

FINAL REPORT

Ecotoxicity of PFAS-Free Fire Fighting Foams: Fish and Aquatic Invertebrate Species

ER20-1518

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14. ABSTRACT The Department of Defense (DoD) is researching both performance and potential health effects of firefighting foams (F3) vs. aqueous film forming foams (AFFFs) that contain per- and polyfluoroalkyl substances (PFAS). This project used a multidisciplinary approach to assess both components of risk: exposure and toxicity. Exposure was assessed via non-targeted analysis of constituents and aging experiments. Toxicity was assessed via acute and long-term tests in freshwater and marine species. Taken together, results can be used to support decision-making with replacement chemistries.					
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Abbreviations

AFFF	Aqueous Film Forming Foams (containing PFAS)
ASTM	American Society for Testing and Materials
F3	Firefighting Foams (PFAS free)
FF	Fluorine Free
DoD	Department of Defense
EC15	Effective Concentration where 15% of individuals show a response
EC50	Effective Concentration where 50% of individuals show a response
ERDC	Engineer Research and Development Center
ESI+ / ESI-	Electrospray ionization (positive) and electrospray ionization (negative)
ESTCP	Environmental Security Technology Certification Program
GSH	Glutathione
h	hour
HC5	Predicted concentration where 5% of the species are expected to be impacted
HRMSMS	High-resolution mass spectrometry
ISO	International Organization for Standardization
LC50	Effective Concentration where 50% of individuals show a lethal response
LOEC	Lowest Observable Effects Concentration
mg/L	Concentration; milligrams per liter
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
NIST	National Institutes of Standards and Technology
NOAA	National Oceanographic and Atmospheric Administration
OECD	Organization for Economic Co-operation and Development

PFAS Per- and Polyfluoroalkylated substances

SERDP Strategic Environmental Research and Development Program

SSD Species Sensitivity Distribution

UPLC-HRMS Ultra-Performance Liquid Chromatography - High-Resolution Mass Spectrometry

US ACoE United States Army Corps of Engineers

US EPA United States Environmental Protection Agency

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Abstract

Introduction and Objectives

The Department of Defense (DoD) is actively engaging in research to identify firefighting foams (F3) that do not contain per- and polyfluoroalkyl substances (PFAS) as replacements for traditional Aqueous Film Forming Foams (AFFF) products that historically contained PFAS. The Strategic Environmental Research and Development Program (SERDP) is currently supporting a number of efforts to help determine environmental bioavailability and toxicity of selected candidate PFAS-free products in relation to traditional PFAS-containing AFFF.

Technical Approach

This project targeted four distinct research tasks: 1) Use non-targeted analysis (NTA Chemical Analysis) to identify multiple ions/fragments for each AFFF and F3 and use this information to develop a targeted LCMS protocol to assist in evaluating exposure; 2) Determine acute toxicity thresholds (LC50 values) for freshwater and marine taxa exposed to F3 products; 3) Use the acute toxicity thresholds to evaluate the possible persistence of toxicity as F3 products age, and; 4) Determine chronic / sublethal AFFF and F3 toxicity thresholds associated with growth or reproduction in marine and freshwater taxa.

Results

Based on acute toxicity thresholds, the marine species were often more sensitive to the F3 products than freshwater species, and the mud snail was the most sensitive species tested. F3-1 was generally observed to be the most toxic F3 product tested. F3-5 was generally the least toxic F3 product tested. Chemical markers were selected based on the non-targeted analysis and were used to demonstrate good correspondence between nominal and measured initial exposure concentrations. Chemical markers were also used to characterize stability of the test compounds over time in the exposures and revealed rapid degradation of F3s over 24-96 h of aqueous exposure, whereas the reference, PFAS-containing AFFF had greater chemical stability.

Two of the F3 products (F3-1 and F3-2) and the Reference AFFF product were tested to evaluate changes in toxicity as the compound aged (degraded). Acute toxicity of both F3 products and the reference decreased with aging. Chronic exposures were used to evaluate the impact of longer exposures and to identify sublethal effects (reductions in growth, development, and reproduction).

Several of the F3s were ranked as “very high” on the hazard scale for chronic impacts to marine algal growth. Juvenile mysids were observed to mature more slowly relative to controls and impacts to shell growth were observed in the clam *M. mercenaria*. Results for these tasks in both freshwater and marine species will be used to support the DoD’s decisions regarding the environmental specifications used to select appropriate replacement F3s.

Benefits

This work is some of the first to test the toxicity of F3 alternatives to marine and freshwater aquatic species. It provides the DoD with information needed for selecting suitable F3 alternatives and conducting ecological risk assessments.

Executive Summary

Introduction

This collaborative project including investigators from the National Oceanic and Atmospheric Administration (NOAA), the United States Army Corps of Engineers (US ACE) and the National Institutes of Standards and Technology (NIST) was selected to support the Strategic Environmental Research and Development Program's (SERDP) research efforts to understand the environmental fate, distribution and impacts of per- and polyfluoroalkyl substances (PFAS), including aqueous film forming foams (AFFF) that are critical tools for fire suppression on Department of Defense (DoD) installations. Based on the documented impacts and hazards associated with PFAS, the DoD mandated the replacement of PFAS containing AFFF products in the 2020 National Defense Authorization Act. In order to facilitate an effective pool of replacement products, a multi-year effort to develop and test (effectiveness and potential environmental hazard) these potential firefighting foam (F3) products was initiated.

A number of potential candidate products were considered and/or developed through SERDP and Environmental Security Technology Certification Program (ESTCP) -funded research to evaluate whether these products meet DoD performance requirements. These products (F3) are intended for use as alternatives to AFFF formulations containing fluoro-surfactants that have demonstrated toxicity to fish and aquatic invertebrates and have the potential for biomagnification in aquatic food webs. The main objectives of this research were to report ecotoxicological data and determine the relative toxicities of candidate F3s compared to a historic AFFF formulation.

Technology Approach

This research project addressed four tasks critical to developing the required acute and chronic toxicity thresholds. Task 1 developed the analytical protocols required to track six F3s (F3-1 through F3-6) and the Reference AFFF using LC-MS/MS. Task 2 assessed the acute toxicity thresholds of the selected F3s to both freshwater and marine fish and aquatic invertebrates. Task 3 assessed the possible persistence of toxicity over time (i.e., to account for effects of aging, transformation and degradation in the environment). Finally, Task 4 evaluated the possible impact of six F3 products and the Reference AFFF product on growth and reproduction of both freshwater and marine fish and aquatic invertebrates when chronically exposed to sublethal concentrations of these products.

Results and Discussion

This document details the results of these acute and chronic toxicity tests with the stated goal of supporting DoD decisions regarding F3 effectiveness and potential environmental hazards posed as F3s are approved for use and fielded.

Based on acute toxicity thresholds, the marine species were often more sensitive to the F3 products than freshwater species, and the mud snail was the most sensitive species tested. F3-1 was generally observed to be the most toxic F3 product tested. F3-5 was generally the least toxic F3 product tested. Chemical markers were selected based on the non-targeted analysis and were used to demonstrate good correspondence between nominal and measured initial exposure concentrations. Chemical markers were also used to characterize stability of the test compounds over time in the exposures and revealed rapid degradation of F3s over 24-96 h of aqueous exposure, whereas the Reference AFFF had greater chemical stability.

Two of the F3 products (F3-1 and F3-2) and the Reference AFFF were tested to evaluate changes in toxicity as the compound aged (degraded). Acute toxicity of both F3 products and the Reference AFFF decreased with aging. Chronic exposures were used to evaluate the impact of longer exposures and to identify sublethal effects (reductions in growth, development, and reproduction).

Several of the F3s were ranked as “very high” on the hazard scale for chronic impacts to marine algal growth. Juvenile mysids were observed to mature more slowly relative to controls and impacts to shell growth were observed in the clam *M. mercenaria*. Results for these tasks in both freshwater and marine species will be used to support the DoD’s decisions regarding the environmental specifications used to select appropriate replacement F3s.

Implications for Future Research

This research is some of the first to test the toxicity of F3 alternatives to marine and freshwater aquatic species, including fish, crustaceans, bivalves, and mollusks. It was designed to address the critical data gaps necessary to understand environmental risk. It provides the DoD with information needed for selecting suitable F3 alternatives and conducting ecological risk assessments. The core outcome of these studies was an improved understanding of the relative toxicity of F3 alternatives compared to short-chain AFFF formulations. A key outcome from this research was Task 3 which investigated the persistence of two F3 formulations and the Reference AFFF along with concurrent toxicity. Notably, acute toxicity of the two F3s and the Reference AFFF decreased with aging. The results from all project tasks are critical to understand the potential risks (including exposure and hazard) that new F3 formulations may pose to the environment. Future research should examine the causes of toxicity of these formulations and examine which components of the formulations are triggering a toxic response to the organisms tested.

1.0 Objectives

A number of F3 products are currently under development through SERDP funded research, and commercially available F3s are being tested under ESTCP to evaluate their ability to meet current DoD performance requirements. These products are intended for use as alternatives to AFFFs containing fluoro-surfactants that have demonstrated toxicity to fish and aquatic invertebrates and have the potential for biomagnification in aquatic food webs.

The main objectives of this research were to develop ecotoxicological data and determine the relative toxicities of candidate F3 products compared to a reference AFFF, by developing empirical data for acute and chronic toxicity to fish and aquatic invertebrates. It is critical to understand the potential risks (including exposure and hazard) that new products may pose to the environment. The benefit of this research is well defined, assisting SERDP in determining the relative hazard of several innovative F3s using a multiple species approach so that the effectiveness of each product can be weighed against its projected environmental hazard. The data generated meet data quality objectives for regulatory thresholds.

To understand potential toxicity of newly developed and novel F3 products, we evaluated the toxicity of six new products, as directed by SERDP, using multiple taxa in both freshwater and estuarine/marine conditions. The formulations tested for this project along with their corresponding code names used in this report are listed in Appendix C.

Toxicity test protocols followed published and widely accepted standard toxicity testing from recognized organizations (e.g., United States Environmental Protection Agency (US EPA), ASTM, Organisation for Economic Co-Operation and Development (OECD), and/or International Organization for Standardization (ISO)) and included one PFAS-containing Reference AFFF.

Exposure during the bioassays was monitored relative to initially spiked aqueous concentration by analysis of selected chemical features via targeted liquid chromatography tandem mass spectrometry (LC-MS/MS). Chemical features for LC-MS/MS analysis were prioritized via non-targeted analyses of fluorine-free AFFF alternatives by LC-high resolution mass spectrometry (HRMS). This research addressed the following technical objectives:

- 1) Determine the acute toxicities of the six F3 products and one Reference AFFF to both freshwater and marine fish and aquatic invertebrates;**
- 2) Determine persistence of toxicity over time (i.e., to account for effects of aging, transformation and degradation in the environment);**
- 3) Determine chronic toxicity to include sublethal endpoints, such as growth and reproduction to both freshwater and marine fish and aquatic invertebrates;**

These objectives were attached to 4 discrete research tasks:

- 1) Identification of F3 and Reference AFFF components via non-target analysis (NTA) and development of chemical protocols for exposure (Task 1)**
- 2) Acute toxicity testing in freshwater and marine species (Task 2)**
- 3) Persistence of F3 and Reference AFFF toxicity (Task 3)**
- 4) Chronic toxicity testing in selected freshwater and marine species (Task 4)**

2.0 Background

Fire suppression of fuels has historically used fluorinated foams that included per- and poly-fluoro alkylated compounds such as perfluorooctane sulfonate (PFOS) and perfluorooctanoate (PFOA). These AFFFs have proven to be effective in the suppression of fuel-based fires. Recent toxicological and epidemiological studies have led regulators and manufacturers to limit and then cease worldwide production of PFOS and PFOA in the early 2000s (European Parliament and Council, 2006; UNEP, 2009). AFFFs now include short-chained PFASs and fluorotelomer-based surfactants but concerns about persistence, bioaccumulation and toxicity remain (Field and Seow 2017; Langberg et al. 2019). These environmental concerns have resulted in new research initiatives aimed at the development of effective fluorine-free foams with reduced potential for ecological and human health impacts (Jia et al. 2019).

Many of these novel chemicals include variations of siloxanes. These products have proven useful in a variety of industrial uses but generally not as a component of AFFF designed to isolate combustible fuel from the atmosphere in the event of a spill or fire. Siloxanes have been cited in the literature to have very low toxicity. Silsesquioxane has been proposed as a potential pharmaceutical carrier due to the low cytotoxicity (Janaszewska et al., 2015; Almutary and Sanderson, 2017). Recently, siloxanes have been suggested as a potential novel class of F3s (Hetzler et al, 2014). There is very little data relating to the environmental fate of this class of compounds which limits our understanding of potential environmental risk associated with proposed new uses for these compounds.

Siloxanes are not the only class of fluorine-free chemicals that have been proposed as replacements for AFFFs. SERDP currently has at least nine active projects investigating development of fluorine-free alternatives and each class or chemical is lacking the requisite environmental toxicity data necessary to understand risk.

This project was designed to address the critical data gaps necessary to understand environmental risk of the candidate formulations under evaluation.

3.0 Materials and Methods

3.1 Preparing F3 and Reference AFFF testing stocks

The consistency and density of the six F3s and Reference AFFF are shown in Table 1. Densities were provided from material safety data sheets, if available. For foams without density data, ~1 g/mL was assumed.

Table 1. Identification and density of tested F3 and AFFF products.

Formulation	Type	Consistency	Density (g/mL)
Reference	AFFF	Liquid	1.01
F3-1	F3	Liquid	1.0 g/mL
F3-2	F3	Liquid	1.01 g/mL
F3-3	F3	Gel	1.03 g/mL
F3-4	F3	Liquid	not determined
F3-5	F3	Liquid	not determined
F3-6	F3	Gel	1.05 g/mL

All solutions were prepared gravimetrically. Foam concentrates and 3% stock solutions (in deionized water 18 M Ω -cm) were stored in sealed plastic (polypropylene; PP) containers at room temperature in the dark. Containers were cleaned prior to use by rinsing 3 times with deionized (DI) water, then 3 times with methanol (ACS or Optima grade), then allowed to dry in a fume hood.

All stocks were made as 3% stock solutions in DI Water (30,000 ppm mass-basis). All stock solutions, other than F3-5, were kept no longer than 12 days; the F3-5 stock solution was used within 1 day of preparation. Concentrations used in all testing were diluted from these 3% stocks.

3.2 Acute Toxicity Tests

Acute toxicity testing using both freshwater and marine taxa represented multiple ecological niches that ranged in freshwater from small invertebrate species (waterflea [*Ceriodaphnia dubia*], midge [*Chironomus dilutus*] and amphipod [*Hyaella azteca*]) to vertebrates (fathead minnow [*Pimephales promelas*] and rainbow trout [*Oncorhynchus mykiss*]). Toxicity testing using marine species included acute toxicity thresholds for sheepshead minnow (*Cyprinodon variegatus*), Eastern mud snail (*Tritia obsoleta*), mysid (*Americamysis bahia*), copepod (*Acartia tonsa*) and the Eastern oyster (*Crassostrea virginica*). Standard protocols and species information used in the determination of acute toxicity thresholds are listed in Table 2.

Table 2. Specific test protocols, duration and endpoints for acute toxicity testing.

Species	Common Name	Life Stage	Endpoints	Duration (d)	Protocol
FRESHWATER					
<i>Ceriodaphnia dubia</i>	daphnia	Juvenile (2 d old)	Survival	2	ASTM E 1295-01 (2013)
<i>Chironomus dilutus</i>	midge	Larvae second-instar (<10 d old)	Survival	10	ASTM E1706-19 (2016) modified for water-only testing (Kunz et al. 2017)
<i>Hyalella azteca</i>	amphipod	Juvenile (approximately 7–10 d old)	Survival	10	ASTM E1706-19 (2016) modified for water-only testing (Kunz et al. 2017)
<i>Oncorhynchus mykiss</i>	rainbow trout	Juvenile (< 3.0 grams)	Survival	4	US EPA 821/R-02/012 (2002); EPA OCSPP 850.1075 (2016)
<i>Pimephales promelas</i>	fathead minnow	Larvae (48 h old)	Survival	4	US EPA 821/R-02/012 (2002); EPA OCSPP 850.1075 (2016)
MARINE					
<i>Acartia tonsa</i>	pelagic copepod	Juvenile (7-9 d old)	Survival	2	Rabalais et al. 2018
<i>Americamysis bahia</i>	mysid	Larvae (24 h old)	Survival	4	USEPA 721-C-16-011 (2016)
<i>Crassostrea virginica</i>	Eastern oyster	Larvae (48 h old veliger)	Survival	2	ASTM E724-98 (2012)
<i>Cyprinodon variegatus</i>	Sheepshead minnow	Larvae (24 - 48 h old)	Survival	2	ASTM E729-96 (2014)
<i>Tritia obsoleta</i>	Eastern mud snail	Larvae (24 - 48 h old)	Survival	2	ASTM E729-96 (2014)

3.3 Chronic Toxicity Tests

The final task within the initial screening of possible F3 formulation toxicity focused on determining chronic toxicity thresholds for sublethal endpoints such as growth and reproduction. Data generated used standardized methods that targeted sublethal and chronic toxicity endpoints that greatly enhanced understanding of the potential environmental risks of these newly formulated foams. To accomplish this task, this project prioritized chronic toxicity endpoints in a variety of organisms. This included one invertebrate and one fish species from marine and freshwater taxa based on relative species sensitivity from the acute toxicity tests. Also, one freshwater alga and one marine alga were used to assess chronic toxicity. A marine bivalve was used to assess shell deposition after chronic exposures to the formulations. Initially, the proposed testing included determining chronic impacts on oyster (*C. virginica*) shell deposition. Consistent and applicable control data were not attainable so shell growth was measured in the larval clam (*Mercenaria mercenaria*). Standard protocols and species information used in the determination of chronic toxicity thresholds are listed in Table 3.

Chronic toxicity assessment using juvenile clams (*Mercenaria mercenaria*) followed methods based on Chung et al. (2007) and Stewart et al (2025). Briefly, treatment media was prepared for each concentration independently along with a control for each of the F3 tested and each treatment consisted of 5 replicates with n=30 clams in each. Water quality (temperature, salinity, dissolved oxygen and pH) were measured daily and maintained throughout the 21-day test. Survival was assessed in each replicate daily and then renewed with fresh exposure media. Shell lengths were determined by digitizing shells from a baseline cohort of *M. mercenaria* and then surviving clams at the end of the test. Baseline shell data was calculated from an initial cohort of 3 replicates (n=30). At the end of the 21-d exposure, all remaining clams were collected and dried and then digitally photographed. Major and minor axis (length) was measured and results were compared across treatments and to the baseline dataset.

Table 3. Specific test protocols, duration and endpoints for chronic toxicity testing.

Species	Common Name	Life Stage	Endpoints	Duration (d)	Protocol
FRESHWATER					
<i>Ceriodaphnia dubia</i>	daphnia	Juvenile (2 d old)	Survival, reproduction	7	ASTM E 1295-01 (2013); US EPA 821/R-02-013 (2002)
<i>Pimephales promelas</i>	fathead minnow	Larvae (48 h old)	Survival, growth	7	US EPA 821/R-02-013 (2002)
<i>Raphidocelis subcapitata</i>	Algae		Survival, Growth	4	OECD 201 (2011); ISO 8692 (2012); US EPA 821/R-02-013 (2002)
MARINE					
<i>Americamysis bahia</i>	mysid	Juvenile (7 d old)	Survival, Growth, Fecundity	7	USEPA-821-R-02-014 (2002)
<i>Cyprinodon variegatus</i>	Sheepshead minnow	Larvae (\leq 24 h old)	Survival, Growth	7	USEPA-821-R-02-014 (2002)
<i>Mercenaria mercenaria</i>	Hard Clam	Juvenile (retained on sieve >1 mm <2 mm)	Survival, Growth	21	Chung et al., 2007; Stewart et al., 2025
<i>Phaeodactylum tricornutum</i>	Diatom	cell densities 10,000 cell/mL	Survival, Growth	3	ISO 10253 (2006)

3.4 Aged F3 and Reference AFFF Toxicity Tests

The conceptual objective of this task was to evaluate the toxic impact of F3 formulations as the products age and undergo likely degradative processes (Figure 1). The taxa selected for the aged F3 studies were based on the data derived from the acute toxicity tests. *Ceriodaphnia dubia* (freshwater) and *Acartia tonsa* (marine) were selected as the species to test for the determination of possible changes in F3 toxicity with age.

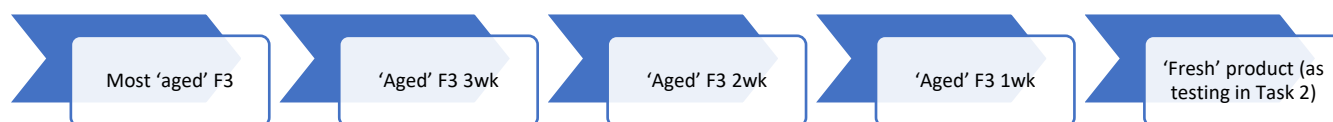


Figure 1. Conceptual model of persistence testing.

The Reference AFFF and the two most toxic F3 formulations (F3-1 and F3-2, relatively), based on the acute toxicity data, were chosen for this assessment. Age-factored toxicity in these formulations were evaluated using two different approaches. First, the toxicity was evaluated weekly for one solution of the highest concentration. Each week, a dilution series matching the concentration exposures used in the acute toxicity tests were created from this media. The second design allowed each formulation treatment level to age independently. Both designs evaluated toxicity with a formulation that was freshly made and then at 7, 14, 21 and 28 days aged.

Protocols following the 48-h acute toxicity tests using *C. dubia* and *A. tonsa* were performed at the end of each ageing interval. Briefly, 10 individuals were placed in each treatment replicate and mortality was recorded every 24 h in a control and 5 additional treatments. All treatment levels included at least 3 replicates. Test concentrations for *C. dubia* were prepared from an initial 3% solution of F3-1, F3-2, or Reference AFFF and diluted across treatments as 0.5X serial dilutions where nominal concentration targets were 29.6 [1X], 14.8, 7.4, 3.7, 1.85 mg/L (F3-1); 103 [1X], 51.5, 25.75, 12.9, 6.4 mg/L (F3-2), and; 416 [1X], 208, 104, 52, 26 mg/L (Reference AFFF).

A second design was also developed where each nominal treatment for the selected formulations was mixed independently and each concentration was then aged over 28 days. Test concentrations for *A. tonsa* were prepared from F3-1, F3-2 and Reference AFFF in a series of 0.5X concentrations: 25.0, 12.5, 6.25, 3.13, 1.56, 0 mg/L for all three formulations with five replicates of each concentration.

The experimental design was developed to evaluate 48-h mortality across a 28-d window. The first ageing framework prepared a bulk volume of the highest nominal concentration for the formulation (freshwater species *C. dubia* only). This bulk solution was held at test conditions across 28 days. An acute 48-h acute toxicity test was performed on day 0 and then again on days 7, 14, 21 and 28. This design effectively ages the highest treatment concentration across 28 days and any change that increases toxicity would result in a decreasing trend in LC50 estimates. A second design was also developed where each nominal treatment for the selected formulations was mixed independently and each treatment was then aged over 28 days. Again, 48-h toxicity tests were run on day 0, 7, 14, 21 and 28 for both a marine (*A. tonsa*) and freshwater (*C. dubia*) species (Figure 2).

During these aging exposures, selected 24-hour intervals were chosen for targeted chemical analysis to ensure consistent dosing. Water samples (10-15 mL) were collected at time 0 and time 24 h, diluted with an equivalent volume of methanol and stored at -20°C until analysis. These water samples were assessed for consistency across both freshwater and marine media as well as from both ageing designs (high treatment aging versus all treatment aging).

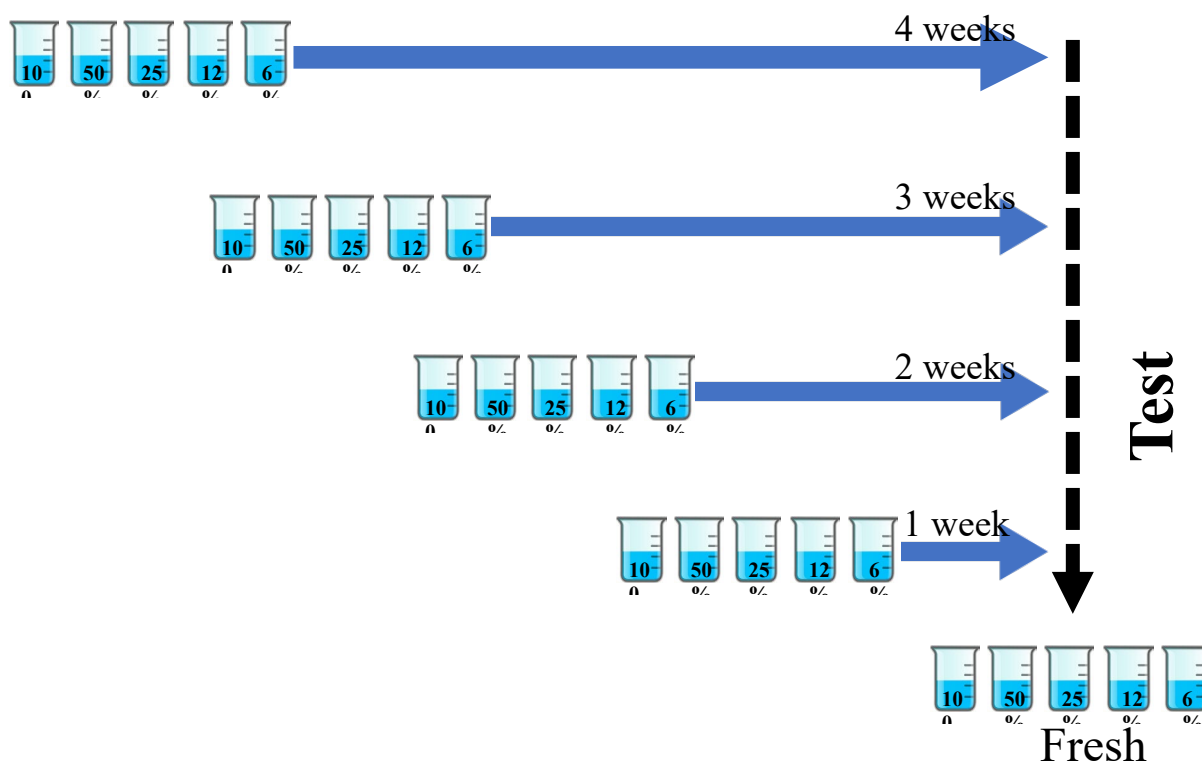


Figure 2. An example Experimental Design for testing the potential persistence of toxicity of F3 formulations and Reference AFFF.

3.5 Chemical Analysis

3.5.1 Chemical analysis – acute and chronic toxicity tests

Novel, targeted compounds and mass fragments from each F3 were assessed to provide a more robust approach to inform researchers and policy makers of the environmental behavior of these new products, in addition to better estimating actual concentrations in controlled exposure experiments. In order to accomplish this task, non-targeted chemical analysis was performed on each F3 using UPLC-HRMS (Vanquish UHPLC, QExactive Orbitrap, Thermo Fisher Scientific) instrumentation following the strategy of Place and Field (2012) for fluorine-containing AFFF products.

For chemical analysis, equal volume aliquots from each replicate exposure tank of the acute tests with *O. mykiss*, *C. variegatus*, and *C. dubia* were collected and composited in a 15-mL polypropylene tube. For example, 1 mL from each of 5 tanks. At least 1 mL was collected in total. The composited aliquots were then diluted 1:1 with methanol. The tubes were capped and sealed with parafilm. Samples were stored in a freezer (-20 °C) until analysis.

Samples from US ACoE/ERDC acute *C. dilutus* and *O. mykiss* (freshwater) tests were received by NCCOS. The samples from ERDC represented exposure media collected at time 0 h and after 96 h of exposure for *C. dilutus* acute testing and 0 h and 48 h for *O. mykiss*. Samples from marine acute testing were collected at time 0 h and 24 h from tests with *C. variegatus*. These samples were received and stored at 4°C in Charleston, SC and represented all 7 formulations received for marine and freshwater toxicity testing. In preparation for analysis, NIST and NCCOS staff diluted these samples as if for use according to the supplied protocols (3% AFFF product) developed under Task 1 (see Appendix B) and target masses were determined using an Agilent 1100 HPLC paired to an AB Sciex API 4000 triple quadrupole mass spectrometer.

3.5.2 Chemical Analysis of aged F3 Formulations

Sample preparation and analysis was based on internal NIST methods described below. A 1 mL sub-aliquot of each sample was transferred to a 15 mL-polypropylene centrifuge tube, centrifuged for 10 min at 13,400 rpm and then 600 µL of the supernatant was transferred to an amber glass autosampler vial. Samples were stored at -20°C until analysis.

Liquid chromatographic separation was performed with a Vanquish ultra-performance liquid chromatograph (UPLC; Thermo Fisher Scientific, Inc., Waltham, MA; NIST# N116479, N116487, and N116483) using an Agilent Zorbax diol guard column (4.6 x 12.5 mm x 6 µm; P.N. 820950-911) coupled to an Agilent Infinity Lab Poroshell 120 EC-C18 analytical column (4.6 x 100 mm x 2.7 6 µm; P.N. 695975-902). The LC mobile phases included 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The flow rate was 0.5 mL/min with a max pressure limit of 600 bar. The mobile phase gradient program was as follows: 95% A, 50% A at 4 min, 5% A from 14-32 min, 95% A at 32.5 min and held at 95% A until 42 min. LC settings are summarized in Table 4.

Table 4. LC settings used for the assessment of non-target analysis of aging formulation media.

UPLC Parameter	Setting
Column temperature	35°C
Eluent A	DI water + 0.1% formic acid (v/v)
Eluent B	Methanol + 0.1% formic acid (v/v)
Eluent flow rate	0.5 mL/min
Injection volume	10 µL
Autosampler temperature	10°C
Gradient	5% B at 0 min, 50% B at 4 min, 95% B 14 – 32 min, 5% B at 32.5 – 42 min

Mass spectrometric detection was performed with a Thermo Q-Exactive Quadrupole-Orbitrap Hybrid Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA; NIST# N116503). Heated electrospray ionization (ESI) was used with the source parameters detailed in Table 5. Samples were all analyzed by a MS1 and MS1 to ddMS2 methods in ESI+ and ESI-mode; experimental settings are provided in Table 6.

Table 5. Mass spectrometer source parameters used for the assessment of non-target analysis of aging formulation media.

Source Parameter	ESI +/-
Fragmentation In-source CID	None
Resolution	35,000
Microscans	1
Lock masses	off
AGC target	1e6
Sheath gas flow rate	53
Aux gas flow rate	14
Sweep gas flow rate	3
Spray voltage (kV)	3.50
Capillary temp. (°C)	269
S-lens RF level	50.0
Aux gas heater temp (°C)	438

Table 6. Mass spectrometry method settings used for the assessment of non-target analysis of aging formulation media in ESI+ and ESI – modes.

Orbitrap Parameter	Setting (Full MS→ddMS²)	
General	ESI +	ESI -
Source type	ESI	ESI
Use lock masses	Best	Best
Chromatographic peak width (FWHM)	8 s	8 s
Runtime	0 to 42 min	0 to 42 min
Polarity	Positive	Negative
Default charge state	1	1
Full MS ESI	ESI +	ESI -
Resolution	35,000	35,000
AGC target	1e6	1e6
Maximum IT	100 ms	100 ms
Scan range	74 to 1110 m/z	74 to 1110 m/z
dd-MS2/dd-SIM	ESI +	ESI -
Resolution	17,500	17,500
AGC target	1e5	1e5
Maximum IT	50 ms	50 ms
Loop count	5	5
Isolation window	1.0 m/z	1.0 m/z
Fixed first mass	--	--
(N)CE/ stepped NCE:	10, 20, 30	10, 20, 30
dd Settings	ESI +	ESI -
Minimum AGC target	8.00e4	8.00e4
Peptide Match	preferred	--
Exclude Isotopes	on	on
Dynamic exclusion	5.0 s	5.0 s

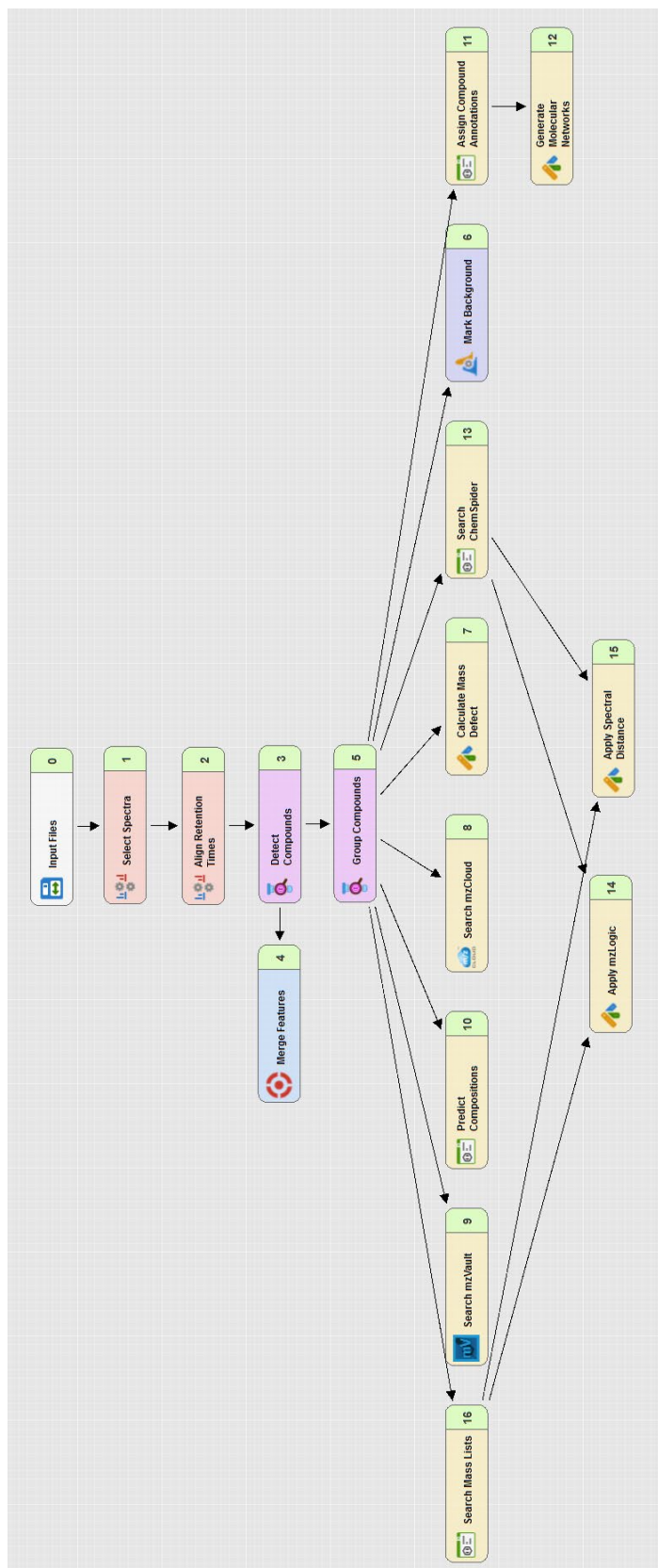


Figure 3. Workflow diagram for Compound Discoverer detailed in Appendix A.

The instrument was calibrated in both polarities using the manufacturer’s calibration solution within 5 days of sample analysis. Non-targeted HRMS data were extracted in Compound Discoverer version 3.1. The workflow steps and associated settings are shown in Figure 3 and Appendix A, respectively.

4.0 Results and Discussion

4.1 Acute Toxicity Tests

The results of the acute toxicity testing are summarized in Table 7. In many cases, effects on mortality were not seen even at the highest exposure concentration of 100 mg/L, and an LC50 could not be calculated. For these results, a '>100 mg/L' entry is represented in this report.

Evaluating the acute LC50 values for these aquatic fish and invertebrate species relative to the other ER20 SERDP projects supports the general findings that the Reference AFFF is often the least acutely toxic formulation tested. As Table 7 shows, LC50 values and a measure of variability (95% Confidence Interval (CI)) were calculated using the Trimmed Spearman Karber method. Acute LC50 values for *H. azteca*, *T. obsoleta*, *P. promelas*, and *C. variegatus* were previously published in Jones et al (2022) using “drm” function of the *drc* package in R. The marine species were often more sensitive to the F3 compounds than freshwater species. F3-1 was generally observed to be the most toxic F3 compound tested. F3-5 was generally the least toxic F3 compound tested. The amphipod, *H. azteca*, and the midge, *C. dilutus*, were the least sensitive species tested. The mud snail, *T. obsoleta*, and the copepod, *A. tonsa*, were often the most sensitive species tested.

Table 7. Summary of acute toxicity thresholds calculated for six F3s and one Reference AFFF in mg/L. LC50 is defined as the concentration calculated to result in a 50% decrease in survival of the test species. LC50 values were calculated using the Trimmed Spearman-Kärber method.

		Reference LC50 (95% CI)	F3-1 LC50 (95% CI)	F3-2 LC50 (95% CI)	F3-3 LC50 (95% CI)	F3-4 LC50 (95% CI)	F3-5 LC50 (95% CI)	F3-6 LC50 (95% CI)
Freshwater	<i>P. promelas</i>	>100	14.1 (12.12-16.24)	35.3 (35.9-36.6)	70.1 (68.85-71.33)	31.97 (29.67-34.45)	>100	35.71 (34.23-37.25)
	<i>O. mykiss</i>	>100	8.92 (8.76-9.07)	27.36 (23.31-32.13)	68.75 (66.52-71.06)	17.84 (17.52-18.15)	>100	35.36 (35.36-35.36)
	<i>H. azteca</i>	>100	36.39 (32.85-40.31)	>100	>100	>100	>100	>100
	<i>C. dilutus</i>	>100	46.96 (38.06-57.93)	>100	>100	>100	>100	>100
	<i>C. dubia</i>	>100	9.11 (9.36-11.27)	40.71 (35.16-47.14)	65.52 (55.67-77.09)	32.42 (29.48-35.65)	>100	68.79 (49.68-95.26)
Marine	<i>C. variegatus</i>	>100	5.91 (5.33-6.57)	13.91 (11.91-16.24)	9.08 (7.04-11.69)	15.01 (13.55-16.75)	31.7 (8.6-54.5)	12.68 (10.38-15.49)
	<i>C. virginica</i>	>100	3.66 (3.3-4.06)	21.4 (14.0-32.7)	41.7 (36.6-47.6)	41.4 (35.7-47.9)	>100	39.6 (34.2-45.8)
	<i>T. obsoleta</i>	19.9 (17.1-22.7)	5.33 (4.76-5.95)	1.40 (1.10-1.78)	2.85 (2.30-3.34)	1.80 (1.49-2.18)	5.01 (4.10-6.11)	1.60 (1.12-2.28)
	<i>A. bahia</i>	>100	16.92 (15.92-17.98)	>100	66.61 (59.34-74.76)	>100	>100	93.89 (75.54-116.71)
	<i>A. tonsa</i>	14.9 (8.18-21.6)	9.23 (6.92-12.30)	16.5 (9.94-23.0)	8.85 (6.77-10.9)	9.98 (0.583-19.4)	6.81 (3.22-14.42)	10.1 (7.38-13.72)

The following figures characterize the response of individual freshwater fish and invertebrate species for the six F3 products and the Reference AFFF using a hazard classification. The US EPA (2025) defines a hazard classification for acute exposures as an LC50 or EC50 >100 mg/L (LOW), 10-100 mg/L (MODERATE), 1-10 mg/L (HIGH) and <1.0 mg/L (VERY HIGH)).

C. dilutus acute survival testing (Figure 4)

- The LC50 (Median Lethal Concentration) for *Chironomus dilutus* was lowest for F3-1.
- The LC50 for F3-1 is ranked as MODERATE hazard.
- For *C. dilutus*, all other tested formulations resulted in LC50s greater than 100 mg/L (or a hazard potential classified as LOW).

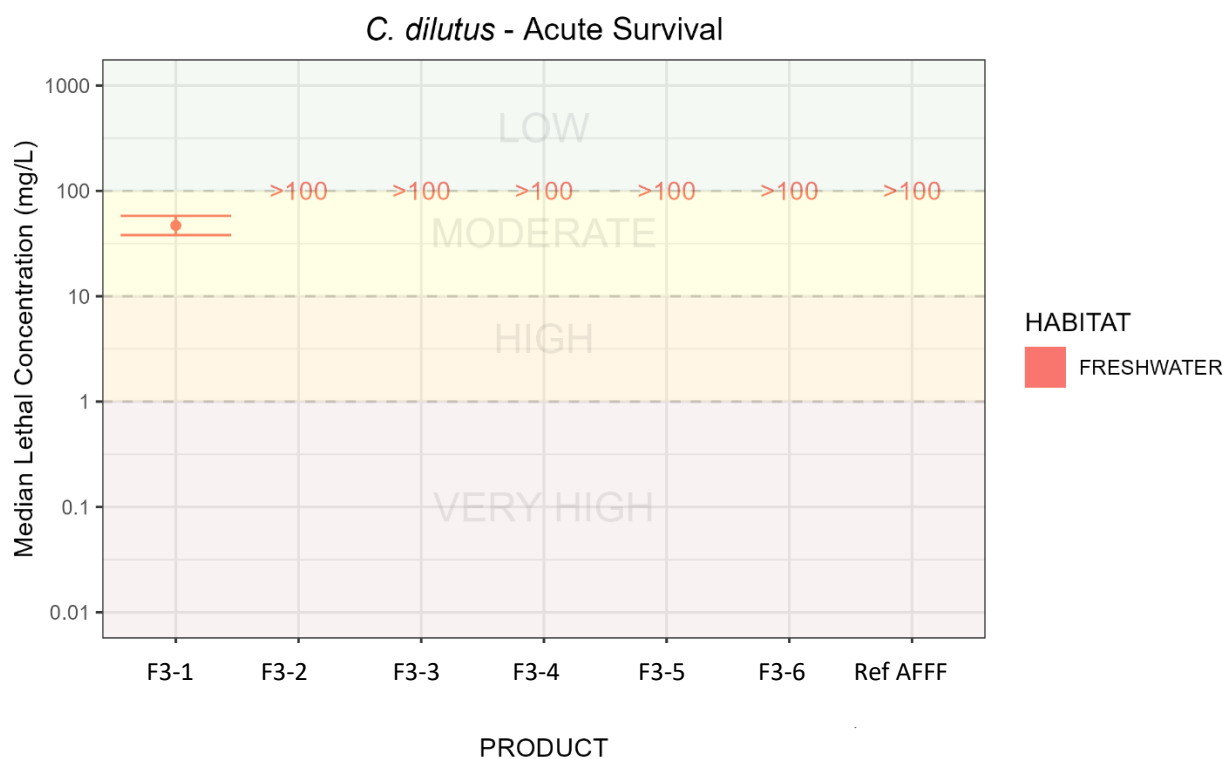


Figure 4. Acute toxicity testing results using the midge larvae *C. dilutus*.

H. azteca acute survival testing (Figure 5)

- Observations from *H. azteca* testing resulted in a similar dataset when compared to *C. dilutus* testing.
- The LC50 was lowest for F3-1, and was ranked as MODERATE hazard.
- All other tested formulations resulted in LC50s greater than 100 mg/L (classified as LOW hazard).

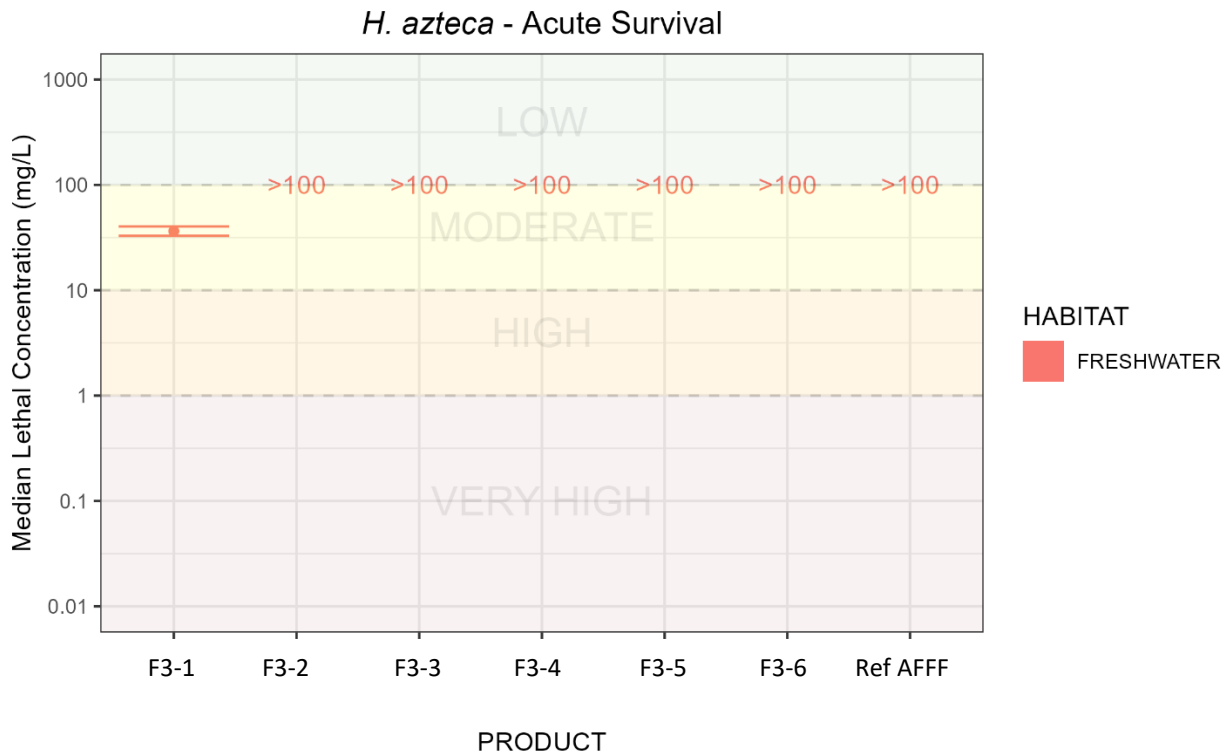


Figure 5. Acute toxicity testing results using the freshwater amphipod *H. azteca*.

C. dubia acute survival testing (Figure 6)

- *C. dubia* 48-h survival testing resulted in LC50 values that ranged from 9.11 mg/L to >100 mg/L.
- The F3-1 formulation was again found to have the lowest calculated LC50 (9.11 mg/L) and is HIGH hazard classification.
- Several formulations were found to have LC50s that would be categorized as MODERATE and these are F3-2, F3-3, F3-4 and F3-6.
- Reference AFFF and F3-5 testing resulted in LC50s that would fall into the LOW category.

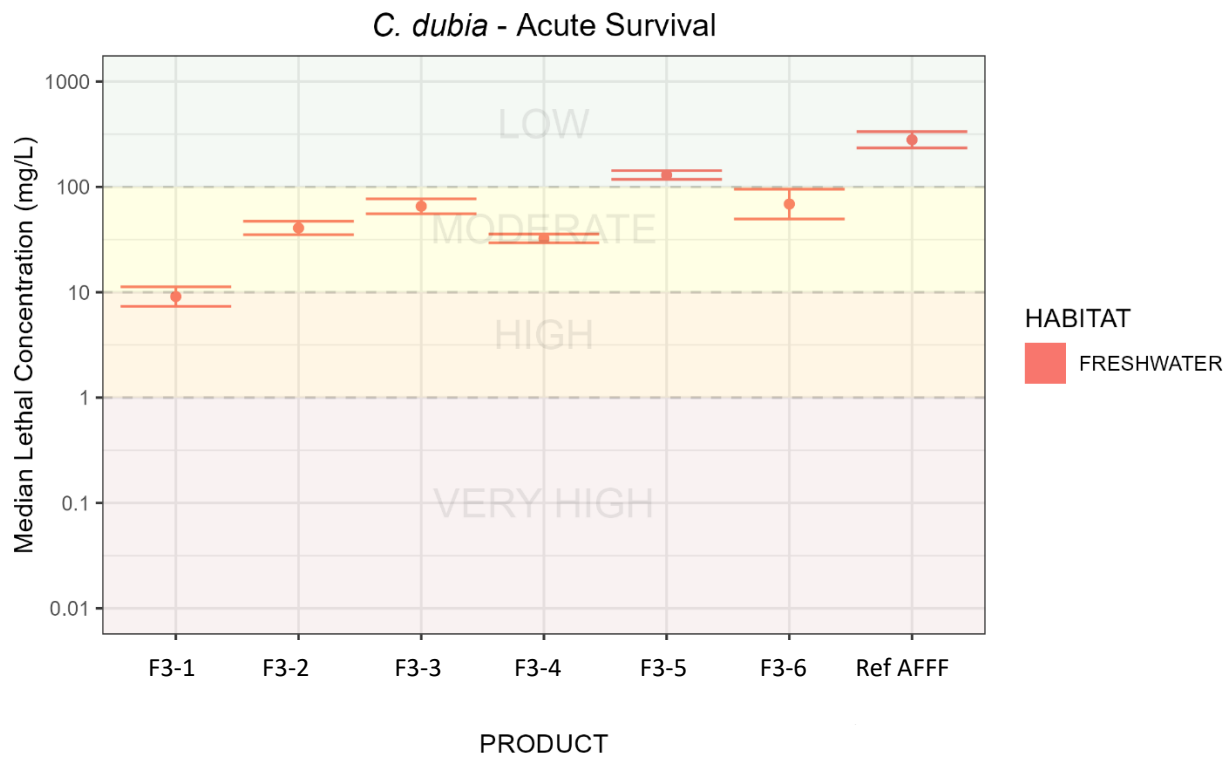


Figure 6. Acute toxicity testing results using the daphnid *C. dubia*.

P. promelas 96-h acute survival testing (Figure 7)

- *P. promelas* acute testing resulted in LC50 values that ranged from 14.1 mg/L to >100 mg/L.
- F3-1 was found to have the lowest LC50 value and was the most toxic compound to *P. promelas*.
- Five of the seven formulations tested would be labeled with a hazard potential of MODERATE.
- Reference AFFF and F3-5 testing resulted in LC50s that exceeded 100 mg/L (classified as LOW hazard).

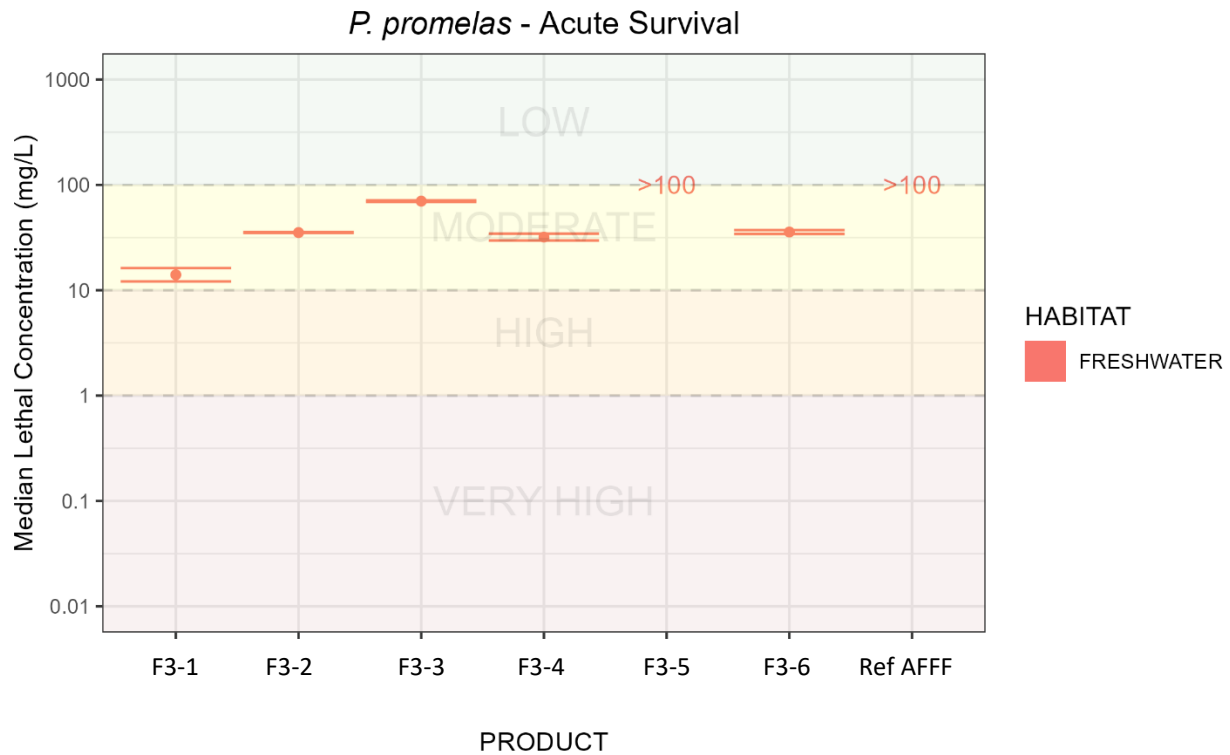


Figure 7. Acute toxicity testing results using the larval freshwater fish *P. promelas*.

O. mykiss 96-h acute survival testing (Figure 8)

- *O. mykiss* testing resulted in a similar pattern of toxicity categorization as *P. promelas* results.
- The LC50 for F3-1 was the lowest calculated for this species and fell just within the upper threshold of the HIGH hazard categorization.
- F3-2, F3-3, F3-4, and F3-6 formulations had LC50s within the MODERATE hazard classification.
- Reference AFFF and F3-5 testing resulted in LC50s that exceeded 100 mg/L and were accordingly labelled with a LOW hazard classification.

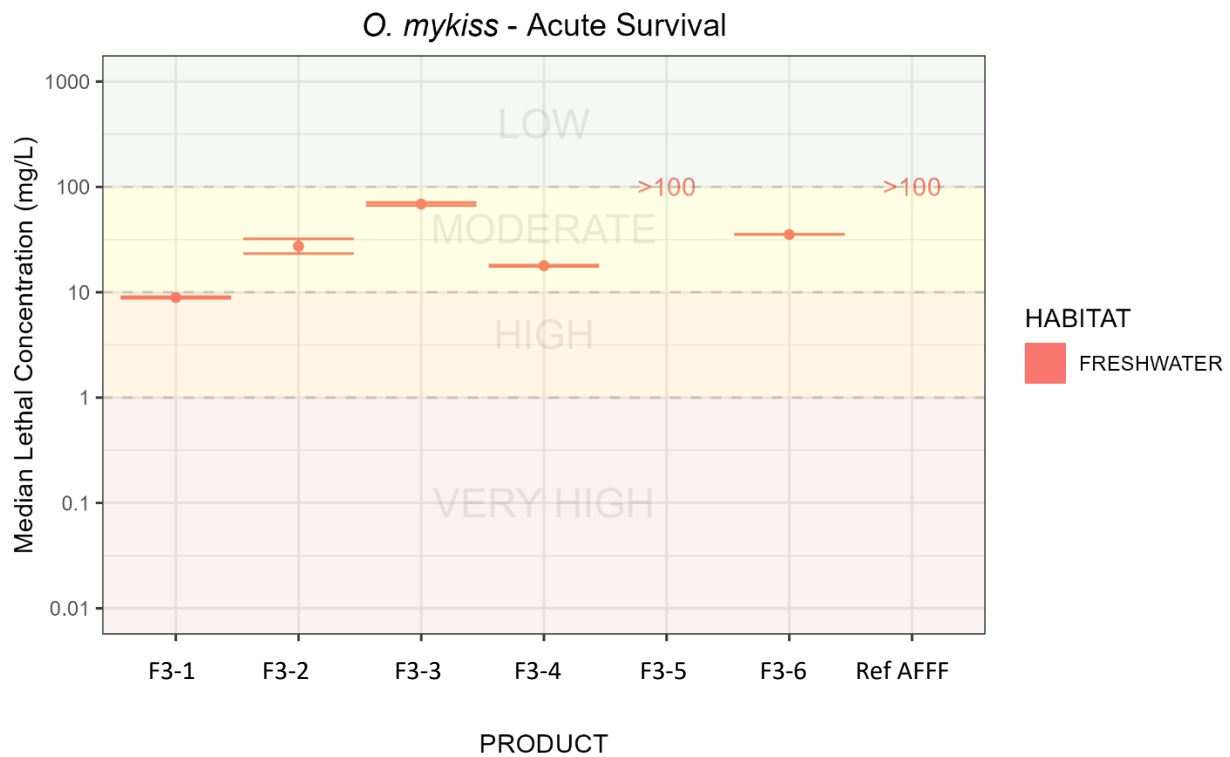


Figure 8. Acute toxicity testing results using the larval freshwater fish *O. mykiss*.

The following figures characterize the response of individual marine/estuarine fish and invertebrate species for the six F3 products and the Reference AFFF using the same EPA hazard classification as before.

Cyprinodon variegatus 48-h larval survival testing (Figure 9)

- Calculated LC50s were found to range from 5.91 mg/L for F3-1 to >100 mg/L for Reference.
- The LC50 from F3-1 testing was the lowest calculated (and classified in the HIGH hazard category, as did F3-3).
- Toxicity thresholds for four F3 products resulted in hazard classification of MODERATE (F3-2, F3-4, F3-5, F3-6), although F3-2, F3-4, and F3-6 had toxicity values near the concentration that delineates the MODERATE and HIGH hazard classification categories.
- The Reference AFFF was the only compound with a classification of “LOW” (LC50 >100).

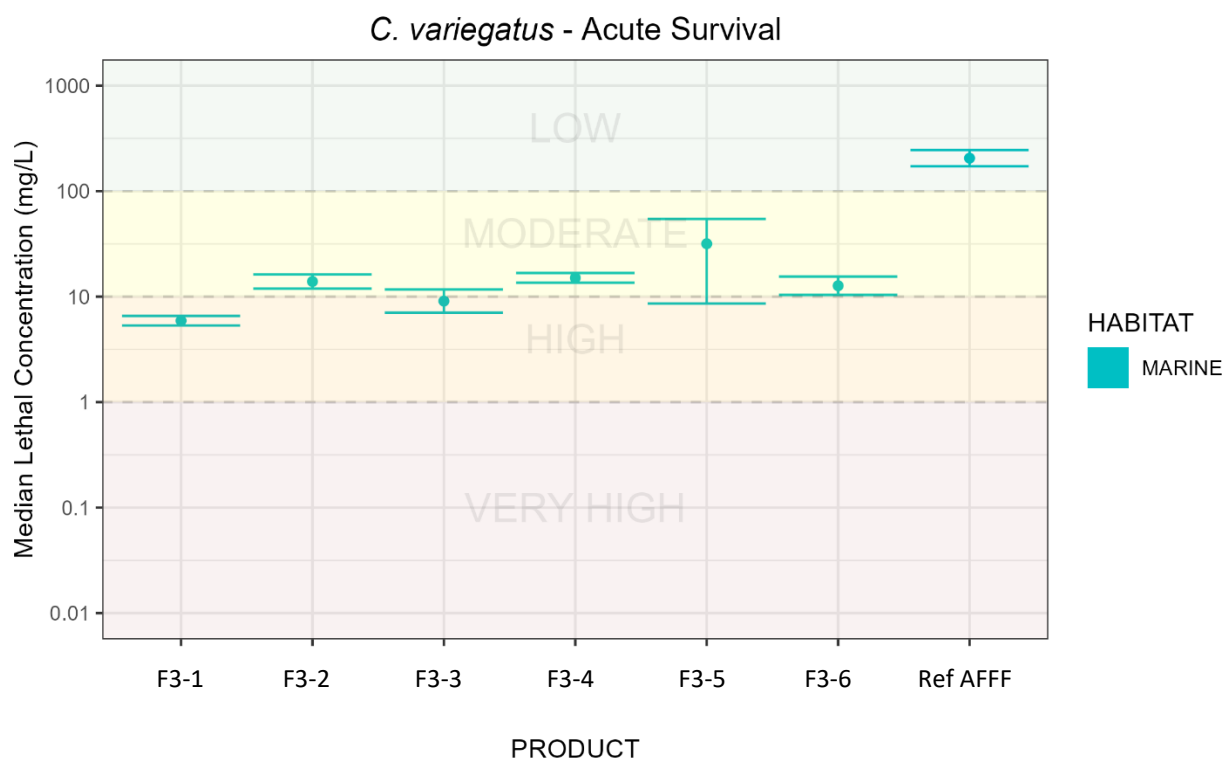


Figure 9. Acute toxicity testing results using the larval marine fish *C. variegatus*.

Americamysis bahia 96-h larval survival testing (Figure 10)

- All calculated LC50s were found to range from 16.92 mg/L for F3-1 up to >100 for F3-2, F3-4, F3-5, and Reference AFFF.
- F3-1 again has the lowest LC50 (most toxic) and Reference AFFF is associated with the highest LC50.
- Five of seven formulations were categorized with a hazard potential of LOW.
- The Reference AFFF resulted in an LC50 categorized as LOW.

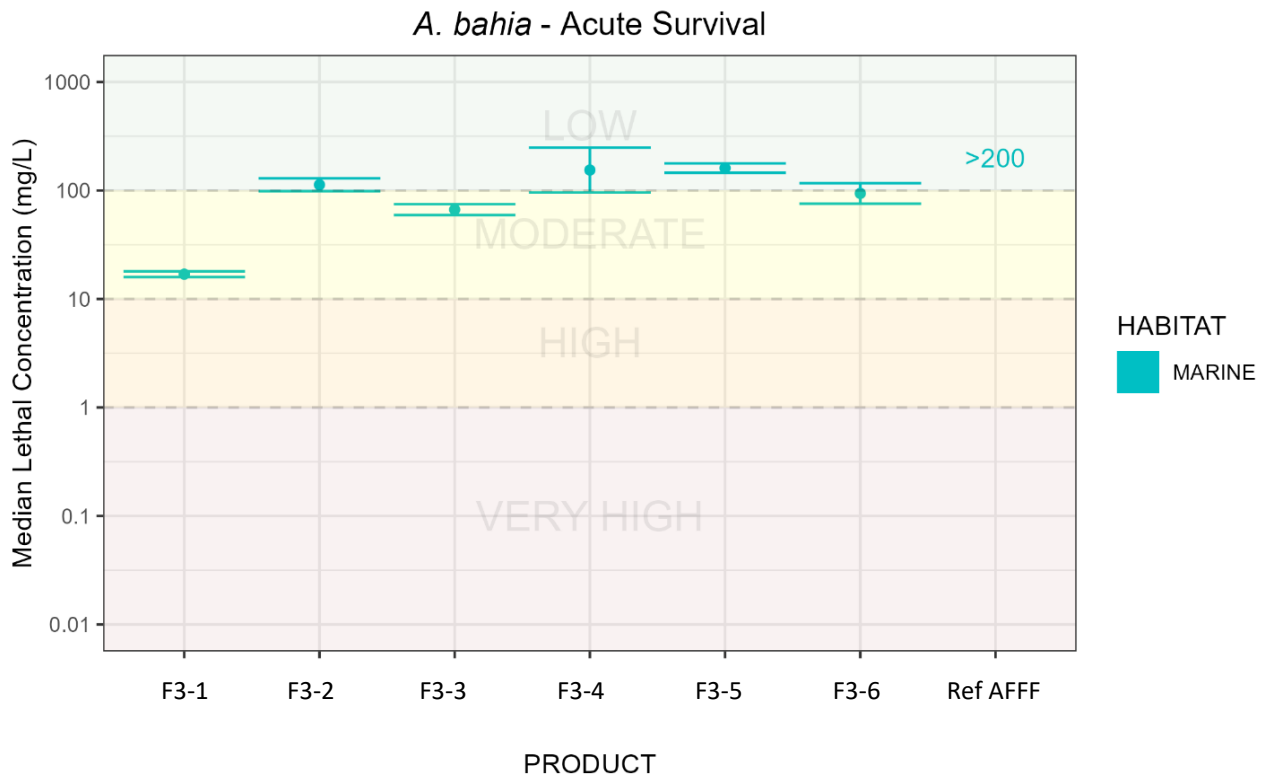


Figure 10. Acute toxicity testing results using the juvenile mysid *A. bahia*.

Tritia obsoleta 48-h larval survival testing (Figure 11)

- All calculated LC50s were found to range from 1.40 mg/L for F3-1 up to 19.9 mg/L for Reference AFFF, making *T. obsoleta* the most sensitive species tested in this study.
- Six of seven formulations were categorized with a hazard potential as “HIGH”.
- The F3-1 formulation was again found to have the lowest calculated LC50 (1.40 mg/L).
- The Reference AFFF resulted in an LC50 categorized as “MODERATE”.

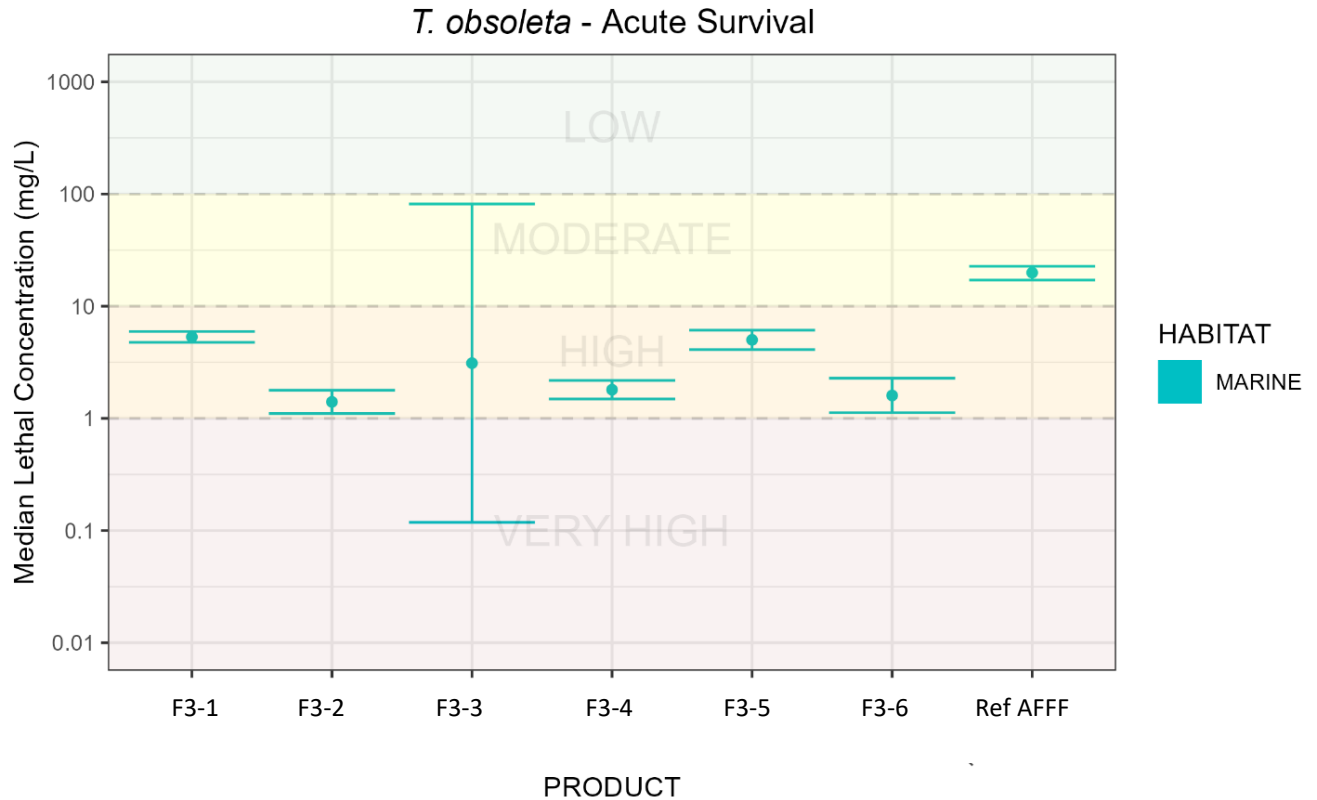


Figure 11. Acute toxicity testing results using the larval mud snail *T. obsoleta*.

Acartia tonsa 48-h survival testing (Figure 12)

- All calculated LC50s were found to range from 6.81 mg/L for F3-5 up to 16.5 for F3-2 making *A. tonsa* the second most sensitive species tested.
- Three of seven formulations were categorized with a hazard potential of HIGH.
- F3-5 is the lowest LC50 and F3-2 is associated with the highest LC50.
- Testing with Reference AFFF resulted in an LC50 categorized as MODERATE.

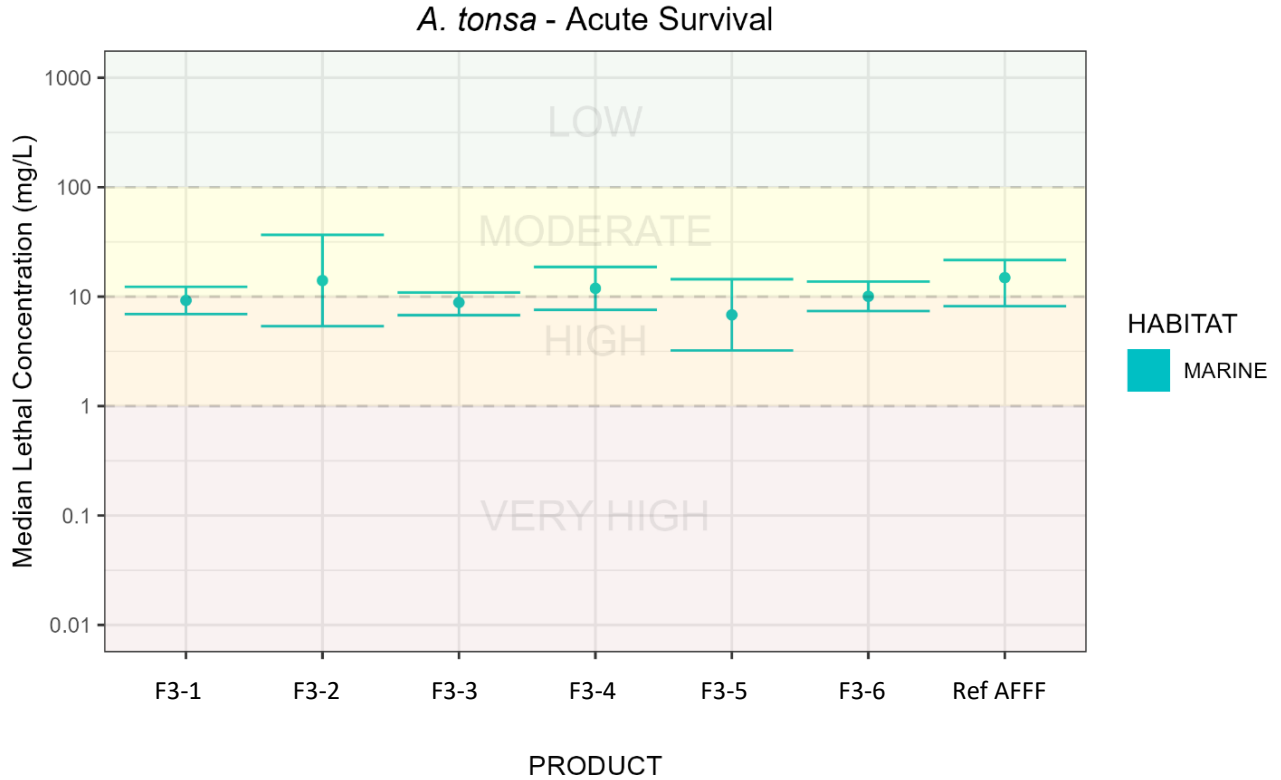


Figure 12. Acute toxicity testing results using the juvenile amphipod *A. tonsa*.

Crassostrea virginica 48-h survival testing (Figure 13)

- All calculated LC50s were found to range from 3.66 mg/L for F3-1 up to >100 mg/L for Reference AFFF and F3-5.
- Four of seven formulations were categorized with a hazard potential of MODERATE, F3-1 having the lowest LC50 was categorized as HIGH.
- Testing with Reference AFFF resulted in an LC50 categorized as LOW.

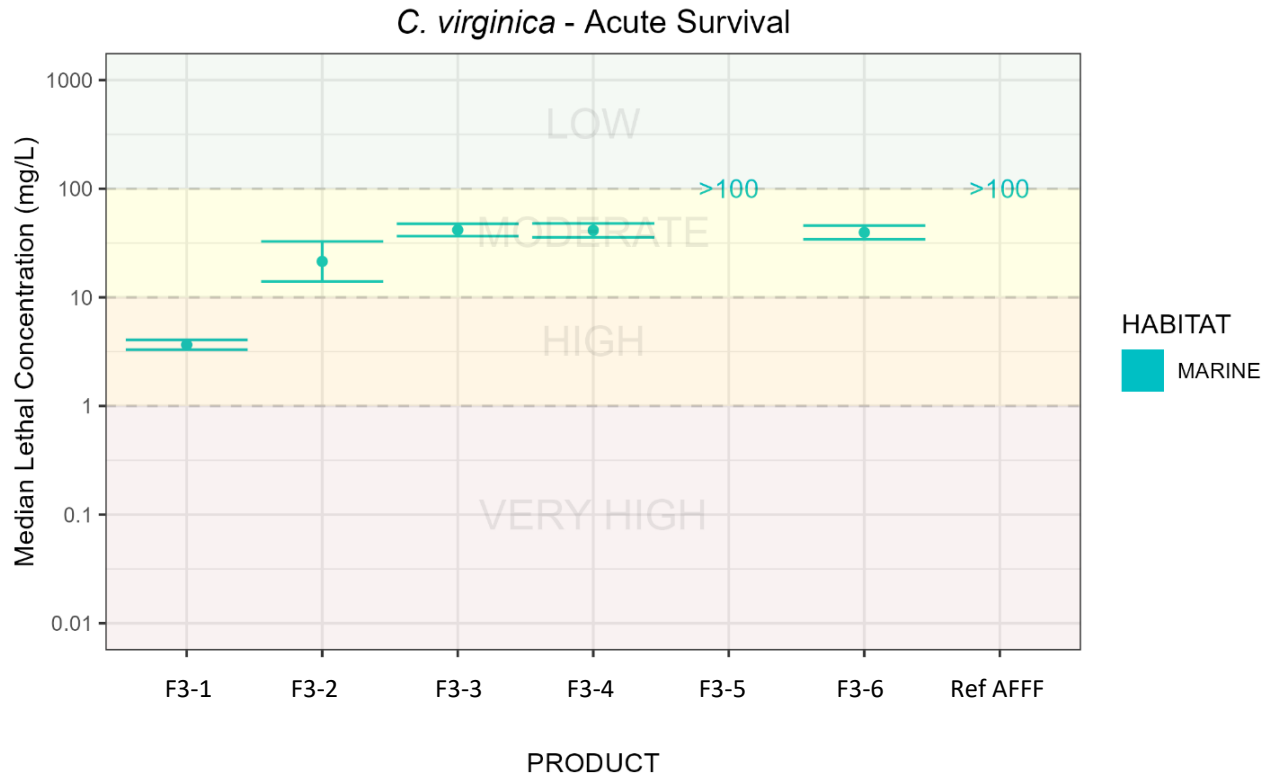


Figure 13. Acute toxicity testing results using the larval oyster *C. virginica*.

Summary of Acute Toxicity Tests

With the acute toxicity testing, three larval fish species were tested (two freshwater and one marine). This multi-species comparison was useful in evaluating potential differential toxicity that may be possible between freshwater and marine waters. The LC50s for the freshwater species, *P. promelas* and *O. mykiss*, when exposed to the Reference AFFF, were both calculated to be >100 mg/L, as was the threshold concentration for the estuarine species, *C. variegatus* (LC50 >100 mg/L) (Table 7). The consistent response of larval fish to the Reference AFFF was encouraging and it is reasonable to assume that there is not a specific toxicity response that is modified when Reference AFFF is found in freshwater versus marine water among these three

species of fish. The lack of significant differences between Reference AFFF LC50s (for fish) was confirmed using the LC50 ratio test (following Wheeler et al., 2006).

While similar, the F3-2 product formulation LC50 values were found to be significantly different between the two freshwater fish species, where the LC50 for *P. promelas* (35.3 mg/L +/- 0.65; mean +/- std. deviation) was higher than the *O. mykiss* LC50 (27.36 mg/L +/- 2.39; Ratio Test, $p < 0.05$). The LC50 for the saltwater species *C. variegatus* (13.91 mg/L +/- 1.17) was found to be significantly lower than the results for both *O. mykiss* and *P. promelas* LC50 ($p < 0.05$).

There were no significant differences in LC50 values between the two freshwater fish species for the remaining formulations tested. The marine *C. variegatus* toxicity thresholds were observed to be significantly lower than the freshwater species for F3-2, F3-3, F3-4, F3-5 and F3-6 (Table 8).

4.2 Chronic Toxicity Tests

Chronic toxicity endpoints (EC50 and EC15) were calculated using the dose response curve package (drc) in R (CRAN, 2016). The EC15 was selected to act as a surrogate for the Lowest Observable Effects Concentration (LOEC). These toxicity thresholds were then categorized using the same US EPA hazard classification system as the acute test results (Table 8). Hazard categories based on chronic exposure were determined by the calculated LOEC where hazard labels were assigned by EC15 (or LOEC) by LOW (>10 mg/L), MODERATE (1-10 mg/L), HIGH (0.1-1 mg/L) and VERY HIGH (<0.1 mg/L).

Table 8. Summary of EC15 values and hazard rankings for chronic toxicity testing with freshwater and marine species.

<i>Freshwater Species</i>				<i>Marine Species</i>			
<i>P. promelas</i>	Measured Endpoint:	individual mass (mg)		<i>C. variegatus</i>	Measured Endpoint:	individual mass (mg)	
	EC15 (mg/L)	Standard Error	Hazard Category		EC15 (mg/L)	Standard Error	Hazard Category
F3-1	0.002	0.019	Very High	F3-1	>4.5	---	---
F3-2	>25	---	---	F3-2	>7.0	---	---
F3-3	>50	---	---	F3-3	>4.5	---	---
F3-4	>50	---	---	F3-4	> 5.0	---	---
F3-5	>100	---	---	F3-5	>4.5	---	---
F3-6	>25	---	---	F3-6	>4.5	---	---
Ref AFFF	>100	---	---	Ref AFFF	> 60	---	---
<i>R. subcapitata</i> *	Measured Endpoint:	Growth rate (divisions/day)		<i>P. tricornutum</i>	Measured Endpoint:	Growth rate (divisions/day)	
	EC15 (mg/L)	Standard Error	Hazard Category		EC15 (mg/L)	Standard Error	Hazard Category
F3-1	4.9	0.45	Moderate	F3-1	0.004	0.002	Very High
F3-2	12.0	0.34	Low	F3-2	0.251	0.017	Very High
F3-3	45.9	3.57	Low	F3-3	0.950	0.133	Very High
F3-4	>100	---	Low	F3-4	0.989	0.141	High
F3-5	47.8	2.3	Low	F3-5	0.042	0.015	Very High
F3-6	78.3	10.0	Low	F3-6	1.088	0.097	Very High
Ref AFFF	>100	---	Low	Ref AFFF	0.840	0.189	High
<i>C. dubia</i>	Measured Endpoint:	Total Neonates		<i>M. mercenaria</i>	Measured Endpoint:	Shell growth	
	EC15 (mg/L)	Standard Error	Hazard Category		EC15 (mg/L)	Standard Error	Hazard Category
F3-1	> 6	---	---	F3-1	1.95	0.58	Moderate
F3-2	2.01	1.06	Moderate	F3-2	10.98	2.63	Low
F3-3	9.48	3.29	Moderate	F3-3	37.35	2.99	Low
F3-4	11.9	3.55	Low	F3-4	8.04	1.20	Moderate
F3-5	48.5	18.1	Low	F3-5	> 100	---	Low
F3-6	30.6	43.2	Low	F3-6	12.38	1.83	Low
Ref AFFF	20.1	12.47	Low	Ref AFFF	>100	---	Low
				<i>A. bahia</i>	Measured Endpoint:	Development (% immature)	
					EC15 (mg/L)	Standard Error	Hazard Category
				F3-1	1.44	0.49	Moderate
				F3-2	>90	---	---
				F3-3	13.05	6.67	Low
				F3-4	10.67	5.05	Low
				F3-5	9.59	3.91	Moderate
				F3-6	>100	---	---
				Ref AFFF	>100	---	---

Freshwater taxa

P. promelas

Growth was used as the chronic endpoint in the 48 h old larval fish *P. promelas* exposed for 7 days. Significant effects on survival after 7-day exposure were only observed for F3-1 and F3-4, which yielded LC50 values (95% C.I.) of 16.1 (10.9-21.3), and 25.1 (16.7-33.5), respectively. The only compound that had a significant effect on growth was F3-1. The EC50 value for growth (mass, dry weight) was 0.81 mg/L (Table 9). The EC15 values calculated for F3-1 was <0.1 mg/L, and thus considered VERY HIGH on the hazard scale, however the standard error was also high (Table 8).

Table 9. Larval *P. promelas* toxicity threshold estimates for chronic impacts where growth (mass of surviving fish in each treatment) would be reduced by 50% 7-day (Fuller et al., 2024).

Formulation	EC50 (mg/L)	95% C.I.
F3-1	0.81	---
F3-2	>25	---
F3-3	>50	---
F3-4	>50	---
F3-5	>100	---
F3-6	>25	---
Reference AFFF	>100	---

C. dubia

The daphnid *C. dubia* was exposed to F3 formulations for 7 days and survival and neonate production was recorded. The resulting datasets were analyzed for the determination of the EC15 for reproduction where the EC15 is a rough estimate for the traditional lowest observable effect concentration (LOEC). The classification of hazard potential was then performed using the EC15 (Figure 14). The chronic hazard classification for F3-2 and F3-3 were found to be MODERATE and the other compounds ranked as LOW hazard (Table 8).

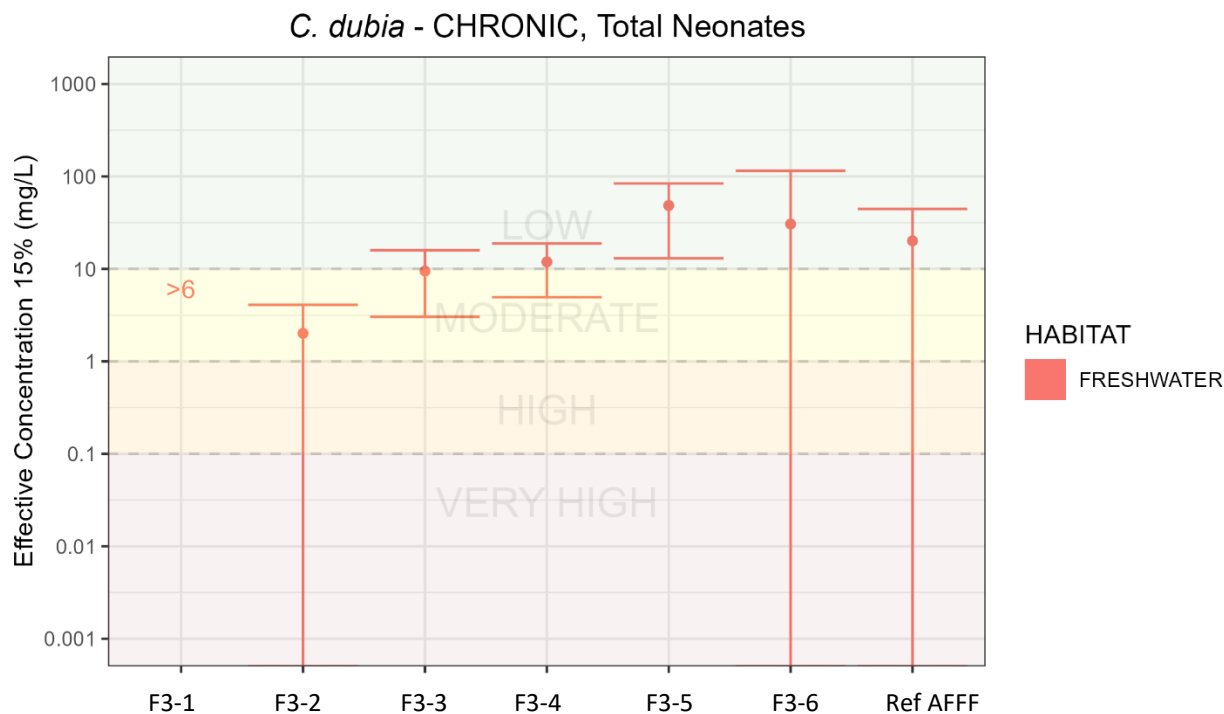


Figure 14. *C. dubia* 7-day EC15 for reproduction (total number of neonates produced) results after exposure to six F3s and a Reference AFFF.

R. subcapitata

Assessing algal taxa allows for population level assessments in a short amount of time. Cell density (cells/mL) were measured across 96 h of F3 exposure and a corresponding EC50 and EC15 were calculated to identify concentrations that would lead to a reduction in population growth rate. For the freshwater algae, growth rate EC50 values were >100 mg/L for the Reference AFFF and F3-4, 100.4 mg/L for F3-6, 82.7 mg/L for F3-3, 70.9 mg/L for F3-5, 14.7 for F3-2, and 13.5 mg/L for F3-1. The EC15 values corresponded to hazard rankings of MODERATE for F3-1 and LOW for the other F3s and the Reference AFFF (Table 8). *R. subcapitata* was the least sensitive freshwater species tested.

Saltwater taxa

C. variegatus

The estuarine fish, *C. variegatus*, was tested for chronic impacts to growth in a 7-day exposure. There were no significant effects on survival or fish growth for any of the tested compounds. The

highest concentrations tested were 60 mg/L for Reference AFFF, 7 mg/L for F3-2, 5 mg/L for F3-4, and 4.5 mg/L for F3-1, F3-3, and F3-5. The range of concentrations was selected to be sublethal based on acute 48 h *C. variegatus* exposures. Because growth was not a more sensitive endpoint than survival, we were not able to calculate chronic effects thresholds or assign hazard rankings for this species (Table 8).

M. mercenaria

Hard clams (*Mercenaria mercenaria*) were exposed for 21 days and survival, lengths of individual shell axes, and wet and dry weights were determined. F3-1, F3-2, F3-3, F3-4, and F3-6 negatively impacted clam growth over the exposure period while F3-5 and the Reference AFFF had no observable effect, with EC50 values for growth (shell length) of 5.81 mg/L +/- 2.13 (est. +/- std. error) for F3-1, 36.4 mg/L +/- 27.2 for F3-2, 13.0 +/- 1.13 for F3-4, 22.7 +/- 3.03 for F3-6, 94.0 mg/L +/- 13.7 for F3-3, and >100 mg/L for Reference AFFF and F3-5 (Figure 15). The EC15 values indicated most compounds would have hazard rankings of LOW, with F3-1 and F3-4 ranking as MODERATE hazard (Table 8).

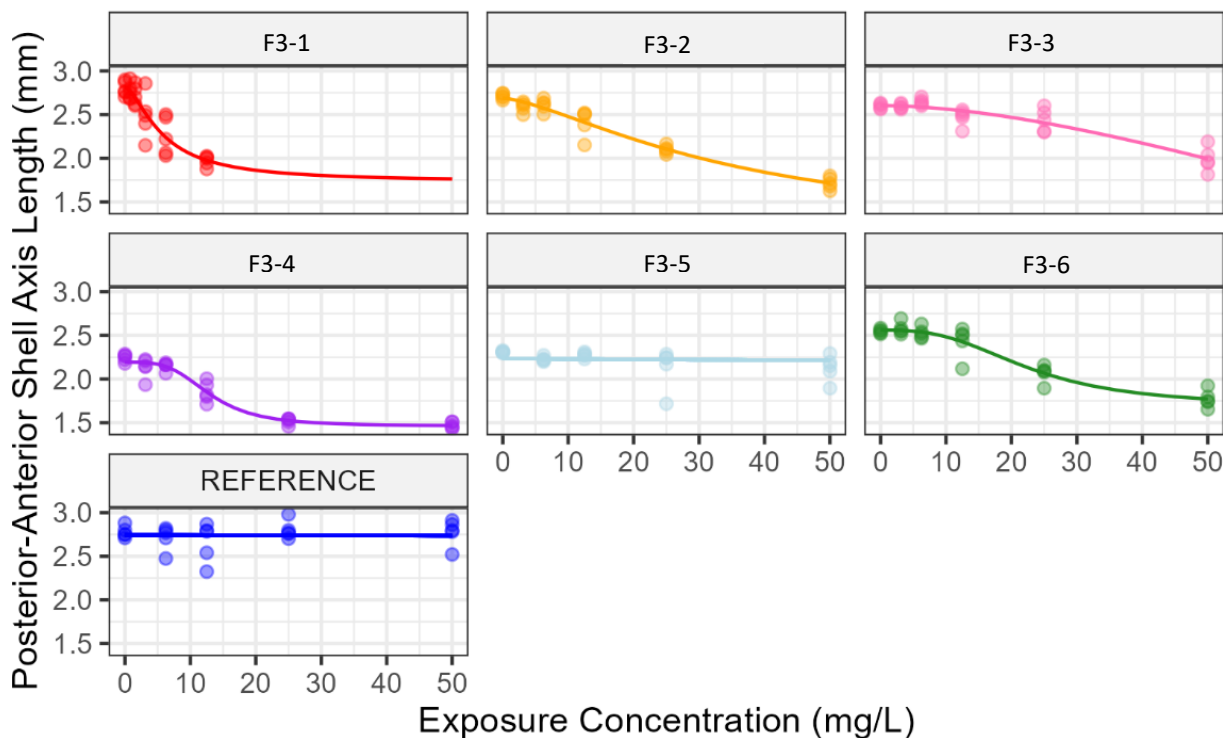


Figure 15. Changes in *M. mercenaria* axis-length as a result of exposure to six F3 products and the Reference AFFF.

A. bahia

Mysids, 7-day old *A. bahia*, were exposed to each of the formulations for 7 days to determine chronic impacts on growth and development. Initial range finding assays were conducted to determine the sublethal concentrations to use in the definitive testing. The definitive testing used the following range of concentrations: 1-16 mg/L (F3-1), 5.625-90 mg/L (F3-2), 4.375-70 mg/L (F3-3), 6.25-100 mg/L (F3-4, F3-5, F3-6, and Reference AFFF). All control survival exceeded the recommended 80% guideline for test acceptability. The minimal survival for any treatment in these chronic assays was 47%. Surviving individuals were assessed at the end of the test in terms of mass and maturity. There was a significant effect of F3 treatment on mysid mass (mg/mysid) after the 7-day exposure at 70 mg/L for F3-3, at ≥ 6.25 mg/L for F3-4, F3-5 and F3-6, at ≥ 5.625 for F3-2, and at ≥ 1 mg/L for F3-1 (ANOVA, Dunnett's test $p < 0.05$). Significant effects on mass were not determined for the Reference AFFF (Figure 16A). The 7-day EC50 values (est. +/- std. error) for mass were 8.6 +/- 4.9 mg/L for F3-4, and 27.7 +/- 7.6 mg/L for F3-6, and 59.1 +/- 10.0 for the Reference AFFF. An EC50 value for mass could not be estimated for F3-1, F3-2, F3-3, F3-5, or the Reference AFFF, and EC15 values were not determined for mass.

There were also significant delays in juvenile to adult development observed in most F3 exposures, with a greater proportion of immature *A. bahia* observed at the end of the test compared to controls. There was a significant effect of F3 treatment on mysid development at ≥ 4 mg/L for F3-1, ≥ 25 mg/L for F3-5, at ≥ 35 mg/L for F3-3, at ≥ 50 mg/L for F3-4 and the Reference AFFF (ANOVA, Dunnett's test $p < 0.05$) (Figure 16B). F3-6 had 31% immatures in the control thus treatment effects were not determined for that F3. The response for F3-2 was variable and no significant effect was determined. The 7 d EC50 values (est. +/- std. error) for development were 6.8 +/- 3.9 mg/L for F3-1, 16.2 +/- 5.4 mg/L for F3-3, 41.2 +/- 20.5 mg/L for F3-4, 62.9 mg/L +/- 170.2 for F3-5. An EC50 value for development was not determined for F3-2, F3-6, or the Reference AFFF. The EC15 values for development are consistent with other chronic endpoints which show F3-1 to be the most toxic F3, and the Reference AFFF to be the least toxic compound tested (Table 8).

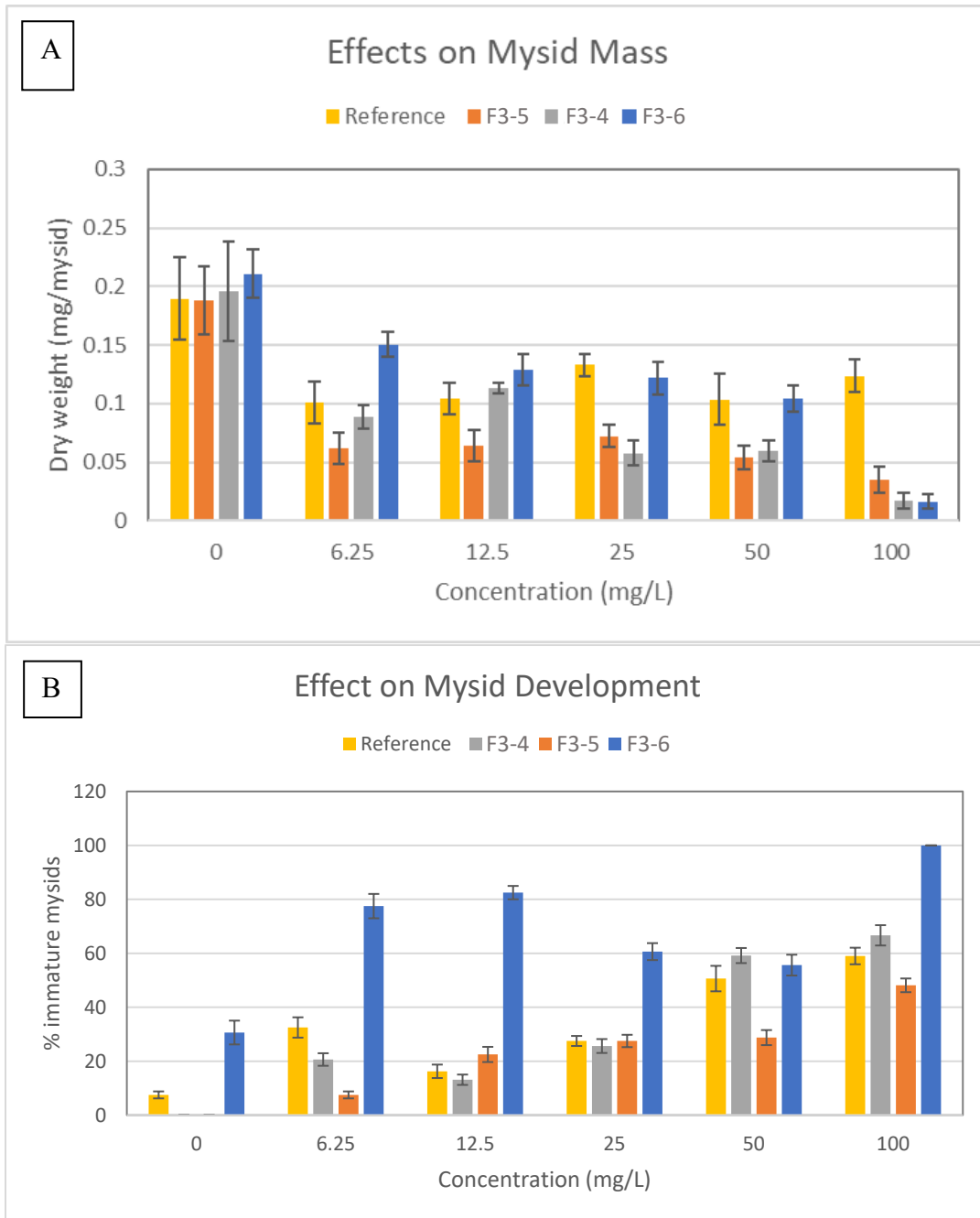


Figure 16. Average mass of surviving mysids after 7-day exposure (A) and percent of immature mysids after 7-day exposure (B) to F3-4, F3-5, F3-6 and Reference AFFF. These four formulations represented graphically because they had the same tested concentrations.

P. tricornutum

The marine diatom *P. tricornutum* was also selected for chronic testing. Initial range finding assays were conducted to determine the concentrations to use in the definitive testing. The

definitive testing used the following range of concentrations: 0.03125-0.5 mg/L (F3-1), 0.1563-2.5 mg/L (F3-5), 0.3125-5.0 mg/L (F3-2), 1.56-25.0 mg/L (F3-3, F3-4, F3-6, and Reference AFFF). Cell density (cells/mL) was measured across 72 h of F3 exposure. The endpoints calculated were the EC50 (50% reduction in growth rate relative to control treatments) (Table 10) and the EC15, which is an estimate of the lowest effects concentration (Table 8). The Reference AFFF was found to have the highest EC50, thus it was the least toxic in terms of algal toxicity, and F3-1 had the lowest EC50 and was thus the most toxic to *P. tricornerutum*. Based on the hazard categories for chronic exposure, Reference AFFF, F3-3, F3-4, and F3-6 were considered MODERATE, while F3-1, F3-2, and F3-5 were considered HIGH. At higher exposure concentrations, several of the F3 compounds (F3-2, F3-3, F3-4, F3-6) caused reductions in cell densities to levels below the starting inoculation of 10,000 cells/mL, thus zero population growth was occurring (Figure 17). Toxicity thresholds for the marine species were significantly lower than those determined with the freshwater algal species tested; indicating greater sensitivity to F3 formulations.

Table 10. *Phaeodactylum tricornerutum* estimated EC50 values [concentrations that correspond to a 50% decrease in growth (divisions/day) relative to controls].

Formulation	EC50 (mg/L)	Standard Error	95% Confidence Interval
F3-1	0.129	0.024	0.082-0.176
F3-2	0.408	0.017	0.374-0.440
F3-3	2.672	0.193	2.289-3.056
F3-4	3.061	0.231	2.604-3.518
F3-5	0.674	0.095	0.485-0.863
F3-6	2.107	0.102	1.905-2.309
Reference AFFF	5.231	0.546	4.149-6.313

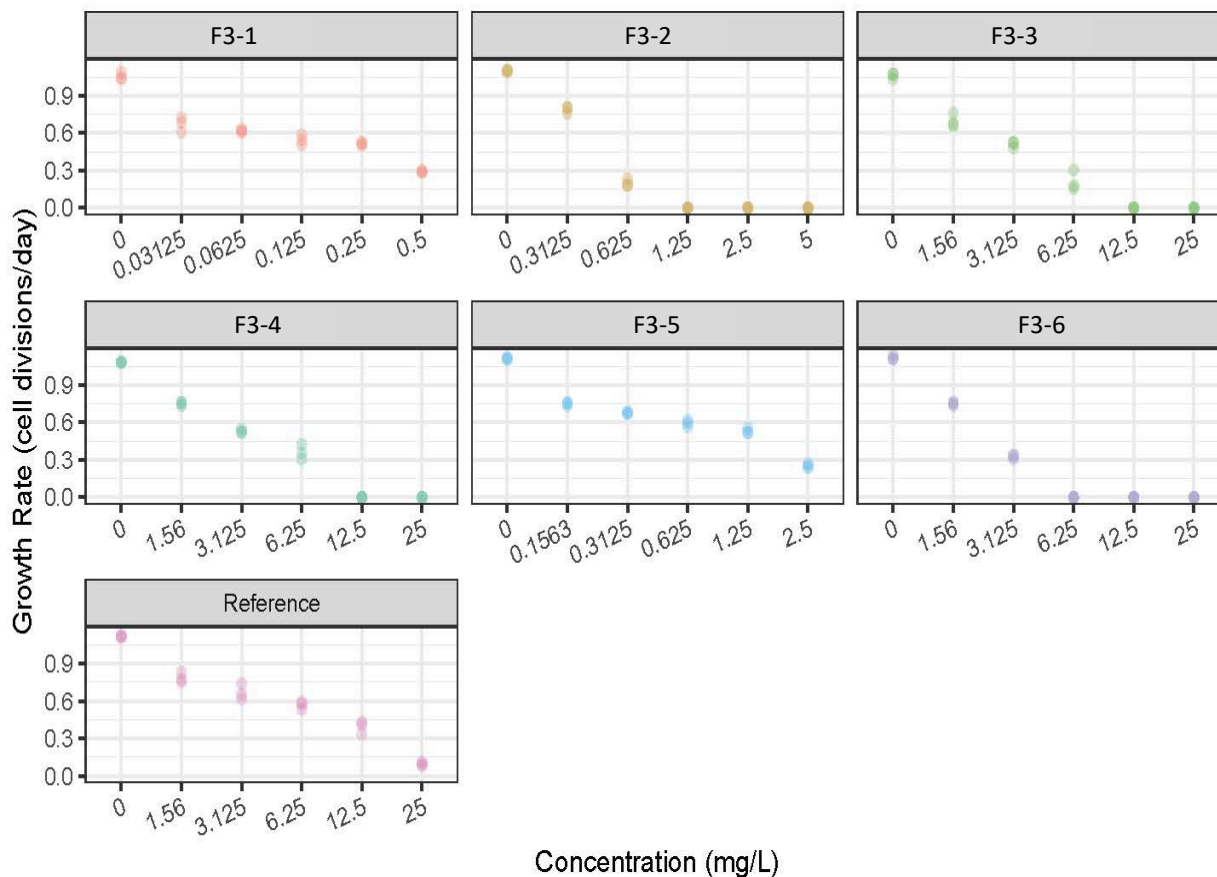


Figure 17. Effect of F3 Exposure on *Phaeodactylum tricornutum* (marine algae) growth rate.

Figure 18 summarizes the range of toxicity thresholds for the ten freshwater and marine species tested. The graph allows comparison of all species and compounds, demonstrating that F3-1 was generally the most toxic compound for most species, while Reference AFFF was generally the least toxic. Only two species, *T. obsoleta* and *A. tonsa*, were acutely sensitive to the Reference AFFF. *C. dilutus* and *H. azteca* were generally the least responsive species to these formulations. The estuarine mud snail (*T. obsoleta*) was typically the most sensitive, based on survival, to F3 formulations relative to other species tested in this project and across other programmatic projects (Jones et al., 2022). Generally, the larval estuarine fish (*C. variegatus*) was more sensitive to F3s than the larval freshwater fish tested (*P. promelas*, *O. mykiss*). For six of the ten species, F3-1 had a hazard potential classification of HIGH based on calculated acute 48-h LC50s.

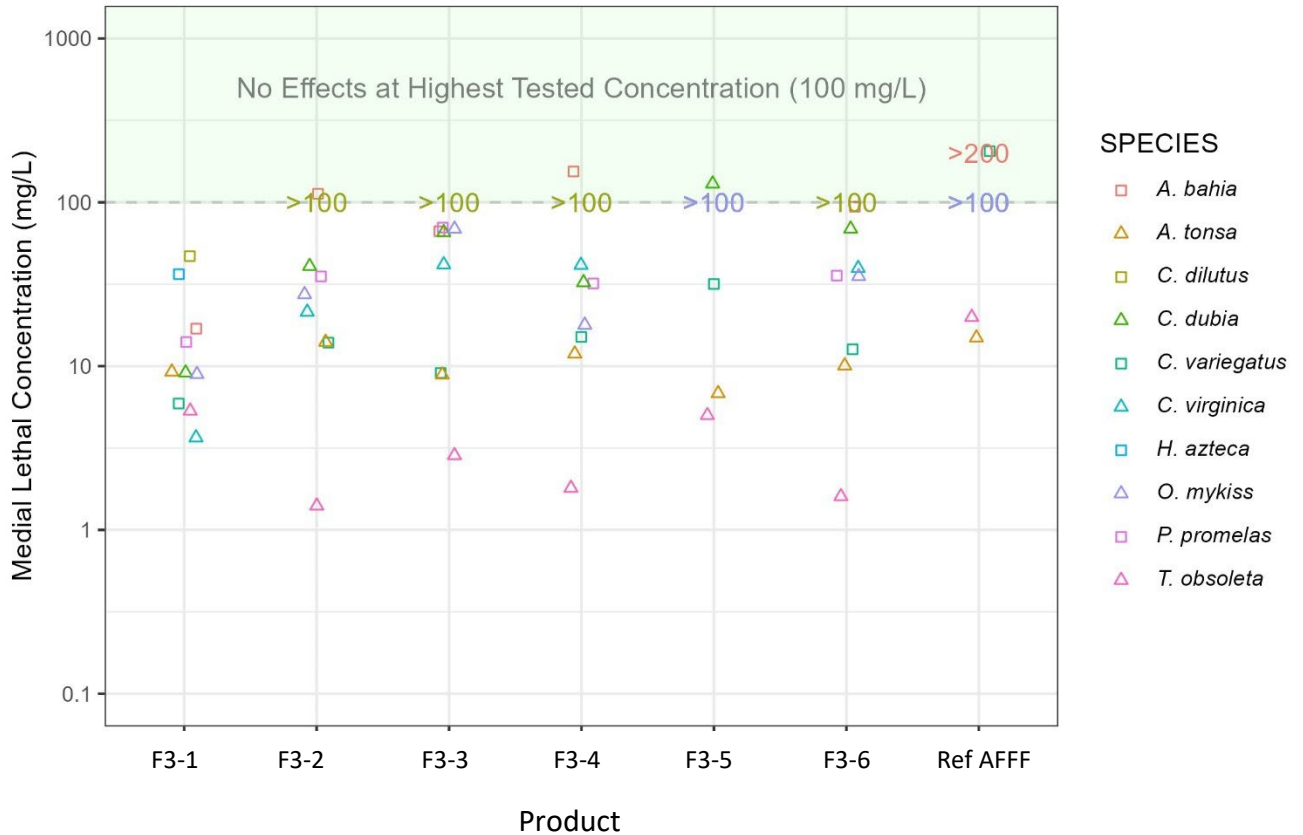


Figure 18. Species sensitivity comparison of acute survival-based toxicity thresholds for six F3 formulations and the Reference AFFF.

Summary of Chronic Toxicity Tests

Chronic results indicate that invertebrate and algal taxa are more sensitive than fish to the F3 formulations (Table 8). In this study, the marine diatom *P. tricornutum* was found to be the most sensitive species using the EC15 (~LOEC) thresholds (Table 8). Freshwater and marine invertebrates did exhibit alterations in growth and development when exposed to these F3s. Juvenile mysids (marine) were observed to mature more slowly relative to controls and these F3s also impacted shell growth in the clam (Table 8). Specifically, the F3s were found to impact clam growth more than the Reference AFFF, with the notable exception of F3-5. Overall, for most species and endpoints, similar or greater chronic toxicity was observed with the F3s than the Reference AFFF.

Figure 19 summarizes the dose response of all taxa and formulations tested. F3-1 had the dose-response curve with the lowest effect concentrations. F3-1 was categorized as VERY HIGH hazard for two species, and MODERATE hazard for three species. F3-4 had the steepest dose-response curve, demonstrating all species effects occurred over a narrow range of exposure concentrations. Formulations with more gradual dose-response slopes that spanned orders of

magnitude in effect concentrations, such as F3-3 and F3-5, indicated a greater difference in sensitivity across taxa. Supporting the conclusions drawn from the acute testing, results of the chronic testing demonstrated that F3-1 was generally the most toxic compound for most species, and that the Reference AFFF was generally the least toxic.

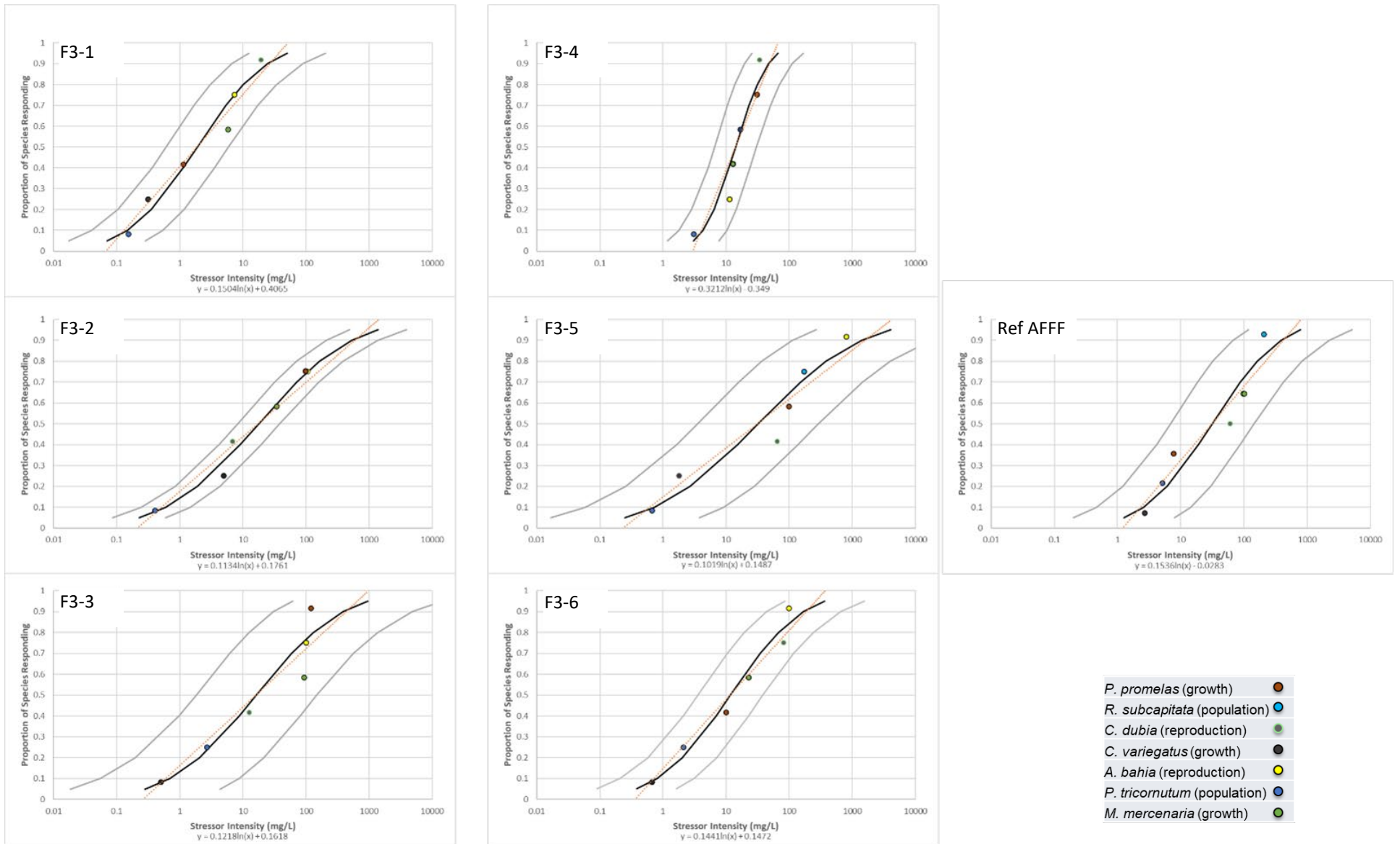


Figure 19. Relative species sensitivity distributions of taxa used in chronic testing.

Only two species, *C. dubia* and *P. tricornutum*, exhibited chronic toxicity with the Reference AFFF. Chronic effects on growth and development tended to occur at lower levels than the effects thresholds for survival, reinforcing the importance of including sublethal assessments in chemical risk assessment.

4.3 Aged F3 and Reference AFFF Toxicity Tests

All three formulations assessed (F3-1, F3-2, and AFFF) showed a decrease in toxicity with aging, often with little to no toxicity across the tested concentration ranges after 3-4 weeks, regardless of aging methodology. Supporting this observation of decreasing toxicity with age, Week 0 determinations of the LC50 were not significantly different (by evaluating overlapping confidence intervals) from the definitive acute toxicity tests as reported in Task 2 for this project (Table 11).

Table 11. LC50 estimates comparing acute toxicity test LC50s with Week 0 LC50s noting the reproducibility of the tests.

Formulation	Species	Initial Acute LC50 (mg/L); Task 2	Initial Test 95% Confidence Interval	Week 0 aged LC50 (mg/L)	Week 0 Test 95% Confidence Interval
Reference AFFF	<i>A. tonsa</i>	14.9	8.18 - 21.6	8.84	3.08 – 25.3
Reference AFFF	<i>C. dubia</i>	>100	-	>100	-
F3-1	<i>A. tonsa</i>	9.23	6.92 - 12.30	5.04	2.52 – 10.05
F3-1	<i>C. dubia</i>	9.11	9.36 - 11.27	12.8	10.7 - 15.4
F3-2	<i>A. tonsa</i>	16.5	9.94 - 23.0	13.63	2.55 – 72.81
F3-2	<i>C. dubia</i>	40.71	35.2 – 47.1	48.05	27.87 – 82.84

Summarized toxicity threshold results for all solutions can be found in Table 12. Qualitative changes in F3 composition can be evaluated using the highlighted feature results from the NTA assessment. For the Reference AFFF, the slopes of the concentration-response curves were relatively similar between the two methods, however, the aged dilution series yielded slightly higher LC50 estimates (less toxic) in both the fresh and Week 1 aged treatments when compared with aged 100% (Figure 20). The decreasing toxicity is indicated by the change in relative linear

slope so that after Week 1 data, there was no measurable dose dependent decrease in organism survival.

Results for F3-1 and F3-2 are found in Figures 21, where decreasing toxicity is indicated by the change in relative linear slope, but toxicity remains for the two F3s chosen relative to the Reference AFFF. For all formulations, there was no difference in survival across treatments by the fourth week of testing. Tabular results for this data and additional features can be found in Appendix B. Results for individual time points are indicated by peak area, which were not subject to normalization with an internal standard and therefore cannot be used for cross comparison or quantification but can be used to indicate presence of the compound and its relationship to time.

Table 12. Weekly calculated median LC50 estimates for F3-1, F3-2 and Reference AFFF aged for up to four weeks. The increase in LC50 thresholds indicates a decreasing toxicity with age of formulation solution. LC50s were calculated using Trimmed Spearman Karber.

Formulation	Aging Time (week)	<i>A. tonsa</i>	95% CI		<i>C. dubia</i>	95% CI	
		LC50 (mg/L)	Lower	Upper	LC50 (mg/L)	Lower	Upper
Reference AFFF	WEEK 0	8.83	3.08	25.32	>100	-	-
	WEEK 1	>25	-	-	>100	-	-
	WEEK 2	>25	-	-	>100	-	-
	WEEK 3	>25	-	-	>100	-	-
	WEEK 4	>25	-	-	>100	-	-
F3-1	WEEK 0	5.04	2.52	10.05	18.04	13.58	23.97
	WEEK 1	10.51	0.52	210.63	>29.6	-	-
	WEEK 2	7.02	0.82	60.11	>29.6	-	-
	WEEK 3	14.03	1.64	120.22	>29.6	-	-
	WEEK 4	>25	-	-	>29.6	-	-
F3-2	WEEK 0	13.63	2.55	72.81	48.05	27.87	82.84
	WEEK 1	>25	-	-	>100	-	-
	WEEK 2	>25	-	-	>100	-	-
	WEEK 3	>25	-	-	>100	-	-
	WEEK 4	>25	-	-	>100	-	-

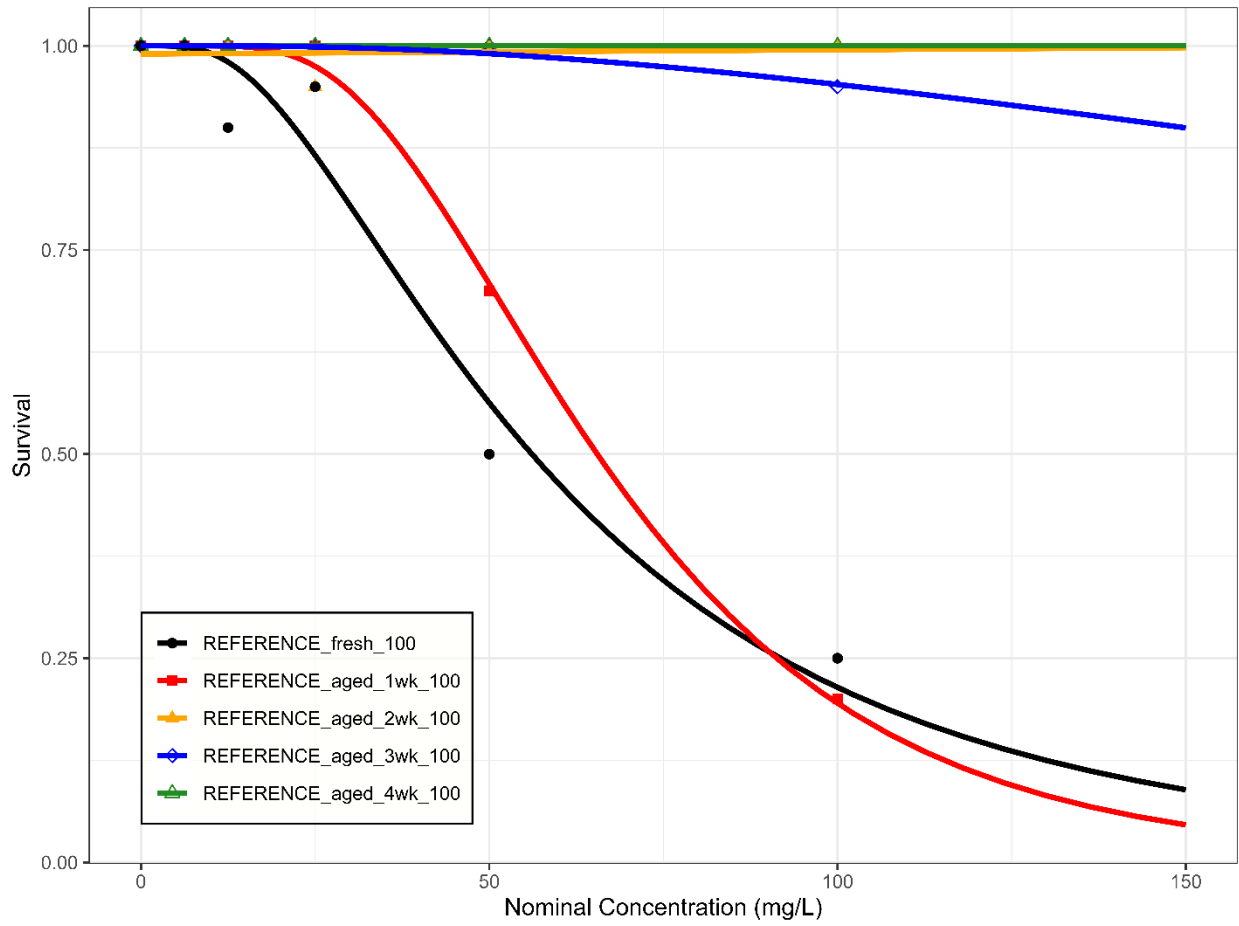
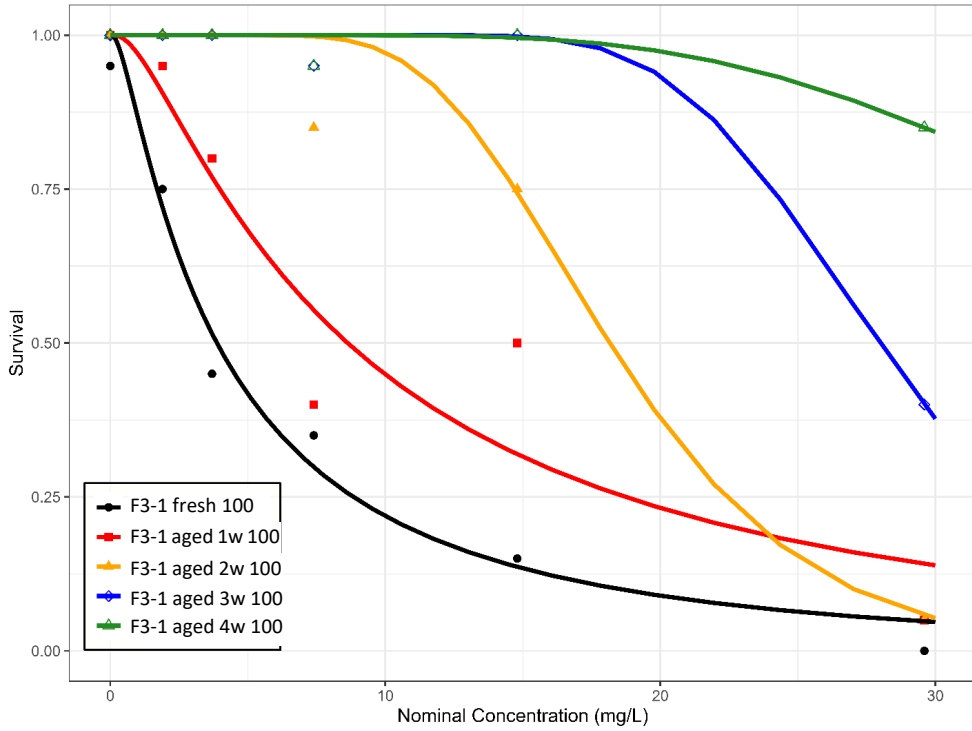


Figure 20. Reference AFFF modelled dose response curves for *C. dubia* survival using a logistic 4-parameter model for each week.

A.



B.

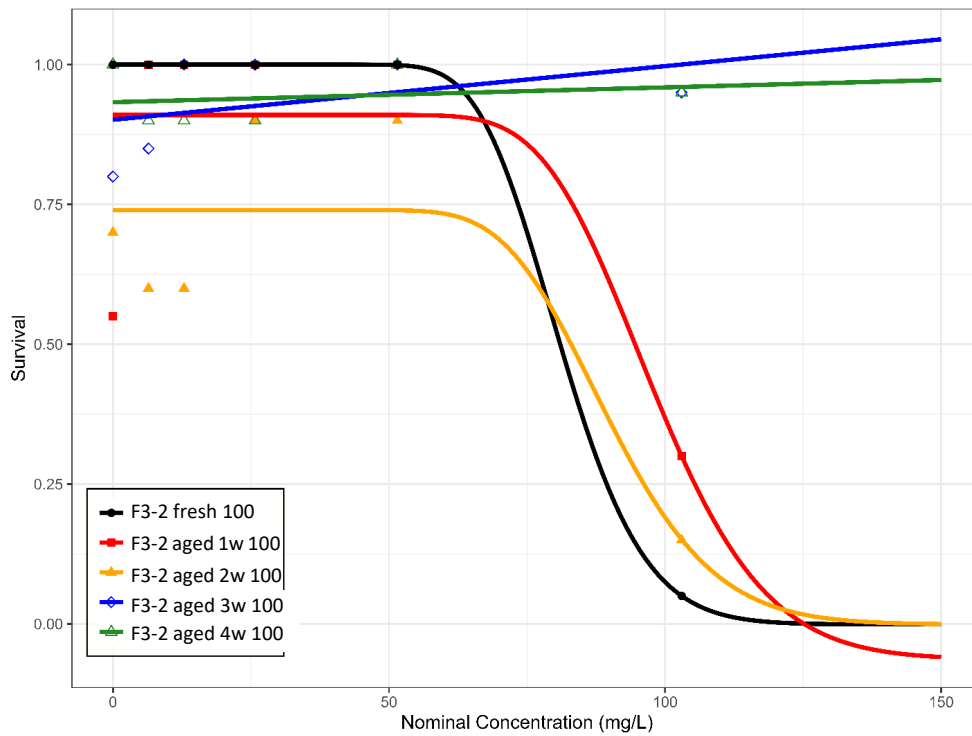


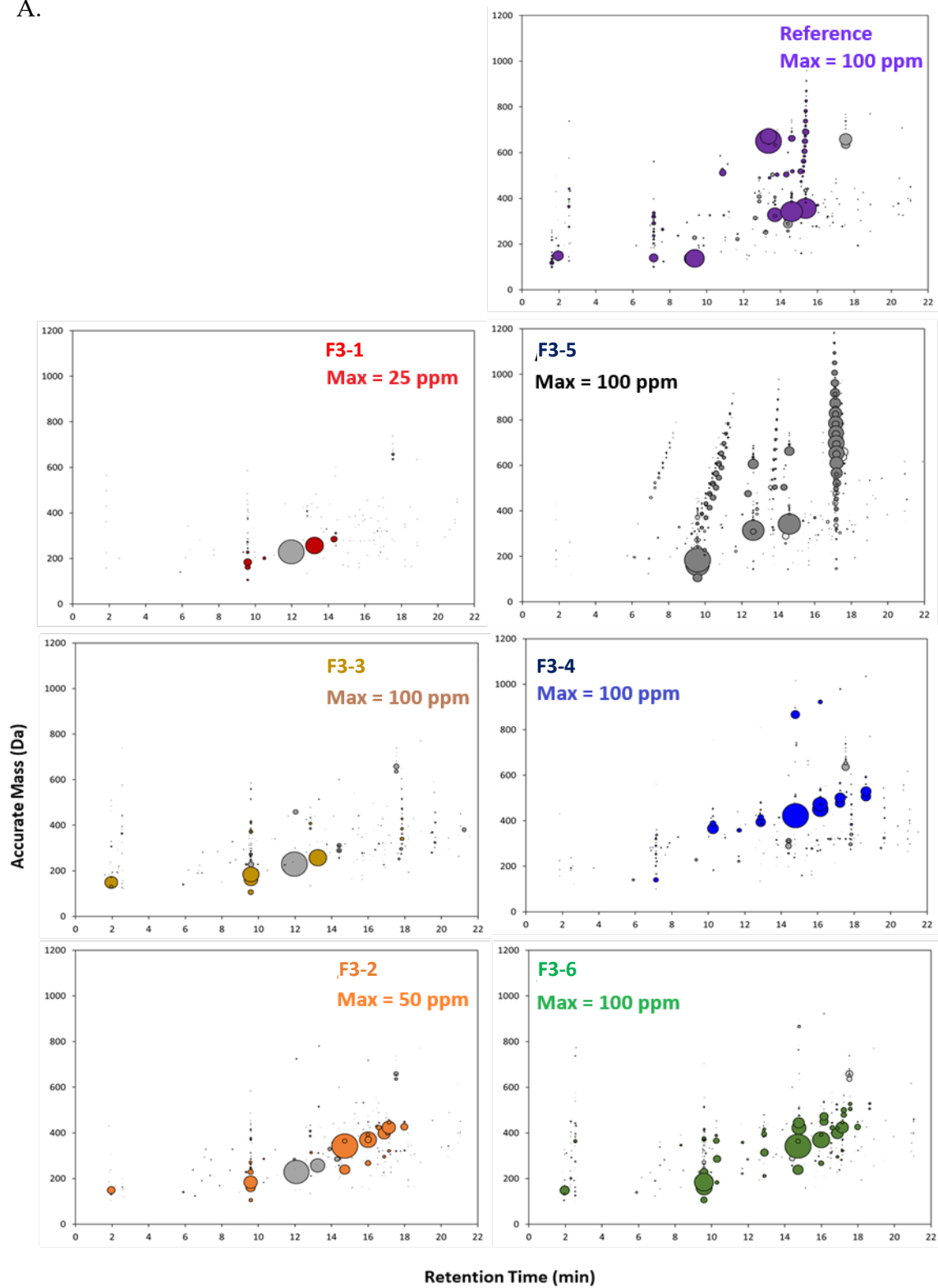
Figure 21. F3-1 (A) and F3-2 (B) modelled dose response curves for *C. dubia* survival using a logistic 4-parameter model for each week.

4.4 Nontarget Chemical Analysis

The results of the non-target analysis (Figure 22) and the resulting features identified (Table 13) indicated between 20 and 200 features for any F3 had potential masses that could be selected for use in a targeted approach to track (or ‘affirm’) F3 exposures relative to other exposures as well as a rough estimation of possible F3 constituents over time in exposure media during toxicity testing.

During non-target analysis, marker compounds were selected via analysis of gravimetrically prepared dilutions of F3s (3 % stock solutions prepared in deionized water; subsequent dilutions in methanol), with data reduction criteria developed to prioritize three to five features per foam that exhibited linear relative abundance with dilution and were stable over time. Based on variability of all HRMS detections in F3 and Reference AFFF, all stocks were stable for 12 days, except for F3-5 - stock stable up to 1 day. The final list of marker compounds used for targeted analysis are found in Table 14.

A.



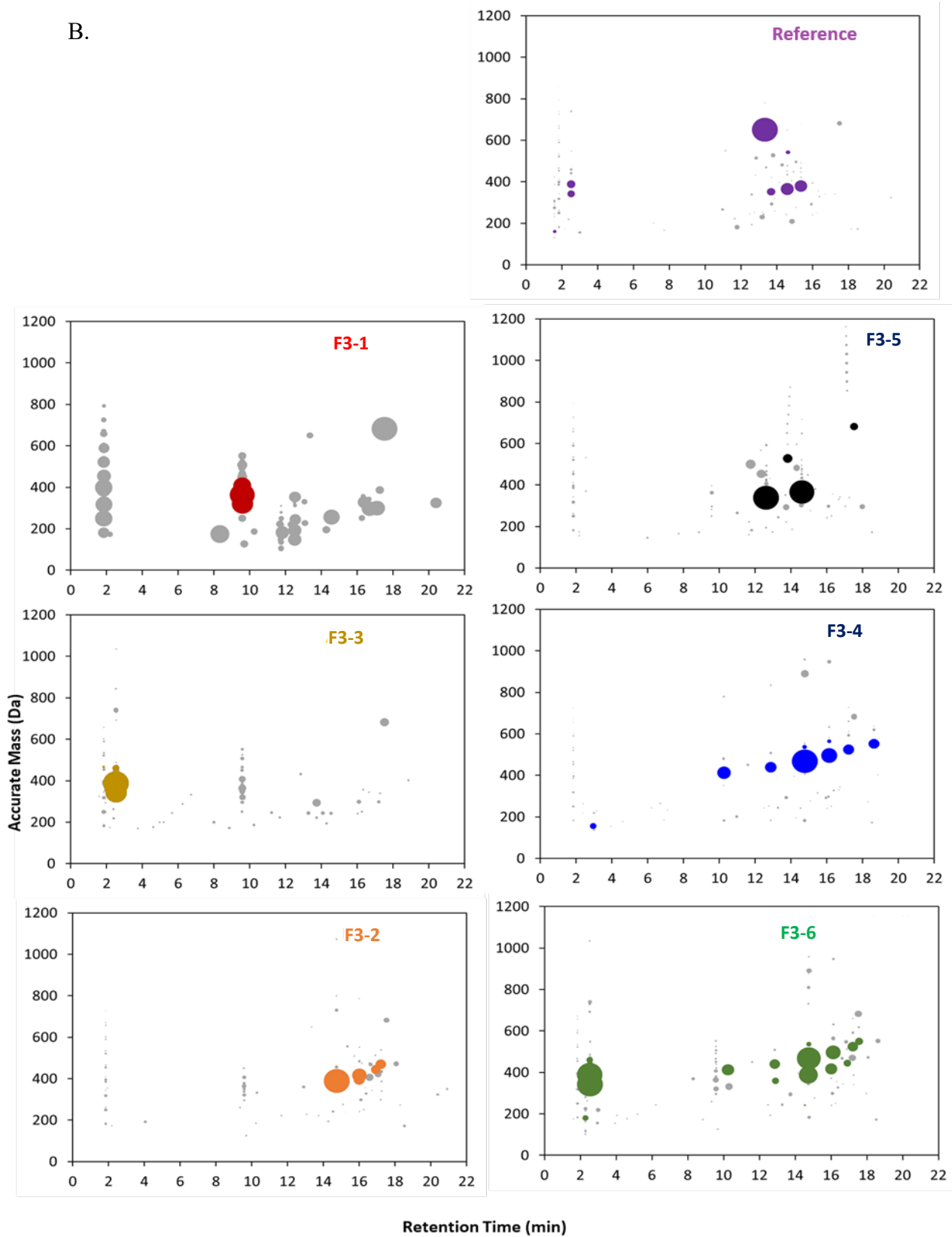


Figure 22. Non-target analysis plots for F3 and Reference AFFF. A) ESI+ and B) ESI- LCS HRMSMS analysis.

Table 13. Number of features identified for each F3 and Reference AFFF formulation evaluated for use during targeted LC-MS/MS analysis.

Formulation	ESI +	ESI -
Reference AFFF	86	9
F3-1	18	4
F3-2	44	4
F3-3	34	5
F3-4	38	10
F3-5	157	4
F3-6	84	18

Table 14. Marker compounds used for target LC-MS/MS analysis of aqueous samples. LC column: Waters XSelect HSS T3 3.5 μm ; 3.0 x 100 mm; Mobile phase: 0.1% formic acid in each DI water (A), MeOH (B); 0.4 mL/min; 0-0.75 min 5% B, 2 min 50% B, 15-23 min 100%

Q1 (m/z)	Q3 (m/z)	ESI	RT	Reference AFFF	F3-1	F3-2	F3-3	F3-4	F3-5	F3-6
163.1325	89.0595	+	8.1		X	X	X		X	
209.085	96.96	-	11.2	X			X			
237.1163	96.96	-	14.4				X			
258.2786	240.2683	+	13.5		X		X			
286.3098	268.2993	+	14.7		X					
338.2531	85.0283	+	13.5	X						
343.2946	240.2318	+	13.7			X			X	
359.326	240.2318	+	11.8					X		X
367.2255	184.1695	+	8.8					X		X
371.326	268.2631	+	15.4			X				
399.3573	296.2946	+	16.6			X				
423.2877	240.2319	+	13.7					X		X
425.3728	322.3102	+	16.9			X				
451.3191	268.2632	+	15.4					X		X
479.3505	296.2946	+	16.7					X		X
649.0717	182.0491	-	12.1	X						
663.4276	343.209	+	13.3						X	
694.4027	147.0471	+	16.6						X	

4.5 Chemical Analysis of Toxicity Tests

Using the targeted LC-MS/MS method, marker compounds were measured in the highest (10-400 mg/L) and lowest doses (0.625-25 mg/L) from 48 h acute static renewal toxicity assays with *C. variegatus* (Figure 23). Average measured concentrations (error bars show standard deviation) indicated agreement within ~10-30% relative to nominal concentrations for all foams tested.

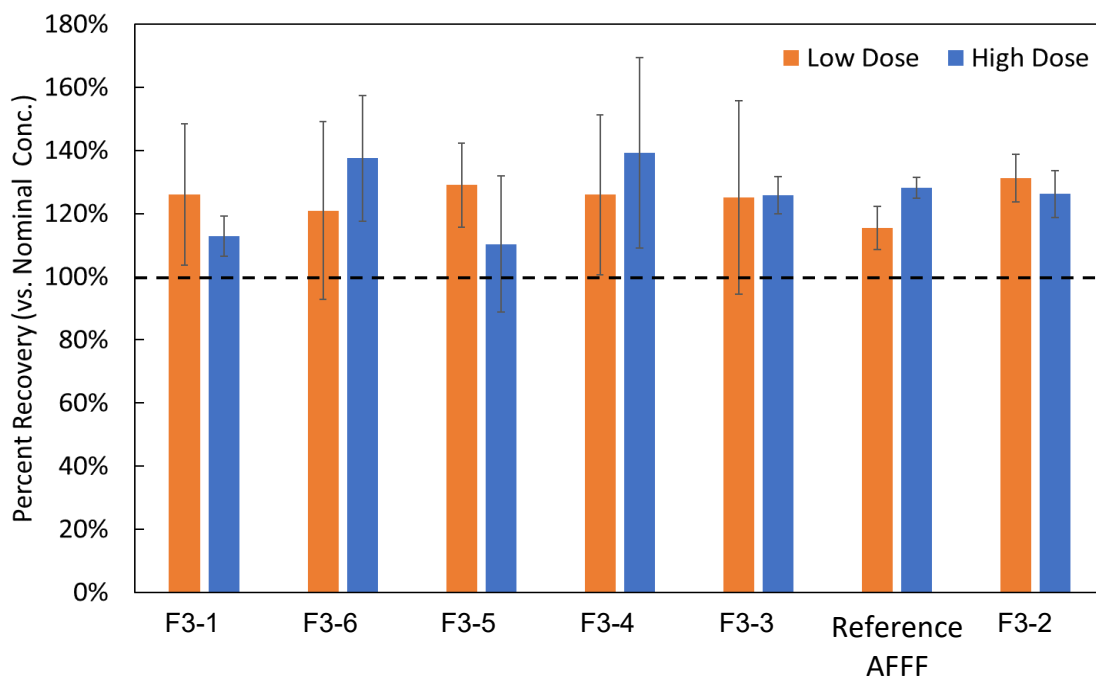


Figure 23. Initial nominal concentration verifications for the F3 and Reference AFFF formulations from exposure samples collected during the definitive *C. variegatus* acute test.

The freshwater invertebrate acute test with *C. dilutus*, showed good agreement between nominal and measured chemical feature concentrations (Table 15; Figure 24). Rapid degradation of chemical markers was seen from $t=0$ to $t=96$ h in the *C. dilutus* test. Some chemical markers, for example F3-2 and F3-4 in the *C. dilutus* test, degraded to non-reportable levels after 96 h. Target and measured chemical marker concentrations in the freshwater fish acute test with *O. mykiss* were within 77-164% of each other (Table 16; Figure 25). Rapid degradation of chemical markers was seen from 0 to 48 h in the *O. mykiss* test. Significant loss in the *O. mykiss* exposure media at 48 h was observed for the highest concentrations for F3-2, F3-4, and F3-6. For the saltwater fish acute test with *C. variegatus*, the measured chemical marker concentrations were within 92-175% of each other (Table 17; Figure 26). Chemical markers remained relatively stable over 24 h in the *C. variegatus* test. The Reference AFFF foam remained stable over 48-96 h of aqueous exposure in freshwater tests and over 24 h in the saltwater fish test.

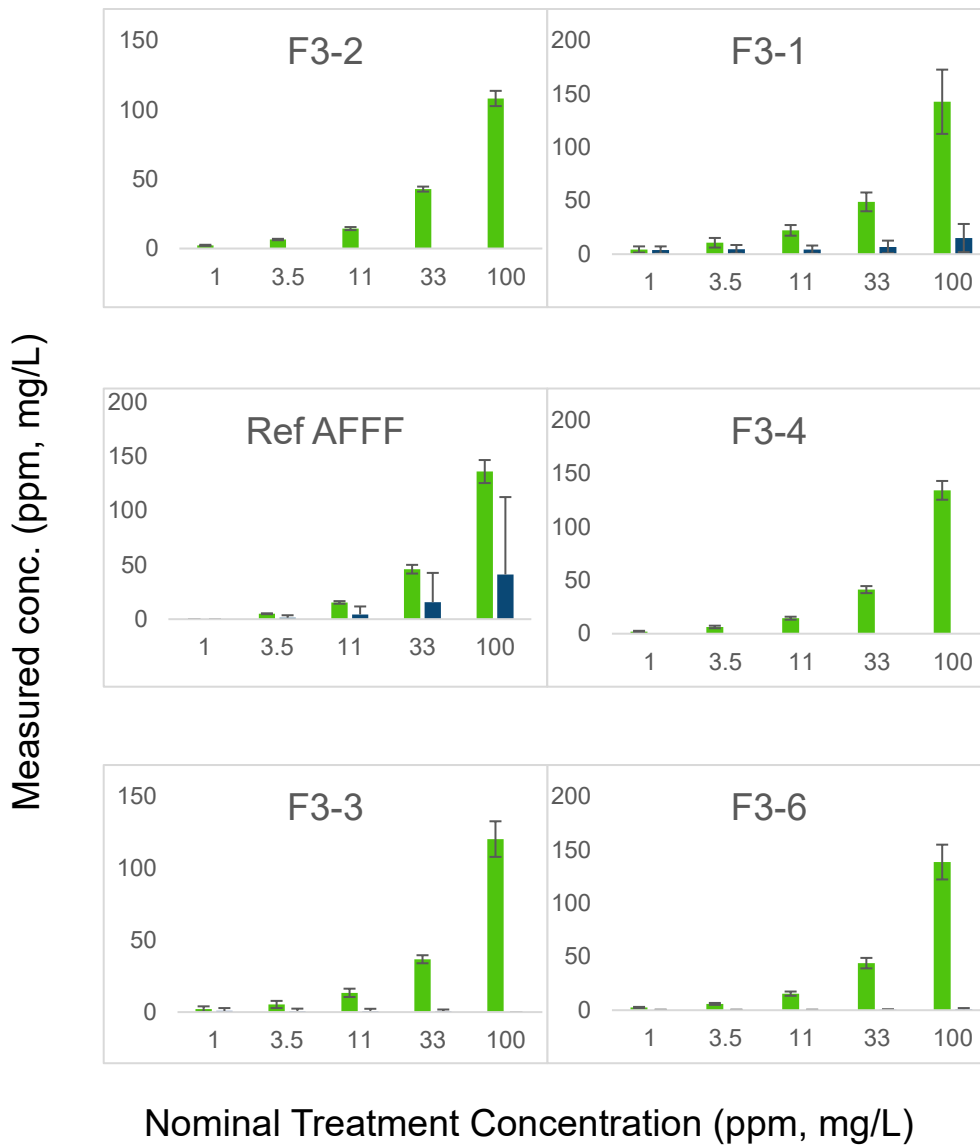


Figure 24. Measured (affirmation or relative to nominal) concentration (mg/L) versus nominal exposure concentration for *C. dilutus* acute testing. Formulation stabilities were affirmed using the average expected recovery of measured mass fragments at 0 (green) and 96 (blue) hours.

Table 15. Average recoveries for multiple mass features for each F3 and Reference AFFF to affirm time 0 and 96 h *C. dilutus* acute assay concentrations. Mass feature specific results are detailed in Appendix B.

Formulation	Date Collected	Exposure Time (h; time media in test chamber)	Nominal Concentration (mg/L)	Average Calculated Affirmation Concentration (mg/L)	SD (mg/L)	Avg % Nominal
F3-1	8/19/2021	0	1	4.4	2.9	439%
	8/19/2021	0	3.5	10.7	4.5	307%
	8/19/2021	0	11	22.3	5	202%
	8/19/2021	0	33	49	8.8	148%
	8/19/2021	0	100	142.8	30.1	143%
	8/23/2021	96	1	3.9	3.4	388%
	8/23/2021	96	3.5	4.6	4	131%
	8/23/2021	96	11	4.3	3.8	39%
	8/23/2021	96	33	6.8	5.9	21%
	8/23/2021	96	100	15.1	13.2	15%
F3-2	8/19/2021	0	1	2.3	0.4	232%
	8/19/2021	0	3.5	6.5	0.5	185%
	8/19/2021	0	11	14.4	1.1	131%
	8/19/2021	0	33	42.9	1.8	130%
	8/19/2021	0	100	108.3	5.5	108%
	8/23/2021	96	1	0	0	0%
	8/23/2021	96	3.5	0	0	0%
	8/23/2021	96	11	0	0	0%
	8/23/2021	96	33	0	0	0%
	8/23/2021	96	100	0	0	0%
F3-3	8/19/2021	0	1	2.1	1.9	215%
	8/19/2021	0	3.5	5.3	2.5	152%
	8/19/2021	0	11	13.4	2.9	122%
	8/19/2021	0	33	36.8	2.8	112%
	8/19/2021	0	100	120.5	12.4	121%

	8/23/2021	96	1	0.9	1.9	94%
	8/23/2021	96	3.5	0.8	1.6	23%
	8/23/2021	96	11	0.8	1.5	7%
	8/23/2021	96	33	0.6	1.2	2%
	8/23/2021	96	100	0	0	0%
F3-4	8/19/2021	0	1	2	0.7	204%
	8/19/2021	0	3.5	6.3	1.2	179%
	8/19/2021	0	11	14.5	1.4	132%
	8/19/2021	0	33	41.3	3.3	125%
	8/19/2021	0	100	134.3	8.8	134%
	8/23/2021	96	1	0	0	0%
	8/23/2021	96	3.5	0	0	0%
	8/23/2021	96	11	0	0	0%
	8/23/2021	96	33	0	0	0%
	8/23/2021	96	100	0	0	0%
F3-6	8/19/2021	0	1	2.5	0.6	252%
	8/19/2021	0	3.5	5.9	0.9	167%
	8/19/2021	0	11	15.5	2	141%
	8/19/2021	0	33	44.1	4.9	134%
	8/19/2021	0	100	138.8	16.3	139%
	8/23/2021	96	1	0.2	0.3	18%
	8/23/2021	96	3.5	0.2	0.3	5%
	8/23/2021	96	11	0.2	0.3	2%
	8/23/2021	96	33	0.4	0.5	1%
	8/23/2021	96	100	0.8	1.2	1%
Reference AFFF	8/19/2021	0	1	0	0	0%
	8/19/2021	0	3.5	5	0.4	143%
	8/19/2021	0	11	15.4	1.2	140%
	8/19/2021	0	33	46	4	139%

	8/19/2021	0	100	135.9	10.6	136%
	8/23/2021	96	1	0	0	0%
	8/23/2021	96	3.5	1.3	2.3	37%
	8/23/2021	96	11	4.3	7.4	39%
	8/23/2021	96	33	15.6	27	47%
	8/23/2021	96	100	41.1	71.3	41%

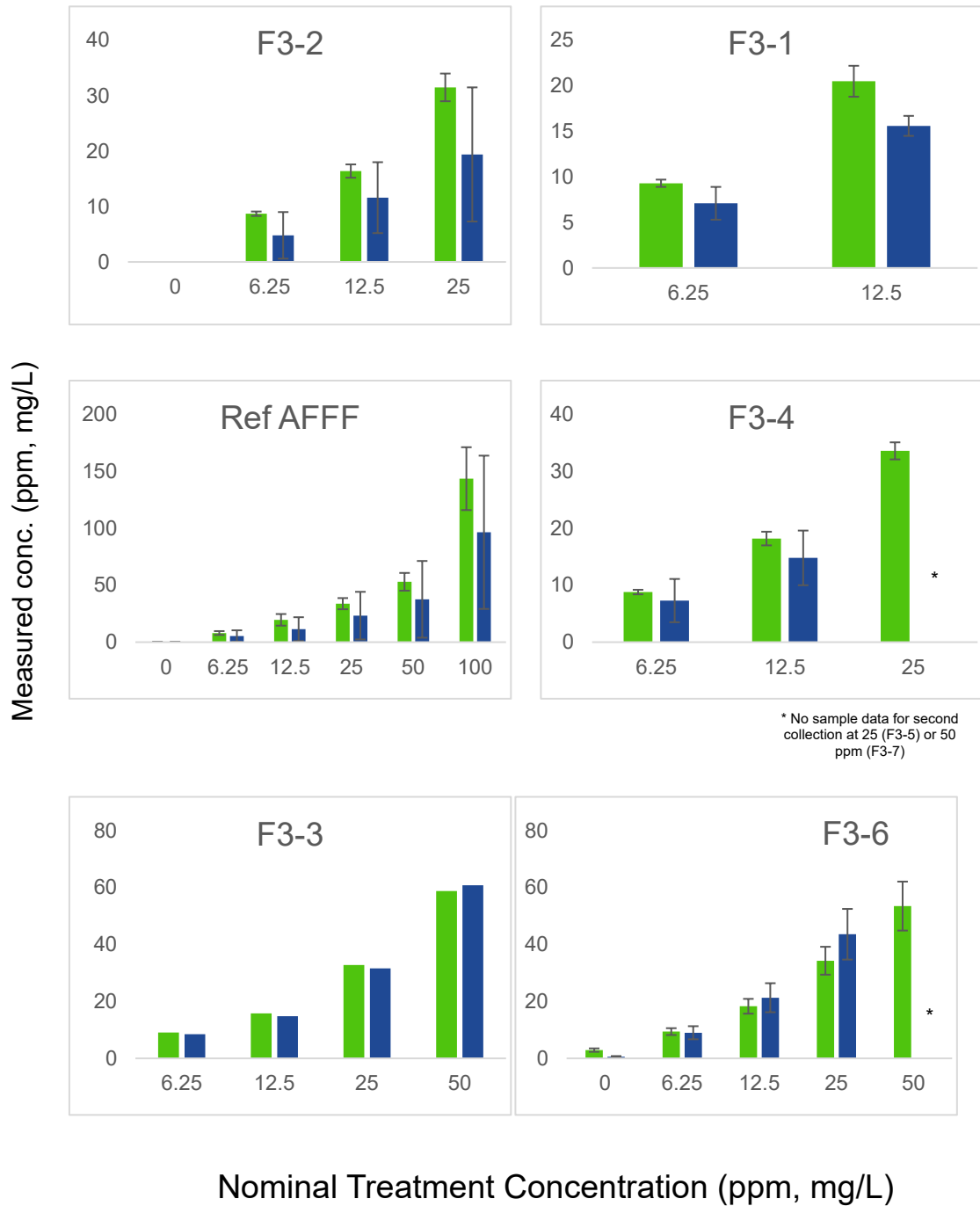


Figure 25. Measured (affirmation) concentrations (mg/L) versus nominal exposure concentrations for *O. mykiss* acute testing. F3 and Reference AFFF stabilities were affirmed using the average expected recovery of measured mass fragments at 0 (green) and 48 (blue) hours.

Table 16. Average recoveries for multiple mass features for those formulations analyzed to affirm 48-h *O. mykiss* acute assay concentrations. Mass feature specific results are detailed in Appendix B.

Formulation	Date Collected	Exposure Time (h; time media in test chamber)	Nominal Concentration (mg/L)	Average Calculated Affirmation Concentration (mg/L)	SD (mg/L)	Avg % Nominal	
F3-1	8/18/2021	0	6.25	9.3	0.4	148%	
	8/18/2021	0	12.5	20.5	1.7	164%	
	8/20/2021	48	6.25	7.1	1.8	113%	
	8/20/2021	48	12.5	15.6	1.1	125%	
F3-2	7/14/2021	0	0	0	0	0%	
	7/14/2021	0	6.25	8.7	0.4	140%	
	7/14/2021	0	12.5	16.4	1.2	131%	
	7/14/2021	0	25	31.5	2.5	126%	
	7/16/2021	48	0	0	0	0%	
	7/16/2021	48	6.25	4.8	4.2	77%	
	7/16/2021	48	12.5	11.6	6.4	93%	
	7/16/2021	48	25	19.4	12.1	78%	
	F3-4	8/18/2021	0	6.25	8.8	0.4	141%
		8/18/2021	0	12.5	18.2	1.2	146%
8/18/2021		0	25	33.6	1.5	134%	
8/20/2021		48	6.25	7.3	3.8	117%	
8/20/2021		48	12.5	14.8	4.8	119%	
F3-6		10/20/2021	0	0	2.9	0.6	
	10/20/2021	0	6.25	9.4	1.2	150%	
	10/20/2021	0	12.5	18.3	2.6	147%	
	10/20/2021	0	25	34.3	4.9	137%	
	10/20/2021	0	50	53.5	8.6	107%	
	10/22/2021	48	0	0.6	0.2		
	10/22/2021	48	6.25	9	2.3	143%	
	10/22/2021	48	12.5	21.3	5.1	171%	

	10/22/2021	48	25	43.6	8.9	175%
Reference AFFF	10/20/2021	0	0	0	0	0%
	10/20/2021	0	6.25	8	1.7	128%
	10/20/2021	0	12.5	19.6	5.1	157%
	10/20/2021	0	25	33.8	4.9	135%
	10/20/2021	0	50	53	7.8	106%
	10/20/2021	0	100	143.6	27.6	144%
	10/22/2021	48	0	0	0	0%
	10/22/2021	48	6.25	5.4	5	86%
	10/22/2021	48	12.5	11.4	10.5	91%
	10/22/2021	48	25	23.3	20.9	93%
	10/22/2021	48	50	37.6	33.7	75%
	10/22/2021	48	100	96.5	67.3	97%
	10/20/2021	0	6.25	9.1		145%
	10/20/2021	0	12.5	15.8		127%
	10/20/2021	0	25	32.8		131%
	10/20/2021	0	50	58.8		118%
	10/22/2021	48	6.25	8.5		135%
	10/22/2021	48	12.5	14.8		118%
	10/22/2021	48	25	31.6		126%
	10/22/2021	48	50	60.8		122%

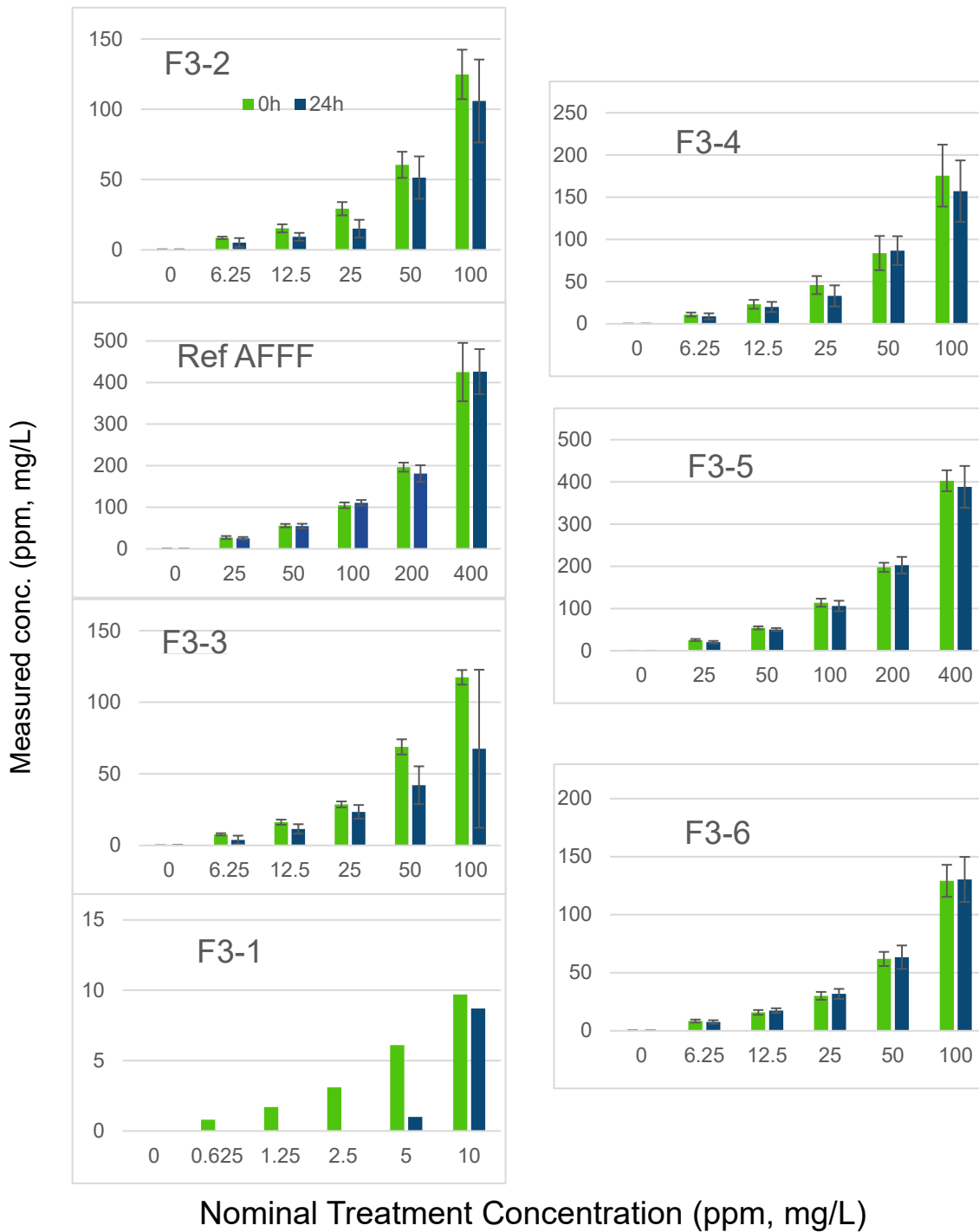


Figure 26. Measured (affirmation) concentrations (mg/L) versus Nominal exposure concentrations for *C. variegatus* acute testing. F3 and Reference AFFF stabilities were affirmed using the average expected recovery of measured mass fragments at 0 (green) and 24 (blue) hours.

Table 17. Average recoveries for multiple mass features for each formulation to affirm *C. variegatus* acute assay concentrations. Concentrations were renewed every 24 h. Mass feature specific results are detailed in Appendix B.

<i>C. variegatus</i>						
Formulation	Date Collected	Exposure Time	Gravimetrically Determined Nominal (mg/L)	Average Calculated Affirmation Concentration (mg/L)	SD (mg/L)	Ave % to Nom Mass
Reference AFFF	3/22/2021	0	0	0	0	0%
	3/22/2021	0	26	27.3	3.6	104%
	3/22/2021	0	53	55.9	4.1	106%
	3/22/2021	0	105	104.8	6.9	100%
	3/22/2021	0	210	196.5	10.9	94%
	3/22/2021	0	420	425.1	70.2	101%
	3/23/2021	24	0	0	0	0%
	3/23/2021	24	26	25.5	3.1	97%
	3/23/2021	24	53	54.8	5.6	104%
	3/23/2021	24	105	110.8	6.7	106%
F3-1	3/23/2021	24	210	181	20.2	86%
	3/23/2021	24	420	426.2	54.2	102%
	3/22/2021	0	0	0	--	0%
	3/22/2021	0	0.7	0.8	--	114%
	3/22/2021	0	1.3	1.7	--	131%
	3/22/2021	0	2.6	3.1	--	119%
	3/22/2021	0	5.2	6.1	--	117%
	3/22/2021	0	10.5	9.7	--	92%
	3/23/2021	24	0	0	--	0%
	3/23/2021	24	0.7	0	--	0%
	3/23/2021	24	1.3	0	--	0%
	3/23/2021	24	2.6	0	--	0%
	3/23/2021	24	5.2	1	--	19%
	3/23/2021	24	10.5	8.7	--	83%

F3-2	3/25/2021	0	0	0.2	0.2	0%
	3/25/2021	0	6.6	8.5	0.9	129%
	3/25/2021	0	13.1	15.3	2.9	117%
	3/25/2021	0	26.2	29.3	4.7	112%
	3/25/2021	0	52.5	60.5	9.3	115%
	3/25/2021	0	105	124.8	17.6	119%
	3/26/2021	24	0	0.2	0.2	0%
	3/26/2021	24	6.6	5.2	3.2	79%
	3/26/2021	24	13.1	9.3	2.8	71%
	3/26/2021	24	26.2	15.1	6.3	58%
	3/26/2021	24	52.5	51.4	15.1	98%
	3/26/2021	24	105	105.9	29.5	101%
F3-3	4/5/2021	0	0	0	0	0%
	4/5/2021	0	6.5	7.7	0.8	118%
	4/5/2021	0	13	16.2	1.8	125%
	4/5/2021	0	26.1	28.6	2.1	110%
	4/5/2021	0	52.2	68.8	5.3	132%
	4/5/2021	0	104.4	117.4	5.1	112%
	4/6/2021	24	0	0.1	0.1	0%
	4/6/2021	24	6.5	3.8	3	58%
	4/6/2021	24	13	11.5	3.3	88%
	4/6/2021	24	26.1	23.4	4.8	90%
	4/6/2021	24	52.2	42.046	13.2	81%
	4/6/2021	24	104.4	67.502	55.2	65%
F3-4	3/25/2021	0	0	0	0	0%
	3/25/2021	0	6.6	11	2.4	167%
	3/25/2021	0	13.2	23.1	5.3	175%
	3/25/2021	0	26.4	45.9	10.7	174%
	3/25/2021	0	52.9	83.8	20.3	158%

	3/25/2021	0	105.7	175.6	36.7	166%
	3/26/2021	24	0	0	0	0%
	3/26/2021	24	6.6	8.9	3.6	135%
	3/26/2021	24	13.2	20	6	152%
	3/26/2021	24	26.4	33.1	12.5	125%
	3/26/2021	24	52.9	86.7	17.1	164%
	3/26/2021	24	105.7	157.2	36.4	149%
F3-5	3/28/2021	0	0	0	0	0%
	3/28/2021	0	26.1	25.4	2.5	97%
	3/28/2021	0	52.3	54.2	3.6	104%
	3/28/2021	0	104.5	113.8	9.6	109%
	3/28/2021	0	209.1	197.7	10.8	95%
	3/28/2021	0	418.1	402.8	24.8	96%
	3/29/2021	24	0	0	0	0%
	3/29/2021	24	26.1	20.5	2.8	79%
	3/29/2021	24	52.3	50.2	3.6	96%
	3/29/2021	24	104.5	106	12.5	101%
	3/29/2021	24	209.1	202.7	19.7	97%
	3/29/2021	24	418.1	388.2	49.6	93%
F3-6	4/5/2021	0	0	0.1	0.2	0%
	4/5/2021	0	6.6	8.4	1.3	127%
	4/5/2021	0	13.2	15.8	2.1	120%
	4/5/2021	0	26.4	30.1	3.4	114%
	4/5/2021	0	52.7	61.9	6.1	117%
	4/5/2021	0	105.4	129.2	13.8	123%
	4/6/2021	24	0	0.1	0.1	0%
	4/6/2021	24	6.6	7.6	1.5	115%
	4/6/2021	24	13.2	17.4	2	132%
	4/6/2021	24	26.4	31.8	4.3	120%

	4/6/2021	24	52.7	63.4	10.1	120%
	4/6/2021	24	105.4	130.4	19.4	124%

4.6 Chemical Analysis of aged F3 Formulations and Reference AFFF

For the analysis of aged formulations, a series of mass features from NTA helps articulate the measurable changes in chemistry over time. Over the 28-d aging of Reference AFFF, three features were observed to have a decreasing response (237.12, 134.07 and 209.12) as prepared solutions aged (Figure 27). Two features were observed to have increased response as the time of solution aging was increased (237.12 and 159.10). An interesting observation is the opposite response for mass 237.12. Chemical library algorithms suggest the following chemical structure C10H22O4S and the feature trend in seawater (decreases with time) and freshwater (increases with time) indicating perhaps that an environmental factor changes how this mass fragment/feature behaves. The F3s also have features that trend with age. Two mass features observed in the NTA from F3-1 testing decreased with time (m/z 265.15, 314.34) while several increased as the solutions aged (265.15, 246.24, 258.28, and 274.27) (Figure 28). In F3-2 solutions, only 4 features exhibited trends across aging (Figure 29). Features at m/z 284.29 and 176.13 increased as solutions aged while m/z features at 101.09 and 371.33 decreased with age. See Appendix B for presumptive chemical formulas for the features noted above.

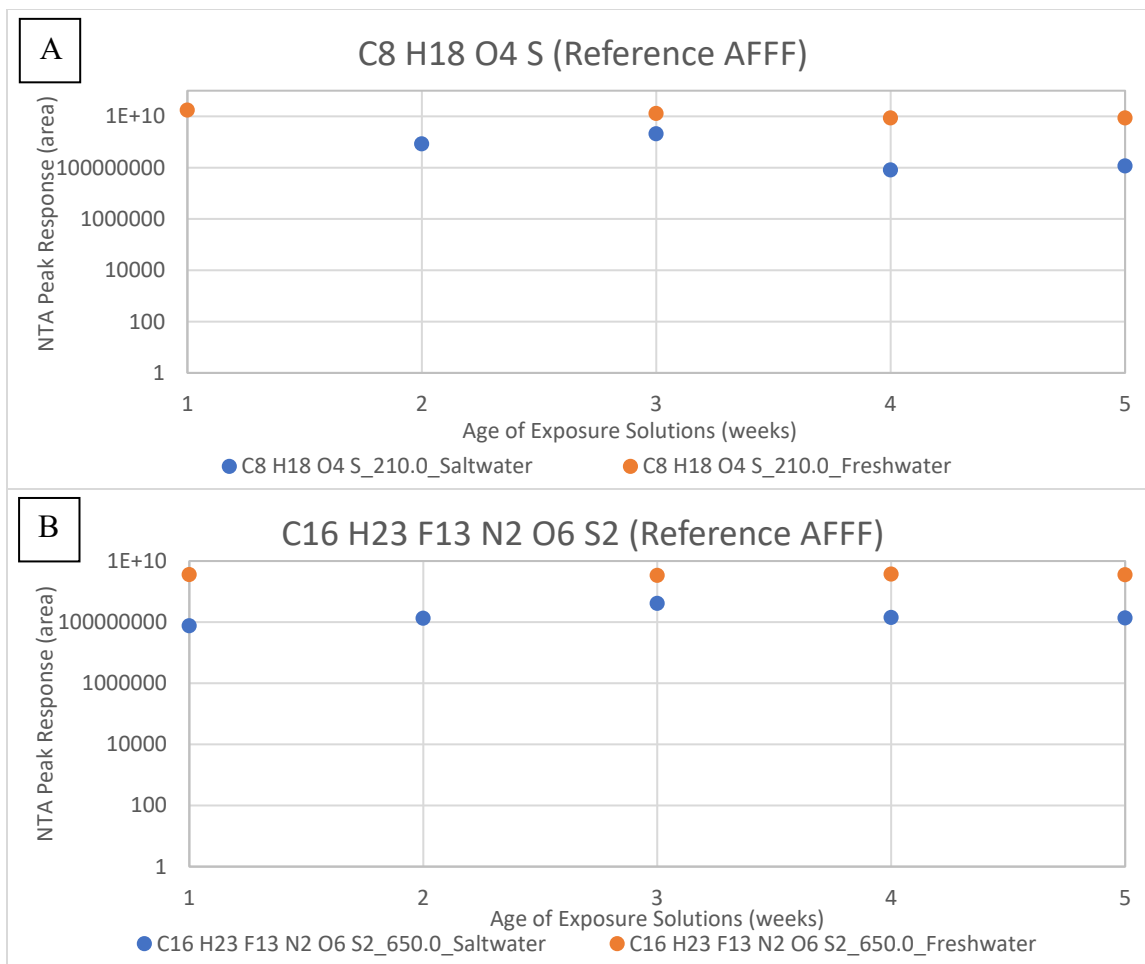


Figure 27. Selected mass fragments from nontarget analysis of Reference AFFF using UPLC/MS matched from NTAs in 2021 and 2024. These plotted m/z areas may qualitatively indicate product stability of these fragments over a 5-week period of testing changes in chemical composition over time using C18- SPE and were observed to change as nominal concentrations changed; Reference AFFF (A) m/z 209.085 and (B) 650.072. Age of Solution 1 is freshly diluted media from a 3% stock and then weekly at test 2 (7-days), test 3 (14-days), test 4 (21-days) and test 5 (28-days).

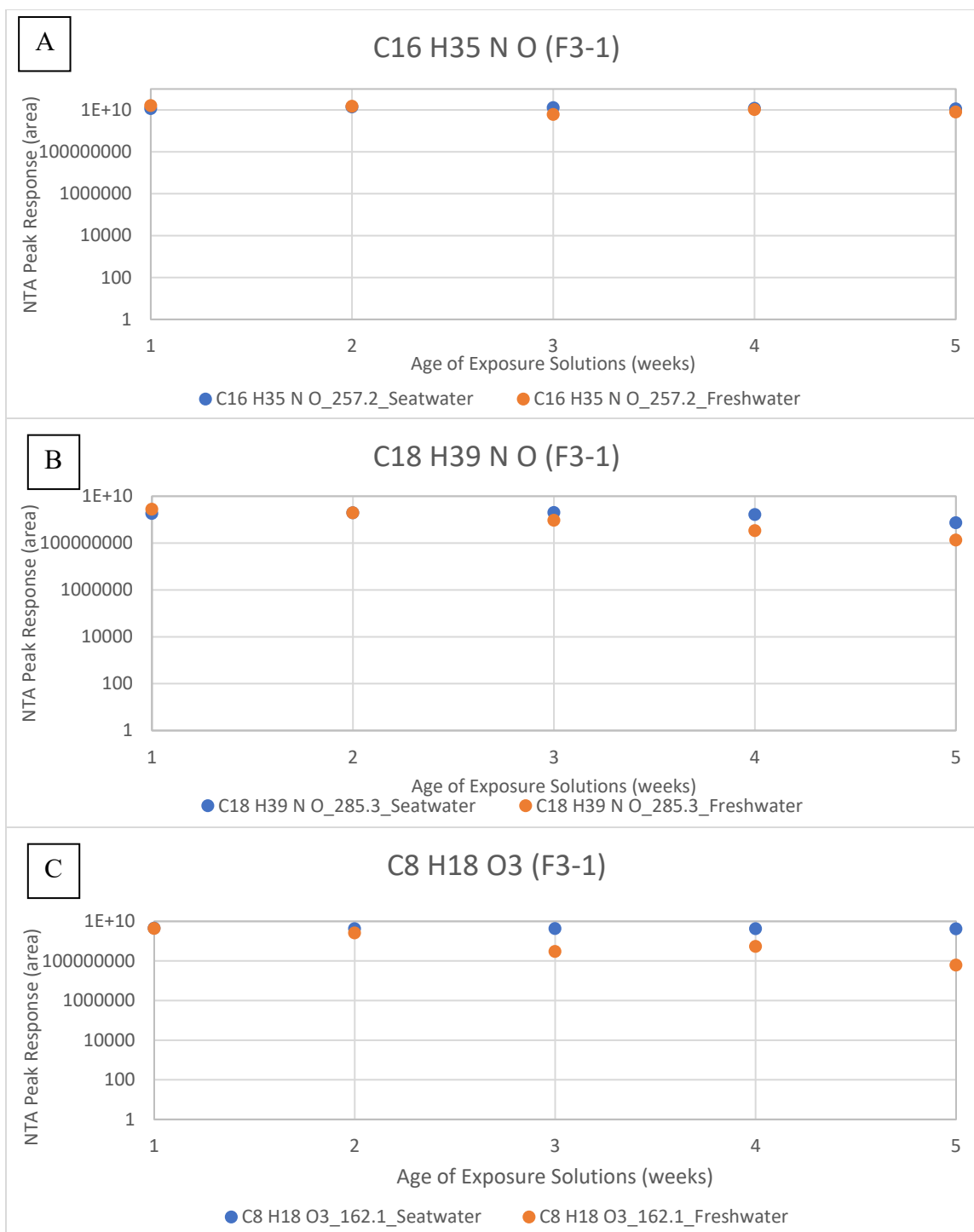


Figure 28. Selected mass fragments from nontarget analysis of F3-1 using UPLC/MS matched from NTAs in 2021 and 2024. These plotted m/z areas may qualitatively indicate product stability of these fragments over a 5-week period of testing changes in chemical composition over time using C18-SPE and were observed to change as nominal concentrations changed; F3-1 (A) m/z 163.133 (B) 258.279 and (C) 286.310. Age of Solution 1 is freshly diluted media from a 3% stock and then weekly at test 2 (7-days), test 3 (14-days), test 4 (21-days) and test 5 (28-days).

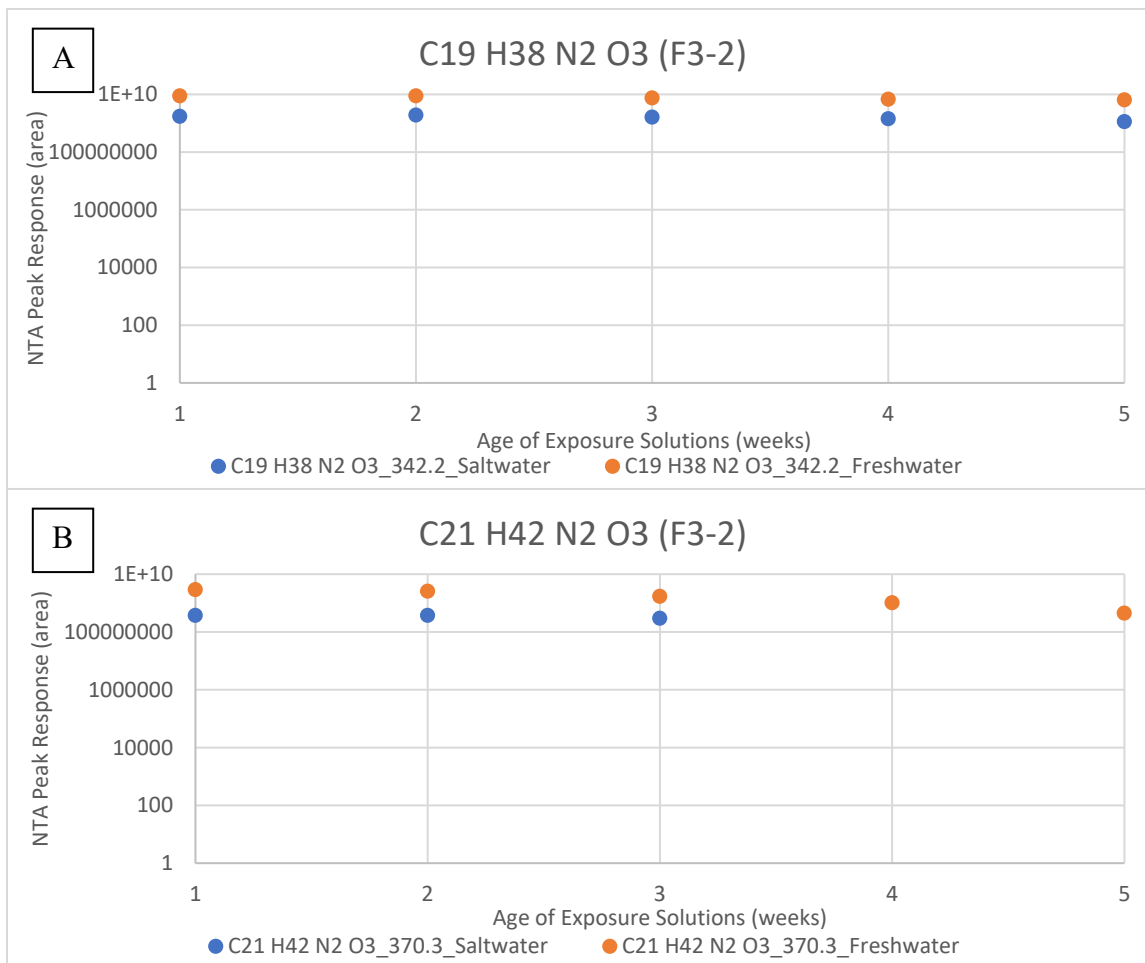


Figure 29. Selected mass fragments from nontarget analysis of F3-2 using UPLC/MS matched from NTAs in 2021 and 2024. These plotted m/z areas may qualitatively indicate product stability of these fragments over a 5-week period of testing changes in chemical composition over time using C18- SPE and were observed to change as nominal concentrations changed; F3-2 (A) m/z 343.295 and (B) 371.326. Age of Solution 1 is freshly diluted media from a 3% stock and then weekly at test 2 (7-days), test 3 (14-days), test 4 (21-days) and test 5 (28-days).

5.0 Conclusions and Implications for Future Research/Implementation

Toxicity tests evaluating selectively aged solutions of F3 and Reference AFFF formulations provided valuable insight into both the potential persistence and toxicity of tested materials including (without additional single chemical exposure testing) any degradation products that may occur as a result of environmental (i.e., photodegradation) or biological (i.e., metabolism) processes. Such tests may be particularly useful when ingredients of tested materials are not known and/or degradation pathways are not known or poorly understood.

In instances where ingredients are known and there is some understanding of potential degradation pathways, test parameters (e.g., UV, temperature, etc.) can be adjusted to evaluate their impact on toxicity/risk of these materials in the environment. In this case; the ability to compare novel or candidate products in relation to a current use product allows for direct comparison of potential toxicity or hazard assessment. In this evaluation of candidate F3 formulations, results of ageing indicate that toxicity decreased with aging. This was also the case for the PFAS containing Reference AFFF formulation. The reasons for decreased toxicity with aging of the material are not clear, but potential contributing factors include:

- Volatilization (though test formulation were maintained covered and without aeration);
- Sorption of toxic components to the test chamber;
- Photolysis, hydrolysis, etc. may have played a role.

Both targeted and non-target chemical analysis could be utilized in conjunction with these tests to help elucidate potential contributing factors to observed changes in toxicity. To date, several of the SERDP sponsored F3/AFFF projects have developed datasets addressing chemical analysis of these products, including this project. For F3-1, F3-2, and AFFF, non-target assessment of the most concentrated exposure solutions across four weeks identified mass features to each formulation that fell into one of three categories (Stable, Degradation, Addition) that may be useful in better understanding chemical changes of these formulations over time.

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7.0 Acknowledgements

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8.0 Appendices

APPENDIX A. Detailed workflow steps and settings for HRMS data extraction and analysis for non-targeted instrumental analysis of F3 and Reference AFFF formulations that supports the relative stability assessment of the solutions used in this study.

Workflow Tree Step	Parameter	Setting
0 Input files		
1 Select Spectra	1. Spectrum properties Filter	
	Lower RT Limit (min)	2
	Upper RT Limit (min)	34
	2. ScanEvent Filters	
	Polarity Mode	Any
2 Align Retention Times	1. General Settings	
	Alignment Model	Adaptive Curve
	Maximum Shift (min)	2
	Mass Tolerance	5 ppm
3. Detect Compounds	1. General Settings	
	Mass Tolerance	5 ppm
	Min. Peak Intensity	8000000
	Use Most Intense Isotope Only	TRUE
	Precursor Mass Tolerance	0.025 Da
	2. Peak Detection	
	Chromatographic S/N Threshold	1.5
	Remove Baseline	FALSE
	3. Isotope Pattern Detection	

	Group Isotopes for	Br; Cl
	4. Compound Assembly	All Selected
4. Merge Features	1. Peak Consolidation	
	Mass Tolerance	5 ppm
	RT Tolerance (min)	0.1
5. Group Compounds	1. General Settings	
	Mass Tolerance	5 ppm
	RT Tolerance (min)	0.1
	Minimum Valley (%)	10
	Align Peaks	FALSE
	Preferred Ions	[M+H] ⁺ +1; [M+NH ₄] ⁺ ; [M-H] ⁻ -1
	Area Integration	Most Common Ion
	2. Peak Rating Contributions	
	Area Contribution	3
	CV Contribution	10
	FWHM to Base Contribution	5
	Jaggedness Contribution	5
	Modality Contribution	5
	Zig-Zag Index Contribution	5
	3. Peak Rating Filter	
	Peak Rating Threshold	5
	Number of Files	2
6. Mark Background	1. General Settings	
	Max. Sample/Blank	5

Max. Blank/Sample	0
Hide Background	TRUE

Workflow Tree Step	Parameter	Setting
7. Calculate Mass Defect	1. Mass Defect	
	Fractional Mass Defect	FALSE
	Standard Mass Defect	FALSE
	Relative Mass Defect	FALSE
	Kendrick Mass Defect	TRUE
	2. Kendrick Formula	
	Formula 1	C2 F4
	Formula 2	C3 F3 O
	Formula 3	C2 H4
	Formula 4	C2 H4 O6
	Formula 5	C8 H8
8. Search mzCloud	1. General Settings	
	Compound Classes	All
	Library	Autoprocessed; Reference
	Search MSn Tree	TRUE
	2. DDA Search	
	Identity Search	NIST
	Match Activation Type	FALSE
	Match Activation Energy	Any
	Activation Energy Tolerance	20

Apply Intensity Threshold	FALSE
Similarity Search	Similarity Forward
Match Factor Threshold	30
3. DIA Search	
Use DIA Scans for Search	FALSE
Max. Isolation Width (Da)	500
Match Activation Type	FALSE
Match Activation Energy	Any
Activation Energy Tolerance	100
Apply Intensity Threshold	FALSE
Match Factor Threshold	20

9. Search mzVault

1. Search Settings

mzVault Library	hr_msms_nist23_1
	hr_msms_nist23_2
	PFAS_CFM_specLibrary_Duke
Compound Classes	All
Match Ion Activation Type	FALSE
Match Ion Activation Energy	Any
Ion Activation Energy Tolerance	20
Match Ionization Method	FALSE
Apply Intensity Threshold	TRUE
Precursor Mass Tolerance	10 ppm
Match Analyzer Type	FALSE
Search Algorithm	NIST
Match Factor Threshold	20

RT Tolerance (min)	2
Use Retention Time	FALSE

Workflow Tree Step	Parameter	Setting
10. Compositions	Predict	
	1. Prediction Settings	
	Mass Tolerance	5 ppm
	Min. Elements Counts	C H
	Max. Elements Counts	C90 H190 Br8 C18 F180 N10 O18 P5 S5
	Min. RDBE	0
	Max. RDBE	40
	Min. H/C	0
	Max. H/C	3.5
	Max. # Candidates	15
	2. Pattern Matching	
	Intensity Tolerance (%)	30
	Intensity Threshold (%)	0.1
	S/N Threshold	3
	Use Dynamic Recalibration	TRUE
	3. Fragments Matching	
	Use Fragments Matching	TRUE
	Mass Tolerance	5 ppm
	S/N Threshold	3

11. Assign Compound Annotations	1. General Settings
---------------------------------	---------------------

Mass Tolerance	5 ppm
2. Data Sources	
Data Source #1	mzCloud Search
Data Source #2	mzVault Search
Data Source #3	ChemSpider Search
Data Source #4	MassList Search
Data Source #5	Predicted Compositions
3. Scoring Rules	
Use mzLogic	TRUE
Use Spectral Distance	TRUE
SFit Threshold	20
Sfit Range	20
4. Reprocessing	
Clear Names	FALSE

12. Generate
Molecular Networks

1. Spectral Similarity	
Use Full MSn Tree	TRUE
Match Mass Shift	TRUE
Match Transformations	TRUE
Variate Transformations	FALSE
S/N Threshold	3
Mass Tolerance	2.5 mmu
Min Fragment m/z	50
2. Transformations	
Phase I	All Selected

Phase II	All Selected
Others	PFAS Chain Shortening (C F2 - ->)
Max. # Phase II	1
Max. # All Steps	3

Workflow Tree Step	Parameter	Setting
12. Generate Molecular Networks	3. Applied View Filters	
	Require Transformation	TRUE
	Require MSn	TRUE
	Min. MSn Score	30
	Min MSn Coverage	30
	Min. Fragments	3
	4. Applied Thresholds	
	Require Transformation	FALSE
	Require MSn	FALSE
	Min. MSn Score	20
	Min. MSn Coverage	20
	Min. Fragments	0
13. Search ChemSpider	1. Search Settings	
	Database(s)	ACToR: Computational Resource DrugBank Aggregated Toxicology

		EAWAG Biocatalyst/Biodegradation Database
		EPA DSSTox
		EPA Toxcast
		FDA UNII - NLM
		NIST
		NIST Spectra
	Search Mode	By Formula or Mass
	Mass Tolerance	5 ppm
	Max. # of results per compound	20
	Max. # of Predicted Compositions to be searched per Compound	3
<hr/>		
14. Apply mzLogic	1. Search Settings	
	Max. # Compounds	0
	Max. # mzCloud Similarity Results to consider per compound	10
	Match Factor Threshold	30
<hr/>		
15. Apply Spectral Distance	1. Pattern Matching	
	Mass Tolerance	5 ppm
	Intensity Tolerance (%)	30
	Intensity Threshold (%)	0.1
	S/N Threshold	3
	Use Dynamic Recalibration	TRUE
<hr/>		
16. Search Mass Lists	1. Search Settings	

Mass Lists

Chemical List PFASSTRUCT-
2022-04-20

Extractables and Leachables
HRAM Compound Database

Natural Products Atlas 2021_08

Natural Products Atlas 2023_06

PFAS_NEG

PFAS_NIST

PFAS_suspectDB_Duke

Workflow Tree Step	Parameter	Setting
16. Search Mass Lists	Use Retention Time	FALSE
	RT Tolerance (min)	0.5
	Mass Tolerance	5 ppm

Data Reduction

After data extraction in Compound Discoverer, lists of feature detections in each foam sample were exported to Excel and further reduced. Features with peak area at least 3-fold greater abundance than the average peak area in blanks were retained; peak area was set to zero for features that did not meet the criteria.

Confidence Scoring

Confidence in molecular formula assignment and library search matching is expressed using the five-level system proposed by Schymanski et al. (2015) which include:

Level 1: Confirmed structure – by reference standard

Level 2: Probable structure

- a. By library spectrum match
- b. By diagnostic evidence

Level 3: Tentative candidate(s) – structure, substituent, class

Level 4: Unequivocal molecular formula – MS isotope/adduct

Level 5: Exact mass of interest

Formula assignments are reported when there is high enough confidence and were removed when there were multiple possible assignments with similar scoring. Level 5 assignments are tentative assignments based on match scoring and should not be considered final. Level 4 assignments have higher certainty, and Level 3 has a library match to MS2 fragmentation providing further evidence. The few Level 2 assignments have multiple libraries matching to the same compound.

All data are stored on the instrument computer located in Room D203 of the Hollings Marine Laboratory, Charleston, SC.

APPENDIX B. Features from NTA (F3-3) that were either 1) matched to task 1 m/z estimates or 2) where area responses were qualitatively observed to change in relation to the length of ageing.

AFFF	Formula	Name	Calc. MW	m/z	Reference Ion	Peak Area Week 0	Peak Area Week 1	Peak Area Week 2	Peak Area Week 3	Peak Area Week 4
REF	C10 H22 O4 S		238.12378	237.1165	[M-H]-1	2180000000	661000000	0	0	0
REF	C10 H22 O4 S		238.12375	237.11648	[M-H]-1	0	0	8090000000	333000000	301000000
REF	C10 H22 O4 S		238.12376	237.11649	[M-H]-1	66200000000	0	11900000000	0	712000000
REF	C10 H22 O4 S		238.12378	237.1165	[M-H]-1	0	17000000000	0	2570000000	0
REF	C16 H23 F13 N2 O6 S2	3-({3-[(2-Hydroxyethyl)(dimethylammonio)propyl]}[(tridecafluorohexyl)sulfonylamino]-1-propanesulfonate	650.07955	649.07233	[M-H]-1	75600000	133000000	409000000	142000000	136000000
REF	C16 H23 F13 N2 O6 S2	3-({3-[(2-Hydroxyethyl)(dimethylammonio)propyl]}[(tridecafluorohexyl)sulfonylamino]-1-propanesulfonate	650.07936	649.07212	[M-H]-1	3600000000	0	3380000000	3700000000	3530000000
REF	C7 H7 N3	4-Methylbenzotriazole	133.06387	134.07114	[M+H] ⁺ ₁	0	50300000	124000000		
REF	C8 H16 O3	2-Butoxyethyl acetate	160.11	161.11728	[M+H] ⁺ ₁	79000000	210000000	124000000		
REF	C8 H16 O3	3-hydroxyoctanoic acid	160.10991	159.10264	[M-H]-1	0	49100000			
REF	C8 H18 O4 S	n-Octyl sulfate	210.09237	209.0851	[M-H]-1	0	845000000			
REF	C8 H18 O4 S	n-Octyl sulfate	210.09246	209.08518	[M-H]-1	17400000000	0			
F3-1	C12 H26 O4 S	Dodecyl sulfate	266.15513	265.14785	[M-H]-1	13300000000	0	0	502000000	891000000
F3-1	C12 H26 O4 S	Dodecyl sulfate	266.15516	265.14789	[M-H]-1	0	16400000000	13000000000	0	0
F3-1	C12 H26 O4 S	Dodecyl sulfate	266.15508	265.14781	[M-H]-1	21100000000	13400000000	824000000	0	0
F3-1	C12 H26 O4 S	Dodecyl sulfate	266.15505	265.14777	[M-H]-1	0	0	0	10100000000	959000000
F3-1	C12 H27 N O	N,N-Dimethyldecylamine N-oxide	201.20901	202.21629	[M+H] ⁺ ₁	814000000	768000000	815000000	781000000	810000000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23511	246.24238	[M+H] ⁺ ₁	0	0	0	180000000	126000000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.2356	246.24287	[M+H] ⁺ ₁	0	0	0	39000000	41300000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23562	246.24289	[M+H] ⁺ ₁	0	44400000	50200000	206000000	78700000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23562	246.2429	[M+H] ⁺ ₁	0	0	44900000	121000000	50200000

F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23557	246.24284	[M+H] ⁺ ₁	0	0	168000000	436000000	110000000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23564	246.24292	[M+H] ⁺ ₁	0	0	0	83100000	64500000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23555	246.24283	[M+H] ⁺ ₁	0	0	48200000	113000000	119000000
F3-1	C14 H31 N O2	1,1'-(Octylazanediy)bis(propan-2-ol)	245.23555	246.24283	[M+H] ⁺ ₁	0	59300000	127000000	54400000	219000000
F3-1	C16 H34 O4 S	cetyl sulfate	322.2179	321.21063	[M-H] ⁻ ₁	949000000	235000000	0	0	0
F3-1	C16 H34 O4 S	n-Hexadecyl sulfate	322.21795	321.21067	[M-H] ⁻ ₁	0	695000000	657000000	0	0
F3-1	C16 H35 N O	2-(Tetradecylamino)ethanol	257.27151	258.27878	[M+H] ⁺ ₁	11500000000	13700000000	12700000000	11800000000	11100000000
F3-1	C16 H35 N O	2-(Tetradecylamino)ethanol	257.27174	258.27901	[M+H] ⁺ ₁	16000000000	14800000000	6020000000	10400000000	7910000000
F3-1	C16 H35 N O2		273.26633	274.27361	[M+H] ⁺ ₁	0	0	0	293000000	109000000
F3-1	C16 H35 N O2		273.26689	274.27416	[M+H] ⁺ ₁	0	113000000	70100000	456000000	71900000
F3-1	C16 H35 N O2		273.26684	274.27412	[M+H] ⁺ ₁	0	0	102000000	452000000	88300000
F3-1	C16 H35 N O2		273.26674	274.27402	[M+H] ⁺ ₁	0	0	0	96300000	62800000
F3-1	C16 H35 N O2		273.26683	274.27411	[M+H] ⁺ ₁	0	0	0	184000000	143000000
F3-1	C16 H35 N O2		273.26687	274.27415	[M+H] ⁺ ₁	0	53200000	0	63700000	93100000
F3-1	C16 H35 N O2		273.26686	274.27414	[M+H] ⁺ ₁	0	0	0	43800000	52000000
F3-1	C18 H39 N O	2-(Hexadecylamino)ethanol	285.30277	286.31004	[M+H] ⁺ ₁	1820000000	1940000000	1990000000	1630000000	733000000
F3-1	C18 H39 N O	2-(Hexadecylamino)ethanol	285.30313	286.31041	[M+H] ⁺ ₁	2750000000	1950000000	934000000	335000000	134000000
F3-1	C20 H43 N O	2-(Octadecylamino)ethanol	313.33434	314.34162	[M+H] ⁺ ₁	114000000	77000000	34900000	0	0
F3-1	C8 H17 N O3	2-Ethylhexyl nitrate	175.12057	176.12785	[M+H] ⁺ ₁	0	0	0	275000000	35000000
F3-1	C8 H17 N O3	2-Ethylhexyl nitrate	175.12083	176.12811	[M+H] ⁺ ₁	160000000	181000000	62000000	191000000	328000000
F3-1	C8 H17 N O3	2-Ethylhexyl nitrate	175.12079	176.12807	[M+H] ⁺ ₁	0	0	62700000	238000000	448000000
F3-1	C8 H18 O3	Dipropylene glycol dimethyl ether	162.12535	185.11458	[M+Na] ⁺ ₁	4500000000	4230000000	4280000000	4230000000	4140000000
F3-1	C8 H18 O3	Dipropylene glycol dimethyl ether	162.12554	163.13277	[M+H] ⁺ ₁	4360000000	2560000000	298000000	535000000	60700000
F3-1	C10 H22 O4 S		238.1237	237.1165	[M-H] ⁻ ₁	0	98700000	103000000	0	0

F3-1	C10 H22 O4 S		238.1238	237.1165	[M-H]-1	103000000	0	0	94000000	94800000
F3-1	C10 H22 O4 S		238.124	237.1167	[M-H]-1	454000000	127000000	136000000	0	0
F3-1	C18 H37 N O	Stearamide	283.2874	284.2947	[M+H] ₁ ⁺	0	0	0	2300000000	2340000000
F3-1	C19 H38 N2 O3	Cocamidopropyl betaine	342.2879	343.2952	[M+H] ₁ ⁺	1720000000	1920000000	1630000000	1430000000	1130000000
F3-1	C19 H38 N2 O3	Cocamidopropyl betaine	342.288	343.2954	[M+H] ₁ ⁺	8820000000	8860000000	7470000000	6820000000	6480000000
F3-1	C21 H42 N2 O3	Myristamidopropyl betaine	370.319	371.3263	[M+H] ₁ ⁺	375000000	373000000	299000000	0	0
F3-1	C21 H42 N2 O3	Myristamidopropyl betaine	370.319	371.3267	[M+H] ₁ ⁺	2900000000	2580000000	1710000000	1030000000	450000000
F3-1	C6 H12 O		100.089	101.0961	[M+H] ₁ ⁺	173000000	145000000	112000000	0	0
F3-1	C8 H17 N O3	2-Ethylhexyl nitrate	175.121	176.1281	[M+H] ₁ ⁺	0	63400000	93100000	1350000000	1550000000
F3-1	C8 H17 N O3	2-Ethylhexyl nitrate	175.121	176.1281	[M+H] ₁ ⁺	0	0	0	162000000	186000000

APPENDIX C.

List of F3 formulations and Reference AFFF tested in this project along with corresponding code name used in this report. The formulation names were the product labels as provided by SERDP in 2020.

Code Name	Formulation name
Reference AFFF	Buckeye Platinum
F3-1	Avio F3 Green KHC
F3-2	BioEx ECOPOL A
F3-3	Fomtec Enviro USP
F3-4	NFD 20-391
F3-5	NRL 502W
F3-6	Solberg Re-Healing RF3