)

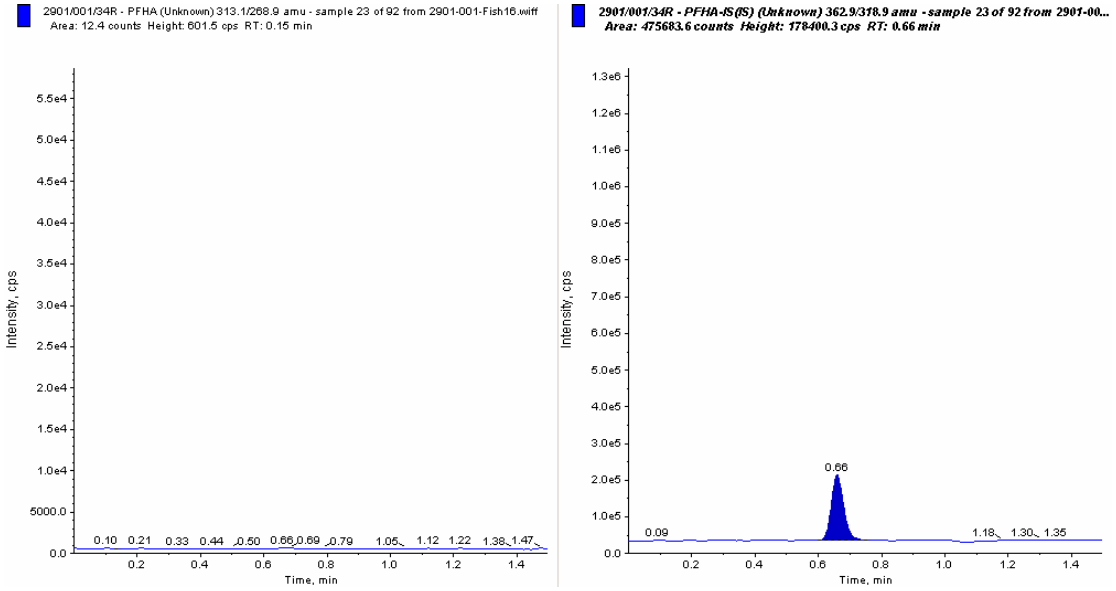


Figure 4
Chromatogram of a 0.2 mg/L Test Water Sample at Day 0
(Dilution factor = 10)

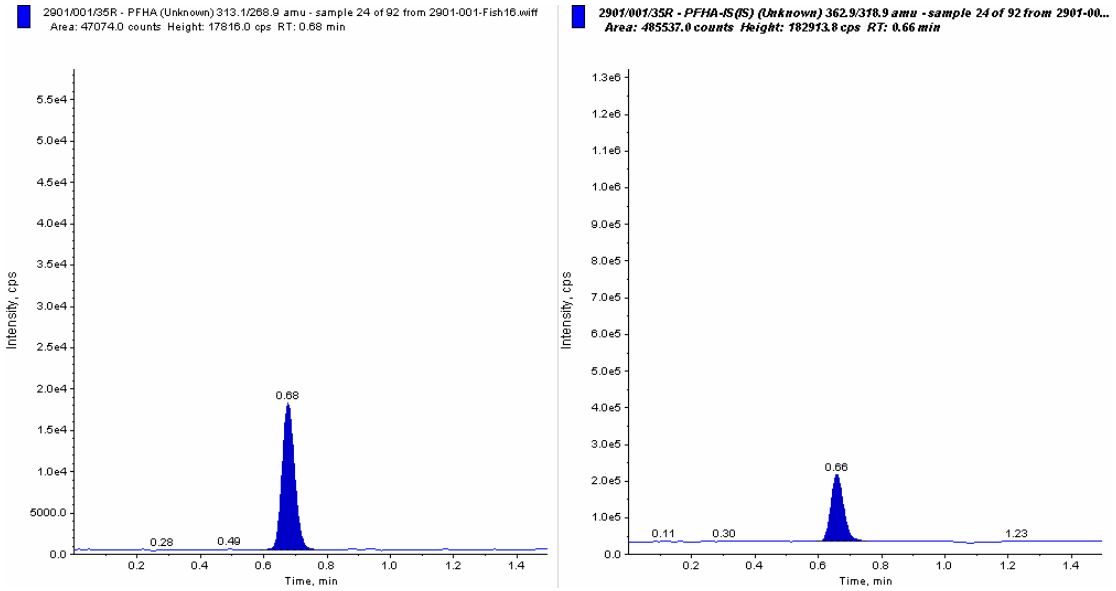


Figure 5
Chromatogram of a 21 mg/L Test Water Sample at Day 0
(Dilution factor = 1000)

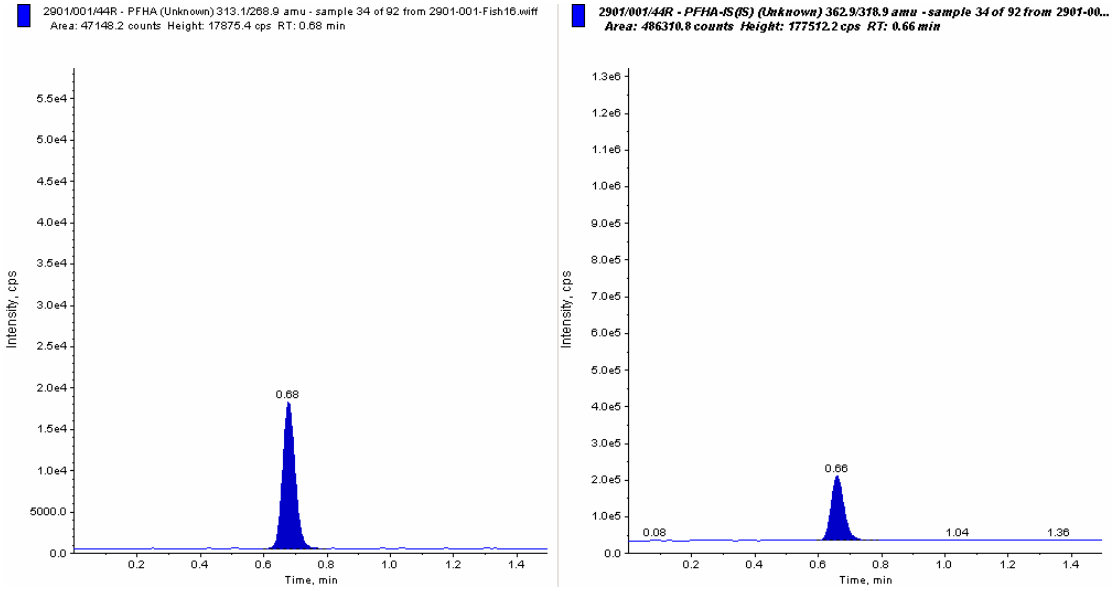


Figure 6
Chromatogram of a 0.2 mg/L Test Water Sample at Day 1
(Dilution factor = 100)

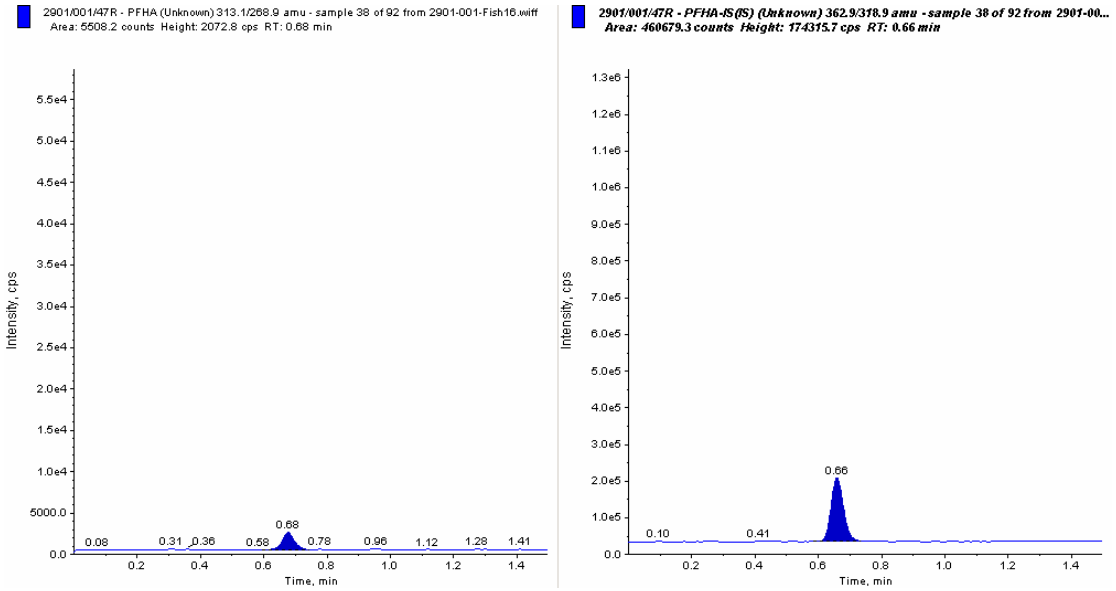


Figure 7
Chromatogram of a 21 mg/L Test Water Sample at Day 1
(Dilution factor =1000)

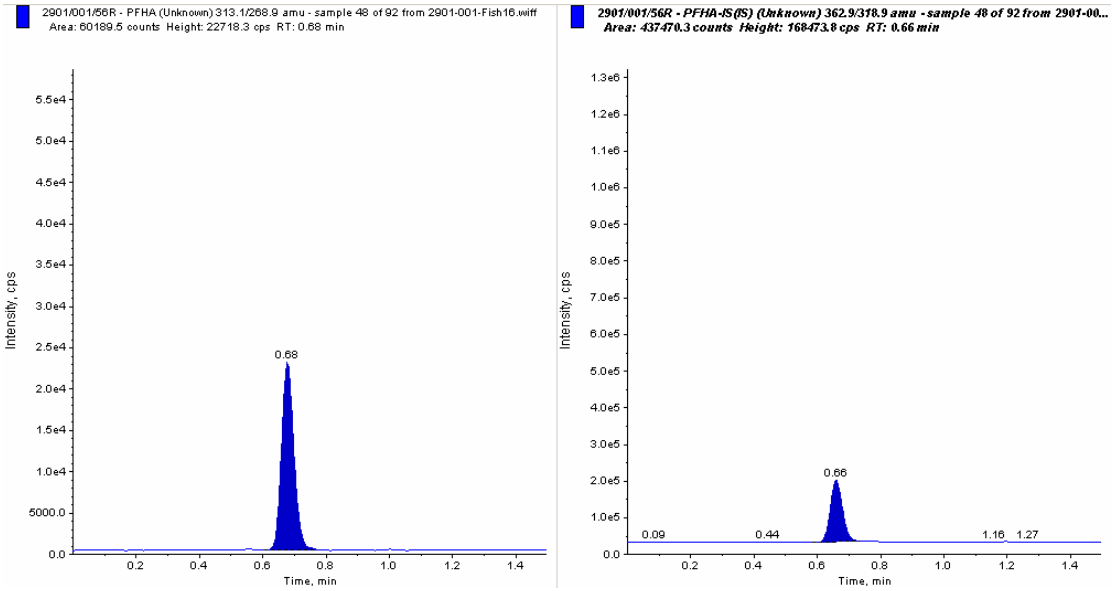


Figure 8
Chromatogram of a 0.2 mg/L Test Water Sample at Day -1 Post Hatch
(Dilution factor = 100)

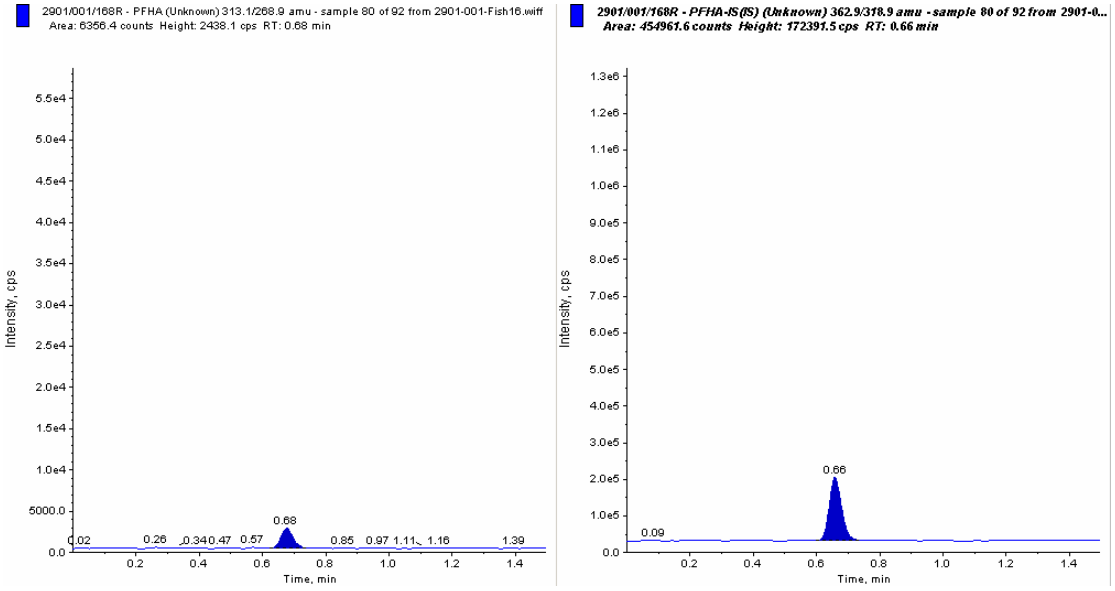


Figure 9
Chromatogram of a 21 mg/L Test Water Sample at Day -1 Post Hatch
(Dilution factor = 1000)

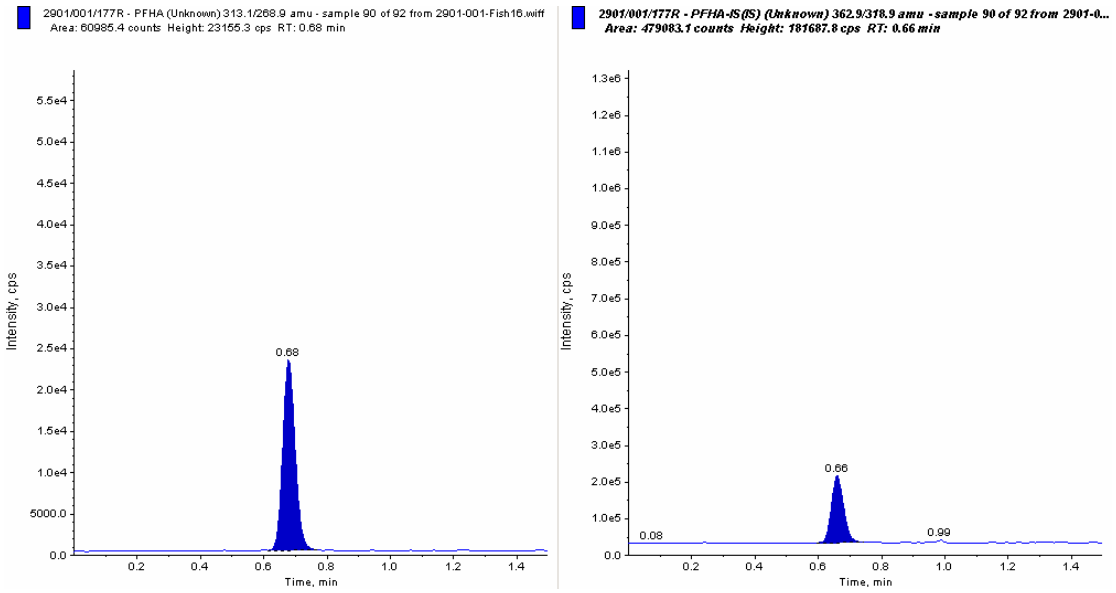


Figure 10
Chromatogram of a 0.2 mg/L Test Water Sample at Day 0 Post Hatch
(Dilution factor = 100)

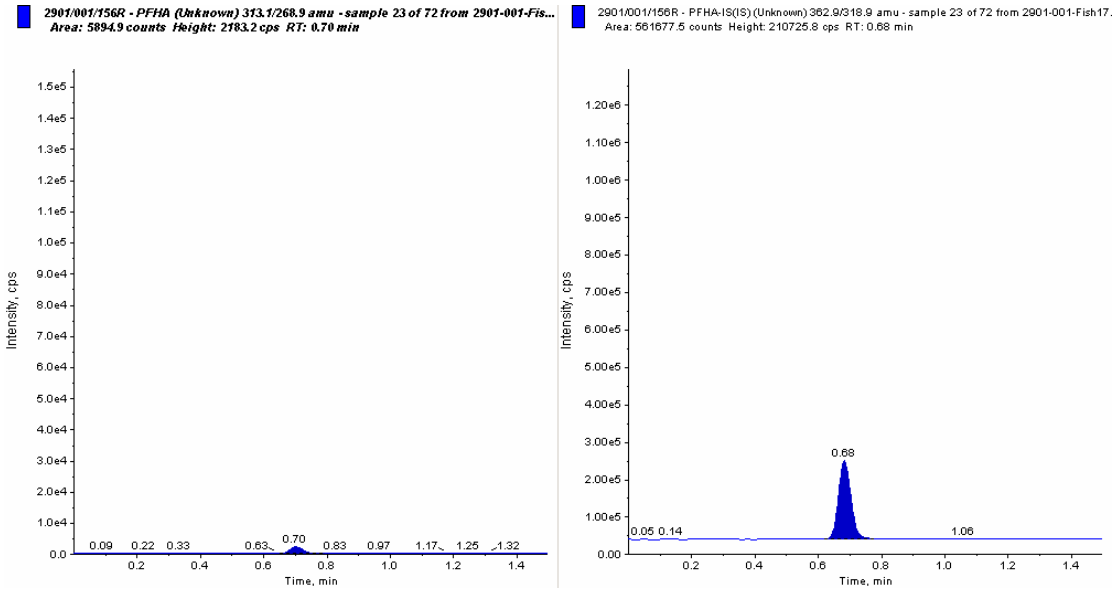


Figure 11
Chromatogram of a 21 mg/L Test Water Sample at Day 0 Post Hatch
(Dilution factor = 1000)

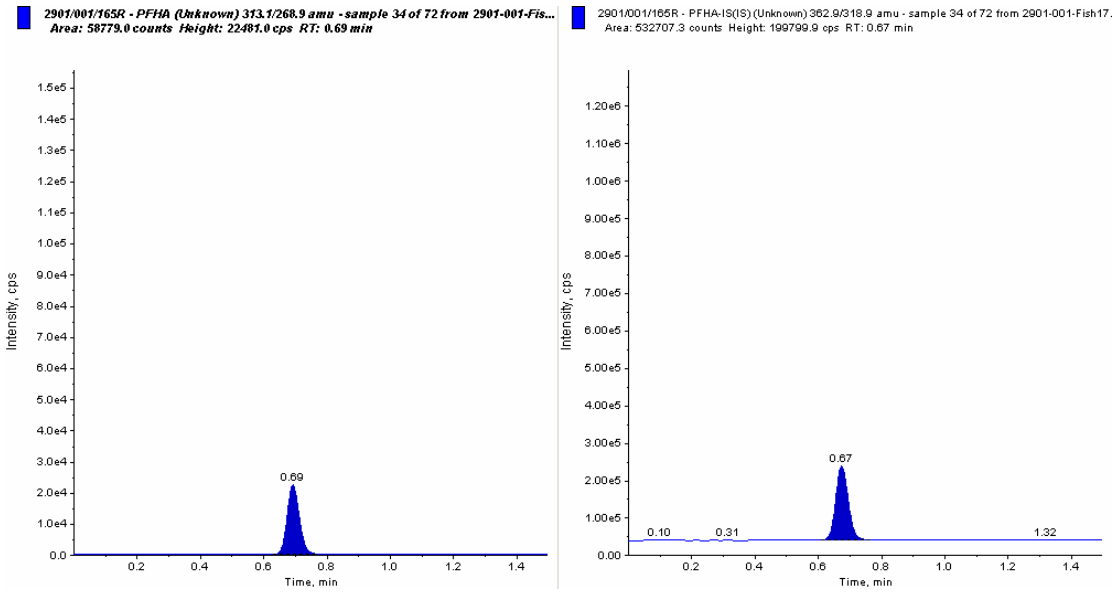


Figure 12
Chromatogram of a Control Test Water Sample at Day 28 Post Hatch
(Dilution factor = 100)

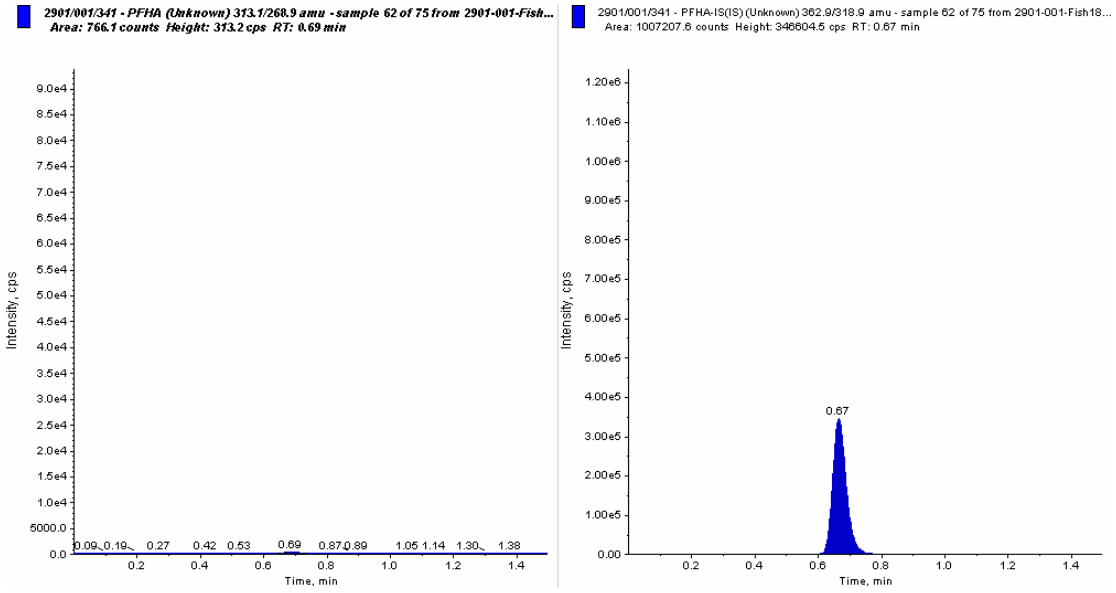


Figure 13
Chromatogram of a 0.2 mg/L Test Water Sample at Day 28 Post Hatch
(Dilution factor = 100)

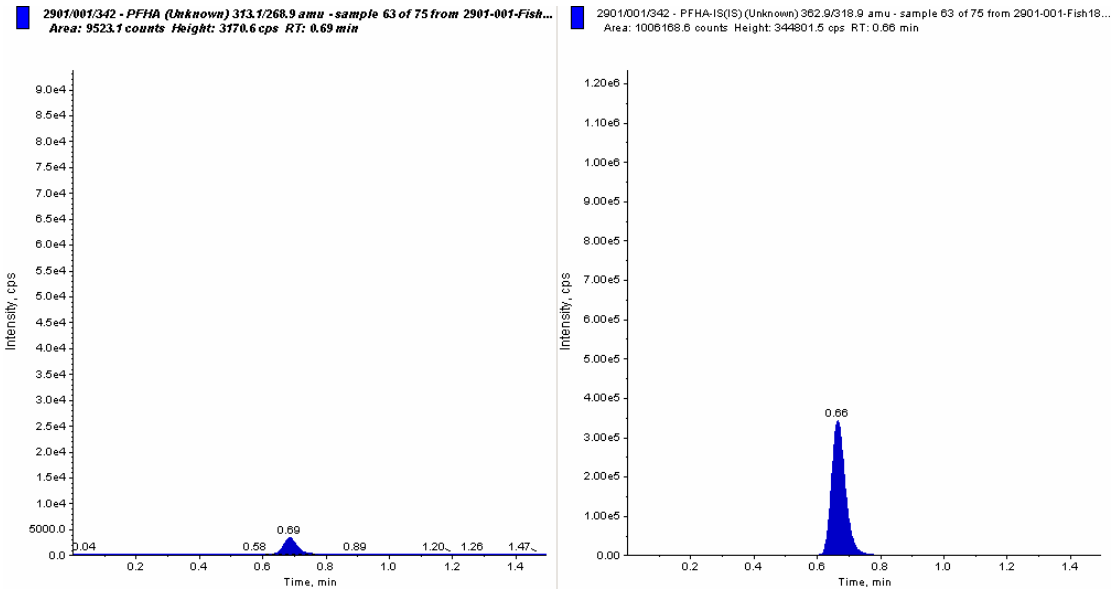


Figure 14
Chromatogram of a 21 mg/L Test Water Sample at Day 28 Post Hatch
(Dilution factor = 1000)

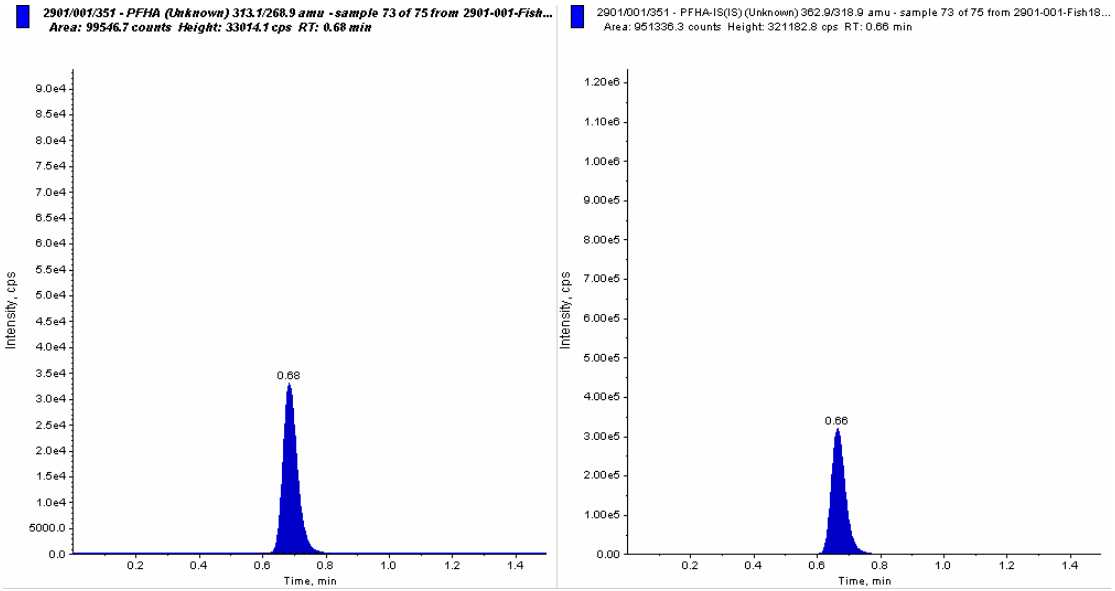


Figure 15
Chromatogram of a New 3.2 mg/L Stock Solution at Day 0
(Dilution factor = 200)

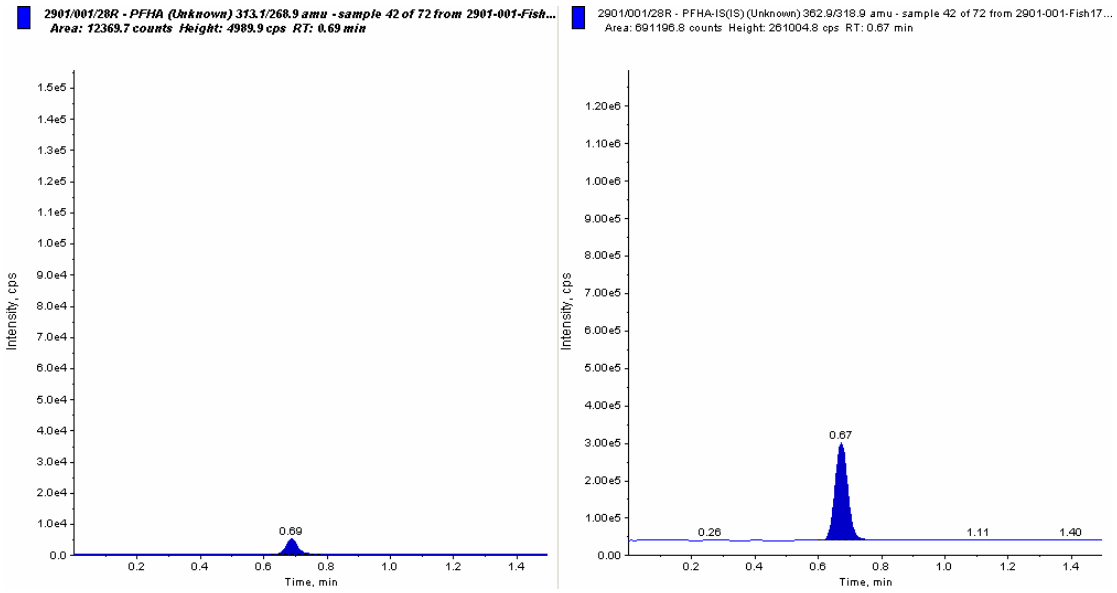


Figure 16
Chromatogram of a New 356 mg/L Stock Solution at Day 0
(Dilution factor = 20000)

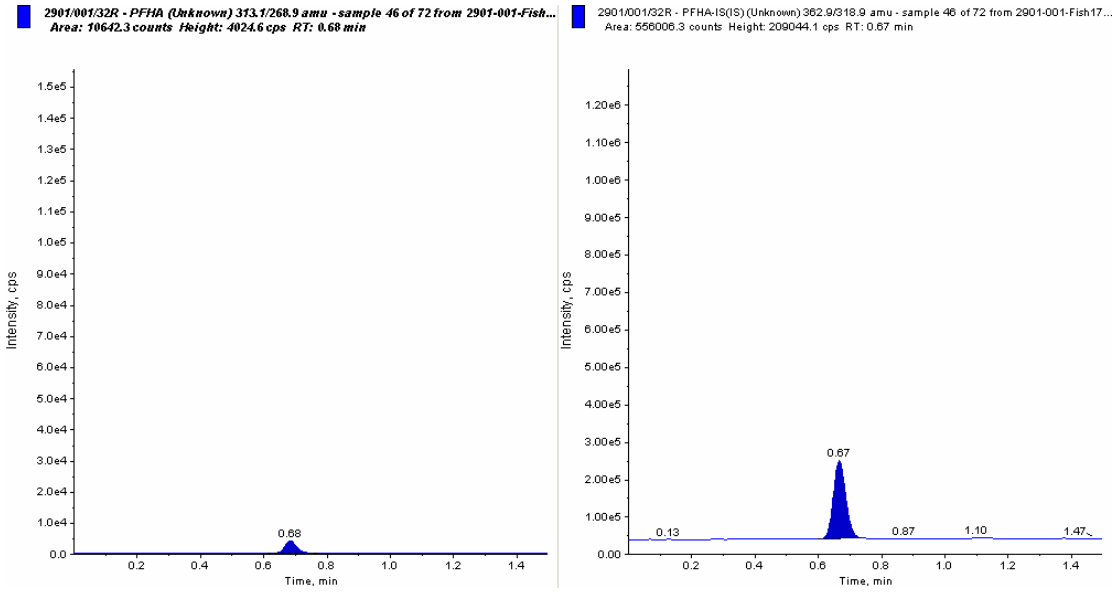


Figure 17
Chromatogram of an Old 3.2 mg/L Stock Solution at Day 4
(Dilution factor = 200)

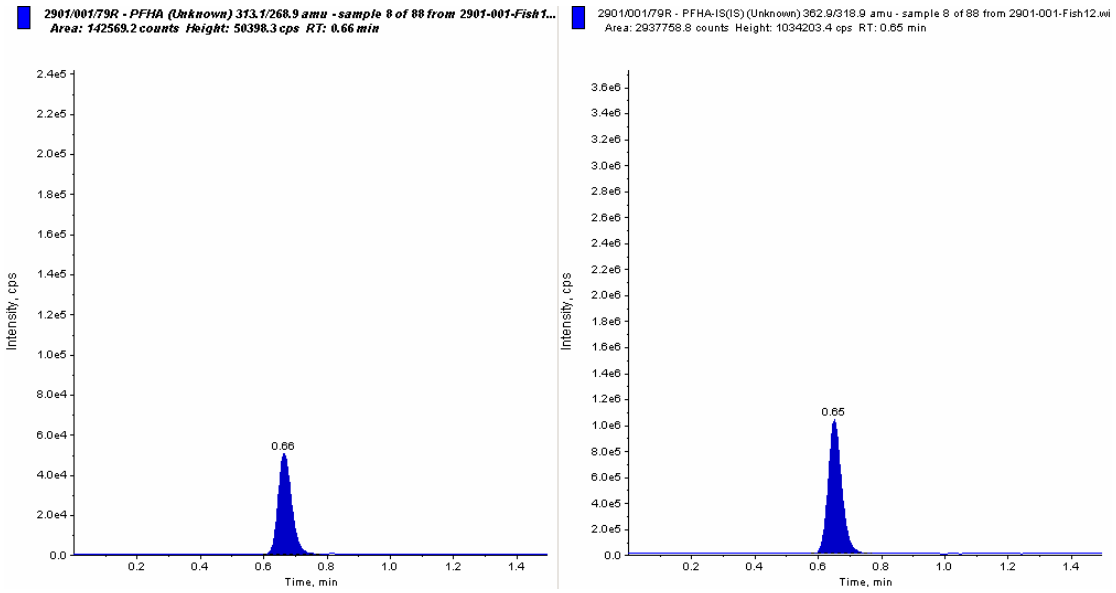


Figure 18
Chromatogram of an Old 356 mg/L Stock Solution at Day 4
(Dilution factor = 20000)

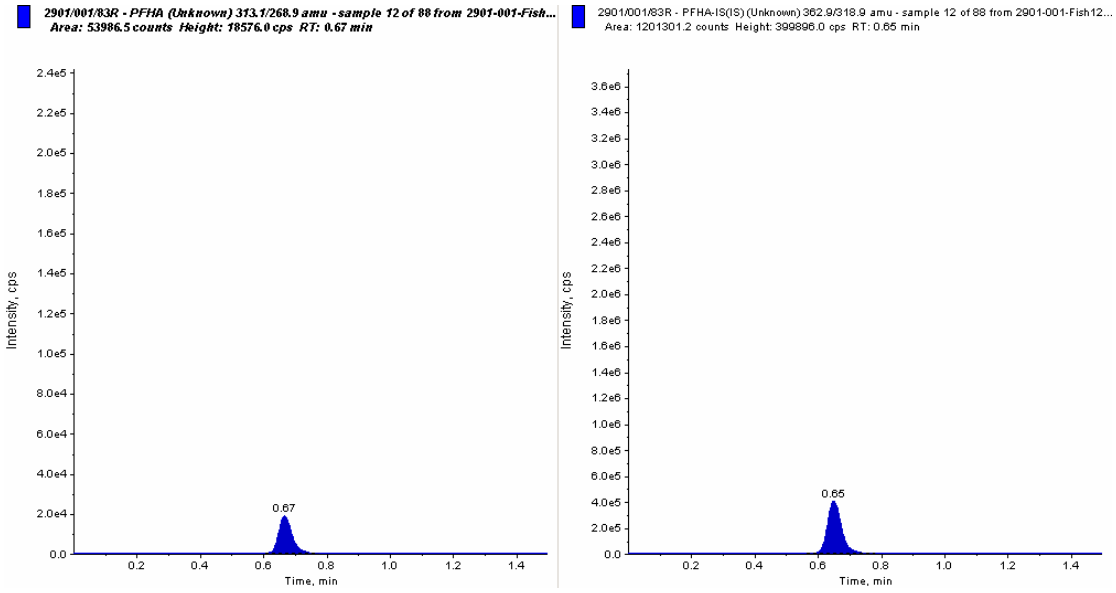
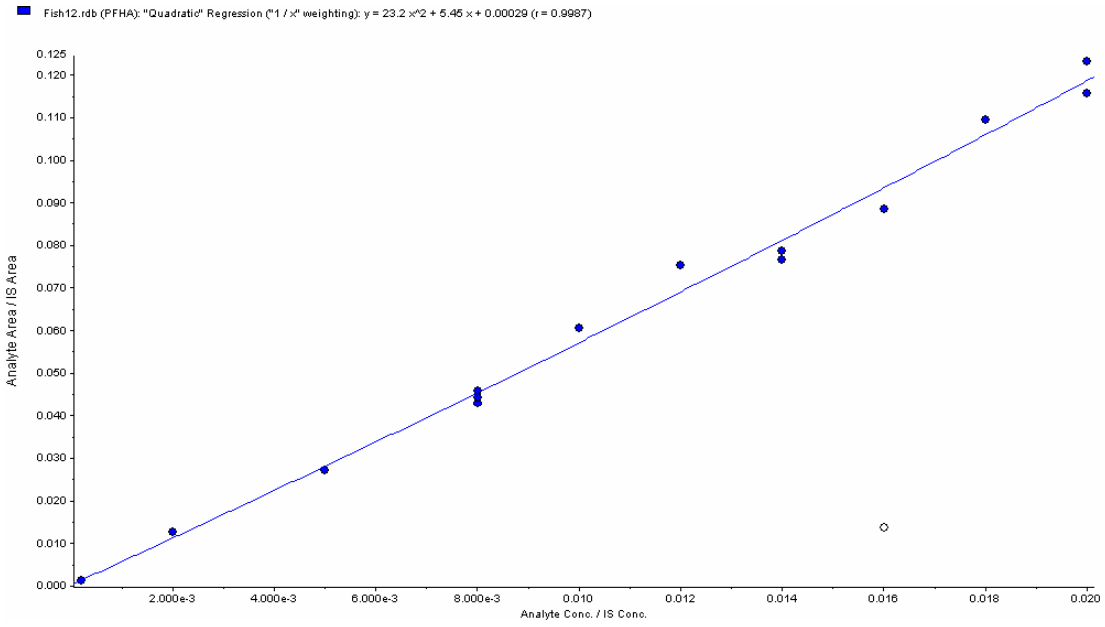


Figure 19
Typical Calibration Line for Perfluorohexanoic Acid
over the range of 0.0002 to 0.02 µg/mL



Reproduced from Analyst batch Fish12

APPENDICES

Appendix 1
Typical Water Characteristics

National Laboratory Service
Science.Service.Solutions.

Analytical Report

Final Report

Report ID - 20007439 - 1

UKAS TESTING
0754

Reported on:
23-Apr-2008

Client: Covance
Folder No: 000633786
Comments: Sample point 2. LB 6.1 Room 16

Project: Potability Analysis
Sampled on: 15-Apr-08 @ 09:18

	Result		MRV	Accred	Lab / TestCode	
Alkalinity to pH 4.5 as CaCO3	16.00	mg/l	5	UKAS	Sx	25
Ammoniacal Nitrogen as N	< 0.0300	mg/l	0.03	UKAS	Sx	25
Chloride	14.20	mg/l	1	UKAS	Sx	25
Nitrogen : Total Oxidised as N	0.910	mg/l	0.2	UKAS	Sx	25
Nitrite as N	< 0.00400	mg/l	0.004	UKAS	Sx	25
Phosphorus : Total as P	1.2600	mg/l	0.02	UKAS	Sx	234
Colour, Filtered	< 5.00	Hazen	5	UKAS	Sx	20
Conductivity at 20C	148.0	uS/cm	10	UKAS	Sx	9
pH	7.4100	pH Units	0.05	UKAS	Sx	9
Turbidity	< 1.00	FTU	1	UKAS	Sx	9
Aluminium	27.0	ug/l	10	UKAS	Sx	34
Cadmium	< 0.100	ug/l	0.1	UKAS	Sx	34
Copper	9.200	ug/l	0.5	UKAS	Sx	34
Lead	< 2.0000	ug/l	0.04	UKAS	Sx	34
Zinc	< 5.00	ug/l	5	UKAS	Sx	34
Calcium	13.70	mg/l	1	UKAS	Sx	38
Iron	< 30.0	ug/l	30	UKAS	Sx	38
Magnesium	2.410	mg/l	0.3	UKAS	Sx	38
Manganese	13.0	ug/l	10	UKAS	Sx	38
Potassium	1.050	mg/l	0.1	UKAS	Sx	38
Sodium	9.00	mg/l	2	UKAS	Sx	38
Sulphate as SO4	30.0	mg/l	10	UKAS	Sx	38
Coliforms, Faecal : Confirmed : Membrane Filtration	< 1	CFU/0.1l	1	UKAS	Sx	948
Coliforms, Faecal : Presumptive : Membrane Filtration	< 1	CFU/0.1l	1	UKAS	Sx	949
Coliforms : Total : Confirmed : Membrane Filtration	< 1	No/100ml	1	UKAS	Sx	785
Coliforms : Total : Presumptive : Membrane Filtration	< 1	No/100ml	1	UKAS	Sx	784
Escherichia coli : Confirmed : Membrane Filtration	< 1	No/100ml	1	UKAS	Sx	789
Escherichia coli : Presumptive : Membrane Filtration	< 1	No/100ml	1	UKAS	Sx	788
Colony count : 3 Days at 22C : Aerobic	30	No/ml	1	UKAS	Sx	815
Colony count : 2 Days at 37C : Aerobic	6	No/ml	1	UKAS	Sx	814
Hardness Total CaCO3	44.10	mg/l	0	None	Sx	864
Nitrate as N	< 0.91	mg/l	0	UKAS	Sx	864
Aldrin	< 0.00100	ug/l	0.001	UKAS	Sx	848
DDT -pp	< 0.00100	ug/l	0.001	UKAS	Sx	848
Dieldrin	< 0.00100	ug/l	0.001	UKAS	Sx	848

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ENVIRONMENT AGENCY

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National Laboratory Service
Science. Service. Solutions.

Analytical Report

Final Report

Report ID - 20007439 - 1

UKAS TESTING
0754

Reported on:
23-Apr-2008

Endrin	< 0.00300	ug/l	*0.003*	UKAS	Sx	848
HCH -gamma	< 0.00300	ug/l	*0.003*	UKAS	Sx	848

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ENVIRONMENT AGENCY

Appendix 2
Analytical Procedure

INTRODUCTION

Concentrations of Perfluorohexanoic Acid in treated mains water, treated with PFH Ammonium Salt were diluted and then determined by LC-MS/MS. The detection of Perfluorohexanoic Acid was achieved by monitoring the ions 313.0 → 268.9.

APPARATUS, MATERIALS, REAGENTS AND SOLUTIONS

Apparatus and Glassware

- HPLC system: Agilent 1100 series HPLC system
- Various laboratory glassware
- Various pipettes

Materials

• Acetonitrile	Rathburn, HPLC grade
• Water	Rathburn, HPLC grade
• Treated Mains Water	In-house
• Ammonium Formate, 98%	Sigma Aldrich Ltd.
• Methanol	Rathburn, HPLC grade
• Perfluoroheptanoic acid, 98%	Sigma Aldrich Ltd.

Reagents and Solutions

50 mM Ammonium Formate

Ammonium Formate (3.153g) was weighed and dissolved in 1 litre of HPLC grade water.

HPLC Mobile Phase (and dilution solution)

Acetonitrile (950 mL) and 50 mM Ammonium Formate (50 mL) were mixed together.

Internal Standard solution

Approximately 10 mg of perfluoroheptanoic acid was weighed and dissolved in 100 mL of HPLC mobile phase to give a solution of 100 µg/mL. This solution was serially diluted to obtain a 0.1 µg/mL solution in the mobile phase.

PROCEDURES

Standard Solution Preparation

Initial Weighing of Stock Solutions

Duplicate analytical standard solutions (A and B), corrected for purity, were prepared in a suitable solvent in volumetric flasks. The amount weighed was at or slightly above the desired weight so that the solvent volume could be corrected to ensure that

these stock solutions and their subsequent dilutions were whole units, (e.g. 100.0 µg/mL).

Dilution into Calibration Range
One standard (A) was diluted over the range of concentrations to be used for the instrument calibration in the chosen injection solvent. The second standard (B) was diluted to a concentration near the mid-point of this calibration range, but was identical to a calibration point.

Standard Correlation
The standard solutions were correlated by injecting each of the two standards (A and B) at a concentration near the mid-calibration point, 5 times into the chosen analytical system. The two solutions were injected alternately in the run sequence. The results for the correlation were between 98 and 102 % of the mean. The calibration line was injected to ensure that the standards met the acceptance criteria.

Calibration Standards
Appropriate calibration standards were prepared in acetonitrile:50 mM Ammonium Formate (95:5 v/v) to cover the range 0.0002 to 0.02 µg/mL, with a lowest calibration level of 0.0002 µg/mL.

Sample Preparation
Samples of test solutions were diluted with the dilution solution to bring the concentrations within the calibration range of 0.0002 to 0.02 µg/mL. Aliquots (1.0 mL) of the final dilutions and the standard solutions were taken for the calibration, 0.1 mL of the 0.1 µg/mL internal standard solution was added to each of them. These solutions were submitted for analysis by LC-MS.

Conditions for LC-MS/MS Analysis

Analytical column	SeQuant, Zic-Hilic, 5µM 200A, 100 x 2.1 mm (id)
Guard column	N/A
In line filter (Acquity)	N/A
Column oven temperature	Nominal 40°C
Autosampler temperature	Nominal 4°C
Mobile phase	50mM Ammoinum Formate : Acetonitrile (5:95, v/v)
Flow rate	0.4 mL/min
Split	N/A
Switching Valve times	N/A
Slave pump solvent	N/A
Slave pump flow rate	N/A
Wash solvent 1	50mM Ammoinum Formate : Acetonitrile (5:95, v/v)
Weak Wash (Acquity)	(Mobile Phase)
Wash solvent 2	Methanol: Water (50 :50 v/v)
Strong Wash (Acquity)	
Injection mode (Acquity)	Partial loop with needle over-fill

Injection loop volume (Acquity)	10 µL (50 µL is our standard, 5 µL is our preferred alternative if lower carry-over required)
Needle placement	1.0 mm from bottom
Injection volume (Recommended)	10 µL (this may vary depending on instrument performance)

Waters Acquity

Weak Wash Volume (µL)	1000 (Range 200 to 5000)
Strong Wash Volume (µL)	1000 (Range 0 to 5000)

Mass Spectrometer Parameters API 4000

Mode of operation	Turbo IonSpray (negative ion) (MS/MS)
Collision gas setting (CAD)	8 (± 5) [Where a setting of 12 is approximately equal to 4.8 x 10-5 Torr]
Curtain gas setting (CUR)	25 psi (± 5)
Ion source gas 1 (GS1)	40 psi
Ion source gas 2 (GS2)	60 psi
IonSpray Voltage (IS)	-4500 V (± 2000 V)
Nebuliser Current (NC)	N/A
Temperature (TEM)	500°C (450-550°C)
Interface Heater Status	On
Analysis time	2 minutes (± 1 minute)

Compound name	Ions monitored (± 0.5 Da)	Dwell time (ms)	Declustering Potential (DP) Volts	Collision Energy (CE) Volts	Collision Cell Exit Potential (CXP) Volts
Perfluorohexanoic Acid	313.0 → 268.9	200	-10	-12	-7
Perfluoroheptanoic Acid	362.9 → 318.9	200	-10	-12	-7

Voltages and dwell times may have required optimisation therefore the above values were used for reference only.

Entrance Potential (EP)	-10 V (± 5 V)
Pause time	5 ms
Collision gas	Nitrogen

Study Specific Instrumentation

Name	Specification (Model, Brand etc)	Supplier/Manufacturer
Pump	Acquity – UPB LC-10ADVP	Waters Shimadzu
Autosampler	Acquity – UPS	Waters
Mass Spectrometer	API 4000	Applied Biosystems
Column oven	Acquity	Waters
Switching Valve	EMMA 10 port-2 position	VICI

A typical chromatogram of a 0.02 µg/mL Perfluorohexanoic Acid calibration standard is shown in Figure 1. The peak at the retention time of approximately 0.56 minutes is due to Perfluorohexanoic Acid. A typical calibration line for standard concentrations between 0.0002 and 0.02 µg/mL is shown in Figure 19.

CALCULATION OF RESULTS

The presence of Perfluorohexanoic acid in a sample was confirmed when the resulting peak, arising from the test sample, had the same chromatographic retention time as the appropriate standard. All peak measurements and calculations were performed using the current version of Analyst data system.

The calibration line was determined by plotting the responses from the calibration solutions (y) against the amount of Perfluorohexanoic acid injected (x) µg/mL to generate a quadratic curve, using a 1/x weighting.

$$y = ax2 + bx + c$$

Where:
a = the quadratic coefficient (curvature)
b = the linear coefficient (gradient)
c = the intercept

Concentrations of Perfluorohexanoic acid (x) µg/mL in the samples are calculated from their response using the equation:

$$x = \frac{-b \pm \sqrt{b^2 + 4ay - 4ac}}{2a} \times \frac{UP3 \times UP2}{UP1}$$

Where:
x = Amount (µg/mL)of Perfluorohexanoic Acid
y = Area of peak due to Perfluorohexanoic Acid
c = y axis intercept on calibration graph
b = linear coefficient (gradient) of calibration graph
a = quadratic coefficient (curvature) of calibration graph
UP1 = Sample Volume (mL)
UP2 = Final Volume (mL)
UP3 = Dilution Factor

Linearity of Response
The linearity of response of the LC-MS/MS system was determined with at least six different concentration standard solutions across the range 0.0002 to 0.02 µg/mL, with the lowest calibration level being 0.0002 µg/mL.

VALIDATION REPORT

This Analytical Procedure was validated using PFH Ammonium Salt (Perfluorohexanoic Acid). This validation was completed on 18 April 2008 and the validation raw data are archived with CLE study number 2901/001.

Linearity Check Procedure
The linearity of detector response to Perfluorohexanoic acid was determined over the concentration range 0.0002 to 0.02 µg/mL. Peak area response was used for the test substance signal, and the linearity correlation was derived from a quadratic least-squares regression, with a 1/x weighting. The coefficient of determination (using a 1/x weighting) was better than 0.99 for Perfluorohexanoic acid.

Precision and Accuracy Procedure
Five replicates of the lowest (0.0002 µg/mL), an intermediate (0.01 µg/mL) and the highest (0.02 µg/mL) calibration standards in (95+5 v/v) acetonitrile + 50mM Ammonium formate were assayed against a calibration line as described above.

The repeatability of the analysis at each standard concentration was evaluated by calculating the coefficient of variation (%) using the formula:

$$\text{Coefficient of Variation (\%)} = \frac{\text{Standard deviation}}{\text{Mean (measured) result of five determinations}} \times 100$$

For an LC-MS/MS method of analysis, a coefficient of variation ≤ 10% was considered acceptable.

The accuracy (%) of the analysis at each standard concentration was evaluated using the formula:

$$\text{Accuracy (\%)} = \frac{\text{[Mean (measured) result of five determinations]}}{\text{nominal concentration}} \times 100$$

For acceptability, the accuracy (%) must be within 90% to 110% of the nominal concentration of the standard at each level.

The results for the precision and accuracy of the instrument are shown in the following table.

Standard Concentration (µg/mL)	Concentration Found (µg/mL)	Recovery (%)	Mean Recovery (%)	Standard Deviation	Coefficient of Variation (%)
0.0002	0.000223	112			
0.0002	0.000199	100			
0.0002	0.000195	97.5	105	5.86	5.61
0.0002	0.000216	108			
0.0002	0.000212	106			
0.01	0.010378	104			
0.01	0.010808	108			
0.01	0.009695	97.0	104	5.38	5.17
0.01	0.011033	110			
0.01	0.010097	101			
0.02	0.021315	107			
0.02	0.020774	103			
0.02	0.020174	101	103	3.09	3.00
0.02	0.020976	105			
0.02	0.019767	98.8			

Injections of 2 Blank solutions showed no peaks for the test substance

METHOD CRITERIA

Analysis by LC-MS/MS will be considered successful only if the following criteria are met.

- At least 6 calibration standards will be used in the determination of each calibration line. A $1/x^2$ weighting should be used.
- A coefficient of determination (r^2) for each calibration line will be ≥ 0.99
- All test samples will be within the appropriate calibration standards range

Appendix 3
Statistical Analysis

COVANCE
THE DEVELOPMENT SERVICES COMPANY

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Date: 03 July 2008

Author: Emma Warriner
Reviewer: Jim Saul

To: Jonathan Burke

Re: 2901/001 : analysis of fish lengths and weights, hatching success and larvae survival

METHODS

Day 28 total lengths and dry weights were analysed using analysis of variance, fitting fixed effects for group and vessel within group. The test concentrations were compared with control using Dunnett's test. Linear contrasts were performed to compare the two vessels within each group. All tests were interpreted with two-sided risk. Where examination of residuals indicated that the untransformed data were not normally distributed, the data were analysed using the same methods after applying a rank transformation.

The number of hatched larvae and the number of larvae surviving at 28 days post-hatch were analysed using Fisher's exact test for pairwise comparisons of test concentrations with control. The tests were interpreted for decreasing incidence with increasing concentration.

The lowest observed effect concentration (LOEC) is defined as the lowest test concentration which produces a statistically significant adverse effect ($P<0.05$) when compared with the control. All test concentrations above the LOEC are required to show an effect that is statistically different from the control.

The no observed effect concentration (NOEC) is defined as the highest test concentration, immediately below the LOEC, which does not produce a statistically significant adverse effect ($P<0.05$) when compared with the control.

RESULTS

The comparison of the two vessels produce significantly different results for nominal concentration 0.0949 mg/L (dry weight, P<0.05). All other comparisons between vessels showed no significant differences (P≥0.05).

Table S1 – Comparisons with control

Nominal concentration (mg/L)	Total length	Dry weight	Hatching success	Larvae survival
0.0949	NS	NS	NS	NS
0.304	*	NS	NS	NS
0.972	NS	NS	NS	NS
3.11	NS	NS	NS	NS
9.96	NS	NS	NS	NS
Statistics	AR	A	f-	f-

* P<0.05
** P<0.01
*** P<0.001
NS not significant

A = ANOVA and Dunnett's
R = rank transformed data
f-= Fisher's exact (lower tail)

	Total length	Dry weight	Hatching success	Larvae survival
NOEC (mg/L)	#	≥9.96	≥9.96	≥9.96
LOEC (mg/L)	#	>9.96	>9.96	>9.96

NOEC and LOEC could not be determined

Appendix 4
Protocol Deviations

The study was conducted within a temperature range of 12 ± 1°C and not at the temperature ranges stated on page 2 of the Protocol.

The method of test media preparation deviated from that described on page 5 of the Protocol. The test media was prepared by combining concentrated stock solutions and dilution water continuously. Appropriate amounts were delivered to a mixing vessel which overflowed via a sidearm directly into each test vessel.

Fish weight measurements were carried out as dry weight measurements and not wet weight measurements as detailed on page 2 of the Protocol.

The experimental end date was greater than one month after the date stated in Protocol Amendment 1.

These deviations do not affect the integrity of the study.