Cytotoxic and transcriptomic effects of the rainbow trout gill (RTgill-W1) cell line exposed to environmentally relevant pesticides

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Abstract

Each year hundreds of new pesticide products are introduced globally with the potential to adversely impact non-target organisms like aquatic wildlife. However, ethical concerns over the use of whole animals and the resources, money, and time required to perform traditional toxicity assessments on these chemicals have called for a paradigm shift away from animal-based *in vivo* testing methods towards *in vitro* mechanism-based tests involving cell lines. Validation and standardization of the Organization for Economic Co-operation and Development (OECD) Test No. 249 involving the rainbow trout gill (RTgill-W1) cell line to characterize cytotoxicity has opened the possibility for what *in vitro* models can achieve from a regulatory stance. The overarching goals of this thesis research were to 1) further our knowledge on the applicability of this assay for chemicals (in this case, pesticides) not previously investigated and 2) determine if the assay can be used to derive transcriptomic points of departure values (tPODs) for these pesticides, which represent benchmark doses (BMDs) at which no adverse effects are observed at the molecular level.

In this thesis, 19 pesticides with diverse mechanisms of toxic action and physiochemical properties were selected based on their relevance and importance to Canadian ecosystems. Chapter 2 saw these 19 pesticides exposed to the RTgill-W1 cells to characterize the cytotoxic response. The OECD Test No. 249 assay was able to be performed in a 96-well plate format seeding 35,000 cells/well without compromising the quality control criteria described in the guideline or cytotoxicity results. Exposure to pesticides caused a decrease in cell viability but given the specific nature of pesticides, some mechanisms of toxic action could not be elicited in the RTgill-W1 cells and responses were not observed. Of the cytotoxic pesticides, results ranged

from one to four orders of magnitudes higher than the *in vivo* lethality data taken from rainbow trout studies. Chapter 3 investigated transcriptome-wide changes in the RTgill-W1 cells following exposure to six of the most cytotoxic pesticides based on the results from Chapter 2 using RNA-sequencing technology. Exposure to pesticides could induce transcriptomic (molecular) changes in the RTgill-W1 cells. tPOD values could be derived from transcriptomic data, which provided more protective values than *in vitro* data and could be used to prioritize pesticides of concern. Pathway level benchmark dose analysis also provided further insights into each chemical's mechanism of action. This thesis provided further insights into the promises of coupling the RTgill-W1 assay with transcriptomic techniques for characterizing a chemical's adverse effects. Moreover, this work advanced our understanding of the molecular mechanism of toxicity for pesticides previously uncharacterized.

Résumé

Chaque année, des centaines de nouveaux pesticides sont introduits dans le monde, avec le potentiel d'avoir un impact négatif sur les organismes non ciblés comme la faune aquatique. Cependant, les préoccupations éthiques concernant l'utilisation d'animaux et les resources, l'argent et le temps nécessaires pour effectuer les méthodes traditionnelles pour évaluer la toxicité de ces produits chimiques, ont appelé à un changement de paradigme des méthodes de test *in vivo* à base d'animaux vers des tests basés sur des mécanismes *in vitro* impliquant des lignes cellulaires. La validation et normalisation de l'essai n°249 de l'Organisation de coopération et de développement économiques (OCDE) impliquant la ligne cellulaire de la truite arc-en-ciel (RTgill-W1) pour caractériser la cytotoxicité ont ouvert la voie à ce que les modèles *in vitro* peuvent réaliser d'un point de vue réglementaire. Les objectifs primordiaux de cette recherche de thèse étaient 1) d'approfondir nos connaissances sur l'applicabilité de ce test pour des produits chimiques (dans ce cas, des pesticides) qui n'avaient pas été étudiés auparavant et 2) de déterminer si le test peut être utilisé pour dériver des valeurs de points de départ transcriptomiques (tPODs), qui représentent des doses de référence (BMD) auxquelles aucun effet indésirable n'est observé au niveau moléculaire.

Dans cette thèse, 19 pesticides avec divers mécanismes d'action toxique et des propriétés physiochimiques ont été sélectionnés en fonction de leur pertinence et de leur importance pour les écosystèmes canadiens. Dans chapitre 2, ces 19 pesticides ont été exposés aux cellules RTgill-W1 afin de caractériser la réponse cytotoxique. L'essai n° 249 de l'OCDE a pu être réalisé dans une plaque à 96 puits avec un ensemencement de 35 000 cellules/puits sans compromettre les critères de contrôle de la qualité décrits dans la directive ou les résultats de la cytotoxicité.

L'exposition aux pesticides a provoqué une diminution de la viabilité cellulaire mais compte tenu de la nature spécifique des pesticides, certains mécanismes d'action toxique n'ont pas pu être déclenchés dans les cellules RTgill-W1 et aucune réponse n'a été observée. Parmi les pesticides cytotoxiques, les résultats variaient d'un à quatre ordres de grandeur supérieurs que les données de létalité *in vivo* extraites des études sur la truite arc-en-ciel. Le chapitre 3 a examiné les changements transcriptomiques dans les cellules RTgill-W1 après exposition à six des pesticides les plus cytotoxiques sur la base des résultats du chapitre 2 en utilisant la technologie de séquençage de l'ARN. L'exposition aux pesticides pourrait induire des changements transcriptomiques (moléculaires) dans les cellules RTgill-W1. Les valeurs tPOD ont pu être dérivées des données transcriptomiques, qui ont fourni des valeurs plus protectrices que les données *in vitro* et qui pourraient être utilisées pour hiérarchiser les pesticides préoccupants. L'analyse de la dose de référence au niveau de la voie a également fourni des informations supplémentaires sur le mécanisme d'action de chaque produit chimique. Cette thèse a permis de mieux comprendre les promesses du couplage de l'essai RTgill-W1 avec des techniques transcriptomiques pour caractériser les effets indésirables d'un produit chimique. En outre, ce travail a permis de mieux comprendre le mécanisme moléculaire de la toxicité de pesticides qui n'avaient pas été caractérisés auparavant.

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Preface and Contributions of the Authors

This thesis is written in the manuscript format, according to McGill University's "*Guidelines for Thesis Preparation.*" Chapter 1 consists of a general introduction of the thesis containing the motivation, objectives, and outlines of this work and a review of the literature related to the experimental work. Chapters 2 and 3 are original research chapters in which the introductions, methods, results, and discussions for each are presented. Chapter 4 summarizes the thesis with a comprehensive discussion and identifies future research needs. The work presented in Chapter 2 will be modified slightly for submission to an academic journal with other work that this author contributed to, and Chapter 3 has been prepared for submission to an academic journal. As first author for each manuscript, I was responsible for the development of study objectives and hypothesis, the literature review, lab work, data and statistical analysis, interpretation and discussion of results, and preparation of manuscripts with constant advice and feedback provided by my supervisor, Dr. Niladri Basu. Both manuscripts were a result of collaboration with my co-authors for the study design and methods.

Manuscript 1. Cytotoxicity of pesticides exposed to rainbow trout gill, liver, and intestinal cell lines. Authors: Sophie Emberley-Korkmaz, Krittika Mittal, Na'im Temlock, and Niladri Basu.

Sophie Emberley-Korkmaz: Conceptualization, methodology, formal analysis, investigation, writing- original draft, writing-review and editing, and visualization.

Krittika Mittal: Conceptualization and methodology.

Na'im Temlock: Assistance with liver and intestinal cell line lab work.

Niladri Basu: Conceptualization, investigation, writing- review and editing, and funding acquisition.

Manuscript 2. Transcriptomic effects of RTgill-W1 rainbow trout gill cells exposed to environmentally relevant pesticides. Authors: Sophie Emberley-Korkmaz, Krittika Mittal, Ke Xu, Niladri Basu.

Sophie Emberley-Korkmaz: Conceptualization, methodology, formal analysis, investigation, writing- original draft, writing-review and editing, and visualization.

Krittika Mittal and Ke Xu: Conceptualization, methodology, and sample collection assistance.

Niladri Basu: Conceptualization, investigation, writing- review and editing, and funding acquisition.

Contribution to Knowledge

This thesis fills important knowledge gaps and contributes to the advancement of understanding as follows:

Chapter 2 investigated the high-throughput ability of the OECD Test No. 249 by adjusting assay conditions so that this method designed for 24-well plates could work in 96-well plates. This chapter validated that exposures could be performed in a 96-well format and then used the derived method to characterize cytotoxicity in 19 pesticides of concern to Canadian ecosystems. Correlation of these *in vitro* results to whole fish studies were not significant when considering the entire group of 19 pesticides, and five pesticides yielded cytotoxic values within one order of magnitude of the corresponding fish LC50 values. This study contributes to the literature available on using a 96-well plate format instead of a 24-well plate, as outlined in OECD Test No. 249, to increase scalability. To the best of our knowledge, this is the first study to explore the cytotoxicity of several pesticides. The findings from this research help increase understanding of OECD Test No. 249's domains of applicability.

Chapter 3 explored transcriptomic-wide changes in the RTgill-W1 cell line following exposure to six pesticides, along with the positive control chemical, 3.4-Dichloroaniline. Transcriptomic point of departure (tPOD) values, which represent a concentration at which concerted molecular changes occur, were derived for each pesticide. This is a relatively novel approach in risk assessment as it: a) provides information on a chemical's mechanism of action versus traditional apical outcome measures (e.g., survival, growth); and b) derives a quantitative point of departure value that is familiar to regulatory toxicologists. Derived tPODs were compared to the *in vitro* EC50s derived in Chapter 2 and *in vivo* whole rainbow trout LC50 values, and overall, the tPODs were found to be more protective or comparable to the *in vivo*

LC50s. Further analysis of pathway level benchmark doses showed new pathways and genes to be dysregulated that were not previously characterized in the literature. To our knowledge, this is the first study to characterize transcriptomic-wide changes in the RTgill-W1 cells by pesticides, and also the first to derive tPOD values using this approach. These results will help prioritize pesticides of concern before apical effects are reached and contribute to the shift toward mechanism-based toxicology risk assessment.

List of Abbreviations

° C	Degrees Celsius
μM	Micromolar
3Rs	Reduce, Replace, Refine
AB	alamarBlue
AMPA	Aminomethylphosphonic acid
ATA	Atrazine
CAR	Carbaryl
CAT	Chlorantraniliprole
CFDA-AM	5-carboxyfluorescein diacetate acetoxy methyl ester
CLN	Clothianidin
CLP	Chlorpyrifos
CMP	Chemical Management Plan
COL	Chlorothalonil
CV	Coefficient of Variation
DIC	Dicamba
DI-P	Dimethenamid-P
DIQ	Diquat
DIZ	Diazinon
EC50	Effective Concentration 50
ECOTOX	Ecotoxicology Knowledgebase
EU	European Union
GLY	Glyphosate

IMI	Imidacloprid
IMZ	Imazethapyr
LC50	Lethal Concentration 50
MBZ	Metribuzin
MET	Metolachlor
mg/L	Milligrams per liter
mM	Millimolar
NAM	New Approach Methods
OECD	Organization for Economic Co-operation and Development
PER	Permethrin
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RNA	Ribonucleic acid
RTgill-W1	Rainbow Trout Gill Cell Line
S-MET	S-metolachlor
SD	Standard Deviation
SE	Standard Error
THX	Thiamethoxam
ToxCast	Toxicity Forecaster
TT21C	Toxicity Testing in the 21st Century
US EPA	United States Environmental Protection Agency
QA	Quality Assurance
QC	Quality Control

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Chapter 1

1. Introduction and Literature Review

1.1 General Introduction

A paradigm shift in toxicity testing is driving research into the exploration and application of alternative approaches to animal toxicity testing. Traditional toxicity testing has been successfully able to measure the risks chemicals pose in hazard assessments for decades by exposing animals to chemicals and measuring apical endpoints. This strategy is no longer feasible to test the increasing number of chemicals that need screening today as new chemicals are consistently being developed and unassessed chemicals are still in use (1, 2). A transformation has thus occurred towards the development of high-throughput alternative approaches based on *in vitro* cell-based methods to further our knowledge and understanding of the negative impacts chemicals can have.

One such development was the standardization of an acute toxicity assay based on the established rainbow trout gill cell line (RTgill-W1) by the Organization for Economic Co-operation and Development (OECD) in 2021 (3). OECD Test No. 249 is a promising assay shown to yield repeatable and reproducible results (4). However, more information is required on how results from this *in vitro* test compare to whole organism studies to determine if it could be used to replace animal testing and the assays focus on cytotoxicity (a lethal outcome measure) is limiting as a significant gap in knowledge are the sublethal effects a chemical can have before apical outcomes are reached (5, 6). While the assay already presents a faster, less expensive alternative to traditional toxicity testing, the current protocol of testing one chemical per 24-well

plate has previously been performed in 96-well plates (7, 8) and was evaluated here to further optimize testing for a larger number of chemicals and increase scalability and feasibility of this approach.

Among the most important chemicals of concern are pesticides due to their well-documented adverse effects on non-target organisms such as fish and their persistence in aquatic environments (9, 10). Since pesticides are a large class of chemicals, high-throughput approaches such as OECD Test No. 249 could be used to screen more of them in a shorter amount of time while providing lethal and sublethal information.

The overall objective of this research was to first optimize the OECD Test No. 249 guideline for 96-well plates, and then use the RTgill-W1 cells following the guideline in this format to study the cytotoxic and transcriptomic effects of exposure to pesticides deemed high priority to Canadian aquatic ecosystems as an alternative approach to animal testing methods.

1.1.1 Specific objectives

Chapter 2: Can the OECD Test No. 249 assay be optimized to 96-well plates and be used to yield *in vitro* cytotoxic results from pesticide exposures that are comparable to *in vivo* lethality values?

Objective 1**:** Determine if the three validation criteria checks described in OECD Test No. 249 for a 24-well plate format can be applied in a 96-well plate format to increase the scalability and efficiency of the assay. The specific goals of this objective were to:

- Determine if seeding 10,000 cells/well or 35,000 cells/well would be better to consistently satisfy the validation criteria.
- Ensure that cytotoxic results from chemical exposures are not to be compromised.

It was hypothesized that validation criteria and chemical exposure results would not be compromised from optimization to a 96-well plate format.

Objective 2: Characterize the cytotoxicity of 19 pesticides considered high priority to Canadian ecosystems in the 96-well plate format and determine the *in vitro-in vivo* correlation. The specific goal of this objective was to compare the cytotoxic values derived for each pesticide from RTgill-W1 exposures to 96-hour rainbow trout fish lethality values from the literature. It was hypothesized that cytotoxic results for pesticide exposures would be comparable to lethality results from the literature.

Chapter 3: How is the transcriptome of the rainbow trout gill cell line RTgill-W1 affected after exposure to pesticides, and are the derived transcriptomics point of departure (tPODs) values protective (i.e., lower) of values associated with adverse outcomes?

Objective 1: Examine the differences in the gill transcriptome of RTgill-W1 cells exposed to six of the most cytotoxic pesticides from Chapter 2 using RNA sequencing. The specific goal of this objective was to:

- Determine the genes (and biological pathways) that are significantly differentially expressed for each pesticide.
- Examine how sublethal toxicity is predictive of or relates to cytotoxicity from Chapter 2 and with *in vivo* literature for each pesticide.

It was hypothesized that we would see more differentially expressed genes in the more cytotoxic pesticides.

Objective 2: Derive tPODs for each pesticide based on benchmark dose (BMD) methods and determine if these values are protective of *in vivo* lethality values.

It was hypothesized that tPODs would be protective of lethality values for pesticides.

1.2 Literature Review

1.2.1 Toxicity Testing

1.2.1.1 Traditional Testing Approaches

Traditional toxicity testing entails the exposure of whole animals to chemicals to determine at what concentration will it cause an adverse effect. This is otherwise referred to as "*in vivo*" testing. Adverse effects in this context are often limited to the impacts on survival, growth, reproduction, and organ pathology in an intact animal (1). The need for toxicity testing became clear in the 1930s when inadequate laws surrounding food and drug safety led to many tragedies in Canada and the United States (US), leading to a push for stronger safety guidelines that included the testing of products on animals before reaching the public (2). The field of toxicity testing has changed a lot since the 1930s with laws continuously being updated not only for human safety but to also include ecological and environmental protection. Environmental toxicity testing (ecotoxicity testing) studies the adverse effects that chemicals have on living organisms. The emergence of this field and public interest in environmental protection began in 1962 with the publication of Silent Springs by Rachel Carson which described the catastrophic impacts that the use of dichlorodiphenyltrichloroethane (DDT) and other pesticides had on bird populations (3).

Traditional toxicity testing has since become the gold standard for chemical hazard identification, producing results instrumental for the development of environmental policy and risk assessment. Exposures to organisms are typically of a water, sediment, or soil sample to evaluate the effects of contamination, or exposure to a chemical standard (4). Some of the main advantages to *in vivo* testing are that since it uses whole living organisms, these models can

investigate the impacts on all physiological and biochemical reactions, multiple generations can be studied, and the metabolism of toxicants can be accounted for (5, 6). Additionally, since these tests have been used for decades, there are large databases available for international collaboration and input, providing harmony for the interpretation of results (5, 6). To note, the Organization for Economic Cooperation and Development (OECD) has developed internationally accepted animal toxicity testing guidelines to reduce the repetition of similar tests conducted by other countries and create harmony for comparisons (2).

Despite the benefits mentioned, a shift is occurring in the field of toxicology where these techniques are quickly becoming outdated. Animal-based toxicity testing is often time-consuming, produces large quantities of waste, requires a considerable number of animals, has lengthy testing times, and requires many additional resources and equipment to test one chemical (7-9). Additionally, the focus on apical endpoints such as mortality or reproduction does not allow for further insights into a chemical's mechanism of action for toxic effects (2, 5). These challenges make it difficult to keep up with testing all the new emergent chemicals introduced each year into the environment that could be causing adverse effects at the apical and sublethal levels.

1.2.1.2 Need for a paradigm shift to alternative testing methods

There is a need for more ethical, less expensive, time- and resource-efficient approaches in toxicity testing. Approximately 23,000 chemicals on the domestic substances list (DSL) in Canada are labeled as "existing substances" that are in use but have not been tested for their potential risks (10). This list only includes chemicals that were introduced before 1994 and does

not include the approximate 600 new chemicals introduced each year into the environment (11). In 2020-2021, 10 new active ingredients and 278 new pesticide products containing new and existing registered active ingredients were registered in Canada (12). A review from Mittal et al. (2022) estimated that the cost to test one chemical using a traditional approach was roughly 118,000 USD, required 135 animals, and took about 8 weeks (7). Based on the high costs and time required, it is not feasible to use this approach to test the thousands of chemicals required in a timely fashion from an ethical or resource-efficiency standpoint.

These limitations coupled with recent legislation in Canada, the US, and the European Union (EU) aiming to reduce or completely stop animal toxicity testing are driving forces towards the need for alternative approaches (13). In 2006, the EU passed new legislation on the regulation of chemicals called the Registration, Evaluation, and Authorization of Chemicals (REACH) Regulation. The mission of REACH was, and still is today, to fill the knowledge gap on approximately 30,000 chemical substances and improve the protection of human and environmental health (14, 15). The legislation placed the burden of proof on industry to prove how the substances they use are safe and ensure proper risk management measures are communicated. In line with the 3R's principles to replace, reduce, and refine animal testing, REACH launched as part of their initiative the exploration of innovative and alternative techniques (genomics, proteomics, metabolomics, *in vitro*, and QSAR (quantitative structure-activity relationships)) to reduce the number of animals tested on and to compliment traditional techniques (16). Following the next year in 2007, the National Research Council (NRC) released the revolutionary report, "Toxicity Testing in the 21st Century: A Vision and a Strategy" (2). This report proposed a framework shift away from whole animal *in vivo* methods towards one

based on high-throughput alternative approaches for chemical concentration-response characterization and hazard assessment (2). It explicitly mentioned that mechanistically based *in vitro* methods using cells or cell lines could drive this transformation by furthering advancements in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology (2). Now in Canada, legislation is working through the senate that proposes amendments to the Canadian Environmental Protection Act (CEPA, 1999) statements on reducing, refining, or replacing (3R's) animal testing and the integration of NAMs to achieve this (13).

Since the release of the NRC report, research into alternative toxicity testing methods has risen significantly in academia, industry, and government. The 2007 report focused on a vision for human health and the application of these ideas to the ecological evaluation of chemical hazards was omitted, however, this has not inhibited the application of such tools for ecological purposes (17). The need for ecologically driven high throughput mechanistic assays is essential for the paradigm shift away from *in vivo* models. Advancements have been made, but more information is needed in two key areas to further the transition away from whole animal models: reliable linkages need to be made between responses measured at the cellular level and apical outcomes traditionally measured, and credible extrapolation techniques must be developed to apply the observed cellular responses to whole organisms (17, 18).

1.2.1.3 Alternatives to *in vivo* toxicity testing

Alternative approaches to *in vivo* toxicity testing can be broadly defined as New Approach Methods (NAMs). NAMs are an umbrella term to describe any technology, methodology, approach, or combination thereof that can be utilized to characterize the risks chemicals pose

without the use of animal testing (19, 20). NAMs also fall in line with the 3R's principle to reduce the number of animals used, replace the use of animals in a study, and refine the study to decrease the severity of inhumane procedures on animals (21, 22). These methods can be further categorized into three main groups. The first method is *in silico* or computational which uses computer and mathematical modeling to determine the toxic effects a chemical could have and the biological processes it could interrupt (2). The second method, which has already been previously mentioned, is *in vitro* methods that involve exposing isolated cells, tissues, or organs to a chemical of interest. The third method is early-life stage methods where embryos are exposed to chemicals. *In vitro* methods using immortalized fish cell lines have shown great potential.

1.2.1.3.1 OECD Test No. 249

The 2007 NRC Report highlighted the potential cell-based *in vitro* approaches have for influencing the paradigm shift away from animal-based testing. Since then, one significant step towards this has been the development of a standardized protocol utilizing rainbow trout gill cells (RTgill-W1). In 2021, the OECD standardized an acute toxicity assay using the immortalized RTgill-W1 cell line focused on characterizing the cytotoxicity of a wide variety of chemicals (23, 24). The assay has been demonstrated to have high repeatability and reproducibility through a round-robin study with 6 different laboratories (25). The gill cell was selected based on its biological relevance as it is the first point of contact with contaminants in the water (9, 26, 27). It also plays a significant role in the uptake of toxicants and is involved in many physiological processes such as gas exchange, osmoregulation, and respiration (26, 28). Thus, damage to the gill cell it is a strong indicator of impaired functioning due to toxicant

exposure. The protocol uses three fluorescent dyes to assess cell viability; alamarBlue to assess metabolic activity, 5-carboxyfluorescein diacetate acetoxy methyl ester to measure cell membrane integrity, and neutral red to evaluate lysosomal activity. OECD Test No. 249 offers a high throughput approach based on a 24-hour exposure to a chemical that improves testing time, reduces the use of resources, and produces less waste (8, 23, 25).

1.2.1.3.2 High-throughput transcriptomic approaches

OECD Test No. 249 is an exciting emergent NAM that could impact the future of toxicity testing. However, its focus on cytotoxicity as an outcome measure is limiting as it does not provide meaningful information on a chemical's mechanism of action. Transcriptomic studies have been used as a tool in toxicology for many years and provide important insights into the ways chemicals impact the molecular signaling networks that underlie the apical endpoints observed in traditional testing approaches (i.e., pathological changes, functional changes, death) (20, 29). High-throughput transcriptomics (HTTr) is a non-targeted approach that uses gene expression profiling to investigate the effects a wide variety of chemicals will have on *in vitro* cell culture systems (20). By performing HHTr under defined treatment conditions (i.e., time, concentration) in a concentration-response model, the concentrations that cause adverse effects in cellular response pathways can be determined (20). Transcriptomic data can be analyzed using the benchmark dose (BMD) method in which toxicological data is fitted into concentration-response models and a predicted no-effect concentration (PNEC) value is derived (29, 30). Thus, resulting in transcriptomic point of departure (tPOD) values. The combination of high-throughput transcriptomics performed in the *in vitro* RTgill-W1 cell systems would help answer

knowledge gaps and better understand how a chemical induces toxicity at low concentrations, providing more protective and conservative measures.

1.2.1.4 Comparing *in vitro* results with traditional results

Given the gold standard for toxicity testing has been whole organism animal exposures since the 1950s, is it crucial to understand how the results from alternative methods compare to intact animal studies. A handful of studies have investigated how the effective concentration 50 (EC50) results from RTgill-W1 exposures compare to lethal concentration 50 (LC50) values from *in vivo* fish exposures (**Table *1-1***).

Table 1-1: Summary of journal articles that have compared *in vitro* results from RTgill-W1 exposures to *in vivo* lethality values from the literature.

Study	Author(s)	Year	Cells	Chemicals	*In vivo* species comparison	How many cytotoxic	Correlation	Sensitivity
1	Tanneberger et al. (9)	2013	RTgill-W1	35 organic chemicals	Fathead minnow	34	73% chemicals within a 5-fold difference of *in vivo* for metabolic activity endpoint	Apart from 5 chemicals, RTgill-W1 cells are more sensitive
2	Natsch et al. (31)	2018	RTgill-W1	38 volatile fragrance chemicals	Fathead minnow	38	Strong correlation (R^2 = 0.90 – 0.94) for all endpoints	Not included
3	Scott et al. (27)	2021	RTgill-W1	14 toxicants (4 organic chemicals, 2 volatiles, and 8 metals and metalloids)	Fathead minnow, Sheepshead minnow	12	Except for 4, values within one order of magnitude and correlation statistically significant for fathead (p= 0.0091) and sheepshead (p=0.0113)	5 of 12 chemicals more sensitive with RTgill-W1 cells
4	Schug et al. (32)	2020	RTgutGC	38 volatile fragrance chemicals (linked to study 2)	Fathead minnow	16	Almost 1:1 correlation between RTgill-W1 results and strong linear relationship with *in vivo* data from fish	Not included

Taken together, these results indicate a strong potential for OECD Test No. 249 to yield comparable results with whole organisms, however, there are hundreds of thousands of chemicals from a range of different chemical classes in the world. Further research is needed into different chemical classes on how the RTgill-W1 cell line differentiates from animal studies.

A few studies have also compared tPODs derived from *in vitro*, *in vivo,* and embryo-based methods with intact fish studies (**Table *1-2***).

Table 1-2: Articles from the literature that compare transcriptomic point of departure (tPODs) values from *in vitro*, *in vivo*, and embryo methodologies to lethality values from fish studies.

Study	Authors	Year	Model	Comparison	Chemicals	Correlation
1	Mittal et al. (30)	2022	*In vitro* - RTgill-W1, RTL-W1, RTG-GC cells	*in vivo* LC50s from a variety of fish species	Methylmercury and fluoxetine	tPODs 10-fold lower than *in vivo* LC50s
2	Pagé-Larivière et al. (33)	2019	*In vivo* - Zebrafish, fathead minnow, rainbow trout fish exposures	Lowest observed effect concentration (LOEC) for each fish	Bisphenol A, diethylstilbestrol, ethinylestradiol	72% tPODs within one order of magnitude of the LOEC for each fish
3	Alcaraz et al. (34)	2021	Rainbow trout embryos acute 4-day exposure	Apical points of departure (PODs)	Ethinylestradiol	tPOD values within the same order of magnitude as derived apical PODs from the literature
4	Alcaraz et al. (35)	2022	Embryo-larval fathead minnows 7-day exposure	Chronic apical benchmark doses (BMD) from literature	Fluoxetine	tPODS were similar to chronic apical BMDs from the literature
5	Martínez et al. (36)	2020	Zebrafish eleuthero embryos 2–5-day acute exposure	*in vivo* LC50	Tributyltin	tPODs one order of magnitude lower than LC50s

These articles represent the potential role tPODs could have in regulatory risk assessment. However, further validation and studies are needed on how *in vitro* approaches could be employed to derive tPODs and if they produce protective results complementary to *in vivo* values.

1.2.2 Pesticides

1.2.2.1 Pesticides in the environment

Pesticides have been investigated for toxicity studies by regulatory agencies, industry, and academics since toxicity testing began decades ago. As discussed, it was initially the deleterious effects DDT had on non-target organisms, specifically birds, which launched the environmental movement in the 1960s (3). Pesticides are manufactured to control pests and stop them from interfering with agricultural practices. Depending on the target pest, pesticides are divided into different classifications including herbicides (kill weeds and other plants that can harm crops), insecticides (kill insects), fungicides (kill parasitic fungus and their spores), and rodenticides (kill rats, mice, and others). These classifications can further be categorized based on their target species. For example, a subclass of herbicides is triazine, which works by inhibiting photosynthesis at photosystem II and includes the active ingredients ametryn, atrazine, cyanazine, prometon, prometryn, propazine, and simazine (37). There are approximately 28 different subclasses of herbicides which insecticides and fungicides are not included (37). Thus, pesticides are widely diverse, and each act in a unique manner that cannot be clumped together.

Pesticides also differ in their solubilities, bioaccumulation abilities, and persistence in the environment which influence how they end up in aquatic ecosystems. The transport of pesticides into aquatic environments can occur in a variety of ways such as misapplications of pesticides directly to water, overspray or drifting during application, leaching processes from agricultural applications after rainfall, and surface runoff picking up pesticides from fields or spills (38). Soluble pesticides will leach through soil layers during precipitation events to reach groundwater, while insoluble pesticides accumulate in the topsoil layer where application occurs to eventually be taken by run-off and erosion to contaminate lakes, streams, and rivers (38). While they are designed to have target organisms, they often lack species selectively which results in adverse effects to non-target organisms (39). Due to the fate of pesticides and this lack of specificity, they can severely impact animal and plant biodiversity, aquatic and terrestrial ecosystems, and food webs (40).

1.2.2.2 Exposure of fish to pesticides

Fishes worldwide spawn in rivers that are contaminated with pesticides from a wide variety of sources (41). They are exposed at all stages of life in three ways; dermally through direct absorption in the skin, through the gills during breathing, or by ingestion of contaminated foods and suspended particle uptakes (40, 42). It was originally thought that since pesticides have target species, the effect on other organisms would be negligent. However, reports on the adverse effects pesticides pose to non-target organisms, particularly fish, are now well-documented (43).

The liver, kidney, brain, and gills are all vulnerable organs to pesticide exposure and are sites of adverse effects (40). Most notably, the release of pesticides into the environment can increase

death and cause mass mortalities of fish (40). Other examples include impacts on the immune system and triggering unwanted sex growth causing the release of the opposite-sex hormones (44). Neurotoxic and behavioural changes are exhibited through instability in swimming, erratic movements, increased anxiety, and can lead to death (45). Pesticides impact protein content and enzyme functionality in key organs such as the liver, kidney, and gills, which severely impede cellular functioning and metabolic activity (46, 47). Lastly, genotoxic effects, have been observed in all organs as well, causing mutations, cancers, and birth defects in fish (48). Thus, pesticides can adversely impact fish and as such are an important chemical group to continuously study.

1.2.2.3 Pesticides and fish cells

Pesticides are a critical group of chemicals used every day all over the world. The Pest Management Regulatory Agency Annual Report 2020-2021 by Health Canada stated that there are over 7600 registered pesticide products and over 600 registered active ingredients in these products (12). Given the need to assess these chemicals and understand their toxic abilities in a timely fashion, pesticides are a relevant group to explore the potential for alternative high-throughput approaches. Only a few studies have explored *in vitro* approaches to characterize pesticide cytotoxicity. Saito et al. (1991) characterized the cytotoxicity of 45 pesticides to GF-Scale (GFS) cells from goldfish using the neutral red assay (assesses lysosomal activity) and found a significant correlation with EC50 values from 48-h *in vivo* carp exposures (49). Babín and Tarazona (2005) exposed rainbow trout liver (RTL-W1) and rainbow trout gonadal (RTG-2) cells to two organophosphates (chlorpyrifos and quinalphos) and four pyrethrinoides (cypermethrin, bifenthrin, cyhalothrin, and λ-cyhalothrin) (50). Cellular effects were measured

by ethoxy resorufin-O-demethylase (EROD) assay activity, β-Gal enzymatic activity, neutral red fluorescent dye assessing lysosomal activity, and the FRAME KB protein assay where EROD was the most sensitive assay for both cell lines and EC50s ranged over several orders of magnitude for each endpoint (50). 18 pesticides from various classes were exposed to the hepatoma cells PLHC-1 from the desert topminnow (*Poeciliopsis lucida*) and characterized for cytotoxicity using the neutral red assay (51). The objective of this study was to determine if chemical lipophilicity (log K_{ow}) would be a good indicator of acute toxicity in fish and results showed a weak relationship ($r^2 = 0.43$) (51). Lastly, Tanneberger et al. (2013), as part of validating the RTgill-W1 cell line, exposed permethrin, lindane, malathion, rotenone, disulfoton, naphthalene, 2,4,6-trichlorphenol, dichlorophene, pentachlorophenol, and parathion ethyl as 10 of the 35 chemicals studied to the RTgill-W1 cells (9). Permethrin and lindane (whole fish over 10-fold more sensitive than *in vitro* results), and pentachlorophenol and rotenone (*in vitro* results over 10-fold more sensitive than whole fish) were outliers of a strong concordance between *in vivo* and *in vitro* values (9). Scott et al. (2019) also studied permethrin exposed to the RTgill-W1 cells and found a similar trend as to the work by Tanneberger et al. (2013) (27). Since pesticides are a highly relevant group of chemicals due to their environmental implications, more studies with the RTgill-W1 cell line could help contribute to the conversation of the potential this alternative approach possesses.

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Preface to Chapter 2

Chapter 2 describes the cytotoxic effects of 19 pesticides considered high priority to Canadian ecosystems exposed to RTgill-W1 cells. Firstly, this study looks to optimize the OECD No. 249 test guideline from 24-well plates to 96-well plates to increase the efficiency and scalability of the assay. This would further increase the high-throughput ability of the assay by allowing to test more chemicals at once. Secondly, this approach in 96-well plates was tested with 19 pesticides and one positive control chemical (3,4-Dichloroaniline) to characterize cytotoxicity with two cell viability endpoints; metabolic activity and membrane integrity. Additionally, it was important to then compare the results from this study with intact fish studies to determine how this alternative to animal testing approach could replace these techniques. To the best of our knowledge, this is the first study to perform a large-scale analysis of pesticides on the RTgill-W1 cells and compare their results to *in vivo* 96h whole rainbow trout exposures.

This chapter is authored by the candidate and co-authored by the candidate's supervisor Dr. Niladri Basu and Dr. Krittika Mittal, a post-doctoral fellow under Dr. Basu's supervision. The optimization to 96-well plates design was carried out by the candidate in collaboration with Dr. Krittika Mittal. The candidate carried out all optimization trials and pesticide exposures, data analysis and correlation, data interpretation, discussion of the results, and preparation of manuscript. Advice throughout was provided by the candidate's supervisor Dr. Niladri Basu. The manuscript is planned to be submitted as part of another paper (candidate as first-author) which exposed the same 19 pesticides to rainbow trout gut and liver cell lines.

Chapter 2

2 Optimization of and cytotoxic effects of RTgill-W1 cells exposed to pesticides following the OECD No. 249 guideline

TITLE
Cytotoxicity of 19 pesticides exposed to rainbow trout gill, liver, and intestinal cell lines

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RUNNING TITLE
Cytotoxicity of 19 pesticides exposed to rainbow trout gill, liver, and intestinal cell lines

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2.1 Abstract

Background: Traditional animal toxicity testing has been the standard for decades, however ethical concerns and resource limitation issues have highlighted the need to move toward alternative testing methods to meet the increasing chemical testing demands. High-throughput alternative approaches to animal testing using *in vitro* cell lines are promising to overcome these issues by reducing the amount of time, resources, and waste produced from testing a larger number of chemicals. This study investigates the applicability of the *in vitro* OECD Test No. 249 assay based on the RTgill-W1 cell line which was recently standardized as an alternative method.

Objectives: The first objective of this work was to optimize the OECD No. 249 test from one chemical/24-well plate into nine chemicals/96-well plate to further increase the efficiency and high-throughput ability of the assay. The second objective of this study was to expose RTgill-W1 cells following OECD Test No. 249 to characterize the cytotoxicity of 19 pesticides considered important to Canadian ecosystems, and to compare these *in vitro* data with lethal concentration 50 (LC50) results from whole animal studies.

Results: Optimization to 96-well plates did not interfere with the quality control checks laid out in OECD Test No. 249 and did not affect pesticide cytotoxicity results based on results from a pilot test with metolachlor (24-well EC50 = 291.2 μM, 96-well EC50 = 311.7 μM). Cytotoxicity results from alamarBlue (assessing metabolic activity) and CFDA-AM (assessing membrane integrity) fluorescent dyes found 11 of the 19 pesticides to be cytotoxic. Spearman ρ correlations with the *in vitro* data obtained and the respective LC50 *in vivo* values from 96h rainbow trout (*Oncorhynchus mykiss*) exposures taken from the literature were measured and showed a significant correlation with the metabolic activity endpoint.

Discussion: The target toxic mode of action, diversity of target receptors in the RTgill-W1 cells, and hydrophobicity of the pesticide all were factors influencing cytotoxicity. Overall, the RTgill-W1 cells show promise for applications in characterizing the toxicity of pesticides but hydrophobicity heavily influences if *in vitro* results will be predictive of *in vivo* toxicity.

2.2 Introduction

The use of fish in acute toxicity testing strategies in Canada (1), the United States (2), and the EU (3) has been essential for characterizing the risk chemical contamination poses to the aquatic environment and organisms. Not only are aquatic ecosystems important sinks for many chemicals, but fish specifically are crucial in food webs for their role in regulating nutrients and acting as indicators for water quality (4). While tests involving fish testing to study chemical toxicity are standardized and validated, they can require a considerable number of fish, are time-consuming, produce large quantities of waste, and require many additional resources to test one chemical (5-7). A bibliometric review conducted in 2022 estimated that traditional *in vivo* tests for one chemical on average cost $118,000 USD, require 135 animals, and takes 8 weeks for the entire process (5). In addition, these tests focus on death as a crude endpoint which brings up the ethicality of this approach (7). These concerns coupled with recent legislation in Canada, the US, and the EU working towards phasing out animal toxicity testing are a driving force towards the development of alternative approaches (8). Given the excessive cost and amount of time needed to simply test one chemical, *in vivo* toxicity testing approaches are not feasible to test the substantial number of chemicals needed ethically or resource-efficiently, and shifts towards alternative approaches are necessary.

Interest in exploring New Approach Methods (NAMs) in compliance with the 3 Rs principle (refinement, reduction, and replacement of animal testing) is a viable option to overcome the limitations presented with traditional approaches. This drive has led to the development/uptake of *in vitro* cellular techniques. As recently as 2021, the Organization for Economic Co-operation

and Development (OECD) standardized a method using gill cells from rainbow trout (RTgill-W1 cells) to assess the cytotoxicity of a wide variety of chemicals (9-11). The gill cell is the first organ to encounter contaminants in the water and plays a key role in the uptake of toxicants (7, 12, 13). It also plays an important physiological role as it is involved in gas exchange, osmoregulation, respiration, and other functions where damage to the gill cell is a strong indicator of impaired functioning due to toxicant exposure (11, 12). The gill cell was therefore selected as the basis for the OECD Test No. 249 given its biological relevance. This assay focuses on cytotoxicity as its outcome measure and has been demonstrated to yield repeatable and reproducible results (14) and has been tested on various chemical groups such as metals (13, 15), polycyclic aromatic hydrocarbons (16), mycotoxins (17), and more.

Considering that traditional animal testing methods have been the gold standard for decades, there is a need to compare the results from OECD Test No. 249 with *in vivo* results. *In vitro-in vivo* comparisons have been made with the RTgill-W1 cell line in which the effective concentration reducing cell viability (EC50) results from cell exposures are compared to lethal concentration 50 (LC50) values from fish exposures. Specifically, comparisons have been made for 35 organic chemicals (7), 38 fragrance chemicals (18), and four organics, two volatiles, and eight metals and metalloids (13). In all studies, *in vitro-in vivo* comparisons were promising but ranged depending on the chemical. Extrapolations from these results therefore cannot be made to other chemical classes as properties and characteristics are distinct.

The desire to shift towards alternative approaches such as the RTgill-W1 cell assay from a regulatory stance means that more information is needed on how the results compare to *in vivo*

fish exposures. To achieve this, studies evaluating various chemicals from the same class are necessary. Since tests with the RTgill-W1 cells have been performed in 96-well plates previously (15) the protocol as presented in the OECD Test No. 249 was altered to adopt a higher throughput approach. Current guidelines use 24-well plates to test one chemical in six concentrations to measure cytotoxicity. It was of interest to explore if equivalent results could be achieved using 96-well plates where 9-10 chemicals could be tested per plate over eight concentrations, as opposed to the one chemical/24-well plate. This would increase the scalability of the assay, further allowing for more chemicals to be tested in a shorter amount of time, reduce costs, produce less waste, and obtain more data at one time. This study selected 19 pesticides from different classes and target modes of action to undergo comparisons with *in vivo* fish EC50 values.

To understand more about the applicability of the RTgill-W1 cell assay as an alternative to current acute toxicity testing methods, 19 pesticides of relevance to Canadian ecosystems were selected and correlated to *in vivo* lethality values (LC50s) taken from the literature. *In vitro* cytotoxicity values (EC50) were derived from exposures to the RTgill-W1 cell line based on nominal concentrations. Comparisons were made with *in vivo* data (LC50) previously reported for rainbow trout fish (*Oncorhynchus mykiss*) following 96h exposures taken from databases and published literature sources. Two fluorescent dyes, alamarBlue assessing metabolic activity, and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) measuring cell membrane integrity were used in combination to measure cell viability.

2.3 Materials and Methods

2.3.1 Quality control checks for optimization

The current guideline tests one chemical per 24-well plate over six concentrations whereas the 96-well plate was optimized to test 10 chemicals over eight concentrations (**Figure *2-1***). It was determined to use one technical replicate per passage, over three passages to obtain three biological replicates. Within the OECD Test No. 249 guideline are three quality control checks to ensure the validity of the test run (9). Thus, it was first required to ensure that the validity criteria would not be compromised due to this transition. The first check pertained to the cell-free control wells in which the variation of raw fluorescence data between the no-cell highest chemical concentration (Chem C1) and the no-cell negative control (L-15/ex media) does not exceed 20%. The second is that the solvent control, in this instance DMSO, does not differ by more than 10% in cell viability from the negative control (L-15/ex) to detect any possible chemical cross-contamination. The last quality check refers to the positive control chemical 3,4-Dichloroaniline (3,4-DCA) in which the measured EC50 value from an experiment should lie in between the two-and-a-half standard deviation ($2\frac{1}{2}$SD) from the EC50 values stated for alamarBlue (EC50 = 43.6 mg/L ± 6.1 mg/L; $2\frac{1}{2}$SD range 28.4 – 58.9 mg/L) and CFDA-AM (EC50 = 62.5 mg/L ± 18.9 mg/L; $2\frac{1}{2}$SD range 15.2 – 109.8 mg/L) to ensure proper assay functioning and performance. The guideline sets a concentration range of 0 – 100 mg/L following a 2-fold serial dilution over five concentrations (6.25, 12.5, 25, 50, 100 mg/L) that should result in 100 – 0% cell viability and is recommended to run a 24-well 3,4-DCA plate every six chemical plates. In the 96-well plate format design, 3,4-DCA was included in every passage tested.

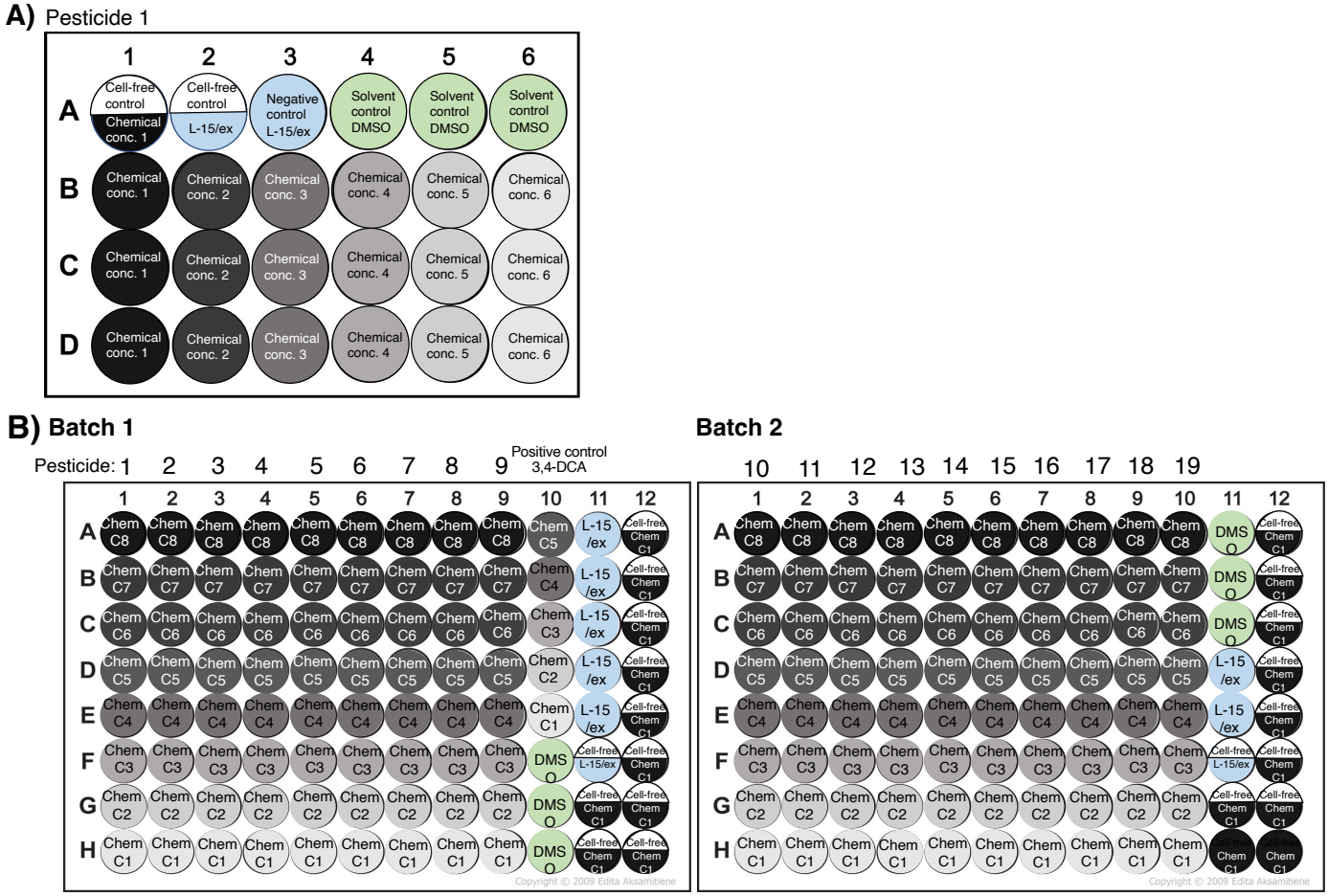

Figure 2-1: A) 24-well pipetting scheme for one chemical over six concentrations and three technical replicates per concentration. Conc. 1 represents the highest concentration tested. B) 96-well pipetting scheme for one biological replicate of batch 1 and batch 2 pesticides tested over eight concentrations and one technical replicate per concentration. Chem C1 represents the highest concentration tested. Tests were performed over three passages (n= 3 biological replicates) to allow for statistical analysis. Positive control chemical, 3,4-DCA tested with each passage.

2.3.2 Pesticide selection

The selection of the 19 pesticides was mostly based on a priority list compiled by a Fisheries and Oceans Canada study (19). After a scope of the published literature, the authors of this paper selected 55 candidate pesticides based on their 2017/2018 sales (kg a.i.), detection

frequency, measured concentrations in Canadian surface waters between 2000-2020, inherent toxicity from guideline values, and regulatory status in other jurisdictions (example; European Union) (19). From this list of 55, pesticides were ranked from highest to lowest priority based on assigned scores for the selected criterion mentioned above between 0 and 3 (19). Points were tallied and pesticides were ranked based on score (high priority to lowest). We chose 17 pesticides from the list of 55 and selected them from different prioritization levels (high, medium, and low) to have a diverse list of test pesticides. Chlorantraniliprole and Aminomethylphosphonic acid (AMPA) were also included to complete the list of 19. Concerning chlorantraniloprole, available fish acute toxicity data are classified as "non-definitive" (20). AMPA is a primary/major metabolite of glyphosate, one of the most used herbicides in the world (21). While glyphosate is found to have no or very low acute toxicity in fish (21-23), there are many gaps in the literature regarding AMPA and its toxicity to non-target organisms (24). AMPA is an important pesticide to observe because it has a lower water solubility and longer soil half-life than glyphosate, meaning it could have a stronger absorption over a longer amount of time (24). A study conducted by de Brito Rodrigues et al. (21) found no acute toxicity at the highest concentration tested of 100 mg/L on zebrafish embryos following the Embryo Acute Toxicity (25) Test, and no acute toxicity for exposures of the same concentrations to Zebrafish larvae and rainbow trout gonad-2 (RTG-2) cells (21).

2.3.3 RTgill-W1 cell culture

The rainbow trout gill cell line (RTgill-W1; CRL-2523) was obtained from American Type Cell Culture (ATCC). Cells were grown in Thermo Scientific™ BioLite™ Cell Culture Treated 100 mm Petri dishes containing 10 ml of complete culture medium (Leibovitz L-15

culture medium supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin), at 21°C. Medium in dishes was replaced twice a week. Once cells reached 80% confluency, they were trypsinized for 3 to 5 min using 1 ml Trypsin-EDTA, and either passaged to continue the culture or counted (using a hemocytometer) and seeded in 96-well plates for exposure experiments (35,000 cells per well). All cell culturing was done by following the OECD Test No. 249 guidelines. The passage numbers of the cells used for the cytotoxicity exposures ranged from p64 to p71.

2.3.4 Chemicals

Protocol for pesticide exposures was followed according to the methods described in OECD Test No. 249 with minor changes. Changes due to optimizing for 96-well plates are described in the following section. Pesticides were purchased from Sigma Aldrich except for metolachlor, diazinon, permethrin, and S-metolachlor purchased from Canadian Life Science (Chem Services). For cytotoxicity studies, stock solutions starting at 200 mM for all 19 pesticides except glyphosate (20 mM) and chlorothalonil (20 mM) were created. Atrazine (>98.0%), clothianidin (≥ 99.9%), imazethapyr (≥ 99.7%), imidacloprid (≥ 99.7%), metolachlor (≥ 99.5%), thiamethoxam (≥ 99.3%), chlorantraniliprole (≥ 96.6%), diazinon (≥ 98.1%), chlorpyrifos (>98.0%), permethrin (≥ 99.5%), S-metolachlor (≥ 99.5%), carbaryl (≥ 99.5%), dicamba (≥ 98.9%), dimethenamid-P (≥ 98.8%), metribuzin (≥ 98.8%), diquat (≥ 99.8%) and aminomethylphosphonic acid (AMPA) (>99.0%) stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma Aldrich) or milli-Q water (diquat and AMPA) and serially diluted (3-fold) in L-15/ex to obtain final concentrations of 0, 0.45, 1.37, 4.12, 12.35, 37.04, 111.11, 333.33, and 1000 μM. Chlorothalonil (≥ 99.7%) and Glyphosate (≥ 98.5%) were prepared in

DMSO and milli-Q water, respectively, and serially diluted (3-fold) in L-15/ex to obtain final concentrations of 0, 0.045, 0.137, 0.412, 1.235, 3.704, 11.11, 33.33, and 100 μM. 3,4-Dichloroaniline (3,4-DCA, positive control, Fisher Scientific) was prepared fresh on the day of exposure in DMSO and diluted in L-15/ex to obtain the following concentrations: 6.25, 12.5, 25, 50, and 100 mg/l. Water and DMSO were added to L-15/ex at a final concentration of 0.5% v/v.

2.3.5 Exposure to pesticides for cell viability

In terms of the overall design of the exposure studies, each pesticide was tested at nine concentrations (including a negative control) spanning ~3.5 orders of magnitude. Three different passages were tested against the pesticides and these exposures were performed in different microplates. Pesticides were exposed in one well per concentration to make up one technical replicate within each passage, and three passages in total to make up three biological replicates. Therefore, six microplates were used to test 19 pesticides over three passages to represent three biological replicates (**Figure *2-1***).

On exposure day 1, RTgill-W1 cells were seeded at a density of 35,000 cells/100 μL of complete medium per well in 96-well plates and incubated for 24 h. Per plate, 11 wells were designated as no-cell controls and contained only the complete medium (**Figure *2-1***).

On day 2, the old complete medium was discarded and replaced with 100 μL of L-15/ex for the negative control (one well), or L-15/ex containing either the pesticide, solvent (DMSO), or positive control (3,4-DCA) at appropriate concentrations (one well/concentration). The highest concentration tested for each pesticide was 1 mM because of a paper published by Harrill

et al. (2019) that described using an 8-concentration dilution dosing system starting at 1 mM as a standardized approach to conducting *in vitro* microplate chemical exposures (26). Pesticides (n=19) were separated into batch 1 (atrazine, clothianidin, glyphosate, imazethapyr, imidacloprid, metolachlor, thiamethoxam, chlorantraniliprole, chlorothalonil, and positive control 3,4-DCA) and batch 2 (diazinon, chlorpyrifos, diquat, permethrin, s-metolachlor, carbaryl, AMPA, dicamba, dimethenamid-P, metribuzin) for exposures to keep consistent formatting (**Figure *2-1***). Pesticide stocks and 3,4-DCA were added to the C1 wells and then serially diluted (3-fold) in L-15/ex. Ten of the eleven no-cell control wells received the highest concentration of chemicals (C1) and the other well-received L-15/ex. The cells were then incubated for 24 h.

On day 3, the exposure medium was discarded, and the alamarBlue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) cytotoxicity assays were conducted immediately to assess metabolic activity and membrane integrity, respectively. Briefly, the cells were rinsed in 100 µL phosphate-buffered saline (PBS) following which alamarBlue (5% v/v in PBS) + CFDA-AM dye was added to the wells, incubated for 30 min and fluorescence was read at 530 nm/595 nm for alamarBlue and 493 nm/541 nm for CFDA-AM using a Synergy HT Gen5 microplate reader (BioTek, Winooski, USA). The alamarBlue + CFDA-AM dye was then discarded. Concentration-response curves of the tested pesticides are reported as percentage viability. Percentage viability is based on the fluorescence units (FU) of the treated cells compared to the DMSO-solvent control fluorescence units based on the equation below:

% cell viability = $\{[FU_{chemical} - FU_{chem.no-cell\ ctrl} \times 100]/[FU_{control} - FU_{ctrl.no-cell}]\}$. The concentration causing 50% cytotoxicity (EC50) was calculated using the drc package for dose-response analysis in R.

2.3.6 *In vitro-in vivo* comparisons

Comparisons were made of the *in vitro* EC50 derived here with *in vivo* LC50 values taken from the scientific literature. *In vivo* LC50 values were taken from 96-h acute toxicity studies with adult rainbow trout (*Oncorhynchus mykiss*) from the United States EPA's Ecotoxicology Knowledgebase (ECOTOX, https://cfpub.epa.gov/ecotox/; (27)), and the EnviroTox database (https://envirotoxdatabase.org/) and peer-reviewed journals if no EPA value was available. Inclusion criteria for the selection of *in vivo* values from the ECOTOX database was a 96h exposure, adult rainbow trout, tests performed with active ingredient (not formulation), the chemical purity >80%, EC50 and mortality were the tested endpoint, and use of flow-through or static systems were accepted. All pesticides except permethrin and carbaryl produced one value from the database. Permethrin produced three values that satisfied the inclusion criteria, and these values were averaged to produce a mean LC50 to compare with *in vitro* cell results. Carbaryl's LC50 was taken from Dwyer et al. (2005) because of the high sample size of adult rainbow trout and satisfying the rest of the inclusion criteria (28). *In vitro-in vivo* correlations were performed using a Spearman's rank ρ coefficient test where alpha = 0.05 (GraphPad Prism (Version 9.5.0 (525)).

2.4 Results

2.4.1 Optimization to 96-well plate format

To ensure valid quality control criteria in the 96-well plate format, trials were conducted with 3,4-DCA. The OECD Test No. 249 guideline calls for seeding 350,000 cells/well in a 24-well format, so it was first tested if 10,000 or 35,000 cells would be more suitable for seeding in the 96-well plates. Quality control criteria did not consistently perform well when seeding 10,000 cells/well whereas seeding with 35,000 cells/well yielded reliable quality control results (**Table *2-1***). Taken together from experiments validating the 96-well plate format and pesticide exposure experiments that included 3,4-DCA, the average EC50 for 3,4-DCA with the alamarBlue dye was 35.2 (21.7 – 48.7) mg/L and 67.3 (47.3 – 87.3) mg/L for the CFDA-AM dye (**Figure *2-2***). The average percentage difference between the solvent control DMSO and unexposed (L-15/ex) control from all optimization tests and pesticide exposures in 96-well plates was 100.2% ± 10.3% and 97.0% ± 9.6.3% for alamarBlue and CFDA-AM, respectively (**Figure *2-3***). Subsequently, the no-cell C1 average variation from the no-cell negative control for alamarBlue was -7.9% ± 8.6%, and for CFDA-AM was -7.2% ± 24.7% **Figure *2-3***). Therefore, quality control criteria were not impacted by the transition to a 96-well plate format and variations in results were still within parameters set out in OECD Test No. 249 which did not compromise the overall quality of the data.

Since the assay was shown to work effectively in a 96-well plate format based on the quality control results, a pilot study was next conducted comparing 24- and 96-well plate results for one test chemical with the alamarBlue fluorescent dye. RTgill-W1 cells were exposed to metolachlor in a 24-well plate over a concentration range of 0.01 – 1000 μM (2-fold serial dilution over 6 concentrations) yielded an EC50 of 291.2 μM (82.6 – 499.7 μM), while RTgill-

W1 cells exposed to metolachlor in a 96-well plate resulted in an EC50 of 311.7 μM (108.9 – 514.4 μM) (**Figure *2-4***). These results demonstrated that the assay was scalable for a 96-well format, thus subsequent experiments were conducted on 19 pesticides of interest.

Table 2-1: Optimization tests comparing the quality control criteria laid out in OECD Test No. 249 for 35,000 cells seeded/well and 10,000 cells seeded/well in a 96-well plate format for alamarBlue and CFDA-AM fluorescent dyes with the positive control chemical 3,4-DCA.

alamarBlue	35,000 cells/well			10,000 cells/well		
Trial #	DMSO within 90-110% of negative control	Variation between no-cell C1 and negative control <20%	DCA EC50 within 28.4 – 58.9 mg/L	DMSO within 90-110% of negative control	Variation between no-cell C1 and negative control <20%	DCA EC50 within 28.4 – 58.9 mg/L
1	107.1	0.7	51.4	**156.9***	-1.4	**10.2***
2	101.5	0.6	51.6	105.9	0.6	**18.4***
3	**59.3***	1.3	**91.3***	**49.7***	-11.8	**99.7***
4	96.8	5.3	37.5	**121.2***	9.7	**23.4***
5[a]	108.8	-3.6	47.2	-	-	-
CFDA-AM	35,000 cells/well			10,000 cells/well		
Trial #	DMSO within 90-110% of negative control	Variation between no-cell C1 and negative control <20%	DCA EC50 within 15.2 – 109.8 mg/L	DMSO within 90-110% of negative control	Variation between no-cell C1 and negative control <20%	DCA EC50 within 15.2 – 109.8 mg/L
1	**114.4***	0	71.2	**108.1***	**-50***	76.2
2	95.9	-3.6	**112.3***	96.0	-2.6	77.2
3	**68.3***	0	**132.9***	**61.9***	0	**109.5***
4	102.6	0	60.3	**111.4***	**-25***	61.2
5[a]	98.9	-1.3	53.6	-	-	-

*Value does not satisfy quality check criteria

[a]Trial 5 did not include seeding 10,000 cells/well and no experiments past trial 4 tested the assay with 10,000 cells/well. All experiments beyond trial 5 focused on pesticide exposures.

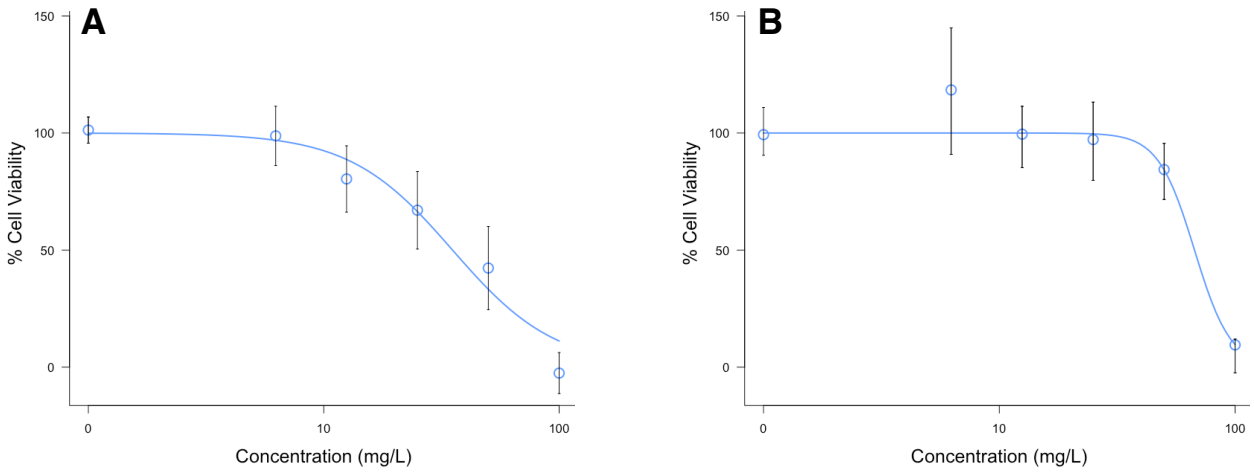

Figure 2-2: Average EC50 for 3,4-DCA in a 96-well plate format for A) alamarBlue and B) CFDA-AM. Error bars represent the standard deviation for each data point for 23 different experiments.

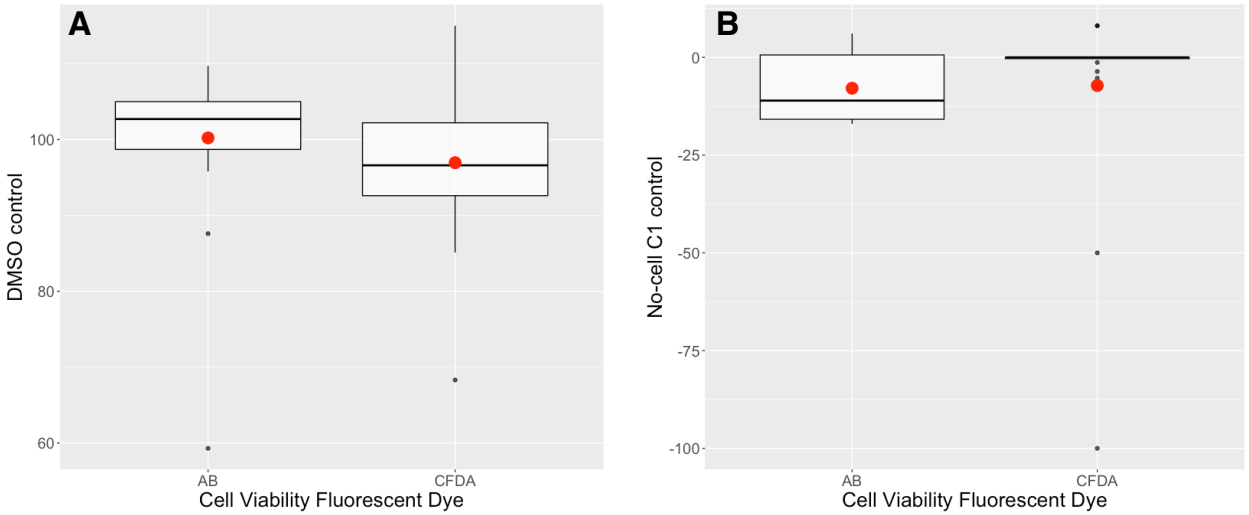

Figure 2-3: Variation of A) DMSO solvent and B) No-cell C1 quality control checks from optimization experiments and pesticide exposures (n=23) for alamarBlue (AB) and CFDA-AM (CFDA) fluorescent dyes. The mean for all experiments is represented by the red circle in each boxplot.

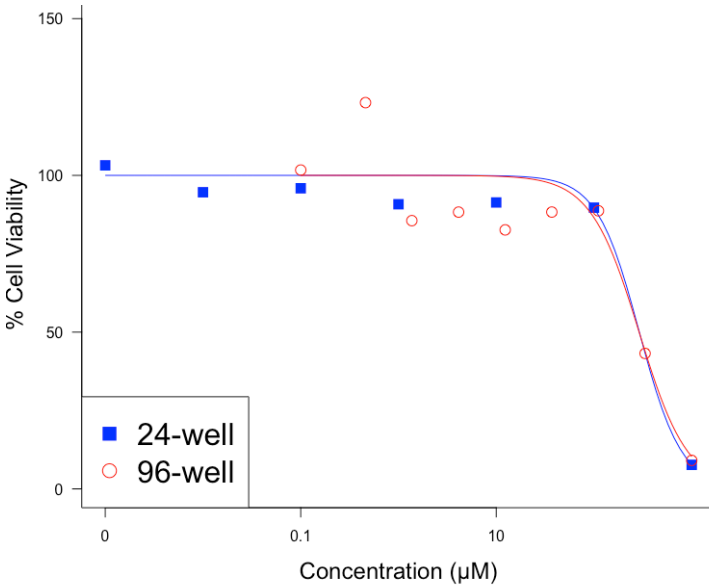

Figure 2-4: Concentration-response curves for metolachlor exposed to RTgill-W1 cells in 24-well and 96-well plates with alamarBlue fluorescent dye assessing metabolic activity.

2.4.2 Pesticide cytotoxicity

The positive control 3,4-DCA was included in each 96-well plate and elicited concentration-response curves with an average EC50 of 33.0 and 62.0 mg/L for the alamarBlue and CFDA-AM cell viability dyes, respectively (**see supplementary Figure S*2-1***). Cell viability of the DMSO solvent control ranged from 100.4% to 104.9% compared to the negative control (**see supplementary Table S*2-2***). The variation in no-cell C1 controls was lower than 20% compared to the no-cell negative control for each pesticide (**see supplementary Table S*2-2***). Combined, these results from tests in the 96-well plates satisfy the quality control criteria outlined in OECD Test No. 249 (9).

Concentration-response curves and corresponding EC50 values were obtained for 10 of the 19 pesticides assessing metabolic activity and seven of the 19 pesticides assessing membrane

integrity assay (**Table *2-2***; **see supplementary Figure S*2-1*** and **Figure S*2-2*** for all 19 concentration-response curves). Diquat, AMPA, dicamba, and imazethapyr were cytotoxic for metabolic activity but not for membrane integrity, whereas the opposite was observed with permethrin. The rank order for most to least toxic based on metabolic activity was chlorothalonil > carbaryl > diquat > chlorpyrifos > dimethenamid-P > metolachlor > s-metolachlor > dicamba > AMPA > imazethapyr (**Table *2-2***). Rank-order for most to least toxic based on membrane integrity was chlorothalonil > chlorpyrifos > carbaryl > permethrin > dimethenamid-P > metolachlor > s-metolachlor. Six pesticides elicited a curve for both cell viability endpoints and values were similar (within 95% confidence intervals) between endpoints except for carbaryl and dimethenamid-P. Metabolic activity was the more sensitive endpoint for pesticide EC50 values that differed outside of the 95% confidence interval except with permethrin.

Table 2-2: Effective concentration 50 (EC50) values from two cell viability endpoints for 19 pesticides and literature *in vivo* median LC50 values from rainbow trout. CI = confidence intervals.

Pesticide	EC50 (μM) ± 95% CI of RTgill-W1 assays		Rainbow Trout LC50 (μM)
	alamarBlue (Metabolic Activity)	CFDA-AM (Membrane integrity)	
Chlorpyrifos	**276.8** (132.0 - 421.6)	**299.1** (165.5 - 432.7)	0.1
Chlorothalonil	**2.6** (1.8 – 3.5)	**3.6** (-0.3 – 7.6)	0.1
Carbaryl	**97.0** (54.5 - 139.6)	**393.1** (219.9 - 566.3)	9.9
Metolachlor	**311.7** (108.9 - 514.4)	**530.2** (17.3-1043.1)	13.7
S-metolachlor	**352.3** (-794.2 - 1498.2)	**565.0** (215.9 - 914.1)	41.9
Dimethenamid-P	**291.7** (185.3 - 398.1)	**497.3 (**319.3 - 675.3)	22.8
Diquat	**100.4** (34.5 - 166.3)	>1000	41
AMPA	**493.1** (307.1 - 679.1)	>1000	2637
Dicamba	**416.3** (250.7 - 581.8)	>1000	612.6
Imazethapyr	**538.5** (-120.5-1197.5)	>1000	1188.9
Permethrin	>1000	**426.7** (141.9 - 711.5**)**	0.01
Diazinon	>1000	>1000	1.3
Atrazine	>1000	>1000	24.6
Chlorantraniliprole	>1000	>1000	28.6
Thiamethoxam	>1000	>1000	342.8
Metribuzin	>1000	>1000	298.7
Clothianidin	>1000	>1000	420.5
Imidacloprid	>1000	>1000	896.1
Glyphosate	>100	>100	792.6

2.4.3 *In vitro-in vivo* correlations

Correlations between the lethality values from rainbow trout fish exposures and the EC50s derived from *in vitro* exposures with metabolic activity and membrane integrity endpoints are shown in **Figure *2-5***. The non-cytotoxic pesticides atrazine, clothianidin, metribuzin, imidacloprid, thiamethoxam, diazinon, and glyphosate were omitted from the correlation analysis as there were no definitive *in vitro* values. Correlation results for metabolic activity (**Figure *2-5***) resulted in a Spearman ρ correlation value was 0.86 with a p-value of 0.0013 (2-

tailed, alpha = 0.05, n = 10) which is a statistically significant correlation (**Table *2-3***). Results for membrane integrity (**Figure *2-5***) derived a Spearman ρ correlation value of 0.74 and p-value of 0.058 (2-tailed, alpha = 0.05, n = 7) which is not statistically significant (**Table *2-3***). An important note is that sample sizes are small (n=10 and n=7) when non-cytotoxic pesticides are excluded from the analysis. Overall, RTgill-W1 cells were more sensitive with the metabolic activity endpoint with permethrin being the only exception.

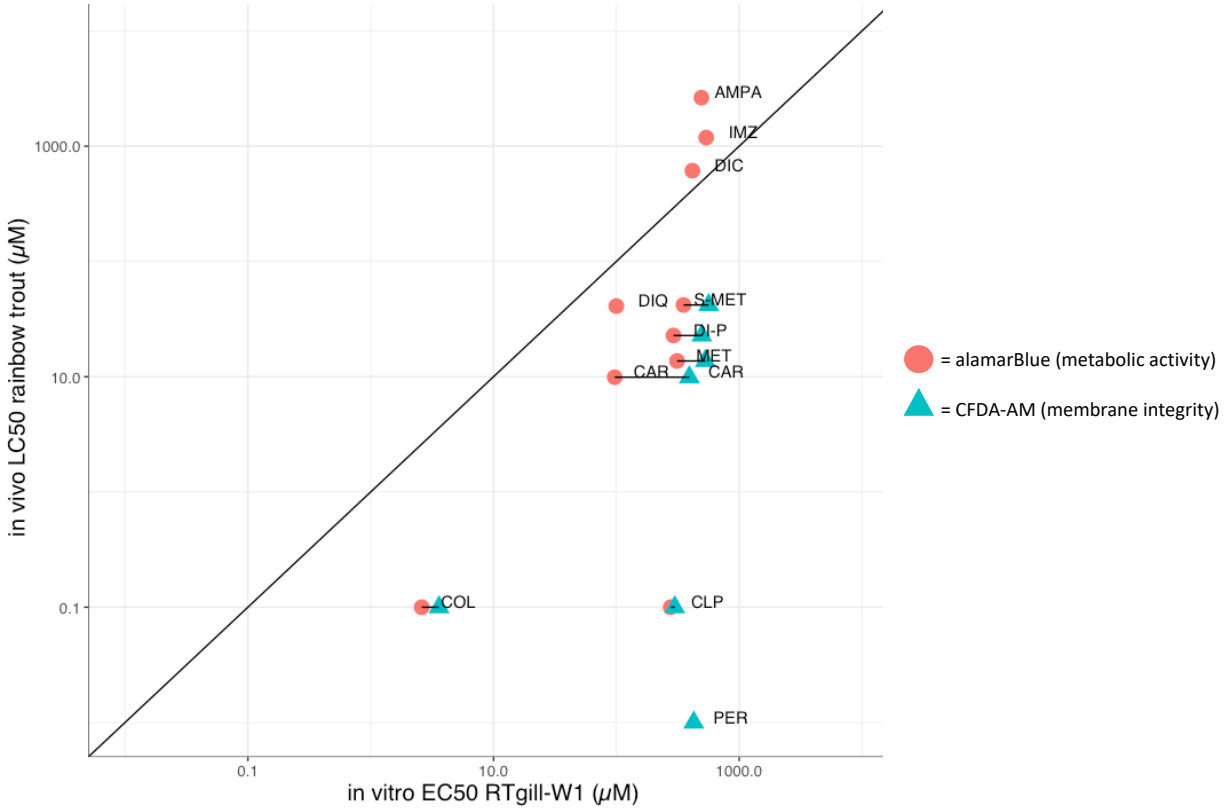

Figure 2-5: Correlation of effective concentration 50 (EC50) values for RTgill-W1 cells based on the A) metabolic activity endpoint (alamarBlue) and B) membrane integrity endpoint (CFDA-AM) for only cytotoxic pesticides with the LC50 values for rainbow trout taken from the EPA database. Solid line represents the line of unity.

Table 2-3: Spearman ρ correlation coefficient, R squared, and p values for comparing *in vitro* EC50s with *in vivo* rainbow trout LC50s.

	R squared	Spearman ρ	P value (alpha<0.05)
AlamarBlue (Metabolic activity) (n=10)	0.46	0.86	0.0013 *
CFDA-AM (Membrane integrity) (n=7)	0.44	0.74	0.058

2.5 Discussion

2.5.1 Optimization to a 96-well plate format

The development of high-throughput screening assays to assist in the prioritization and regulation of environmental chemicals for regulatory agencies was a driving force behind the standardization and validation of the cell-based *in vitro* RTgill-W1 assay. In this study, seeding 35,000 cells/well in a 96-well plate format proved to be sufficient for meeting the three quality control checks in optimization studies and did not influence pesticide results exhibited through a pilot test with metolachlor (**Table *2-1***, **Figure *2-2***, **Figure *2-3***, **Figure *2-4***). This is not surprising as tests with the RTgill-W1 cells in 96-well plates have been done in the past, before the standardization of OECD Test No. 249(15, 29). Initial concerns with scaling the assay to 96-well plates were that the final DMSO concentration in the exposure concentration needed to be 0.5% (v/v) to ensure it would not cause a cytotoxic response (9). This consideration was maintained in a 96-well plate format. Validating that the specific quality controls set out in OECD Test No. 249 would still apply in a 96-well plate format was important to include before

any experiments took place. The results represent the potential to scale up the assay to efficiently screen large numbers of chemicals for prioritization and address knowledge gaps.

2.5.2 Cytotoxicity and correlation results for pesticides

The pesticides selected for this study differed in their class (10 herbicides, 8 insecticides, and one fungicide), target receptors and/or mechanisms of action, hydrophobicity (log Kow from -3.75 to 6.1), volatility (log H -15.5 to -1.3), and water solubility (from 0.2 mg/L to $1.5x10^6$ mg/L). The present research determined that 11 of the 19 pesticides tested were cytotoxic to RTgill-W1 cells (**Table *2-2***). Of the 11 pesticides, 7 herbicides (diquat, metolachlor, s-metolachlor, dicamba, imazethapyr, dimethenamid-P, and AMPA) and one fungicide (chlorothalonil), for a total of 8 pesticides, yield *in vitro* values that were within one order of magnitude of *in vivo* LC50 values from rainbow trout fish. Between all 11 cytotoxic pesticides, *in vitro* EC50s ranged between 0 to 4 orders of magnitude from the *in vivo* LC50s with insecticides chlorpyrifos and permethrin yielding the least predictive *in vitro* EC50s. While the results suggest that there was a significant correlation between *in vitro* and *in vivo* studies with the metabolic activity endpoint, some pesticides that are known to be very acutely toxic *in vivo*, had no effect on the RTgill-W1 cells (for example, atrazine). Since pesticides are a broad term to describe a class of chemicals that kill, repel, or control plants or animals deemed as pests, each pesticide is distinct in its compositions and mechanisms of action, and this affects their toxicity to the RTgill-W1 cells uniquely.

2.5.2.1 Physiochemical properties

Physiochemical properties such as hydrophobicity, volatility, and solubility can impact cytotoxicity results in microplate exposures. A common trend was not observed between volatility and hydrophobicity of the chemicals and which ones yielded cytotoxic results. Moreover, the assay system was observed to not be as predictive of toxicity *in vivo* for the more hydrophobic (log Kow > 3) pesticides (**see supplementary Table S2-1**). For example, chlorpyrifos, which is hydrophobic (log Kow = 4.7) and volatile (log H = -1.3) was cytotoxic, but the resulting *in vitro* cell values were 3 orders of magnitude higher than the *in vivo* lethality value observed in rainbow trout fish (**Figure *2-5***). Similarly, permethrin (log Kow = 6.1) yielded cytotoxic values 4 orders of magnitude higher than the *in vivo* LC50 from rainbow trout fish. Interestingly, metolachlor (log Kow = 3.4) and s-metolachlor (log Kow = 3.1), which are both hydrophobic, were within one order of magnitude of the LC50 values from rainbow trout for the RTgill-W1 cells. Hydrophobicity and volatility have been previously observed with the RTgill-W1 and RTgutGC cells and other microplate studies to impact *in vitro* results (7, 15, 31, 32). It has been demonstrated with hydrophobic chemicals that there is a higher potential for stability and toxicity estimation issues because of chemicals partitioning out of solution and sorbing to the plastic well, reducing the exposure to cells (33-37). Stadnicka-Michalak et al. (2014) showed with the RTgill-W1 cells in 24-well plates that chemicals with a higher log Kow (more hydrophobic) are more likely to absorb to the plastic well (37). A way to account for this in *in vitro* microwell experiments is by considering the internal exposure concentration through measuring concentration at time of exposure (C_{0h}) and 24 hours later (C_{24h}) and could be more comparable to LC50s from *in vivo* acute lethality exposures (35). The present research only considered nominal concentrations in yielding EC50s. Accounting for chemical loss could be

beneficial in the future when studying pesticides using *in vitro* cell-based assays to understand the discrepancies in cytotoxicity values and potentially yield results that could be more comparable to *in vivo* fish lethality values.

Studies using some of the same pesticides that did perform chemical analysis can be employed to help corroborate the results obtained. Scott et al. (2021) measured the concentration of permethrin at $T_{0h \text{ and}}$ T_{24h} and found an over 80% decrease in the concentration based on the initial nominal concentrations (38). Permethrin is a hydrophobic chemical, as discussed, and the chemical partitioning out of the assay system could explain the low cytotoxicity observed in this study. Secondly, Dupraz et al. (2019) exposed 16 pesticides, including s-metolachlor, glyphosate, AMPA, chlorpyrifos, and imidacloprid to marine microalgae in 48-well plastic microplates and performed chemical analysis. In terms of chemical recovery, this study found that the herbicides and imidacloprid were stable throughout the exposure time, chlorpyrifos had a large decrease in concentration, and α-cypermethrin, which is closely related to permethrin, saw a less than 10% recovery (30). As well, the percentage of chemical recovered was found to be more linked to hydrophobicity rather than volatility, in which the more hydrophobic the pesticide is, the lower the concentration percentage recovered (30). These findings help to explain the current study results, as the noted stability and similarity between tested and nominal concentrations for s-metolachlor in the 48-well test system could explain why hydrophobicity did not appear to impact results as much, and since metolachlor is similar physiochemically to s-metolachlor, these findings could be extended to it as well. It is also possible that the extent of hydrophobicity (ie. how high the log Kow is) could also impact chemical stability since metolachlor and s-metolachlor are not as hydrophobic as chlorpyrifos and permethrin.

2.5.2.2 Herbicides

Of the herbicides, dimethenamid-P, imazethapyr, metolachlor, s-metolachlor, diquat, dicamba, and AMPA elicited concentration-response curves. The ones that did not were atrazine, metribuzin, and glyphosate. Atrazine and metribuzin are from the chemical family in which atrazine is of the symmetrical triazines and metribuzin is of the asymmetrical triazines (alternatively, triazinones) and have the same target site of action in which they inhibit photosystem II in photosynthesis to cause oxidative damage and plant cell death by starving the cells (19, 31). It is not surprising that atrazine and metribuzin did not cause cytotoxicity as their target mode of action, inhibiting photosystem II in photosynthesis, is a structure not found in animal cells (19, 31). Atrazine has been observed to alter the detoxification of enzymes, change the concentration of hormones, and induce oxidative stress in the livers of fish (32, 33). Only one study has been conducted on fish cells regarding atrazine cytotoxicity. In this study, atrazine was exposed to the ZC7901 cell line from grass carp (*Ctenopharyngodon idellus*) and yielded a 12h IC50 (concentration inhibiting 50% of *in vitro* cell growth) of 199.0 mg/L and a 72h IC50 of 11.6 mg/L following the MTT (3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2-H-tetrazoliumbromide) assay (34). It is possible that the highest concentration used in this study was not toxic enough to cause cell death. Metribuzin exposure to fish has also been shown to induce oxidative stress and increase activities of biotransformation and antioxidant defense enzymes in common carp (*Cyprinus carpio*) and exposure to a commercial herbicide Sencor, whose active ingredient is metribuzin, decreased the activities of catalase, glutathione, peroxidase, glutathione reductase, and Ache enzymes in the kidneys and livers of goldfish (*Carassius auratus*)(35-37). Interestingly, exposure to Sencor on goldfish exhibited no effect on

free radicals processed in gills and the glutathione-dependent antioxidants protected proteins and lipids from oxidation(38). In a study by Plhalova et al. (2012), the histopathological changes to the gill, liver, and kidney of zebrafish (*Danio rerio*) were observed after metribuzin exposure and revealed no morphological changes to the gills and only pathological lesions in the liver (39). No studies have examined the toxicity of metribuzin on fish cells. Atrazine and metribuzin appear to target toxicity in the livers and kidneys of fish instead of the gills. This, combined with the fact that their target proteins are plant-specific, could explain the lack of toxicity to the RTgill-W1 cells. An acute cytotoxic response for glyphosate exposed to the RTgill-W1 cells was not expected as glyphosate has low acute toxicity to fish. Only a couple of other studies have characterized the cytotoxic response of glyphosate to fish cells but used concentrations much higher than the present study (highest concentration tested = 1 mM, equivalent to 169.1 mg/L). For example, exposure of glyphosate to rainbow trout liver cells (RTL-W1) yielded a 24h EC50 of 730 mg/L but cytotoxic responses were only observed at concentrations above 250 mg/L (tested concentration range 0.05 to 1000 mg/L) (40). As well, concentrations of 0, 80, 240, 400, and 560 mg/L glyphosate exposed to diploid and triploid fin cell lines from *Misgurnus anguillicaudatus* yielded EC50s of 315.34 and 371.77 mg/L, respectively. While glyphosate was not cytotoxic, its metabolite AMPA was (EC50 = 493.1 μM), which highlights that glyphosate is important to keep monitoring at acute levels since it indirectly has adverse effects.

2.5.2.3 Insecticides

Of the insecticides, permethrin, chlorpyrifos, and carbaryl derived EC50 values. Permethrin is a neurotoxic pyrethroid insecticide found to be neurotoxic to insects and non-target organisms by interfering with voltage-gated sodium channels and disrupting neuron functioning

(41, 42). It only elicited a response with CFDA-AM, which assesses membrane integrity. This is consistent with previous studies by Scott et al (2021) and Tanneberger et al. (2013) where permethrin exposed to RTgill-W1 cells exhibited higher sensitivity with membrane integrity than with metabolic activity (7, 13). The non-cytotoxic insecticides, clothianidin, imidacloprid, thiamethoxam, and diazinon, also have neurotoxic modes of action so it is not as surprising that they did not induce toxicity in the RTgill-W1 cells (**Table *2-2***). Chlorpyrifos and diazinon are both organophosphate insecticides and share a common mechanism of toxicity. Despite this similarity, only chlorpyrifos was found to be highly toxic in this study (**Table *2-2***). A study exposing chlorpyrifos and diazinon to human bronchial epithelial (A549) cells and immortalized T-lymphocytes (jurkat) cells showed that chlorpyrifos was cytotoxic at concentrations ≥ 250 μM but diazinon was not toxic up to 1 mM, leading the authors to concluded that diazinon must not directly affect cell viability and instead down-regulates normal synthesis pathways in a cell at higher concentrations (43). These findings are strikingly similar to the results from this study with the RTgill-W1 cells (**Table *2-2***), where chlorpyrifos was cytotoxic at concentrations ≥ 111 μM and diazinon was not toxic at concentrations up to 1 mM. Diazinon and chlorpyrifos have been reported in other studies to differ in their toxic responses in cells. One example is when both pesticides were exposed to primary cortical cultures, chlorpyrifos induced toxicity through an entirely different mechanism of neurotoxicity than diazinon, and neither involved acetylcholine (44). The OECD No. 249 assay has been described to be less sensitive compared to whole organisms for neurotoxic chemicals (9) but was only tested with a select few, including permethrin. The present results indicate that the RTgill-W1 cells also likely have limited use for other neurotoxicant chemicals. Alternatively, chlorantraniliprole binds to the muscle-specific ryanodine receptor that causes muscles to leak calcium and leads to paralysis and death (20). It

has been demonstrated that chlorantraniliprole has a greater than 500-fold and 2,000-fold selectivity for the ryanodine receptor in insects over the receptor in mammals by Cordova et al. (2006) and Sattelle et al. (2008) respectively (45, 46). This potentially explains why there is a lack of response observed in the RTgill-W1 cells since gill cells do not contain the ryanodine receptor and chlorantraniliprole binds tighter to the receptor in insects. The use of two different endpoint dyes to assess cell viability proved to be important for eliciting cytotoxic responses that otherwise would have been missed for pesticides. The metabolic activity endpoint (alamarBlue) was more sensitive than membrane integrity (CFDA-AM) except for one pesticide, permethrin, in which the inverse is true. Therefore, both viability dyes should be used together to help elicit different responses with pesticides.

2.5.3 General limitations

In this study, *in vitro* cytotoxicity results were less sensitive than the corresponding *in vivo* lethality by one to four orders of magnitude, except for AMPA, dicamba, and imazethapyr (**Table *2-2***). This trend has been reported in other studies in which rainbow trout gut cells (RTG-2) were exposed to 16 common pollutants including pesticides and compared to *in vivo* 96h rainbow trout results where the *in vitro* test was 20 – 200 times less sensitive than the *in vivo* results determined by the neutral red dye assessing lysosomal integrity (47). Similarly, the fish hepatoma cell line PLHC-1 exposed to 18 pesticides produced *in vitro* cytotoxicity values 3 - 3000 times higher from neutral red assessment than the corresponding 96h acute toxicity LC50 in rainbow trout fish (48). It is likely that since the RTgill-W1 cell line is a single culture, it could not account for the diverse target sites needed to elicit cytotoxicity by all the selected pesticides (49). The low toxicity of certain pesticides could thus be explained by the fact that

RTgill-W1 cells do not possess enough AChE or contain the target nAChRs for the neurotoxic insecticides, the target ryanodine receptor for chlorantraniliprole, or the plant-specific proteins in photosystem II for atrazine and metribuzin. This was observed in Saito et al. (1991) in which the low cytotoxicity of carbamates (a class of neurotoxic insecticides that cause carbamylation of acetylcholinesterase) exposed to goldfish GF-Scale cells could be explained by the cells not possessing enough acetylcholinesterase activity to permit their inhibition by the chemicals (50). Although the RTgill-W1 cells elicited a cytotoxic response with carbaryl and the Saito et al. (1991) study did not, a similar idea can be applied in which responses from cell-based studies are limited to the metabolic pathways and target sites they possess. Two other established cell lines from the liver (RTL-W1) and gut epithelial (RTgutGC) of rainbow trout are available and could be used in conjunction with the RTgill-W1 cell line to represent a wider diversity of target sites and potentially elicit more cytotoxic responses. It has been determined that the OECD No. 249 assay could be performed with the RTgutGC cell line (51) and the RTL-W1 cell line (52).

2.6 Conclusion

Through this study, an increased understanding of the scalability and applicability of the RTgill-W1 cell assay OECD Test No. 249 tested with 19 pesticides was gained. Optimization tests to a 96-well plate format indicated that the assay could be performed in a more high-throughput manner. The three quality control checks; solvent control (DMSO), no-cell control (no cell C1), and positive test chemical control (3,4-DCA), were consistently satisfied. It was also established that the shift would not compromise or impact chemical exposure results. Upon satisfying these conditions, exposure of 19 pesticides to the RTgillW1 cells in this 96-well

format elicited EC50s for 10 pesticides with the metabolic activity endpoint and 7 pesticides with the membrane integrity endpoint for a total of 11 cytotoxic pesticides. The metabolic activity endpoint was more sensitive than membrane integrity for 10 of the 11 pesticides, eliciting concentration-response curves for four more chemicals and yielding lower EC50s for the pesticides that overlapped between the two endpoints. One test pesticide permethrin was cytotoxic for only the membrane integrity endpoint. A Spearman's correlation test comparing the *in vitro* RTgill-W1 results to literature lethality values from *in vivo* 96h rainbow trout exposures established a significant correlation for the metabolic activity endpoint and not for the membrane integrity endpoint. However, five pesticides yielded values approximately within one order of magnitude of their associated lethality value. Discrepancies between *in vitro* and *in vivo* values could be due to a pesticide's hydrophobicity (ie. increased hydrophobicity = decreased cytotoxicity) and target toxic mode of action mode which reduces their sensitivity to RTgill-W1 cells. Further studies with pesticides using *in vitro* cell-based models could better correlate results to *in vivo* values by measuring initial and final concentrations of test chemicals after the 24h exposure and using different fish cell lines such as the liver (RTL-W1) or gut (RTgut-GC) to represent a wider range of target sites.

2.7 Acknowledgements

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Research Chairs program, NSERC Discovery Grants program, and the Department of Fisheries and Oceans grant from the National Contaminants Advisory Group.

2.8 Supplementary Tables and Figures

Table S2-1: Test pesticides and their molecular weight, chemical family, mode of toxic action, hydrophobicity ($LogK_{ow}$), volatility (logH), and water solubility values. Pesticides with a log Kow > 3 were considered hydrophobic and chemicals with a log H > -5.6 volatile.

Pesticide	Abbrev.	CAS	Molecular weight (g/mol)	Chemical Family[a]	Mode of action[a,b]	$logK_{ow}$[a]	logH [a,d]	$logK_{aw}$[e]	Water solubility (mg/L) a
Chlorpyrifos	CLP	2921-88-2	350.58	Organophosphate insecticide	Acetylcholinesterase inhibitor	4.7	-1.3	-3.9	1.05
Chlorothalonil	COL	1897-45-6	265.91	Fungicide	Prevents spore germination and zoospore motility	2.9	-6.6	-5.5	0.81
Carbaryl	CAR	63-25-2	201.22	Carbamate insecticide	Cholinesterase inhibitor	2.4	-8.0	-7.3	9.1
Metolachlor	MET	51218-45-2	283.83	Chloroacetamide herbicide	Inhibition of VLCFA (cell division)	3.4	-7.6	-6.2	530
S-metolachlor	S-MET	87392-12-9	283.83	Chloroacetamide herbicide	Inhibition of VLCFA (cell division)	3.1	-7.7	-6.2	480
Dimethenamid-P	DI-P	163515-14-8	275.79	Chloroacetamide herbicide	Fatty acid inhibitor	1.9	-8.3	-7.5	1499
Diquat dibromide monohydrate[c]	DIQ	6385-62-2	362.06	Quaternary ammonium herbicide	Photosystem I (electron transport) inhibitor	-3.8	-3.9	-4.2	535908
Aminomethyl-phosphonic acid	AMPA	1066-51-9	111.04	Metabolite of glyphosate	N/A	-1.6	-5.8	-13.3	1466561
Dicamba	DIC	1918-00-9	221.04	Benzoic acid herbicide	Synthetic auxin	-1.8	-9.3	-6.6	250000

Imazethapyr	IMZ	81335-77-5	289.33	Imidazolinone herbicide	Inhibits plant amino acid synthesis	1.5	-6.9	-10.6	1400
Permethrin	PER	52645-53-1	391.31	Pyrethroid insecticide	Sodium channel modulator, neurotoxic	6.1	-5.7	-4.1	0.2
Diazinon	DIZ	333-41-5	304.38	Organophosphate insecticide	Acetylcholinesterase inhibitor	3.7	-6.2	-5.2	60
Atrazine	ATA	1912-24-9	215.68	Triazine herbicide	Inhibition photosynthesis (photosystem II)	2.7	-8.8	-6.4	35
Chlorantraniliprole	CAT	500008-45-7	483.15	Diamide insecticide	Ryanodine receptor modulator	2.9	-13.5	-16.0	0.88
Thiamethoxam	THX	153719-23-4	291.71	Neonicotinoid insecticide	Nicotinic acetylcholine receptor competitive modulator	-0.1	-14.3	-10.4	4100
Metribuzin	MBZ	21087-64-9	214.29	Triazinone herbicide	Inhibits photosynthesis (photosystem II)	1.8	-9.5	-10.7	10700
Clothianidin	CLN	210880-92-5	249.68	Neonicotinoid insecticide, metabolite of thiamethoxam	Nicotinic acetylcholine receptor competitive modulator	0.9	-15.5	-12.1	340
Imidacloprid	IMI	138261-41-3	255.66	Neonicotinoid insecticide	Nicotinic acetylcholine receptor competitive modulator	0.6	-14.8	-10.8	610
Glyphosate	GLY	1071-83-6	169.07	Organophosphate herbicide	Inhibition of EPSP synthase	-6.3	-12.7	-10.1	100000

[a] Physio-chem properties were taken from PPDB (Pesticide Properties DataBase): Pesticide Properties DataBase (http://sitem.herts.ac.uk/aeru/ppdb/en/index.htm)

[b] VLCFA (Very Long Chain Fatty Acids), EPSP synthase (5-enolpyruvylshikimate-3-phosphate synthase)

[c] Diquat properties from Comptox EPA (Environmental Protection Agency) database https://comptox.epa.gov/dashboard/chemical/properties/DTXSID7031248

[d] Henry's Law constant is given in units $atm \cdot m^3 \cdot mol^{-1}$

[e] air-water partition coefficient (LogKaw) was taken from Eas-E Suite Beta database (https://beta.eas-e-suite.com/) or calculated based on ideal gas constant, temperature, and Henry's Law constant

Table S2-2: Test validity criteria for the average of the three biological replicates for batch 1 and batch 2 pesticides with alamarBlue and CFDA-AM**.**

	Batch 1 p69, p70, p71 (n=3 plates)				Batch 2 p64, p65, p67 (n=3 plates)			
Endpoint	**DMSO within 90-110% of negative control**	**Variation between no-cell C1 and negative control <20%**		**DCA EC50 within range**	**DMSO within 90-110% of negative control**	**Variation between no-cell C1 and negative control <20%**		**DCA EC50 within range**
alamarBlue (Metabolic Activity)	101.7	ATA	-1.2	Range: 28.4 - 58.9 mg/L	104.9	DIZ	-3.8	Range: 28.4 - 58.9 mg/L
		CLN	-6.5			CLP	-6.3	
		GLY	**-23.9***			DIQ	**-24.0***	
		IMZ	-11.4			PER	-12.1	
		IMI	-11.2			SMET	-13.1	
		MET	-9.6	33.0		CAR	-9.4	-
		THX	-9.2			AMPA	-12.0	
		CAT	-10.6			DIC	-11.3	
		COL	**-25.2***			DIP	-12.8	
		DCA	0.72			MBZ	-15.6	
CFDA-AM (Membrane Integrity)	102.4	ATA	0	Range: 15.2 – 109.8 mg/L	100.4	DIZ	0	Range: 15.2 – 109.8 mg/L
		CLN	0			CLP	-16.7	
		GLY	-19.1			DIQ	-16.7	
		IMZ	-15.1			PER	-16.7	
		IMI	-5.6			SMET	-16.7	
		MET	-15.1	62.0		CAR	-16.7	-
		THX	-9.5			AMPA	-16.7	
		CAT	-9.5			DIC	0	
		COL	-15.1			DIP	-16.7	
		DCA	3.6			MBZ	-16.7	

*Test pesticides consistently out of range for each test performed

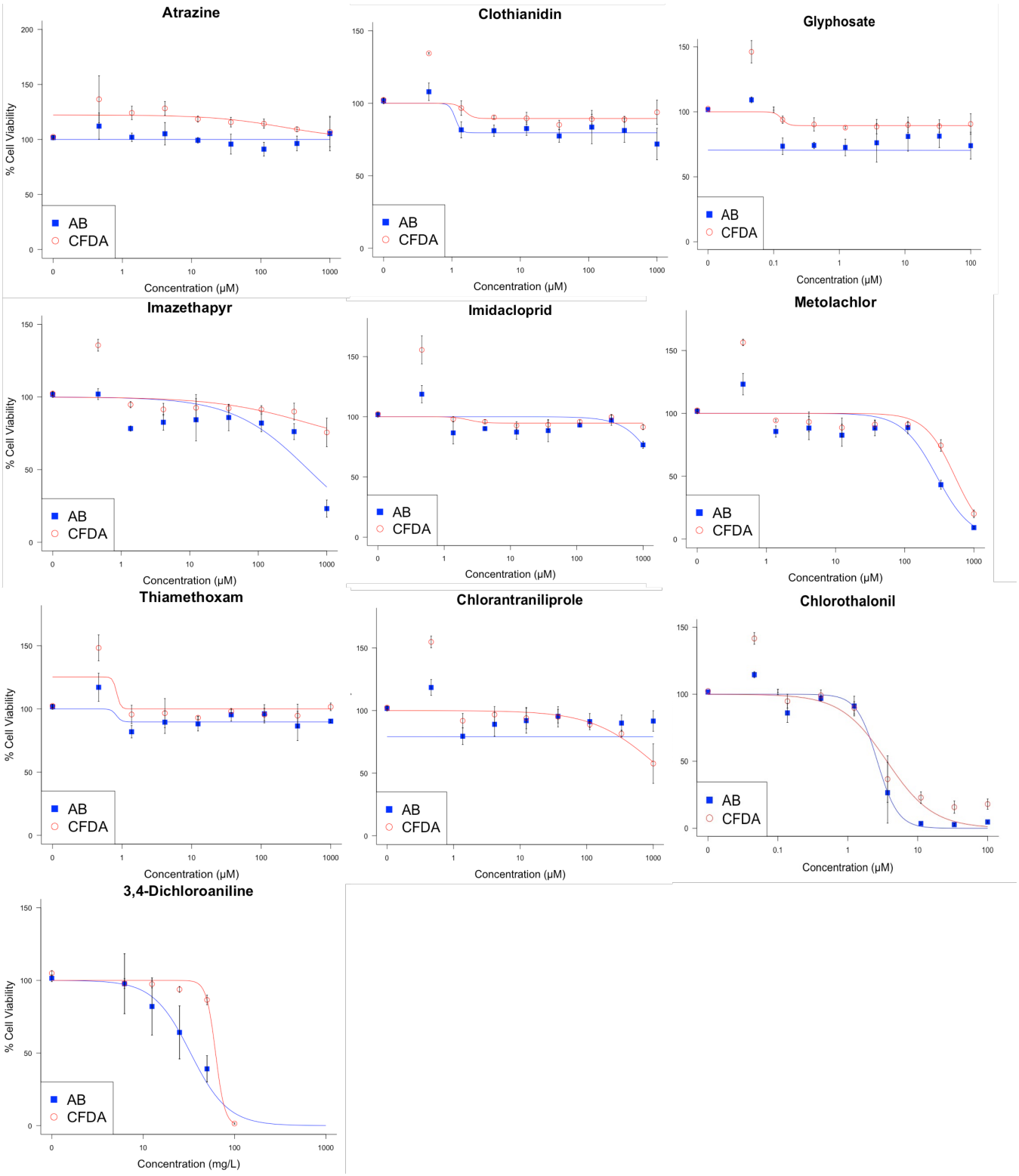

Figure S2-1: Concentration-response curves for batch 1 pesticides based on nominal concentrations for metabolic activity (square) and membrane integrity (open circle). Data points indicate the mean of trials conducted in triplicate and error bars represent the standard deviation for these trials.

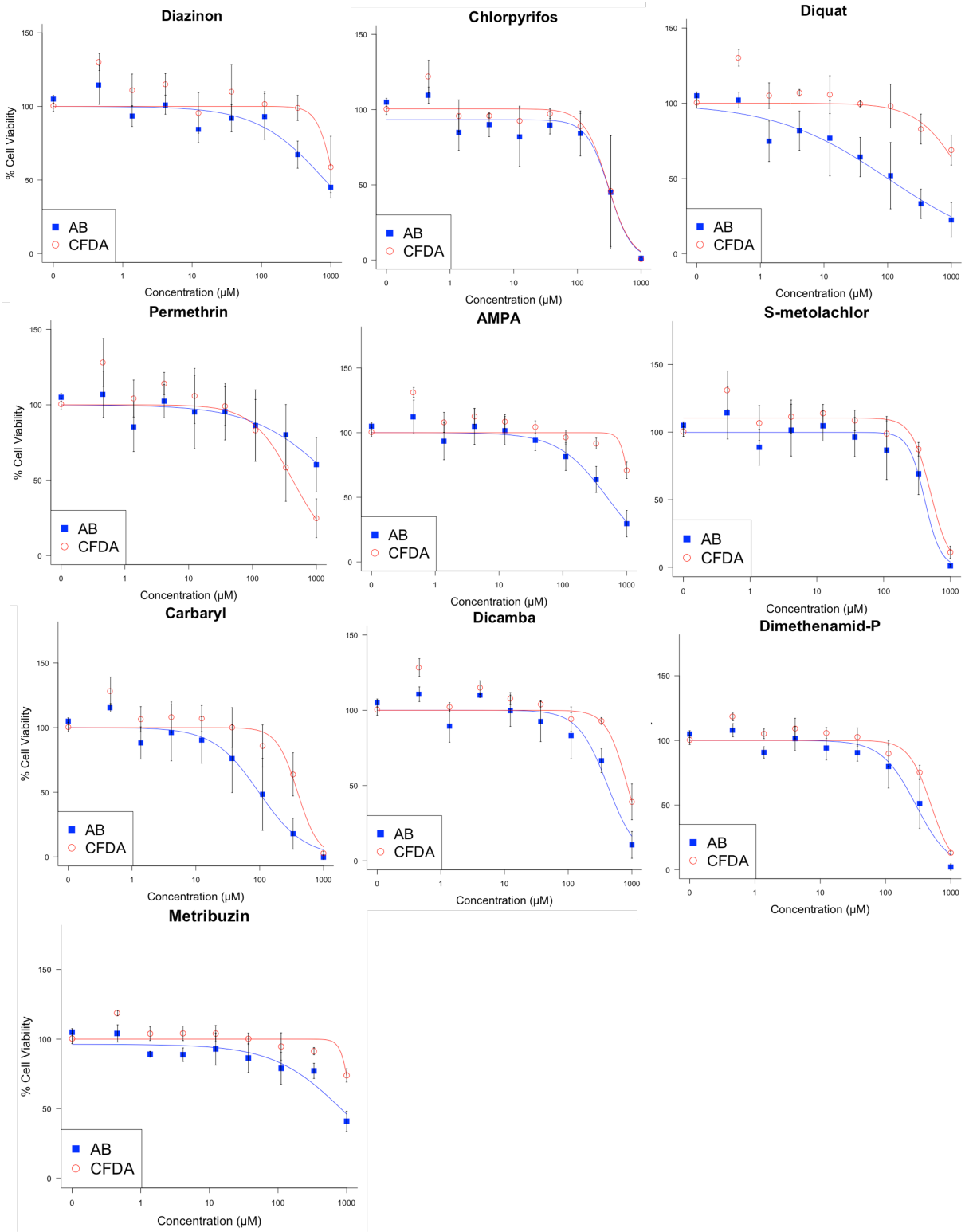

Figure S2-2: Concentration-response curves for batch 2 pesticides based on nominal concentrations for metabolic activity (square) and membrane integrity (open circle). Data points indicate the mean of trials conducted in triplicate and error bars represent the standard deviation for these trials.

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Preface to Chapter 3

Chapter 2 studied the applicability of the RTgill-W1 cells following the OECD No. 249 assay to characterize the cytotoxicity of 19 pesticides. The high-throughput ability of the assay and the correlation between the *in vitro* results from gill cell exposures and *in vivo* values taken from lethality studies in the literature were examined. There was a significant correlation between these two groups with values between one to four orders of magnitude.

Chapter 3 investigates the molecular effects that some of the more cytotoxic pesticides (n=6) impose on the transcriptome of the RTgill-W1 cells. This was realized through high-throughput RNA sequencing approaches from which I could derive transcriptomic point of departure (tPOD) values for each pesticide. Taken together with apical outcome information from Chapter 2, a full range of toxicity from the sublethal molecular impacts (in Chapter 3) to lethal effects (ie. cell death from Chapter 2) will be described. This chapter will add valuable molecular information, that to the best of our knowledge does not exist yet for the majority of these pesticides, and also determine if the tPOD approach can work with RTgill-W1 cells following the OECD No. 249 assay.

This chapter is authored by the candidate and co-authored by the candidate's supervisor Dr. Niladri Basu, and Drs. Krittika Mittal and Ke Xu who are post-doctoral fellows under Dr. Niladri's supervision. The study design for RNA extraction and library preparation was carried out by the candidate and both fellows; the candidate was responsible for all exposures, sample preparation, data analysis and interpretation, discussion of the results, and preparation of the

manuscript. Advice throughout was provided by the candidate's supervisor Dr. Niladri Basu. The manuscript is planned for submission to Environmental Toxicology and Chemistry.

Chapter 3

3 Transcriptomic effects of RTgill-W1 cells exposed to pesticides

TITLE
Transcriptomic effects of RTgill-W1 cells exposed to environmentally relevant pesticides

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Transcriptomic effects of RTgill-W1 cells exposed to environmentally relevant pesticides

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DISCLAIMERS
The authors declare no competing financial interests or other conflicts of interest.

3.1 Abstract

Background: The demand for more efficient and ethical testing strategies in ecological risk assessments has called for the development of New Approach Methods (NAMs). Advancements in sequencing technologies have granted the ability to combine high-throughput transcriptomic techniques with *in vitro* cell-based assays, offering a promising animal-free approach.

Objective: Using six environmentally relevant pesticides, the objective of this work was to derive transcriptomic points of departure (tPOD) values from dose-response RNA sequencing data.

Methods: The RTgill-W1 cell line, based on the Organization for Economic Co-operation and Development (OECD) Test No. 249, was exposed to one fungicide (chlorothalonil), two herbicides (metolachlor and diquat), and three insecticides (chlorpyrifos, permethrin, and carbaryl).

Results: $tPOD_{mode}$ values were derived for each pesticide ranging from 0.4 to 39 µM. Pathway analysis revealed the ribosomal pathway as the most sensitive for chlorothalonil, chlorpyrifos, and metolachlor, and identified other dysregulated pathways relating to cell cycle, signaling, oxidative stress, and synthesis of biomolecules across pesticides. tPOD values were consistently lower than corresponding *in vitro* cytotoxicity values from exposure to RTgill-W1 cells and comparable to *in vivo* lethality values from whole fish studies.

Discussion: This study highlights the abilities of *in vitro* high-throughput methods in yielding values protective of adverse apical outcomes and providing deeper insights into mechanisms of action without animal testing.

3.2 Introduction

Chemical and environmental risk assessment has always heavily relied on live animal traditional toxicity testing methods to derive threshold concentrations or doses at which no adverse effects are observed (points of departure; PODs). A benchmark dose (BMD) analysis has been used for extrapolating such a value from experimental dose-response data for apical endpoints (ie. death, reproduction, development) (1, 2). Obtaining such values is protective of, and therefore invaluable, to ecological species health. However, such experiments are low-throughput, resource-, and time-intensive, making these methods unusable for producing POD toxicity data for the increasing number of chemicals in the environment that are of concern (1, 3, 4). Consequently, there is a paradigm shift happening in toxicology testing towards mechanism-based approaches to support environmental risk assessments (5). Advancements in high-throughput transcriptomic sequencing technologies have provided the ability to characterize the sublethal impacts of chemicals and deepen our understanding of their adverse effects while simultaneously reducing costs, resources, and waste, and testing a broader range of chemicals in a shorter amount of time (1, 5).

Shifting away from traditional techniques towards whole transcriptomic analysis also has largely to do with the support for developing New Approach Methods (NAMs) to replace, reduce, and refine (3R's) animal testing techniques. NAMs using high-throughput transcriptomic analyses have the potential to assist environmental risk assessments by increasing screening of chemicals that lack *in vivo* toxicity knowledge and filling in mechanistic knowledge gaps for chemicals where *in vivo* toxicity data is available (6). Moreover, transcriptomic points of departure (tPODs) values would identify the first site of molecular disruptions before apical

effects and base the level of risk on these early insights not normally observed in traditional acute toxicity testing (1, 7). Therefore, the combination of NAMs with high-throughput transcriptomics would produce more efficient testing methods that yield faster results and are more protective of ecological species.

The Organization of Economic Co-operation and Development (OECD) recently standardized an acute toxicity assay utilizing rainbow trout gill cells (RTgill-W1) to characterize the cytotoxicity of chemicals (OECD Test No. 249) (8). Thus far, the guideline protocol has gone through a robust validation study (9) and the RTgill-W1 cells have been tested with a wide range of chemical classes (10-15). Mittal et al., (2022) determined that following guideline OECD No. 249 for chemical exposures, tPODs could successfully be derived for methylmercury and fluoxetine and these values were protective of *in vitro* cytotoxicity and *in vivo* lethality values (16). This study also highlighted that meaningful information at the gene and pathway levels could be obtained from *in vitro* acute exposures, suggesting that this alternative to animal testing approach warrants further attention. Taken together, using OECD Test No. 249 to derive tPODs for chemicals that previously have not been well characterized could contribute to environmental risk assessments in ways not previously explored.

Pesticides specifically are an important class of chemicals given their persistence in aquatic ecosystems and adverse effects on non-target organisms (17-21). In a comprehensive literature review by Anderson et al. (2021), pesticides were prioritized for risk to aquatic ecosystems in Canada, and the authors identified sublethal toxicity as a major knowledge gap for many pesticides (21). Moreover, fish are an impacted species due to pesticide contamination in

bodies of water which makes them an excellent model of water health (15, 22, 23). Thus, there is a need for more information about pesticide mechanisms of toxic action in fish. Exploring alternative methods such as OECD No. 249 and high-throughput transcriptomic analysis to do this would allow for screening more pesticides while being cost-effective and requiring fewer resources.

The main objective of this study was to estimate tPOD values and characterize the mechanisms of action for six cytotoxic pesticides after exposure to the RTgill-W1 cells. The resulting tPODs were compared to the cytotoxic EC50s taken from Chapter 2 and apical LC50s taken from the US EPA's database Ecotoxicology Knowledgebase (ECOTOX). Conducting a transcriptomic analysis of *in vitro* RTgill-W1 cells exposed to pesticides will further our understanding of the opportunities possible with this technique under the guise that coupling OECD Test No. 249 with high-throughput transcriptomics may enable researchers to test large numbers of chemicals and yield more information that is predictive and protective of *in vivo* traditional approach results.

3.1 Materials and Methods

3.1.1 RTgill-W1 cell culture

The rainbow trout gill cell line (RTgill-W1; CRL-2523) was obtained from American Type Cell Culture (ATCC). Cells were grown in Thermo Scientific™ BioLite™ Cell Culture Treated 100 mm Petri dishes containing 10 ml of complete culture medium (Leibovitz L-15 culture medium supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin), at

21°C. Medium in dishes was replaced twice a week. Once cells reached 80% confluency, they were trypsinized for 3 to 5 min using 1 ml Trypsin-EDTA, and either passaged to continue the culture or counted (using a hemocytometer) and seeded in 96-well plates for exposure experiments (35,000 cells per well). All cell culturing was done following OECD Test No. 249 guidelines. The passage numbers of the cells used for the transcriptomic exposures were p80, 82, and 83.

3.1.2 Chemicals

Protocol for pesticide exposures was followed according to the methods described in Chapter 2. Briefly, pesticides were purchased from Sigma Aldrich except for metolachlor, diazinon, permethrin, and S-metolachlor purchased from Canadian Life Science (Chem Services) and chlorpyrifos from Toronto Research Chemicals (TRC). Stock solutions starting at 200 mM were created for all pesticides except chlorothalonil (20 mM). Metolachlor, chlorpyrifos, permethrin, carbaryl, chlorothalonil, and diquat, stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma Aldrich) or milli-Q water (diquat). 3,4-Dichloroaniline (3,4-DCA, positive control, Fisher Scientific) was prepared fresh on the day of exposure in DMSO and diluted in L-15/ex to obtain the following concentrations: 1.5625, 3.125, 6.25, 12.5, and 25 mg/l. Water and DMSO were added to L-15/ex at a final concentration of 0.5% v/v. Concentrations for cell exposures were based on LC20s calculated from the alamarBlue (metabolic activity) assay findings from Chapter 2 cytotoxicity exposures. If a pesticide was only cytotoxic with CFDA-AM (ie. permethrin), that corresponding LC20 was used. Concentrations followed a 3-fold serial dilution over 8 concentrations to ensure that there would be living cells to extract RNA from **(Table *3-1*).**

Table 3-1: EC20 values for each cytotoxic pesticide calculated from Chapter 2 results.

Chemical	EC20 (µM)
Permethrin	153
Chlorpyrifos	173
Chlorothalonil	15
Carbaryl	31
Metolachlor	199
Diquat	6
	EC20 (mg/L)
3,4-DCA	25

3.1.3 Pesticide exposures for library preparation

Overall, each pesticide was tested at nine concentrations (including a negative control) spanning ~3.5 orders of magnitude. Three different passages were tested against the pesticides and these exposures were performed in different microplates.

On day 1, RTgill-W1 cells were seeded at a density of 35,000 cells/100 µL of complete medium per well in 96-well plates and incubated for 24 h. No-cell C1 control or negative control wells were not included in the plate design and the positive control chemical (3,4-DCA) was included in each plate for each passage.

On day 2, the old complete medium was discarded and replaced with 100 µL of L-15/ex, or L-15/ex containing either the pesticide, solvent (DMSO), or positive control (3,4-DCA) at appropriate concentrations (one well/concentration). The cells were then incubated for 24 h.

On day 3, the lysis buffer premix consisting of UPX 3' cell lysis buffer (13.33 µL, one reaction), RNA inhibitor (3.33 µL, one reaction), and nuclease-free water (23.33 µL, one

reaction) following the manufacturers (Qiagen) protocol was prepared. The exposure medium in each 96-well was discarded and cells were rinsed with 100 µL of phosphate-buffered saline (PBS). Then, 40 µL of lysis buffer was added and pipetted up and down vigorously 10 times in each well to facilitate cell lysis and placed on an orbital shaker for 10 minutes. After this, cells were pipetted up and down 10 more times to ensure proper cell lysis and put in a -80°C freezer until library preparation.

3.1.4 Library Preparation

Cell lysis, fast select rRNA removal, reverse transcription, library amplification (and indexing), and bead clean-up steps were performed using the QIAseq UPXome RNA library kit for whole transcriptome RNA-sequencing following the manufacturer's protocol with some modifications. All materials were obtained from Qiagen unless otherwise specified. With each set of libraries prepared from a 96-well plate, three wells were designated for isolated RNA (2 ng/µL) from unexposed RTgill-W1 cells using the RNeasy mini kit from Qiagen. This was included as an internal control and referred to as XpressRef.

3.1.4.1 QIAseq Fast Select rRNA removal

Briefly, Fast Select reagent (0.1X; 1 µL, one reaction) was prepared and then added to US RT (Reverse Transcription) Buffer (5X; 4 µL, one reaction) to create the Fast Select master mix. Next, for one reaction, 5 µL of master mix, 4 µL cell lysate, and 2 µL of nuclease-free water were added to one well in a 384-well plate and incubated in the BIO-RAD thermal cycler (CFX384 Real-time system) at 75°C for 2 minutes, 70°C for 2 minutes, 65°C for 2 minutes, 60°C

for 2 minutes, 55℃ for 2 minutes, 37℃ for 2 minutes, 25℃ for 2 minutes, and 4℃ for 2 minutes.

3.1.4.2 Reverse transcription and first bead clean-up

Reverse transcription master mix consisting of DTT (100 mM), dNTP (10 mM), nuclease-free water, N6-T RT primer, ODT-T RT primer, EZ reverse transcriptase, and RNase inhibitor was added to the reverse transcription plate (SID-TS-96S) containing lyophilized primers that are incorporated into the cDNA during the reserve transcription process. The entire product from the Fast Select step (11uL) was added to corresponding wells in the SID-TS-96S plate and incubated in a BIO-RAD thermal cycler (CFX96 Real-time system) at 4℃ for 1 minute, 42℃ for 90 minutes, 70℃ for 10 minutes, and 4℃ for 1 minute. Once the incubation was complete, up to 24 wells of cDNA were pooled together into one tube for bead clean up. The XpressRef control RNA wells were pooled separately into one tube. To each tube of cDNA, 1.1x volume of QIAseq beads were added, mixed by pipetting, and incubated at room temperature for 2 minutes. The cDNA pools were then placed onto a magnetic rack until the solution had cleared. Leaving the tubes on the magnetic rack, the supernatant was discarded, and beads in each tube were then washed twice with 200 µL of 80% ethanol and left to air-dry at room temperature until cracks became visible in the bead pellet. The tubes were then removed from the rack and 22 µL of nuclease-free water was added to elute DNA. Tubes were centrifuged briefly and returned to the magnetic rack until the solution cleared. 20 µL of the supernatant was transferred to a new tube to which 22 µL of QIAseq beads were added and the ethanol wash steps were repeated until the drying step. 25 µL of nuclease-free water was added to the dried bead pellet and gently

mixed, centrifuged, and incubated for 2 minutes at room temperature. The tubes were placed back on the rack and 23 µL of the supernatant was transferred to a new tube.

3.1.4.3 Library amplification, indexing, and second bead clean-up

With the pooled samples in 5 tubes, 25 µL of the QIAseq 2x HiFi MM and 2 µL of index from the QIAseq UX index plate were added to each tube. Reactions were briefly centrifuged, vortexed, and centrifuged again. Samples were then placed in a BIO-RAD thermal cycler (CFX384 Real-time system) and incubated for 98℃ for 30 seconds, 18 cycles of 1) 98℃ for 5 seconds, 2) 55℃ for 10 seconds, and 3) 72℃ for 20 seconds, then 72℃ for 2 minutes, and 4℃ for 1 minute. Following this, 1.1x volume of QIAseq beads (40 µL) were added to each cDNA pool and mixed well by pipetting before incubating at room temperature for 2 minutes. The cDNA pools were then placed onto a magnetic rack until the solution had cleared. Leaving the tubes on the magnetic rack, the supernatant was discarded, and beads in each tube were then washed twice with 200 µL of 80% ethanol and left to air-dry at room temperature until cracks became visible in the bead pellet. The tubes were then removed from the rack and 22 µL of nuclease-free water was added to elute DNA. Tubes were mixed and centrifuged briefly and returned to the magnetic rack until the solution cleared. 20 µL of the supernatant was transferred to a new tube and quality control of the prepared libraries was assessed using a Bioanalyzer (2100 Bioalanyzer; Aligent).

3.1.5 Quantification (library quant assay kit) and library dilution

The UPXome cDNA libraries were quantified according to the to the QIAseq Lib Quant Kit. The qPCR master mix (RNase and DNase-free water, SYBR Green mastermix, primer mix,

and template) and two dilutions (1:2000 and 1:20000) of each cDNA library were prepared. 25 µL of the qpCR master mix plus diluted libraries was tested in triplicate and the qPCR reaction was run on the BIO-RAD thermal cycler (CFX384 Real-time system) at 95℃ for 10 minutes and then for 30 cycles of 95℃ for 15 seconds, 60℃ for 30 seconds, and 72℃ for 2 minutes. Ct values were obtained after the run was complete. From this, the appropriate dilution was used to dilute libraries.

3.1.6 RNA-sequencing and Data Analysis

All samples were shipped to the Genome Quebec Innovation Centre (McGill University, Montreal, QC, Canada) for messenger RNA sequencing. Libraries were sequenced using a NovaSeq 6000 S4 PE100 to generate 100 bp paired end reads. Raw RNA Sequencing fastq files were submitted to Qiagen CLC Genomic Workbench (24). First, files were demultiplexed to yield individual reads files for each sample (well) and then mapped to the rainbow trout genome (Omyk_1.1, version 100) to yield raw counts. Second, the counts were submitted to ExpressAnalyst (https://www.expressanalyst.ca/) for differential expression analysis and benchmark dose-response analysis (25). The data uploaded to ExpressAnalyst were summarized by the sum with filtering for variance set to 30 and filtering for abundance set to 15. Counts were converted to log2-counts/million (log-CPM) and differential expression analysis was performed with the EdgeR package to filter genes that were not significantly differentially expressed following pesticide exposure. Genes were considered significantly differentiated if the false discovery rate (FDR) adjusted p-value was < 0.05 and the abs(log2FC) was >1 for chlorothalonil, chlorpyrifos, and metolachlor, or if the FDR adjusted p-value was < 0.2 and the abs(log2FC) was >1 for diquat, carbaryl, and permethrin. Benchmark dose-response analysis was

performed on the differentially expressed genes (DEGs) with all models except for Poly3 and Poly4, and models were kept that passed the lack-of-fit p-value threshold of 0.1. A BMR factor of 1 was used to calculate gene BMDs from the fitted curves. tPOD values were derived for each treatment group. Next, pathway-level BMDs were computed using the KEGG pathways database and only kept if the adjusted $p < 0.05$.

3.1.7 Comparisons with literature values

Comparisons were made of the *in vitro* EC50 derived here with *in vivo* LC50 values taken from the scientific literature. The geometric mean of the metabolic activity and membrane integrity EC50 results from Chapter 2 for each pesticide were used for comparisons. *In vivo* LC50 values were taken from 96-h acute toxicity studies with adult rainbow trout (*Oncorhynchus mykiss*) from the United States EPA's Ecotoxicology Knowledgebase (ECOTOX, https://cfpub.epa.gov/ecotox/; (26)) and peer-reviewed journals if no EPA value was available. Inclusion criteria for the selection of *in vivo* values from the ECOTOX database was a 96h exposure, adult rainbow trout, tests performed with active ingredient (not formulation), the chemical purity >80%, LC50 and mortality were the tested endpoint, and use of flow-through or static systems were accepted. All pesticides except permethrin and carbaryl produced one value from the database. Permethrin produced three values that satisfied the inclusion criteria, and these values were averaged to produce a mean LC50 to compare with *in vitro* cell results. Carbaryl's LC50 was taken from Dwyer et al. (2005) because of the high sample size of adult rainbow trout and satisfying the rest of the inclusion criteria (27). All figures were plotted with the R software (R v4.1.3, RStudio, v2023.08.01).

3.2 Results and Discussion

3.2.1 Overview of transcriptomic results

The number of raw reads per sample ranged from 0.7 – 8.3 million. After trimming, the percent of reads per sample ranged from 84.6 – 99.5% and 42 – 79% of these were mapped to gene IDs for downstream analysis. Overall, 8,600 to 9,800 genes were identified across all samples.

3.2.2 Transcriptomic Benchmark Dose-Response Analysis

3.2.2.1 3,4-DCA

Since 3,4-DCA is the recommended positive control chemical in OECD Test No. 249 (8), transcriptomic analysis was run on this chemical in addition to the pesticides. In the current study, 3,4-DCA was exposed in each microplate (n=6), so all 6 replicates were pooled. Curve fitting for 3,4-DCA resulted in gene BMDs for 64 DEGs, and from this tPOD values were calculated which ranged from 1.45 – 4.07 mg/L (**Table 3-2**, **Error! Reference source not found.**). To our knowledge, this is the first study to derive a tPOD value for this chemical.

3.2.2.2 Pesticides

Transcriptomic analysis was run for chlorothalonil, diquat, carbaryl, metolachlor, chlorpyrifos, and permethrin (**Table 3-2**). Chlorothalonil, diquat, and carbaryl were the three most cytotoxic pesticides based on the RTgill-W1 exposures from Chapter 2. Chlorothalonil induced differential expression in the greatest number of genes with 1498 genes and yielded the lowest tPOD values ($tPOD_{mode}$ = 0.4 μM) (**Error! Reference source not found.**, **Figure 3-1**).

Chlorpyrifos induced differential expression in the second-highest number of genes to obtain 863 gene BMDs with a calculated $tPOD_{mode}$ value of 36 μM. Metolachlor induced differential expression in the third highest number of genes with 216 DEGs and obtained a $tPOD_{mode}$ of 13 μM. Diquat-exposed cells only induced differential expression in 49 genes to obtain 15 transcriptomic BMDs but obtained the second lowest $tPOD_{mode}$ value of 0.8 μM. Similarly, carbaryl and permethrin only produced 11 and 10 gene BMDs from exposure to RTgill-W1 cells, respectively. The corresponding rank order for lowest to highest $tPOD_{mode}$ is chlorothalonil < diquat < carbaryl < metolachlor < chlorpyrifos < permethrin (**Error! Reference source not found.**, **Figure 3-1**). Permethrin yielded the lowest tPOD values ($tPOD_{mode}$ of 39 μM). To our knowledge, this is the first study to derive tPODs for the six pesticides evaluated from an *in vitro*, cell-based exposure model with fish. Otherwise, a tPOD for chlorpyrifos was determined following exposures to Japanese quail liver tissue ($tPOD_{mode}$ value of 21.52 μg/g, equivalent to 61.4 μM) (28), which is within 1 order of magnitude of the derived $tPOD_{mode}$ value from chlorpyrifos-exposed RTgill-W1 cells presented here (36 μM).

Table 3-2: Summary of the number of differentially expressed genes (DEGs), number of DEGs with benchmark doses (gene BMDs), and transcriptomic point of departure (tPOD) values in RTgill-W1 cells exposed to 3,4-DCA and pesticides.

Chemical	# of DEGs	# of gene BMDs	tPOD (mg/L)		
			20th gene	Mode	10th percentile
3,4-DCA	65	64	2.25	4.07	1.45
			tPOD (µM)		
			20th gene	Mode	10th percentile
Chlorothalonil	1498	989	0.08	0.38	0.23
Diquat	49	15	N/A	0.81	0.20
Carbaryl	17	11	N/A	3.43	1.29
Chlorpyrifos	967	863	3.77	36.28	25.19
Metolachlor	216	188	10.75	13.35	29.46
Permethrin	28	10	N/A	38.59	26.22

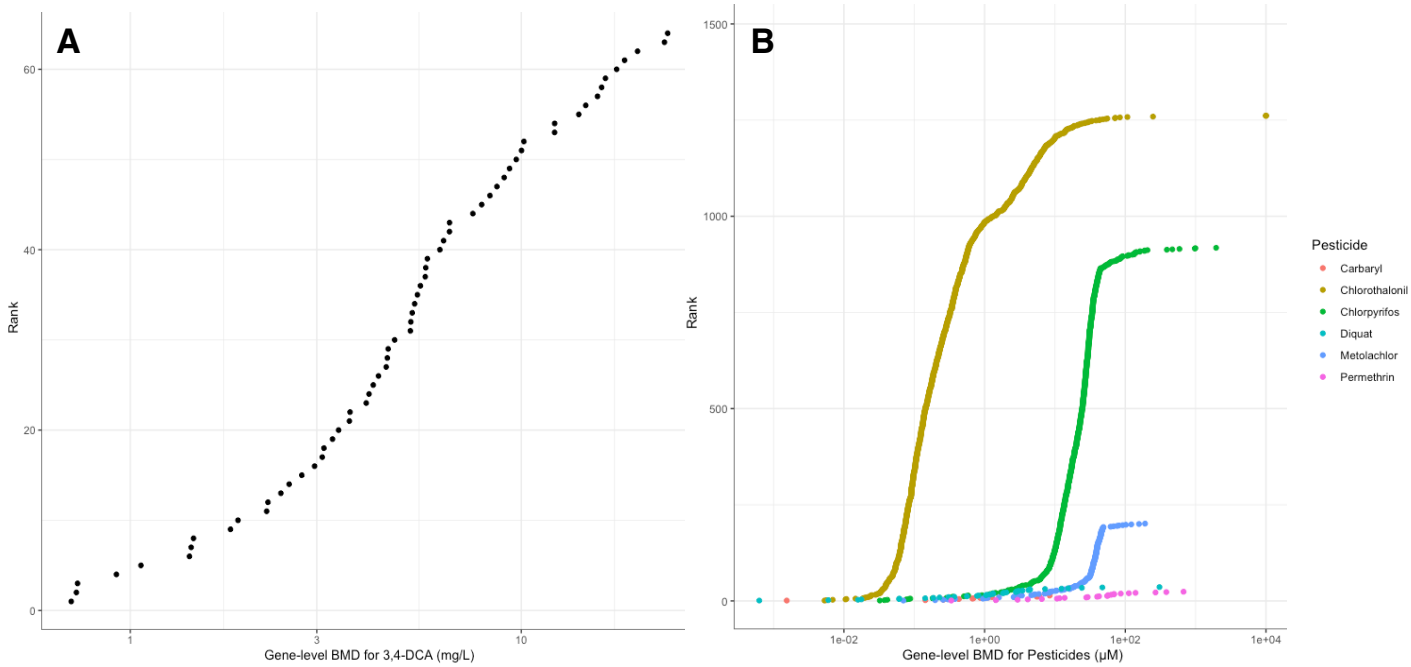

Figure 3-1: Gene-level accumulation plots for A) 3,4-DCA and B) Pesticides.

3.2.3 Pathway BMD Enrichment Analysis

3.2.3.1 3.4-DCA

Pathway level BMD analysis was also performed for 3,4-DCA to better understand the mechanism of action in RTgill-W1 cells. The NF-Kappa B and IL-17 signaling pathways (n=2, BMD values 2.76 mg/L) were significantly enriched when a significant p-value < 0.05 was applied (**Table 3-3**, **Figure *3-2***). These pathways have not been identified in previous transcriptomic studies regarding 3,4-DCA exposure in zebrafish embryos(29-31), larvae (29, 31), and adults (32, 33), rare minnow embryos and larvae (34), and RTgill-W1, RTL-W1, and PLHC-1 cells (35). However, this work was the first to perform whole-transcriptomic analysis with 3,4-DCA, whereas previous works had focused on specific genes relating to biotransformation and detoxification (i.e., *cyp1a*, *ahr2*) (29, 30), reproductive hormone regulation (i.e., *stAR, cyp17, cyp19a*) (32), cell cycle, and stress response (i.e., *hsp70*, *hmox1*)(31), or focused on specific tests such as a Fpg-modified comet assay (35). Furthermore, the same genes mentioned above were not significantly up-or down-regulated with the 3,4-DCA exposed cells (data not known). However, a review of the data for 3,4-DCA in the Comparative Toxicogenomic Database (CTD) (ctdbase.org) revealed the immune system as the third most dysregulated pathway. The results presented from this work provide new insights into 3,4-DCA's mechanism of action not previously explored in rainbow trout.

3.2.3.2 Pesticides

To further investigate the transcriptomic differences across pesticides, enrichment analysis on the DEGs was performed to derive pathway BMDs (**Table 3-3**). Pathways were considered significantly enriched if the p-value < 0.05 for chlorothalonil, chlorpyrifos, and

metolachlor, or p-value < 0.2 for carbaryl, diquat, and permethrin. The top 5 significantly enriched pathways are presented for each pesticide if available (**Table 3-3**, **Figure *3-2***; see **Supplementary Table S3-1** for full list). The ribosomal pathway was the most sensitive pathway for chlorothalonil, chlorpyrifos, and metolachlor (0.41, 131.55, and 138.02 μM, respectively). Chlorothalonil significantly enriched pathways (n=22) relating to cell death, stress response, cellular functioning and growth, protein functioning, metabolism, muscle contraction, energy production, immune response, and more with BMDs ranging from 0.34 to 1.2 μM. The lowest pathway BMD was associated with oxidative phosphorylation (0.34 μM), with all pathway BMDs below 1 μM except for protein processing in the endoplasmic reticulum (1.20 μM) and NOD-like receptor signaling pathway (1.09 μM). The upregulated pathways relating to cell death, xenobiotic metabolism, oxidative stress response, and immune response, have all been shown to be dysregulated in other transcriptomic studies with chlorothalonil exposed to guppy (36), olive flounder (37), potato beetle (38), grass carp kidney cells (39, 40), estuarine polychaete (41), white mouth croaker (42), zebrafish embryos and larvae (43), and adult zebrafish livers (44). This is the first study to link ribosome, phagosome, and proteasome pathways to chlorothalonil, however, these pathways have been shown to be dysregulated by other factors in the gills of juvenile marbled flounder and rainbow trout (45, 46).

Chlorpyrifos enriched the greatest number of pathways (n=25) with BMDs ranging from 43.56 to 80.6 μM. Pathways dysregulated by chlorpyrifos exposure to RTgill-W1 cells relating to oxidative stress, muscle contraction (regulation of actin cytoskeleton, cardiac muscle contraction), immune responses including inflammation, cell cycle and cellular degradation, glutathione metabolism, and apoptosis were also identified in the transcriptomic literature to

impact juvenile and adult rainbow trout (47-50) and common carp (51-54). Evaluation of the CTD also displayed dysregulation in similar pathways following chlorpyrifos exposure.

Metolachlor significantly enriched three pathways, namely ribosomal, proteasomal, and arachidonic acid metabolism ranging from 98.2 to 138.0 μM. The CTD also showed that the ribosomal pathway was significantly enriched after exposure to metolachlor as it was the third most significant pathway. However, not a lot of transcriptomic information is available for metolachlor with fish exposures. Experiments exposing metolachlor to human liver HepG2 cells found significant enrichment of pathways relating to cell cycle, cellular response, and DNA replication and repair (55). Our results indicated significant enrichment of the proteasome pathway (BMD of 137 μM), which plays important roles in cellular processes, cycling, and proliferation, and DNA repair (56, 57). The significantly enriched pathway with the lowest BMD was arachidonic acid metabolism (98 μM). This indicates that metolachlor also targets metabolic pathways of fatty acid synthesis in non-target organisms such as fish, as this is part of metolachlors mechanism of action in plants (21).

Analysis on carbaryl, diquat, and permethrin did not produce any significantly enriched biological pathways when p-value < 0.2, likely due to small gene sets of 15 or fewer gene BMDs. To explore the dataset for these three pesticides further, pathways with an unadjusted p-value < 0.2 were considered and I present these results with caution. It is important to note that these results are not necessarily reliable due to the small number of hits (n=1 or n=2) for all pathways. For a brief overview of these results, the identified pathways were focused on cell cycle and death, stress responses, synthesis and secretion of steroids and hormones, and immune

responses for carbaryl. Permethrin induced differential pathway expression for pathways relating to metabolism, homeostasis, and protein functions. It may not be surprising that limited molecular effects are observed for carbaryl and permethrin as they are both neurotoxic insecticides that inhibit the acetylcholinesterase (AChE) enzyme and interfere with voltage-gated sodium channels to disrupt neuron functioning, respectively. Diquat mainly enriched pathways related to the synthesis, degradation, and metabolism of amino acids and biomolecules. Diquat is known to cause oxidative stress and affect homeostasis in fish (58-60), however likely due to low DEG and hit numbers, these responses were not observed in the present study.

Table 3-3: Top pathway BMD concentrations in RTgill-W1 cells exposed to 3,4-DCA and pesticides.

Chemical	Name of pathway	Pathway BMD (mg/L)	P-Value	Adjusted P-value	Number of hits
3,4-DCA	NF-kappa B signalling pathway	2.76	2.5e-4	3.7e-2	4
	IL-17 signalling pathway	2.76	3.9e-4	3.7e-2	4
		Pathway BMD (µM)			
Chlorothalonil	Ribosome	0.41	2.8e-17	5.2e-15	49
	Antigen processing and presentation	0.56	1.1e-7	1.0 e-5	16
	Proteasome	0.53	1.7e-7	1.0 e-5	22
	Ribosome biogenesis in eukaryotes	0.38	7.3e-7	3.4 e-5	19
	Phagosome	0.41	1.0e-6	3.9 e-5	25
Chlorpyrifos	Ribosome	75.50	1.6e-66	2.9e-64	90
	Proteasome	58.30	5.7e-14	5.4e-12	28
	Spliceosome	46.92	1.3e-8	8.3e-7	36
	Oxidative phosphorylation	69.65	5.9e-8	2.8e-6	28
	Antigen processing and presentation	67.43	7.6e-7	2.9e-5	14
Metolachlor	Ribosome	138.02	3.0e-37	5.7e-35	39
	Proteasome	137.28	2.3e-5	2.2e-3	8
	Arachidonic acid metabolism	98.21	7.4e-4	4.7e-2	4
Carbaryl	MAPK signaling pathway	3.83	2.5e-2	1	2
	Insulin secretion	3.33	3.7e-2	1	1
Diquat	Complement and coagulation cascades	3.13	3.2e-2	1	1
	Valine, leucine, and isoleucine degradation	0.002	3.8e-2	1	1
Permethrin	Homologous recombination	74.69	2.5e-2	1	1
	Jak-STAT signaling pathway	140.94	7.0e-2	1	1

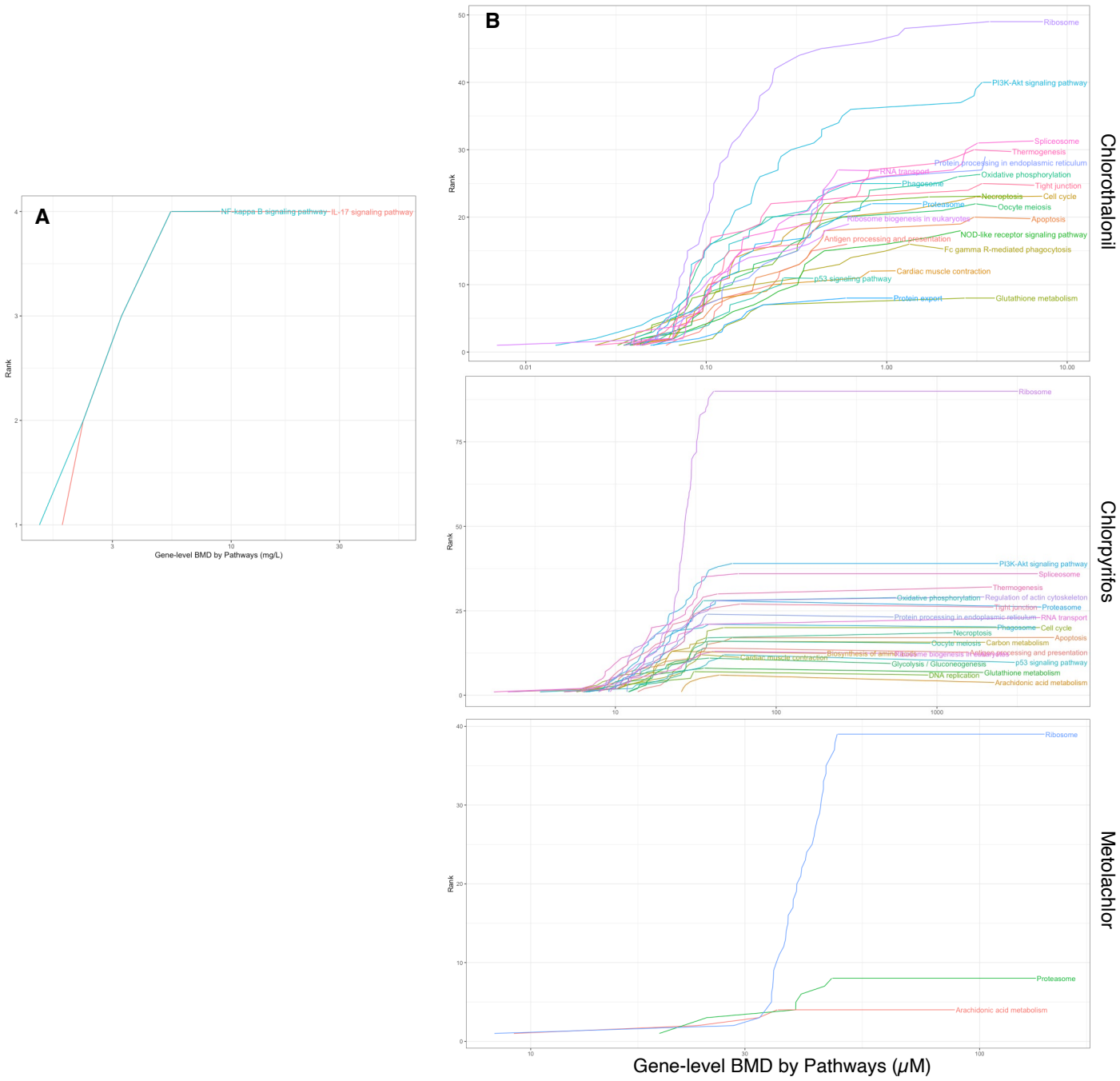

Figure 3-2: Pathway level accumulation plots for RTgill-W1 cells exposed to A) 3,4-DCA and B) significantly enriched (p-value < 0.05) pesticides (chlorothalonil, chlorpyrifos, and metolachlor).

3.2.4 Benchmarking *in vitro* tPOD and pathway BMD values with lethality values

3.2.4.1 3,4-DCA

The $tPOD_{mode}$ result from this work was compared to the EC50 value from RTgill-W1 cell viability assays in Chapter 2 and LC50 values from *in vivo* 96h exposures to rainbow trout fish (**Figure 3-3**). The $tPOD_{mode}$ value was approximately 10-fold lower than the *in vitro* RTgill-W1 EC50 and within one order of magnitude of the *in vivo* LC50 from rainbow trout ($tPOD_{mode}$ is 4.1 mg/L, and *in vivo* LC50 is 2.2 mg/L) (**Table 3-4**).

3.2.4.2 Pesticides

Values derived from this present work were compared to the derived EC50 values from cytotoxicity experiments with the RTgill-W1 cells in Chapter 2 and LC50 values from *in vivo* 96h exposures to rainbow trout fish (**Figure 3-3**). The $tPOD_{mode}$ results were approximately 7- to 123-fold lower than the corresponding EC50 values from the RTgill-W1 cell viability assays (**Table 3-4**). The largest difference is observed for diquat with an approximate 123-fold difference between these two groups. Chlorpyrifos and permethrin yielded $tPOD_{mode}$ values 363- and 385-fold higher than the corresponding *in vivo* LC50s from rainbow trout, respectively. However, chlorothalonil was within a one order of magnitude difference of the *in vivo* LC50. Diquat, carbaryl, and metolachlor yielded $tPOD_{mode}$ values approximately 0.02- to 50-fold lower than acute *in vivo* LC50s (**Table 3-4**).

Overall, there was a consistent trend between all chemicals that the $tPOD_{mode}$ values were lower than the cytotoxic results from RTgill-W1 cell exposures. This makes sense as measuring adverse effects at the molecular level should yield more protective (i.e., lower) values compared

to apical endpoints (16, 61). Interestingly, the *in vitro* results from RTgill-W1 exposures were the least predictive of *in vivo* lethality, ranging from 1 to 4 orders of magnitude higher for all pesticides (**Figure 3-3**). The $tPOD_{mode}$ results, apart from chlorpyrifos and permethrin, were more comparable to *in vivo* toxicity as values were within one order of magnitude difference or more conservative. This suggests the power of deriving tPODs from *in vitro* cell-based exposures in which these values serve as references for molecular changes before apical outcomes are reached.

Table 3-4: Overview of $tPOD_{mode}$, *in vitro* geometric mean EC50 (μM) from RTgill-W1 exposures in Chapter 2, and *in vivo* LC50 (μM) values from rainbow trout 96h exposures taken from the literature for 3,4-DCA and pesticides.

Chemical	**$tPOD_{mode}$ from RTgill-W1 cells (mg/L)**	***In vitro* RTgill-W1 geometric mean EC50 (mg/L)**	***In vivo* Rainbow trout LC50 (mg/L)**
3,4-DCA	4.07	45.2	2.2
	$tPOD_{mode}$ from RTgill-W1 cells (μM)	***In vitro* geometric mean RTgill-W1 EC50 (μM)**	***In vivo* Rainbow trout LC50 (μM)**
Chlorothalonil	0.38	3.1	0.1
Diquat	0.81	100.4	41.0
Carbaryl	3.43	195.3	9.9
Chlorpyrifos	36.28	287.7	0.1
Metolachlor	13.35	406.5	13.7
Permethrin	38.59	426.7	0.01

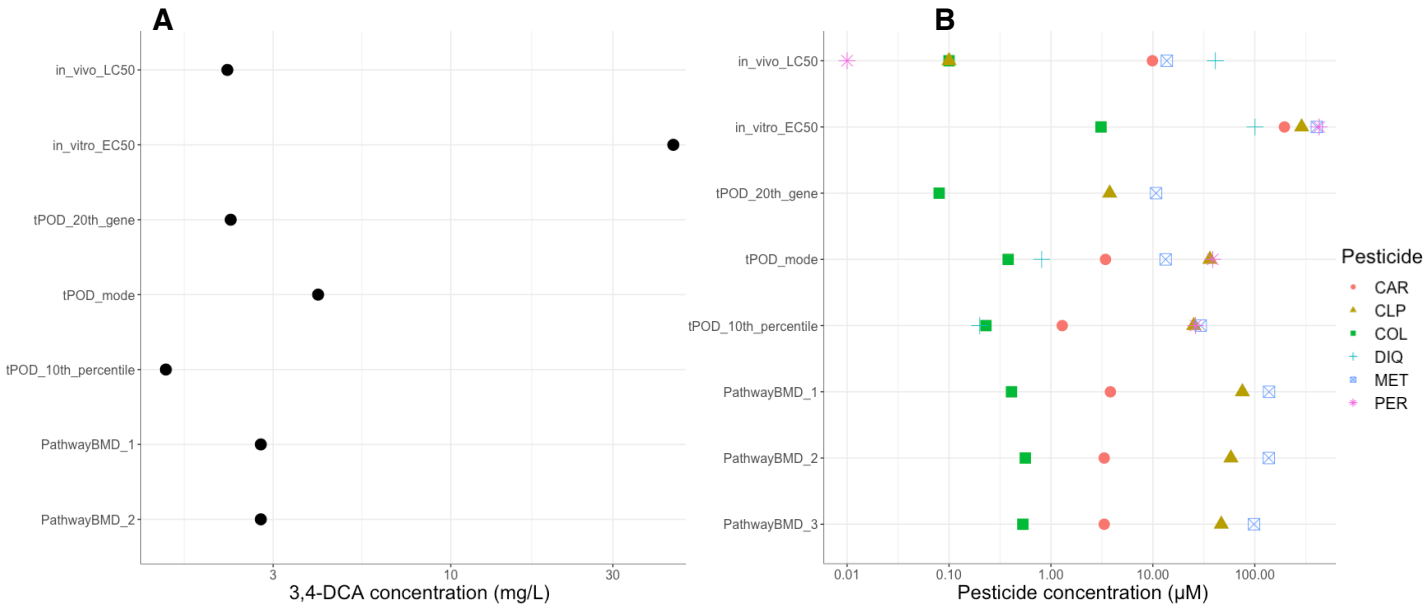

Figure 3-3: Comparison of *in vitro* tPOD and pathway BMD values for A) 3,4-DCA (mg/L) and B) pesticides (μM) from the present study with *in vitro* EC50 cytotoxicity values from Chapter 2 and *in vivo* LC50 values from the literature. The top 3 pathway BMD values were included in the comparison, if available, for each pesticide.

3.2.5 Limitations

The use of the UPXome RNA library kits with ecological samples was a novel approach as it was previously designed and used for human samples. The use of this technology offers a high-throughput approach for obtaining tPOD and pathway BMD values strongly associated with *in vivo* lethality values. The UPXome approach calls for pooling samples together and labeling each with a unique index to allow for samples to be sequenced simultaneously during a single sequencing run. This advancement has significantly reduced costs and time while increasing data output and run times (62, 63). However, improper identification and sorting of pooled libraries in the initial data analysis phase can lead to index misassignment, known as 'index hopping'(62-64). The result of incorrectly assigning libraries to a different index can lead to misalignment and

inaccurate sequencing results. Efforts were made to reduce the levels of index hopping in this work, such as including a reference unexposed RNA sample (Xpressref), pooling similar samples together, storing the prepared libraries at -20 ℃, performing a double washing step during bead clean-up to remove as many impurities as possible, and using unique indexes for each sample, but it is not possible to entirely remove the risk of it occurring (63).

A continuing limitation with transcriptional information obtained from non-model species such as the rainbow trout, is that it is not a core species in the KEGG database of organisms (16). Thus, the interpretation of the gene and pathway information obtained can be more difficult as the functional annotations available are more limited (3, 4). While the molecular information obtained here is still highly relevant and applicable, there is an urgent need for the rainbow trout genome to be fully annotated and incorporated into the database since rainbow trout is an important species for environmental risk assessments (65).

Benchmarking the tPOD and pathway information with results from *in vivo* exposures was useful in determining that transcriptomic information derived from *in vitro* cell-based assays is comparable to lethal and molecular findings from *in vivo* exposures. This highlights the promise of high-throughput transcriptomics when coupled with *in vitro* assays to reduce the use of animal testing methods. However, tPODs derived from *in vivo* rainbow trout studies are lacking, and this makes benchmarking results more challenging as this would be the most ideal comparison. Furthermore, the use of only one cell line (RTgill-W1) does not capture the complex multi-cellular environments found in whole organisms (66). Comparisons of transcriptomic information between exposures of methyl mercury and fluoxetine to RTgill-W1, RTL-W1, and

RTgutGC cell lines found cell-specific differences with the gut cells being the most notably different (16). The next steps would be to perform the pesticide exposures in the liver and gut rainbow trout cells to compare how tPOD and pathway-level BMDs could differ. The development of a neuron cell line would also greatly benefit the efforts to derive tPODs as many of the pesticides studied have neurotoxic modes of action which could not be explored in depth.

3.3 Conclusion

To our knowledge, this is the first study to derive tPODs from an *in vitro* rainbow trout gill cell model for environmentally relevant pesticides. The calculated tPODs were generally lower than *in vitro* cytotoxicity and *in vivo* lethality values, indicating the promise of using *in vitro* systems to derive more protective values. Insights into each pesticide's mechanisms of action and pathway-level perturbations also revealed comparable findings to *in vivo* whole organism studies as well as new pathways previously unreported. Overall, these findings support the idea that transcriptomic information derived from short-term *in vitro* assays provide meaningful and rich data that support risk assessments and the transition away from cost- and time-consuming animal-based methods.

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3.5 Supplementary Tables and Figures

Table S3-1: Full list of significantly enriched pathway BMD values for each pesticide.

Pesticide	Name of pathway	Pathway BMD (µM)	P-Value	Adjusted P-value	Number of hits
Chlorothalonil	Ribosome	0.41	2.8e-17	5.2e-15	49
	Antigen processing and presentation	0.56	1.1e-7	1.0 e-5	16
	Proteasome	0.53	1.7e-7	1.0 e-5	22
	Ribosome biogenesis in eukaryotes	0.38	7.3e-7	3.4 e-5	19
	Phagosome	0.41	1.0e-6	3.9 e-5	25
	Oxidative phosphorylation	0.34	9.6e-6	3.0 e-4	26
	Cell cycle	0.53	1.3e-5	3.5 e-4	23
	Oocyte meiosis	0.60	2.3 e-5	5.4 e-4	22
	Spliceosome	0.60	6.6 e-5	1.4 e-3	31
	Necroptosis	0.60	1.0e-4	1.9 e-3	23
	RNA transport	0.56	1.1 e-4	1.9 e-3	28
	Protein processing in endoplasmic reticulum	1.20	2.0 e-4	3.1 e-3	29
	PI3K-Akt signaling pathway	0.53	1.0 e-3	1.5 e-2	40
	Thermogenesis	0.38	1.4 e-3	1.9 e-2	30
	Glutathione metabolism	0.56	1.6 e-3	2.0 e-2	8
	Tight junction	0.49	2.0 e-3	2.4 e-2	25
	NOD-like receptor signaling pathway	1.09	2.6 e-3	2.9 e-2	18
	Apoptosis	0.71	2.8 e-3	2.9 e-2	20
	Protein export	0.53	2.9 e-3	2.9 e-2	8
	Cardiac muscle contraction	0.38	3.8 e-3	3.5 e-2	12
	Fc gamma R-mediated phagocytosis	0.41	4.1 e-3	3.6 e-2	16
	p53 signaling pathway	0.49	5.5 e-3	4.7 e-2	11
Chlorpyrifos	Ribosome	75.50	1.6e-66	2.9e-64	90
	Proteasome	58.30	5.7e-14	5.4e-12	28
	Spliceosome	46.92	1.3e-8	8.3e-7	36
	Oxidative phosphorylation	69.65	5.9e-8	2.8e-6	28

	Antigen processing and presentation	67.43	7.6e-7	2.9e-5	14
	Tight junction	47.92	2.3e-5	7.3e-4	27
	Phagosome	43.33	2.9e-5	7.7e-4	21
	P13K-Akt signaling pathway	57.13	6.5e-5	1.5e-3	39
	Thermogenesis	74.13	1.5e-4	3.0 e-3	30
	Regulation of actin cytoskeleton	40.25	1.6e-4	3.0 e-3	28
	Cell cycle	79.95	1.8e-4	3.0 e-3	20
	Biosynthesis of amino acids	49.20	3.4e-4	5.4 e-3	13
	P53 signaling pathway	66.92	4.5e-4	6.5 e-3	12
	Glutathione metabolism	45.89	5.9e-4	7.9 e-3	8
	Ribosome biogenesis in eukaryotes	58.44	7.7e-4	9.6 e-3	13
	Cardiac muscle contraction	72.19	1.0-e3	1.2e-2	12
	Protein processing in endoplasmic reticulum	57.82	1.3e-3	1.4 e-2	24
	Glycolysis/gluconeogenesis	49.20	1.4e-3	1.4 e-2	11
	Carbon metabolism	51.71	2.9e-3	2.9 e-2	16
	DNA replication	48.83	3.1e-3	2.9 e-2	7
	Oocyte meiosis	62.32	3.7e-3	3.3 e-2	16
	Necroptosis	78.44	3.9e-3	3.3 e-2	17
	Arachidonic acid metabolism	80.61	5.0e-3	4.0 e-2	6
	RNA transport	43.56	5.3e-3	4.0 e-2	21
	Apoptosis	57.50	5.4e-3	4.0 e-2	17
Metolachlor	Ribosome	138.02	3.0e-37	5.7e-35	39
	Proteasome	137.28	2.3e-5	2.2e-3	8
	Arachidonic acid metabolism	98.21	7.4e-4	4.7e-2	4
Carbaryl	MAPK signaling pathway	3.83	2.5e-2	1	2
	Insulin secretion	3.33	3.7e-2	1	1
	Cortisol synthesis and secretion	3.33	3.7e-2	1	1
	Thyroid hormone syntehsis	3.33	4.1e-2	1	1
	Aldosterone synthesis and secretion	3.33	4.8e-2	1	1
	p53 signaling pathway	1.29	5.5e-2	1	1
	NF-Kappa B signaling pathway	6.36	5.6e-2	1	1
	RNA degradation	4.27	5.9e-2	1	1
	Parathyroid hormone synthesis, secretion, and action	3.33	8.5e-2	1	1
	Longevity regulating pathway	3.33	9.2e-2	1	1
	TNF signaling pathway	3.33	9.3e-2	1	1
	C-type lectin receptor signaling	6.36	1.0e-1	1	1
	Osteoclast differentiation	6.36	1.0e-1	1	1
	Cell cycle	1.29	1.1e-1	1	1
	Apoptosis	1.29	1.1e-1	1	1
	Estrogen signaling pathway	3.33	1.1e-1	1	1

	Relaxin signaling pathway	3.33	1.1e-1	1	1
	Adrenergic signalling in cardiomyocytes	3.33	1.2e-1	1	1
	Dopaminergic synapse	3.33	1.2e-1	1	1
	FoxO signaling pathway	1.29	1.3e-1	1	1
	cGMP-PKG signaling pathway	3.33	1.4e-1	1	1
	Protein processing in endoplasmic reticulum	3.33	1.5e-1	1	1
	Cellular senescence	1.29	1.5e-1	1	1
	P13K-Akt signaling pathway	3.33	2.4	1	1
Diquat	Complement and coagulation cascades	3.13	3.2-3	1	1
	Valine, leucine, and isoleucine degradation	0.002	3.8-2	1	1
	Cysteine and methionine metabolism	0.00	5.2e-2	1	1
	Glycerolipid metabolism	5.30	6.9e-2	1	1
	Biosynthesis of amino acids	0.002	7.5e-2	1	1
	ErbB signaling pathway	0.05	1.0e-1	1	1
	mRNA surveillance pathway	0.89	1.2e-1	1	1
	Purine metabolism	3.81	1.7e-1	1	1
	RNA transport	0.89	1.8e-1	1	1
Permethrin	Homologous recombination	74.69	2.5e-02	1	1
	Jak-STAT signaling pathway	140.94	7.0e-02	1	1
	Ubiquitin mediated proteolysis	16.63	9.9e-02	1	1
	Apoptosis	140.94	9.9e-02	1	1
	Purine metabolism	71.32	1.2e-01	1	1
	Protein processing in endoplasmic reticulum	35.06	1.4e-01	1	1
	PI3K-Akt signaling pathway	140.94	2.2e-01	1	1

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Chapter 4

4 Discussion

4.1 Discussion

4.1.1 Shift toward alternative approaches to animal testing

The list of chemicals entering the environment is growing at an accelerated rate and has raised considerable concerns over the abilities of traditional animal methods to meet testing expectations. The time, costs, resources, and animals required for traditional testing coupled with the influential 2007 National Research Council (NRC) report "Toxicity Testing in the 21st Century: A Vision and Strategy" which described a paradigm shift away from animal-based toxicity testing methods and towards mechanism-based *in vitro* methods, were major drivers for the development of NAMs (1). Given the fact that traditional approaches are anchored in apical outcomes such as mortality, it is the goal to eventually replace these techniques with NAMs that focus more on pathway-level responses before adverse apical outcomes are reached (2, 3).

A key feature of the change happening in toxicology is the implementation of *in vitro* high-throughput screening approaches to make NAMs more efficient (4). One way to do this is to increase the scalability of existing *in vitro* cell-based microplate assays (5). By moving from 6- and 24-well microplates towards 96- and 384-well plates, the volumes of reagents, amount of waste, and time required to perform tests all reduce significantly and become more high-throughput (6). This can yield more data in a shorter amount of time. In this thesis, exposures were conducted in 96-well plates instead of 24-well plates as described in OECD Test No. 249, which allowed for testing more chemicals over a larger concentration range and significantly

reduced the volumes of media, time, and waste produced. The transition to 96-well plates in Chapter 2 did not compromise the quality control criteria described in OECD Test No. 249 or impact cytotoxic outcomes. The use of 96-well plates coupled with the UPXome RNA library kits also increased scalability as this technology allows for individual samples (wells) to be pooled together through the process of indexing. This decreased the number of samples from 576 down to 25 for library preparation and sequencing. Thus, this high-throughput transcriptomic approach was able to provide important insights into affected pathways from the molecular level (Chapter 3).

Another driving factor for NAMs is the need for a deeper understanding of subcellular mechanisms of toxicity (4, 7). Utilizing *in vitro* cell-based systems for high-throughput transcriptomic analyses can provide insights into alterations in gene expression that are indicative of adverse outcomes in whole organisms (8). More recently, the potential of toxicogenomic techniques has been extended beyond gene expression profiling and towards using transcriptomics information to derive point-of-departure values (PODs) based on the benchmark dose approach. The shift away from apical endpoints towards molecular analyses applying the tPODs approach serves to identify a marker for which molecular changes begin and yields results more protective of ecological health (8). Chapter 3 in this thesis looked at deriving tPODs and characterizing molecular changes using whole transcriptome RNA sequencing (RNA-seq) which was only made possible for a couple of reasons. First, the increasing accessibility and affordability of high-throughput analysis by RNA-seq allowed for a whole transcriptomic approach to be applied in a high-throughput manner. RNA-seq was coupled with the UPXome RNA library kits by Qiagen which labels each well with a unique sample ID during the reverse

transcription step to allow for samples to be pooled together for all subsequent steps instead of having to prepare each sample individually, significantly improving preparation time. To our knowledge, this is the first example of applying the UPXome protocol to ecological samples (rainbow trout gill cells) as it was designed for human and mammalian samples. Second, the increasing abilities of computational tools in analyzing transcriptomic results were also realized. In Chapter 3, the RNA-sequencing data was demultiplexed and mapped to the rainbow trout genome using Qiagen's online software CLC Genomics Workbench (9) and analyzed by the online tool ExpressAnalyst for DEG analysis and tPOD derivation. Taken together, this thesis was able to combine microplate assay methods to characterize cellular and sub-cellular changes that could be linked to *in vivo* apical outcomes and contribute insight into the capabilities of NAMs in the shift away from animal testing.

4.1.2 Regulatory Acceptance

Collaborative efforts and scientific evidence are required to obtain regulatory acceptance of *in vitro* toxicity testing methods. In many cases, regulators need trusted evidence that alternative approaches can be applied to chemicals of priority while also producing biologically relevant results (10). At the beginning of this work, the Department of Fisheries and Oceans (DFO) released a paper on the list of priority pesticides for Canadian ecosystems in 2021 (11). In particular, the publication noted that sublethal impacts are a major gap in knowledge of these chemicals (11). Based on this publication, 17 of the 19 pesticides tested were selected to perform cytotoxic and transcriptomic studies. Chapters 2 and 3 contribute to filling important knowledge gaps about the molecular modes of toxic action and are in line with the goals of the DFO in evaluating pesticides and screening them for prioritization.

A main challenge associated with regulatory uptake is how the results from an *in vitro* cell-based exposure relate to the whole organism and if these results can be extrapolated (12, 13). In the standardization of OECD Test No. 249, comparisons between the results from RTgill-W1 cells and whole fathead minnow 96h exposures showed almost a 1:1 correlation for 35 organic chemicals (14, 15). Further comparisons of *in vitro* RTgill-W1 cell viability results with literature *in vivo* lethality values indicate the promise this method holds (16, 17). Chapter 2 contributes novel information to this discussion and builds confidence that the RTgill-W1 acute assay could be applied to other groups of chemicals such as pesticides. However, similar issues that were raised during the development and validation of OECD Test No. 249 were still prominent and discussed in this work (Chapter 2). Physiochemical properties (hydrophobicity and volatility) are continuously shown to influence a chemical's predictiveness of *in vivo* results in the microplate format (18, 19), in part because these tests are done in plastic microwell plates (20, 21). The implementation or use of glass micro-well plates or aluminum/adhesive foil covering methods could help overcome these issues for hydrophobic and volatile chemicals, respectively (16, 19). Moreover, in Chapter 2 some pesticides known to be very acutely toxic *in vivo* were observed to not cause cell death with the RTgill-W1 results (for example, atrazine). For chemicals with large databanks to compare *in vitro* results with, reasons for these differences can be investigated, but for new or uncharacterized chemicals it is still too early to confidently say that this assay could completely replace *in vivo* animal testing right now. However, Chapter 3 was able to derive tPODs more closely related to *in vivo* lethality from whole organisms, indicating that coupling OECD Test No .249 with transcriptomic analysis will potentially be able

to account for adverse effects that the RTgill-W1 cells alone might not be sensitive or specific enough for.

Another key consideration in the regulatory uptake of NAMs is the repeatability and reproducibility of data (22). This work was anchored in the standardized OECD Test No. 249 which was validated through a ring test for its reproducible and repeatable cytotoxic results (12). In Chapter 2, exposures for cytotoxicity were conducted in triplicate and displayed low variability between replicates over different days and passages. Displaying the ability of a high-throughput *in vitro* cell-based assay to yield reliable results is important because the cytotoxicity outcome measure is akin to apical endpoints from *in vivo* studies, and thus the best way to apply a direct comparison. Transcriptomic approaches, while newer, already show promise for reliably detecting alterations in gene expression and consistently displaying the links between tPODs and conventional points of departure from traditional studies (8, 23). Comparisons of the detected dysregulated pathways in Chapter 3 were similar to whole fish transcriptomic studies in the literature. Furthermore, to ensure the quality of data used in Chapter 3, quality control measures (positive and negative controls) and reference cDNA from unexposed RTgill-W1 cells were included in the experiment and the quality of cDNA at the end of library preparation was checked. This work highlights the importance of considering results from different biological levels together when interpreting toxicity results. It also emphasizes that transparency in reporting why certain methods were used and linking them to standardized protocols will support regulator acceptance.

4.1.3 Limitations and future works

In this thesis, adverse effects at the cellular and molecular levels could be elicited for pesticides and highlighted the possibility of using *in vitro* high-throughput methods to do so. However, this work is still limited by the choice of cell models and the environmental relevancy of the concentration tested. First, this work only tested the RTgill-W1 cell line which is only representative of the rainbow trout gill, but given the target specificities of pesticides it is possible that certain pathways and/or receptors were not present, and this is why toxicity was undetected for some pesticides (24). Also, the selection of just one cell line is not representative of multi-cell environments or other tissues in the whole rainbow trout (21, 25). Future studies should look to include the established liver (RTL-W1) and gut epithelial (RTgutGC) cell lines from rainbow trout (21, 26) when conducting exposures. Furthermore, a major limitation of OECD Test No. 249 is the lack of predictivity for neurotoxicants, but there is no neural cell line commercially available for trout which greatly limits the testing that can be done on these chemicals. Second, the concentrations used in this thesis were not based on environmental concentrations but were consistent for all pesticides for cytotoxicity characterization in Chapter 2. This was done to promote a high-throughput approach as the use of repeatable concentrations will allow for easier comparisons to be drawn with future studies (27). Lastly, the UPXome approach is a newer approach in general, and this study was the first to employ it for ecological sample testing. It required a large amount of troubleshooting and validation in the protocol development as naturally some steps had to be changed from the mammalian protocol. The quality control measures indicated good quality data overall, but there was some variability in the quality of output data from sequencing. Since this thesis, the protocol has been updated to

verify the method's reproducibility and repeatability for future studies that could be used in regulatory decision-making.

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Chapter 5

5 Conclusion

5.1 Conclusion

This master's thesis performed laboratory studies to advance knowledge of the effects of pesticides on the cellular and molecular level of the RTgill-W1 cell line as an alternative to animal testing approach. It aimed to improve our understanding of the scalability of OECD Test No. 249, how it compares to whole organism lethality values, and what is the tPOD value and subsequent mechanism of action for each pesticide. Significant advancements in biotechnologies allowed for a high-throughput, whole transcriptomic strategy to be applied and provided further insides into pesticides' sublethal effects. Anchoring transcriptomic analyses with *in vitro*, cell-based methods has strong implications in being able to screen a wider range of priority chemicals while gaining mechanistic toxicity information, thus potentially influencing decision-making at the regulatory level.

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