Research Article

A Validated Algorithm for Selecting Non-Toxic Chemical Concentrations

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Summary

The maximal chemical concentration that causes an acceptably small or no effect in an organism or isolated cells is an often-sought-after value in toxicology. Existing approaches to derive this value have raised several concerns; thus, it is often chosen case-by-case based on personal experience. To overcome this ambiguity, we propose an approach for choosing the non-toxic concentration (NtC) of a chemical in a rational, tractable way. We developed an algorithm that identifies the highest chemical concentration that causes no more than 10% effect ($\leq$ EC10) including the modeled 95% confidence intervals and considering each of the measured biological replicates; and whose toxicity is not significantly different from no effect. The developed algorithm was validated in two steps: by comparing its results with measured and modeled data for 91 dose-response experiments with fish cell lines and/or zebrafish embryos; and by measuring actual effects caused by NtCs in a separate set of experiments using a fish cell line and zebrafish embryos. The algorithm provided an NtC that is more protective than NOEC (no-observed-effect-concentration), NEC (modeled no-effect concentration), EC10 and BMD (benchmark dose). Despite focusing on small-scale bioassays here, this study indicates that the NtC algorithm could be used in various systems. Its application to the survival of zebrafish embryos and to metabolic activity in cell lines showed that NtCs can be applied to different effect measurements, time points, and levels of biological organization. The algorithm is available as Matlab and R source code, and as a free, user-friendly online application.

Keywords: fish cell lines and embryos, *in vitro*, bioassays, toxicology, animal testing alternatives

1 Introduction

1.1 Non-toxic concentrations in ecotoxicology

Selecting the maximum chemical concentration that causes an acceptably small or no toxic effect in a test object, such as an organism or isolated cells, is often required or recommended for environmental toxicology purposes (OECD, 2006; US EPA, 1991; Shao, 2000). Currently, two basic approaches are commonly used, although there is no agreement in the scientific community on which of these approaches, if either, is appropriate to summarize toxicity (Green et al., 2013). The no observed effect concentration (NOEC) is statistically determined as the highest tested concentration that did not cause an effect significantly different from the control (OECD, 2006), whereas the effective concentration (ECx) is the regression-based concentration at which there is an x% effect for the measured endpoint. The NOEC has been criticized in several studies (Landis and Chapman, 2011; Laskowski, 1995; Van Der Hoeven, 1997), e.g., due to its inac-

curacy, dependence on tested concentrations and lack of confidence intervals. A common disadvantage of ECx is that it is often not clear which effective concentration (EC1, EC10, EC20…) to use (Green et al., 2013; Murado and Prieto, 2013). The same concern is often raised regarding the benchmark dose (BMD) method (Crump, 1984), which requires setting of a benchmark risk (BMR) in order to estimate the lower confidence limit of the benchmark dose (BMDL) (Crump, 1995). Thus, in order to avoid having to pre-set a concrete ECx or BMR, several threshold models have been introduced (Cox, 1987; Kooijman, 1996; Kooijman and Bedaux, 1996; Pires et al., 2002) that can be used to determine the so called "no effect concentration" (NEC), i.e., the highest modeled parametric concentration that does not cause an effect. These models may, however, not fit the measured data very well and they are often criticized due to their marginalization of hormesis (Calabrese, 2007, 2009). Finally, Jager (2011) argued that, because toxicity is the response of a dynamic biological system, a simplified representation of reality should be created, e.g.,

by applying mechanistic toxicokinetic-toxicodynamic (TK-TD) models (Ashauer et al., 2011; Jager, 2011). Yet, this approach requires time-resolved experiments and is much more complex than regression-based, and most NEC, models.

1.2 Non-toxic concentrations in *in vitro* systems

While these different approaches have mostly been discussed with regard to organism responses in the context of ecotoxicological risk assessment, the issue of deriving a no effect concentration applies in principle to all test objects studied in toxicology, including small scale assays, such as with isolated cells (*in vitro*), aiming to reduce or replace the use of animals in toxicology. A common problem in such assays is to identify a chemical concentration that allows measuring the toxicokinetics or molecular responses in cells whose plasma membrane and general biochemical machinery are still intact. For instance, it would be difficult to study the uptake or protein and gene expression of chemicals that cause death of 20% of the tested cells. Moreover, in dynamic live-cell assays, such as with alamarBlue® (resazurin dye), used to determine cell metabolic activity (Schirmer et al., 1997), the variability in the control samples depends not only on the activity of the cells *per se* but also on technical aspects, like the exact timing of the measurements. For this reason, using measures like NOEC might be ill-advised. Thus, if such a non-toxic concentration can be rationally derived, it will help to improve reproducibility and provide greater certainty to experimental design, both factors which not only make the application of *in vitro* systems more efficient but can also foster the reduction or replacement of testing on animals.

1.3 Problem formulation and study overview

All above concerns regarding different approaches for the determination of the non-toxic concentration result in a need for a robust algorithm that would allow choosing a chemical concentration that could be safely used in downstream experiments. Instead, given the lack of a unified and broadly accepted method or framework to determine concentrations causing no effect, scientists generally choose this concentration case by case. The selection is often based on the modeled dose-response curve, quality of the measured data and the aim (e.g., to be above the limit of quantification (LOQ) available for chemical analysis), but mostly on personal experience. To overcome this ambiguity, we here propose an algorithm for identifying the highest non-toxic concentration (NtC) of a chemical in a rational, tractable way. The idea is to select the highest chemical concentration that meets the following criteria: i) it is not more toxic than EC10 (including its confidence intervals; EC10 was chosen based on the OECD guideline (OECD, 2006)), ii) its toxicity is not significantly different from the control and iii) it is not higher than the tested concentration that caused at least 10% toxicity in any of the biological replicates. In this way, we use the advantages of already existing approaches by including both measured and modeled data.

The developed algorithm was then validated in two steps: i) by comparing its results with measured and modeled data for 91 dose-response experiments obtained on chemical exposure of fish cell lines and/or zebrafish embryos; and ii) by measuring actual effects caused by NtCs in a separate set of experiments using a fish cell line and again zebrafish embryos. Moreover, we compared NtCs with the NOEC values for a sub-set of the dose-response data and with the modeled NECs for all dose-response curves. The algorithm is available as scripts in different programming languages, as well as in a free, user-friendly online application. Given its applicability to dose-response data of various origins, we envision its broad use in toxicology but also specifically in support of the development of alternatives to animal research. For one, a robust approach for the determination of non-toxic concentrations can facilitate *in vitro* experiments that are developed to be used as alternatives to animal testing. In addition, the proposed algorithm can potentially reduce the number of animal experiments needed, for instance, for toxicokinetic purposes (e.g., to determine chemical bioconcentration and biotransformation). In such tests, it is often required to identify and apply non-toxic concentrations as using a toxic concentration may influence toxicokinetic processes. A wrongly determined non-toxic concentration can result in a need for the experiment to be repeated and in unnecessary harm to animals.

2 Materials and methods

NtC algorithm assumptions
The algorithm for the determination of the highest chemical concentration that is not yet toxic (NtC) was developed based on the following assumptions: i) a dose-response curve can be fitted to measured data and confidence intervals can be determined, ii) NtC does not cause an effect significantly different from the control ("NtC$_{upperCI}$"), iii) the effect of NtC (including confidence intervals) does not exceed EC10 ("NtC$_{lowerCI}$"), iv) tested concentrations lower than NtC do not cause effects greater than 10% ("NtC$_{measured}$"). In addition, an algorithm correction was implemented for very narrow confidence intervals for which even very low effects would be significantly different from the control.

NtC algorithm development and implementation
The algorithm for NtC was implemented in Matlab (Matlab with Statistics and Optimization Toolboxes, Release 2015b, The MathWorks, Inc., Natick, Massachusetts, United States), as well as in R[1] in which also a GUI web application has been created. This app is available online for free[2]. The scripts are available in the supporting information[3] as StadnickaNtC.m and StadnickaNtC.R for Matlab and R, respectively (DataExample.csv is an example of prepared dose-response data).

Dose-response data are used as the algorithm input. They are loaded as a matrix in which the first column represents in-

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creasing chemical concentrations and all other columns are the biological replicates of the measured respective effects (in %) on testing objects. The regression-based dose-response curve (in our case Equation 1, chosen based on previous studies with fish cells and embryos (Knöbel et al., 2012; Tanneberger et al., 2013; Yue et al., 2015)) is fitted to the data by applying the Matlab "fit" function using the non-linear least-squares method.

$$\text{Endpoint} = \frac{100}{1+10^{(logEC50-logconc)\cdot slope}}, \quad \% \qquad \text{(Eq. 1)}$$

where: Endpoint (between 0 and 100%) is the effect endpoint (survival, metabolic activity, etc.) of a certain chemical concentration (conc), logEC50 and slope are the fitted parameters that represent log10-normalized EC50 and the slope of the curve, respectively, and logconc is the log10-normalized chemical concentration.

The fitted curve is plotted together with 95% confidence intervals (95%CI, details for their estimation are provided in the statistical analysis section) and measured data. Then, the following parameters are determined: $NtC_{lowerCI}$, $NtC_{upperCI}$ and $NtC_{measured}$. $NtC_{lowerCI}$ is the highest fitted chemical concentration that is lower than the concentration at the intersection of the lower confidence interval and the 10% effect. $NtC_{upperCI}$ is the highest fitted chemical concentration that is lower than the concentration at the intersection of the upper confidence interval and the 0% effect. $NtC_{measured}$ is the lowest tested chemical concentration that caused at least 10% effect. Due to the simplicity of this approach, it is possible that the fitted 95%CI are so narrow that already very
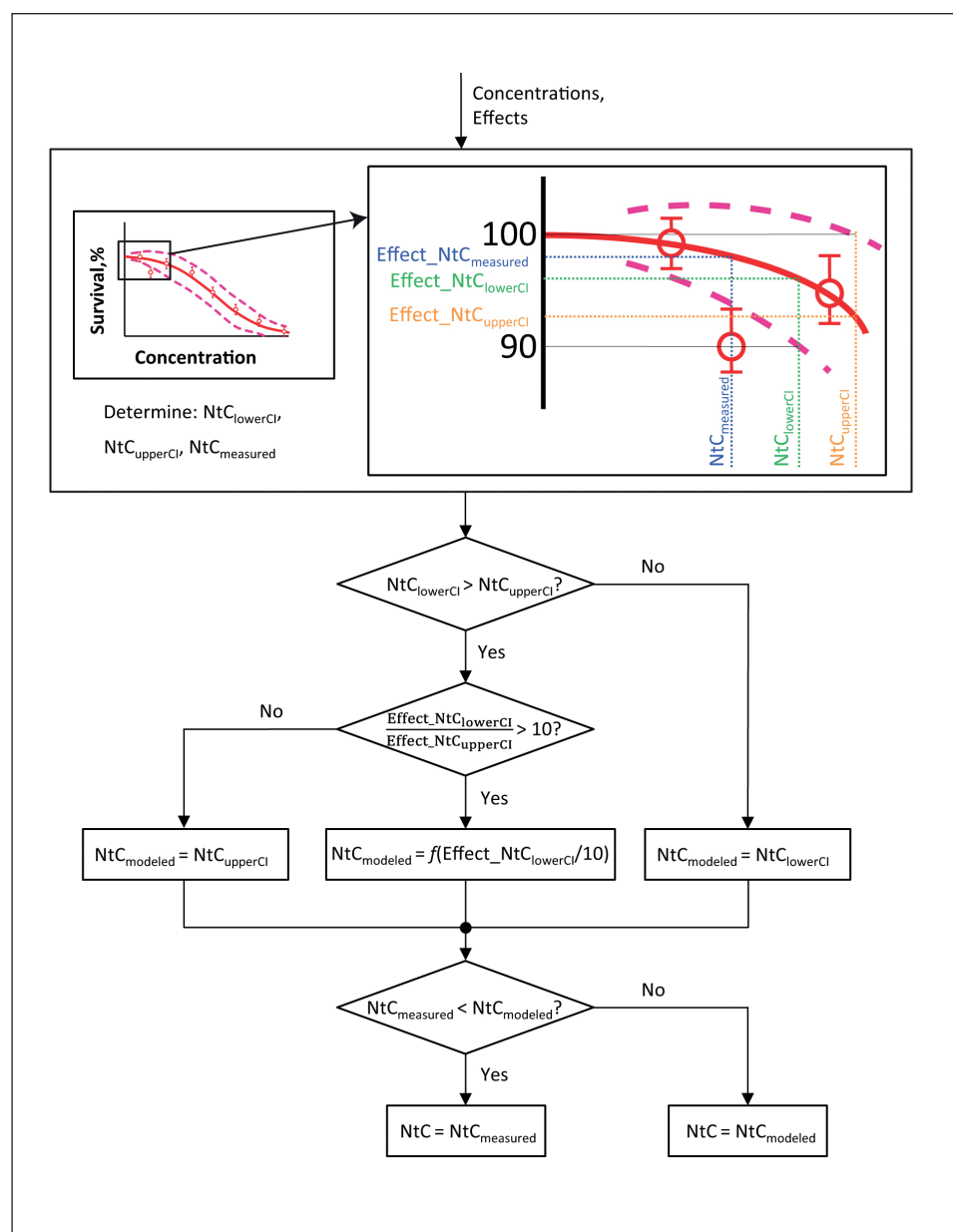

Fig. 1: Overview of the algorithm for an example survival effect endpoint
"f" indicates the function that is based on the transformation of Equation 1.

low chemical concentrations appear significantly different from the control ($NtC_{upperCI}$, see examples in Fig. S1A-B[4]). To avoid this over-protectiveness, an algorithm correction was applied in a way that, if the estimated effect caused by $NtC_{lowerCI}$ is at least one order of magnitude larger than the effect caused by the $NtC_{upperCI}$, the effect of the chosen modeled NtC is set to the effect of $NtC_{lowerCI}$ divided by 10. Then the actual modeled NtC is calculated based on this effect using Equation 1. One order of magnitude limit has been chosen because it assures that such corrected NtC cannot cause an effect larger than EC1, thus still being very protective. Finally, the NtC is determined as the lowest of $NtC_{lowerCI}$, $NtC_{upperCI}$ (including the algorithm correction) and $NtC_{measured}$. The algorithm overview is presented in Figure 1.

Algorithm validation
The algorithm was validated in two steps. First, NtCs were determined for 91 dose-response experiments and compared with the measured and modeled data. These experiments were carried out with fish cell lines and zebrafish embryos in our laboratories in the past. In the second step, the determined NtCs of a subset of chemicals were applied anew to one cell line (RTgill-W1, twelve chemicals) and to zebrafish embryos (five chemicals, one the same as with RTgill-W1) in order to test if their measured effects also fulfill the algorithm's criteria. A detailed description of the experimental procedures used for both steps of the algorithm validation is presented in the supplementary file[4].

First-step validation data
The algorithm was tested based on 91 dose-response experiments stemming from 61 inorganic and organic chemicals and their impact on cell viability in three rainbow trout (*Oncorhynchus mykiss*) cell lines: a gill cell line – RTgill-W1 (Bols et al., 1994), an intestinal cell line – RTgut-GC (Kawano et al., 2011) and a liver cell line – RTL-W1 (Lee et al., 1993) and/or on survival of zebrafish (*Danio rerio*) embryos. All experiments with fish cell lines presented in this study were essentially performed as described previously (Tanneberger et al., 2013; Schirmer et al., 1997), but different well plates, cell number and medium volumes were used in some cases (see detailed experimental set-ups and procedure in Tab. S1[4]). Cell viability was quantified in a live cell, dynamic bioassay by measuring fluorescence of the dye alamarBlue® (AB, Invitrogen, Basel, Switzerland), which indicates cellular metabolic activity (O'Brien et al., 2000). Measurements were made on the Infinite M200 microplate reader (TECAN, Männedorf, Switzerland; excitation: 530 nm, emission: 595 nm). Fluorescent readings from cell viability assays were presented as relative to the solvent control ("% of solvent control"), where the solvent control was set to 100% cell survival. Experiments with zebrafish embryos were performed as described in Knöbel et al. (2012) and in agreement with the ISO 15088 (ISO, 2007). Survival of embryos was expressed as the % of alive embryos compared to the starting point.

An overview of the chemicals and testing objects is presented in Table 1.

Tab 1: Testing objects and chemicals used for the first-step model validation

No.	Chemical	Acronym	CAS	Testing object	Reference
1	silver nanoparticles	Ag-NP	7440-22-4	RTgill-W1, RTgut-GC	Yue et al., 2015; Internal data
2	silver nitrate	AgNO^3	7761-88-8	RTgill-W1, RTgut-GC	Yue et al., 2015; Internal data
3	PVP coated silver nanoparticles	PVP-Ag	7440-22-4	RTgut-GC	Internal data
4	gold nanoparticles	AuNP	7440-57-5	RTgut-GC	Internal data
5	sodium tetrachloroaurate	NaAuCl^4	15189-51-2	RTgut-GC	Internal data
6	CuO nanoparticles	CuO-NP	1317-38-0	RTgut-GC	Internal data
7	ZnO nanoparticles	ZnO-NP	1314-13-2	RTgut-GC	Internal data
8	2,4-di-tert-butylphenol	DTBP	96-76-4	RTgut-GC	Internal data
9	4-nonylphenol	NPH	104-40-5	RTgut-GC	Internal data
10	ibuprofen	IBP	15687-27-1	RTgut-GC	Internal data
11	diclofenac	DCF	15307-86-5	RTgut-GC, RTL-W1	Internal data; Internal data
12	propanolol	PPL	525-66-6	RTgut-GC, RTL-W1	Internal data; Internal data
13	pentachlorophenol	PCP	87-86-5	RTL-W1, RTgill-W1	Internal data; Tanneberger et al., 2013
14	Acid Blue 62	AcB	4368-56-3	RTgill-W1	Internal data

No.	Chemical	Acronym	CAS	Testing object	Reference
15	Reactive Blue 19	ReB	2580-78-1	RTgill-W1	Internal data
16	Acid Black 210	AcBk	99576-15-5	RTgill-W1	Internal data
17	Direct Blue 71	DiB	4399-55-7	RTgill-W1	Internal data
18	difenoconazole	DFZ	119446-68-3	RTgill-W1	Internal data
19	2-mercaptobenzothiazole	MBT	149-30-4	RTgill-W1	Internal data
20	topramezone	TPZ	210631-68-8	RTgill-W1	Internal data
21	cyproconazole	CPZ	94361-06-5	RTgill-W1	Stadnicka-Michalak et al., 2015
22	propiconazole	PPZ	60207-90-1	RTgill-W1	Stadnicka-Michalak et al., 2015
23	ethanol	EtOH	64-17-5	RTgill-W1	Tanneberger et al., 2013
24	4-decylaniline	4DA	37529-30-9	RTgill-W1	Tanneberger et al., 2013
25	aniline	ANI	62-53-3	RTgill-W1	Tanneberger et al., 2013
26	4-chlorophenol	4CP	106-48-9	RTgill-W1	Tanneberger et al., 2013
27	N-methylaniline	NMA	100-61-8	RTgill-W1	Tanneberger et al., 2013
28	acrolein	ACR	107-02-8	RTgill-W1	Tanneberger et al., 2013
29	2,4-dinitrophenol	DNP	51-28-5	RTgill-W1	Tanneberger et al., 2013
30	permethrin	PER	52645-53-1	RTgill-W1	Tanneberger et al., 2013
31	lindane	LIN	58-89-9	RTgill-W1	Tanneberger et al., 2013
32	2-aminoethanol	2AE	141-43-5	RTgill-W1	Tanneberger et al., 2013
33	dibutyl maleate	DBM	105-76-0	RTgill-W1	Internal data
34	6-methyl-5-hepten-2-one	MHO	110-93-0	RTgill-W1	Internal data
35	bisphenol-A	BPA	80-05-7	RTgill-W1	Internal data
36	2,2,2-trichloroethanol	TCE	115-20-8	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
37	diethylphthalate	DEP	84-66-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al, 2012
38	di-n-butylphthalate	DBP	84-74-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
39	naphthalene	NAP	91-20-3	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
40	1,2-dichlorobenzene	DCB	95-50-1	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
41	dichloromethane	DCM	75-09-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
42	tetrachloroethylene	TeCE	127-18-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
43	1,2,4-trichlorobenzene	TCB	120-82-1	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
44	2,4,6-trichlorphenol	TCP	88-06-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
45	3,4-dichloroaniline	DCA	95-76-1	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
46	allyl alcohol	AAL	107-18-6	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012

No.	Chemical	Acronym	CAS	Testing object	Reference
47	ethanal	ETH	75-07-0	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
48	menadione	MED	58-27-5	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
49	2,3-dimethyl-1,3-butadiene	DMBD	513-81-5	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
50	dichlorophene	DCP	97-23-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
51	4-fluoroaniline	4FA	371-40-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
52	hexachlorophene	HCP	70-30-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
53	malathion	MAL	121-75-5	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
54	disulfoton	DIS	298-04-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
55	rotenone	ROT	83-79-4	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
56	caffeine	CAF	58-08-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
57	parathion ethyl	PAR	56-38-2	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
58	hexamethylenetetramine	HMT	100-97-0	RTgill-W1, zebrafish embryos	Tanneberger et al., 2013; Knöbel et al., 2012
59	azinphosmethyl	APM	86-50-0	zebrafish embryos	Knöbel et al., 2012
60	4-nitrolphenol	4NP	100-02-7	zebrafish embryos	Knöbel et al., 2012
61	sodium dodecyl sulfate	SDS	151-21-3	RTgill-W1, RTgut-GC, zebrafish embryos	Tanneberger et al., 2013; internal data (Knöbel et al., 2012)

Data from Knöbel et al. (2012), Tanneberger et al. (2013) and Yue et al. (2015) are based on the measured concentrations at the beginning and the end of experiments while for all other chemicals nominal concentrations (measured data not available) were used.

Second-step validation data

The effects on survival of the determined NtCs were measured for the following twelve chemicals with the RTgill-W1 cell line according to the test procedures established by Tanneberger et al. (2013): DFZ, TPZ, DEP, PAR, TCP, AAL, MED, DNP, 2AE, SDS, DBP and ANI (Tab. 1). Moreover, the following five chemicals were tested in triplicates for impact on zebrafish embryo survival according to Knöbel et al. (2012) but with 20 (instead of 10) eggs per chemical concentration as recommended in the OECD TG 236 of 2013 (OECD, 2013): TCP, 4NP, DCA, DIS and HCP. Chemicals were selected from the available compound list assuring that their NtCs were chosen based on different algorithm criteria (see Tab. 2).

NtC algorithm false positive rate

In order to further analyze the algorithm, the NtC's false positive rate was investigated. First, NtCs were determined for all 91 dose-response curves used in the first-step of the algorithm validation. Then, resampling of the data within each chemical concentration and for all curves was performed (10,000 resamplings per curve) and new dose-response curves were created for the resampled data. Finally, each NtC from the original evaluation of the algorithm (i.e., the first step) was compared with each resampled dose-response curve in order to check if it still fitted the non-toxic class set by the algorithm criteria. The false positive rate was determined by dividing the number of events in which NtC would be more toxic than 10% by the total number of resamplings.

Tab. 2: NtCs of chemicals selected for the second step of the algorithm validation and their estimated effects

Chemical	NtC equal to	NtC (mg/l)	Effect_NtC (%)	Testing object
DNP	$NtC_{upperCI}$	0.071	2.16	RTgill-W1
SDS	$NtC_{upperCI}$	0.159	3.66	RTgill-W1
DBP	$NtC_{upperCI}$	15.38	2.84	RTgill-W1
DFZ	$NtC_{upperCI}$	0.165	0.91	RTgill-W1
TPZ	$NtC_{upperCI}$	9.204	0.82	RTgill-W1
AAL	$NtC_{upperCI}$	171.0	2.41	RTgill-W1
TCP	$NtC_{upperCI\_corrected}$	2.063	0.91	zebrafish embryos
TCP	$NtC_{lowerCI}$	0.144	3.98	RTgill-W1
MED	$NtC_{lowerCI}$	0.025	4.73	RTgill-W1
2AE	$NtC_{lowerCI}$	53.95	4.55	RTgill-W1
ANI	$NtC_{lowerCI}$	39.81	3.90	RTgill-W1
DEP	$NtC_{measured}$	11.52	3.34	RTgill-W1
PAR	$NtC_{measured}$	0.108	1.14	RTgill-W1
4NP	$NtC_{measured}$	1.000	0.00	zebrafish embryos
DCA	$NtC_{measured}$	0.500	0.42	zebrafish embryos
DIS	$NtC_{measured}$	1.170	0.46	zebrafish embryos
HCP	$NtC_{measured}$	0.020	0.01	zebrafish embryos

NEC (no effect concentration) approach

NtC values were compared with NEC values calculated in this study by applying the approach proposed by Pires et al. (2002). In this threshold model, NEC can be determined by fitting the following equation to the measured data:

$$\text{Effect} = \min(100 \cdot \exp(\text{slope} \cdot (\text{logconc} - \text{logNEC}) \, f(\text{logconc} - \text{logNEC}); 100),$$

$$(\text{Eq. 2})$$

where: slope and logNEC are the fitted parameters that represent the slope of the curve and log10-normalized NEC, respectively, logconc is the log10-normalized chemical concentration and f(logconc) is the indicator function:

$$f(\text{logconc}) = \begin{cases} 1, & \text{logconc} > 0 \\ 0, & \text{logconc} \leq 0 \end{cases}, \qquad (\text{Eq. 3})$$

NOEC determination

In order to determine the NOEC for dynamic bioassays that are used to determine cell metabolic activity, a consistent study, i.e., following the exact same assay procedure throughout the experiments that were, preferably, done by the same person, must be used. For this reason, a study on the cytotoxicity of 35 organic chemicals in the RTgill-W1 cell line (Tanneberger et al., 2013) was chosen, and the determined NOEC values were compared with the NtCs.

Statistical analysis and goodness of fit

Statistical analysis was performed in Matlab using the Statistics and Optimization toolboxes.

Coefficient of determination (r^2) was used to describe the goodness of fit of the sigmoidal dose-response curve (Eq. 1) as well as of the NEC model (Eq. 2 and 3). In the present study, r^2 refers to the square of the correlation coefficient between measured and modeled values, and compares the model behavior with the data characteristic as described in the FOCUS guidance document (FOCUS, 2006).

Pearson correlation coefficient (r) was used to evaluate the NtCs' correlation between different testing objects, as well as between $LogK_{OW}$ and effect caused by the NOEC. It describes the strength of a linear correlation (association) between two variables. r = -1 represents a perfect negative correlation, while r = 1 means a perfect positive correlation.

Confidence intervals were used to determine $NtC_{lowerCI}$ and $NtC_{upperCI}$, and to compare NtCs with respective NEC values. They were plotted as 95% non-simultaneous functional prediction bounds ("functional", "off" in Matlab) as from our experience the highest variability in measured effects occurs when the sigmoidal slope is the steepest. Thus, the confidence intervals were determined based on the following equation:

$$95\%\text{CI} = Effect \pm t\sqrt{xSx^T}, \qquad (\text{Eq. 4})$$

where: t depends on the confidence level, and is computed using the inverse of Student's t cumulative distribution function, S is the covariance matrix of the coefficient estimates, $(X^TX)^{-1}s^2$ and x is a row vector of the design matrix or Jacobian evaluated at a specified predictor value.

ANOVA and the multi-comparison post-hoc test were used for the evaluation of the second-step validation data and for the determination of NOEC values following the instructions from the OECD guidance (OECD, 2006). The ANOVA was implemented in Matlab, in which the "anova1" function was used with the significance level set to 0.05, and the post-hoc test was performed using the "multcompare" function.

3 Results

3.1 NtC algorithm analysis (first-step validation data)

In general, the chosen sigmoidal dose-response model fitted the measured data very well. Inclusion of all data sets, i.e., whether or not they were very well described by the dose-response model, led to r^2 values between 0.62 and 0.99.
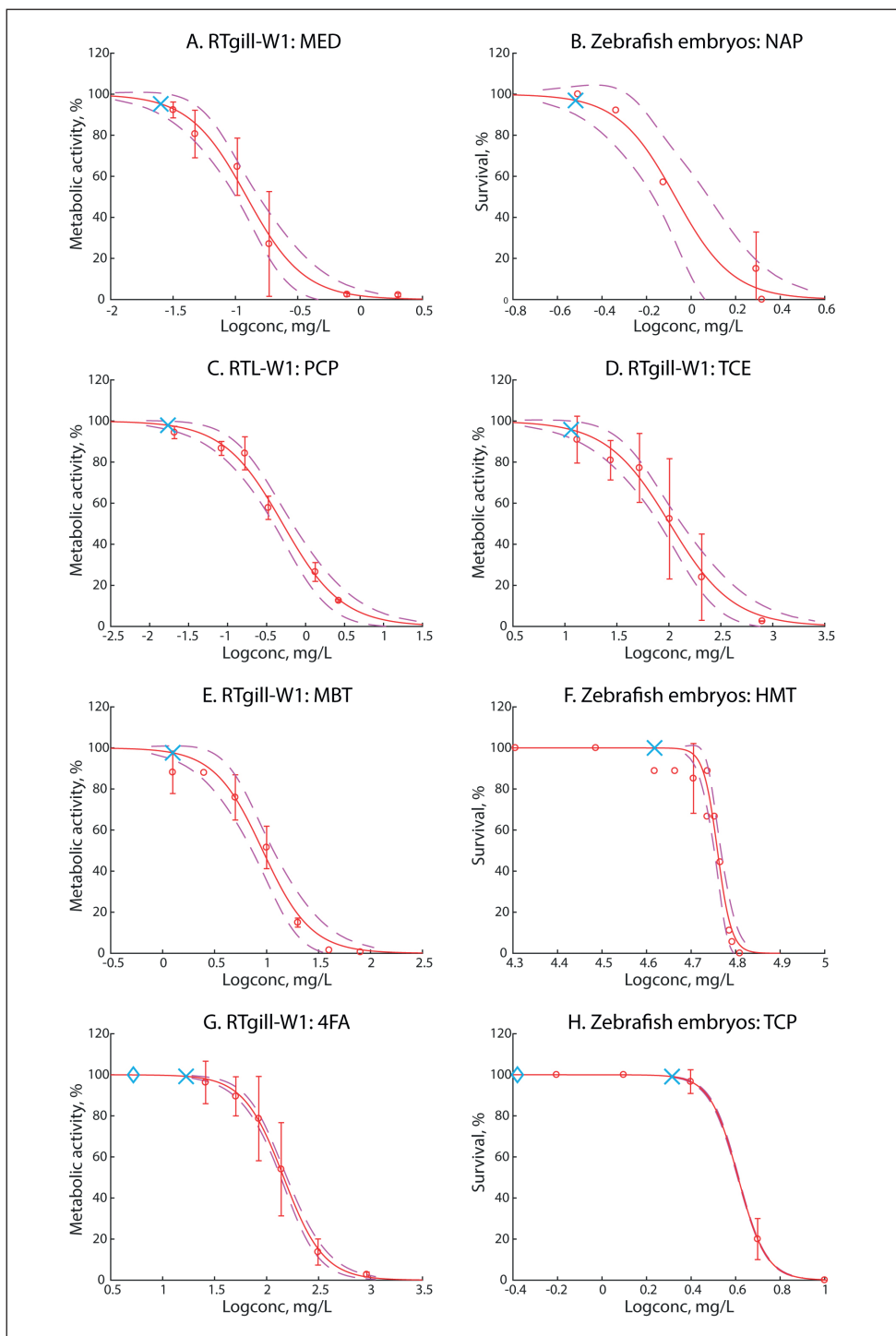
Fig. 2: Selection criteria of NtCs
A-B, lower limit constraint; C-D, upper limit constraint; E-F, measurement constraint; G-H, upper limit constraint with correction. Blue crosses indicate NtCs, circles represent measured effect caused by the respective concentration, solid lines are the fitted sigmoidal dose-response curves and dashed lines are 95% CI. Diamonds are NtCs without the corrections for narrow CI.

For all 91 dose-response data, NtCs were above zero. Of the three model assumptions, the lower limit constraint ($NtC_{lowerCI}$) was the most conservative for 30 out of 91 dose-response experiments (see examples in Fig. 2A-B), the upper limit constraint ($NtC_{upperCI}$) for 24 experiments (Fig. 2C-D), and the measured constraint ($NtC_{measured}$) for 37 experiments (Fig. 2E-F, all NtCs$_i$ for these compounds are shown in Fig. S1-S2[4]). The correction for very narrow confidence intervals (also $NtC_{upperCI}$) was applied in ten dose-response curves (Fig. 2G-H). In general, $NtC_{upperCI}$ was the most protective criterion when the sigmoidal dose-response curve fitted the measured data best (comparison of r^2 values).

3.2 Estimated effect caused by NtC (first-step validation)

Non-toxic concentrations, determined for all 91 dose-response experiments, were included in the sigmoidal model in order to estimate their respective effect (ECx). These estimated effects are presented in Figure 3. No NtC would cause an effect larger than 10% (including 95%CI). On average, the effect caused by NtCs was 1.85% (95%CI: 0-4.39%). No effect (EC0) caused by NtC was found for EtOH in the RTgill-W1 and 4NP in zebrafish embryo experiments and the highest effect was for DiB in the RTgill-W1 experiment (EC4.8). There was no correlation between effects caused by NtCs for different testing objects and the same chemicals; however, the effects caused by NtCs in embryo experiments were, in general, lower than those in cell line experiments (on average EC0.72 compared to EC2.35).

3.3 Measured effect caused by NtC (second-step validation)

Of all 17 experiments performed with the RTgill cell line and zebrafish embryos, the NtC was equal to the $NtC_{upperCI}$ for seven chemicals (including one with the algorithm correction), to the $NtC_{measured}$ for six chemicals, and to the $NtC_{lowerCI}$ for four chemicals (see details in Tab. 2).

The comparison between estimated and measured effects of determined NtCs is presented in Figure 4. None of the measured NtC effects was larger than EC10 and none of the measured effects was significantly larger than the control for each biological replicate (ANOVA, post-hoc test). For three chemicals
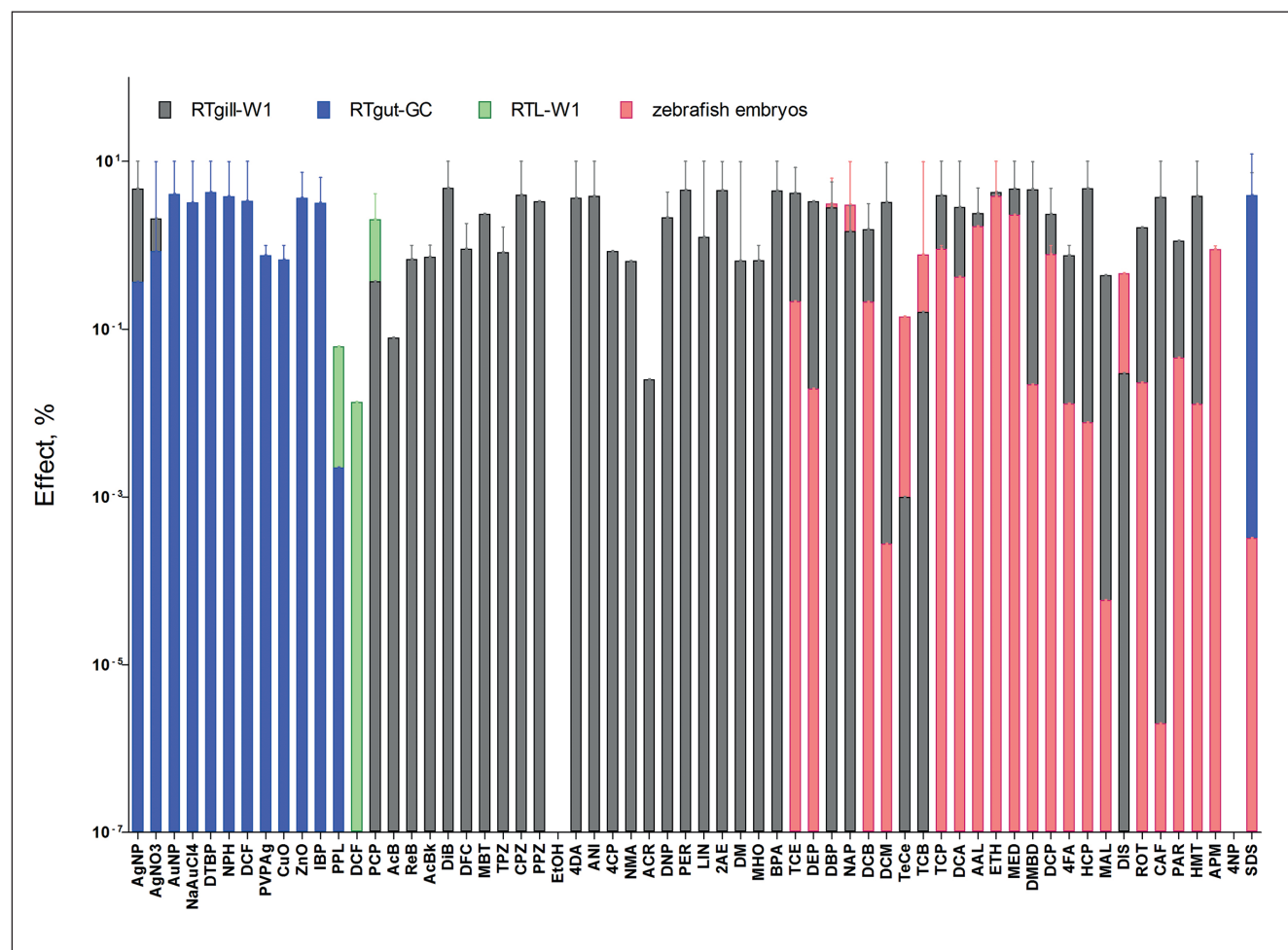

Fig. 3: Effects of non-toxic concentrations determined in our study on cell metabolic activity and embryo survival
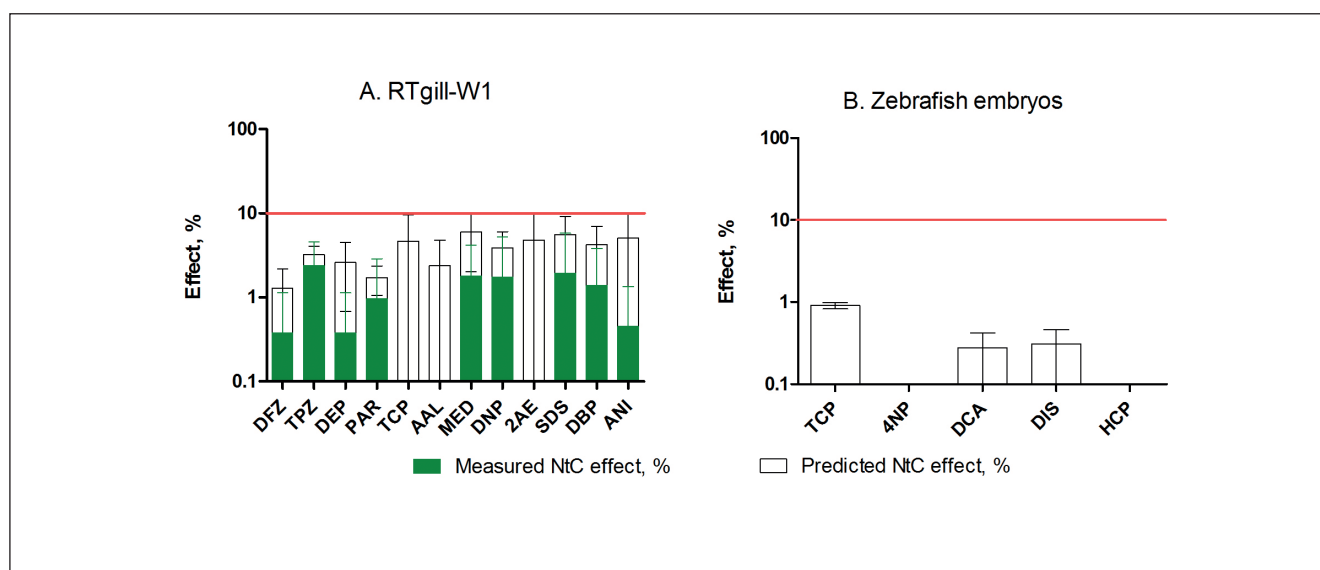
Fig. 4: Measured and predicted effects of NtCs
A, RTgill-W1 and B, zebrafish embryos. No effect of selected NtCs on zebrafish embryos was detected.

for RTgill-W1 and all five for zebrafish embryos, no effect was measured at all.

3.4 NtC algorithm false positive rate

The false positive rate of NtCs for all tested 91 dose-response curves was 1.54%. One study (for 4NP in zebrafish embryos) was responsible for 15.97% of all FPs – this study included only two biological replicates for the most crucial chemical concentration (i.e., lying on the steepest part of the sigmoid curve) and one of the replicates indicated a 10% effect while the second replicate indicated an 80% effect.

3.5 NtC *vs* NEC

The comparison between NtC and NEC values is presented in Figure 5. NtC values varied from 0.012 nM (NEC = 0.27 nM) for ROT in RTgill-W1 experiments to 296 mM

Fig. 5: Comparison between NtC and NEC values
Each symbol represents one concentration with 95%CI; the red line is the line of unity.

(NEC = 383 mM) for HMT in zebrafish embryos. 8% of all data were equally well described by both approaches (comparison of coefficients of determination) and 81% were better described by the sigmoidal dose-response curve used for the determination of NtCs. For all chemicals except for AgNO$_3$ in RTgut-GC (for which NtC = NEC = 3.87 μM), NEC values were higher than NtCs (above the line of unity in Fig. 5) and for 68% of all dose-response curves the difference between NEC and NtC was significant (comparison of 95%CI). For experiments with TCE and TeCE with zebrafish embryos, NEC values had very wide confidence intervals despite a good coefficient of determination ($r^2 = 0.9$ in both cases).

3.6 NtC *vs* NOEC

The comparison of the estimated NtC effects on the RTgill-W1 cell line with the respective measured effects of NOEC values is presented in Figure 6. For 33 (out of 35) chemicals, the effect caused by the NtC would be lower than that caused by the NOEC. For two chemicals, HMT and DMBD, Effect_NOEC was 0% and 0.5%, respectively, while Effect_NtC was 3.33% and 1.67%, respectively.

For eleven chemicals the effect caused by the NOEC was larger than 20% (e.g., for LIN – EC52, HCP – EC50, 4FA – EC46), while for all NtCs the effects were below 10% including 95%CI. No strong correlation between LogK$_{OW}$ and effect caused by the NOEC was found (r = 0.47).

4 Discussion

4.1 NtC algorithm performance and analysis

The algorithm, which is available as a free and user-friendly online app[2] and in the supplementary material[3] as Matlab and R source code, provided NtCs that were located in the flat
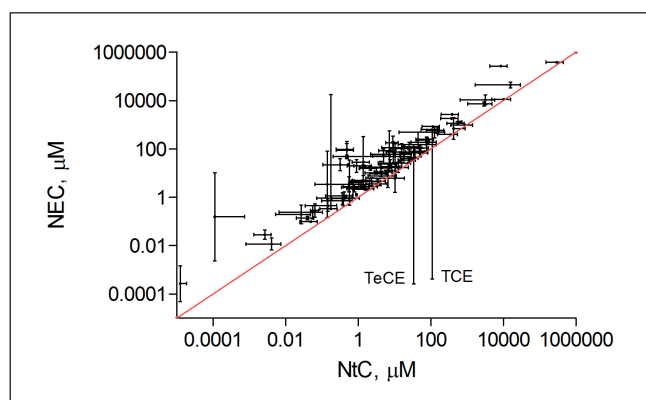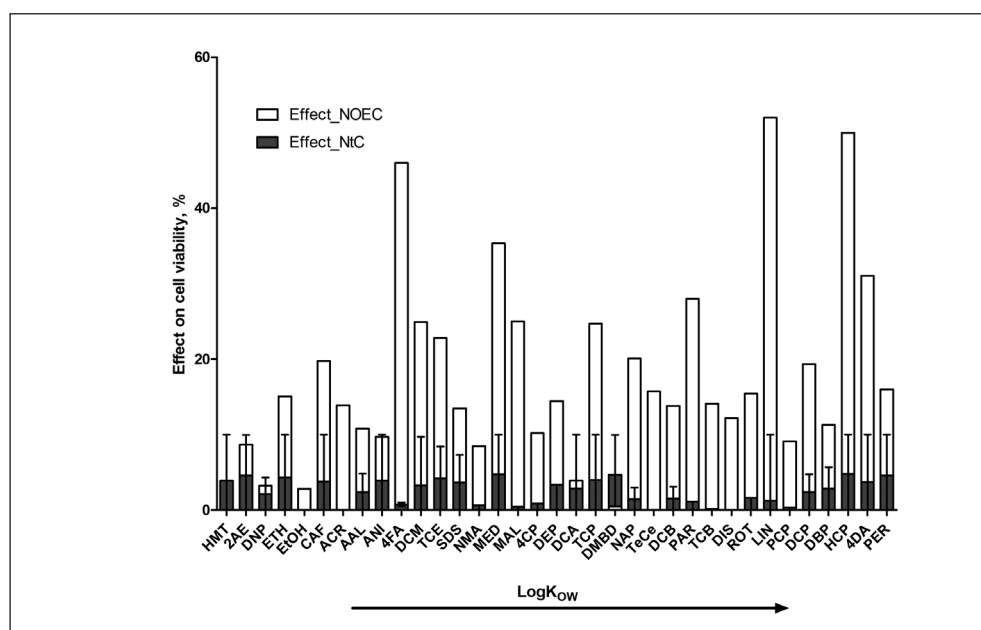
Fig. 6: Comparison of the effects of estimated NtCs with the measured effects of NOECs on the RTgill-W1 cell line

part of the sigmoidal-dose response curve for all 91 considered dose-response cases. In addition, out of 17 experiments performed to test the NtC algorithm, all measured effects of selected NtCs fulfilled the criteria selected for defining the non-toxic concentration regardless of which algorithm criterion was the most protective (Tab. 2, Fig. 4). While for *in vitro* experiments with the RTgill-W1 cell line, a small effect of the selected NtC was detected for most of the chemicals, none of the chosen NtCs caused an effect on embryo survival. This finding can be explained by two important differences between these two systems, namely, the measured effect endpoints and the number of testing objects. Firstly, opposite to the metabolic perturbation measured in experiments with cell lines, the survival endpoint that was measured in zebrafish embryos is a binary (i.e., more downstream) measure. Secondly, more than a million cells were used per chemical concentration in one biological replicate of *in vitro* experiments compared to 20 zebrafish embryos per chemical concentration (as recommended by the OECD guideline 236 (OECD, 2013). Thus, the lowest detectable effect in the experiments with zebrafish embryos was 5%, which resulted in no detected effect caused by the selected NtCs.

Despite its simplicity, it is important to perform the analysis carefully and to be aware of how the algorithm works. For instance, no effect of NtC was estimated for EtOH in RTgill-W1 and 4NP in zebrafish embryos (Fig. 3). This was because for these two compounds, one biological replicate caused the toxicity of at least 10% already for the first (i.e., the lowest) tested concentration, while even the fourth tested concentration caused the same or lower toxicity. In other words, the first part of the sigmoidal dose-response curve was almost horizontal for these two chemicals and their estimated effects of NtCs (= NtC$_{measured}$) were close to zero. In case one would want to apply the algorithm without considering the NtC$_{measured}$,

a switch is included to turn on/off the NtC$_{measured}$ in our web application.

The choice of the model to fit the data and the confidence intervals can influence the results. In our case, the sigmoidal dose-response curve, which is commonly used in ecotoxicology (Haanstra et al., 1985; Kerr and Meador, 1996), fitted the measured data very well; however, it is important to note that there are different variations of sigmoidal curves (different parameter number, constraints, etc.) (Ritz, 2010; Meddings et al., 1989). In addition, in case of hormesis, a U-shaped curve may have to be applied (Calabrese, 2009; Calabrese and Baldwin, 1999). Although we did not test the variety of dose-response curves, the algorithm code provided in Matlab and R can be easily adapted by users if a different fitting equation is desired. In our opinion, the algorithm assumptions should work for other models as long as they provide confidence intervals.

Regarding the confidence intervals, based on our experience and for our goals, the non-simultaneous functional confidence intervals were chosen but different methods could possibly be applied. In case of using observation bounds, however, it could happen that already the lowest possible chemical concentration (including its prediction intervals) would be more toxic than EC10, so further algorithm adaptations might then be needed.

The width of confidence intervals and r^2, i.e., the model goodness of fit, turned out to be an important factor influencing which of the model constraints – lower (NtC$_{lower}$) or upper (NtC$_{upper}$) – was more protective. In general, NtC$_{upper}$ was lower than NtC$_{lower}$ when the sigmoidal model fitted the measured data better (Fig. 2A-D). In some cases, when the confidence intervals happened to be extremely narrow, coincidentally (Fig. 2G) or due to a non-optimal concentration range (Fig. 2H), the selected NtC would be very low (close to zero) if the algorithm correction was not applied. Not correcting the

algorithm might lead to its over-protectiveness. However, in case a user does not want to apply this rather artificial model correction, we installed a switch to turn on/off this function in the online application.

4.2 NtC *vs* other approaches

In general, the sigmoidal dose-response model used in our study for the determination of NtCs fitted the measured data better than the threshold approach that delivered NEC values (Pires et al., 2002). In addition, in 90 out of 91 cases (in one NtC = NEC), NtCs were more protective than NECs. One might argue that the NtC is too conservative and its use is not realistic because of the limit of detection for chemical quantification (LOQ) that often must be considered. For this reason, we compared the NtC values with the respective LOQs reported in the study with the RTgill-W1 cell line (Tanneberger et al., 2013); 80% of the NtCs for RTgill-W1 were above the respective LOQs. In addition, for half of the remaining 20%, even EC50s would be below the reported LOQ. Thus, in general, NtCs are high enough to be determined by already existing quantification methods originally developed for chemical screening purposes in small scale assays.

The comparison between NtCs and NOECs (Fig. 6) not only indicates that NtCs are more protective but also illustrates the problems with using NOECs. When the effects caused by chemical concentrations were compared with the effect in a control sample, the toxicity of the NOEC was in some cases higher than the EC50. Together with the lack of the NOECs' confidence intervals, this results in the non-reliability of applying NOECs in *in vitro* experiments. Especially, if the concentration range is not chosen optimally, the NOEC might be equal to zero, which has no application in the experimental work.

It is difficult to compare NtCs with EC10, EC20 or other effect concentrations because one of the algorithm criteria is that the effect caused by the non-toxic concentration should not exceed 10% (including the confidence intervals). Thus, NtCs are always lower than EC10 and EC20. It might be worthwhile to highlight that if EC10 or EC20 are used without confidence intervals and the dose-response slope is steep, even small variability in the measurements could result in an increase of toxicity beyond EC50. This is possible especially for experiments with fewer organisms. For instance, in our study, we noticed that the slope of the sigmoidal dose-response curve was, in general, steeper for zebrafish embryos than for cell lines. While ECx does not account for the slope, the NtC toxicity was lower (below EC2) if the slope of the fitted sigmoidal dose-response curve was steep (i.e., lower than -5).

We did not look for correlations between NtCs and ECx or NOECs, even though there are studies that reported correlation between the NOEC and ECx (Beasley et al., 2015; Radix et al., 2000). We believe that as these values originate from completely different approaches, they should not be used interchangeably.

While the NOEC and ECx are still the most often used measures for ecotoxicological purposes, the benchmark dose (BMD) is frequently used in human toxicology and health risk assessment (Allen et al., 1994; Filipsson et al., 2003). However, this approach is not yet widely used in environmental toxicology. One reason could be that, regarding the regulatory context, as Travis et al. (2005) stated, the BMD is a relatively complex interpolation tool that delivers little more than the no-observed-adverse-effect level (NOAEL); it will never entirely replace the latter. Another concern might result from the study of Izadi et al. (2012), which showed that, in general, the BMD was higher than the NOAEL determined in 50 different studies received by the New Substances Assessment and Control Bureau of Health Canada. Since the BMDL is determined in the same way as our lower limit constraint (see e.g., Fig. 1 from EFSA guidance 2017 (Hardy et al., 2017)), and the benchmark risk is usually set to 10% (US EPA, 2012), which is in agreement with the cut-off value used in our study, our approach is more protective by default. On the other hand, it is important to note that, while our approach is much easier to use, the BMD software released by the US EPA now contains 30 different dose-response models, which are not yet implemented in the NtC algorithm.

Another interesting approach to deliver chemical non-toxic concentrations would be the use of TK-TD modeling, e.g., using the GUTS model (Jager, 2011), which provides a threshold value that should be valid over time. This approach has already been applied in *in vitro* experiments with fish cell lines (Stadnicka-Michalak et al., 2015); however, it requires elaborate time resolved experiments with several different chemical concentrations. Thus, except for some dedicated studies, not enough data are generally available for this approach. Nevertheless, it should be possible to carry out such experiments in small scale bioassays in high throughput mode. Therefore, investigating the NtC values over time and comparing them with the threshold parameter obtained by the GUTS model might be an interesting future project.

5 Conclusions

The purpose of this study was to develop an algorithm for choosing the non-toxic concentration of a chemical in a rational, tractable way, so that it does not depend on subjective experience. The algorithm, which considers both measured and modeled data, provides an NtC that is more protective than the NOEC, NEC and EC10 but is still above the already existing chemical LOQs.

Despite focusing on small scale bioassays, the NtC algorithm can be used in various systems as dose-response models underlying our approach are widely used also in biomedical science, mammalian toxicological or environmental research. Its applicability to the survival endpoint for organisms (i.e., zebrafish embryos) and to metabolic activity of isolated cells showed that NtCs can successfully be applied for different effect measures, time points and levels of biological organization. In addition, broad and consistent application of NtCs for down-stream applications, such as toxicokinetic explorations or integrative analyses of the genome, proteome or metabolome, has the potential to advance the robustness, interpretation and comparability of responses preceding a toxicological outcome. Thus, it

can result in the improvement of alternative methods to animal testing as well as reduce the number of still necessary animal experiments.

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Conflict of interest

The authors declare no potential conflict of interests.

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