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Cytotoxicity of 19 pesticides in rainbow trout gill, liver, and intestinal cell lines

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Abstract

The rainbow trout gill cell line (RTgill-W1), via test guideline 249 of the Organisation for Economic Co-operation and Development, has been established as a promising New Approach Methodology, although to advance confidence in the method more case studies are needed that: 1) expand our understanding of applicability domains (chemicals with diverse properties); 2) increase methodological throughput (96-well format); and 3) demonstrate biological relevance (in vitro to in vivo comparisons; gill vs. other cells). Accordingly, the objective of our study was to characterize the cytotoxicity of 19 pesticides against RTgill-W1 cells, and also liver (RTL-W1) and gut epithelial (RTgutGC) cell lines, and then to compare the in vitro and in vivo data. Of the 19 pesticides tested, 11, 9, and 8 were cytotoxic to the RTgill-W1, RTL-W1, and RTgutGC cells, respectively. Six pesticides (carbaryl, chlorothalonil, chlorpyrifos, dimethenamid-P, metolachlor, and S-metolachlor) were cytotoxic to all three cell lines. Aminomethylphosphonic acid, chlorantraniliprole, dicamba, diquat, imazethapyr, and permethrin exhibited cell-line–specific toxicity. No cytotoxic responses were observed for three herbicides (atrazine, glyphosate, and metribuzin) and four insecticides (clothianidin, diazinon, imidacloprid, and thiamethoxam). When cytotoxicity was measured, there was a strong correlation ($r_s = 0.9$, $p < 0.0001$) between in vitro median effect concentration (EC50) values (based on predicted concentrations using the In Vitro Mass Balance Model Equilibrium Partitioning (IV-MBM EQP) Ver. 2.1) derived from RTgill-W1 and RTL-W1 cells with in vivo median lethal concentration (LC50) values from 96-h acute toxicity studies with trout. In all 28 cases, the in vitro EC50 was within 18-fold of the in vivo LC50. These data help increase our understanding of the ecotoxicological domains of applicability for in vitro studies using cultured rainbow trout cells, while also demonstrating that these assays performed well in a 96-well format and have promise to yield data of biological relevance.

Keywords: pesticides, in vitro toxicology, aquatic toxicology, new approach methods

Introduction

Toxicity testing regulations in jurisdictions such as Canada (Environment and Climate Change Canada [ECCC], 2007), the United States (US Environmentl Protection Agency [USEPA], 1994), and the European Union (Hengstler et al., 2006) have long relied on fish to characterize the risk posed by chemical contaminants to aquatic ecosystem health. Although many toxicity tests involving fish are standardized and validated, they often require a considerable number of animals, are time-consuming, and produce contaminated waste (Basu et al., 2019; Norberg-King et al., 2018; Tanneberger et al., 2013). In addition, standardized tests tend to focus on death as an endpoint, which provides little information on a chemical's mechanism of action and also raises ethical concerns. Such concerns, coupled with recent legislation in Canada, the United States, and the European Union working toward phasing out animal toxicity testing, are driving the development of alternatives to animal approaches.

Interest in New Approach Methodologies (NAMs) compliant with the three Rs principles (refinement, reduction, and replacement of animal testing) holds promise to overcome the limitations associated with animal-based toxicity testing. Toward this aim, in 2021 the Organization for Economic Co-operation and Development (OECD, 2021) standardized and published test guideline 249 using gill cells from rainbow trout (RTgill-W1 cells) to assess the cytotoxicity of chemicals (International Organization for Standardization, 2019; Lee et al., 2009). The gill cell was selected as the basis for OECD test guideline 249 because it plays a key role in the uptake of toxicants from water (Bury et al., 2014; Scott et al., 2021; Tanneberger et al., 2013). It also plays an important physiological role given its involvement in osmoregulation and respiration (Bury et al., 2014; Lee et al., 2009). The assay has been demonstrated to be repeatable and reproducible (Fischer et al., 2019). Furthermore, studies with 35 organic chemicals and 38 fragrance chemicals showed that most median effect concentration (EC50) values were comparable to lethality values from studies on whole fish (Natsch et al., 2018; Tanneberger et al., 2013).

Despite the promise of this test method, there remain outstanding questions that warrant further research. For one, RTgill-W1 cells represent a single tissue and may not reflect responses in other tissues or in the whole organism. In a study of three rainbow trout cell lines (RTgill-W1, along with liver

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[RTL-W1] and intestine [RTgutGC]) exposed to benzo[*a*]pyrene, metabolic capacity was evident in all cells although biotransformation was the weakest in the RTgill-W1 cells (Stadnicka-Michalak et al., 2018). Cytotoxic responses differed between RTgill-W1, RTL-W1, and RTgutGC cells following exposure to the synthetic dye Acid Blue 80 (Tee et al., 2011), although in a study of all three cell lines exposed to fluoxetine and methylmercury, many cytotoxic and transcriptomic responses were comparable (Mittal et al., 2022). Similarly, the RTgutGC cell line predicted cytotoxicity of 16 fragrance chemicals with an almost 1 to 1 relationship to the RTgill-W1 cells and in vivo results (Natsch et al., 2018; Schug et al., 2020).

Another aspect that warrants more consideration is the number of chemicals evaluated by rainbow trout cell lines. The published literature suggests that several dozens of chemicals have been tested for cytotoxicity in RTgill-W1 cells, such as fragrance chemicals (Natsch et al., 2018), polycyclic aromatic hydrocarbons (Bussolaro et al., 2019), nanobiomaterials (Hernández-Moreno, Navas, et al., 2022), microplastics (Boháčková et al., 2023), and biocides (Hernández-Moreno, Blázquez, et al., 2022). However, there is sparse or no representation from notable chemical classes such as pesticides.

Further increasing scientific confidence in OECD test guideline 249 will need to consider several factors (van der Zalm et al., 2022) including how this NAM performs: 1) under certain applicability domains (i.e., exposure to chemicals with diverse physicochemical properties and mechanisms of action); 2) technically with advances to the method, including resource efficiency considerations (e.g., shifting from 24-well to 96-well microplates, using two cell viability endpoints instead of three); and 3) in terms of biological relevance (i.e., how in vitro values compare with those in vivo; responses in RTgill-W1 cells vs. other rainbow trout cell lines). Accordingly, the overall goal of our study was to characterize the cytotoxicity of 19 pesticides with comparable whole animal data, and diverse potencies and mechanisms of action in three rainbow trout cell lines (RTgill-W1, RTL-W1, and RTgutGC). Selected pesticides are of concern in Canadian ecosystems (Anderson et al., 2021), and include herbicides (aminomethylphosphonic acid [AMPA], atrazine, dicamba, dimethenamid-P, diquat, glyphosate, imazethapyr, metolachlor, S-metolachlor, and metribuzin), insecticides (carbaryl, chlorantraniliprole, chlorpyrifos, clothianidin, diazinon, imidacloprid, permethrin, and thiamethoxam), and a fungicide (chlorothalonil). The first study objective was to compare intercell line cytotoxic responses, and the second objective was to correlate the in vitro results with in vivo ones available from the literature.

Methods

Pesticide selection

Selection of the 19 pesticides was motivated by a priority list compiled by a Fisheries and Oceans Canada study (Anderson et al., 2021) that includes compounds used worldwide. Anderson et al. (2021) identified a list of 55 pesticides and ranked them based on criteria such as 2017/2018 sales, detection frequency, measured concentrations in Canadian surface waters, inherent toxicity from guideline values, and regulatory status in other countries. For each criterion, pesticides were given a score between 0 and 3, with a total of 33 points possible. Eight pesticides scored 23 points or more and were thus categorized as top priority; all eight are included in our study (atrazine, chlorothalonil, diazinon, clothianidin, chlorpyrifos, diquat, permethrin, and S-metolachlor). Nine pesticides with scores ranging from 14 to 22 were selected to cover a range of chemical families, physicochemical properties, and mechanisms of action (Supporting Information, Table S1). We also included chlorantraniliprole and AMPA. Chlorantraniloprole was selected because available fish acute toxicity data are classified as "nondefinitive" (USEPA, 2008). Aminomethylphosphonic acid was selected because it is a major metabolite of glyphosate, one of the most widely used herbicides worldwide (de Brito Rodrigues et al., 2019). Although glyphosate has been found to have no (or low) acute toxic in fish (Antón et al., 1994; de Brito Rodrigues et al., 2019; Folmar et al., 1979), literature gaps remain regarding AMPA and its toxicity to nontarget organisms (Tresnakova et al., 2021). Furthermore, AMPA has a lower water solubility and a longer soil half-life than glyphosate, suggesting that it may be more persistent (Tresnakova et al., 2021).

Rainbow trout cell culture

The rainbow trout gill cell line (RTgill-W1; CRL-2523) was obtained from American Type Cell Culture. The liver and intestinal cell lines (RTL-W1 and RTgutGC, respectively) were a kind donation from Dr. Lucy Lee (University of the Fraser Valley, Abbotsford, BC, Canada). All cell culturing was done in accordance with OECD test guideline 249 designed for RTgill-W1 cells. The culture of these RTL-W1 and RTgutGC cell lines following the OECD guideline was previously evaluated (Mittal et al., 2022; Schug et al., 2020) and deemed to be appropriate. In brief, cells were grown at 21 °C in 100-mm Petri dishes containing 10 mL of complete culture medium (L-15; Leibovitz L-15 culture medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin). The culture medium was replaced twice a week. Once cells had reached 80% confluency, they were trypsinized for 3 to 5 min using 1 mL of trypsin-ethylenediaminetetraacetic acid, and either passaged to continue the culture or counted (using a hemocytometer) and seeded in 96-well plates (Falcon 96-well, Cat. No. 08-772-2C; Fischer Scientific) for exposure experiments (35,000 cells/well). The cell passage number ranged from 64 to 71 for RTgill-W1 cells, 77 to 79 for the RTgutGC cells, and 82 to 84 for the RTL-W1 cells.

Chemicals

The cells were exposed to pesticides according to the methods described in OECD test guideline 249 with minor changes (largely due to optimizing the assay for 96-well plates, and these are described in the following section, *Exposure*). Pesticides were purchased from Sigma-Aldrich except for metolachlor, diazinon, permethrin, and S-metolachlor, which were purchased from Canadian Life Science, and chlorpyrifos, which was purchased from Toronto Research Chemicals (see Supporting Information, Table S1 for details such as CAS and lot numbers, chemical classifications, and physicochemical properties). For cytotoxicity studies, 200-mM stock solutions were created for all pesticides except glyphosate (20 mM) and chlorothalonil (20 mM), which were created at lower concentrations due to solubility issues. All stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), except for diquat, AMPA, and glyphosate, which were prepared in Milli-Q water. The stock solutions were serially diluted (threefold) in L-15 exposure medium (L-15/ex; a specifically designed protein- and animal-component–free buffer that provides optimal pH and osmolality between 7 and 7.5 and 290 and 320 mmol/kg, respectively, for the cells during their chemical exposures) to obtain final test concentrations of 0, 0.45, 1.37, 4.12, 12.35, 37.04, 111.11, 333.33, and 1000 μM for chlorothalonil, or 0, 0.05, 0.14, 0.41, 1.24, 3.7, 11.11, 33.33, and 100 μM for glyphosate (due to their stock solutions being made 10-fold lower than

the other pesticides). The OECD test guideline 249 positive control chemical (3,4-dichloroaniline [3,4-DCA]; 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.

Exposure

The OECD test guideline 249 protocol was modified slightly in a few ways to increase efficiency. First, the test provides guidance on testing one chemical in a 24-well plate over six concentrations. To increase the throughput potential of this method, we scaled the method to perform in a 96-well plate so that it could test 10 chemicals over eight concentrations in a single plate while also retaining quality control wells. Performance of this OECD test in 96-well plates has previously been done (Hernández-Moreno, Navas, et al., 2022). Second, two cell viability dyes (alamarBlue for assessing metabolic activity and 5-carboxyfluorescein diacetate acetoxymethyl ester [CFDA-AM] for assessing cell membrane integrity) were used to assess cytotoxicity as opposed to the three dyes outlined in the OECD test (including Neutral Red for assessing lysosomal membrane integrity). This was decided because alamarBlue and CFDA-AM solutions can be combined and applied to cells as one solution (OECD, 2021), which represents a more resource-efficient option. Furthermore, previous results have shown high intercorrelation among the three dyes, and that the use of one dye (alamarBlue) might be sufficient (Natsch et al., 2018; Tanneberger et al., 2013). Third, as previously mentioned, the applicability of OECD test guideline 249 has been explored with liver and gut rainbow trout cell lines (Mittal et al., 2022; Schug et al., 2020), and these cells were included in the present study alongside the gill cells for comparative purposes and to further explore biological relevance and domains of applicability. Finally, OECD test guideline 249 calls for exposure solutions to be taken to verify actual chemical concentrations, although the transition to a 96-well plate limits the ability to perform chemical analysis due to the lower volume (1.5 mL in a 24-well plate vs. 100 μL in a 96-well plate), so this aspect was omitted. However, attempts were made to model the disposition of chemicals in exposure media using the In Vitro Mass Balance Model Equilibrium Partitioning (IV-MBM EQP) Ver. 2.1 as described further in the *Mass balance modeling* section.

A series of pilot tests was conducted to ensure that the quality control criteria described in OECD test guideline 249 as well as the expected cytotoxicity results would not be affected by this transition. It was determined that seeding 35,000 cells/well in a 96-well plate format (as opposed to 350,000 cells/well in a 24-well plate format) would satisfy three quality control checks while not changing the cytotoxicity results (Supporting Information, Figure S1 and Table S2). In addition, a pilot test with metolachlor in 24- and 96-well plates with the alamarBlue fluorescent dye was performed to ensure that results would not differ in the latter format (Supporting Information, Figure S2). The RTgill-W1 cells exposed to metolachlor in a 24-well plate over a concentration range of 0.01 to 1000 μM (twofold serial dilution over six concentrations) yielded a median lethal concentration (LC50) of 291.2 μM (82.6–499.7 μM), and the RTgill-W1 cells exposed to metolachlor in a 96-well plate resulted in an LC50 of 311.7 μM (108.9–514.4 μM; Supporting Information, Figure S2). These results provide confidence that the assay can be scalable for a 96-well format.

In terms of the exposure study design, each pesticide was tested in each of the three cell lines at eight concentrations (as well as a negative control) spanning approximately 3.5 orders of magnitude. For each of the cell lines, three different passages were tested against each pesticide and these exposures were performed in different microplates. To keep the exposure design consistent, the 19 pesticides were split into "batch 1" chemicals consisting of 9 pesticides and 3,4-DCA, and "batch 2" chemicals consisting of the subsequent 10 pesticides (Supporting Information, Figure S1B).

On exposure Day 1, cells were seeded at a density of 35,000 cells/100 μL of complete medium/well in 96-well plates and incubated for 24 h. In each plate, 11 wells were designated as no-cell controls and contained only the complete medium. On Day 2, the 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 concentration/well). The highest concentrations of the pesticide stocks and 3,4-DCA were added to the wells and then serially diluted (threefold) in L-15/ex. Ten of the 11 no-cell control wells received the highest concentration of chemicals, 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 (CFDA-AM) cytotoxicity assays were conducted immediately to assess metabolic activity and membrane integrity, respectively. Briefly, the cells were rinsed in 100 μL of phosphate-buffered saline (PBS) following which alamarBlue (5% v/v in PBS) + CFDA-AM dye was added to the wells, and the cells were incubated for 30 min. Next, 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). 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 with the DMSO-solvent control fluorescence units based on the equation below:

$$\%\ \text{cell viability} = \{[FU_{\text{chemical}} - FU_{\text{chem.no-cell ctrl}} \times 100]/[FU_{\text{control}} - FU_{\text{ctrl.no-cell}}]\}$$

The EC50 value was calculated using the drc package for dose–response analysis in R. All EC50 data are reported as mean ± SD values.

Mass balance modeling

Physicochemical properties, specifically hydrophobicity and volatility, of test chemicals complicate the comparison of toxicity values between in vitro and in vivo assay results (Dupraz et al., 2019; Schug et al., 2020; Stadnicka-Michalak et al., 2014; Tanneberger et al., 2013). To help overcome this challenge, OECD test guideline 249 recommends performing chemical analysis at the start and end of an experiment. However chemical measures are not always possible due to technical, logistical, and financial barriers (such as in the present study), and so modeling approaches have emerged to help calculate biologically effective doses in vitro (Armitage et al., 2021; Stadnicka-Michalak et al., 2021). A modeling framework was developed by Stadnicka-Michalak et al. (2021) for RTgill-W1 cells but could not be used here because the model was built for 24- and 48-well plates, and our study used 96-well plates. Instead, we used IV-MBM EQP Ver. 2.1, which is a mass balance model compatible with 96-well in vitro studies that predicts chemical concentrations in the medium and intracellular environment (Armitage et al., 2021). A good correlation was observed between IV-MBM EQP–predicted concentrations and analytically measured concentrations from four different in vitro cell-based studies involving a variety of

chemicals (R^2 between 0.69 and 0.84; Armitage et al., 2021), including S-metolachlor, glyphosate, AMPA, chlorpyrifos, and imidacloprid. The model is implemented as an Excel/VBA spreadsheet tool and is available for download at ARC Arnot Research and Consulting (2023).

In vitro–in vivo comparisons

Comparisons were made between the in vitro EC50 data we derived (both nominal and predicted concentrations) with in vivo LC50 values drawn from the scientific literature. The OECD test guideline 249 states that the EC50 of the most sensitive endpoint (i.e., the lower EC50 between metabolic activity and membrane integrity endpoints) can be used to predict the in vivo LC50 (OECD, 2021), so this value was determined for each pesticide and used for in vitro–in vivo comparisons. In vivo LC50 values were taken from 96-h acute toxicity studies with juvenile rainbow trout (*Oncorhynchus mykiss*) from the USEPA's (2023) Ecotoxicology Knowledgebase (ECOTOX), the EnviroTox (2023) database, and peer-reviewed journals if no database value was available. Inclusion criteria for the selection of in vivo values included: 96 h exposure, tests performed with active ingredient (not formulation), a chemical purity of more than 80%, LC50 value calculated, and use of flow-through or static systems. All pesticides, except for permethrin and carbaryl, returned one LC50 value from the databases. Three database LC50 values were obtained for permethrin, and these were averaged. Carbaryl's LC50 value was taken from a published study that satisfied inclusion criteria (Dwyer et al., 2005). In vivo–in vitro correlations were performed using a Spearman's rank ρ coefficient test where $\alpha = 0.05$.

Results

Quality control

Quality control data are outlined in the Supporting Information (Tables S3 and S4) with key findings summarized in the present study. Across all batches of all cell lines, cell viability of the DMSO solvent control ranged from 90.5% to 109.0% compared with the negative (unexposed) controls except for RTgutGC cell Batch 1 exposures (87.4%). The variation in the cell-free control well was below 20% for most pesticides (except for glyphosate, diquat, and chlorothalonil). The positive control 3,4-DCA was tested with every passage and elicited concentration–response curves for the RTgill-W1 cells with aaverage EC50 values of 57.7 ± 4.5 and 62.0 ± 6.3 mg/L for the alamarBlue and CFDA-AM cell viability dyes, respectively (Supporting Information, Figure S3). Combined, these results satisfy most quality control criteria outlined in OECD test guideline 249, thus indicating that the assay (which we adapted in our study for a 96-well format) was performing well.

Mass balance modeling

Using the IV-MBM EQP Ver. 2.1 model, and in consideration of the test chemicals and in vitro assay parameters, the predicted concentration of chemicals in the culture medium ranged from 0.01% to 99.8% of the nominal concentration (Supporting Information S1, Table S5). For seven pesticides (glyphosate, diquat, AMPA, thiamethoxam, imidacloprid, clothianidin, and imazethapyr), the predicted concentration was within 10% of the nominal one. Dicamba, metribuzin, and dimethenamid-P were predicted to have 82% to 89% of chemical available in the test system. Five pesticides (carbaryl, atrazine, S-metolachlor, metolachlor, and diazinon) were predicted to have between 19.5% and 55% of the test concentration available in the medium. Finally, levels of chlorantraniliprole, chlorothalonil, chlorpyrifos, and permethrin were predicted to be <3% of the nominal value. With increasing hydrophobicity (i.e., log octanol/water partition coefficient [K_{OW}]) of a particular chemical, the predicted concentration in the cell culture media decreased (Supporting Information S1, Figure S4). Throughout the present study, the exposure data are presented and discussed in terms of both nominal and model-predicted concentrations.

Cell line results

RTgill-W1 results

For RTgill-W1 cells, concentration–response curves and corresponding EC50 values were obtained for 10 of the 19 pesticides (Table 1) assessing metabolic activity (Figure 1) and 7 of the 19 pesticides assessing membrane integrity (Figure 2), for a total of 11 pesticides being classified as cytotoxic to these gill cells. Diquat, AMPA, dicamba, and imazethapyr (all herbicides) were cytotoxic for metabolic activity but not for membrane integrity, whereas the opposite was observed with permethrin (insecticide). Six pesticides elicited a response for both cell viability endpoints, and of these six, all except carbaryl and dimethenamid-P produced similar values (within 95% confidence intervals [CI]). The rank-order, for most to least cytotoxic based on the most sensitive EC50 value calculated from the nominal concentrations, was: chlorothalonil > carbaryl > diquat > chlorpyrifos > dimethenamid-P > metolachlor > S-metolachlor > dicamba > permethrin > AMPA > imazethapyr. However, when the EC50 data were considered using the predicted concentrations, the order changed so that permethrin, chlorothalonil, and chlorpyrifos were ranked most potent, with EC50 values below approximately 1 μM followed by carbaryl and diquat, with EC50 values below 100 μM (Table 1).

RTL-W1 results

In the liver cells, EC50 values were obtained for nine pesticides (Table 1). Seven of the nine pesticides (chlorpyrifos, chlorothalonil, carbaryl, metolachlor, S-metolachlor, dimethenamid-P, and diquat) were cytotoxic with both cell viability endpoints (Figures 1 and 2). Aminomethylphosphonic acid was only cytotoxic with metabolic activity, and permethrin was only cytotoxic with membrane integrity. The rank order, for most to least toxic based on the lowest EC50 values calculated from the nominal concentrations, was: chlorothalonil > diquat > carbaryl > chlorpyrifos > metolachlor > S-metolachlor > dimethenamid-P > AMPA > permethrin. However, when the EC50 data were considered using the predicted concentrations, the order changed in a manner nearly identical to that of the RTgill-W1 cells given in the previous section, *RTgill-W1 cell results*.

RTgutGC results

In the intestinal cells, eight pesticides (chlorpyrifos, chlorothalonil, carbaryl, metolachlor, S-metolachlor, dimethenamid-P, diquat, and chlorantraniliprole) elicited concentration–response curves and LC50 values for both cell viability assays (Figures 1 and 2). The rank-order for most to least toxic, based on the most sensitive EC50s calculated from the nominal concentrations, was: chlorothalonil > diquat > carbaryl > dimethenamid-P > metolachlor > S-metolachlor > chlorpyrifos > chlorantraniliprole (Table 1). However, when the EC50 data were considered using the predicted concentrations, the order changed so that chlorothalonil, chlorpyrifos, and chlorantraniliprole were ranked most potent, with EC50 values below approximately 1.5 μM followed by carbaryl and diquat, with values below 100 μM.

Table 1. Mean median effect concentration values based on nominal concentrations and model predicted values for three rainbow trout cells.

	Mean EC50 (μM) ± 95% CI values based on nominal conc.										
	RTgill-W1		RTL-W1		RTgutGC			EC50 (μM) based on predicted conc.			
Pesticide	Metabolic activity	Membrane integrity	Metabolic activity	Membrane integrity	Metabolic activity	Membrane integrity	Predicted compared with nominal conc. (%)	RTgill-W1	RTL-W1	RTgutGC	In vivo RT LC50 (μM)
Chlorpyrifos	276.8 (132.0–421.6)	299.1 (165.5–432.7)	304.7 (−143.1 to 752.6)	212.9 (66.0–359.8)	364.0 (−56.2 to 784.2)	353.2 (−64.5 to 771.0)	0.4	1.1	0.8	1.4	0.1
Chlorothalonil	2.6 (1.8–3.5)	3.6 (−0.3 to 7.6)	1.8 (0.5–3.0)	1.7 (0.4–3.0)	0.7 (0.04–1.3)	0.8 (0.2–1.5)	3.0	0.1	0.1	0.02	0.1
Carbaryl	97.0[a] (54.5–139.6)	393.1[a] (219.9–566.3)	121.8[a] (−2.4 to 246)	454.1[a] (−31.0 to 939.2)	146.0 (−408.3 to 700.2)	298.0 (−15.6 to 611.5)	39.4	38.2	48.0	57.5	9.9
Metolachlor	311.7 (108.9–514.4)	530.2 (17.3–1043.1)	407.0[a] (87.2–726.7)	736.1[a] (−106.0–1578.2)	301.1	209.0 (10.4–407.7)	43.3	134.9	176.1	90.4	13.7
S-metolachlor	352.3 (−794.2 to 1498.2)	565.0 (215.9–914.1)	431.5 (122.7–740.3)	502.8 (173.5–832.1)	301.6 (−376.8 to 980)	289.2 (14.5–563.8)	55.0	193.8	237.3	159.1	41.9
Dimethenamid-P	291.7[a] (185.3–398.1)	497.3[a] (319.3–675.3)	559.8 (18.5 –1101.2)	467.0 (23.0–911.1)	202.4 (−2299.4 to 2704.1)	315.2	87.4	254.9	408.1	176.9	22.8
Diquat	100.4[a] (34.5–166.3)	>1000	13.8[a] (−1.9 to 29.4)	913.7[a] (−496.3 to 2323.7)	13.3[a] (7.2–19.5)	105.2[a] (−48.6 to 258.9)	97.4	97.8	13.4	13.0	41.0
AMPA	493.1[a] (307.1–679.1)	>1000	618.1[a] (−1335 to 2571.1)	>1000	>1000	>1000	99.7	491.7	616.3	>1000	2637.4
Dicamba	416.3[a] (250.7–581.8)	>1000	>1000	>1000	>1000	>1000	81.8	340.7	>1000	>1000	612.6
Imazethapyr	538.5[a] (−120.5 to 1197.5)	>1000	>1000	>1000	>1000	>1000	92.3	496.8	>1000	>1000	1188.9
Permethrin	>1000	426.7[a] (141.9–711.5)	>1000	643.7[a] (−314.0 to 1601.3)	>1000	>1000	0.002	0.008	0.01	>1000	0.01
Chlorantraniliprole	>1000	>1000	>1000	>1000	376.6[a] (15.2– 738)	757.8[a] (−276.3 to 1791.8)	0.3	>1000	>1000	1.0	>28.6
Diazinon	>1000	>1000	>1000	>1000	>1000	>1000	19.5	>1000	>1000	>1000	1.3
Atrazine	>1000	>1000	>1000	>1000	>1000	>1000	25.2	>1000	>1000	>1000	24.6
Thiamethoxam	>1000	>1000	>1000	>1000	>1000	>1000	95.8	>1000	>1000	>1000	>342.8
Metribuzin	>1000	>1000	>1000	>1000	>1000	>1000	88.8	>1000	>1000	>1000	298.7
Clothianidin	>1000	>1000	>1000	>1000	>1000	>1000	96.3	>1000	>1000	>1000	420.5
Imidacloprid	>1000	>1000	>1000	>1000	>1000	>1000	97.4	>1000	>1000	>1000	896.1
Glyphosate	>100	>100	>100	>100	>100	>100	99.8	>100	>100	>100	792.6

[a] Values between endpoints within a cell line are different (not within 95% CI).
[b] Values between cell lines are different (not within 95% CI).

Note: Cells are gill (RTgill-W1), liver (RTL-W1), and gut (RTgutGC) cells exposed to 19 pesticides, along with LC50 values from the literature on in vivo studies from rainbow trout.The EC50s of cell viability are calculated from assays of metabolic activity (alamarBlue fluorescent dye) and membrane integrity (CFDA-AM fluorescent dye). The predicted EC50s are calculated from the most sensitive cell viability endpoint. In vivo data points are based on one data point for pesticides except for permethrin, which was an average of three data points.

AMPA = aminomethylphosphonic acid; CFDA-AM = carboxyfluorescein diacetate-acetoxymethyl ester; EC50 = median effect concentration; LC50 = median lethal concentration; RT = rainbow trout.

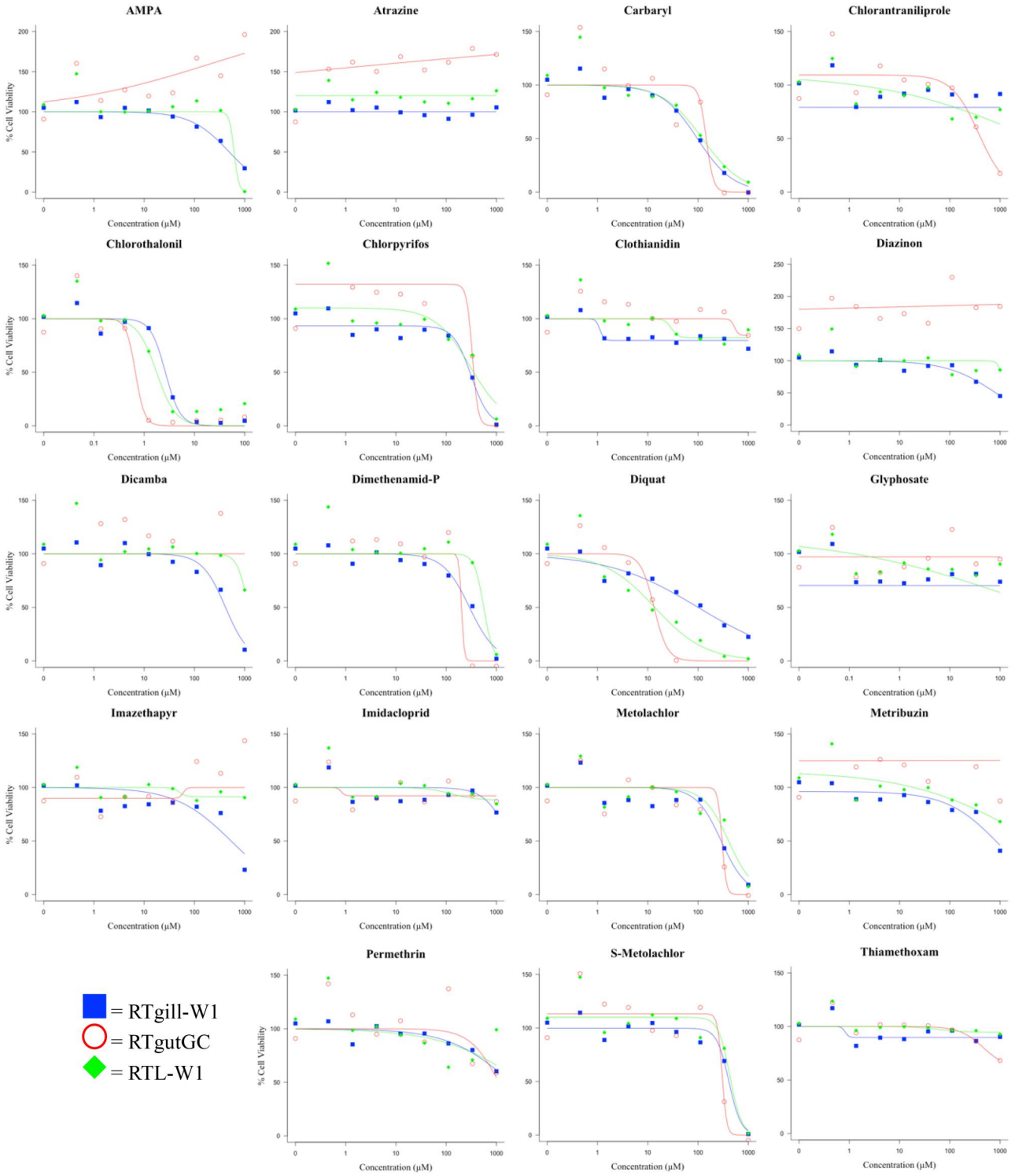

Figure 1. Concentration–response curves for all three cell lines with the metabolic activity (alamarBlue fluorescent dye) endpoint. The x-axis is the nominal test concentration. Each data point represents the mean of three biological replicates for the rainbow trout (RT)gill-W1 (blue squares), RTgutGC (red circle), and RTL-W1 (green diamond) cell lines to create fitted concentration–response curves. AMPA = aminomethylphosphonic acid.

Intercell line comparisons

Some common trends in cytotoxicity were observed across the three cell lines. First, a visual inspection of a principal components analysis plot shows separation between the cytotoxic and noncytotoxic pesticides (Supporting Information S1, Figure S5). Second, seven pesticides (chlorpyrifos, chlorothalonil, carbaryl, metolachlor, S-metolachlor, dimethenamid-P, and diquat) were cytotoxic to all three cell lines (Table 1). Six of these pesticides (chlorpyrifos, chlorothalonil, carbaryl, metolachlor, S-metolachlor, and dimethenamid-P) were cytotoxic across all the cell lines according to both cell viability endpoints, whereas diquat did not yield a concentration–response curve in the RTgill-W1 cell line via the membrane integrity assay. Third, the top three toxic pesticides in all three cell lines (based on the predicted

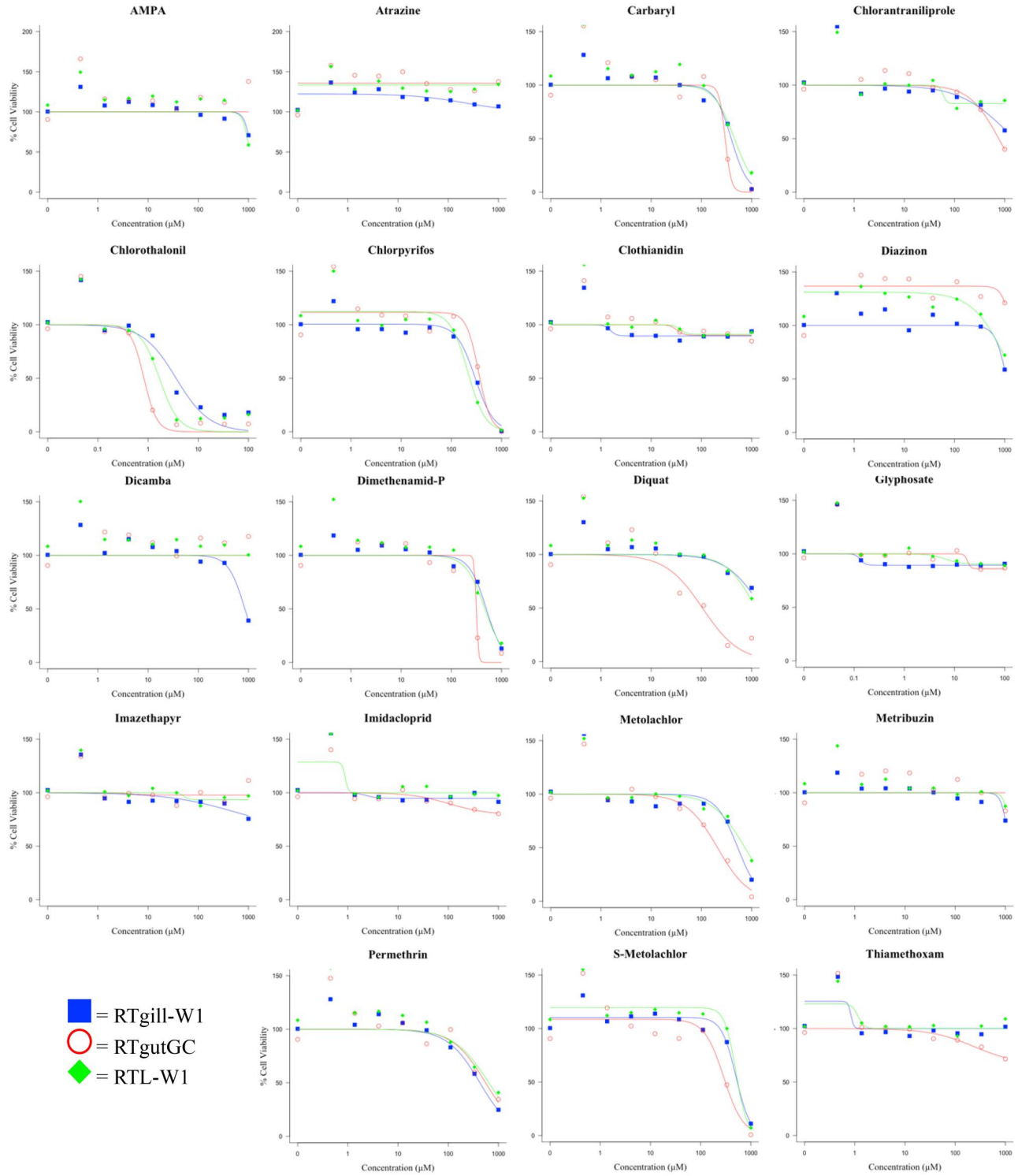

Figure 2. Concentration–response curves for all three cell lines with the membrane integrity (carboxyfluorescein diacetate-acetoxymethyl ester [CFDA-AM] fluorescent dye) endpoint. The x-axis is the nominal test concentration. Each data point represents the mean of three biological replicates for the rainbow trout (RT)gill-W1 (blue squares), RTgutGC (red circle), and RTL-W1 (green diamond) cell lines to create fitted concentration–response curves. AMPA = aminomethylphosphonic acid.

concentrations) always included chlorpyrifos and chlorothalonil. When the EC50 data were examined based on nominal concentrations, the top three toxic pesticides in all three cell lines were consistently ranked differently as chlorothalonil > diquat > carbaryl. Fourth, of the seven pesticides that were cytotoxic in all three cell lines, chlorpyrifos, chlorothalonil, carbaryl, and S-metolachlor ($n = 4$) produced EC50 (µM) values from metabolic activity and membrane integrity endpoints within 95% CI of each other. Lastly, no cytotoxic responses were observed in any cell line for seven pesticides (three herbicides: atrazine, glyphosate, and metribuzin; and four insecticides: diazinon, clothianidin, thiamethoxam, and imidacloprid).

There were also notable differences among the cell lines and endpoints with key examples provided in our study.

The RTgill-W1 cell line yielded the most concentration–response curves (with 11 of the 19 pesticides yielding EC50 values), followed by the RTL-W1 (9 pesticides) and RTgutGC cell lines (8 pesticides). Aminomethylphosphonic acid and permethrin demonstrated tissue and endpoint sensitivity in which they only elicited EC50 values with the metabolic activity and membrane integrity endpoints, respectively, for the RTgill-W1 and RTL-W1 cell lines. Neither of these pesticides caused cytotoxicity in the RTgutGC cell line. Tissue specificity was also observed with dicamba, imazethapyr, and chlorantraniliprole. Dicamba and imazethapyr only caused a cytotoxic response in the RTgill-W1 cells, and chlorantraniliprole was only cytotoxic to the RTgutGC cell line. Finally, across all cell lines, diquat was found to be more cytotoxic according to the metabolic activity endpoint versus the membrane integrity endpoint.

In vitro–in vivo comparisons

In the dataset, 57 cell line–pesticide combinations were tested of which 28 yielded cytotoxic responses up to test concentrations that were either 100 or 1000 μM (Table 1). Across these 28 cytotoxicity cases, 13 yielded in vitro EC50 values based on nominal concentrations that were within 1 order of magnitude of the corresponding in vivo LC50 values, 10 were within 30-fold, and chlorpyrifos and permethrin yielded results >2000-fold. However, when the in vitro EC50 values were adjusted to reflect their predicted concentrations, 23 of them yielded in vitro EC50 values that were within 1 order of magnitude of the corresponding in vivo LC50 values, and all 28 cases yielded EC50 values that were within 18-fold of the in vivo value.

Correlations between the EC50 values we derived in vitro with lethality values from rainbow trout fish exposures (derived from the literature) were calculated. Although correlations using in vitro EC50 values based on nominal concentrations did not return statistically significant results, with the exception of some borderline associations within RTgill-W1 cells (Figure 3 and Supporting Information, Figures S6 and S7 and Table S6), correlation calculations between in vivo LC50 and in vitro EC50 values from the most sensitive endpoint based on predicted concentrations were significant for the RTgill-W1 (Spearman ρ value = 0.94, p value < 0.0001, $r^2 = 0.88$) and RTL-W1 (Spearman ρ value = 0.86, p value = 0.0028, $r^2 = 0.84$) cell lines (Figure 3 and Supporting Information, Figure S8). For the RTgutGC, the Spearman p correlation was not of statistical significance (Spearman ρ value of 0.46, p value = 0.26, $r^2 = 0.54$). Although our results focus on concentrations reported on a molar basis, corresponding in vitro and in vivo values on a mass basis (mg/L) are also provided (Supporting Information, Table S7).

Using model predicted concentrations (vs. nominal ones) that consider physicochemical variables resulted in EC50 values that were more closely aligned with mortality data from 96-h rainbow trout bioassays. In the present study, when cytotoxicity was measured, we observed that the log air/water partition coefficient (K_{AW}) correlated with EC50 values in RTgill-W1 and RTL-W1 but not RTgutGC cell lines (Supporting Information, Figure S9). Weak nonsignificant correlations (i.e., $p = 0.1$) were also observed in some cases for log H, water solubility, and log K_{OW} (Supporting Information, Figures S10–S12), although, overall, when data were considered for all chemicals (cytotoxic and noncytotoxic ones), there were no clear patterns.

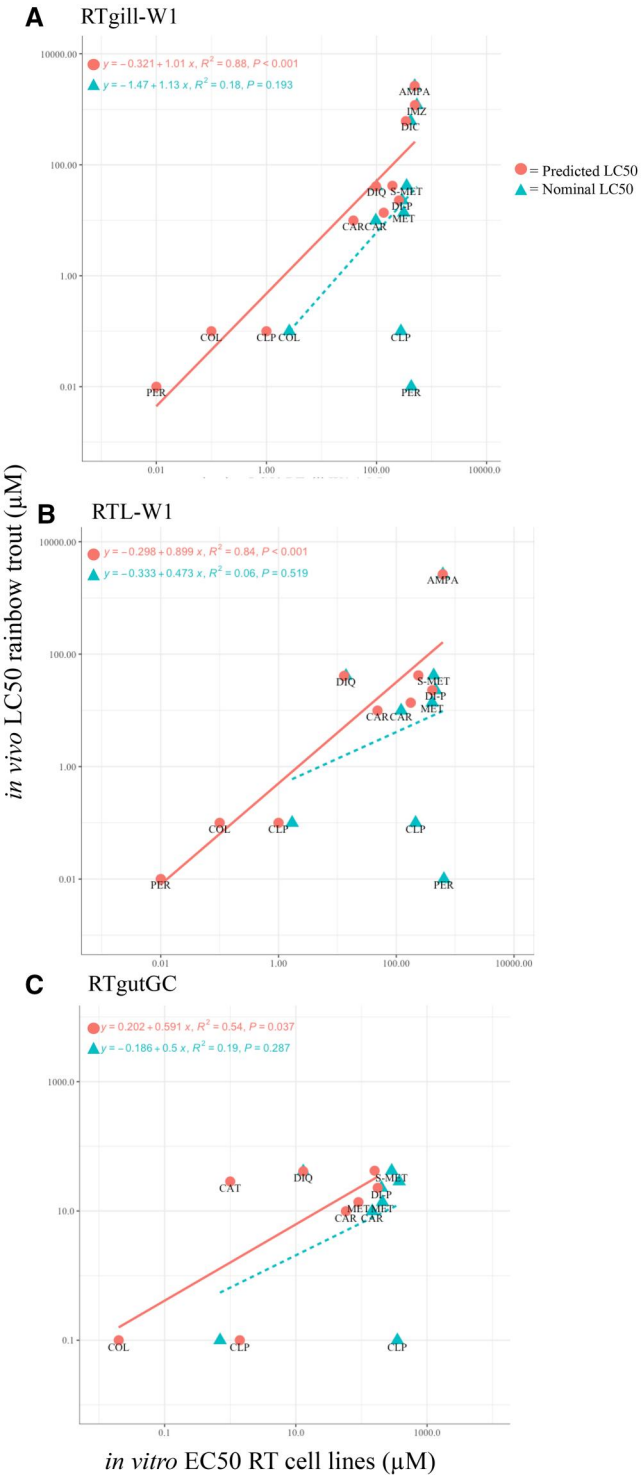

Figure 3. Correlation of nominal (blue triangle) median effect concentration (EC50) values and model predicted (pink circle) EC50s from the most sensitive endpoint for (**A**) rainbow trout (RT)gill-W1; (**B**) RTL-W1, and (**C**) RTgutGC cell lines. Only cytotoxic pesticides were included in our study. The in vivo median lethal concentration (LC50) values (y-axis) for rainbow trout were obtained from database and literature sources. The solid pink line (predicted EC50s) and dashed blue line (nominal EC50s) represent the lines of best fit. The equations that best represent the nominal and predicted EC50 correlations are presented on each plot for each cell line.

Discussion

The overall objective of our study was to increase an understanding of the ecotoxicological domains of applicability (i.e., performance against pesticides with varying potencies and mechanisms of action) for cultured rainbow trout cells, while also determining whether the assay performed well technically

in a 96-well format and yielded data of biological relevance (i.e., how do in vitro values compare with those in vivo). This was addressed through a study of diverse pesticides of ecological concern that differed in their class (10 herbicides, eight insecticides, and one fungicide), target receptors and/or mechanisms of action, hydrophobicity ($\log K_{OW}$ from −3.75 to 6.1), volatility ($\log H$ −15.5 to −1.3), and water solubility (from 0.2 mg/L to 1.5×10^6 mg/L). In doing so, we demonstrate that 11 of the 19 pesticides tested were cytotoxic to RTgill-W1 cells, whereas 9 and 8 were cytotoxic to the RTL-W1 and RTgutGC cells, respectively. Select herbicides (metolachlor, S-metolachlor, dimethenamid-P, and diquat), insecticides (chlorpyrifos and carbaryl), and a fungicide (chlorothalonil) were cytotoxic to all three cell lines.

In all cases in which we measured cytotoxicity, EC50 values (based on model-predicted concentrations in the culture media) were within 18-fold of the in vivo LC50 values. In 82% of these cases, the EC50 value was within 10-fold (1 order of magnitude) of the corresponding in vivo LC50 value. This level of concordance is comparable to a past RTgill-W1 cell study of 35 organic chemicals (with diverse physicochemical properties and modes of action) in which approximately 73% of the test chemicals yielded EC50 values that were within fivefold of the in vivo one (Tanneberger et al., 2013); for comparative purposes, approximately 57% of derived EC50s in the present study were within fivefold of in vivo values, although this was 73% for the RTgill-W1 cell results. Reasons for discrepancies between in vitro and in vivo results are detailed below, and may relate to the class of chemical under investigation, mechanisms underpinning bioavailability and toxic action, study design (e.g., 96-well plates with model-predicted concentrations vs. 24-well plates with measured concentrations in Tanneberger et al., 2013), and variability with the in vivo LC50 values, which largely hail from single studies.

Most of the cytotoxic pesticides in the present study were classified as herbicides. All 10 herbicides tested, except for atrazine, metribuzin, and glyphosate, caused cytotoxicity in at least one experiment. Perhaps it is not surprising that atrazine and metribuzin did not cause cytotoxicity in any of the three cell lines because their target mode of action (inhibiting photosystem II in photosynthesis) is a structure not found in animal cells (Anderson et al., 2021; Retzinger & Mallory-Smith, 1997). For glyphosate, some studies have characterized cytotoxicity in fish cells, although at concentrations much higher than in the present study. (The highest concentration tested in our study was 1 mM, which is equivalent to 169.1 mg/L.) For example, in RTL-W1 cells, cytotoxic responses were observed at concentrations above 250 mg/L following exposure to technical-grade glyphosate (Santos et al., 2019). Although we did not find glyphosate to be cytotoxic, its metabolite AMPA caused cytotoxicity in the gill and liver cells at approximately 500 μM.

Of the eight insecticides we studied, chlorpyrifos and carbaryl yielded EC50 values in all cell lines, with permethrin and chlorantraniliprole yielding selective responses. Permethrin disrupts neuronal function in insects and nontarget organisms by interfering with voltage-gated sodium channels (USEPA, 2007; Silver et al., 2014). In the present study, this compound only elicited a response with the membrane integrity endpoint (CFDA-AM) in RTgill-W1 and RTL-W1 cell lines. This is consistent with previous RTgill-W1 cell studies of permethrin in which membrane integrity was a more sensitive endpoint than metabolic activity (metabolic activity of 426.7 μM; Scott et al., 2021; Tanneberger et al., 2013). Divergent responses were also found for two organophosphate insecticides (chlorpyrifos and diazinon) with a common mechanism of toxicity (Harper et al., 2009). In the present study, chlorpyrifos was cytotoxic in all three trout cell lines at concentrations of ≥100 μM, while diazinon was not cytotoxic in any of the cells at concentrations up to 1 mM. Similarly, in a study of human bronchial epithelial (A549) cells and immortalized T-lymphocytes (jurkat) cells, chlorpyrifos was cytotoxic at concentrations of ≥250 μM, but diazinon was not toxic up to 1 mM (Oostingh et al., 2009). The remaining insecticides classified as noncytotoxic in the present study (clothianidin, imidacloprid, and thiamethoxam) are neonicotinoids with high selectivity for insect neural nicotinic acetylcholine receptors (Ihara & Matsuda, 2018). This specificity may not be captured by gill, liver, and gut cell lines. This sentiment is one that is reflected in the OECD test guideline 249 document, in which the gill cell has been described as not being well suited to study neurotoxic chemicals that act through specific ion channels or receptors typically found in the brain (OECD, 2021).

The one fungicide we studied, chlorothalonil, was among the top three most toxic compounds tested, with EC50 values that ranged from approximately 1 to 3 μM across all cell lines. Chlorothalonil has been determined to be acutely toxic to fish in vivo (Anderson et al., 2021; Stebbins & Lin, 2020). It acts as a protectant fungicide that deactivates thiols, in particular glutathione, to prevent fungal spore germination, respiration, and glycolysis (Stebbins & Lin, 2020). Glutathione is a well-conserved antioxidant across phyla, so toxicity in nontarget species is not surprising.

Of the 57 cell line–pesticide combinations studied here, 28 yielded cytotoxic responses (up to test concentrations that were either 100 or 1000 μM). However, this also means that approximately 50% of the cell line–pesticide combinations did not yield a cytotoxicity value even though all the pesticides in our study have lethality data in vivo. Many of these exceptions were discussed previously. The presence of false negatives could be driven by the lack of in vivo biological processes, physicochemical properties of the test compounds, or study design considerations. Of the seven pesticides that caused no cytotoxicity in the three cell lines, five (glyphosate, metribuzin, clothianidin, thiamethoxam, and imidacloprid) had in vivo LC50 values that were above approximately 300 μM or within one-third of the highest test concentration. Perhaps testing higher concentrations in vitro would have yielded cytotoxic outcomes values. Regardless, for assays like the ones studied here to be confidently applied, there needs to be a global understanding of when it may yield accurate and relevant data, and when it may not.

Our results indicate divergent cell-line–specific results for several pesticides. This is in contrast to Schug et al. (2020), who found a nearly 1 to 1 correlation between gill and gut results for 16 fragrance chemicals with similar modes of action. Gill cells represent an important site for toxicant uptake via water, whereas the gut cell has been discussed as a viable model for hydrophobic chemicals that sorb to organic matter and could enter the fish through a dietary route. The pesticides we evaluated have differing modes of toxic action and vast physicochemical properties that could affect cells differently, which could explain the more variable nature of our study versus that of Schug et al. (2020). The relationships among physicochemical properties ($\log K_{AW}$, $\log H$, $\log K_{OW}$) and EC50 values for the pesticides studied varied across the three cell lines, and the data may be repurposed by others to help increase our understanding of chemical domains of applicability for different trout cells.

The present study was motivated by the establishment of OECD test guideline 249 using RTgill-W1 cells to assess the

cytotoxicity of chemicals, along with growing regulatory and scientific interest in NAMs. Assay formalization under the OECD is a rigorous undertaking, and so it is not surprising that this assay is technically sound (Fischer et al., 2019) and yields cytotoxicity data that are comparable to lethality values in vivo (Tanneberger et al., 2013). Although the method has been approved by the OECD, for it to gain widespread implementation more demonstrative case studies, such as the present study, are needed. Building scientific confidence in a NAM must be viewed as an evolving and iterative process, and thus needs to consider several factors, as outlined by van der Zalm et al. (2022). In terms of technical performance, we have demonstrated that the RTgill-W1 cytotoxicity assay performed well in a 96-well microplate format by comparing several quality control measures with expectations outlined in OECD test guideline 249, and comparing 96-well plate cytotoxicity results with results from 24-well plates. Thus our study highlights the potential of this assay for higher throughput applications. We further extended this analysis to also show that technically good measures can also be yielded from the RTL-W1 and RTgutGC cell lines studied in 96-well microplates. Such results with these cell lines are not surprising given that several researchers have previously demonstrated that cytotoxicity assays in these cell lines yield quality and robust data (Dayeh et al., 2005; Mittal et al., 2022; Natsch et al., 2018; Schug et al., 2020).

An ultimate question in the present study was whether the in vitro data could be used to predict in vivo outcomes. When cytotoxicity was measured and considered in terms of nominal concentration in the microplate well, the associations with in vivo adverse outcomes (from databases and the literature) were weak or nonexistent. Ideally, chemical concentrations in the well should be determined, although this it was not possible in the present study due to technical, logistical, and financial barriers further reinforced by the relatively small volumes in 96-well microplates. This remains a major limitation of our study that warrants future attention. Nonetheless, when our in vitro concentration data were modeled using the IV-MBM EQP Ver. 2.1 model (Armitage et al., 2021) to yield predicted values within the cell culture media, the resulting comparisons between the in vitro and in vivo data were considerably strengthened. Other researchers studying rainbow trout cells have highlighted the importance of considering physicochemical properties and modeling approaches when comparing in vitro and in vivo data (Dupraz et al., 2019; Schug et al., 2020; Stadnicka-Michalak et al., 2014; Tanneberger et al., 2013), which is reinforced by our present analysis.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

All data are accessible in the article and its Supporting Information.

Author contributions

Sophie Emberley-Korkmaz: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing—original draft; Writing—review & editing. Krittika Mittal: Investigation; Methodology; Writing—review & editing. Na'im Temlock: Investigation; Methodology; Writing—review & editing. Jessica Head: Funding acquisition; Writing—review & editing. Niladri Basu: Conceptualization; Funding acquisition; Methodology; Project administration; Supervision; Writing—original draft; Writing—review & editing.

Disclaimer

The authors declare no competing financial interests.

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