

# A Validated Algorithm for Selecting Non-Toxic Chemical Concentrations

## Supplementary Data

### Supplementary methods

#### *In vitro* experiments with rainbow trout cell lines

Cells were routinely cultured at 19°C in 75 cm<sup>2</sup> cell culture flasks (TPP, Trasadingen, Switzerland) with Leibovitz's L-15 culture medium (w/o phenol red; Invitrogen, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS, Eurobio, Les Ulis, France). Experiments with RTgill-W1 were carried out as explained in detail in Tanneberger et al. (2013). For exposure to test chemicals, cells in confluent flasks were washed twice with versene (Invitrogen, Basel, Switzerland), detached with trypsin (0.25% in PBS w/o calcium and magnesium; PAN Biotech, Aidenbach, Germany) and re-suspended in the culture medium. Cell number in the resulting suspension was then determined using the electric field multi-channel cell counting system (CASY1 TCC, Schärfe System, Reutlingen, Germany).

The exposure medium, volume and number of seeded cells is given below for the RTgill-W1 cell line; for values used in other experiments, please refer to Table S1.

One ml of L-15 medium supplemented with 5% FBS and containing 350,000 cells was added into the wells of 24-well plates (Greiner Bio-One, Frickenhausen, Germany) in order to seed cells for exposure.

Chemical stock solutions were prepared by dissolving selected chemical concentrations in DMSO (Dimethyl sulfoxide). The final solvent concentration of DMSO in L-15/ex medium (Schirmer et al., 1997) within the well was 0.5% v/v. The dosing stock was freshly prepared for each experiment. 72 h after seeding, cells were washed with 1 ml L-15/ex before the chemical or control (with and without solvent) in L-15/ex was added. Experiments were carried out three times (i.e. three biological replicates) with cells from different passage numbers. Each biological replicate consisted of three technical replicates.

Cell survival was quantified 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 relative to the solvent control ("% of solvent control"), where the solvent control was set to 100% cell survival.

#### *In vitro* experiments with zebrafish embryos

Experiments with zebrafish embryos were carried out for the second step of the validation as explained in detail in Knöbel et al. (2012) and according to OECD TG 236 (OECD, 2013). The evening before fertilized eggs were needed, glass trays covered with a metal mesh (3 mm pore size) were placed in the maintenance tanks. Eggs were collected from the spawning trays about 1 h after onset of light, pooled into a plastic mesh (0.1 mm pore size) and carefully rinsed with tank water. Embryo exposure medium was prepared by adding the chemical stock solution in an appropriate amount to the embryo medium (ISO, 2007). Each well of a 24-well plate was loaded with 1 egg and 2 ml exposure medium (with a chemical or control) and covered with an adhesive foil. Experiments were carried out in technical triplicates using 3 24-well plates with 20 embryos per chemical (or control) sample, each. Chemical exposures were performed for 48 h. Coagulation of fertilized eggs, lack of detachment of the tail bud from the yolk sack, nonformation of somites, and lack of heart beat were considered indicators of lethality (OECD, 2013). Thus, if any of these effects was observed, an embryo was declared dead.



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[doi:10.14573/altex.1701231s1](https://doi.org/10.14573/altex.1701231s1)

**Tab. S1: Experimental set-up used in experiments for the first-step model validation**

	Chemical	Testing object	Well plate	Exposure medium	Medium volume	Cell/embryo number
1	Ag-NP	RTgill-W1, RTgut-GC	24-well plate 24-well plate	L-15/ex L-15/ex	1 ml 1 ml	300,000 150,000
2	AgNO <sub>3</sub>	RTgill-W1, RTgut-GC	24-well plate 24-well plate	L-15/ex L-15/ex	1 ml 1 ml	300,000 150,000
3	PVP-Ag	RTgut-GC	24-well plate	L-15/ex	1 ml	150,000
4	AuNP	RTgut-GC	24-well plate	d-L-15/ex	1 ml	150,000
5	NaAuCl <sub>4</sub>	RTgut-GC	24-well plate	d-L-15/ex	1 ml	150,000
6	CuO-NP	RTgut-GC	24-well plate	L-15/ex	1 ml	150,000
7	ZnO-NP	RTgut-GC	24-well plate	L-15/ex	1 ml	150,000
8	DTBP	RTgut-GC	24-well plate	L-15/ex	2 ml	150,000
9	NPH	RTgut-GC	24-well plate	L-15/ex	2 ml	150,000
10	IBP	RTgut-GC	24-well plate	L-15/ex	2 ml	150,000
11	DCF	RTgut-GC, RTL-W1	24-well plate 24-well plate	L-15/ex L-15/ex	2 ml 2 ml	150,000 150,000
12	PPL	RTgut-GC, RTL-W1	48-well plate 48-well plate	L-15/ex L-15/ex	0.5 ml 0.5 ml	75,000 75,000
13	PCP	RTL-W1, RTgill-W1	48-well plate 24-well plate	L-15/ex L-15/ex	0.5 ml 2 ml	75,000 300,000
14	AcB	RTgill-W1	48-well plate	L-15/ex	0.5 ml	75,000
15	ReB	RTgill-W1	48-well plate	L-15/ex	0.5 ml	75,000
16	AcBk	RTgill-W1	48-well plate	L-15/ex	0.5 ml	75,000
17	DiB	RTgill-W1	48-well plate	L-15/ex	0.5 ml	75,000
18	DFZ	RTgill-W1	24-well plate	L-15/ex	2 ml	350,000
19	MBT	RTgill-W1	24-well plate	L-15/ex	2 ml	350,000
20	TPZ	RTgill-W1	24-well plate	L-15/ex	2 ml	350,000
21	CPZ	RTgill-W1	24-well plate	L-15/ex	2 ml	400,000
22	PPZ	RTgill-W1	24-well plate	L-15/ex	2 ml	400,000
23	EtOH	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
24	4DA	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
25	ANI	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
26	4CP	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
27	NMA	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
28	ACR	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
29	DNP	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
30	PER	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
31	LIN	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
32	2AE	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
33	DBM	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
34	MHO	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
35	BPA	RTgill-W1	24-well plate	L-15/ex	2 ml	300,000
36	TCE	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
37	DEP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
38	DBP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
39	NAP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
40	DCB	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
41	DCM	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
42	TeCE	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
43	TCB	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
44	TCP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
45	DCA	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
46	AAL	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10

47	ETH	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
48	MED	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
49	DMBD	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
50	DCP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
51	4FA	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
52	HCP	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
53	MAL	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
54	DIS	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
55	ROT	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
56	CAF	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
57	PAR	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
58	HMT	RTgill-W1, zebrafish embryos	24-well plate 24-well plate	L-15/ex Embryo medium	2 ml 2 ml	300,000 10
59	APM	zebrafish embryos	24-well plate	Embryo medium	2 ml	10
60	4NP	zebrafish embryos	24-well plate	Embryo medium	2 ml	10
61	SDS	RTgill-W1, RTgut-GC, zebrafish embryos	24-well plate 24-well plate 24-well plate	L-15/ex L-15/ex Embryo medium	2 ml 2 ml 2 ml	300,000 150,000 10

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

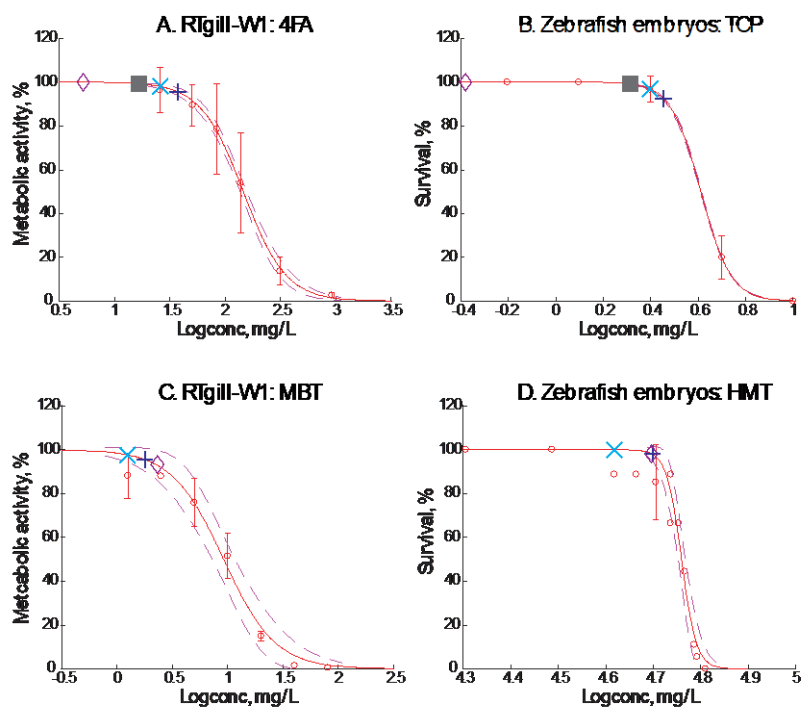


Figure S1. Selecting  $NtC$ s based on different algorithm criteria. Green pluses represent  $NtC_{lowerCI}$ , purple diamonds:  $NtC_{upperCI}$ , blue crosses:  $NtC_{measured}$  and grey squares: algorithm correction due to narrow confidence intervals.

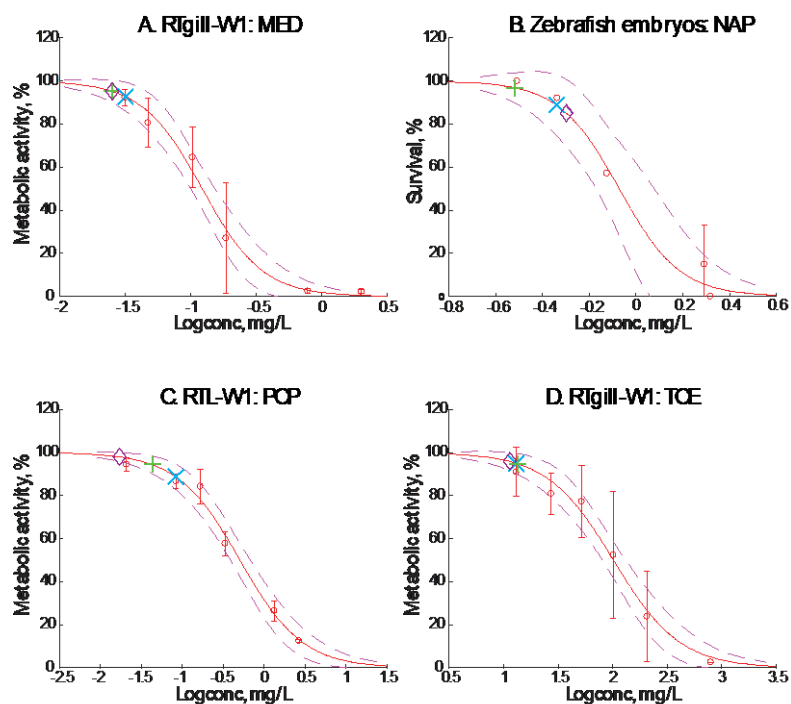


Figure S2. Selecting  $NtC$ s based on different algorithm criteria. Green pluses represent  $NtC_{lowerCI}$ , purple diamonds:  $NtC_{upperCI}$  and blue crosses:  $NtC_{measured}$ .