CDFW Renewable Diesel Toxicity Testing
Project-End Report: CDFW-OSPR_2026_UCD-GC

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<u>Test/Project IDs</u>:

- 100% Renewable Diesel (RND) Toxicity Testing:
    - AA RF – 100 RND (*Atherinops affinis*, 06/12/24), Rangefinder
    - AA RF – 100 RND_REDO (*Atherinops affinis*, 4/8/25), Rangefinder
    - AA Def – 100 RND (*Atherinops affinis*, 09/24/24), Definitive
    - MG RF – 100 RND (*Mytilus galloprovincialis,* 9/26/24), Rangefinder
    - PP RF – 100 RND (*Pimephales promelas*, 2/05/25), Rangefinder
    - PP Def – 100 RND (*Pimephales promelas*, 3/26/25), Definitive
    - CD RF – 100 RND (*Ceriodaphnia dubia,* 5/27/25), Rangefinder
    - CD Def – 100 RND (*Ceriodaphnia dubia,* 7/1/25), Definitive

- 100% Conventional Diesel (CND) Toxicity Testing:
    - AA RF – 100 CND (*Atherinops affinis*, 12/10/24), Rangefinder
    - AA Def – 100 CND (*Atherinops affinis*, 1/21/25), Definitive
    - PP RF – 100 CND (*Pimephales promelas*, 3/5/25), Rangefinder
    - PP Def – 100 CND (*Pimephales promelas*, 4/30/25), Definitive
    - CD RF – 100 CND (*Ceriodaphnia dubia,* 6/17/25), Rangefinder
    - CD Def – 100 CND (*Ceriodaphnia dubia,* 7/11/25), Definitive
    - MG RF – 100 CND (*Mytilus galloprovincialis,* 1/30/25), Rangefinder
    - MG Def – 100 CND (*Mytilus galloprovincialis,* 4/29/25), Definitive
    - MG Def – 100 CND (*Mytilus galloprovincialis*, 2/6/26), Definitive

- Blend 1 (20 % CND : 80% RND) Toxicity Testing:
    - CD RF – 20 CND (*Ceriodaphnia dubia,* 8/7/25), Rangefinder
    - CD Def – 20 CND (*Ceriodaphnia dubia,* 8/18/25), Definitive
    - PP RF – 20 CND (*Pimephales promelas*, 8/27/25), Rangefinder
    - PP Def – 20 CND (*Pimephales promelas*, 9/11/25), Definitive

- Blend 2 (5% CND : 95% RND) Toxicity Testing:
    - CD RF – 5 CND (*Ceriodaphnia dubia,* 10/06/25), Rangefinder
    - CD Def – 5 CND (*Ceriodaphnia dubia,* 10/17/25), Definitive
    - PP RF – 5 CND (*Pimephales promelas*, 10/29/25), Rangefinder

Table of Contents

Introduction

The California Department of Fish and Wildlife, Office of Spill Prevention and Response (CDFW-OSPR) is California's lead agency dedicated to spill prevention, response, and restoration in terrestrial and marine environments. The CDFW-OSPR Natural Resource Damage Assessment (NRDA) unit is responsible for quantifying potential damage to natural resources by oil spills or discharge. Renewable diesel is the most commercially available renewable form of fuel in California, and shipment of this fuel in pure form is internationally sent to major marine terminals across California, blended with conventional diesel at coastal facilities, and shipped across the state for use. The independent and synergistic effects of these blends on the environment are largely unknown and require toxicity data to assess the impacts on marine and freshwater larval fish and invertebrates.

To address these data gaps, the UC Davis Marine Pollution Studies Laboratory at Granite Canyon (MPSL) prepared high-energy water accommodated fractions (HEWAF) and conducted toxicity tests with four types of diesel fuels provided by CDFW-OSPR using *Atherinops affinis* (a marine fish), *Pimephales promelas* (a freshwater fish), *Mytilus galloprovincialis* (a marine invertebrate), and *Ceriodaphnia dubia* (a freshwater invertebrate). The overall objective of this work is to compare toxicity profiles and determine potential environmental hazards of conventional and renewable diesels, and their blends using a suite of model organisms exposed to HEWAF preparations of the test fuels. Toxicity point estimates were calculated by generating dose response curves with HEWAF dilution series. Toxicity endpoints were further contextualized using total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) in the HEWAF preparations measured by the CDFW-OSPR Petroleum Chemistry Laboratory.

MPSL is a state-certified laboratory, and all analyses, sample management, and data management comply with internal laboratory Quality Assurance Documentation, Surface Water Ambient Monitoring Program (SWAMP) protocols, and the best management practices for toxicity analysis as certified by the California Environmental Laboratory Accreditation Program (CA ELAP). Additionally, MPSL followed all established Institutional Animal Care and Use Committee (IACUC) protocols for larval fish work.

Materials and Methods

HEWAF Preparation

All stock diesel fuels were provided to MPSL by CDFW-OSPR in 4L amber jugs prior to testing and were stored in a refrigerator at 4°C. Toxicity testing was conducted using four diesel types – Conventional Diesel (CND), Renewable Diesel (RND), Diesel Blend 1 (20 % CND : 80 % RND), and Diesel Blend 2 (5 % CND : 95 % RND). HEWAF was prepared daily in a Waring CB15 commercial food blender in accordance with an adapted HEWAF protocol developed during the Deepwater Horizon Oil Spill (Carney et al 2016). A pre-cleaned stainless-steel blender was filled with 3.5 L of freshwater (MPSL well water) or seawater (pumped directly from California coast on MPSL property; filtered to one µm). Fuel was weighed in a glass graduated cylinder to achieve a loading rate of 20 g fuel/L water and added to the blender. The loading rate for the 3-brood chronic *Ceriodaphnia* test was reduced to 10 g fuel/L water after it was determined that 20 g fuel/L water HEWAF caused the organism to become trapped in the surface tension and resulted in unreliable toxicity results. The blender was closed with a hexane-rinsed, aluminum-lined lid. The fuel-water mixture was blended on low for 30 seconds. The contents were added to a 5 L separatory funnel and allowed to separate for one hour in a temperature-controlled room. The bottom layer of the HEWAF was collected into a 4 L glass beaker without disturbing or collecting the surface oil slick. The volume collected in the 4 L glass beaker was noted and labeled as 100% HEWAF. Serial dilutions were made from this 100 % HEWAF using control water per the test requirements (Table 1).

All oiled glassware and blenders were cleaned using a detailed cleaning procedure. Glassware was rinsed three times with 99.9% hexane (ACS Grade) and allowed to completely evaporate before being washed with hot water and Micro90® cleaning solution. Glassware was then rinsed three times with DI water, three times with 99.5% acetone (ACS Grade), and three times with hexane, in that order, allowing for complete evaporation between solvents. Before use, the blender was rinsed once with either freshwater or seawater.

Toxicity Testing and Chemical Analysis

Four toxicity tests were conducted in this study, including two marine tests- the 7-day chronic larval topsmelt (*Atherinops affinis)* test (U.S. EPA 1995), the 48-hour chronic mussel (*Mytilus galloprovincialis)* test (U.S. EPA 1995), and two freshwater tests- the 7-day chronic larval fathead minnow (*Pimephales promelas)* test (U.S. EPA 2002), and the 3-brood chronic daphnid (*Ceriodaphnia dubia)* test (U.S. EPA 2002). Protocol summary and test details can be found in Table 1. Standard operating procedures (SOPs, Appendix A), the scanned laboratory test packets and culture logs (Appendix B), and chains of custody documentation and analytical reports (Appendix C) for all tests are included at the end of this document.

Larval fish were obtained from Aquatic Biosystems (Fort Collins, CO), and mussels were acquired from Taylor Shellfish Farms (Shelton, WA). The daphnid culture was originally obtained from Aquatic Biosystems and maintained in-house throughout the year. All organisms were acclimated according to MPSL SOPs, Surface Water Ambient Monitoring Program (SWAMP) measurement quality objectives (www.waterboards.ca.gov/water_issues/programs/swamp/mqo.html), and IACUC protocols (when applicable).

Positive reference toxicant tests were conducted alongside fuel tests using dilutions of copper chloride (*Ceriodaphnia,* larval fathead minnow, and larval topsmelt) and cadmium chloride (mussels). These tests were utilized to track organismal health and laboratory performance (U.S. EPA 1995) by preparation of control charts that display toxicity point estimate values and variability among test batches.

Rangefinder tests (RF) were run with HEWAF preparations of 0.0, 1.0, 5.0, 10.0, 50.0, and 100.0 (%). Toxicity results from the rangefinder tests were used to determine the exposure series for definitive test to best capture median lethal and inhibitory concentrations. Toxicity point estimates discussed in the

Results and Discussion section below were derived for each test species from the definitive tests. No definitive tests were conducted in instances of no measurable response in the rangefinder test.

On Day 0 of a definitive test, 1 L HEWAF subsamples from each concentration and control were collected in amber glass bottles and sent to CDFW-OSPR Petroleum Chemistry Laboratory (PCL) (Rancho Cordova, CA) for chemical analysis. Samples were analyzed using Gas-Chromatography with Flame Ionization Detector (GC-FID) to measure diesel range organics with a carbon range from C10-C28, referred to as total petroleum hydrocarbons (TPHs). Gas-Chromatography with Mass Spectrometry in Selected Ion Monitoring (GC-MS (SIM)) was used to measure polycyclic aromatic hydrocarbons (PAHs). A summation procedure of 45 individual PAHs was performed to generate a total PAH_{45} value. On Day 7 of the definitive fish tests, composite samples from the test chambers were collected for each HEWAF concentration in 1 L amber glass jars and sent to PCL for chemical analysis. In instances where a rangefinder test showed no toxicity at the highest HEWAF concentration (100%), the fuel was considered nontoxic for that test type, and a definitive test was deemed unnecessary. Quantitation of TPHs and PAHs in such instances was conducted using samples collected on Day 0 and Day 7 of the rangefinder test, when possible, (e.g., larval fathead, Blend 2 test). Only Day 0 chemistry was collected for daphnid and mussel tests as the replicate sizes were too small in volume to collect adequate sample for chemistry analysis at the end of the test.

The first set of tests conducted for this project (rangefinder and definitive larval topsmelt tests with renewable diesel) were prepared by making HEWAF every other day and storing it in the fridge between preparations. Initial analysis of the chemistry subsamples from these tests showed a ~10-fold decrease in total TPH concentration over 24 hours. Further analyses on Day 6 samples confirmed consistent concentrations relative to Day 0 in freshly prepared HEWAF, and potential TPH loss to beaker surfaces during the exposure over 24 hours. Following this finding, it was decided to renew the test with freshly made HEWAF every day. Given this change, the rangefinder test for larval topsmelt with renewable diesel was repeated using daily HEWAF preparation. Toxicity results from the first set of tests are included only for comparison in the Results and Discussion section below, but were not utilized for statistical analyses and conclusions for topmselt. It must be noted that the mussel renewable diesel test shared Day 0 HEWAF with the topsmelt test, but did not require to be repeated because it is a non-renewal 48-hr test. HEWAF preparation records for each test are included in the scanned test data packets. The 24-hr reduction in TPH and PAH_{45} in the fish exposures was calculated by subtracting concentrations measured in the test beakers on Day 7 from freshly prepared HEWAF concentrations measured on Day 0 or Day 6 (in some instances).

Toxicity point estimates were calculated using the Comprehensive Environmental Toxicity Information System (CETIS™) software (v.1.9.6.9). These point estimates include median lethal concentrations (LC_{50}), no observed effect concentrations (NOEC), and lowest observed effect concentrations (LOEC) for each rangefinder and definitive test. For tests with a growth or reproduction endpoint, the median effective concentration (EC_{50}) was also calculated.

Table 1. Summary of toxicity test conditions showing acceptable ranges of parameters required for the test (DO = dissolved oxygen).

Details	Chronic *A. affinis*	Chronic *P. promelas*	Chronic *M. galloprovincialis*	Chronic *C. dubia*
Common Name	Topsmelt	Fathead minnow	Mussel	Daphnid
Test Type	Daily Static Renewal	Daily Static Renewal	Static Non-Renewal	Daily Static Renewal
Duration	7 days	7 days	48 hours	6-8 days
Salinity (if applicable)	5 – 36 ppt ± 2 ppt	N/A	28 – 34 ppt ± 2 ppt	N/A
Temperature	20 °C ± 1 °C	25 °C ± 1 °C	15 °C or 18 °C ± 1 °C	25 °C ± 1 °C
Chamber Size	600 mL	600 mL	20 mL	20-40 mL
Replicate Volume	200 mL	250 mL	10 mL	15 mL
Organism Supplier	Aquatic Biosystems	Aquatic Biosystems	Taylor Shellfish Farms	Cultured in-house (original culture from Aquatic Biosystems)
Organism age	9-15 days	Newly-hatched larvae <24 hours old; if shipped, <48 hours old with a 24-hour age range	Within 4 hours of fertilization	<24 hours old and within <8 hours of each other
No. of organisms per replicate	5 larvae	10 larvae	150-200 larvae	1 neonate
Feeding Regime	40 *Artemia* nauplii per larvae, fed twice daily	*Artemia* nauplii ad libidum	Do not feed	200 uL 3:1 Selenastrum:YCT
Aeration	None unless DO drops below 4.0 mg/L	None unless DO drops below 4.0 mg/L	None unless initial DO below 4.0 mg/L	None unless DO drops below 4.0 mg/L
Endpoints	Survival and Biomass	Survival and Biomass	Survival with Normal Development	Survival and Reproduction
Acceptability Criteria	≥80% mean survival and larval weight = 0.85 mg average in controls	≥80% mean survival and larval weight of ≥0.25 mg average in controls	≥50% mean survival and ≥90% mean normal shell development average in controls	≥80% mean survival in controls, 60% of the surviving control females must produce 3 broods with an average of 15 or more neonates per female

Results and Discussion

Each test has a set of test acceptability criteria (TAC) that are related to test-specific endpoints (Table 1). All test controls met TAC for survival. For the topsmelt tests used for statistical analyses, average dry weight for the controls ranged between 0.624 mg and 0.753 mg per individual larval fish, which was below the TAC (≥ 0.85 mg/individual). Exposure variables that can affect the weight endpoint, such as water quality and food consumption, were noted to be acceptable for these tests. Spawning differences across fish batches could also affect control weights as noted in dry weight per individual larval fish (13 days old) at the beginning of the test (ranging from 0.376 to 0.456 mg per fish). Despite these findings, the health of the fish was deemed optimal given their survival response and batch consistency across controls. The health of the fish is also tracked via their response in reference toxicant tests. Both survival and growth responses for topsmelt reference toxicant tests (with $CuCl_2$ dilution series) were within the acceptable range (± 2 standard deviations of the running mean between 2003 and 2025) which is tracked as a Quality Assurance/Quality Control (QA/QC) measure. Given the lack of significant differences across all test concentrations with renewable diesel HEWAF and relative to controls, the lower weights didn't seem to affect the test outcome. For conventional diesel exposures, the data provided clear dose responses and were acceptable given the consistent control response across batches. The minimum significant difference (MSD) for survival endpoint in the larval topsmelt definitive test for conventional diesel was 27.61% which slightly exceeded the TAC OF <25%. However, the dose response was well-defined, and the test results were accepted. The percentage of mussel larvae with normal shell development in the conventional diesel rangefinder and definitive tests were 89% and 87% respectively, which does not meet the TAC minimum of 90%. Additionally, the dose responses between the two tests resulted in conflicting point estimates. Therefore, these tests were removed from further analysis, and a third test was conducted which passed all TAC.

Organismal responses in positive reference toxicant tests ran alongside HEWAF tests were within two standard deviations of the running mean, indicating that the organisms were healthy and responded as expected and thereby passed MPSL's internal QA/QC criteria. Given the role of pH and ionic strength in fathead minnow response to copper, changes were made to reference toxicant concentrations and dilution water preparation over the course of these tests. In one instance, a larval topsmelt reference toxicant test control response failed TAC and therefore, was subsequently re-ran. As a note, the reference toxicant tests associated with *Ceriodaphnia* were ran mostly non-concurrently, as is acceptable by the EPA protocol for in-house cultures of test organisms (U.S. EPA 2002).

All water quality parameters were within recommended ranges except for minor deviations in some salinity and temperature values (Table 2). Some salinity values in larval topsmelt tests reached a maximum of 36.91 ‰, which is slightly outside the recommended range of 34 ‰ ± 2‰. All larval topsmelt tests had a minimum temperature that ranged between 18.0°C and 18.6°C, which is slightly outside the recommended range of 20°C ± 1°C. Additionally, one mussel test had a maximum temperature of 16.2°C and one larval fathead minnow test had a minimum temperature of 23.4°C, which is just outside the recommended temperature ranges of 15°C ± 1°C, and 25°C ± 1°C, respectively. These minor deviations are generally not expected to affect the results of the test, as topsmelt can tolerate a wide range of salinities, and the temperature variations are not substantial. It must be noted that these deviations represent few points in the entire duration of a given test and do not reflect consistent exposure conditions. One larval fathead minnow test had a final dissolved oxygen concentration below the required threshold of 4.0 mg/L. The sample was not aerated as the test was terminated at the time of measurement.

Table 2. A summary of the minimum and maximum measurements for the water quality parameters monitored during each test. DO = dissolved oxygen, RND = Renewable Diesel, CND = Conventional Diesel, Blend 1 = 20% CND : 80% RND, Blend 2 = 5% CND : 95% RND.

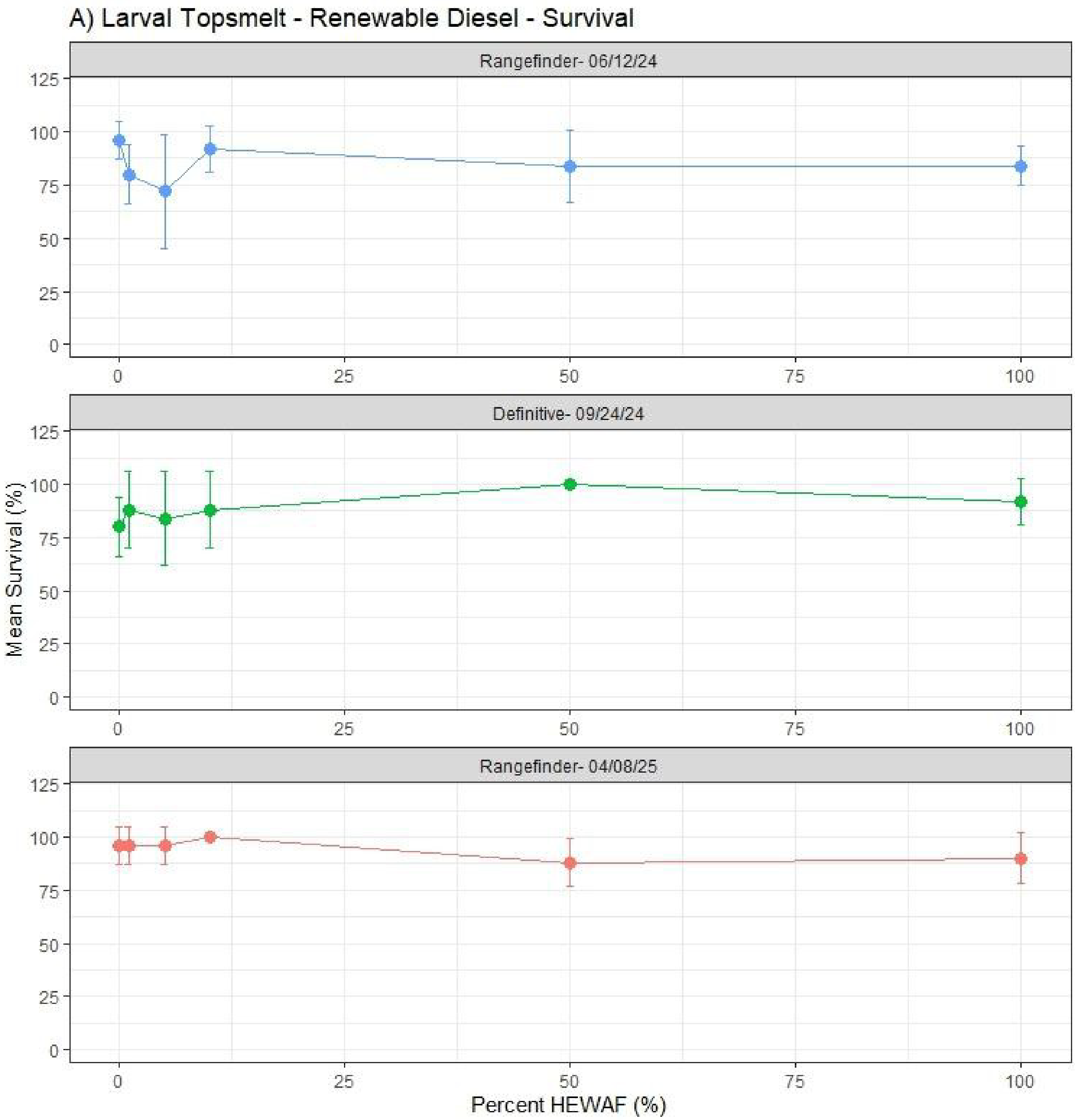
Fuel	Species	Test	Start Date	DO Min/Max (mg/L)	pH Min/Max	Salinity Min/Max (‰)	Conductivity Min/Max (µS/cm)	Temperature Min/Max (°C)
RND	*A. affinis*	7-day Rangefinder	06/12/2024	5.46/7.89	7.62/8.03	32.97/36.04	NA	18.0/20.4
RND	*A. affinis*	7-day Rangefinder	04/08/2025	4.51/7.45	7.78/8.09	33.44/36.16	NA	19.6/20.4
RND	*A. affinis*	7-day Definitive	09/24/2024	5.63/7.86	7.73/8.05	34.49/36.14	NA	18.4/20.7
RND	*M. galloprovincialis*	48-hour Rangefinder	09/26/2024	6.60/7.15	7.92/8.02	34.00/35.00	NA	14.4/16.2
RND	*P. promelas*	7-day Rangefinder	02/05/2025	3.99/9.24	7.82/8.66	N/A	989.3/933.0	23.4/25.4
RND	*P. promelas*	7-day Definitive	03/26/2025	5.49/8.74	7.90/8.63	N/A	720.1/914.8	24.4/25.7
RND	*C. dubia*	7-day Rangefinder	05/27/2025	7.38/8.39	8.45/8.59	N/A	775.1/837.7	24.3/25.3
RND	*C. dubia*	7-day Definitive	07/01/2025	7.08/9.38	8.05/8.58	N/A	743.0/835.0	24.1/25.1
CND	*A. affinis*	7-day Rangefinder	12/10/2024	5.59/7.62	7.85/8.08	33.86/34.98	NA	18.6/20.5
CND	*A. affinis*	7-day Definitive	01/21/2025	6.42/7.57	7.87/8.12	32.72/36.91	NA	18.3/20.6
CND	*P. promelas*	7-day Rangefinder	03/05/2025	5.94/8.49	8.08/8.70	N/A	873.1/920.0	24.3/25.7
CND	*P. promelas*	7-day Definitive	04/30/2025	5.23/8.63	8.07/8.58	N/A	778.6/812.9	24.3/25.2
CND	*C. dubia*	7-day Rangefinder	06/17/2025	7.58/8.52	8.07/8.58	N/A	731.4/773.0	24.3/25.6
CND	*C. dubia*	7-day Definitive	07/11/2025	7.39/9.11	8.38/8.59	N/A	714.7/741.8	24.0/25.4
CND	*M. galloprovincialis*	48-hour Definitive	02/06/2026	6.99/7.56	7.96/8.10	32.90/34.00	NA	15.1/16.0
Blend 1	*C. dubia*	7-day Rangefinder	08/07/2025	6.38/8.70	8.08/8.56	N/A	731.3/784.4	24.2/25.3
Blend 1	*C. dubia*	7-day Definitive	08/18/2025	7.07/8.69	7.07/8.58	N/A	693.4/758.1	24.1/25.2
Blend 1	*P. promelas*	7-day Rangefinder	08/27/2025	4.99/9.00	7.63/8.48	N/A	695.5/737.2	24.4/26.0
Blend 1	*P. promelas*	7-day Definitive	09/11/2025	4.88/8.81	7.83/8.53	N/A	689.2/750.8	24.0/25.6
Blend 2	*C. dubia*	7-day Rangefinder	10/06/2025	6.52/8.56	8.34/8.58	N/A	743.0/746.6	24.0/25.4
Blend 2	*C. dubia*	7-day Definitive	10/17/2025	7.60/9.48	8.46/8.56	N/A	701.0/782.1	24.0/25.1
Blend 2	*P. promelas*	7-day Rangefinder	10/29/2025	5.37/9.00	7.90/8.56	N/A	742.3/766.2	24.0/25.5

Objective 1: Conduct toxicity tests using a marine fish – Atherinops affinis

For all larval topsmelt tests, samples were collected on Day 0 and Day 6 to measure TPH and PAH_{45} concentrations in freshly made HEWAF before it was introduced to the exposure beakers. Samples were also collected on Day 7 to measure TPH and PAH_{45} concentrations at the end of exposure after the HEWAF had been in the exposure beakers for a 24-hr period.

The first set of larval topsmelt tests with renewable diesel were conducted using HEWAF prepared every other day and stored in the fridge between preparations. Therefore, once it was determined that HEWAF was to be made fresh every day instead, a third renewable diesel rangefinder was conducted to ensure the toxicity results did not significantly change. All three tests showed no significant effect on larval topsmelt survival or biomass at the highest test concentration of 100% HEWAF (7-day NOEC = 100 % HEWAF and 310 mg/L TPH), and LC_{50} value was above this concentration (Figure 1, Tables 3 and 5). It must be noted that there was a 95% decrease in this TPH concentration over a 24-hr period after renewal as seen in the HEWAF samples collected on Day 6 (256 mg/L) and Day 7 (11.7 mg/L) (Table 3). Although it is unclear what caused this reduction, it is possible that the hydrocarbons moved out of the solution into the waxy layer observed on top of the water column (see Photos: 'Waxy build-up in larval topsmelt test containing HEWAF prepared with 100% renewable diesel'), thereby not being detected in the water column. Another reason behind this loss could be adherence to beaker surfaces thus removing them from the water column. It is likely that during the course of the 7-day test, the TPH concentrations declined over every 24-hr period and were replenished with every subsequent HEWAF renewal. Given the lack of toxicity observed in the larval tests with renewable diesel HEWAF, a definitive test was deemed unnecessary. Additionally, it was determined to not proceed with testing with Blend 1 and Blend 2 fuels with this fish model as both blends contain less than 100 % renewable diesel.

As expected, the larval topsmelt was much more sensitive to HEWAF prepared with conventional diesel (Figures 2 and 3, Table 4), with a 7-day LC_{50} of 1.71 % HEWAF and a 7-day EC_{50} of 2.32 % HEWAF (Table 5). The 7-day LOEC was found to be 2.5 % HEWAF which contained 19.2 mg/L TPH and 89.98 µg/L PAH_{45} in freshly prepared HEWAF (Figure 3). At this HEWAF dilution, a 97% reduction in TPH (19.2 mg/L to 0.607 mg/L) and a 96 % reduction in PAH_{45} (90 µg/L to 3.25 µg/L) concentrations was observed between freshly prepared and 24-hr old HEWAF (Table 4). The 7-day NOEC was found to be 1.25% HEWAF which had 4.22 mg/L TPH and 22.5 µg/L PAH_{45} (Figure 3; Tables 4 & 5). A comparable decrease in TPH (95%) and PAH_{45} (96%) concentrations was also observed at 1.25% HEWAF (Table 4). Larval fish in HEWAF made with conventional diesel appeared more distressed and often displayed erratic or abnormal swimming behavior, such as swimming upside down, swimming on their side, becoming momentarily immobilized and drifting head down, or periodic thrashing.


A) Larval Topsmelt - Renewable Diesel - Survival
Rangefinder- 06/12/24
Definitive- 09/24/24
Rangefinder- 04/08/25
Mean Survival (%)
Percent HEWAF (%)
0
25
50
75
100
125

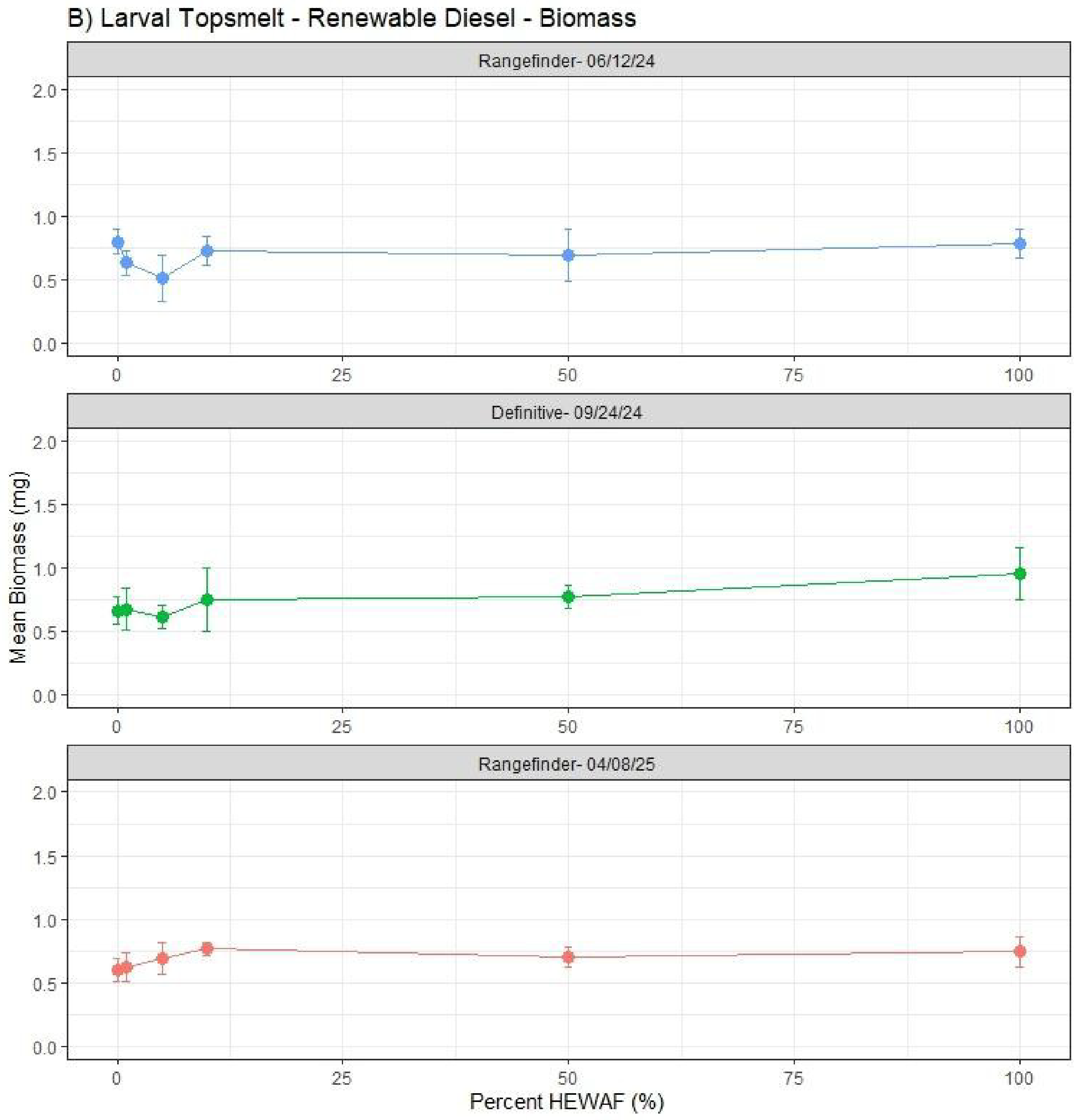
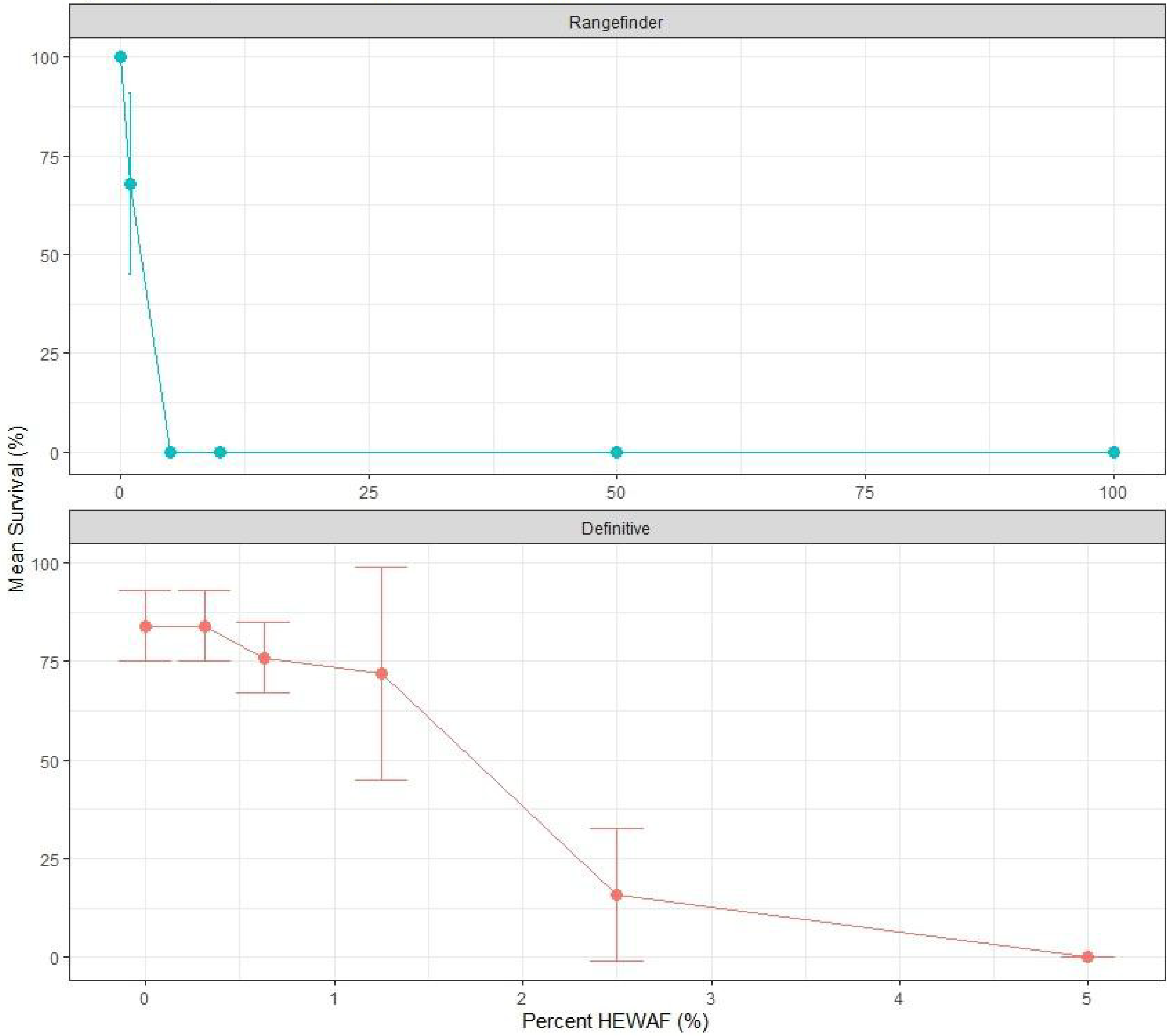


Figure 1. Rangefinder and definitive results from the chronic 7-day larval topsmelt toxicity tests with renewable diesel. The first two graphs in both panels (A and B) show results from the first set of tests wherein HEWAF was prepared on alternate days, and the bottom graph shows results from tests conducted with daily HEWAF preparations. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 3. Toxicity results and TPH concentrations (mg/L) associated with each HEWAF preparation from the renewable diesel toxicity test for larval topsmelt. ND = non detect. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 6 TPH Concentration (mg/L)	Day 7 TPH Concentration (mg/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	0.0388 DNQ	0.0423 DNQ	< 0.0476 ND	96 ± 9.0	0.6 ± 0.091
1	3.12	2.7	0.0859 DNQ	96 ± 9.0	0.624 ± 0.113
5	12.6	12.4	0.728	96 ± 9.0	0.692 ± 0.127
10	27	29.6	2.38	100 ± 0.0	0.768 ± 0.05
50	136	141	5.81	88 ± 11.0	0.708 ± 0.079
100	310	256	11.7	90 ± 12.0	0.745 ± 0.119

A) Larval Topsmelt - Conventional Diesel- Survival



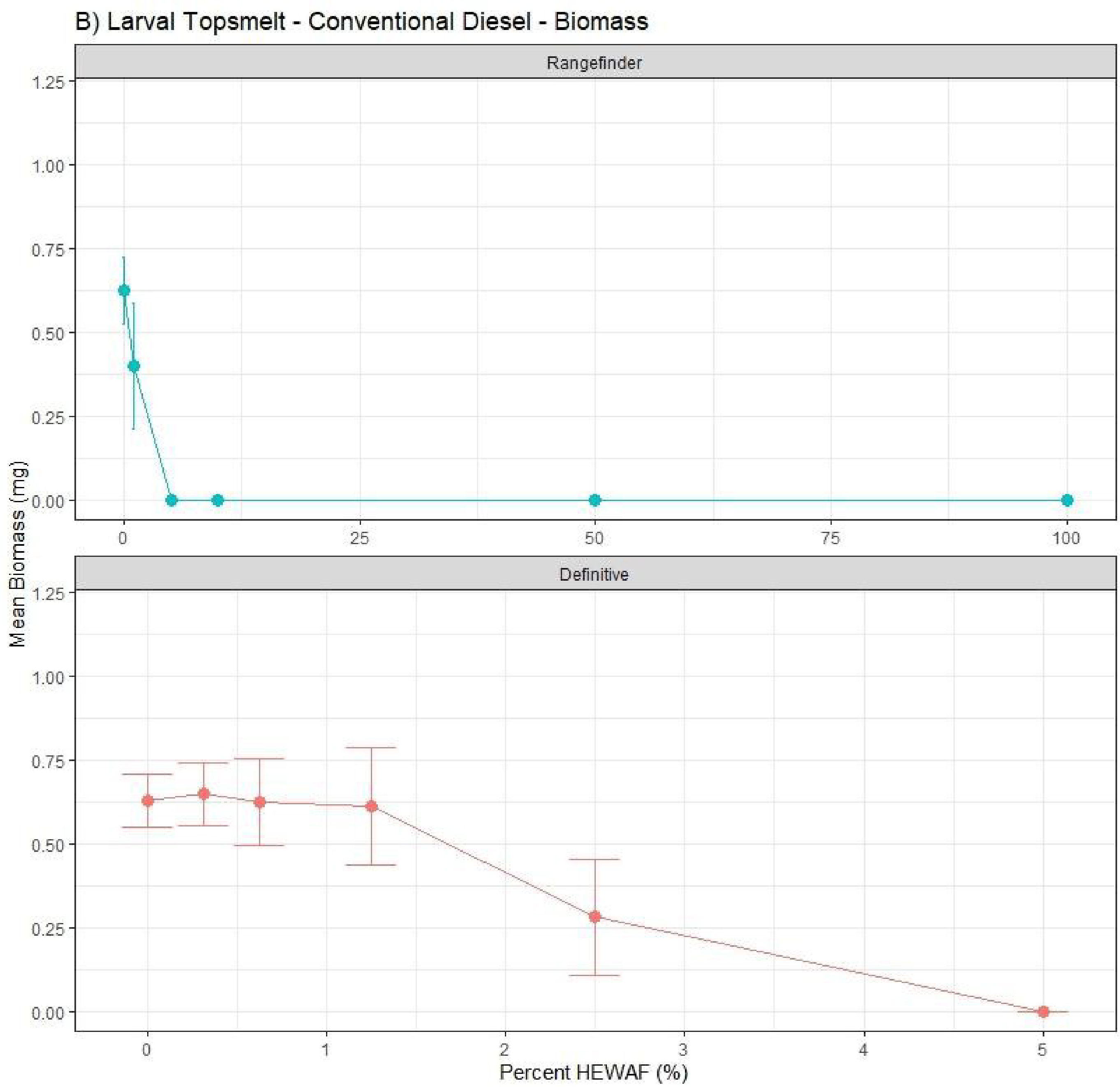
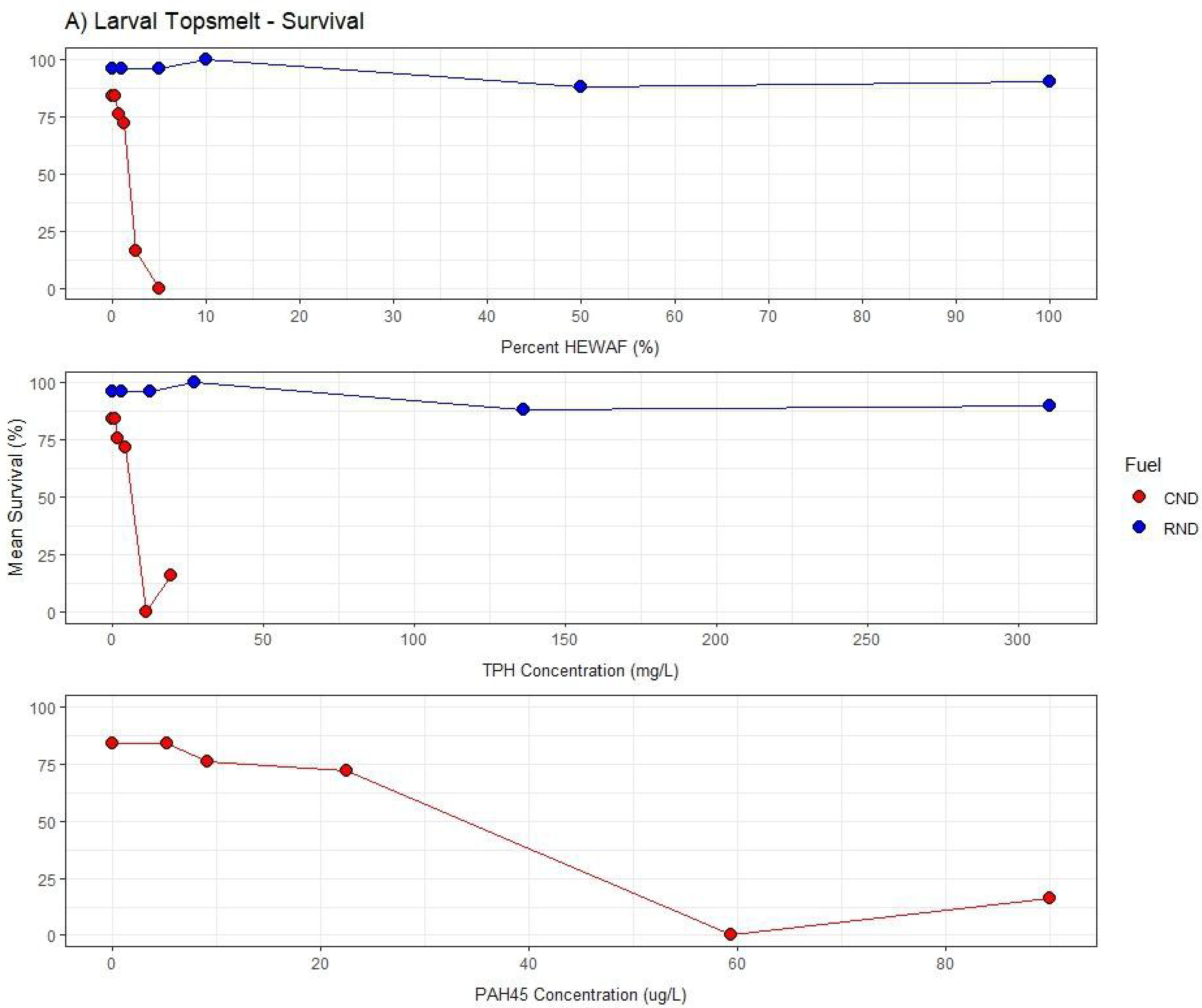


Figure 2. Rangefinder and definitive results for the chronic 7-day larval topsmelt toxicity tests with conventional diesel. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 4. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations (ug/L) associated with each HEWAF preparation from the conventional diesel definitive test for larval topsmelt. ND = non detect, NA = No analyses, DNQ = Detected, not quantified All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 6 TPH Concentration (mg/L)*	Day 7 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Day 7 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	< 0.0362 ND	0.0541 DNQ	0.0456 DNQ	0.0023	0.0581	84 ± 8.9	0.628 ± 0.079
0.3125	1.06	0.075 DNQ	0.0902 DNQ	5.3	0.351	84 ± 8.9	0.648 ± 0.094
0.625	1.74	0.095 DNQ	0.114 DNQ	9.17	0.449	76 ± 8.9	0.624 ± 0.131
1.25	4.22	0.168	0.199	22.5	0.844	72 ± 26.8	0.612 ± 0.175
2.5	19.2	0.339	0.607	90	3.25	16 ± 16.7	0.280 ± 0.174
5	11.3	1.15	NA	59.4	NA	0 ± 0.0	0 ± 0.0

* Day 6 chemistry was not conducted on fresh HEWAF.


A) Larval Topsmelt - Survival
Mean Survival (%)
Percent HEWAF (%)
TPH Concentration (mg/L)
PAH45 Concentration (ug/L)
Fuel
CND
RND

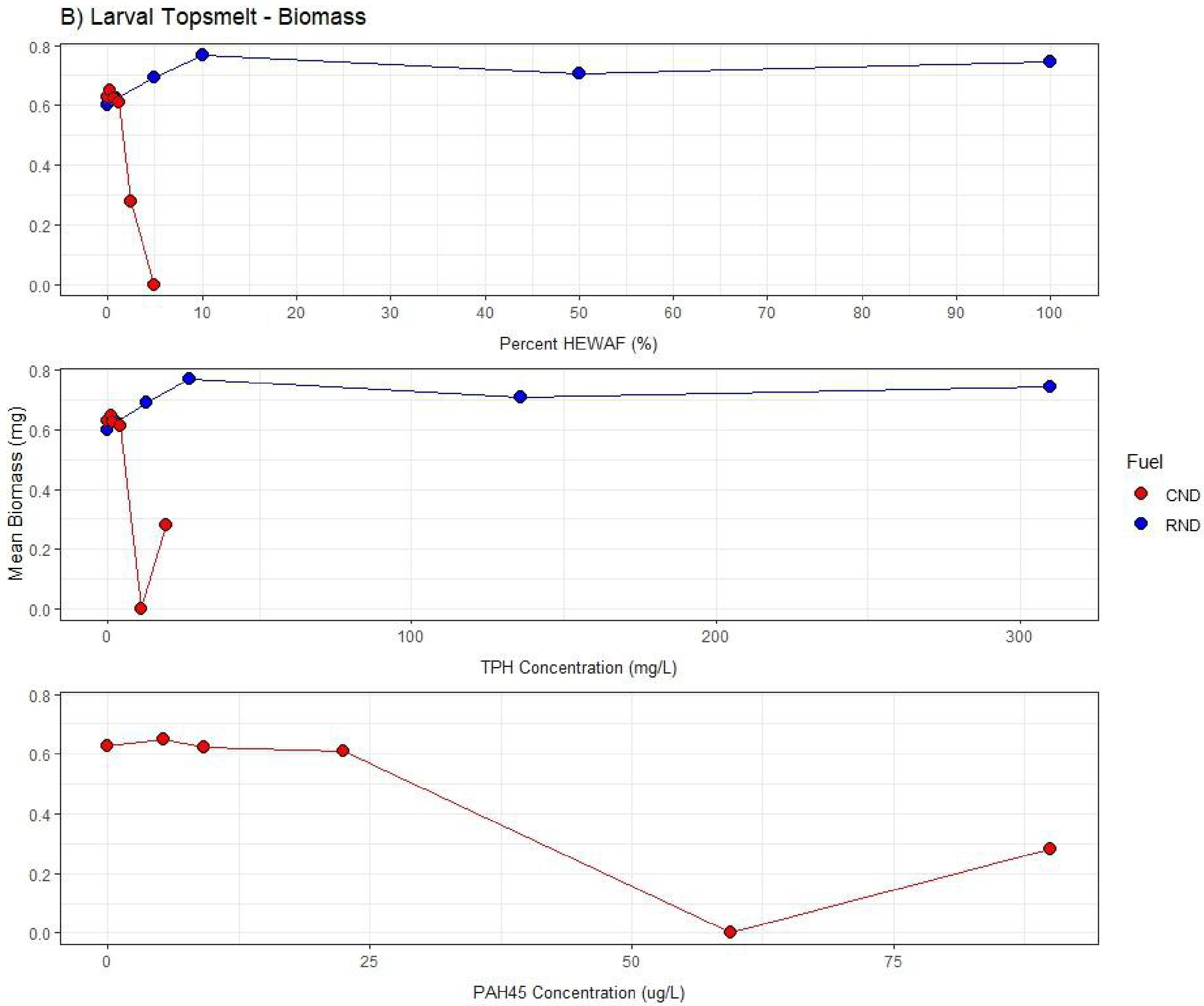


Figure 3. Definitive 7-day chronic larval topsmelt toxicity test A) mean percent survival on y-axis and B) mean biomass on y-axis, both compared to percent HEWAF, TPH concentration (mg/L), and PAH_{45} concentration (ug/L) for each fuel type. RND = Renewable Diesel. CND = Conventional Diesel. Total PAH_{45} concentration was not measured in renewable diesel. Error bars are included on Figures 1 and 2 for each fuel.

Table 5. Toxicity test point estimates (% HEWAF) and statistical results for the chronic 7-day larval topsmelt test in renewable diesel and conventional diesel. LC_{50} = Median lethal concentration for the survival endpoint. EC_{50} = Median effect concentration for the biomass endpoint. NOEC = no observed effect concentration. LOEC = Lowest observed effect concentration. LCL and UCL = lower and upper confidence intervals around the point estimate. *These tests were conducted with HEWAF prepared every other day.

Diesel Type	Test Type	Date	Point Estimate Type	Point Result	NOEC	LOEC	95% LCL	95% UCL
Renewable Diesel	Rangefinder*	06/12/2024	LC_{50}	>100	100	>100	N/A	N/A
Renewable Diesel	Rangefinder*	06/12/2024	EC_{50}	>100	100	>100	N/A	N/A
Renewable Diesel	Definitive*	09/24/2024	LC_{50}	>100	100	>100	N/A	N/A
Renewable Diesel	Definitive*	09/24/2024	EC_{50}	>100	100	>100	N/A	N/A
Renewable Diesel	Rangefinder	04/08/2025	LC_{50}	>100	100	>100	N/A	N/A
Renewable Diesel	Rangefinder	04/08/2025	EC_{50}	>100	100	>100	N/A	N/A
Conventional Diesel	Rangefinder	12/10/2024	LC_{50}	1.531	<1	1	1.107	2.119
Conventional Diesel	Rangefinder	12/10/2024	EC_{50}	N/A	N/A	N/A	N/A	N/A
Conventional Diesel	Definitive	01/21/2025	LC_{50}	1.71	1.25	2.5	1.447	2.021
Conventional Diesel	Definitive	01/21/2025	EC_{50}	2.323	1.25	2.5	1.674	N/A

Objective 2: Conduct toxicity tests using a marine invertebrate – Mytilus galloprovincialis

The endpoint for the 48-hr chronic larval mussel test is the percentage of larvae that survived with normal shell development (U.S. EPA 1995). All larvae are considered alive except for those with completely empty shells (U.S. EPA 1995). Normal shell development is defined as larvae in the prodissoconch ‘D-hinge’ developmental stage (U.S. EPA 1995). For this protocol, the toxicity endpoint combines the proportion of survival with normal development into a combined proportion calculation which is adjusted using Abbott’s formula to account for natural mortality in the controls. The combined proportion approach is a powerful statistical tool that integrates two sequential events - survival and normal development - into a sensitive marker of toxicant-driven impairments at early stages of bivalve development.

Due to the small test volumes used for the 48-hr chronic larval mussel development test, chemistry samples collection was only possible on Day 0 from freshly prepared HEWAF before it was introduced to the exposure beakers. The Day 0 chemistry sample for the renewable diesel HEWAF mussel test was shared with the first topsmelt test (09/24/24) and TPH concentrations from this test are listed in Table 6.

Only a rangefinder was conducted for mussels in HEWAF prepared with renewable diesel, as no dose response was observed at 100 % HEWAF, and the statistical results showed a 48-hr EC_{50} of >100 % HEWAF, a 48-hr NOEC of 100 % HEWAF, and a 48-hr LOEC of >100 % HEWAF (Figure 4, Table 8). In the 100 % HEWAF, 224 mg/L TPH were measured on Day 0 (Figure 6; Table 6) and, with no observed toxicity at this concentration, the rangefinder test was accepted as the definitive result.

HEWAF prepared with conventional diesel resulted in a clear dose response (Figure 5, Table 7) and a 48-hr EC_{50} of 25.12 % HEWAF, and a 48-hr LOEC of 25 % HEWAF. At 25 % HEWAF, 112 mg/L TPH and 437 ug/L PAH_{45} were measured in freshly prepared HEWAF on Day 0 (Figure 6; Tables 7 & 8). The 48-hr NOEC was found to be 12.5 %, which contained 51.5 mg/L TPH and 225 ug/L PAH_{45} measured in freshly prepared Day 0 HEWAF (Figure 6; Tables 7 & 8). HEWAF prepared with conventional diesel had a stronger effect on normal shell development than mussel survival (Figure 5). For example, at 25 % HEWAF, larval mussel survival was 96.2 %, while only 27.3 % of larval mussels had normal shell development (Figure 5; Table 7).

Similar to larval topsmelt, given the lack of sensitivity of mussels to HEWAF prepared with renewable diesel, further toxicity testing with Blends 1 and 2 was removed for both organisms from the experimental design.

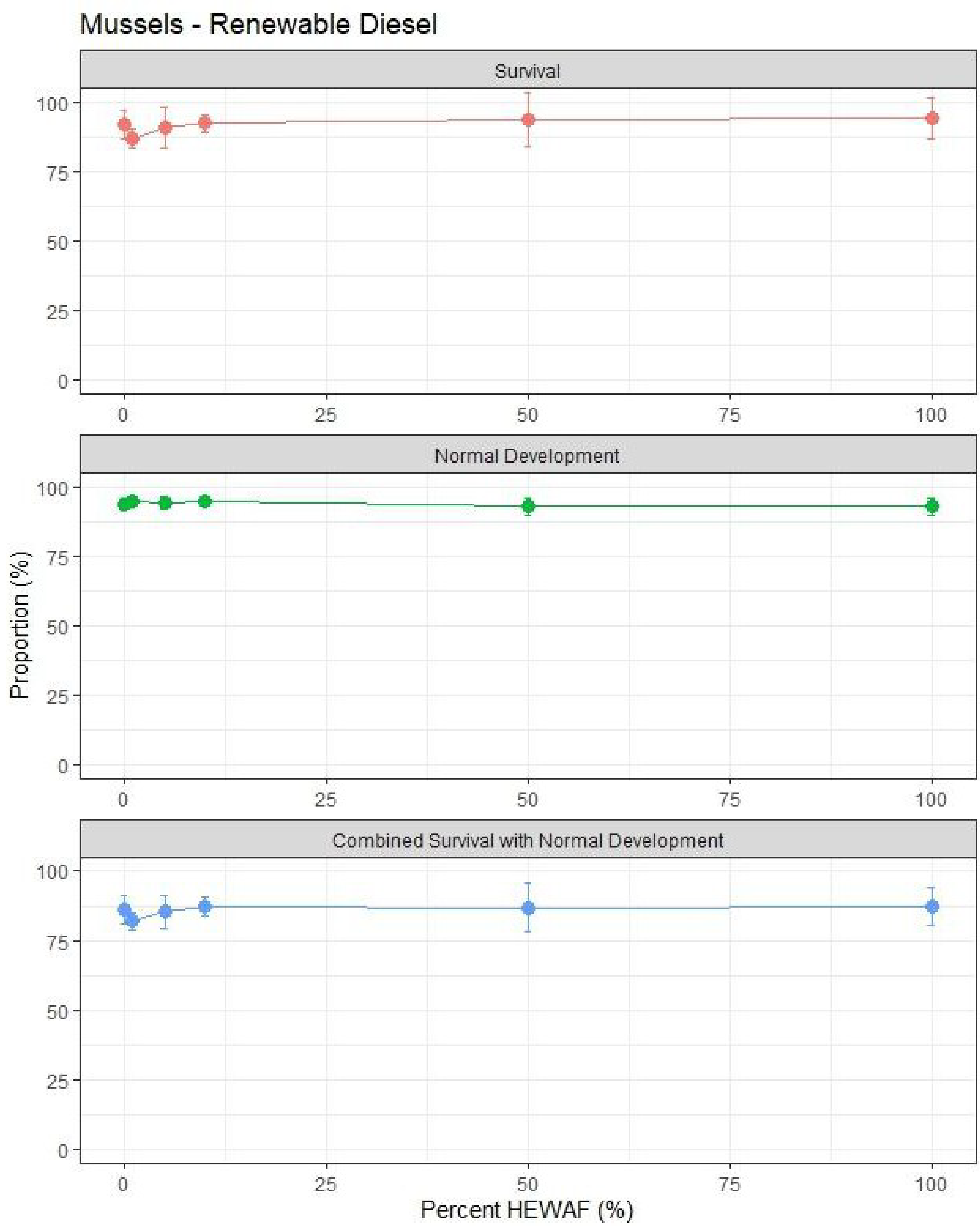


Figure 4. Definitive results for the 48-hour chronic larval mussel toxicity test with renewable diesel. The proportion of survival, normal development, and combined survival with normal development are shown on the y-axes and percent HEWAF on the x-axis. Error bars indicate +/- standard deviations.

Table 6. Toxicity results and TPH concentrations (mg/L) associated with HEWAF preparation from the renewable diesel definitive test for larval mussels. ND = non detect.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Percent Survival (mean ± SD)	Normal Development (mean ± SD)	Combined Proportion Normal (mean ± SD)
0	<0.0358 ND	91.9 ± 5.2	93.7 ± 2.2	86.1 ± 5.2
1	0.872	86.7 ± 3.4	94.4 ± 1.7	81.9 ± 3.3
5	6.00	90.7 ± 7.6	94.3 ± 2.3	85.4 ± 6.0
10	15.5	92.2 ± 3.2	94.8 ± 1.2	87.4 ± 3.4
50	97.1	93.7 ± 9.7	92.8 ± 3.2	86.9 ± 8.9
100	224	94.2 ± 7.2	92.7 ± 3.0	87.3 ± 6.6

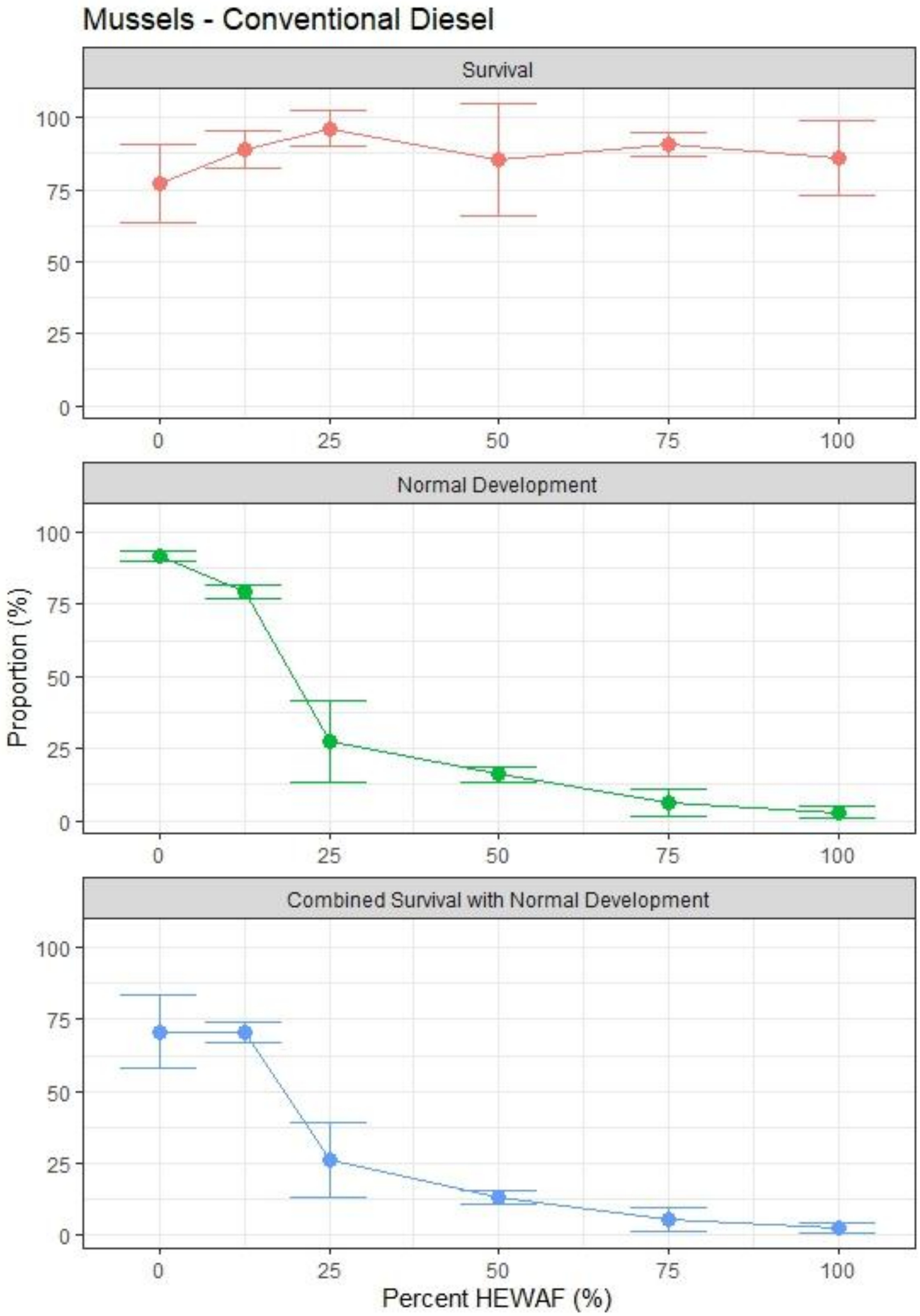


Figure 5. Definitive results for the 48-hour chronic larval mussel toxicity test with conventional diesel. The proportion of survival, normal development, and combined survival with normal development are shown on the y-axes and percent HEWAF on the x-axis. Error bars indicate +/- standard deviations.

Table 7. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations (ug/L) associated with HEWAF preparation from the conventional diesel definitive test for larval mussels. ND = non detect.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Normal Development (mean ± SD)	Combined Proportion Normal (mean ± SD)
0	< 0.0348 ND	0.0133	77.2 ± 13.6	91.5 ± 1.8	70.7 ± 12.6
12.5	51.5	225	89.0 ± 6.6	79.4 ± 2.6	70.6 ± 3.7
25	112	437	96.2 ± 6.0	27.3 ± 14.2	26.1 ± 12.9
50	266	966	85.5 ± 19.5	16.0 ± 2.7	13.4 ± 2.3
75	489	1491	90.7 ± 4.3	6.2 ± 4.7	5.5 ± 4.1
100	875	1763	85.7 ± 12.9	2.9 ± 1.9	2.6 ± 1.8

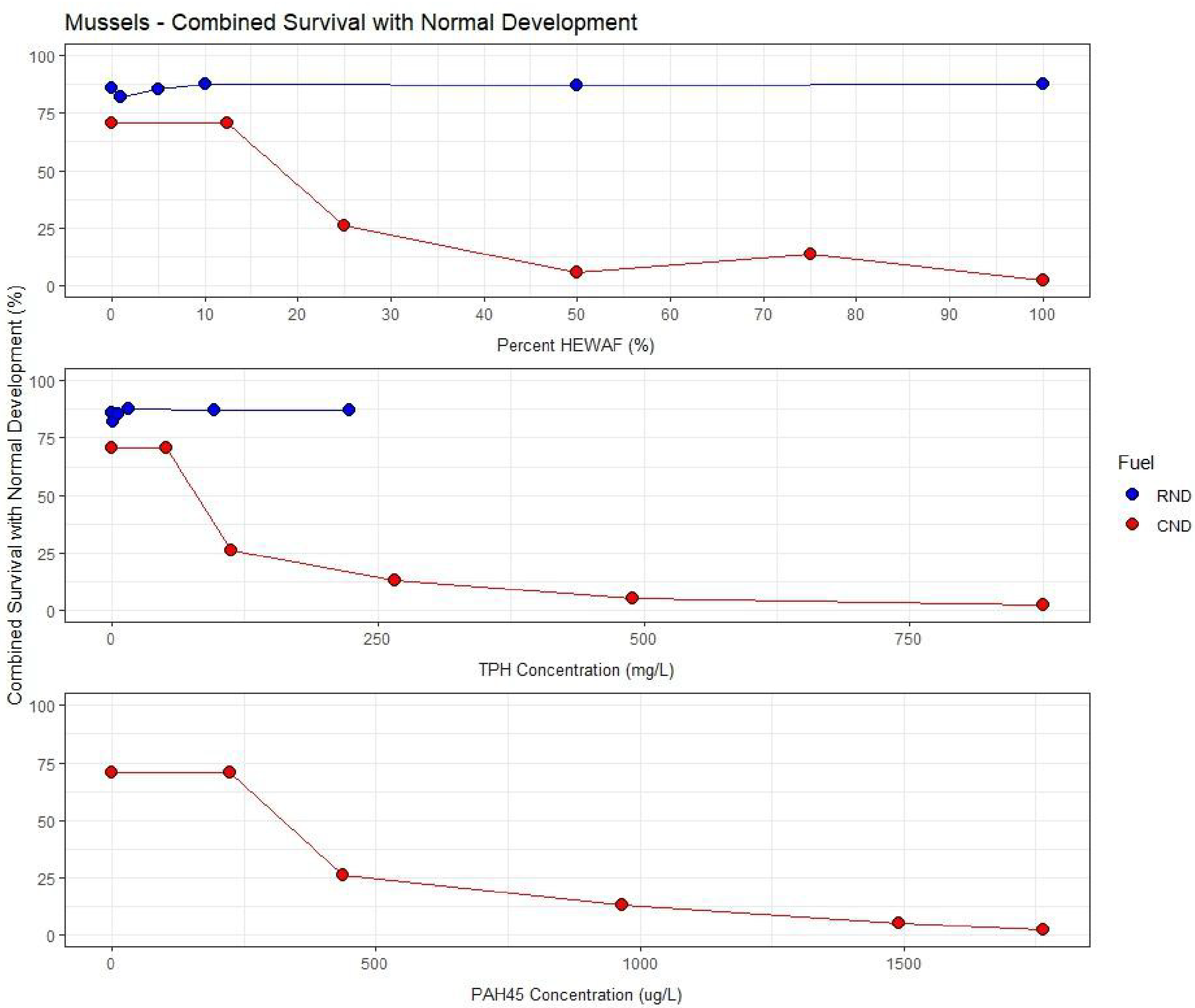


Figure 6. Definitive 48-hour chronic larval mussel toxicity test mean proportion of combined survival with normal development on the y-axis and percent HEWAF, TPH concentration (mg/L), and PAH_{45} concentration (ug/L) on the x-axes for larval mussels for each fuel type. RND = Renewable Diesel. CND = Conventional Diesel. PAH_{45} concentration was not measured for renewable diesel. Error bars are included on Figures 4 and 5 for each fuel.

Table 8. Toxicity test point estimates (% HEWAF) and statistical results for the chronic 48-hour larval mussel test in renewable diesel and conventional diesel. Results are calculated for the combined proportion of larvae survival with normal development endpoint. EC_{50} = Median effect concentration. NOEC = no observed effect concentration. LOEC = Lowest observed effect concentration. LCL and UCL = lower and upper confidence intervals around the point estimate.

Diesel Type	Test Type	Date	Point Estimate Type	Point Estimate Result	NOEC	LOEC	95% LCL	95% UCL
Renewable Diesel	Rangefinder	09/26/2024	EC50	>100	100	>100	N/A	N/A
Conventional Diesel	Definitive	02/06/2026	EC50	25.12	12.5	25	24.53	25.72

Objective 3: Conduct toxicity tests using a freshwater fish – Pimephales promelas

For all larval fathead tests, samples were collected on Day 0 to measure TPH and PAH_{45} concentrations in freshly made HEWAF before it was introduced to the exposure beakers. Samples were also collected on Day 7 to measure TPH and PAH_{45} concentrations at the end of exposure after the HEWAF had been in the exposure beakers for 24 hours. An extra sample of freshly prepared HEWAF was collected on Day 6 prior to the daily renewal of the test for the renewable diesel series (Table 9).

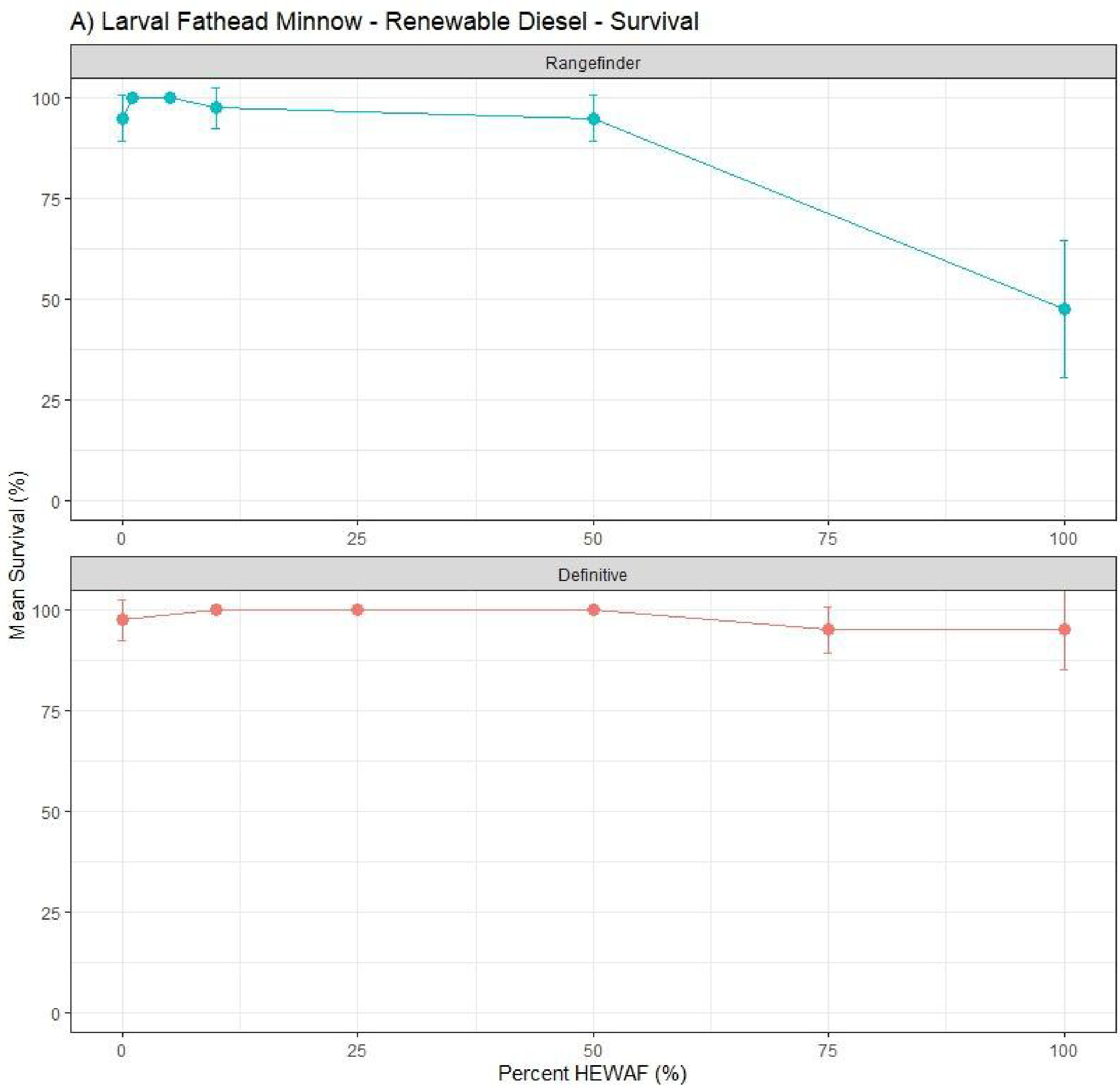
The definitive test for HEWAF prepared with renewable diesel resulted in a 7-day LC_{50} and EC_{50} of 100 % HEWAF, a 7-day NOEC of 100 % HEWAF, and a 7-day LOEC of >100 % HEWAF (Table 13). 100 % HEWAF contained 621 mg/L TPH in freshly prepared HEWAF on Day 0 (Table 9), with a measured 98 % reduction in TPH concentration (1120 mg/L on Day 6 to 27.3 mg/L on Day 7) after 24 hours. Notably, results from the rangefinder and definitive tests with larval fathead minnow and HEWAF prepared with renewable diesel had differences in exposure and measured toxicity effects (Figure 7, Table 13). While the definitive test had point estimates of >100 % HEWAF for both LC_{50} and EC_{50}, the rangefinder had a 7-day LC_{50} of 97.6 % HEWAF, and a 7-day EC_{50} of 69.61 % HEWAF (Table 13). For the survival endpoint, the 7-day NOEC and LOEC in these tests was found to be 50 % and 100% HEWAF, respectively. The biomass endpoint was more sensitive with a 7-day NOEC and LOEC of 10 % and 50 % HEWAF, respectively. During the rangefinder test, larval fathead minnows were observed consuming and excreting the waxy layer that formed at the top of the beakers with higher exposure concentrations. This waxy layer increased in thickness over the course of the test. Towards the end of the 7-day rangefinder test, the fish appeared to have increased difficulty consuming their food (*Artemia* nauplii), and their gut color changed from orange to white, possibly reflecting consumption and retention of waxy materials accumulated in the exposure beaker. On Days 6 and 7 of the rangefinder test, larval fathead minnow mortality was noted in the fish exposed to 100 % HEWAF. Similar observations were noted in the definitive test but on a delayed timeline during the exposure which could explain the higher toxicity point estimates calculated from this test (LC_{50} , EC_{50}, and LOEC >100 % HEWAF; Table 13). Although these findings provide insights into the differences between the rangefinder and definitive tests, it must be noted that the current study was not designed to capture feeding behaviors and their relationships with apical endpoints such as mortality. Therefore, given the qualitative nature of the above-mentioned chance observations, further investigations are recommended to quantify these effects.

HEWAF prepared with conventional diesel had accelerated lethal effects on larval fathead minnow (Figure 8, Table 10), with major die-offs occurring within 2 days of the rangefinder and definitive test initiation. The definitive test resulted in a 7-day LC_{50} of 0.221 % HEWAF with a 7-day NOEC of <0.15625 % HEWAF for the survival endpoint (Table 13). The biomass endpoint showed a 7-day EC_{50} of 0.241 % HEWAF with a 7-day NOEC of 0.15625 % HEWAF (Table 13). The LOEC for survival and biomass was 0.15625 % HEWAF and 0.3125 % HEWAF, respectively (Table 13). At 0.15625 % HEWAF, 1.3 mg/L TPH and 5.35 ug/L PAH_{45} were measured in freshly prepared HEWAF (Figure 11; Table 10). Over 24 hrs, there was an 82% reduction (1.3 mg/L to 0.239 mg/L) in TPH and a 75% reduction (5.35 ug/L to 1.33 ug/L) in PAH_{45} at this HEWAF dilution. At 0.3125 % HEWAF, 3.2 mg/L TPH and 13.24 ug/L PAH_{45} was measured in freshly prepared HEWAF (Figure 11; Table 10), with an 86 % reduction in TPH (3.2 mg/L to 0.443 mg/L) and a 71 % reduction (13.24 mg/L to 3.79 mg/L) in PAH_{45} over 24-hrs. Qualitative observations in conventional diesel exposures included instances of abnormal swimming behavior, such as swimming upside down, swimming on their side, becoming momentarily immobilized and drifting head down, or periodic thrashing.

Similar to topsmelt and mussels, larval fathead minnow did not appear to be particularly sensitive to HEWAF prepared with renewable diesel. However, it was decided to move forward with testing this organism in Blend 1 and Blend 2 fuels given the results discussed above. No dose responses were observed in the rangefinder and definitive tests of Blend 1 (Figure 9, Table 11), with a 7-day LC_{50} and EC_{50} of >100 % HEWAF, 7-day NOECs of 100 % HEWAF, and 7-day LOECs of >100 % HEWAF calculated for survival and biomass for both tests (Table 13). In this blend, 1270 mg/L TPH and 247 ug/L PAH_{45} were measured in freshly prepared 100% HEWAF (Figure 11; Table 11). This TPH concentration was the highest measured in this study and reflects TPHs from both renewable and conventional diesels mixed in 20:80 ratio. Over 24 hours, there was a 95 % reduction (1270 mg/L to 57.7 mg/L) in TPH and 92% reduction (247 ug/L to 18.8 ug/L) in PAH_{45} at the 100 % HEWAF dilution.

The rangefinder test with Blend 2 also had no dose responses, with a 7-day LC_{50} and EC_{50} of >100 % HEWAF, 7-day NOECs of 100 % HEWAF, and 7-day LOECs of > 100 % HEWAF calculated for survival and biomass for both tests (Table 13). In Blend 2, 100 % HEWAF contained 908 mg/L TPH and 194.4 ug/L PAH_{45} in freshly prepared HEWAF (Figure 11; Table 12). Over 24 hours, there was a 96% reduction in both TPH (908 mg/L to 36.4 mg/L) and PAH_{45} (194.4 ug/L to 7.30 ug/L) concentrations in 100 % HEWAF. Due to the lack of dose response in the rangefinder, no definitive test was performed (Figure 10, Table 12). It must be noted that in conventional diesel HEWAF exposures, effects were observed for exposures with 1.3 mg/L TPH and 5.35 µg/L PAH_{45}. However, despite the high TPH and PAH_{45} concentrations in both blends, no significant exposure effects were observed. Differences in swimming behavior and likely ingestion of any waxy materials accumulated in the top layer of the beaker could result in increased variability in fish toxicity endpoints across replicates. Lack of fathead toxicity in both blends warrants further investigations into exposure chemistry as well as physiological and behavioral responses of exposed fish.

In Blend 1, there were two recorded instances of a fish swimming abnormally (once during the rangefinder, and once during the definitive test). In Blend 2, larval fathead generally appeared healthy over the course of the test, with no visual signs of distress. It is important to note that Blend 1 has a higher proportion of conventional diesel than Blend 2, as the abnormal swimming behavior periodically seen in Blend 1 was very similar to that seen in the conventional diesel test. Across all fuel types, larval fathead minnow survival and biomass was most sensitive to HEWAF prepared with conventional diesel (Figure 11).



A) Larval Fathead Minnow - Renewable Diesel - Survival
Rangefinder
Definitive
Mean Survival (%)
Percent HEWAF (%)
0
25
50
75
100

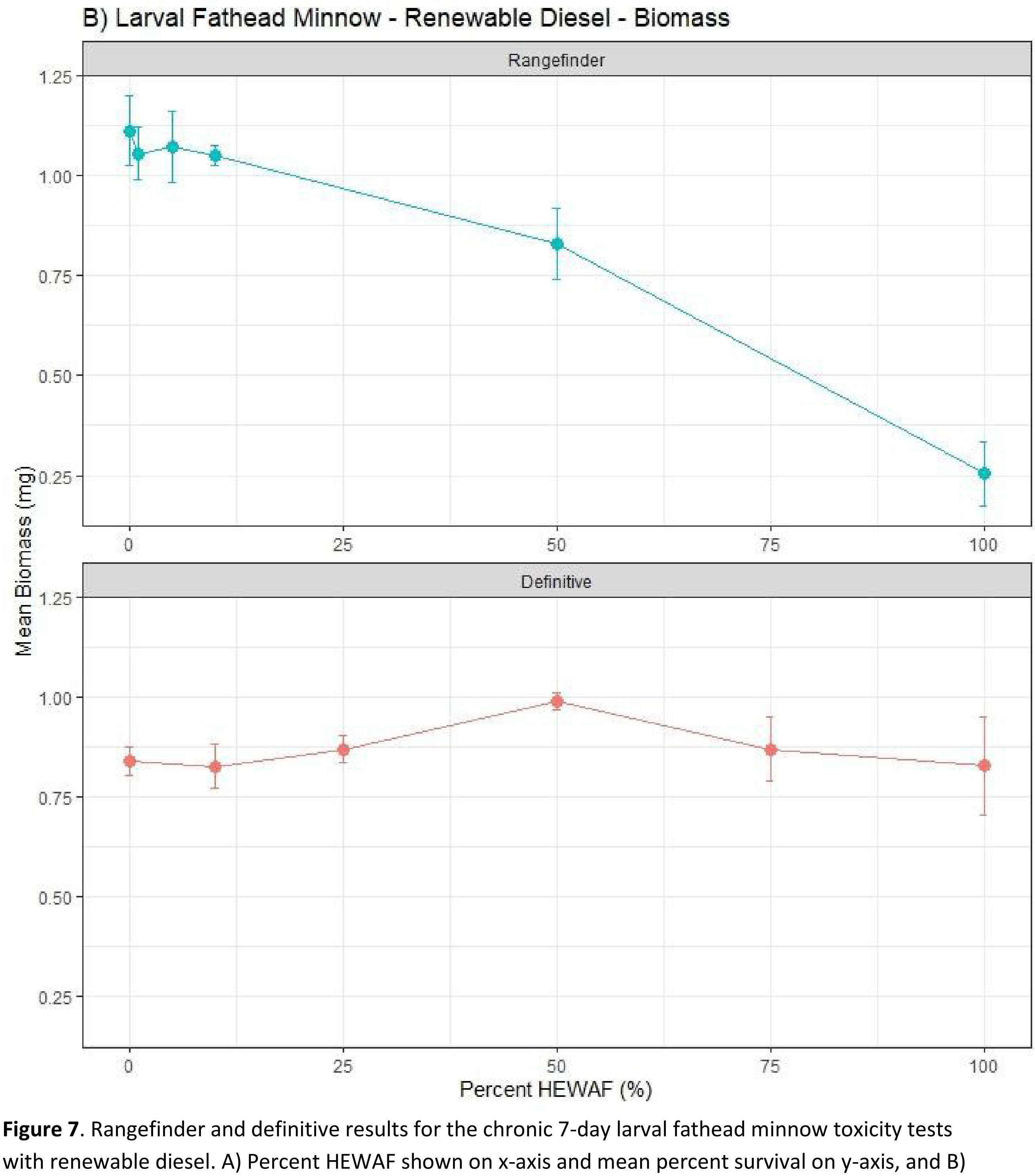
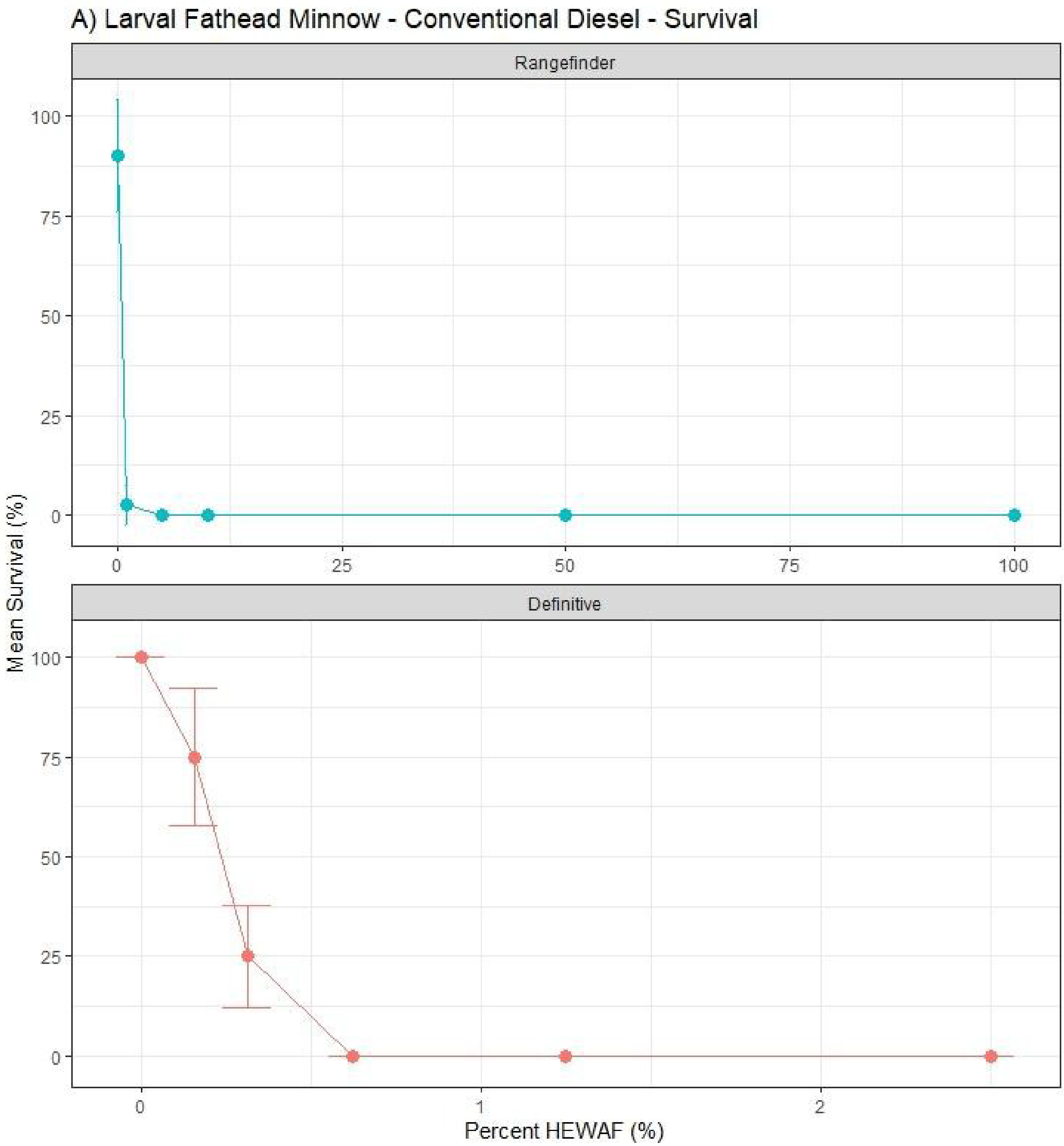


Figure 7. Rangefinder and definitive results for the chronic 7-day larval fathead minnow toxicity tests with renewable diesel. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 9. Toxicity results and TPH concentrations (mg/L) associated with HEWAF preparation from the renewable diesel definitive test for larval fathead minnow. ND = non detect. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 6 TPH Concentration (mg/L)	Day 7 TPH Concentration (mg/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	0.0518 DNQ	< 0.0362 ND	0.0806 DNQ	97.5 ± 5.0	0.840 ± 0.035
10	18.7	45.9	3.96	100 ± 0.0	0.828 ± 0.054
25	48.4	101	11.4	100 ± 0.0	0.870 ± 0.034
50	99.4	307	19.5	100 ± 0.0	0.990 ± 0.022
75	216	1020	20.6	95 ± 5.8	0.870 ± 0.080
100	621	1120	27.3	95 ± 10.0	0.828 ± 0.123


A) Larval Fathead Minnow - Conventional Diesel - Survival
Rangefinder
Definitive
Mean Survival (%)
Percent HEWAF (%)
100
75
50
25
0
0
25
50
75
100
0
1
2

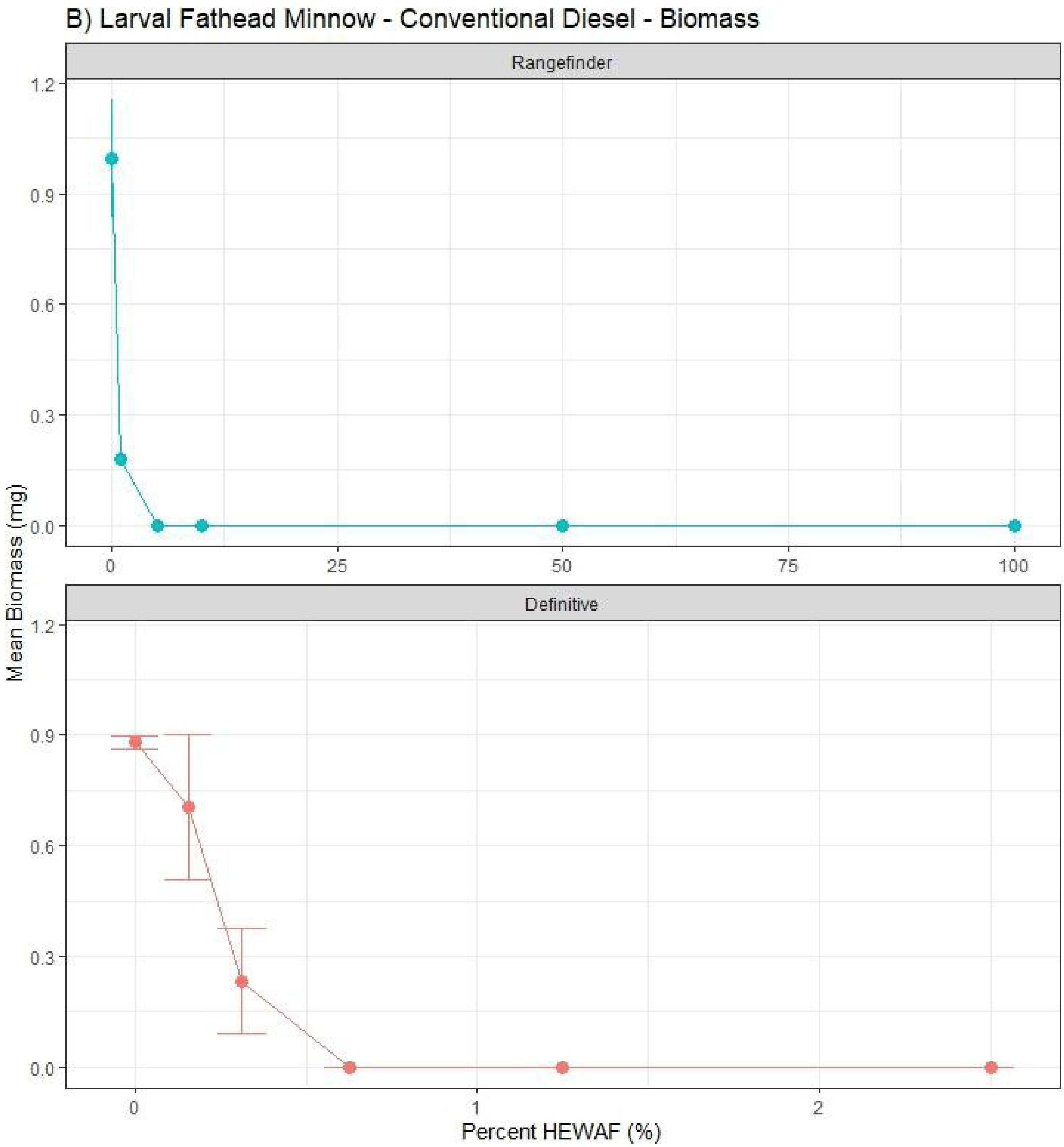
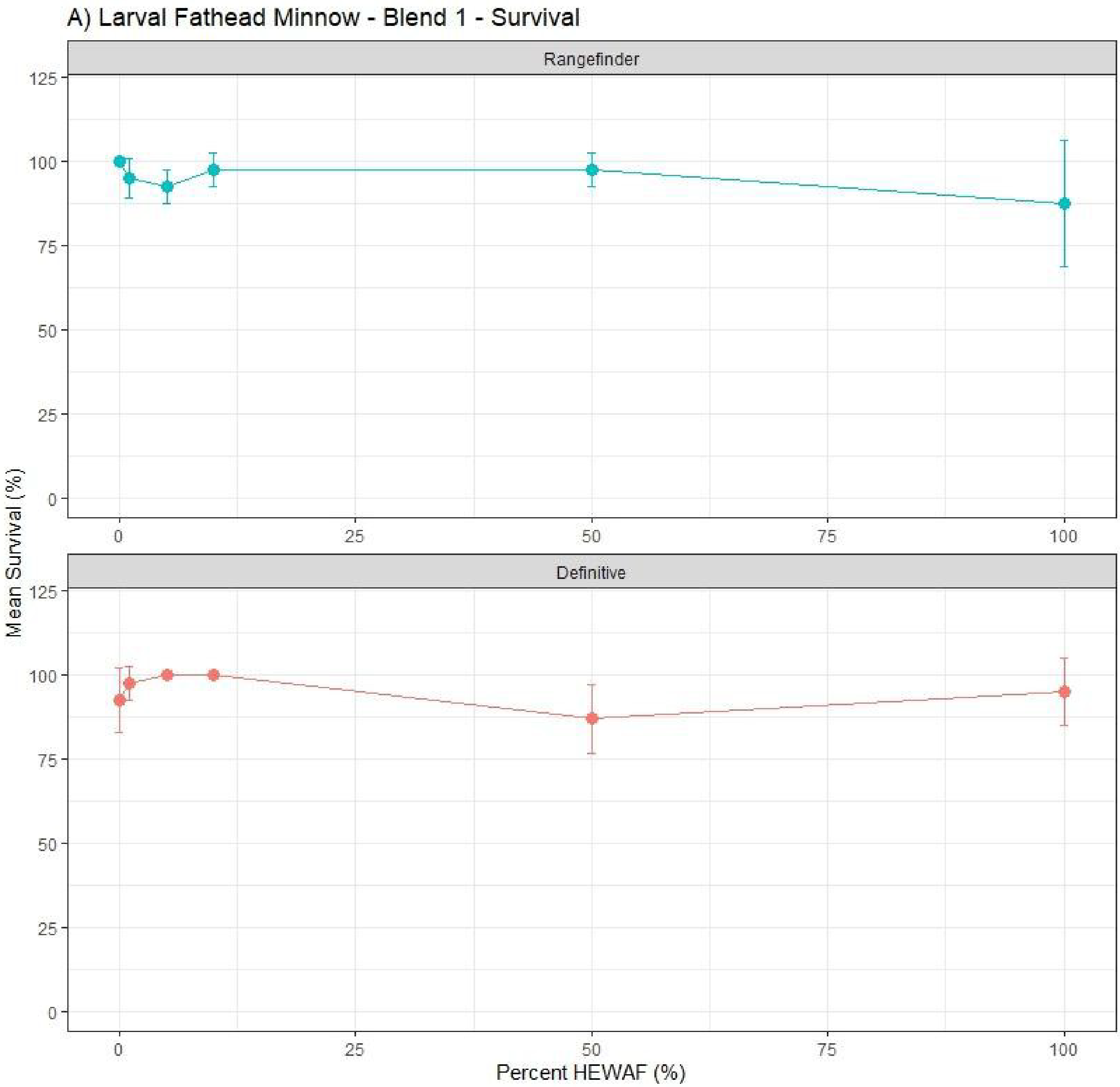


Figure 8. Rangefinder and definitive results for the chronic 7-day larval fathead minnow toxicity tests with conventional diesel. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 10. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations (ug/L) associated with HEWAF preparation from the conventional diesel definitive test for the larval fathead minnow. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 7 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Day 7 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	0.0549 DNQ	0.0461 DNQ	0.21	0.0241	100 ± 0.0	0.880 ± 0.018
0.15625	1.3	0.239	5.35	1.33	75 ± 17.3	0.705 ± 0.195
0.3125	3.21	0.443	13.24	3.79	25 ± 12.9	0.232 ± 0.143
0.625	10.6	1.83	46.9	8.14	0 ± 0.0	0 ± 0.0
1.25*	7.03	4.97	32.22	21.9	0 ± 0.0	0 ± 0.0
2.5	26.6	3.6	106.33	35.7	0 ± 0.0	0 ± 0.0

*Lost some sample during TPH/PAH extraction.


A) Larval Fathead Minnow - Blend 1 - Survival
Rangefinder
Definitive
Mean Survival (%)
Percent HEWAF (%)
0
25
50
75
100
125

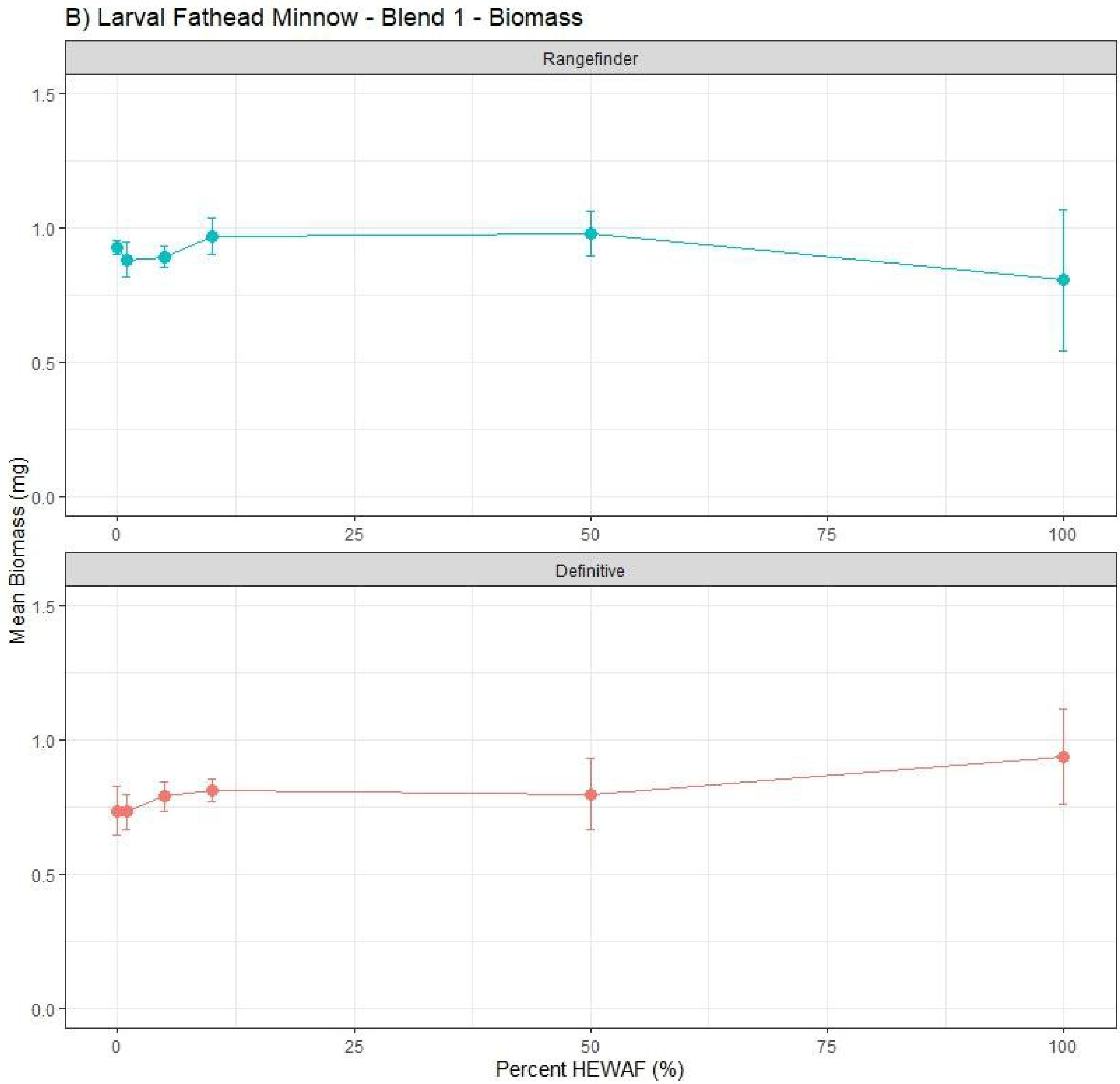
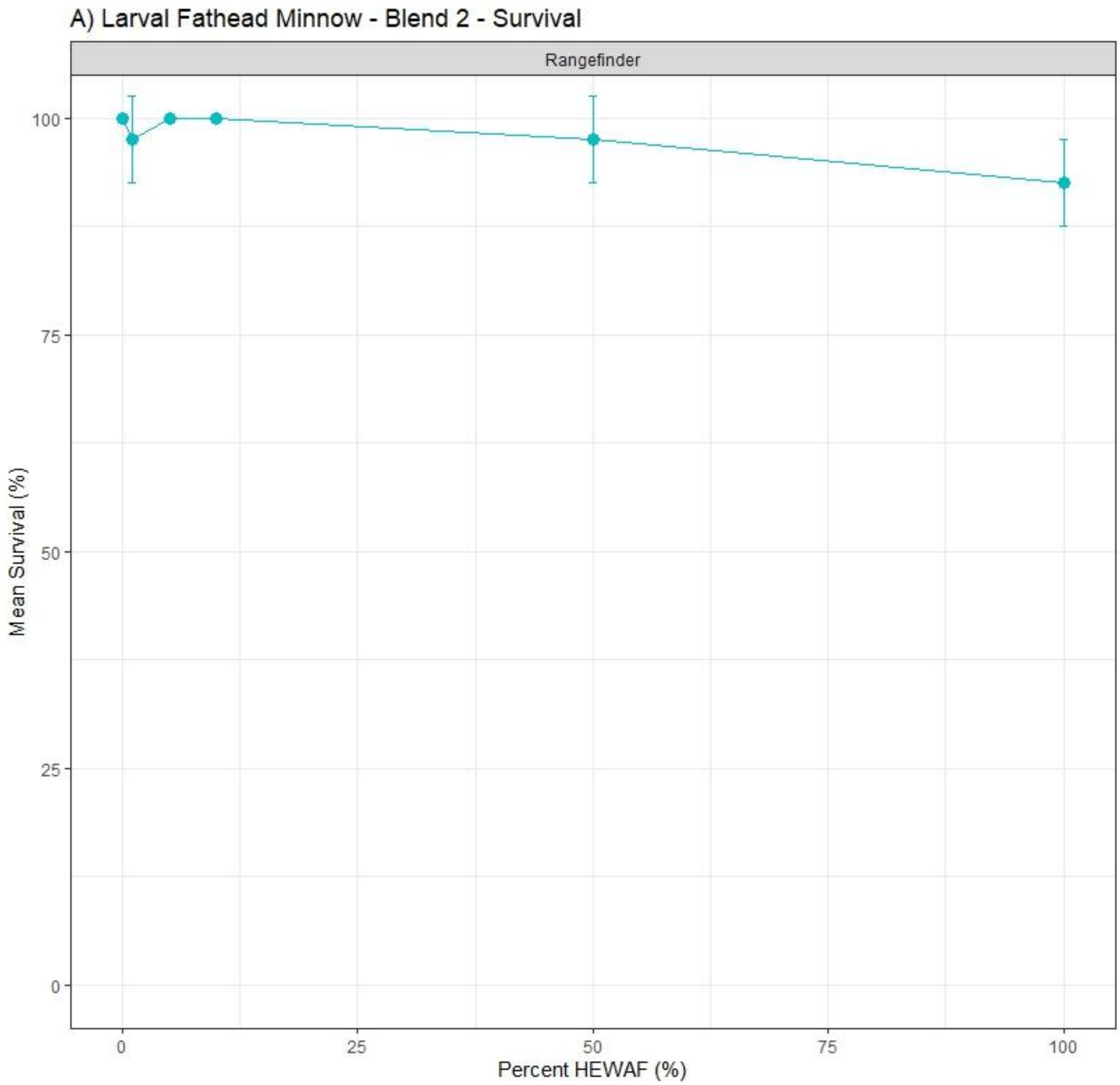


Figure 9. Rangefinder and definitive results for the chronic 7-day larval fathead minnow toxicity tests with Blend 1 (20 % CND : 80 % RND). A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 11. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations (ug/L) associated with each HEWAF preparation from Blend 1 (20 % CND : 80 % RND) definitive test for the larval fathead minnow. ND = non detect. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 7 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Day 7 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	< 0.0348 ND	0.0561 DNQ	0.021	0.12	92.5 ± 9.6	0.735 ± 0.090
1	12.1	1.41	3.89	0.792	97.5 ± 5.0	0.733 ± 0.066
5	39.5	5.03	12.3	1.48	100 ± 0.0	0.790 ± 0.055
10	73.7	7.72	25	2.74	100 ± 0.0	0.813 ± 0.040
50	572	20.5	142	8.62	86.9 ± 10.2	0.798 ± 0.133
100	1270	57.7	247	18.8	95.0 ± 10.0	0.937 ± 0.177


A) Larval Fathead Minnow - Blend 2 - Survival
Rangefinder
Mean Survival (%)
100
75
50
25
0
0
25
50
75
100
Percent HEWAF (%)

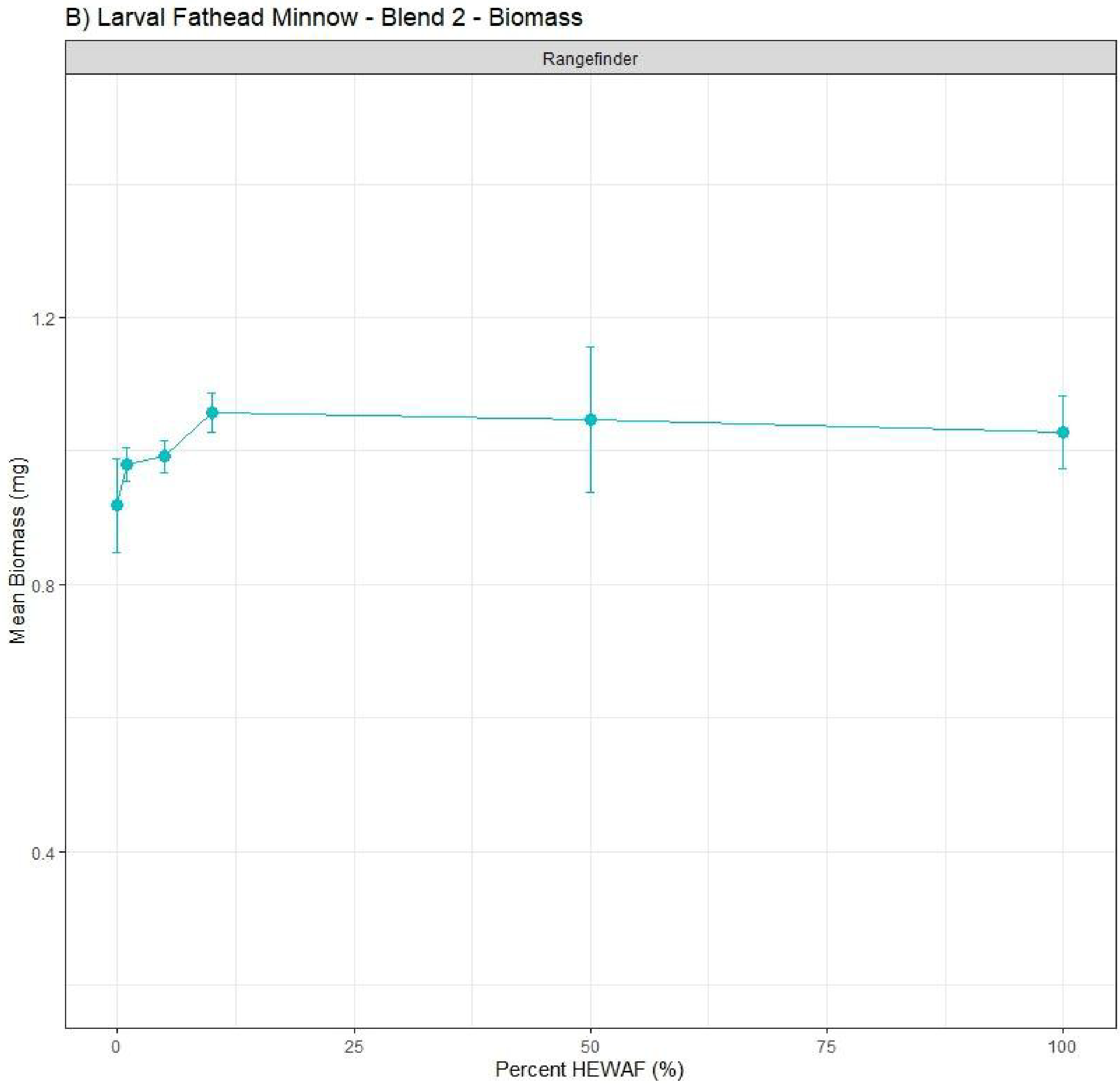
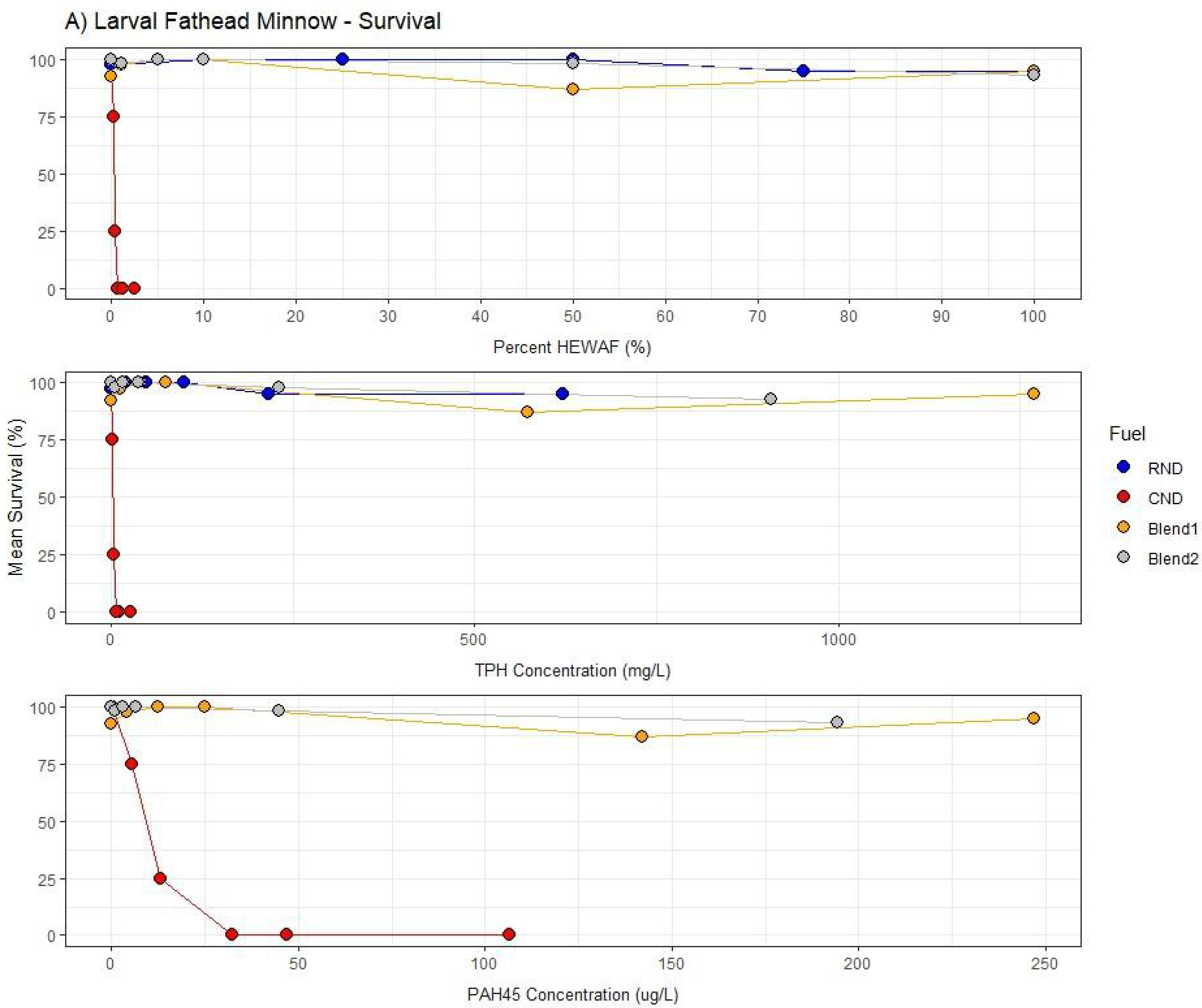


Figure 10. Rangefinder results for the chronic 7-day larval fathead minnow toxicity tests with Blend 2 (5 % CND : 95 % RND). A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean biomass on y-axis. Error bars indicate +/- standard deviations.

Table 12. Toxicity results, TPH concentrations (mg/L), and total PAH_{45} concentrations (ug/L) associated with each HEWAF preparation from the diesel Blend 2 (5 % CND: 95 % RND) test for the larval fathead minnow. ND = non detect.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 7 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Day 7 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Biomass (mg) (mean ± SD)
0	< 0.0348 ND	< 0.0414 ND	0.025	0.043	100 ± 0.0	0.919 ± 0.070
1	3.99	1.42	0.837	0.465	97.5 ± 5.0	0.980 ± 0.026
5	15	4.54	2.9	1.01	100 ± 0.0	0.992 ± 0.024
10	36	5.82	6.62	1.5	100 ± 0.0	1.058 ± 0.030
50	230	16.3	44.9	4.47	97.5 ± 5.0	1.048 ± 0.109
100	908	36.4	194.4	7.3	92.5 ± 5.0	1.028 ± 0.055


A) Larval Fathead Minnow - Survival
Mean Survival (%)
Percent HEWAF (%)
TPH Concentration (mg/L)
PAH45 Concentration (ug/L)
Fuel
RND
CND
Blend1
Blend2

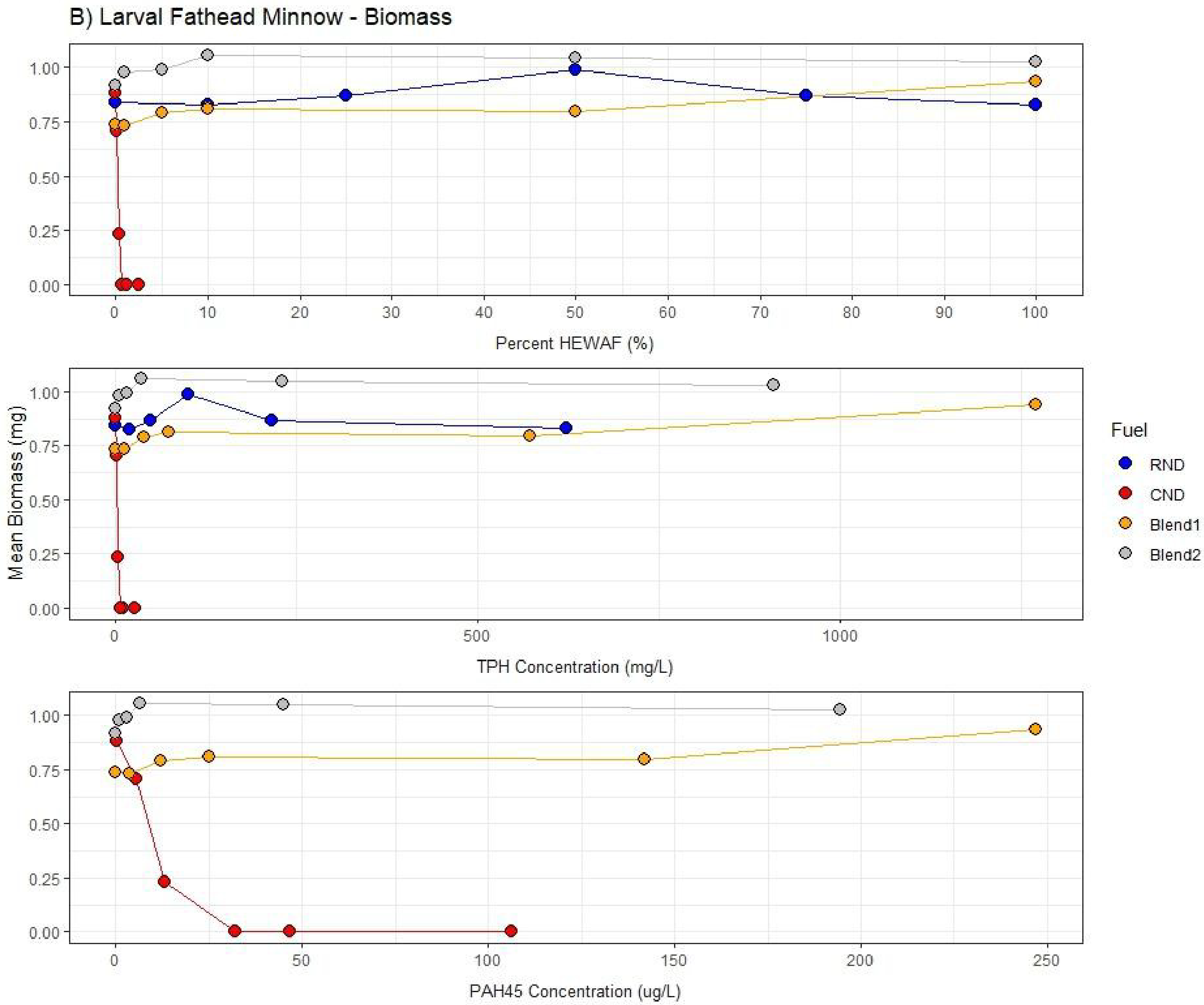


Figure 11. Definitive 7-day chronic larval fathead minnow toxicity test A) mean percent survival on the y-axis and B) mean biomass on the y-axis, both compared to percent HEWAF, TPH concentration (mg/L), and PAH_{45} concentration (ug/L) on the x-axes for each fuel type. RND = Renewable Diesel. CND = Conventional Diesel. Blend 1 = 20 % CND : 80 % RND. Blend 2 = 5 % CND : 95 % RND. PAH_{45} concentration was not measured for renewable diesel. Error bars are included on Figures 7, 8, and 9 for each fuel type.

Table 13. Toxicity test point estimates (% HEWAF) and statistical results for the chronic 7-day larval fathead minnow test in all diesel types. LC_{50} = Median lethal concentration for the survival endpoint. EC_{50} = Median effect concentration for the biomass endpoint. NOEC = no observed effect concentration. LOEC = Lowest observed effect concentration. LCL and UCL = lower and upper confidence intervals around the point estimate. Blend 1 = 20% CND : 80% RND. Blend 2 = 5% CND : 95% RND.

Diesel Type	Test Type	Date	Point Estimate Type	Point Estimate Result	NOEC	LOEC	95% LCL	95% UCL
Renewable Diesel	Rangefinder	02/05/2025	LC50	97.6	50	100	78.42	121.5
Renewable Diesel	Rangefinder	02/05/2025	EC50	69.61	10	50	61.47	76.2
Renewable Diesel	Definitive	03/26/2025	LC50	>100	100	>100	N/A	N/A
Renewable Diesel	Definitive	03/26/2025	EC50	>100	100	>100	N/A	N/A
Conventional Diesel	Rangefinder	03/05/2025	LC50	0.4283	<1	1	0.4058	0.4829
Conventional Diesel	Rangefinder	03/05/2025	EC50	N/A	N/A	N/A	N/A	N/A
Conventional Diesel	Definitive	04/30/2025	LC50	0.221	<0.15625	0.15625	0.1932	0.2527
Conventional Diesel	Definitive	04/30/2025	EC50	0.2414	0.15625	0.3125	0.1826	0.2878
Blend 1	Rangefinder	08/27/2025	LC50	>100	100	>100	N/A	N/A
Blend 1	Rangefinder	08/27/2025	EC50	>100	100	>100	N/A	N/A
Blend 1	Definitive	09/11/2025	LC50	>100	100	>100	N/A	N/A
Blend 1	Definitive	09/11/2025	EC50	>100	100	>100	N/A	N/A
Blend 2	Rangefinder	10/29/2025	LC50	>100	100	>100	N/A	N/A
Blend 2	Rangefinder	10/29/2025	EC50	>100	100	>100	N/A	N/A

Objective 4: Conduct toxicity tests using a freshwater invertebrate – Ceriodaphnia dubia

The endpoints for the 3-brood chronic daphnid test are survival and reproduction (U.S. EPA 2002). Reproduction is calculated as the mean number of neonates produced by the female daphnid across three broods. The test is terminated when ≥60% of the surviving control females have produced their third brood, or at the end of 8 days, whichever occurs first (U.S. EPA 2002). All daphnid tests conducted for this study ended on Day 7 of the test.

Due to the low volumes required for the 3-brood chronic daphnid test, chemistry samples were only collected on Day 0 from freshly prepared HEWAF before it was introduced to the exposure beakers.

There was a strong dose response observed for the daphnids exposed to HEWAF prepared with renewable diesel in both rangefinder and definitive tests (Figure 12, Table 14). The survival endpoint was greatly affected, as the definitive test resulted in a 7-day LC_{50} of 1.745 % HEWAF, a 7-day NOEC of 2.5 % HEWAF, and a 7-day LOEC of 5 % HEWAF (Table 18). In the LOEC of 5 % HEWAF, 17.2 mg/L TPH was measured in freshly prepared HEWAF (Figure 16; Table 14) where 80 % daphnid mortality was observed (20% survival). No effects were seen on survival at or below 8.64 mg/L TPH (2.5% HEWAF). The reproductive endpoint was affected at even lower percent HEWAF exposures, with a 7-day EC_{50} of 0.241 % HEWAF, a 7-day NOEC of <0.3125 % HEWAF, and a 7-day LOEC of 0.3125 % HEWAF. In 0.3125 % HEWAF, TPH concentration was 1.74 mg/L (Figure 16; Table 14) and 63 % reproductive impairment was observed relative to controls. In exposures containing ≥1 % HEWAF in both the rangefinder and definitive tests, daphnids visually appeared stunted in growth around Day 4. It is important to note that these effects were observed in HEWAF exposures prepared with half the loading rate (10 g fuel/L water) compared to HEWAF preparations for other test organisms used in this study. These results show that daphnids are sensitive to TPH concentrations as low as 1.74 mg/L in renewable diesel.

HEWAF prepared with conventional diesel produced a similar pattern of dose response as seen with daphnids in renewable diesel, though at lower concentrations than with renewable diesel (Figures 13 & 16, Table 15). Organism survival was greatly affected, with a 7-day LC_{50} of 0.1701 % HEWAF, a 7-day NOEC 0.125 % HEWAF, and a 7-day LOEC of 0.25 % HEWAF (Table 18). At 0.25 % HEWAF, 1.92 mg/L TPH and 12.46 ug/L PAH_{45} were measured (Figure 16; Table 15) and 100 % mortality was recorded. No effects on survival were recorded at or below 1.01 mg/L TPH and 7.24 ug/L PAH_{45} (0.125 % HEWAF). Reproduction was affected at lower HEWAF dilutions, with a 7-day EC_{50} of 0.08736 % HEWAF, a 7-day NOEC of <0.0625 % HEWAF, and a 7-day LOEC of 0.0625 % HEWAF) (Table 18). At 0.0625 % HEWAF, TPH and PAH_{45} concentrations were 0.486 mg/L and 4.23 ug/L, respectively, (Figure 16; Table 15) and 30% reproductive impairment was observed relative to controls. The sensitivity of daphnids to conventional diesel is higher as effects on survival are seen at TPH concentrations as low as 1.92 mg/L and reproductive effects are noted at 0.486 mg/L. In addition to the TPH toxicity as seen in renewable diesel daphnid exposures, this increased sensitivity to conventional diesel is likely due to the effects of PAH_{45}.

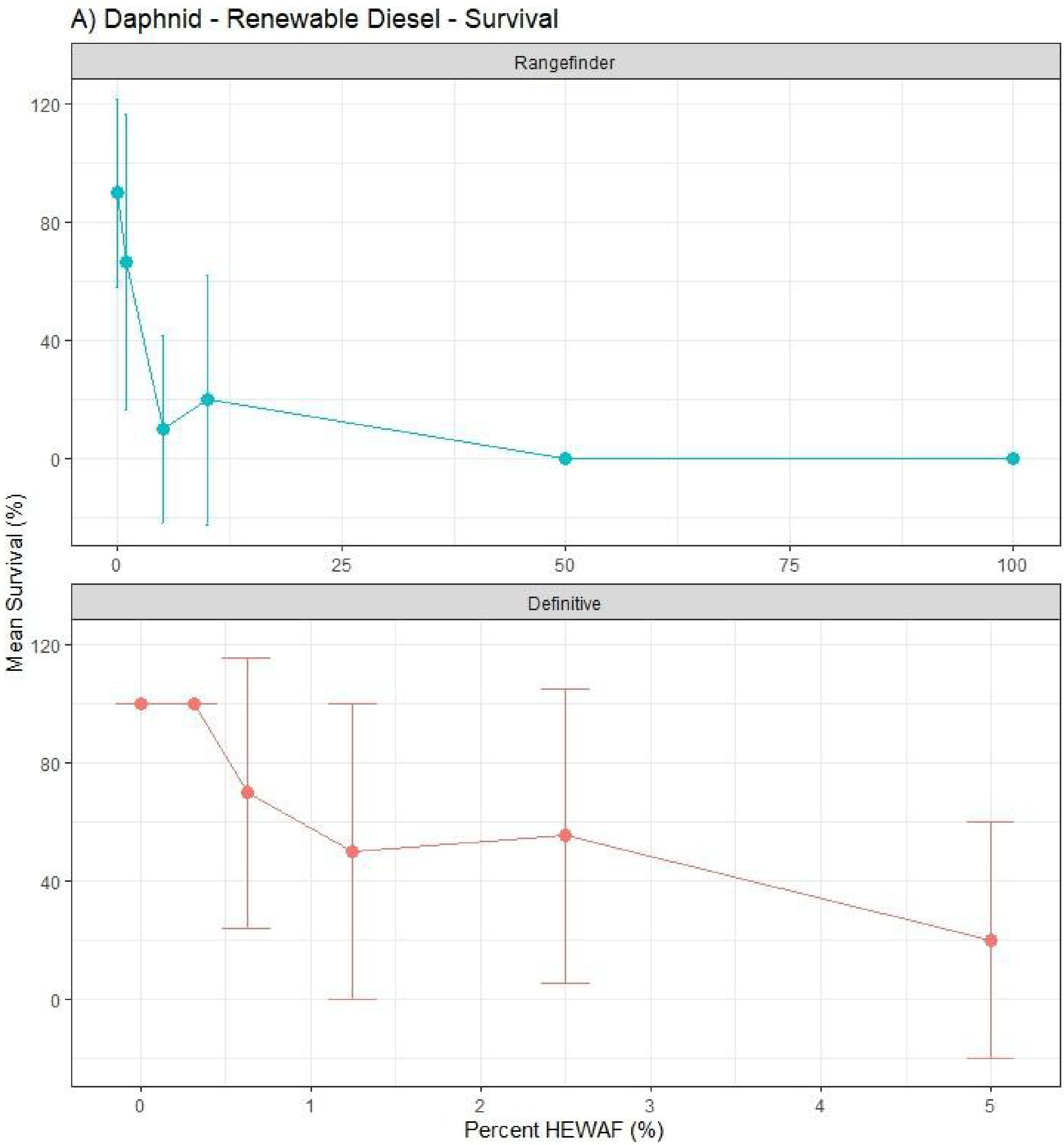
The reproductive endpoint was also found to be more impacted than survival in HEWAF prepared with Blend 1 and Blend 2 fuels (Figures 14 & 15, Tables 16 and 17). For the survival endpoint, Blend 1 HEWAF had a 7-day LC_{50} of 0.5955 % HEWAF, a 7-day NOEC of 0.5 % HEWAF, and a 7-day LOEC of 1 % HEWAF (Table 18). At 1 % HEWAF, 15.2 mg/L TPH and 5.56 ug/L PAH_{45} were measured in freshly prepared HEWAF (Figure 16; Table 16) and 56 % mortality was observed (44% survival). No effects on survival were recorded at or below 7.7 mg/L TPH and 2.95 ug/L PAH_{45} (0.5 % HEWAF). For the reproductive endpoint, Blend 1 HEWAF had a 7-day EC_{50} of 0.1721 % HEWAF, a 7-day NOEC of <0.125 % HEWAF, and a 7-day LOEC of 0.125 % HEWAF (Table 18). At 0.125 % HEWAF, 1.84 mg/L TPH and 0.924 ug/L PAH_{45} was measured in freshly prepared HEWAF, and 31% reproductive impairment was observed relative to controls.

In Blend 2 HEWAF, the survival results showed a 7-day LC_{50} of 0.5119 % HEWAF, a 7-day NOEC of 0.3125 % HEWAF, and a 7-day LOEC of 0.625 % (Table 18). At this LOEC, 3.64 mg/L TPH and 1.49 PAH_{45} were measured and 50 % mortality was observed (Table 17). No effects on survival were recorded at or below 1.44 mg/L TPH and 0.72 ug/L PAH_{45} in freshly prepared 0.3125% HEWAF (Figure 16; Table 17). For the reproductive endpoint, Blend 2 had a 7-day EC_{50} of 0.1006 % HEWAF, a 7-day NOEC of <0.15625 % HEWAF, and a 7-day LOEC of 0.15625 % HEWAF (Table 18). At 0.15625 % HEWAF, 0.813 mg/L TPH and 0.425 ug/L PAH_{45} were measured in freshly prepared HEWAF (Figure 16; Table 18) and 76 % reproductive impairment was observed relative to controls.

The lowest concentrations of TPH and PAH_{45} at which reproductive effects are noted are slightly higher in Blend 1 (1.84 mg/L and 0.924 µg/L, respectively) than Blend 2 (0.813 mg/L and 0.425 ug/L, respectively);

however, Blend 2 exposures with 5 % conventional and 95 % renewable mix showed 76 % reproductive impairment in daphnids at these concentrations versus 31 % in Blend 1 (Tables 16-18). When this comparison is extended to the survival endpoint, LOECs for TPH and PAH_{45} were noted to be greater in Blend 1. In Blend 1, 56 % mortality was observed at 15.2 mg/L TPH and 5.56 µg/L PAH_{45} versus a comparable 50 % mortality in Blend 2 at lower concentrations of 3.64 mg/L TPH and 1.49 µg/L PAH_{45}. It must be noted these exposures often showed high variability in toxicity endpoint measurements across replicates which are each placed in individual exposure chambers. The control variability was often much lower, and the reference toxicant test response was within acceptable range thereby confirming the validity of these tests. It is possible that, in the low volume exposures used for the daphnid test, small quantities of waxy material formed over 24 hours and affected swimming behavior and neonate production. Physical impairments and physiological changes related to these events could affect the survival and reproductive endpoints.

The dose-response curves show differences across four fuel types and highlight the differences in TPH and PAH_{45} relationships with both toxicity endpoints (Figure 16). Organisms in both the rangefinder and definitive tests for Blend 1 and Blend 2 appeared stunted in growth and were often seen twitching at the bottom of the cup before dying the following day. Across all fuel types, the daphnid was most sensitive to HEWAF prepared with conventional diesel (Figure 16 and Table 17).


A) Daphnid - Renewable Diesel - Survival
Rangefinder
Definitive
Mean Survival (%)
Percent HEWAF (%)
120
80
40
0
0
25
50
75
100
0
1
2
3
4
5

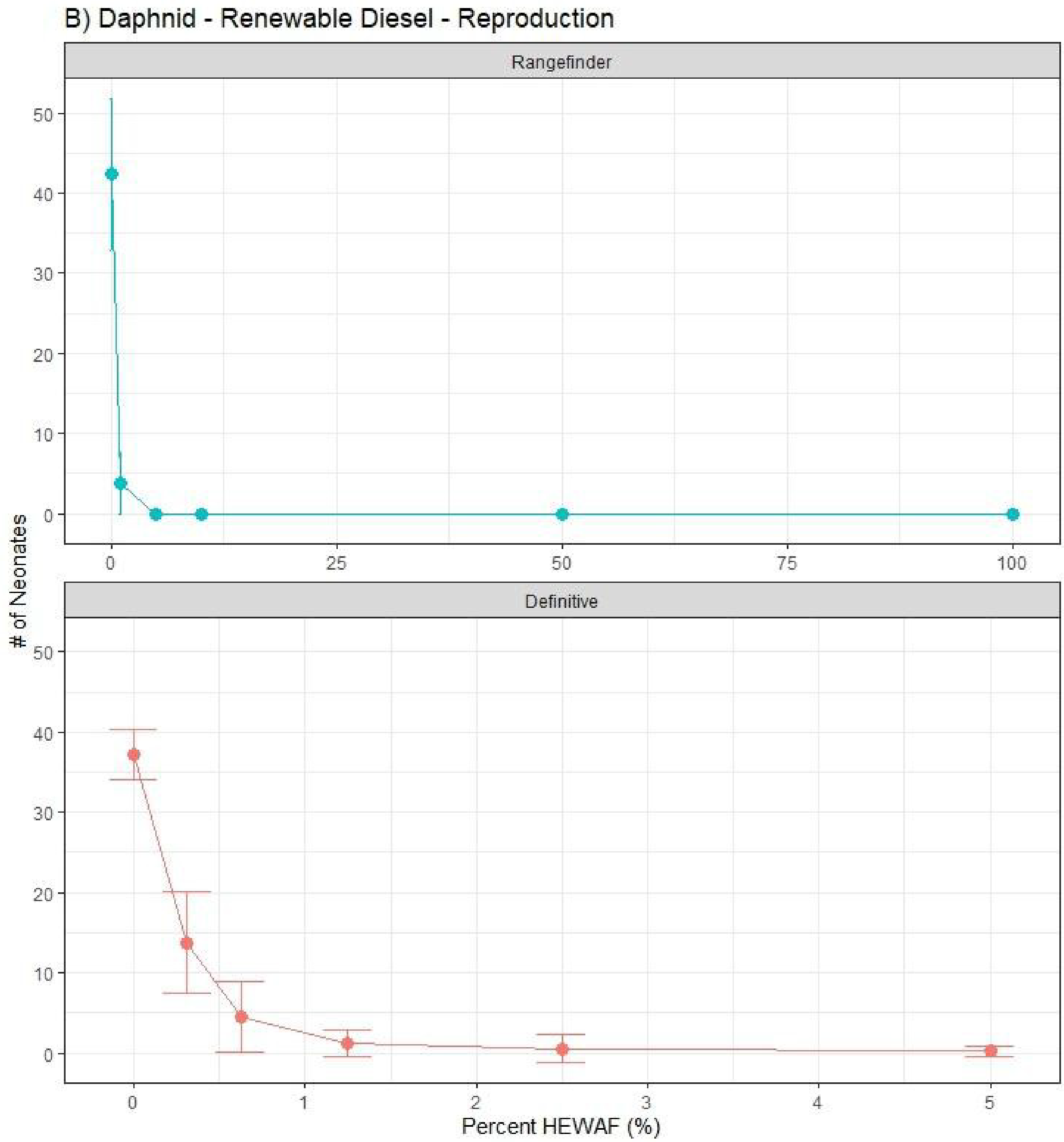
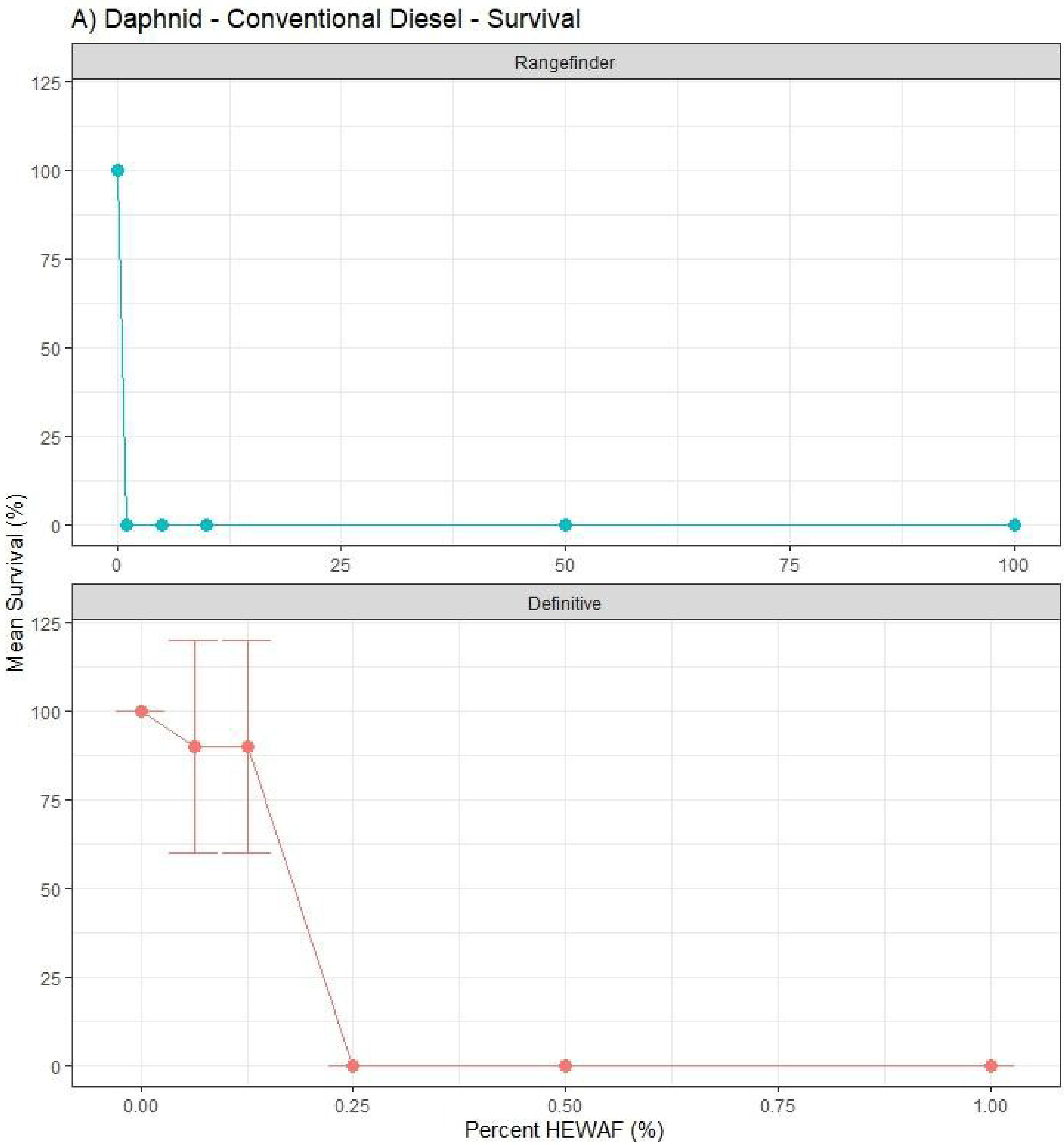


Figure 12. Rangefinder and definitive results for the chronic 3-brood daphnid toxicity tests with renewable diesel. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean number of neonates (reproduction) on y-axis. Error bars indicate +/- standard deviations.

Table 14. Toxicity results and TPH concentrations (mg/L) associated with HEWAF preparation from the renewable diesel definitive test for the daphnid. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 Total Concentration (mg/L)	Percent Survival (mean ± SD)	Reproduction (mean neonates ± SD)
0	0.0484 DNQ	100 ± 0.0	37.2 ± 3.2
0.3125	1.74	100 ± 0.0	13.8 ± 6.3
0.625	3.42	70.0 ± 45.8	4.5 ± 4.5
1.25	6.42	50.0 ± 50.0	1.2 ± 1.7
2.5	8.64	55.6 ± 49.7	0.6 ± 1.7
5	17.2	20.0 ± 40.0	0.2 ± 0.6


A) Daphnid - Conventional Diesel - Survival
Rangefinder
Definitive
Mean Survival (%)
125
100
75
50
25
0
0
25
50
75
100
0.00
0.25
0.50
0.75
1.00
Percent HEWAF (%)

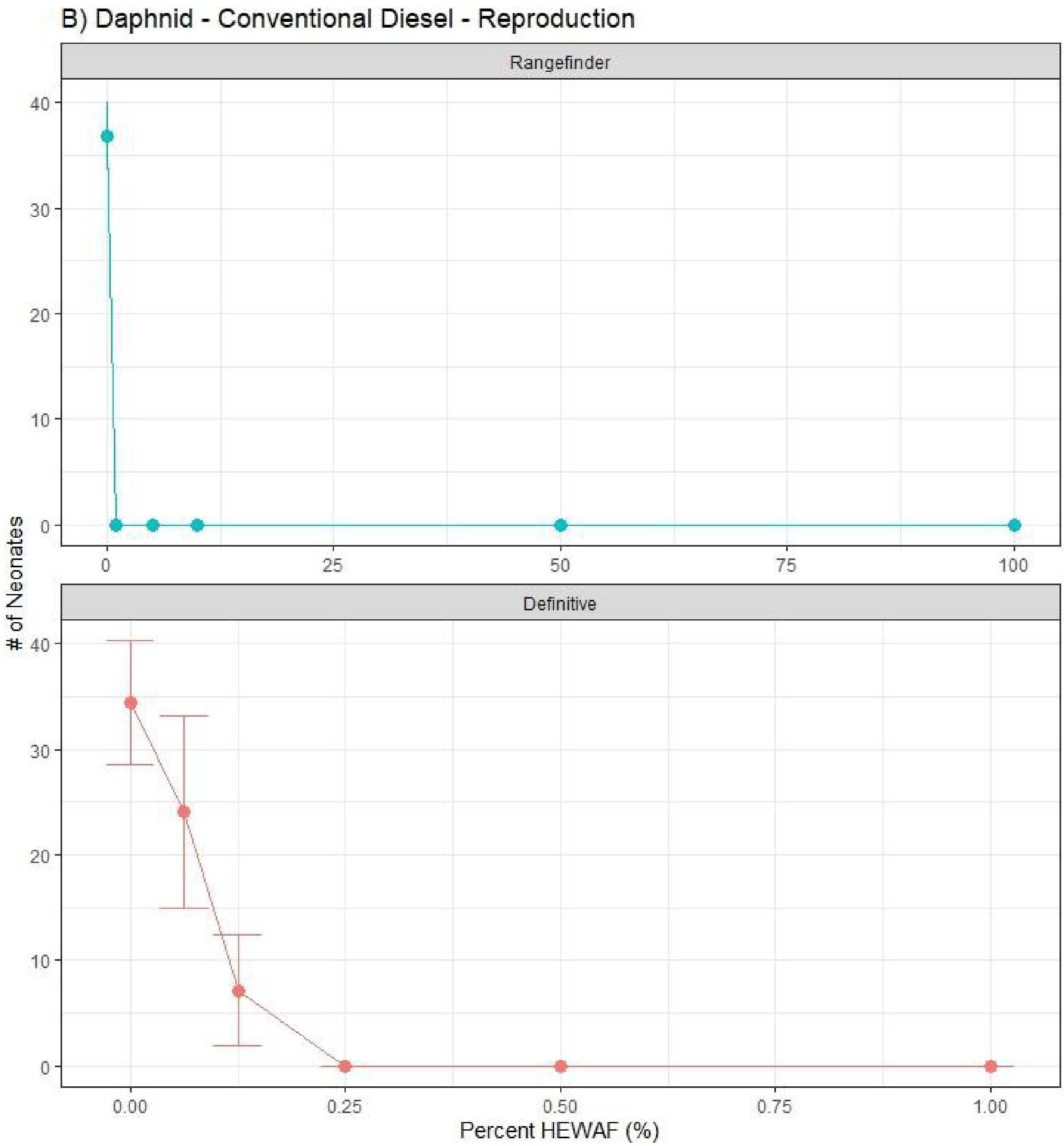
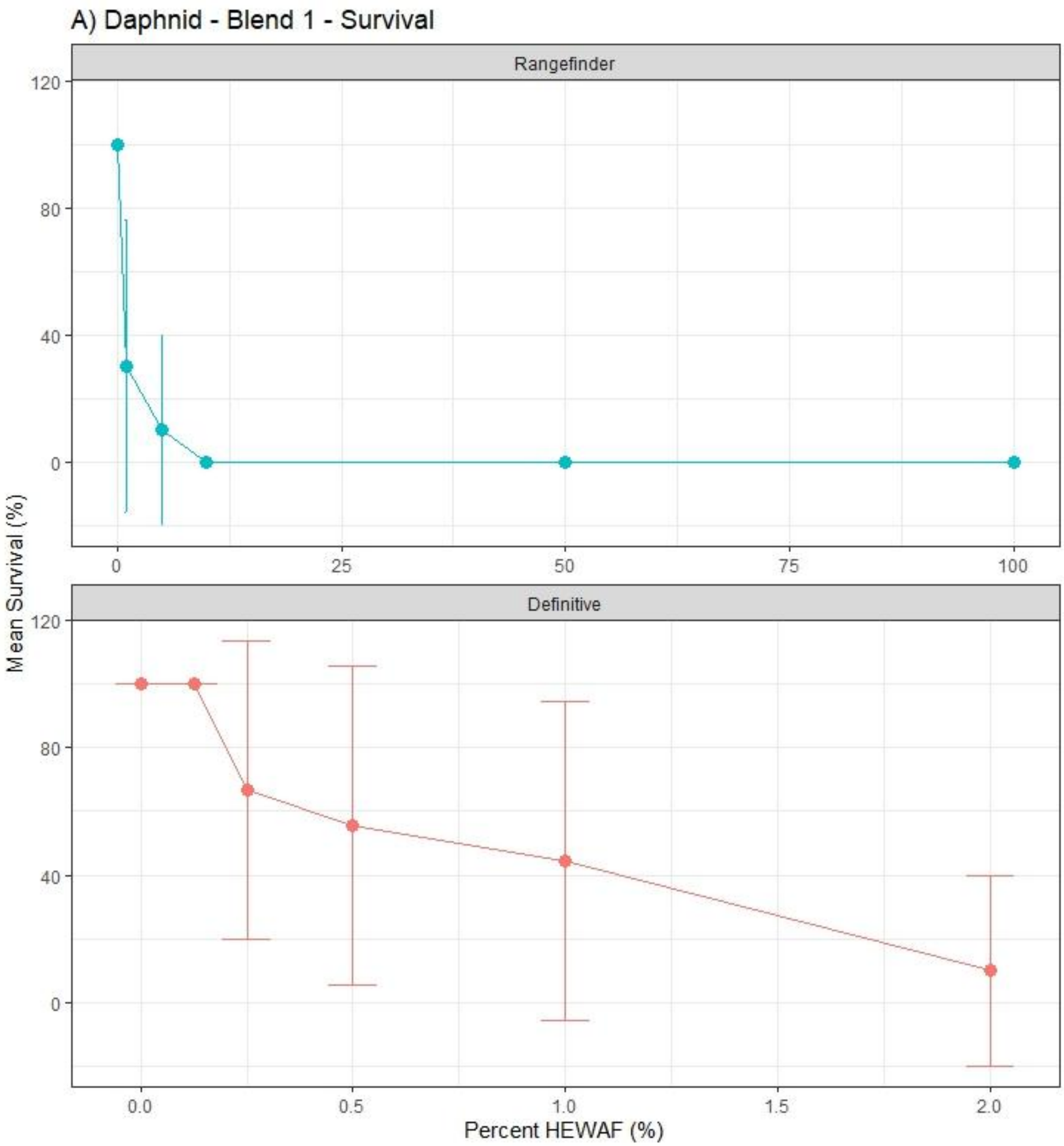


Figure 13. Rangefinder and definitive results for the chronic 3-brood daphnid toxicity tests with conventional diesel. A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean number of neonates (reproduction) on y-axis. Error bars indicate +/- standard deviations.

Table 15. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations associated with HEWAF preparation from the conventional diesel definitive test for the daphnid. DNQ = Detected, not quantified. All DNQ values show concentrations above the Method Detection Limit but below the Laboratory Reporting Limit and should be considered as estimated values.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Reproduction (mean neonates ± SD)
0	0.0459 DNQ	0.04	100 ± 0.0	34.4 ± 5.9
0.0625	0.486	4.23	90.0 ± 30.0	24.1 ± 9.1
0.125	1.01	7.24	90.0 ± 30.0	7.1 ± 5.3
0.25	1.92	12.46	0.0 ± 0.0	0.0 ± 0.0
0.5	4.93	29.68	0.0 ± 0.0	0.0 ± 0.0
1	8.24	47.76	0.0 ± 0.0	0.0 ± 0.0


A) Daphnid - Blend 1 - Survival
Rangefinder
Definitive
Mean Survival (%)
Percent HEWAF (%)
120
80
40
0
0
25
50
75
100
0.0
0.5
1.0
1.5
2.0

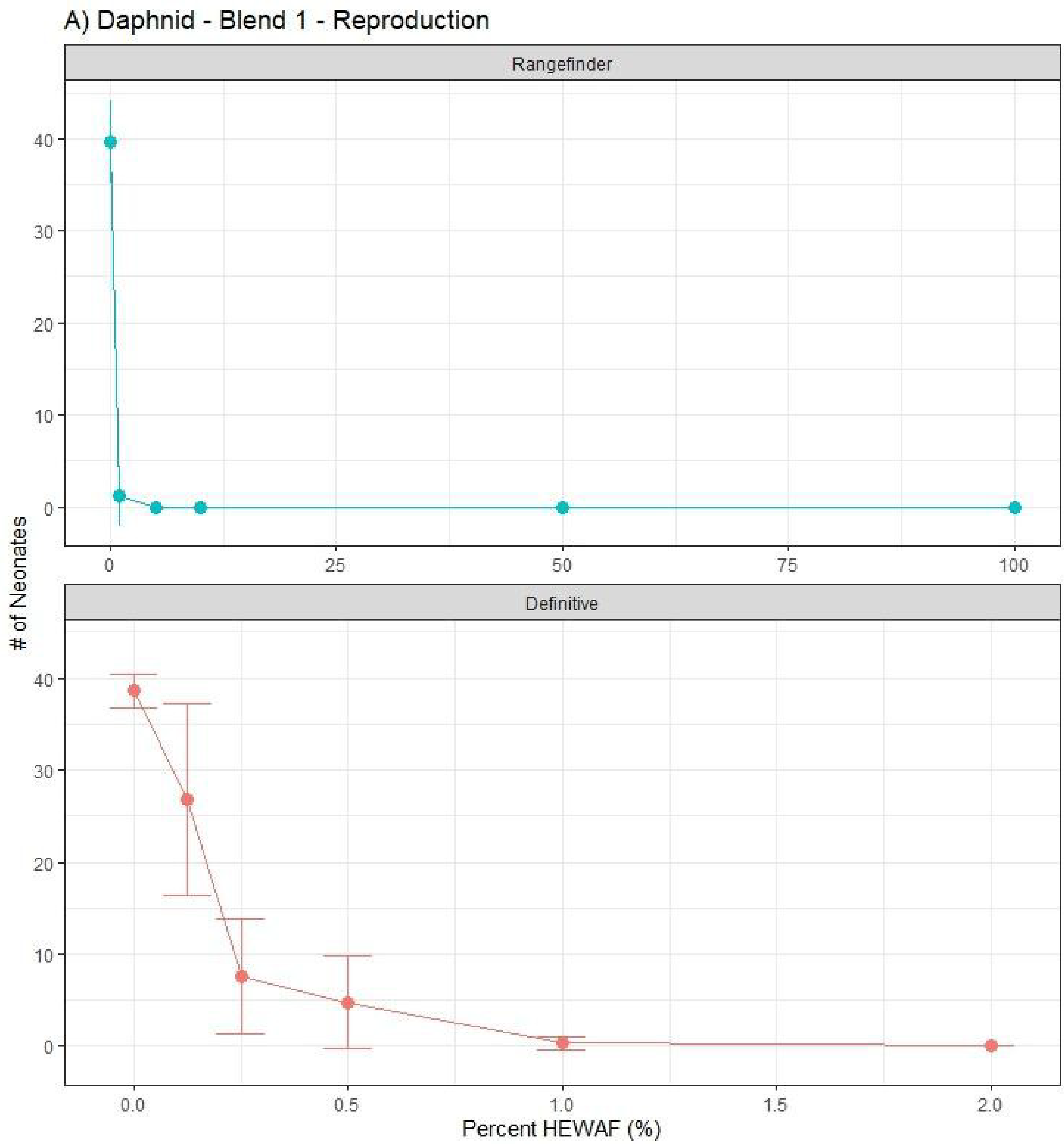
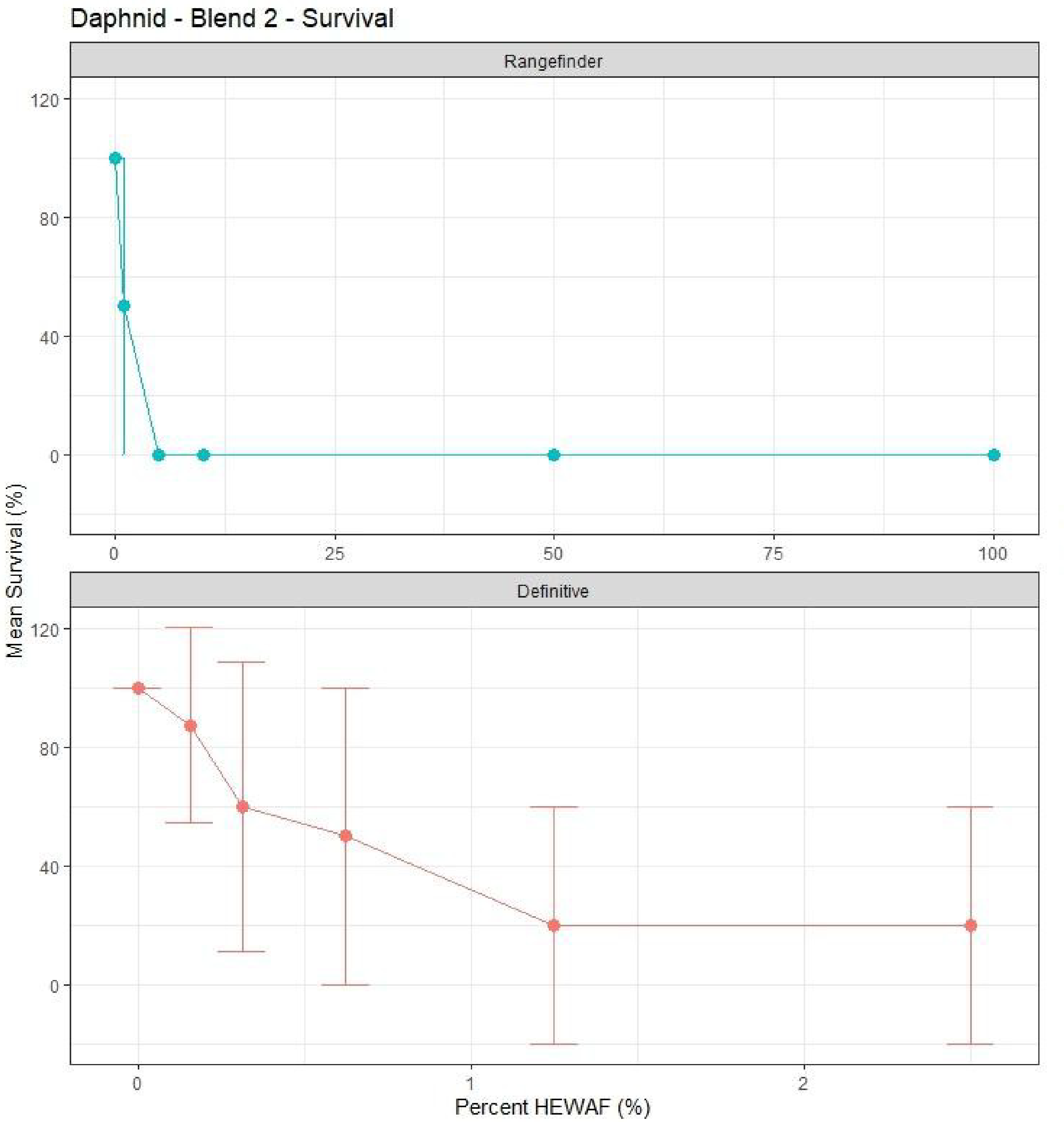


Figure 14. Rangefinder and definitive results for the chronic 3-brood daphnid toxicity tests with Blend 1 (20 % CND : 80 % RND). A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean number of neonates (reproduction) on y-axis. Error bars indicate +/- standard deviations.

Table 16. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations associated with HEWAF preparation from the Blend 1 diesel (20 % CND : 80 % RND) definitive test for the daphnid. ND = non detect.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 0 PAH Concentration (ug/L)	Percent Survival (mean ± SD)	Reproduction (mean neonates ± SD)
0	< 0.0356 ND	0.066	100 ± 0.0	38.6 ± 1.9
0.125	1.84	0.924	100 ± 0.0	26.78 ± 10.40
0.25	3.84	1.53	66.67 ± 47	7.56 ± 6.23
0.5	7.68	2.95	55.56 ± 50	4.78 ± 5.02
1	15.2	5.56	44.44 ± 50	0.33 ± 0.71
2	33.6	11.8	10 ± 30	0.0 ± 0.0


Daphnid - Blend 2 - Survival
Rangefinder
120
80
40
0
0
25
50
75
100
Mean Survival (%)
Definitive
120
80
40
0
0
1
2
Percent HEWAF (%)

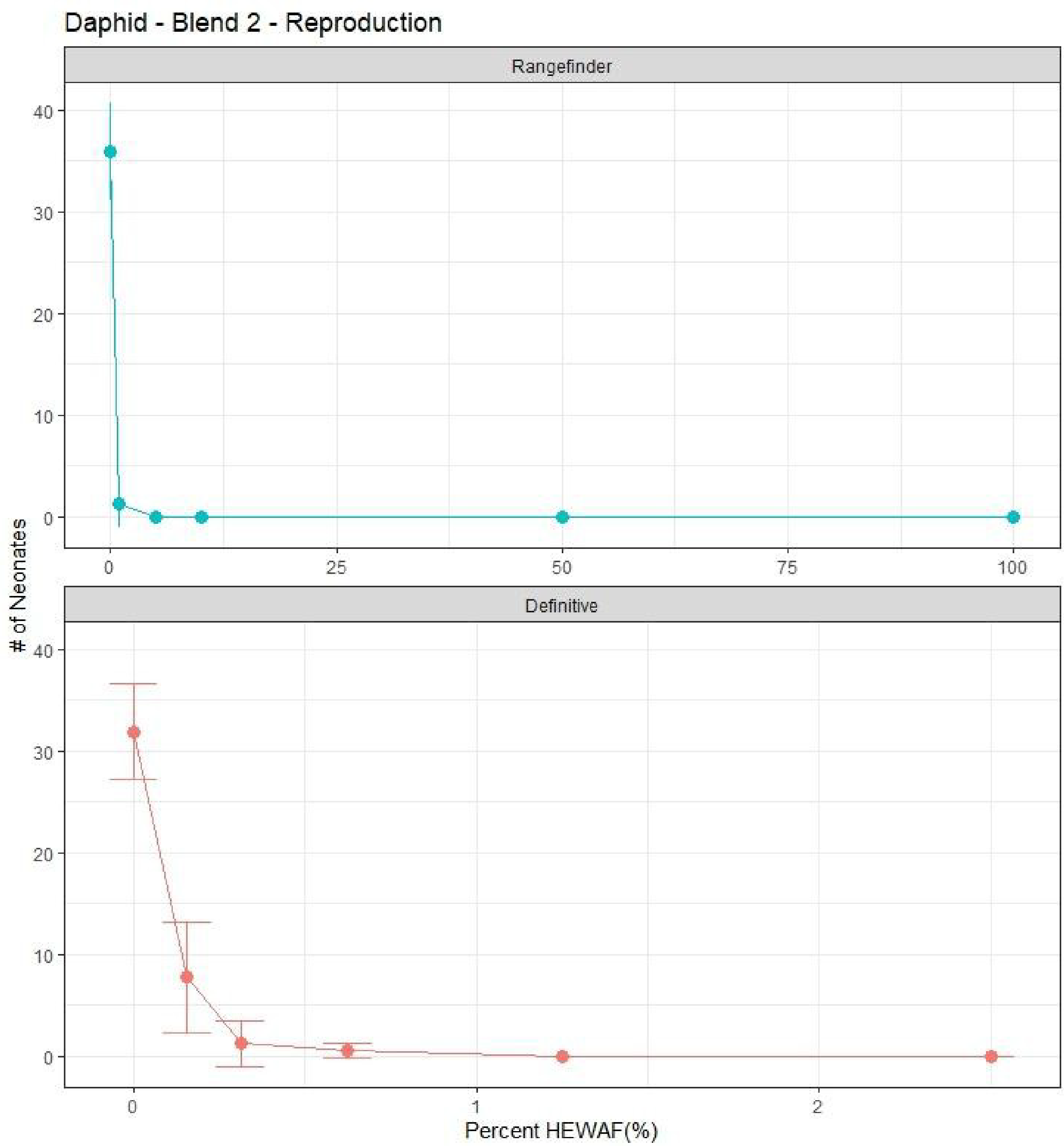
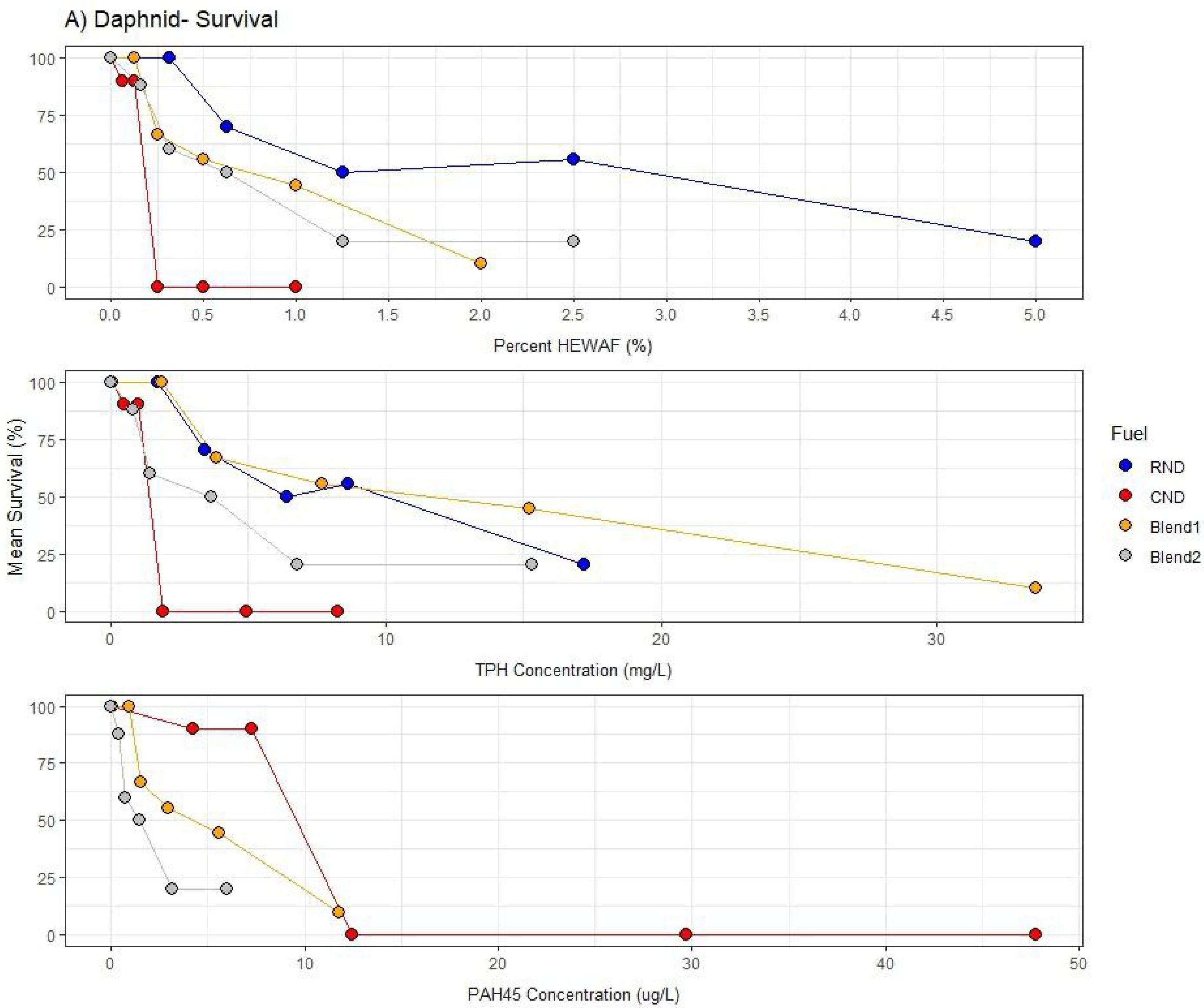


Figure 15. Rangefinder and definitive results for the chronic 3-brood daphnid toxicity tests with Blend 2 (5 % CND : 95 % RND). A) Percent HEWAF shown on x-axis and mean percent survival on y-axis, and B) Percent HEWAF shown on x-axis and mean number of neonates (reproduction) on y-axis. Error bars indicate +/- standard deviations.

Table 17. Toxicity results, TPH concentrations (mg/L), and PAH_{45} concentrations associated with HEWAF preparation from the Blend 2 diesel (5 % CND : 95 % RND) definitive test for the daphnid. ND = non detect.

Percent HEWAF (%)	Day 0 TPH Concentration (mg/L)	Day 0 PAH45 Concentration (ug/L)	Percent Survival (mean ± SD)	Reproduction (mean neonates ± SD)
0	< 0.0348 ND	0.029	100 ± 0.0	31.9 ± 4.7
0.15625	0.813	0.425	87.5 ± 33	7.8 ± 5.5
0.3125	1.44	0.72	60 ± 49	1.2 ± 2.2
0.625	3.64	1.49	50 ± 50	0.5 ± 0.7
1.25	6.79	3.15	20 ± 40	0.0 ± 0.0
2.5	15.3	6	20 ± 40	0.0 ± 0.0



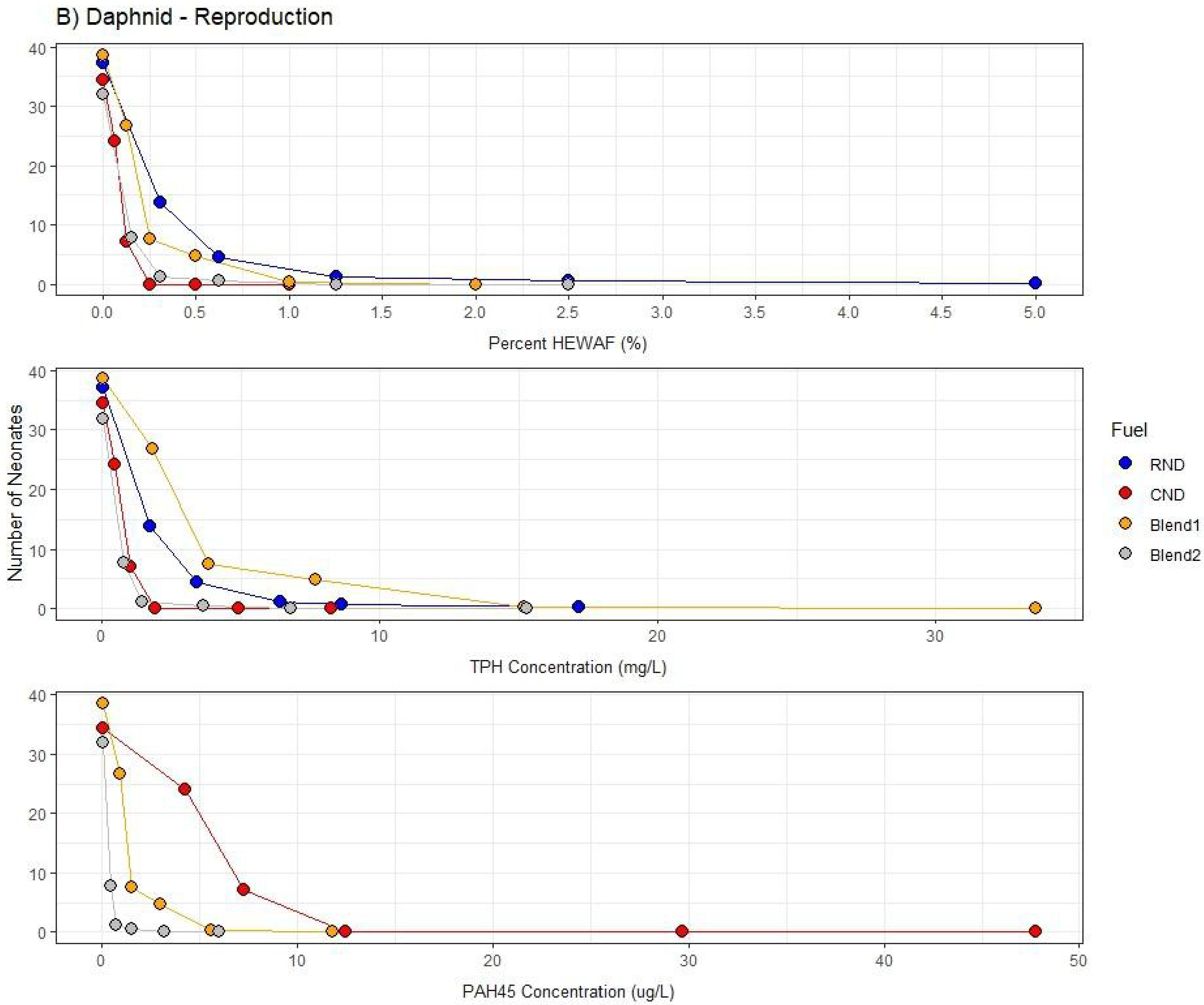


Figure 16. Definitive chronic 3-brood daphnid toxicity test A) mean percent survival on the y-axis, and B) and mean number of neonates produced (reproduction) on the y-axis, both compared to percent HEWAF, total TPH concentration (mg/L), and PAH_{45} concentration (ug/L) on the x-axes for each fuel type. RND = Renewable Diesel. CND = Conventional Diesel. Blend 1 = 20 % CND : 80 % RND. Blend 2 = 5 % CND : 95 % RND. PAH_{45} concentration was not measured for renewable diesel.

Table 18. Toxicity test point estimates (% HEWAF) and statistical results for the chronic 3-brood daphnid test in all diesel types. LC_{50} = Median lethal concentration for the survival endpoint. EC_{50} = Median effect concentration for the reproduction endpoint. NOEC = no observed effect concentration. LOEC = Lowest observed effect concentration. LCL and UCL = lower and upper confidence intervals around the point estimate. Blend 1 = 20 % CND : 80 % RND. Blend 2 = 5 % CND : 95 % RND.

Diesel Type	Test Type	Date	Point Estimate Type	Point Estimate Result	NOEC	LOEC	95% LCL	95% UCL
Renewable Diesel	Rangefinder	05/27/2025	LC50	1.964	1	5	1.133	3.405
Renewable Diesel	Rangefinder	05/27/2025	EC50	0.4631	<1	1	0.4316	0.5129
Renewable Diesel	Definitive	07/01/2025	LC50	1.745	2.5	5	0.9499	3.207
Renewable Diesel	Definitive	07/01/2025	EC50	0.241	<0.3125	0.3125	0.2060	0.2984
Conventional Diesel	Rangefinder	06/17/2025	LC50	0.4142	<1	1	0.4142	0.4142
Conventional Diesel	Rangefinder	06/17/2025	EC50	0.4142	N/A	N/A	0.4142	0.4142
Conventional Diesel	Definitive	07/11/2025	LC50	0.1701	0.125	0.25	0.1568	0.1845
Conventional Diesel	Definitive	07/11/2025	EC50	0.08736	<0.0625	0.0625	0.06843	0.1016
Blend 1	Rangefinder	08/07/2025	LC50	0.6407	<1	1	0.4402	1.402
Blend 1	Rangefinder	08/07/2025	EC50	0.4296	N/A	N/A	0.4142	0.4566
Blend 1	Definitive	08/18/2025	LC50	0.5955	0.5	1	0.3652	0.971
Blend 1	Definitive	08/18/2025	EC50	0.1721	<0.125	0.125	0.1383	0.1945
Blend 2	Rangefinder	10/06/2025	LC50	1	<1	1	0.4860	1.884
Blend 2	Rangefinder	10/06/2025	EC50	0.432	N/A	N/A	0.4160	0.451
Blend 2	Definitive	10/17/2025	LC50	0.5119	0.3125	0.625	0.3020	0.8679
Blend 2	Definitive	10/17/2025	EC50	0.1006	<0.15625	0.15625	0.0890	0.1178

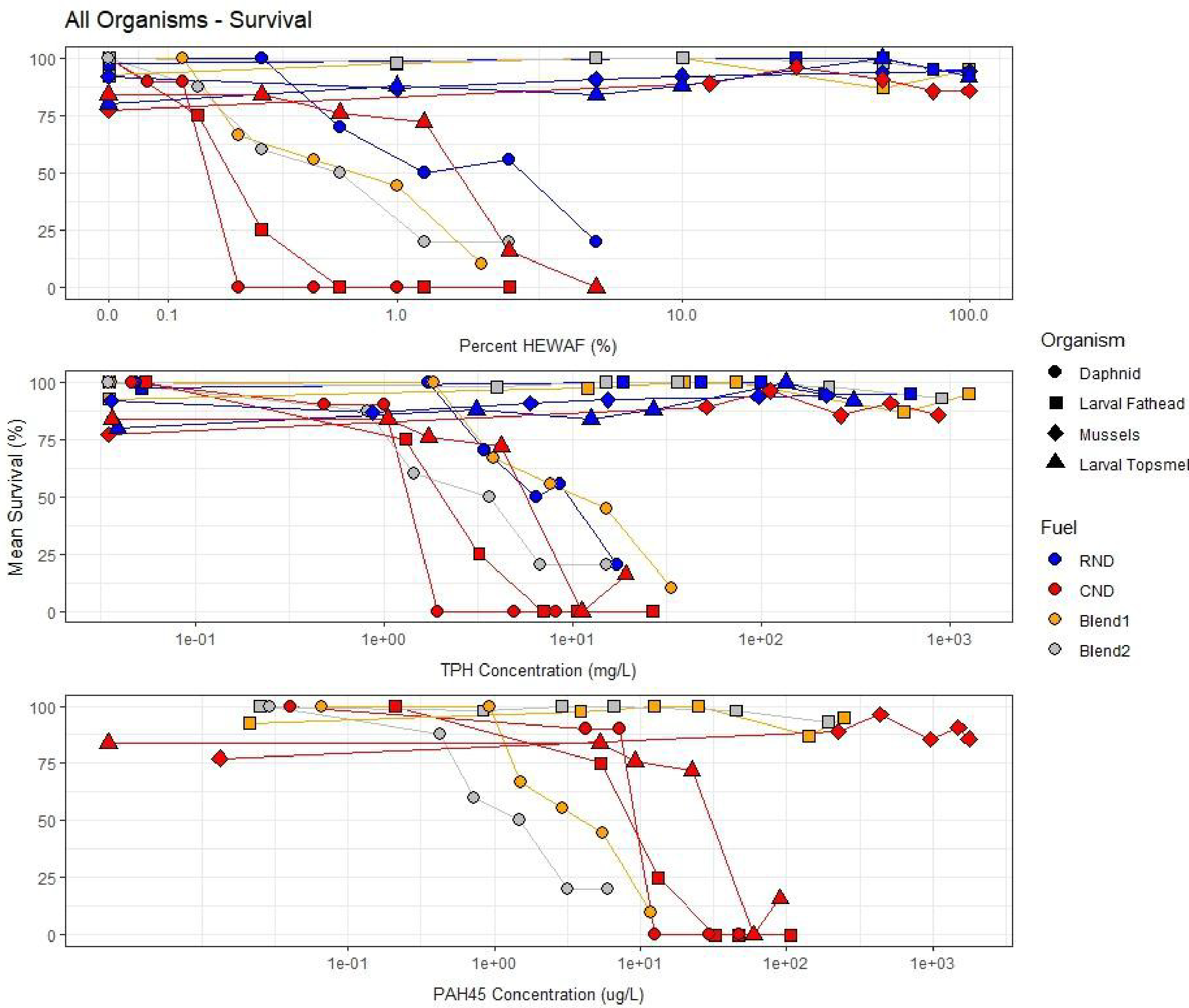


Figure 17. Definitive toxicity test results for all organisms and all fuel types tested. Mean percent survival is on the y-axis, and is compared to percent HEWAF, TPH concentration (mg/L), and PAH_{45} concentration (ug/L) on the x-axes. The x-axis was scaled using $\log^{10}$ for visual clarity between tests. RND = Renewable Diesel. CND = Conventional Diesel. Blend 1 = 20 % CND : 80 % RND. Blend 2 = 5 % CND : 95 % RND. PAH_{45} concentration was not measured for renewable diesel.

Conclusions

Ceriodaphnia dubia was the most sensitive organism to renewable diesel HEWAF exposures in this study (Figure 17), and toxic effects on its reproduction were the most significant. The daphnid was the only organism to have a strong dose response to HEWAF prepared with renewable diesel and the two fuel blends tested in this study, even with half the loading rate compared to tests conducted with other organisms. As seen in Figure 17, larval topsmelt, larval fathead minnow, and larval mussels showed no survival response to HEWAF prepared with renewable diesel. However, there were observations related to fish feeding, swimming behaviors, and possible physical impairments in HEWAF exposures that need further investigations. Significant losses of TPH and PAH_{45} were recorded over 24-hr periods in exposure chambers. Therefore, lack of toxicity should be considered alongside these losses from the water column to beaker walls and surface layers. Petroleum hydrocarbons are known to have adverse effects on aquatic organisms (Dupuis and Francisco 2015, Hobbs et al 2020, Kuppusamy et al 2019), as observed in our toxicity tests with conventional diesel. In this study, conventional diesel was toxic to all test organisms (the daphnid, larval fathead, larval topsmelt, and larval mussels – listed in order from most sensitive to least sensitive for the survival endpoint). Notably, although *Mytilus* larval survival was not affected in the conventional diesel exposures, larval development was significantly impacted indicating high sensitivity of invertebrate development markers to petroleum hydrocarbons.

References

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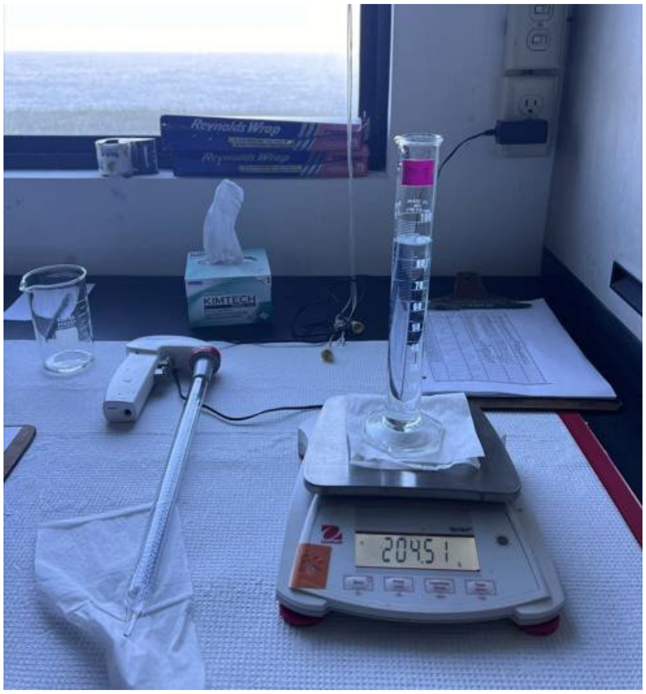
Kuppusamy et al (2019). Total Petroleum Hydrocarbons: Environmental Fate, Toxicity, and Remediation. Springer.

U.S. EPA (1995) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to west coast marine and estuarine organisms. EPA/600/R-95-136.

U.S. EPA (2002) Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA-821-R-02-013. Office of Water, Washington DC, USA

Photos

HEWAF Preparation – Weighing fuel to make HEWAF:

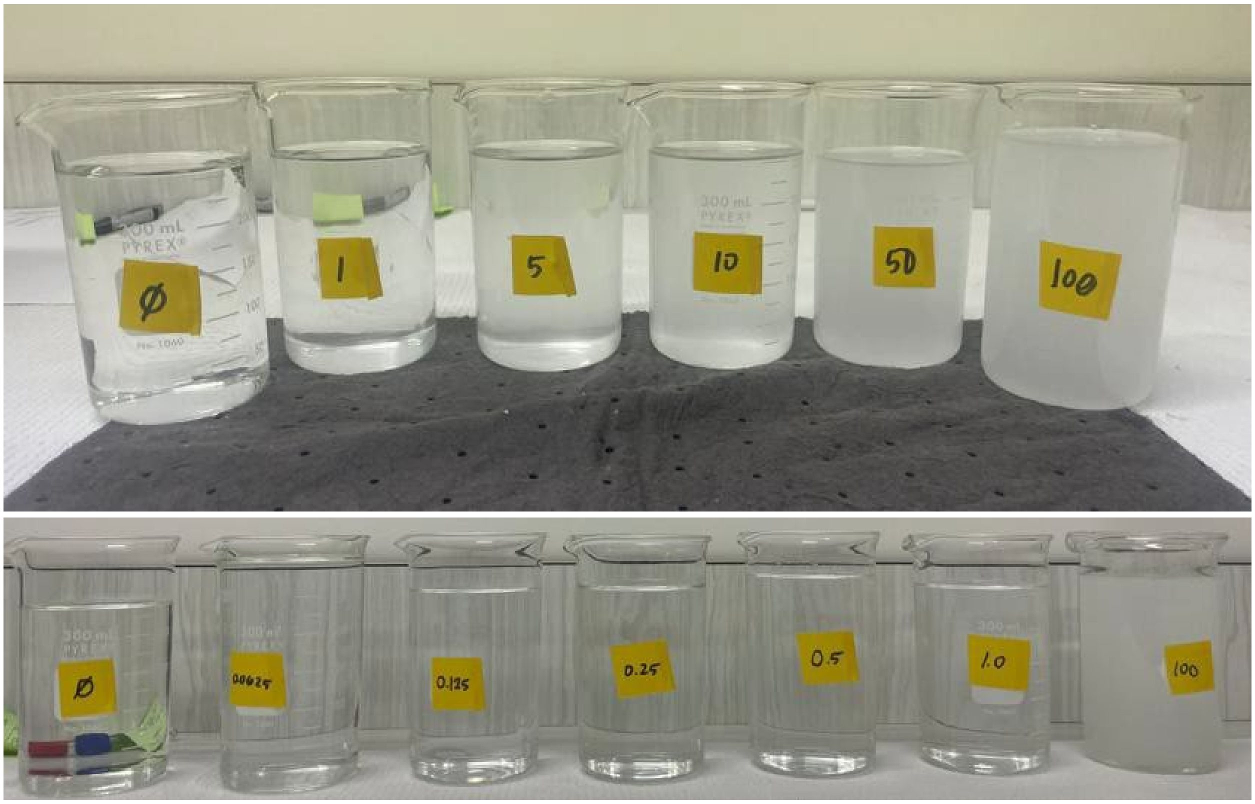


HEWAF Preparation- HEWAF being poured into separatory funnel for one hour separation period:

Renewable Diesel HEWAF after one hour separation period:

Conventional Diesel HEWAF after one hour separation period:

***Conventional Diesel HEWA*F** – Rangefinder (top) and definitive (bottom) exposure series made with 100% HEWAF.



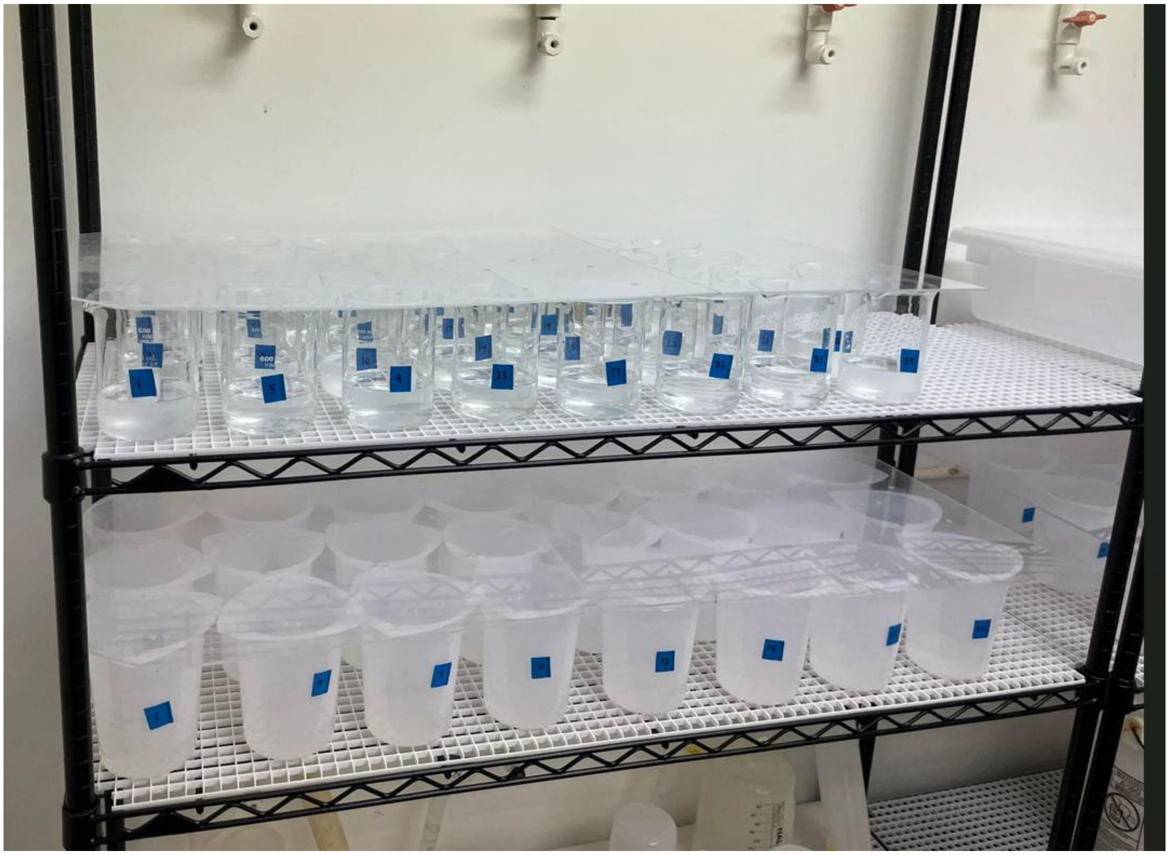
Blend 1 HEWAF after one hour separation period:

Blend 2 HEWAF after one hour separation period:

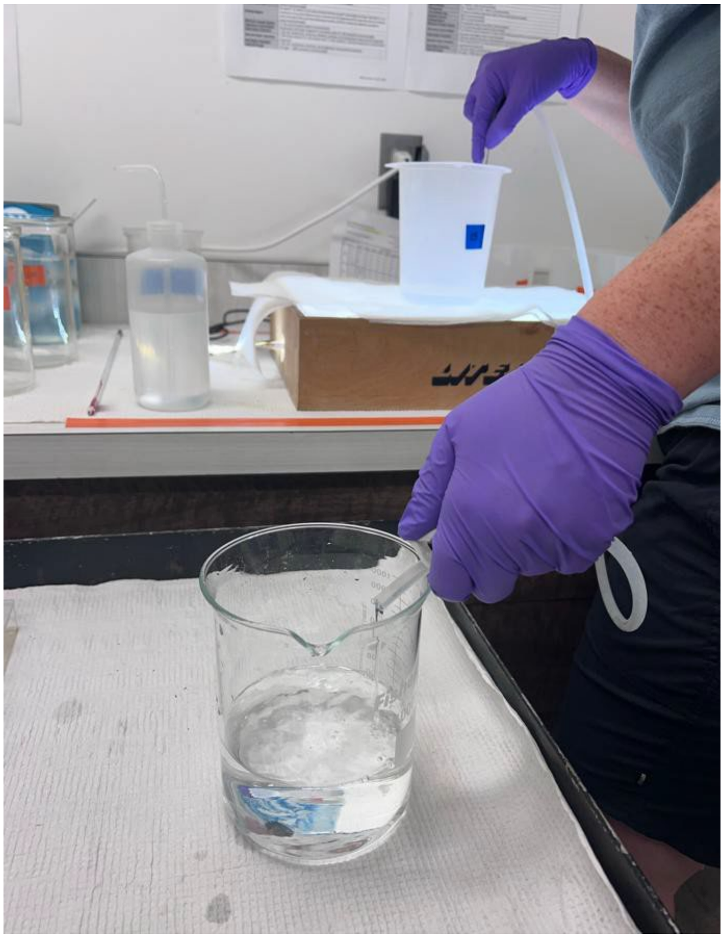
Larval topsmelt culture upon arrival:

Technicians loading a test with larval fish:

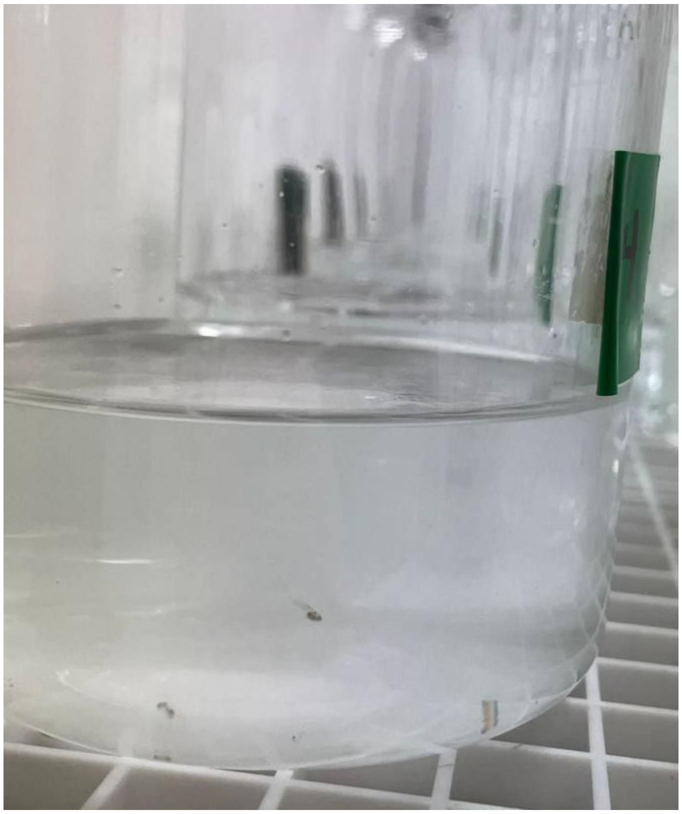
General test and reference toxicant test setup for larval fish:



Technician conducting daily renewal of fish test using a siphon:



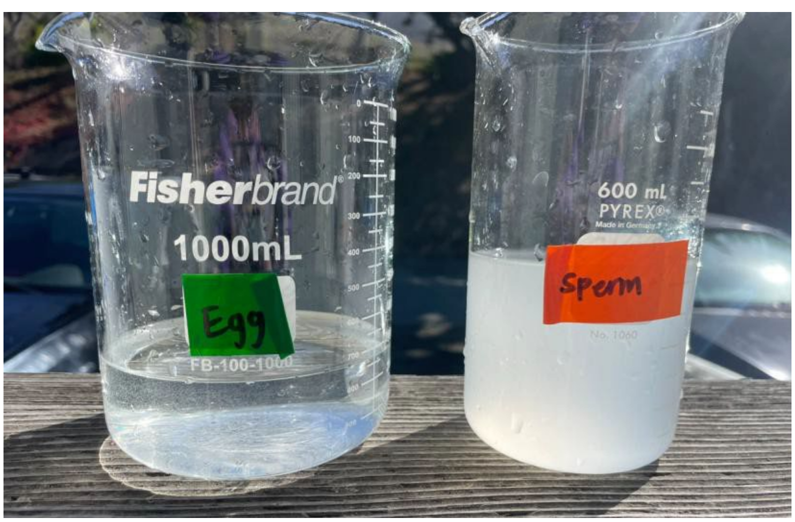
Larval topsmelt in test containing HEWAF prepared with 100% renewable diesel:



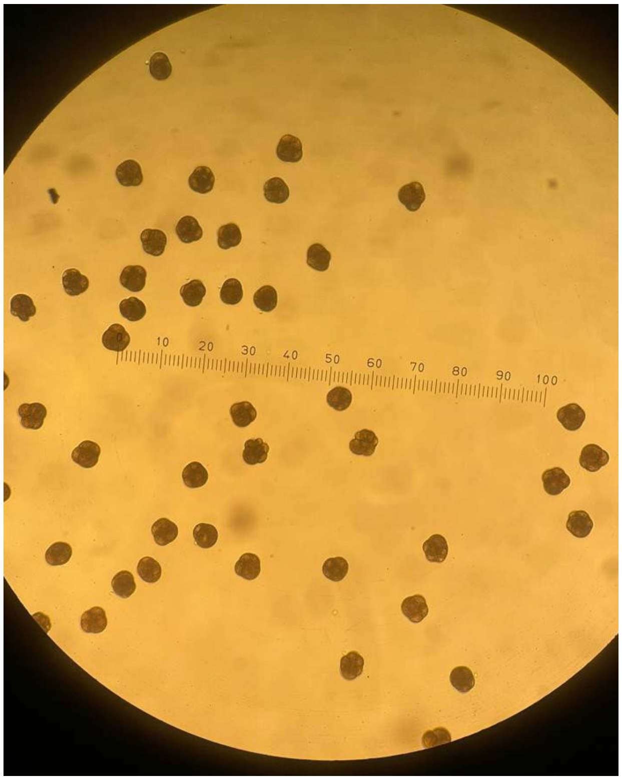
Waxy build-up in larval topsmelt test containing HEWAF prepared with 100% renewable diesel:

Mussels in heat shock set-up for spawning:

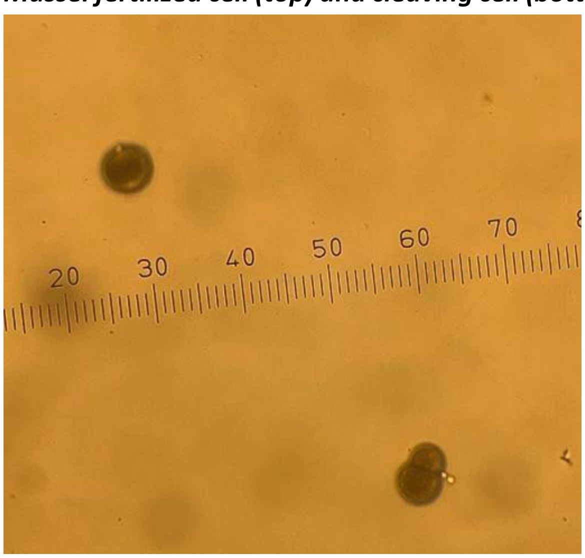
Mussel eggs and sperm post-spawn:



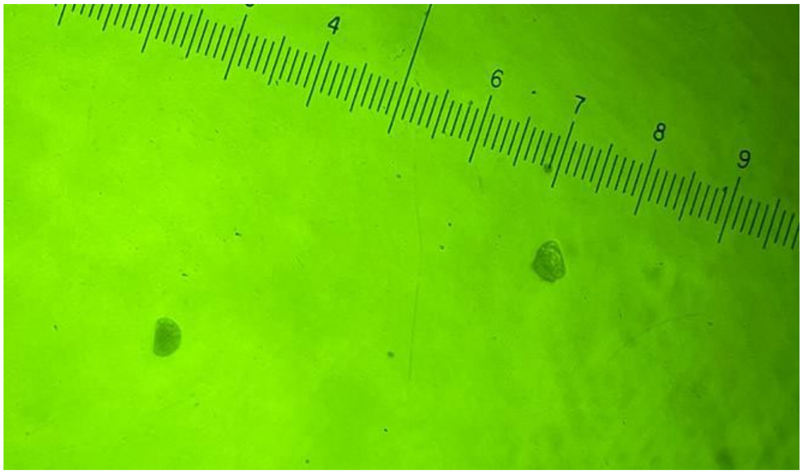
Mussel cleaving cells after fertilization:



Mussel fertilized cell (top) and cleaving cell (bottom):

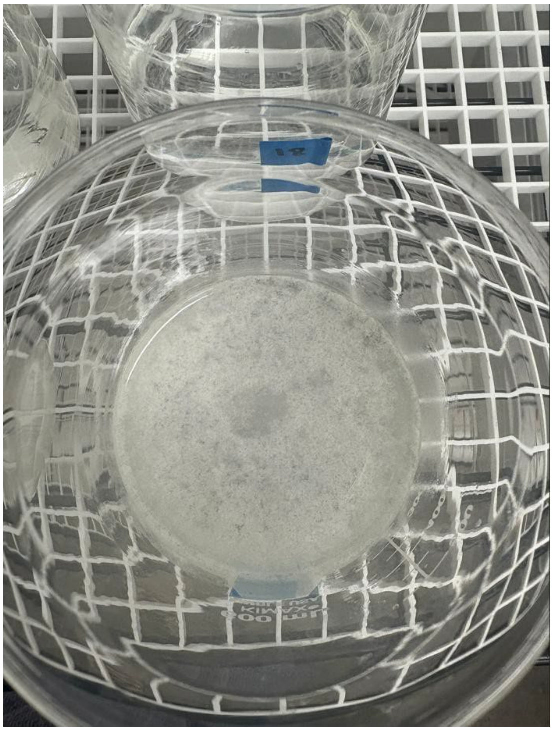


Normal mussel veliger larvae (left) vs. abnormal mussel larvae (right):

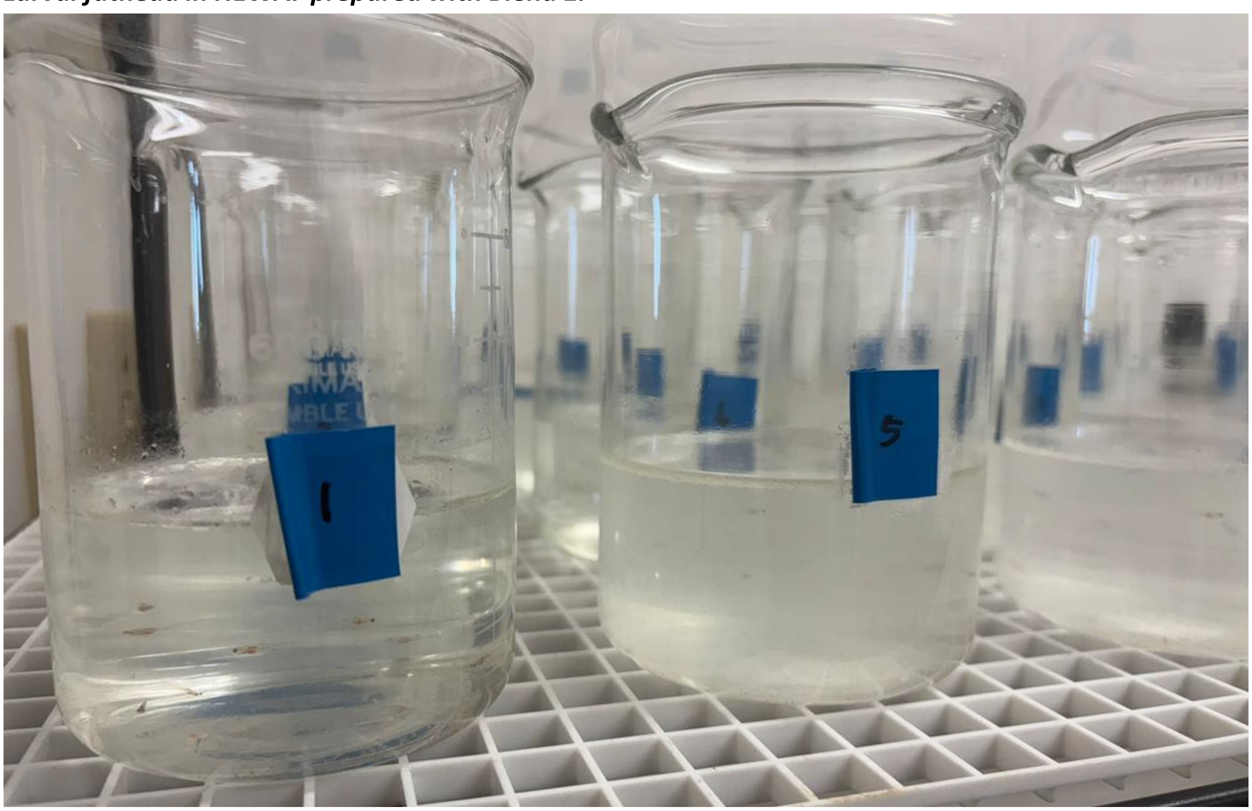


Larval fathead in HEWAF prepared with 100% renewable diesel:

Photo showing the waxy build-up in the larval fathead Blend 1 test:



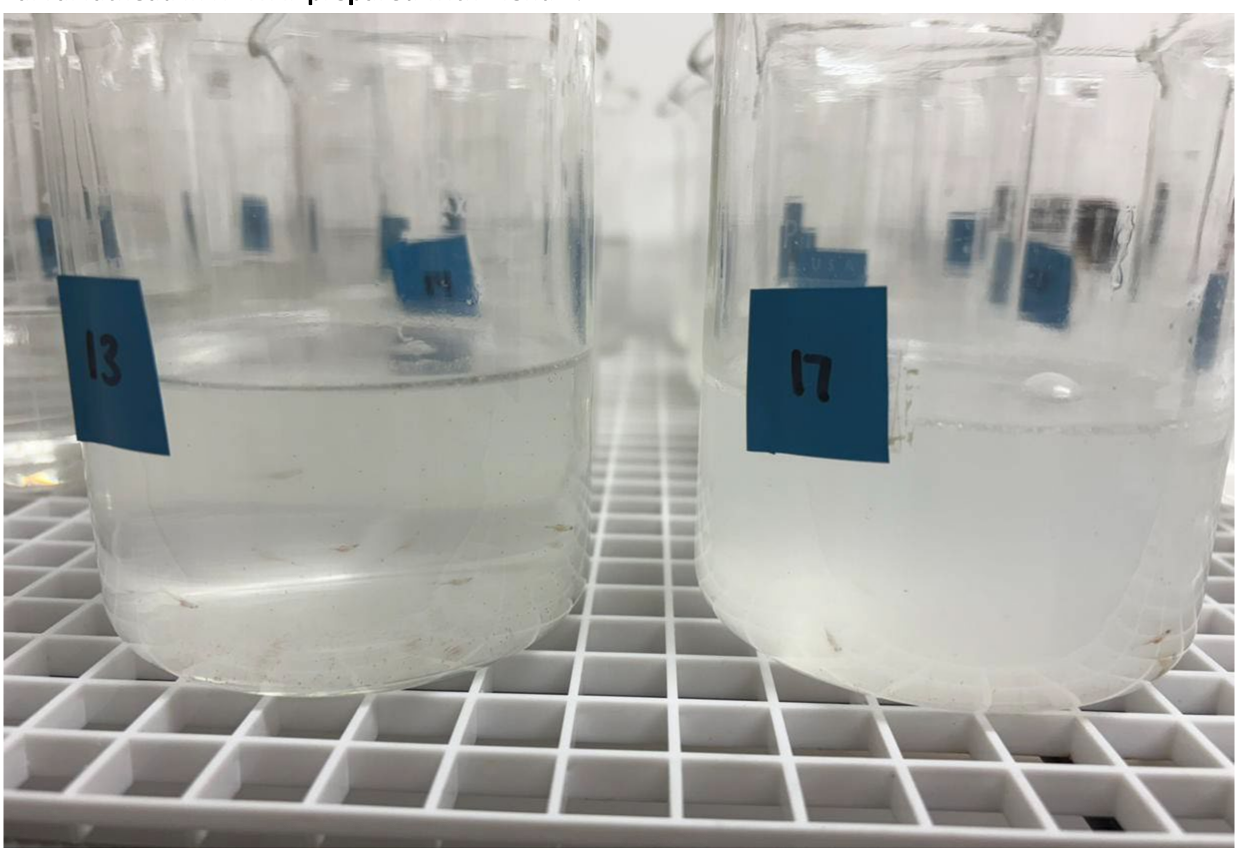
Larval fathead in HEWAF prepared with Blend 1:



Larval fathead in HEWAF prepared with Blend 2:

Photo showing the waxy build-up in the larval fathead Blend 2 test (right):

Larval fathead in HEWAF prepared with Blend 2:



Larval fathead collected for growth measurements at the end of a test ran with Blend 2:

Larval fathead collected for growth measurements at the end of a test ran with Blend 2:

Example of a healthy daphnid adult in test control:

Example of a stunted daphnid adult found in a test conducted with Blend 1: