Title

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Publication Date

2022-05-01

DOI

10.1016/j.vascn.2022.107173

Peer reviewed

Journal of Pharmacological and Toxicological Methods 115 (2022) 107173

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Diisopropylfluorophosphate (DFP) volatizes and cross-contaminates wells in a common 96-well plate format used in zebrafish larvae toxicology studies

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ARTICLE INFO

Keywords:
Acetylcholinesterase
Diisopropylfluorophosphate
Organophosphate
96-well plates
Zebrafish

ABSTRACT

Diisopropylfluorophosphate (DFP) is an organophosphate (OP) that is commonly used as a surrogate of OP nerve agents to study the neurotoxic effects of acute OP intoxication. In preliminary studies, we discovered abnormally high incidence of deaths in DMSO control zebrafish larvae housed in the same 96-well plate as DFP-exposed larvae and hypothesized that DFP volatilizes and cross-contaminates wells when using static waterborne exposures. Survivability and acetylcholinesterase activity assays were indicative of the presence of DFP in the tissues of zebrafish ostensibly exposed to DMSO only. These findings are consistent with DFP cross-contamination, which raises concerns for the experimental design of studies evaluating the toxicity of volatile and semi-volatile substances in zebrafish using medium-to-high throughput approaches.

1. Introduction

Organophosphate (OPs) cholinesterase inhibitors represent a class of neurotoxic compounds used extensively as insecticides around the world (DiBartolomeis, Kegley, Mineau, Radford, & Klein, 2019; Kumar, Kaushik, & Villarreal-Chiu, 2016). Self-poisonings with OP pesticides are estimated to occur at a rate of approximately 200,000 per year in developing countries (Eddleston, Buckley, Eyer, & Dawson, 2008; Gunnell, Eddleston, Phillips, & Konradsen, 2007), and OPs weaponized as chemical threat agents have been used in wars and acts of terrorism (Haley, 2018; Vogel, 2013). Non-fatal outcomes of acute OP poisonings can be severe and long-lasting, and include the development of epilepsy and impaired learning and memory in humans (Chen, 2012; Jett et al., 2020) and experimental rat (González et al., 2020; Guignet et al., 2020; Hobson et al., 2019) and mouse (Calsbeek et al., 2021) models. Thus, there is great interest in characterizing and understanding OP neurotoxicity to identify novel, mechanistically relevant therapeutic targets for mitigating adverse effects.

Diisopropylfluorophosphate (DFP) is widely used to study acute OP intoxication in the laboratory (Reddy, Zaayman, Kuruba, & Wu, 2021). The primary mechanism of action (MOA) of DFP and most other OPs is acetylcholinesterase (AChE) inhibition, which leads to the accumulation of acetylcholine in cholinergic synapses within the central and peripheral nervous systems (Hulse, Davies, Simpson, Sciuto, & Eddleston, 2014). Due to challenges of analyzing the highly reactive and short-lived DFP, AChE inhibition is often used as a surrogate marker to determine whether DFP has effectively penetrated target tissue and engaged its molecular target (Ferchmin et al., 2014; González et al., 2020).

The neurotoxic effects of DFP have been investigated in zebrafish (ZF), a common teleost model, via waterborne exposure (Brenet et al., 2020) and intraperitoneal injection (Faria et al., 2018). Zebrafish are increasingly used for medium-to-high-throughput toxicological assays (Cassar et al., 2020; Tal, Yaghoobi, & Lein, 2020). Static waterborne exposures of ZF larvae in 96-well plates is used extensively, particularly when screening large chemical libraries or phenotyping mutant models (Colón-Rodríguez et al., 2020; Dach et al., 2019; Tal et al., 2020; Truong et al., 2020). Microinjection is a possible alternative to aqueous exposure, but it is more time-consuming and not as amenable to medium-to-high throughput screening (Schubert, Keddig, Hanel, & Kammann, 2014). Alternatively, efficient plate sealers could allow exposure in 96-well plates; however, these are not amenable to the collection of some endpoints. For example, filming behavioral assays, which is a common endpoint in studies of ZF neurotoxicity, would be particularly difficult in plates with plate sealers as the camera is typically positioned above the plate.

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https://doi.org/10.1016/j.vascn.2022.107173
Received 9 November 2021; Received in revised form 23 March 2022; Accepted 1 May 2022
Available online 8 May 2022

Prompted by the discovery of abnormal patterns of death in vehicle controls in preliminary studies of DFP using ZF larvae exposed via static waterborne exposure, we assessed the possibility that DFP volatilizes and cross-contaminates wells in a 96-well plate format. This possibility was supported by the log Kow of 1.13, vapor pressure of 0.579 mmHg at 20 °C, and low boiling point of 62 °C at 9 mmHg for DFP (National Center for Biotechnology, 2021). Considering that the concentration of a dissolved gas is directly proportional to the partial pressure of that gas above the liquid (Henry's law), a compound with a positive log Kow and a low vapor pressure can be predicted to have volatile properties. To test this hypothesis, we conducted survivability assays and measured AChE specific activity in ZF exposed to DMSO and DFP on the same plates in comparison to larvae exposed to DMSO only on separate plates lacking DFP-exposed larvae.

2. Materials and methods

2.1. Zebrafish husbandry

Fish husbandry, spawning, and experiments were performed with the approval of the University of California, Davis (UC Davis), Institutional Animal Care and Use Committee. All experiments complied with the NIH Guide for the Care and Use of Laboratory Animals (National Research Council (US), 2011) and ARRIVE guidelines (du Sert et al., 2020). Fish husbandry and spawning were conducted according to previously described methods (Mundy et al., 2021). Tropical 5D wild type ZF were obtained from Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University, Corvallis, OR, and subsequent generations were raised at UC Davis. The Tropical 5D wild type ZF line is generally considered an appropriate choice of wild type strain for chemical stressor experiments, considering its status as a heterogenous population is supported by the finding of significantly more variants compared to other strain populations via whole genome sequencing (Balik-Meisner, Truong, Scholl, Tanguay, & Reif, 2018). Spawning was conducted overnight in false bottom chambers, with embryos subsequently collected and transferred to plastic petri dishes containing embryo medium (EM) (pH 7.2–7.3) (Westerfield & ZFIN., 2000). All experiments were conducted using 5 days postfertilization (dpf) larvae, and each experiment included replicates using embryos obtained from at least three separate spawning events.

2.2. Chemical source information

DFP was purchased from Sigma-Aldrich (St. Louis, MO, USA) (~90 ± 7% purity), and stored as neat stock in −80 °C. DFP was dissolved in either 100% DMSO (Sigma) or embryo media to create a 1000× stock the day of use. Further dilution to 2× sub-stocks was conducted using embryo medium immediately before exposure. Vehicle was 0.1% DMSO for all exposure paradigms except the paradigm specifically including no vehicle carrier.

2.3. Survivability assays

Zebrafish larvae were raised in petri dishes until 4 dpf. At 4 dpf, individual larvae were randomly chosen and transferred into a single well of a 12 × 8 96-well plate (Falcon® polystyrene treated plates, REF 353075, Corning Inc., Corning, NY) containing 50 μL EM, covered with Parafilm® between the wells and the provided lid to limit evaporation, and allowed to acclimate overnight in a 29 °C incubator. For experiments specifically testing non-plasma treated plates, Costar polystyrene non-treated plates (REF 3370, Costar, Kennebunk, ME) were used. At 5 dpf, larvae were exposed to DFP or vehicle via the addition of 50 μL of 2× sub-stock in embryo media. The plates were covered with Parafilm® and placed in 29 °C incubator.

For all survivability assays except the control plates and randomized experiment, larvae were exposed to vehicle (0.1% DMSO) or varying concentrations of DFP (0.01, 0.03, 0.1, 0.3, or 1 mM) in the first six columns of the plate and repeated in columns 7–12 (Fig. 1A and S1). In the randomized experiment, larvae were exposed to vehicle or DFP (0.01, 0.03, 0.1, 0.3, or 1 mM) in randomized groups of four across the entire plate (Fig. S2). Control fish in a separate plate were exposed to DMSO-only or one concentration of DFP in two columns of the plate only (e.g., DMSO-only in column 1 and 7, 0.01 mM DFP in columns 2 and 8, etc.) (Fig. 1A).

At 6 h and 24 h, larvae were observed under a light microscope. Deceased larvae were counted and their position on the plate recorded. The conditions tested in the survivability assays included 1) increasing DFP concentrations across wells, 2) increasing DFP concentrations across wells using a brand of 96-well plate that is not treated with plasma-gas (Costar), 3) increasing DFP concentrations across wells using embryo media only as a carrier for DFP, and 4) randomizing the exposures across the plate. Larvae lacking a heartbeat were considered dead. Each experiment was conducted in triplicate ($n = 3$ separate plates) using 16 larvae per exposure group ($n = 48$ larvae per experimental group).

2.4. Acetylcholinesterase (AChE) activity assay

Larvae were exposed according to the exposure paradigm described above ("Survivability assays" section). Separate plates of control fish were exposed to vehicle in columns 1 and 7. After 1 h of exposure, larvae were removed from the wells and processed in pools of two, such that each column provided four samples of the same exposure group. In total, 56 samples were collected from each exposure plate, and 8 samples from each control plate (Fig. 2A). Exposure time of 1 h was chosen because 1) no significant amount of death was observed at this timepoint, and 2) it is within the reasonable range of a typical behavioral assay performed after acute exposure (i.e., 40 min photomotor assay performed after acute exposure (Mundy et al., 2021)). The experiment was done in triplicate (n = 3 larval pairs per spatial position).

The AChE activity assay was conducted as previously described in (Yang et al., 2011).

The larvae were rinsed with DI water, euthanized on ice, and then homogenized in 120 μL of cold 1× phosphate-buffered saline (PBS) containing 1% TritonX-100 using a handheld homogenizer with disposable pestle. Lysates were snap-frozen in liquid nitrogen and stored at −80 °C until use. Lysates were thawed and spun at 12,000 X g for 1 min. The supernatant was added to a 96-well plate in triplicate, and AChE activity was measured using the standard Ellman assay (Ellman, Courtney, Andrew, & Featherstone, 1961) with 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) and acetylthiocholine iodide (ASChI) as substrates (Sigma-Aldrich). All plates contained three blanks with DTNB. Plates were equilibrated at room temperature for 5 min, and the reaction was initiated by the addition of ASChI (final concentration of 5% *v*/v). Absorbance at 405 nm was measured every two minutes for 30 min at a constant temperature of 37 °C on a Synergy H1 hybrid reader (BioTek, Winooski, VT). The average change in absorbance (ΔOD) per min was collected. AChE activity was normalized to total protein concentration of each sample as determined by a BCA assay (Pierce, Rockford, IL). The specific activity (μM substrate formed/min/mg protein) was calculated as follows:

$$\text{Specific activity} = ((\text{Avg } \Delta\text{OD}/min \text{ sample} – \text{Avg OD}/min \text{ blank})*\text{X})$$

where X is equal to:

$$\left[0.014\ (\text{extinction coefficient}, \mu\text{M}^{-1}\ \text{cm}^{-1})*0.01\ (\text{sample volume}, \text{mL})\right]$$

2.5. Statistical analysis

2.5.1. Survivability assays

To specifically measure the effect of exposing larvae to DMSO or DFP

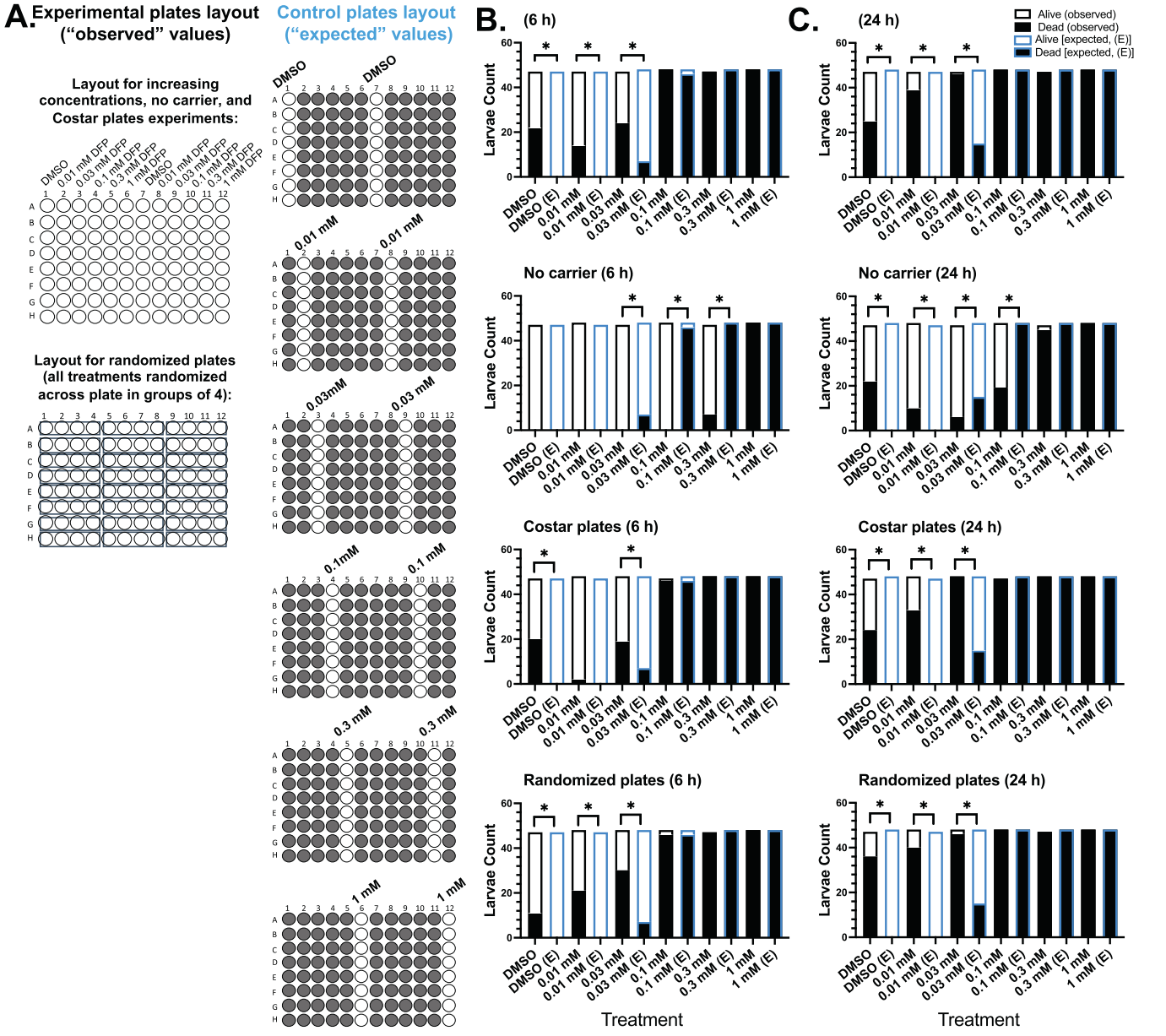

Fig. 1. Survivability of zebrafish larvae exposed to DFP. A) Diagrams of positional experimental design for experimental plates and control plates. All plates (experimental and control) were completed in triplicate. Grey circles represent wells not used. B) Mortality measured at 6 h of exposure. C) Mortality measured at 24 h of exposure. $n = 47$–48 per group. * $p < 0.05$ in Fischer's exact-test, in comparison to expected values for each group.

in the same plate (referred to as the "experimental plate") in comparison to using separate plates for each exposure (the "control" plates), the total number of dead larvae in the control plates (plates that contained only one exposure concentration) were considered the "expected" values (Fig. 1A). Each "observed" value (the total number of dead larvae in the experimental plates) was compared to the corresponding concentration of DMSO or DFP on the control plates (the "expected" values) using two-sided Fischer's exact test ($\alpha < 0.05$). All experimental plates including visual representation of spatial position of death at 6 and 24 h are shown in Figs. S1 and S2. The number of surviving larvae at 6 h and 24 h for control and experimental plates is shown in Fig. S3, and the percentage of survival over time for control and experimental plates is shown in Fig. S4. Kaplan-Meier survival analysis was conducted across exposure concentrations within each plating experiment. Results of Log-Rank (Mantel-Cox) test are shown in Table S1, reporting *p*-values of multiple comparison tests adjusted using the Bonferroni method. All survivability assay statistics were completed using GraphPad Prism (Version 9.1.0).

2.5.2. AChE activity assay

To ensure the control plates did not have spatial differences in AChE activity, a non-parametric Kruskal Wallis ANOVA and post-hoc Dunn's pairwise comparison were completed to compare each spatial position with the other. Because no significant differences were found (Fig. S5), the values from all control samples ($n = 24$) were combined and used as the control for comparison to each position on the exposed plates. Each spatial position ($n = 3$) was compared to the control samples in non-parametric Kruskal Wallis ANOVA and post-hoc Dunnett's multiple *t*-test ($\alpha < 0.05$). AChE activity assay statistics were completed using R (version 4.0.3) (R Core Team, 2021), and packages rstatix (Kassambara, 2021) and DescTools (Signorell, 2021). Code for analysis and graphing is available at https://github.com/insideafish. Raw data is available upon

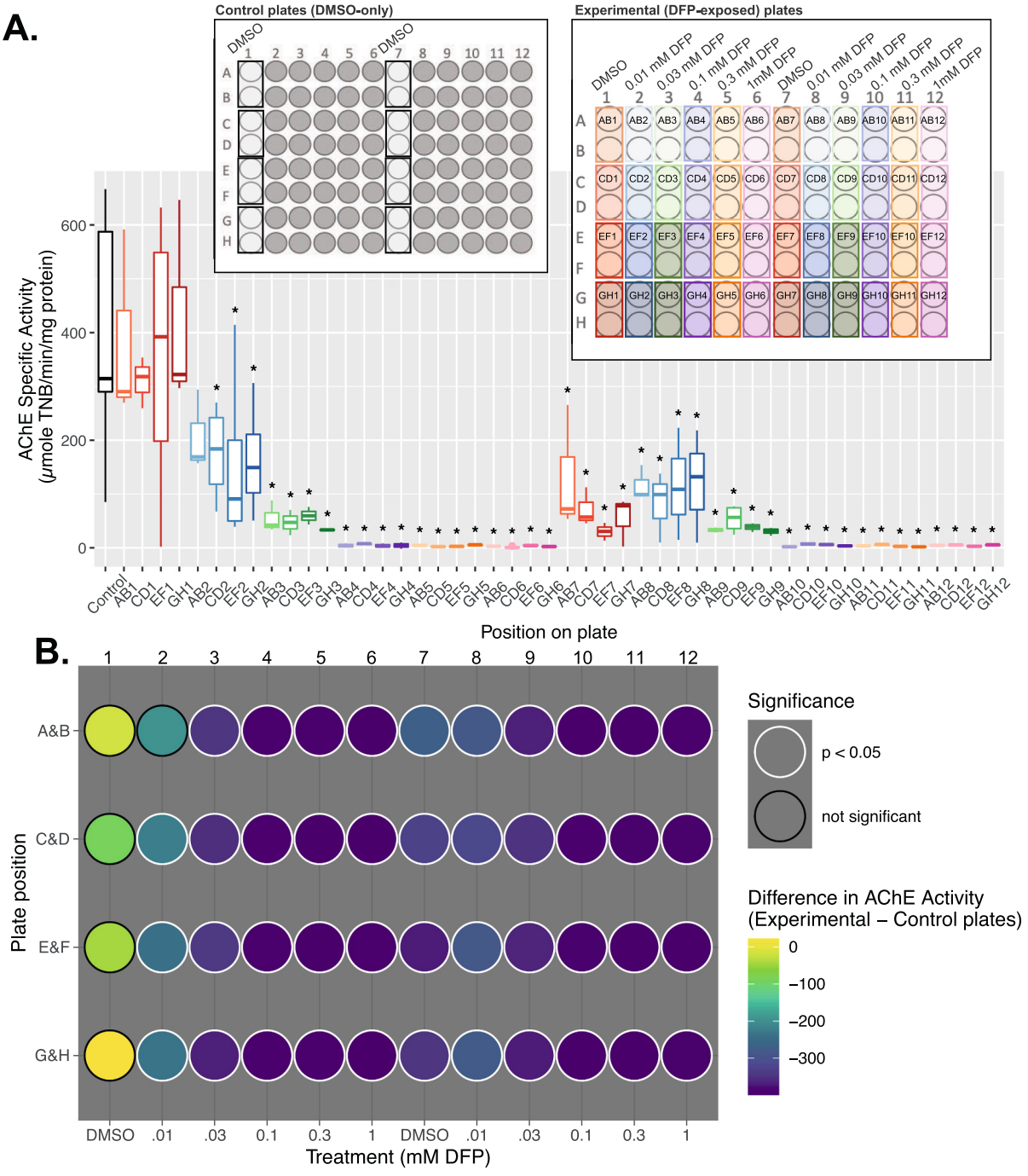

Fig. 2. Acetylcholinesterase activity in zebrafish larval tissue. A) Boxplot represents AChE specific activity. Schematics show positional exposure paradigm. Boxes within the schematics represent pairs of larvae that were pooled for AChE activity assay, and labels within the boxes correspond to the labels on the x axis of the boxplot. $n = 3$, * $p < 0.05$ in Dunnett's multiple t-test in comparison to control. B) Heatmap shows the difference of AChE activity in exposed plates subtracted from control plates. $p < 0.05$ in Dunnett's multiple t-test in comparison to control ($n = 3$).

request.

3. Results

3.1. Survivability assays

To investigate whether the cause of death in the vehicle control ZF observed in preliminary studies was due to DFP cross-contamination, we first conducted survivability assays. The conditions tested in the survivability assays were 1) increasing DFP concentrations across wells, 2) increasing DFP concentrations across wells using a brand of 96-well plate ot treated with plasma-gas (Costar brand), 3) increasing DFP concentrations across wells using embryo media only as a carrier for DFP, and 4) randomizing the exposure groups across the plate.

Larvae exposed to increasing concentrations of DFP on the same plate exhibited significantly more death in the DMSO, 0.01 mM, and 0.03 mM DFP exposed wells in comparison to control at 6 h and 24 h of exposure (Fig. 1B, C). Controls in this experiment are the "expected" rate of death of larvae exposed to the corresponding concentration of DFP on a separate control plate. When the same experiment was conducted with

no carrier (using DFP that was not prepared in DMSO), the larvae on the control plates (separated exposures) exhibited significantly more death at 6 h of exposure to 0.03, 0.1, and 0.3 mM DFP (Fig. 1B). At 24 h of exposure, the larvae exposed on the same plate exhibited significantly more death compared to control plates in the vehicle and 0.01 mM DFP exposed ZF (Fig. 1C). When using a different brand of plate (Costar), larvae exposed to increasing concentrations of DFP on the same plate exhibited significantly more death in vehicle DMSO and 0.03 mM DFP exposed wells at 6 h, and vehicle, 0.01, and 0.03 mM DFP exposed wells at 24 h in comparison to control plates (Fig. 1B, C). Finally, when larvae were exposed to randomized concentrations of DFP on the same plate, significantly more death was observed in the vehicle, 0.01 mM, and 0.03 mM DFP wells at 6 h and 24 h of exposure in comparison to control plates (Fig. 1B, C).

3.2. AChE activity assays

To evaluate whether ZF were exposed to DFP, AChE activity was measured in ZF tissue in experimental plates in which larvae were exposed to vehicle and increasing concentrations of DFP on the same plate for 1 h in comparison to separate control plates containing vehicle-only exposed larvae. Each position on the experimental plate was compared to the control values. AChE activity was found to be significantly decreased in all wells except the first column of vehicle control larvae and the top two wells of 0.01 mM DFP-exposed larvae. Most notably, all vehicle control larvae in column 7 (in the middle of the plate) exhibited significantly decreased AChE activity.

4. Discussion

Zebrafish are a powerful tool for toxicologic and pharmacologic screening (Tal et al., 2020). For most medium-to-high throughput assays that use ZF larvae, animals are exposed via static waterborne exposures. Here, based on preliminary studies of higher-than-expected deaths of vehicle control larvae plated in the same 96-well plate as DFP-exposed larvae, we investigated whether the common 96-well plate exposure method is appropriate for testing larval ZF exposure to semi-volatile chemicals, such as DFP.

In the survivability assays, the most notable observation was the consistent and significant death of the vehicle control larvae on experimental plates that also contained wells of ZF exposed to increasing concentrations of DFP. Further, the vehicle control larvae on the experimental plates spatially positioned in the middle of the plate showed more and earlier death compared to vehicle control larvae in the leftmost column (Fig. S1) that were furthest away from the DFP containing wells.

To test whether the pretreatment (plasma gas coating) of the polystyrene Falcon Corning plates affected survivability, we repeated the experiment using non-treated polystyrene Costar plates. Polystyrene tissue culture plates are typically pretreated with plasma gas to deposit a negative charge at the bottom of the wells to enhance cell attachment. While we are not aware of any literature suggesting that the pretreatment of culture plates with plasma gas has negative impacts on ZF larvae survival, we tested whether the pretreatment of polystyrene Falcon Corning plates is a confounding factor by repeating the experiment with non-treated polystyrene 96-well plates (Costar brand). Because the results were almost identical to the Falcon Corning plates (Fig. 1A, B), we concluded that plasma gas pretreatment of the 96-well plate is not an important factor in whether DFP volatilizes and cross-contaminates wells.

In order to test whether exposure position on the plate had an effect on death, we plated vehicle and DFP-exposed larvae in a randomized fashion and found that location in the plate did not influence the results. These results indicate that the spatial positioning on the plate alone does not influence the rate of death. Rather, the proximity to high concentrations of DFP appears to influence the incidence of death. The exact directional movement of DFP when volatilizing and depositing into wells is unknown as we did not perform experiments to test where exactly DFP travels. However, survivability analysis suggests that all wells on the plate were affected by the volatilization of DFP, not just the wells ostensibly exposed to DMSO (Fig. S4, Table S1).

Interestingly, the larvae exposed to DFP without carrier (DFP was dissolved in embryo media directly without DMSO) exhibited significantly lower death than larvae exposed on separate plates to DFP dissolved in DMSO. These results suggest that (1) DMSO increases the volatilization of DFP, (2) DFP may not be fully soluble in embryo media and coming out of solution, and/or (3) the presence of DMSO facilitates better delivery to ZF tissues. Distinguishing between these possibilities would require analysis of DFP levels in ZF tissues, but this is impractical given the short biological half-life of DFP (National Center for Biotechnology, 2021). Rather, DFP exposure was confirmed by analyzing AChE activity in ZF, which confirmed that vehicle control larvae had significant AChE activity inhibition. While DMSO is a highly soluble and non-volatile compound with a log Kow of −1.35 and a boiling point of 189 °C (National Center for Biotechnology, 2021), it is known to be reduced to dimethyl sulfide (DMS) (a volatile organic solvent) in mammalian tissues (Li et al., 2016). Although further investigation would be needed, this could explain an increase in volatility of a substance such as DFP when combined with DMSO.

Collectively, our observations suggest that DFP volatilizes and cross-contaminates wells within the same 96-well plate. Temperature and vehicle may be two factors that play an important role in the volatility of DFP. Considering the design of all experiments presented here, it is important to note that all tests were conducted at 29 °C, and all experiments (excluding the tests specifically using no carrier) used DMSO as the vehicle carrier. It may be worth exploring the possibility of exposing ZF to DFP using the 96-well plate method under different conditions (i.e., using a lower temperature or different vehicle such as methanol or acetone) in order to maintain the high-throughput ability of the assay while minimizing cross contamination.

In summary, our data support the hypothesis that DFP volatilizes and cross-contaminates wells when placed in embryo media in a 96-well plate with lid and Parafilm® at 29 °C using DMSO as a vehicle. These observations serve to inform other investigators of the possibility of cross-contamination with compounds of similar chemical properties to DFP when using waterborne exposures of ZF larvae in multi-well plate formats. Our recommendation is that when using static waterborne exposure of ZF larvae to semi-volatile and volatile reagents, experimenters consider separating exposure groups so that each exposure group is in a separate multi-well plate as opposed to designing all exposure conditions on one plate.

Credit author statement

P.C. Mundy: Conceptualization, Methodology, Data Curation, Investigation, Writing-Original Draft, Visualization; Supervision; R. Mendieta: Investigation; P.J. Lein: Conceptualization, Writing-Review & Editing, Project Administration, Funding Acquisition.

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.

Acknowledgments

We gratefully acknowledge Donald Bruun (UC Davis) and Cris Grodzki (UC Davis) for consulting on the acetylcholinesterase inhibition assays. We also thank Dr. Suzette Smiley-Jewell (UC Davis CounterACT Center) and Dr. Heike Wulff (UC Davis Dept. of Pharmacology) for reviewing and editing this manuscript. This work was supported by the

National Institute of Neurological Disorders and Stroke CounterACT program [grant number U54 NS079202 to PJL]. The sponsors were not involved in the study design, the collection, analysis, and interpretation of data, in the writing of the report or in the decision to submit the paper for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vascn.2022.107173.

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