ECOTOXICOLOGY

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Toxicology across scales: Cell population growth in vitro predicts reduced fish growth

Julita Stadnicka-Michalak,[1,2]* Kristin Schirmer,[1,2,3] Roman Ashauer[4]

Environmental risk assessment of chemicals is essential but often relies on ethically controversial and expensive methods. We show that tests using cell cultures, combined with modeling of toxicological effects, can replace tests with juvenile fish. Hundreds of thousands of fish at this developmental stage are annually used to assess the influence of chemicals on growth. Juveniles are more sensitive than adult fish, and their growth can affect their chances to survive and reproduce. Thus, to reduce the number of fish used for such tests, we propose a method that can quantitatively predict chemical impact on fish growth based on in vitro data. Our model predicts reduced fish growth in two fish species in excellent agreement with measured in vivo data of two pesticides. This promising step toward alternatives to fish toxicity testing is simple, inexpensive, and fast and only requires in vitro data for model calibration.

INTRODUCTION

Every day, about 15,000 new substances are registered with the Chemical Abstracts Service (CAS), and of the ~100 million chemicals thus far registered, very few are being regulated, and even fewer are assessed for their safety (*1*, *2*). Safety assessment of chemicals is a daunting task. Only about 10 high-production volume chemicals (that is, >1000 tons/year) were tested per year in the past, and an ~300-fold increase in throughput is required to comply with the European legislation REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) (*3*). The main goals of assessing the risk of chemicals are to prevent environmental pollution and to ensure safe and sustainable use of chemicals by keeping a balance between the benefits to humanity and dangers of introducing synthetic compounds into Earth's ecosystems. Thus, comprehensive testing and risk assessment are required by environmental regulations before any new man-made chemical can be approved for use. Every year, more than a million fish are used for experimental and other scientific purposes in the European Union (*4*, *5*). In 2011 alone, this number included almost 180,000 fish that were used for regulatory toxicological and other safety evaluations. Between 3 and 6 million fish per year are currently used for whole effluent testing in the United States (*6*). Moreover, at least 400 fish are used per one Fish Early Life Stage (FELS) test (*7*), which is often required by environmental agencies because it covers different developmental stages from fertilization to juvenile stage, at which fish are more sensitive to chemicals than adult fish (*8*). Thus, there is consensus among scientists, regulators, and industry that a paradigm shift in risk assessment is needed because the current approach is too slow and expensive and consumes millions of animals per year, which is ethically questionable and therefore controversial. For these reasons, methods other than in vivo fish toxicity testing are urgently sought to be included in an integrated testing framework.

Here, we propose that the inhibition of cell population growth under chemical stress, measured over the course of a few days, can be taken as proxy for chemical effects on fish growth, which takes weeks to emerge. By cell population growth, we mean the increase of total cell number determined by cell death and cell proliferation over time. The increase of cell number is directly linked to animal size because some animals are bigger than others not because their cells are much bigger but because they have more cells (*9*).

GILL CELLS AS PROXY FOR TOXICITY TO FISH TISSUE

Specifically, we aimed to link information on cell survival and proliferation of a fish gill cell line (in vitro) to the impact of chemical stress on fish growth (in vivo) because cell lines have been repeatedly shown to be a very good predictor for at least fish acute toxicity (*10*, *11*). The well-standardized (*10*, *12*, *13*) and commercially available rainbow trout gill cell line [RTgill-W1; American Type Culture Collection (ATCC), reference: ATCC CRL-2523] used in our study was obtained from rainbow trout (*Oncorhynchus mykiss*) gills (*14*). The premise to prefer a fish cell line over a mammalian cell line was discussed in previous studies (*12*, *15*): not only do piscine cell cultures better reflect the properties of a fish (for example, in terms of genetics and biochemical properties) than a mammalian cell line, but they are, at least in the case of the RTgill-W1 cell line, also much more tolerant to simple culture conditions that better mimic exposure to water. In addition, the RTgill-W1 cell line stems from a normal gill of a healthy rainbow trout, whereas most permanent mammalian cell lines are cancer-derived. Thus, when selecting the cell line, it was assumed that the rainbow trout gill cells exemplify all types of cells in a fish with regard to sensitivity to the chemicals. Although we recognize that this assumption may not apply to all kinds of toxicity in all types of tissue, it is supported by at least two lines of thought: first, our work focuses on two basic cellular responses, survival and proliferation, which can be expected to be broadly similarly affected throughout the fish body; and second, the gill is representative of well-perfused tissues, which are in general more prone to chemical exposure than poorly perfused tissues and among which the kinetics of chemical accumulation is comparable (*16*).

INTERNAL EXPOSURE AND REVERSE DOSING

To predict chemical impact on fish growth based on cell survival and proliferation, we selected two fungicides that can impair fish growth: cyproconazole and propiconazole. Both are characterized by the same mechanism of action, that is, they inhibit fungal sterol biosynthesis,

[1]Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland. [2]School of Architecture, Civil and Environmental Engineering, École Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland. [3]ETH (Eidgenössische Technische Hochschule) Zürich, Institute of Biogeochemistry and Pollutant Dynamics, 8092 Zürich, Switzerland. [4]Environment Department, University of York, Heslington, York YO10 5DD, UK. *Corresponding author. E-mail: julita.stadnicka-michalak@epfl.ch; julita.stadnicka@eawag.ch

but they differ in molecular weights and octanol-water partition coefficients (K_{OW}) (table S1).

To test our hypothesis, we first estimated the internal concentrations of chemicals in fish gills, following the paradigm that it is the internal concentration that gives rise to biologically effective doses (*17*). Calculations were based on a well-established physiologically based toxicokinetic (PBTK) model (*18*–*20*), which simulates a chemical's distribution into various fish tissues and organs, so that the chemical concentrations in fish gills could be predicted on the basis of external exposure concentrations. Assuming that the chemical concentration in fish gills in vivo is the same in each cell of the gills, we then projected which chemical concentration to use in the exposure medium for in vitro experiments to achieve the same internal concentrations (Table 1). For that purpose, we used measurements on chemical distribution in the in vitro exposure system and then applied a previously developed toxicokinetic model for cells (*16*). This one-compartment toxicokinetic model was developed to describe concentrations of organic chemicals in gill cells; however, in this study, we applied it backwards to calculate how much of the chemical had to be added to the culture medium to achieve its target cell internal concentration.

IN VITRO EXPERIMENTS

In vitro experiments were performed to study the impact of cyproconazole and propiconazole on survival and cell proliferation over time. Cell survival was measured under conditions allowing to quantify survival without interference by cell proliferation. However, because this was only possible for up to 72 hours (see Materials and Methods: Cell survival), we applied the General Unified Threshold Model of Survival–Stochastic Death (GUTS-SD) (*21*) on cell populations to predict cell survival under chemical exposure for up to 120 hours, which is the same time needed to detect a change in cell population growth. Indeed, 120 hours was the shortest period that allowed discrimination of differences in cell proliferation caused by different chemical concentrations (see Materials and Methods: Cell proliferation).

At the same time, the lowest chemical concentrations used in experiments for cell survival (1.5 mg/liter for cyproconazole and 2.3 mg/liter for propiconazole) were the highest concentrations used in the cell proliferation experiments, which are then corresponding to the highest concentrations used in the FELS test (Table 1). These concentrations did not cause any effect on cell survival within 120 hours [see Fig. 1 and table S8; confidence intervals (CIs) for these concentrations included 100% cell survival]. In support of these experimental data, the threshold values for the initiation of cell death were 1063 mg/kg wet weight for cyproconazole (corresponding to 16.9 mg/liter in exposure medium) and 713.6 mg/kg wet weight for propiconazole (corresponding to 6.78 mg/liter in exposure medium) according to the GUTS-SD model.

Table 1. Chemical concentrations in water and medium, their corresponding predicted internal concentrations in fish gills (in vivo) and gill cells (in vitro), and their respective effects on fish and fish cell weight.

Concentration in water (mg/liter) in FELS	Concentration in fish gills and gill cells (μmol/g)	Concentration in medium (mg/liter) in cell proliferation experiments	Fish weight* (% of control)	Modeled cell weight† (% of control)
Cyproconazole (log K_{OW} = 2.9; molecular weight, 291.78 g/mol)				
2.4	0.296	1.5	All dead	47.9 (±9.62)
1.2	0.148	0.75	51.2 (±23.1)	53.1 (±11.1)
0.6	0.074	0.375	70.9 (±15.6)	66.4 (±15.9)
0.3	0.037	0.1875	74.3 (±13.4)	74.1 (±13.2)
0.15	0.019	0.09375	78.2 (±12.5)	85.9 (±14.2)
Propiconazole (log K_{OW} = 3.72; molecular weight, 342.22 g/mol)				
1.0	0.403	2.3	23.2 (±13.2)	29.6 (±5.67)
0.5	0.202	1.15	74.1 (±22.3)	60.5 (±8.62)
0.25	0.101	0.575	83.2 (±20.4)	77.5 (±13.0)
0.125	0.050	0.2875	93.9 (±20.7)	87.9 (±11.3)
0.0625	0.025	0.14375	96.1 (±24.6)	93.6 (±11.8)

*Measured in FELS studies at day 62 for cyproconazole (rainbow trout) and at day 31 for propiconazole (fathead minnow).
†Predicted with von Bertalanffy growth model on the basis of in vitro cell proliferation experiments (5-day exposure).

MODELING CHEMICAL IMPACTS ON FISH GROWTH

Next, the von Bertalanffy growth model (*22*) was implemented to predict the inhibition of cell proliferation caused by the chemicals during long-term exposure. In this model, organism growth is presented as body length over time; however, we focused on the increase of fish weight and not length because it was shown that the former is more sensitive to chemical exposure (*23*). The model was also used to interpolate fish weight reduction to other chemical concentrations (Fig. 2; Materials and Methods: Interpolation to other chemical concentrations), so that the inhibition of cell population growth could be compared with the inhibition of fish growth caused by the chemical concentrations in the FELS tests. The comparisons were made for the exposure times used in the FELS tests at the only time points available, which were 31 days for fathead minnow (*Pimephales promelas*) and 62 days for rainbow trout (*O. mykiss*).

The close correspondence between inhibited cell proliferation (in vitro) and reduced fish growth (in vivo) supports our hypothesis that the modeled inhibition of cell population growth under chemical stress can be taken as proxy for chemical effects on fish growth (Table 1 and Fig. 3; for model parameters and equations, see Materials and Methods). The impact of four concentrations of cyproconazole on the growth of rainbow trout (62 days) and five concentrations of propiconazole on the growth of fathead minnow (31 days) were predicted on the basis of in vitro data. The predictions agreed very well with the observed reduction in fish growth, especially when considering the variation in fish growth data (Fig. 3).

APPLICABILITY FOR DIFFERENT FISH AND CHEMICALS

When generalizing these results, one could argue that influences of cell line choice and exposure conditions during cell population growth matter, especially with respect to extrapolating rainbow trout gill cell proliferation to fathead minnow growth. The optimal temperature for growth of the fathead minnow and its cells is 24° to 26°C, whereas rainbow trout cells are cultured at 19°C. A 10°C increase in temperature causes a twofold increase of cell population growth (*24*); thus, to

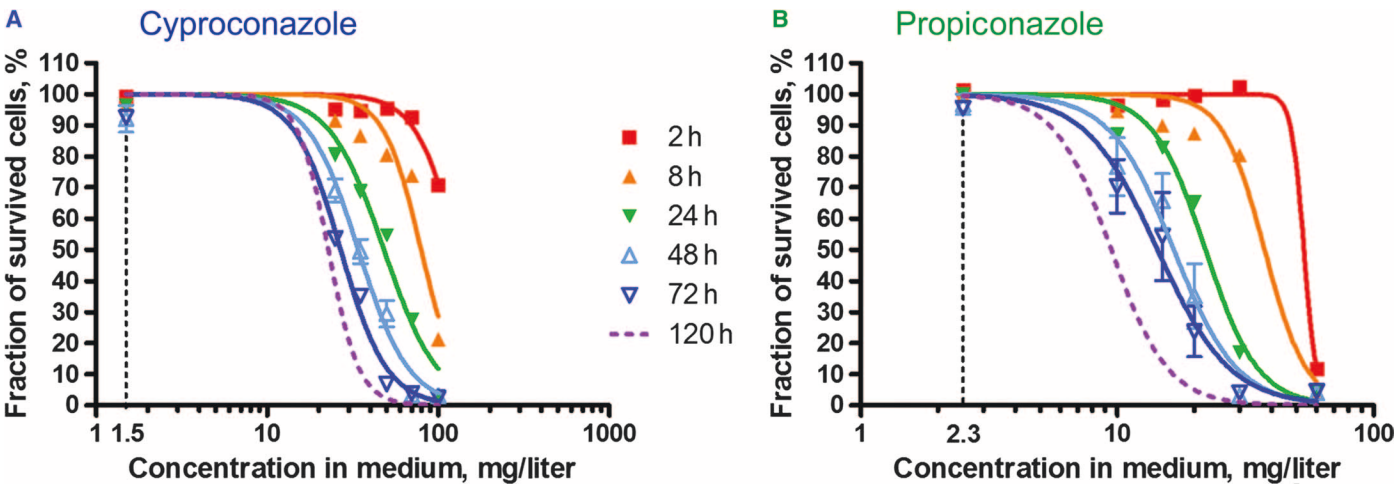

Fig. 1. Time-dependent chemical concentration-response curves. (**A** and **B**) Results for cyproconazole (A) and for propiconazole (B). Symbols, measured values; solid lines, values described by sigmoidal concentration-response curves. Data points for 120-hour exposure (dashed lines) were predicted by the GUTS-SD model, and the sigmoidal concentration-response curve was fitted to these values. CIs are given in table S8.

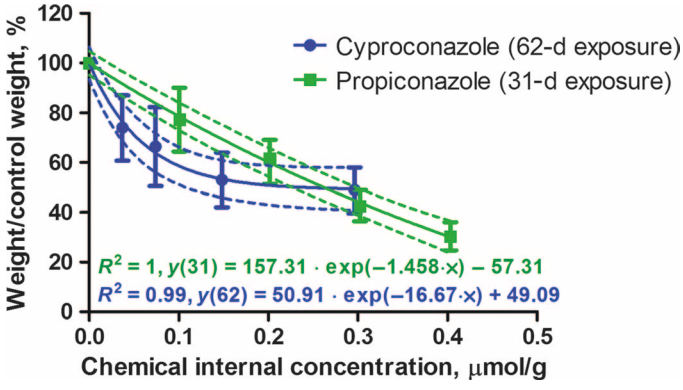

Fig. 2. Interpolation of the predicted fish weight reduction to other chemical concentrations. Symbols represent predictions with model uncertainty (Materials and Methods: Linking cell population growth to whole-organism growth) obtained on the basis of measured in vitro data and the von Bertalanffy growth model, and lines represent fitted model with 95% CIs (Materials and Methods: Interpolation to other chemical concentrations).

account for potential temperature-related differences in cell population growth, we also applied our model for linking cell proliferation to whole-organism growth to twofold longer and twofold shorter doubling times than measured in our study. These calculations should also account for potential differences in cell proliferation caused by different cell characteristics (for example, size) or if, for example, another protein (serum) content would have been applied. In general, a 5 to 10% serum content is optimal for fish cell population growth with differences in population doubling times in the exponential growth phase being clearly less than twofold (*14*, *25*). Results show that the impact of doubling time on the inhibition of cell population growth increased with the increase of chemical concentration. For cyproconazole and propiconazole, the inhibition predicted for the highest respective chemical concentration used in our study changed by ±10 and ±13.5% for twofold varied doubling times.

We have shown that the mechanistic approach presented here can be successfully applied to at least two freshwater fish species differing in size, lipid content, and temperature preferences. It has been tested for two nonvolatile fungicides characterized by K_{OW} values differing by about one order of magnitude. However, the toxicokinetic submodels that we used here have been tested for a wider range of organic compounds. The toxicokinetic submodel for the cultured gill cells has been tested for chemicals with log K_{OW} values between 0.5 and 7 (including cyproconazole and propiconazole) (*16*), and the PBTK model for fish has been tested for chemicals with log K_{OW} values between 1.5 and 7 in our previous study (*20*) and also for other fish species (*26*–*28*). Thus, the suggested approach may also be suitable for a wider range of chemicals. However, the toxicokinetic model for in vitro gill cells was not suitable for volatile compounds because of unreliable measurements of their concentrations (*16*). Thus, for volatile chemicals, we suggest to adapt in vitro experiments by, for example, the use of passive dosing (*29*).

Regarding the chemical mechanism of action, both cyproconazole and propiconazole inhibit fungal sterol biosynthesis, and they may disrupt sex steroids, vitellogenin, and cholesterol synthesis in fish (*30*). The influence of chemicals characterized by this mechanism of action on fish growth was very well predicted by our approach. Moreover, we expect that our model will also be suitable for baseline and many reactive toxicants. However, further work with other chemicals is required to generalize the approach outlined here.

Another biological process that did not have to be considered in this study, but which might be important for other chemicals, is biotransformation. Thus, for compounds that are biotransformed in fish, we recommend further in vitro experiments to measure biotransformation rates in liver and/or other important organs. An interesting approach regarding the estimation of chemical biotransformation in fish based on modeling and measured in vitro biotransformation rates has been proposed by Nichols and colleagues (*31*).

IMPLICATIONS FOR CHEMICAL SAFETY ASSESSMENT

We can quantitatively predict sublethal toxicity at organism level based on in vitro data. The presented in vitro–to–in vivo toxicity extrapolation requires experiments only with a representative cell line. It comprises a very promising step toward alternatives to whole-organism

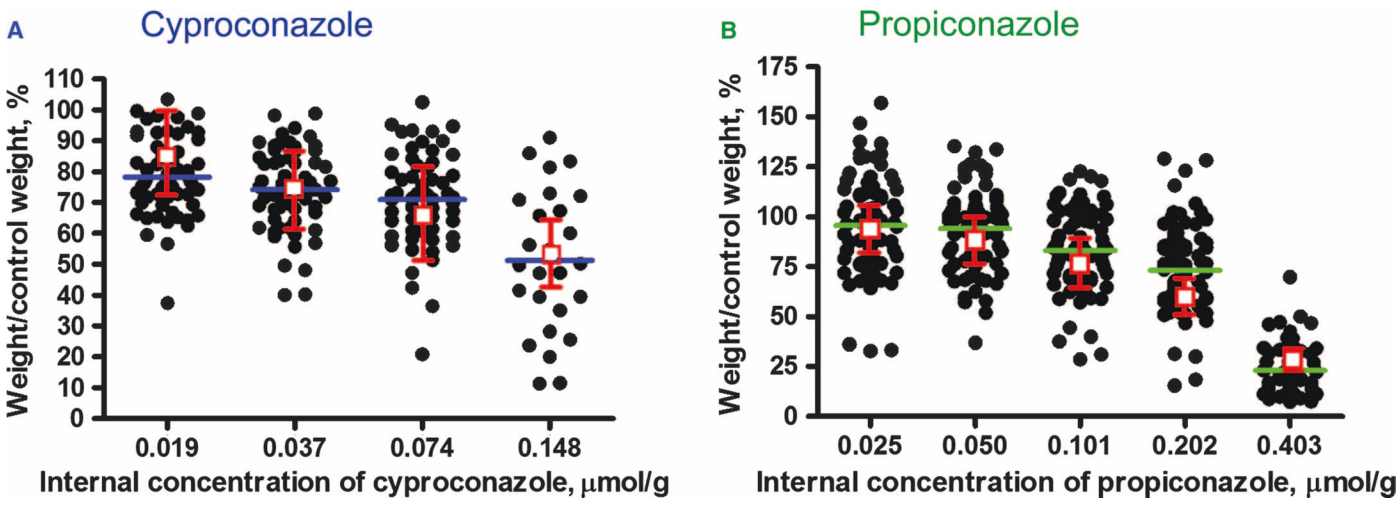

Fig. 3. Measured reduction of fish weight ["•" (each dot represents one fish); mean, "–"] and predictions including model uncertainty (Materials and Methods: Linking cell population growth to whole organism growth) based on in vitro cell population growth data ("▭") for different chemical concentrations. (**A** and **B**) Results for cyproconazole (A) and for propiconazole (B). Weight reduction is presented as percent of weight of control samples after 62-day exposure for cyproconazole and 31-day exposure for propiconazole. We assumed that the total mass of all fish cells is the same as fish weight. For cyproconazole, all tested concentrations caused a significant effect on fish weight, whereas for propiconazole, the three highest concentrations caused significant effect on fish weight [$P < 0.05$, analysis of variance (ANOVA) and Dunnett's multiple comparison test, performed in GraphPad Prism].

toxicity testing, especially taking into consideration the simplicity, rapidity, and low costs of this method. Today, the combination of in vitro experiments and computer modeling cannot yet completely substitute the use of live animals in environmental risk assessment. However, this study shows that despite making several assumptions and simplifications, the combination of in vitro experiments with a fish cell line and a modeling approach could lead to an effective strategy for screening chemicals to predict impacts on fish. Our approach can already not only deliver information required by regulatory agencies, for instance, various effect concentrations (that is, EC_x), but also additional knowledge regarding the influence of chemicals on organisms, such as morphological or metabolic changes in cells. It is also important to note that when envisioning a tiered approach to high-throughput testing, alternative tests, even for just one end point, can be extremely valuable long before the FELS test can potentially be completely replaced (*32*).

The in vitro–to–in vivo toxicity extrapolation demonstrated here is a small but important step because of the concept used. Toxicokinetic models of in vivo and in vitro systems enable informed reverse dosing experiments in vitro. Moreover, the use of the same toxicodynamic model in vitro and in vivo, here the van Bertalanffy model, facilitates the toxicity extrapolation. We hope that our very encouraging results inspire further work on alternatives to animal testing, for instance, with other fish, chemicals, cell culture models, and effect end points. Thus, we expect that our approach will fundamentally advance toxicity testing of chemicals because it demonstrates the predictive power of a model-driven approach—as opposed to the traditional "test first interpret later" risk assessment work flow.

MATERIALS AND METHODS

Test substances

Experiments were carried out with two pesticides: propiconazole (CAS# 60207-90-1) and cyproconazole (CAS# 94361-06-5). A mixture of ^{14}C-labeled and unlabeled chemicals was used. This allowed measuring the chemical concentration in the exposure medium based on the chemical's radioactivity. The unlabeled compounds (propiconazole: chemical purity, 98.4%; cyproconazole: chemical purity, 99.8%) were purchased from Sigma-Aldrich, the labeled propiconazole ([dioxolane-4-^{14}C], 99.8%) was purchased from the Institute of Isotopes Co., Ltd., and the labeled cyproconazole ([triazylol-U-^{14}C]-cyproconazole, 98.9%) was a gift from Syngenta. Stock solutions were prepared using methanol (99.9%, Acros Organics). The final concentration of methanol in the dosed system was ≤ 0.16% (v/v). Physicochemical properties of the test substances (log K_{OW}, log H, molecular weight, and specific radioactivity) are presented in table S1.

Fish growth data

We used fish growth data from the FELS studies for cyproconazole (*33*) and propiconazole (*34*) provided by Syngenta as an example of typical industrial studies for regulatory risk assessment. Fathead minnow (*P. promelas*) embryos and larvae were continuously exposed for 35 days (31 days after hatch) to five propiconazole concentrations (1.0, 0.5, 0.25, 0.125, and 0.0625 mg/liter), whereas rainbow trout (*O. mykiss*) embryos and larvae were continuously exposed for 89 days (62 days after hatch) to five cyproconazole concentrations (2.4, 1.2, 0.6, 0.3, and 0.15 mg/liter). Observations were made on the survival of organisms at hatch, time to swim up (only for rainbow trout), and survival and growth (wet weight and total length) of larvae after 31 days (fathead minnow) or 62 days (rainbow trout) post-hatch exposure. Both these studies were conducted in compliance with good laboratory practices as published by the U.S. Environmental Protection Agency (40 CFR, part 160). In addition, despite the fact that the studies had been performed before the newest Organisation for Economic Co-operation and Development guideline (*7*) was introduced, they fulfilled important requirements stated in this guideline, including water temperature, dissolved oxygen concentration, analytical measurements, and survival of fertilized eggs in the controls.

Derivation of target chemical concentrations

We designed our in vitro experiments such that the concentrations in the RTgill cells should be the same as the concentrations in gills of fish that caused effects in FELS toxicity tests. Target chemical concentrations in the exposure medium were derived from FELS studies with propiconazole and cyproconazole. From these studies, we chose the three highest chemical concentrations in water (propiconazole: 1, 0.5, and 0.25 mg/liter; cyproconazole: 2.4, 1.2, and 0.6 mg/ml) because they caused the greatest effects on fish (see Table 1). In addition, one more concentration of cyproconazole (0.3 mg/liter) and propiconazole (0.75 mg/liter) was chosen to find the correlation between chemical concentration and predicted effects on fish growth. For all chosen aqueous concentrations, we predicted the corresponding chemical concentrations in the gills of rainbow trout (for cyproconazole) and fathead minnows (for propiconazole) using the PBTK model for fish developed by Nichols and colleagues (*18*, *19*, *35*, *36*) and further described and adapted for fathead minnow in our previous study (*20*). We assumed chemical concentrations in gills (in vivo) and gill cells (in vitro) to be the same as in other richly perfused tissues in the PBTK model. Next, we calculated the exposure concentrations in the cell culture medium that would result in the same internal concentration in the RTgill cell line as those in the fish gills during the FELS study. For that, we used the toxicokinetic model for the in vitro cell line system previously developed (*16*). Essentially, we back-calculated the target concentrations in medium for our in vitro cell toxicity tests from predicted internal effect concentrations in FELS studies.

Determination of chemical concentrations in exposure medium

During the experiments where we tested the effect of cyproconazole and propiconazole on cell survival and proliferation, chemical concentrations in exposure medium at each time point were determined by measuring the radioactivity of samples. Each sample, containing 100 µl of exposure medium, was taken from the respective well and added into a vial filled with 10 ml of Ecoscint A scintillation cocktail (Chemie Brunschwig). All samples were shaken and measured using a liquid scintillation counter (Tri-Carb 2200CA, Packard). The results were corrected for the background activity by subtracting the average activity in vials containing 10 ml of Ecoscint A and 100 µl of control exposure medium. Chemical concentrations measured in the exposure medium for each experiment are presented in tables S2 to S7.

Our approach of confirming chemical concentrations based on total radioactivity in the culture medium over time did not account for any potential biotransformation reactions of cyproconazole and propiconazole in the applied gill cell line. Little knowledge thus far exists with regard to the capacity of fish cell lines to biotransform chemicals in general, and no previous study has attempted to study the survival and growth of a fish cell line in the presence of the two pesticides investigated here. Very little data are available on the bioaccumulation and biotransformation of these pesticides in fish. Despite the fact that Konwick *et al.* (*37*) showed that both cyproconazole and propiconazole are biotransformed in rainbow trout, their transformation products might not change the toxicity to fish in comparison to toxicity caused by parental compounds. This is due to the triazole ring, the active group of these pesticides, which is also present in metabolites in fish and mammals (*38*, *39*). The main difference between parental compounds and their biotranformation products was the added hydroxyl groups. These groups would likely make transformation products more soluble in water but would not be expected to change the toxic mechanism. Changes in water solubility may influence chemical bioconcentration in fish or fish cells; however, Syngenta used a flow-through exposure system in the FELS tests that replenishes the parent compound. All these considerations support the use of total ^{14}C concentrations as a proxy for cyproconazole and propiconazole in the in vitro experiments.

Cell line culture

We used the commercially available rainbow trout gill cell line RTgill-W1 (ATCC, reference: ATCC CRL-2523). This cell line was obtained from rainbow trout (*O. mykiss*) gills; its basic characterization, including growth characteristics and karyotype, is presented by Bols *et al.* (*14*). Cells were routinely cultured at 19°C in 75-cm^2 cell culture flasks (TPP) with L15 culture medium (Invitrogen) supplemented with 5% fetal bovine serum (Biochrom) and 1% penicillin-streptomycin solution (10 mg/ml; Bioswisstec AG), which we termed "complete L15." For exposure to test chemicals, confluent cells were washed twice with Versene (Invitrogen), detached with trypsin (0.25% in phosphate-buffered saline without calcium and magnesium; Biowest), and resuspended in the complete L15 (cell splitting during the cell culture: every 2 weeks; ratio, 1:2). Gill cells with different passage numbers (from P-57 to P-98) have been used for the experiments, and mycoplasma tests (using Lonza MycoAlert Mycoplasma Detection Kit) were carried out six times during this study (in all cases, test results showed no bacterial contamination). Cells in the resulting suspension were counted using the electric field multichannel cell counting system (CASY1 TCC, Schärfe System).

The starting cell density for all experiments was 400,000 cells/ml. For experiments to determine the cell survival upon chemical exposures, 1 ml of that suspension was seeded into 24-well culture plates (cell density, ~181,000 cells/cm^2; Huber and Co.). After 24 hours of attachment, the cells were used for testing. For experiments on cell population growth, 5 ml of the cell suspension in the mixture of cell culture medium and chemical solution was seeded into 25-cm^2 cell culture flasks (cell density, ~14,000 cells/cm^2).

Cell survival

The method for determining effect concentrations in the RTgill-W1 cell line was described in detail by Tanneberger *et al.* (*10*). In an identical manner, in our study, chemical stock solutions were prepared by dissolving and serially diluting pesticides in dimethyl sulfoxide (DMSO). The final solvent concentration of DMSO in L15/ex medium (*40*) within the well was 0.5% (v/v). L15/ex is a modified Leibovitz medium and only includes galactose, sodium pyruvate, and salts; thus, in this medium, cells are viable but no longer proliferate, so the cell culture system remains stable. For each experiment, stock solution and dilution series (that is, six different chemical concentrations + control) were freshly prepared. Twenty-four hours after seeding, cells from all well plates were washed with 1 ml of L15/ex before the chemical or control (solvent) in L15/ex was added. For each concentration and control, triplicates ($n = 3$) were dosed on each test plate. The fraction of living cells was quantified 0, 2, 8, 24, 48, and 72 hours after dosing because longer-term exposure was not possible because of the L15/ex exposure medium (*40*). In this modified Leibovitz medium, it is possible to separate effects on cell survival from effects on proliferation. However, we noticed a decline in metabolic activity of cells in L15/ex medium in controls after 72 hours, likely due to depletion of cell internal nutrients and energy reserves (*41*). One well plate was used for each time point

and contained wells with cells and six different chemical concentrations or the control. Each experiment was carried out twice ($N = 2$) with cells from different passage numbers.

For each time point, cell survival was quantified by measuring fluorescence of the dye alamarBlue (Invitrogen), which is a measure for cellular metabolic activity (*42*). Measurements were made on the Infinite M20 microplate reader (Tecan; excitation, 530 nm; emission, 595 nm). Fluorescence readings from cell viability assays were presented relative to the solvent control ("% of solvent control"), where the solvent control was set to 100% cell survival. Concentrations leading to 50% reduction in cell viability (EC_{50}) were determined by fitting the sigmoidal concentration-response curve (Hill slope equation and its parameters for all concentration-response curves are available in eq. S1 and table S8) using nonlinear regression in GraphPad Prism (GraphPad Software).

Cell proliferation

Unlike experiments on cell survival, experiments on cell proliferation were carried out in complete L15 medium because this allows cells to proliferate. Chemical concentrations for experiments on cell population growth were initially chosen on the basis of chemical effective concentrations for fish (see section "Derivation of target chemical concentrations" above), and our cell survival experiments confirmed that these concentrations were not lethal for cells (see results for cell survival experiments and Fig. 1).

Experiments were carried out in 25-cm^2 cell culture flasks. Experiments lasted 120 hours, which we determined as the shortest possible time for cells to be well in the exponential growth phase and allow determination of population doubling times. Cell number was determined daily by using the electric field multichannel cell counting system. For each chemical concentration and control, triplicates ($n = 3$) were dosed and measured. In addition, experiments with the highest chemical concentrations were repeated ($N = 2$) with cells from different passage numbers, which gave essentially similar results.

Modeling cell survival

The fraction of surviving cells was modeled using internal concentrations of the chemicals as exposure variable and scaled damage as dose metric in the GUTS (*21*). Internal concentrations were calculated by a model for toxicokinetics in cells (*16*). The GUTS model takes into consideration two different approaches: one based on the assumption of "individual tolerance" (GUTS-IT) and one based on the assumption of "stochastic death" (GUTS-SD). According to the first approach, each individual has its own sensitivity threshold (that is, it immediately dies after reaching a certain internal damage), whereas in the stochastic death assumption, individuals are identical and mortality is treated as a stochastic process at the level of the individual (*21*). The model based on the assumption of stochastic death (GUTS-SD) was used in our study because this is more appropriated for cultured cells.

As the mortality of cells for each time point was independently determined (that is, it did not depend on the cells' mortality measured for a previous time point) and the numbers of cells were very large, least-squares calibration was carried out for the GUTS-SD model. Parameters were fitted to measured survival fractions by minimizing the sum of squares between measured and modeled values using the Levenberg-Marquardt algorithm. Parameter estimates are provided in table S9.

The model was implemented and solved using ModelMaker (version 4.0, Cherwell Scientific Ltd.). Details about model equations, implementation, and calibration are presented in the Supplementary Materials.

Linking cell population growth to whole-organism growth

To link cell population growth to fish growth in both the absence and presence of chemical exposure, the cell number in each flask was converted to the cell mass based on average measured cell diameter (15 μm) and cell density (set to 1 kg/liter). The increase of the cells' weight in time was modeled on the basis of the von Bertalanffy growth model (*22*) because this approach could be applied for both the cell and organism scale. According to the von Bertalanffy model, growth can be described with the following equation:

$$L(t) = L_\infty \cdot (1 - e^{-K \cdot (t - t_0)}) \quad (1)$$

where $L(t)$ is length (mm) at time t (day), L_∞ is the theoretical maximum length (asymptotic, mm), K is a growth coefficient (1/day), and t_0 is the theoretical age (day) at $L = 0$.

On the basis of the assumption that a cell has a spherical shape, its weight is proportional to the cube of the length. Thus, the cells' growth expressed by the increase in their weight, and the ratio between the mass of cells (or fish weight) exposed to a certain chemical concentration and the mass of cells (or fish weight) in the control sample, can be described by the following equations (*43*):

$$W(t) = W_\infty \cdot (1 - e^{-K \cdot (t - t_0)})^3 \quad (2)$$

$$\frac{W_{\text{concentration}}(t)}{W_{\text{control}}(t)} = \frac{W_\infty \cdot (1 - e^{-K_{\text{concentration}} \cdot (t - t_{0_{\text{concentration}}})})^3}{W_\infty \cdot (1 - e^{-K_{\text{control}} \cdot (t - t_{0_{\text{control}}})})^3} \quad (3)$$

where $W(t)$ is weight (mg) at time t (day), W_∞ is the theoretical maximum weight (asymptotic, mg), K is a growth coefficient (1/day), and t_0 is the theoretical age (day) at $W = 0$.

The advantage of this approach is that only one parameter (K) has to be fitted to the measured data if we know the organism's hatching time (or cell population dynamics) and the theoretical maximum organism length or weight (these data can be taken from literature). On the other hand, it was shown that the von Bertalanffy equation might not be the most suitable model to estimate growth of juvenile organisms (*44*). For this reason, we fitted this model to the measured data to test how well it describes the growth of rainbow trout and fathead minnow and of the RTgill cell population.

In theory, cell populations in in vitro studies with continuous cell lines can grow indefinitely; however, because we aimed to model and compare the impact of chemicals on cell population growth with that on fish, we assumed the asymptotic weight of cells to be the same as for fish in the von Bertalanffy model. We focused on the increase of fish weight and not length because it was shown that fish weight as an end point is more sensitive than length (*23*). In addition, the impact on cell population growth by chemical concentrations also lower than those measured in our study was modeled. To do so, three additional chemical concentrations (one for cyproconazole and two for propiconazole), taken from the FELS studies, were taken into account (see section "Interpolation to other chemical concentrations" below). The von Bertalanffy model was run for each concentration for 31 days (for propiconazole, fathead minnow) or for 62 days (for cyproconazole, rainbow trout) as the impact of chemicals on fish growth was measured for these time points in the FELS study. Finally, the modeled inhibition of cell population growth caused by each chemical concentration was compared with the measured inhibition of fish growth. The rationale behind our comparison

was that the concentrations in the cells, whether in vitro or in vivo, cause the toxic effect. Therefore, our comparison was made for the same internal concentrations, calculated with the toxicokinetic model for cells and the PBTK model for fish, rather than concentrations in the test medium.

Model uncertainty was calculated in "R" (*45*) on the basis of frequentist inference and by using the following packages: deSolve (*46*), lpridge (*47*), and lokern (*48*). The results are presented in Figs. 2 and 3 and figs. S2 and S3 as model 90% CIs.

Interpolation to other chemical concentrations

The impact of chemical concentrations on fish growth over time was simulated on the basis of the von Bertalanffy model. However, the reduction in fish growth for different chemical concentrations after a certain exposure time (for example, in this study, 62 days for cyproconazole and 31 days for propiconazole) is well described with exponential decay curves and calculated on the basis of the following equation:

$$y(t,x) = (y0 - \text{Plateau}) \cdot \exp(-A \cdot x) + \text{Plateau} \quad (4)$$

where $y(t,x)$ is the fraction of control fish weight (%) at time t (day) and for internal chemical concentration x (μmol/g), $y0 = 100$ is the fraction of control fish weight (%) for concentration 0 (μmol/g), and A and Plateau are parameters fitted on the basis of in vitro data (Fig. 2).

The same equation could be used to extrapolate model predictions to other chemical concentrations also for different exposure times.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/1/7/e1500302/DC1

Table S1. Physicochemical properties of cyproconazole and propiconazole.

Table S2. Measured concentrations of cyproconazole in exposure medium during the cell survival experiment (±SD): experiment 1, three technical replicates.

Table S3. Measured concentrations of cyproconazole in exposure medium during the cell survival experiment (±SD): experiment 2, three technical replicates.

Table S4. Measured concentrations of propiconazole in exposure medium during the cell survival experiment (±SD): experiment 1, three technical replicates.

Table S5. Measured concentrations of propiconazole in exposure medium during the cell survival experiment (±SD): experiment 2, three technical replicates.

Table S6. Measured concentrations of cyproconazole in exposure medium during cell proliferation experiments (0±SD) with three technical replicates per experiment.

Table S7. Measured concentrations of propiconazole in exposure medium during cell proliferation experiments (±SD) with three technical replicates per experiment.

Table S8. Parameters of the Hill slope equations for different time points (*49*).

Table S9. Estimates of GUTS-SD parameters for cell survival (in vitro) (lower/upper 95% confidence limit) (*21*, *50*).

Table S10. Estimated parameters of the von Bertalanffy growth model.

Fig. S1. Survival fraction of RTgill-W1 cells exposed to a range of concentrations of the two pesticides over time.

Fig. S2. Proliferation of RTgill-W1 cells exposed to different cyproconazole concentrations (left column) and predicted reduction of fish weight caused by the respective concentration (right column: dashed lines represent the model uncertainty).

Fig. S3. Proliferation of RTgill-W1 cells exposed to different propiconazole concentrations (left column) and predicted reduction of fish weight caused by the respective concentration (right column: dashed lines represent the model uncertainty).

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Julita Stadnicka-Michalak, Kristin Schirmer and Roman Ashauer

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