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Assessing the effects of fluorine-free and PFAS-containing firefighting foams on development and behavioral responses using a zebrafish-based platform

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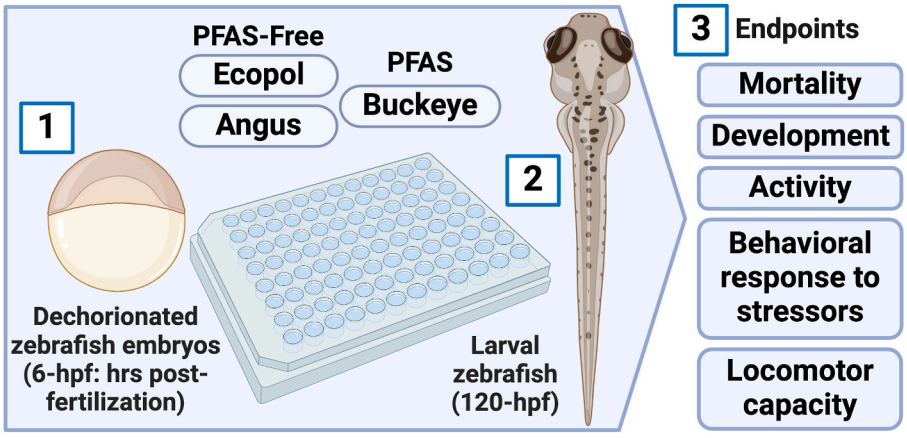
HIGHLIGHTS

- F3s are potential alternatives, but risk assessment is crucial.
- F3s more toxic than PFAS-containing AFFFs in acute and developmental toxicity assessments.
- F3s significantly affect activity and behavioral stress responses.
- Our findings highlight the utility of the proposed toxicity assessment platform.

GRAPHICAL ABSTRACT



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ABSTRACT

Significant progress has been made in developing fluorine-free firefighting foams (F3) as alternatives to per-fluoroalkyl substances (PFAS)-containing aqueous film-forming foams (AFFF) to help eliminate the health and environmental concerns linked to PFAS exposure. However, developing viable F3 options hinges on a thorough assessment of potential risks alongside the technical performance evaluations. This study showcases the capability of a zebrafish-based platform to discern the developmental and behavioral toxicities associated with exposure to one AFFF and two F3 formulations. To facilitate direct exposure to the chemicals, embryos were

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enzymatically dechorionated and then exposed to the diluted formulations (6–120 hours post fertilization (hpf)) at concentrations folding from 0.1% of the manufacturer-recommended working concentrations. The exposure regimen also included daily automated media changes (50%) and mortality assessments (24 and 120 hpf). At 120 hpf, a comprehensive assessment encompassing overall development, prevalence of morphological defects, and behavioral responses to acute stressors (visual, acoustic, and peripheral irritant) was conducted. Exposure to both F3s significantly increased larval mortalities to percentages exceeding 90%, whereas AFFF exposures did not cause any significant effect. Overall development, marked by total larval length, was significantly impacted following exposures to all foams. Behavioral responses to acute stressors were also significantly altered following exposures to both F3s, whereas the AFFF did not alter behavior at the concentrations tested. Our findings demonstrate toxicities associated with tested F3 formulations that encompass several endpoints and highlight the utility of the proposed platform in evaluating the developmental toxicities of current and future foam formulations.

1. Introduction

Aqueous film-forming foam (AFFF) is a specialized foam formulation comprising hydrocarbon and fluorinated surfactants developed in the 1970s. Its formulation is designed to extinguish Class B fires involving flammable liquids such as oil and gasoline by forming a film that suffocates flames and prevents them from reigniting (Dlugogorski and Schaefer, 2021; Yang et al., 2022; Ateia et al., 2023). The commendable firefighting efficiency of AFFF facilitated their widespread adoption across diverse domains, including military training sites, airports, and firefighter training facilities, both in the United States and globally. Nevertheless, this extensive use has raised substantial human and environmental health concerns due to the inclusion of per- and poly-fluoroalkyl substances (PFAS) in the composition of AFFF. This has triggered a concerted effort to develop or adopt fluorine-free alternatives capable of effectively addressing fire-related risks while concurrently mitigating the health risks inherent in PFAS-containing AFFF formulations (Ateia and Scheringer, 2024; Jahura et al., 2024).

The drive to shift to fluorine-free foams (F3) began in the early 2000s, with a surge in activity recently due to heightened health concerns surrounding PFAS. In industry, companies have been creating innovative halogen-free surfactants and additives as potential substitutes for PFAS (Sheng et al., 2021; Ateia et al., 2023). The U.S. Department of Defense has committed to halting the procurement of PFAS-containing AFFF in 2023 and, in compliance with Congressional mandates, established a new military specification for F3 formulations (MIL-PRF-32725) (Ateia et al., 2023). As of October 2023, they started listing F3 products on the qualified products database that adhere to the military specifications for F3 in land-based and freshwater firefighting applications. The European Union is also diligently formulating regulations to restrict the use of PFAS across a multitude of sectors (Brennan et al., 2021; Spyrakis and Dragani, 2023) and Australia has effectuated the use of an AFFF substitute across all 27 principal airports, reflecting a concerted transcontinental initiative toward mitigating PFAS-related risks and fostering the adoption of ecologically benign alternatives. As a result of these efforts, the F3's novel alternative active components are utilized in larger quantities as compared to their PFAS-containing counterparts in AFFF (Yu et al., 2020; McDonald et al., 2022).

Laboratory investigations into PFAS have consistently highlighted the environmental persistence of this group of chemicals and their potential to disrupt various systems and organs (Fenton et al., 2021). This persistence prompted an initial strategic shift from long-chain PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), to short-chain PFAS like perfluorohexane sulfonic acid (PFHxS) and perfluorohexanoic acid (PFHxA), which exhibit a reduced potential for bioaccumulation. However, due to concerns about the properties of short-chain PFAS and the extreme persistence of their final degradation products (Brendel et al., 2018), the decision was made to completely phase out this group of chemicals. Simultaneously, laboratory investigations demonstrated effects of different PFAS on the neuroendocrine systems, causing adverse effects that span across the reproductive, thyroid, and adrenal axes (Godfrey et al., 2017; Gaballah et al., 2020; Beans, 2021; Wasel et al., 2021; Beale et al., 2022; Rickard et al., 2022; Tomei Torres and Masten, 2023; Bline et al., 2024). These findings were further substantiated by field investigations on various vertebrate species as well as epidemiological studies on human subjects (Street et al., 2018; Canova et al., 2020; Itoh, 2020; Li et al., 2020; Liu et al., 2020; Radke et al., 2022; Dunder et al., 2023; India-Aldana et al., 2023). Echoing the patterns observed with other neuroendocrine disruptors, the adverse effects of PFAS are particularly profound and enduring as exposures occur during developmentally sensitive periods (Szilagyi et al., 2020; Fenton et al., 2021).

Concurrent toxicological research on F3 formulations is still in its early stages, with studies mainly limited to acute toxicity investigations conducted on a limited number of species, often without clear demarcation between toxicity tests conducted on the foam, formulation, product itself, or individual components (Gharehveran et al., 2022). The initial findings from investigations conducted in aquatic species suggest that available F3 products may exhibit comparable or even greater acute toxicities than PFAS-containing AFFF. Moreover, sublethal investigations on F3 formulations conducted in worms and plants have also highlighted their adverse effects on growth and reproduction (Montagnolli et al., 2017; Fuller et al., 2024). Additionally, toxicological studies on specific components of F3 formulations, such as surface tension-lowering surfactants, demonstrated their potential for cellular membrane damage and cytotoxicity (Denkov et al., 2020; Deotale et al., 2023; Pan et al., 2018). Sodium lauryl sulfate, a common surfactant in F3 formulations, was found to irritate the skin, trigger immunological reactions, induce oxidative stress, and in rare circumstances, cause endocrine disruption (Bondi et al., 2015; Havelka-Rivard et al., 2022). Stabilizers such as ethanolamines have also been shown to affect aquatic life through bioaccumulation and long-term pollution (Libralato et al., 2010). These findings underscore the necessity for ongoing research to fully understand the environmental implications of proposed F3 formulations, particularly when compared to the developmental and long-term effects of AFFF.

Zebrafish (*Danio rerio*) is increasingly recognized as an exemplary model for chemical safety evaluation, particularly pertinent to developmental exposure effects (Shen and Zuo, 2020). The extrauterine nature of their transparent embryos, coupled with their high fecundity and ease of maintenance, render them ideal for developmental and drug discovery toxicology research (Bambino and Chu, 2017; Cassar et al., 2019). Their genetic, anatomic, and physiological similarities to humans render research using the zebrafish model to hold great translational relevance to human health (Adhish and Manjubala, 2023). Zebrafish also offers high-throughput screening capabilities as well as expedited and comprehensive assessment across a multitude of endpoints. Furthermore, their capacity for high-throughput behavioral analysis adds a subtle dimension to toxicity assessments. Finally, from an ethical standpoint, the use of zebrafish embryos aligns with the 3Rs principles, reducing the reliance on traditional vertebrate models and minimizing potential harm by enabling non-invasive observation of developmental progressions (Clark, 2018).

In this study, we employed a zebrafish-based platform to assess and

compare the safety profiles of current and proposed firefighting foams: A PFAS-containing AFFF (Buckeye Platinum Plus C6 - 3% Mil-Spec) and two proposed F3 formulations (Angus JetFoam 3% and BioEx Ecopol A3+). We evaluated the developmental and behavioral impacts associated with early developmental exposure to these chemicals at dilutions folding from their manufacturer-recommended working concentrations (See Section S1 for details on the tested formulations). The results of our study delineate toxicological concerns associated with F3 formulations across multiple endpoints and underscore the efficacy of assessing the developmental toxicities of formulations using the proposed zebrafish-based platform.

2. Materials and methods

2.1. Zebrafish husbandry

Wild-type zebrafish (*Danio rerio*, AB strain) breeders used in these investigations were maintained in an aquatic recirculating housing system (Iwaki Aquatics, Inc.) following a 14:10 h light–dark cycle and temperature of 28 ± 1 °C, the pH level of 7.2, and conductivity of 500 μS/cm (Newell and Brocca, 2022). Adult fish were fed twice daily with a combination of brine shrimp (Great Lake Artemia nauplii) and groundfish flakes (TetraMin Flakes, Tetra USA). The water quality parameters, including ammonia, nitrate, nitrite, and chlorine levels were continuously checked and maintained within permissible levels. The breeding process was conducted in 1.7 L sloped spawning tanks (Tecniplast) by setting breeders in the afternoon at a male-to-female ratio of 1:3. The embryos were collected 2 h after the commencement of the light cycle in the following morning and maintained in E3 embryo medium until experimentation. The E3 embryo medium was prepared using a 60x stock solution consisting of 34.5 g NaCl, 1.6 g KCl, 5.8g $CaCl_2$–H_2O, and 9.78 g $MgCl_2$–$6H_2O$ dissolved in 2 L of Millipore water and pH adjusted to 7.2. The zebrafish care and experimental work were carried out at Louisiana State University with procedures undergone a thorough review and approval from the Louisiana State University – Institutional Animal Care and Use Committee (IACUC; protocol reference # IACUCAM-22-022).

2.2. Chemical treatments

Stock solutions corresponding to the manufacturer-recommended working concentrations of the chemicals – 3% of Buckeye Platinum Plus C6 - 3% Mil-Spec AFFF, Angus Fire JetFoam, and Ecopol A3+ F3 – were prepared in millipore water without noting any solubility issues. These solutions were then further diluted in pH-adjusted E3 medium (pH = 7.2–7.4) to the target concentrations of 0.1% of the manufacturer-recommended working concentrations and subsequent 5-fold dilutions (0.02%, 0.004%, 0.0008%, 0.00016%) were prepared as concentration gradient using a robotic liquid handling system (OT-2, Opentrons). To facilitate direct chemical exposure, embryos were enzymatically dechorionated at 4 h post fertilization (hpf) using 0.05% pronase (Sigma Aldrich) as previously delineated (Mandrell et al., 2012). The protective outer membrane known as the chorion may function as a barrier, decreasing the effectiveness of chemical penetration. Pores inside the chorion have the capacity to limit the absorption of substances based on their size, as demonstrated with fluorescent dextrans larger than 3 kDa (Henn and Braunbeck, 2011; Pelka et al., 2017). By removing it, we can ensure that the embryos are exposed to our target chemicals in a more precise and regular manner, thereby enhancing the accuracy and rigor of our toxicity evaluations. The dechorionated embryos were then individually plated in 96-square well plastic plates (Whatman) prefilled with the corresponding exposure media (n = 128–160 larvae/treatment). The plates were sealed with parafilm to minimize evaporation, orbitally shaking at 75 rpm until 6 hpf to ensure uniform exposure to the target chemicals, then incubated at 28 ± 0.5 °C. Throughout the exposure period (6–120 hpf), the embryos were maintained in the dark, with intermittent daily exposures to light, to mitigate circadian influences on behavior and limit chemical photodegradation. Previous research demonstrated that zebrafish can acclimatize to low-light environments and undergo regular growth during early developmental stages (Villamizar et al., 2014). Daily media changes (50%) were performed using the OT-2 robotic system to ensure appropriate water quality and continuous exposure to the target chemicals. The control group mortality rate of less than 10% at 24 hpf was established as the criterion for overall batch health, following the Organisation for Economic Co-operation and Development (OECD) guideline number 236 fish embryonic acute toxicity (FET) test acceptability criteria (OECD, 1994). It is also noteworthy that experiments were performed at different times using batches of embryos collected from a minimum of six breeding groups and divided over a number of plates (8–10 plates per experiment).

2.3. Mortality and developmental changes

At 24 and 120 hpf, zebrafish embryos and larvae were subjected to mortality assessments, with death determined by either tissue disintegration or the absence of a heartbeat. Concurrently, at 120 hpf, the larvae were screened for developmental defects, including jaw, eye, pigmentation, swim bladder defects, yolk sac and pericardial edema, body axis deformities, and truncated bodies. To enhance visualization, the larvae were anesthetized using tricaine-s (75 mg/L) and examined under a Stemi 508 stereoscope (Carl Zeiss Inc.) for developmental abnormalities. The detection of any visual malformation in any of the categories emphasized was recorded using a custom-built Microsoft Excel sheet, purposefully created to allow for both the direct graphical display and analytical interpretation of the results.

A subset of the exposed larvae was imaged at 120 hpf using the Stemi 508 stereoscope paired with a camera (AxioCam 208 color, Zeiss). Their overall development was evaluated by measuring their total length – the apex to the caudal tip of the tail – using ImageJ software.

2.4. Behavioral assessments

The overall activity of larval zebrafish (120 hpf) maintained in the dark was evaluated, and then they were exposed to auditory, visual, and peripheral irritant stimuli, and the distances traveled as a stress response to these stimuli were recorded. In brief, zebrafish larvae individually housed in 96-square well plates were moved in the dark to a behavioral tracking chamber equipped with infrared illumination (ZebraBox; ViewPoint Behavior Technology). Toward the end of a 20 min acclimatization period in the dark, the larval baseline activity was assessed. Subsequently, the larvae were sequentially exposed to three different acute stimuli; a visual stimulus (bright light of 1200 lux for 10 min), acoustic stimuli (100 Hz pulses at 100 ms intervals for 1 min), and a peripheral irritant (25 μM Mustard oil; Sigma). In agreement with the product's Material Safety Data Sheet, mustard oil completely dissolves in the embryo media and leaves a noticeable smell. The larvae were allowed a 10 min acclimatization period before the introduction of the next stimuli; a timespan established in previous investigations as adequate for the larvae to revert to baseline activity levels (McAtee and Abdelmoneim, 2024). The temperature during the behavioral assays was consistently regulated at 28 ± 0.5 °C using a temperature regulator and verified with a digital probe thermometer.

The startle responses for acoustic and peripheral stimuli were immediate following the initiation of the stimuli, whereas the response to the visual stimulus was prompted by the cessation of the light stimulus, marking the transition from light to dark. The larval activity was measured as the distance traveled (mm) over 2 s intervals to quantify the acoustic motor response (AMR) and 60 s intervals to quantify the visual (VMR) and peripheral motor responses (PMR). The evaluation of the stimuli-induced behavioral responses involved calculating both peak and total larval activity during the stress response periods. Peak activity

is defined as the utmost distance traveled within a defined interval (2 s for AMR and 1 min for VMR and PMR), recorded over a span of 1 min for acoustic stimuli or 5 min for visual and peripheral stimuli. Total activity represents the summative distance covered across these specified periods.

2.5. Statistical analysis

The data was statistically analyzed using GraphPad Prism 10.1.2 (GraphPad Software Inc.), and the findings were expressed as percentages or as mean ± standard error of the mean (SEM). To compare mortality and developmental defect evaluations against the control groups, we used Chi-square test and two-tailed Fischer's exact test for post-hoc analysis. For overall development and behavioral data, one-way analysis of variance (ANOVA) followed by LSD test for post-hoc analysis were performed. If ANOVA assumptions failed when testing the data for normality using the Shapiro-Wilk test or lognormality using the Kolmogorov-Smirnov test, the Kruskal-Wallis test followed by Dunn's test as post-hoc were employed. Statistical significance was attributed to the differences between treatments and controls with $p < 0.05$.

3. Results

3.1. Mortality and developmental defects

Percent mortalities in larvae were evaluated at both 24 and 120 hpf, revealing no significant increases in mortality in association with Buckeye (PFAS-containing AFFF) exposures, whereas Angus and Ecopol (PFAS-free F3; 0.02% and 0.1%) exposures induced significant increases in mortalities that exceeded 90% ($p < 0.0001$, Fig. 1A, B, and 1C). When we fitted the mortality data in ORIGINPRO®2024b, the 114-h LC_{50} (lethal concentration 50) values for Angus and Ecopol were respectively determined as 0.00629% ± 0.00529% and 0.00557% ± 0.00085%. The LC_{50} for Buckeye could not be calculated, as the observed mortalities did not exceed 6.33%. Additionally, to ensure minimal variability between experimental plates, we analyzed the mortality rate of larval zebrafish at 120 hpf in control groups from all 96-well plates within the same experiment and recorded no statistically significant differences between these groups (Fig. S1).

Buckeye and Angus exposures evaluated did not elicit any significant increases in the array of developmental defects investigated (Fig. 1D). On the contrary, Ecopol (0.004%) induced significant increases in the prevalence of body axis deformities ($p < 0.01$), pigmentation defects (loss of pigmentation, $p < 0.05$), and swim bladder defects (swim bladder deflation, $p < 0.05$). Due to mortalities surpassing 90%, Angus and Ecopol (PFAS-free F3; 0.02% and 0.1%) were excluded from developmental defects investigations and subsequent sublethal endpoint assessments. The remaining Ecopol exposure concentrations examined did not induce any significant increases in the prevalence of developmental defects investigated (Fig. 1D).

3.2. Larval development

The total larval length was measured in a subset of larvae to evaluate change in overall larval development in association with exposures to the target formulations and concentrations (Fig. 2). Exposures to Buckeye (0.02%; $p < 0.01$), Angus (0.004%; $p < 0.01$), and Ecopol (0.004%; $p < 0.05$) significantly reduced larval length. The remaining treatments examined were not significantly altered.

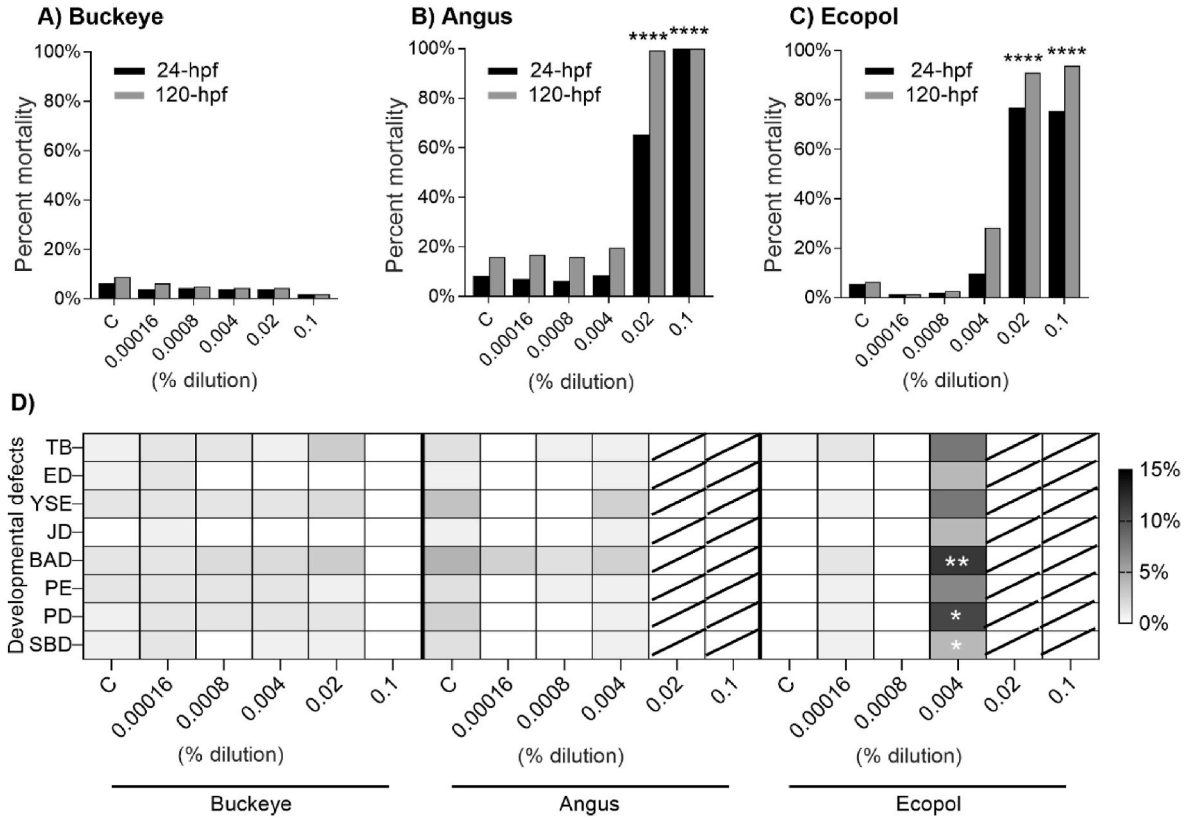

Fig. 1. Represent 24 and 120 hpf mortality of developing zebrafish embryos/larvae following exposures (6–120 hpf) to C (E3 media only), 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of Buckeye (PFAS-containing AFFF, **A**), Angus (PFAS-free F3, **B**), and Ecopol (PFAS-free F3, **C**). **D**) Represent a heat map of the prevalence of developmental defects recorded. Data points represent percent mortality or developmental defects (n = 141–158/treatment). Significant differences from the control group are denoted with asterisks ($p < 0.05$, *; $p < 0.01$, **; and $p < 0.0001$, ****). Abbreviations: hpf, hour post-fertilization; TB, truncated body; ED, eye defect; YSE, yolk sac edema; JD, jaw defect; BAD, body axis deformity; PE, pericardial edema; PD, pigmentation defect; SBD, swim bladder defect.

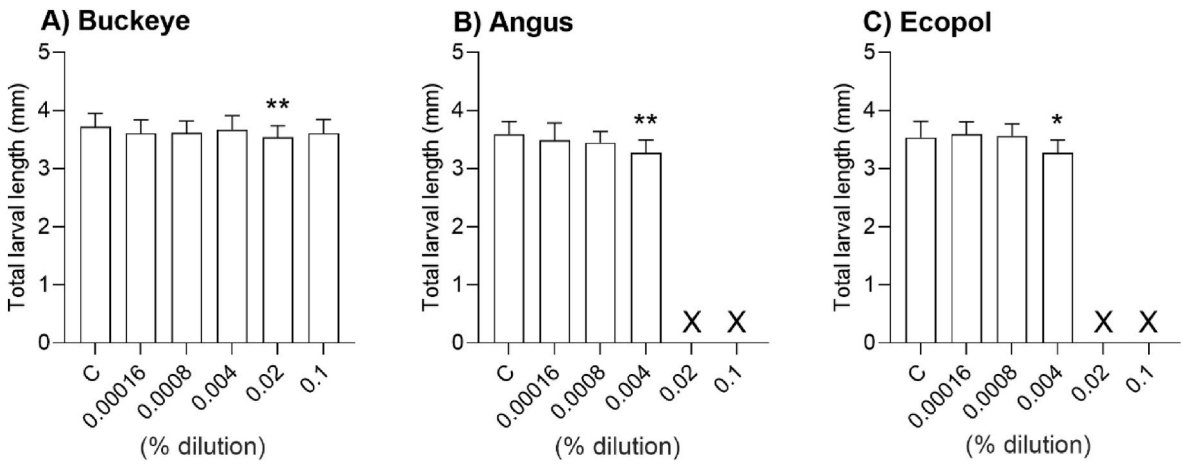

Fig. 2. Total larval length in mm measured at 120 hpf following exposure to C, 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of Buckeye (PFAS-containing AFFF, **A**), Angus (PFAS-free F3, **B**), and Ecopol (PFAS-free F3, **C**). Data points represent mean ± SEM (Sample size per treatment; Buckeye - C = 30; 0.00016% = 31; 0.0008% = 28; 0.004% = 28; 0.02% = 24; and 0.1% = 22, Angus - C = 30%; 0.00016% = 30%; 0.0008% = 29; and 0.004% = 29, and Ecopol - C = 27; 0.00016% = 28; 0.0008% = 33; and 0.004% = 29). Significant differences from the control group are denoted with asterisks ($p < 0.05$, * and $p < 0.01$, **). Abbreviation: hpf, hour post-fertilization.

3.3. Larval activity

When we examined larval activity in the dark and in the absence of intended stimuli, exposure to Angus (0.004%) and Ecopol (0.004%) significantly depressed larval activity ($p < 0.0001$; Fig. 3B and C). The remaining Angus and Ecopol concentrations examined, as well as all Buckeye concentrations examined, did not significantly change larval activity (Fig. 3).

3.4. Behavioral responses to acute stimuli

Larval zebrafish from each treatment group were exposed to three different acute stimuli – visual, acoustic, and peripheral irritant – in order to evaluate their behavioral stress responses. Exposure to the acoustic stimuli, which consisted of sound frequencies of 100 Hz at 100 ms intervals for 1 min, initially triggered a peak of activity (within the first 2 s) that quickly tapered off, indicating habituation (McAtee and Abdelmoneim, 2024). Exposures to Buckeye (0.01%; $p < 0.05$), Angus (0.00016% and 0.004%; $p < 0.05$), and Ecopol (0.0008% and 0.004%; $p < 0.05$) significantly depressed the peak response of larval zebrafish to the acoustic stimuli. The total activity during the larval response to the acoustic stimuli was depressed following larval exposures to Angus (0.004%; $p < 0.0001$) and Ecopol (0.004%; $p < 0.05$). The remaining Buckeye, Angus, and Ecopol exposure concentrations examined did not alter the larval response to the acoustic stimuli (Fig. 4).

The visual stimulus, consisting of bright light at an intensity of 1200 lux for 10 min, elicited a stress response following the cessation of the stimulus that lasted more than 5 min. With the peak response occurring within the initial 5 min interval, our evaluation of this stress response was confined to this duration. During this duration, both peak and post-stimulus total activity were significantly depressed in larvae exposed to Angus and Ecopol (0.004%; $p < 0.01$). No significant changes in larval responses to the visual stimulus were observed in the remaining Angus and Ecopol exposure concentrations, nor any of the Buckeye treatments (Fig. 5).

Exposure to mustard oil at a concentration of 25 μM provoked a peripherally driven motor response, which was distinctly observed within the first 5 min following the application of the irritant. In this

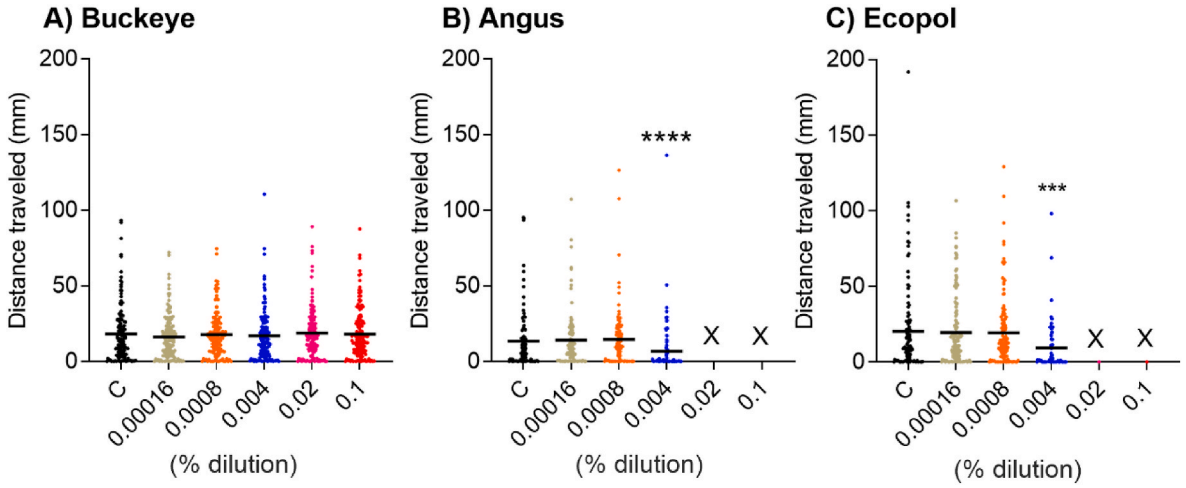

Fig. 3. Baseline activity of 120 hpf larval zebrafish following exposure to C, 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of Buckeye (PFAS-containing AFFF, **A**), Angus (PFAS-free F3, **B**), and Ecopol (PFAS-free F3, **C**). Data points represent individual values and lines represent the mean distance traveled in 1 min (Sample size per treatment Buckeye - C = 143; 0.00016% = 144; 0.0008% = 150; 0.004% = 152; 0.02% = 150; and 0.1% = 151, Angus - C = 148; 0.00016% = 150; 0.0008% = 159; and 0.004% = 148, and Ecopol - C = 128; 0.00016% = 136; 0.0008% = 134; and 0.004% = 61). Significant differences from the control group are denoted with asterisks ($p < 0.001$, *** and $p < 0.0001$, ****). Abbreviation: hpf, hour post-fertilization.

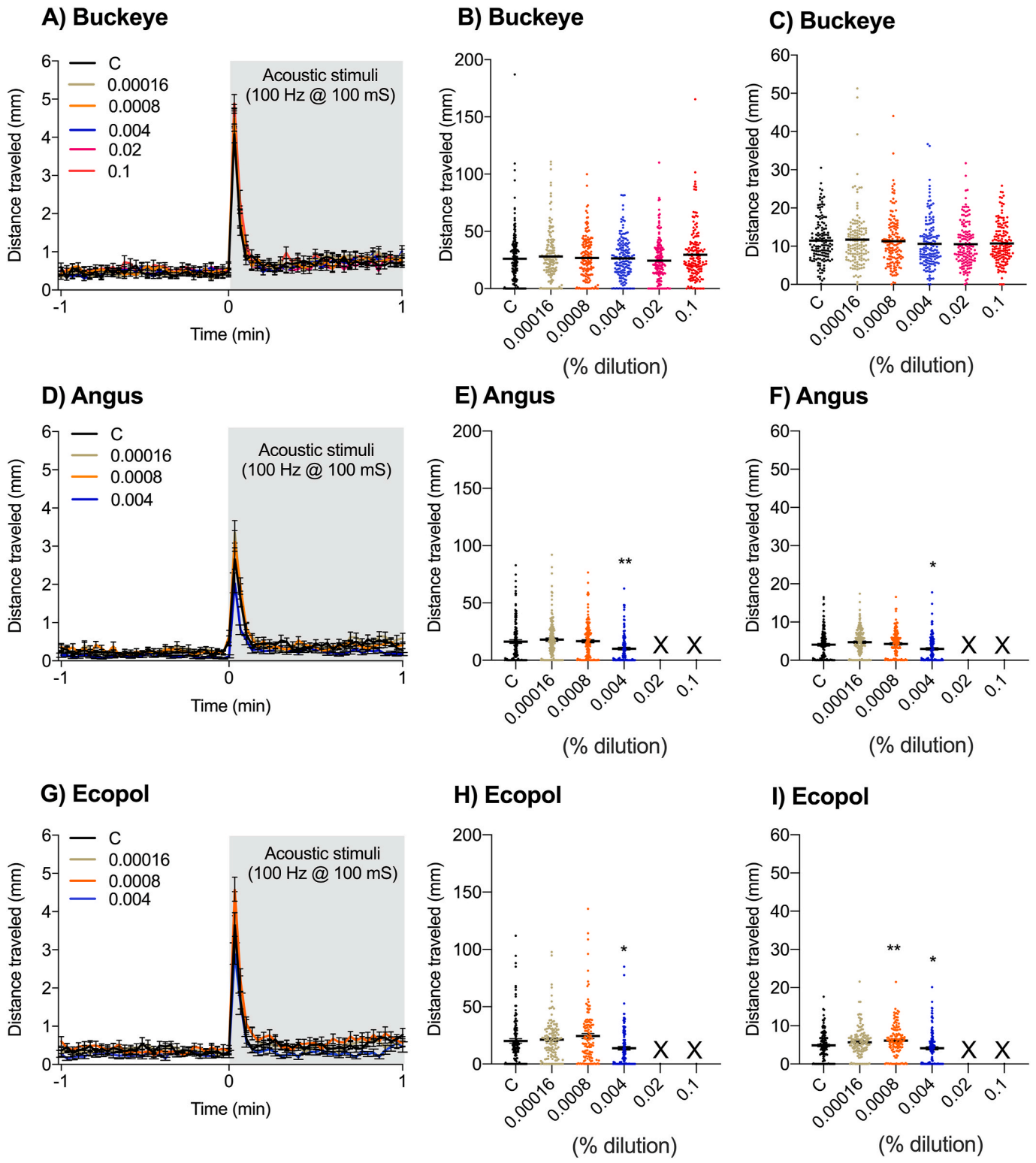

Fig. 4. Larval zebrafish (120-hpf) response to acoustic stimuli (100Hz@ 100 ms intervals for 1-min) following exposure to C, 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of Buckeye (PFAS-containing AFFF, **A, B, C**), Angus (PFAS-free F3, **D, E, F**), and Ecopol (PFAS-free F3, **G, H, I**). Chromatogram demonstrating changes in distance traveled (mm/2 s) before and during larval exposure to acoustic stimuli (**A**, **D**, and **G**). Distance traveled during stimuli (1 min; **B**, **E**, and **H**) and peak activity recorded during the stimuli (**C**, **F**, and **I**) were recorded. Data points represent the mean distance traveled and individual responses (Sample size per treatment for Buckeye - C = 143; 0.00016% = 144; 0.0008% = 150; 0.004% = 152; 0.02% = 150; and 0.1% = 151, Angus - C = 148; 0.00016% = 150; 0.0008% = 159; and 0.004% = 148, and Ecopol - C = 128; 0.00016% = 136; 0.0008% = 134; and 0.004% = 61). Significant differences from the control group are denoted with asterisks ($p < 0.05$, *; $p < 0.01$, **, and $p < 0.0001$, ****). Abbreviations: hpf, hour post-fertilization; mm, millimeter; min, minute; Hz. Hertz; and ms, millisecond.

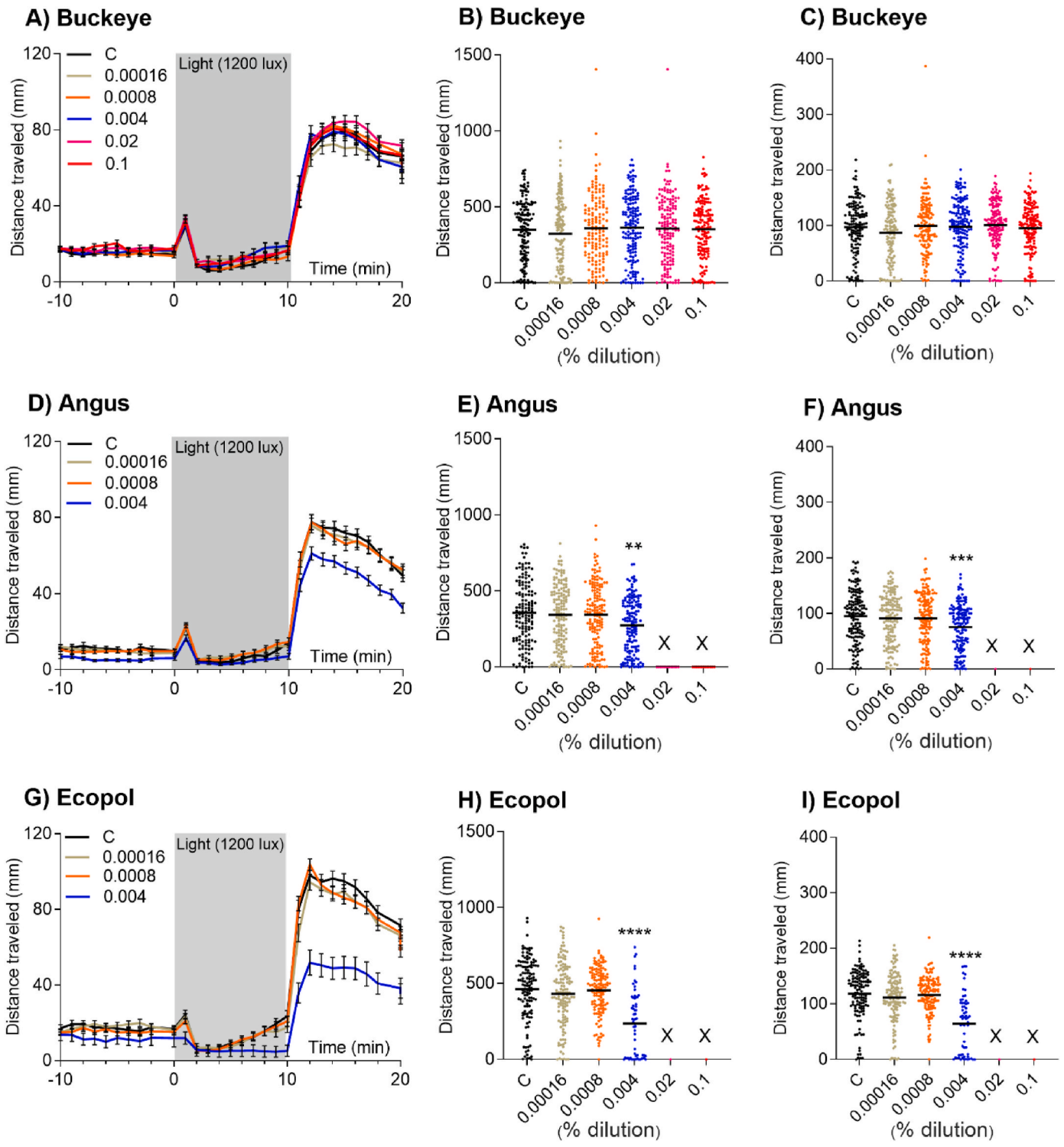

Fig. 5. Larval zebrafish (120 hpf) response to light stimulus (1200 lux for 10 min) following exposure to C, 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of Buckeye (PFAS-containing AFFF, **A, B, C**), Angus (PFAS-free F3, **D, E, F**), and Ecopol (PFAS-free F3, **G, H, I**). Chromatogram demonstrating changes in distance traveled (mm/min) before, during, and after larval exposure to the light stimulus (**A**, **D**, and **G**). Distance traveled post-stimulus (5 min; **B**, **E**, and **H**) and peak activity recorded within the 5 min post-stimulus (**C**, **F**, and **I**) were recorded. Data points represent the mean distance traveled and individual responses (Sample size per treatment for Buckeye - C = 143; 0.00016% = 144; 0.0008% = 150; 0.004% = 152; 0.02% = 150; and 0.1% = 151, Angus - C = 148; 0.00016% = 150; 0.0008% = 159; and 0.004% = 148 per treatment, and Ecopol - C = 128; 0.00016% = 136; 0.0008% = 134; and 0.004% = 61). Significant differences from the control group are denoted with asterisks ($p < 0.01$, **; $p < 0.001$, ***; and $p < 0.0001$, ****). Abbreviations: hpf, hour post-fertilization; mm, millimeter; and min, minute.

time frame, our analyses revealed that Angus at 0.004% showed significant depression in peak activity ($p < 0.001$). Similarly, data showed that larvae exposed to Ecopol (0.004%) showed significant depression in peak and total response to the exposure to the peripheral irritant ($p <$ 0.0001). In contrast, larval responses to the peripheral irritant remained unchanged across the other Ecopol exposure concentrations as well as all tested Buckeye and Angus treatments (Fig. 6).

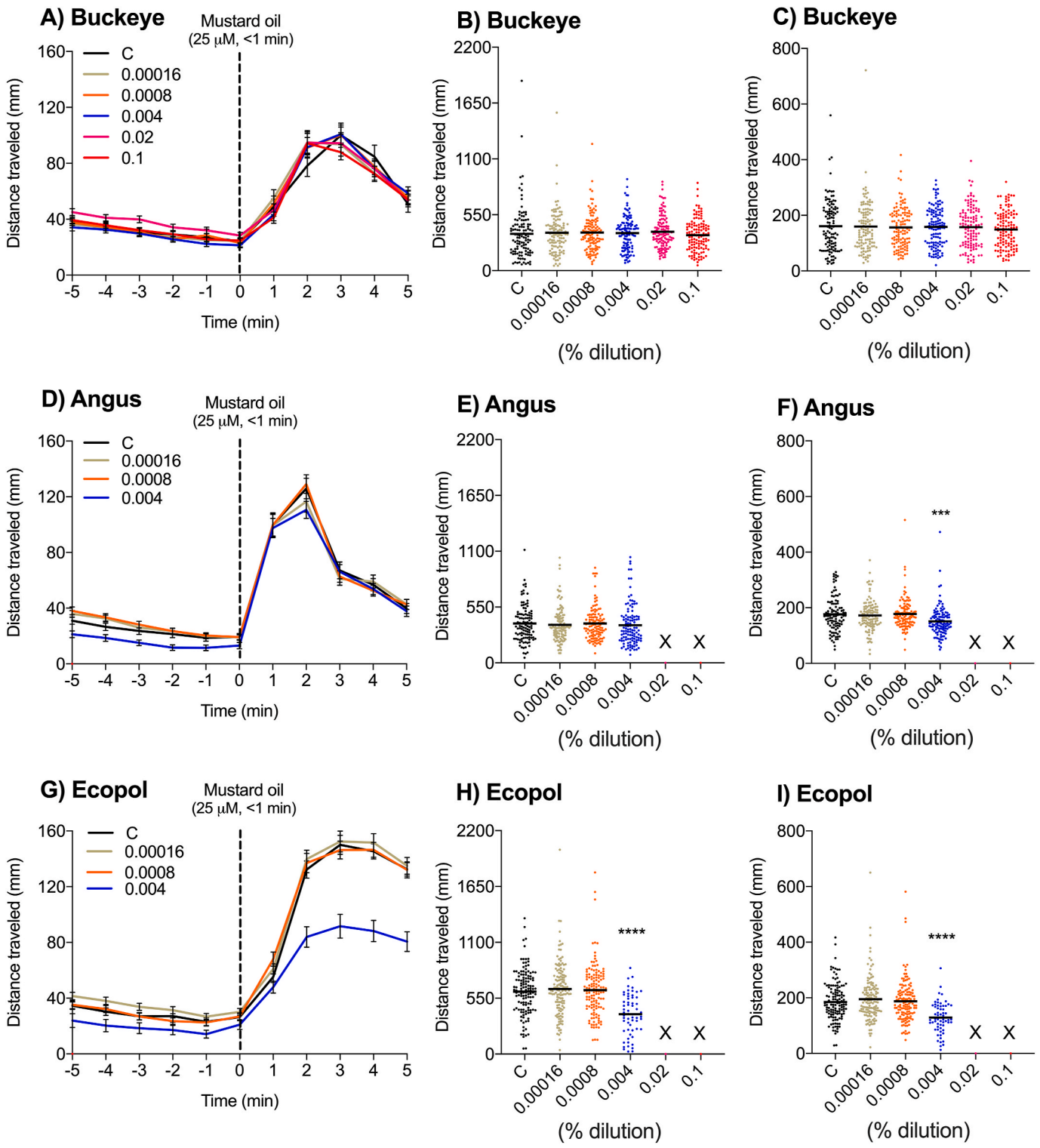

Fig. 6. Larval zebrafish (120 hpf) response to a peripheral irritant (mustard oil 25 μM) following exposure to C, 0.00016%, 0.0008%, 0.004%, 0.02%, and 0.1% of PFAS-containing AFFF (Buckeye; **A, B, C**), Angus (PFAS-free F3, **D, E, F**), and Ecopol (PFAS-free F3, **G, H, I**). Chromatogram demonstrating changes in distance traveled (mm/min) before and after larval exposure to the irritant (**A**, **D**, and **G**). Distance traveled post-stimulus (5 min; **B**, **E**, and **H**) and peak activity recorded within the 5 min post-stimulus (**C**, **F**, and **I**) were recorded. Data points represent the mean distance traveled and individual responses (Sample size per treatment for Buckeye - C = 143; 0.00016% = 144; 0.0008% = 150; 0.004% = 152; 0.02% = 150; and 0.1% = 151, Angus - C = 148; 0.00016% = 150; 0.0008% = 159; and 0.004% = 148, and Ecopol - C = 128; 0.00016% = 136; 0.0008% = 134; and 0.004% = 61). Significant differences from the control group are denoted with asterisks ($p < 0.001$, *** and $p < 0.0001$, ****). Abbreviations: hpf, hour post-fertilization; mm, millimeter; and min, minute.

4. Discussion

The present study contributes to the growing body of research that seeks to investigate the safety profiles of F3 formulations, which are being advanced as replacements for existing PFAS-containing counterparts. We are the first to leverage a zebrafish-based platform to assess the developmental and behavioral toxicities associated with early developmental exposure to F3 formulations. We did this by comparing the toxicological impacts of two proposed F3 formulations (Angus and Ecopol) to a reference PFAS-containing AFFF (Buckeye) through a multifaceted evaluation covering a spectrum of developmental and behavioral endpoints.

In order to have a more realistic insight into the developmental and behavioral toxicities resulting from exposure to the proposed F3 formulations in relation to PFAS-containing AFFF, we targeted assumptive but plausible exposure concentrations starting at 0.1% of the manufacturer-recommended working concentrations of all three AFFF formulations and folding down to 0.00016% of the manufacturer-recommended working concentrations. Other toxicological investigations have investigated different exposure ranges, surpassing our selected range, and evaluated their effects on various aquatic, rodent, avian, and *in vitro* models (Snow et al., 2017; East et al., 2022; Hossain et al., 2022; Jones et al., 2022). To the best of our knowledge, this study represents the first attempt to investigate the developmental and behavioral toxicity of these F3 chemicals using the zebrafish model. A substantial body of evidence demonstrates that toxicological investigations using this model can provide valuable insights into potential environmental and human health risks associated with chemical exposures (Dai et al., 2014).

The first endpoint we evaluated within our developmental investigations was mortality. We observed markedly elevated mortality rates among larvae exposed to Angus and Ecopol (0.1% and 0.02%), marking their 114 h LC_{50}, respectively, at 0.00629% ± 0.00529% and 0.00557% ± 0.00085%. In contrast, there was no significant increase in mortalities among all tested Buckeye concentrations, suggesting higher levels of acute toxicity among the F3 formulations under evaluation. This finding aligns with previous studies that reported increased acute toxicity of F3 formulations compared to PFAS-containing AFFF (Allcorn et al., 2018; Kuperman et al., 2023). In a toxicological investigation using mice, the LD_{50} of Buckeye and Ecopol exceeded 2000 mg/kg/day (East et al., 2022), and the acute LD_{50} for these formulations surpassed 1597 mg/kg in adult quail (Hossain et al., 2022). Another study highlighted a higher acute toxicity of Buckeye (LC_{50}; 3124 mg/L) in contrast to Ecopol (LC_{50}; 4858 mg/kg), when tested in adult white worms (*Enchytraeus crypticus*) (Kuperman et al., 2023). A study evaluated the composition and the acute toxicity of an AFFF formulation obtained from Massachusetts Department of Environmental Protection (MA-DEP) and reported a 96 hpf LC_{50} of 0.000741% in zebrafish. After identifying several components in this AFFF formulation, the researchers assessed their acute toxicities in zebrafish. The mixtures of the two primary PFAS components, PFOS and PFKxS, had an LC_{50} of 29.63 mg/L, with these components present in the formula at a concentration of 2.69 mg/L (Annunziato et al., 2020). Additionally, the acute toxicity of several non-PFAS surfactants was assessed, revealing LC_{50} values of 0.66 mg/L for sodium tetradecyl sulfate (TDS) and 3.67 mg/L for sodium dodecyl sulfate (SDS). The AFFF formula exhibited higher toxicity to zebrafish compared to the individual components, suggesting possible additive or synergistic effects (Annunziato et al., 2020).

Developmentally, among the treatment groups evaluated, only larvae exposed to Ecopol (0.004%) displayed significant increases in the prevalence of several developmental defects, suggesting possible alterations in osmoregulation and endocrine disruption. Edema, a developmental defect recorded in association with Ecopol exposures, is indicative of disrupted osmotic regulation (Sant and Timme-Laragy, 2018). In embryonic zebrafish, the yolk sac exhibits limited solute and water permeability and is highly lipophilic. A synchronized "water barrier" sustains an osmotic gradient with the surrounding aquatic environment (Hill et al., 2004), regulated by the digestive system and gills until the kidney develops into a fully functioning organ. Truncated bodies and body axis deformities observed can signify alterations in overall growth, whether induced by musculoskeletal defects or endocrine disruption (Dubińska-Magiera et al., 2016; Tomak, 2023; Yan et al., 2023). Altered pigmentation is another indicator of potential endocrine disruption. The thyroid hormones (TH) play an essential role in the development of subcutaneous melanocyte precursors (McMenamin et al., 2014; Saunders et al., 2019; Dong et al., 2023). Reduced T4 concentration and alterations in TH-related gene expression have been correlated with reduced eye pigment (Walpita et al., 2009), suggesting that altered pigmentation may serve as an indicator of disruption in thyroid hormone regulation. Swim bladder deflation, a commonly observed developmental endpoint, was recorded in association with Ecopol exposures. Some studies have linked swim bladder deflations with alterations in thyroid regulations as well (Godfrey et al., 2017). Others reported aberrant swim bladder inflation and an inverted tail phenotype in sox2 mutant zebrafish, suggesting a potential link to vestibular dysfunction (Cao et al., 2023). It is, however, important to highlight that larvae exposed to concentrations of each of the three AFFF formulations: Buckeye (0.02%), Ecopol (0.004%), and Angus (0.004%), exhibited shorter body length, suggesting potential developmental effects that may be associated with thyroid disruption. The activity of TH plays a particularly critical role during the early stages of development in fish and amphibians. The thyroid toxicity profiles of zebrafish and humans are quite similar, given the conserved thyroid endocrine system in zebrafish and the production of THs under the regulation of the hypothalamic-pituitary-thyroid (HPT) axis (Raldúa et al., 2012; Tang et al., 2015).

Our investigations also extended to evaluate the effects of exposure to AFFF formulation on behavioral endpoints. Behavioral alterations can be observed at levels of exposure not associated with morphological effects and can have drastic effects on the living quality and possibly the survival of organisms. In these investigations, we observed significant reductions in the activity of larval zebrafish at baseline stress levels following exposures to Angus (0.004%) and Ecopol (0.004%) (Fig. 3). This reduced activity could be a result of impaired larval development (Fig. 2) or a significant contributing factor to stunted growth. Organisms with reduced activity may struggle to obtain sufficient energy to support their growth and survival. However, since the larvae in our investigations were not free-feeding and relied primarily on their yolk sac, the latter theory is unlikely (Sant and Timme-Laragy, 2018).

Additionally, in these investigations, we subjected larvae to two acute sensory stimuli, visual and acoustic, to detect behavioral alterations and segregate centrally driven deficits from deficits relating to specific sensory organs (Deeti et al., 2014; McAtee and Abdelmoneim, 2024). Larval zebrafish exposed to Angus (0.004%) and Ecopol (0.0008% and 0.004%) showed significant alterations in their behavioral responses to the acoustic stimuli, but Buckeye-exposed larvae didn't (Fig. 4). Acoustic stimuli are perceived by hair cells in the inner ear and lateral lines of zebrafish larvae, and the AMR is mediated through the activation of Mauthner cells; prominent reticulospinal neurons situated in the hindbrain recognized for their function in triggering swift escape reactions to stimuli (Tabor et al., 2014; Shimazaki et al., 2019). Longer latency responses involve neuronal populations in the hindbrain, cranial relay neurons, and reticulospinal neurons (Chong and Drapeau, 2007; Koyama et al., 2011; Wu et al., 2021). Investigations also suggest that the non-genomic release of cortisol is involved in increasing Mauthner cell excitability via a post-synaptic effector mechanism. Visual motor responses were also significantly altered in larval zebrafish exposed to Angus (0.004%) and Ecopol (0.004%) but not Buckeye, confirming that the altered stress responses we are observing with F3 exposures are likely due to central deficits over deficits in specific sensory organs. Studies focusing on PFHxA, the second most prevalent PFAS in buckeye, reported alterations to behavioral responses

to visual and acoustic stimuli in larval zebrafish following exposure concentrations exceeding what was adopted in this study (Gaballah et al., 2020; Hamed et al., 2024). Responses to visual stimuli rely heavily on the hypothalamic-pituitary-adrenal axis, as demonstrated by rising cortisol levels and disruptions caused by glucocorticoid receptor modulations (Lee et al., 2019). We deemed it acceptable to expose zebrafish larvae to bright light of 1200 lux intensity as an acute stimulus as studies found no significant change in zebrafish photomotor response to different light intensities ranging between 335 and 3500 lux (Hill et al., 2022; Keever et al., 2023). Studies investigating light-induced damage to the retina usually employ far greater levels of light intensity, ranging between 13,000 lux and20,000 lux, in order to detect any harmful impacts on the retina and overall visual function (Kawase et al., 2016; Rosa et al., 2023).

The use of peripheral irritants excludes the possibility that depressed behavioral responses observed following chemical exposures are due to locomotor impairments (Fig. 6H and I). These irritants are recognized by transient receptor potential ankyrin 1 (TRPA1) ion channel receptors on sensory neurons that innervate skin cells and elicit a peripherally-driven rapid escape response through the spinal reflex arc (Jordt et al., 2004). The selective targeting of nociceptors by peripheral irritants such as mustard oil allows for a more focused understanding of pain perception and response processes compared to touch responses, which involve several types of sensory neurons and pathways (Dubin and Patapoutian, 2010; Rostock et al., 2018). Exposure to Buckeye was not associated with any changes in baseline activity or with behavioral alterations, whether elicited by acute sensory stimuli or peripheral irritants. Both PFAS-free F3 that are Angus and Ecopol at 0.004% showed depressed baseline activity and depressed responses to visual and acoustic stimuli but were also associated with significant depression in peripheral motor responses to the exposure to the peripheral irritant. These findings suggest that the behavioral defects we observed are associated, at least in part, with a locomotor deficit where the larvae are unable to move freely regardless of the integrity of their central nervous system and stress circuitry. The lack of significant alterations in response to the larval exposure to the peripheral irritant suggests the lack of locomotor deficit and that the depression is likely to be driven by disruption in the central circuitry regulating behavioral responses (Emran et al., 2008; Basnet et al., 2019; Albers et al., 2024; Hamed et al., 2024).

5. Conclusion

In conclusion, this study employed a zebrafish-based platform to assess and compare the safety profiles of three AFFFs; a PFAS-containing AFFF (Buckeye) and two proposed F3 formulations (Angus and Ecopol). We reported various developmental and behavioral impacts associated with early developmental exposure to these chemicals at dilutions folding from their manufacturer-recommended working concentrations and highlighted the higher acute and behavioral toxicities of the proposed F3 formulations. The results of our study delineate toxicological concerns associated with proposed F3 formulations across multiple endpoints, highlight the need for comprehensive toxicological assessments in order to develop safer alternatives, and underscore the efficacy of our platform in determining the developmental toxicities of F3 formulations.

Disclaimer

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CRediT authorship contribution statement

Kamal Niaz: Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Demetrius McAtee:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Pranup Adhikari:** Methodology, Data curation. **Patrik Rollefson:** Methodology, Data curation. **Mohamed Ateia:** Writing – review & editing, Visualization, Validation, Supervision, Data curation. **Ahmed Abdelmoneim:** Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.143361.

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