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Human Exposure and Developmental Effects of Organophosphate Esters

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All supplementary materials reported in this dissertation are available through ProQuest Dissertations & Theses.

Dedication

I would like to dedicate this dissertation to my mother, Malavika Bayanagari, my father Raghuram Reddiam, and my brother, Akarsh Reddiam. Thank you for being my safe harbor during all the storms; everything I am is because of you. I would also like to dedicate this dissertation to Amala Neelamraju for bringing so much light into my life.

ABSTRACT OF THE DISSERTATION

Human Exposure and Developmental Effects of Organophosphate Esters

by

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Doctor of Philosophy, Graduate Program in Environmental Toxicology
University of California, Riverside, March 2021
Dr. David C. Volz, Chairperson

Organophosphate esters (OPEs) are a class of semi-volatile organic compounds that are used as flame retardants and plasticizers. Due to their ubiquitous use and ability to migrate out of end-use products, elevated concentrations of OPEs have been detected within indoor dust, a prevalent source for human exposure. Similar to the built environment, organophosphate esters are introduced into vehicles as plasticizers and flame retardants and have the potential to migrate and accumulate within vehicle dust. While human exposure to OPEs via indoor dust is well characterized, more information is needed to address the extent of OPE exposure and subsequent risk with time spent in vehicles and understand mechanisms underlying OPE-specific toxicity during early development. Therefore, the primary objectives of this dissertation are to 1) increase our understanding of the mechanisms of developmental toxicity for triphenyl phosphate (TPHP), a high-production volume OPE, 2) measure human exposure to OPEs within a population that spends a significant amount of time in personal transportation, and 3) calculate the risk associated with ingestion of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP), another high-production volume OPE, within car interiors. For Aim 1, using whole-embryo exposures and a combination of mRNA-sequencing, phenotypic assessments, neutral lipid staining, and metabolomics, TPHP was shown to result in adverse effects on the

liver, lipid abundance, and osmoregulation within the developing zebrafish. For Aim 2, using silicone wristbands to monitor personal OPE exposure within undergraduate students at the University of California, Riverside (UCR), we identified that longer commutes were associated with increased TDCIPP exposure. Building on the results of Aim 2, in Aim 3 we demonstrated that a partial reduction in car dust does not affect increased TDCIPP exposure as a result of longer time spent in vehicles. Lastly, for Aim 4, a risk assessment identified that while the ingestion of TDCIPP from car interior dust does not pose a cancer risk, inhalation of benzene and formaldehyde from car interior air does pose a cancer risk for commuters in California. Overall, our findings contribute to our understanding of human OPE exposure in relation to commute times and how TPHP – a ubiquitous OPE – may alter embryonic development.

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Chapter 1: Introduction

1.1 Organophosphate Esters in the Indoor Environment

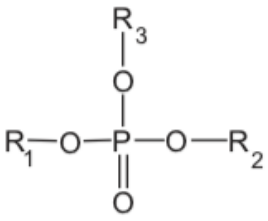

Figure 1.1. General structure of organophosphate esters

Organophosphate esters (OPEs) are a class of semi-volatile organic compounds (SVOCs) that contain a phosphate and an ester group (Figure 1.1). The properties of OPEs vary and depend on the type of R groups bonded to the phosphate core; these could be either a halogenated, alkyl or aryl side chain (van der Veen and de Boer, 2012). OPEs are commonly used as plasticizers, flame retardants, anti-foaming agents and wetting agents (Marklund et al., 2003; van der Veen and de Boer, 2012), and are being increasingly used as flame retardants to meet the demand for alternatives to brominated flame retardant (BFR) mixtures (Bollmann et al., 2012). BFRs are currently being banned or voluntarily phased-out of numerous commercial products around the world due to concerns about bioaccumulation, persistence, and toxicity (BSEF., 2011, EU., 2003, UNEP., 2009). In North America alone, the annual production of OPEs tris(2-chloroisopropyl)phosphate (TCIPP), tris(1,3-dichloro-2-isopropyl)phosphate (TDCIPP) and tris(2-cholorethyl)phosphate (TCEP) increased from less than 14,000 tonnes per year in 1986 to 38,000 tonnes per year in 2012 (Schreder et al., 2016).

Similar to BFRs, the majority of OPEs are added and not chemically bound to their end-use products. Therefore, with time and use, OPEs migrate out of end-use products via volatilization, leaching, or abrasion (Marklund et al., 2003). Due to the ability to partition out of end-use products, OPEs are found within several different environments. OPEs have been detected in wastewater, surface water, ground water (Reemtsma et al., 2008; Rodil et al., 2005), sediments (Cao et al., 2017), and within indoor air, outdoor air and particulate matter (Reemtsma et al., 2008). Unlike standard

air pollutants, SVOCs can readily partition from air to dust, contributing to persistent residues within the indoor environment (Adamkiewicz et al., 2011). Thus, in addition to other environmental media, OPEs have been ubiquitously detected within indoor dust of several areas of the built environment such as homes, offices, school buildings, hotels, and day care centers (Carignan et al., 2013; Fromme et al., 2014; Stapleton et al., 2009; Wei et al., 2015).

1.2 Organophosphate Esters in Personal Vehicles

Interestingly, based on studies within the published literature, the concentrations of OPEs within indoor dust are consistently lower than OPE levels detected within car dust (Abdallah and Covaci, 2014; Ali et al., 2013; Brandsma et al., 2014; Brommer et al., 2012; Brommer and Harrad, 2015; Carignan et al., 2013a; Harrad et al., 2016; Zhou et al., 2017). Commonly detected OPEs include TCIPP, TDCIPP, triphenyl phosphate (TPHP), tributyl phosphate (TBP), tris(2-butoxyethyl)phosphate (TBOEP), TCEP, and ethylhexyl diphenyl phosphate (EHDPP). The mean or median of the highest detected OPE in each of these studies is compiled within Table 1.1. Interestingly, the concentrations of OPEs were higher than BFRs, indicative of the decreasing use of these compounds within commercial products (Ali et al., 2013; Brommer et al., 2012; Brommer and Harrad, 2015).

Study	Country	N	Predominant OPE (Concentration)
Brommer et al., (2012)	Germany	12	TDCIPP (Mean: 130 μg/g)
Ali et al., (2013)	Kuwait Pakistan	15 15	TCIPP/TDCIPP (Mean: 36 μg/g) TPHP (Mean: 0.665 μg/g)
Brandsma et al., (2014)	Netherlands	8	TDCIPP (Median 110 μg/g)
Abdallah and Covaci (2014)	Egypt	20	TCIPP (Mean 0.513 μg/g)
Brommer and Harrad (2015)	U.K.	21	TDCIPP (Mean 110 μg/g)
Harrad et al., (2016)	Australia Germany	39 19	TDCIPP (Mean: 64 μg/g) TDCIPP (Mean: 94 μg/g)
Zhou et al., (2017)	Germany	5	TCIPP (Mean: 0.084 μg/m^3)
Tokumura et al., (2017)	Japan	12	TBOEP (Mean: 40 μg/g)
Christia et al., (2018)	Greece	25	TCIPP (Mean: 8 μg/g)

Table 1.1. Concentration of top OPE detected within interior car dust among different studies.

A common trend among the studies is the substantial contribution from TDCIPP and TCIPP. These two compounds are widely used in flexible and rigid polyurethane foam (PUF) (Stapleton et al., 2009) which is often used in the upholstery of permanently installed car seats. Studies also reported a significant association between the dominant OPE and car use since last car vacuum (Abdallah and Covaci, 2014; Brommer et al., 2012). This suggests that intensive usage of the car leads to greater abrasion of vehicle upholstery and consequent release of OPEs. Furthermore, as TDCIPP and TCIPP are common replacements to phased-out BFRs, high concentrations of OPEs may reflect increased fire standards specific to automobiles.

The detection of other compounds can also be explained by their application in different polymers used in car interiors. TPHP is used as a plasticizer in polyvinyl chloride (PVC), cellulosic polymers, thermoplastic matrices, and synthetic rubbers as well as a flame retardant in Firemaster 550 (FM550). TBP is used in hydraulic fluids and as plasticizers in polyester resins, PVC, acrylonitrile butadiene styrene (ABS), and synthetic rubber. TBOEP is often used as floor polish and in rubber stoppers, and EHDPP is applied to hydraulic fluids and PVC (Stapleton et al., 2009; van der Veen and de Boer, 2012). These compounds are extensively applied in parts of instrument panels, electronic equipment, floor mats, and textiles in the car interior (Marklund et al., 2003; Wei et al., 2015). Thus, the presence of these OPEs within interior car dust

suggests that there is a high potential for volatilization and/or leaching from end-use automotive products.

1.3 Commuting Patterns in Southern California

The American Community Survey (ACS) by the U.S. Census Bureau reported that a little more than 87% of the American population use their personal vehicles to commute. On average, a single American spends 53.8 minutes commuting per day (U.S. Census Bureau, 2017). In California, the commute time per day is higher, with the average worker spending around approximately 60 minutes commuting per day (U.S. Census Bureau, 2017). However, out of this population, 2.9% and 9.75% of the working population commute over 3 hours and 2 hours per day, respectively (Transportation Research Board, 2006). Throughout the country, the Riverside-San Bernardino-Ontario area has the fourth highest percent of workers commuting for more than 90 minutes one way (Transportation Research Board, 2006). Furthermore, 11.15% of the workers in the Los Angeles-Riverside-Orange County area and 6.40% in the San Diego area spend more than 60 minutes one way (Transportation Research Board, 2006). These statistics indicate that residents of Southern California spend a significant time in their vehicles when commuting to work.

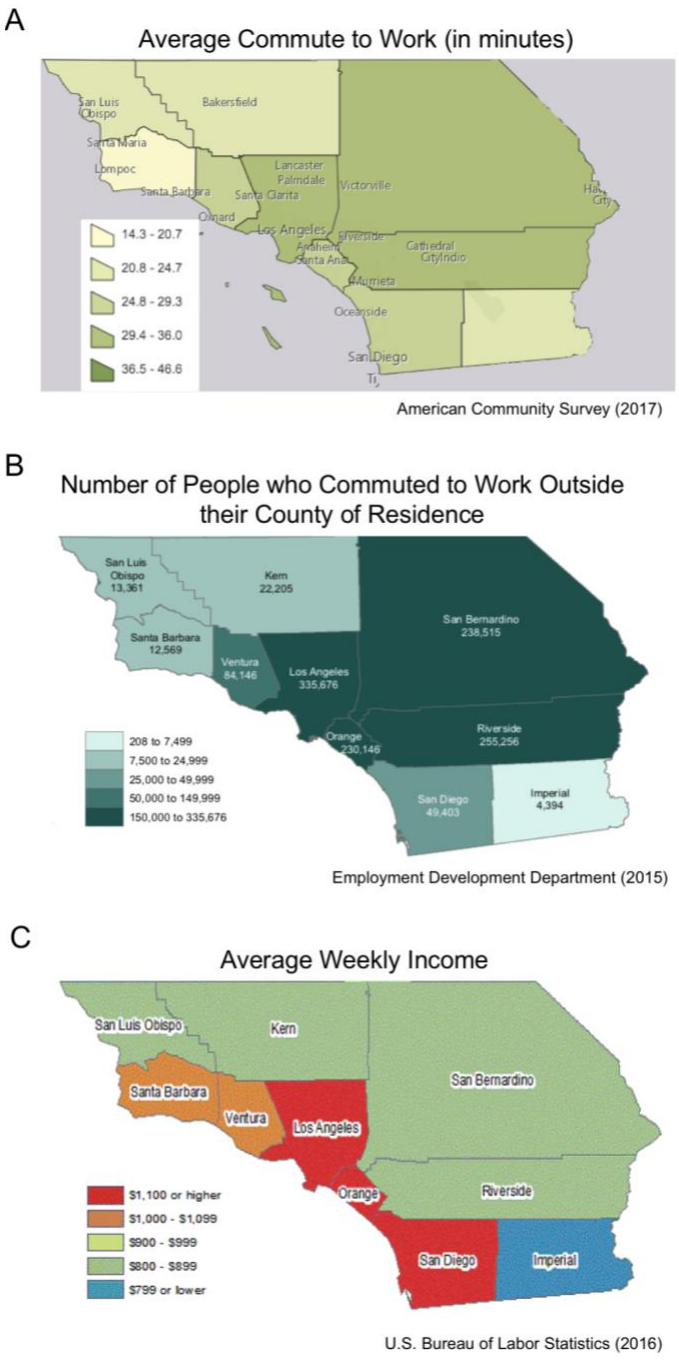

Figure 1.2. Variation in commuting (A,B) and income (C) demographics in the different counties in Southern California.

Large commutes for Southern Californian residents is most likely due to the improvement of the national economy, which has caused the housing market to expand, making it more expensive to own a house (Richardson, 2019). As a consequence, 55% of the American population live in the suburbs (Parker et al., 2018) and a significant portion of them commute to urban areas for work. Furthermore, people from lower income communities tend to live further away from cities (Wiener, 2018), and therefore spend a longer time commuting. In Figure 1.2, there is a strong relationship between the average income, number of people who work outside their county of residence and average commute time, especially within the counties of Riverside and San Bernardino.

Several studies have examined the relationship between lower socio-economic status and increased chemical exposure. In America, people of low socio-economic status experience higher concentrations of air pollutants and increased exposure to lead, pesticides, and polybrominated diphenyl ethers (PBDEs) (Adamkiewicz et al., 2011; Gaitens et al., 2009; Hajat et al., 2015; Zota et al., 2017). As increased commuting within this population could lead to an increase in OPE exposure, this has the potential to add an additional environmental burden on an already stressed community (i.e., as a result of lower incomes, food insecurity, immigration status, increased chemical exposure, etc.). Therefore, there is a need to evaluate exposure in relation to the amount of time spent in a personal vehicle.

1.4 Exposure Routes for Organophosphate Esters

Due to the ubiquitous presence in indoor environments and the potential for exposure to large and already stressed population, there is an increasing body of research investigating how humans are exposed to OPEs. Human exposure to OPEs usually occurs via 1) ingestion of food, water or dust containing OPEs, 2) inhalation of gaseous OPEs or OPEs bound to particulate matter, or 3) ingestion, inhalation and dermal contact with dust.

Within food samples, OPEs were detected in different food groups depending on the country of origin. In China and Belgium, cereals and fats/oils were most contaminated (Poma et al., 2018), compared to Sweden where fats/oils and desserts contained the highest concentrations of OPEs (Poma et al., 2017). Interestingly, in China, rice contributed to 60% of the total intake of OPEs (Zhang et al., 2016). In Australia, plant-based foods such as vegetables, fruits and cereals were most contaminated (He et al., 2018b), whereas in the United States the highest median OPE concertation was found in meat and fish (Han et al., 2019; Xu et al., 2015). Several

OPEs have been detected in tap water samples, although concentrations were lower compared to surface water, seawater, and rain water (Kim and Kannan, 2018). Ingestion of dust is another important route of OPE exposure. Several studies have highlighted that toddlers have higher exposure to OPEs compared to their mothers (Butt et al., 2014; Gibson et al., 2019). This has been attributed to being closer to the floor or behaviors such as hand-to-mouth activity (Sugeng et al., 2017), resulting in ingestion of OPE-contaminated dust.

OPEs are ubiquitously found on handwipes, suggesting that dermal exposure to these compounds is constantly occurring (Liu et al., 2017; Stapleton et al., 2014; Sugeng et al., 2017). Depending on the structure, some OPEs such as TCIPP and TCEP can penetrate through the skin rapidly, whereas TDCIPP and TPHP tend to accumulate on skin tissue (Frederiksen et al., 2018). However, the location of exposure plays an important role, as studies have reported that the application of nail polish is a significant source of TPHP exposure (Mendelsohn et al., 2016). This could possibly be attributed to thinner skin near the cuticles which provides a shorter distance for OPE absorption.

As discussed above, OPEs are ubiquitously detected within indoor dust. Therefore, inhalation of dust is a significant exposure route. Using active personal air samplers, higher levels of OPEs were detected within the inhalable particulate fraction (Schreder et al., 2016). Intake of OPEs through the inhalation exposure route was estimated to exceed intake via dust ingestion. On the whole, a comprehensive assessment of human exposure to OPEs through inhalation, dust ingestion, and dermal absorption reported that estimated total OPE exposure was highest with stationary air inhalation, followed by dust ingestion, floor dust ingestion, personal air inhalation, and dermal absorption (Xu et al., 2016).

1.5 Uptake of Organophosphate Esters

Once adsorbed into the body, OPEs undergo various forms of biotransformation. The two major metabolites of TBOEP are products of O-dealkylation and hydroxylation, whereas TPHP is mainly transformed through O-dearylation into its diester metabolite, diphenyl phosphate (DPHP). For chlorinated OPEs, TCEP is poorly metabolized by oxidative dehalogenation into its diester, and the major metabolite of TCIPP is a product of oxidative dehalogenation. TDCIPP is mainly transformed into either its diester, bis(1,3-dichloro-2-propyl) phosphate (BDCIPP), or a glutathione S-conjugate (Van den Eede et al., 2013).

As humans are constantly exposed to OPEs, several studies have detected OPE metabolites in human blood, urine, and breast milk, confirming uptake and intake of OPEs from drinking water, dietary sources, and/or surrounding environmental media such as dust. Other studies have reported significant correlations between OPE concentrations in indoor dust and their respective urinary metabolites (Cequier et al., 2015; Meeker et al., 2013). Moreover, significant correlations have been identified between OPEs detected on hand wipes and silicone wristbands as well as OPE metabolites in the participant's urine and serum (Gibson et al., 2019; Hammel et al., 2016; Hoffman et al., 2014). These metabolites are also seen to be reliable over several consecutive days, suggesting that their parent compounds may come from a continuous source of exposure. A cross sectional study by Hoffman et al., (2017) reported that, compared to samples collected in 2002 and 2003, urinary BDCIPP concentrations are 15.28 times higher in samples collected in 2014 and 2015 and urinary DPHP concentrations are 2.98 times higher in samples collected in 2010 and 2011. Most, if not all, of these studies have correlated exposure to OPEs in indoor dust with corresponding urinary metabolites. Indeed, Cequier et al., (2015) reported that urinary concentrations of OPE metabolites within pregnant women were lower at

a time when women were likely to be outside the household. This further highlights the significance of OPE exposure through indoor dust and raises the need for more research to determine whether these levels of exposure are related to adverse health outcomes, particularly within pregnant women.

1.6 Maternal Exposure to Organophosphate Esters

Toxicology data have suggested that certain OPEs might be developmental toxicants, leading to increasing concern about the uptake of OPEs by pregnant mothers and transfer to developing fetuses. Studies have shown that a vast majority of pregnant women have detectable levels of OPE metabolites in their urine samples (Castorina et al., 2017; Hoffman et al., 2017b, 2014; Romano et al., 2017). In fact, Hoffman et al. reported that BDCIPP and DPHP levels tend to be higher among pregnant women (Hoffman et al., 2017b). Moreover, urinary OPE metabolite levels were shown to have moderate to strong consistency throughout different stages of pregnancy and after giving birth, indicating that there is a continuous source of exposure to these chemicals (Hoffman et al., 2014; Romano et al., 2017).

Higher OPE exposure in pregnant women has been associated with higher levels of oxidative stress (Ingle et al., 2020). Both oxidative stress and prenatal exposures to certain OPEs have been associated with preterm birth and preeclampsia (Ferguson et al., 2014; Hoffman et al., 2018), suggesting a relationship between oxidative stress, prenatal exposures, and pregnancy-related complications. Moreover, OPEs have shown to significantly alter progesterone secretion and promote human chorionic gonadotropin (hCG) production. This could affect the synthesis of progesterone in the placenta and consequently affect fetal development (Hu et al., 2017).

Studies have shown that chemicals taken up by pregnant women may be transferred to the developing embryo and fetus across the placenta (Mitro et al., 2015). Therefore, pollutant levels in the placenta are not only a biomarker of maternal exposure, but also for prenatal exposure of the fetus throughout pregnancy (Myren et al., 2007). OPEs have been detected in placental tissue (Ding et al., 2016) as well as chorionic villi, suggesting a direct exposure to early human embryos prior to placental formation (Zhao et al., 2017). Embryonic stages are a critical window for development of the nervous system, heart and circulatory system. The presence of OPEs in chorionic villi suggests a risk of *in utero* exposure to these chemicals (Zhao et al., 2017). Considering the potential developmental toxicity of OPEs, to OPEs may pose a risk to normal embryonic development.

1.7 Developmental Effects of Chlorinated Phosphate Esters

Due to concerns regarding exposure of OPEs to the developing embryo, there are a growing number of studies examining the developmental effects of OPEs. This introduction will focus on two classes of OPEs – chlorinated phosphate esters (CPEs) and aryl phosphate esters (APEs). In humans, prenatal exposure to TDCIPP is associated with adverse behaviors such as withdrawal, attention problems, depression, hyperactivity and aggression (Doherty et al., 2019). In other mammalian models, prenatal exposure to TDCIPP in rats led to a decrease in offspring weight gain, liver weights and weaning times (Moser et al., 2015).

Among non-mammalian models, embryonic exposure to TCEP decreased body length in Japanese Medaka (Sun et al., 2016), and exposures to TDCIPP resulted in developmental effects in *Caenorhabditis elegans* (*C. elegans*) (Behl et al., 2015). In cultured chicken embryos, TCPP exposure delayed pipping and reduced tarsus length in chickens (Farhat et al., 2013). TDCIPP exposure to chicken embryos

caused a significant decrease in head length, bill length, embryo mass, gallbladder size, and plasma-free T_4 and cholesterol levels (Farhat et al., 2013). Genes associated with immune function, lipid and steroid metabolism, and liver/biliary function were also disrupted (Farhat et al., 2014).

As zebrafish are commonly used to model developmental toxicity, there are several studies that have looked at the effects of CPEs on zebrafish embryos. Following parental exposure in zebrafish, CPEs are detected within the offspring. The F1 generation also displayed decreased survival, body length and heart rate as well as thyroid disruption, generation of reactive oxygen species (ROS) and delayed nervous system development (Wang et al., 2015; Yu et al., 2017). Embryonic exposure to TDCIPP led to a delay in epiboly (Dasgupta et al., 2018, 2017; McGee et al., 2012), which led to abnormal development of the embryo (Fu et al., 2013). Early-life exposures to TDCIPP as well as TCIPP also altered photomotor response, larval swimming activity, and predator escape behavior (Dishaw et al., 2014; Noyes et al., 2015; Oliveri et al., 2015). Embryonic exposures were also associated with endocrine disrupting activity, where TDCIPP decreased T_4 and increased T_3 concentrations. The embryonic exposures to TDCIPP also led to disruption in genes associated with muscle development, thyroid hormone synthesis, thyroid development, nuclear receptors, and dorsoventral patterning (Dasgupta et al., 2017; Fu et al., 2013; Liu et al., 2012; Wang et al., 2013).

1.8 Developmental Effects of Aryl Phosphate Esters

As aryl phosphate esters do not have halogenated side chains, the developmental effects are different from CPEs. Several studies were also conducted using the FM 550 mixture which is 62% APEs (17% TPHP and 45% isopropylated triaryl phosphates (ITPs)). In humans, prenatal exposure to TPHP was also associated

with adverse behaviors in children such as decreased full-scale intelligence quotient and working memory (Castorina et al., 2017). In rats, prenatal exposure to FM 550 resulted in advanced female puberty, weight gain, male cardiac hypertrophy, insulin resistance and altered exploratory behaviours in offspring (Patisaul et al., 2013).

In non-mammalian models, embryonic exposure to TPHP affected hatchability, increased incidences of gross abnormalities and decreased body length and heart rate in Japanese Medaka (Sun et al., 2016). In *C. elegans*, TPHP, isopropylated phenyl phosphate (IPP), and tert-butylphenyl diphenyl phosphate (tBPDP) inhibited development, disrupted mitochondrial function and affected reproduction (Behl et al., 2015).

Similar to CPEs, zebrafish have been used to study the effects of APEs on development. Embryonic exposure to TPHP, IPP, BPDP and tricresyl phosphate (TCP) resulted in a higher incidence of mortality, decreased hatch success, and malformations such as curved spine, edema, smaller head, and smaller eyes (Behl et al., 2015). Exposures to TPHP, EHDPP, IPP and TCP also affected heart rate, locomotor activity, spontaneous motor function, short-term motility and long-term impairment of anxiety related behavior and exploration (Glazer et al., 2018; Jarema et al., 2015; Noyes et al., 2015; Oliveri et al., 2015; Shi et al., 2018). Moreover, developmental exposures to TPHP and ITP resulted in pericardial edema as well as effects on cardiac looping during embryogenesis which lead to bradycardia and reduction of myocardium (Du et al., 2015; McGee et al., 2013; Mitchell et al., 2018). This disruption, which is AhR-independent, may be mediated by the zebrafish retinoic acid receptors (RARs) (Isales et al., 2015; McGee et al., 2013). The pericardial edema effect is mitigated by pre-treatment with fenretinide, a pan-RAR agonist (Mitchell et al., 2018), further suggesting the potential role of RAR in TPHP-induced cardiotoxicity. TPHP exposure to zebrafish embryos also disrupted genes associated with the retinoid

X receptor (RXR), central nervous system, nuclear receptors and thyroid hormone synthesis (Kim et al., 2015; Liu et al., 2013; Mitchell et al., 2018; Shi et al., 2018).

1.9 Overview of Research Aims

As discussed above, OPEs are high-production volume chemicals that are being increasingly used to meet flammability standards. Due to the ubiquitous presence of OPEs within indoor environments and personal vehicles, there is concern regarding exposure to these compounds as well as the potential for developmental toxicity. Therefore, the overall goals of this research are to 1) increase our understanding about the mechanisms of developmental toxicity for one of these OPEs, TPHP, 2) measure exposure to OPEs within a population that spends a significant amount of time in their personal vehicles, and 3) identify the risk associated with ingestion of tris(1,3-dichloro-2-isopropyl)phosphate (TDCIPP) – another high production volume OPE – within personal vehicles. Within Chapter 2, the TPHP-induced effect on the transcriptome and the respective phenotypes are examined. Moreover, we assess if these effects may be mitigated by pre-treatment with an RAR agonist (fenretinide). Within Chapter 3, we examine human exposure to organophosphate esters in relation to the amount of time spent in a personal vehicle. Within Chapter 4, we examine the contribution of dust located on interior parts at the front of the vehicle to personal exposure to OPEs. Within Chapter 5, we examine the risk associated with TDCIPP ingestion from car interior dust compared to other chemicals used in personal vehicles. Overall, our findings increase our understanding of human OPE exposure in relation to commute times and how TPHP – a ubiquitous OPE – may alter embryonic development.

Chapter 2: mRNA-Sequencing Identifies Liver as a Potential Target Organ for Triphenyl Phosphate in Embryonic Zebrafish

2.0 Abstract

Triphenyl phosphate (TPHP) is a commonly used organophosphate flame retardant and plasticizer in the United States. Using zebrafish as a model, the overall objective of this study was to identify potential organs that might be targeted by TPHP during embryonic development. Based on mRNA-sequencing, TPHP exposure from 24 to 30 h post fertilization (hpf) and 24 to 48 hpf significantly affected the abundance of 305 and 274 transcripts, respectively, relative to vehicle (0.1% DMSO) controls. In addition to minor effects on cardiotoxicity- and nephrotoxicity-related pathways, Ingenuity Pathway Analysis (IPA) of significantly affected transcripts within 30- and 48-hpf embryos revealed that hepatotoxicity-related pathways were strongly affected following exposure to TPHP alone. Moreover, while pre-treatment with fenretinide (a retinoic acid receptor agonist) mitigated TPHP-induced pericardial edema and liver enlargement at 72 hpf and 128 hpf, respectively, IPA revealed that fenretinide was unable to block TPHP-induced effects on cardiotoxicity-, nephrotoxicity-, and hepatotoxicity-related pathways at 48 hpf, suggesting that TPHP-induced effects on the transcriptome were not associated with toxicity later in development. In addition, based on Oil Red O staining, we found that exposure to TPHP nearly abolished neutral lipids from the embryonic head and trunk and, based on metabolomics, significantly decreased the total abundance of metabolites – including betaine, a known osmoprotectant – at 48 and 72 hpf. Overall, our data suggest that, in addition to the heart, TPHP exposure during early development results in adverse effects on the liver, lipid utilization, and osmoregulation within embryonic zebrafish.

2.1 Introduction

Triphenyl phosphate (TPHP) is an unsubstituted aryl phosphate ester that is widely used as an additive flame retardant and plasticizer (van der Veen and de Boer, 2012). Within the built environment, TPHP migrates out of end-use products via leaching and volatilization, resulting in accumulation within indoor dust (Stapleton et al., 2009). As such, TPHP is ubiquitous within indoor dust samples around the world, where concentrations ranging from 0.24 to 1,789,000 ng/g have been detected within indoor dust samples in the United States, Australia, Egypt, Canada, Germany, Finland, Kazakhstan, United Kingdom, and China (Harrad et al., 2016; Khairy and Lohmann, 2019; Rantakokko et al., 2019; Stapleton et al., 2009; Sun et al., 2018). Due to the presence of TPHP within indoor dust, humans are exposed to TPHP through ingestion, inhalation, and dermal absorption, resulting in frequent detection of the primary metabolite of TPHP – diphenyl phosphate (DPHP) – in urine, blood serum, breast milk, and placental tissue (Ding et al., 2016; He et al., 2018a; Kim et al., 2014; Ospina et al., 2018; Sun et al., 2018).

Prior studies conducted *in vitro* have shown that TPHP induces cytotoxicity in chicken embryonic hepatocytes (Su et al., 2014), and induces intracellular reactive oxygen species generation, apoptosis, and cell cycle arrest in HepG2 cells (Zheng et al., 2018). Within invertebrates, TPHP disrupts the metabolome in both earthworms and freshwater algae, leading to downstream effects on membrane integrity (Wang et al., 2019b; Wang et al., 2018). TPHP also disrupts lipid metabolism through inhibition of carboxylesterase, resulting in an increase in body weight, liver weight, and hepatic steatosis in male mice (Morris et al., 2014; D. Wang et al., 2019a). In aquatic vertebrates, TPHP induces neurotoxicity in Chinese rare minnows and reproductive toxicity in Japanese medaka (Hong et al., 2018; Li et al., 2018), and, in zebrafish, induces thyroid endocrine disruption, developmental neurotoxicity, ocular toxicity, and

hepatotoxicity (Du et al., 2016; Jarema et al., 2015; Kim et al., 2015; X. Liu et al., 2016; Shi et al., 2018, 2019).

Our prior studies have shown that exposure of developing zebrafish embryos to TPHP reliably induces cardiotoxicity and pericardial edema in a concentration-dependent manner (Isales et al., 2015; McGee et al., 2013; Mitchell et al., 2018). Although TPHP-induced cardiotoxicity is independent of the aryl hydrocarbon receptor (McGee et al., 2013), TPHP-cardiotoxicity is enhanced in the presence of non-toxic concentrations of a pan-retinoic acid receptor (RAR) antagonist (BMS493) (Isales et al., 2015) and mitigated by pre-treatment with non-toxic concentrations of a pan-RAR agonist (fenretinide) (Mitchell et al., 2018), suggesting that RAR may be involved in mediating the effects of TPHP within developing zebrafish embryos. Retinoic acid signaling regulates development and differentiation of many tissues, organs, and processes within zebrafish, including but not limited to hindbrain patterning, heart morphogenesis, lipid abundance, and kidney/liver development (Begemann et al., 2004; Samarut et al., 2015; Stafford and Prince, 2002; Stainier and Fishman, 1992; Wingert et al., 2007; Yutzey and Bader, 1995). Therefore, in addition to the heart, RAR-mediated TPHP toxicity may be impacting other organs in the zebrafish embryo.

Using zebrafish as a model, the overall objective of this study was to identify other potential organs that might be targeted by TPHP during embryonic development. To accomplish this objective, we relied on whole-embryo exposures and a combination of mRNA-sequencing, phenotypic assessments, neutral lipid staining, and metabolomics to test the hypothesis that TPHP interferes with normal development of other organ systems – effects that, similar to our findings with the heart, may be mitigated by pre-treatment with an RAR agonist (fenretinide).

2.2 Materials and Methods

Animals. Adult wildtype (5D) zebrafish were maintained and bred on a recirculating system using previously described procedures (Mitchell et al., 2018). Adult breeders were handled and treated in accordance with Institutional Animal Care and Use Committee-approved animal use protocol (#20150035 and #20180063) at the University of California, Riverside.

Chemicals. TPHP (99.5% purity) was purchased from ChemService, Inc. (West Chester, PA, USA), whereas fenretinide (>99.3% purity) was purchased from Tocris Bioscience (Bristol, UK). All stock solutions were prepared by dissolving chemicals in high performance liquid chromatography-grade dimethyl sulfoxide (DMSO). TPHP and fenretinide were stored at room temperature and 4°C, respectively, in 2-ml amber glass vials with polytetrafluoroethylene-lined caps. Working solutions were freshly prepared by spiking stock solutions into particulate-free water from our recirculating system (pH and conductivity of ~7.2 and ~950 µS, respectively), resulting in 0.1% DMSO within all vehicle control and treatment groups. Tricaine methanesulfonate (MS-222) (Western Chemical, Inc., Ferndale, WA, USA) solutions were freshly made by dissolving MS-222 into particulate-free water from our recirculating system. Propylene glycol (>99.5% purity) and Oil Red O (>75% dye content) were purchased from Fisher Scientific (Hampton, NH, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

Phenotypic assessments. Newly fertilized eggs were collected immediately following spawning and incubated in groups of approximately 100 per 100 X 15 mm polystyrene petri dish within a light- and temperature-controlled incubator until 24 h post fertilization (hpf). Viable embryos were transferred to 100 X 15 mm polystyrene petri dishes containing 10 ml of either vehicle (0.1% DMSO) or 10 µM TPHP, resulting

in 30 initial embryos per dish (three dishes per treatment). All dishes were then covered with a plastic lid and incubated under static conditions at 28°C under a 14:10-h light-dark cycle until either 30, 48, or 72 hpf. Figure 2.1 provides an overview of exposure scenarios used for all experiments throughout this study.

At 30, 48, or 72 hpf, embryos were removed from the incubator and anesthetized with 100 mg/l MS-222 by adding 5 ml of 300 mg/l MS-222 to each petri dish; 30-hpf embryos were dechorionated by incubating in 0.3 mg/ml pronase for 10 min. Surviving embryos (≥80% survival across all treatment groups) were then transferred to black 384-well microplates with 0.17-mm glass-bottom wells (Matrical Bioscience, Spokane, WA, USA), and each plate was centrifuged at 200 rpm for 3 min to orient hatched embryos in right or left lateral recumbency. Embryos were then imaged under transmitted light using automated image acquisition protocols and parameters previously optimized for our ImageXpress Micro XLS Widefield High-Content Screening System (Molecular Devices, Sunnyvale, CA, USA) (Yozzo et al., 2013). Following image acquisition, embryos were then euthanized by placing the plate at 4°C for 30 min. Body length (μm) and pericardial area (μm^2) were quantified within MetaXpress 6.0.3.1658 (Molecular Devices) using previously described protocols (Yozzo et al., 2013).

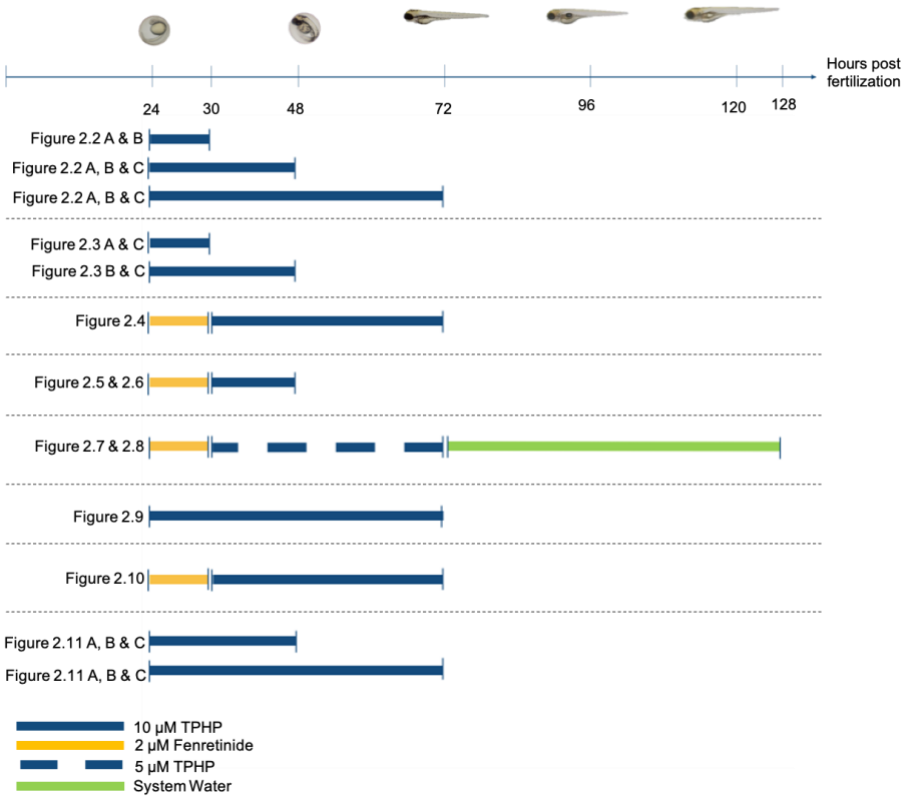

Figure 2.1. Overview of exposure scenarios associated with Figures 2.2-2.11 within the main text. Solid blue line = 10 µM TPHP; dashed blue line = 5 µM TPHP; solid yellow line = 2 µM TPHP; and solid green line = system water.

To identify potential effects of TPHP on liver morphology, embryos (30 per dish; three dishes per treatment) were exposed to either vehicle (0.1% DMSO) or 5 µM TPHP from 30 to 72 hpf as described above, and then transferred to system water from 72 to 128 hpf (Figure 2.1). As exposure to 10 µM TPHP from 30 to 72 hpf resulted in significant mortality at 128 hpf (<50% survival), we relied on 5 µM TPHP as the maximum tolerated concentration in the absence of significant mortality (≥80% survival) for liver morphology assessments. At 128 hpf, all embryos were fixed overnight in 4% paraformaldehyde, transferred to 1X phosphate buffer solution (PBS), oriented in right lateral recumbency, and imaged under transmitted light at 3.2X magnification using a Leica MZ10 F stereomicroscope equipped with a DMC2900 camera. Liver area (μm^2) was then quantified within ImageJ.

Fenretinide pre-treatments. Embryos were pre-treated with 10 ml of vehicle (0.1% DMSO) or 2 μM fenretinide from 24 to 30 hpf as described above, and then transferred to clean petri dishes and exposed to 10 ml of vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf or 30-72 hpf as described above (Figure 2.1). Procedures for phenotypic assessments were identical to those described above.

mRNA-sequencing. To assess the potential effects of TPHP on the embryonic transcriptome at 30 and 48 hpf (experiment #1), 24-hpf embryos (30 embryos per petri dish; six petri dishes per treatment) were exposed to 10 ml of vehicle (0.1% DMSO) or 10 μM TPHP as described above (Figure 2.1). At 30 and 48 hpf, two petri dishes containing up to 30 embryos each were pooled into one 2-ml cryovial and snap-frozen in liquid nitrogen, resulting in up to 60 embryos per vial and three replicate 2-ml cryovials per treatment per time-point. To determine whether fenretinide pre-treatment mitigated TPHP-induced effects on the embryonic transcriptome at 48 hpf (experiment #2), 24-hpf embryos (30 embryos per petri dish; six petri dishes per treatment) were pre-treated with 10 ml of vehicle (0.1% DMSO) or 2 μM fenretinide from 24 to 30 hpf, and then transferred to clean petri dishes and exposed to 10 ml of vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf as described above. At 48 hpf, two petri dishes containing up to 30 embryos each were pooled into one 2-ml cryovial and snap-frozen in liquid nitrogen, resulting in up to 60 embryos per vial and three replicate 2-ml cryovials per treatment per time-point. All samples (24 total) were stored at -80°C until total RNA extraction.

Embryos were homogenized in 2-ml cryovials using a PowerGen Homogenizer (Thermo Fisher Scientific, Waltham, MA, USA), resulting in a total of 24 samples. Following homogenization, an SV Total RNA Isolation System (Promega, Madison, WI, USA) was used to extract total RNA from each replicate sample following the

manufacturer's instructions. RNA quantity and quality were confirmed using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA), respectively. Based on sample-specific Bioanalyzer traces, the RNA Integrity Number (RIN) was >8 for all RNA samples used for library preparations. Libraries were prepared using a QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen, Vienna, Austria) and indexed by treatment replicate following the manufacturer's instructions. Library quality and quantity were confirmed using a Qubit 4.0 Fluorometer and 2100 Bioanalyzer system, respectively. Libraries were then pooled by experiment number, diluted to a concentration of 1.3 pM (with 1% PhiX control), and single-read (1X75) sequenced on our Illumina MiniSeq Sequencing System (San Diego, California, USA) using three separate 75-cycle High-Output Reagent Kits (two replicate kits for experiment #1; one kit for experiment #2).

All sequencing data were uploaded to Illumina's BaseSpace in real-time for downstream analysis of quality control. Raw Illumina (fastq.gz) sequencing files (36 files) are available via NCBI's BioProject database under BioProject ID PRJNA529921, and a summary of sequencing run metrics are provided in Tables S2.1, S2.4, and S2.11 (>83% of reads were ≥Q30 across all runs). All 36 raw and indexed Illumina (fastq.gz) sequencing files were downloaded from BaseSpace and uploaded to Bluebee's genomics analysis platform (https://www.bluebee.com) to align reads against zebrafish genome assembly GRCz10. After combining treatment replicate files, a DESeq2 application within Bluebee (Lexogen Quantseq DE 1.2) was used to identify significant treatment-related effects on transcript abundance (relative to vehicle controls) based on a false discovery rate (FDR) *p*-adjusted value < 0.01. Using DESeq2-identified transcripts with a FDR *p*-adjusted value <0.01, downstream analyses were run using Qiagen's Ingenuity Pathway Analysis (IPA) (Germantown, MD, USA). Statistically significant transcripts were uploaded to IPA, and human, rat,

and mouse homologs were automatically identified within IPA using NCBI's HomoloGene. A Tox Analysis was then performed using a Fisher's Exact Test *p*-value threshold of 0.05 as the basis for identifying statistically significant pathways; the algorithm considered both direct and indirect relationships using Ingenuity Knowledge Base (genes only) as the reference set. In addition, for experiment #2, significantly affected transcripts were imported into the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 for Gene Ontology (GO) enrichment analysis, using a count of 2 and an EASE of 1.

Oil Red O staining. Embryos were exposed to either vehicle (0.1% DMSO) or TPHP (2.5, 5, or 10 μM) from 24-72 hpf as described above. In addition, embryos were pre-treated with 2 μM fenretinide and then exposed to 10 μM TPHP as described above (Figure 2.1). At 72 hpf, embryos were fixed in 4% paraformaldehyde for 24 h and then transferred to 1X PBS. Fixed embryos were stained with Oil Red O (ORO) using previously described protocols (Passeri et al., 2009). Briefly, whole fixed embryos were infiltrated with a graded series of propylene glycol and then stained with 0.5% ORO in 100% propylene glycol overnight. Stained embryos were washed with decreasing concentrations of propylene glycol, rinsed several times with 1X PBS, and then stored in 1X PBS at 4°C. Stained embryos were imaged using under transmitted light at 4X magnification using a Leica MZ10 F stereomicroscope equipped with a DMC2900 camera. Mean color intensity within stained embryos was quantified using the mean gray value within ImageJ.

Untargeted metabolomics. Embryos (30 embryos per petri dish; three petri dishes per treatment) were exposed to 10 ml of vehicle (0.1% DMSO) or 10 μM TPHP from 24-48 hpf or 24-72 hpf as described above (Figure 2.1). At 48 and 72 hpf,

embryos from replicate dishes were transferred to 1.5-ml microcentrifuge tubes, snap-frozen in liquid nitrogen, and stored at -80°C until extractions. For all extractions, 500 µl of ice-cold solvent (30:30:20:20 MeOH:ACN:IPA:water) was added to each tube, and tubes were then sonicated for 15 min, vortexed for 15 min, sonicated for 15 min, and centrifuged at 16,000 x g at 4°C for 15 min. The supernatant was then transferred to glass HPLC vials for LC-MS analysis.

LC-MS was performed on a Synapt G2-Si quadrupole time-of-flight mass spectrometer (Waters, Milford, MA, USA) coupled to a I-class UPLC system (Waters, Milford, MA, USA). Separations were carried out on a CSH C18 column (2.1 x 100 mm, 1.7 µM) (Waters, Milford, MA, USA). The mobile phases were (A) 60:40 acetonitrile:water with 10 mM ammonium formate and 0.1% formic acid, and (B) 90:10 isopropanol:acetonitrile with 10 mM ammonium formate and 0.1% formic acid. The flow rate was 350 µl/min and the column was held at 50°C. The injection volume was 4 µl. The following gradient program (with respect to mobile phase B) was used: 0 min, 10% B; 1 min, 10% B; 3 min, 20% B; 5 min, 40% B; 16 min, 80% B; 18 min, 99% B; 20 min 99% B; 20.5 min, 10% B. Prior to LC-MS analysis, samples were diluted 20-fold to prevent detector saturation. The MS was operated in positive ion mode (50 to 1200 m/z) with a 100-ms scan time. Source and desolvation temperatures were 120°C and 500°C, respectively. Desolvation and cone gas were set to 1000 l/hr and 150 l/hr, respectively. All gases were (generated by pooling equal aliquots of each sample) was analyzed every 4-5 injections to monitor system stability and performance. Samples were analyzed in random order. Leucine enkephalin was infused and used for mass correction.

Untargeted data processing (peak picking, alignment, deconvolution, integration, normalization, and spectral matching) was performed in Progenesis Qi software (Nonlinear Dynamics, Durham, NC, USA). Data were normalized to total ion

abundance. Features with a CV greater than 20% or with an average abundance less than 200 in the quality control injections were removed (Dunn et al., 2011). To help identify features that belong to a single metabolite, features were assigned a cluster ID using RAMClust (Broeckling et al., 2014). An extension of the metabolomics standard initiative guidelines was used to assign annotation level confidence (Schymanski et al., 2014; Sumner et al., 2007). Several MS/MS metabolite databases were searched against including Metlin, Mass Bank of North America, Lipidblast, and an in-house database.

Liver histology. To identify potential effects of TPHP on hepatic tissue with or without fenretinide pre-treatment, embryos were 1) pre-treated with 10 ml of vehicle (0.1% DMSO) or 2 µM fenretinide from 24 to 30 hpf as described above; 2) transferred to clean petri dishes and exposed to 10 ml of vehicle (0.1% DMSO) or 5 µM TPHP from 30-72 hpf and; 3) transferred to system water from 72 to 128 hpf (Figure 2.1). At 128 hpf, embryos were fixed overnight in 4% paraformaldehyde, transferred to 1X PBS, and immediately shipped to HistoWiz Inc. (Brooklyn, NY, USA) for histology procedures. Samples were then oriented in right lateral recumbency, paraffin-embedded, and 4-µm thick-step sections through the whole body were mounted on glass slides and stained with hematoxylin and eosin (H&E). After staining, sections were dehydrated and film cover-slipped using a Tissue-Tek Prisma and Coverslipper (Sakura Finetek USA, Inc., Torrance, CA, USA). Whole-slide scanning was performed on an Aperio AT2 (Leica Biosystems, Wetxlar, Germany) using a 40X objective, and digital slides were transferred from HistoWiz to our lab for qualitative assessments.

Statistical analyses. For all phenotypic data, a general linear model (GLM) analysis of variance (ANOVA) ($\alpha = 0.05$) was performed using SPSS Statistics 24, as

these data did not meet the equal variance assumption for non-GLM ANOVAs. Treatment groups were compared with vehicle controls using pair-wise Tukey-based multiple comparisons of least square means to identify significant differences. For metabolomics data, Q values were generated by performing the Benjamini-Hochberg correction of p-values generated from an ANOVA in R using the aov function. Z-scores were calculated by subtracting mean relative abundance values for each treatment group from individual replicate relative abundance values, and then dividing this number by the standard deviation for each treatment group.

2.3 Results

mRNA-sequencing reveals that TPHP exposure affects transcripts associated with cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways

Our previous studies demonstrated that exposure to 5, 10, and 20 μM TPHP from 24 to 72 hpf reliably induced pericardial edema in zebrafish embryos (Mitchell et al., 2018). Therefore, we selected 10 μM TPHP as an optimal concentration for the majority of exposures and pre-treatment experiments. Relative to vehicle controls, initiation of exposure to 10 μM TPHP at 24 hpf resulted in 1) a significant decrease in body length at 48 and 72 hpf (but not 30 hpf) (Figures 2.2A and 2.2B) and 2) a significant increase in pericardial area at 72 hpf (but not 48 hpf) (Figures 2.2A and 2.2C).

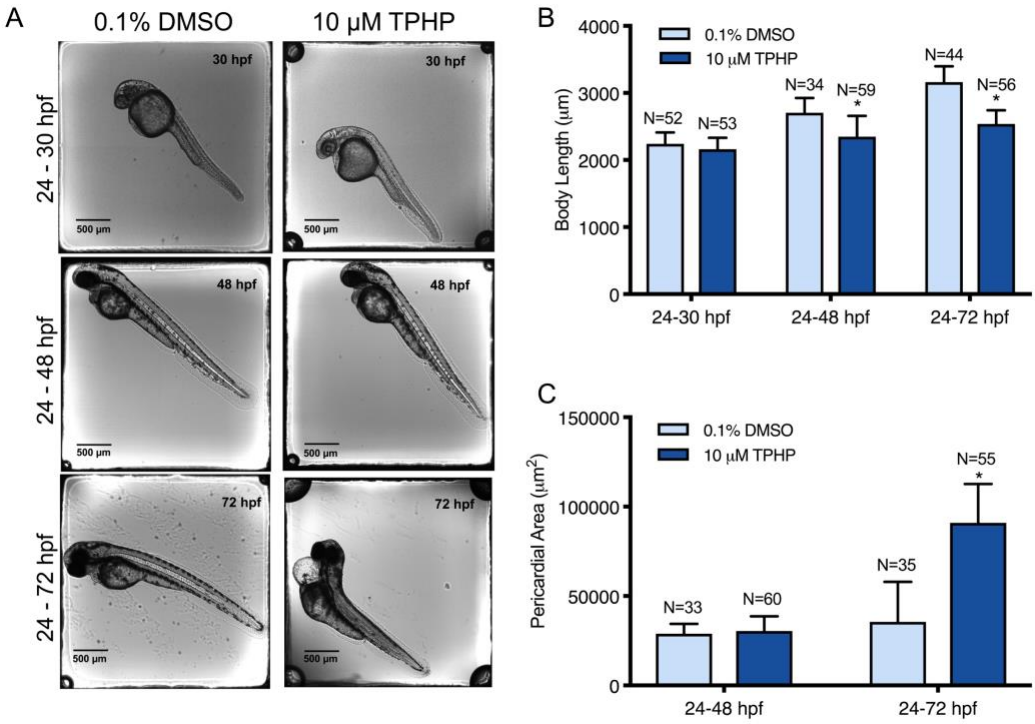

Figure 2.2. Representative images (A), mean body length (± standard deviation) (B), and mean pericardial area (± standard deviation) (C) of 30-, 48-, and 72-hpf embryos treated with either vehicle (0.1% DMSO) or 10 µM TPHP from 24-30 hpf, 24-48 hpf, or 24-72 hpf.

Individual raw DESeq2 outputs following exposure to 10 µM TPHP from 24-30 hpf and 24-48 hpf are provided within Tables S2.2-S2.3 and Tables S2.5-S2.6 for sequencing replicates #1 and #2, respectively. Combined raw DESeq2 outputs for exposures from 24-30 hpf and 24-48 hpf are provided within Tables S2.7 and S2.8, respectively. Following exposure from 24-30 hpf, TPHP exposure resulted in a significant decrease and increase in the abundance of 213 and 92 transcripts (305 total), respectively, at 30 hpf (Figure 2.3A). Following exposure from 24-48 hpf, TPHP exposure resulted in a significant decrease and increase in the abundance of 73 and 201 transcripts (274 total), respectively, at 48 hpf (Figure 2.3B).

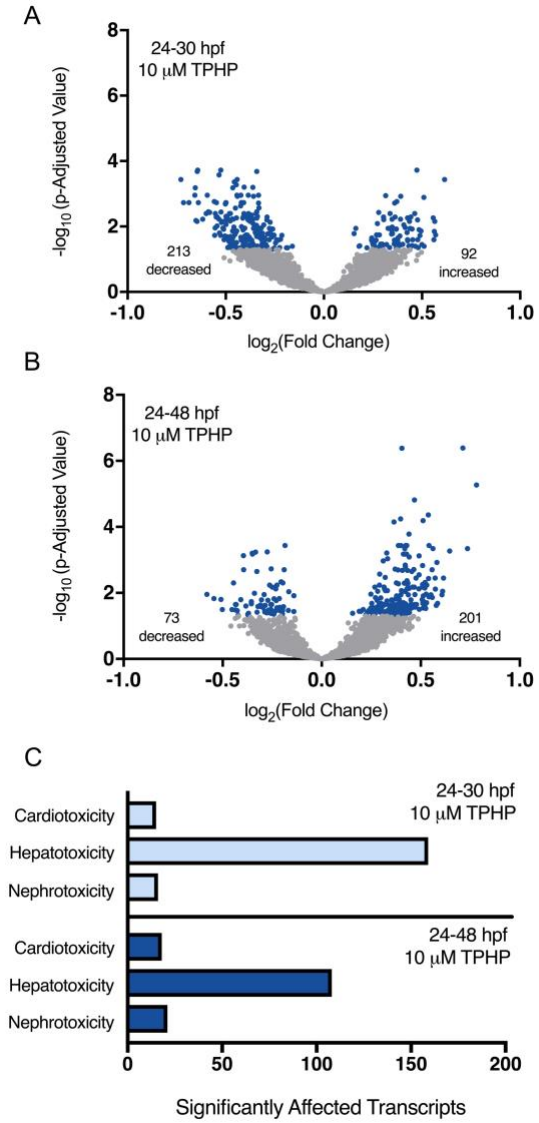

Figure 2.3. Volcano plots showing the number of significantly different transcripts (blue circles) following treatment with 10 μM TPHP from 24-30 hpf (A) or 24-48 hpf (B) relative to vehicle (0.1% DMSO) controls. Log_2-transformed fold change is plotted on the x-axis and log_{10}-transformed p-adjusted value is plotted on the y-axis. Transcripts associated with cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways were identified by Ingenuity Pathway Analysis's (IPA) toxicity analysis using a Fisher's exact p-value of ≤0.05 (C).

Statistically significant transcripts with human, rat or mouse homologs were included within IPA's Tox Analysis. Approximately 50% of statistically significant transcripts were included in IPA's Tox Analysis; the remaining statistically significant transcripts were excluded by IPA's Tox Analysis due to the absence of human, rat, and/or mouse orthologs within NCBI's Homologene database. A list of significantly affected pathways identified by IPA's Tox Analysis for 24-30 hpf and 24-48 hpf are provided within Tables S2.9 and S2.10, respectively. Based on this analysis, pathways associated with cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways were significantly affected, where the abundance of transcripts associated with

hepatotoxicity was >5-fold higher than the abundance of transcripts associated with cardiotoxicity and nephrotoxicity pathways (Figure 2.3C).

Pre-treatment with fenretinide does not mitigate TPHP-induced effects on cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways

Our previous studies demonstrated that pre-treatment with 2 μM fenretinide reliably blocked pericardial edema (but not body length) following exposure to 20 μM TPHP (Mitchell et al., 2018). Likewise, within this study pre-treatment with 2 μM fenretinide from 24-30 hpf significantly mitigated TPHP-induced pericardial edema (but not effects on body length) following exposure to 10 μM TPHP from 30-72 hpf (Figures 2.4A and 2.4B).

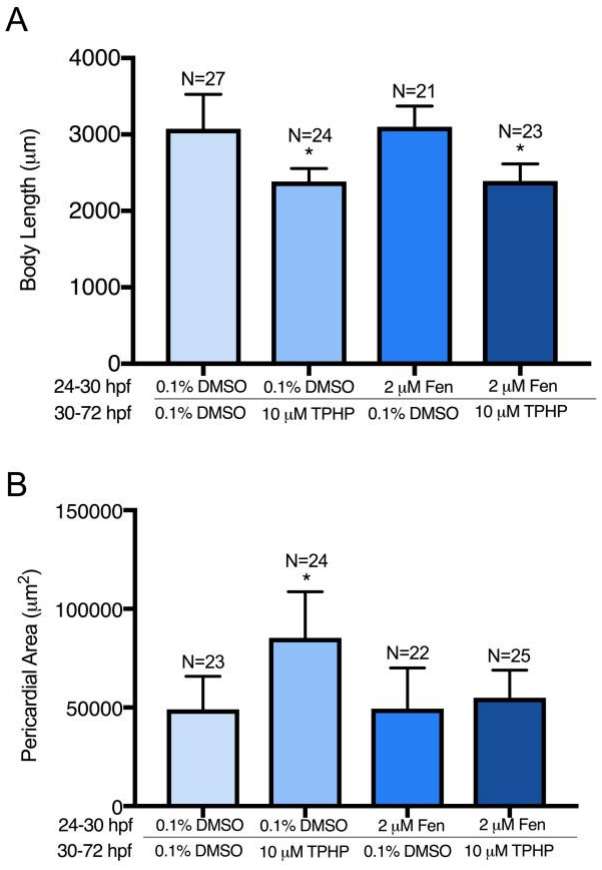

Figure 2.4. Mean body length (± standard deviation) (A) and mean pericardial area (± standard deviation) (B) of embryos pre-treated with either vehicle (0.1% DMSO) or 2 μM fenretinide (Fen) from 24-30 hpf and then treated with vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf (A) or 30-72 hpf (A,B,C).

Raw DESeq2 output are provided within Tables S2.12-S2.14. Based on these data, TPHP exposure from 30-48 hpf resulted in a significant effect on the abundance of 150 transcripts (14 decreased and 136 increased) at 48 hpf relative to vehicle controls (Figure 2.5A). While pre-treatment with fenretinide from 24-30 hpf only affected 12 transcripts (3 decreased and 9 increased) at 48 hpf (Figure 2.5B), pre-treatment with fenretinide from 24-30 hpf followed by TPHP exposure from 30-48 hpf significantly affected the abundance of 134 transcripts (24 decreased and 110 increased) at 48 hpf (Figure 2.5C).

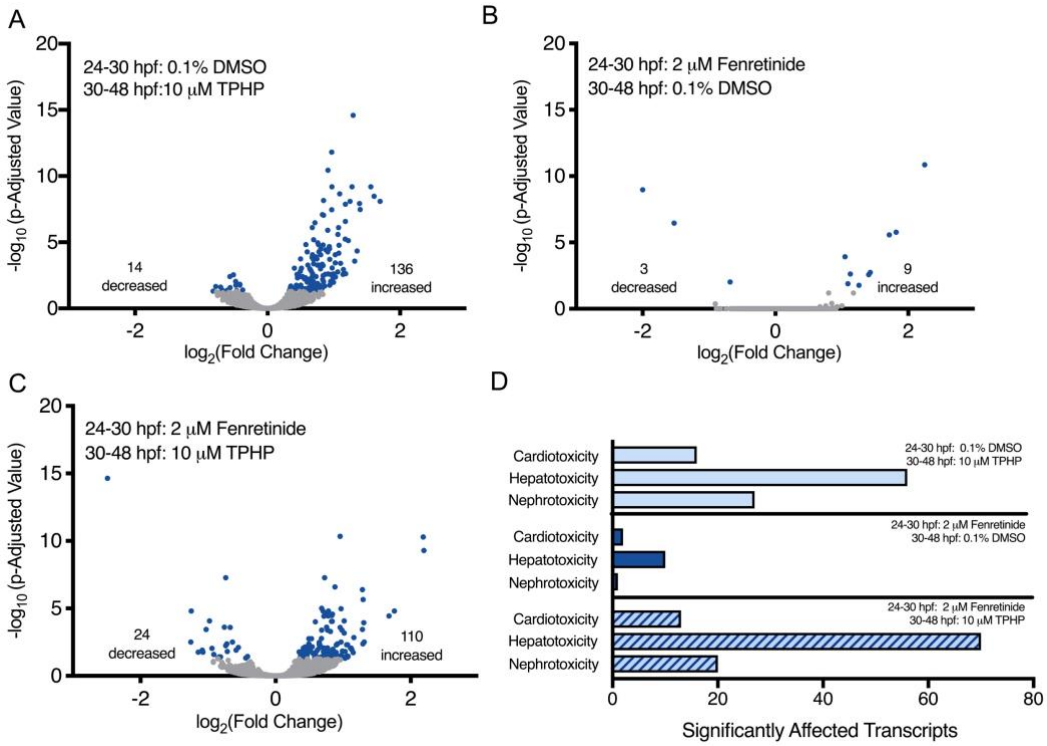

Figure 2.5. Volcano plots (A, B, and C) showing the number of significantly different transcripts (blue circles) at 48 hpf following pre-treatment with vehicle (0.1% DMSO) or 2 μM fenretinide from 24-30 hpf followed by treatment with vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf; all three plots are relative to treatment with vehicle (0.1% DMSO) from 24-48 hpf. Log_2-transformed fold change is plotted on the x-axis and log_{10}-transformed p-adjusted value is plotted on the y-axis. Transcripts associated with cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways were identified by Ingenuity Pathway Analysis's (IPA) toxicity analysis using a Fisher's exact p-value of ≤0.05 (D).

Statistically significant transcripts were included in DAVID's Biological Process analysis, and available human, rat or mouse homologs were included in IPA's Tox Analysis. A list of significantly affected biological processes identified by DAVID for all three treatment groups (relative to vehicle controls) are provided within Tables S2.15-S2.17. While pre-treatment with fenretinide from 24-30 hpf did not alter the majority of TPHP-induced biological processes identified by DAVID (Figure 2.6), fenretinide blocked TPHP-induced effects on regulation of cell cycle and ion transport.

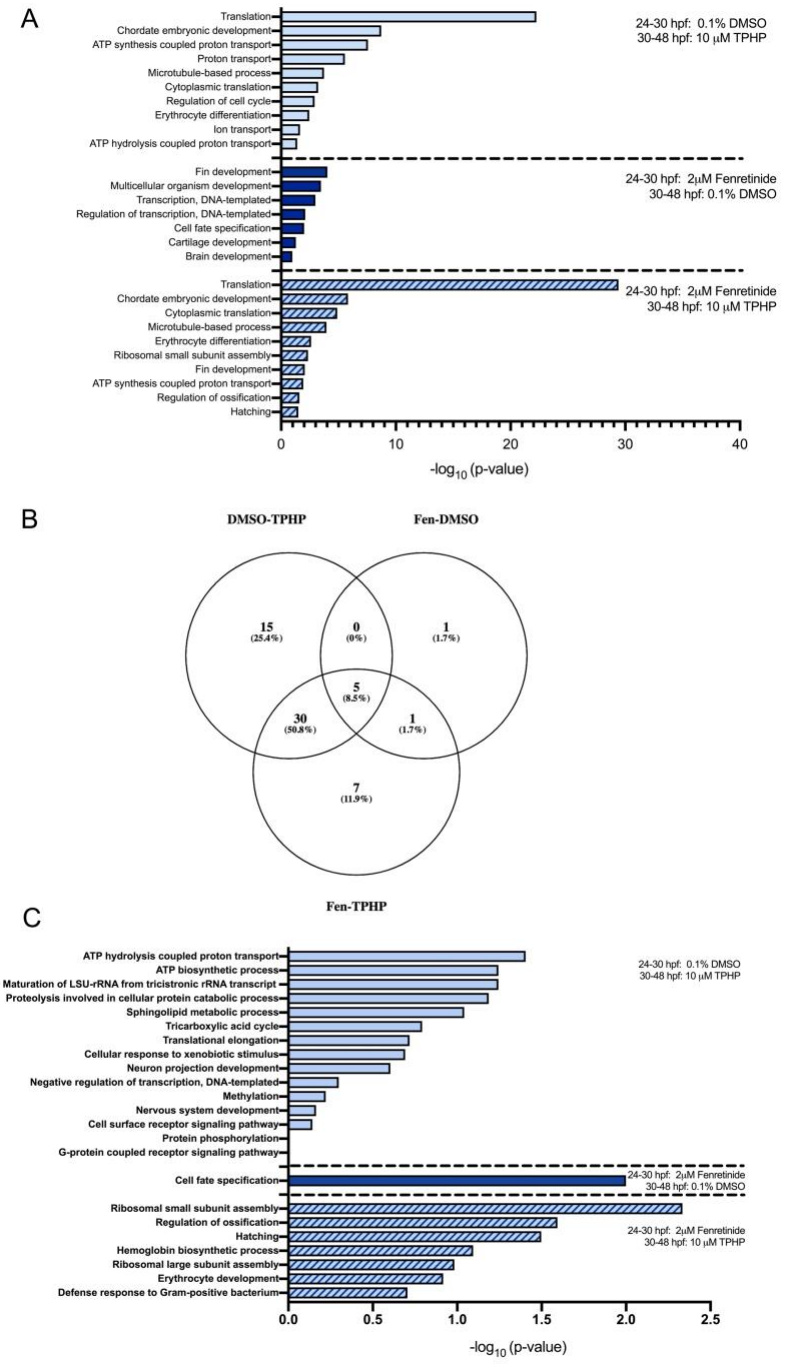

Figure 2.6. Top 10 DAVID-identified biological processes (based on significantly different transcripts) at 48 hpf following pre-treatment with vehicle (0.1% DMSO) or 2 μM fenretinide from 24-30 hpf followed by treatment with vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf (A); within the fenritinide-alone treatment, only seven biological processes were identified by DAVID. Venn diagram showing the number and percent of significantly different biological processes among treatment groups (B). Biological processes unique to each treatment group (C).

Similar to experiment #1, approximately 50% of statistically significant transcripts were included in IPA's Tox Analysis; the remaining statistically significant transcripts were excluded by IPA's Tox Analysis due to the absence of human, rat, and/or mouse orthologs within NCBI's Homologene database. A list of significantly affected pathways identified by IPA's Tox Analysis for all three treatment groups (relative to vehicle controls) are provided within Tables S2.18-S2.20. Similar to embryos treated with TPHP-alone from 24-30 hpf and 24-48 hpf (Figure 2.3C), cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways were significantly affected in all three treatment groups (Figure 2.5D). Interestingly, although fenretinide mitigated TPHP-induced pericardial edema (Figure 2.3C), fenretinide did not mitigate the effect of TPHP on cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways (Figure 2.5D).

TPHP induces liver enlargement in the absence of hepatocellular toxicity

Exposure to 5 μM TPHP from 30-72 hpf resulted in a significant decrease in body length at 128 hpf relative to vehicle controls (Figure 2.7B). This effect was not mitigated by pre-treatment with 2 μM fenretinide, as pre-treatment with 2 μM fenretinide from 24-30 hpf followed by exposure to vehicle (0.1% DMSO) from 30-72 hpf also resulted in a significant decrease in body length at 128 hpf (Figure 2.7B). In addition, exposure to 5 μM TPHP from 30-72 hpf resulted in an increase in liver area in 128-hpf embryos relative to vehicle controls (Figures 2.7A and 2.7C) – an effect that was mitigated by pre-treatment with 2 μM fenretinide from 24-30 hpf. When liver area was normalized to body length, exposure to 5 μM TPHP resulted in a significantly higher liver area to body length ratio, an effect that was also mitigated by pre-treatment with 2 μM fenretinide (Figure 2.7D).

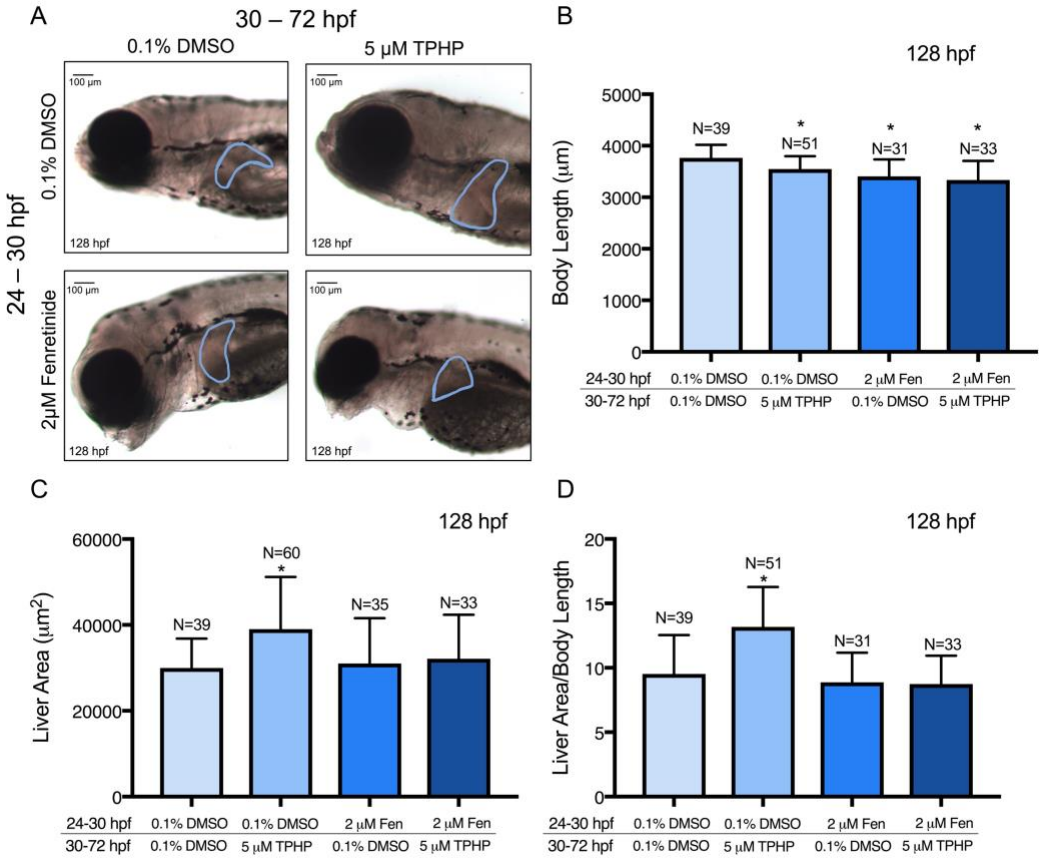

Figure 2.7. Representative brightfield images (A), mean body length (± standard deviation) (B), mean liver area (± standard deviation) (C), and mean body length-normalized liver area (± standard deviation) (D) of 128-hpf embryos pre-treated with either vehicle (0.1% DMSO) or 2 µM fenretinide (Fen) from 24-30 hpf, treated with vehicle (0.1 % DMSO) or 5 µM TPHP from 30-72 hpf, and then reared in clean system water until 128 hpf. Within Panel A, the blue border denotes an outline of the liver on the left lateral side of a 128-hpf embryo.

Interestingly, although liver area at 128 hpf was increased following exposure to TPHP, H&E-stained histologic sections from TPHP-exposed livers were qualitatively similar to livers from vehicle controls and embryos pre-treated with 2 µM fenretinide followed by exposure to vehicle or TPHP (Figure 2.8). Overall, these data suggest that, at 128 hpf, TPHP exposure from 30-72 hpf resulted in liver enlargement in the absence of hepatocellular toxicity (based on histology), an effect that, similar to pericardial edema, was mitigated by pre-treatment with fenretinide.

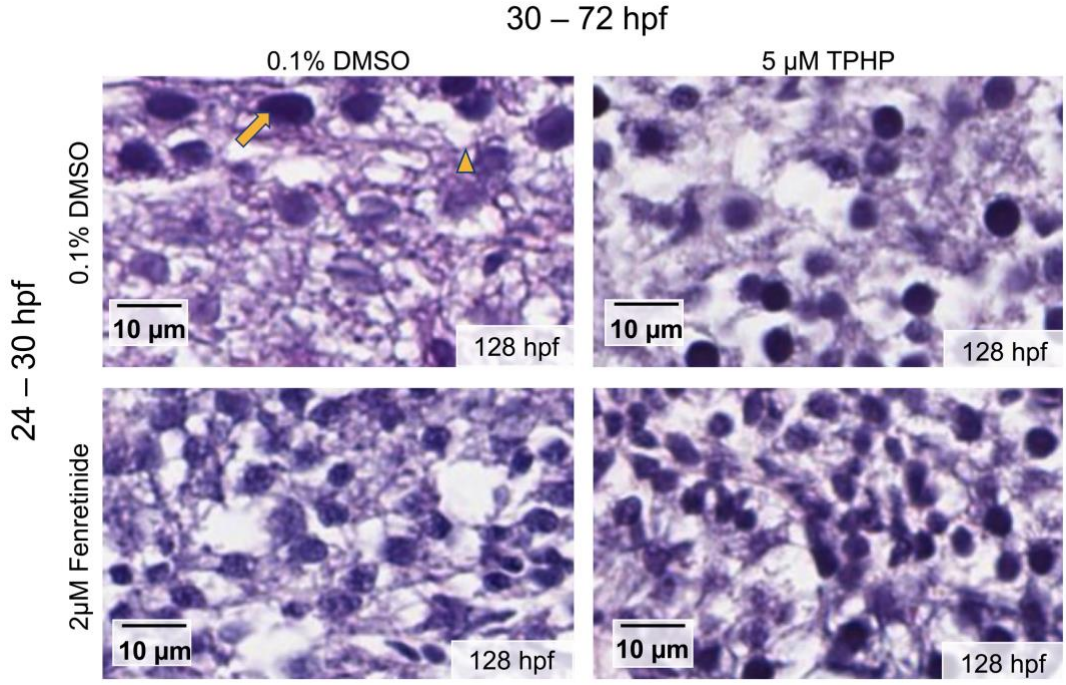

Figure 2.8. Representative histologic liver sections from 128-hpf embryos pre-treated with vehicle (0.1% DMSO) or 2 µM fenretinide (Fen) from 24-30 hpf, treated with vehicle (0.1% DMSO) or 5 µM TPHP from 30-72 hpf, and then reared in clean system water until 128 hpf. Nuclei (dark purple) are stained with hematoxylin (yellow arrow), proteins (light purple) are stained with eosin, and empty spaces (white) denote vacuolization (yellow arrowhead) within the embryonic liver.

TPHP induces significant changes in embryonic lipids and metabolites

Neutral lipid abundance within the whole embryo was measured using Oil Red O staining; representative images are provided in Figure 2.10A. Exposure to 2.5, 5, and 10 µM of TPHP from 24-72 hpf resulted in a concentration-dependent decrease in the color intensity of Oil Red O within the total body (Figure 2.9A), head (Figure 2.9B), yolk sac (Figure 2.9C), trunk (Figure 2.9D) and pericardial region (Figure 2.9E). Interestingly, exposure to 10 µM TPHP from 30 to 72 hpf affected biological processes associated with tricarboxylic acid cycle and sphingolipid metabolic process (Figure 2.6), an effect that was driven by four transcripts (*cs*, *sdhc*, *serinc1*, and *psap*) (Table S2.12).

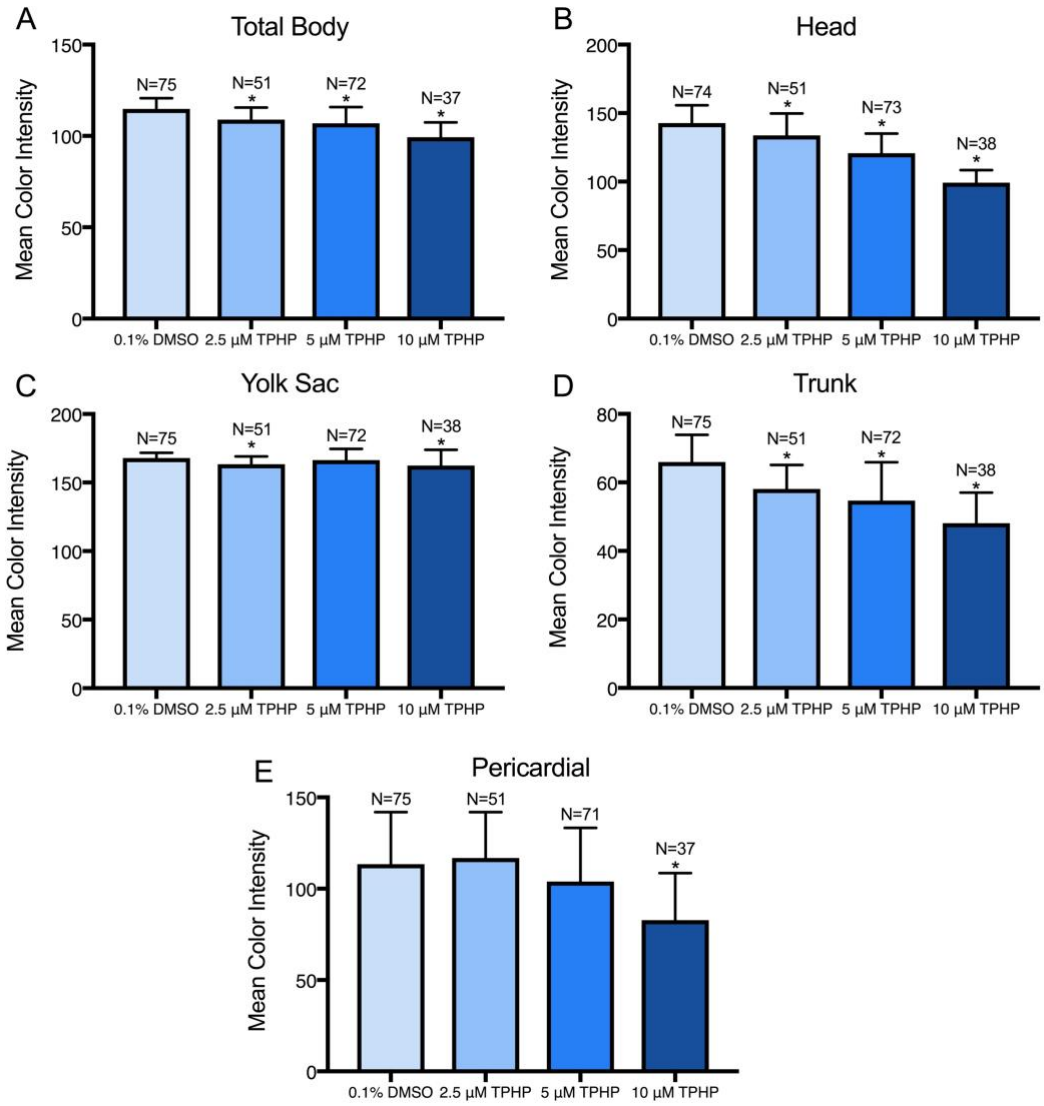

Figure 2.9. Mean color intensity (± standard deviation) of the total body (A), head (B), yolk sac (C), trunk (D), and pericardial (E) of 72-hpf embryos treated with either 1) vehicle (0.1 % DMSO) or TPHP (2.5, 5, or 10 µM) from 24-72 hpf

Interestingly, pre-treatment with 2 µM fenretinide from 24-30 hpf did not block TPHP-induced effects on Oil Red O-specific color intensity within the whole embryo (Figure 2.10B) and the embryonic head (Figure 2.10C), pericardial (Figure 2.10D), trunk (Figure 2.10E) and yolk sac (Figure 2.10F) region.

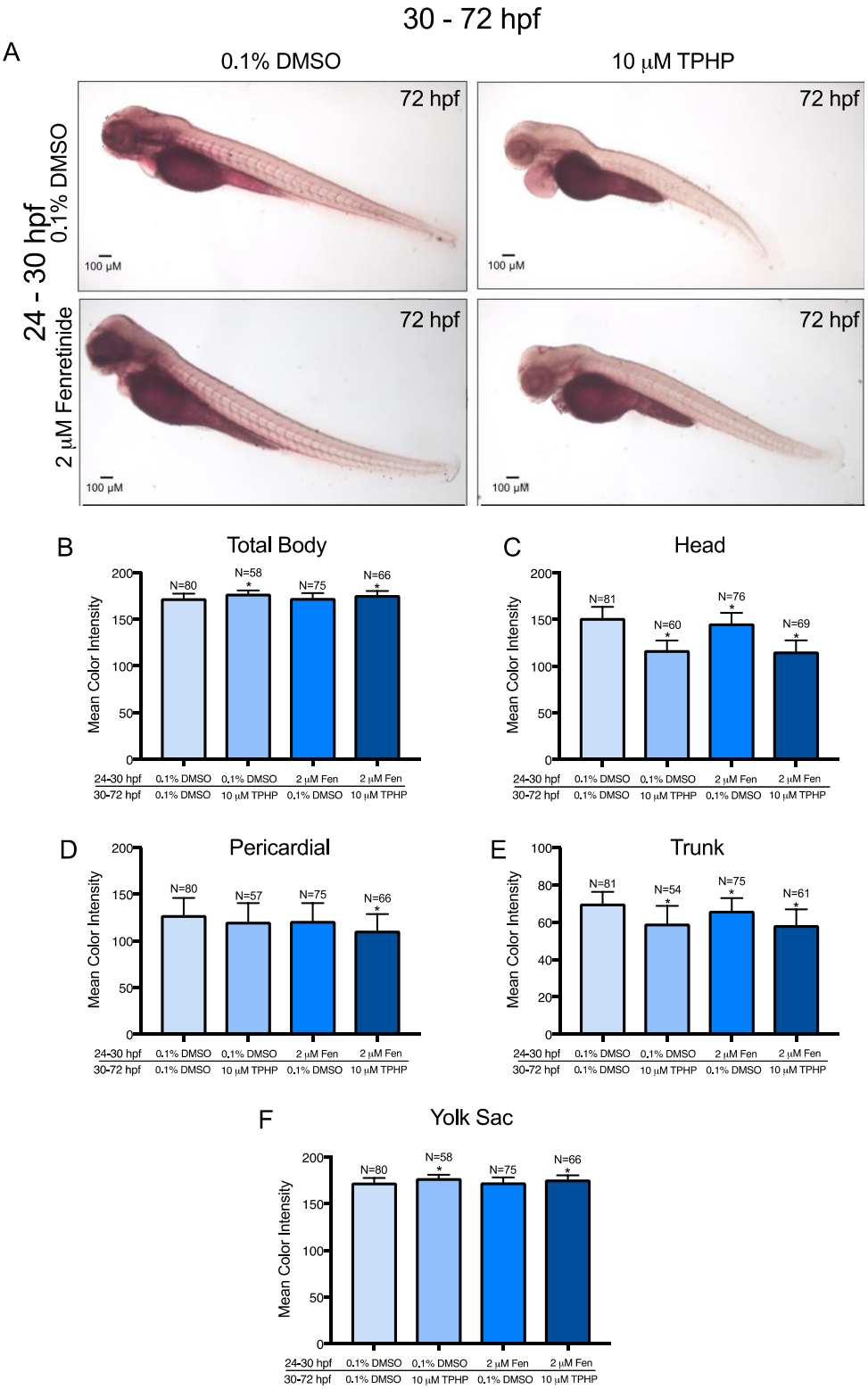

Figure 2.10. Oil Red O-stained representative images (A), mean color intensity (± standard deviation) of the head (B,D) and trunk (C,E) of 72-hpf embryos treated with either 1) vehicle (0.1 % DMSO) or TPHP (2.5, 5, or 10 μM) from 24-72 hpf or 2) pre-treated with vehicle (0.1% DMSO) or 2 μM fenretinide (Fen) from 24-30 hpf and then treated with vehicle (0.1 % DMSO) or 10 μM TPHP from 30-72 hpf.

All metabolites quantified using untargeted metabolomics are provided within Table S2.21. Despite strong stage-dependent differences in metabolite abundances between 48 and 72 hpf (Figure 2.11A), the total abundance of all metabolites was significantly decreased following exposure to 10 μM TPHP from 30-48 hpf and 30-72 hpf (Figure 2.11B). Out of 102 metabolites that were identified, betaine was the most significantly affected metabolite at 48 and 72 hpf following exposure to vehicle (0.1% DMSO) or 10 μM TPHP (Figure 2.11C).

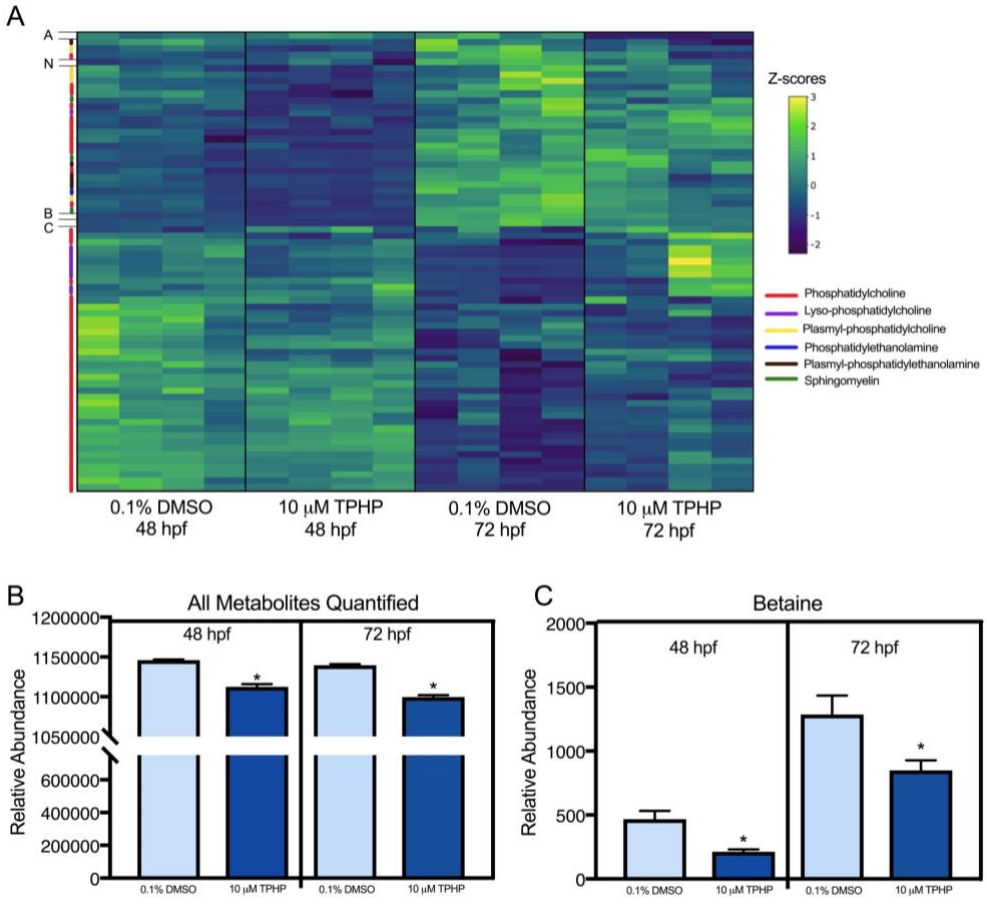

Figure 2.11. Untargeted metabolomics analysis (A) of 48- and 72-hpf embryos treated with either vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf or 30-72 hpf, respectively. Data shown within the heat map are z-scored. Each column represents an individual biological replicate, each row represents an individual metabolite, and each color on the y-axis represents a different metabolite class represented within the legend to the right. Acylcarnitine 22:6 (A), N-acetylhistidine (N), Betaine (B) and Cholesterol/Lathosterol (C) did not fall within any of the broader metabolite classes. Relative abundance (± standard deviation) of all metabolites (B) and betaine (C) within 48- and 72-hpf embryos treated with either vehicle (0.1% DMSO) or 10 μM TPHP from 30-48 hpf or 30-72 hpf, respectively.

2.4 Discussion

Although TPHP is known to disrupt cardiac looping and induce pericardial edema in zebrafish embryos (Isales et al., 2015; McGee et al., 2013; Mitchell et al., 2018), little is known about the target and mechanism of TPHP-induced cardiotoxicity during early development. Although cardiac looping occurs between 36 and 48 hpf (Bakkers, 2011), we found that initiation of TPHP exposure at 24 hpf did not result in cardiotoxicity before (30 hpf) or immediately after (48 hpf) cardiac looping, suggesting that TPHP-induced cardiotoxicity (detected at 72 hpf) occurs between 48-72 hpf. However, IPA's toxicity analysis revealed that transcripts associated with cardiotoxicity, hepatotoxicity and nephrotoxicity were significantly affected by TPHP at 30 and 48 hpf, suggesting that effects on the transcriptome occurred in the absence of detectable effects on cardiac development.

Interestingly, while fenretinide (a pan-RAR agonist) blocked TPHP-induced pericardial edema, pre-treatment with fenretinide did not block TPHP-induced effects on cardiotoxicity-related pathways, a finding consistent with our recent study demonstrating that fenretinide does not mitigate TPHP-induced effects on the distance between the sinus venosus and bulbus arteriosis (SV-BA) (Mitchell et al., 2019). Fenretinide is a synthetic retinoid and analogue of retinol (vitamin A) with known anti-inflammatory properties (Kanagaratham et al., 2014). In mice, fenretinide decreases and increases the concentration of arachidonic acid (an initiator of inflammation) and docosahexaenoic acid (anti-inflammatory compound) (López-Vales et al., 2010), respectively, resulting in a net decrease in inflammation and edema formation. As retinoic acid blocks edema formation within embryonic zebrafish following alcohol and paclobutrazol exposure (Marrs et al., 2010; Wang et al., 2017), fenretinide may have blocked TPHP-induced pericardial edema formation from 48-72 hpf by decreasing inflammation and/or fluid accumulation. Interestingly, based on biological processes

identified by DAVID, ion transport was significantly altered after exposure to 10 μM TPHP – a biological process that was mitigated by pretreatment with 2 μM fenretinide. These data suggest that fenretinide may play a role in blocking TPHP-induced effects on ion transport and, as a result, edema formation.

TPHP induces cytotoxicity within hepatocytes derived from chicken embryos (Su et al., 2014), and increases levels of reactive oxygen species in HepG2 cells (Zheng et al., 2018). Moreover, the rate of TPHP metabolism within human hepatocytes is slower compared to certain organophosphate flame retardants (e.g., tris(2-butoxyethyl) phosphate), resulting in the potential for bioaccumulation and persistent toxicity within the liver (Van den Eede et al., 2016). Within this study, IPA revealed that the majority of transcripts significantly affected by TPHP at 30 and 48 hpf were associated with hepatotoxicity pathways. Indeed, exposure to TPHP from 30-72 hpf resulted in an increase in liver area at 128 hpf. This effect occurred in the absence of detectable histologic changes (e.g., necrosis, fatty liver, and inflammation) within the liver, suggesting that enlargement of the liver was not due to hepatocellular hypertrophy, hyperplasia, lipid accumulation, nor inflammation. In addition, TPHP-induced liver enlargement was blocked by pre-treatment with fenretinide, suggesting that, similar to pericardial edema, fenretinide may block fluid accumulation within the liver.

Prior studies have shown that TPHP disrupts hepatic carbohydrate and lipid metabolism in adult zebrafish and mice (Du et al., 2016; D. Wang et al., 2019a), an effect that may be driven by activation of peroxisome proliferator-activated receptor γ (PPARγ) (Pillai et al., 2014). Using Oil Red O staining, we found that TPHP exposure resulted in a concentration-dependent decrease in neutral lipids within the embryo (particularly within the head and trunk), an effect that was not blocked by pre-treatment with fenretinide. Similarly, untargeted metabolomics revealed that TPHP resulted in a

significant decrease in the total abundance of lipid-specific metabolites at 48 and 72 hpf. Within zebrafish, previous studies have demonstrated that a decrease in yolk sac-associated lipids from 48 to 72 hpf is accompanied by an increase in body-associated lipids (Fraher et al., 2015). Therefore, TPHP may have an impact on the abundance, movement, and/or utilization of lipids from the yolk sac to the body from 48 to 72 hpf.

Among all of the metabolites quantified, betaine was the most significantly affected metabolite following TPHP exposure. In mice and humans, betaine plays an important role in liver and kidney function. Human patients with non-alcoholic liver diseases have significantly decreased hepatic betaine concentrations, suggesting that decreased betaine levels are associated with liver toxicity (Sookoian et al., 2017). Within kidneys, betaine acts as an osmoprotectant to ensure that osmolarity is balanced to allow water reabsorption (Kempson et al., 2013). Osmoprotectants raise the osmotic pressure in the cytoplasm by stabilizing enzyme and protein structures in order to maintain the structure of membranes when exposed to stress (McNeil et al., 1999). Within our study, decreased betaine concentrations following TPHP exposure may have precluded adaptation to osmotic stress, leading to disruption of osmotic balance and fluid accumulation throughout the entire embryo. As liver enlargement did not appear to be a result of hepatocellular hypertrophy, it is possible that an increase in liver area was due to accumulation of fluid within or surrounding the liver. Indeed, our recent study demonstrated that co-exposure to TPHP and mannitol (an osmoprotectant) resulted in a significant decrease in pericardial edema and SV-BA length relative to TPHP-alone (Mitchell et al., 2019), suggesting that the presence of an osmoprotectant in the surrounding water mitigates TPHP-induced fluid accumulation within zebrafish embryos.

In summary, our data collectively suggest that, in addition to cardiotoxicity, TPHP adversely affects hepatotoxicity-related pathways, liver morphology, neutral

lipid abundance, and metabolite abundance within developing zebrafish embryos. Moreover, our data suggest that TPHP decreases baseline betaine concentrations, leading to potential direct effects on osmoregulation and indirect effects on organ development. However, additional research is needed to (1) determine if the addition of excess betaine mitigates TPHP-induced effects on the developing embryo and (2) determine whether PPARγ (a known target for TPHP) is required for TPHP-induced effects on osmoregulation.

Chapter 3: Longer Commutes are Associated with Increased Human Exposure to Tris(1,3-dichloro-2-propyl) phosphate

3.0 Abstract

Organophosphate esters (OPEs) are a class of semi-volatile organic compounds (SVOCs) used as flame retardants, plasticizers, and anti-foaming agents. Due to stringent flammability standards in vehicles and the ability of OPEs to migrate out of end-use products, elevated concentrations of OPEs have been found in car dust samples around the world. As many residents of Southern California spend a significant amount of time in their vehicles, there is potential for increased exposure to OPEs associated with longer commute times. As approximately 70% of the University of California, Riverside's undergraduate population commutes, the objective of this study was to use silicone wristbands to monitor personal exposure to OPEs and determine if exposure was associated with commute time in a subset of these students. Participants were asked to wear wristbands for five continuous days and complete daily surveys about the amount of time spent commuting. Data were then used to calculate a participant-specific total commute score. Components of Firemaster 550 (triphenyl phosphate, or TPHP, and isopropylated triaryl phosphate isomers) and Firemaster 600 (TPHP and tert-butylated triaryl phosphate isomers) – both widely used commercial flame retardant formulations – were strongly correlated with other OPEs detected within participant wristbands. Moreover, the concentration of tris(1,3-dichloro-2-propyl) phosphate (TDCIPP) was significantly correlated with the concentration of several Firemaster 500 components and tris(2-chloroisopropyl) phosphate (TCIPP). Finally, out of all OPEs measured, TDCIPP was significantly and positively correlated with total commute score, indicating that longer commutes are associated with increased human exposure to TDCIPP. Overall, our findings raise concerns about the potential for chronic TDCIPP exposure within vehicles and other forms of

transportation, particularly within densely populated and traffic-congested areas such as Southern California.

3.1 Introduction

Organophosphate esters (OPEs) are a class of semi-volatile organic compound (SVOCs) used as flame retardants, plasticizers, and anti-foaming agents (Wei et al., 2015). OPEs are used in a wide range of products including cosmetics, textiles, polyurethane foam, upholstery, electric cables, lubricants, floor waxes/polishes, construction materials, and vehicles (Marklund et al., 2003; Wei et al., 2015). As OPEs are not chemically bound to these products, OPEs have the potential to readily migrate into surrounding environmental media such as indoor air and dust. Interestingly, OPE concentrations within indoor air are significantly higher relative to outdoor air, suggesting that the built environment represents a primary source of OPEs (Carlsson et al., 1997). Indeed, OPEs are ubiquitous within indoor dust around the world, with concentrations ranging from 1 to 577 µg/g within industrialized countries such as the United States, Switzerland, Denmark, Sweden, China, and Japan (Araki et al., 2014; Bergh et al., 2011; Hartmann et al., 2004; He et al., 2015; Langer et al., 2016; Stapleton et al., 2009). The wide range of concentrations may be attributed to different country-specific flammability standards and approaches to meet these standards.

In addition to buildings and homes, dust samples collected from vehicles also have OPE concentrations ranging from 1.1 ng/g to 1100 µg/g (Abdallah and Covaci, 2014; Ali et al., 2013; Brandsma et al., 2014; Harrad et al., 2016). Interestingly, the total mass of OPEs is higher in car dust samples compared to indoor dust samples from buildings and homes (Ali et al., 2016; Brandsma et al., 2014; Brommer and Harrad, 2015; Christia et al., 2018; Zhou et al., 2017). As OPEs are commonly used

as flame retardants (Wei et al., 2015), this finding is likely attributed to more stringent, vehicle-specific flammability standards. For example, Federal Motor Vehicle Safety Standard (FMVSS) No. 302 – the federal-level flammability standard for car interiors in the United States – is more rigorous than the California-specific smolder resistance test for upholstered furniture (Technical Bulletin 117-2013). Within FMVSS No. 302, the ignition source (a Bunsen burner) is placed 19 mm below interior materials tested (e.g., seat cushions, seat backs, seat belts, etc.), and these materials fail if any are burned or transmit a flame across its surface (>102 mm/minute).

While prior studies have used active samplers on lapels (Tsai and Vincent, 2001) and within backpacks (Nethery et al., 2012) to monitor individual human exposure to environmental chemicals, these samplers often require a battery-powered pump to force air through the sampling module (Bohlin et al., 2007). Biological samples such as urinary and serum biomarkers are also used to measure human exposure, but biological sample collection may be invasive and result in smaller sample sizes due to lower study participation rates (Aerts et al., 2018; Needham et al., 2005). Alternatively, passive sampling represents another method of measuring human exposure, where air-borne SVOCs diffuse into the lipophilic membrane of passive samplers (Anderson et al., 2017).

Silicone wristbands are non-invasive and have been rapidly adopted to support human exposure assessments within the United States and abroad. For example, wristbands have been used to measure 1) brominated flame retardants (BFRs) and OPEs in preschool children (Kile et al., 2016); 2) pesticides, environmental chemicals, and personal care products in agricultural and urban communities (Bergmann et al., 2017); and 3) polycyclic aromatic hydrocarbons (PAHs) in pregnant women (Dixon et al., 2018). Interestingly, PAHs detected on wristbands were more strongly associated with urinary metabolites compared to active air monitoring samplers (Dixon et al.,

2018). Furthermore, when comparing measurements of OPEs on hand wipes and metabolites in pooled urine samples, OPEs measured on wristbands were more strongly associated with urinary metabolites compared to hand wipes (Hammel et al., 2016). Likewise, polybrominated diphenyl ethers (PBDEs) measured within wristbands were positively associated with serum biomarkers from the same participants (Hammel et al., 2018), and OPEs detected in wristbands were positively associated with hand wipes, brooches, and active air samples (Wang et al., 2019). Therefore, wristbands have the potential to account for both inhalation and dermal routes of exposure and represent a cost-effective and non-invasive tool to measure biologically relevant human exposure.

In Southern California (particularly within the Inland Empire), residents spend an average of 62.45 minutes per day commuting to and from work (Sirotnik et al., 2018). As approximately 19% of commuters spend two or more hours within their vehicles per commute (Sirotnik et al., 2018), this raises concerns about the potential health risks associated with spending extended periods of time in their personal vehicles over multiple days, weeks, years, and possibly decades. While elevated urinary concentrations of the primary TDCIPP metabolite (bis(1,3-dichloro-2-propyl) phosphate, or BDCIPP) were previously associated with spending more than one hour per day within vehicles (Hammel et al., 2016), to our knowledge human OPE exposure as a function of total commute time has not been evaluated to date. Therefore, the overall objective of the study was to leverage silicone wristbands to monitor personal OPE exposure within a subset of commuter vs. non-commuter undergraduate students at the University of California, Riverside (UCR). As 70% of UCR's undergraduate population commutes to campus multiple times a week from all over Southern California, we hypothesized that OPE exposure is elevated for students who spend longer amounts of time commuting to and from UCR.

3.2 Materials and Methods

Study Design. Study participants (N=88) were recruited in January/February 2019 from UCR. Participants were eligible for the study if they were 1) at least 18 years old; 2) willing to wear a silicone wristband continuously for five days; and 2) willing to complete five, one-minute online questionnaires. All study protocols and materials used for this study were approved by UCR's Institutional Review Board (IRB Number: HS-18-162), and each participant provided informed consent prior to enrolling in the study.

Wristband Collection. Blue wristbands were purchased in a single size from 24HourWristbands.Com (https://24hourwristbands.com/) (Houston, TX, USA) and, using previously described procedures (Hammel et al., 2016), cleaned with two, 12-h Soxhlet extractions with 1:1 ethyl acetate/hexane (v/v) and 1:1 ethyl acetate/methanol (v/v) as well as passive drying in a fume hood. Wristbands were then wrapped in pre-baked (at 450°C) aluminum foil and placed in ziplock bags. Participants were asked to wear wristbands continuously for five days, including during bathing, sleeping, and other daily activities. During the 5-d study period, weather conditions did not significantly vary, with an average outdoor temperature ranging from ~4.4-13.3°C (~40-56°F), average precipitation of 0.19 in, and average humidity of 60%. At the end of the study period, participants re-wrapped their wristband in either the originally provided aluminum foil or a clean (i.e., pre-baked) piece of aluminum foil, and then placed the wrapped wristband in the originally provided ziplock bag. Six solvent-rinsed wristbands were used as field blanks to account for potential OPEs present within wristbands as well as OPE exposure during shipping and handling. Field blanks were un-wrapped for 30 s at room temperature on the third floor of the Science Laboratories 1 Building at UCR, and then re-wrapped with the same aluminum foil. Participant

wristbands and field blanks were stored at -20°C until overnight shipment on dry ice to Duke University (Durham, NC, USA) for extraction and analysis.

Questionnaires and Calculation of Commute Scores. Study participants completed an initial recruitment survey. In this survey, participants were asked to provide demographic information (age, gender, ethnicity, and household income) as well as whether they use transportation to commute to campus. During the study, participants completed five short online surveys (one survey per day). Participants were asked if they had used a form of transportation on that day (personal vehicle, rideshare, vanpool, public transport, or other) and, if so, how much time they spent in each mode of transportation (<30 min, 30-60 min, 60-180 min, or >180 min). For this study, we did not collect personal vehicle information (year, make, and model), as prior studies have shown that OPE concentrations in car dust are not significantly correlated with these characteristics (Abdallah and Covaci, 2014; Brommer and Harrad 2015; Christia et al., 2018; Harrad et al., 2016). While we did collect information about the participant's residence ZIP code, we were unable to rely on these data since, after completion of the study, we discovered that some study participants reported ZIP codes for permanent (e.g., family) addresses rather than addresses for current residences.

The amount of time a participant spent in a mode of transportation was used to calculate their total commute score. Each time bracket was assigned a daily commute score: <30 min was assigned 1; 30-60 min was assigned 2; 60-180 min was assigned 3; and >180 min was assigned 4. The daily commute scores were then summed to obtain the total commute score for each participant. For participants who did not complete all five surveys, averages were calculated based on surveys that were completed (90.9% of participants completed all five surveys) and multiplied by five (for

each day of the study). For the purpose of this study, we assumed that a longer time spent in a mode of transportation was associated with a longer commute time.

Extraction and Analysis of OPEs from Wristbands. Wristbands were extracted and analyzed using a modified version of the method reported by Hammel et al. (2016) and Hammel et al. (2018). Each wristband (samples and field blanks) was cut into ~1-in fragments and the mass of each sample was recorded (~0.8 g per wristband). The wristband fragments were transferred to a clean glass centrifuge tube, spiked with isotopically-labeled compounds (Table S3.1), and extracted via sonication in 10 mL of a 50:50 (v:v) mixture of hexane:dichloromethane for 15 min. The extraction was repeated three times and the extracts were combined. Each sample extract was concentrated to ~1.0 mL using purified nitrogen gas prior to column chromatography. Extracts were purified using 8 g of deactivated, 100-200 mesh Acros Organics Florisil (Thermo Fisher Scientific, Waltham, MA, USA). An F1 fraction (40 mL hexane) and F2 fraction (40 mL ethyl acetate) were eluted and collected. The F1 and F2 extracts were combined and concentrated to 1 mL. Sample extracts were then concentrated to near dryness and reconstituted in 1 mL of hexane. Isotopically labelled recovery standards (Table S3.2) were spiked into each sample prior to mass spectrometry analysis.

Samples were analyzed for OPEs using a Q Exactive GC Hybrid Quadrupole-Orbitrap GC-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA) operated in full scan Electron Ionization (EI) mode. Field blanks (clean wristbands; N=6) and lab blanks (solvent; N=5) were processed and analyzed with each batch of wristbands for quality assurance and quality control. No significant differences were detected between field and lab blanks; therefore, field blanks were used to estimate method detection limits (MDLs). MDLs were three times the standard deviation of the field blank responses, or a value equal to ten times the signal-to-noise if the analyte was

not detected in the field blanks. MDLs for all target OPEs are available in the supporting information (Table S3.6)and were generally at or below 1 ng/g for all target OPEs. Recoveries for all OPE surrogate standards are reported in Table S3.6.1, and the average across all OPE surrogate standards was 105 ± 44% (median: 100%). Analyte concentrations (in ng of analyte per g wristband) were calculated based upon standard calibration curves and all samples were recovery-corrected and blank-subtracted.

Statistical Analyses. A general linear model (GLM) and Tukey's post-hoc test (α=0.05) was performed using SPSS Statistics 24 (IBM, Armonk, NY, USA) to identify significant differences between total commute scores and demographic data (age, gender, ethnicity, or household income). As OPE concentrations across all participant wristbands displayed a log-normal distribution, a heat map based on $\log_{10}$-transformed concentrations for all OPEs was generated within Morpheus (Broad Institute, Cambridge, MA, USA), and hierarchical clustering was performed using the Euclidean distance and complete linkage method. Within Prism v8.0.2 (GraphPad, San Diego, CA, USA), Spearman's correlation coefficients (r_s) were calculated to examine potential associations between OPEs. Within SPSS Statistics 24 (IBM, Armonk, NY, USA), GLM (α=0.05) was performed to determine whether total commute scores were predictive of $\log_{10}$-transformed concentrations for OPEs detected on at least 62 wristbands (70% detection rate). Based on these results, statistically significant OPEs were then reanalyzed within SPSS Statistics 24 (IBM, Armonk, NY, USA) using an adjusted GLM (α=0.05) in order to correct for age as a covariate.

3.3 Results and Discussion

Longer commutes are associated with older study participants

Demographic data for all study participants are included within Table S3.3. A summary of demographic data demonstrates that, for the most part, study participants (Figure 3.1) represented demographics of UCR's entire undergraduate population (Figure 3.2). While our study did not recruit participants younger than 18 years old, 89% of participants were between 18 to 21 years old (Figure 3.1A) – a characteristic that was similar to the age distribution within UCR's undergraduate population (Figure 3.2A). On the other hand, the gender of our study population was skewed towards a higher proportion of females compared to males (Figure 3.1B) – a characteristic that was not reflective of UCR's undergraduate population (Figure 3.2B) – since there were fewer male participants who had an initial interest in our study despite our diverse recruitment methods. Similar to age, the distribution of study participant ethnicity (Figure 3.1C) was similar to UCR's undergraduate population (Figure 3.2C), where the top four ethnicities were Hispanic, Asian, White, and Multi-Racial. The "other" ethnicity category within the undergraduate population (Figure 3.2C) includes ethnicities that, despite various attempts to recruit campus-wide, were not represented within our final study population (Black or African American; Native Hawaiian or Pacific Islander; or Unknown). Finally, the average household income – either reported as parental or personal income from dependent or independent students, respectively – for our study population contained a higher proportion of students within the average household income of <$30,000 and $75,001-$110,000 groups (Figure 3.1D), a characteristic that was not similar to UCR's undergraduate population (Figure 3.2D).

While the distribution of certain demographics was not identical to UCR's undergraduate population, the total commute score of study participants did not vary by gender, ethnicity, nor household income (Figures 3.3B, 3.3C, and 3.3D). As

described within Section 2.3, total commute score was calculated based on the amount of time the participant spent in all forms of transportation – which was primarily personal vehicles – during each day of the study (Tables S3.4 and S3.5. Interestingly, total commute score was significantly (~2-3 times) higher among all age groups relative to participants that were 18 years old (Figure 3.3A), a finding that was likely due to a larger number of 18-year-old freshman students that live within residential halls or are within walking distance to campus. As a result, this subset of the student population likely spends less time within personal vehicles during a typical week of the academic year.

Study Population

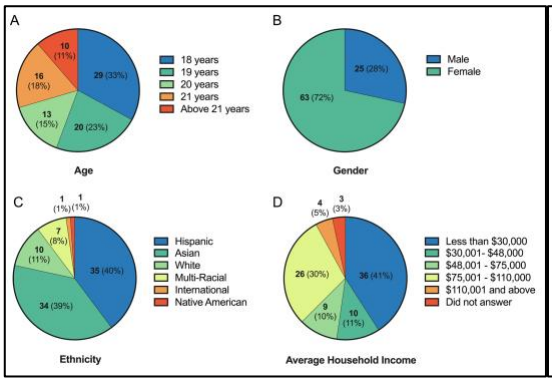

Figure 3.1. Demographics of study participants (N=88) grouped by age (A), gender (B), ethnicity (C), or average household income (D). Bold numbers denote the number of participants within each category, and numbers within parentheses denote the percent of total study participants.

UCR Undergraduate Population

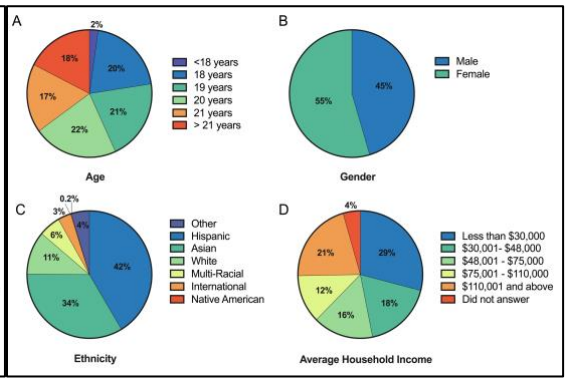

Figure 3.2. Demographics of the entire UCR undergraduate student population (N=20,581 based on Fall 2018 headcount) grouped by age (A), gender (B), ethnicity (C), or average household income (D). Bolded numbers denote the percent of the total undergraduate student population.

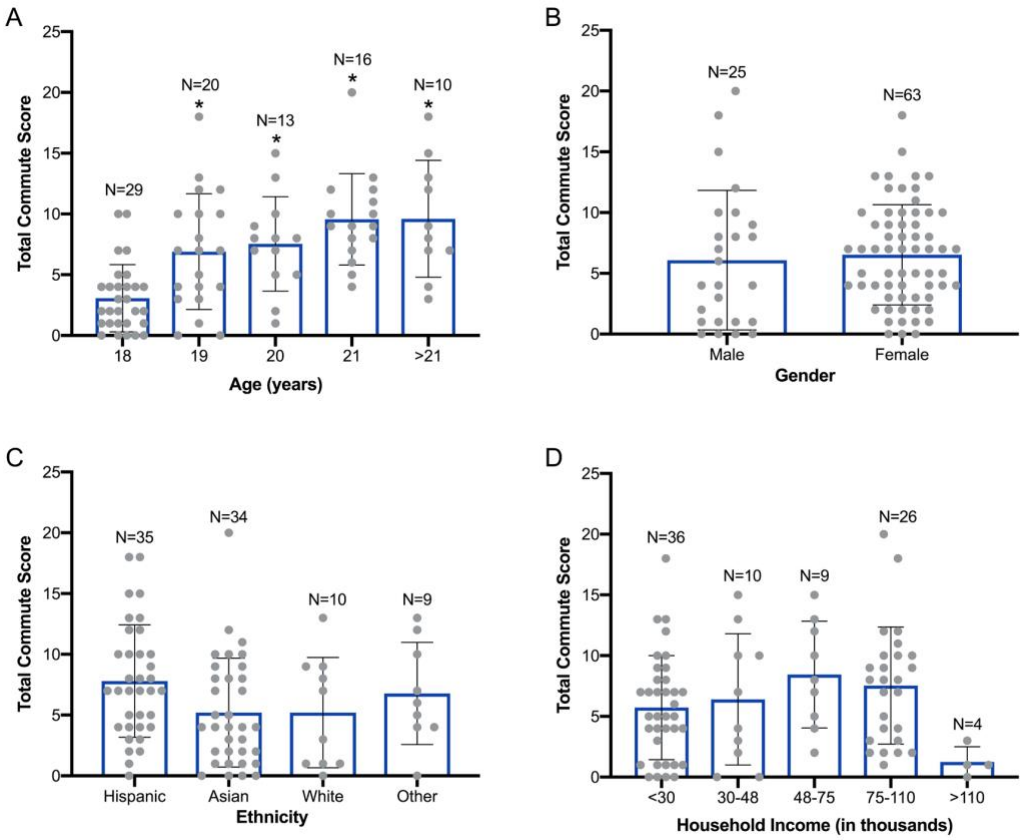

Figure 3.3. Total commute score of study participants grouped by age (A), gender (B), ethnicity (C), and household income (D). N=88 for age, gender, and ethnicity; N=85 for household income as a result of three participants not responding (Table S3.1). Asterisk (*) denotes significant difference ($p<0.05$) relative to the 18-year-old age group within Panel A.

OPE mixtures detected on study participant wristbands are likely driven by overlapping use patterns

Descriptive statistics for all OPEs measured on study participant wristbands are provided within Table S3.6 and summarized within Figure 3.4A. Although total OPE concentrations across all participants ranged from 0.06 to 7604 ng/g, the average concentration by OPE class did not significantly vary by commute score (Figure 3.4B). Spearman's correlation coefficients were calculated for OPEs detected on at least 62 wristbands (70% detection rate) (Figure 3.5). Within the correlation matrix, OPEs were ranked by $-\log_{10}$(p-value) derived from unadjusted GLM-based analyses of total

commute scores and OPE concentrations (Figure 3.5 inset; Table S3.7), with TDCIPP representing the only OPE that was significantly affected by total commute score.

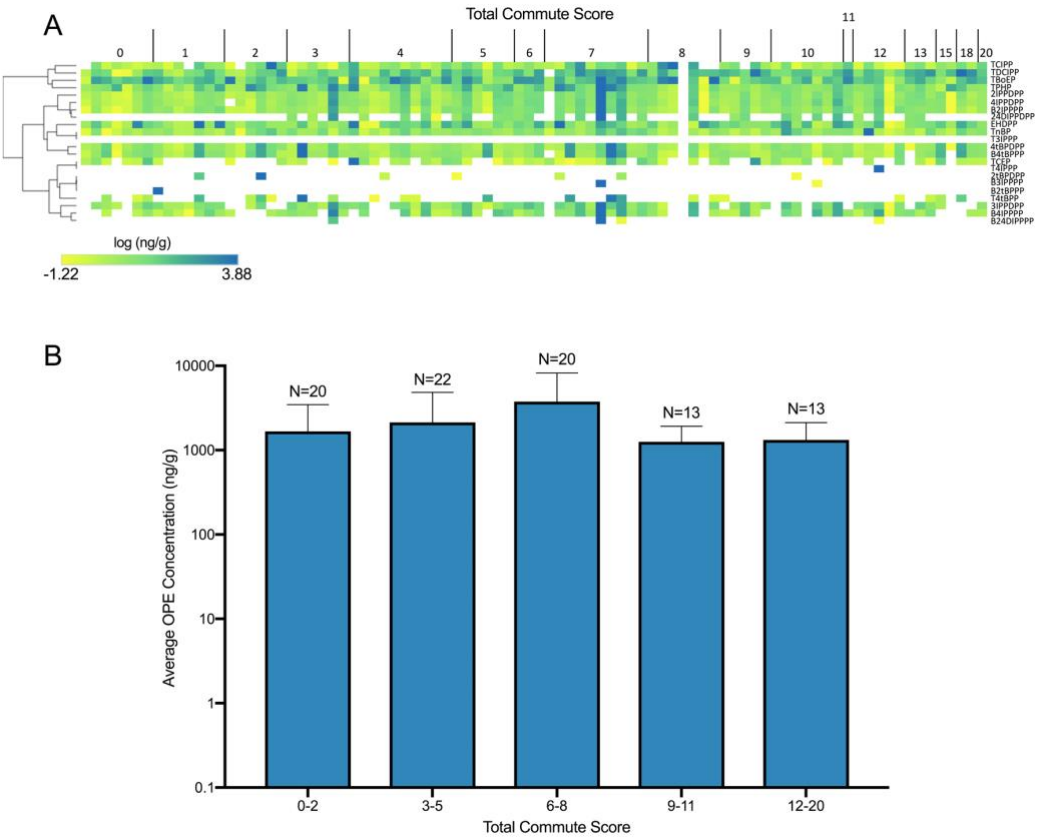

Figure 3.4. Heat map representing wristband concentrations of individual OPEs vs. total commute scores (A). OPE concentration data shown within the heat map were log_{10}-transformed, and hierarchical clustering was performed using the Euclidean distance and complete linkage method. Average concentration (± standard deviation) of all OPEs and total commute score (B).

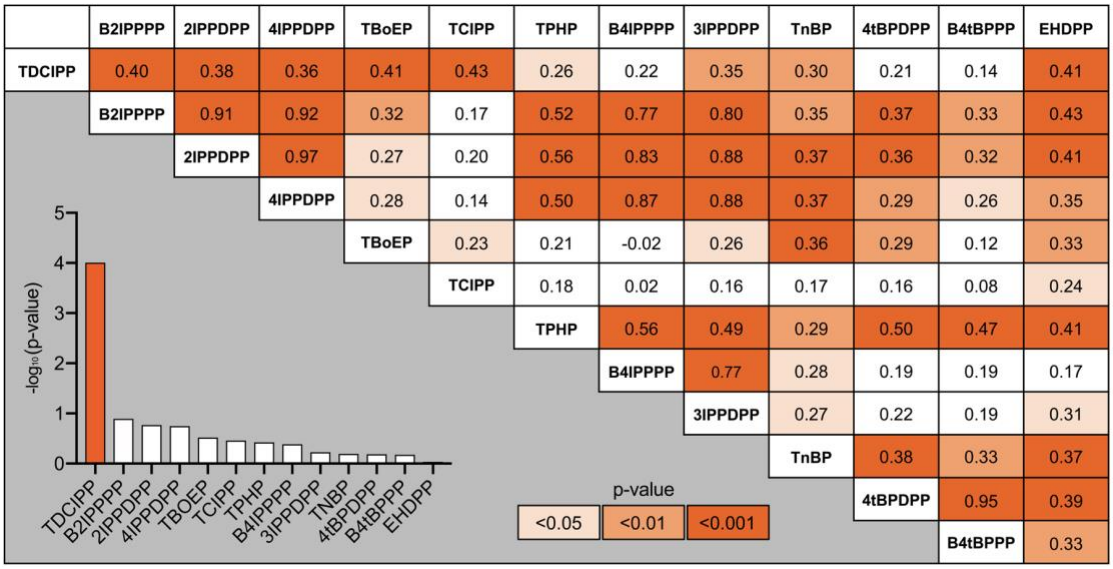

Figure 3.5. Correlation matrix showing Spearman's correlation coefficients for OPEs with >70% detection (at least 62 wristbands) relative to all study participants. OPEs within the matrix are ordered in descending order of $-\log_{10}$(p-value). Bar chart within the inset shows the $-\log_{10}$(p-value) derived from unadjusted GLM-based analyses of total commute scores and OPE concentrations. Darker orange denotes a lower p-value.

Not surprisingly, a large number of OPEs were positively correlated with each other, as multiple OPEs may be used within the same products as well as among different products within the same location. For example, OPEs found within Firemaster 550 (TPHP; 2-isopropylphenyl diphenyl phosphate, or 2IPPDPP; 3-isopropylphenyl diphenyl phosphate, or 3IPPDPP; 4-isopropylphenyl diphenyl phosphate, or 4IPPDPP; bis(2-isopropylphenyl) phenyl phosphate, or B2IPPPP; and bis(4-isopropylphenyl) phenyl phosphate, or B4IPPPP) (Phillips et al., 2017) – an additive flame retardant formulation introduced into automotive headliners as flame lamination and polyurethane foam – were positively correlated with each other (r_s = 0.49-0.97), with isopropylated triaryl phosphate (ITP) isomers showing the strongest and most significant correlations with each other (r_s = 0.77-0.97). Moreover, OPEs found within Firemaster 600 (TPHP; 4-*tert*-butylphenyl diphenyl phosphate, or 4tBPDPP; and bis(4-*tert*-butylphenyl) phenyl phosphate, or B4tBPPP) (Phillips et al., 2017) – a formulation with similar applications as Firemaster 550 – were correlated

with each other (r_s = 0.47-0.94), with tert-butylated triaryl phosphate (TBPP) isomers (B4tBPPP and 4tBPDPP) showing the strongest and most significant correlation with each other (r_s = 0.94). Interestingly, 4tBPDPP (a component of Firemaster 600) was also positively correlated with B2IPPPP (r_s = 0.37) and 2IPPDPP (r_s = 0.36) (both components of Firemaster 550). Similarly, ITP and TBPP isomers within indoor dust are dominated by 2IPPDPP and 4tBPDPP, respectively (Guan et al., 2019; Phillips et al., 2018).

TDCIPP concentrations were also significantly and positively correlated with other OPEs detected on study participant wristbands (Figure 3.5). In addition to Firemaster 550, TDCIPP was, until being added to California's Proposition 65 List in 2011, one of the primary PentaBDE replacements recommended and used to meet flammability standards for upholstered furniture (i.e., California's Technical Bulletin 117 and 117-2013) within residential and commercial environments (EPA, 2015). Therefore, a strong correlation of TDCIPP with certain Firemaster 550 components (4IPPDPP, B2IPPDPP and 21PPDPP) (r_s = 0.36-0.40) suggests that TDCIPP may be applied to products located within the same environment as Firemaster 550-containing products (EPA, 2015; Stapleton et al., 2009). Finally, TDCIPP was significantly correlated with TCIPP (r_s = 0.43), suggesting that, consistent with previously published studies (Stapleton et al., 2012, 2011), both OPEs are used within polyurethane foam-containing products (e.g., baby products and sofas) located within the same environment. Indeed, prior studies found that TDCIPP and TCIPP accounted for the majority of OPE mass in car dust, a finding that was likely due to co-application of both OPEs within vehicles (Brandsma et al., 2014; Brommer and Harrad, 2015; Christia et al., 2018; Zhou et al., 2017).

Longer commutes are predictive of increased TDCIPP concentrations on study participant wristbands

Out of 22 OPEs analyzed (Table S3.6), 13 OPEs were detected on at least 62 wristbands (70% detection rate) (Table S3.7). Among these 13 OPEs, TDCIPP was the only OPE that was significantly predicted by total commute score – even after adjusting for age as a covariate (Table S3.8) – suggesting that longer commutes are associated with increased human exposure to TDCIPP (Figure 3.6). In general, OPE concentrations are higher in dust from vehicles compared to residential and commercial environments (Ali et al., 2016; Brandsma et al., 2014; Brommer and Harrad, 2015; Christia et al., 2018; Zhou et al., 2017), and TDCIPP is prevalent within dust of personal vehicles in the United Kingdom (Brommer et al., 2012). Moreover, Harrad et al. (2016) found that TDCIPP concentrations are consistently high within cars, suggesting that polyurethane foam may be a primary source of TDCIPP exposure within vehicles around the world. Indeed, TDCIPP is commonly detected within dashboard dust (Brandsma et al., 2014), suggesting that, similar to residential and commercial environments, TDCIPP readily migrates from vehicle components over time.

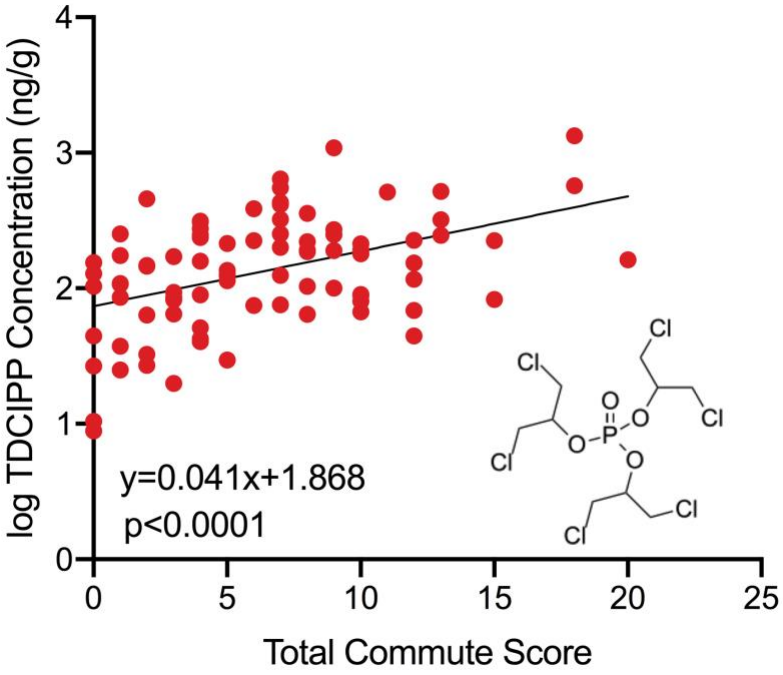

Figure 3.6. Log TDCIPP concentration as a function of total commute score. Corrected model p-value was derived from an adjusted GLM (α=0.05) in order to correct for age as a covariate. N=86 wristbands (out of 88 total) with detectable TDCIPP.

Following the addition of TDCIPP to California's Proposition 65 List in 2011 and revision of Technical Bulletin 117 in 2013, the use of TDCIPP to comply with furniture-specific flammability standards has declined within California and across the United States (Cooper et al., 2016). However, as vehicles already contain numerous hazardous chemicals within California's Proposition 65 List, there is a possibility that TDCIPP may continue to be added to permanently installed seats as well as plastic and electronics within a vehicle's dashboard and console, respectively, within the United States in order to comply with a stringent, federal-level flammability standard (FMVSS No. 302) adopted by the National Highway Traffic Safety Administration (U.S. Department of Transportation) in 1971. As a result, compared to upholstered furniture within residential and commercial environments, the use and concentration of TDCIPP is likely higher in vehicle components, a recent trend that may explain why we identified a strong relationship between TDCIPP concentrations and total commute score even after adjusting for age.

3.4 Conclusions

In summary, our findings suggest that longer commutes are associated with increased human exposure to TDCIPP. While previous studies have detected high TDCIPP concentrations within vehicle dust samples, to our knowledge this is the first study to uncover a strong association between the amount of time spent in a vehicle and human TDCIPP exposure. Although we did not collect urine from participants within this study, silicone wristbands have previously been shown to be significantly correlated with urinary biomarkers of exposure for TDCIPP (Hammel et al., 2016), suggesting that urinary BDCIPP (the primary TDCIPP metabolite) concentrations may also be associated with longer commutes. However, since we didn't measure TDCIPP nor other OPEs within vehicle dust, it is unknown whether vehicle dust was the primary source of TDCIPP and other OPEs detected within wristbands. Moreover, since we did not collect personal vehicle information (vehicle year, make, and model), it is unknown whether vehicle year, make, and/or model was significantly associated with TDCIPP concentrations and/or commute score.

As TDCIPP is a SVOC, the primary routes of exposure within vehicles and other forms of transportation are likely inhalation, ingestion, and transdermal permeation following dermal exposure to contaminated surfaces (Hou et al., 2016; Schreder and La Guardia, 2014; Weschler and Nazaroff, 2012). TDCIPP is known to disrupt epigenetic reprogramming, embryogenesis, behavior, liver development, and reproduction in zebrafish (Dasgupta et al., 2019, 2018, 2017; Jarema et al., 2015; Kupsco et al., 2017; C. Liu et al., 2016; Liu et al., 2013; McGee et al., 2012; Volz et al., 2016), and was added as a probable carcinogen on California's Proposition 65 List. Moreover, TDCIPP has also been associated with abnormal pregnancy outcomes such as a decline in fertilization and a shorter gestational period (Carignan et al., 2018; Hoffman et al., 2018). Given that a large fraction of the human population within

Southern California – as well as other densely populated regions across the United States – spend one or more hours commuting on a near-daily basis, our study raises concerns about the potential for chronic TDCIPP exposure within vehicles and possibly other forms of transportation.

Chapter 4: Partial dust removal in vehicles does not mitigate human exposure to organophosphate esters

4.0 Abstract

Organophosphate esters (OPEs) have been detected within car interior dust, suggesting that the indoor microenvironment of vehicles may represent a potential route of human exposure to OPEs. We recently showed that people with longer commutes are exposed to higher concentrations of tris(1,3-dichloro-2-isopropyl)phosphate (TDCIPP) – a widely used OPE – and other studies have suggested that dust removal may lead to lower exposure to chemicals. Therefore, the overall objective of this study was to determine if a decrease in interior car dust results in mitigation of personal OPE exposure. Participants (N=49) were asked to wear silicone wristbands, and a subset of them wiped interior parts at the front of their vehicles prior to one study week (N=25) or both study weeks (N=11). There were no significant differences in total OPE concentrations (77.79-13,660 ng/g) nor individual OPE concentrations (0.04-4852.81 ng/g) across the different wiping groups nor in relation to participant residence ZIP codes and AC/Heater usage. These findings suggest that higher exposure to TDCIPP for participants with longer commutes may be independent of dust located on interior parts at the front of the vehicle. Therefore, our study demonstrates that there is a need for research on the potential contribution of other sources of TDCIPP exposure within car interiors.

4.1 Introduction

The American Community Survey (ACS) by the US Census Bureau reported that approximately 87% of Americans use their personal vehicles to commute to work, and studies have shown that higher commute times are associated with urban population density (Zhu et al., 2017). While the average American spends 53 min

commuting per day, areas with higher population density – such as New Jersey, Massachusetts, Maryland, New York, and California – experience commute times of at least 60 min per day (U.S. Census Bureau). Therefore, a large part of the American population spends a significant amount of time within their vehicles, potentially spanning over many years.

The interior of a personal vehicle is an indoor microenvironment and, due to its limited space, chemicals emitted from the interior of a vehicle have the potential to be concentrated (Faber and Brodzik, 2017). Indeed, volatile organic compounds, flame retardants, perfluoroalkyl compounds, phthalates and particulate matter (Chien, 2007; Geiss et al., 2009; Goosey and Harrad, 2011; Stuart et al., 2008; Zulauf et al., 2019) have been detected in car interior air and dust, suggesting that people who spend a large amount of time in their vehicles may be exposed to elevated concentrations of these contaminants relative to non-commuters. Although the use of chemicals in products, including vehicles, is regulated by the Toxic Substances Control Act (TSCA) in the United States, the release of chemicals from vehicle parts and subsequent impact on air quality within car interiors are not monitored nor regulated. Furthermore, while the US Environmental Protection Agency (EPA) and Occupational Safety and Health Administration (OSHA) regulate outdoor (ambient) air quality and indoor air quality within the workplace, respectively, vehicles are, similar to residences, considered personal space and lack regulations controlling indoor air quality (US EPA, *WHO*, 2010). As a result, there is a large gap in our knowledge about personal exposure within vehicles and more research is needed to measure human exposure to chemicals within indoor microenvironments of car interiors.

Silicone wristbands have been used as passive samplers to measure exposure to chemicals within indoor environments (Hammel et al., 2016; Levasseur et al., 2021) and have shown to be positively correlated with both hand wipes and active air

samplers (Hammel et al., 2020; S. Wang et al., 2019). As wristband chemical levels correlate well with both urinary and serum biomarkers, wristbands enable assessment of both inhalation and dermal routes of exposure and, as such, are useful for measuring human exposure to chemicals via air and dust (Hammel et al., 2018, 2016). This is particularly relevant for semi-volatile organic compounds (SVOCs) that readily partition from the gas phase to particulates (e.g., dust and airborne particles) and vice versa, contributing to persistence within the indoor environment (Adamkiewicz et al., 2011). Organophosphate esters (OPEs) represent a class of SVOCs that are used as flame retardants to meet Federal Motor Vehicle Safety Standard (FMVSS) No. 302 – a vehicle-specific federal flammability standard – and, as a result, are frequently detected within car interior dust (Abdallah and Covaci, 2014; Ali et al., 2013; Brandsma et al., 2014; Brommer et al., 2012; Harrad et al., 2016). A number of studies have also found that OPEs may induce carcinogenesis, neurotoxicity, endocrine disruption, and developmental/reproductive toxicity (Behl et al., 2015; Farhat et al., 2014; McGee et al., 2013; Moser et al., 2015; van der Veen and de Boer, 2012). As such, there is increasing concern regarding elevated exposure to OPEs as a function of spending longer times within a vehicle.

Previously, we showed that longer commutes are associated with increased human exposure to tris(1,3-dichloro-2-isopropyl)phosphate (TDCIPP), a widely used OPE that is carcinogenic and listed on California's Proposition 65 list. However, to our knowledge, no studies have evaluated the contribution of interior car dust to personal OPE exposure. As removal of dust within indoor environments may lead to lower exposure to chemicals (Dixon et al., 1999; Gibson et al., 2019; Roberts et al., 2009), the US EPA recommends wiping and vacuuming the interior of cars to reduce personal OPE exposure (EPA, 2016). Therefore, the overall objective of this study was to 1) characterize the potential contribution of interior car dust to personal OPE exposure

using silicone wristbands and 2) determine if a decrease in interior car dust results in mitigation of personal OPE exposure (including TDCIPP) for participants who spend a significant amount of time in their personal vehicles.

4.2 Materials and Methods

Study Design. Study participants (N=49) were recruited in January and February 2020 from the University of California, Riverside. Participants were eligible for the study if they 1) were at least 19 years old; 2) commuted between one to two hours roundtrip to campus using their personal vehicle; 3) did not use other forms of transportation to commute to campus; 4) were willing to wear a silicone wristband continuously for two separate five-day durations; and 5) were willing to complete ten one-minute online questionnaires. All study protocols and materials used for this study were approved by UCR's Institutional Review Board (IRB Number: HS-19-309), and each participant provided informed consent prior to enrolling in the study.

Once participants enrolled in the study, they were distributed across four groups that determined when the participants wiped their car interiors. The participants were distributed to ensure limited demographic, vehicle, and commuting variability across the different study groups. The participants were instructed to use 10 wipes to clean the dashboard cover, air vents, steering wheel, instrument panel, wiper switch/turn signal, temperature and radio controls, outside the glove compartment, and gear shift according to a "wiping checklist" (Table S4.1), and participants were provided with one pack of identical Armor-All Cleaning Wipes (20/participant). Participants were only asked to wipe interior parts at the front of the vehicle rather than wipe and vacuum the remaining parts of the vehicle interior (e.g., car seats, windows, carpet, etc.) since 1) wristbands were expected to be in close proximity to dust at the front of the vehicle during both study weeks and 2) our objective was to streamline and standardize a

wiping protocol rather than asking study participants to clean and vacuum the entire vehicle interior once or twice within a two-week study period. Participants within Group 1 did not wipe for both weeks; participants within Group 2 did not wipe the first week but did wipe the second week; participants within Group 3 wiped the first week but not the second week; and participants within Group 4 wiped for both weeks. For Groups 2-4, participants wiped on the Friday before initiation of study Week 1 and/or 2.

Wristband Collection. Green wristbands were purchased as a single size from 24HourWristbands.Com (https://24hourwristbands.com/) (Houston, TX, USA) and, using previously described procedures (Hammel et al., 2016; Reddam et al., 2020), wristbands were solvent-cleaned with multiple Soxhlet extractions before being wrapped in combusted aluminum foil and placed in Ziploc bags (one wristband per bag). Participants were asked to wear wristbands continuously for a 5-d period, including during bathing, sleeping, and other daily activities, for two consecutive weeks (one wristband per week per participant). At the end of each week, participants re-wrapped their wristband in aluminum foil, and then placed the wrapped wristband in the originally provided Ziploc bag. Six cleaned wristbands were used as field blanks to account for potential OPEs present within wristbands as well as OPE exposure during shipping and handling. Field blanks were un-wrapped for 30 s at room temperature on the third floor of the Science Laboratories 1 Building at UCR, and then re-wrapped with the same aluminum foil. Field blanks were only un-wrapped for 30 s to mimic the same shipping and handling experience as the participant wristbands without allowing significant interaction with the surrounding environment. Participant wristbands and field blanks were stored at -20°C until overnight shipment on dry ice to Duke University (Durham, NC, USA) for extraction and analysis.

Questionnaires and Calculation of Commute and Ventilation Scores. Study participants completed an initial recruitment survey. In this survey, participants were asked to provide demographic information (age, gender, ethnicity, residence ZIP codes, and household income), commuting information (length of commute and method of commuting), and personal vehicle information (upholstery material, year, mileage, make and model of car and time of last car interior cleaning). During the study, participants completed ten short online surveys (one daily survey, resulting in five daily surveys per study week). Participants were asked how long they drove their vehicle; if they were the passenger or the driver; if they used any other forms of transportation; and if they used the AC/heater (and, if so, for how long) within their car.

The amount of time a participant spent in a mode of transportation was used to calculate their total commute score. Each time bracket was assigned a daily commute score: <30 min was assigned 1; 30-60 min was assigned 2; 60-90 min, 90-120 min and 120-180 min were assigned 3; and >180 min was assigned 4. 60-90 min, 90-120 min and 120-180 min were all assigned 3 in order to ensure that calculation of the commute score was consistent with our previously conducted study (Reddam et al., 2020). Daily commute scores were then summed to obtain the total commute score for each participant. The amount of time a participant used the AC/heater was used to calculate their total ventilation score. Similar to the total commute score, each time bracket was assigned a daily ventilation score: no AC/heater use was assigned 0; <30 min was assigned 1; 30-60 min was assigned 2; 60-180 min was assigned 3; and >180 min was assigned 4. The daily ventilation scores were then summed to obtain the total ventilation score for each participant.

Extraction and Analysis of OPEs from Wristbands. Wristbands were extracted and analyzed using previously described protocols (Reddam et al., 2020). Briefly,

wristbands were cut into ~1-in fragments, spiked with isotopically-labelled internal standards, and extracted via sonication. Extracts were then purified using 8 g of water deactived 100-200 mesh Acros Organics Florisil (Thermo Fisher Scientific, Waltham, MA, USA). Samples were then concentrated to 1 mL and spiked with an additional set of isotopically labelled standards prior to mass spectrometry analysis in order to measure recovery of internal standards. Samples were analyzed for OPEs using a Q Exactive GC Hybrid Quadrupole-Orbitrap GC-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA) operated in full scan Electron Ionization (EI) mode. Field blanks (clean wristbands; N=6) and lab blanks (solvent; N=5) were processed and analyzed with each batch of wristbands for quality assurance and quality control. No significant differences were detected between field and lab blanks; therefore, field blanks were used to estimate method detection limits (MDLs). MDLs were calculated using three times the standard deviation of the field blank responses, or a value equal to ten times the signal-to-noise if the analyte was not detected in the field blanks. MDLs for all target OPEs are available in the supporting information (Table S4.7) and were generally at or below 3 ng/g for OPEs with the exception of TCIPP and EHDPP. Recoveries for all OPE surrogate standards are reported in Table S4.7.1, and the average across all OPE surrogate standards was 91.5 ± 15.5% (median: 92.25%). Analyte concentrations (in ng of analyte per g wristband) were calculated based upon standard calibration curves and all samples were recovery-corrected and blank-subtracted

Statistical Analyses. A general linear model (GLM) and Tukey's post-hoc test (α=0.05) was performed using RStudio (PBC, Boston, MA, https://www.rstudio.com) to identify significant differences among study groups/weeks and demographic data (age, gender, ethnicity, or household income), commuting information (length of

commute and commute score), or personal vehicle information (upholstery material, mileage, and last car interior cleaning). As OPE concentrations across all participant wristbands displayed a log-normal distribution, a heat map based on log_{10}-transformed concentrations for all OPEs was generated within Morpheus (Broad Institute, Cambridge, MA, USA), and hierarchical clustering was performed using the Euclidean distance and complete linkage method for the rows. Using RStudio, a GLM and Tukey's post-hoc test (α=0.05) were performed to determine if there was a significant difference in OPE concentrations for compounds having a detection frequency greater than 70% among study groups and weeks, and if wiping interior parts at the front of the vehicle resulted in a significant effect on personal exposure. Furthermore, all participants were pooled into either a wipe or no-wipe group to enhance statistical power, and a GLM and Tukey's post-hoc test (α=0.05) were used to determine if there was an overall effect of wiping on total OPE concentrations and individual OPE concentrations.

ArcGIS (Esri, Redlands, CA, USA) was used to generate maps with concentrations of total OPEs and TDCIPP in weeks 1 and 2 in relation to the participants' residence ZIP code. Furthermore, for compounds with a detection frequency greater than 70% (67 wristbands total), GLM and Tukey's post-hoc test (α=0.001) were performed to identify significant differences between total (week 1 + week 2) OPE concentrations and personal vehicle information (upholstery material, mileage, year of car, and last car interior cleaning). A lower p-value (α=0.001) was selected to account for false positives as a result of multiple comparisons. Participants (N=3) who did not turn in their Week 2 wristbands were excluded from this analysis. Lastly, GLM (α=0.001) was performed within RStudio to determine whether total ventilation scores were predictive of log_{10}-transformed concentrations for OPEs detected on at least 67 wristbands (70% detection rate).

4.3 Results and Discussion

Participant demographics and commuting characteristics are consistent across different groups

Demographic data and car characteristics for all study participants are presented in Table S4. Participants were divided among the different intervention groups in a manner that minimized differences in participant demographics (Figures 4.1A-4.1D), commute time (Figure 4.1E), and car characteristics (Figures 4.1F-4.1H) between groups (Table S4.5). In addition, mapping of participant's residence ZIP codes within ArcGIS revealed that there were no groups clustered in a particular area of Southern California (Figure 4.2A).

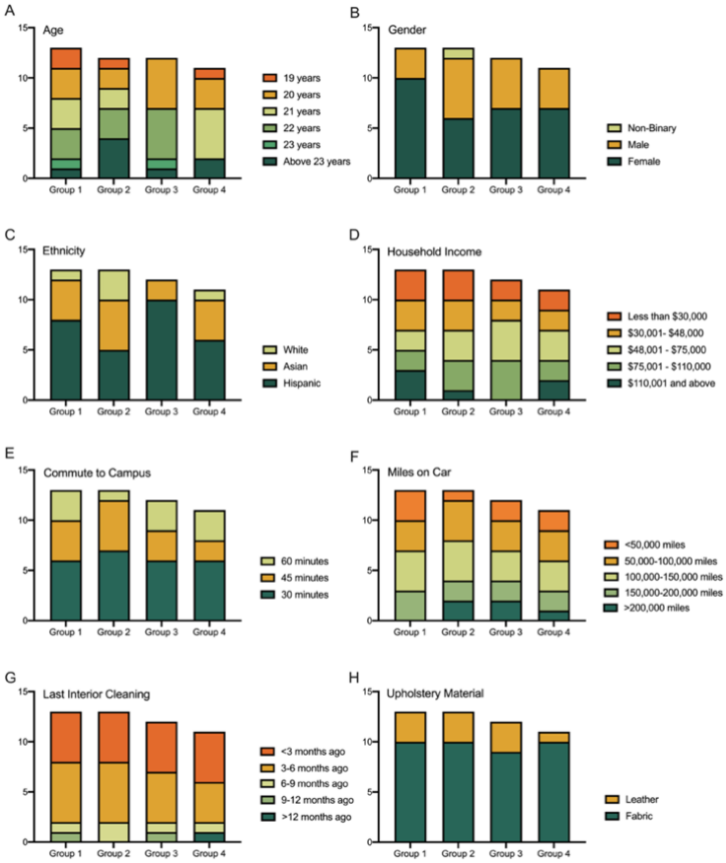

Figure 4.1. Distribution of demographics and car characteristics of study participants in Group 1 (N = 13), Group 2 (N = 13), Group 3 (N = 12) and Group 4 (N = 11) by age (A), gender (B), ethnicity (C), household income (D), commute to campus (E), miles on car (F), last interior cleaning (G) and upholstery material (H). Group 1 = no wipe in Week 1 + no wipe in Week 2; Group 2 = no wipe in Week 1 + wipe in Week 2; Group 3 = wipe in Week 1 + no wipe in Week 2; Group 4 = wipe in Week 1 + wipe in Week 2.

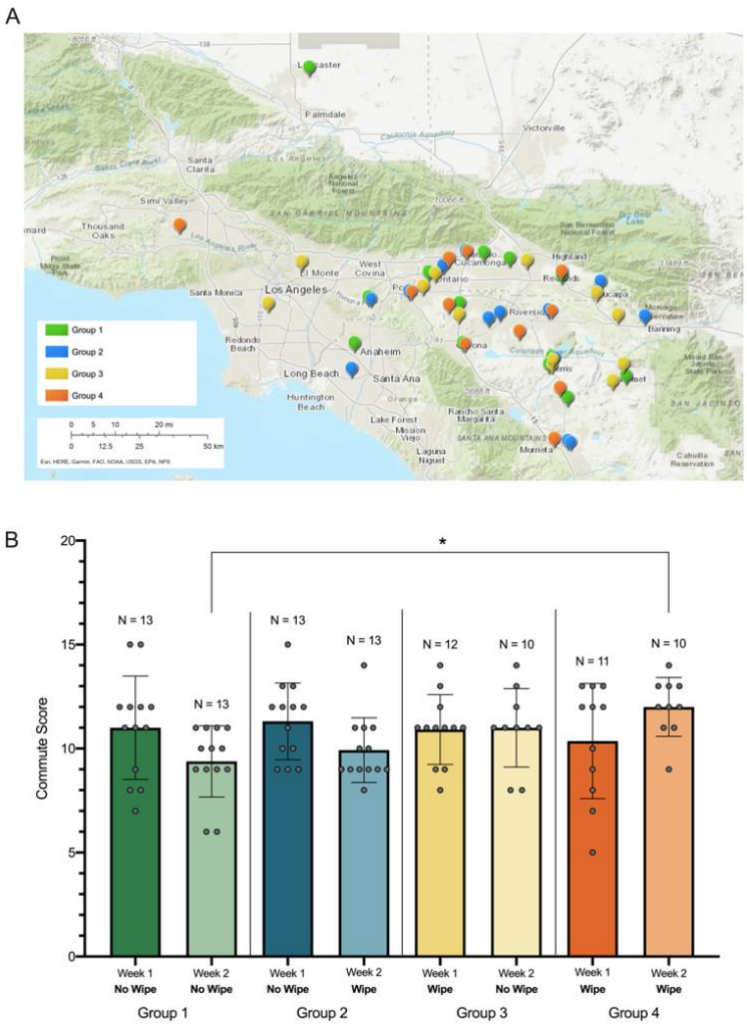

Figure 4.2. Distribution of study participants across Southern California (A). Total commute score of study participants grouped by different study groups and weeks. Asterisk (*) denotes significant difference ($p<0.05$) between participants in Groups 1 and 4 in Week 2.

The total commute score was calculated based on the amount of time the participant spent in their personal vehicle for the duration of the study (Tables S4.6.1 and S4.6.2). One of the eligibility criteria was that participants commuted between 1-2 h roundtrip to campus. Therefore, the range of commute times was intentionally narrowed to a subset of the commuter population to ensure that commute scores of study participants did not significantly vary across the study groups and weeks (Table S4.5). However, there was a significant difference in the average commute score of participants in Group 1 vs. Group 4 during the second week of the study (Figure 4.2B).

This may have been a result of participants using their vehicles for purposes other than commuting to campus, resulting in varied commute scores between these two groups within Week 2 only.

OPE concentrations did not significantly vary as a function of wiping, demographics, and car characteristics

Descriptive statistics for all OPEs measured on study participant wristbands are provided within Table S4.7 and summarized within Figure 4.3A. Although total OPE concentrations across all participants ranged from 194 to 13,573 ng/g in Week 1 and 78 to 8,219 ng/g in Week 2, the total concentration of all OPEs did not significantly vary by group nor week (Figure 4.3B). Furthermore, none of the individual OPEs were significantly different across the different groups and weeks (Table S4.8), suggesting that wiping interior parts at the front of the vehicle did not affect OPE concentrations. In order to determine if there were any trends associated with participant residence ZIP code and total OPE concentration, an ArcGIS map was generated to assess whether location was associated with total OPE concentration (Figure 4.3C). While some participants who drove longer distances tended to have higher concentrations of OPEs on their wristbands, there were no significant differences between commute score and total OPE concentration for all participants regardless of group (Table S4.9). Therefore, higher total OPE concentrations for people who live further away may have been attributed to other OPE sources such as indoor dust or air derived from the home or workplace.

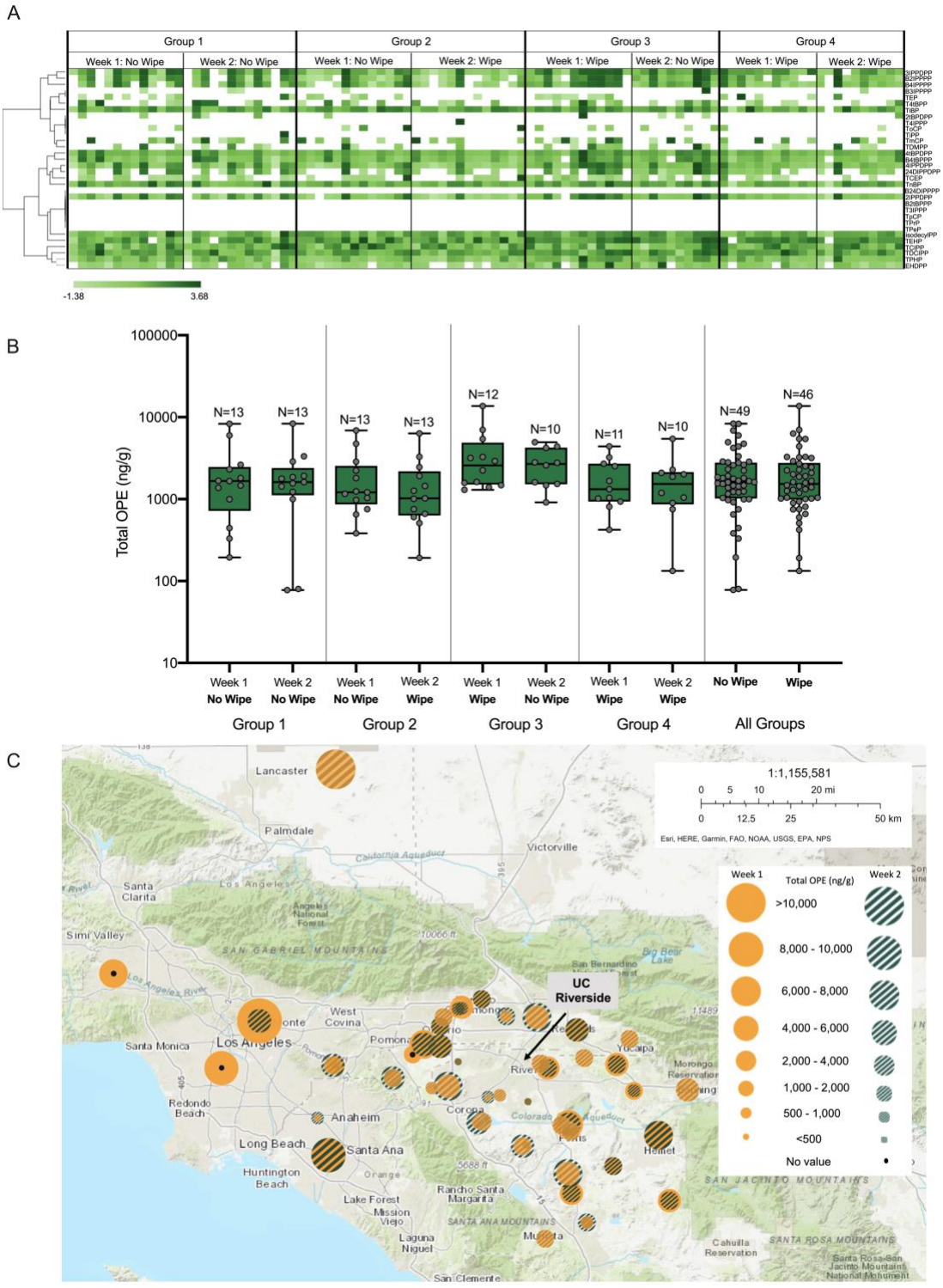

Figure 4.3. Heat map representing wristband concentrations of individual OPEs vs. different study groups (A). OPE concentration data shown within the heat map were log_{10}-transformed, and hierarchical clustering was performed using the Euclidean distance and complete linkage method. Total concentration of all OPEs for each participant divided by different study groups and wiping intervention (B) and by location of participants' residence ZIP code (C).

As there were no significant differences in OPE concentrations across study groups, we performed correlations to identify potential trends between OPE concentrations and car characteristics. There were no significant differences between total nor individual OPE concentrations (Figure 4.4; Table S4.9) and vehicle mileage, time of last cleaning, upholstery material, and year of the car. These results are consistent with other studies that found no significant differences in OPE concentration in vehicular dust relative to age of car (Abdallah and Covaci, 2014; Brommer and Harrad, 2015; Christia et al., 2018) and upholstery material (Christia et al., 2018). Brommer et al. (2012) found that the most dominant OPE in samples was correlated with mileage since last cleaning; however, this correlation was only positive for the most contaminated samples in each case and the inclusion of all samples resulted in an insignificant relationship (Brommer et al., 2012).

Among all participants, wristbands derived from two participants driving/riding within a 2005 Toyota Corolla and 2016 Mazda CX-5 had the highest total OPE concentrations (16,428 and 16,150 ng/g, respectively). However, without additional replication, we are unable to draw any conclusions about the potential relationship between car make/model and OPE concentrations detected on wristbands. Furthermore, we found different total OPE concentrations in cases where two vehicles had the same make, model and year. Specifically, we found that wristbands derived from two participants driving/riding within a 2017 Honda Civic had total OPE concentrations of 1710 and 3465 ng/g, and wristbands derived from two participants driving/riding within a 2005 Toyota Matrix had total OPE concentrations of 9186 and 2037 ng/g. Therefore, there does not appear to be an influence of vehicle manufacturer on total OPE concentrations.

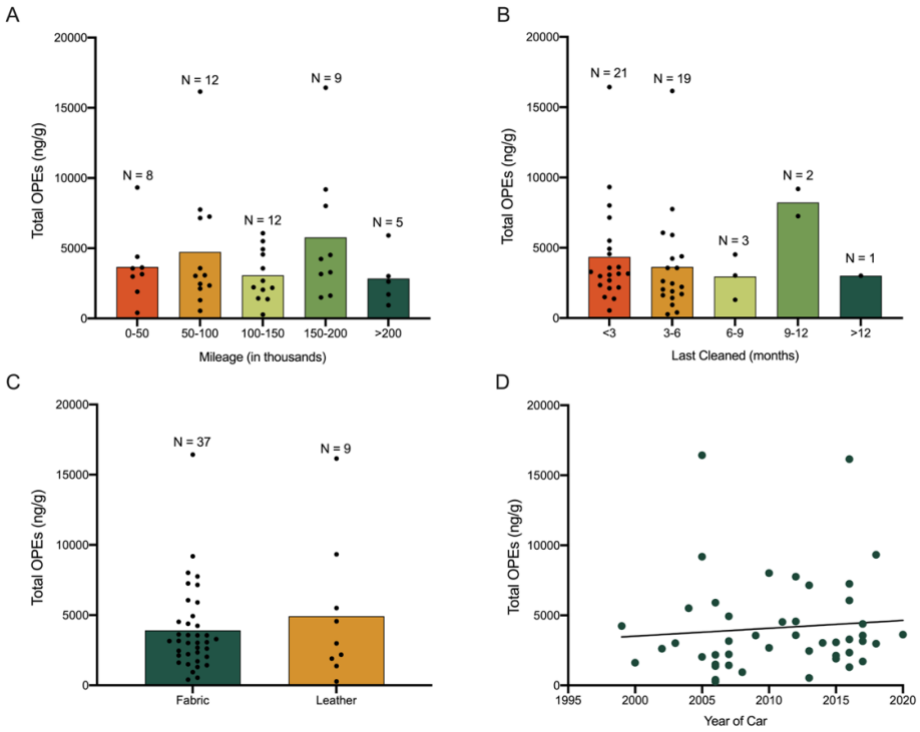

Figure 4.4. Total OPE concentrations for each participant distributed by mileage (A), time since last car interior cleaning (B) and upholstery material (C). Total OPE concentration as a function of year of the car (D).

TDCIPP concentrations did not significantly vary as a function of wiping, residence ZIP code nor ventilation

Using a different participant cohort, we previously found that higher TDCIPP concentrations on participant wristbands were associated with longer commutes (Reddam et al., 2020). As SVOCs released from end-use products are equilibrated among the gas phase and particulates (surface dust and airborne particles) (Weschler and Nazaroff, 2012), elevated concentrations of TDCIPP detected within wristbands may be due to 1) airborne TDCIPP directly released from vehicle parts, 2) TDCIPP partitioning from air to car interior dust after being released from vehicle parts, and/or 3) movement of TDCIPP from outdoor air through a vehicle's ventilation system. Although prior studies have primarily detected OPEs within car interior dust (Abdallah and Covaci, 2014; Brommer et al., 2012; Harrad et al., 2016; Tokumura et al., 2017), there was no significant difference in wristband TDCIPP concentrations among the

different wiping groups (Figure 4.5A) despite concentrations being higher than those of non-commuters (Figure 4.5B) (Reddam et al., 2020). Moreover, when all of the no-wipe and wipe data were pooled to enhance statistical power, we detected no significance difference in TDCIPP concentrations between these two groups (Figure 4.5A, Table S4.8).

The commute score and respective TDCIPP concentrations of the current study are in agreement with our previous study (Figure 4.5B). However, due to one of the eligibility criteria for this study (i.e., all participants commuted between 1-2 h roundtrip to campus), there was no significant difference between commute score and TDCIPP concentrations within this study. Moreover, similar to total OPE concentrations, there were no apparent trends in TDCIPP concentrations and residence ZIP codes of participants (Figure 4.5C). Despite dust removal from interior parts at the front of the vehicle, continued exposure to TDCIPP suggests that the correlation between TDCIPP exposure and longer commutes within our previous study may have been attributed to other sources of TDCIPP within the vehicle. We also calculated ventilation scores (Tables S4.10.1 and S4.10.2) to identify the potential contribution of air circulation to TDCIPP exposure. Some studies have shown that the presence of recirculation air conditioners reduces the concentration of airborne contaminants within vehicles by improving ventilation rate and filtering efficiency (Chan et al., 1991a; Riediker et al., 2003; Wu et al., 2013). However, we did not find a significant difference between any of the OPEs (including TDCIPP) on participant wristbands and ventilation scores (Figures 4.5D and 4.5E; Table S4.11), suggesting that TDCIPP exposure within car interiors may be independent of ventilation within vehicles. Indeed, studies have shown that SVOCs persist for longer durations within the indoor environment if the only removal mechanism is ventilation (Weschler and Nazaroff, 2012).

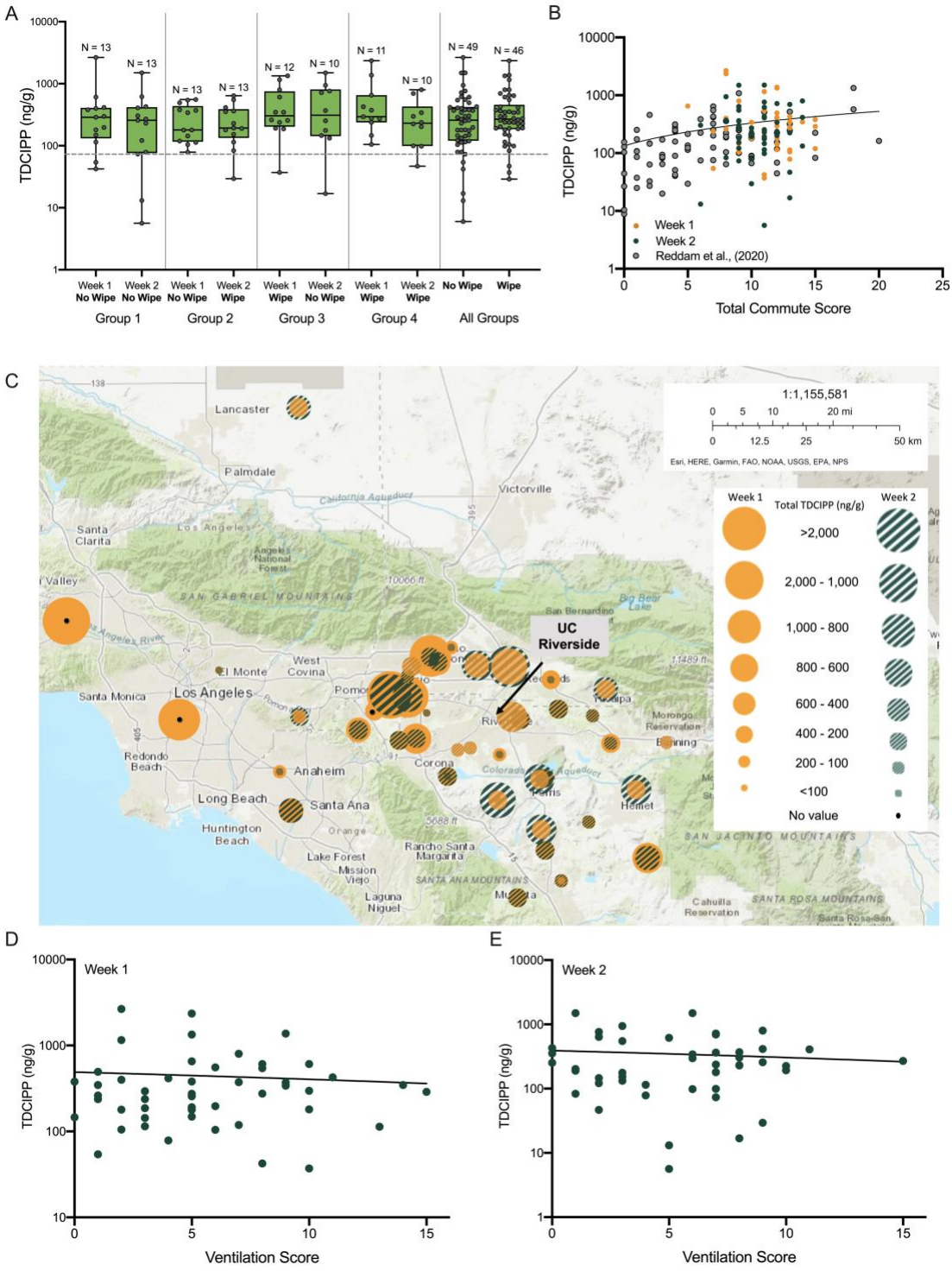

Figure 4.5. TDCIPP concentration for each participant divided by different study groups and wiping intervention where the grey dotted line represents the average TDCIPP concentration for non-commuters based on Reddam et al., (2020) (A). TDCIPP concentrations as a function of total commute score from participants from the current study vs. Reddam et al. (2020) (B). Week 1 and 2 TDCIPP concentrations in relation to the location of participants' residence ZIP code (C) and as a function of ventilation score (D and E).

As TDCIPP is an SVOC, it has the potential to partition from vehicle parts directly into wristbands via car interior air. A limited number of studies have measured the concentration of TDCIPP within car interior air (Kim et al., 2019; Tokumura et al., 2017). For example, Kim et al. (2019) reported that higher concentrations of TDCIPP were found in air compared to dust in indoor environments. Interestingly, concentrations of OPEs in wristbands have not been consistently correlated with concentrations in dust (Hammel et al., 2020) but have been correlated with urine biomarkers (Gibson et al., 2019; Hammel et al., 2020, 2016) and active air samplers (S. Wang et al., 2019). This suggests that larger TDCIPP concentrations may be within air, and that wristbands may reflect partitioning from airborne TDCIPP. Moreover, TDCIPP circulating within the car interior air may result in inhalation and dermal absorption based on its properties as an SVOC (Schreder et al., 2016; Weschler and Nazaroff, 2012). Indeed, Schreder et al. (2016) suggested that the total intake of chlorinated OPEs via inhalation was estimated to exceed intake via dust. Overall, this highlights an important exposure route to consider when quantifying exposure to TDCIPP and underscores the need for more studies examining the concentration of airborne TDCIPP within the indoor microenvironment of vehicles. Although TDCIPP is on California's Proposition 65 list based on its potential to cause cancer following ingestion, there is a need for more studies examining the potential toxicity of TDCIPP following inhalation.

While previous studies have detected TDCIPP concentrations within vehicle dust and air samples, to our knowledge this is the first study to evaluate the potential contribution of dust on interior parts at the front of the vehicle to TDCIPP exposure while commuting in cars. Although we did not collect biomarkers from participants, several studies have shown that wristbands are significantly correlated with urinary

biomarkers for TDCIPP (Gibson et al., 2019; Hammel et al., 2020, 2016). Furthermore, as the participants in our study only wiped dashboard dust and did not remove dust from all sources (e.g., roof upholstery, car seats, floor mats, windows, etc.), we are unable to draw any definite conclusions between complete, in-vehicle dust elimination and TDCIPP exposure. Nevertheless, given that a large percentage of Americans spend a significant amount of time within their vehicles, our study identifies a potential knowledge gap that should be addressed in order to increase our understanding of the mechanisms of TDCIPP exposure within vehicles.

Chapter 5: Inhalation of two Prop 65-listed chemicals within vehicles may be associated with increased cancer risk

5.0 Abstract

Chemicals are listed on California's Proposition 65 (Prop 65) for their potential to cause cancer, birth defects or other reproductive harm, and certain chemicals from this list are often detected within interior vehicle dust and air. Therefore, this study examined the potential risk associated with five Prop 65-listed chemicals detected within vehicle interiors: benzene, formaldehyde, di (2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and tris(1,3-dichloro-2-propyl)phosphate (TDCIPP). Exposure estimates based on time spent within a vehicle were derived from a meta-analysis of estimated concentrations from the literature. Regulatory levels established by the California Office of Environmental Health Hazard Assessment (OEHHA) were then used to generate percent reference doses (%RfDs) for chemical-specific daily doses as well as determine the probability of risk (exceedance probability) as a function of %RfD for each chemical-specific daily dose. Based on our meta-analysis, benzene and formaldehyde were detected in vehicle interior air whereas DEHP, DBP and TDCIPP were detected in vehicle interior dust. Benzene and formaldehyde were the only two chemicals with an estimated %RfD > 100 across any of the commute times. For commute times of 20 min or longer, the %RfD was >100 for maximum exposures based on the "maximum allowable daily level" for benzene, and for 95th-percentile exposures based on the "no significant risk level" for benzene and formaldehyde. Furthermore, the probability of exceeding 100% RfD was highest for cancer risks associated with benzene, followed by cancer risks associated with formaldehyde and the risk of reproductive and developmental toxicity associated with benzene. Lastly, within the entire state of California, the percent of commuters with a 10% probability of exceeding cancer risk associated with benzene or formaldehyde exposure was 78%

and 63%, respectively. Overall, our study raises concerns about the potential risk associated with inhalation of benzene and formaldehyde for people who spend a significant amount of time in their vehicles, an issue that is especially pertinent to traffic-congested areas where people have longer commutes.

5.1 Introduction

California's Proposition 65 (Prop 65), also known as the Safe Drinking Water and Toxic Enforcement Act of 1986, requires businesses to inform Californians about exposure to chemicals known to cause cancer, birth defects or other reproductive harm. Prop 65-listed chemicals represent a wide range of naturally occurring and synthetic chemicals that include additives or ingredients in pesticide formulations, common household products, food, drugs, dyes, or solvents. In some cases, Prop 65-listed chemicals that are used in indoor products have the potential to migrate, abrade, or off-gas from end-use products and accumulate in indoor environments (Mitro et al., 2016). The presence of Prop 65-listed chemicals in indoor air and dust has been well documented (Greco et al., 2020; Hwang et al., 2008; Lucattini et al., 2018; Rudel et al., 2003), suggesting that people may be exposed to these chemicals through inhalation of air and ingestion of dust. While several studies have evaluated the potential risk to Prop 65-listed chemicals detected within indoor environments (Ali, 2019; Ao et al., 2019; Kang et al., 2012; W. Wang et al., 2013; Zhou et al., 2019), there is minimal information available about the potential risk of Prop 65-listed chemicals due to exposure within personal vehicles.

The interior of a personal vehicle is considered an indoor microenvironment and, due to its small, confined space, chemicals emitted from the interior of the vehicle have the potential to be concentrated (Faber and Brodzik, 2017). Chemicals such as phthalates, volatile organic compounds (VOCs), flame retardants and hydrocarbons –

some of which are Prop 65-listed – are commonly detected within interior vehicle dust (Müller et al., 2011; Riediker et al., 2003; Zulauf et al., 2019). Furthermore, prior studies have demonstrated that the concentration of certain chemicals within vehicle interiors were 2- to 3-fold higher compared to indoor concentrations within the built environment (Faber and Brodzik, 2017), suggesting that vehicle interiors are an important indoor microenvironment to consider when evaluating exposure to chemicals.

American adults spend an average of 6% of their time within an enclosed vehicle (Klepeis et al., 2001), a large amount of which is spent commuting. In the United Sates, a person spends an average of 52.8 minutes per day commuting to work (U.S. Census Bureau, 2017). Longer commute times are known to be strongly associated with negative health outcomes such as shorter sleep, obesity, and poor physical/mental health (Ding et al., 2014; Hansson et al., 2011; Oliveira et al., 2015; Sugiyama et al., 2013). Moreover, people who spend a longer amount of time in vehicles are exposed to higher concentrations of particulate matter, carbon monoxide, VOCs, ozone, and flame retardants (Huang et al., 2012; Ramos et al., 2016; Reddam et al., 2020), suggesting that people experiencing long commutes over years and, in some cases, decades likely represent a sub-population vulnerable to excess exposure to vehicle-borne chemicals. Therefore, it is important to evaluate the potential risk associated with exposure to vehicle-specific chemicals as a function of commute time.

The aim of this study was to assess the potential human health risk of Prop 65-listed compounds found in personal vehicles; cumulative risks resulting from other stressors associated with long commutes (e.g., shorter sleep, obesity, etc.) were not considered within this study. We first summarized the estimated concentrations of five Prop 65-listed chemicals in interior vehicle air and dust, and then derived exposure estimates based on time spent within a vehicle. The potential human health risks resulting from exposure to these compounds as a function of commute time were then

evaluated using regulatory levels established by the California Office of Environmental Health Hazard Assessment (OEHHA).

5.2 Methods

Figure 5.1 outlines the four phases (chemical identification, exposure assessment, hazard identification, and risk assessment) that were followed to measure the potential risk of Prop 65-listed chemicals to commuters. All four phases are described in detail within Sections 2.1 and 2.2.

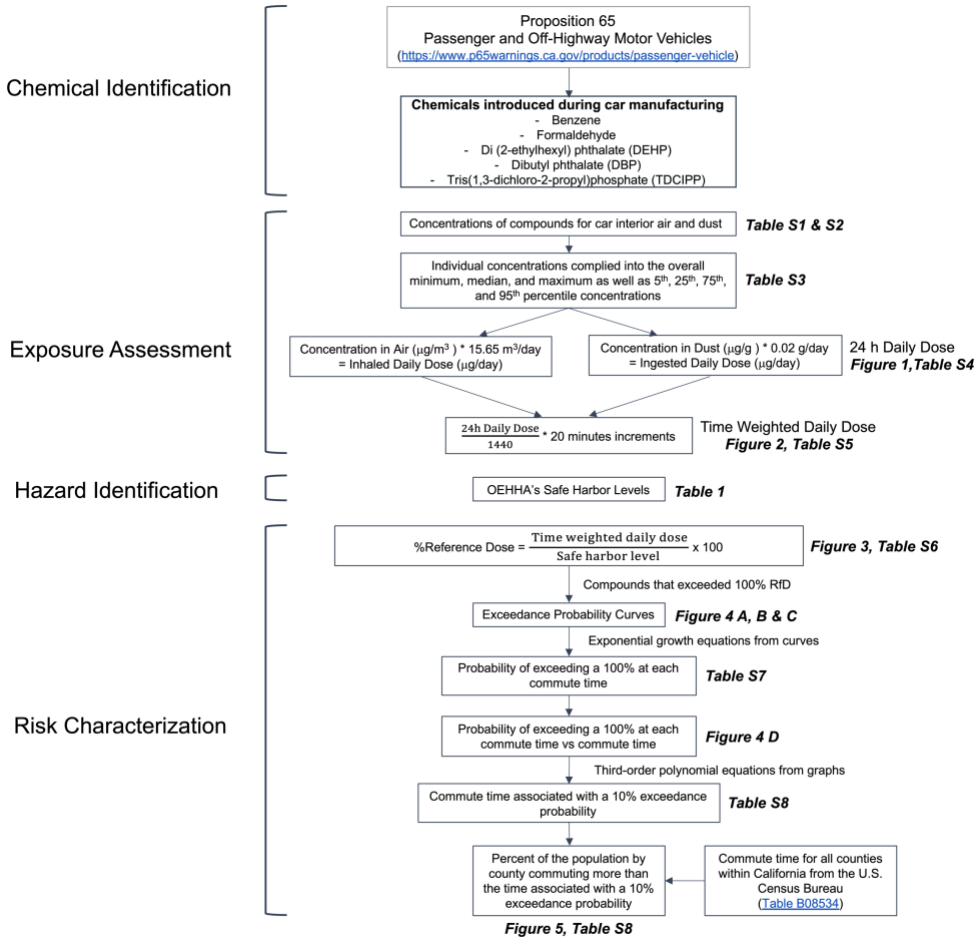

Figure 5.1. Methods used for chemical identification, exposure assessment, hazard identification, and risk assessment to measure risk associated with Prop 65-listed chemicals within vehicles

Identification of Prop 65-listed chemicals introduced within vehicles. Based on OEHHA's Prop 65 Fact Sheet (dated June 2019) entitled "Passenger Vehicles and Off-Highway Motor Vehicles", benzene, carbon monoxide, diesel and gasoline engine exhaust, lead, formaldehyde, and phthalates [Dibutyl phthalate (DBP), Di (2-ethylhexyl) phthalate (DEHP), Diisodecyl phthalate (DIDP), and Diisononyl phthalate (DINP)] were identified as Prop 65-listed chemicals either introduced within or generated by vehicles. For the purpose of this study, only chemicals introduced within vehicles during manufacturing (benzene, formaldehyde, and phthalates) were assessed for potential human health risks. In addition, while not currently on OEHHA's fact sheet dated June 2019, tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) was assessed in this study since TDCIPP is a Prop 65-listed chemical that has been detected within indoor vehicle dust (Ali et al., 2013; Brandsma et al., 2014; Brommer and Harrad, 2015; Harrad et al., 2016) and exposure to TDCIPP is significantly associated with longer commute times (Reddam et al., 2020). Therefore, we conducted a meta-analysis of the peer-reviewed literature in order to synthesize measured concentrations of benzene, formaldehyde, phthalates (DBP, DEHP, DIDP and DINP), and TDCIPP that have been detected within dust and air collected within vehicle interiors. Studies selected for review and risk assessment met the following three inclusion criteria: 1) evaluated concentrations of benzene, formaldehyde, phthalates, and TDCIPP in interior car dust or air; 2) published before or during July 2020; and 3) published in English.

Data collection and analysis

Exposure assessment. Based on dust and air samples collected within vehicle interiors, measured concentrations of benzene, formaldehyde, phthalates and TDCIPP were compiled from the following studies that met all three inclusion criteria listed

above: Abdallah and Covaci, 2014; Albar et al., 2017a; Ali et al., 2013; Brandsma et al., 2014; Brodzik et al., 2014; Brommer et al., 2012; Brommer and Harrad, 2015; Buters et al., 2007; Carignan et al., 2013a; Chan et al., 1991; Chen et al., 2014; Christia et al., 2018; Faber et al., 2014; Fujii et al., 2003; Geiss et al., 2009; GLOBAL 2000., 2005; Harrad et al., 2016; Hoehner et al., 2012; Liang et al., 2019; Lv et al., 2020; Staaf and Östman, 2005; Tokumura et al., 2017, 2016; Wensing, 2009; Xiong et al., 2015; Yoshida et al., 2006; You et al., 2007; Zhou et al., 2017. When compiling concentrations of benzene, formaldehyde, phthalates and TDCIPP, this study did not consider the type and age of vehicles, ventilation conditions, ambient temperatures, sampling methods, and time of sample collection.

For studies where chemical concentrations were reported as a distribution (rather than raw data for individual samples), the minimum, median, and/or maximum (depending on what was reported) were used for estimating the exposure distribution within this study. Using all available data identified from our meta-analysis, the overall minimum, median, and maximum as well as 5^{th}, 25^{th}, 75^{th}, and 95^{th} percentile concentrations were then identified and used to calculate daily doses for benzene, formaldehyde, phthalates, and TDCIPP based on ingestion and inhalation within adults. As DIDP and DINP were not detected within interior vehicle dust nor air, daily doses were not calculated for these chemicals. Daily doses were calculated using adult ingestion and inhalation rates derived from the U.S. Environmental Protection Agency's Exposure Factors Handbook. The dust ingestion rate associated with 12 years through adult was acquired from Table 5-1 (under "Dust: General Population Central Tendency") whereas the inhalation rate was calculated by averaging the "Mean" rate from 16 to <71 years from Table 6-1. Our risk analysis assumed that the average ingestion and inhalation rate from the EPA Exposure Factors Handbook was applicable to the general population. These rates were multiplied by the overall

minimum, median, and maximum as well as 5^{th}, 25^{th}, 75^{th}, and 95^{th} percentile concentrations to produce a distribution of daily doses for each chemical. Time-weighted daily doses were then calculated from 20 to 240 min (using 20-min increments) by first dividing the total daily dose by the number of minutes in one day (1440 min) and then multiplying by the commute time (which ranged from 20 to 240 min). Time-weighted daily doses for oral or inhalation routes of exposure were not calculated for chemicals that did not have corresponding OEHHA-generated safe harbor levels. For example, daily doses based on inhalation of TDCIPP, DBP, and DEHP were not calculated since, as of August 2020, inhalation-based safe harbor levels for TDCIPP, DBP, and DEHP were not determined by OEHHA.

Hazard identification. Safe harbor levels were obtained directly from OEHHA (https://oehha.ca.gov/proposition-65/proposition-65-list). If OEHHA concluded that a chemical is a known carcinogen, the "no significant risk level" (NSRL) was used; the NSRL is defined as the daily intake level posing a 10^{-5} lifetime risk of cancer. If OEHHA concluded that a chemical is known to cause birth defects or other reproductive harm, the "maximum allowable daily level" (MADL) was used; the MADL is derived from No Observable Effect Levels (NOELs) or Lowest Observable Effect Levels (LOELs). The NSRL and MADL were reported for chemicals that are known to cause cancer and reproductive/developmental toxicity, respectively, based on OEHHA's conclusions. Values associated with both oral and inhalation routes of exposure were also reported when available.

Risk characterization. Percent reference doses (%RfDs) were calculated by dividing chemical-specific daily doses by chemical-specific safe harbor levels (NSRLs or MADLs) and then multiplying by 100. Chemicals detected within the air of vehicle

interiors were divided by safe harbor levels specific to inhalation exposure, whereas chemicals detected within dust of vehicle interiors were divided by safe harbor levels specific to oral exposure. Exceedance probability curves were generated for chemicals with %RfDs that exceeded 100% (the regulatory threshold of concern), where %RfDs calculated for each chemical were assigned exceedance probabilities (i.e., 0.99, 0.95, 0.75, 0.5, 0.25, 0.05 and 0.01). After plotting exceedance probabilities, exponential growth curve equations were then generated for each commute time in order to calculate the probability of exceeding 100% RfD as a function of commute time. In addition, we plotted exceedance probabilities at 100% RfD as a function of commute time to generate third-order polynomial equations and estimate the probability of exceeding 100% RfD at different commute times for each chemical.

Finally, a 10% exceedance probability threshold was selected as a benchmark of concern for estimating the percent of California commuters (by county) that may be at risk from elevated exposure to Prop 65-listed chemicals within vehicles. For each chemical, the commute time associated with a 10% exceedance probability was calculated based on third-order polynomial equations as described above. Commute time for all counties within California were acquired from the U.S. Census Bureau (Table B08534). The percent of the population by county commuting more than the time associated with a 10% exceedance probability was then calculated and plotted on a map using mapchart.net.

5.3 Results

Estimated daily doses of benzene and formaldehyde are orders of magnitude higher than TDCIPP, DEHP and DBP

Based on our meta-analysis, concentrations of benzene, formaldehyde, phthalates (DBP, DEHP, DIDP and DINP), and TDCIPP detected within interior vehicle dust and air are reported in Tables S5.1 and S5.2, respectively. The overall minimum, median, and maximum as well as 5^{th}, 25^{th}, 75^{th}, and 95^{th} percentile concentrations for each chemical are reported within Table S5.3. The median concentration of DBP, DEHP, and TDCIPP within interior vehicle dust was 11.8, 488.5, and 3 µg/g, respectively, and the median concentrations of benzene, DBP, DEHP, TDCIPP and formaldehyde within interior car air were 10.35, 198.5, 370, 0.014 and 24.25 $\mu g/m^3$, respectively. Concentrations of DIDP and DINP in interior car dust and air were not reported within any studies included within our meta-analysis.

As described in the exposure assessment subsection, an ingestion and inhalation rate of 0.02 g/day and 15.65 m^3/day, respectively, were used for calculation of daily doses (Table S5.4). Based on a 24-h exposure scenario, the daily doses for TDCIPP, DBP, DEHP, benzene, and formaldehyde are summarized within Figure 5.2; chemicals that did not have corresponding OEHHA-generated safe harbor levels (e.g., inhalation-specific safe harbor levels for TDCIPP, DBP, and DEHP) were not included within Figure 5.2. The median daily doses of DEHP, DBP, and TDCIPP based on ingestion of interior vehicle dust was 9.77, 0.236, and 0.06 µg/day, respectively, and the median daily doses of formaldehyde and benzene based on inhalation of interior vehicle air were 379.51 and 161.97 µg/day, respectively.

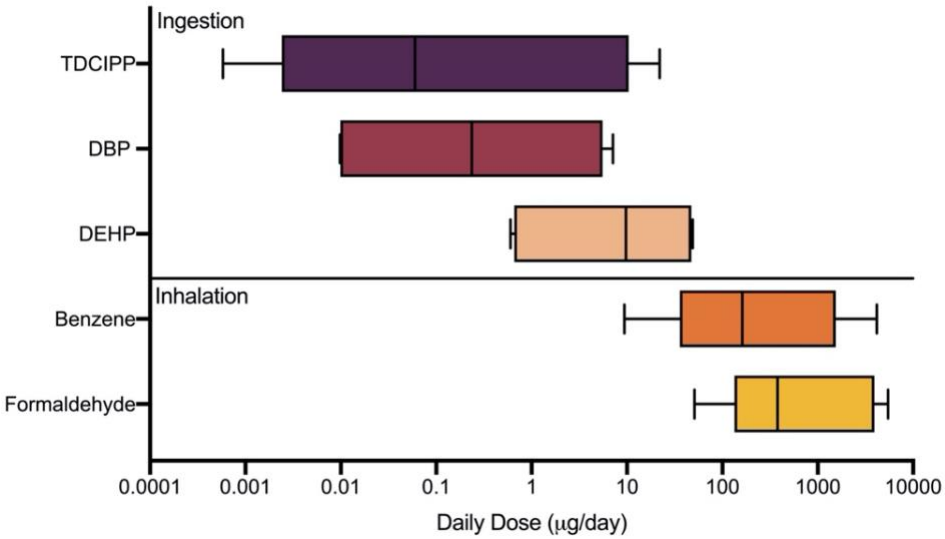

Figure. 5.2. Estimated daily dose (μg/day) for TDCIPP (N=117), DBP (N=10) and DEHP (N=10) based on ingestion of interior vehicle dust (top), and estimated daily dose (μg/day) for benzene (N=74) and formaldehyde (N=52) based on inhalation of interior vehicle air for 24 h (bottom). N = number of data points based on the meta-analysis (Tables S5.1 and S5.2).

Time-weighted exposures were calculated for all five chemicals in increments of 20 min (Figure 5.3 and Table S5.5). The estimated median dose of formaldehyde, benzene, DEHP, DBP and TDCIPP for an adult spending 20 min within a car per day was 5.27, 2.25, 0.14, 0.003, and 0.0008 μg/day, respectively – a dose that increases from 20 min to the highest exposure scenario tested (240 min, or 4 h). The estimated median dose of formaldehyde, benzene, DEHP, DBP and TDCIPP for an adult who spent 240 min within a car per day was 63.25, 27, 1.63, 0.04, and 0.01 μg/day, respectively. Similar to the 24-h exposure scenario, chemicals present within interior vehicle air resulted in a higher daily dose – in some cases by five orders of magnitude – relative to chemicals present within interior vehicle dust.

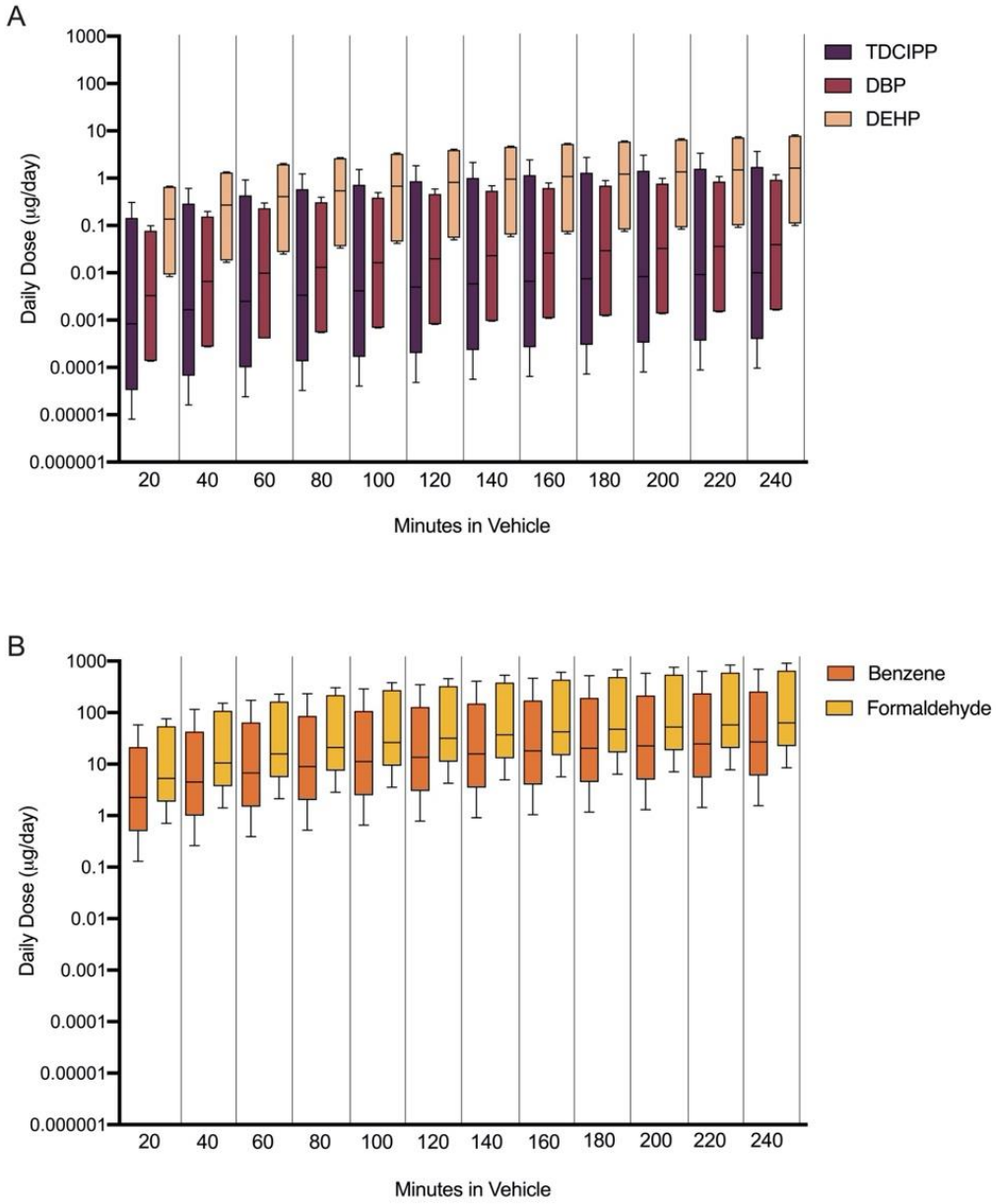

Figure 5.3. Distribution of time-weighted daily doses (µg/day) of chemicals found within interior vehicle dust (A) and air (B) as a function of commute time.

TDCIPP has the lowest safe harbor level out of all five Prop 65-listed chemicals introduced into vehicles during manufacturing

A summary of OEHHA's safe harbor levels is presented within Table 5.1. A NSRL was used for chemicals with the potential to cause cancer whereas a MADL was used for chemicals with the potential to cause reproductive and developmental toxicity. For TDCIPP, the NSRL for oral exposure is 5.4 µg/day and was derived based

on results from a 2-year chronic toxicity study using rats (Freudenthal and Henrich, 2000). In this study, daily dietary exposure to TDCIPP for 24 months resulted in a dose-dependent increase in the incidence of liver, kidney and testicular tumors, and the tumor incidence data were used to derive a cancer potency estimate of 0.13 $(mg/kg-day)^{-1}$ that served as the basis for the NSRL.

For DBP, the MADL for oral exposure is 8.7 μg/day and was based on a LOEL of 1.5 mg/kg-day (Lee et al., 2004). Dietary maternal exposure of rats to DBP during pregnancy and lactation adversely affected reproductive development of male and female offspring (Lee et al., 2004). OEHHA derived a NSRL and MADL for DEHP, as this chemical has the potential to cause cancer as well as developmental and reproductive toxicity. The NSRL for oral exposures is 310 μg/day and was derived from a cancer potency estimate of 0.0022 $(mg/kg-day)^{-1}$ based on rodent studies conducted by the NTP (1982) and David et al. (1999). In both studies, oral exposure to DEHP resulted in a higher incidence of hepatocellular carcinomas. Additionally, the MADL for oral exposures for DEHP is 410 μg/day for adults and was derived from a NOEL of 5.8 mg/kg-day based on male reproductive effects in the form of testicular damage (David et al., 2000).

OEHHA derived a NSRL and MADL for benzene, as this chemical has the potential to cause cancer as well as developmental and reproductive toxicity. The NSRL for oral and inhalation routes of exposure are 6.4 and 13 μg/day, respectively; these two NRSLs were derived from cancer potency estimates of 0.054 $(mg/kg-day)^{-1}$ and 0.11 $(mg/kg-day)^{-1}$ for oral and inhalation routes of exposure, respectively. These estimates were derived from two different cohorts – the Pliofilm Cohort (Paxton et al., 1994; Rinsky, 1989) and Chinese Worker Cohort (Hayes et al., 1997) – that developed leukemia following occupational exposure to benzene. The MADL for oral and inhalation routes of exposure are 24 and 49 μg/day, respectively, and were derived

from a LOEL of 5 ppm based on effects on hematopoiesis within a developmental toxicity study in mice (Keller and Snyder, 1988). For formaldehyde, the NSRL for an inhalation route of exposure is 40 μg/day and was derived from a cancer potency estimate of 0.021 $(mg/kg\text{-}day)^{-1}$ based on histopathological changes within the nasal cavity and upper respiratory tract of rats and mice (Kerns et al., 1983).

Endpoint	Cancer (NSRL)		Developmental and Reproductive Toxicity (MADL)	
Route of Exposure	Oral	Inhalation	Oral	Inhalation
TDCIPP	5.4 μg/day	N.C.	N.C.	N.C.
DBP	N.C.	N.C.	8.7 μg/day	N.C.
DEHP	310 μg/day	N.C.	410 μg/day	N.C.
Benzene	6.4 μg/day	13 μg/day	24 μg/day	49 μg/day
Formaldehyde	N.C.	40 μg/day	N.C.	N.C.

Table 5.1. OEHHA's safe harbor levels for TDCIPP, DBP, DEHP, benzene, and formaldehyde. N.C. = not calculated by OEHHA as of August 2020.

Benzene and formaldehyde concentrations are predicted to exceed safe harbor levels following a 20-min commute

Percent RfD (%RfD) was calculated for benzene, formaldehyde, DEHP, DBP, and TDCIPP to evaluate the potential risk associated with exposure to these chemicals from 20-240 min (Figure 5.4 and Table S5.6). Each %RfD was calculated by dividing the daily dose by the safe harbor level (NSRL or MADL) and then multiplying by 100; therefore, a %RfD > 100 indicates that the daily dose exceeds levels considered safe by OEHHA.

Benzene and formaldehyde were the only two chemicals with an estimated %RfD > 100 across any of the commute times. Two different %RfDs were calculated for each safe harbor level since a NSRL and MADL were available for benzene. Based on the NSRL for benzene, the %RfD was >100 resulting from exposures at 1) the 25th percentile or higher combined with commute times of 200 min or longer and 2) the 95th percentile or higher combined with commute times of 20 min or longer (Figure 5.4). On

the other hand, based on the MADL for benzene, the %RfD was >100 resulting from exposures at 1) the 75th percentile or higher combined with commute times of 200 min or longer and 2) the maximum combined with commute times of 20 min or longer (Figure 5.4). Based on the NSRL for formaldehyde, the %RfD was >100 resulting from exposures at 1) the 25th percentile or higher combined with commute times of 240 min or longer and 2) the 95th percentile or higher combined with commute times of 20 min or longer (Figure 5.4).

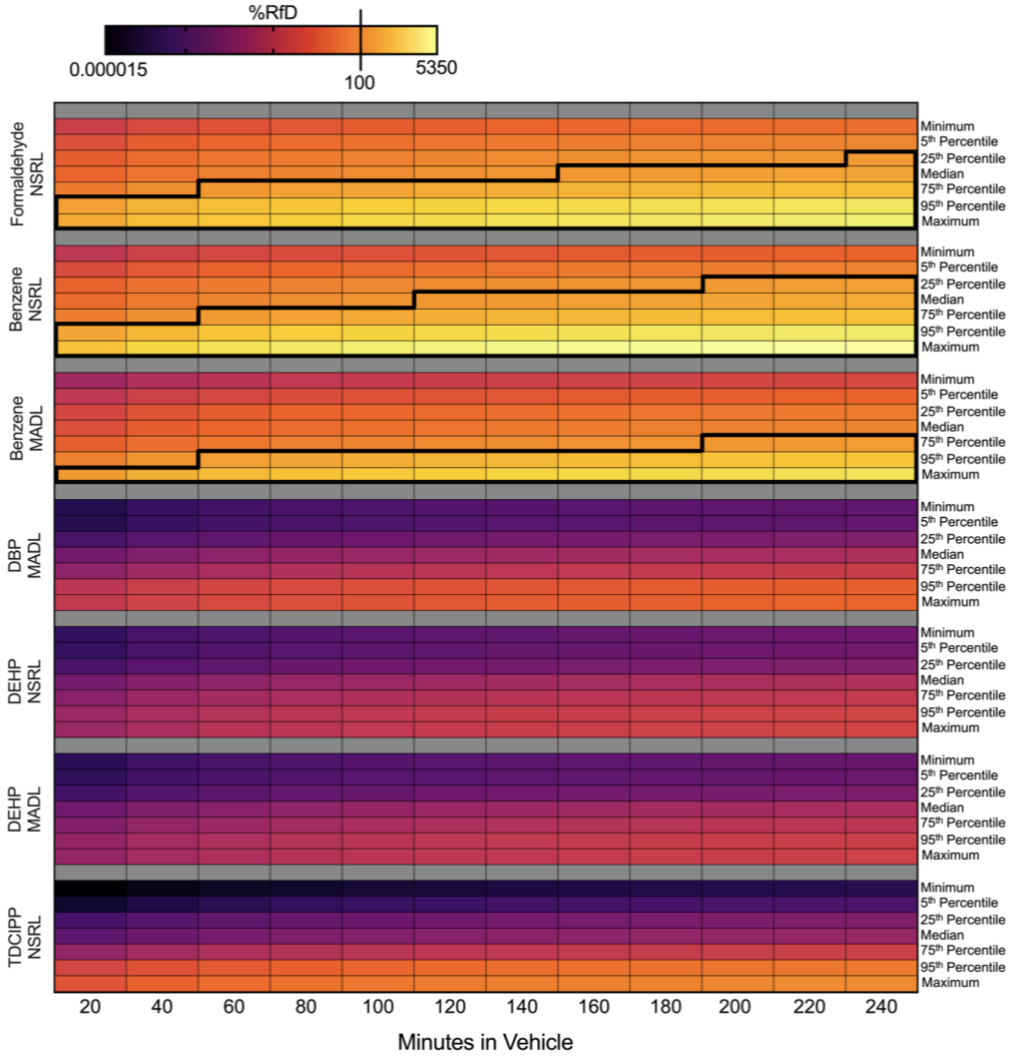

Figure 5.4. Heat map showing %RfDs for formaldehyde, benzene (NSRL and MADL), DBP, DEHP (NSRL and MADL) and TDCIPP as a function of exposure distribution and commute time. The %RfD values shown within the heat map were $\log_{10}$-transformed. Cells with %RfD>100 are outlined with a black solid line.

Predicted cancer risks associated with benzene and formaldehyde exposure are higher than the risk of reproductive and developmental toxicity associated with benzene exposure

For benzene and formaldehyde, exceedance probability curves (Figures 5.5A-5.5C) were then generated to estimate the probability of risk (exceedance probability) as a function of %RfD (Table S5.7). The probability of exceeding 100% RfD was dependent on both the chemical and commute time. For cancer risks associated with benzene exposure, the probability of exceeding 100% RfD ranged from 0.024 to 0.775 for commute times of 20-240 min. Similarly, for cancer risks associated with formaldehyde exposure, the probability of exceeding 100% RfD ranged from 0.009 to 0.744 for commute times of 20-240 min. However, the risk of reproductive and developmental toxicity following benzene exposure was substantially lower than cancer risks associated with benzene or formaldehyde exposure (Figure 5.5D), as the probability of exceeding 100% RfD ranged from 0.000001 to 0.322 for commute times of 20-240 min.

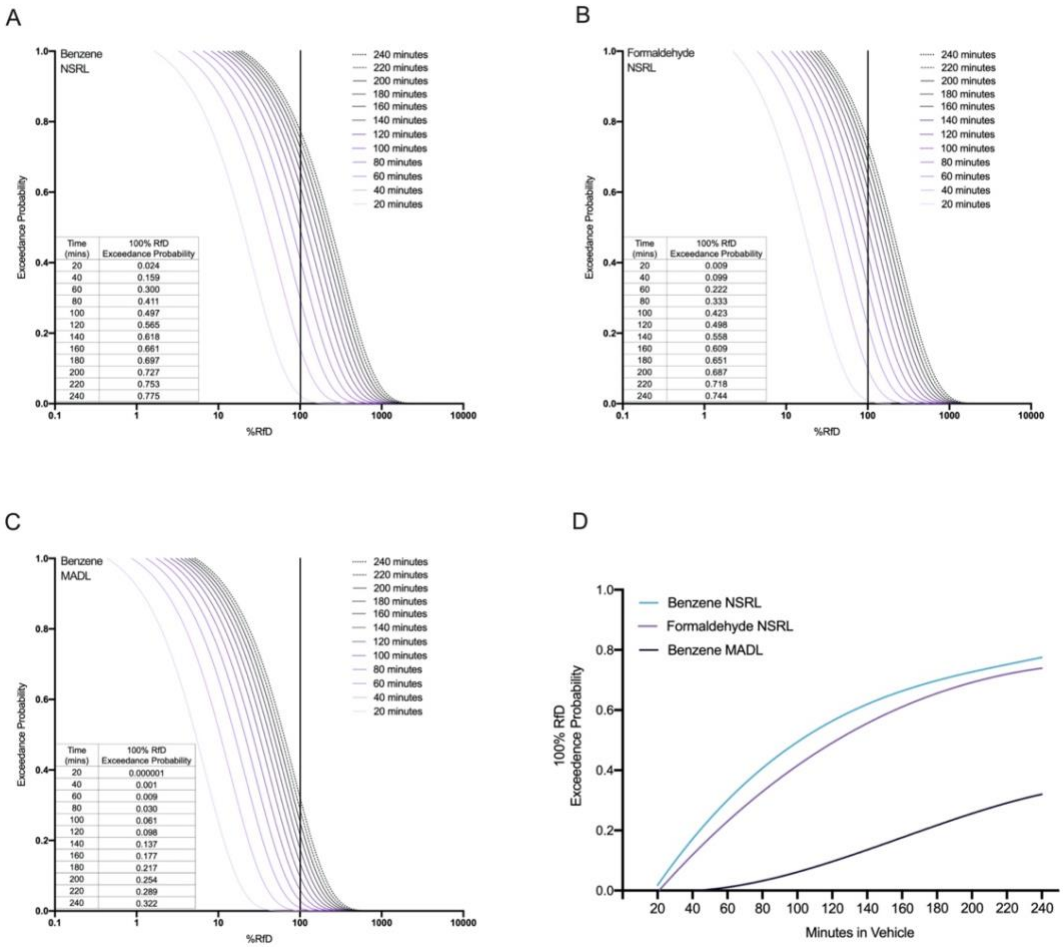

Figure 5.5. Exceedance probability curves for cancer risk (NSRL) associated with benzene (A) or formaldehyde (B) exposure as well as risk of reproductive and developmental toxicity (MADL) associated with benzene (C) exposure as a function of %RfD. The inset within panels A, B, and C show the probability of exceeding 100% RfD as a function of commute time. Curves representing the probability of exceeding 100% RfD as a function of commute time for all three different chemical risk scenarios (D).

For all California counties, the percent of commuters with a 10% probability of exceeding 100% RfD (Table S5.8) is represented in Figure 5.6. Within the entire state of California, the percent of commuters with a 10% probability of exceeding cancer risk associated with benzene or formaldehyde exposure was 78% and 63%, respectively, whereas the percent of commuters with a 10% probability of exceeding the risk of reproductive and developmental toxicity associated with benzene exposure was 11%. Across all three risk scenarios and counties, San Francisco County had the highest

percentage of commuters with a 10% chance of exceeding risk associated with benzene or formaldehyde exposure.

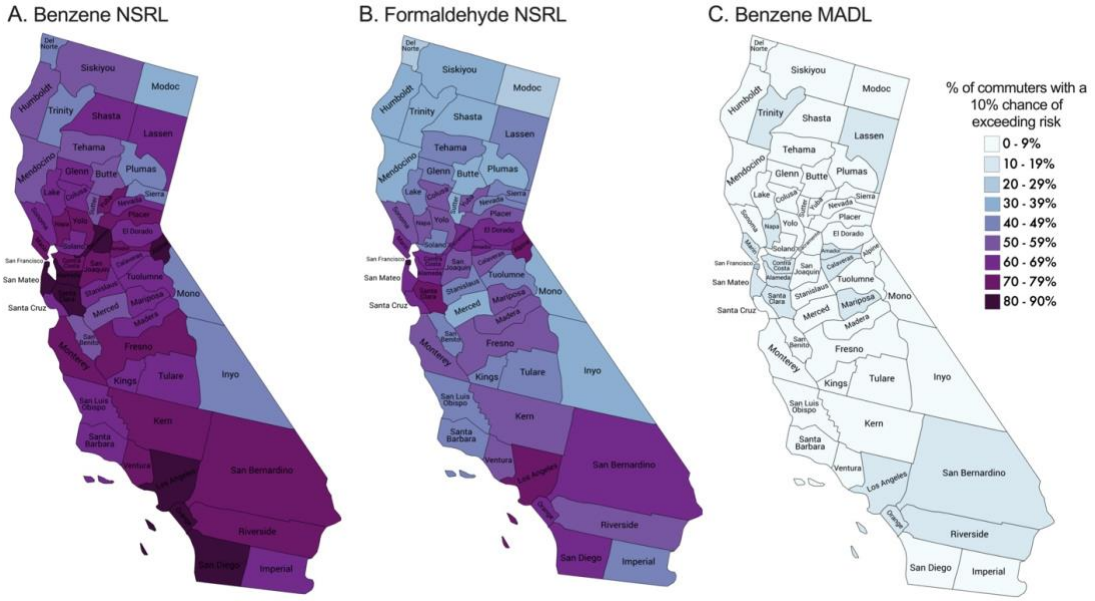

Figure 5.6. Maps of California counties showing the percent of commuters with a 10% probability of exceeding cancer risk (NSRL) and/or the risk of reproductive and developmental toxicity (MADL) for benzene or formaldehyde.

5.4 Discussion

Although the risk associated with Prop 65-listed chemicals within indoor environments is well characterized (Ali, 2019; Ao et al., 2019; Zhou et al., 2019), there is limited information on the risk that these chemicals within vehicle interiors pose as a function of commute time. Based on our meta-analysis, benzene, formaldehyde, DBP, DEHP and TDCIPP have all been previously detected within the interior of vehicles. While DBP, DEHP and TDCIPP were previously detected within interior car air and dust, benzene and formaldehyde were only found within the air of vehicle interiors – a finding that is linked to the high volatility of both chemicals. As benzene and formaldehyde are both VOCs, these chemicals are readily emitted into air and, as

such, exist almost entirely in the gaseous state. While DBP, DEHP and TDCIPP have been detected in the air of vehicle interiors, based on our meta-analysis these chemicals have been primarily found within dust of vehicle interiors. As DBP, DEHP and TDCIPP are semi-volatile organic compounds (SVOCs), these chemicals are more likely to adsorb onto surfaces of dust particles, furnishing materials, plastics, etc. (Harrad and Abdallah, 2011).

The presence of these compounds within vehicles can be attributed to extensive use in different vehicle parts. Formaldehyde is used in carpets, leather and paints within vehicles, resulting in off-gassing and high concentrations within indoor air (Pang and Mu, 2007). Furthermore, formaldehyde is also used as an adhesive and binder in the production of synthetic fibers, fiberboards, plastics, and textile finishing treatments, products that are commonly present in vehicles (Public Health England, 2017). The high concentration of benzene in vehicles has been attributed to fuel- and exhaust-related emissions that accumulate in the cabin of operating vehicles (Fedoruk and Kerger, 2003). However, several studies have also detected benzene within brand new cars under static conditions, suggesting that interior components are also off-gassing benzene into the air of vehicle interiors (Brodzik et al., 2014; Faber et al., 2013; Yoshida et al., 2006; Zhang et al., 2008). Benzene is used to produce styrene, nylon, and phenol which are, in turn, used to produce plastics, resins, and synthetic fibers (Hahladakis et al., 2018; CDC, 2018). Benzene is also used extensively in rubber, dyes, and lubricants and, from these products, benzene residue can off-gas and accumulate within indoor air. Phthalates such as DEHP and DBP are predominantly used as plasticizers in soft plastics, such as in a large variety of polyvinyl chloride (PVC) products including car seat fabric, cable insulation and interior and exterior trim in vehicles (Heudorf et al., 2007; Patil et al., 2017). TDCIPP is a commonly used flame retardant that is used within polyurethane foam of permanently installed seats as well

as plastics and electronics present in the vehicle's dashboard and console (Brandsma et al., 2014; Harrad et al., 2016).

Out of the five different Prop 65-listed chemicals assessed in this study, benzene and formaldehyde were the only two chemicals with estimated %RfDs exceeding 100. While this may be partially a result of lower safe harbor levels for benzene and formaldehyde relative to chemicals such as DEHP, the primary drivers are higher airborne concentrations relative to dust combined with higher inhalation rates relative to ingestion. Therefore, our study suggests that the presence of benzene and formaldehyde within air of vehicle interiors pose a higher risk to commuters relative to chemicals detected within dust of vehicle interiors. For benzene and formaldehyde, none of the commute times associated with the minimum or 5th percentile of the exposure distribution resulted in a %RfD that exceeded 100, suggesting that, if a commuter is on the lower end of the exposure spectrum, the daily dose will not exceed safe harbor levels associated with benzene and formaldehyde despite spending up to 4 hours in a vehicle. On the other hand, all of the commute times associated with the maximum daily dose exceeded a 100% RfD, underscoring the importance of estimating where a commuter lies within the exposure distribution.

Prior studies have shown that different factors such as interior temperature, ventilation rate and mode, humidity, solar radiation, vehicle age and grade, cabin value, car upholstery material, and travel distance influence the concentrations of benzene and formaldehyde detected within a vehicle (Chen et al., 2014; Xiong et al., 2015; Xu et al., 2016). Lower concentrations of aromatic hydrocarbons, such as benzene, are associated with fabric seats compared to leather seats and vehicles with larger volume cabins (Xu et al., 2016). Furthermore, off-gassing of VOCs may decrease with car age, total car travel mileage, increased ventilation rate, and lower in-car temperature or relative humidity (Chen et al., 2014; Xiong et al., 2015; Xu et al.,

2016). These different factors suggest that measures can be taken to reduce a commuter's daily dose and, as a result, decrease the probability of exceeding 100% RfD irrespective of time spent in the vehicle.

Based on our exceedance probability curves, cancer risks associated with exposure to benzene and formaldehyde are substantially higher than the risk associated with reproductive and developmental effects due to benzene exposure. Previous epidemiology studies in professional drivers (i.e., taxi drivers) have found significant associations between their profession and different forms of cancer, including lung, bladder, esophageal, stomach, and rectal cancer (Gubéran et al., 1992; Hansen et al., 1998; Ole Jensen et al., 1987; Tsoi and Tse, 2012). Moreover, additional studies have demonstrated that taxi drivers have a higher risk of cancer resulting from exposure to formaldehyde (Hadei et al., 2019; Pang and Mu, 2007) and benzene (Chen et al., 2016). While studies have previously examined associations between taxi drivers and cancer risks, there are virtually no studies that have investigated the potential association between cancer risk and commute time within the general population. A recent study by Patterson et al. (2020) found that commuting by personal vehicles has been associated with an increased rate of incident cancer compared to commuting by bicycle, rail or walking. Therefore, more research is needed to study the potential role of benzene and formaldehyde exposure in higher cancer incidence associated with longer commutes.

In California, more than 1.5 million people commute for more than 2 hours a day, with 3% of the population commuting for more than 3 hours a day (U.S. Census Bureau, 2017). Therefore, based on our study, it is possible that a substantial proportion of the population within California may exceed 100% RfD for benzene and formaldehyde on a daily basis. Interestingly, a study by Mapou et al. (2013) found that concentrations of in-vehicle formaldehyde in California communities were about twice

as high as New Jersey and Texas communities. This suggests that exposure to benzene and formaldehyde through interior car air is a pertinent issue, especially in California where a large percentage of the population is commuting by personal vehicles.

5.5 Conclusion

While this study was able to evaluate the potential risks associated with benzene and formaldehyde, risks for other chemicals detected within the air of vehicle interiors were not assessed due to the lack of inhalation-specific safe harbor levels established by OEHAA (TDCIPP, DBP and, DEHP). Moreover, while daily doses were calculated using intake rates, our risk assessment is based on the assumption that chemicals being inhaled and ingested are 100% bioavailable. Despite these limitations, this study highlights the potential risk associated with inhalation of benzene and formaldehyde for people who spend a significant amount of time in their vehicles. Furthermore, while the variability in chemical concentrations from countries with diverse climates may not be directly applicable to the state of California, this study provides a starting point for additional risk analyses. As benzene and formaldehyde are on the Prop 65 list due to cancer and reproductive/developmental toxicity concerns, there is a need for more information on the potential association between commute time within vehicles and exposure to both of these chemicals. As people with long commutes are an already vulnerable sub-population, additional measures may need to be implemented in order to mitigate potential cancer risks associated with benzene and formaldehyde exposure.

Chapter 6: Summary and Conclusions

6.1 Summary

Organophosphate esters (OPEs) are ubiquitously used as flame retardants, plasticizers and anti-foaming agents. Due to the nature of their use, OPEs are added to end-use products and are loosely associated with their matrix. OPEs can then volatilize, leach or abrade from the products and migrate into different environmental media such as indoor and outdoor air, water, soil and dust. In fact, studies have found OPEs in high concentrations within these environments and have detected their metabolites in human blood, urine and breast milk confirming exposure and intake of these chemicals. Therefore, while there is evidence suggesting that OPEs are being taken up by humans, more information is needed to understand the mechanisms underlying OPE-specific toxicity during early development and the different factors that influence human exposure to this class of compounds.

The findings and data presented in this dissertation 1) demonstrates the developmental effects of triphenyl phosphate (TPHP), a high production volume OPE, 2) measures human exposure to OPEs within a population that spends a significant amount of time within their vehicle, and 3) calculates the risk associated with tris(1,3-dichloro-2-isopropyl)phosphate (TDCIPP) – another high production volume OPE – following ingestion within personal vehicles. Regarding the developmental toxicity of OPEs, the data presented in Chapter 2 demonstrates that TPHP adversely affects hepatotoxicity-related pathways, liver morphology, neutral lipid abundance, and metabolite abundance within developing zebrafish embryos. Regarding human exposure to OPEs, in Chapter 3, we demonstrated that longer commutes are associated with increased exposure to TDCIPP within UCR undergraduates. Furthermore, in Chapter 4, we revealed that while TDCIPP is often detected in interior car dust, its removal from dust located on interior parts at the front of the vehicle does

not significantly mitigate TDCIPP exposure in people with longer commutes. Lastly, in Chapter 5, we determined that among five chemicals detected in car interior air and dust, exposure to TDCIPP through dust ingestion does not pose a risk for the average commuter. Overall, these data highlight the developmental effects of TPHP, the role of commuting and car dashboard wiping on TDCIPP exposure, and the risk associated with ingestion of TDCIPP within car interiors.

6.2 Triphenyl Phosphate as a Developmental Toxicant

Triphenyl phosphate (TPHP) is widely used as a flame retardant and plasticizer, and several studies have demonstrated its potential as a developmental toxicant. Past studies have shown that TPHP induces cardiotoxicity within the developing zebrafish embryo and this phenotype is mitigated by pre-treatment with non-toxic concentrations of a pan-retinoic acid receptor agonist (RAR), fenretinide. In Chapter 2, we aimed to identify other potential organs that might be targeted by TPHP during embryonic development and observe if these effects were mitigated by pre-treatment with fenretinide. Using mRNA-sequencing, we found pathways associated with cardiotoxicity, hepatotoxicity and nephrotoxicity in TPHP-treated embryos. In addition, exposure to TPHP also resulted in an increase in liver area in embryos relative to vehicle controls. However, while fenretinide mitigated TPHP-induced pericardial edema and liver enlargement, it did not mitigate the effect of TPHP on cardiotoxicity-, hepatotoxicity-, and nephrotoxicity-related pathways. Fenretinide is a pan-RAR agonist, however it is also a synthetic retinoid with known anti-inflammatory properties. Therefore, a fenretinide-induced decrease in pericardial edema and liver enlargement may be due to fenretinide blocking fluid accumulation within the pericardial region and liver. This suggests that TPHP-induced developmental effects

may not be RAR-mediated and may be due to another mechanism in the developing embryo.

Staining with Oil Red O, a neutral lipid stain, demonstrated that TPHP exposure resulted in a concentration-dependent decrease in neutral lipids, an effect that was not blocked by pre-treatment with fenretinide. This was supported by untargeted metabolomics that revealed a significant decrease in lipid-specific metabolites in TPHP-treated zebrafish embryos. Furthermore, among all metabolites, betaine was the most significantly affected metabolite. Within kidneys, betaine acts as an osmoprotectant to ensure osmolarity is balanced to allow water reabsorption. These results suggest that TPHP-induced toxicity may be due to a decrease in betaine which could potentially be affecting osmoregulation within the embryo and, as a result, may cause downstream effects on the pericardial region and liver (Figure 6.1).

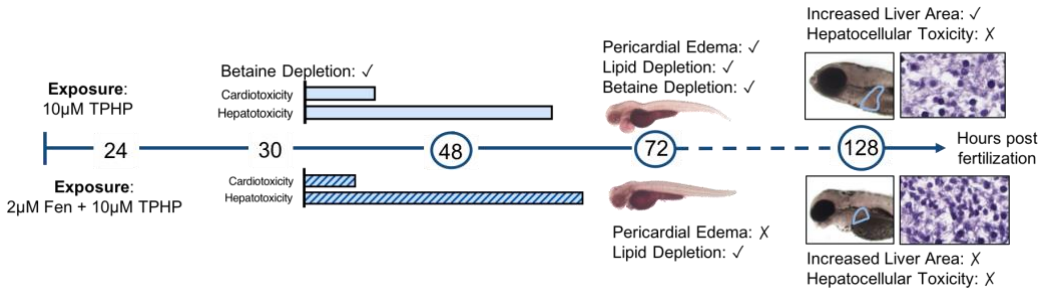

Figure 6.1. Conceptual diagram demonstrating the different experimental endpoints and results discussed in Chapter 2.

Betaine is produced by the choline cycle and mRNA-sequencing revealed that genes that regulate the choline cycle (CCT genes) were significantly downregulated at both 30- and 48-hours post-fertilization (hpf), suggesting that TPHP might be affecting CCT genes which may have downstream effects on choline levels within the embryo and consequently disrupt betaine production. As betaine is an osmoprotectant, its depletion may affect osmoregulation within the embryo. This disruption in osmoregulation can also explain the affected nephrotoxicity pathways identified by

mRNA-sequencing. Overall, this work elucidates the mechanisms behind TPHP-induced developmental toxicity within zebrafish embryos and potentially points towards disruption of osmoregulation as a key mechanism. Given the increasing use of TPHP as a flame retardant and plasticizer, future studies are needed to 1) identify the effect of TPHP on different osmoregulation mechanisms in the zebrafish embryos, 2) observe the effect of TPHP on other by-products of the choline cycle, 3) determine whether similar effects on embryonic development occur within rodent models, and 4) examine correlations between TPHP exposure on embryo implantation and maternal fertility within human populations.

6.3 Human Exposure of Tris(1,3-dichloro-2-propyl) phosphate in Vehicles

High concentrations of OPEs have been consistently detected within car interior dust, and with a large number of people in Southern California spending a significant amount of time within their vehicles, there is a need to characterize the relationship between commute time and OPE exposure. Within Chapter 3, we identified that longer commutes within undergraduate students at the University of California, Riverside were associated with increased TDCIPP exposure. TDCIPP, along with other OPEs, was used as an alternative flame retardant after the phase-out of brominated flame retardant mixtures. However, in 2011, TDCIPP was included in California's Proposition 65 list for its potential to cause cancer. Furthermore, in 2013, Technical Bulletin 117 was revised to include physical barriers as a way to comply with flammability in furniture. These two events may have led to a decline in the use of TDCIPP within indoor furniture and, consequently, a reduction in the detection of TDCIPP within the indoor environment. However, vehicles have higher flammability standards and several other Prop 65 chemicals within them. Therefore, vehicle manufacturers continue to use TDCIPP within permanently installed seats and

electronics in the car dashboard and console. This may be the potential explanation for higher exposures to TDCIPP for participants who spent a longer amount of time within their vehicle.

Our study relied on silicone wristbands to measure exposure to TDCIPP, and while research has shown a strong correlation between wristband OPE concentrations and respective urine metabolites, further research is needed to examine the relationship between longer commutes and concentrations of BDCIPP (TDCIPP's primary metabolite) within human urine and serum samples to validate our results. Furthermore, research has shown that TDCIPP is a carcinogen as well as reproductive and developmental toxicant within animal models. Therefore, further studies are needed to identify the relationships between longer commutes, TDCIPP exposure and human health outcomes such as infertility and cancer rates in the general adult population. Lastly, a larger volume of flame retardants are used in vehicles due to higher flammability standards. Similar to alternatives used within indoor furniture (e.g., physical barriers), other alternatives should be explored to meet the stringent, federal-level flammability standard in vehicles to reduce human exposure to TDCIPP.

As TDCIPP has been primarily detected within car interior dust, we hypothesized that a temporary solution to reduce human exposure to it within vehicles would be to reduce dust within the car interior. Therefore, within Chapter 4, our aim was to identify if a reduction in car dashboard dust would result in a significant decrease in TDCIPP exposure. Our research demonstrated that neither TDCIPP, nor any of the other OPEs measured on the wristbands, were significantly affected by wiping the dashboard dust. Furthermore, none of the OPE concentrations were affected by participant residence ZIP codes nor their variable AC/Heater usage. These findings suggest that elevated TDCIPP concentrations for people with longer commutes detected within Chapter 3 may be independent of dashboard dust, ambient

outdoor air concentrations, and ventilation within vehicles. As TDCIPP is a semi-volatile organic compound, TDCIPP released from end-use products may either settle within the dust and/or circulate within the air. Therefore, elevated TDCIPP exposure for people who spend longer amounts of time in their vehicle may be a function of circulating TDCIPP within car interior air. Overall, this work highlights an important exposure route to consider when quantifying exposure to TDCIPP as a function of time spent in vehicles. Further studies are needed to examine concentrations of airborne TDCIPP within the car interior air. Finally, a limitation within this study was that only the car dashboard dust was wiped, therefore a future study looking at entire car dust removal (i.e., from car seats and floor mats) is important to characterize the overall contribution of dust on increased TDCIPP exposure as a function of longer commutes.

6.4 Risk Associated with Tris(1,3-dichloro-2-propyl) phosphate Exposure within Vehicles

As we identified that longer commute times were associated with increased TDCIPP exposure, in Chapter 5 our aim was to characterize the potential risk associated with TDCIPP ingestion from dust within vehicles. Our work showed that ingestion of TDCIPP from car interior dust does not pose a cancer risk for people who spend between 20-240 minutes in their vehicle. However, research conducted within Chapter 4 highlighted the possible importance of inhalation of TDCIPP as an exposure route within vehicles. Due to limited data looking at the concentration of TDCIPP within car interior air and the absence of inhalation safe harbor levels by Prop 65, we were unable to examine the risk posed by inhalation of TDCIPP within car interiors. This is additionally important because the rate of inhalation (15.65 m^3/day) is significantly higher than ingestion of dust (0.02 g/day), suggesting that inhalation is an important exposure route that isn't being characterized.

California's Proposition 65 establishes a list of chemicals known to cause cancer, reproductive toxicity, or developmental toxicity. TDCIPP is included within this list for its potential to cause cancer, however its respective safe harbor level is only associated with ingestion. Preliminary literature suggests that TDCIPP has the potential to cause inhalation-mediated toxicity, and therefore there is a need for an inhalation-based safe harbor level for TDCIPP. If the safe harbor level for inhalation of TDCIPP is comparable to either its oral level or to benzene and formaldehyde's inhalation level, a significant population in California (especially in counties where people spend a large amount of time in their vehicles) may be exposed to TDCIPP concentrations within their vehicles that may pose cancer risk. Without sufficient research looking at both the concentrations of TDCIPP circulating within car interior air and inhalation toxicity there is a large data gap in the potential human health effects of TDCIPP following exposure within vehicles. Furthermore, while there are few studies looking at effects of chemical exposure on professional drivers (i.e., taxi drivers), there is no research, to our knowledge, looking at the effects of chemical exposure on commuters with long commutes, a question that is becoming increasingly relevant as commute times increase across the United States.

6.5 Organophosphate Esters: Further Directions and Considerations

As OPEs are continuing to be used in larger amounts, concentrations in environmental media and human exposure to them are bound to increase. Therefore, there is a need to efficiently and accurately characterize the various mechanisms of OPE exposure and toxicity. The toxicology-based research conducted within Chapter 2 utilized zebrafish embryos, a vertebrate model commonly used to assess the effects of chemical exposure on embryonic development. Due to its small size, rapid development, and overlap with other vertebrate developmental processes, the

zebrafish allows for rapid developmental toxicity testing. Furthermore, the exposure-based research conducted within Chapters 3 and 4 leveraged silicone wristbands to measure exposure to OPEs. Research has shown strong correlations between concentrations of OPEs on silicone wristbands and their respective metabolites in human serum and urine. Therefore, wristbands are being increasingly used as a cost-effective and non-invasive way to characterize biologically relevant exposures to OPEs. Using these techniques, our research has demonstrated complementary methods to characterize developmental toxicity and human exposure to OPEs.

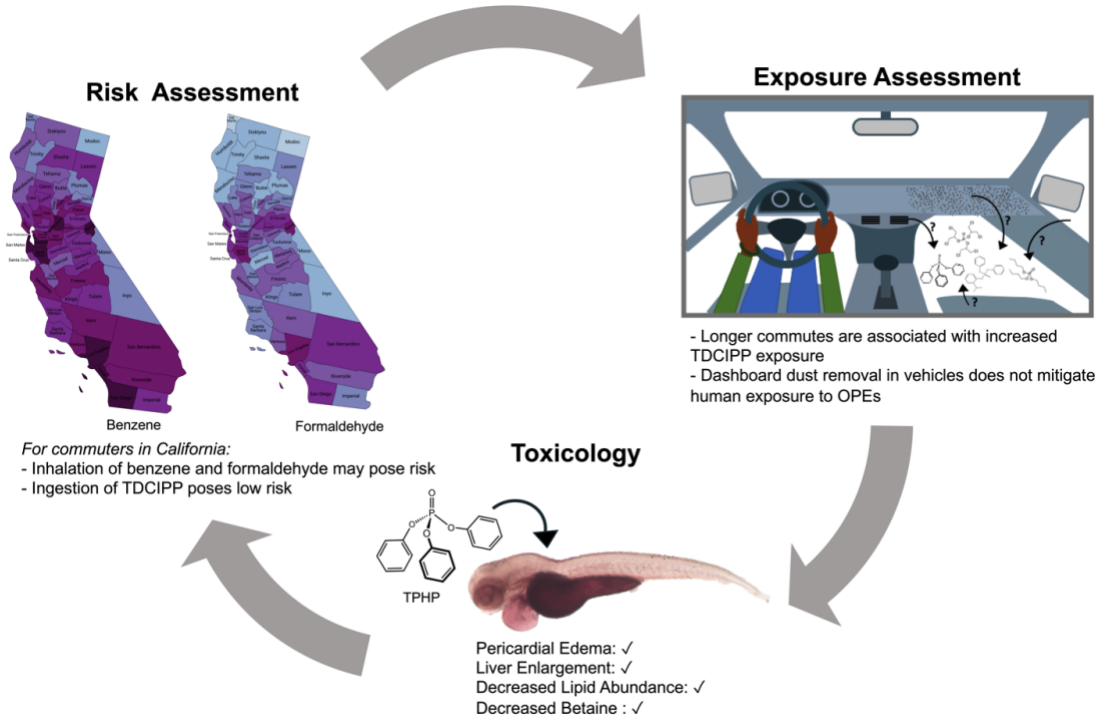

Figure 6.2. Schematic outlining the different research outcomes discussed within this dissertation.

The three core disciplines in environmental health that are used for risk assessment are exposure assessment, toxicology and epidemiology. The research presented within this dissertation has aimed to fill some of the gaps associated with the influence of OPEs on environmental health (Figure 6.2). Our research addresses 1) the toxicological mechanisms of TPHP, a highly used OPE, 2) the exposure assessment of OPEs as a function of increased commuting and partial removal of car dust, and, lastly, 3) the risk assessment of ingestion of TDCIPP within car interior dust. The data generated from this research can be used in addition to current and future studies to characterize and mitigate the risk posed by OPEs on the human population.

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