DEVELOPMENT OF A QUANTITATIVE ADVERSE OUTCOME PATHWAY (qAOP) NETWORK TO ASSESS DEVELOPMENTAL NEUROTOXICITY (DNT) OF PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS) MIXTURE IN *CAENORHABDITIS ELEGANS*

by

SETH DANIEL CURRIE

(Under the Direction of Lili Tang)

ABSTRACT

Per- and Polyfluoroalkyl Substances (PFAS) are a diverse class of industrial chemicals that have been utilized for decades in various applications due to their unique water- and grease-resistant properties. These substances have become ubiquitous in the environment, primarily as a result of widespread use in consumer products, food packaging, and industrial processes. Their persistence in the environment and bioaccumulation in living organisms has raised significant concerns regarding human health and ecological impacts, particularly as PFAS have been consistently detected in human bloodstreams, indicating a direct link between environmental exposure and potential health risks. Numerous studies have associated PFAS exposure with a range of adverse health outcomes, including hepatotoxicity, immunotoxicity, endocrine disruption, tumorigenicity, and neurotoxicity. Developmental neurotoxicity (DNT) has garnered particular attention due to the alarming increase in neurodevelopmental disorders observed in children linked to both pre- and postnatal exposure to PFAS. The growing prevalence of conditions such as attention deficit hyperactivity disorder (ADHD), autism spectrum disorders,

and learning disabilities in children raises critical questions about the long-term impacts of PFAS on neurodevelopment. In this dissertation, we aimed to develop a quantitative adverse outcome pathway (qAOP) network for assessing the developmental neurotoxicity (DNT) of PFAS mixtures using the model organism *Caenorhabditis elegans*. To explore these pathways, we employed the nematode *Caenorhabditis elegans* as a model organism, which is particularly well-suited for neurotoxicologically studies due to its simplicity, possessing only 302 neurons and a fully mapped network of chemical and electrical connections, enabling detailed examination of neurotoxic effects while facilitating high-throughput screening (HTS) for assessing cognitive impairments. The qAOP framework elucidates how PFAS exposure initiates neurodevelopmental disruptions through a sequence of biologically relevant key events (KEs). The qAOP integrates findings from *C. elegans* model systems, highlighting critical exposure windows and the impact of PFAS mixtures on neurodevelopment. By systematically linking these KEs and KERs, this framework advances the understanding of PFAS-induced neurotoxicity and supports more informed risk assessments. The dynamic nature of the qAOP framework ensures it can accommodate new scientific data and advancements, enhancing its applicability for regulatory science and public health protection.

INDEX WORDS: Per- and Polyfluoroalkyl Substances (PFAS), Neurodevelopment, *Caenorhabditis elegans*, Quantitative Adverse Outcome Pathway (qAOP), Developmental Neurotoxicity (DNT)

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DEDICATION

Dedicated to my parents, whose steadfast support and encouragement have been the cornerstone of my success. Your belief in me and constant encouragement have made this journey possible, and I am forever grateful.

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CHAPTER 1

INTRODUCTION

1.1 Background

Per- and Polyfluoroalkyl Substances (PFAS) represent a diverse class of synthetic industrial chemicals that have been widely employed for their exceptional water- and grease-resistant properties over several decades (Peritore, Gugliandolo, Cuzzocrea, Crupi, & Britti, 2023). These compounds, often referred to as "forever chemicals" due to their remarkable persistence in the environment, have infiltrated various environmental matrices, including soil, water, and air, primarily as a result of their extensive use in consumer products, food packaging, stain-resistant textiles, firefighting foams, and industrial applications (Jha et al., 2021). This widespread application has led to the accumulation of PFAS in the environment, where they can remain for years without significant degradation. Their bioaccumulation in living organisms has raised substantial concerns regarding their potential impacts on human health and ecological systems (De Silva et al., 2021). Notably, PFAS have been detected in human bloodstreams, establishing a direct correlation between environmental exposure and associated health risks (Fenton et al., 2021). This pervasive presence in biological tissues underscores the urgency of understanding their effects on health. A growing body of research has linked PFAS exposure to an array of adverse health outcomes, including hepatotoxicity (Kashobwe et al., 2024), immunotoxicity (Janssen et al., 2024), endocrine disruption (Rosen et al., 2022), developmental outcomes (Saha et al., 2025) and developmental neurotoxicity (DNT) (Carstens et al., 2023; (Xie et al., 2022)). Increasing evidence suggests that early life exposure to PFAS can interfere with normal neurodevelopmental processes, potentially leading to lasting cognitive and behavioral

deficits (Oh et al., 2022; Yao et al., 2023). However, nearly all current studies focused on PFOA and PFOS, the DNT data of most other PFAS are largely unavailable. Moreover, the most existing data derived from exposure to individual PFAs may not adequately represent or predict the risk from combined exposure in real world scenario. Considering that the developing brain is extremely sensitive to toxic chemicals, studies taking real- life exposure to PFAS mixtures into account for assessment of adverse effects of DNT are critical and urgent.

The nematode *Caenorhabditis elegans* has been employed as an excellent candidate for studying neurotoxicity with its simple yet well-mapped nervous system consisting of approximately 302 neurons, provides an exceptional framework for investigating neuronal structure, function, and connectivity (Haley & Chalasani, 2024). Laboratory *C. elegans* assays are usually rapid, of low cost, and amenable to high-throughput analysis. Additionally, the worms' transparency and the ease of making reporter gene fusions (e.g., with green fluorescent protein, GFP) enable visualization of cell morphology and protein expression patterns in vivo. Thus, this model organism allows us to combine the study of neuronal development, connectivity, physiology and behavior across a wide range of dosing, which would serve as a suitable intermediate between cell culture and higher order animal models for DNT of PFAS mixture testing. Additionally, *C. elegans* neurotoxicity testing will not only be instrumental to replace mammalian testing in neurological hazard assessment but also offers a compelling framework for developing a Quantitative Adverse Outcome Pathway (qAOP), with a sequential set of causally linked key events (KEs) at different levels of biological organization, beginning with a molecular initiating event (MIE) and culminating in an adverse outcome (AO). The developed qAOP framework can be used for in-deep mechanistic understanding and toxicity prediction, informing regulatory risk assessments, guiding policy decisions and public health

interventions aimed at mitigating the adverse effects of PFAS on neurodevelopment (Li et al., 2024).

1.2 Purpose of this Study

The overall goal of this study is to use a non-mammalian in vivo model organism, *C. elegans*, combining with high-throughput and high-content (HTHC) platform, transcriptomic technology, and a qAOP framework to investigate the DNT of PFAS single and mixture at different levels of biological organization following early-life exposure. Results from this study will yield comprehensive insights into the DNT of PFAS mixtures at multi-level understanding from molecular to organismal scales. Database generated by multi-dimensional approaches in this study will bridge gaps in our understanding of PFAS mixture exposure, revealing critical mechanistic pathways and predicting potential early-life impacts, which will facilitate the targeted interventions and policies to protect the health of children.

The specific aims of this study are as follows:

1. To construct an environmentally relevant and representative PFAS reference mixture and access the general toxicity of individual PFAS.
2. To investigate the developmental neurotoxicity (DNT) of PFAS in *C. elegans* model organism.
3. To develop a quantitative AOP (qAOP) network for developmental neurotoxicity (DNT) of PFAs in *C. elegans* model organism.

The hypothesis guides the dissertation the DNT of whole mixture of PFAs is sufficiently similar to reference mixture. The dissertation fulfilled the goal of establishing a quantitative

adverse outcome pathway to assess developmental neurotoxicity of per- and polyfluoroalkyl substances (PFAS) mixtures in *Caenorhabditis elegans*.

1.3 Outline of Dissertation

This dissertation examines the developmental neurotoxicity of PFAS mixtures using a non-mammalian model and a mechanistic framework. It begins with an overview of PFAS exposure, their persistence in the environment, and concerns regarding their potential neurotoxic effects. This is followed by investigations into toxicity at multiple biological levels, integrating high-throughput approaches and mechanistic insights. The results are systematically presented to illustrate the progression of effects across different levels of biological organization. The dissertation concludes by summarizing key findings and their implications for advancing neurotoxicity assessment and guiding future research directions.

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CHAPTER 2

LITERATURE REVIEW

[1] Currie, S. D., Wang, J. S., & Tang, L. (2023). *Environments 11, 188*
This chapter is a slightly modified version of [1] and has been reproduced here with the permission of the publisher.

2.1 PFAS and Health Impacts

Per- and polyfluoroalkyl substances (PFAS) are a group of synthetic chemicals that have been widely used in industrial and consumer products due to their unique water- and grease-resistant properties (Peritore, Gugliandolo, Cuzzocrea, Crupi, & Britti, 2023). These chemicals have been incorporated into a wide range of products, including nonstick cookware, food packaging, waterproof clothing, stain-resistant carpets, and firefighting foams (Ramírez Carnero et al., 2021). The exceptional durability of PFAS makes them highly effective in these applications, but it also means that they do not break down easily in the environment (Dimitrakopoulou et al., 2024). As a result, PFAS have accumulated in various environmental media, including soil, water, and air, where they can persist for decades. The widespread use of PFAS, combined with their ability to resist degradation, has led to significant environmental contamination. PFAS can enter human systems through multiple exposure routes, including contaminated drinking water, food, and air (Holder et al., 2023). In addition, individuals may be exposed to PFAS through occupational settings, especially in industries that manufacture or use these chemicals, such as the aerospace, automotive, and textile industries (Paris-Davila, Gaines, Lucas, & Nylander-French, 2023). Once in the human body, PFAS accumulate in various tissues, including the blood, liver, and kidneys, and can persist for years, raising concerns about their long-term health impacts (Fenton et al., 2021). Unlike essential metals or nutrients, which have specific biological roles, PFAS have no known beneficial effects on human health and are instead associated with harmful toxicological outcomes.

Exposure to PFAS has been linked to various adverse health outcomes, including liver damage (Zhang et al., 2023), immune system suppression (Bline et al., 2024), endocrine disruption (Li et al., 2024), and developmental toxicity (Bharal et al., 2024). Of particular

concern is their impact on the nervous system, especially during early development. Studies suggest that prenatal and early-life exposure to PFAS may contribute to cognitive impairments, behavioral changes, and neurodevelopmental disorders (Ames, Sharma, & Lyall, 2025). These effects may result from disruptions in key biological pathways involved in brain growth, synaptic function, and neurotransmitter regulation. While research continues to uncover the full extent of PFAS-related health risks, their widespread presence in the environment and the human body highlights the need for ongoing monitoring and assessment to better understand their long-term consequences.

2.2 PFAS and Developmental Neurotoxicity

2.2.1 Introduction

According to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), neurodevelopmental disorders (NDD) are defined as a group of conditions with onset in the developmental period, inducing deficits that produce impairments of functioning (Morris-Rosendahl et al., 2020). Impairments of cellular growth and metabolism during critical periods of prenatal brain development may result from the effects of environmental toxins, nutritional deficits, maternal illnesses, and genetic disorders, alone or in combination (Graf et al., 2013). It has been established that the environment plays a crucial role in influencing juvenile health, with an increased risk of negatively affecting neurodevelopment (Ijomone et al., 2020). Additionally, the significant increase in the occurrence of neurodevelopmental disorders suggests that environmental factors could be a major contributing cause (Landrigan et al., 2012). There are approximately 200 chemicals which have been found to be neurotoxic in humans, and for many more there is at least some evidence of neurotoxicity deriving from animal studies (Grandjean et

al., 2006; Giordano et al., 2012). However, of over 80,000 chemicals on the market, only a handful (about 200) have undergone developmental neurotoxicity testing according the established guidelines (Giordano et al., 2012; Makris et al., 2009). One class of chemicals that is of increasing concern for neurodevelopmental disorders are per- and polyfluoroalkyl substances.

Per- and polyfluoroalkyl substances (PFAS) are a varied collection of synthetic compounds defined by their chemical structure, which includes one or more carbon atoms bonded to fluorine atoms, forming fully fluorinated groups like −CF3 (perfluorinated methyl) or −CF2− (perfluorinated methylene). These substances differ in their carbon chain lengths, the degree of fluorination, and the types of additional chemical groups they may contain (Panieri et al., 2022). The development of PFAS began in the 1930s, and their unique property of interacting with both hydrophobic and hydrophilic substances has driven their widespread adoption in a range of industrial and commercial uses (Di Nisio et al., 2022). PFASs are often used for their "non-stick" and surface-tension lowering properties, which makes them useful for repelling oil and water (preventing stains) and modifying surface chemistry (Sunderland et al., 2019). Humans are exposed to PFAS primarily through consuming contaminated food and water, inhaling or ingesting dust and fumes from PFAS-containing items found in residential and office environments, and through occupational contact in industries that manufacture or utilize PFAS (Spratlen et al., 2020; Trudel et al., 2008). The most significant source of human exposure to PFAS is dietary intake (food and water) (Death et al., 2021). In certain situations, the intake of PFAS through drinking water can be just as significant as dietary sources of these chemicals (Ericson et al., 2008). Several studies have detected PFASs in surface- and groundwater worldwide; both of which are important sources for drinking water production and as a result public concern has arisen over human exposure risks to PFASs (Banzhaf et al., 2017).

Despite the extensive and widespread use of PFAS over recent decades, it was only in the past few years that significant attention was paid to human exposure to these chemicals and their potential negative health impacts (Domingo et al., 2019). PFAS pose numerous environmental and health risks. While some PFAS are regarded as having minimal health impact, others are linked to harmful effects in both humans and wildlife at present environmental exposure levels (Cousins et al., 2020). PFAS has been associated with adverse effects in many organs and systems, including reproductive (Cui et al., 2020; Gardener et al., 2021), immune (Stein et al., 2016; Papadopoulou et al., 2021), endocrine (Alderete et al., 2019; Chen et al., 2020; Fan et al., 2020; Blomberg et al., 2021), hepatic (Ojo et al., 2021), cardiovascular (Lind et al., 2017; Bjorke-Monsen et al., 2020), and neurodevelopmental effects (Carstens et al., 2023; Chen et al., 2014; Oh et al., 2022; Yao et al., 2023). Neurodevelopment begins shortly after conception, around three weeks into pregnancy, and progresses through the stages of infancy and into puberty (Rock et al., 2018). Various pieces of evidence indicate that the nervous system in development may be more vulnerable, or differently affected, by toxic exposures compared to the adult nervous system (Costa et al., 2019).

Extensive research shows that various PFAS are frequently found in pregnant women (Hamm et al., 2010; Szilagyi et al., 2020; Erinc et al., 2021), and that the placenta is a reasonable target for PFAS (Blake et al., 2020; Liu et al., 2024). PFAS accumulate in the placenta and pass the placental barrier, affecting the developing embryo (McAdam et al., 2023). Studies have shown that PFAS can cross the placental barrier and are associated with fetal growth restriction, immunosuppression, neurotoxicity, and some other health effects (Cai et al., 2020; Gutzkow et al., 2012). The ability of PFAS to pass through the placenta varies depending on factors such as the length of the carbon-fluorine chain, the presence of functional groups, and the overall

chemical structure. This process is largely influenced by the interaction of PFAS with serum carrier proteins and placental transport mechanisms (Bloom et al., 2022). Furthermore, It has been observed that young children tend to reach their highest PFAS concentrations before the age of two (Liu et al., 2020), possibly due to cumulative exposure via breastfeeding (Brantsaeter et al., 2013; Liew et al., 2018). The combination of exposure routes from gestation through adolescence makes PFAS an agent of neurodevelopmental toxicity. Increasing evidence from epidemiological research suggests that exposure to PFAS during pregnancy might influence neurodevelopment in children. This could affect various cognitive functions, including learning, IQ, and memory, and may also be related to behavioral disorders such as ADHD and autism spectrum disorders (ASD) (Spratlen et al., 2020; Gao et al., 2023). While IQ, ASD, and ADHD constitute prominent endpoints in the study of neurodevelopmental disorders, the broader category of neurodevelopmental disorders encompass a range of complex conditions marked by impairments in cognitive function, communication abilities, behavioral patterns, and motor skills, all stemming from atypical brain development (Mullin et al., 2013).

The US Centers for Disease Control and Prevention (CDC) has reported, based on findings from the National Health and Nutrition Examination Survey (NHANES), that PFAS have been found in the bloodstream of 97% of people in the United States (Lewis et al., 2015). With the omnipresence of PFAS in humans, there is an increasing concern of PFAS exposure throughout prenatal and postnatal development. Given the increasing concerns, through this review, we explore epidemiological studies examining the association between early-life PFAS exposure and childhood neurodevelopmental disorders, specifically focusing on IQ, ADHD, and ASD.

2.2.2 Material and Methods

2.2.2.1 Data Sourcing

To investigate the neurodevelopmental toxicity of per- and polyfluoroalkyl substances, a comprehensive literature search was conducted using PubMed. The search strategy included a combination of keywords related to PFAS and neurodevelopmental outcomes. The primary search terms used were: "PFAS," "per- and polyfluoroalkyl substances," "neurodevelopment," "IQ," "intelligence quotient," "ASD," "autism spectrum disorder," "ADHD," and "attention deficit hyperactivity disorder." These terms were combined using the Boolean operators “AND” and “OR” to form the search string: ("PFAS" OR "per- and polyfluoroalkyl substances") AND ("neurodevelopment" OR "IQ" OR "intelligence quotient" OR "ASD" OR "autism spectrum disorder" OR "ADHD" OR "attention deficit hyperactivity disorder"). Studies had to include clear exposure assessment for PFAS in humans. We included all primary epidemiological studies that presented quantitative measures of the association between at least one type of PFAS and at least one neurodevelopmental disorder. Specifically, this encompassed research that provided statistical estimates of how PFAS exposure correlates with neurodevelopmental outcomes. We excluded studies that were reviews or focused on non-epidemiological aspects, such as mechanistic studies, in vitro experiments, or animal research. Additionally, we did not consider human studies that failed to provide quantitative estimates of the relationship between PFAS and neurodevelopmental disorders (Figure 2.1).

2.2.2.2 Exposure Assessment

Exposure assessment in the included studies primarily involved measuring concentrations of PFAS compounds in biological samples, such as serum or plasma, from participants. These

measurements were typically obtained through high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS), a highly sensitive and specific analytical method (Da Silva et al., 2020; Marra et al., 2020). However, a subset of studies employed liquid chromatography (LC), indicating a variability of results. The studies varied in the specific PFAS compounds assessed, with common ones including perfluorooctanoic acid (PFOA), perfluorooctanesulfonic acid (PFOS), and perfluorohexanesulfonic acid (PFHxS). Additionally, the timing of exposure assessment ranged from prenatal (e.g., maternal blood or cord blood) to postnatal periods, reflecting critical windows of neurodevelopmental susceptibility (Henn et al., 2017). These observations highlight the diversity in methods and compounds analyzed, as well as the broad range of timing the exposure assessments, which collectively contribute to our understanding of PFAS exposure in relation to neurodevelopmental disorders.

2.2.2.3 Outcomes

The assessment of neurodevelopmental outcomes such as Intelligence Quotient (IQ), Autism Spectrum Disorder (ASD), and Attention deficit hyperactivity disorder (ADHD) in the included studies utilized standard and accepted means of evaluating neurodevelopmental disorders. Intelligence Quotient (IQ) was evaluated using widely recognized tests including Wechsler Intelligence Scale for Children-Fourth Edition (WISC-IV) and the Full Scale Intelligence Quotient (FSIQ) (Gansler et al., 2017; Smelror et al., 2023). For the diagnosis of ASD, studies often relied on established diagnostic criteria from the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), employing tools like Mullen Scales of Early Learning (MSEL) and the Behavioral Assessment System for Children-2 (BASC-2) (Chatham et al., 2018; Burns et al., 2013). Finally, ADHD was assessed and diagnosed using the criteria laid out in the

DSM-5. These diagnostic tools included ADHD-Rating Scale (ADHD-RS) and the Child Behavior Checklist (CBCL) (Tsujii et al., 2021; Biederman et al., 2021). These assessment tools ensure that the neurodevelopmental outcomes were accurately assessed to allow comparison between studies.

2.2.2.4 Covariates

Throughout the various studies between PFAS and neurodevelopment, several covariates were considered to control for potential confounding variables within each study. Commonly adjusted covariates included sociodemographic variables such as child's age, sex, parental education, household income, and maternal age at childbirth. Additionally, studies accounted for smoking, country of birth, and quality of the child's home environment. By controlling for certain covariates, the studies reduced bias and confounding variables to more accurately assess the relationship between PFAS and neurodevelopmental endpoints, such as IQ, ASD, and ADHD.

2.2.2.5 Data Extraction

Data extraction was conducted systematically using a standardized form to ensure consistency and comprehensiveness across all included studies. Key information collected included the following: authors' names, publication year, study design, sample size, and population characteristics such as age, sex, and geographic location. PFAS exposure assessment were recorded, including the types of PFAS compounds measured, biological sample type, and concentration levels. For neurodevelopmental outcomes, data on assessment methods for IQ, ASD, and ADHD were extracted, specifying the tools/criteria used for evaluation, such as

standardized intelligence tests, diagnostic interviews, and rating scales. Further, sample sizes, confidence intervals, and significance was also noted. Finally, any covariates that were adjusted for throughout the studies were recorded. Using a systematic data extraction process, all necessary information was collected in detail for accurate comparison between studies.

2.2.3 Results

2.2.3.1 Intelligence Quotient (IQ)

Within a literature search (Table 2.1), ten studies focusing on the association between PFAS exposure throughout childhood and intelligence quotient were included (Spratlen et al., 2020; Goodman et al., 2023; Beck et al., 2023; Vuong et al., 2021; Wang et al., 2015; Harris et al., 2018; Skogheim et al., 2020; Zhang et al., 2024). In the total, 14 individual PFAS were included, but only PFOS, PFHxS, and PFOA were seen in every study. Cognitive assessments were performed using various standardized tests, primarily the Wechsler Preschool and Primary Scale of Intelligence (WPPSI) and the Full Scale Intelligence Quotient (FSIQ). Most studies used either one of these main tests or a combination of them. However, Harris et al., 2018, chose to use the Kaufman Brief Intelligence Test (KBIT-2), while Skogheim et al., 2020, employed the Stanford-Binet Intelligence Test. While each study has different testing parameters, an extensive body of research has demonstrated that IQs obtained from different intelligence tests substantially correlate at the group level (Bunger et al., 2021). The results of the included studies showed conflicting results, but some studies indicated that there was some significant evidence that PFAS could have an inhibitory effect on intelligence quotient. Some of the inconsistent results could be associated with the several covariates such as PFAS exposure levels, child's age, and child's sex.

2.2.3.2 Attention Deficit Hyperactivity Disorder (ADHD)

As a results of the literature search (Table 2.2), nine primary research studies were identified that focused on PFAS exposure on attention deficit hyperactivity disorder (ADHD) (Vuong et al., 2021; Skogheim et al., 2020; Forns et al., 2020; Dalsager et al., 2021; Kim et al., 2023; Liew et al., 2015; Skogheim et al., 2021; Itoh et al., 2022; Quaak et al., 2016). While eleven PFAS were examined throughout the different studies, PFOS and PFOA were the only PFAS that were included in every study. While there is not a current standardized ADHD test, various tests with the main ADHD criteria being outlined in the Diagnostic and Statistical Manual of Mental Disorders were utilized in the different studies. These include commonly used diagnostic exams such as Attention Syndrome Scale of the Child Behavior Checklist (CBCL-ADHD) and Behavioral Assessment System for Children-2 (BASC-2). Most studies did not show any association between PFAS exposure and ADHD. However, a few studies showed conflicting results. Skogheim et al., 2021 and Itoh et al., 2022 indicated that there could be an inverse, protective effect of PFAS. Furthermore, Kim et al., 2023 and Vuong et al., 2021 indicated that there could be a positive association between PFAS exposure and ADHD. These conflicting results could be associated with the inability to conduct consistent ADHD diagnosis.

2.2.3.3 Autism Spectrum Disorder (ASD)

Through a systematic literature review (Table 2.3), six primary clinical studies were identified that focus on associating PFAS exposure on autism spectrum disorder (ASD) (Oh et al., 2022; Skogheim et al., 2021; Oh et al., 2021; Choi et al., 2024; Shin et al., 2020; Lyall et al., 2018). While ten different PFAS were analyzed, PFOA PFOS, PFHxS, and PFNA appeared in

every study. The diagnosis of autism spectrum disorder was conducted through several different standardized exams. The Mullen Scales of Early Leaning (MSEL) or the International Classification of Diseases (ICD)-8 were used in diagnosis in every study, except for Lyall et al., 2018 which used the DSM-5 criteria for diagnosis. Additionally, the Vineland Adaptive Behavioral Scale (VABS) and the Autism Diagnostic Observation Schedule (ADOS) were utilized in corroborating the results of the MSEL and the ICD-8. A majority of the studies showed significant association between PFAS exposure and increased odds of ASD. However, two studies that showed a low protective effect were Lyall et al., 2018 and Skogheim et al., 2021. This conflicting result could be due to the difference in diagnostic method utilized in the study. The results of these studies indicate that PFAS could have an inhibitory effect on neurodevelopment leading to ASD.

2.2.4 Discussion

In this review, we systematically gathered the current available evidence on the effects of early development PFAS exposure with respect to the outcome of neurobehavioral defects in children. Our results indicated that there is a potential effect of PFAS exposure throughout development on intelligence quotient, but the results are inconclusive. Furthermore, recent evidence (since 2022) highlights that PFAS exposure throughout gestation and early childhood has significant adverse effects on both ASD and ADHD. Although some studies have produced conflicting results, the latest research underscores the serious impact of PFAS on these neurodevelopmental disorders, emphasizing the importance of continued investigation to better understand these associations.

Within the past decade, researchers have considered PFAS exposure during gestation and childhood and its association with a variety of neurodevelopmental disorders. There are data supporting the plausibility of PFAS exposure as a risk factor on neurodevelopment through various routes of exposure. Initial exposure of PFAS begins with exposure of the developing fetus (Hoadley et al., 2023). This is due to the ability for PFAS to cross the placenta barrier from a pregnant woman to her fetus (Mamsen et al., 2019; Hanssen et al., 2013; Chen et al., 2017). PFAS has also been shown to expose developing children through breast milk (Macheka-Tendenguwo et al., 2018; Timmermann et al., 2023; Jian et al., 2018; Ragnarsdottir et al., 2022). These early exposure pathways have been positively associated with cardiovascular (Lin et al., 2013), immunologic (Grandjean et al., 2012; Granum et al., 2013), sexual maturation (White et al., 2011; Yang et al., 2009), thyroid function (Lopez-Espinosa et al., 2012; Tsai et al., 2017; Lin et al., 2013), kidney function (Kataria et al., 2015; Qin et al., 2016), and neurodevelopment outcomes (Fei et al., 2008; Goudarzi et al., 2016; Stein et al., 2013). Neurodevelopmental Disorders are a class of disorders affecting brain development and function and are characterized by wide genetic and clinical variability (Parenti et al., 2020). According to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), NDDs comprise of autism spectrum disorder, attention-deficit/hyperactivity disorder, and intellectual disabilities (Antolini et al., 2023).

Environmental chemicals and toxins have been correlated with a higher risk of neurodevelopmental impairments (Bragg et al., 2022). Epidemiological research supports these conclusions by documenting higher rates of birth defects, developmental disabilities, and reduced IQ levels in areas where mothers and children are exposed to various environmental pollutants (Brabhukumr et al., 2020). Intelligence quotient (IQ) is a measure of the progress an

individual had made in mental or cognitive development compared to same-aged peers (Carroll et al., 1997). For groups with neurodevelopmental disorders, mean IQ scores are generally below the population normative mean (Iqubal et al., 2020). Intelligence quotient was utilized to assess generalized intellectual disabilities to PFAS exposure. Studies using the Wechsler Preschool and Primary scales of Intelligence (WPPSI) and the Full Scale Intelligence Quotient (FSIQ) showed conflicting results on early PFAS exposure to intelligence disabilities. Some studies indicated that there was no association between PFAS and inhibition of IQ (Spratlen et al., 2020; Wang et al., 2023; Liew et al., 2018; Skogheim et al., 2020; Vuong et al., 2019). However, other studies found associations with multiple PFAS and their adverse effects on intelligence (Goodman et al., 2023; Beck at al., 2023; Wang et al., 2015; Harris et al., 2018; Zhang et al., 2024). There were a few confounding variables that adjusted the association. Goodman et al., 2023 noted that there was a significant association difference between the sex of the child with intellectual deficiencies and that this could be due to sex-specific effect by one or more mechanisms. This suggests that sex could act as an effect modifier rather than just a confounder. While some studies controlled for sex, they may have missed important variations by not examining how sex modifies the association between PFAS exposure and neurodevelopmental outcomes. Future studies should consider stratified analyses or interaction terms to better understand how sex influences these associations.

Attention-Deficit Hyperactivity Disorder (ADHD) is among the most prevalent neurodevelopmental disorders in children, marked by difficulties with attention, excessive activity, and impulsive behavior [108]. Various epidemiological studies have explored the link between early exposure to PFAS and the onset of ADHD during childhood (Vuong et al., 2021; Skogheim et al., 2020; Forns et al., 2020; Dalsager et al., 2021; Kim et al., 2023; Liew et al.,

2015; Skogheim et al., 2021; Itoh et al., 2022; Quaak et al., 2016). Early studies did not find any associations between PFAS and ADHD, however more recent studies have discovered that there is a significant association. In 2022, there were significant changes within the DSM-5 for the diagnosis of ADHD to make the diagnosis more accurate (Koutsoklenis et al., 2022). It was previously presumed that any symptoms of inattention and/or hyperactivity–impulsivity was secondary to ASD and not due to an additional ADHD diagnosis (DSM-5, 2000). This could lead to misdiagnosis in ADHD diagnosis, potentially distorting the results of earlier studies on the link between PFAS and ADHD. However, newer research has found a relationship between PFAS exposure at age 2 and the risk of developing ADHD by age 8 (Kim et al., 2023).

Over the past two decades, the prevalence of autism spectrum disorders has progressively increased (Wang et al., 2023). Autism spectrum disorder (ASD), characterized by deficits in social communication and restricted, repetitive behaviors or interests, affects approximately 2.3% of 8-year-old children in the US (Hirota et al., 2023). Studies using the Mullen Scales of Early Learning (MSEL) determined that PFOA had the strongest association with risk of ASD with an odds ratio [OR] per ng/mL increase: 1.99 (95% confidence interval [CI]: 1.20-3.29) (Oh et al., 2022). PFOS and PFHxS exhibited borderline associations with elevated risks of autism spectrum disorder (ASD). Specifically, PFOS had an odds ratio of 1.46 (95% CI: 0.98-2.18), suggesting a potential increase in risk, while PFHxS had an odds ratio of 1.03 (95% CI: 0.99-1.08), indicating a similar, though less pronounced, association (Shin et al., 2020). However, the remaining PFAS investigated did not show any significant relationship with ASD. The inability to determine association between some PFAS and ASD could be due to the inability to measure PFAS in biological samples over the limit of detection (LOD). Furthermore, evaluating co-occurring conditions in autism spectrum disorder (ASD) is difficult due to the overlap of

symptoms with other disorders, the risk of diagnostic overshadowing, and the often unclear presentation of symptoms. These factors make it challenging to accurately assess and differentiate additional conditions in individuals with ASD (Rosen et al., 2018).

Previous studies have considered neurodevelopment as one of the most sensitive endpoints for PFAS exposure. The findings of this extensive review have found significant associations between early-life PFAS exposure and the prevalence neurodevelopmental disorders. Given there is a ubiquitous exposure to PFAS, investigating the association between early-life exposure and neurodevelopmental disorders provides valuable information in understanding PFAS toxicity. Furthermore, the increasing prevalence and improved diagnostic techniques for neurodevelopmental disorders makes it essential to understand the detrimental impacts of environmental pollutants on human health.

2.2.5 Conclusion

In this review, PFAS exposure through neurodevelopment was strongly associated with neurodevelopmental disorders, such as ADHD and ASD, and potentially an inhibitory effect on IQ. Importantly, these findings indicate that the ubiquitous exposure of PFAS throughout gestation and into early childhood development could lead to neurodevelopmental disorders. However, there are notable data gaps that need addressing. For instance, high limits of detection (LODs) may have hindered the identification of associations between PFAS and NDD. Developing more sensitive analytical methods for detecting PFAS in biological matrices could enable the identification of lower levels of PFAS and provide crucial data. Further investigation using larger prospective cohort studies with standardized diagnostic methods is essential to confirm these results and elucidate the relationships between PFAS structures and associated

risks. Expanding research to fill these data gaps will offer valuable insights into the potential biological mechanisms underlying these adverse effects and guide future studies in this field.

2.2.6 Credit Author Statement

Seth D. Currie: investigation, formal analysis, writing—original draft, and writing—reviewing and editing. Jia-Sheng Wang: writing—reviewing and validation and writing. Lili Tang: resources, supervision, funding acquisition, and writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

2.3 *Caenorhabditis elegans (C. elegans)* and Neurotoxicity

Caenorhabditis elegans (C. elegans) is a small, transparent nematode that has become a powerful model organism for studying neurotoxicity and various aspects of toxicology (Melnikov, Kucharíková, Bárdyová, Botek, & Kaiglová, 2023). Their simple yet well-characterized nervous system consists of approximately 302 neurons, making it an ideal model for examining neuronal structure, function, and connectivity (Roussos, Kitopoulou, Borbolis, & Palikaras, 2023). *C. elegans* has several advantages as a model organism, including its rapid life cycle, ease of maintenance, and genetic tractability, allowing for high-throughput studies and the ability to manipulate its genome (Giunti, Andersen, Rayes, & De Rosa, 2021). The transparency of the worm enables direct visualization of cellular processes, including neuronal development and behavior, using fluorescent markers or other imaging techniques (Shah, Bao, & Zaidel-Bar, 2022). Additionally, the ability to expose *C. elegans* to a wide range of environmental toxins in a controlled laboratory setting provides valuable insights into how these substances affect neuronal function and development.

C. elegans is particularly useful for investigating neurotoxicity due to its well-mapped neural circuits and the similarity of many of its biological pathways to those in humans, including neurotransmitter signaling, synaptic function, and neurodevelopment (Ruszkiewicz et al., 2018). The model has been widely employed to study the effects of various neurotoxic substances, such as heavy metals, pesticides, and other environmental pollutants, on the nervous system. Through behavioral assays and molecular analyses, researchers can assess changes in motor function, learning, and memory, as well as alterations in gene expression related to neuronal health (Rahmani, McMillen, Allen, Minervini, & Chew, 2024). Because of its simplicity and high sensitivity to neurotoxicants, *C. elegans* serves as a valuable intermediate model between in vitro studies and more complex vertebrate models. It provides a cost-effective, efficient, and ethically sound approach for investigating the mechanisms underlying neurotoxicity and evaluating the potential risks of environmental chemicals, including their effects on neurodevelopment and long-term neurological health.

2.4 Adverse Outcome Pathways and Developmental Neurotoxicity

Adverse Outcome Pathways (AOPs) are a conceptual framework used to understand and describe the chain of events that lead from a molecular initiating event (MIE) to an adverse outcome (AO) at the organismal or population level (Jeong, Gasparyan, & Choi, 2025). In the context of neurodevelopmental toxicity, AOPs help elucidate the underlying biological mechanisms through which environmental toxicants, such as per- and polyfluoroalkyl substances (PFAS), can disrupt normal brain development and function (Rim, 2020). These pathways typically involve a series of sequentially linked key events (KEs), which may begin with molecular interactions, such as receptor binding or enzyme inhibition, and progress to more

complex biological processes, including neuronal differentiation, synaptic function, and behavioral outcomes (Brescia, Alexander-White, Li, & Cayley, 2023). AOPs provide a structured approach for understanding how chemical exposures can lead to adverse effects in the nervous system, aiding in the prediction of potential risks associated with neurodevelopmental toxicity.

The development and application of AOPs in neurodevelopmental toxicity research are particularly important for assessing the impact of environmental chemicals on the developing brain. During critical windows of neurodevelopment, exposure to toxicants can interfere with processes such as neurogenesis, axon guidance, synaptogenesis, and neural circuit formation, leading to long-term cognitive and behavioral deficits (Rock & Patisaul, 2018). By mapping out the key events and biological processes involved in neurodevelopmental toxicity, AOPs help identify potential biomarkers for early detection of adverse effects, guide the design of more targeted toxicological studies, and improve risk assessment practices. AOPs are increasingly being used to link molecular and cellular-level disturbances to functional impairments, offering insights into how specific chemicals or mixtures affect neurodevelopment (Bajard et al., 2023). This framework not only advances our understanding of the mechanisms of neurotoxicity but also supports regulatory efforts to identify and mitigate risks associated with exposure to harmful substances, ultimately contributing to better protection of human health and the environment.

2.5 Summary

PFAS are persistent environmental contaminants with significant implications for human health, particularly in relation to neurodevelopmental toxicity. These chemicals, commonly found in water, food, and air, have been shown to disrupt various biological systems, leading to

potential cognitive and behavioral impairments. The toxicological impacts of PFAS on the developing nervous system are a growing concern, as evidence suggests that they can interfere with neural development, synaptic function, and neurotransmitter signaling. The *Caenorhabditis elegans* model, with its well-defined neural circuits and genetic tractability, has emerged as a valuable tool for investigating these neurotoxic effects. Its simplicity, rapid life cycle, and ability to respond sensitively to neurotoxicants make it an ideal model for studying the mechanisms of neurotoxicity, particularly in the context of PFAS exposure. Through a combination of behavioral assays and molecular analyses, the effects of PFAS on neuronal function, motor skills, learning, and memory can be assessed, providing insights into how these pollutants may influence neurodevelopment. Adverse outcome pathways (AOPs) further contribute to understanding the progression of neurotoxic effects, linking molecular disruptions to functional impairments in the nervous system. This integrated approach helps clarify the potential risks posed by PFAS exposure and emphasizes the need for more research into the long-term impacts on human neurodevelopment.

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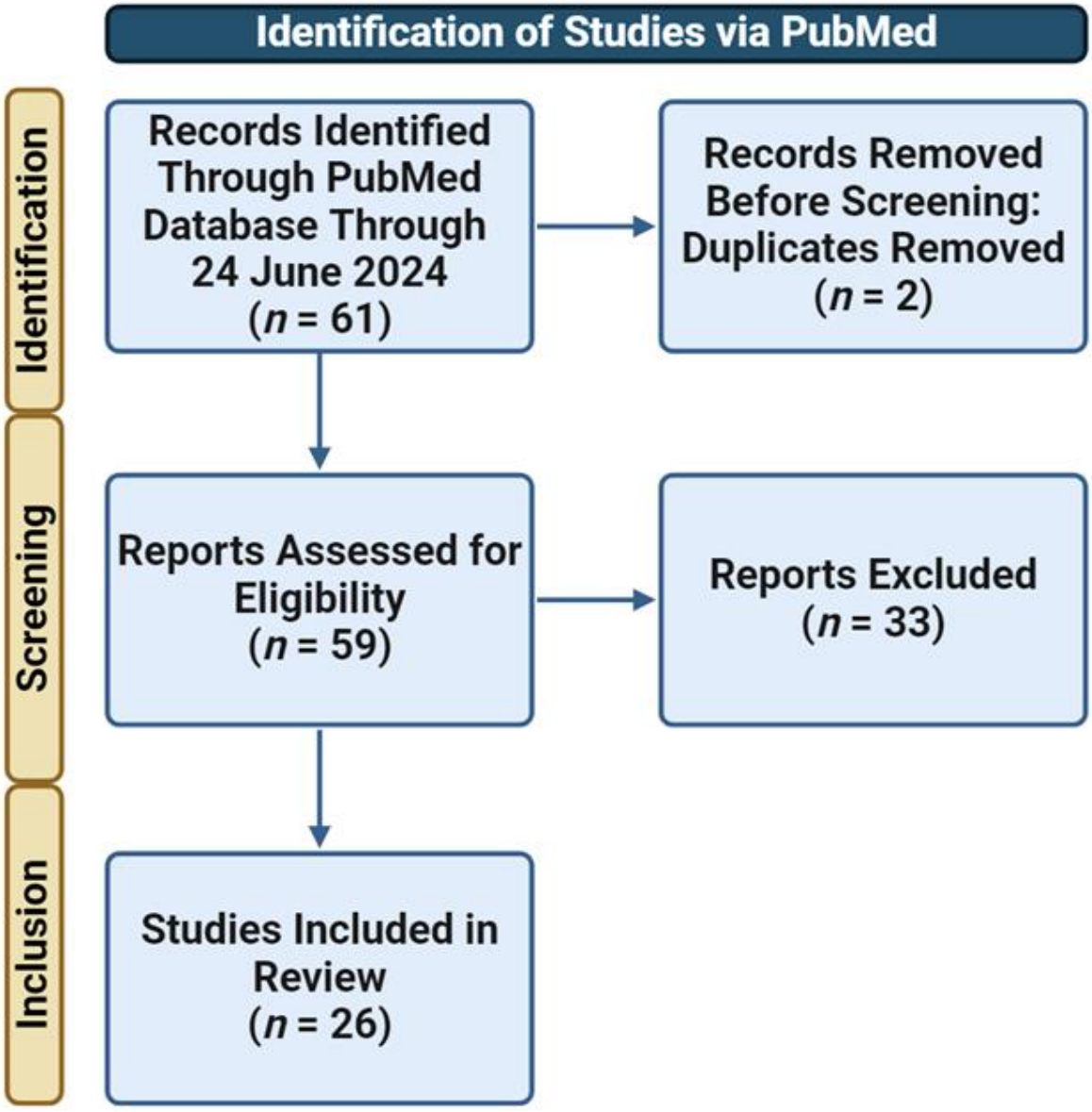

Figure 2.1: Study Flow Design

Table 2.1: Summary of articles, results, and evidence on PFAS exposure to Intelligence Quotient (IQ)

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Carly V Goodman/2023/ Canada [59]	Cohort Study	$n = 522$	Between 3 and 4	PFOA, PFOS, and PFHxS	Plasma/ UHPLC–MS/MS	PFOA: 1.68 (1.10–2.50), PFOS: 4.97 (3.20–6.20), PFHxS: 1.09 (0.67–1.60) (µg/L)	Wechsler Preschool and Primary Scale of Intelligence, Third Edition (WPPSI-III), composite full-scale IQ (FSIQ), performance IQ (PIQ), and verbal IQ (VIQ) scores	Gestational week of blood sampling, maternal age, pre-pregnancy BMI, country of birth (Canadian born, foreign born), maternal level of education (trade school diploma or lower, bachelor's degree or higher), parity (0, 1, 2+), maternal smoking during pregnancy (current smoker, former smoker, never smoked), study site, and the Home Observation Measurement of the Environment (HOME) score, a continuous measure of the quality of the child's home environment	Each doubling of PFHxS levels corresponded to a reduction of 2.0 points (95% CI: −3.6, −0.5) in FSIQ and 2.9 points (95% CI: −4.7, −1.1) in PIQ in males. However, in females, PFHxS showed no association with FSIQ or PIQ. PFOA and PFOS were also linked to lower PIQ scores in males (PFOA: B = −2.8, 95% CI: −4.9, −0.7; PFOS: B = −2.6, 95% CI: −4.8, −0.5), while in females, they were slightly positively associated with PIQ, but not FSIQ
Iben Have Beck/2023/ Denmark [60]	Cohort Study	$n = 967$	7 years old	PFOS, PFOA, PFHxS, PFNA, and PFDA	Serum/ LC–MS	PFOS: 4.61 (3.08–7.08), PFOA: 2.48 (1.58–3.49), PFHxS: 0.33 (0.21–0.50), PFNA: 0.57 (0.40–0.78), PFDA: 0.18 (0.13–0.24) (ng/mL)	Abbreviated version of the Danish WISC-V, Full-Scale Intelligence Quotient (FSIQ) score, and Verbal Comprehension Index (VCI) score	Maternal educational level, BMI, and sex	PFOS and PFNA exposure and FSIQ remained significant, with β coefficients of −1.7 (95% CI: −3.0, −0.3) and −1.7 (95% CI: −3.0, −0.4)
Ann M Vuong/2019/ United States [69]	Cohort Study	$n = 221$	3 and 8 years old	PFOA, PFOS, PFHxS, and PFNA	Serum/ HPLC–MS/MS	PFOA: 2.4, PFOA: 3.9, PFHxS: 1.4, PFNA: 0.8 (ng/mL)	Wechsler Intelligence Scale for Children-Fourth Edition (WISC-IV) and Full Scale IQ (FSIQ)	Maternal sociodemographic, behavioral factors, and biological measurements of environmental chemical	Findings do not support that PFAS are adversely associated with cognitive function
Hui Wang/2023/ China [62]	Cohort Study	$n = 2031$	4 years old	PFOA, PFOS, PFNA, PFUA, PFDA, PFHxS, PFBS, PFDoA, PFHpA, and PFOSA	Plasma/ HPLC–MS/MS	PFOA: 13.12 (9.36–15.50), PFOS: 11.3 (6.66–13.68), PFNA: 2.05 (1.27–2.49), PFDA: 2.16 (1.18–2.67), PFHxS: 0.62 (0.42–0.69) (ng/mL)	Wechsler Preschool and Primary Scales of Intelligence-Fourth Edition (WPPSI-IV)	Maternal age at delivery, maternal educational level, maternal pre-pregnancy body mass index, parity, maternal folic acid intake during pregnancy, maternal place of birth, maternal active/passive smoking status during pregnancy, maternal freshwater fish intake during pregnancy, and self-reported economic status	No significant associations between ln-transformed nine individual PFAS and child full scale IQ (FSIQ) or subscale IQ after adjusting for potential confounders
Zeyan Liew/2018/ Norway [63]	Cohort Study	$n = 1592$	5 years old	PFOS, PFOA, PFHxS, PFNA, PFHpS, PFDA, and PFOSA	Plasma/ LC–MS/MS	PFOS: 28.10 (21.60–35.80), PFOA: 4.28 (3.51–5.49), PFHxS: 1.07 (0.76–1.38), PFNA: 0.46 (0.36–0.57), PFHpS: 0.37 (0.27–0.49), PFDA: 0.17 (0.14–0.22), PFOSA: 2.32 (1.38–4.16) (ng/mL)	Wechsler Primary and Preschool Scales of Intelligence–Revised (WPPSI-R)	Maternal age at delivery, parity, maternal IQ, socioeconomic status, maternal smoking during pregnancy, maternal alcohol consumption during pregnancy, maternal prepregnancy BMI, child's sex	There is no reliable evidence establishing a connection between prenatal exposure to PFAS and IQ scores in children at the age of five

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Yan Wang/2015/ United States [64]	Cohort Study	n = 120	5 years old	PFHxS, PFOA, PFOS, PFNA, PFDeA, PFUnDA, PFDoDA, PFHpA, and PFHxA	Serum/ HPLC–MS/MS	PFHxS: 0.45 (0.35–0.57), PFOA: 2.00 (1.72–2.33), PFOS: 11.5 (10.2–13.07), PFNA: 1.33 (1.12–1.59), PFDeA: 0.39 (0.34–0.44), PFUnDA: 3.05 (2.37–3.94), PFDoDA: 0.29 (0.25–0.34) (ng/mL)	Full-Scale Intelligence Quotient (FSIQ), verbal IQ (VIQ) and performance IQ (PIQ)	Maternal age, maternal education, previous live births, family income, and maternal fish consumption during pregnancy	Exposure to two types of long-chain PFAS during pregnancy has been linked to lower IQ scores in children
Maria H Harris/2018/ United States [65]	Cohort Study	n = 1226	3 years old	PFOA, PFOS, PFHxS, PFNA, MeFOSAA, and PFDeA	Plasma/ HPLC–MS/MS	PFOA: 4.4 (3.1–6.0), PFOS: 6.2 (4.2–9.7), PFHxS: 1.9 (1.2–3.4), PFNA: 1.5 (1.1–2.3), MeFOSAA: 0.3 (<LOD −0.6), PFDeA: 0.3 (0.2–0.5) (ng/mL)	Peabody Picture Vocabulary Test (PPVT-III), Wide Range Assessment of Visual Motor Abilities (WRAVMA), Kaufman Brief Intelligence Test (KBIT-2), and Visual Memory Index of the Wide Range Assessment of Memory and Learning (WRAML2)	Child sex, age at cognitive testing, maternal race/ethnicity, age, maternal and paternal education, socioeconomic status and maternal intelligence scores	Prenatal PFAS were associated with both better and worse cognitive scores
Miranda J. Spratlen/2020/ United States [11]	Cohort Study	n = 110	Children ages 3–7 years	PFOS, PFOA, PFHxS, PFNA, PFDS, PFBS, PFOSA, PFHxA, PFHpA, PFDA, PFUnDA, and PFDoDA	Plasma/ HPLC–MS/MS	PFOS: 6.27 (1.05, 33.7), PFOA: 2.37 (0.18, 8.14), PFNA: 0.45 (<LOQ, 10.3), PFHxS: 0.69 (<LOQ, 15.8), PFDS: 0.13 (<LOQ, 0.64) (ng/mL)	Bayley Scales of Infant Development (BSID-II), Mental Development Index (MDI), Psychomotor Development Index (PDI), and Wechsler Preschool and Primary Scale of Intelligence (WPPSI)	Maternal age; material hardship during pregnancy; pre-pregnancy BMI; maternal IQ; maternal race; maternal education; home smoking exposure; marital status; parity; child's gestational age at birth; exact child age on test date; child's sex; maternal demoralization score; and child breastfeeding history	Findings on prenatal PFAS exposure and child neurodevelopment are inconsistent
Thea S. Skogheim/2020/ Norway [66]	Longitudinal Prospective Study	n = 944	3.5 years old	PFOA, PFNA, PFDA, PFUnDA, PFHxS, PFHpS, and PFOS	Plasma/ LC–MS/MS	PFOA: 2.50 (1.77–3.21), PFNA: 0.41 (0.29–0.53), PFDA: 0.15 (0.10–0.23), PFUnDA: 0.22 (0.14–0.32), PFHxS: 0.65 (0.46–0.88), PFHpS: 0.15 (0.10–0.20), PFOS: 11.51 (8.77–14.84) (ng/mL)	The Preschool Age Psychiatric Assessment interview, Child Development Inventory and Stanford–Binet (5th revision)	Maternal age, maternal education, maternal fish intake, parity, maternal ADHD symptoms, child sex, premature birth, birth weight, maternal BMI, maternal smoking, maternal alcohol consumption, maternal anxiety/depression and maternal iodine intake	No consistent evidence to conclude that prenatal exposure to PFAS are associated with cognitive dysfunctions in preschool children aged three and a half years
Boya Zhang/2024/ China [67]	Cohort Study	n = 327	7 years old	PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS, PFHpS, PFOS, PFDS, and PFOSA	Serum/ UHPLC–MS/MS	PFHpA: 0.27 (0.23–0.30), PFOA: 3.51 (3.29–3.75), PFNA: 0.32 (0.28–0.36), PFDA: 0.86 (0.76–0.96), PFUnDA: 0.61 (0.57–0.65), PFDoDA: 0.13 (0.12–0.14), PFBS: 0.08 (0.07–0.09), PFHxS: 0.09 (0.08–0.10), PFHpS: 0.06 (0.05–0.07), PFOS: 2.10 (1.98–2.22) (ng/mL)	Wechsler Intelligence Scale for Children-Chinese Revised (WISC-CR)	Maternal age at delivery, parity, maternal educational level, child's sex, annual household income, pet ownership, changes in marital status, pre-pregnancy BMI	Increased prenatal exposure to PFAS negatively affected the IQ of school-aged children

Table 2.2: Summary of articles, results, and evidence on PFAS exposure to Attention Deficit Hyperactivity Disorder (ADHD)

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Joan Forns/2020/ Norway [70]	Cross-Sectional Study	$n = 518$	3, 6, 12, and 24 months of age	PFOS and PFOA	Serum/ HPLC–MS/MS	PFOS: 20.19 (4.1–87.3), PFOA: 1.83 (0.5–5.1) (ng/mL)	Attention Syndrome Scale of the Child Behavior Checklist (CBCL-ADHD), Hyper-activity/Inattention Problems subscale of the Strengths and Difficulties Questionnaire (SDQ-Hyperactivity/Inattention), and ADHD Criteria of Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (ADHD-DSM-IV)	Maternal prepregnancy body mass index, maternal age at delivery, maternal education, maternal smoking during pregnancy, maternal parity, duration of total breastfeeding, and child sex	Exposure to PFOS or PFOA early in life was not linked to ADHD during childhood, with odds ratios (ORs) varying between 0.96 (95% CI: 0.87, 1.06) and 1.02 (95% CI: 0.93, 1.11). Analysis using stratified models indicates that the impact of PFAS may vary based on the child's sex and the mother's level of education
Louise Dalsager/2021/ Denmark [71]	Cohort Study	$n = 1138$	2.5–5 years old	PFHxS, PFOS, PFOA, PFNA, and PFDA	Serum/ LC–MS/MS	PFOS: 4.65 (11.22), PFOA: 2.43 (6.40), PFHxS: 0.32 (0.81), PFNA: 0.58 (1.24), PFDA: 0.18 (0.37), Median (95th percentile) (ng/mL)	Child Behavior Checklist 1.5–5	Parity, maternal educational level, parental psychiatric diagnosis, child sex	No correlation has been found between PFAS levels in mothers or children and symptoms of ADHD
Johanna Inhyang Kim/2023/South Korea [72]	Prospective Cohort Study	$n = 521$	2, 4, and 8 years old	PFOA, PFNA, PFDA, PFUnDA, PFHxS, and PFOS	Serum/ HPLC–MS/MS	PFOA: 3.61 (1.91–6.72), PFNA: 0.99 (0.45–2.96), PFDA: 0.34 (0.12–0.94), PFUnDA: 0.45 (0.17–0.94), PFHxS: 1.01 (0.54–1.95), PFOS: 3.94 (1.80–7.47) (ng/mL	ADHD Rating Scale IV (ARS)	Mother's age during pregnancy, mother's educational attainment, father's educational background, socioeconomic conditions, maternal smoking during pregnancy, use of assisted reproductive technologies, maternal stress levels during pregnancy	PFAS exposure at age 2 was associated with ADHD development at age 8
Ann M Vuong/2021/ United States [61]	Cohort Study	$n = 240$	5 and 8 years old	PFOA, PFHxS, PDNA, and PFOS	Serum/ HPLC–MS/MS	PFOA: 5.3 (1.7), PFOS: 12.8 (1.7), PFHxS: 1.5 (0.8), PFNA: 0.90 (1.5), mean (SD) (ng/mL)	The Behavioral Assessment System for Children-2 (BASC-2) and the Diagnostic Interview Schedule for Children–Young Child (DISC-YC) were used to evaluate ADHD symptoms and diagnostic criteria	Maternal age, race/ethnicity, education, family income, ln-maternal serum cotinine (ng/mL), maternal depression, marital status, maternal IQ, parity, and child sex	PFOS and PFNA were consistently linked to hyperactive-impulsive ADHD traits across two validated assessment tools

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Thea S. Skogheim/2021/ Norway [74]	Cohort Study	$n = 821$	3 years old	PFOA, PFNA, PFDA, PFUnDA, PFHxS, PFHpS, and PFOS	Plasma/ LC–MS/MS	PFOA: 2.46 (3.46–2.86), PFNA: 0.42 (0.20–0.49), PFDA: 0.19 (0.15–0.23) (ng/mL)	Adult ADHD Self-Report Scale (ASRS screener)	Child sex, birth weight, and small for gestational age (SGA); maternal age at delivery, education, parity, pre-pregnancy body mass index (BMI, kg/m^2), self-reported smoking and alcohol intake during pregnancy, as well as FFQ-based estimates of seafood (g/day), and dietary iodine intake (µg/day)	Several PFAS (PFUnDA, PFDA, and PFOS) were inversely associated with odds of ADHD and/or ASD
Sachiko Itoh/2022/ Japan [75]	Prospective Cohort Study	$n = 770$	8 years old	PFHxS, PFOS, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFTrDA, and PFTeDA	Plasma/ UHPLC–MS/MS	PFHxS: 0.32 (0.22–0.41), PFOS: 6.66 (4.92–8.31), PFOA: 2.48 (1.50–3.00), PFNA: 1.16 (0.79–1.38), PFDA: 0.53 (0.34–0.62), PFUnDA: 1.37 (0.73–1.73), PFDoDA: 0.18 (0.12–0.23), PFTrDA: 0.35 (0.24–0.44) (ng/mL)	ADHD Rating Scale (ADHD-RS)	Age of the mother at delivery, number of previous pregnancies, level of education, body mass index before pregnancy, alcohol consumption during pregnancy, smoking habits during pregnancy, and the sex of the child	Higher the maternal PFAS levels, lower the risk of ADHD symptoms at 8 y of age
Ilona Quaak/2016/The Netherlands [76]	Cohort Study	$n = 76$	18 months	PFOA, PFOS, PFHxS, PFHpS, PFNA, PFDA, and PFUnDA	Plasma/ LC–MS/MS	PFOA: 905.6 (437.1), PFOS: 1583.6 (648.3), PFHxS: 140.0 (69.2), PFHpS: 35.6 (21.3), PFNA: 140.0 (61.8), PFDA: 52.2 (20.9), PFUnDA: 32.05 (11.9), Mean (SD) (ng/L)	Child Behavior Checklist 1.5–5 (CBCL)	Family history, educational level, smoking, alcohol use and illicit drug use during pregnancy	Prenatal exposure to PFAS showed no significant associations with ADHD scores
Thea S. Skogheim/2020/ Norway [66]	Cohort Study	$n = 944$	3.5 years old	PFHpS, PFOS, PFHxS, PFOA, PFDA, PFUnDA, and PFNA	Plasma/ LC–MS/MS	PFOA: 2.61 (1.77–3.21), PFNA: 0.45 (0.29–0.53), PFDA: 0.19 (0.10–0.23), PFUnDA: 0.25 (0.05–0.32), PFHxS: 0.79 (0.46–0.88), PFHpS: 0.16 (0.10–0.20), PFOS: 12.32 (8.77–14.84), (ng/mL)	The Preschool Age Psychiatric Assessment interview, Child Development Inventory and Stanford–Binet (5th revision)	Maternal age, maternal education, maternal fish intake, parity, maternal ADHD symptoms, child sex, premature birth, birth weight, maternal BMI, maternal smoking, maternal alcohol consumption, maternal anxiety/depression and maternal iodine intake	Consistent evidence was not found to link prenatal PFAS exposure with ADHD symptoms or cognitive impairments in preschool children around three and a half years old
Zeyan Liew/2015/ United States [73]	Cohort Study	$n = 220$	Average 10.7 years old	PFOS, PFOA, PFHxS, PFHpS, PFNA, and PFDA	Plasma/ LC–MS/MS	PFOS: 26.80 (19.20, 35.00), PFOA: 4.06 (3.08, 5.50), PFHxS: 0.84 (0.61, 1.15), PFHpS: 0.30 (0.20, 0.40), PFNA: 0.42 (0.34, 0.52), PFDA: 0.15 (0.11, 0.20), (ng/mL)	ICD-10 codes F90.0	Maternal age at delivery, socioeconomic status, maternal smoking, alcohol drinking during pregnancy, mother's self-reported psychiatric illnesses, child's birth year, child's sex	Evidence does not consistently support a link between prenatal PFAS exposure and an increased risk of ADHD

Table 2.3: Summary of articles, results, and evidence on PFAS exposure to Autism Spectrum Disorder (ASD)

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Thea S. Skogheim/2021/ Norway [74]	Cohort Study	$n = 400$	3 years old	PFOA, PFNA, PFDA, PFUnDA, PFHxS, PFHpS, and PFOS	Plasma/ LC–MS/MS	PFOA: 2.46 (3.46–2.86), PFNA: 0.42 (0.20–0.49), PFDA: 0.19 (0.15–0.23) (ng/mL)	Diagnoses of "pervasive developmental disorders" were identified using ICD-10 codes F84.0, F84.1, F84.5, F84.8, or F84.9	Child's sex, birth weight, and status as small for gestational age (SGA); maternal age at delivery, education level, number of previous births, pre-pregnancy body mass index (BMI, kg/m^2), self-reported smoking and alcohol consumption during pregnancy, as well as estimates of seafood intake (g/day) and dietary iodine intake (µg/day) based on a food frequency questionnaire (FFQ).	An increased risk of Autism Spectrum Disorder (ASD) was observed in the second quartile of PFOA exposure [OR = 1.71 (95% CI: 1.20, 2.45)]. Conversely, PFUnDA, PFDA, and PFOS were associated with a reduced likelihood of ADHD, and the overall PFAS mixture showed a decreased risk of ASD [OR = 0.76 (95% CI: 0.64, 0.90)].
Jiwon Oh/2022/ United States [31]	Case–control Study	$n = 551$	2–5 years old	PFOS, PFHxS, PFNA, PFDA, PFPeA, PFUnDA, PFBS, PFHxA, MeFOSAA, and EtFOSAA	Serum/ HPLC–MS/MS	PFOA: 2.20 (0.91, 6.30), PFOS: 2.01 (0.81, 8.01), PFHxS: 0.59 (0.20, 3.05), PFNA: 0.71 (0.26, 2.49), PFDA: 0.14 (0.06, 0.49), PFPeA: 0.51 (0.20, 1.33), PFHpA: 0.23 (0.03, 1.00), PFUnDA: 0.03 (<LOD, 0.13), PFBS: <LOD (<LOD, 0.10), PFHxA: <LOD (<LOD, 0.43), MeFOSAA: 0.10 (<LOD, 1.56), EtFOSAA: <LOD (<LOD, 0.06) (ng/mL)	Mullen Scales of Early Learning (MSEL) and Vineland Adaptive Behavior Scales (VABS) are combined to generate an Early Learning Composite (Composite) score	Child's sex, age at sampling, recruitment regional center; sampling year; gestational age at delivery, maternal factors, parity, breastfeeding duration, race/ethnicity, and socioeconomic status.	PFOA was linked to higher odds of ASD, with an odds ratio (OR) of 1.99 per log ng/mL increase (95% CI: 1.20, 3.29). PFHpA also showed increased odds of ASD with an OR of 1.61 (95% CI: 1.21, 2.13). Conversely, perfluoroundecanoic acid (PFUnDA) was associated with lower odds of ASD, showing an OR of 0.43 (95% CI: 0.26, 0.69). Additionally, mixtures of PFAS were associated with increased odds of ASD, with an average OR of 1.57 and a range from the 5th to 95th percentile of 1.16 to 2.13.
Jiwon Oh/2021/ United States [77]	Cohort Study	$n = 57$	3 years old	PFOA, PFOS, PFHxS, PFNA, PFDA, PFUnDA, PFDoDA, MeFOSAA, and EtFOSAA	Serum/ Reverse-Phase LC–MS/MS	PFOA: 0.9 (0.3–2.3), PFOS: 3.0 (1.1–6.8), PFHxS 0.4 (0.2–1.6), PFNA 0.5 (0.2–1.0), PFDA 0.1 (<LOD −0.4), PFUnDA 0.1 (<LOD −0.3), PFDoDA: <LOD (<LOD −0.1), MeFOSAA: 0.1 (<LOD −0.8), EtFOSAA <LOD (<LOD-<LOD) (ng/mL)	Autism Diagnostic Observation Schedule (ADOS) and Mullen Scales of Early Learning (MSEL)	Child's sex, birth year, maternal vitamin intake in the first month of pregnancy, maternal education, and homeownership.	PFOA and PFNA were positively associated with ASD risk, with relative risks (RR) of 1.20 (95% CI: 0.90, 1.61) and 1.24 (95% CI: 0.91, 1.69), respectively, for each 2-fold increase in concentration. In contrast, PFHxS was negatively associated with ASD risk, showing an RR of 0.88 (95% CI: 0.77, 1.01).

First Author/ Year/Country	Design	Sample Size	Age of Children	PFAS	Sample/ Measuring Method	Exposure Measure	Test Type and Indicator	Adjustment of Covariates	Conclusion
Jeong Weon Choi/2024/ United States [78]	Cohort Study	$n = 280$	3 years old	PFHxS, PFOS, PFOA, PFNA, and PFDA	Serum/ Reverse-Phase LC–MS/MS	PFHxS: 0.45 (0.2–1.60), PFOS: 2.93 (1.10–7.00), PFOA: 0.87 (0.35–2.10), PFNA: 0.48 (0.20–1.00), PFDA 0.14 (<LOD −0.40) (ng/mL)	Autism Diagnostic Observation Schedule and Mullen Scales of Early Mullen Scales of Early Learning	Child sex, child age at assessment, year of birth, gestational age at delivery, maternal age at delivery, parity, maternal pre-pregnancy BMI, maternal race/ethnicity, maternal education, breastfeeding duration, homeownership, maternal smoking status during pregnancy, and child ASD outcome group.	PFOS, PFNA, and PFDA were associated with several behavioral problems among children diagnosed with ASD.
Hyeong-Moo Shin/2020/ United States [79]	Case–control Study	$n = 239$	2–5 years old	PFOA, PFOS, PFHxS, and PFNA	Plasma/ Reverse-Phase HPLC–MS/MS	PFOA: 1.07 (0.37–3.40), PFOS: 3.10 (1.08–10.03), PFHxS: 0.50 (0.20–1.63), PFNA: 0.50 (<LOD −1.23) (ng/mL)	Mullen Scales of Early Learning (MSEL), the Vineland Adaptive Behavior Scales (VABS), Autism Diagnostic Interview-Revised (ADI-R), Autism Diagnostic Observation Schedules-Generic (ADOS-G)	Age and sex of the child at the time of assessment, year of birth, regional center of recruitment, number of previous pregnancies, gestational age at birth, maternal race/ethnicity, place of maternal birth, mother's age at delivery, maternal BMI before pregnancy, vitamin intake around conception, duration of breastfeeding.	Increases in PFHxS and PFOS levels were tentatively connected to a higher risk of ASD diagnosis in children. For each nanogram per milliliter increase, PFHxS had an odds ratio of 1.46 (95% CI: 0.98, 2.18) and PFOS had an odds ratio of 1.03 (95% CI: 0.99, 1.08).
Kristen Lyall/2018/ United States [80]	Case–control Stude	$n = 553$	15–19 weeks gestational age	Et-PFOSA, Me-PFOSA, PFDeA, PFHxS, PFNA, PFOA, PFOS, PFOSA	Serum/ Negative-ion Turbo Ion Spray–tandem mass spectrometry	Et-PFOSA: 0.68 (0.63, 0.73), Me-PFOSA: 1.14 (1.07, 1.23), PFDeA: 0.17 (0.16, 0.18), PFHxS: 1.39 (1.29, 1.49), PFNA: 0.60 (0.57, 0.63), PFOA: 3.58 (3.41, 3.76), PFOS: 17.5 (16.8, 18.3), PFOSA: 0.11 (0.10, 0.11) (ng/mL)	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR) criteria	Child sex, month and year of birth, maternal age, country of maternal birth, maternal race/ethnicity, parity, and maternal education.	While most PFAS prenatal concentrations were not significantly linked to ASD, notable inverse associations were observed for perfluorooctanoate (PFOA) and perfluorooctane sulfonate (PFOS). Specifically, the adjusted odds ratios for the highest versus lowest quartiles were 0.62 (95% CI: 0.41, 0.93) for PFOA and 0.64 (95% CI: 0.43, 0.97) for PFOS.

CHAPTER 3

THE STAGE-SPECIFIC TOXICITY OF PER- AND POLYFLUROALKYL SUBSTANCES IN NEMATODE *CAENORHABDITIS ELEGANS* [2]

[2] Currie, S. D., Doherty, J. P., Xue, K. S., Wang, J. S., & Tang, L. (2023). *Environmental pollution 336*, 122429.
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Abstract:

Per- and Polyfluoroalkyl Substances (PFAS) are a diverse class of industrial chemicals that have been used for decades in industrial and commercial applications. Due to widespread use, and stability in the environment, and potential impacts on human health, PFAS have raised significant concerns. In this study, ten PFAS were selected according to their occurrence in different water bodies. The wild-type worms were exposed to individual PFAS at 0, 0.1, 1 ,10, 100 and 200 µM, and the toxic effects of PFAS on development, fecundity, and behavior at different life stages were investigated using a high-throughput screening (HTS) platform. Our findings revealed that PFOS, NEtFOSAA, PFBS, and PFHxS exhibited a significant inhibited the growth in as early as the L4 stages with concentrations ranging from 1a 00 to 200 µmol/L. PFOS and PFBS significantly decreased the brood size of worms across all tested concentrations ($p < 0.05$), and the most sensitive PFAS is PFOS with BMC of 0.02013 µM (BMCL, 1.6e-06 µM). During adulthood, all PFAS resulted in a significant reduction in motility ($p< 0.01$), while only PFOS can significantly induced the behavior alteration at early larvae stage. Furthermore, the larval stages were found to be the most susceptible to the effects of PFAS exposure. These findings provide valuable insights into the potential adverse effects associated with PFAS exposure and show the importance of considering developmental stages in toxicity assessments.

3.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are a class of structurally diverse synthetic organic chemicals characterized by the replacement of carbon-hydrogen bonds with carbon-fluorine bonds, either partially (polyfluorinated) or completely (perfluorinated). PFAS have been described as the “forever chemicals”(Kwiatkowski et al., 2020) for their chemically stability, resistant to degradation , and persistent in terrestrial and aquatic environments (Starnes et al., 2022). The synthesis of PFAS dates back to the 1930s, and their amphiphilic properties have led to their widely used in various industrial and commercial applications (Di Nisio et al., 2022). While some PFAS, such as perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), have been voluntarily phased out or regulated, exposure to PFAS remains ubiquitous(Bach et al., 2022). PFAS have been found in the environment and in the blood of humans and animals worldwide. According to a report by the Centers for Disease Control and Prevention (CDC), which using data from the National Health and Nutrition Examination Survey (NHANES), PFAS were detected in the blood of 97% of Americans(Lewis et al., 2015). Exposure in humans occurs through various routes, including the ingestion of contaminated food and water, inhalation or ingestion of dust and fumes from PFAS-containing products in homes and offices, and occupational exposure in workplaces that produce or use PFAS (Poothong et al., 2020; Spratlen et al., 2020). PFAS have been associated with several adverse effects , such as immune function (Stein et al., 2016; von Holst et al., 2021), thyroid function (Coperchini et al., 2020), liver disease & cancer (Cave, 2020; Filgo et al., 2015), lipid and insulin dysregulation (Birru et al., 2021; Chen et al., 2020; Rosen et al., 2022), kidney disease & cancer (Blake et al., 2018; Stanifer et al., 2018; Steenland and Winquist, 2021) reproductive and developmental

outcomes (Apelberg et al., 2007; Haervig et al., 2022; Velez et al., 2015; Waterfield et al., 2020), and neurodevelopment (Carstens et al., 2023; Chen et al., 2014; Oh et al., 2022; Yao et al., 2023).

The extensive human exposure to PFAS, along with the evidence indicating that some of these chemicals can cause adverse health effects, has raised global public concern. (Cousins et al., 2020). While PFOS and PFOA human exposure has been declining in western countries over the last decade due to regulatory interventions, scientists have identified more than 12,000 PFAS, which largely lack comprehensive toxicity data (Salvatore et al., 2022). Evaluating the toxicity of thousands of PFAS compounds presents a great challenge. Traditional mammalian toxicity assays are generally costly and time consuming, and it would be very difficult to test multiple chemicals and various concentrations of chemicals in parallel. Additionally, it is difficult to understand the lifetime exposure effects on complex mammalian models. Therefore, the use of a rapid *in vitro and alternative non-mammalian species* and High-throughput methodologies are highly justified.

Caenorhabditis elegans (C. elegans) are a nematode of small size (approximately 1 mm). They are known for their short lifespan, ease of culturing and maintenance, and large brood size make it a viable animal model for lab studies (Meyer and Williams, 2014). *Laboratory C. elegans* assays are usually rapid, of low cost, and amenable to high-throughput analysis (O'Reilly et al., 2014). *C. elegans* is well placed to be an alternative model in reducing use of the mammal animals (rodents) in traditional toxicity testing for its similar genetic make-up and whole-body design (e.g. skin, intestine, neurons) to humans(Leung et al., 2008; Schmeisser et al., 2017; Tejeda-Benitez and Olivero-Verbel, 2016; Xiong et al., 2017). Worms express homologues to approximately 80% of human genes with the basic biological functions and many biochemical pathways being highly conserved with higher organisms (Culetto and Sattelle, 2000).

Additionally, the transparent bodies allow unobstructed observation of all cells in mature and developing animals (Leung et al., 2008). Moreover, the use of non-*mammalian* models, such as *C. elegans* would enable mechanistic understanding of chemically induced adverse effects in different levels to define the adverse outcome pathways (AOPs) of PFAS and mixtures in the future. Using this model, our laboratory has established a high-throughput screening platform to assess the toxicity of mycotoxins and metals in *C. elegans* (Tang et al., 2019; Yang et al., 2015).

In this study, ten PFAS were selected according to their occurrence in different water bodies (Wang et al., 2022), which represent a wide range of typical PFAS structures, including perfluoroalkyl carboxylic acids (PFBA, PFHxA, PFOA), sulfonic acids (PFBS, PFHXS, PFOS), sulfonamides and derivatives (PFOSA, NetFOSAA), fluorotelomers (6:2 FTS), and new substitutes (HFPO-DA, the acid form of GenX). The toxic effect of PFAS on development, reproduction and behavior were assessed at the different life stages in *C. elegans* using a high-throughput screening platform. Further, the most sensitivity stage was predicting stage-classified matrix model to predict the critical stages that are most vulnerable to PFAS.

3.2 Material and Methods

3.2.1 Chemicals

The occurrence of PFASs in different water bodies based on the data in 23 studies conducted identified the most abundant PFAS (Wang et al., 2022) . Breaking down the information further into classifications, the most abundant PFAS in each category was identified with some additional alternative PFAS used as replacements for legacy PFAS. The 10 PFAS selected for this study include perfluorobutanoic acid (PFBA), perfluorohexanoic acid (PFHxA), perfluorooctanoic acid (PFOA), perfluorobutanesulfonic (PFBS), perfluorohexanesulfonic acid

(PFHxS), perfluorooctanesulfonic acid (PFOS), 1H,1H, 2H, 2H-perfluorooctanesulfonamidoacetic acid (NEtFOSAA), 6:2 fluorotelomer sulfonic acid (6:2 FTS), perfluorooctanesulfonamide (PFOSA), and hexafluoropropylene oxide dimer acid (HFPO-DA).

Analytical grade PFBA and PFOA were purchased from Sigma-Aldrich (St. Louis, MO). Analytical grade NEtFOSAA, PFHxA, and PFBS were purchased from Astatech Inc. (Bristol, PA). Analytical grade PFOS, PFHxS, HFPO-DA, 6:2 FTS, and PFOSA was purchased from Synquest Laboratories, Inc. (Alachua, FL). Stock solutions at 1M were prepared in dimethyl sulfate (DMSO), and working solutions were diluted using k-medium (32mM KCl and 51mM NaCl) (Williams and Dusenbery, 1990) containing OP50 at 1 mg/ml as food source and 0.1% DMSO (Brenner, 1974).

3.2.2 *C. elegans Culture and Exposure*

The nematodes, wild-type N2, along with the *Escherichia coli* strain OP50 were obtained from Caenorhabditis Genetics Center (Minneapolis, MN, USA). *C. elegans* were maintained at 25°C on solid nematode growth medium (NGM) plates seeded with OP50 (Brenner, 1974). By using sodium-bleach hydroxide isolation of eggs, synchronized L1 growth-arrested larvae were collected by hatching eggs in K-medium overnight with shaking (Lewis and Fleming, 1995). The L4-stage nematodes were obtained through allowing L1 synchronized worms to grow on NGM for 28-30 hours. For all toxicity testing, age synchronized worms were washed off the NGM plates using K-medium and centrifuged at 2500xg for 10 minutes to concentrate the worms,and exposed to different concentrations of individual PFAS. The exposure concentrations were 200, 100, 10, 1, 0.1 µmol/L.

3.2.3 High-Throughput Screening Platform for Growth, Brood Size, and Behavior in C. elegans

As described previously, high-throughput screening assays testing for growth, brood size, and behavior using COPAS BIOSORT and WMicrotracker-One™ (PhylumTech)(Tang et al., 2019; Tang et al., 2020). Wild-type *C. elegans* were tested in each assay to understand a general toxicity of each individual PFAS. Each experiment was performed independently 3 times with 6 replicates each time. Testing solutions contained K-medium and bacteria food, and an individual PFAS was added before dispensing the age-synchronized worms to designated wells (96-well plates) using the COPAS BIOSORT. The plates were incubated with plate shaking at the desired temperature for the predetermined time periods. For measurements of brood size and growth, each well was aspirated using the ReFLx module within the COPAS BIOSORT and analyzed for six parameters: extinction (EXT), time of flight (TOF), number of objects, as well as green, yellow, and red fluorescence. For measurements of behavior, the number of infrared laser beam interferences calculated an activity score for each well using the WMicrotracker-One™. Data was read, recorded, processed, and plotted using R software.

3.2.3.1 Growth

Fifty age-synchronized L1-stage worms were selected and distributed into each well containing 100 µL of test solution. Following an incubation period at 25°C, the plates were loaded into COPAS BIOSORT for measurement at specific time points, which were based on the normal life cycle of wild-type C. elegans at 25°C. These time points included the following stages: L2 larvae(12h), L3 larvae (20 h), L4 larvae (28 h), young adult (38 h), adult (46 h) and aging adult (60 h) after the L1 stage. Before conducting the measurement 10µL of 10% formalin solution was introduced to each well to effectively immobilize and align the worms for accurate

measurement. The EXT and TOF values for each event were recorded as size indictors for each worm.

3.2.3.2 Brood Size

A total of five L4-stage worms were selected and distributed into individual wells, each containing 100 µL of the test solution. The plates were then incubated at 25°C for a period of 72 hours. Following the incubation period, the plates were prepared for measurement. The event counts per well, which corresponded to the brood sixe per replicate, were recorded. The number of eggs produced during this specific timeframe served as a valuable indicator to assess the impact of PFAS on reproduction.

3.2.3.3 Behavior

Fifty age-synchronized L1 were selected and distributed into individual wells containing 100 µL of test solution. The wells were incubated at 25°C. Subsequently, the worms were loaded into the wMicrotracker for a 30-minute behavioral assessment. The assessment was conducted at specific time points based on the normal life cycle of wild-type *C. elegans* at 25°C (same as growth assay). The activity scores were recorded for each well and time point. These activity scores served as a valuable indicator to evaluate the effects of PFAS on behavior.

3.2.4 Calculation of the Benchmark Dose (BMD)

The benchmark concentration at 10% (BMC 10%) and lower confidence limit (BMCL) were calculated in PROASTweb software (version 70.1; https://proastweb.rivm.nl/). The data from each assay were summarized in Excel, containing information on the concentration levels

of PFAS and corresponding response effects then imported into the PROASTweb software (version 70.1). The software fits the selected model to the provided data using a maximum likelihood estimation (MLE). BMC calculations were performed using default settings: confidence level: 95, constant variance was assumed. The model selection parameter was set to lowest Akaike Information Criterion (AIC), which is a statistical measure used for model selection based on goodness-of-fit and a p-value cutoff of 0.05 was used. BMC and BMCL estimates were reported for all samples along with associated model parameters and fit statistics. For each PFAS, BMC and BMCL were calculated for all assays after exposure. This resulted in a dose of the PFAS (BMC or BMCL) that marks the beginning of the predetermined change in the response rate for the investigated assay. This quantitative calculation allows both a ranking of the assays for a PFAS using the parameters (TOF for growth, number of progeny for brood size, and activity score for behavior).

3.2.5 Statistical Analysis

All data were processed with the statistical program R, version 3.3.4 and were expressed as means ± standard deviation (SD). The level of statistical significance relative to control was calculated by using the analysis of variance (ANOVA) with Tukey's post-hoc test as a statistical method. The significance is represented in the graphs by asterisks (*, $p \leq 0{,}05$; **, $p \leq 0{,}01$; ***, $p \leq 0{,}001$). The sensitivity analysis was conducted using stage-classified matrix model to predict the critical stages that are most vulnerable to PFAS (Vindenes et al., 2021). To capture the dynamics of the population, the stage-specific parameters, including developmental, reproductive and motility rates for individuals in each stage, are organized into a matrix. The elasticities(ε) of

each stage-specific parameter output with respect to changes in vital rates (e.g., survival, fecundity, and growth rates) were calculated using the equation (1):

$$\varepsilon_i = \left(\frac{\partial \ln \lambda}{\partial \ln \lambda_i}\right) \times \left(\frac{\lambda_i}{w_i}\right) \quad (1)$$

where ε_i represents the elasticity of a variable or parameter of stage i, λ_i is the stage-specific reproductive or developmental output of stage i, and w_i denotes the proportion of individuals in stage i. ln is the natural logarithm, $\partial \ln \lambda / \partial \ln \lambda_i$ is the partial derivative of the natural logarithm of population growth rate with respect to the natural logarithm of stage-specific reproductive output (Caswell, 2001). The R package "*popbio"* was employed to calculate the ε values, the stage with the highest ε value is the most sensitive stage.

3.3 Results

3.3.1 Toxic Effects of Individual PFAS on Growth

For investigate the toxic effects of PFAS on development, the synchronized L1 were exposed to varying concentrations of 200, 100, 10, 1, and 0.1 µmol/L. The body length of the worms, represented as time of flight (TOF), was measured at different life stages, L2-L4 larvae, young adult, adult, and aging adult. The benchmark dose was calculated and listed in Table 3.1.

As shown in Figure 3.1, the PFAS exhibited a significant inhibited the growth in adult and aging-adult stages within the concentration range of 100 to 200 µmol/L. NEtFOSAA, PFBS, PFHxS, and PFOS exhibited a significant inhibited the growth in as early as the L4 stages at concentrations between 100 to 200 µmol/L**.** However, none of PFAS induced significant reduction at L2 and L3 stages. According to the BMC value, the toxicity ranks of ten PFAS in this adult stage was as follows: PFOS > PFOSA > PFBS > NEtFOSAA > 6:2 FTS > HFPO-DA > PFHxA > PFOA > PFBA > PFHxS.

3.3.2 Toxic Effect of Ten PFAS on Reproduction

Brood size, which was used as an indicator of reproductive toxicity, was measured by exposing age-synchronized L4-stage worms to 200, 100, 10, 1, and 0.1 μmol/L PFAS for 72 h, and then counting the number of offspring (brood size).As shown in Figure 3.2, compared to untreated controls, PFOS and PFBS significantly decreased the brood size of worms for all tested concentrations ($p < 0.05$). At concentrations as low as 0.1 μmol/L, PFOS was able to exert a 7.69 % of inhibition on brood size. At concentrations of 200 μmol/L, all PFAS showed significant reduction in brood size. The inhibition rates were 33.7% for 6:2 FTS, 80.7% for HFPO-DA, 50.7% for NEtFOSAA, 33.9% for PFBA, 38.8% for PFBS, 68.5% for PFHxA, 41.9% for PFHxS, 54% for PFOA, 69.3% for PFOS, and 21.4% for PFOSA, respectively. The BMC value with BMCL was estimated from the concentration-effect curve of each treated PFAS and listed in Table 3.2. The most sensitive PFAS is PFOS with BMC of 0.02013 μM (BMCL, 1.6e-06 μM).

3.3.3 Toxicity of PFAS on Behavior in Wild-type C elegans

The synchronized L1 were exposed to ten PFAS at concentrations of 200, 100, 10, 1, and 0.1 μmol/L, and the effects of PFAS on behavior were assessed by monitoring the motility using WMicrotracker. As shown in Figure 3.3, the behavior was significantly affected by PFAS in a concentration- and life-dependent manner ($p < 0.05$). During the adulthood, all PFAS resulted in a significant reduction in motility ($p< 0.01$), except for the 6:2 FTS which didn't show any reduction in motility at young adult stage. As expected, PFOS is the most toxic substance among the ten PFAS, causing significant behavior impairment at all life stages ($p<0.01$). At the L2 stage, only PFOS induced the behavior alteration with the BMC value of 0.1406 μM (BMCL:

0.000747 µM). NEtFOSAA, HFPO-DA, and HFPO-DA exhibited motility reduction starting from L3 life stage. However, PFBS, PFOA and PFHxA didn't impair behavior at larvae stage until adulthood. According to the BMC, the toxic rank at adult stage was as follows: PFOS > NEtFOSAA > PFBS > 6:2 FTS > PFHxA > HFPO-DA > PFBA > PFHxS > PFOA > PFOSA.

3.3.4 Sensitivity Analysis

To predict the stage specific sensitivity, a population matrix was extrapolated, and the elasticity was calculated using the "popbio" package in R software. The most sensitive parameter motility data was used, and the activity score was standardized to the control at each stage. These results are illustrated in Figure 3.4. The larval stage is the most sensitive phase when exposed to higher concentrations of PFAS, excluding PFOS and HFPO-DA. During the L2 stage, PFOS exhibited vulnerability at all concentrations. HFPO-DA showed susceptibility at low concentrations, not only at the larval stage but also in the young adult stage.

3.4 Discussion

In this study, ten PFAS were selected, and the general toxic effect such as development, reproduction and behavior were assessed at the different life stage in *C. elegans* using a high-throughput screening platform. Our results showed that PFAS exhibited a significant inhibited the growth in adult and aging adult stages within the concentration range of 100 to 200 µmol/L, except for NEtFOSAA, PFBS, PFHxS, and PFOS, which had inhibited effect as early as L4 stage. All PFAS showed significant reduction in brood size at the concentration of 200 µmol/L. The most toxic PFAS is PFOS with BMC of 0.02013 µM (BMCL, 1.6e-06 µM). For the behavior, all PFAS resulted in a significant reduction in motility ($p < 0.01$) at adult and aging

adult. The toxic rank at adult stage was as follows: PFOS > NEtFOSAA > PFBS > 6:2 FTS > PFHxA > HFPO-DA > PFBA > PFHxS > PFOA > PFOSA. Further sensitivity analysis showed that the larval stage is the most sensitive phase when exposed to higher concentrations of PFAS with the exception of PFOS and HFPO-DA, which exhibited vulnerability at low concentrations.

The presence of thousands of PFAS and the growing body of evidence linking some of them to adverse health effects has created immense need to better understand PFAS toxicity (Sonne et al., 2023). However, a significant amount of toxicological data currently is available for a limited number of PFAS, such as the legacy PFAS, PFOS and PFOA(Perez et al., 2023). In this present study, the ten PFAS were selected from the top abundant PFAS in each of the major categories in water systems within the United States (Wang et al., 2022). The PFAS were investigated in a liquid form independently present in k-medium and a bacteria food source. The concentrations used allow for the investigation at both an environmentally relevant (0.1 µM) and a value significantly higher (200 µM), along with values in between. This range gives for an insight of both the environment level and direct ingestion exposure.

C. elegans is a highly valuable model organism due to its physiologically and genetically traceability. Its low maintenance and short life cycle make it advantageous for studying the toxicological effects of environmental toxicants across different developmental stages(Leung et al., 2008). Using our lab established High-throughput screening platform(Tang et al., 2019; Tang et al., 2020), three endpoints, growth, brood size, and motility, were selected to explore the toxicity of PFAS. Of which, growth is an indicator of development and overall health status of worms, brood size refers to reproductive system, while motility reflects the neuronal status in *C. elegans*(Leung et al., 2008; Ruszkiewicz et al., 2018).Our results were consistent with

previous findings, all tested PFAS caused developmental, reproduction and behavioral toxicity (Conley et al., 2022; Conley et al., 2019; Perez et al., 2023; Rericha et al., 2022; Ulhaq and Tse, 2023; Wasel et al., 2022; Yue et al., 2020), and PFOS is the most toxic substance among them. Notably, it is the first time found that PFOSA and 6:2FTS induced toxicity in *C. elegans*. Moreover, our study found a significant inhibitory effect of PFAS during the adult and aging adult stages, primarily attributed to the bioaccumulative nature of PFAS. PFAS are known to be bioaccumulative in human tissue(Pérez et al., 2013), environment (Ghisi et al., 2019; Munoz et al., 2022), aquatic species (Burkhard, 2021), lab animals (Bangma et al., 2022) , including *C. elegans*(Sammi et al., 2019; Yue et al., 2020). This indicated that early exposure and subsequent bioaccumulation of PFAS could lead to cumulative damage over time, which resulted in chronic health conditions, such as neurodegeneration. Our study found that all tested PFAS resulted in a significant behavioral impairment during adult and aging adult, with statistical significance ($p<0.01$).

To predict the susceptible population, a population matrix model was used for extrapolation. Our study found that the larval stage is the most sensitive phase when exposed to higher concentrations of PFAS, excluding PFOS and HFPO-DA which exhibited susceptibility at very low concentrations. This could be due to several factors such as functional groups, carbon length and rate of ingestion. PFOS and GenX have a unique ability to have a high transfer efficiency increasing their rate of ingestion by increasing permeability of epithelial cells (Wang et al., 2011). This gives them a higher toxicity at lower concentrations. Growing concerns have been raised about the potential health effects of PFAS exposure, particularly on children. As the development of the brain and nervous system in children is a dynamic process, any disruptions or alterations in neurodevelopment have the potential to significantly impact their health throughout

development, and potentially even in later stages of life. While research is still ongoing, several studies have suggested that exposure to PFAS may have adverse effects on child health (Anderko and Pennea, 2020; Beck et al., 2023; Feng et al., 2022; Roth and Wilks, 2014; Sunderland et al., 2019). Research has revealed that PFAS have the ability to cross the placenta barrier , leading to potential exposure of children during breastfeeding (Szilagyi et al., 2020); Higher prenatal PFAS exposure was associated with lower performance IQ in males(Goodman et al., 2023); Johansson, et al. (Johansson et al., 2008) demonstrated neurobehavioral deficits in adult mice after neonatal PFOA and PFOS exposure, where spontaneous behavior of adult mice manifested as hyperactivity and inability to habituate. Our study also observed a decrease in body size and behavior with an increase of concentration throughout the developmental stages.

Taking together, using nematode model with combination with the High-throughput screen platform provided many advantages for evaluating the toxicity of PFAS. The findings obtained from this study will contribute to bridging the existing gap in our understanding of PFAS toxicity. Given that the larval stages exhibited the highest sensitivity, our future research will focus on investigating the developmental neurotoxicity of PFAS. Furthermore, it is important to consider that humans, especially children, are exposed to multiple PFAS simultaneously and/or sequentially from various sources (Bjerregaard-Olesen et al., 2019; Ojo et al., 2021).Considering the co-occurrence of numerous PFAS in environmental samples, it becomes essential to conduct studies that account for real life exposure to PFAS mixtures when assessing the developmental neurotoxicity.

3.5 Conclusion

Using a high-throughput screening platform, we investigated the toxicity of selected 10 PFAS on development, reproduction, and behavior in wild-type *C. elegans*. Our findings suggested that individual PFAS have an impact on all three endpoints at varying concentration levels. Chemicals with similar functional groups have similar exposure results. Furthermore, larval stages exhibited the highest sensitivity to PFAS. These observations shed light on the potential adverse effects of PFAS exposure and emphasize the importance of considering developmental stages in toxicity assessment.

3.6 Credit Author Statement

Seth Currie: Methodology, Investigation, Formal analysis, Writing- Original Draft, Writing-Reviewing and Editing. Joseph P. Doherty: Investigation. Kathy S. Xue: Validation, Writing-Reviewing and Editing, Jia-Sheng Wang: Validation, Writing-Reviewing and Validation, Writing Lili Tang: Resources, Supervision, Funding acquisition, Writing-Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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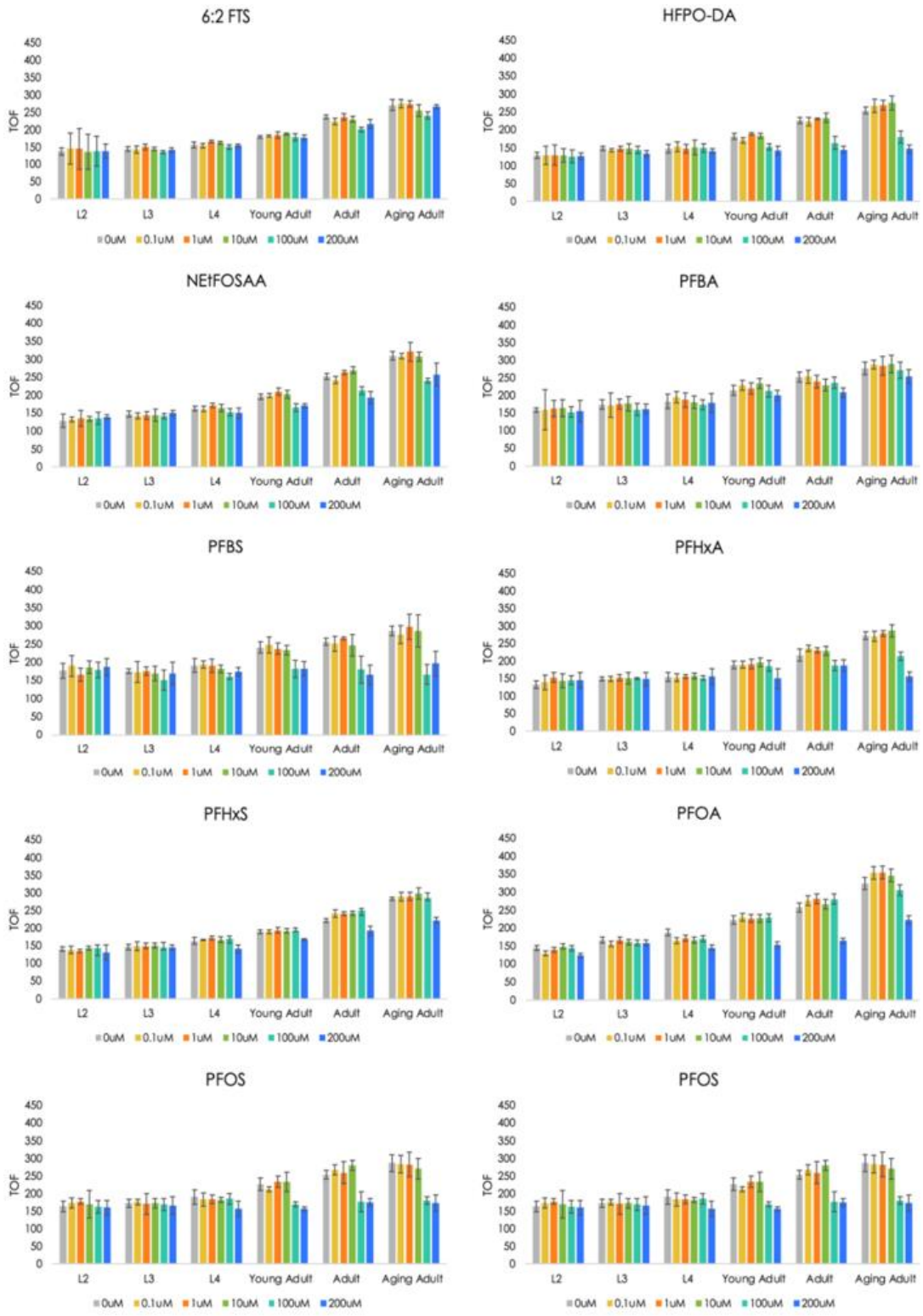

Figure 3.1: Effects of PFAS on Growth (Body Length) on N2 (wild-type) *C. elegans* after Exposure. All Values are represented as a Time of Flight (TOF).

Table 3.1: The Benchmark Concentration 10% of Individual PFAS on Growth at Each Stage of the Life Cycle

PFAS	Life Stage	PROAST BEST Model	BMC (BMCL) [µM]
6:2 FTS	Adult	Hill m3-	132.7 (12.2)
	Aging Adult	Hill m3-	116.3 (10.3)
HFPO-DA	YA	Expon. m3-	64.53 (26.5)
	Adult	Hill m5-	52.84 (23.6)
	Aging Adult	Hill m5-	52.29 (18.7)
NEtFOSAA	L4	Expon. m3-	196 (98.2)
	YA	Hill m5-	44.26 (21.1)
	Adult	Hill m5-	72.5 (36)
	Aging Adult	Expon. m5-	13.3 (10.6)
PFBA	YA	Expon. m3-	186.3 (117)
	Adult	Expon. m3-	188.6 (87.9)
	Aging Adult	Hill m3-	41.12 (0.648)
PFBS	L4	Hill m3-	48.24 (0.806)
	YA	Expon. m5-	18.8 (4.75)
	Adult	Expon. m3-	13.24 (1.51)
	Aging Adult	Expon. m5-	18.57 (10.5)
PFHxA	Adult	Hill m5-	42.79 (19.8)
	Aging Adult	Expon. m3-	49.22 (29.6)
PFHxS	L4	Hill m3-	171.7 (160)
	YA	Hill m3-	186.1 (168)
	Adult	Hill m3-	162 (149)
	Aging Adult	Hill m3-	150.8 (129)
PFOA	Adult	Hill m3-	202 (103.6)
	Aging Adult	Hill m3-	162 (52.8)
PFOS	L4	Hill m3-	177.5 (139)
	YA	Hill m5-	50.19 (15.4)
	Adult	Expon. m5-	61.43 (12.4)
	Aging Adult	Expon. m5-	17.87 (6.57)
PFOSA	YA	Hill m3-	249.3 (156)
	Adult	Expon. m3-	196.6 (168)
	Aging Adult	Hill m3-	103.5 (7.17)

Table 3.2: The Benchmark Concentration 10% of the Individual PFAS on Reproduction at Each Stage of the Life Cycle

PFAS	PROAST Best Model	BMC (BMCL) [µM]
6:2 FTS	Hill m5-	22.19 (9.31)
HFPO-DA	Hill m5-	35.8 (2.48)
NEtFOSAA	Hill m5-	10.39 (0.0504)
PFBA	Hill m3-	127 (52.9)
PFBS	Hill m3-	0.01084 (1.81e-06)
PFHxA	Hill m5-	41.99 (9.17)
PFHxS	Expon. m3-	86.41 (68)
PFOA	Expon. m3-	51.52 (0.18)
PFOS	Hill m3-	0.02013 (1.6e-06)
PFOSA	Expon. m3-	45.96 (2.59)

P-Value ≤ 0.05

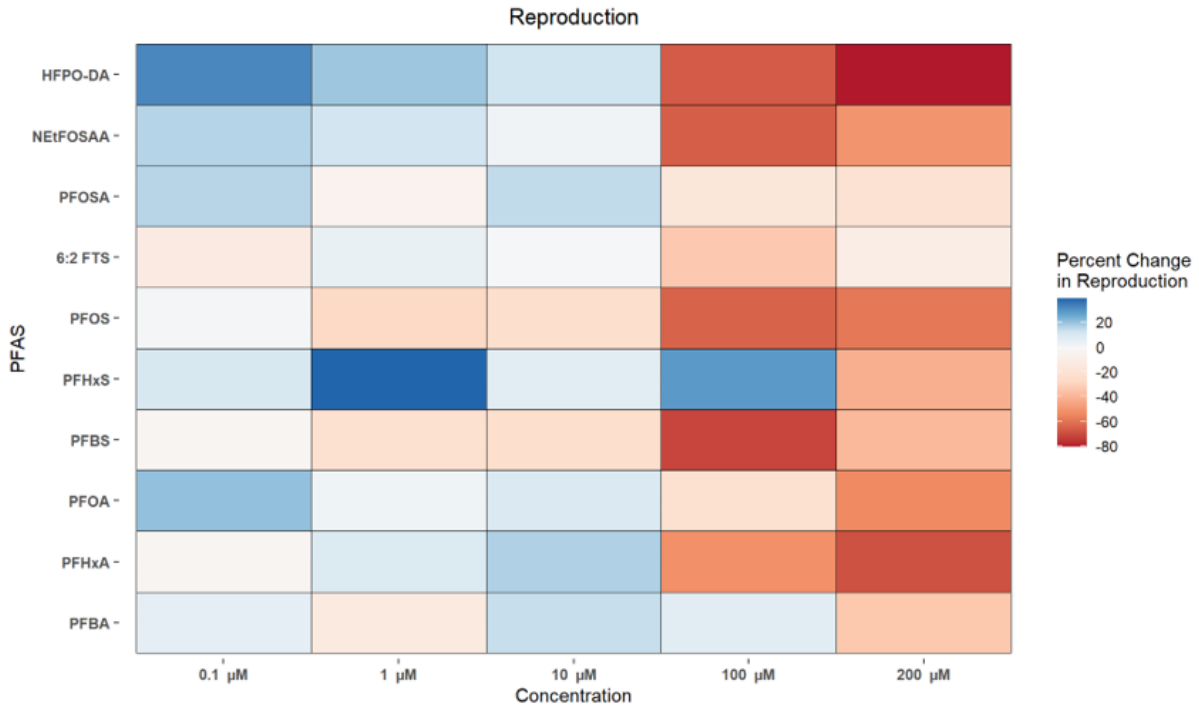

Figure 3.2: Effects of PFAS on Reproduction (Brood Size) on N2 (wild-type) *C. elegans* after 72hr of Exposure. All Values are Represented as a Percent Change.

Table 3.3: The Benchmark Concentration 10% of Individual PFAS on Behavior at Each Stage of the Life Cycle

PFAS	Life Stage	PROAST BEST Model	BMC (BMCL) [µM]
6:2 FTS	YA	Expon. m5-	42.02 (8.03)
	Adult	Expon. m5-	17.57 (4.95)
	Aging Adult	Expon. m5-	35.24 (5.8)
HFPO-DA	L3	Expon. m5-	37.18 (6.43)
	L4	Expon. m5-	56.06 (7.34)
	YA	Expon. m5-	52.85 (6.68)
	Adult	Expon. m5-	51.82 (4.9)
	Aging Adult	Expon. m5-	42.83 (5.46)
NEtFOSAA	L3	Expon. m5-	5.556 (0.654)
	L4	Expon. m5-	4.639 (1.03)
	YA	Expon. m5-	35.78 (6.07)
	Adult	Expon. m5-	11.78 (9.03)
	Aging Adult	Expon. m5-	14.15 (9.42)
PFBA	Adult	Expon. m3-	83.8 (46.5)
	Aging Adult	Expon. m3-	85.42 (37.6)
PFBS	YA	Expon. m5-	42.87 (4.46)
	Adult	Expon. m5-	16.53 (6.82)
	Aging Adult	Expon. m5-	12.57 (6.4)
PFHxA	YA	Hill m3-	105.1 (17.8)
	Adult	Hill m5-	40.2 (14.8)
	Aging Adult	Expon. m5-	22.42 (6.22)
PFHxS	L3	Expon. m3-	138.4 (23.7)
	L4	Expon. m3-	82.85 (47.1)
	YA	Hill m3-	98.72 (72.4)
	Adult	Hill m3-	91.4 (68.3)
	Aging Adult	Hill m3-	87.73 (75.3)
PFOA	YA	Hill m3-	59.72 (0.00408)
	Adult	Hill m3-	101.9 (35.7)
	Aging Adult	Hill m3-	93.9 (38.7)
PFOS	L2	Expon. m3-	0.1406 (0.000747)
	L3	Expon. m5-	7.42 (3.51)
	L4	Expon. m3-	2.403 (0.375)
	YA	Expon. m5-	4.886 (1.9)
	Adult	Hill m3-	1.504 (0.67)
	Aging Adult	Expon. m5-	12.76 (5.99)
PFOSA	L4	Expon. m3-	108.9 (32.7)
	YA	Expon. m3-	121.2 (45)
	Adult	Hill m3-	138.3 (63.3)
	Aging Adult	Expon. m3-	139.2 (67.3)

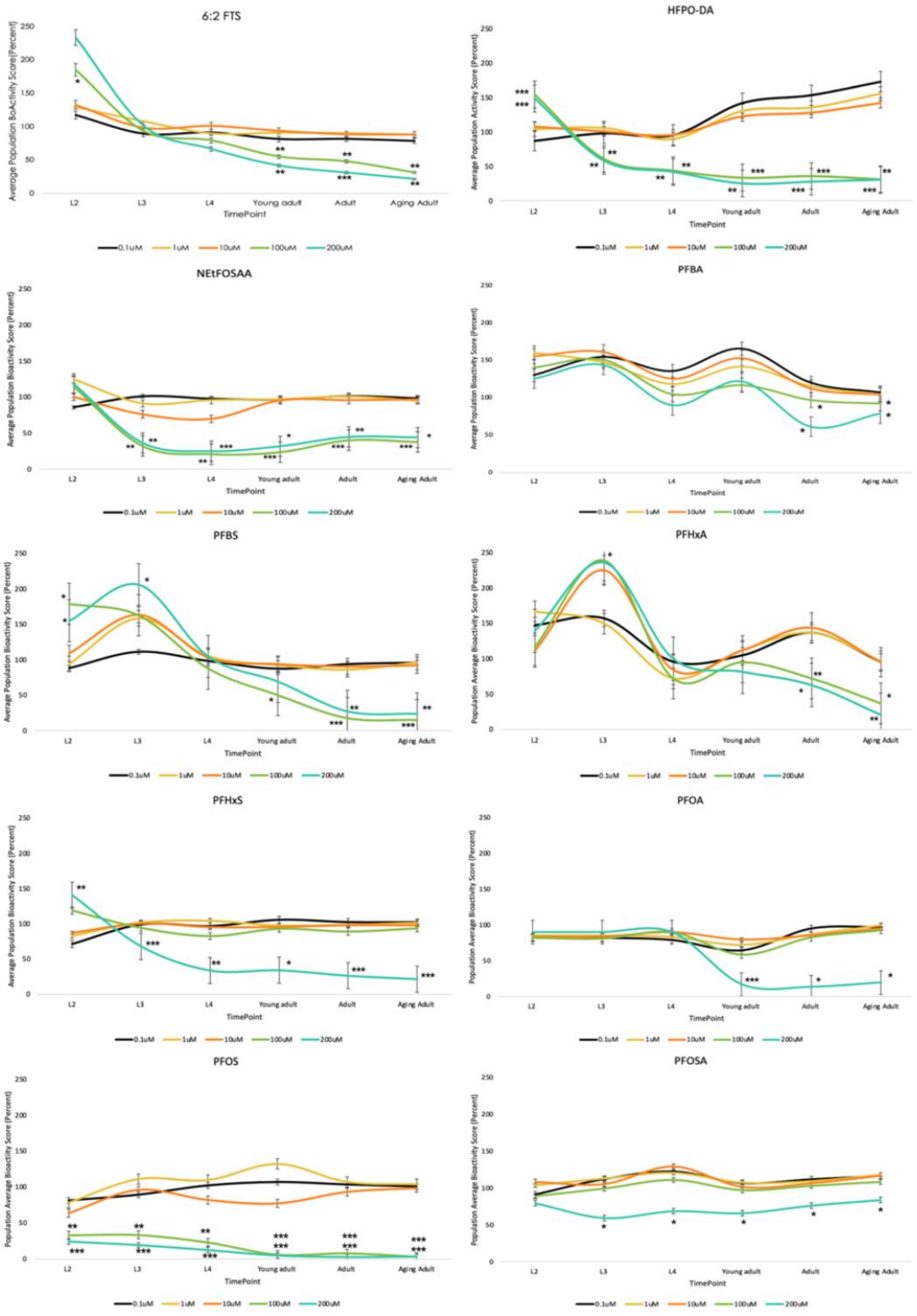

Figure 3.3: Effects of PFAS on Motility (Activity Score) on N2 (Wild-type) *C. elegans* after Exposure. All Values are Represented as an Activity Score.

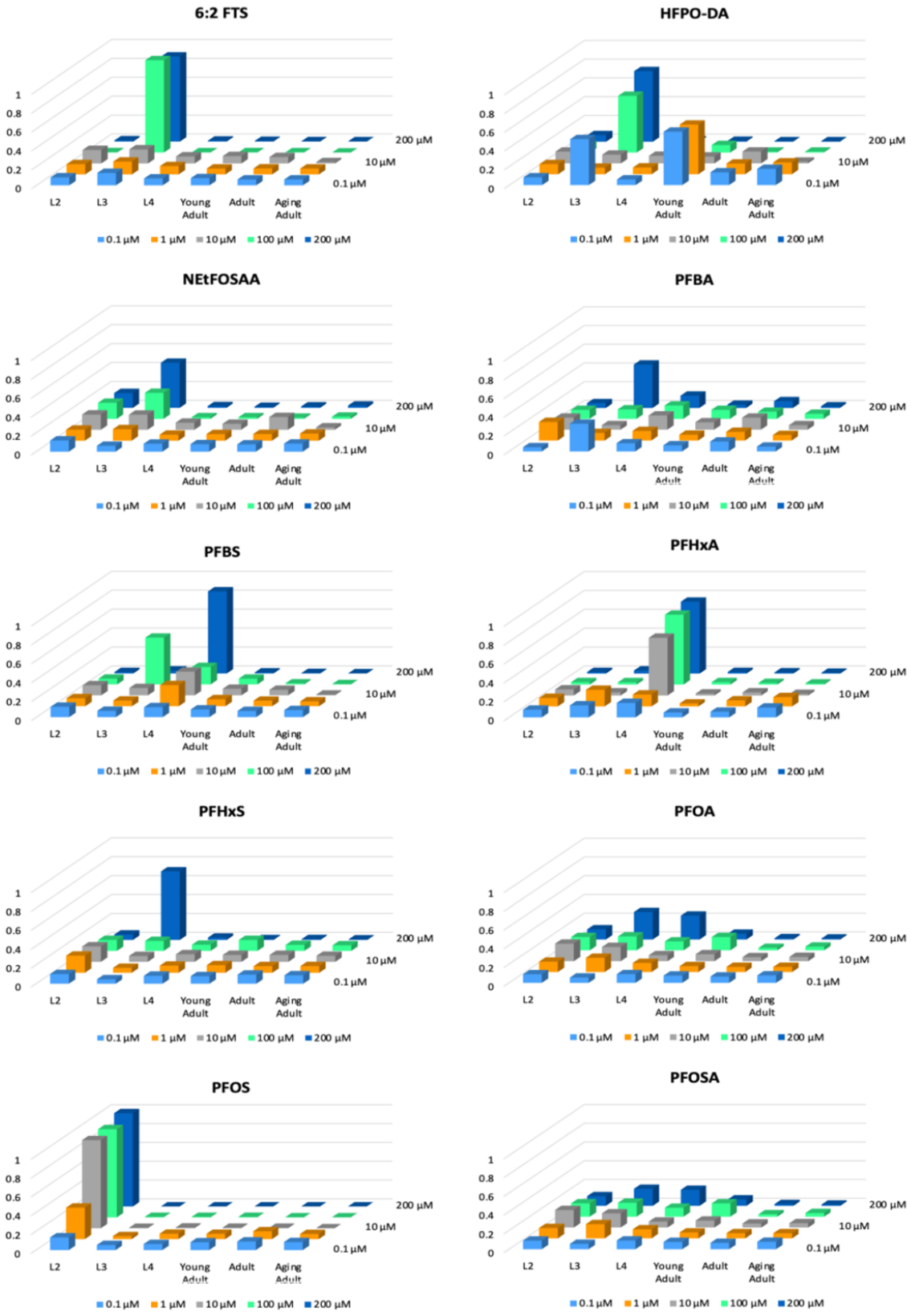

Figure 3.4: Stage-Specific Sensitivity Analysis of PFAS on N2 (wild-type) *C. elegans*

CHAPTER 4

TRANSCRIPTOMIC INSIGHTS INTO PFAS-INDUCED NEURODEVELOPMENT TOXICITY IN *CAENORHABDITIS ELEGANS*[3]

[3]Currie, S. D., Wang, J. S., & Tang, L. To be submitted to a peer-reviewed journal.

Abstract:

Per- and Polyfluoroalkyl Substances (PFAS) represent a diverse class of industrial chemicals widely utilized in various applications over the past several decades. Due to their extensive use and consequent environmental bioaccumulation, PFAS are consistently detected in human blood. These compounds have been associated with several adverse health outcomes, including hepatotoxicity, immunotoxicity, endocrine disruption, tumorigenicity, and notably, neurotoxicity, particularly in relation to developmental neurotoxicity (DNT). The rising prevalence of neurodevelopmental disorders among children has been linked to pre- and postnatal exposure to PFAS; however, the underlying mechanisms remain largely uncharacterized. This study selected five PFAS compounds, representing typical daily exposure levels in the United States, including perfluoroalkyl carboxylic acids (PFBA, PFOA) and sulfonic acids (PFBS, PFHxS, PFOS). Wild-type *C. elegans* at the first larval stage (L1) were exposed to either individual PFAS compounds or a mixture of all five compounds at concentrations of 0 ppm, 5 ppm, and 50 ppm for 48 hours. Subsequent analyses focused on alterations in neural behavior and transcriptomic signatures. All PFAS compounds significantly reduced locomotion ($p < 0.01$), with PFAS mixtures exhibiting an additive effect. Pathway enrichment analyses underscored a significant association the MAPK signaling pathway and highlights its critical role in neurodevelopment. Our findings provide novel evidence of the neurodevelopmental toxicity of PFAS and elucidate potential mechanisms of toxicity, contributing valuable insights for assessing the adverse health impacts of these emerging environmental pollutants.

4.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) are a group of human-made chemicals that have been in widespread use since the 1940s across various industries and commercial applications. Known for their persistence in the environment, they are often referred to as "forever chemicals." This persistence is largely attributed to the strong bonds between carbon and fluorine in their chemical structure, making them resistant to degradation (Buck et al., 2011). Due to their widespread use and stability, PFAS are now found globally in environmental samples, as well as in human and animal tissues (Dey, Shafi, Chowdhury, Dubey, & Sen, 2024). Exposure to these chemicals occur through multiple pathways, including contaminated drinking water, food, inhalation, and contact with consumer products (DeLuca, Minucci, Mullikin, Slover, & Cohen Hubal, 2022; Sunderland et al., 2019; Wee & Aris, 2023). Data from the National Health and Nutrition Examination Survey (NHANES), analyzed by the Centers for Disease Control and Prevention (CDC), revealed that PFAS were present in the blood of 98% of Americans (R. C. Lewis, Johns, & Meeker, 2015).

The growing concern over PFAS is due to their association with numerous adverse health effects. These include disruptions in immune (DeWitt, Peden-Adams, Keller, & Germolec, 2012; Grandjean & Budtz-Jorgensen, 2013), thyroid (Coperchini et al., 2020; Freire et al., 2023), metabolic (Chen et al., 2020; Sen et al., 2022), and reproductive systems (Green, Harvey, Finger, & Tarulli, 2021; Qin et al., 2023; Rickard, Rizvi, & Fenton, 2022). Furthermore, Recent studies have raised concerns about the neurodevelopmental toxicity of PFAS, highlighting the increased susceptibility of developing nervous systems to these substances (Carstens et al., 2023; Eick et al., 2021; Varsi et al., 2022). Increasing epidemiological evidence suggests that PFAS exposure during critical developmental windows can lead to neurodevelopmental disorders (NDDs) such

as attention deficit hyperactivity disorder (ADHD) and autism spectrum disorders (ASD), as well as deficits in cognitive functions such as memory and learning (Seth D. Currie, Wang, & Tang, 2024; Gao et al., 2023).

Neurodevelopmental toxicity refers to the adverse effects on the developing nervous system from exposure to harmful substances during critical growth periods, manifesting as neurodevelopmental disorders (NDD) (Morris-Rosendahl & Crocq, 2020). Alarmingly, PFAS can accumulate in the placenta, crossing the placental barrier and potentially affecting fetal development, with prenatal exposure linked to adverse outcomes such as fetal growth restriction and neurotoxicity (Cai et al., 2020; Gutzkow et al., 2012; McAdam & Bell, 2023). Infants and young children are particularly susceptible to PFAS, with exposure levels peaking during early life, especially due to breastfeeding (Liu, Li, Buchanan, & Liu, 2020). Research continues to reveal that prenatal and early life PFAS exposure may negatively affect brain development, including impairments in cognitive function, motor skills, and synaptic plasticity, which are foundational for learning and memory (Johansson, Fredriksson, & Eriksson, 2008; Starnes, Rock, Jackson, & Belcher, 2022). In vitro studies further confirm that PFAS disrupt neuronal differentiation and synaptogenesis, leading to neurodevelopmental toxicity (Tukker et al., 2020).

Caenorhabditis elegans (*C. elegans*), a nematode species measuring just 1mm in length, is an invaluable model organism for studying the effects of toxicants on neurodevelopment. With a rapid life cycle of 2-3 days from egg to adult, a short lifespan, and the ability to sustain large populations in a small space, *C. elegans* is cost-effective and amenable to high-throughput analysis (Gonzalez-Moragas, Roig, & Laromaine, 2015). Its ease of use makes it ideal for investigating how early exposure to chemicals impacts neurodevelopment. Studies have demonstrated that PFAS can accumulate in *C. elegans*, disrupting development, behavior, and

reproduction (Chowdhury, Sana, Panneerselvan, Sivaram, & Megharaj, 2022; Sana, Chowdhury, Logeshwaran, Dharmarajan, & Megharaj, 2021). Our research has shown that exposure to certain PFAS compounds, such as PFOS and PFBS, results in neurodevelopmental toxicity, even at early stages of life such as the L2 larval stage, leading to altered neuronal function and behavioral impairments (S. D. Currie, Doherty, Xue, Wang, & Tang, 2023). Additionally, we have explored the bioconcentration of PFAS in *C. elegans* while also assessing their effects on cognitive functions, such as learning and memory, providing a more comprehensive understanding of how PFAS exposure affects neurodevelopment. Despite these findings, the specific molecular mechanisms underlying these effects remain poorly understood and warrant further investigation.

This study aims to explore the mechanisms underlying the varying risks of neurodevelopmental disorders associated with typical PFAS exposures in the United States. Specifically, we aim to utilize transcriptomic analyses to examine how environmentally relevant PFAS mixtures impact neurodevelopment. Five PFAS compounds were selected based on their prevalence in water sources within the Carolina Region of the United States (Smalling et al. 2023). These compounds represent a range of common chemical structures, including perfluoroalkyl carboxylic acids (perfluorobutanoic acid [PFBA], perfluorooctanoic acid [PFOA]) and sulfonic acids (perfluorobutanesulfonic acid [PFBS], perfluorohexanesulfonic acid [PFHxS], perfluorooctanesulfonic acid [PFOS]). The transcriptomic profiles of each individual PFAS, as well as their mixture, were assessed to understand their impacts on the developing nervous system within *C. elegans*. The research goals include elucidating the neurodevelopmental effects of these substances, identifying potential toxicity mechanisms, and assessing how exposure to PFAS mixtures may influence developmental outcomes. Ultimately, this study will contribute to a deeper understanding of the health risks associated with PFAS exposure.

4.2 Materials and Methods

4.2.1 Chemicals

The occurrence of PFAS in tap water of unregulated private wells and regulated public supply across the United States (Smalling et al., 2023). Based on the relative concentrations of PFAS in these water sources, the most abundant PFAS were selected for this study. The five PFAS selected for this study include PFBA (95%, BCCF2984, COA), PFOA (95%, WXBD6815, COA), PFBS (98%, P151-08994, COA), PFHxS (95%, 751400, COA), PFOS (98%, 830800, COA).

Analytical grade PFBA and PFOA were purchased from Sigma Aldrich (St. Louis, MO). Analytical grade PFBS was purchased from Astatech Inc. (Bristol, PA). Analytical grade PFOS and PFHxS, were purchased from Synquest Laboratories, Inc. (Alachua, FL). Stock solutions at 1M were prepared in dimethyl sulfoxide (DMSO), and working solutions were diluted with K-medium (containing 32mM KCl and 51mM NaCl). OP50 bacteria (1 mg/mL) were used as the food source for *C. elegans*. The final concentration of DMSO in the solutions was kept at 0.1% to prevent toxicity (Brenner, 1974; Williams & Dusenbery, 1990).

4.2.2 Mixture Selection

To construct an environmentally relevant mixture of PFAS for this study, five PFAS compounds representing the most commonly detected in U.S. water supplies were selected (Smalling et al., 2023). A reference mixture was formulated based on the relative concentration ratios found in the full mixture: PFOS (30%), PFBA (20%), PFOA (20%), PFHxS (15%), and PFBS (15%). approximates the typical PFAS exposure found in U.S. drinking water. The final concentration of the mixture will vary depending on the experimental condition, but the

proportions of each compound will remain constant. This approach allows for the investigation of patterns in the toxicity profiles of PFAS mixtures in comparison to individual compounds (East, Anderson, & Salice, 2021).

4.2.3 C. elegans Culture and Exposure

The nematodes, wild-type N2, along with *Escherichia coli* strain OP50 were obtained from Caenorhabditis Genetics Center (Minneapolis, MN, USA). The nematodes were cultured at 25°C on solid nematode growth medium (NGM) agar plates seeded with OP50 bacteria as their food source (Brenner, 1974). Synchronized L1 growth-arrested larvae were obtained using a sodium hypochlorite isolation method and then incubated overnight in an unseeded K-medium solution at 25°C with shaking (J. A. Lewis & Fleming, 1995; Pires da Silva, 2005). For all subsequent experiments, age-synchronized worms were exposed to individual PFAS compounds or the reference PFAS mixture at concentrations of 0, 5, and 50 ppm. These concentrations, though higher than typical environmental levels, were selected based on previous studies that identified the necessary PFAS levels to elicit measurable responses in *C. elegans* related to development, reproduction, and behavior (S. D. Currie et al., 2023). The nematodes exposure was performed in K-medium for 48 hours.

4.2.4 High-throughput Screening Platform for Behavior

High throughput screening assays testing for behavior using COPAS BIOSORT (Union Biometrica, Inc., Massachusetts, USA) and WormLab system (MBF Bioscience, Vermont, USA). Wild-type *C. elegans* were tested to understand specific behavioral endpoint toxicity of individual PFAS and with a reference mixture. Testing solutions contained k-medium and

bacteria food, and either the individual PFAS or the mixture. Age-synchronized worms were dispensed into 96-well plates using the COPAS BIOSORT, then incubated with shaking for 24h and 48h. For behavioral measurements, worms were aspirated from individual wells and placed on unseeded solid nematode growth medium (NGM) plates. These plates were rested for 60 minutes to assimilate the worms to their new environment. Plates were recorded for 60 seconds with a minimum track duration of 30 seconds with a resolution of 1280 x 960 pixels taken at 7.5 frames/s using a digital camera (Nikon DSLR, Tokyo, JPN). For measurements of behavior, the absolute peristatic speed was calculated not takin direction of movement into account using WormLab system as previously described(Angstman, Frank, & Schmitz, 2016). Data was read, processed, and plotted using GraphPad Prism.

4.2.5 Behavior Toxicity Assessment

Fifty age-synchronized L1-stage worms were selected and distributed into each well containing 100 µL of testing solution. The plates were incubated at 25°C for a period of 48 hours. At 24h and 48h, triplicates were transferred to solid NGM plates and recorded using the WormLab System. The absolute peristaltic speed and amplitude were calculated for each well and time point. *C. elegans* have evolved as a useful model for determining the neurotoxicity of substances by observing changes in locomotor behavior, neural architecture, and gene expression levels, making these behavioral parameters critical for assessing neurodevelopmental function (Wang et al., 2023).

4.2.6 RNA Extraction and Sequencing

Following PFAS exposure, RNA was extracted from the nematodes to assess changes in gene expression. Replicate samples of nematodes were washed three times with M9 buffer (Stiernagle 2006) and centrifuged at 5,000 rpm. The supernatant was discarded, and 500µl of Trizol was added to lyse the worms. These samples were then stored at -80°C. Subsequently, the samples were sent to Arraystar Inc. (Rockville, USA) for total RNA isolation and sequencing services. RNA samples were quantified using a NanoDrop ND-1000 instrument and assessed for integrity via agarose electrophoresis.

For RNA-seq library preparation, 1-2 µg of total RNA from each sample was used. mRNA was isolated using the NEBNext® Poly(A) mRNA Magnetic Isolation Module. The resulting libraries were evaluated on an Agilent 2100 Bioanalyzer to confirm concentration, fragment size distribution (400-600 bp), and the absence of adapter dimer contamination. For sequencing, the libraries underwent denaturation using 0.1M NaOH to generate single-stranded DNA molecules. These molecules were loaded onto flow cell channels at a concentration of 8 pM and amplified in situ using the NovaSeq 6000 S4 Reagent Kit (300 cycles). Sequencing was conducted on the Illumina NovaSeq 6000 according to the manufacturer's instructions (150 cycles).

4.2.7 Gene Expression Profiling and Bioinformatics Analysis

The raw sequencing data produced by the Illumina NovaSeq 6000, after passing the Illumina chastity filter, are used for subsequent analysis. Trimmed reads, which have had their 5' and 3'-adaptor bases removed, are aligned to a reference genome. Arraystar Inc. (Rockville, USA) evaluates the alignment results through statistical analysis, including mapping ratio,

rRNA/mtRNA content, and fragment sequence bias, to assess suitability for further analysis. If deemed appropriate, the analysis includes expression profiling, identification of differentially expressed genes and transcripts, and prediction of novel genes and transcripts. Differential expression is determined using fold change (cutoff 1.5), p-value (≤ 0.05), and FPKM (≥ 0.5 mean in one group) for filtering differentially expressed genes and transcripts. Statistical techniques such as Principal Component Analysis (PCA), Correlation Analysis, and Hierarchical Clustering, as well as functional analyses such as Gene Ontology (GO) and Pathway Analysis, are performed using scatter plots, volcano plots, and tools in either the R or Python environment for computational and graphical analysis.

4.2.8 Quantitative real-time PCR (qPCR)

To validate the results of transcriptome sequencing, the remaining RNA was used for real-time PCR (RT-qPCR) to determine the relative expression levels of related genes as our previously described (Yang et al., 2018). TaqMan™ Real-Time PCR (Thermo Fischer Scientific, Waltham, MA) was performed on TaqMan Fast Virus one step Master Mix for qPCR. The calculation involved determining the relative quantification of the target genes (*rict-1, osm-10, glna-2, egl-43,* and *che-13*) compared to the reference gene (*rps-23*) expressing the results as the ratio of target gene expression to reference gene expression in the treatment group relative to the control group. The selection of these target genes was based on their known involvement in neurodevelopmental processes and neurotoxic responses. Specifically, genes like *rict-1* are linked to neuroplasticity, *egl-43* is involved in synaptic signaling, and *glna-2* plays a role in neuronal development and function, making them valuable biomarkers for assessing neurotoxic

effects. The sequences of the primers used in this study are provided in Supplementary Table 4.S1.

4.2.9 Correlation Analysis Between Behavior and mRNA

The correlation between behavior and mRNA expression levels was thoroughly investigated to illuminate the relationship between PFAS exposure and its impact on gene expression related to neurodevelopment. Pearson correlation analysis was utilized to quantify the relationship between behavioral endpoints and mRNA expression levels in C. elegans ($p<0.05$). The resulting correlations were visualized using a heatmap, highlighting patterns within the potential relationships between behavior and gene expression. These correlations provide valuable insight into the underlying mechanisms by which PFAS may influence neurodevelopmental processes. The R package "mice" was employed to calculate the correlation coefficients.

4.2.10 Statistical Analysis

The data analysis utilized either GraphPad Prism, version 10.1.2 (La Jolla, CA), or the statistical program R, version 3.3.4. Results were presented as means ± standard deviation (SD). Statistical significance relative to the control group was assessed using analysis of variance (ANOVA) followed by Tukey's post-hoc test to compare multiple groups. In the graphical representations, statistical significance levels are denoted by asterisks (* for $p\leq0.05$, ** for $p\leq0.01$, and *** for $p\leq0.001$).

4.3 Results

4.3.1 Toxic Effects of PFAS on Behavior

Synchronized L1 *C. elegans* were exposed to five individual PFAS and a reference mixture at concentrations of 0, 5, and 50 ppm. Behavioral toxicity was assessed by measuring absolute peristaltic speed using the WormLab system. As shown in Figure 4.1, PFAS exposure significantly disrupted behavior in a concentration- and time-dependent manner (*p* < 0.05). A notable interaction between time and dose was observed, where the effects of higher concentrations became more pronounced over longer exposure periods, suggesting that prolonged exposure amplifies the toxicity of PFAS.

After 48 hours of exposure, all PFAS and the mixture resulted in a significant reduction in motility at the highest concentration ($p < 0.05$). As expected, PFOS was the most toxic individual PFAS at both concentrations and time points. Interestingly, the reference mixture was the only other chemical that experienced a significant reduction with respect to concentration and time ($p < 0.01$). The mixture decreased absolute peristaltic speed by as much as 68.8%, compared to 45.3% in PFOS (Table 4.S2). This suggests an additive toxic effect among the various PFAS. However, PFBS and PFHxS did not cause any behavioral reduction until 48 hours of exposure. Based on the percentage decrease after 48 hours, the mixture exhibited greater toxicity compared to the individual compounds.

4.3.2 Identification of Differentially Expressed Genes in PFAS-Exposed Nematodes

To elucidate the mechanisms underlying PFAS toxicity, RNA sequencing was performed on *C. elegans* exposed to individual PFAS and the mixture at 0, 5, and 50 ppm for 48 hours. Differential expression analysis revealed substantial changes in gene expression with thresholds

set based on p-values ($p<0.05$) and mean fold changes (≥ 1.5). PFBA exposure resulted in the highest number of differentially expressed genes (DEGs), with 1,284 (318 upregulated, 966 downregulated) at 5 ppm and 4,519 (827 upregulated, 3,692 downregulated) at 50 ppm (Figure 4.2).

Hierarchical clustering revealed distinct gene expression patterns across treatments, with PFBA and the mixture displaying clear separation from other PFAS exposures (Figure 4.3). These results indicate that individual PFAS and mixtures elicit unique transcriptional responses, with the mixture inducing the most significant gene expression changes.

4.3.3 Functional Enrichment Analysis of Differentially Expressed Genes (DEGs) Induced by PFAS

Functional enrichment analysis using Gene Ontology (GO) terms was conducted to explore the biological processes, cellular components, and molecular functions affected by PFAS exposure. As shown in the Venn diagrams, there was a clear overlap in enriched GO terms between individual PFAS and the mixture (Figure 4.4). The commonly enriched GO terms among PFBA, PFBS, PFHxS, PFOA, and PFOS at all exposures compared to the control, were 27, 7, 4, 6, and 18, respectively. Notably, the mixture shared several GO terms with individual PFAS, indicating common biological processes impacted by both single compounds and their combination.

Specific GO terms associated with PFBS are detailed in Figure 4.5**,** categorized into biological processes (BP), cellular components (CC), and molecular functions (MF). For PFBS, the major alterations in BP were related to neuropeptide signaling and cell communication, while changes in MF were associated with neuropeptide receptor binding. Alterations in CC were

predominantly located in the neuron projection, gap junctions, and dendrites, as highlighted by fold enrichment. Similar GO term enrichments for other PFAS are presented in Figure 4.S1**,** facilitating comparison across exposures.

4.3.4 Pathway Analysis of Differentially Expressed Genes in PFAS-Exposed Nematodes

Pathway enrichment analysis using the KEGG database revealed several signaling pathways associated with neurodevelopmental toxicity following exposure to PFAS and their mixtures (Table 4.1**).** PFBS, PFOS, PFHxS, PFOA, and PFBA influenced 2, 12, 8, 6, and 9 pathways, respectively. Common pathways among individual PFAS exposures included glutathione metabolism, xenobiotic metabolism via cytochrome P450, and drug metabolism. These pathways indicate a cellular response to oxidative stress, which can interfere with neurodevelopment by disrupting redox signaling and neuronal integrity. Such oxidative stress may contribute to the observed alterations in neuronal gene expression, reinforcing the connection between PFAS exposure and potential neurotoxicity.

The mixture exposure uniquely downregulated critical neurodevelopmental pathways, including the MAPK signaling pathway, neuroactive ligand-receptor interactions, and metabolic pathways (Table 4.S3). The MAPK signaling pathway, which plays a pivotal role in neuronal growth, differentiation, and synaptic plasticity, was notably suppressed in the mixture, suggesting a direct impact on neuronal development and function. Additionally, the downregulation of neuroactive ligand-receptor interactions indicates impaired synaptic signaling and neurotransmitter activity, further highlighting the disruption of neuronal communication. These findings emphasize the potential for PFAS mixtures to induce unique and more profound

neurotoxic effects compared to individual compounds, potentially due to additive or synergistic interactions.

The differences in pathway enrichment between the mixture and individual PFAS exposures suggest that while some pathways are affected by individual PFAS, the mixtures induce more pronounced and differentiated disruptions, amplifying toxicity beyond what is observed with single compounds. The downregulation of neurodevelopmentally significant pathways by the mixture highlights its ability to disrupt critical processes essential for maintaining neuronal structure and function. These results emphasize the importance of studying chemical mixtures in the context of neurodevelopmental toxicity to understand the broader impacts of environmental PFAS exposure on nervous system health.

4.3.5 Analysis of the Common DEGs Induced by PFAS

To identify common transcriptional responses, neuronal-related DEGs at 50 ppm were categorized based on GO terms (Figure 4.6). A range of neuronal genes were downregulated following PFAS exposure, including those associated with synaptic development and function, such as *aex-1*, *glna-2*, *unc-112*, and *lgc-43 (Xiang et al., 2013)*. Additionally, genes involved in neuronal structure *(Blackwell, Sewell, Wu, & Han, 2019; Lapierre, 2023)*, such as *rict-1*, *dyf-1*, and *hlh-30*, along with genes related to sensory neuron function (Caldwell, Willicott, & Caldwell, 2020), including *che-13* and *osm-10*, were also identified. This widespread alteration in gene expression underscores the significant impact of PFAS exposure on neuronal development and function.

Interestingly, the mixture exposure consistently downregulated all identified neuronal-related DEGs, suggesting that combined PFAS exposures may exacerbate disruptions in neuronal

structure and function compared to individual compounds. Notably, *rict-1* was associated with the mTOR signaling pathway, *glna-2* with the MAPK signaling pathway, and *hlh-30* with the autophagy pathway, providing mechanistic insights into how PFAS mixtures may impair neuronal development and signaling. These results underscore the potential for PFAS mixtures to uniquely alter neurodevelopmental processes, amplifying toxicity in ways that single compounds do not, and highlight the need for further investigation into their combined effects on neuronal health.

4.3.6 Quantitative real-time PCR Confirmation of Neurodevelopment Related Gene Change

To validate transcriptomic findings, quantitative real-time PCR (qPCR) was conducted on key neurodevelopmental genes (*rict-1*, *che-13*, *glna-2*, *osm-10*, and *egl-43*). Exposure to 50 ppm of individual PFAS and the mixture resulted in significant downregulation of *rict-1* expression, with fold changes ranging from 1.6-fold (PFOS) to 4.6-fold (mixture). Similarly, *che-13* expression was significantly reduced, with the most pronounced decrease observed in PFBA (9.2-fold). Expression of *glna-2*, *osm-10*, and *egl-43* was also significantly reduced, further supporting the RNA sequencing findings and highlighting the broad impact of PFAS exposure on neurodevelopment-related genes.

The qPCR results were consistent with the RNA-sequencing data, confirming that PFAS exposure significantly alters the expression of genes critical for neurodevelopment. These findings suggest that PFAS exposure may disrupt essential pathways that play important roles in neuronal development and signaling. These results provide valuable mechanistic insights into how PFAS exposure may contribute to neurotoxicity and highlight the need for further investigation into the long-term effects on neurodevelopment.

4.3.7 Correlation Analysis Between Behavior and mRNA

To assess the relationship between behavior and mRNA expression levels, the Pearson Correlation Coefficient was calculated using the "mice" package in R software. This analysis compared mRNA expression levels with behavioral outcomes 48 hours after exposure. The results, shown in Figure 4.8, demonstrate strong correlations between neurodevelopment-related genes and behavior. The most significant positive correlations were observed for all genes exposed to the PFAS mixture, suggesting a synergistic effect when several individual PFAS compounds are combined. However, exposure to PFHxS and PFBS showed weaker or even negative correlations with behavior, indicating a lesser impact on neurodevelopmental processes. Interestingly, *egl-43* exhibited a slight negative correlation with behavior specifically following exposure to the sulfonic acids (PFBS and PFHxS), leading to a weaker overall correlation between this gene and behavior in the mixture group. Our results underscore the complexity of the relationship between neuronal gene expression and behavior, demonstrating the varied effects of PFAS exposure.

4.4 Discussion

The present study provides novel insights into the molecular mechanisms underlying PFAS-induced neurodevelopmental toxicity, offering a comprehensive analysis of gene expression alterations and their behavioral consequences in *C. elegans*. While previous research has investigated the effects of individual PFAS compounds, our study is the first to integrate transcriptomic analysis with behavioral assays to reveal the complex disruption of critical signaling pathways, particularly the MAPK pathway, in response to PFAS exposure. This work contributes to a deeper understanding of how environmental toxins like PFAS may interfere with

neurodevelopmental processes and emphasizes the need for further investigation into the molecular pathways involved.

Our transcriptomic analysis identified the MAPK signaling pathway as one of the most significantly altered pathways in *C. elegans* exposed to PFAS. This pathway is crucial for a variety of cellular processes, including neuronal differentiation, synaptic plasticity, and the establishment of neural circuits (Huang & Reichardt, 2001; Pearson et al., 2001). The disruption of MAPK signaling is consistent with previous studies that have linked alterations in MAPK function to neurodevelopmental defects (Pastuhov, Hisamoto, & Matsumoto, 2015). In this study, we observed that PFAS exposure led to a significant downregulation of genes such as *glna-2*, which is involved in MAPK-dependent cellular processes critical for neuronal growth and synaptic maturation. The gene expression change is likely to contribute to the observed behavioral impairments, such as altered locomotion and reduced learning and memory capacity, which reflect the disrupted neurodevelopment in response to PFAS.

The MAPK pathway plays a key role in regulating neuronal morphology, synaptic function, and plasticity, processes that are essential for proper neurodevelopment (Fukunaga & Miyamoto, 1998). Our findings suggest that PFAS-induced disruption of MAPK signaling impairs these processes in *C. elegans*, potentially leading to long-term behavioral deficits. Additionally, PFAS exposure is known to affect membrane fluidity (Kim et al., 2020), which may further exacerbate the disruption of MAPK signaling. The downregulation of *glna-2*, a gene involved in synaptic plasticity and neuronal growth, is particularly noteworthy. Previous studies have demonstrated that dysregulation of *glna-2* can result in impaired synaptic transmission and reduced neuronal connectivity, which likely contributes to the observed behavioral changes (Li, Yu, Gao, & Yin, 2020). Similarly, other genes implicated in neuronal signaling, such as *hlh-30* (a

transcription factor involved in cellular stress responses) and *rict-1* (a key regulator of cellular growth and metabolism), may also play important roles in mediating the neurodevelopmental effects of PFAS exposure (Blackwell et al., 2019; Wong, Ryan, Bonal, Mills, & Lapierre, 2023). Together, these findings highlight the centrality of MAPK signaling and membrane fluidity in the neurodevelopmental toxicity of PFAS and its potential role in the observed behavioral impairments.

The *glna-2* gene produces GLUN2A, which plays a key role in PFAS-induced neurotoxicity, particularly through the MAPK signaling pathway. GLUN2A integrates signals involved in neuronal growth, differentiation, and stress responses (Xu et al., 2019), acting as a scaffold that facilitates protein assembly and signaling (Figure 4.9A) (Tian et al., 2021). It regulates critical processes like axonal growth, dendritic development, and synaptic connectivity, essential for proper neuronal architecture (Deep, Seelig, Paul, & Poddar, 2024). As a central molecular hub, GLUN2A orchestrates the translation of proteins vital for neuronal maturation, which directly influences the establishment and maintenance of functional neural circuits (Figure 4.9B) (Swanger, He, Richter, & Bassell, 2013). However, our transcriptomic analysis revealed *glna-2* dysregulation following PFAS exposure, disrupting MAPK signaling and compromising neurodevelopmental processes (Figure 4.9C). This positions *glna-2* as a primary target of PFAS-induced developmental neurotoxicity. Additionally, disruptions in genes related to neuronal growth and synaptic formation suggest PFAS exposure undermines neurodevelopment through MAPK dysfunction. Observed disruptions in several genes associated with neuronal growth and synaptic formation further suggest that PFAS exposure undermines the neurodevelopmental integrity of *C. elegans* through various potential pathways including glna-2-mediated MAPK dysfunction.

The implications of PFAS-induced dysregulation of MAPK signaling extend beyond the C. *elegans* model. Given the conserved nature of MAPK signaling across species, the disruptions observed in this study are likely to have relevance to human neurodevelopment (Xing et al., 2016). The MAPK pathway is known to regulate key processes such as neuronal differentiation, axonal growth, and synaptic formation—all of which are crucial for the development of functional neural circuits (Albert-Gascó, Ros-Bernal, Castillo-Gómez, & Olucha-Bordonau, 2020). Disruptions in these processes are implicated in various neurodevelopmental disorders, including autism spectrum disorder, attention deficit hyperactivity disorder (ADHD), and other cognitive and behavioral impairments (Iroegbu, Ijomone, Femi-Akinlosotu, & Ijomone, 2021). Our findings suggest that PFAS exposure could contribute to the pathogenesis of such disorders by disrupting MAPK signaling during critical periods of neurodevelopment. Notably, exposure to PFAS mixtures likely compounds disruptions, as their synergistic effects may target overlapping or distinct molecular pathways. This can intensify neurodevelopmental impacts, particularly through pathways like MAPK signaling and synaptic function. These findings highlight the potential for PFAS mixtures to contribute to neurodevelopmental disorders, with broader implications for human health beyond the effects of individual PFAS compounds.

Future research should aim to validate these findings in mammalian models, where the impact of PFAS exposure on MAPK signaling can be further explored. In particular, studies should focus on examining the effects of PFAS on synaptic plasticity and neuronal connectivity in higher organisms, as well as assessing the long-term behavioral consequences of early-life exposure. Additionally, given the potential therapeutic relevance of MAPK signaling in neurodevelopmental disorders, future studies may investigate whether MAPK pathway modulators could mitigate the neurotoxic effects of PFAS exposure. Identifying specific

inhibitors or activators of MAPK signaling could offer novel therapeutic strategies for preventing or treating PFAS-related neurodevelopmental disorders.

This study provides compelling evidence that PFAS exposure disrupts MAPK signaling and other critical pathways involved in neurodevelopment. These findings not only contribute to our understanding of PFAS-induced toxicity but also highlight the potential risks posed by PFAS exposure to human neurodevelopment. Moving forward, future research should focus on the translational implications of these findings, particularly in the context of human health. By examining the long-term effects of PFAS on neurodevelopment in more complex models, researchers can gain a clearer understanding of the risks posed by PFAS exposure and develop strategies to mitigate its impact on public health.

4.5 Conclusion

The study findings indicate that exposure to PFAS in *C. elegans* disrupts neuronal system processes, particularly influencing nervous system function. Significant alterations in behavior, such as changes in locomotor activity, further point to neurological impacts, suggesting that PFAS exposure may impair key neurodevelopmental processes. At the molecular level, gene expression analyses revealed widespread changes in pathways related to neurodevelopment, including synaptic formation, neurotransmitter signaling, and stress response mechanisms. These alterations underscore the complex mechanisms through which PFAS compounds may exert neurodevelopmental toxicity and highlight the relevance of these findings for understanding potential human health risks, particularly during critical developmental periods. The evolutionary conservation of the impacted biological pathways supports the broader implications of PFAS-induced neurotoxicity. By utilizing *C. elegans* as a model organism, this study offers a robust

platform for further investigating the molecular basis of PFAS toxicity in neurodevelopment. Future research should focus on identifying specific molecular targets and key events in the neurodevelopmental pathway that are disrupted by PFAS exposure and explore the long-term neurological effects of these chemicals. Additionally, studies should aim to integrate findings from *C. elegans* with human-relevant models to better inform regulatory policies and health risk assessments related to PFAS contamination

4.6 Credit Author Statement

Seth Currie: Methodology, Investigation, Formal analysis, Writing- Original Draft, Writing-Reviewing and Editing. Jia-Sheng Wang: Writing-Reviewing and Validation, Writing. Lili Tang: Resources, Supervision, Funding acquisition, Writing-Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

4.7 References

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Table 4.S1: List of primers and their sequences used in this study.

Gene	Primer Sequence (5'- 3')	Gene ID
rict-1	F- TCGGCCTTTTCCTGGAGTTC R- GGAAGGCTTCGTTTCTTAAAGCAT	WBGene00009245
egl-43	F- AATTGCACTCGGAAGGACCA R- GACTTTGACACGTTGGGCAC	WBGene00001207
che-13	F- AATTGCACTCGGAAGGACCA R- GACTTTGACACGTTGGGCAC	WBGene00000492
glna-2	F- AAAGATCGTTGGGGACGGAC R- GGAGGTCAAACGTATCACTCAA	WBGene00008435
osm-10	F- CCACCTCGTGACTCTGGTTC R- GCTTTCCCGGCTTTCTTGTG	WBGene00003890
rps-23	F- ACGCCAAGGGAATCGTTCTT R- AATATCTCCGACGGCGTGAC	WBGene00004492

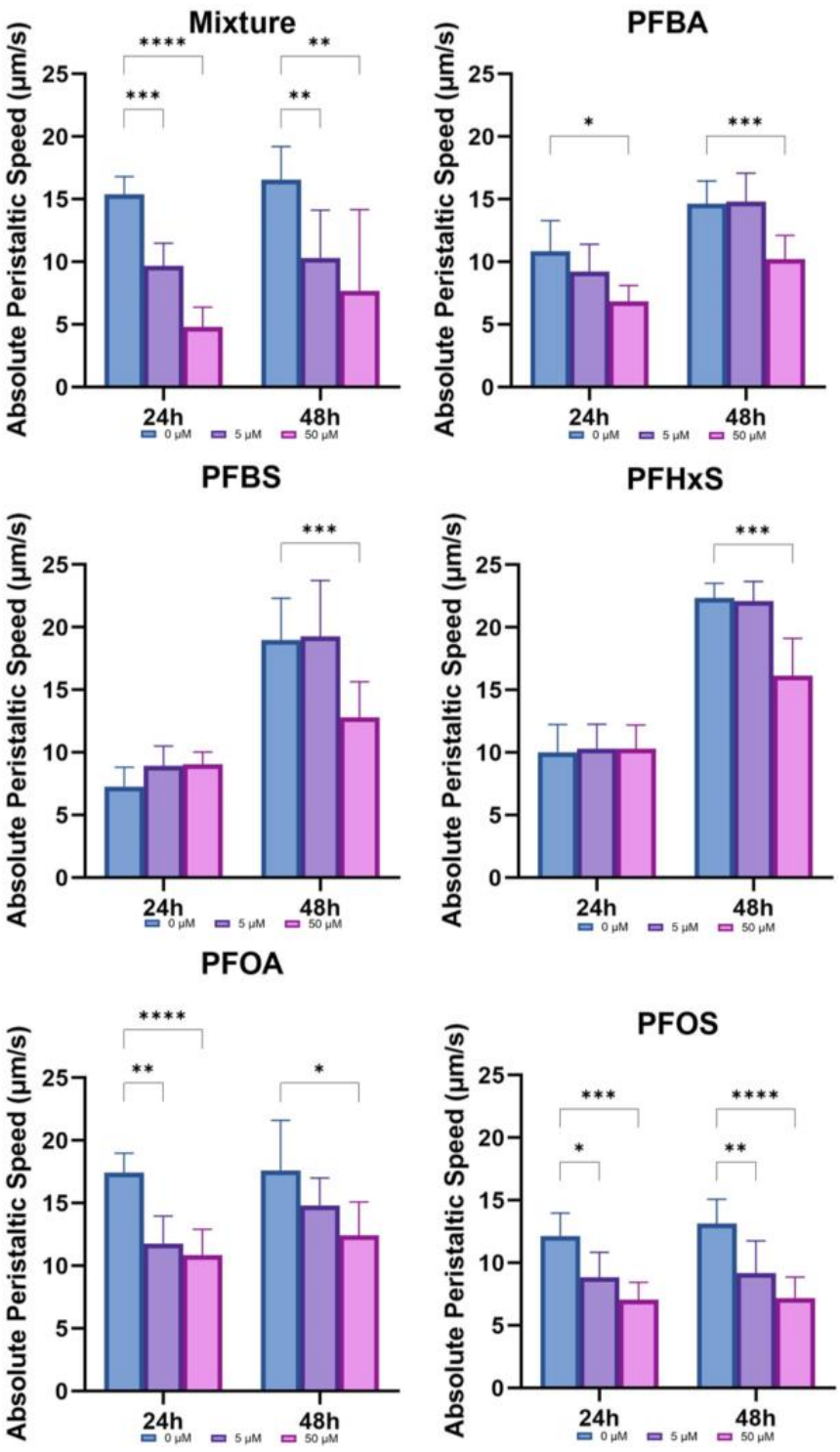


Figure 4.1: Effects of PFAS on Behavior (Absolute Peristatic Speed) on N2 (wild type) *C. elegans* after exposure. All Values are represented as an Absolute Peristatic Speed (µm/s). * for p≤0.05, ** for p≤0.01, and *** for p≤0.001.

Table 4.S2: The Mean (95% CI) of PFAS on Behavior (Absolute Peristatic Speed) on N2 (wild-type) *C. elegans* after exposure. All values are represented as an Absolute Peristatic Speed (µm/s)

Absolute Peristatic Speed						
	24h			*48h*		
	0 ppm	5 ppm	50 ppm	0 ppm	5 ppm	50 ppm
Mixture	15.42 (14.04-16.80)	9.68 (7.91-11.46)***	4.81 (3.26-6.35)****	16.57 (14.02-19.12)	10.32 (6.57-14.06)**	7.70 (1.60-13.80)**
PFBA	10.84 (8.42-13.25)	9.22 (7.09-11.36)	6.84 (5.57-8.11)*	14.64 (12.84-16.45)	14.79 (12.53-17.06)	10.21 (8.33-12.09)***
PFBS	7.29 (5.78-8.80)	8.92 (7.35-10.49)	9.07 (8.12-10.02)	7.29 (5.78-8.80)	19.27 (14.90-23.64)	12.80 (10.01-15.59)***
PFHxS	10.02 (7.83-12.22)	10.33 (8.43-12.22)	10.33 (8.49-12.16)	10.02 (7.83-12.22)	22.09 (20.54-23.64)	16.13 (13.24-19.02)***
PFOA	17.42 (15.86-18.97)	11.78 (9.65-13.91)**	10.87 (8.86-12.88)****	17.42 (15.86-18.97)	14.81 (12.65-16.98)	12.43 (9.82-15.04)*
PFOS	12.17 (10.37-13.96)	8.85 (6.87-10.82)*	7.05 (5.66-8.44)***	12.17 (10.37-13.96)	9.18 (6.64-11.72)**	7.19 (5.55-8.84)****

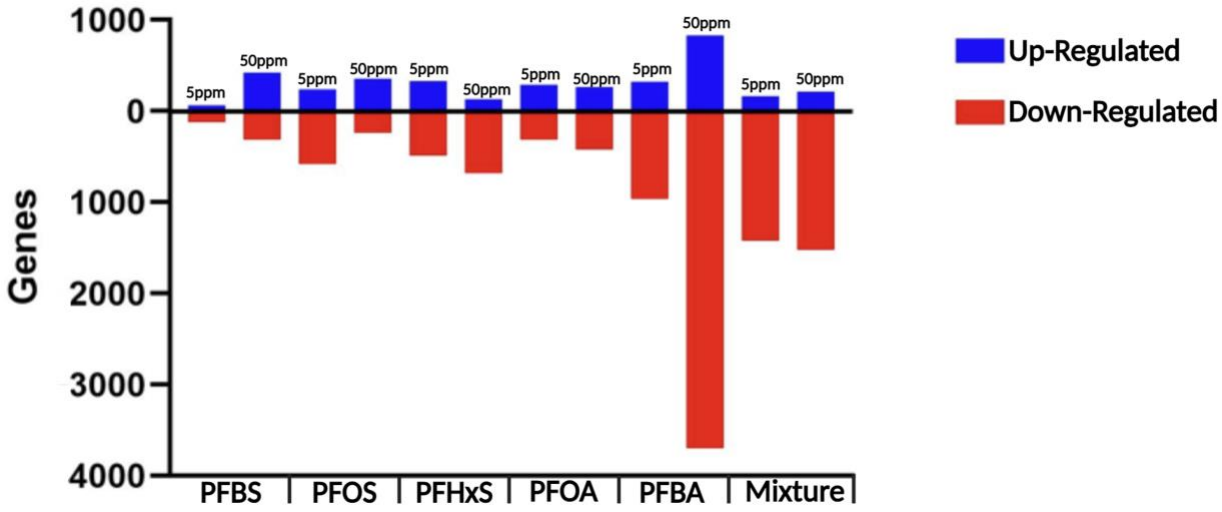


Figure 4.2: Differential Gene Expression Profiles of *C. elegans* Exposed to Various PFAS Compounds at Different Concentrations

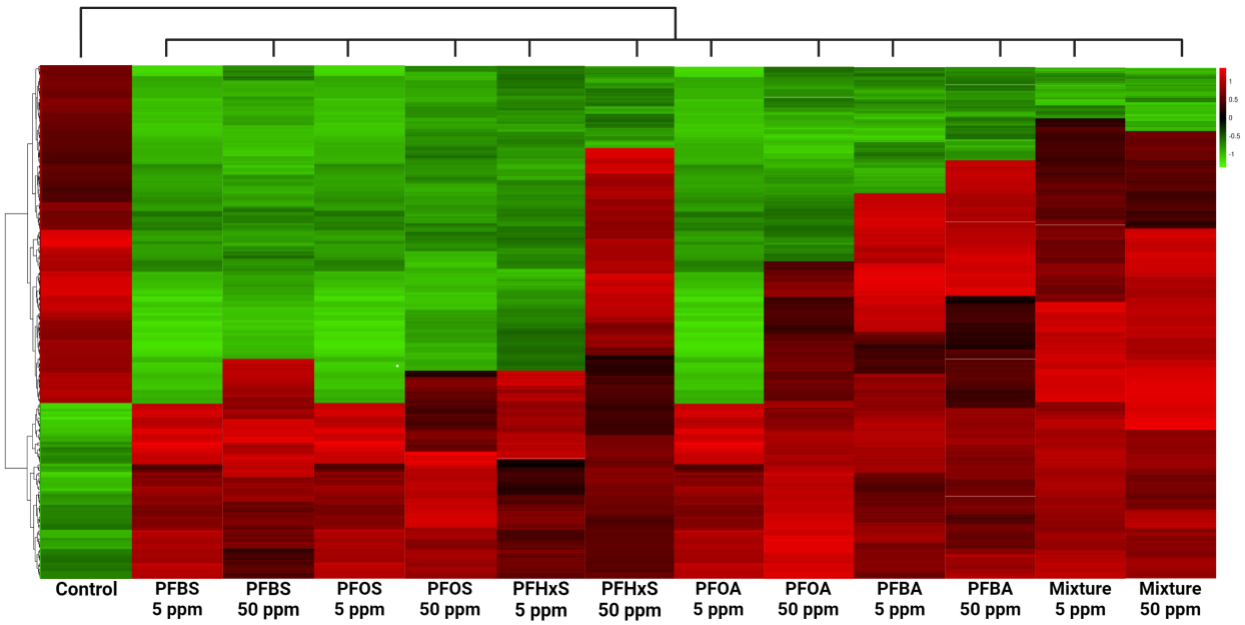


Figure 4.3: The Clustering Analysis Heatmap for Genes Differentially Expressed in all PFAS Exposures and Concentrations.

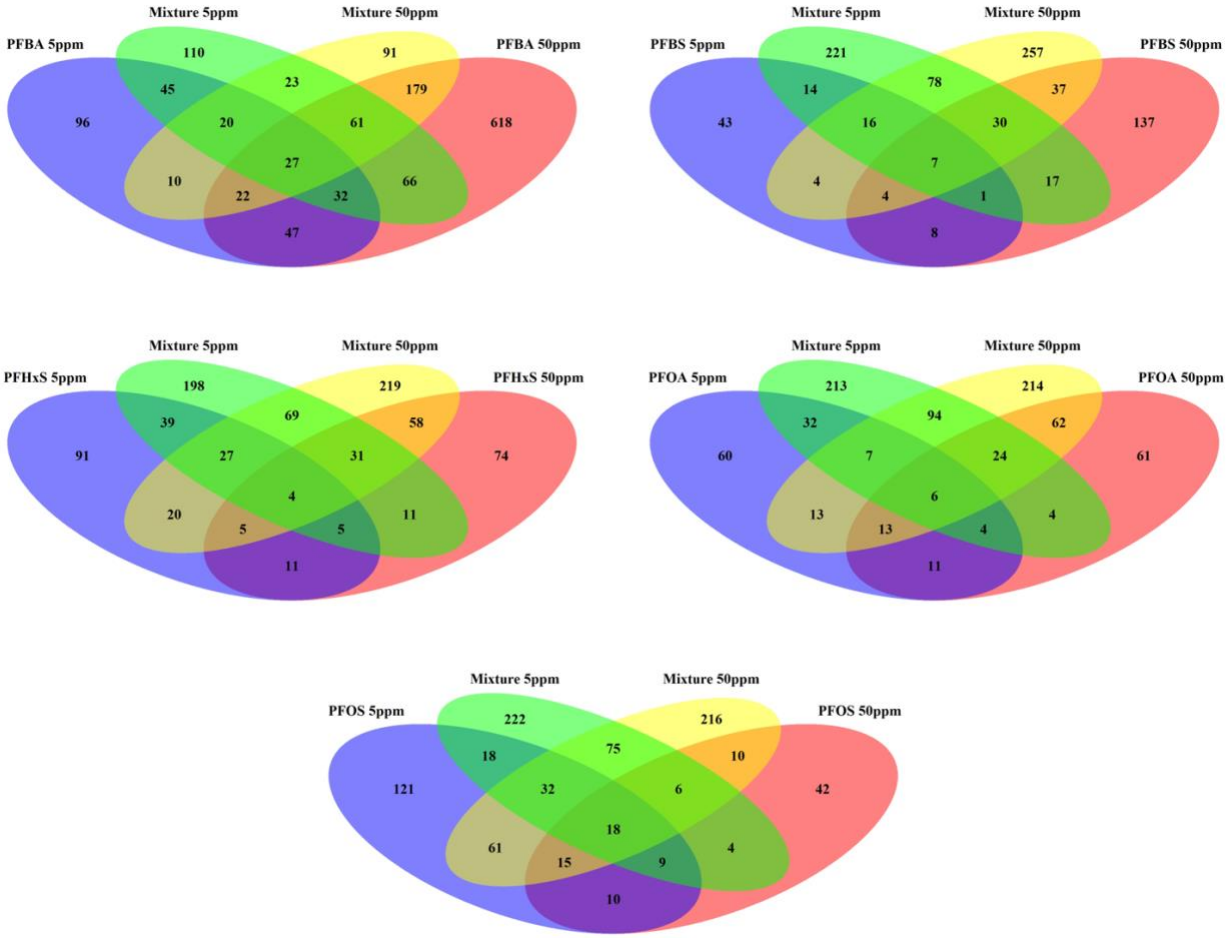


Figure 4.4: Comparison of Significantly expressed GO terms number from differentially expressed genes at each PFAS and exposure compared to the reference mixture.

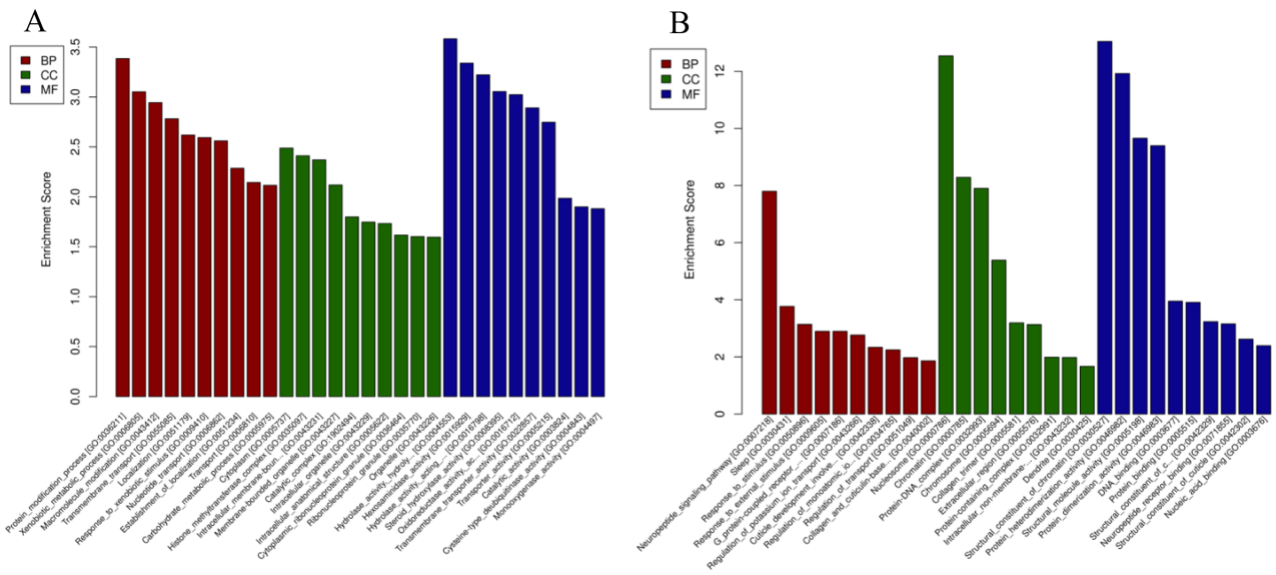


Figure 4.5: Major Gene Ontology (GO) terms represented in the PFBS at 5ppm. BP= Biological Process; CC = Cellular Component; MF = Molecular Function. (A) Up Differentially Expressed Genes. Ordered from left to right by p-value (-log10 scaled), with the most significant GO terms on the left. (B) Down Differentially Expressed Genes. Ordered from left to right by p-value (-log10 scaled), with the most significant GO terms on the left.

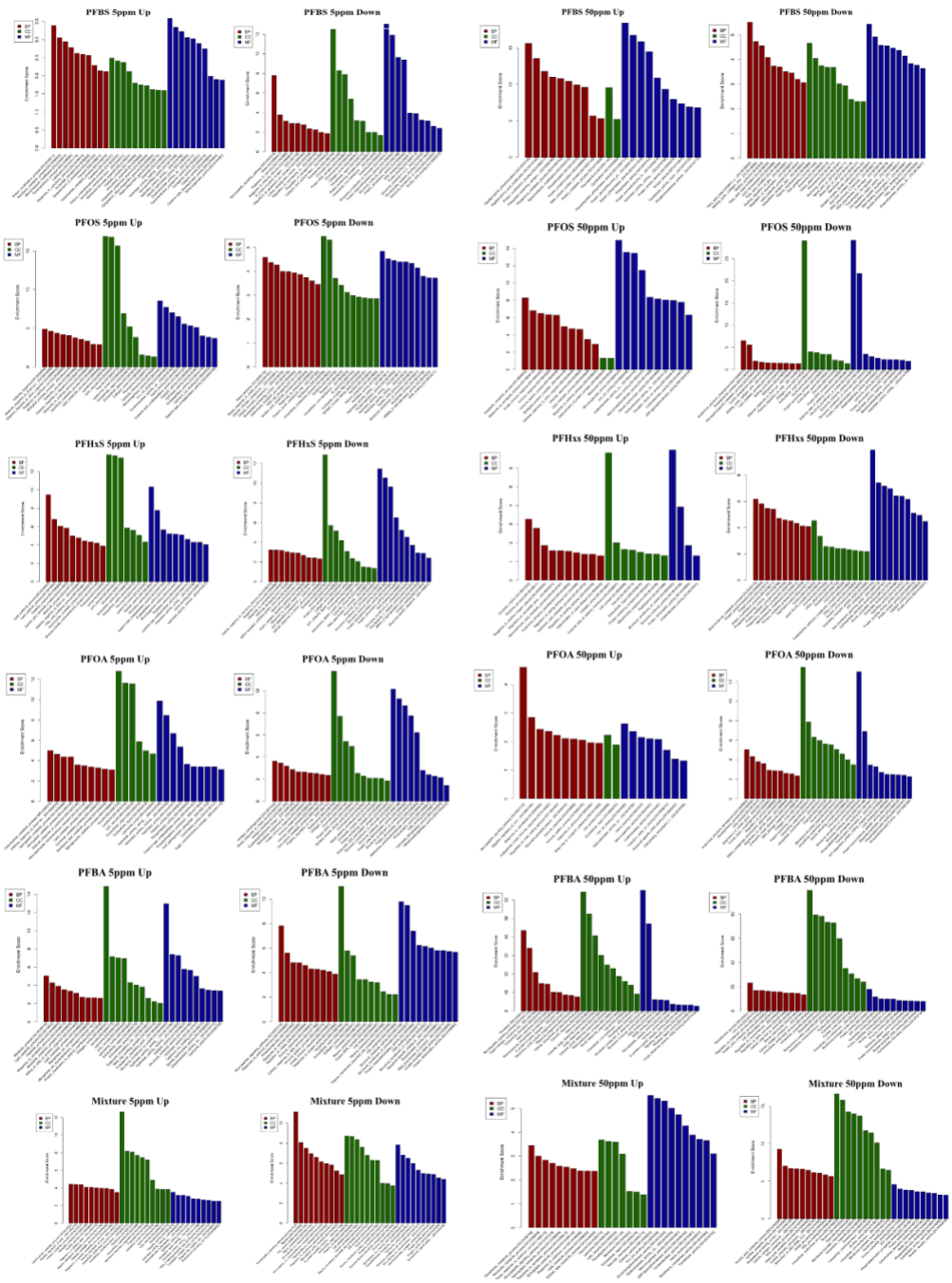


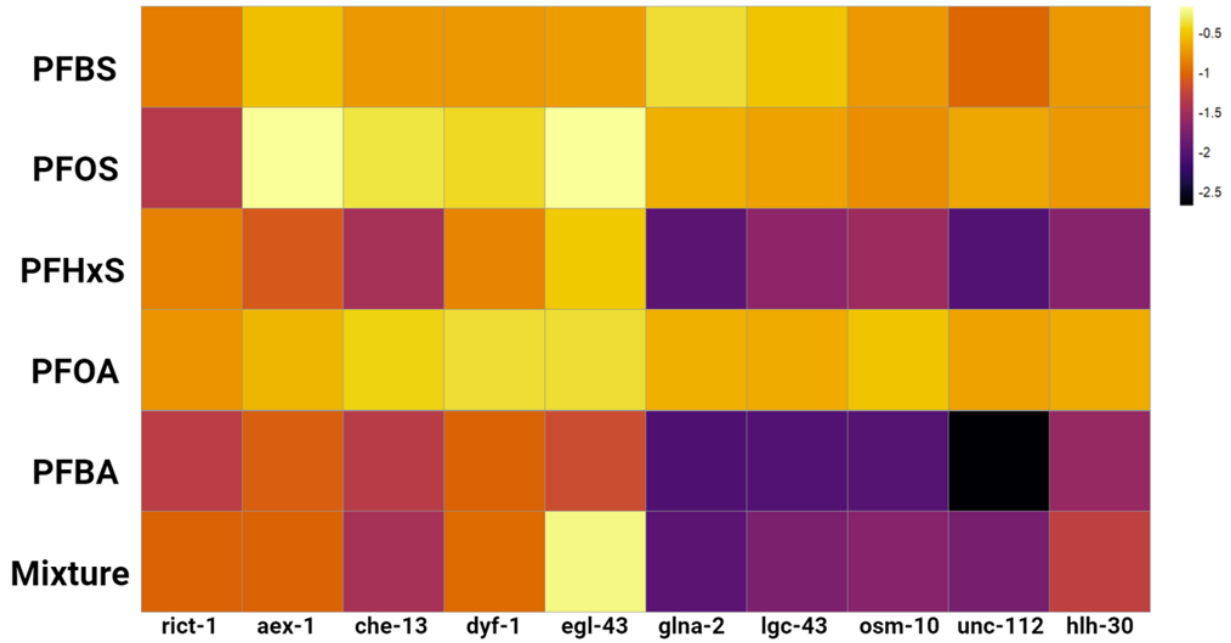
Figure 4.S1: Major Gene Ontology (GO). BP= Biological Process; CC = Cellular Component; MF = Molecular Function. (A) Up Differentially Expressed Genes. Ordered from left to right by p-value (-log10 scaled), with the most significant GO terms on the left. (B) Down Differentially Expressed Genes. Ordered from left to right by p-value (-log10 scaled), with the most significant GO terms on the left.

Table 4.1: Enriched KEGG pathways following PFAS exposure in *C. elegans*. Up-Regulated pathways is represented by a positive enrichment score, while Down-Regulated pathways are represented by a negative enrichment score.

	Pathway ID	Pathway	P-Value	Enrichment Score
PFBS				
	cel04142	Lysosome	0.010	-1.982
	cel03015	mRNA Surveillance Pathway	0.010	1.996
PFOS				
	cel00983	Drug Metabolism-Other Enzymes	1.685e-08	7.773
	cel00980	Metabolism of Xenobiotics by Cytochrome P450	2.529e-08	7.597
	cel00053	Ascorbate and Aldarate Metabolism	0.002	2.690
	cel00982	Drug Metabolism-Cytochrome P450	5.825e-08	7.235
	cel04212	Longevity Regulating Pathway-worm	4.761e-05	4.322
	cel00600	Sphingolipid Metabolism	0.001	3.021
	cel00040	Pentose and Glucuronate Interconversions	0.002	2.690
	cel00830	Retinol Metabolism	0.002	-2.635
	cel00860	Porphyrin Metabolism	0.002	-2.635
	cel00480	Glutathione Metabolism	0.001	-3.419
	cel01100	Metabolic Pathways	0.003	-2.597
	cel00511	Other Glycan Degradation	0.015	1.812
PFHxS				
	cel04142	Lysosome	0.013	1.895
	cel00040	Pentose and Glucuronate Interconversions	0.002	-2.777
	cel00830	Retinol Metabolism	0.002	-2.705
	cel00053	Ascorbate and Aldarate Metabolism	0.015	-1.820
	cel00860	Porphyrin Metabolism	0.017	-1.770
	cel00980	Metabolism of Xenobiotics by Cytochrome P450	0.022	-1.653
	cel00982	Drug Metabolism-Cytochrome P450	0.031	-1.509
	cel00983	Drug Metabolism-Other Enzymes	0.036	1.443
PFOA				
	cel04010	MAPK Signaling Pathway	0.009	-2.024
	cel00500	Starch and Sucrose Metabolism	0.015	-1.819
	cel01100	Metabolic Pathways	0.048	-1.314
	cel04142	Lysosome	0.019	1.718
	cel04212	Longevity Regulating Pathway-worm	0.012	1.922
	cel00010	Glycolysis/Gluconeogenesis	0.022	1.666
PFBA				
	cel04980	Cobalamin Transport and Metabolism	0.029	-1.541
	cel00500	Starch and Sucrose Metabolism	0.010	-2.016
	cel03460	Fanconi Anemia Pathway	0.001	-3.949
	cel00970	Aminoacyl-tRNA Biosynthesis	0.001	-3.488
	cel04010	MAPK Signaling Pathway	0.002	-2.752
	cel00053	Ascorbate and Aldarate Metabolism	0.003	-2.584
	cel00860	Porphyrin Metabolism	0.014	-1.869
	cel00040	Pentose and Glucuronate Interconversions	0.026	-1.584
	cel04512	ECM-Receptor Interaction	0.034	-1.423

Table 4.S3: Enriched KEGG pathways unique to PFAS mixture exposure in *C. elegans* compared to individual PFAS exposure, Upregulated pathways is represented by a positive enrichment score, while downregulated pathways are represented by a negative enrichment score.

Pathway ID	Pathway	P-Value	Enrichment Score
cel04080	Neuroactive Ligand-Receptor Interaction	0.005	-2.333
cel00590	Arachidonic Acid Metabolism	0.0156	-1.809
cel04361	Axon Regeneration	0.037	-1.43
cel00052	Galactose Metabolism	0.001	3.117
cel03050	Proteasome	0.023	1.642



Gene Symbol	Description
rict-1	Expressed in several structures, including head neurons and ventral nerve cord
aex-1	Plays a crucial role in synaptic transmission and is required for normal axonal guidance
che-13	Plays a role in the formation and maintenance of sensory cilia
dyf-1	Involved in cilia structure and function
egl-43	Known for its involvement in neurodevelopment
glna-2	Involved in neurotransmitter synthesis
lgc-43	Encodes a ligand-gated ion channel
osm-10	Involved in osmotic avoidance behavior and functions in sensory neurons
unc-112	Plays a key role in muscle and neuron function
hlh-30	Expressed in several structures, including nerve ring

Figure 4.6: Top 10 of commonly differentially regulated genes induced by PFAS at 0, 5, and 50 ppm. All values represent the fold-change compared to the control.

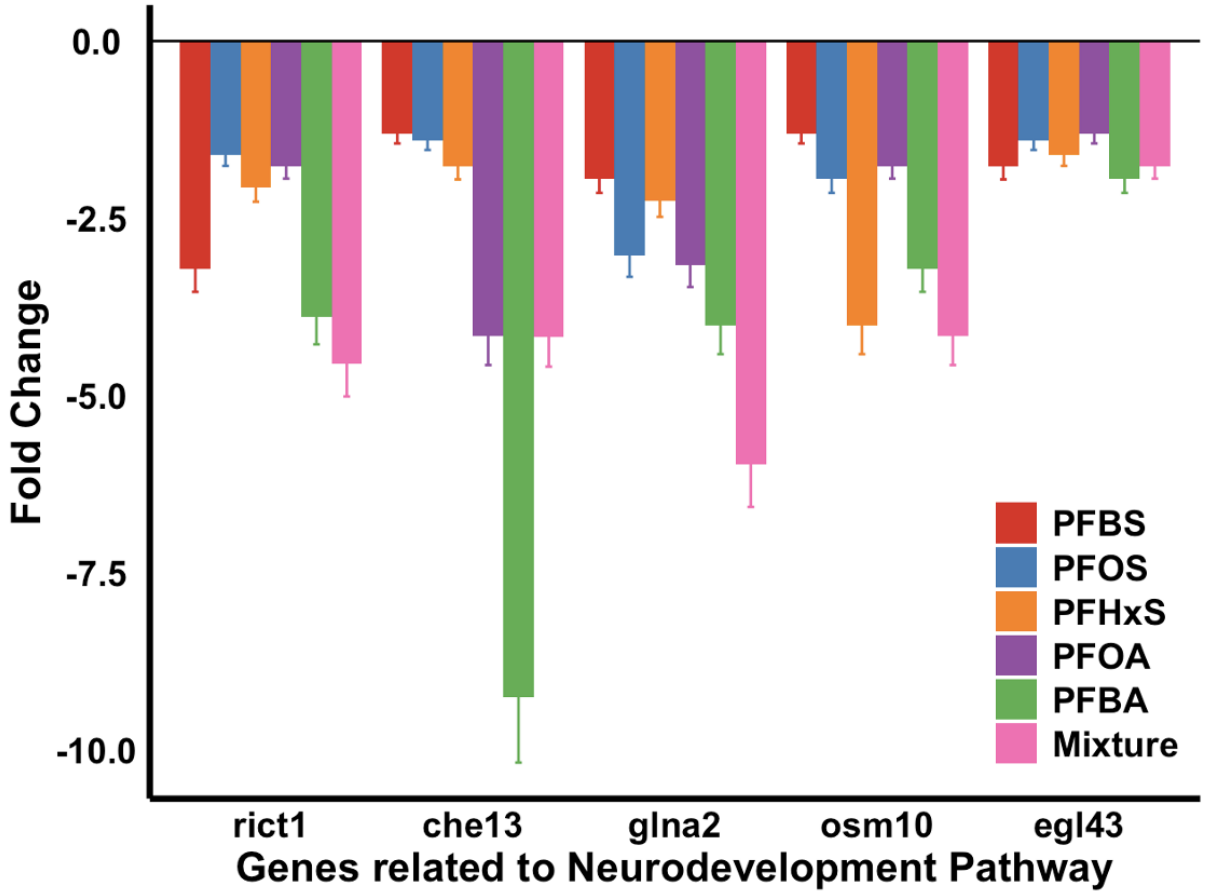


Figure 4.7: Validation of gene expression pattern using real-time quantitative RT PCR. Data are presented in arbitrary unit compared to control (control = 1, mean ± standard error of mean; n = 5).

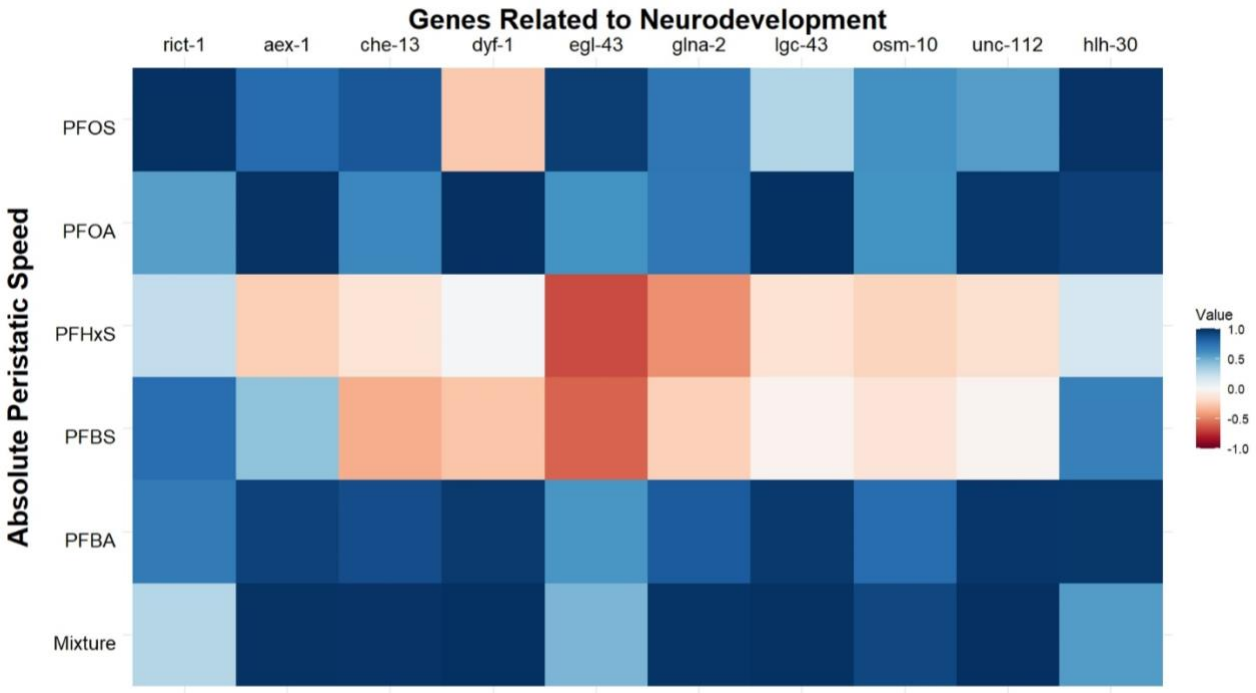


Figure 4.8: Correlation between Behavior and mRNA Expression levels. All values are represented as a Pearson Correlation Coefficient.

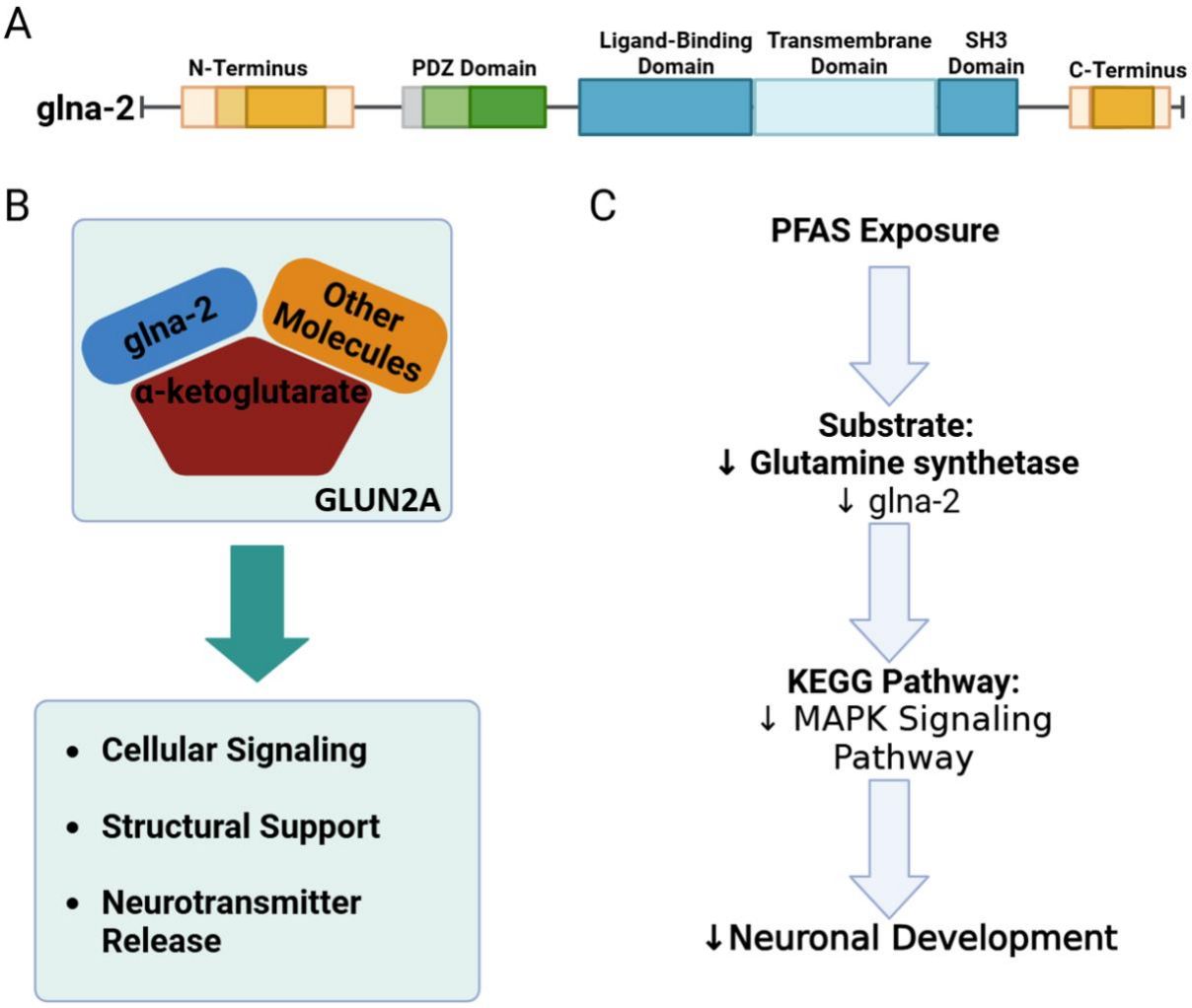


Figure 4.9: *C. elegans* neuronal development based on target of MAPK Signaling Pathway. (A) glna-2 domain structure. The glutamate protein has a characteristic structure of three unique domains. (B) Model of glutamate ammonia ligase protein complex. Glutamate ammonia ligase is believed to play several integral roles in the neuronal system. (C). Proposed mechanism of neurodevelopmental toxicity induced by PFAS in *C. elegans.*

CHAPTER 5

UTILIZATION OF ARTIFICIAL INTELLIGENCE COUPLED WITH A HIGH-THROUGHPUT, HIGH-CONTENT PLATFORM IN THE EXPLORATION OF NEURODEVELOPMENT TOXICITY OF INDIVIDUAL AND COMBINED PFAS[4]

[4] Currie, S. D., Benson, D.B., Xie, Z.R., Wang, J. S., & Tang, L. (2025). *J. Xenobiotics. 15, 24* This chapter is a slightly modified version of [4] and has been reproduced here with the permission of the publisher.

Abstract:

Per- and Polyfluoroalkyl Substances (PFAS) are synthetic chemicals used in various products, such as firefighting foams and non-stick cookware, due to their resistance to heat and degradation. However, these same properties make them persistent in the environment and human body, raising public health concerns. This study selected eleven PFAS commonly found in drinking water and exposed *Caenorhabditis elegans* to concentrations ranging from 0.1 to 200 µM to assess neurodevelopmental toxicity using a high-throughput, high-content screening (HTS) platform coupled with artificial intelligence for image analysis. Our findings showed that PFAS such as 6:2 FTS, HFPO-DA, PFBA, PFBS, PFHxA, and PFOS inhibited dopaminergic neuron activity, with fluorescence intensity reductions observed across concentrations from 0.1 to 100 µM. PFOS and PFBS also disrupted synaptic transmission, causing reduced motility and increased paralysis in aldicarb-induced assays, with the most pronounced effects at higher concentrations. These impairments in both neuron activity and synaptic function led to behavioral deficits. Notably, PFOS was one of the most toxic PFAS, affecting multiple neurodevelopmental endpoints. These results emphasize the developmental risks of PFAS exposure, highlighting the impact of both individual compounds and mixtures on neurodevelopment. This knowledge is essential for assessing PFAS-related health risks and informing mitigation strategies.

5.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) represent a broad category of human-made fluorinated compounds that are prevalent environmental contaminants leading to frequent human exposure (Ding, Harlow, Randolph, Loch-Caruso, & Park, 2020). These compounds are highly fluorinated aliphatic substances where all hydrogen atoms attached to one or more carbon atoms have been replaced by fluorine atoms (Buck et al., 2011). The robust carbon-fluoride bonds in PFAS contribute to their exceptional stability (Langenbach & Wilson, 2021). This stability makes them resistant to degradation by high temperatures, natural light, chemical reactions, and biological processes (Yi et al., 2023). Toxicologist have deemed PFAS as 'forever chemicals' due to their high stability. Many PFAS are exploited for their surfactant characteristics, leading to their intentional inclusion in a wide array of products such as cookware, packaging materials, clothing, carpets, and foams used in firefighting (Roth et al., 2021). Biomonitoring studies have shown that PFAS are found in the blood over 98% of humans within the United States (Lewis, Johns, & Meeker, 2015; Rappazzo, Coffman, & Hines, 2017). PFAS exposure occurs through multiple channels, such as consuming food and water, breathing air, using personal care products, and coming into contact with common items like outerwear, rugs, cleaning supplies, paper products, and indoor dust (Berhanu et al., 2023; Ragnarsdottir, Abdallah, & Harrad, 2022; Sunderland et al., 2019).

Epidemiological evidence links PFAS to a broad spectrum of health concerns, including thyroid dysfunction (Lee & Choi, 2017; Lopez-Espinosa, Mondal, Armstrong, Bloom, & Fletcher, 2012), immune system impairments (Buser & Scinicariello, 2016; Dong et al., 2013; Grandjean et al., 2012), liver disease and cancer (C. Y. Lin et al., 2010), disturbances in lipid and insulin levels (Frisbee et al., 2010; Wen et al., 2016), kidney disease and cancer (Ferrari,

Orlando, Ricci, & Ronco, 2019; Watkins et al., 2013), reproductive and developmental issues (Slotkin, MacKillop, Melnick, Thayer, & Seidler, 2008; X. Wang et al., 2018), and impacts on neurodevelopment (Butenhoff, Ehresman, Chang, Parker, & Stump, 2009; Liew, Goudarzi, & Oulhote, 2018; Zhou et al., 2023). Among these, developmental neurotoxicity (DNT) from PFAS exposure is a significant concern, as it has been associated with neurodevelopmental disorders, including autism spectrum disorder (ASD), attention-deficit/hyperactivity disorder (ADHD), and intellectual disabilities (Seth D. Currie, Wang, & Tang, 2024). PFAS chemicals disrupt critical processes in brain development, such as synaptic formation, neurotransmission, and neural connectivity (Brown-Leung & Cannon, 2022). The mechanisms underlying these effects include oxidative stress, mitochondrial dysfunction, inflammation, and disruption of the blood-brain barrier, all of which compromise neuronal integrity and brain function (Starnes, Rock, Jackson, & Belcher, 2022). These findings highlight the need for the development of more effective models to better understand PFAS's impact on neurodevelopment and investigate its effects on neuronal processes.

Caenorhabditis elegans (C. elegans) has emerged as a powerful model organism for studying neurotoxicity, particularly due to its well-known and rapid developmental cycle. Its utility is particularly evident when investigating the impacts of per- and polyfluoroalkyl substances on dopaminergic neurons and overall neuronal health. These small, transparent nematodes offer numerous advantages for toxicological studies, including a short lifespan, ease of cultivation, and ability to produce many offspring make them an excellent model for laboratory research (Meyer & Williams, 2014). The optical transparency of *C. elegans* provides a unique advantage for researchers, allowing them to examine neuronal cells and features without dissection, which is further enhanced by the use of fluorescent protein reporters to monitor

changes in neuronal morphology and function in real time (Ma et al., 2018). Importantly, nematodes possess homologous genes for approximately 80% of those found in humans (Culetto & Sattelle, 2000). This strong conservation makes *C. elegans* an ideal platform for evaluating the neurotoxic effects of PFAS, which are known to impair neuronal structure, disrupt synaptic connectivity, and cause deficits in neurobehavioral responses. In particular, the well-characterized dopaminergic system of *C. elegans* has been well-characterized, making it a suitable model for studying the impacts of environmental exposures on DA neurons (Leung et al., 2008; Sammi et al., 2019). Furthermore, the disruption of dopaminergic function in *C. elegans* exposed to PFAS has direct consequences on behavior, particularly in tasks that require learning and memory (S. D. Currie, Doherty, Xue, Wang, & Tang, 2023; Seth D. Currie, Ji, Huang, Wang, & Tang, 2024).

The vast number of PFAS, along with their structural diversity, presents a significant challenge for traditional toxicity testing approaches. With thousands of PFAS compounds in the environment, many of which lack comprehensive toxicity data, there is an urgent need for high-throughput, high-content platforms capable of efficiently assessing the neurotoxic potential of these chemicals. These platforms enable the rapid evaluation of neurotoxic endpoints, such as changes in neuronal morphology, synaptic function, and behavior, at a scale and speed that traditional methods cannot achieve (Fryer et al., 2024). Using the *C. elegans* model, our laboratory has created high-throughput screening platforms to evaluate the toxicity of PFAS and various neurotoxins (S. D. Currie et al., 2023; Tang et al., 2019; Yang et al., 2018). To maximize the effectiveness of these platforms, artificial intelligence (AI) tools are increasingly being integrated to enhance data analysis. AI-driven approaches allow for the processing of large datasets, identification of subtle neurotoxic effects, and automated assessments, thus reducing

analysis time and improving consistency (Bacon et al., 2024). By enabling advanced image analysis and pattern recognition, AI technologies support faster identification of hazardous PFAS, facilitating more efficient regulatory decision-making and risk assessment.

The integration of artificial intelligence (AI) in toxicological research offers a novel approach to streamline data analysis and improve the accuracy and efficiency of assessing chemical toxicity. AI encompasses the creation of computer systems designed to perform tasks traditionally requiring human intelligence (Briganti, 2023). These tasks include recognizing patterns, learning from data, and making decisions. By leveraging advanced algorithms and machine learning techniques, AI systems can process vast amounts of information, identify trends, and adapt to new data. The advancements in AI and the life sciences are deeply interconnected. AI technologies, such as machine learning and data analytics, are revolutionizing the life sciences by enabling more efficient data processing, complex pattern recognition, and predictive modeling (Xu et al., 2021). Neuroscience has been significantly accelerated with the utilization of AI (Hassabis, Kumaran, Summerfield, & Botvinick, 2017). AI techniques have revolutionized the analysis of neuroimaging datasets, offering significant potential to expedite connectomic analysis (Glasser et al., 2016). This technology can be applied to investigate the impacts of PFAS on developing dopaminergic neurons through morphological alterations, synaptic formation, and neuronal activity in *C. elegans*.

Deep learning, a subset of machine learning, has been particularly transformative, utilizing artificial neural networks inspired by the structure and functioning of the human brain (Sarker, 2021). These networks consist of multiple stacked layers, allowing AI systems to interpret complex, high-dimensional data and extract intricate patterns across diverse fields such as computer vision, language processing, and biomedicine (Barragán-Montero et al., 2021). This

layered approach has accelerated advances in life sciences by facilitating detailed image analysis, disease prediction, and drug discovery, enabling scientists to make strides in understanding human health and biology at unprecedented scales (Filipp, 2019). Deep learning's impact on neuroimaging is profound, as algorithms designed for massive data analysis allow researchers to identify subtle neural pathways and interactions between neurons, bringing new insights into neurodegenerative diseases and cognitive functions (Avberšek & Repovš, 2022). The adaptability of deep learning models is especially advantageous for studying environmental effects on neural development. By enhancing our analytical capabilities, deep learning technologies not only deepen our understanding of life sciences and neuroscience but also create actionable insights that support efforts in environmental safety and public health, illustrating the profound synergy between AI and scientific discovery.

In this study, eleven PFAS were selected according to their widespread occurrence in different water bodies (Y. Wang et al., 2022), which represent a wide range of typical PFAS structures, including perfluoroalkyl carboxylic acids (perfluorobutanoic acid [PFBA], perfluorohexanoic acid [PFHxA], perfluorooctanoic acid [PFOA], perfluorononanoic acid [PFNA]), sulfonic acids (perfluorobutanesulfonic acid [PFBS], perfluorohexanesulphonic acid [PFHxS], Perfluorooctanesulfonic acid[PFOS]), sulfonamides and derivatives (perfluorooctanesulfonamide [PFOSA], [NetFOSAA]), fluorotelomers (6:2 fluorotelomer sulfonic acid [6:2 FTS]), and new substitutes (hexafluoropropylene oxide dimer acid [HFPO-DA], the acid form of GenX). The goal is to investigate the impact of PFAS exposure on dopaminergic (DA) neurons in *C. elegans*, with a specific focus on morphological alterations, synaptic formation, and behavior (neuron activity). By focusing on these aspects of dopaminergic neuron development, we aim to understand the mechanisms through which PFAS

affect neurodevelopment. Additionally, this study utilized AI tools to enhance data analysis, providing fast and accurate results that will facilitate high-throughput screening of PFAS toxicity. Through these combined approaches, this study contributes valuable insights into the neurotoxic effects of PFAS and help improve methodologies for assessing the environmental and health risks of these contaminants.

5.2 Materials and Methods

5.2.1 Chemicals

A review of data from 23 studies investigated the prevalence of PFAS in various water bodies to determine the most common PFAS (Y. Wang et al., 2022). The analysis also highlighted the primary PFAS in each category and identified several alternatives used to replace legacy PFAS. The 10 PFAS chosen for this study were PFBA (95%, BCCF2984, COA), PFHxA (98%, P102-28684, COA), PFOA (95%, WXBD6815, COA), PFNA (97%, 394459, COA), PFBS (98%, P151-08994, COA), PFHxS (95%, 751400, COA), PFOS (98%, 830800, COA), NEtFOSAA (95%, P102-28720, COA), 6:2 FTS (95%, 754100, COA), PFOSA (96%, CDS010729, COA), and HFPO-DA (96%, 00022309, COA).

Analytical grade PFBA, PFNA, and PFOA were sourced from Sigma Aldrich (St. Louis, MO). NEtFOSAA, PFHxA, and PFBS were obtained from Astatech Inc. (Bristol, PA). PFOS, PFHxS, HFPO-DA, 6:2 FTS, and PFOSA were procured from Synquest Laboratories, Inc. (Alachua, FL). Stock solutions at a concentration of 1M were prepared using dimethyl sulfoxide (DMSO), and working solutions were further diluted with K-medium (32mM KCl and 51mM NaCl) supplemented with OP50 as a 1mg/ml food source, resulting in a final DMSO concentration of 0.1% (Brenner, 1974).

5.2.2 Mixture Selection

We identified five PFAS that predominantly contribute to the PFAS load in U.S. water sources (Smalling et al., 2023). From these chemicals, we created a reference mixture based on their relative concentrations in the overall mixture. The estimated proportions were PFOS (30%), PFBA (20%), PFOA (20%), PFHxS (15%), and PFBS (15%). This mixture will serve as a model for typical daily exposure in the U.S. While the total concentration of the mixture may vary, the proportions will remain fixed. Evaluating mixture profile patterns helps in assessing specific PFAS compounds that are frequently found at higher concentrations (East, Anderson, & Salice, 2021).

5.2.3 C. elegans Culture and Exposure

BZ555 nematodes (dat-1p::GFP) and the Escherichia coli strain OP50 were sourced from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). The *C. elegans* were cultivated at 25°C on nematode growth medium (NGM) plates with OP50 as their food. To obtain synchronized L1 larvae, we performed alkaline lysis (10M NaOH, 2% sodium hypochlorite), followed by overnight hatching in k-medium (Brenner, 1974). For individual neuron imaging experiments, exposure concentrations were set at 100, 1, and 0 μmol/L, while for synaptogenesis studies, the concentrations were 200, 100, 10, 1, 0.1 μmol/L. These concentrations were chosen based on our previous findings (S. D. Currie et al., 2023) and the U.S. EPA's ToxCast program recommendations for in vivo assays (Rowan-Carroll et al., 2021). Considering *C. elegans* is an in vivo model with interspecies variations, we selected 200 μmol/L as the highest concentration to effectively observe the elicited effects and responses.

5.2.4 High-throughput, High Content Platform for Dopaminergic Neurons in Nematodes

As previously described, a high-throughput platform was utilized using the COPAS BIOSORT (Union Biometrica, Inc., Massachusetts, USA) to investigate the impacts of PFAS on dopaminergic neurons (S. D. Currie et al., 2023; Polli et al., 2015; Tang et al., 2019). Nematode worms were sorted and distributed into 96-well plates by the COPAS BIOSORT, each well filled with k-medium, a bacterial food source, and varying levels of PFAS. The experiments were conducted in triplicate, with three separate plates, each containing 10 technical replicates per exposure level. Negative controls were included on each plate. To ensure accuracy, the experiments were repeated three times. The plates were incubated at 25°C with continuous shaking, and assessments were conducted at both 24 and 48 hours. At time of assessment, the vivoChip-2x technology (vivoVerse, Texas, USA) was employed to analyze the impacts of PFAS on individual dopaminergic neurons, providing high-resolution data on their physiological changes (Ben-Yakar, 2019; Mondal et al., 2016). Moreover, the Cytation 5 Cell Imaging Multi-Mode Reader (Agilent, California, USA) was used to capture detailed images of the nematodes, allowing for precise visual documentation and analysis of their condition from exposure. Images were captured using a Sony CMOS, 16-bit color camera (SONY, Tokyo, JPN) at 60x magnification. Integrating the vivoChip-2x and the Cytation imaging system creates a high-throughput, high-content platform for investigating the effects of potential neurotoxins. Data was read, processed, and plotted using GraphPad Prism.

5.2.5 Artificial Intelligence

Images were analyzed using DeepImageJ, an open-source framework that integrates deep learning models for image analysis within the ImageJ ecosystem. This innovative tool allows for

the seamless incorporation of advanced machine learning techniques into traditional image analysis workflows, enhancing both accuracy and efficiency. For this study, we employed the HPA cell segmentation model, specifically designed for identifying and quantifying cellular structures within complex biological images. This is accomplished through the anaylsis of both neuronal size and fluorescence intensity. Prior to analysis, all images underwent preprocessing to standardize dimensions, formats, and contrast levels, ensuring optimal input for the model. After importing the images into DeepImageJ, the HPA cell segmentation model was applied, utilizing a convolutional neural network (CNN) trained on an extensive dataset of annotated cellular images. The workflow for the image analysis is outlined in figure 5.1. This model excels at accurately segmenting cells based on their morphological characteristics, thus enabling detailed quantification of cellular parameters such as size, shape, and density. To validate the accuracy of the segmentation results, we conducted a series of performance assessments, comparing the model outputs with manually annotated reference images and through traditional fluorescence analysis. Significant changes in morphological structure could indicate that exposure to PFAS impacts neurodevelopment, reflecting disruptions in cellular processes or alterations in neuronal development.

5.2.6 Synaptogenesis Assay

Fifty age-synchronized *C. elegans* L1-stage worms were allocated into individual wells containing 90µl of testing solution, which consisted of k-medium, a bacterial food source, and varying concentrations of PFAS. The plates were incubated at 25°C for periods of 24 and 48 hours. The experiments were conducted in triplicate, with three separate plates, each containing 10 technical replicates per exposure level. Negative controls were included on each plate. After

incubation, the impacts on synaptogenesis were assessed using an aldicarb-sensitive assay to determine the time to paralysis upon aldicarb exposure (Izquierdo, O'Connor, Green, Holden-Dye, & Tattersall, 2021). Aldicarb (Toronto Research Chemicals, 98%, X11437815, COA) was added into each well with a final concentration of 500μM. Paralysis was measured with the WMicrotracker-One™ (PhylumTech) over a 3-hour period, with motility loss monitored at fifteen-minute intervals. The aldicarb-induced paralysis assay is a quick and simple method to evaluate alterations in synaptic transmission in *C. elegans* by correlating the rate of neurotransmitter release with the onset of paralysis (Oh & Kim, 2017).

5.2.7 Area Under the Curve

The area under the curve (AUC) is a quantitative measure that represents the total cumulative effect of a variable over a specified range, capturing the overall magnitude of response or activity in a given dataset (Scheff, Almon, Dubois, Jusko, & Androulakis, 2011). The area under the curve for synaptogenesis was meticulously calculated using the "pracma" package in R to measure the cumulative synaptic response to varying concentrations of test substances, integrating time-course data of synaptic activity to capture the dynamics of synaptic development over time. Higher AUC values indicate a more pronounced synaptic response, reflecting enhanced synaptogenesis, while lower values suggest reduced activity. To facilitate accurate comparisons, AUC values were normalized to those of a control group, allowing for a direct assessment of treatment effects by expressing the AUC of each condition relative to the control. This normalization process ensures that baseline variations are accounted for, providing a clear basis for evaluating how different concentrations impact synaptogenesis. By analyzing these normalized AUC values, researchers can effectively compare the extent of synaptic

development across different treatments, offering a robust quantitative framework for ranking substances based on their efficacy in promoting or inhibiting synaptic growth and development.

5.2.8 Calculation of Benchmark Dose

Benchmark concentrations at 10% (BMC 10%) and their lower confidence limits (BMCL) were estimated using PROASTweb software (version 70.1; https://proastweb.rivm.nl/). The software processes assay data, including PFAS concentration levels and their effects, by fitting the data to a model using maximum likelihood estimation (MLE), optimized according to the lowest Akaike Information Criterion (AIC). The AIC is used to select the most appropriate model for the data. The BMC was calculated with a 95% confidence interval and a p-value threshold of 0.05, based on the model with the lowest AIC. The BMC and BMCL values, derived for the synaptogenesis assay, represent the point at which significant changes of 10% occurs in response rates compared to the control occur, allowing for the ranking of PFAS compounds according to their impact on the neuronal system.

5.2.9 High-throughput Screening Platform for Behavior

To perform high-throughput behavior assays, we employed the WormLab system (Version 2023.1.1, MBF Bioscience, Vermont, USA) in conjunction with the COPAS BIOSORT (Union Biometrica, Inc., Massachusetts, USA) as previously described (S. D. Currie et al., 2024) . Age-synchronized L1-stage nematodes were allocated into individual wells of 96-well plates, each containing 100 µl of the varying concentrations of PFAS along with k-medium and a bacterial food source. The worms were exposed to varying concentrations of individual PFAS compounds, and each experimental condition was performed in triplicate. The experiments were

conducted in triplicate, with three separate plates, each containing 10 technical replicates per exposure level. Negative controls were included on each plate. The plates were incubated at 25°C for either 24 or 48 hours with continuous shaking. After incubation, the worms were transferred to NGM plates and allowed to acclimate for 1 hour before being analyzed. The WormLab system was used to analyze behavioral endpoints, recording the worms' movement for 60 seconds with a minimum track duration of 30 seconds. Images were captured using a Nikon DSLR camera (Tokyo, JPN) at a resolution of 1280 x 960 pixels and a frame rate of 7.5 frames per second. Center point speed was assessed as an indicator of locomotion and overall motor function in *C. elegans.* The nematodes' movement was tracked to evaluate their speed and coordination, reflecting their neural and muscular integrity (Ke, Prince, Bowman, & Aschner, 2021). Efficient movement is crucial for the worms' ability to respond to environmental changes and avoid unfavorable conditions (Goodman & Sengupta, 2019). Data on center point speed were collected, analyzed, and visualized using GraphPad Prism.

5.2.10 Correlation Analysis Between Toxicity and Neurodevelopment

The investigation into the connection between PFAS toxicity and its effects on neurodevelopment sought to clarify how varying levels of toxicity can impact the development of neural structures and functions. To quantify this relationship in *C. elegans*, Pearson correlation analysis was performed ($p<0.05$), allowing for a detailed assessment of the effects. The findings were illustrated using a heatmap, which visually represented the intricate patterns of interaction between toxicity and neurodevelopmental outcomes. These correlations shed light on the underlying mechanisms that may link PFAS exposure to disruptions in neurodevelopment. For the analysis, the "mice" package in R was utilized to compute the correlation coefficients,

enabling a robust statistical evaluation of the data. This comprehensive approach not only enhances our understanding of PFAS's impact on neurodevelopment but also highlights the significance of examining toxicity in relation to cognitive processes.

5.2.11 Statistical Analysis

Data analysis was performed using GraphPad Prism version 10.1.2 (La Jolla, CA) or R version 3.3.4, and the results were reported as means ± standard deviation (SD). Statistical comparisons between experimental groups and controls were conducted using analysis of variance (ANOVA) to determine overall differences, with subsequent Tukey's post-hoc test applied for pairwise comparisons to identify specific group differences. Significance levels are indicated in the graphs with asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

5.3 Results

5.3.1 Dopaminergic Neurons

To evaluate the developmental toxicity of PFAS, synchronized L1-stage worms were subjected to different concentrations: 100, 1, and 0 μmol/L. As seen in Fig. 5.2, a notable decrease in fluorescence intensity of dopaminergic neurons (CEP: Cephalic Sensilla Neurons and ADE: Anterior Deirids Neurons) can be visually observed with increasing concentrations of PFOS after 48 hours of exposure, highlighting the compound's impact on neuronal morphological alterations. Additionally, the presence of blebs in the images serves as a morphological marker of neurodegeneration and is associated with impaired neuronal function. However, visual observations alone cannot be quantified, necessitating further quantitative analysis to accurately assess the extent of the fluorescence changes. The fluorescence intensity

and size of individual dopaminergic neurons were measured at different time points, including 24 and 48 hours, using deep learning techniques to analyze and quantify the size and intensity levels. This approach provides a quantitative assessment of the dopaminergic neurons and enables precise measurement of the effects of PFAS on neuronal morphology, facilitating the identification of dose-dependent changes and the comparison of toxicity among different compounds.

As illustrated in Fig. 5.3 and 5.S1, the quantitative fluorescence measurements determined through deep learning allows for a detailed comparison of dopaminergic neuron across various PFAS concentrations. Among the PFAS tested, 6:2 FTS, HFPO-DA, PFBA, PFBS, PFHxA, NEtFOSAA, and PFOS were identified as the most toxic, as they consistently caused significant reductions in intact neurons at both 24 and 48 hours, indicating a substantial and immediate impact on dopaminergic neuron morphology. In contrast, PFOA and PFNA only showed significant toxic effects on neurons only at high concentration. The highest levels of inhibitions were 26.61% for 6:2 FTS, 24.58% for HFPO-DA, 20.90% for PFBA, 26.44% for PFBS, 15.53% for PFHxA, and 25.81% for PFOS. In contrast, PFHxS, showed significant decreases in intact neurons at only one of the measured time points, suggesting variations in the rate at which these compounds exert their effects or their ability to induce delayed cellular responses. This temporal specificity highlights that the mechanisms of action for these PFAS compounds may differ, potentially involving complex interactions with neuronal processes that manifest at different stages. Notably, PFOSA did not exhibit any significant decreases in neuronal morphology, indicating a lower or negligible impact on dopaminergic neurons compared to the other PFAS compounds tested.

5.3.2 Synaptogenesis

To examine the developmental toxicity of PFAS, synchronized L1-stage organisms were exposed to a range of concentrations: 200, 100, 10, 1, and 0.1 μmol/L. Synapse formation was assessed using an aldicarb-sensitive assay, measuring the time to paralysis upon aldicarb exposure at 24 and 48 hours. The Area Under the Curve (AUC) for paralysis was calculated to quantify the synaptic response, with detailed values of benchmark dose provided in Table 5.1. This assessment offered insights into how different concentrations affect synaptic development and enabled a comparison of the efficacy of various test substances in modulating synaptogenesis.

As shown in Fig. 5.4, the aldicarb-induced paralysis assay revealed significant alterations in synaptogenesis across different concentrations at 48 hours. The most pronounced effects on synaptic transmission were observed at concentrations of 100–200 μmol/L, where there was a notable decrease in the time to paralysis, indicating enhanced disruption of synaptic function (Figure 5.4 and 5.S2). Specifically, PFOS and PFBS exhibited significant changes in synaptic response, as evidenced by a marked reduction in motility and increased time to paralysis at both 24 and 48 hours. This suggests that these compounds strongly impact synaptic transmission over time. The assay demonstrated that higher concentrations of these PFAS compounds lead to more severe impairments in synaptic activity, highlighting their potent effects on neurotransmission. The maximum inhibition rates were 45.7% for 6:2 FTS, 73.8% for HFPO-DA, 91.4% for NEtFOSAA, 33.2% for PFBA, 72.2% for PFBS, 48.6% for PFHxA, 48.5% for PFHxS, 72.8% for PFOA, 88.9% for PFOS, 48.2%for PFNA, 71.9% for PFOSA, and 72.3% for the mixture. According to the BMC value, the toxicity ranks of ten PFAS in the adult stage was as follows:

PFBA> NEtFOSAA> HFPO-DA> PFOSA> Mixture> PFOS> 6:2 FTS> PFHxS> PFOA> PFHxA> PFNA> PFBS.

5.3.3 Behavior

To investigate the effects of PFAS on behavioral performance, synchronized L1-stage *C. elegans* were exposed to varying concentrations of PFAS: 200, 100, 10, 1, and 0.1 μmol/L. Center point speed was measured to evaluate the impact on locomotion and motor function. This behavioral assessment was conducted at 24- and 48-hours' post-exposure. Changes in center point speed provided insights into how different PFAS concentrations affect the worms' ability to move and respond to their environment. Detailed results, including the benchmark concentration of the mean center point speed for each concentration, are presented in Table 5.2. This analysis allowed for a comparative evaluation of the effects of various PFAS on nematode locomotion and motor coordination.

As illustrated in Fig. 5.5, the assessment of center point speed revealed significant effects on locomotion across different PFAS concentrations. The most notable reductions in speed were observed at concentrations of 100–200 μmol/L, indicating a marked impairment in the worms' movement ability (Figure 5.5). Specifically, PFOS and PFBS caused substantial decreases in center point speed, with pronounced effects evident at both 24 and 48 hours. This indicates that these compounds have a strong influence on nematode motility over time. The analysis demonstrated that higher concentrations of these PFAS compounds lead to greater reductions in center point speed, underscoring their significant impact on locomotor function. The maximum reductions in speed were observed with PFOS, PFBS, and PFNA, with the highest inhibition rates at 54.13% for PFOS, 57.06% for PFBS, and 49.31% for PFNA. According to the BMC

value, the toxicity ranks of ten PFAS in the adult stage was as follows: Mixture> HFPO-DA> PFBA> PFOS> NEtFOSAA> PFBS> 6:2 FTS> PFNA> PFHxA> PFHxS> PFOSA> PFOA.

5.3.4 Correlation Analysis Between Toxicity and Neurodevelopment

To assess the relationship between PFAS toxicity and its effects on neurodevelopment and synaptogenesis, a comprehensive analysis was conducted using the Pearson Correlation Coefficient, calculated through the "mice" package in R software. This evaluation took into account both the behavioral toxicity and the impacts on neural development processes in addition to the synaptogenesis observed after 48 hours of exposure to various PFAS compounds. These results, illustrated in Figure 5.6, reveled distinct correlations between PFAS toxicity and neurodevelopment. PFOS, PFBS and PFHxS exhibited the strongest negative correlations with neurodevelopment and synaptogenesis, indicating that these substances are particularly harmful and may inhibit normal neural growth and function. In contrast, 6:2 FTS and PFHxA showed minimal correlation, which may reflect differences in their mechanisms of action, despite both chemicals affecting neurodevelopment in the assays.

5.4 Discussion

In this study, individual PFAs and a reference mixture were selected to examine the impacts on neurodevelopment and the neuronal system in *C. elegans*. A high-throughput, high-content screening platform, enhanced by AI, was employed for this comprehensive assessment. Our findings revealed that PFAS significantly hindered the neurodevelopment of *C. elegans* at concentrations ranging from 100 to 200 μM after 48 hours of exposure during their developmental stages. Our results demonstrated that 6:2 FTS, HFPO-DA, PFBA, PFBS, PFHxA,

and PFOS significantly inhibited dopaminergic neuron activity in synchronized L1-stage worms, with notable reductions in size and fluorescence intensity observed across a concentration range of 1 to 100 μmol/L. This decline in neuronal fluorescence indicates a dose-dependent impact of these PFAS compounds on neuronal health, with PFOS and PFBS showing the most significant effects.

Further analysis through aldicarb-induced paralysis assays revealed that PFAS not only affected dopaminergic neurons but also significantly disrupted synaptic transmission. This disruption was characterized by decreased motility and increased paralysis times, particularly at higher concentrations, indicating a profound impact on the worms' neuromuscular function as seen in the evaluation of behavior. Although both neuron-specific inhibition and synaptic inhibition were observed, the slight differences in their effects suggest that these processes may be influenced by similar but slightly divergent mechanisms. These nuances in how PFAS compounds interact with neuronal and synaptic functions underscore the complexity of their toxicity. Among the tested PFAS, PFOS emerged as one of the most potent, exhibiting significant toxicity across all neurodevelopmental endpoints.

The widespread occurrence of PFAS in human populations, coupled with evidence of potential adverse effects, has led to growing apprehension about the possible implications of the abundant usage (Hamm, Cherry, Chan, Martin, & Burstyn, 2010). In response to findings from dose-dependent toxicity studies, epidemiologist have increasingly concentrated on investigating legacy PFAS, such as PFOS and PFOA, and their impact on human adverse effect (Olsen, Butenhoff, & Zobel, 2009). However, due to the bioaccumulation nature of PFAS, there has been increasing international regulatory efforts on legacy compounds (Lilienthal, Dieter, Holzer, & Wilhelm, 2017). In response to regulations, alternative PFAS have been introduced obtain

similar goals (Lindstrom, Strynar, & Libelo, 2011). However, a majority of the alternative PFAS are not well-documented or identified due to their nature as proprietary compounds or by products of manufacturing processes (Ateia, Maroli, Tharayil, & Karanfil, 2019). In the present study, we identified eleven PFAS from the most commonly found compounds in major U.S. water systems (Y. Wang et al., 2022). Additionally, a reference mixture was prepared from the identified compounds, employing their observed ratios to facilitate the simultaneous testing of multiple substances. We observed that the toxicity of the mixture differed from the individual components. The mixture had a higher concentration than some of the individual PFAS, potentially leading to interactions between the substances that may have influenced the overall toxicity. However, this requires further investigation to fully understand the underlying mechanisms.

The nematode, *Caenorhabditis elegans*, has been an invaluable model organism in scientific research for almost fifty years (Marsh & May, 2012). *C. elegans* offers numerous advantages as a model organism, including its ease of genetic manipulation, small size, and low-cost maintenance, while its transparent body enables the direct visualization of fluorescently labeled cells (Gammon, 2017). *C. elegans* have been extensively studied for in vivo toxicity to assess how exposure affects individual cells and organs (Wu, Xu, Liang, & Tang, 2019). *C. elegans* possesses four key organ systems—neural, digestive, immune, and reproductive—that are analogous to those found in vertebrates, making its findings both reliable and significant for comparative studies (Hunt, 2017). Employing high-throughput, high-content screening platform developed in our laboratory (S. D. Currie et al., 2023; Tang, Williams, Xue, Wang, & Tang, 2020), the neurodevelopmental toxicity of PFAS was assessed by analyzing their impact on individual neurons and the entire neural network, taking into account localized as well as

systemic effects. The neuropeptide signaling in nematodes, despite their limited number of neurons and simpler anatomy, shows an unexpected level of conservation and diversity similar to the signaling observed in the human brain (Ripoll-Sanchez et al., 2023). The findings from this part of the study were in agreement with previous research, revealing that most PFAS and their mixtures had detrimental effects on both neuronal development and synaptic transmission (Sammi et al., 2019). While limited to only PFOS, the previous research laid the foundation for further investigation into PFAS.

Furthermore, the investigation of dopaminergic (DA) neurons is critical for understanding neurotoxic impacts because these neurons are highly involved in controlling movement, mood regulation, and reward mechanisms, all of which are crucial for proper neurological function (Juárez Olguín, Calderón Guzmán, Hernández García, & Barragán Mejía, 2016). Disruptions to DA neuron activity are known to be associated with neurodevelopmental disorders, including Parkinson's disease and other neurodegenerative conditions (Taoufik, Kouroupi, Zygogianni, & Matsas, 2018). As such, studying PFAS compounds effect on DA neurons can provide valuable insights into their potential role in the development of neurological diseases. Furthermore, *C. elegans* provides an ideal model for such investigations due to its well-characterized dopaminergic system and the ability to directly observe changes in DA neuron function, morphology, and behavior. Dopaminergic (DA) neurons play a pivotal role in neurodevelopment, as they are involved in essential processes such as neuronal differentiation, synaptic formation, and the establishment of neural circuits, making them critical targets for studying developmental neurotoxicity (Nass and Blakely, 2003).

Building on this groundwork, our research is crucial as it expands the scope by including a broader array of PFAS compounds and their mixtures, allowing us to explore their diverse

effects on dopaminergic (DA) neurons, neurodevelopment, and overall health. By examining additional PFAS, we aim to provide a more comprehensive understanding of their neurotoxic effects and environmental impact, addressing gaps left by earlier studies and contributing valuable insights for regulatory and public health considerations. Previous studies in mammalian and non-mammalian models have shown that PFAS disrupt crucial neurotoxic targets for neurotransmission (Brown-Leung & Cannon, 2022). These studies have demonstrated that PFAS disrupt neurotransmitter levels (Foguth et al., 2020), interfere with the expression and function of synaptic proteins (Mshaty et al., 2020), and alter calcium signaling pathways (Zhang et al., 2016). By employing a diverse range of model organisms, researchers have gained deeper insights into how PFAS influence neural communication and signaling, particularly with regard to DA neurons. These findings underscore the need for further investigation into how PFAS disrupt these essential neural circuits. Although there is extensive research on PFAS in mammalian models, these studies have limitations, and many PFAS chemicals still lack mammalian data (Carlson, L.M., et al., 2022). Additionally, these studies come with significant drawbacks, including ethical concerns, high expenses, extended study durations due to the long lifespans of the animals, and limited sample sizes, which can pose challenges for achieving statistically significant results (Alvarez, Alvarez-Illera, Santo-Domingo, Fonteriz, & Montero, 2022). In contrast, *C. elegans* present a cost-effective and efficient alternative offering a practical solution for studying PFAS neurodevelopmental toxicity, with the added advantage of a well-characterized dopaminergic system, making it an ideal platform for examining the impact of PFAS on DA neuron function and overall neurodevelopment.

As experimental platforms continue to generate increasingly large and complex datasets, there is a rising need for real-time processing and high-performance computing to manage and

analyze the vast amounts of data being produced (H. Wang et al., 2023). This growing reliance on advanced computing technologies is essential for efficiently storing, processing, and interpreting data at the high speeds required by modern research. The growth of AI technology and its utilization is transforming how we analyze various data sets, greatly enhancing our ability to explore and understand intricate biological systems. High-throughput, high-content screening platforms often generate extensive datasets, including high-content imaging data (S. Lin et al., 2011). Despite technological advances, visual inspection of phenotypes and tissue features remains a fundamental method. Typically, this involves manually reviewing microscopic images to identify and differentiate between normal and abnormal phenotypic characteristics (N. Wang, Dong, Qiao, Yin, & Lin, 2024). However, manual investigation of image data sets comes with several limitations. As the volume of data increases, detecting subtle changes in morphology and performing detailed quantitative evaluations becomes progressively more difficult, even for seasoned professionals. Previous research has utilized AI-based image analysis to investigate neural network development in zebrafish (Shang, Long, Lin, & Cong, 2019). Leveraging AI for image analysis in *C. elegans* neural network studies can deliver a more detailed and extensive insights into the impacts of neurotoxins. Furthermore, these programs can lead to significant advancements in neurodevelopment and environmental toxicology research.

The combination of a nematode model with sophisticated high-throughput, high-content screening coupled with AI technology enhances the capability to study PFAS toxicity and its impact on neurodevelopment. These results are poised to advance our comprehension of PFAS-induced neurodevelopmental toxicity by addressing critical gaps in current knowledge. Specifically, this study examines the impacts of PFAS on the dopaminergic neuron morphological alterations, synaptic formation, and neuron activity. However, a limitation of this

study is the limited range of exposure levels assessed, which restricts a more comprehensive evaluation of the impacts on neuronal development. Understanding the mechanisms behind PFAS toxicity is crucial for refining risk assessments and safety evaluations related to these substances. Additionally, it is important to recognize that individuals are frequently exposed to multiple PFAS simultaneously from diverse sources, with the specific mixtures and concentrations varying significantly by geographical region. This variability underscores the complexity of PFAS exposure and the need for thorough investigation into its cumulative effects of several combinations. Although evaluating every possible PFAS combination is unfeasible, it is crucial to conduct research that reflects real-world exposure scenarios, specifically in high population areas, to better understand developmental neurotoxicity.

5.5 Conclusion

By employing a high-throughput, high-content screening approach integrated with AI, we assessed the effects of individual PFAS and a reference mixture on both neurodevelopment and the neuronal system. Our findings indicate that PFAS have a detrimental impact on neurodevelopment in *C. elegans*. This study found that PFAS compounds with higher toxicity in our assays were associated with more significant neuronal damage, although the relationship with mammalian toxicity remains to be explored. Additionally, exposure to PFAS mixtures appears to exacerbate neurodevelopmental issues. These results provide crucial insights into the potential harmful effects of PFAS exposure and underscore the need for comprehensive studies on developmental exposure.

5.6 Credit Author Statement

Seth Currie: Methodology, Investigation, Formal analysis, Writing- Original Draft, Writing-Reviewing and Editing. David Benson: Investigation. Zhong-Ru Xie: Validation. Jia-Sheng Wang: Writing-Reviewing and Validation, Writing. Lili Tang: Resources, Supervision, Funding acquisition, Writing-Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

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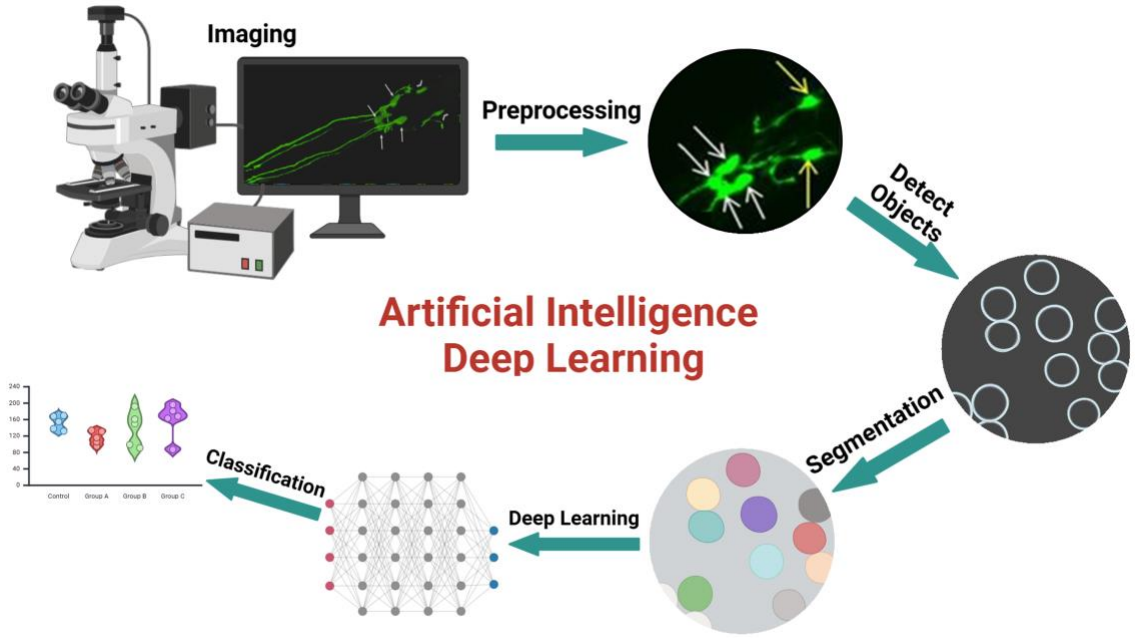


Figure 5.1: Deep learning image analysis workflow. Image analysis utilizes preprocessing, object detection, segmentation, and classification to determine key features and patterns within the data.

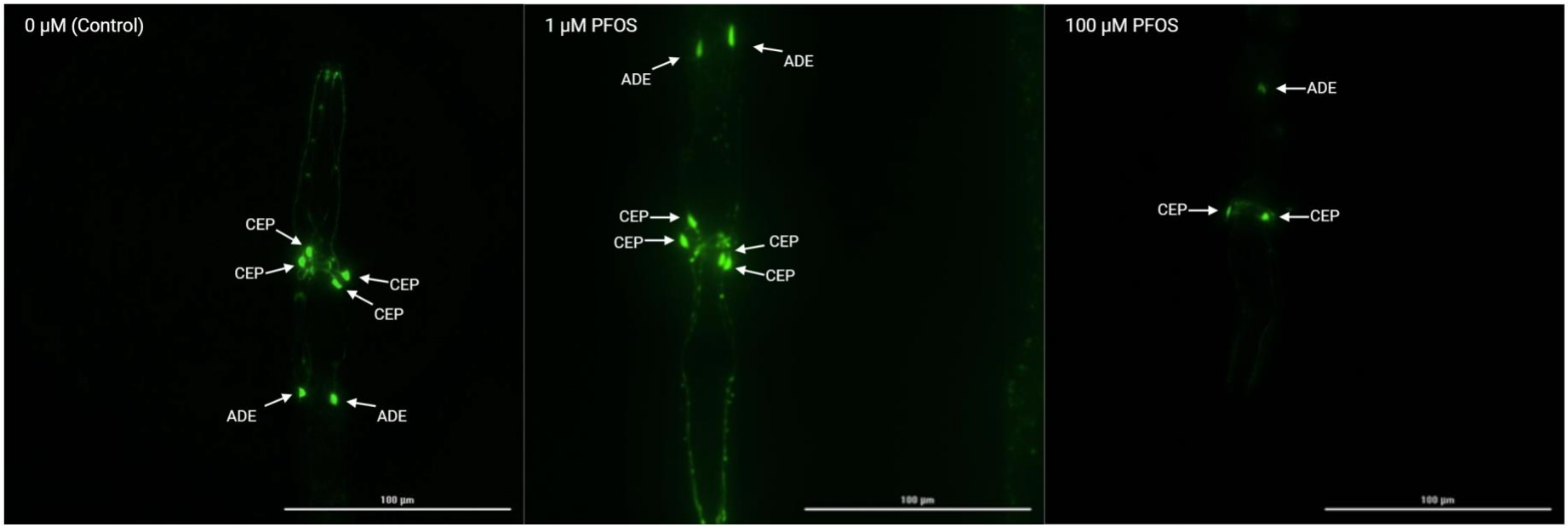
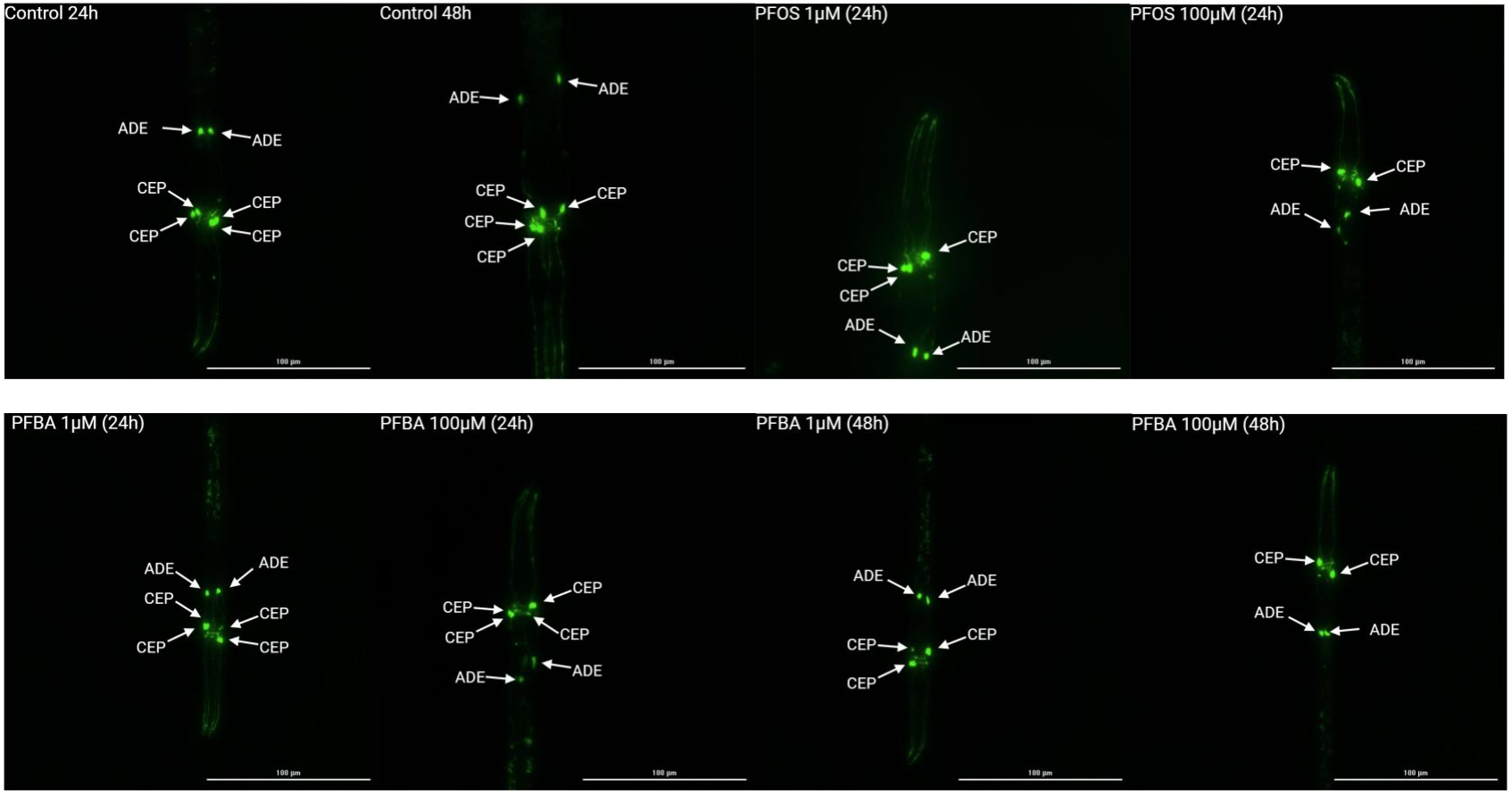
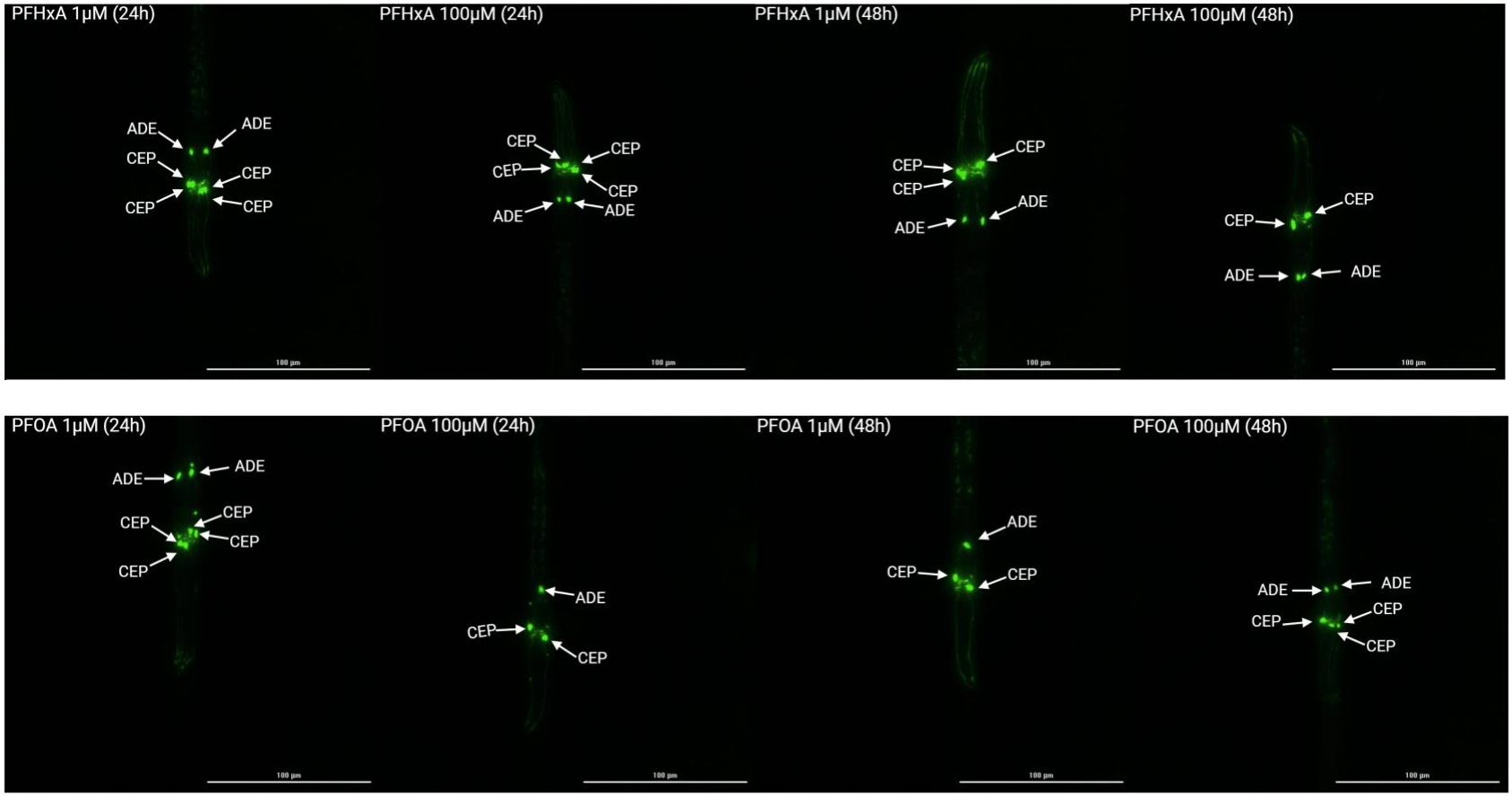


Figure 5.2: Impacts of PFOS on Dopaminergic Neurons on BZ555 (dat-1p::GFP) *C. elegans* after 48 hours of exposure utilizing the Cytation5 Imaging Multi-Mode Reader at 60x magnification. CEP: Cephalic Sensilla Neurons. ADE: Anterior Deirids Neurons.

Figure 5.S1: Impacts of PFAS on Dopaminergic Neurons on BZ555 (dat-1p::GFP) *C. elegans* after 48 hours of exposure utilizing the Cytation5 Imaging Multi-Mode Reader at 60x magnification. CEP: Cephalic Sensilla Neurons. ADE: Anterior Deirids Neurons.




PFHxA 1µM (24h)
ADE
ADE
CEP
CEP
CEP
CEP
PFHxA 100µM (24h)
CEP
CEP
CEP
CEP
ADE
ADE
PFHxA 1µM (48h)
CEP
CEP
CEP
ADE
ADE
PFHxA 100µM (48h)
CEP
CEP
ADE
ADE
PFOA 1µM (24h)
ADE
ADE
CEP
CEP
CEP
CEP
PFOA 100µM (24h)
ADE
CEP
CEP
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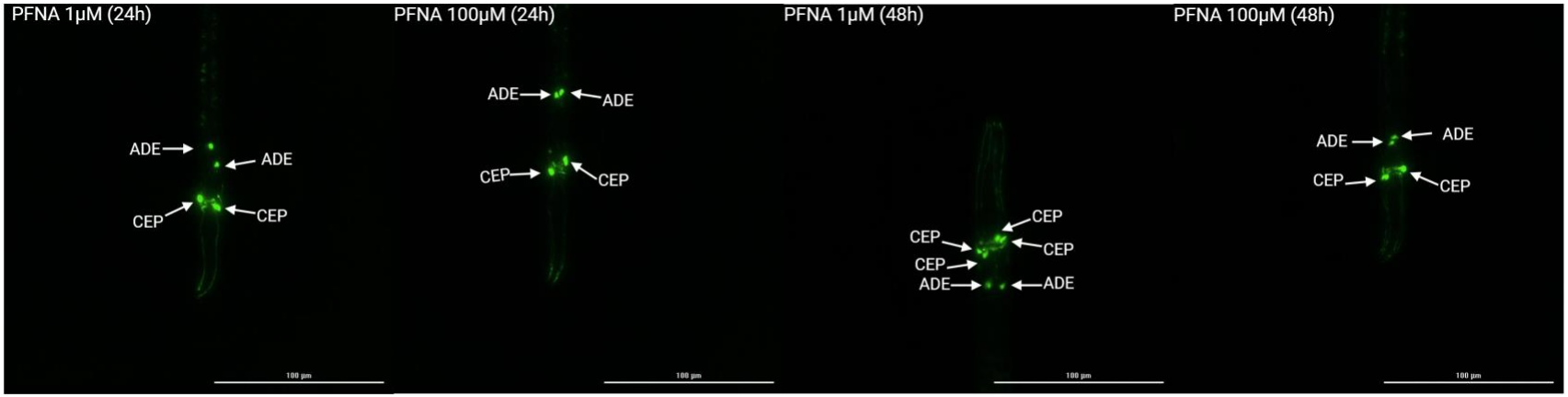
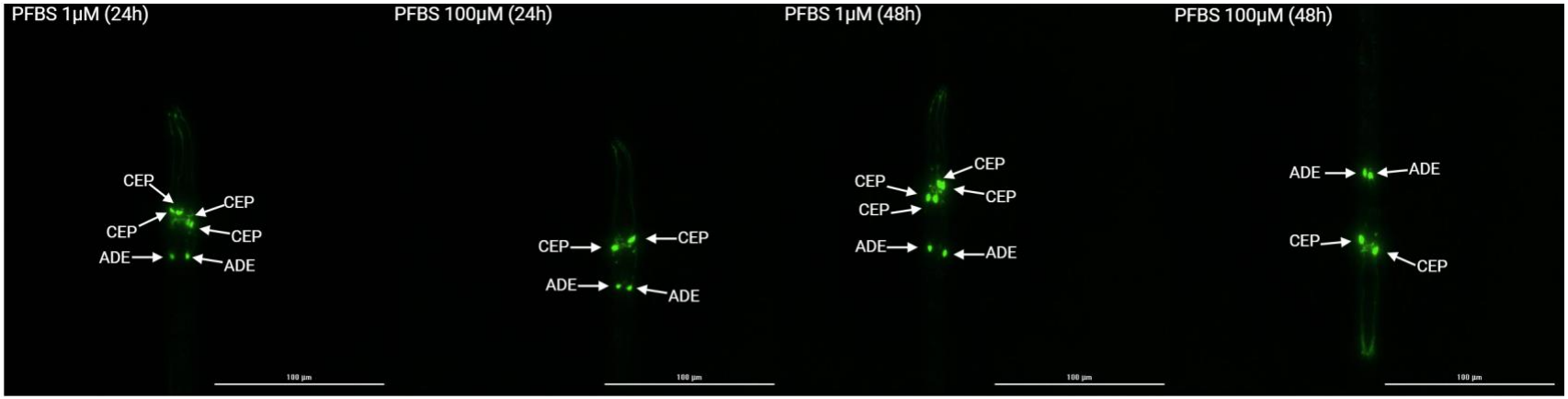
PFOA 100µM (48h)
ADE
ADE
CEP
CEP
CEP


PFNA 1μM (24h)
ADE
ADE
CEP
CEP
PFNA 100μM (24h)
ADE
ADE
CEP
CEP
PFNA 1μM (48h)
CEP
CEP
CEP
CEP
ADE
ADE
PFNA 100μM (48h)
ADE
ADE
CEP
CEP


PFBS 1μM (24h)
CEP
CEP
CEP
CEP
ADE
ADE
PFBS 100μM (24h)
CEP
CEP
ADE
ADE
PFBS 1μM (48h)
CEP
CEP
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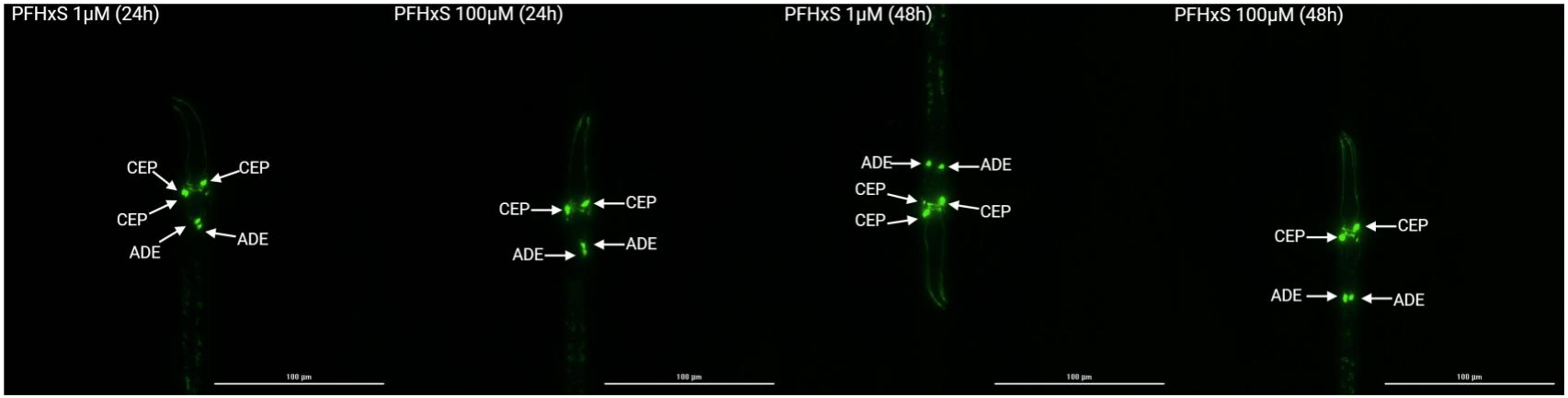
ADE
ADE
PFBS 100μM (48h)
ADE
ADE
CEP
CEP


PFHxS 1µM (24h)
CEP
CEP
CEP
ADE
ADE
PFHxS 100µM (24h)
CEP
CEP
ADE
ADE
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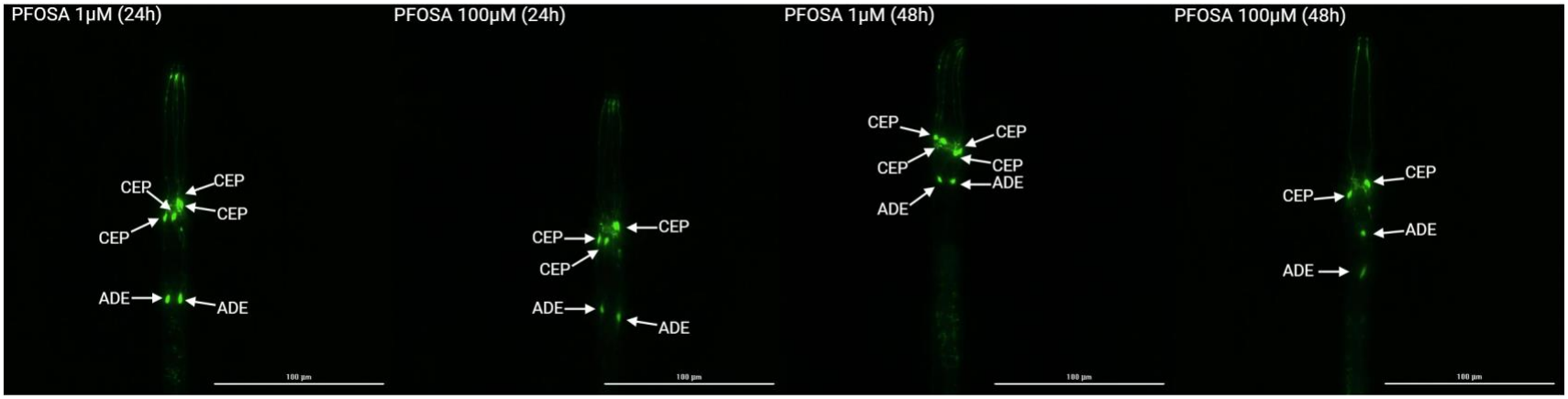
PFHxS 100µM (48h)
CEP
CEP
ADE
ADE


PFOSA 1µM (24h)
CEP
CEP
CEP
CEP
ADE
ADE
PFOSA 100µM (24h)
CEP
CEP
CEP
ADE
ADE
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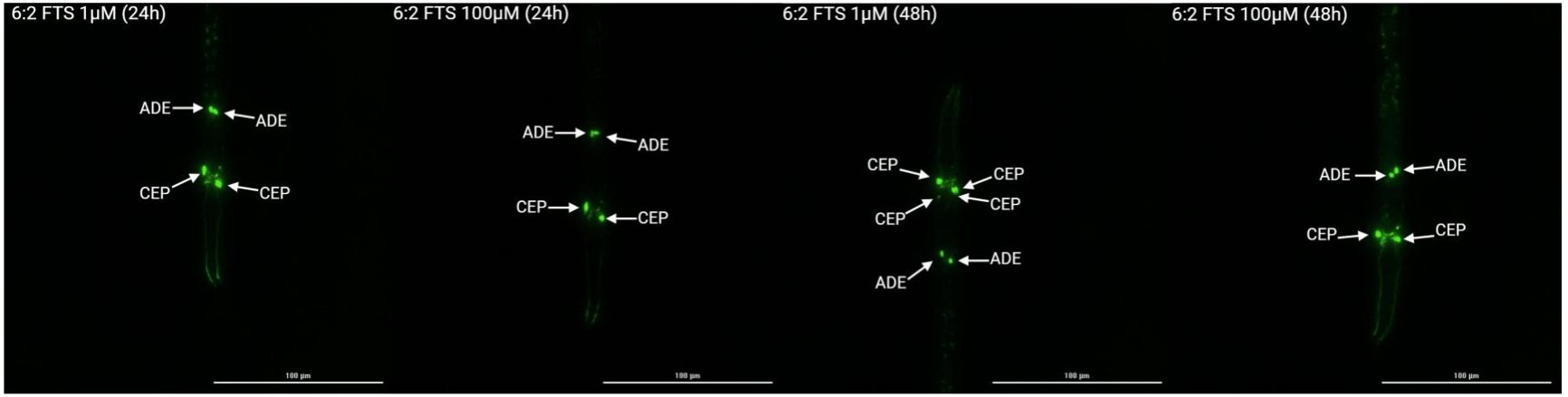
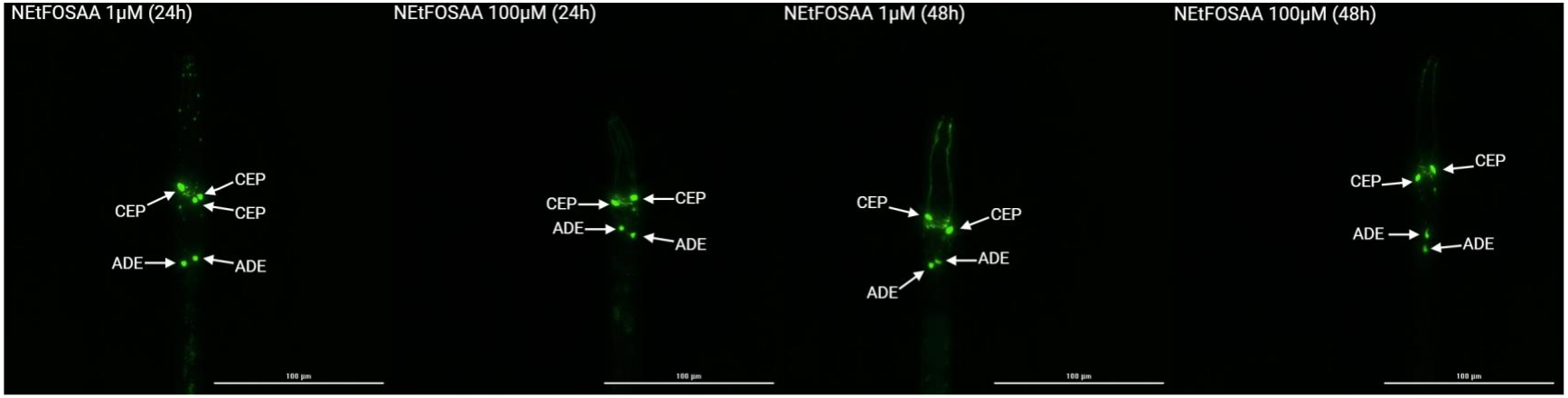
ADE
PFOSA 100µM (48h)
CEP
CEP
ADE
ADE


6:2 FTS 1µM (24h)
ADE
ADE
CEP
CEP
6:2 FTS 100µM (24h)
ADE
ADE
CEP
CEP
6:2 FTS 1µM (48h)
CEP
CEP
CEP
ADE
ADE
6:2 FTS 100µM (48h)
ADE
ADE
CEP
CEP


NEtFOSAA 1µM (24h)
CEP
CEP
CEP
ADE
ADE
NEtFOSAA 100µM (24h)
CEP
CEP
ADE
ADE
NEtFOSAA 1µM (48h)
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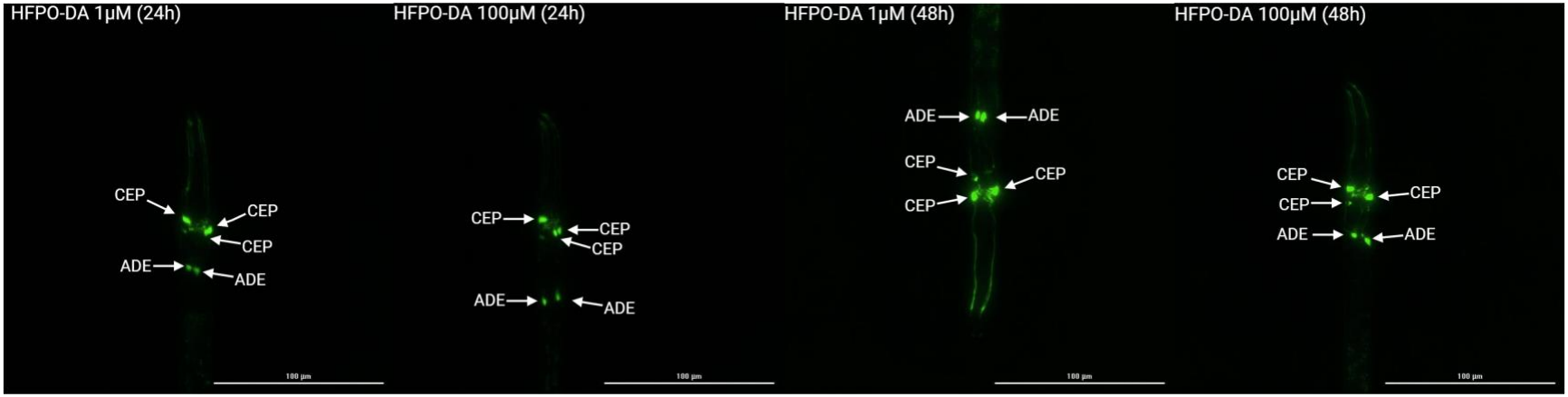
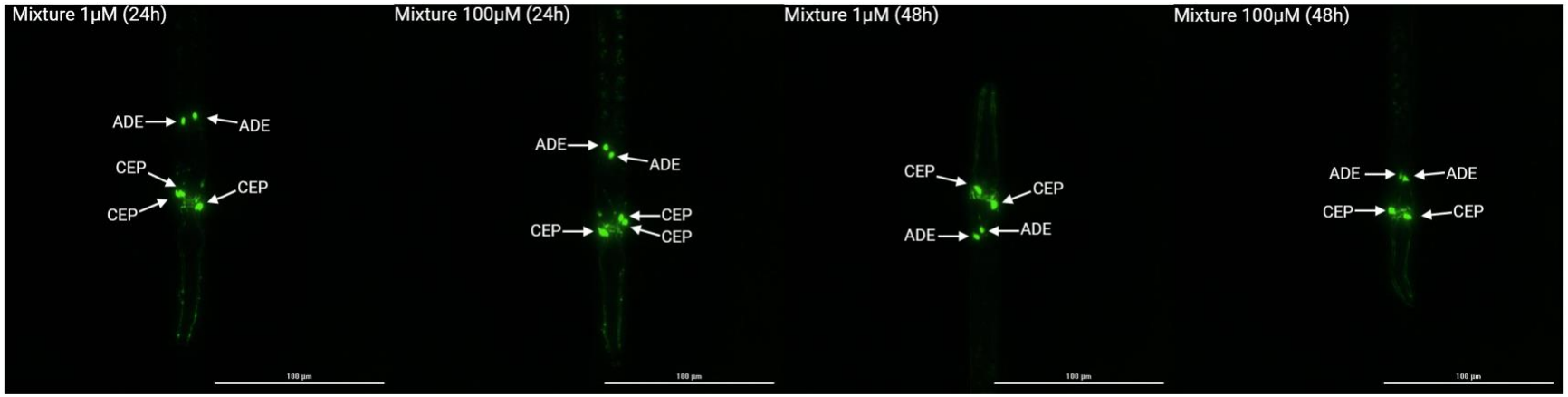
ADE
NEtFOSAA 100µM (48h)
CEP
CEP
ADE
ADE


HFPO-DA 1μM (24h)
HFPO-DA 100μM (24h)
HFPO-DA 1μM (48h)
HFPO-DA 100μM (48h)
CEP
ADE
100 μm


Mixture 1μM (24h)
Mixture 100μM (24h)
Mixture 1μM (48h)
Mixture 100μM (48h)
CEP
ADE
100 μm

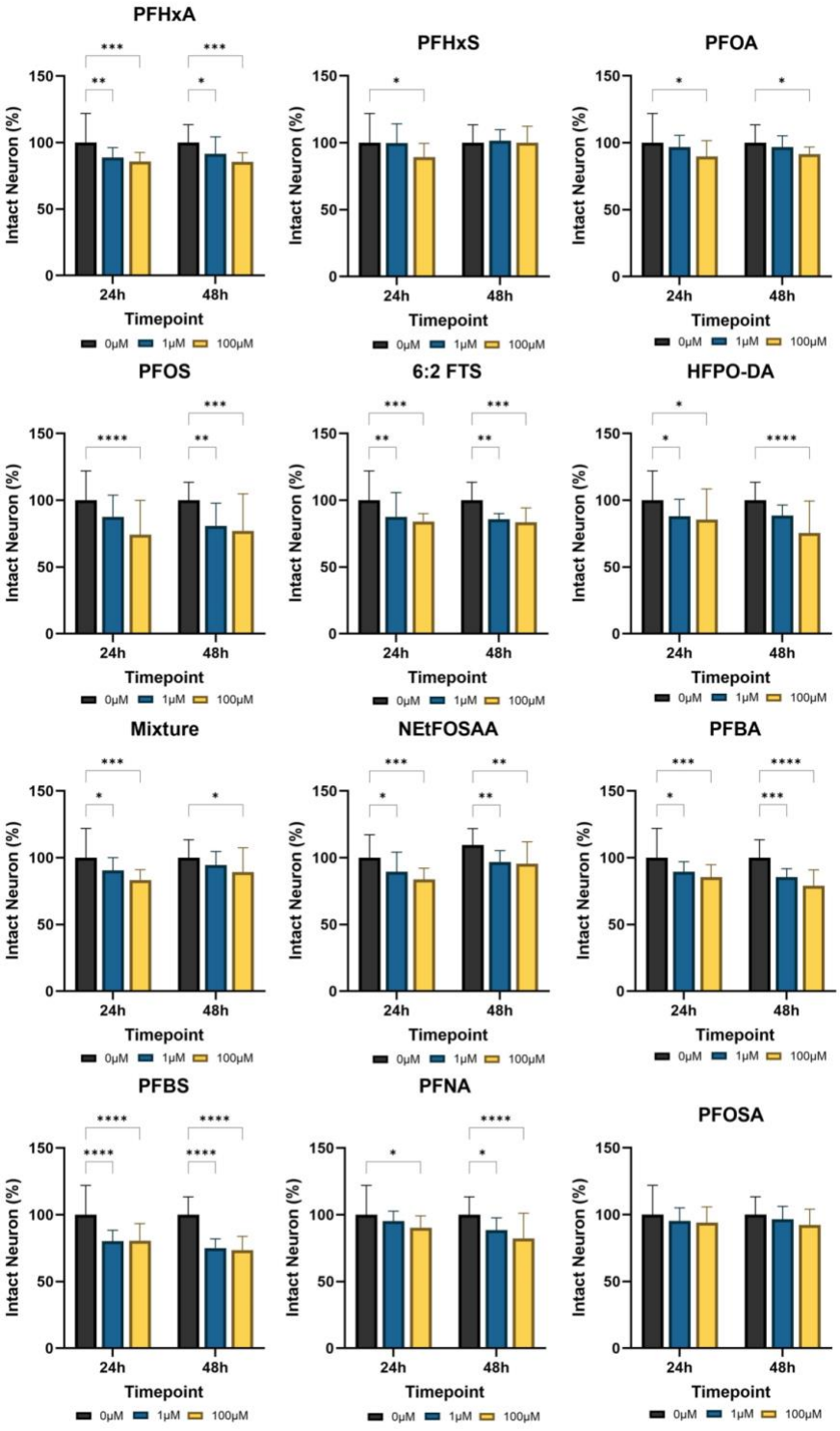


Figure 5.3: Effects of PFAS on Dopaminergic Neurons on BZ555 (dat-1p::GFP) *C. elegans* after exposure. All values are represented as Percent of Intact Neuron (n = 30, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$)

Table 5.1: The Benchmark Concentration 10% PFAS on synaptogenesis at different timepoints

PFAS	Timepoint	PROAST BEST Model	BMC (BMCL) [µM]
6:2 FTS	24h	Expon. m3-	32.28 (21.2)
	48h	Hill m5-	23.21 (13.3)
HFPO-DA	24h	Expon. m5-	4.963 (2.58)
	48h	Expon. m5-	0.6213 (0.259)
Mixture	24h	Expon. m3-	26.3 (17.4)
	48h	Hill m3-	5.17 (1.94)
NEtFOSAA	24h	Expon. m5-	0.6453 (0.146)
	48h	Expon. m5-	1.359 (0.107)
PFBA	24h	Expon. m5-	0.2181 (0.00654)
	48h	Hill m3-	0.3951 (0.0107)
PFBS	24h	Expon. m5-	52.39 (8.17)
	48h	Hill m5-	45.25 (9.33)
PFHxA	24h	Hill m3-	0.8323 (0.000163)
	48h	Expon. m3-	37 (21.2)
PFHxS	24h	Expon. m3-	111.4 (70.1)
	48h	Expon. m3-	34.93 (24.9)
PFNA	24h	Expon. m3-	99.77 (70.2)
	48h	Expon. m3-	40.12 (11.2)
PFOA	24h	Hill m3-	0.05857 (0.0000481)
	48h	Expon. m3-	35.8 (15.9)
PFOS	24h	Expon. m3-	20.37 (11.9)
	48h	Expon. m3-	17.56 (10.8)
PFOSA	24h	Expon. m3-	0.4424 (0.0215)
	48h	Expon. m5-	10.14 (4.81)

BMC: Benchmark Concentration; BMCL: Benchmark Concentration Lower-Confidence Limit

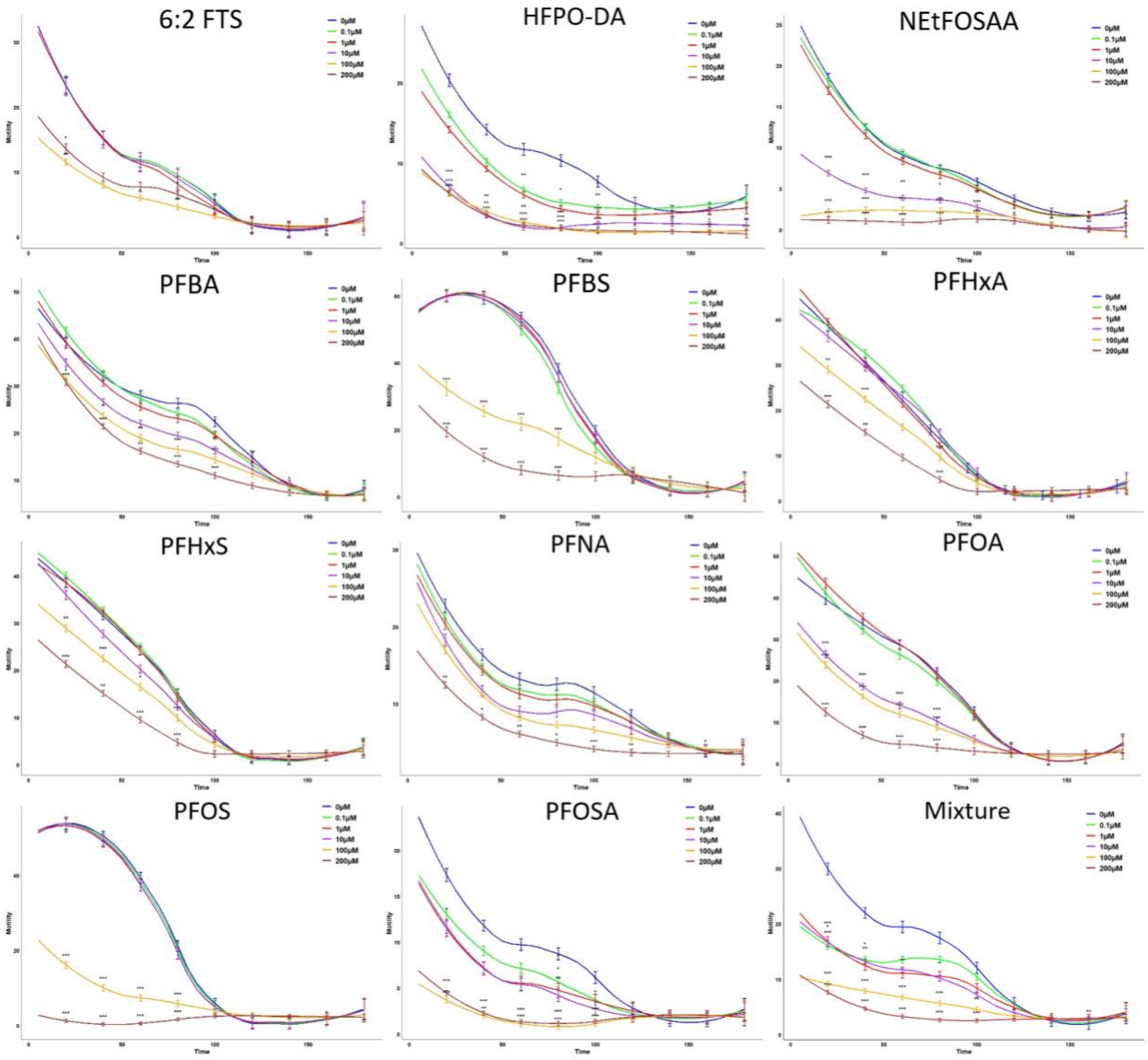


Figure 5.4: Impacts of PFAS on Synaptogenesis of *C. elegans* after 48 hours of exposure. All values are represented as motility (n = 30, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

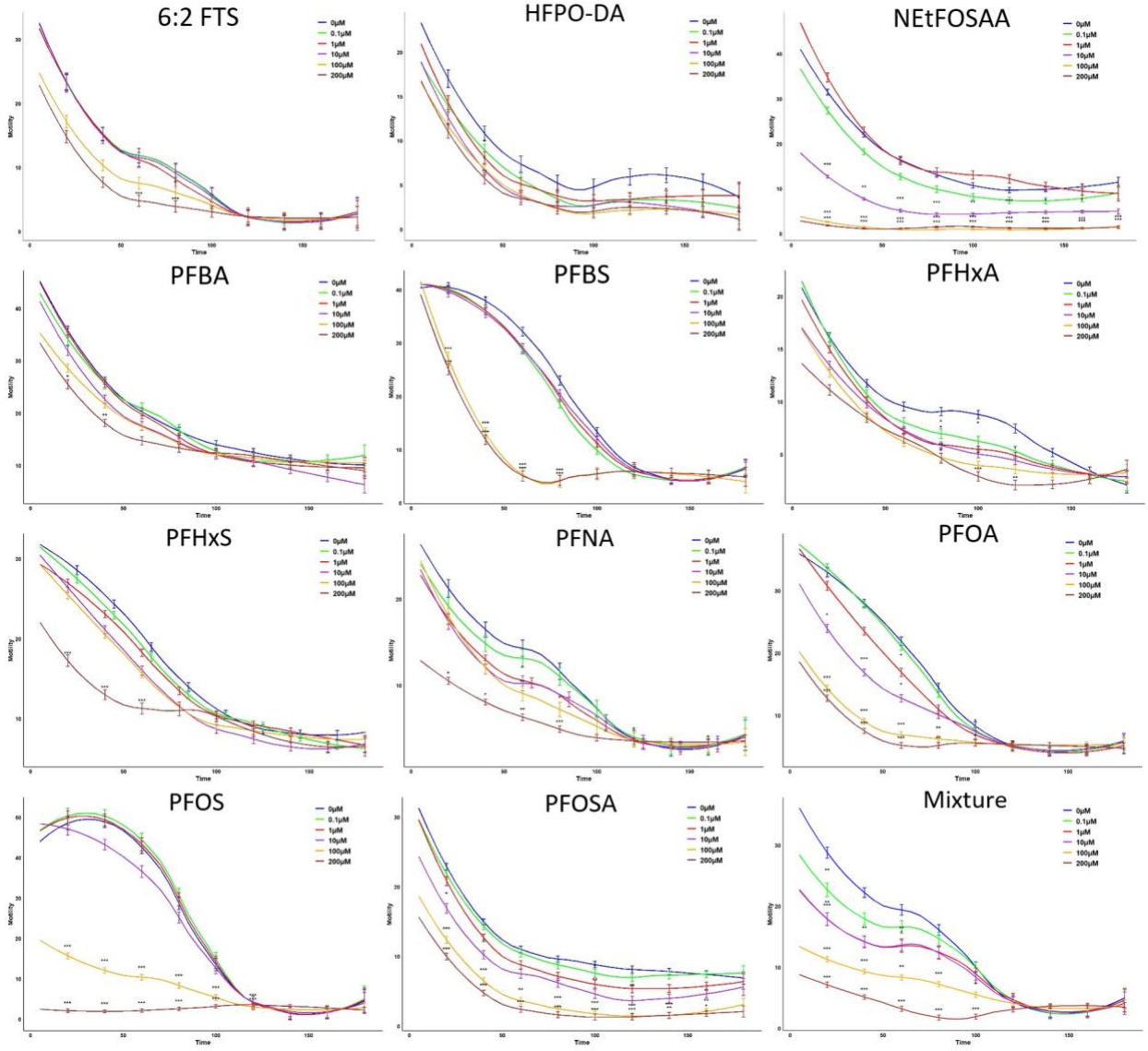


Figure 5.S2: Impacts of PFAS on Synaptogenesis of *C. elegans* after 24 hours of exposure. All values are represented as motility (n = 30, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Table 5.2: The Benchmark Concentration 10% PFAS on behavior (center point speed) at different time points.

PFAS	Timepoint	PROAST BEST Model	BMC (BMCL) [μM]
6:2 FTS	48h	Expon. m3-	11.51 (3.42)
HFPO-DA	24h	Expon. m3-	0.8936 (0.00804)
	48h	Expon. m5-	0.2197 (0.128)
Mixture	48h	Hill m3-	0.004287 (0.0000239)
NEtFOSAA	24h	Expon. m5-	50.59 (12.7)
	48h	Hill m5-	5.868 (0.668)
PFBA	24h	Hill m3-	122.7 (84.4)
	48h	Hill m5-	0.72 (0.1572)
PFBS	24h	Expon. m3-	41.63 (9.0)
	48h	Expon. m5-	8.031 (1.51)
PFHxA	24h	Expon. m3-	94.12 (59.5)
	48h	Expon. m5-	41.7 (7.57)
PFHxS	24h	Expon. m5-	0.07352 (0.0000646)
	48h	Expon. m3-	48.55 (14.1)
PFNA	24h	Hill m5-	32.04 (13.9)
	48h	Expon. m5-	28.79 (6.72)
PFOA	48h	Expon. m3-	82.48 (41.1)
PFOS	24h	Expon. m3-	29.4 (7.12)
	48h	Hill m5-	4.62 (1.21)
PFOSA	24h	Hill m3-	148.3 (103)
	48h	Expon. m3-	56.56 (8.14)

BMC: Benchmark Concentration; BMCL: Benchmark Concentration Lower-Confidence Limit

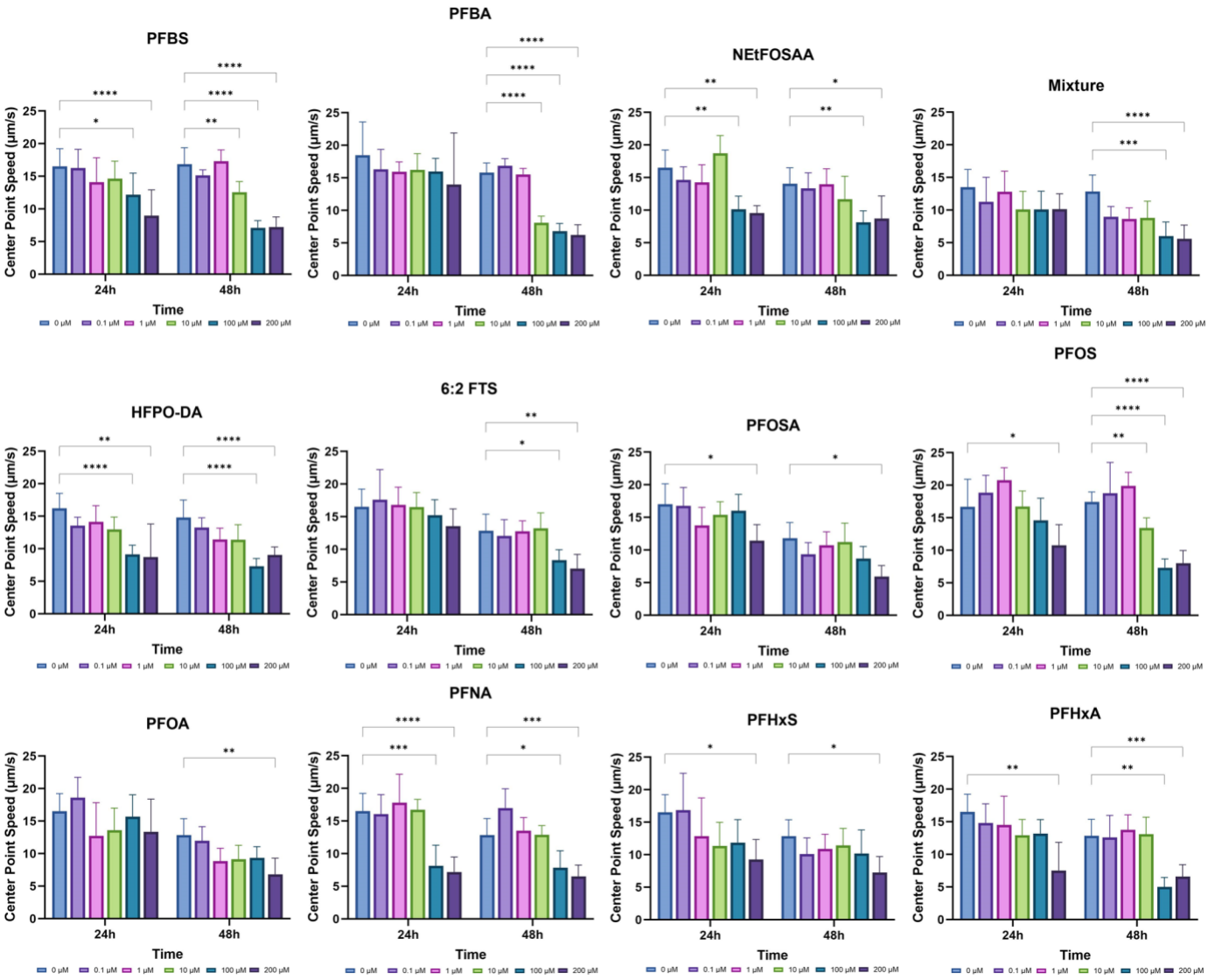


Figure 5.5: Effects of PFAS on behavior (Center Point Speed) on N2 (wild type) *C. elegans* after exposure. All values are represented as Center Point Speed (µm/s) (n = 30, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

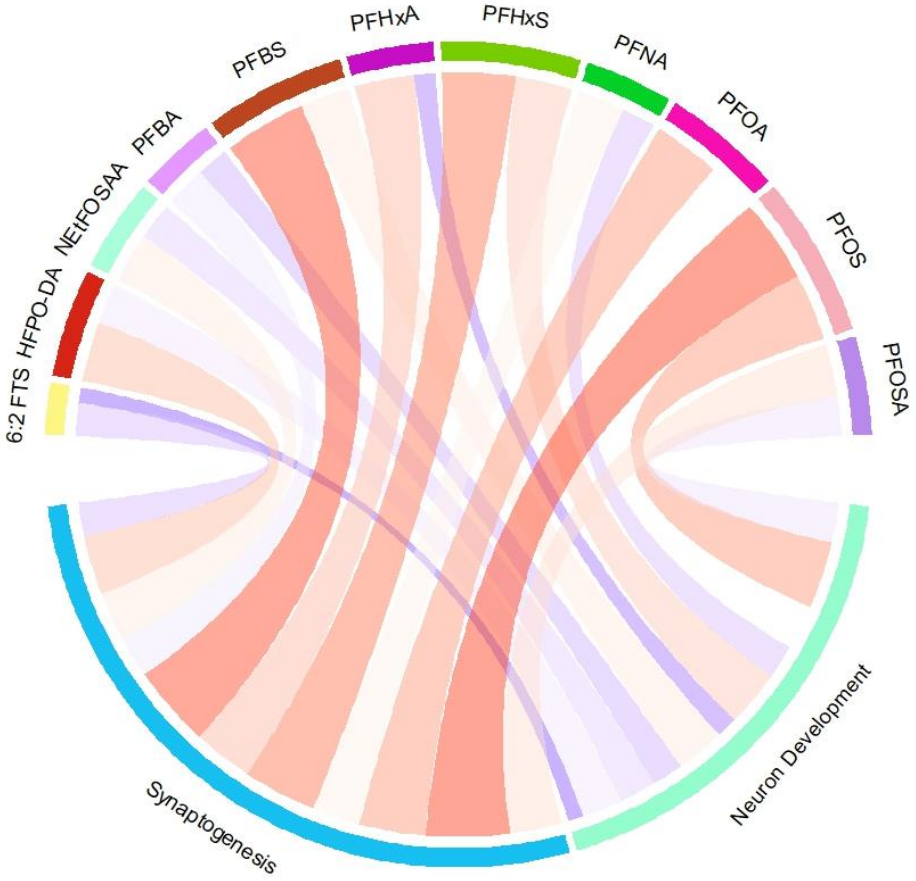


Figure 5.6: Correlation between toxicity and neurodevelopment: Blue indicates a positive and red indicates negative correlation. All values are represented as a Pearson Correlation Coefficient. The correlation circle was plotted using the “chord” package in R (version 3.3.4)

CHAPTER 6

THE IMPACT OF EARLY LIFE EXPOSURE TO INDIVIDUAL AND COMBINED PFAS ON LEARNING, MEMORY, AND BIOACCUMULATIN IN *C. ELEGANS*[5]

[5]Currie, S. D., Yuqing, J., Huang, Q., Wang, J. S., & Tang, L. (2023). *Environmental pollution 363*, 125257.
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Abstract:

Per- and Polyfluoroalkyl Substances (PFAS) are a group of water-soluble chemicals used for decades with important industrial and commercial applications. Due to their chemical and thermal stability, persistence in the environment, and widespread human exposure, PFAS become an important concern for public health. In this study, eleven highly prevalent PFAS and a reference mixture were selected according to various drinking water sources. The nematode, *Caenorhabditis elegans*, were exposed to PFAS at 0.1, 1, 10, 100, and 200µM, and the toxic effects on learning & memory along with the bioaccumulation were investigated using a high-throughput screening (HTS) platform. Our results showed that perfluorooctanesulfonic acid (PFOS) and perfluorobutanesulfonic acid (PFBS) exhibited significant inhibitory effects ($p<0.05$) on learning and memory in both time points at concentrations between 100-200 µmol/L. After 48 hours of exposure, every PFAS resulted in an inhibition of learning and memory with a concentration of 200 µmol/L. Furthermore, the PFOS and PFBS had the highest bioaccumulation levels after 48 hours of exposure. These findings provide valuable insight into the developmental adverse effects associated with exposure and the bioaccumulation of both individual and mixtures of PFAS.

6.1 Introduction

Per- and polyfluoroalkyl substances (PFAS) constitutes a class of water-soluble chemical compounds extensively employed over the past six decades due to their diverse range of applications (Domingo and Nadal 2019). Due to their chemical and thermal stability, PFAS are very useful for industrial purposes. Sometimes called 'forever chemicals,' they break down very slowly, if at all, in the environment (Cousins et al. 2016). The extensive use of PFAS has led to the circulation of thousands of different PFAS compounds in the global market, resulting in their widespread presence in the environment and pervasive human exposure (Zhang et al. 2022). Human exposure to PFASs occurs through ingestion of contaminated drinking water and seafood, inhalation of indoor air, and contact with other contaminated media (Trudel et al. 2008). Previous studies have demonstrated geographical variability in PFAS serum concentrations, with the U.S. exhibiting higher levels than other countries (Jian et al. 2018). With the addition of high serum levels, PFAS have shown to have a low elimination rate in the human body therefore potentially causing various adverse health effects (Olsen et al. 2007). Exposure to PFASs can affect a variety of organs and systems, resulting in numerous health consequences such as reduced antibody response to vaccinations, increased susceptibility to infections, reduced fertility in adult males, increased serum levels of cholesterol and liver enzymes (Rosato et al. 2022). Moreover, PFAS has been linked to impaired cognitive function and neurodevelopmental disorders (Grandjean and Landrigan 2006; Liu et al. 2024; Johansson, Fredriksson, and Eriksson 2008).

Neurodevelopmental toxicity induced by PFAS has been associated in several epidemiological studies conducted across the United States (Donauer et al. 2015; Stein, Savitz, and Bellinger 2014; Vuong et al. 2016; Braun et al. 2014). The Diagnostic and Statistical Manual

of Mental Disorders (DSM-5) defines neurodevelopmental disorders as a set of conditions that begin during the developmental period, inducing deficient that produce impairments of functioning (Morris-Rosendahl and Crocq 2020). Neurodevelopmental disorders linked to the exposure of PFAS can include intellectual disabilities (ID) (Lyall et al. 2018), autism spectrum disorder (ASD) (Shin et al. 2020; Skogheim et al. 2021), attention-deficit/hyperactivity disorder (ADHD) (Lien et al. 2016; Piekarski, Diaz, and McNerney 2020), and inhibition of learning and memory (Nannaware, Mayilswamy, and Kandasubramanian 2024; Anderko and Pennea 2020). The mechanism of action for neurodevelopmental toxicity is currently unknown, it is believed to be related to PFAS's ability to bioaccumulate in the human body. PFAS predominantly accumulate in protein-rich tissues like the liver and serum, but they have also been identified in human nervous tissues (Starnes et al. 2022). The bioaccumulation in nervous tissues, specifically the brain, have been strongly associated with neurodevelopmental disorders at varying levels (Cao and Ng 2021).

The buildup of PFAS in nervous tissue has been directly linked to neurodevelopmental toxicity, affecting various functions such as synaptic signaling (Liao et al. 2009), neurotransmitter levels (Austin et al. 2003), and neurotransmitter metabolism (Salgado et al. 2016). These mechanisms suggest that PFAS may induce lasting changes in neuronal connectivity and increase the risk of neurological dysfunction (Brown-Leung and Cannon 2022). A few studies have induced neurodevelopmental deficiencies using PFAS, reporting proportionality between accumulation and deficiencies (Foguth, Sepulveda, and Cannon 2020; Chang et al. 2009; Wang et al. 2015). The exposure and accumulation of PFAS during phases of development provide valuable insights into neurodevelopmental deficiencies.

Legacy PFOS and PFOA exposure and accumulation in humans and animals has been extensively investigated (Stylianou et al. 2019). However, PFOS and PFOA human exposure have been declining in western countries over the last decade due to regulatory interventions; scientists have identified more than 12,000 PFAS, which largely lack comprehensive toxicity data (Salvatore et al. 2022). Evaluating the extensive combinations of potential toxins presents a great challenge for traditional toxicology research in human health evaluation. Scientific organizations and government regulatory agencies are increasingly acknowledging that alternative methods could substitute animal testing, aiming to enhance human health (Van Norman 2019). A valuable method for assessing toxicity involves studying sentinel species-organisms that are naturally exposed to the chemicals of interest and can reveal signs of toxicity before they are detectable in humans (Foguth, Sepulveda, and Cannon 2020).

Caenorhabditis elegans (C. elegans) served as a valuable model for studying diverse biological phenomena, resulting in a wealth of genotypic and phenotypic data accessible to researchers (Marsh and May 2012). Maintaining *C. elegans* is cost-effective due to their tiny size (around 1 mm in length), their diet of affordable bacteria such as Escherichia coli, and their ability to be stored long-term at −80°C or in liquid nitrogen (Park, Jung, and Lee 2017). *C. elegans* is a highly relevant model organism for studying neurodevelopment toxicity, owing to its full sequenced genome and ease of genetic manipulation and mutant screening (Bouyanfif et al. 2019). Worms express counterparts to roughly 80% of human genes, with essential biological functions and numerous biochemical pathways being well conserved across higher organisms (Culetto and Sattelle 2000). Additionally, *C. elegans* provide data from a whole organism, showcasing fully operational and metabolically active digestive, reproductive, endocrine, sensory, and neuromuscular systems (Hunt 2017). Using this model, our lab has investigated the

general toxicity of PFAS and showed that the larval stages were the most vulnerable to PFAS exposure (Currie et al. 2023).

Current PFAS research primarily focuses on individual compound exposure. It is important to consider that humans, especially children, are exposed to multiple PFAS simultaneously and/or sequentially from various sources (Ojo, Peng, and Ng 2021). Considering the co-occurrence of numerous PFAS in environmental samples, it is essential to conduct studies that account for real life exposure of multiple PFAS when assessing neurodevelopmental toxicity. There is still limited knowledge about how PFAS mixtures interact and what their combined effects might entail (Wang et al. 2017). A few in vitro studies have investigated PFAS mixtures (Zhou et al. 2017; Wolf et al. 2014; Hu et al. 2014) and reported both additive and synergistic. exposure using zebrafish has demonstrated a diverse range of interactions, varying from additive and antagonistic to synergistic as the PFOS molar ratio increases (Ding et al. 2013). While there are infinite combinations of PFAS, utilization of reference mixtures can be optimal for determining the inhibitory effects of mixtures.

In this study, eleven PFAS were selected according to their occurrence in different water bodies (Wang et al. 2022), which represent a wide range of typical PFAS structures, including perfluoroalkyl carboxylic acids (perfluorobutanoic acid [PFBA], perfluorohexanoic acid [PFHxA], perfluorooctanoic acid [PFOA], perfluorononanoic acid [PFNA]), sulfonic acids (perfluorobutanesulfonic acid [PFBS], perfluorohexanesulphonic acid [PFHxS], Perfluorooctanesulfonic acid[PFOS]), sulfonamides and derivatives (perfluorooctanesulfonamide [PFOSA], [NetFOSAA]), fluorotelomers (6:2 fluorotelomer sulfonic acid [6:2 FTS]), and new substitutes (hexafluoropropylene oxide dimer acid [HFPO-DA], the acid form of GenX). The

neurodevelopmental toxic effects of PFAS were assessed through the determination and association of the bioconcentration factor (BCF) and impairment to learning and memory.

6.2 Materials and Methods

6.2.1 Chemicals

Based on data from 23 studies, the presence of PFAS in different water bodies was examined to identify the most abundant PFAS (Wang et al. 2022). By further categorizing the information, the most abundant PFAS in each classification was identified, along with some alternatives used as replacements for legacy PFAS. The 10 PFAS selected for this study include PFBA (95%, BCCF2984, COA), PFHxA (98%, P102-28684, COA), PFOA (95%, WXBD6815, COA), PFNA (97%, 394459, COA), PFBS (98%, P151-08994, COA), perfluorohexanesulfonic acid (PFHxS, 95%, 751400, COA), PFOS (98%, 830800, COA), 1H,1H, 2H, 2H-perfluorooctanesulfonamidoacetic acid (NEtFOSAA, 95%, P102-28720, COA), 6:2 fluorotelomer sulfonic acid (6:2 FTS, 95%, 754100, COA), PFOSA (96%, CDS010729, COA), and HFPO-DA (96%, 00022309, COA).

Analytical grade PFBA, PFNA, and PFOA were purchased from Sigma Aldrich (St. Louis, MO). Analytical grade NEtFOSAA, PFHxA, and PFBS were purchased from Astatech Inc. (Bristol, PA). Analytical grade PFOS, PFHxS, HFPO-DA, 6:2 FTS, and PFOSA were purchased from Synquest Laboratories, Inc. (Alachua, FL). Stock solutions (1M) were prepared in dimethyl sulfoxide (DMSO), and working solutions were diluted with K-medium (32mM KCl and 51mM NaCl), supplemented with OP50 as a 1mg/ml food source, resulting in a final DMSO concentration of 0.1%. (Brenner 1974).

6.2.2 Mixture Selection

We identified five PFAS that accounted for a majority of the PFAS load within United States water source (Smalling et al. 2023). Using these five chemicals, a reference mixture was constructed using the relative concentration ratios found in the full mixture. We approximated the ratios as PFOS (30%), PFBA (20%), PFOA (20%), PFHxS (15%), and PFBS (15%). This mixture will be used to represent the typical daily exposure in the United States. The final concentration of the mixture will vary with the proportions remaining constant. Analyzing mixture profile patterns offers a way to assess certain PFAS compounds that are often detected at higher concentrations compared to others (East, Anderson, and Salice 2021).

6.2.3 C. elegans Culture and Exposure

Wild-type N2 nematodes and Escherichia coli strain OP50 were obtained from the Caenorhabditis Genetics Center (Minneapolis, MN, USA). The *C. elegans* were grown at 25°C on nematode growth medium (NGM) plates supplemented with OP50 as their food source. Synchronized L1 larvae were obtained by alkaline lysis (10M NaOH, 2% sodium hypochlorite), then hatched in k-medium overnight (Brenner 1974). The exposure concentrations were 200, 100, 10, 1, 0.1 μmol/L for learning & memory and the equivalence of 10, 5, and 0 ppm for the bioaccumulation represented in μmol/L. The exposure concentrations were selected based on our previous research (Currie et al. 2023) and based on the U.S EPA's ToxCast program's recommended concentrations for in vivo assays (Rowan-Carroll et al. 2021). Although these concentrations exceed those typically found in environmental settings, using higher levels is crucial for effectively characterizing the toxicological effects of PFAS compounds and understanding the underlying mechanisms of action. Recognizing that *C. elegans* is an *in vivo*

model and there are interspecies variations, we chose 200 µmol/L as the maximum concentration to reliably observe the effects and responses elicited.

6.2.4 High-Throughput Screening Platform for Learning and Behavior

As previously described high-throughput screening assays using WormLab system (Version 2023.1.1, MBF Bioscience, Vermont, USA) and COPAS BIOSORT (Union Biometrica, Inc., Massachusetts, USA) (Currie et al. 2023; Polli et al. 2015; Tang et al. 2019). Nematode worms were sorted and dispensed into designated wells (96-well plates) using COPAS BIOSORT containing k-medium, bacteria food source, and varying concentration of individual PFAS. Each experiment was performed in triplicates. The plates were incubated for 24h and 48h periods with shaking at 25°C. Worms were transferred to NGM plates and normalized for 1 hour prior to recording on the WormLab system. Plates were recorded for 60 seconds with a minimum track duration of 30 seconds with a resolution of 1280 x 960 pixels taken at 7.5 frames/s using a digital camera (Nikon DSLR, Tokyo, JPN). Data was read, processed, and plotted using GraphPad Prism.

6.2.5 Learning

Fifty age-synchronized L1-stage worms were allocated into individual wells containing 100 µl of testing solution. The plates were incubated at 25°C for a period of 24 and 48 hours. After the desired incubation time, the worms were measure on the WormLab system for learning and memory endpoints simultaneously. The ability for the worms to learn was measured using the frequency of pirouette previously stated (Pierce-Shimomura, Morse, and Lockery 1999; Huang, Cosman, and Schafer 2006; Roussel et al. 2014). *C. elegans* demonstrate an impressive

ability to learn and recall environmental cues that signal the presence of good food, bad food, no food, or adverse stimuli. This enables the worms to navigate toward more favorable environments through chemotaxis, thermotaxis, or aerotaxis (Ardiel and Rankin 2010). Data was recorded, processed, and plotted using GraphPad Prism.

6.2.6 Memory

Fifty age-synchronized L1-stage worms were allocated into individual wells containing 100 µl of testing solution. The plates were incubated at 25°C for a period of 24 and 48 hours. After the desired incubation time, the worms were measure on the WormLab system for learning and memory endpoints simultaneously. Memory was assessed based on the absolute peristatic speed, disregarding the direction of movement (Angstman, Frank, and Schmitz 2016). Exploring memory mechanisms in *C. elegans* contributes to our understanding of fundamental memory processes across the animal kingdom, bridging insights that apply to more complex organisms, including humans (Wong and Rankin 2019). Data was recorded, processed, and plotted using GraphPad Prism.

6.2.7 Calculation of the Benchmark Dose

The benchmark concentration at 10% (BMC 10%) and its lower confidence limit (BMCL) were computed using PROASTweb software (version70.1; https://proastweb.rivm.nl/). The software takes the assay data, which includes PFAS concentration levels and their corresponding response effects, and utilizes the maximum likelihood estimation (MLE) to fit the data to a selected model based on the lowest Akaike Information Criterion (AIC). The AIC is a statistical measurement for determining the best model. The benchmark concentration (BMC)

was calculated using a 95% confidence interval, a p-value cut off of 0.05, and the lowest AIC. The BMC and BMCL were produced for each learning and memory endpoint which marks the beginning of the predetermined change in the response rate. The finalized values can be used to rank the PFAS in each assay based the desired endpoint.

6.2.8 Estimation of Internal Dose of PFAS

For estimation of internal levels of PFAS, approximately 10,000 worms were treated with 0, 5, or 10 ppm of each individual PFAS, standardized across all chemicals for consistency. However, the concentrations are presented in µM, adjusted based on the molecular weight of each compound. The supernatant was removed and wet weight for each pellet was calculated as previously detailed (Sammi et al. 2019). The samples were normalized using a mass-labelled PFAS extraction standards solution (MPFAC-HIF-ES, 99%, MPFACHFES1023, COA). Samples were stored at -80°C until use.

6.2.9 PFAS Analysis

The PFAS levels were quantified using Ultra-High-Performance Liquid Chromatography (UHPLC) coupled with triple-stage quadrupole mass spectrometer equipped with an eclipse plus C18 column (4.6 mm internal diameter, 3.5 mm particle size, and 100mm length). The column over was kept at 40°C and the flow rate was 0.3 mL/min. A 16-minute gradient run was operated with a mobile phase A: 5 mM ammonium acetate in HPLC water; mobile phase B: 5 mM ammonium acetate in methanol/acetonitrile mixture (80:20, v:v mixture) (Tang et al. 2023; Wang, Munir, and Huang 2023). Final PFAS values were calculated using the internal standard,

followed with the calculation of the bioconcentration factor. Data was calculated, processed, and plotted using GraphPad Prism.

6.2.10 Calculation of Benchmark Concentration Factor (BCF)

Using the wet weight calculated during the extraction process and the quantification values for each PFAS, the bioconcentration factor (BCF) was calculated. As previously outlined, the ratio of exposure levels in the tissues (wet weight) to those in the medium represents the bioconcentration factor for the exposed worms (Arnot and Gobas 2006). The formula for BCF is as follows:

$$BCF = \frac{C_{Organism}}{C_{Environment}} \qquad (1)$$

Where bioconcentration factor (BCF) indicates the degree to which a substance can accumulate in an organism, $C_{Organism}$ represents the concentration of the chemical in the organism and the $C_{Environment}$ represents the concentration of the chemical in the surrounding environment. The bioconcentration factor is a critical parameter in determining the ability for the PFAS to accumulate with the living tissue compared to the surrounding environment. These values can be ranked to determine which individual PFAS has a tendency to bioaccumulate within the C. *elegans*.

6.2.11 Correlation Analysis Between Toxicity and Bioaccumulation

The correlation between toxicity and bioaccumulation was thoroughly investigated to illuminate the relationship between PFAS's bioaccumulation potential and its toxicity on learning and memory. Pearson correlation analysis was utilized to quantify the relationship between bioaccumulation and toxicity in *C. elegans* ($p<0.05$). The resulting correlations were

visualized using a heatmap, which identifies patterns within potential relationships between bioaccumulation and toxicity. Correlations between bioaccumulation and toxicity provides valuable insight into the potential mechanisms between PFAS and learning and memory. The R package “mice” was employed to calculate the correlation coefficient.

6.2.12 Statistical Analysis

Data was processed with the statistical program GraphPad Prism, version 10.1.2 (La Jolla, CA) or statistical program R, version 3.3.4 and were expressed as means ± standard deviation (SD). The statistical significance relative to the control was determined using analysis of variance (ANOVA) followed by Tukey’s post-hoc test. The level of statistical significance relative to the control was determined using a one-way analysis of variance (ANOVA) with an unpaired t-test test for the gene expression as a statistical method. The significance is represented in the graphs by asterisks (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$).

6.3 Results

6.3.1 Toxic Effect of PFAS on Memory in Nematodes

Absolute peristatic speed, which was used as indicators of memory, was measured by exposing age-synchronized L1 worms to 200, 100, 10, 1, and 0.1 μmol/L, then recording the movement of worms at 24 and 48 hours. WormLab system initially analyzes the speed of the worms in both the forward and reverse direction (Figure 6.1). Furthermore, the system calculates the absolute peristatic speed without taking direction into account. As shown in Figure 6.2, the absolute peristatic speed was significantly decreased with a concentration and time dependent manner ($p<0.05$). After 48 hours of exposure, all PFAS resulted in a significant reduction in

absolute peristatic speed ($p<0.05$). Furthermore, NEtFOSAA, PFBS, PFHxA, PFHxS, PFNA, PFOS, and PFOSA had significant inhibition as early as 24 hours. However, PFOA only experienced a significant inhibition in absolute peristatic speed after 48 hours with an exposure concentration of 200 μmol/L having a BMC value of: 104.026μM (BMCL: 100.31) (Table 6.1). According to the BMC (Table 7.1), the toxic rank after 48 hours of exposure is as follows: PFHxS > HFPO-DA > PFBA > PFBS > NEtFOSAA > PFOS > PFNA > PFHxA > PFOSA > 6:2 FTS > PFOA.

6.3.2 Toxic Effect of PFAS on Learning in Nematodes

For investigating the toxic effects of PFAS on learning, age-synchronized L1 worms were exposed to varying concentrations of 200, 100, 10, 1, and 0.1 μmol/L. The number of pirouettes, which was used as an indicator of learning, was calculated by recording the movement of the worms after 24 and 48 hours of exposure. After 48 hours, every PFAS displayed significant inhibition of learning ability at the highest concentration ($p<0.01$). As shown in figure 6.3, PFOS and PFBS were the most toxic for substances, causing significant learning impairment at both time points ($p<0.05$). PFOA showed the least amount of toxicity with a BMC of 108.5μM (BMCL: 70.5μM) (Table 6.2). After 24 hours of exposure, PFBA, PFHxA, and PFOA were the only ones that did not has a significant impairment in learning. According to the BMC, the toxic rank after 48 hours of exposure was as follows: HFPO-DA > PFBA > PFBS > PFOS > PFNA > 6:2 FTS > PFHxA > PFHxS > NEtFOSAA > PFOSA > PFOA.

6.3.3 Accumulation of PFAS in Nematodes

To determine the bioconcentration factor of PFAS, ten thousand age-synchronized L1 worms exposed to the equivalence 0, 5, and 10 parts per million (ppm), standardized across all PFAS and expressed in µM based on each compound's molecular weight, for 48 hours. The worms were then collected and processed through a UHPLC-MS to determine the bioaccumulation of PFAS. The final internal levels were normalized with the wet weight of the exposed worms (Figure 6.4). As shown in figure 6.4, the bioaccumulation was significant in every exposure ($p<0.05$), except for NEtFOSAA at 8.544 µM. As expected, PFBS and PFOS had the highest internal levels at the lowest concentrations, which correlates with their high toxicity levels, 1822 and 97.77 µM, respectively (Table 6.3). Taking the internal levels, the bioconcentration factor (BCF) can be calculated for determining the likelihood of bioaccumulation within *C. elegans*. As shown in table 6.4, PFBS has the highest BCF which could be attributed to its relatively small size. Furthermore, as seen in figure 6.5, 6:2 FTS and PFBS showed a significant decrease in BCF at the higher exposure compared to the lower exposure concentration. This represents that the *C. elegans* reached a maximum intake value which is the reason that the BCF decreased. Knowing and understanding the BCF is critical in determining the risk of PFAS intake within the environment for *C. elegans*. According to table 6.4, the BCF rank at the equivalence of 10 ppm is as follows: PFBS > PFOS > PFOSA > PFNA > PFHxS > PFOA > NEtFOSAA > 6:2 FTS > HFPO-DA > PFBA > PFHxA.

6.3.4 PFAS Mixture Effect

For investigating the toxic effects of PFAS mixtures on learning and memory, age-synchronized L1 worms were exposed to varying concentrations of 200, 100, 10, 1, and 0.1

μmol/L. The absolute peristatic speed (memory) and number of pirouettes (learning) were calculated by recording the movement of the worms after 24 and 48 hours of exposure. After 48 hours, the mixture significant impact on memory in exposure concentrations greater than 10 μM ((p<0.001) (Figure 6.6A). Furthermore, the mixture had an inhibitory effect on learning as early as 24 hours (p<0.05) (Figure 6.6B). The mixture appears to have an additive inhibitory effect on learning and memory because the overall inhibitory effect observed is consistent with the sum of the individual effects of each PFAS component, Specifically, while the mixture does not show a greater inhibition compared to PFOS and PFBS, individually, the combined inhibitory effect aligns with the expected outcome if each PFAS's effects is added together. This indicates that the PFAS mixture's inhibition is the result of the individual PFAS components' without exceeding the level of inhibition observed from individual exposures.

To further understand the impact of PFAS mixtures on *C. elegans* ten thousand age-synchronized L1 worms exposed to the equivalence of 0, 5, and 10 parts per million (ppm) for 48 hours. The worms were then collected and processed through a UHPLC-MS to determine the bioaccumulation of PFAS mixtures. The final internal levels were normalized with the wet weight of the exposed worms. Every PFAS in the mixture, except for PFBA, showed a significant increase (p<0.0001) in internal levels as the exposure concentration increased (Figure 6.6C). However, the BCF for PFOS decreases in the mixture at a higher concentration which indicates that there could be an inhibitory effect on the PFOS intake in the presence of additional PFAS (Figure 6.6D). The BCF for each PFAS is decreased in the mixture compared to the individual PFAS. PFBS had a 5-fold decrease which was the biggest decrease amongst the different PFAS, followed by PFOS having a 2-fold decrease at the highest exposure level. This could indicate an antagonistic effect in uptake between PFAS at different exposure levels.

6.3.5 Correlation Between Toxicity and Bioaccumulation

To predict the correlation between bioaccumulation and toxicity, a Pearson Correlation Coefficient was calculated using the "mice" package in R software. The bioconcentration factor and the impact on learning and memory after 48 hours of exposure were used to calculate the correlation coefficient. These results are illustrated in Fig. 6.7 and show conflicting results depending on the individual PFAS. The highest negative correlation between bioaccumulation and PFAS was seen in PFBS and PFOS, while PFBA showed no correlation. However, 6:2 FTS, PFHxS, and PFOA had slight positive correlation indicating a potential protective effect. Finally, HFPO-DA and PFHxA showed a negative association with memory, but a positive association with learning.

6.4 Discussion

In this study, individual PFASs and a reference mixture were selected, and the toxic effects on learning and memory were assessed along with determining the bioaccumulation in *C. elegans* using a high-throughput screening platform. Our results showed that PFAS exhibited a significant inhibition on the learning and memory of *C. elegans* within a concentration range of 100-200µM after 48 hours of exposure in the developmental life stages. The most toxic PFAS on memory were PFOS and PFBS with a BMC of 17.36 and 3.972, respectively. For learning, all PFAS showed significant inhibition ($P<0.05$) after 48 hours. The bioaccumulation was determined to significantly increase in the presence of each PFAS ($p<0.05$). Furthermore, the bioconcentration factor was calculated for each exposure. The BCF after 48 hours and exposure at the equivalence of 10 ppm was as follows: PFBS > PFOS > PFOSA > PFNA > PFHxS >

PFOA > NEtFOSAA > 6:2 FTS > HFPO-DA > PFBA > PFHxA. Finally, an increase in BCF strongly correlates with an impact in both learning and memory.

Epidemiologist and toxicologist around the world have labeled PFAS as “forever chemicals” precisely because of their ability to persist over time in the hydrosphere (Peritore et al. 2023). Exposure to PFAS may result in numerous adverse health effects (Pelch et al. 2022). Through critical reviews of PFAS exposure and adverse health effects are centered around legacy compounds, PFOS and PFOA (Jane et al. 2022). However, a significant amount of toxicological data currently unavailable for a majority of PFAS outside of the legacy PFAS (Perez et al. 2023). In the present study, the eleven most abundant PFAS were selected in each major category in water systems in the United States (Wang et al. 2022). Additionally, a reference mixture was created using the top five most abundant PFAS found in drinking water sources (Smalling et al. 2023). The PFAS were investigated in a liquid form present in k-medium with a bacteria food source. The concentrations selected allowed for the investigation of learning and memory at both an environmentally relevant value (0.1µM) and a value significantly higher (200µM), along with values. The concentrations selected for determining bioaccumulation were at the equivalence of 5 and 10 ppm for each PFAS allowing for detection above the machinery’s limit of detection. The concentration selected for this study were based on the US. EPA’s ToxCast Program (Rowan-Carroll et al. 2021), which used top concentrations of 100µM for in vitro studies.

Higher exposure levels were selected in this study due to the *C. elegans* being an in vivo model with known species variations to PFAS exposure on toxicokinetics and toxicodynamics (Angstman, Frank, and Schmitz 2016). Using higher concentrations is essential not only for detecting clear and measurable effects but also for understanding the full spectrum of

toxicological responses. This approach facilitates the identification of critical thresholds and mechanisms that may not be apparent at lower, environmentally relevant concentrations. Observing toxic effects at these elevated levels, while not directly applicable to typical environmental exposures, underscores the potential risks associated with PFAS. Currently, regulatory levels for PFAS, such as the U.S. EPA's guidelines, recommend a maximum contaminant level of 0.00016 µM for PFOA and 0.000139 µM for PFOS in drinking water, highlighting how these levels are significantly lower than those used in our study (Cordner et al. 2019). However, some states allow for maximum levels up to 2.415 µM (Garnick et al. 2021). In contrast, typical environmental levels of PFAS can range from about 0.0001 to 0.1 µM, depending on the location and source of contamination, indicating how real-world values exceed regulatory values. Importantly, the benchmark concentration (BMC10), representing a 10% reduction in learning and memory, calculated for each endpoint in our study, falls within both regulatory and environmentally relevant concentrations. This demonstrates that the adverse effects observed in our study have relevance to real-world exposure scenarios. Additionally, by employing a range of exposure levels, including significantly elevated concentrations, we can better elucidate the impacts of PFAS on learning and memory functions and bioaccumulation, ultimately providing insights that are relevant for risk assessment and regulatory considerations. Understanding these toxicological mechanisms at higher concentrations is critical for developing protective measures and informing policies aimed at mitigating the adverse effects of PFAS on human health and the environment.

Caenorhabditis elegans is a reliable toxicological model that is sensitive to a wide range of contaminants at environmentally relevant concentrations with established toxicity testing methods (Wang et al. 2024). *C. elegans* have a short life cycle (approx. 3 days at 25°C) with the

developmental stages occurring in the first 48 hours (Ruszkiewicz et al. 2018). Their advantageous traits encompass its compact size, prolific breeding capability, ease of cultivation, low maintenance cost, long-term cryopreservation, rapid generation time, transparency, consistent cell count and development, and capacity for gene activity reduction through feeding RNAi (Corsi, Wightman, and Chalfie 2015). With their small size and ease of use, several high-throughput screening platforms have been developed to analyze toxicants (Kinser and Pincus 2017; Boyd et al. 2010; Ai et al. 2014). Using high-throughput screening platform established in out laboratory (Tang et al. 2019; Tang et al. 2020; Currie et al. 2023), learning & memory along with bioaccumulation were selected to explore the toxicity of PFAS throughout development. Learning and memory are indicators of development and the neuronal status in *C. elegans* (Amano and Maruyama 2011; Rankin, Beck, and Chiba 1990; Giles, Rose, and Rankin 2006). Results from this portion of the study were consistent with previous findings, all PFAS and the mixtures had an adverse effect on both learning and memory (Feng et al. 2022; Currie et al. 2023; Brown-Leung and Cannon 2022; Gaballah et al. 2020; Wang et al. 2015; Mshaty et al. 2020; Long et al. 2013). The most toxic individual substances were PFBS and PFOS. The bioaccumulation of PFAS in *C. elegans* has only been investigated in a few studies. The results of this study are consistent with previous studies (Sammi et al. 2019; Ma et al. 2023). However, it should be noted that the bioaccumulation has only been determined for PFOS, PFOA, PFBS, and PFBA in previous studies. The bioaccumulation of PFAS is likely influenced by their long half-lives, which allow these substances to persist for extend periods, leading to higher internal concentrations. Bioaccumulation in *C. elegans* remains understudied, yet factors such as half-lives in liquid, bacteria, and worms, as well as variations in absorption, can significantly influence responses to specific toxicant exposure levels (Sammi et al. 2019). Remarkably, this is

the first time that the bioaccumulation has been investigated for PFHxA, PFNA, PFHxS, 6:2 FTS, PFOSA, NEtFOSAA, HFPO-DA, and a mixture in *C. elegans*. Moreover, our study found that these additional PFAS have similar, if not greater, bioconcentration factors compared to the legacy PFAS, indicating a high incidence of bioaccumulation. This leads to indications that the bioaccumulation of PFAS leads to inhibition in learning and memory when exposed to the developmental stages. Our study found that high bioconcentration factors correlated with significant learning and memory impairment with a statistical significance ($p<0.05$). Additionally, mammalian models (Flynn et al. 2021; Narizzano et al. 2022) and non-mammalian models (Kim et al. 2021; Dasgupta et al. 2020) have identified PFAS as causing developmental delays. However, there is limited data on the alternative PFAS compared to the legacy compounds when using mammalian models due to time and cost. Additionally, there is abundance of PFAS literature in non-mammalian models, but they come with limitations. Zebrafish are frequently used as an alternative to mammalian models, but their biological complexity is somewhat limited, particularly in studying early-stage neurodevelopmental defects (Babin, Goizet, and Raldua 2014). Considering the alternatives, *C. elegans* provide an excellent model in investigating developmental defects on learning and memory.

Everything considered, using the nematode model coupled with a high-throughput screening platform provides numerous advantages for evaluating PFAS toxicity on learning and memory. The findings obtained from this study correlate the bioaccumulation of PFAS and its inhibitory effects on learning and memory. These findings will contribute to bridging the existing gap in our understanding of PFAS neurodevelopmental toxicity. The limitation is that we could establish a wider range of exposure for calculating the BCF to see the maximum amount of bioaccumulation in *C. elegans*. Given the known adverse effects initiated by PFAS exposure, the

underlying mechanisms could yield valuable insight into risk assessment and safety evaluation of PFAS exposure. Furthermore, it is important to recognize that individuals are often exposed to multiple PFAS compounds simultaneously from various sources, such as contaminated water, food, and consumer products. These compounds can be found in a wide range of combinations, which may vary significantly based on geographical location due to local industrial practices, environmental contamination, and regulations. This variability can influence the specific types and concentrations of PFAS present in a given area, affecting the overall exposure risk for individuals living in different regions. These variations reflect the diverse environmental conditions and sources of contamination across different regions (Miranda et al. 2022). While it is impossible to evaluate every combination of PFAS, it is essential to conduct studies that account for developmental neurotoxicity using environmentally relevant samples.

6.5 Conclusion

Utilizing high-throughput screening, we investigated both individual PFAS along with a reference mixture on learning and memory along with the bioaccumulation. Our results suggest that there is a correlation between bioaccumulation and impact on the learning and memory within *C. elegans*. Chemicals with known toxicities have been determined to have the highest bioaccumulation levels. Furthermore, exposure to mixtures of PFAS appears to have a compounding effect on learning and memory. These observations contain valuable insight on the potential adverse effects of PFAS exposure and emphasizes the importance of bioaccumulation specifically with exposure throughout development.

6.6 Credit Author Statement

Seth D. Currie: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. Yuqing Ji: Methodology, Investigation. Qingguo Huang: Resources, Methodology. Jia-Sheng Wang: Writing – review & editing, Validation. Lili Tang: Writing – review & editing, Supervision, Resources, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Table 6.1: The Benchmark Concentration 10% PFAS on memory (absolute peristatic speed) at different time points.

PFAS	Timepoint	PROAST BEST Model	BMC (BMCL) [µM]
6:2 FTS	48h	Expon. m5-	62.14 (7.09)
HFPO-DA	24h	Expon. m5-	0.66 (0.10)
	48h	Expon. m5-	0.19 (1.91e-2)
NEtFOSAA	24h	Expon. m5-	54.92 (10.30)
	48h	Expon. m3-	7.80 (0.52)
PFBA	24h	Hill m3-	119.70 (84.80)
	48h	Hill m5-	2.48 (0.95)
PFBS	24h	Hill m5-	34.13 (9.87)
	48h	Hill m5-	3.972 (1.41)
PFHxA	24h	Hill m3-	100.90 (72.60)
	48h	Hill m5-	41.84 (8.68)
PFHxS	24h	Hill m5-	0.076817 (2.38e-3)
	48h	Hill m3-	0.004809 (2.42e-6)
PFNA	24h	Hill m5-	33.80 (13.20)
	48h	Hill m5-	23.59 (10.20)
PFOA	48h	Hill m3-	104.03 (100.31)
PFOS	24h	Hill m5-	39.16 (7.71)
	48h	Expon. m5-	17.36 (6.30)
PFOSA	24h	Hill m3-	149.9 (106)
	48h	Expon. m3-	45.27 (2.81)

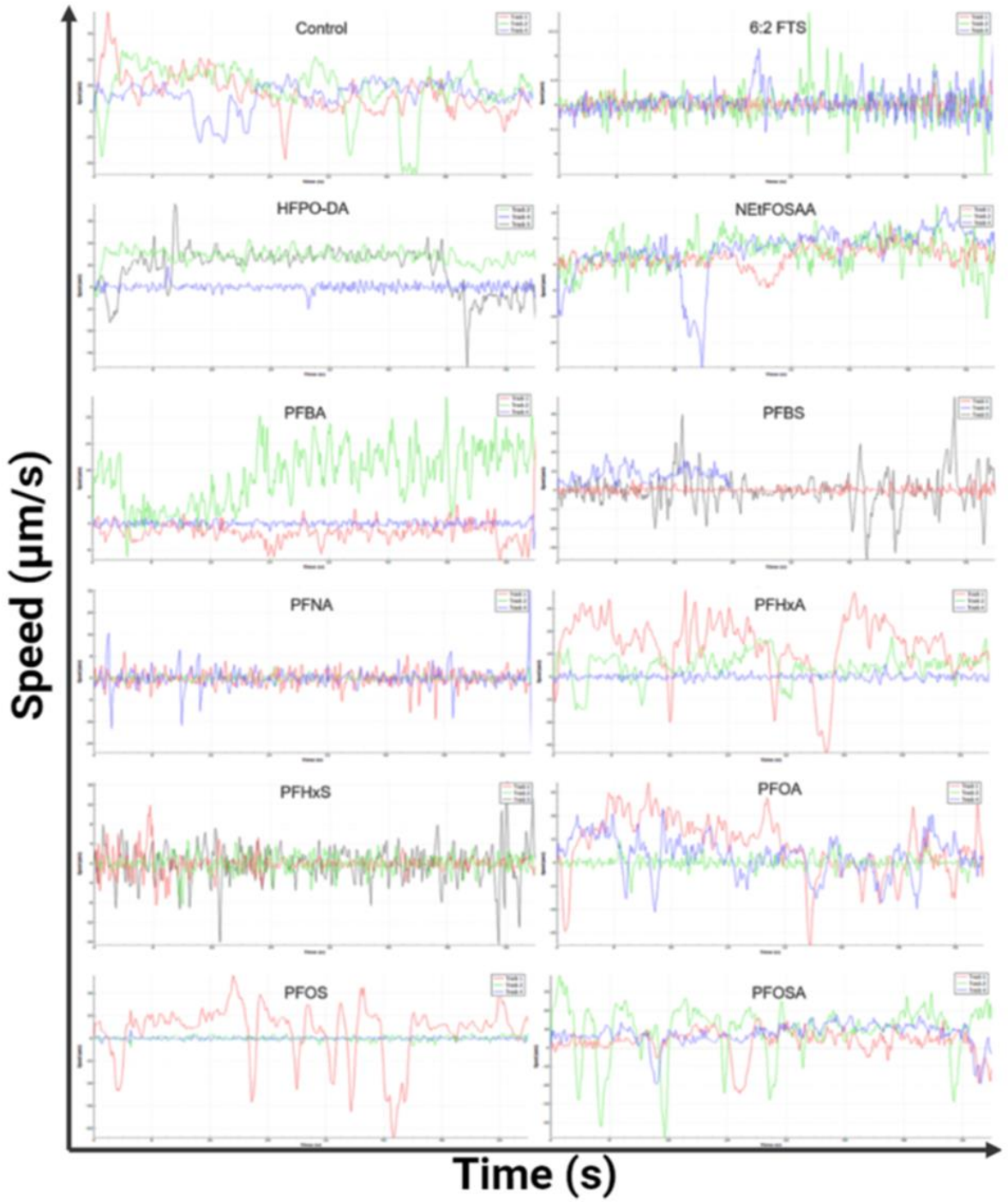

Figure 6.1: Effects of PFAS on speed on N2 (wild-type) *C. elegans* after exposure. Each color (red, blue, green, black) curve represents speed of a *C. elegans* selected at random. The speed sign is positive, indicating the forward movement along the head direction and the speed sign is negative, indicating the negative movement along the tail.

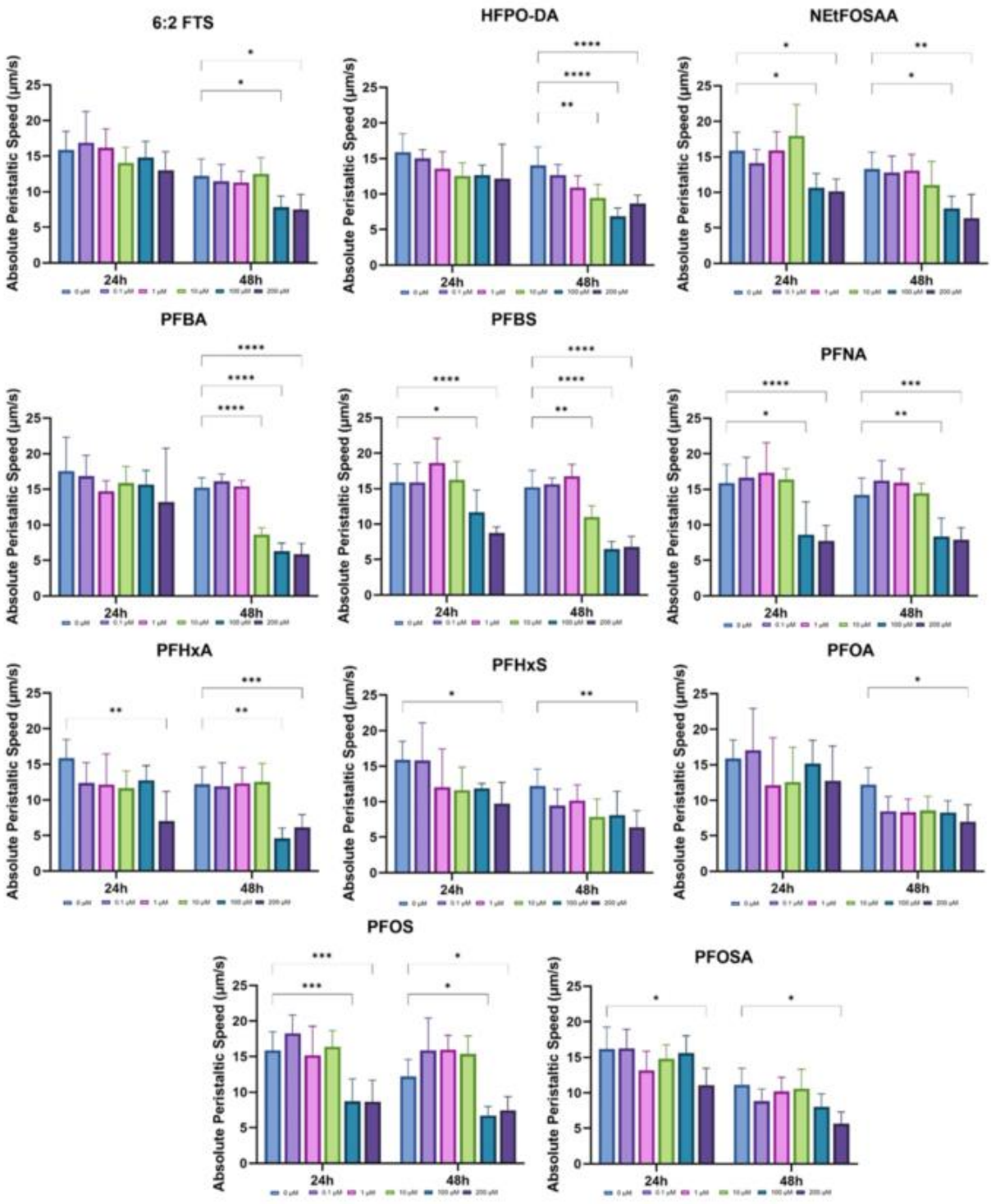

Figure 6.2: Effects of PFAS on memory (Absolute Peristatic Speed) on N2 (wild type) *C. elegans* after exposure. All values are represented as an Absolute Peristatic Speed.

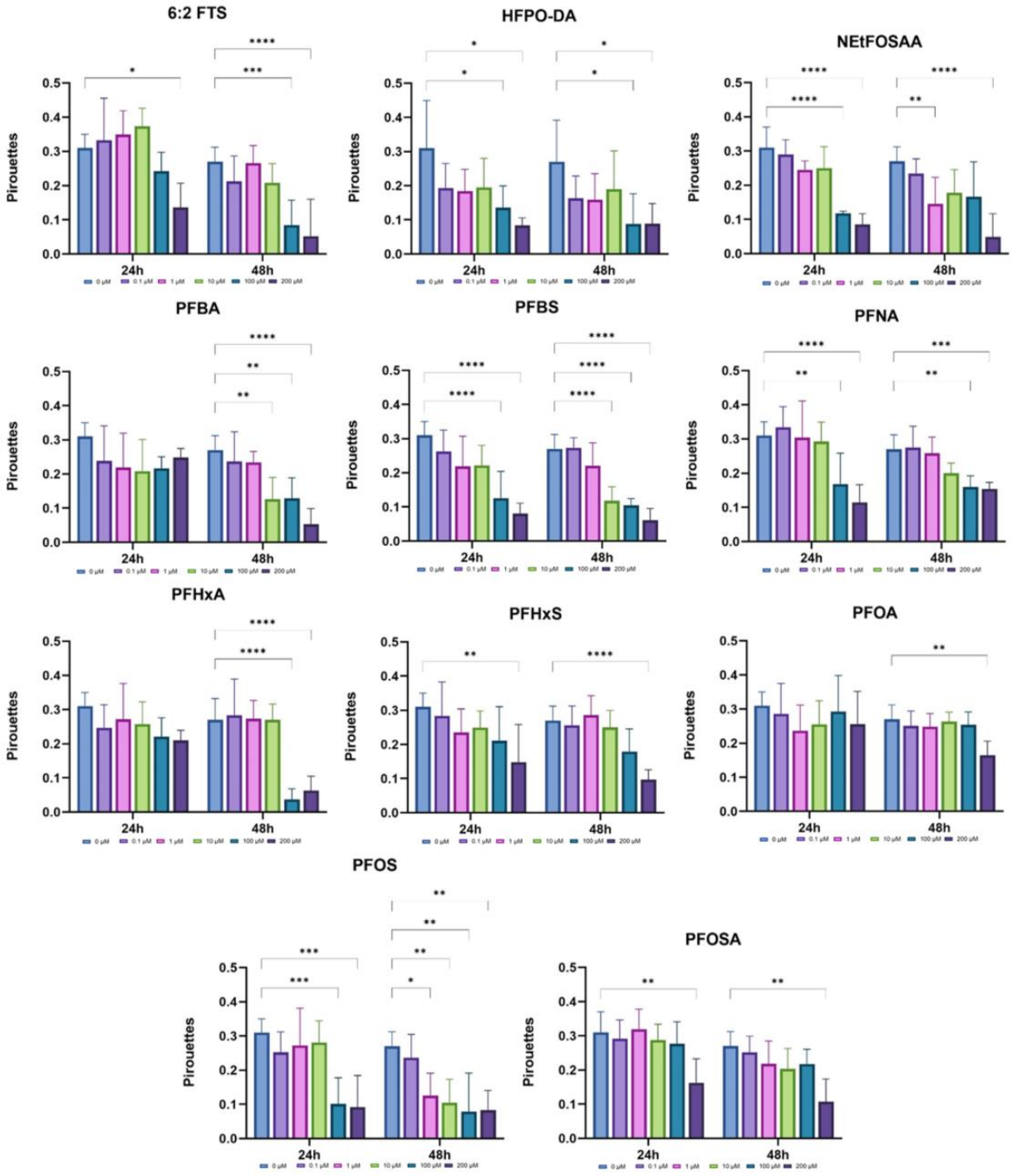

Figure 6.3: Effects of PFAS on learning (Pirouette) on N2 (wild type) *C. elegans* after exposure.

All values are represented as a number of pirouettes.

Table 6.2: The Benchmark Concentration 10% PFAS on learning (pirouette)

at different time points.

PFAS	Timepoint	PROAST BEST Model	BMC (BMCL) [µM]
6:2 FTS	24h	Expon. m3-	52.30 (31.60)
	48h	Expon. m5-	3.19 (0.59)
HFPO-DA	48h	Expon. m3-	2.91e-4 (5.94e-5)
NEtFOSAA	24h	Expon. m3-	13.61 (3.08)
	48h	Expon. m3-	44.38 (22.60)
PFBA	48h	Expon. m5-	2.13e-3 (7.52e-5)
PFBS	24h	Expon. m3-	0.0293 (8.66e-4)
	48h	Expon. m3-	0.0121 (7.82e-4)
PFHxA	48h	Hill m5-	12.19 (8.42)
PFHxS	24h	Expon. m3-	14.53 (1.33)
	48h	Expon. m3-	16.17 (5.26)
PFNA	24h	Expon. m3-	2.346 (0.451)
	48h	Hill m3-	0.036 (1.62e-4)
PFOA	24h	Hill m3-	8.25e-3 (4.20e-4)
	48h	Expon. m3-	108.5 (70.5)
PFOS	24h	Expon. m5-	8.94 (2.51)
	48h	Expon. m5-	0.073 (5.29e-4)
PFOSA	24h	Hill m3-	103.7 (60.5)
	48h	Hill m3-	96.25 (73.4)

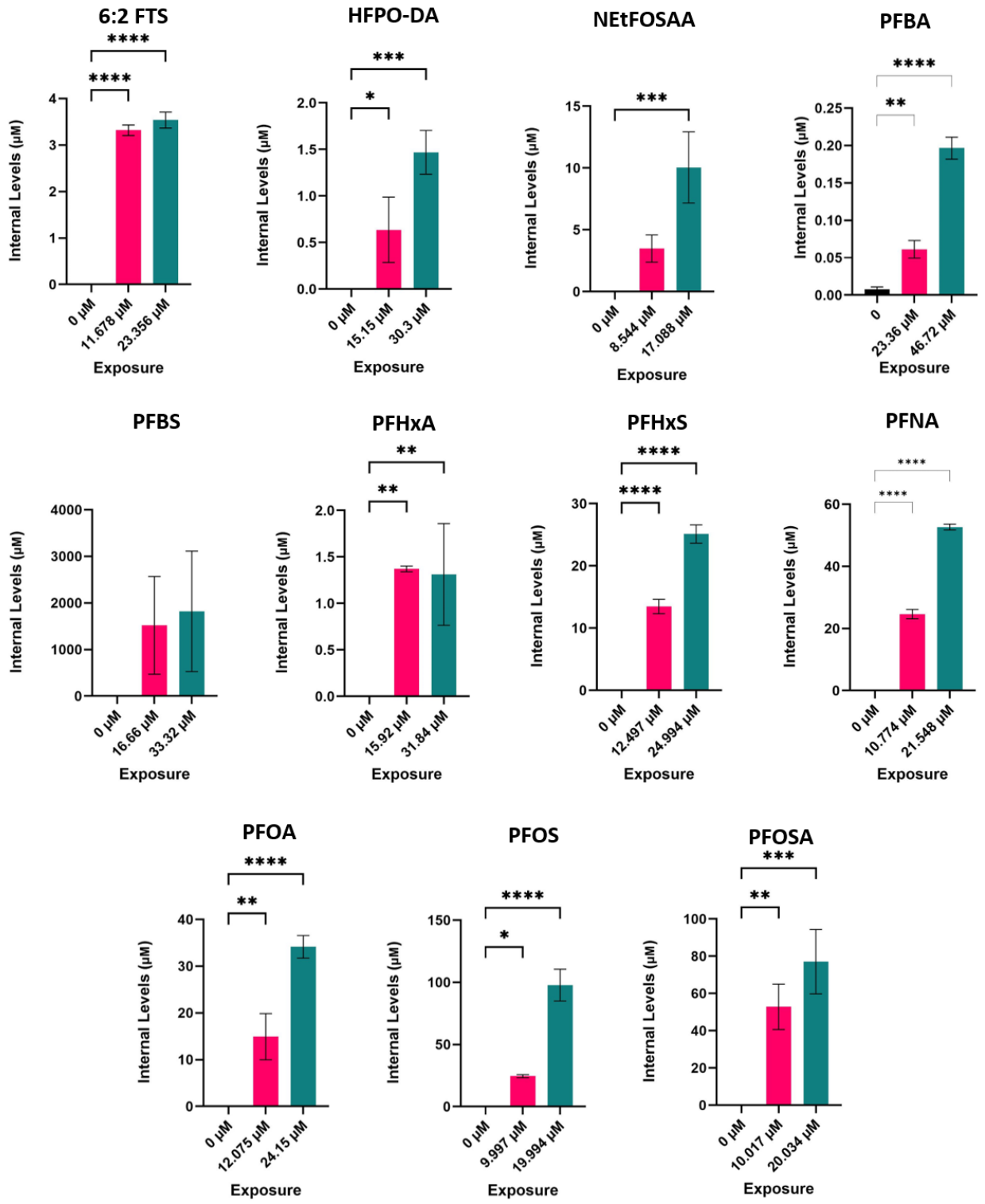

Figure 6.4: Bioaccumulation levels of PFAS within N2 (wild-type) *C. elegans* after 48 hours of exposure. All values are represented as internal levels.

Table 6.3 The Internal Levels of PFAS in *C. elegans*

PFAS	Exposure	Internal Levels, ppb	P-Value
PFBA			
	0 ppm	1.609 (0.7221)	
	5 ppm	13.1 (2.509)	0.0018 **
	10 ppm	42.06 (3.157)	P ≤ 0.0001 ****
PFHxA			
	0 ppm	>LOD (>LOD)	
	5 ppm	430.6 (9.941)	0.0033**
	10 ppm	411.9 (171.9)	0.0041**
PFOA			
	0 ppm	31.45 (8.929)	
	5 ppm	6177 (2038)	0.0022**
	10 ppm	14135 (1002)	<0.0001****
PFNA			
	0 ppm	>LOD (>LOD)	
	5 ppm	11425.4 (575.2)	<0.0001****
	10 ppm	24440.3 (359.4)	<0.0001****
PFBS			
	0 ppm	>LOD (>LOD)	
	5 ppm	456085 (314655)	<0.0001****
	10 ppm	546802 (388334)	<0.0001****
PFHxS			
	0 ppm	>LOD (>LOD)	
	5 ppm	5387 (459.2)	<0.0001****
	10 ppm	10053 (586.5)	<0.0001****
PFOS			
	0 ppm	>LOD (>LOD)	
	5 ppm	12263 (578.1)	0.0122*
	10 ppm	48896 (6422)	<0.0001****
6:2 FTS			
	0 ppm	>LOD (>LOD)	
	5 ppm	1421 (48.41)	<0.0001****
	10 ppm	1515 (73.30)	<0.0001****
PFOSA			
	0 ppm	>LOD (>LOD)	
	5 ppm	26373 (6096)	0.0033**
	10 ppm	38475 (8650)	0.0005***
NEtFOSAA			
	0 ppm	>LOD (>LOD)	
	5 ppm	2036 (644.4)	0.0920 ns
	10 ppm	5882 (1684)	0.0008***
HFPO-DA			
	0 ppm	>LOD (>LOD)	
	5 ppm	210.1 (115.6)	0.0327*
	10 ppm	485.0 (77.68)	0.0006***

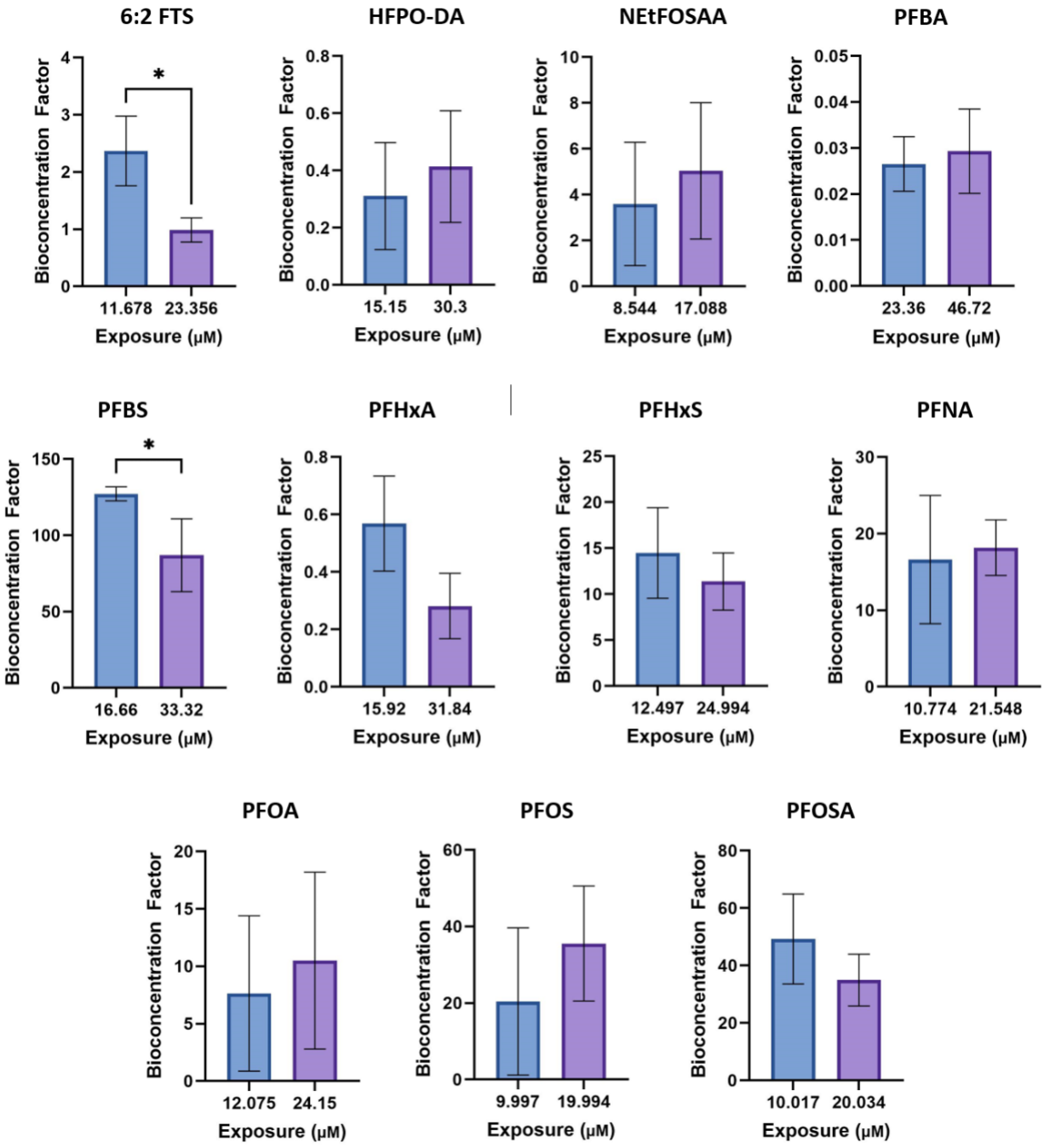

Figure 6.5: Bioconcentration Factor of PFAS in N2 (wild-type) *C. elegans* after 48 hours of exposure. All values are represented as internal levels. All values are represented as Bioconcentration Factor.

Table 6.4: The Bioaccumulation Factor for PFAS on *C. elegans*

PFAS	Exposure	Bioconcentration Factor	P-Value
PFBA			
	5 ppm	0.02932 (0.005950)	
	10 ppm	0.02932 (0.009157	0.685 ^{ns}
PFHxA			
	5 ppm	0.5684 (0.1656)	
	10 ppm	0.2812 (0.1137)	0.0685 ^{ns}
PFOA			
	5 ppm	7.637 (6.756)	
	10 ppm	10.49 (7.700)	0.6550 ^{ns}
PFNA			
	5 ppm	16.603 (6.838)	
	10 ppm	18.164 (2.953)	0.5174 ^{ns}
PFBS			
	5 ppm	127.2 (4.663)	
	10 ppm	86.95 (23.83)	0.0454*
PFHxS			
	5 ppm	14.48 (4.927)	
	10 ppm	11.38 (3.108)	0.4090 ^{ns}
PFOS			
	5 ppm	20.41 (19.22)	
	10 ppm	35.54 (15.03)	0.3433 ^{ns}
6:2 FTS			
	5 ppm	2.367 (0.6085)	
	10 ppm	0.9881 (0.2119)	0.0207 ^{ns}
PFOSA			
	5 ppm	49.25 (15.66)	
	10 ppm	34.94 (9.010)	0.2418 ^{ns}
NEtFOSAA			
	5 ppm	3.597 (2.694)	
	10 ppm	5.038 (2.975)	0.5679 ^{ns}
HFPO-DA			
	5 ppm	0.3107 (0.1871)	
	10 ppm	0.4136 (0.1953)	0.5459 ^{ns}

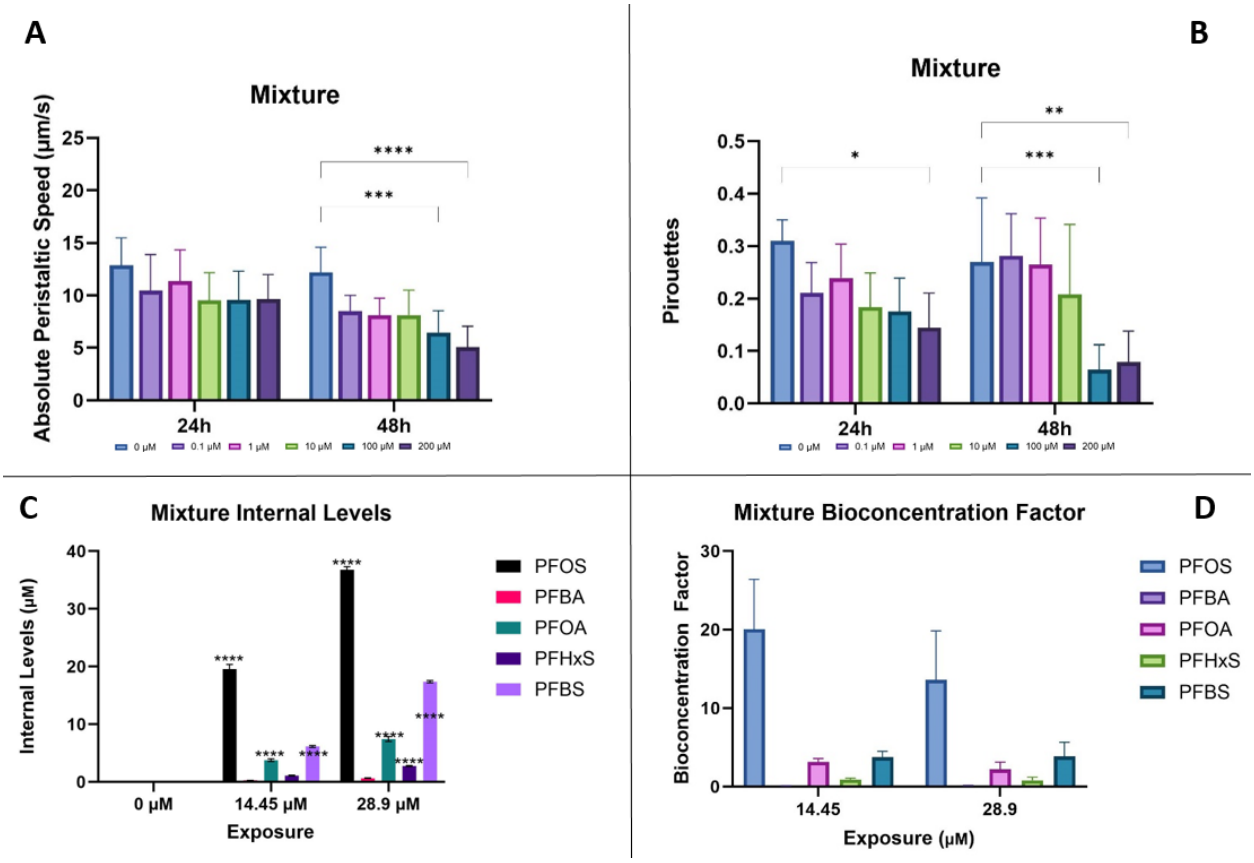

Figure 6.6: Toxic response in N2 (wild-type) *C. elegans* nematodes to PFAS Mixture. (A) Effects on memory (Absolute Peristatic Speed). All Values are represented as an Absolute Peristatic Speed (µm/s). (B) Effects on learning (Pirouette). All values are represented as a number of pirouettes. (C) Bioaccumulation levels after 48h of exposure. All Values are represented as internal PFAS levels (µM). (D) Bioconcentration Factor after 48h of exposure. All values are represented as Bioconcentration Factor.

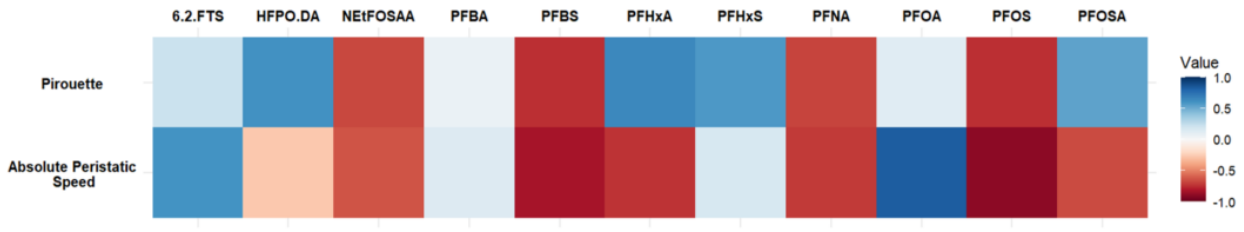

Figure 6.7: Correlation between bioaccumulation and toxicity. All values are represented as a Pearson Correlation Coefficient.

CHAPTER 7

THE QUANTITATIVE ADVERSE OUTCOME PATHWAY TO ASSESS DEVELOPMENTAL NEUROTOXICITY (DNT) OF PER- AND POLYFLUOROALKYL SUBSTANCES (PFAS) MIXTURE IN *CAENORHABDITIS ELEGANS*[6]

[6]Currie, S. D., Simon, T., Wang, J. S., & Tang, L. To be submitted to a peer-reviewed journal

Abstract

An Adverse Outcome Pathway (AOP) is a conceptual framework that organizes existing knowledge of biological processes leading from an identified molecular initiating event (MIE) through a series of key events (KEs) to an adverse outcome (AO) of regulatory concern. This study focuses on developing a Quantitative Adverse Outcome Pathway (qAOP) leading to developmental neurotoxicity (DNT) in *Caenorhabditis elegans* (*C. elegans*) starting with the MIE of decreased MAPK signaling pathway. Per- and Polyfluoroalkyl substances (PFAS) may be a stressor that can trigger this MIE sufficiently to initiate neurodevelopmental disruptions through a sequence of biologically relevant KEs. Key events in this AOP include alterations in neuronal growth, synaptic function, and behavioral changes. Data from mechanistic studies and statistical analyses using Bayesian network models provide both qualitative and quantitative insights into these key event relationships (KERs). This approach predicts the probability and severity of adverse neurodevelopmental outcomes from PFAS exposure by integrating *C. elegans* model findings, highlighting critical exposure windows, and linking key events (KEs) and key event relationships (KERs) to advance understanding of PFAS-induced neurotoxicity and support informed risk assessments.

7.1 Introduction

Adverse Outcome Pathways (AOPs) provide a systematic model for describing how chemical exposures lead to harmful effects lining the chain of biological processes, starting from molecular initiating events and passing through key events, to the final adverse outcomes (Saarimaki et al., 2023). Proposed in 2010, this organizational framework was developed to enhance the integration and interpretation of mechanistic toxicity data, serving as a key component in a more predictive approach to chemical safety assessment (Ankley et al., 2010). Instead of detailing the entire complexity of molecular and cellular activities, AOPs offer streamlined toxicity pathways that emphasize crucial events serving as landmarks on the way to an adverse outcome (Draskau, Spiller, Boberg, Bowles, & Svingen, 2020; Villeneuve et al., 2014). The process of developing AOPs relies on a weight of evidence (WoE) approach that aggregates multiple types of evidence to establish direct causal relationships among measurable biological events (Bal-Price & Meek, 2017). While the AOP framework organizes qualitative knowledge systematically, mathematical models that describe key event relationships at different scales allow for the quantitative synthesis of biological understanding, facilitating both interpretation and extrapolation, and helping to fill data gaps (Paini et al., 2022). This quantitative approach, known as a quantitative adverse outcome pathway (qAOP), can serve to predict both the probability or severity of an adverse outcome from the activation of an MIE (Conolly et al., 2017).

The process of developing an AOP requires determining the biological responses and causal linkages induced by stressors, including both chemical and non-chemical types, with the key challenge being to establish the mechanistic links among the molecular initiating event, intermediate events, and adverse outcomes (Lizano-Fallas, Carrasco Del Amor, & Cristobal,

2023). Following the molecular initiating event (MIE), the pathway progresses through a sequence of key events (KEs) leading to the adverse outcome, with scientific evidence establishing the causal and predictive relationships between each KE (KER; Key Event Relationships) (Villeneuve et al., 2018). The modular nature of AOPs promotes the reuse of key events and relationships across different pathways, while collaborative development ensures that AOPs are regularly updated and refined, incorporating new scientific data to enhance their robustness and adaptability as scientific knowledge and regulatory needs evolve (Chauhan et al., 2021; Oki, Nelms, Bell, Mortensen, & Edwards, 2016). AOPs are living documents, continuously evolving as new scientific insights and data become available, integrating new methodologies and technologies like high-throughput screening and computational modeling (Vieira, Souza, & Farias, 2024). This evolving nature helps AOPs remain relevant for understanding toxicological processes and addressing emerging challenges in risk assessment and regulatory science, supporting evidence-based decision-making and policy development (Coady et al., 2019).

Per- and polyfluoroalkyl substances (PFAS) are persistent environmental contaminants that accumulate in *Caenorhabditis elegans* (*C. elegans*), posing long-term exposure risks that can significantly affect biological functions (Foguth, Sepulveda, & Cannon, 2020). Chronic exposure to PFAS in *C. elegans* has been shown to result in a variety of adverse effects, including disruptions in neuronal development, behavior impairments, and interference with critical cellular processes such as mitochondrial function and oxidative stress response (Ma, Pan, Wang, Li, & Luo, 2023). The complexity of PFAS toxicity is significantly heightened when considering mixtures of these compounds rather than single substances alone. PFAS mixtures, often encountered in environmental and occupational settings, present unique challenges for toxicity

assessment due to the potential for interactions between different PFAS compounds (Ankley et al., 2021). Interactions between different PFAS compounds in *C. elegans* may alter their individual toxicities, leading to synergistic or antagonistic effects that could amplify or mitigate the observed outcomes (Lagunas-Rangel et al., 2022; Silins & Hogberg, 2011). To accurately assess the combined toxicity of PFAS mixtures, it is crucial to develop adverse outcome pathways (AOPs) in *C. elegans* that capture the molecular and systemic impacts of these compounds. This approach is essential for advancing our understanding of PFAS-related health risks and improving the accuracy of risk assessments and regulatory strategies in environments with complex PFAS exposure (Iulini et al., 2024).

The link between PFAS exposure and neurodevelopmental effects is an emerging area of concern, with studies in *Caenorhabditis elegans* (*C. elegans*) highlighting disruptions during critical stages of development. Research indicates that these substances can affect neuronal growth, synaptic function, and behavior (Sammi et al., 2019). These disruptions in *C. elegans* may manifest as behavioral abnormalities, motor impairments, and deficits in learning and memory (Chowdhury, Sana, Panneerselvan, Dharmarajan, & Megharaj, 2021). The simplicity of the *C. elegans* nervous system allows for real-time observation of these effects, which provides valuable insights into the molecular mechanisms of PFAS-induced neurodevelopmental toxicity (Ruszkiewicz et al., 2018). By utilizing this model organism, researchers can identify key pathways affected by PFAS and help build adverse outcome pathways (AOPs) that will enhance our understanding of PFAS toxicity. These findings highlight the utility of *C. elegans* as a model for investigating PFAS toxicity, providing insights that can inform certain aspects of human biology and contribute to the development of risk assessments and regulatory policies.

Caenorhabditis elegans (*C. elegans*) offers a powerful model system for investigating the neurodevelopmental effects of PFAS exposure, particularly when considering the complexity of PFAS mixtures (Roussos, Kitopoulou, Borbolis, & Palikaras, 2023). Due to its well-characterized nervous system, which shares many similarities with more complex organisms, *C. elegans* provides a unique opportunity to study the molecular and cellular mechanisms underlying neurodevelopmental toxicity (Ruszkiewicz et al., 2018). The simplicity of the *C. elegans* nervous system, combined with advanced genetic tools, allows researchers to explore how PFAS exposure affects neuronal development, synaptic function, and behavior (Hobert, 2010). Moreover, the transparency of *C. elegans* enables real-time observation of neurodevelopmental processes, facilitating the identification of key events and pathways disrupted by PFAS (Corsi, Wightman, & Chalfie, 2015). By exposing *C. elegans* to PFAS and PFAS mixtures, researchers can investigate how these chemicals interact with neurotransmitter systems, alter neuronal growth, and interfere with neurotrophic signaling, all critical for brain development. This model organism also allows for the examination of how PFAS affect key stages of neurodevelopment, from embryogenesis to adulthood, offering insights into the timing and duration of exposure that are most detrimental. Furthermore, *C. elegans* can be used to screen for potential neurodevelopmental effects of PFAS mixtures, helping to elucidate the combined impacts of multiple compounds on the nervous system (Starnes, Rock, Jackson, & Belcher, 2022). The integration of various *C. elegans* studies can enhance our understanding of PFAS toxicity and contribute to the development of adverse outcome pathways (AOPs) relevant to exposure assessment and regulatory considerations.

The following sections will offer a comprehensive, step-by-step examination of each component within the Adverse Outcome Pathway (AOP) (Figure 8.1). As scientific research

continues to advance, enhancing our knowledge of the intricate molecular and cellular mechanisms, updates to the key events (KEs) and key event relationships (KERs) related to the effects of per- and polyfluoroalkyl substances (PFAS) on neurodevelopment in *Caenorhabditis elegans* are anticipated. The AOP Wiki is designed to accommodate and integrate these evolving insights, ensuring that the framework remains current and reflective of the latest scientific understanding. This dynamic capability allows for continuous refinement and improvement of the pathway as new data and discoveries emerge.

7.2 Statistical Analysis

Bayesian network models were employed to quantitatively assess the adverse outcome pathway (qAOP) for neurodevelopmental toxicity induced by per- and polyfluoroalkyl substances (PFAS), utilizing data exclusively from research conducted within our laboratory. While relevant studies and external data were referenced for comparative and contextual purposes, the statistical analysis was conducted solely on the experimental dataset generated in our laboratory.

The selection of a Bayesian framework was motivated by its unique advantages in addressing the inherent uncertainties and complexities associated with toxicological data, especially in the context of neurodevelopmental investigations. Neurodevelopment is a multifaceted process characterized by intricate interactions among genetic, environmental, and biochemical factors that influence neuronal growth, differentiation, and connectivity (Budday, Steinmann, & Kuhl, 2015). Bayesian network models, which rely on directed acyclic graphs (DAGs), have gained increasing utility in biological research, enabling improved representation of causal relationships in complex systems. Bayesian modeling further facilitates the

incorporation of prior knowledge and the integration of evidence from various sources (Vilares & Kording, 2011), thereby enhancing our understanding of the mechanisms underlying neurodevelopmental toxicity.

The Bayesian network model was specifically designed to estimate conditional dependencies among variables such as gene expression, neuronal activity, and behavioral outcomes, thereby facilitating the identification of critical biomarkers for neurotoxicity. By graphically representing these relationships, the Bayesian approach enhanced interpretability, allowing for a clearer visualization of how disruptions in neurodevelopment propagate through the network to influence overall outcomes. Additionally, Bayesian methods enabled the quantification of uncertainty, supporting probabilistic inferences about neurodevelopmental outcomes based on observed data (Matsumori, Koike, & Matsumoto, 2018). This was particularly useful given the limited sample sizes and variable data quality inherent in neurodevelopmental toxicology studies.

The analysis was performed using the 'bnlearn' R package, which provides specialized tools for structure learning, parameter estimation, and inference within Bayesian networks. Structure learning was conducted using the Hill-Climbing algorithm with Bayesian Information Criterion (BIC) scoring to determine the optimal DAG representing the relationships among variables. This approach ensured that the network structure was both statistically sound and biologically plausible. Once the network structure was identified, parameter estimation was conducted using Bayesian parameter learning methods to derive conditional probability distributions for each node. These probability distributions were used to infer the likelihood of various neurodevelopmental outcomes under different exposure conditions.

Model validation was achieved through k-fold cross-validation, wherein predictive performance was assessed by comparing observed and inferred outcomes. The performance metrics included log-likelihood scores, posterior predictive checks, and robustness tests under varying data assumptions. Additionally, sensitivity analyses were performed to assess the influence of different parameters and assumptions on model predictions. These methodological steps ensured robustness in capturing the complex interplay among key biological variables such as gene expression, neuronal activity, and behavioral outcomes. Further, we performed Markov blanket analysis to determine the most influential variables within the network, enhancing interpretability and reducing potential model overfitting.

7.3 Stressor (Chemical Properties)

Per- and Polyfluoroalkyl Substances (PFAS) represent a diverse class of synthetic chemicals characterized by their unique carbon-fluorine bonds, which are among the strongest in organic chemistry (Grgas, Petrina, Stefanac, Beslo, & Landeka Dragicevic, 2023). This strong bonding imparts exceptional chemical stability to PFAS, making them highly resistant to environmental degradation processes such as hydrolysis, photolysis, and microbial action (Shittu et al., 2023; Verma, Lee, Sahle-Demessie, Ateia, & Nadagouda, 2022). The core structure of PFAS consists of a carbon chain fully fluorinated with fluorine atoms, which may be terminated by functional groups like carboxylic acids or sulfonic acids (Buck et al., 2011). This configuration gives PFAS their hydrophobic and lipophilic properties, making them effective as surfactants in a variety of applications, including stain-resistant textiles, non-stick cookware, and firefighting foams (Meegoda, Kewalramani, Li, & Marsh, 2020).

PFAS are categorized based on the length and branching of their fluorinated carbon chains, resulting in a wide range of chemical properties within this class (Evich et al., 2022). Long-chain PFAS, such as perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), are particularly notable for their environmental persistence and bioaccumulation potential (Wee & Aris, 2023). These compounds are used in products requiring durable, water- and oil-repellent properties, which contributes to their widespread presence in the environment and biological systems (Bonato et al., 2020; Kurwadkar et al., 2022). In contrast, short-chain PFAS, like perfluorobutanoic acid (PFBA), exhibit different environmental behaviors and generally have lower persistence but still pose challenges due to their mobility and widespread use (Brendel, Fetter, Staude, Vierke, & Biegel-Engler, 2018; Zhan et al., 2023).

Furthermore, PFAS exhibit diverse physical and chemical properties based on their molecular structures. The degree of fluorination and the length of the carbon chain affect their solubility, surface tension, and interactions with other substances (Haug, Dunder, Lind, Lind, & Salihovic, 2023). The chemical stability of PFAS enables them to persist in the environment and accumulate in living organisms, where they can be difficult to remove or remediate (Ellen Mantus, 2020). Advanced analytical techniques, such as high-resolution mass spectrometry and chromatographic methods, are often required to detect and quantify these substances due to their low concentrations and persistence (Kirkwood-Donelson, Dodds, Schnetzer, Hall, & Baker, 2023). Understanding the chemical properties of PFAS is critical for developing effective environmental monitoring methods, designing appropriate remediation strategies, and crafting regulatory policies to manage their impacts and ensure public health and environmental safety.

In addition to their inherent chemical stability, PFAS also exhibit varying degrees of environmental mobility and bioavailability. The chemical properties of PFAS, such as their

affinity for water and lipid phases, influence their distribution and persistence in different environmental compartments, including soil, water, and air (Nayak et al., 2023). The interaction of PFAS with particulate matter and their potential to form complex mixtures further complicate their environmental behavior (G. Feng et al., 2024). The ability of PFAS to migrate through soil and water systems can lead to widespread contamination, impacting both terrestrial and aquatic ecosystems (Ma, Ye, Wang, Li, & Luo, 2022). Furthermore, the long-range transport of PFAS in the atmosphere and their potential for global distribution highlight the need for international cooperation in monitoring and regulating these substances (Z. Zhao et al., 2012). A thorough understanding of these dynamics is necessary to gauge the full scale of PFAS contamination and to formulate effective strategies for their management and reduction, thereby ensuring the safety of public health and the environment.

7.4 Molecular Initiating Event

The molecular initiating event (MIE) is essential for understanding how certain chemicals, such as per- and polyfluoroalkyl substances (PFAS), trigger harmful biological effects. The MIE for PFAS involves several interconnected stages: exposure, bioconcentration, and cellular interactions. Each of these stages is crucial for unraveling the mechanisms by which PFAS impact biological systems.

7.4.1 Exposure

The exposure stage is the initial point where PFAS interact with biological systems, setting the foundation for potential health impacts. PFAS are a group of highly stable chemicals widely used in consumer and industrial products such as firefighting foams, non-stick cookware,

stain-resistant fabrics, and water-repellent items (Xia et al., 2022). Due to their resistance to degradation, these substances persist in the environment, resulting in contamination of soil, water, and air (Shahsavari et al., 2020). As environmental pollutants, they have become a significant concern due to their potential to bioaccumulate in both the environment and living organisms. The widespread distribution of PFAS makes them an ever-present threat to ecological and human health.

In the model organism *Caenorhabditis elegans* (*C. elegans*), PFAS exposure occurs through various routes similar to those in more complex organisms. These nematodes can ingest PFAS directly from contaminated soil or water (Han, Oyeyemi, Zenobio, Salawu, & Adeleye, 2023; Leuthner, Zhang, Kohrn, Stapleton, & Baugh, 2024). Given the small size of *C. elegans*, these exposure pathways are essential to understanding how PFAS may enter and accumulate in their bodies. The worms can absorb these chemicals through their gut, particularly if PFAS are present in the surrounding environment or experimental media (Stylianou, Björnsdotter, Olsson, Ericson Jogsten, & Jass, 2019).

Understanding how PFAS accumulate and affect *C. elegans* is crucial for assessing the broader environmental and health impacts of PFAS exposure. Factors such as concentration, duration of exposure, and specific PFAS compounds can influence the efficiency of these exposure routes (Queiros et al., 2019). Research on PFAS exposure dynamics reveals the molecular mechanisms of PFAS toxicity and their interaction with biological systems. This knowledge aids in understanding PFAS contamination in the environment and informs public health strategies and regulatory actions to mitigate associated risks.

7.4.2 Bioaccumulation

Bioaccumulation is a critical stage in the molecular initiating event, where PFAS accumulate in the tissues of organisms following environmental exposure. The chemical stability enables PFAS to accumulate within *C. elegans*, allowing them to remain within the organism's tissues (F. Chen et al., 2018; Currie, Ji, Huang, Wang, & Tang, 2024; Sammi et al., 2019). The molecular structure of PFAS, such as the length of the fluorinated carbon chain and the presence of functional groups (e.g., carboxylates or sulfonates), influences their bioaccumulation potential. This accumulation is exacerbated by the fact that PFAS are not easily metabolized or excreted, leading to their persistence in biological systems over time (Leuthner et al., 2024).

The efficiency of PFAS bioaccumulation in *C. elegans* depends on various factors, including the concentration of PFAS in their environment, the duration of exposure, and the specific PFAS compounds involved (Queiros et al., 2019). While long-chain PFAS are more likely to accumulate in higher concentrations, short-chain PFAS, though less bioaccumulative, have greater mobility and can more easily contaminate water and soil, raising concerns about their spread in the environment (Ehsan et al., 2023). Understanding how PFAS accumulate *in C. elegans* is necessary for evaluating the environmental risks of these substances and the potential impact on ecosystems. By studying bioaccumulation in *C. elegans*, researchers gain insights into the persistence and movement of PFAS in the environment, contributing to the development of more effective monitoring and regulatory strategies (de Souza & Meegoda, 2024).

7.4.3 Cell Interactions

The interactions of PFAS at the cellular level in *Caenorhabditis elegans* are critical for understanding the molecular mechanisms underlying their neurotoxicity. Once accumulated in

biological tissues, PFAS disrupt cellular processes, leading to structural and functional changes in neuronal systems that may manifest as cognitive or behavioral deficits (Bharal et al., 2024; Cao & Ng, 2021; Xie et al., 2024). The interactions of PFAS at the cellular level are crucial for understanding their toxic effects in *C. elegans.* Recent omics data (Table 7.1) provide a detailed look at the Molecular Initiating Events (MIEs) that underpin these toxic effects. These early molecular disruptions help identify the pathways by which PFAS impact neuronal function, through MAPK signaling pathway, specifically influencing the behavior of ion channels, receptors, and neurotransmitter release. By investigating these changes at the molecular level, we can gain insights into how PFAS exposure leads to neurodevelopmental abnormalities.

Ion channels are fundamental to the proper functioning of neurons, as they regulate the flow of ions across the cell membrane, a process that is essential for generating action potentials and synaptic signaling (Voglis & Tavernarakis, 2006). PFAS exposure has been shown to directly impact MAPK signaling in higher organisms, influencing the function of ion channels in various animal model (Bharal et al., 2024). *Caenorhabditis elegans* have homologs of ion channels found in higher organisms, including voltage-gated sodium and calcium channels (Hobert, 2010). When PFAS integrate into the lipid bilayer, their resemblance to fatty acids disrupts lipid packing (Roth, Imran, Liu, & Petriello, 2020). This disruption can affect the gating and conductance of ion channels, altering neuronal excitability and reducing signaling efficiency. Kim et al., 2020 demonstrated in *C. elegans* that PFAS exposure alters the lipid composition, specifically affecting the polyunsaturated fatty acids in phospholipids that are critical for maintaining membrane integrity and cell signaling (H. M. Kim et al., 2020). These changes in membrane fluidity compromise the function of ion channels, which are necessary for proper signal transmission and neuronal activity. This disruption in ion channel function contributes to

impaired communication between neurons, affecting overall neuronal network activity and function (Coburn & Bargmann, 1996).

Similarly, changes caused by PFAS exposure also affect membrane-bound receptors that are essential for neurotransmitter signaling (J. M. Brown-Leung & Cannon, 2022). Genomic insights from Li et al. (2020) suggested that PFAS-induced changes have the potential to disrupt signaling, particularly in neurotransmitter receptors involved in synaptic plasticity (Z. Li, Yu, Gao, & Yin, 2020). Genomic insights from other model organisms, such as zebrafish and mice, which share homologous neurotransmitter receptors with *C. elegans*, have revealed similar disruptions in receptor signaling pathways following PFAS exposure, further supporting the relevance of these findings across species (Ding et al., 2009; Ruiz et al., 2018). These receptors play a crucial role in detecting extracellular signals and triggering intracellular responses that are essential for regulating neuronal function, as observed in alternative model organisms (L. Zhao et al., 2023). These alterations can hinder synaptic communication, further compounding the impact on neuronal network activity and leading to broader functional impairments.

Additionally, the disruption of synaptic communication is particularly evident in the process of neurotransmitter release, which depends on the precise interaction between synaptic vesicles and the presynaptic membrane (Hendi, Kurashina, & Mizumoto, 2019). This process is tightly regulated by ion channels, particularly those responsible for controlling calcium influx, which triggers vesicle fusion and neurotransmitter release (Calahorro & Izquierdo, 2018). Sammi et al., 2019 indirectly investigated the impact of PFOS and PFOA on neurotransmitter release in *C. elegans*, demonstrating how PFAS exposure affects neuronal signaling (Sammi et al., 2019). This occurs through impairment in neurotransmitter release, disrupting synaptic transmission, which is a conserved pathway that aligns with similar disruptions observed in other model

organisms (Taoufik, Kouroupi, Zygogianni, & Matsas, 2018). Transcriptomic analyses further revealed a significant downregulation of the MAPK signaling pathway, which plays a crucial role in regulating neurotransmitter release and synaptic function (Currie et al., 2025). Disruptions in MAPK signaling intensify PFAS-induced alterations in lipid composition and membrane stability, further impairing synaptic vesicle function and neurotransmitter release. This combined disruption exacerbates neuronal dysfunction, underscoring the essential role of MAPK signaling in maintaining synaptic integrity. PFAS exposure highlights how environmental toxins can interfere with these critical processes in *C. elegans* and other organisms.

The changes in the MAPK signaling pathway induced by PFAS exposure have significant implications for neuronal function in *C. elegans*, affecting ion channels, receptors, and neurotransmitter release. These alterations at the cellular level disrupt key aspects of neuronal communication, leading to impaired neural signaling and synaptic activity (Poole, Flames, & Cochella, 2024). The changes in signaling induced by PFAS exposure are driven by a multifaceted cellular response leading to developmental neurotoxicity in *C. elegans* (Figure 7.2). These disruptions contribute to a cascade of effects that compromise the integrity of neuronal networks, leading to broader functional impairments within the nervous system. Understanding these mechanisms is essential for identifying potential biomarkers of neurotoxicity and for developing strategies to mitigate the adverse effects of PFAS exposure on neuronal health.

7.5 Cellular Key Event Responses

Exposure to PFAS during development induces a spectrum of cellular responses, including shifts in gene expression and disruptions in biochemical pathways that ultimately affect cellular function. The nature and extent of these changes depend on the concentration of

PFAS and the duration of exposure, with effects ranging from initial adaptive responses to more severe toxic outcomes. Early exposure periods may alter the formation of cellular structures, while sustained or high levels of PFAS can exacerbate these disruptions, leading to functional impairments. This variability underscores the critical role of key events in determining the overall impact of PFAS on neurodevelopment.

7.5.1 KE #1: Disruption in Neurotransmitter Dynamics

In *Caenorhabditis elegans*, exposure to PFAS has been shown to disrupt neurotransmitter dynamics, particularly in GABA neurons, which are essential for maintaining the balance of excitatory and inhibitory signaling in the nervous system (Josephine M. Brown-Leung & Cannon, 2023; Sammi et al., 2019). GABAergic signaling plays a critical role in regulating neural activity, controlling muscle movement, and maintaining proper synaptic transmission (Barbagallo et al., 2017). Alterations to this delicate system can result in neurodevelopmental defects, including changes in motor behavior, learning, and synaptic function. PFAS exposure has been found to interfere with key processes in GABA neurons, such as neurotransmitter synthesis, vesicular release, and reuptake, ultimately impairing the function of these neurons and affecting overall neural circuit integrity (Gendrel, Atlas, & Hobert, 2016).

These alterations in GABAergic signaling, resulting from PFAS exposure, disrupt the normal function of neurons, contributing to broader neurodevelopmental impairments in *C. elegans* (Figure 7.3) (Currie et al., 2025). Specifically, the disruption of neurotransmitter dynamics in GABA neurons leads to an imbalance in excitatory and inhibitory signaling, which in turn triggers cellular stress responses (Wirak, Florman, Alkema, Connor, & Gabel, 2022). One of the key consequences of this imbalance is an increase in oxidative stress within neural cells

(Tönnies & Trushina, 2017). While the direct link between neurotransmitter disruption and oxidative stress has been extensively reported in other organisms, similar pathways are implicated in *C. elegans*. The disruption of neurotransmitter release and reuptake, combined with impaired neuronal function, generates an excess of reactive oxygen species (ROS), overwhelming the antioxidant defense systems of neurons (G. Li, Gong, Lei, Liu, & Xu, 2016). This heightened oxidative stress further exacerbates cellular damage, impeding normal neurodevelopment and potentially contributing to neurotoxic effects that disrupt neural circuit formation and function.

7.5.2 KE #2: Oxidative Stress

In *C. elegans*, exposure to PFAS has been shown to significantly increase oxidative stress by elevating the levels of reactive oxygen species (ROS) within cells (Sammi et al., 2019; Smith, Latta, Denver, & Estes, 2014). These highly reactive molecules can damage various cellular components, including lipids, proteins, and DNA (Schieber & Chandel, 2014). The increased ROS production overwhelms the organism's antioxidant defenses, which are crucial for neutralizing these damaging molecules (Ayuda-Durán, González-Manzano, González-Paramás, & Santos-Buelga, 2020). As a result, oxidative stress disrupts normal cellular processes, impairing neuronal function, synaptic communication, and overall neurodevelopment (K. W. Kim & Jin, 2015).

The excess ROS generated by PFAS exposure also places a strain on the mitochondria, the energy-producing organelles essential for maintaining cellular function. Mitochondria are particularly vulnerable to oxidative damage due to their role in energy metabolism, which produces ROS as a byproduct (Dilberger et al., 2019). Under normal conditions, mitochondria

manage this by utilizing antioxidant systems, but when ROS levels exceed the capacity of these systems, mitochondrial function becomes impaired (Campbell & Zuryn, 2024). This dysfunction reduces the cell's ability to produce energy efficiently, leading to further neuronal impairments and contributing to the neurotoxic effects observed in PFAS-exposed *C. elegans* (Table 7.2). The damage to mitochondria exacerbates oxidative stress, creating a vicious cycle that negatively impacts neurodevelopment, ultimately influencing neuronal health and behavior.

7.5.3 KE #3 Mitochondrial Dysfunction

In *C. elegans*, mitochondria play a central role in energy production and maintaining cellular homeostasis (Onraet & Zuryn, 2024). They are particularly important in neurons, where energy demands are high (Campbell & Zuryn, 2024). When ROS levels exceed the cell's antioxidant capacity, mitochondrial integrity becomes compromised, disrupting the normal function of these organelles (Gualtieri et al., 2021). This damage contributes to the impairment of cellular energy production, which in turn affects neuronal function and survival.
Neurons are particularly vulnerable to accumulating damage from mitochondrial dysfunction, which is critical for their survival and function (Rea, Ventura, & Johnson, 2007). As a result, mitochondrial dysfunction is strongly associated with numerous neurodegenerative disorders (Mao et al., 2019). These disruptions result in an overall decline in cellular efficiency and can impair neural communication by reducing energy availability for synaptic transmission and neuronal signaling (Roussos et al., 2023). The compromised mitochondrial function contributes to neurodegenerative processes and can affect various neuronal populations, including those involved in movement and behavior (Table 7.3). As the cellular energy production pathways in *C. elegans* become disrupted by mitochondrial dysfunction, neuronal degeneration begins to

emerge (W. Chen, Zhao, & Li, 2023). Neurodegeneration caused by PFAS exposure leads to progressive loss of neuronal function, disrupting neural circuits and impairing critical behaviors (von Mikecz, 2023). Dopaminergic neurons are particularly vulnerable due to their high metabolic demand and sensitivity to oxidative stress, making them key indicators of PFAS-induced neurotoxicity (J. M. Brown-Leung & Cannon, 2022).

7.5.4 KE #4 Dopaminergic Neurodegeneration

Dopaminergic neurodegeneration in *Caenorhabditis elegans* following PFAS exposure is characterized by the damage and loss of dopamine-producing neurons (Table 7.4). This damage is initially observed through changes in neuron structure, such as the shrinkage of cell bodies and the fragmentation of axons (L. Chen & Chisholm, 2011). Over time, these neurons lose their characteristic morphology, with the cell bodies becoming smaller and less able to support vital cellular processes, such as energy production and protein synthesis (Harald Hutter, Wacker, Schmid, & Hedgecock, 2005). As a result, the neurons' ability to communicate with other cells is impaired, disrupting normal neural circuitry and contributing to observed motor and behavioral defects.

Axonal degeneration is another critical feature of dopaminergic neurodegeneration. The axons, which are essential for transmitting electrical signals between neurons, begin to break down and retract, significantly impeding neuronal communication (H. Hutter, 2000). This leads to a reduction in the number of functional synapses, with fewer and weaker connections forming between neurons. The remaining synapses often display abnormalities, such as irregular shapes and reduced density, which further disrupts neuronal function (H. Hutter, 2004). These changes in synaptic integrity contribute to the decline in motor behavior and cognitive functions,

highlighting the widespread impact of PFAS exposure on the neural network (Abraham et al., 2006).

As dopaminergic neurons continue to deteriorate, additional signs of cellular stress, such as vacuolation and the accumulation of protein inclusions, become apparent (Joshi, Matlack, & Rongo, 2016). Vacuoles form within the cell as a result of disrupted autophagy and impaired cellular processes, leading to gaps in the cytoplasm where essential organelles and components are missing. Protein inclusions, which consist of misfolded or damaged proteins, accumulate within the neurons, further exacerbating the stress on cellular machinery. These features are indicative of a breakdown in the neuron's ability to maintain normal function and homeostasis, contributing to the eventual death of the neurons. The loss of dopaminergic neurons directly correlates with motor dysfunction and behavioral impairments in *C. elegans*, underscoring the detrimental effects of PFAS exposure on neural development and movement (Apfeld & Fontana, 2017).

7.6 Organ-Level Response

7.6.1 KE #5 Decreased Neuronal Network Function

Following PFAS exposure in *Caenorhabditis elegans*, a critical aspect of neurodevelopmental impairment is the reduction in synaptogenesis (Figure 7.4) (Currie et al., 2025), the process by which new synapses are formed between neurons. This decrease in synaptogenesis severely hampers the formation of functional neuronal networks. Synapses are the primary sites of communication between neurons, and their formation and maintenance are essential for proper signaling, learning, and memory (Kennedy, 2013). In response to PFAS exposure, neurons display a marked reduction in the density and complexity of synaptic

connections, leading to weakened network functionality. Fewer synapses mean that neurons struggle to efficiently transmit signals, ultimately leading to diminished communication between different regions of the neural circuit (Pereda, 2014). The process of synaptogenesis in *C. elegans* has been shown to closely resemble that in other animal models, suggesting that the mechanisms underlying synaptic formation and the effects of PFAS exposure may be conserved across species (Mizumoto, Jin, & Bessereau, 2023).

The reduction in synaptogenesis also impacts the quality of neuronal signaling. With fewer synapses, the transmission of electrical signals between neurons becomes less reliable and less effective (Lepeta et al., 2016). In PFAS-exposed neurons, the synapses that do form are often weak or unstable, preventing the establishment of a well-functioning neural network. This disruption in synaptic connectivity compromises the coordination of neural circuits, leading to long-term deficits in motor control, learning, and sensory processing (Cardon, 2018). This impairment in synaptic connections during critical stages of neurodevelopment can have lasting effects on neural function, hindering the organism's ability to adapt and respond to environmental stimuli (McMillen & Chew, 2024).

In addition to hindering synapse formation, PFAS exposure also disrupts synaptic plasticity, the process by which synapses are strengthened or weakened in response to neural activity (Lohmann & Kessels, 2014). This disruption affects the maturation and maintenance of synapses, leading to premature pruning or weakening of synaptic connections. As a result, neurons in PFAS-exposed *C. elegans* fail to strengthen their synapses and form stable connections, further impairing network integrity (S. Kim, Kim, Kralik, & Jeong, 2016). Through the application of simple assays, the effects of PFAS exposure on synapse formation can be quantified, with dose-response curves illustrating a decline in synaptic density and plasticity.

This disruption in synaptic development leads to compromised neural network formation, which manifests as behavioral deficits, such as impaired locomotion and reduced responsiveness to environmental changes (Kunert, Maia, & Kutz, 2017). These impairments in synaptic function indicate broader disruptions in neural communication, affecting motor skills, learning, and behavior during the organism's development.

7.7 Adverse Outcome

7.7.1 Impairment on Learning and Memory

In addition to its other health effects, PFAS exposure has been shown to negatively impact neuronal function, with significant consequences for learning and memory (Chowdhury et al., 2021; Chowdhury, Sana, Panneerselvan, Sivaram, & Megharaj, 2022; Currie, Ji, et al., 2024; Z. Feng et al., 2022; Sana, Chowdhury, Logeshwaran, Dharmarajan, & Megharaj, 2021; Sana, Chowdhury, Logeshwaran, & Megharaj, 2023; Yue et al., 2020). The impairment of cognitive functions is primarily attributed to disruptions in critical neuronal processes, such as synaptic plasticity, neuronal differentiation, and network connectivity (Cowen, Raizen, & Hart, 2024). PFAS can interfere with the normal development and function of neurons, leading to deficits in learning and memory by affecting neuronal regions essential for these behavioral processes.

Studies have demonstrated that PFAS exposure can alter neuronal signaling pathways and impair synaptic function, which are vital for effective communication between neurons (Josephine M. Brown-Leung & Cannon, 2023; Sammi et al., 2019). Such disruptions can impair neuronal performance in *C. elegans*, leading to deficits in learning and memory capabilities (Brandel-Ankrapp & Arey, 2023). Currie et al. (2024) examined the effects of multiple PFAS

compounds on cognitive function in *C. elegans*, including long-chain PFAS such as PFOA and PFOS, and short-chain alternatives like PFHxA and PFBS. Worms were exposed to concentrations ranging from 0.1 µM to 100 µM for 48 hours, with behavioral assays like pirouette frequency and absolute peristaltic speed used to assess learning and memory. The study found dose-dependent cognitive impairments, with more severe effects observed for long-chain PFAS, particularly PFOA and PFOS, compared to their short-chain counterparts. This highlights that PFAS chain length and chemical properties significantly influence neurotoxicity.

Additional studies by Chowdhury et al. (2022) and Sana et al. (2021) investigated single PFAS compounds at lower exposure levels, using chemotaxis plasticity assays to assess cognitive function. These studies found no significant cognitive impairments, suggesting that higher concentrations or cumulative exposure may be necessary to observe notable effects on learning and memory. Additionally, the degree of cognitive impairment is associated with the level and duration of PFAS exposure, indicating that specific thresholds of exposure are required to observe significant effects on neuronal health and cognitive function (Table 7.5). These findings underscore the importance of considering PFAS exposure levels when assessing potential risks to cognitive development and brain function.

7.8 Bayesian Model Analysis

The Bayesian network model developed for evaluating the neurodevelopmental toxicity of PFAS revealed significant dependencies among biological variables, providing a detailed map of interactions that drive adverse neurodevelopmental outcomes (Figure 7.5). The model identified decreased MAPK signaling pathway activity as the primary molecular initiating event (MIE) within the adverse outcome pathway (AOP). This MIE influenced downstream key events

(KEs), including oxidative stress, mitochondrial dysfunction, dopaminergic neurodegeneration, and disruptions in synaptogenesis, ultimately contributing to behavioral impairments. The models provided probabilistic estimates of the likelihood of adverse outcomes across various PFAS exposure scenarios, offering insights into the differential impacts of individual compounds and their mixtures. Sensitivity analyses indicated that specific events, such as oxidative stress and dopaminergic neurodegeneration, played a disproportionately large role in driving neurodevelopmental toxicity, underscoring their significance within the pathway.

To enhance clarity regarding the derivation of values in Figure 7.5, we explicitly detail the computational process utilized to generate these estimates. The values in Figure 7.5 represent marginal probabilities, calculated by integrating information from each node's parent variables. This integration was performed using belief propagation algorithms within the Bayesian network, ensuring that each node's probability reflected the cumulative influence of upstream events. Specifically, we applied junction tree inference to efficiently compute posterior probability distributions, allowing for a comprehensive assessment of how perturbations in upstream molecular events influence downstream neurodevelopmental outcomes.

To further refine our model, we employed Monte Carlo simulations to assess the stability of the probability estimates. By running multiple simulations with different input parameter distributions, we ensured that our predictions were robust under varying biological conditions. The Bayesian framework also facilitated Bayesian model averaging (BMA), which allowed us to account for model uncertainty by integrating results from multiple plausible network structures, thereby improving the reliability of our findings.

Furthermore, the model underwent rigorous validation through posterior predictive checks, wherein simulated data generated from the Bayesian network were compared against

observed experimental data to assess model fidelity. The probabilistic framework also enabled uncertainty quantification, identifying areas of high-confidence predictions and highlighting regions where further data collection would be beneficial. This approach allowed us to refine our understanding of neurodevelopmental toxicity mechanisms, ensuring that the Bayesian network accurately captured the causal structure of the qAOP. The network's inference results were cross-validated using alternative Bayesian frameworks, such as Bayesian hierarchical modeling, to confirm consistency and reliability.

Overall, the integration of the Bayesian network model in this investigation contributes to advancing the understanding of neurodevelopmental toxicity mechanisms, offering valuable insights that may inform future research directions and risk assessment strategies for PFAS and other environmental contaminants. By combining pathway-based insights with exposure scenarios, the model provides a predictive, probabilistic framework for evaluating the potential neurodevelopmental risks associated with PFAS exposure and advancing efforts in environmental toxicology.

7.9 Species Specificity and Relevance to Humans

Overall, the specificity of *Caenorhabditis elegans* (*C. elegans*) as a model organism is well-established, particularly in the context of genetic, developmental, and neurobiological research (Hunt, 2017; Leung et al., 2008; Ruszkiewicz et al., 2018). The nematode's genome is fully sequenced, and its developmental processes, including neurodevelopment, are highly conserved, making it an invaluable tool for studying basic biological functions (Basyoni & Rizk, 2016; Coghlan, 2005; Wilson, 1999). *C. elegans* offers unique advantages due to its simple anatomy, transparency, and well-documented cell lineage, enabling researchers to observe

developmental processes and neuronal differentiation in real time (Corsi et al., 2015; Kimble & Nusslein-Volhard, 2022). However, the evolutionary distance between *C. elegans* and humans introduces limitations regarding its applicability to human neurobiology. While certain key events (KEs), such as apoptosis and neurogenesis, are conserved across species, the absence of complex nervous systems and higher-order brain structures in *C. elegans* restricts its utility in modeling human-specific neural processes, particularly those related to cognitive functions and complex behaviors (Hobert, 2010; Randi & Leifer, 2020).

Despite these limitations, the relevance of *C. elegans* to human neurodevelopment and neurobiology is underscored by the conservation of numerous molecular pathways involved in neuronal differentiation, synaptic function, and neural signaling (Caldwell, Willicott, & Caldwell, 2020; Godini, Fallahi, & Pocock, 2022). Many genes and signaling pathways identified in *C. elegan*s have homologs in humans, making it possible to extrapolate findings from the nematode to human neural systems (Apfeld & Alper, 2018). The discovery of genes involved in axon guidance and synapse formation in *C. elegans* has been instrumental in understanding similar processes in human neurodevelopment, with direct implications for neurological disorders (Chisholm, Hutter, Jin, & Wadsworth, 2016; Mizumoto et al., 2023). Furthermore, *C. elegans* has been used to study mechanisms of neurodegeneration, providing insights into diseases such as Alzheimer's and Parkinson's (Alexander, Marfil, & Li, 2014). The ability to manipulate the genome of *C. elegans* easily and observe the outcomes of such manipulations in a living organism allows for a level of experimental control that is often not feasible in higher organisms (Sugi, 2016). However, it is important to recognize that while these conserved pathways offer significant insights, they represent only a subset of the complexities of human neurobiology. The absence of direct evidence linking certain KEs observed in *C. elegans*

to more intricate human neural networks highlight the need for cautious interpretation when applying findings from this model organism to human neurodevelopmental and neurodegenerative conditions.

In contrast to more complex model organisms, *C. elegans* lacks the sophisticated brain structures and neuronal diversity found in humans, which limits its utility in certain areas of neurodevelopmental research (Van Pelt & Truttmann, 2020). For instance, the simplicity of its nervous system, while advantageous for studying basic neural functions, does not fully capture the intricacies of human brain development, such as the formation of the cerebral cortex or the complex interactions between different brain regions (Barbulescu, Mestre, Oliveira, & Silveira, 2023). This limitation is particularly relevant when studying neurodevelopmental disorders, where the underlying causes often involve disruptions in higher-order neural processes and brain connectivity. As a result, while *C. elegans* serves as a powerful model for understanding fundamental aspects of neurodevelopment, its relevance to human neurobiology, particularly in the context of complex neural disorders (Melnikov, Kucharikova, Bardyova, Botek, & Kaiglova, 2023; Rapti, 2020), may require validation in more complex organisms, such as rodents, that possess more anatomically and functionally similar neural architectures. Integrating findings from *C. elegans* with data from higher organisms can provide a more comprehensive understanding of the underlying mechanisms driving neurodevelopment and neurodegeneration in humans, thereby enhancing the translational value of these research efforts.

7.10 Scientific Confidence in AOP

Scientific confidence in the Adverse Outcome Pathway (AOP) linking PFAS exposure to neurodevelopmental impairment is established through a rigorous evaluation of biological plausibility, essentiality, and empirical evidence, which are critical for regulatory frameworks utilized by agencies like the U.S. Environmental Protection Agency (EPA). The EPA uses these assessments to determine the relevance and applicability of AOPs for guiding risk management and regulatory decisions (Fenner-Crisp & Dellarco, 2016; Jacobs et al., 2020). To enhance this evaluation, a Bayesian model was employed to systematically integrate and synthesize diverse sources of evidence, allowing for probabilistic updates as new data emerges and uncertainties are addressed (Bujkiewicz et al., 2013; Paini et al., 2022; Spinu, Cronin, Enoch, Madden, & Worth, 2020). This approach improves the robustness and reliability of the conclusions regarding key event relationships and the overall AOP.

Below, we provide an overview of the evidence assessment, focusing on how AOP-specific considerations of biological plausibility, essentiality, and empirical support are applied to the evaluation of PFAS-related neurodevelopmental effects.

7.10.1 Support for Biological Plausibility of the KERs

Biological plausibility is defined by the presence of a mechanistic relationship between key events (KEs) that aligns with established biological knowledge (Organisation for Economic & Development, 2016; E. P. o. P. P. Products et al., 2017). In the context of the Adverse Outcome Pathway (AOP) linking PFAS exposure to neurodevelopmental impairments in *C. elegans*, the biological plausibility of the key event relationships (KERs) is strongly supported by a substantial body of mechanistic evidence. This includes extensive research showing how PFAS

affects neuronal development at various stages (Chowdhury et al., 2021; Di Nisio et al., 2022; Z. Feng et al., 2022; Sammi et al., 2019; Yue et al., 2020). Studies have consistently demonstrated that PFAS exposure leads to disruptions in key processes such as neural stem cell differentiation and neuronal maturation (N. Chen, Li, Li, Yang, & He, 2014). These disruptions are critical because they set the stage for subsequent adverse effects on neuronal function and cognitive performance, thereby validating the mechanistic links outlined in the AOP.

The first key event, PFAS-induced disruptions in neuronal development (KE#1), is well-supported by evidence indicating that PFAS impairs neural stem cell differentiation and neuronal maturation (Bose, Spulber, & Ceccatelli, 2023; Wu et al., 2024). Research has shown that PFAS can alter the expression of genes and proteins involved in these processes, leading to developmental abnormalities (Rericha et al., 2024; Zhuchen, Wang, Liu, & Shi, 2023). These disruptions have cascading effects on synaptic plasticity and neuronal connectivity (KE#2), both of which are essential for learning and memory functions (Bozorgmehr, Ardiel, McEwan, & Rankin, 2013). The final adverse outcome (AO), which is a decline in learning and memory capabilities in *C. elegans*, is directly linked to these earlier key events. The evidence consistently aligns with known neurobiological mechanisms, reinforcing the connection between PFAS exposure and cognitive impairments (O'Shaughnessy et al., 2023). This alignment supports the notion that the described KERs effectively capture the underlying biological processes leading to the observed adverse outcomes.

Additionally, variations in the sensitivity of *C. elegans* to PFAS exposure may uncover interactions with other biological pathways or mechanisms that contribute to the observed cognitive effects. PFAS may interact with neuronal signaling pathways or stress response mechanisms, influencing the extent of cognitive impairment (Leuthner et al., 2024). Exploring

these interactions can provide a more comprehensive understanding of how PFAS impacts neurodevelopment. Such insights further support the biological plausibility of the KERs by highlighting the complex interplay between PFAS exposure and various biological processes. The consistency of these findings across different experimental conditions and models strengthens the overall validity of the AOP, demonstrating that the KERs are grounded in well-established neurobiological principles.

7.10.2 Support for Essentiality of KEs

The concept of essentiality within the AOP framework is pivotal in establishing the credibility of the pathway. Essentiality is defined by whether the downstream key events (KEs) and/or the adverse outcome (AO) can be averted if an upstream KE is blocked or inhibited (Vinken et al., 2017). In the context of PFAS-induced impairments in learning and memory, the evidence supporting the essentiality of specific key events is robust. For instance, experimental studies have shown that when early disruptions in neurodevelopment caused by PFAS exposure are prevented, subsequent impairments in synaptic function and cognitive abilities do not manifest (Sciences, 2022). This finding strongly suggests that early key events are crucial for the progression to the final adverse outcome, making them indispensable in the sequence of events leading to neurodevelopmental deficits.

Further support for the essentiality of these key events comes from studies where key molecular pathways involved in neurodevelopment, particularly those impacted by PFAS exposure, are inhibited. In these cases, the prevention of early disruptions in signaling pathways protects against downstream effects on neuronal maturation and synaptic plasticity, ultimately preserving learning and memory functions (Jha et al., 2022). Additionally, genetic studies using

model organisms like *C. elegans* reinforce this conclusion (Ma et al., 2023). When specific genes responsible for the neuronal response to PFAS are knocked out or altered, the associated cognitive impairments fail to occur (Nisar et al., 2022). The consistency of these findings across various experimental models underscores the critical role of these key events in driving the adverse outcomes observed, further validating their essentiality within the AOP framework.

7.10.3 Empirical Evidence

The empirical evidence supporting the key events (KEs) and adverse outcome pathway (AOP) for PFAS-induced neurodevelopmental impairments in *C. elegans* is robust and multifaceted (Paini et al., 2022; Serafini et al., 2024). This model's strength lies in its ability to quantify the relationships between various biological events, allowing for a detailed understanding of how PFAS exposure leads to specific neurodevelopmental outcomes (Sohn & Narain, 2021). By analyzing large datasets, the Bayesian model identified significant associations between PFAS exposure and key neuronal disruptions, providing a statistical foundation for the proposed AOP (E. Panel o. P. P. Products et al., 2021).

Complementing the quantitative analysis, experimental data provide qualitative evidence that further validates the model's predictions. Behavioral assays in *C. elegans* exposed to PFAS consistently reveal impairments in learning and memory, key indicators of neurodevelopmental health (Sasakura & Mori, 2013). These behavioral changes are linked to molecular disruptions observed in critical signaling pathways involved in neuron function and synaptic formation (Harris et al., 2023). For instance, PFAS exposure has been shown to alter gene expression related to neurotransmitter release and synaptic plasticity (Foguth et al., 2020), both of which are

essential for proper learning and memory formation. The replication of these effects across various studies underscores the reliability of the empirical evidence.

Moreover, the consistency of the observed effects across different experimental settings and conditions strengthens the empirical support for this AOP. The alignment of behavioral deficits with molecular disruptions in *C. elegans* exposed to PFAS provides a coherent narrative that connects the mechanistic understanding of PFAS toxicity with observed neurodevelopmental impairments. This comprehensive body of evidence, both quantitative and qualitative, firmly establishes the link between PFAS exposure and neurodevelopmental deficits in *C. elegans*, reinforcing the biological plausibility and essentiality of the proposed KEs within this AOP.

7.10.4 Discussion of Likelihood of Alternative Modes of Action

When assessing the adverse outcome pathway (AOP) for PFAS-induced neurodevelopmental impairments in *C. elegans*, it is essential to consider the potential for alternative modes of action (MOAs) that might account for the observed effects. Although the primary focus has been on disruptions in key neuronal signaling pathways and synaptic functions, it's important to recognize that PFAS could also interfere with other biological processes that may contribute to neurodevelopmental deficits. For example, PFAS are known to interact with various cellular receptors and ion channels (Azhagiya Singam et al., 2024; Liao, Cui, Zhou, Duan, & Jiang, 2009; Yue et al., 2020), which could indirectly affect neuronal function or even activate alternative pathways that result in impaired learning and memory.

Moreover, the specific effects observed in *C. elegans* exposed to PFAS lend additional support to the plausibility of the proposed AOP as the primary explanation for neurodevelopmental deficits. The consistent patterns of behavioral and molecular changes

documented across various studies closely align with disruptions in well-characterized neuronal pathways, diminishing the likelihood that alternative MOAs are the primary cause of these outcomes. Furthermore, any alternative MOAs would need to account for the precise and reproducible nature of the effects seen in *C. elegans*, which currently aligns more convincingly with the proposed AOP.

In summary, the weight of evidence strongly favors the primary MOA involving direct neuronal disruption as the principal cause of PFAS-induced neurodevelopmental impairments in *C. elegans*. While alternative MOAs remain a possibility, they are less supported by the existing research and do not provide a more compelling explanation for the specific outcomes observed in this model organism.

7.11 Discussion: Applications of this AOP

The OECD guidelines for developing Adverse Outcome Pathways (AOPs) highlight the diverse applications these frameworks can have in toxicological research and regulatory practices (Ankley & Edwards, 2018). The utility of this particular AOP depends on the scientific confidence established in its predictions and relevance to specific contexts. Even when quantitative predictions regarding adverse outcomes from molecular initiating events (MIEs) are uncertain, the AOP can still provide valuable insights that inform various applications in risk assessment and hazard identification. In the sections that follow, we will discuss the confidence associated with this AOP and explore its potential applications in understanding the neurodevelopmental impacts of PFAS exposure in *C. elegans*.

7.11.1 Which KEs Can Be Used to Predict the Adverse Outcome

Currently, several key events (KEs) have been identified that may serve as indicators for adverse neurodevelopmental outcomes resulting from PFAS exposure in *C. elegans*. One critical KE is the activation of genes involved in neuron development, synaptic connectivity and plasticity, which play essential roles in establishing functional neural networks. Changes in the expression of these genes can provide valuable insights into the potential impacts on neuronal development and function.

Additionally, alterations in the structural integrity of neurons represent another significant KE. The presence and morphology of these neurons directly influence synaptic strength and transmission efficacy (Udvary et al., 2022). Research indicates that PFAS exposure may lead to a reduction in both the number and quality of dopaminergic neurons, which can impair synaptic communication and hinder the formation of robust neuronal circuits (S. Li et al., 2024). Moreover, disturbances in signaling pathways are relevant KEs that can influence neuronal growth and differentiation. These pathways are crucial for the cellular responses needed for normal neurodevelopment. Disruption in these signaling mechanisms may not only alter gene expression but also compromise neuronal resilience and adaptability.

While the correlation between these KEs and adverse neurodevelopmental outcomes is evident, more extensive research is needed to establish quantitative predictive models. The temporal dynamics and sustained effects of PFAS exposure on these KEs are critical factors that must be considered. A comprehensive understanding of the linkages between these KEs and downstream adverse outcomes is essential for developing reliable predictive frameworks that can guide risk assessment and regulatory decision-making concerning PFAS exposure and neurodevelopmental health.

7.11.2 Using this AOP for PFAS Mixtures

The complexity of assessing the neurodevelopmental impacts of per- and polyfluoroalkyl substances (PFAS) is heightened by the ubiquitous presence of multiple PFAS compounds in both environmental and biological contexts (Luo et al., 2022). This AOP framework serves as a critical tool for evaluating the cumulative neurodevelopmental effects of PFAS mixtures on *Caenorhabditis elegans*, a model organism that facilitates the study of genetic and environmental interactions in a well-mapped neuronal network. By delineating the key events (KEs) that characterize neurodevelopmental toxicity, this AOP allows researchers to identify potential interactions among various PFAS compounds. Understanding how different PFAS might coalesce to disrupt neuronal function is essential, as synergistic effects could amplify the neurotoxic potential beyond what individual compound assessments might reveal. For example, simultaneous exposure to multiple PFAS could lead to greater impairments in synaptogenesis and neuronal signaling, potentially resulting in severe long-term deficits in motor coordination and cognitive functions.

The framework provided by this AOP also enables a more nuanced risk assessment of PFAS mixtures by clarifying the role of each component in contributing to neurodevelopmental toxicity. By analyzing the relative potencies and mechanisms of action of various PFAS within the context of the AOP, researchers can prioritize specific mixtures for further investigation based on their predicted neurotoxic potential. For instance, the identification of certain PFAS as more potent disruptors of synaptic connectivity could guide targeted studies aimed at understanding their mechanisms of action. Furthermore, such an approach can inform public health strategies and regulatory policies by pinpointing the most hazardous PFAS mixtures that

require immediate attention, thereby enhancing our capacity to protect vulnerable populations, particularly during critical developmental windows.

Finally, the application of this AOP to PFAS mixtures supports the integration of data from high-throughput screening studies, enabling researchers to derive robust conclusions about the neurodevelopmental consequences of environmental exposure (Table 7.6). By systematically compiling and analyzing data on the effects of various PFAS combinations on neurodevelopmental endpoints in C. elegans, this AOP framework provides valuable insights into the complex interactions at play. Such integration not only enhances our understanding of the cumulative impact of PFAS exposure but also aids in the refinement of testing methods and guidelines aimed at evaluating chemical safety. Ultimately, the insights gained from employing this AOP for PFAS mixtures will pave the way for more informed regulatory decisions and protective measures, safeguarding neural health across populations.

7.11.3 Using this AOP for Integrating Approaches to Testing

The application of this AOP within an integrated approach to testing and assessment (IATA) offers a robust framework for evaluating the neurodevelopmental hazards associated with PFAS exposure (Hernández-Jerez et al., 2021). One of the primary utilities of this AOP is to assess the potential of various PFAS compounds to disrupt critical neurodevelopmental processes, particularly through mechanisms that involve sustained activation of signaling pathways. In this context, the AOP can guide the development of decision trees that prioritize testing strategies based on the likelihood of PFAS compounds to induce neurodevelopmental toxicity. Initial steps in the IATA can involve the use of high-throughput screening techniques and in silico models to evaluate the capacity of these substances to interact with key receptors,

leading to transcriptional changes across multiple cell types (Schmeisser et al., 2023). Such early-stage assessments are vital for identifying compounds that warrant further investigation.

While the current understanding of PFAS-induced neurodevelopmental effects is still evolving, the AOP facilitates the identification of key events that are predictive of adverse outcomes. Compounds demonstrating significant activity in preliminary assays can be subjected to more rigorous testing, such as in vitro neurodevelopmental assays, which can further elucidate their impact on neuronal function and connectivity (Dravid, Raos, Svirskis, & O'Carroll, 2021). For example, assays measuring alterations in synaptogenesis or neuronal morphology can provide critical data on the potential for a given PFAS to cause lasting neurodevelopmental damage. By systematically evaluating the effects of PFAS on these endpoints, researchers can gather valuable insights that help determine the relevance of specific compounds to neurodevelopmental disorders.

Furthermore, integrating exposure data with the AOP enhances the predictive power of the assessment process (Tollefsen et al., 2014). For instance, information on environmental concentrations of PFAS and their bioavailability can be combined with in vitro findings to model potential risks to human health (Lu, Li, Yang, & Yu, 2021). This integrated approach can also inform decisions about the need for in vivo studies, such as chronic exposure models in *C. elegans* or other relevant organisms, to validate the effects observed in vitro. By employing a comprehensive decision framework that accounts for both mechanistic data and exposure scenarios, this AOP can significantly improve our capacity to predict the neurodevelopmental risks posed by PFAS mixtures, ultimately informing regulatory strategies and public health initiatives.

7.11.4 Using this AOP to Inform Test Method Development or Refinement

Applying this AOP to inform test method development is essential for enhancing our understanding of PFAS-induced neurodevelopmental toxicity. An integrated testing approach, incorporating both *in vitro* and *in vivo* assays, can effectively differentiate between PFAS compounds that pose risks to neuronal health and those that do not (Crofton et al., 2022). By developing a suite of assays that evaluate key neurodevelopmental endpoints—such as transcriptional activation and neurodevelopmental outcomes—researchers can create predictive models that assess the potential for specific PFAS compounds to induce adverse effects during critical developmental periods. This integrated approach can provide a more comprehensive assessment of PFAS toxicity and its implications for neurodevelopment.

Furthermore, within the framework of OECD test guidelines, establishing performance criteria for in vitro assays, particularly those assessing gene interactions and their effects on neuronal differentiation, is crucial (Juberg et al., 2023). By refining these assays, researchers can improve their reliability and ensure that they yield meaningful insights into the neurodevelopmental impacts of PFAS. This includes optimizing assay conditions, standardizing exposure durations, and enhancing the relevance of endpoints assessed. The development of validated test guidelines focused on neurodevelopmental outcomes will not only streamline regulatory assessments of PFAS but also enhance scientific rigor in toxicity testing. By integrating this AOP into method development, researchers can elucidate the mechanisms underlying PFAS-induced neurodevelopmental effects, ultimately supporting more effective regulatory measures to protect human health.

7.11.5 Taxonomic Application: Using this AOP for Screening-Level Evaluations of Hazard and Risk to Humans

The exploration of hazards and risks posed by per- and polyfluoroalkyl substances (PFAS) has become increasingly sophisticated, particularly regarding their neurodevelopmental impacts. Utilizing the quantitative adverse outcome pathway (AOP) framework allows for a comprehensive assessment of how PFAS exposure can disrupt critical biological processes that govern neuronal development (Kaiser et al., 2022). In this context, *C. elegans* serves as a powerful model organism, providing insights into the cellular and molecular mechanisms through which PFAS may induce neurotoxic effects. The simplicity of the *C. elegans* nervous system, coupled with its well-defined behavioral assays, enables researchers to investigate the consequences of PFAS exposure on locomotion, learning, and memory (Ruszkiewicz et al., 2018). These behavioral endpoints not only reflect the organism's neurodevelopmental integrity but also serve as indicators for potential adverse outcomes in humans, given the evolutionary conservation of many neurobiological pathways (van Thriel et al., 2012).

Employing the AOP framework facilitates a detailed understanding of the effects of PFAS at various biological levels, thereby illuminating the multifaceted nature of neurodevelopmental toxicity. Research has demonstrated that PFAS can interfere with critical pathways involved in neurogenesis, synaptic plasticity, and neurotransmitter signaling (Cao & Ng, 2021). These disruptions can lead to oxidative stress, inflammation, and altered gene expression, all of which are known to negatively impact neuronal maturation and function (Uttara, Singh, Zamboni, & Mahajan, 2009). The use of *C. elegans* not only allows for high-throughput screening of multiple PFAS compounds but also provides an opportunity to elucidate the dose-response relationships that characterize neurodevelopmental outcomes. By assessing

behavioral alterations in response to different concentrations of PFAS, researchers can identify specific thresholds and critical windows of exposure that are particularly susceptible to neurotoxic effects (Ingber & Pohl, 2016).

However, it is crucial to recognize the inherent differences between *C. elegans* and humans when interpreting findings derived from AOP-based assessments (Table 7.7). While *C. elegans* offers a relevant model for studying neurodevelopmental processes, species-specific variations in sensitivity and response to toxicants must be carefully considered (Hunt, 2017). For instance, differences in metabolism, cellular signaling pathways, and developmental timelines can influence how PFAS exposure translates to human health risks. Therefore, while the AOP framework provides a robust platform for screening-level evaluations, integrating data from *C. elegans* studies with human epidemiological data and other relevant research is essential for more accurately predicting the potential hazards posed by PFAS. This holistic approach will enhance our understanding of developmental neurotoxicity and inform regulatory decisions aimed at mitigating the risks associated with PFAS exposure.

7.12 Conclusion

This review provides a comprehensive foundation for understanding the neurodevelopmental effects of per- and polyfluoroalkyl substances (PFAS), highlighting the utility of *C. elegans* as a model organism in this field of study. PFAS are a diverse group of synthetic chemicals known for their persistence in the environment and their potential to disrupt various biological processes. Research has demonstrated that exposure to PFAS can adversely affect neuronal development and function, potentially leading to long-term cognitive and behavioral deficits (Currie, Wang, & Tang, 2024). By examining the mechanisms underlying

these effects, this review aims to establish a quantitative adverse outcome pathway (AOP) that captures the critical events associated with PFAS-induced neurotoxicity. This framework will facilitate a better understanding of the dose-response relationships and interspecies differences in sensitivity to PFAS, paving the way for more effective risk assessments and regulatory measures aimed at protecting human health and the environment. As the body of evidence grows, it will be essential to refine this AOP to incorporate emerging data and insights, ultimately enhancing our ability to evaluate the impacts of PFAS exposure on neurodevelopment.

7.13 Credit Author Statement

Seth Currie: Methodology, Investigation, Formal analysis, Writing- Original Draft, Writing-Reviewing and Editing. Ted W. Simon: Writing-Review and Editing Jia-Sheng Wang: Writing-Reviewing and Editing, Writing. Lili Tang: Resources, Supervision, Funding acquisition, Writing-Reviewing and Editing. All authors have read and agreed to the published version of the manuscript.

7.14 References

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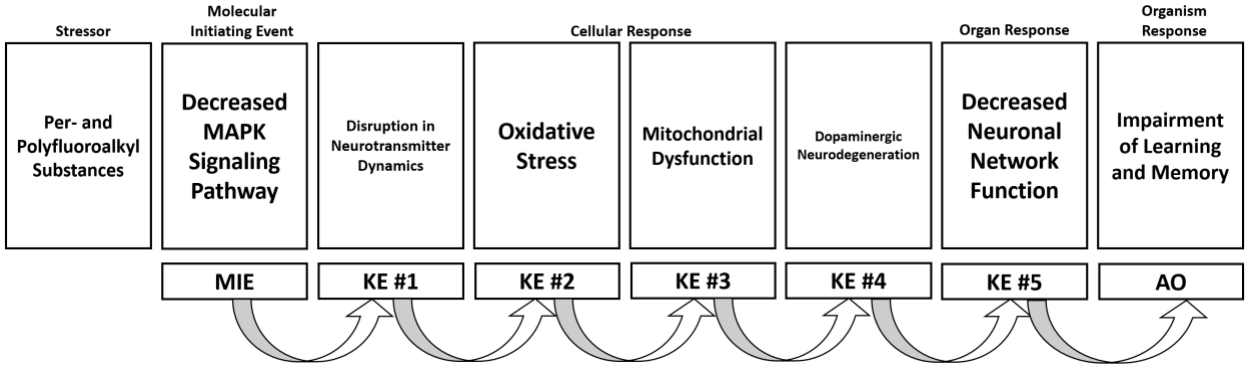


Figure 7.1. Schematic of the Neurodevelopmental Inhibition AOP showing the molecular initiating event (MIE), intermediate key events (KE), and the adverse outcome (AO).

Table 7.1: Omics-Based Insights into the exposure of per- and polyfluoroalkyl substances (PFAS) in *C. elegans* for suggesting potential molecular mechanisms for neurodevelopmental toxicity.

Study	PFAS Types	Exposure Conditions	Omics Platform	Mechanism/Pathways Involved	Key Findings
Currie et, al., 2025	PFBA, PFOA, PFBS, PFHxS, PFOS	48-hour Exposure, 5ppm – 50ppm	Transcriptomics	MAPK Signaling Pathway	PFAS led to disruption in both metabolism and neuronal system processes.
Li et al., 2020	PFOA	48-hour Exposure 1.0 ng/L	Genomics	MAPK Signaling Pathway, fatty acid degradation and TGF-β	PFAS disrupted important signaling pathways, affecting key genes involved in neuronal membrane function.
Kim et al., 2020	PFOA, PFOS	24-hour Exposure 0.5 mg/L (PFOS) 2 mg/L (PFOA)	Lipidomics	aminoacyl-tRNA biosynthesis, valine, leucine, and isoleucine biosynthesis, and phenylalanine, tyrosine and tryptophan biosynthesis pathways	PFAS disrupted amino acid and lipid composition, resulting in changes to neuronal membrane function and neurotransmitter biosynthesis.

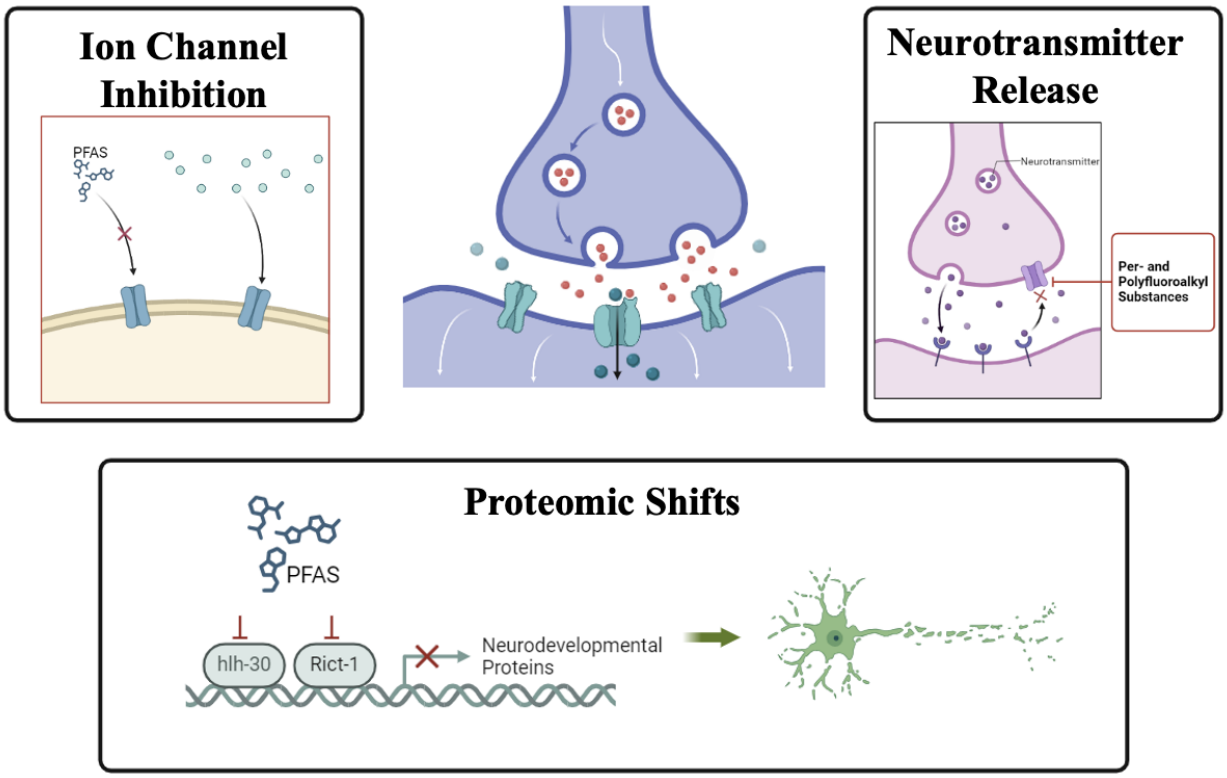


Figure 7.2: Cellular Interactions of Per- and Polyfluoroalkyl Substances (PFAS) Impacted Through Disruptions in MAPK Signaling Pathway as a Potential Molecular Initiating Event (MIEs)

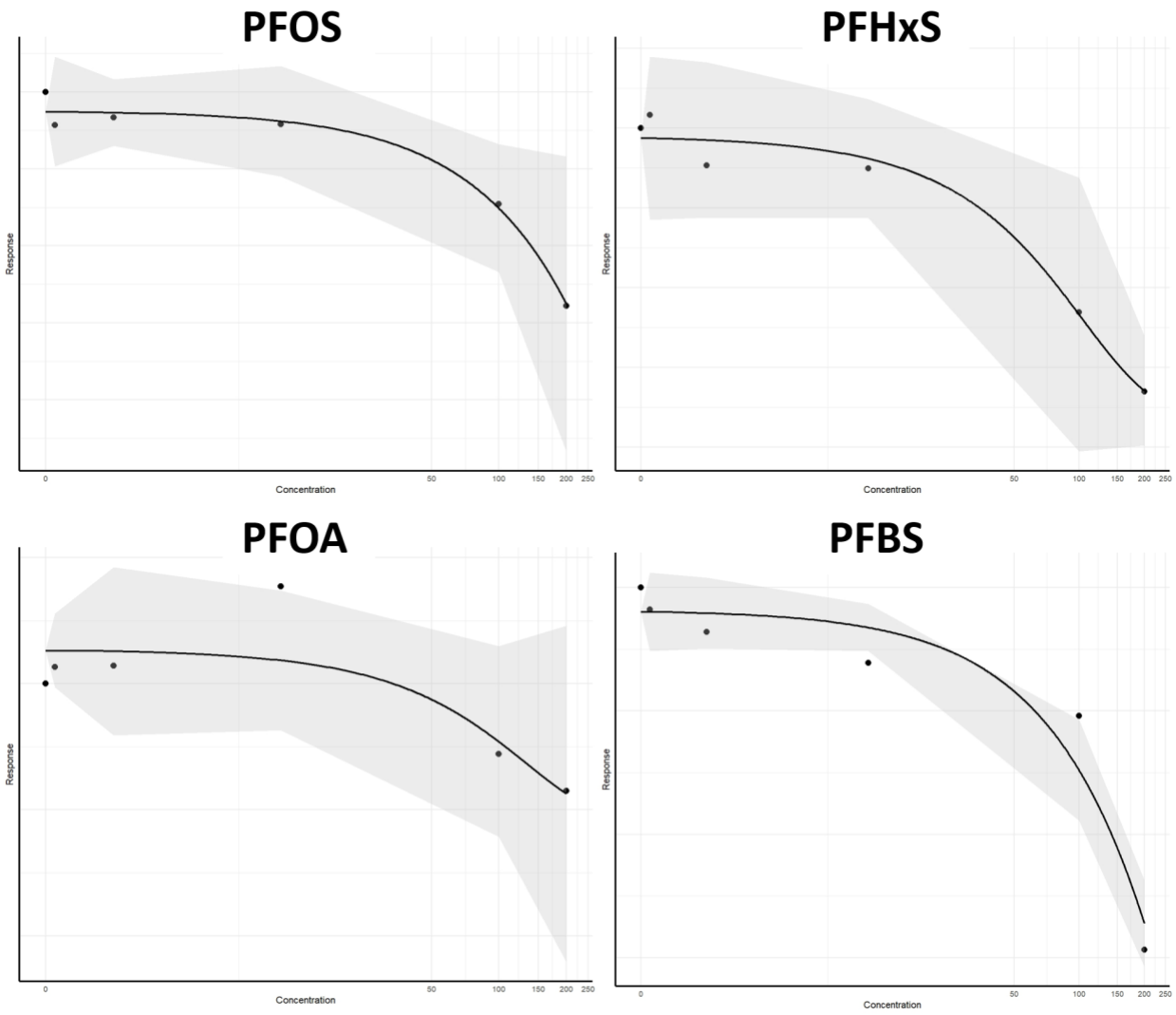


Figure 7.3: Impacts of Per- and Polyfluoroalkyl Substances on GABA Neurotransmitters within *C. elegans* after 48-hours of exposure. Benchmark Concentration 10% (BMC) represented as µM.

Table 7.2: Effects of PFAS Exposure on Oxidative Stress Levels Generation in *Caenorhabditis elegans*

Study	PFAS	Key Observations	Supporting Evidence
Currie et al., 2025	PFOS, PFBS, PFHxS, PFOS	This study found that exposure to PFAS, particularly PFBS and PFOS, induced significant oxidative stress. The effects were most pronounced at higher concentrations (0.1–200 µM) and after 48 hours of exposure. The study highlights the heightened sensitivity of developmental stages to oxidative stress caused by PFAS exposure.	The study provided evidence that PFAS exposure, specifically PFBS and PFOS, led to significant increases in oxidative stress at higher concentrations. These increases in oxidative stress was linked to reduced motility, with developmental stages of *C. elegans* showing particular sensitivity to oxidative stress. This underscores the need to focus on early-life exposure in toxicity assessments.
Sammi et al., 2019	PFOS	PFOS exposure induced significant oxidative stress in C. elegans, even at low concentrations (≥2 µM), leading to altered ROS levels. The study found that dopaminergic neurotoxicity was associated with oxidative stress.	The study observed elevated oxidative stress levels in *C. elegans* exposed to PFOS, with oxidative stress being evident at concentrations as low as 2 µM. Neuroprotective approaches, including antioxidants and PFAS-protein dissociation, mitigated the effects of oxidative stress.

Table 7.3: Mitochondrial Dysfunction following PFAS Exposure in *C. elegans*

Study	PFAS	Key Observations	Supporting Evidence
Currie et al., 2025	PFOS, PFBS, PFHxS, PFOA	Significant mitochondrial dysfunction, especially at higher concentrations. Mitochondrial function impaired at 48 hours.	Exposure to PFAS, such as PFBS and PFOS, caused a reduction in motility and significant oxidative stress at higher concentrations. Mitochondrial dysfunction was notably observed in wild-type *C. elegans* at 0.1-200 µM concentrations, with more pronounced effects at 48 hours.
Sammi et al., 2019	PFOS	Mitochondrial content affected at low exposure levels (≥1 ppm). Oxidative stress increased with PFOS exposure.	At exposures ≥2 µM, PFOS reduced mitochondrial content and increased oxidative stress. Superoxide dismutase mutation in *C. elegans* exacerbated these effects, suggesting a mitochondrial dysfunction pathway. Protective antioxidants mitigated damage, supporting mitochondrial involvement in PFOS neurotoxicity.
Wei et al., 2021	PFOS	Disruption of fatty acid metabolism and mitochondrial function. Reduced ATP synthesis observed.	PFOS exposure led to a reduction in ATP synthesis, as measured by the luciferase method, and a dysregulation of fatty acid metabolism. The decrease in ATP production highlights the mitochondrial dysfunction associated with PFOS exposure.
Feng et al., 2022	HFPO-DA	Developmental delay and transcriptional changes in mitochondrial-related genes.	Exposure to HFPO-DA (1.25 × 10-5–4 g/L) caused developmental delays, and differential gene expression linked to detoxification and mitochondrial functions was observed. Despite inconclusive transcriptional data, the low-dose transcriptional changes indicate possible mitochondrial involvement.

Table 7.4: Consequences of PFAS Exposure on Dopaminergic Neurodegeneration in *C. elegans*

Study	Exposure Conditions	PFAS	Key Observations
Currie et al., 2025	24-hour exposure, 0.1 µM – 100 µM	6:2 FTS, HFPO-DA, NEtFOSAA, PFBA, PFBS, PFHxA, PFHxS, PFNA, PFOA, PFOS, PFOSA	Dose-dependent neurotoxic effects observed, with more severe effects for long-chain PFAS like PFOA and PFOS. Significant reductions in neuronal function across exposure times.
Sammi et al., 2019	72-hour exposure, 25-200 ppm	PFOS	High LC50 value, indicating relatively low toxicity at the tested concentrations.
Chen et al., 2014	48-hour exposure, 2 µM and 20 µM	PFOS	No significant cognitive impairments observed at these concentrations.

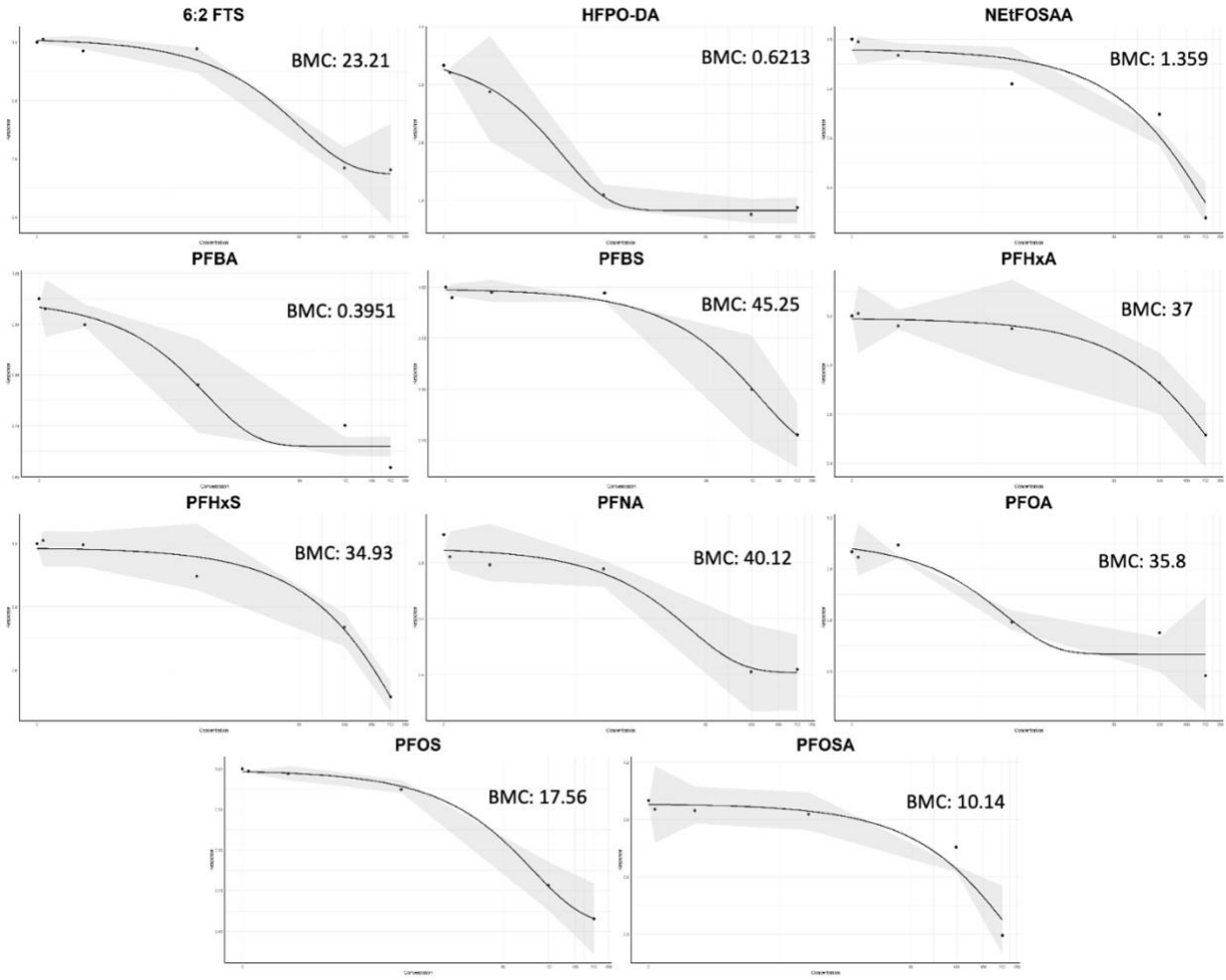
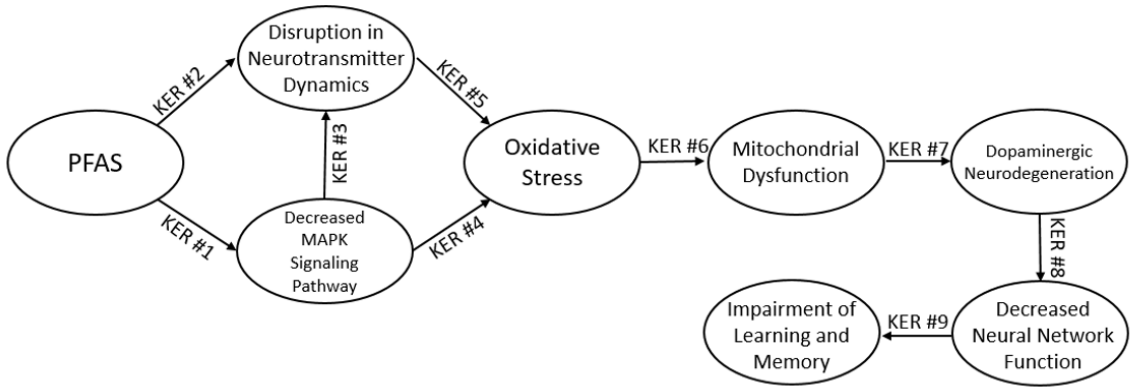


Figure 7.4: Impacts of Per- and Polyfluoroalkyl Substances on Neuronal Network Function within *C. elegans* after 48-hours of exposure. Benchmark Concentration 10% (BMC) represented as µM.

Table 7.5: Impacts of learning and memory induced by per- and polyfluoroalkyl substances (PFAS) with *C. elegans*.

Study	Endpoint [Learning/Memory]	PFAS Types	Exposure Conditions	Assessment Methods	Cognitive Response Measures
Currie et al., 2024	Learning	6:2 FTS, HFPO-DA, NEtFOSAA, PFBA, PFBS, PFHxA, PFHxS, PFNA, PFOA, PFOS, PFOSA	48-hour exposure, 0.1 µM – 100 µM	Pirouette	Dose-dependent reductions in pirouette frequency for most PFAS, indicating impaired learning capabilities, with stronger effects observed for PFOA and PFOS.
	Memory	6:2 FTS, HFPO-DA, NEtFOSAA, PFBA, PFBS, PFHxA, PFHxS, PFNA, PFOA, PFOS, PFOSA	48-hour exposure, 0.1 µM – 100 µM	Absolute Peristatic Speed	Decreased speed correlated with PFAS exposure, with the most pronounced impairments observed for PFOS and PFOA, demonstrating memory-related deficits.
Chowdhury et al., 2022	Learning	PFOS	48-hour exposure, 0.0001 µM – 2 µM	Chemotaxis Plasticity	Not significant compared to the control group
Sana et al., 2021	Learning	PFOA	48-hour exposure, 1 µM and 2 µM	Chemotaxis Plasticity	No Significant Changes



KER	Predictor Variable	Response Variable	Probabilistic Value
KER #1	PFAS	Decreased MAPK Signaling Pathway	0.587858
KER #2	PFAS	Disruption in Neurotransmitter Dynamics	0.26429
KER #3	Decreased MAPK Signaling Pathway	Disruption in Neurotransmitter Dynamics	0.50312
KER #4	Decreased MAPK Signaling Pathway	Oxidative Stress	0.51547
KER #5	Disruption in Neurotransmitter Dynamics	Oxidative Stress	0.58881
KER #6	Oxidative Stress	Mitochondrial Dysfunction	0.378576
KER #7	Mitochondrial Dysfunction	Dopaminergic Neurodegeneration	0.56677
KER #8	Dopaminergic Neurodegeneration	Decreased Neural Network Function	0.70414
KER #9	Decreased Neural Network Function	Impairment of Learning and Memory	0.46018

Figure 7.5: Bayesian Network Model of the Quantitative Adverse Outcome Pathway for Developmental Neurotoxicity of Per- and Polyfluoroalkyl Substances in *Caenorhabditis elegans*. This network represents the relationships between key events (KEs) involved in PFAS-induced neurodevelopmental toxicity. Nodes Correspond to key biological processes, while arcs indicate a causal relationship.

Table 7.6: Effects of Per- and Polyfluoroalkyl Substances (PFAS) Mixtures on Key Events Associated with Developmental Neurotoxicity within *C. elegans*.

Study	**Key Event**	**Key Findings**
Currie et al., 2025	Decreased MAPK Signaling Pathway	PFAS mixtures had a significant impact on the MAPK signaling pathway, leading to decreased phosphorylation of key MAPK proteins. This decrease was greater in the mixture than in individual PFAS exposure.
Currie et al., 2025	Disruption in Neurotransmitter Dynamics	PFAS mixtures alter neurotransmitter release and uptake, resulting in cumulative effects on synaptic communication compared to individual PFAS exposure.
Bonilla et al., 2024	Oxidative Stress	While PFAS mixtures have been shown to increase oxidative stress in other models, no studies to date have directly examined their combined effects in C. elegans.
Bonilla et al., 2024	Mitochondrial Dysfunction	Although PFAS mixtures exacerbate mitochondrial dysfunction in other organisms, there is no current research on their combined effects in C. elegans.
Currie et al., 2025	Dopaminergic Neurodegeneration	Exposure PFAS mixtures revealed a compounded impact on dopaminergic neurons, with greater neuronal damage observed compared to some individual PFAS.
Currie et al., 2025	Decreased Neuronal Network Function	Exposure to PFAS mixtures revealed a synergistic impact on neuronal network function, with greater functional impairments observed compared to some individual PFAS.
Currie et al., 2024	Impairment of Learning and Memory	Mixtures appears to have an additive inhibitory effect on learning and memory due to the overall inhibitory effect observed is consistent with the sum of the individual effects of each PFAS component.

Table 7.7: Concordance of KEs in the AOP between *C. elegans* for the purpose of assessing human relevance

Key Event	Qualitative Concordance	Quantitative Concordance
MIE Decreased MAPK Signaling Pathway	Deactivation of the MAPK signaling pathway is observed in both *C. elegans* and humans following PFAS exposure, particularly in neural tissues. The pathway's activation is consistent across species, though the response may vary depending on species-specific signaling mechanisms.	The extent of MAPK pathway deactivation is concentration-dependent in both *C. elegans* and humans, though exact levels differ due to physiological and metabolic differences.
KE #1 Disruption in Neurotransmitter Dynamics	Altered neurotransmitter release, uptake, and signaling are evident in C. elegans and humans following PFAS exposure, disrupting neural communication.	Quantitative differences exist in neurotransmitter alterations, but both species exhibit dose-dependent effects on neural dynamics.
KE #2 Oxidative Stress	Elevated oxidative stress markers are consistently observed in C. elegans and human cell lines after PFAS exposure, indicating a conserved response.	Both species show dose- and time-dependent increases in oxidative stress, though humans may experience additional environmental modifiers.
KE #3 Mitochondrial Dysfunction	PFAS exposure impairs mitochondrial function in C. elegans and humans, leading to energy deficits and disrupted cellular homeostasis.	Quantitative effects, such as reductions in ATP production and mitochondrial membrane potential, are dose-dependent but vary between species.
KE #4 Dopaminergic Neurodegeneration	There is a strong connection between C. elegans and humans in the loss of dopaminergic neurons after PFAS exposure, contributing to motor dysfunction and neurodegeneration in both species.	Quantitative effects vary, but both species show dose-dependent neuron loss, with some differences in sensitivity and threshold doses.
KE #5 Decreased Neuronal Network Function	Impairment in synaptic communication and neuronal connectivity is observed in both C. elegans and humans as a consequence of PFAS exposure, affecting overall network function.	With dose-dependent decreases in neuronal network activity, although the specific extent of functional loss may vary between species.
AO Impairment of Learning and Memory	PFAS-induced learning and memory deficits are consistent across C. elegans and humans, affecting cognitive processes similarly due to compromised neuronal signaling.	Similar patterns of cognitive impairment observed at comparable exposure levels, although exact PFAS sensitivity differs between species.

CHAPTER 8

CONCLUSIONS AND FUTURE DIRECTIONS

The primary objective of this dissertation was to develop a quantitative adverse outcome pathway (qAOP) network to assess the developmental neurotoxicity (DNT) of PFAS mixtures in *Caenorhabditis elegans*. The importance of this research is underscored by the persistent environmental presence of PFAS and the rising concerns about their neurodevelopmental impacts on vulnerable populations, including children. By leveraging *C. elegans* as a model organism, this study aimed to bridge critical knowledge gaps in understanding the neurotoxicologically mechanisms of PFAS exposure. Emerging evidence demonstrates that PFAS compounds can disrupt key neurodevelopmental pathways, leading to cognitive and behavioral impairments. However, comprehensive analysis of the developmental neurotoxicity of PFAS mixtures, particularly through a qAOP framework, has been limited. This dissertation contributes to closing this gap by investigating both individual PFAS and a constructed PFAS reference mixture to develop a qAOP network that integrates molecular, cellular, and behavioral data to examine whole-mixture effects to reflect realistic exposure scenarios. These efforts provide valuable insights into the dose-response relationships and mechanistic pathways through which PFAS impacts neurodevelopment. A high-throughput approach, along with innovative techniques and behavioral assessments in *C. elegans*, enabled a systematic evaluation of key neurodevelopmental events and associated adverse outcomes. The findings suggest that PFAS-induced disruptions in neuronal growth, synaptic function, and behavioral responses may be

sufficiently similar across individual and mixture-based PFAS exposures. Reflecting on the dissertation's specific goals, we conclude that:

1. Exposure to PFAS has been shown to correlate with an increased risk of neurodevelopmental disorders, including impairments in cognitive functions such as memory and IQ, as well as behavioral issues like ADHD and ASD, underscoring the significant impact these chemicals have on brain development.
2. PFAS demonstrated significant toxicity on growth, reproduction, and behavior in *C. elegans*, with the larval stages showing the highest sensitivity to exposure. These findings underscore the importance of considering developmental stages in toxicity assessments and highlight the potential risks of PFAS exposure to neurodevelopment.
3. PFAS exposure demonstrated a significant correlation between bioaccumulation and impaired learning and memory in *C. elegans*. These findings highlight the compounded effects of PFAS mixtures on cognitive function and stress the importance of accounting for bioaccumulation during developmental exposure in toxicity assessments.
4. PFAS exposure, assessed through high-throughput screening integrated with artificial intelligence, has been shown to significantly impact neurodevelopment and the neuronal system. Toxicity was identified as being more pronounced in mixtures which exacerbated neurodevelopmental adverse effects, highlighting the need for further research on mixture exposure.
5. PFAS exposure in *C. elegans* caused significant disruptions in metabolism and neuronal system processes, particularly affecting nervous system activity and behavior. These findings highlight the complex mechanisms of PFAS toxicity and underscore the potential human

health implications, emphasizing the need for further investigation using *C. elegans* to inform regulatory policies and mitigate the risks of these persistent environmental pollutants.

6. The development of the quantitative adverse outcome pathway (qAOP) for PFAS-induced neurodevelopmental toxicity in *C. elegans* provides a valuable framework for understanding the molecular and behavioral effects of PFAS exposure. This approach enhances our ability to predict potential risks, supporting more informed risk assessments and regulatory decisions aimed at protecting human and environmental health.

In light of these conclusions, the following suggestions for future exploration on relevant topics are outlined below:

1. Further research is needed to incorporate additional PFAS compounds and mixtures to explore their effects on neurodevelopment, considering different exposure concentrations and chemical structures. This will help refine the mechanistic understanding of PFAS-induced developmental neurotoxicity.
2. Future studies should focus on the long-term, transgenerational effects of PFAS exposure, specifically investigating how early-life exposure impacts adult worms and their subsequent generations, including neurodevelopmental outcomes and behavior. Investigating the effects of PFAS exposure on adult worms and the next generation's development will provide valuable insights into the enduring consequences of PFAS on neurodevelopment.
3. Future research should focus on combining transcriptomic, proteomic, and metabolomics analyses to uncover the full spectrum of molecular alterations caused by PFAS exposure. This approach will help identify potential biomarkers and molecular signatures of

neurotoxicity, improving our ability to assess the risks of PFAS-induced neurodevelopmental disruptions and guiding more effective regulatory measures.

4. To enhance the translational potential of *C. elegans* findings, future research should prioritize the development of human-based in vitro models, such as stem cell-derived neurons, which can more closely mimic human neurodevelopmental processes. These models would provide a more accurate representation of how PFAS exposure affects neurodevelopment in humans, allowing for better risk prediction and a deeper understanding of the molecular mechanisms underlying PFAS-induced neurotoxicity.
5. To make toxicity findings more relevant, it is important to integrate real-world exposure data, such as concentrations of PFAS in drinking water, food, and the environment, with laboratory-based results. Testing real-world water samples for PFAS levels and their impact on neurodevelopment in model organisms will provide more accurate insights into human exposure risks and improve the accuracy of risk assessments and regulatory decisions.