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Binary and complex mixture toxicity tests with Daphnia magna and Danio rerio using the Cremona and Bologna groundwater

Table of Contents

1. WP4: Ecotoxicology

1.1 Summary

The present report aims at describing the status of the work performed within the scope of WP4: Ecotoxicology, which main objective is to assess the toxicity of groundwater samples from the two field sites, Cremona and Bologna, using *Daphnia magna* and *Danio rerio* as surrogate species. The D4.3 report follows the deliverable D4.1 and D4.2, completed at the end of month 12, which included a literature review on the toxicities of several Emerging Contaminants (ECs) considered as priority and further testing of additional anthropogenic contaminants known to be relevant within the project case studies (Bologna and Cremona aquifers) using standard protocols, and month 18, where toxicity tests using the synthetic groundwaters were performed for each selected compounds (boric acid, ammonium hydroxide and sodium fluoride) in order to compare the ecotoxicological patterns of each compound under several media and we evaluated the performance of *Daphnia magna* and *Danio rerio* in the synthetic groundwater samples of Bologna and Cremona (with and without the ECs, according to concentrations measured in the field), respectively. Results from the toxicity assessment of the two synthetic waters mimicking the chemical composition of the two Italian aquifers have shown that both waters can be used by adapting the already established international protocols since they fulfill the validity criteria set.

After the first approach, where the toxicity of the two synthetic waters (Bologna and Cremona) as well as single toxicity of the selected compounds (Acetaminophen, Triclosan, PFOS and PFOA) in those waters was assessed to both model organisms, *D. magna* and *D. rerio*, binary and ternary mixtures were performed using selected chemicals (boric acid, ammonium hydroxide, sodium fluoride, acetaminophen, triclosan, PFOS and PFOA) in an attempt to mimic a real scenario exposure. The first step, was to assess with the use of the Concentration Addition model, whether the binary mixtures, referring to the compounds found in the ground water (boric acid, ammonium hydroxide, sodium fluoride) along with a pharmaceutical (acetaminophen) commonly found in high concentrations in surface waters, followed additivity or showed deviations to synergism or antagonism to a standard medium (ASTM) in one model organism (*Daphnia magna*) exposed assuming possible exposure scenarios (section 1.2 Binary mixtures approach in Cremona and Bologna groundwaters). As a second step, with the use of the Concentration Addition model, binary mixtures composed by the four selected compounds (acetaminophen, triclosan, PFOS and PFOA) were performed for each synthetic water and standard medium using both model organisms (section 1.2 Binary

mixtures approach in Cremona and Bologna groundwaters). Subsequently, we evaluated the performance of *D. magna* in the two synthetic groundwater samples of Bologna and Cremona under ternary mixtures exposure (section 1.3 Complex mixtures approach in Cremona and Bologna groundwaters).

Additional (bibliographical) information as well as a summary of results on the compounds tested within this framework in the two model species (*Daphnia magna* and *Danio rerio*) and in other species and types of cells can be found in **Annex 1** and **2**.

1.2 Binary mixtures approach in Cremona and Bologna groundwaters

1.2.1 *Daphnia magna*

Within the scope of WP4: Ecotoxicology, a total of 15 binary mixtures were performed with the standard model organism *Daphnia mag*na using Cremona and Bologna synthetic waters as well as ASTM medium, a standard culture medium for comparison reasons. For the binary approach, the conceptual model, Concentration Addition (CA), was chosen as it is a more robust and conservative model in effects prediction and one of the preferred models by risk assessors for Risk Assessment Management. Additionally, with the help of the MIXTOX tool [1], any deviations to the CA model, such as Synergism/Antagonism (S/A), Dose Ratio (DR) and Dose Level (DL), were also determined. Part of this work was presented in the SETAC Conference in May 2018 in Rome, Italy.

Initially, the toxicity patterns of the mixtures with the chemicals (boric acid, ammonium hydroxide, sodium fluoride) already identified in Cremona and Bologna groundwater were scrutinized using daphnids immobilization (following a standardized protocol) [2] as endpoint and **Table 1** from Jonker et al [1] for parameter interpretation.

Table 1. Interpretation of additional parameters, a and b, which define the functional from the deviation patterns from the reference model Concentration Addition (CA). Adapted from Jonker et al. [1].

Deviation pattern	Parameter a (CA)	Parameter b (CA)
Synergism (S)/ Antagonism (A)	a>0 antagonism a<0 synergism	-
Dose-ratio dependent (DR)	a>0 antagonism except for mixture ratios where negative b value indicate synergism a<0 synergism except for mixture ratios where positive b value indicate antagonism	bi>0 antagonism where the toxicity of the mixture is caused mainly by toxicant i bi<0 synergism where the toxicity of the mixture is caused mainly by toxicant i
Dose-level dependent (DL)	a>0 antagonism at low dose level and synergism at high dose level a<0 synergism at low dose level and antagonism at high dose level	bDL>1 change at lower EC50 level bDL=1 change at EC50 level 0< bDL<1 change at higher dose level than the EC50 bDL<0 no change, but the magnitude is dose level dependent

Data from the binary mixture of boric acid with ammonium hydroxide was well adjusted to the reference model, CA model, however continuing in the nested framework for assessing potential deviations, the best fitted deviation was the DR deviation, this is supported by the $p(\chi 2)$ <0.05, R^2 value= 0.92 and by the lowest sum of squared residuals (SS) value= 42.36 comparing with all the others deviations in the CA model (**Table 2, a**). The parameter a_{DR} was positive, and b_{DR} was negative, denoting antagonism (a>0), except for mixture ratios where synergism was observed and caused mainly by one of the chemicals, in this case boric acid (b<0) (Please check **Table 1** for additional information on parameters interpretation). The synergism caused by boric acid is well observed in the isobologram (red arrow in **Figure 1, a**). Deviations from the additive model, CA model, were found.

Antogonism (a>0) was the best fitted deviation observed for the binary mixture of boric acid with sodium fluoride ($p(\chi 2)$<0.05, R^2 =0.80, SS=91.07; **Table 2, b**), this is supported by the isobologram as well (**Figure 1, b**). During the growth of crustaceans, more specifically due

to the moulting process (which in daphnids occurs for the first time after 24h), some of the fluoride could be eliminated, contributing to a slow bioaccumulation of the compound and consequently reducing the toxic effects.

When testing the boric acid in binary mixture with the pharmaceutical acetaminophen, the DL deviation was the best fitted one ($p(\chi 2)<0.05$, R^2 =0.89, *SS*=50.25; **Table 2, c**). In this DL deviation, antagonism was found in low doses and synergism at high doses of the chemicals and this change in pattern occurs at higher levels than the EC_{50} value (parameter $a_{DL}>0$ and parameter $0<b_{DL}<1$, please check **Table 1** for additional information on parameters interpretation). The antagonistic pattern at low doses in clearly observed in Figure 2, c. When organisms are under stress, due to for instance environmental contaminants, and in order to minimize oxidative damage, organisms have evolved several antioxidant defenses (*e.g.* antioxidant enzyme: catalase). Acetaminophen was already found to cause oxidative stress in microcrustaceans; this oxidative stress provides valuable information to evaluate damage in proteins, lipids and DNA.

In addition, a DL deviation was the best fitted deviation for the ammonium hydroxide with sodium fluoride ($p(\chi 2)<0.05$, R^2 =0.78, *SS*=108.2; **Table 2, d**). The additional parameters were $a_{DL}>0$ and $b_{DL}<0$, meaning antagonism at low doses and synergism at high doses, but the magnitude is dose level dependent; therefore only antagonism was observed in the isobologram and synergism cannot be depicted (**Figure 1, d**).

For the binary mixture with ammonium hydroxide with acetaminophen a DL deviation was the best fitted model ($p(\chi 2)<0.05$, R^2 =0.88, *SS*=49.03), with synergism observed at low dose level and at high dose level antagonism ($a_{DL}<0$) and this change occurs at lower EC50 level ($b_{DL}>1$) (**Table 2, e; and Table 1**). At low doses, higher toxicity than expected (synergism) occurred, thus being of great concern for risk assessors and general public.

Finally, for sodium fluoride with acetaminophen, a DR deviation was found ($p(\chi 2)<0.05$, R^2 =0.85, *SS*=65.74; Table 2 f); with antagonism observed except when one of the chemicals, in this case acetaminophen, is the main responsible for synergism ($a_{DR}>0$ and $b_{DR}<0$). This pattern is well observed in **Figure 1, f.**

Table 2. Summary of the mixture analysis of *Daphnia magna* (immobilization data - 48h) exposed to the mixture of **a)** boric acid with ammonium hydroxide **b)** boric acid with sodium fluoride **c)** boric acid with acetaminophen **d)** ammonium hydroxide with sodium fluoride **e)** ammonium hydroxide with acetaminophen **f)** sodium fluoride and acetaminophen.

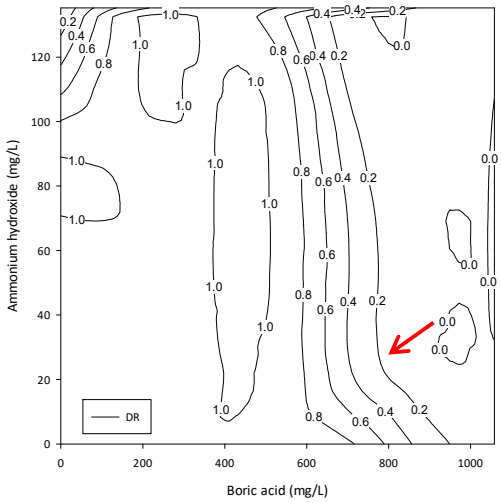
	a) Boric acid with ammonium hydroxide			
		Deviations from CA model		
	CA	S/A	**DR**	DL
R^2	0.78	0.81	0.92	0.73
SS	109.8	95.57	42.36	137.7
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	**<0.05**	n.d.
max	0.98	0.98	0.98	0.98
a	-	2.37	4.75	-0.01
b	-	-	-5.65	136.6
	b) Boric acid with sodium fluoride			
		Deviations from CA model		
	CA	S/**A**	DR	DL
R^2	0.71	0.80	0.80	0.81
SS	134.5	91.07	90.80	89.72
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	>0.05	>0.05
max	0.98	0.98	0.98	0.98
a	-	0.77	0.93	0.099
b	-	-	-0.28	-5.02
	c) Boric acid with acetaminophen			
		Deviations from CA model		
	CA	S/A	DR	**DL**
R^2	0.78	0.87	0.87	0.89
SS	100.5	58.37	58.37	50.25
***p*(*F*-test)**	**<0.05**	-	-	-

$p(\chi^2)$	-	**<0.05**	>0.05	**<0.05**
max	0.97	0.98	0.98	0.98
a	-	2.42	2.45	6.48
b	-	-	-0.06	0.36
	d) Ammonium hydroxide with sodium fluoride			
		Deviations from CA model		
	CA	S/A	DR	**DL**
R^2	0.58	0.74	0.76	**0.78**
SS	202.6	126.2	117.9	**108.2**
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	**<0.05**	**<0.05**
max	0.98	0.98	0.98	0.98
a	-	2.10	2.30	0.62
b	-	-	-0.10	-1.53
	e) Ammonium hydroxide with acetaminophen			
		Deviations from CA model		
	CA	S/A	DR	**DL**
R^2	0.73	0.84	0.84	0.88
SS	110.8	66.54	65.97	49.03
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	>0.05	**<0.05**
max	0.98	0.98	0.98	0.98
a	-	1.87	1.21	-1.85
b	-	-	1.30	1.21
	f) Sodium fluoride and acetaminophen			
		Deviations from CA model		
	CA	S/A	**DR**	DL
R^2	0.57	0.83	0.85	0.84
SS	193.97	74.77	65.74	73.74
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	**<0.05**	>0.05

max	0.96	0.96	0.96	0.96
a	-	3.08	1.18	2.54
b	-	-	3.82	-0.11

R^2 represents the coefficient of determination; SS represents the sum of squared residuals; p(F-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, a and b represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

a)



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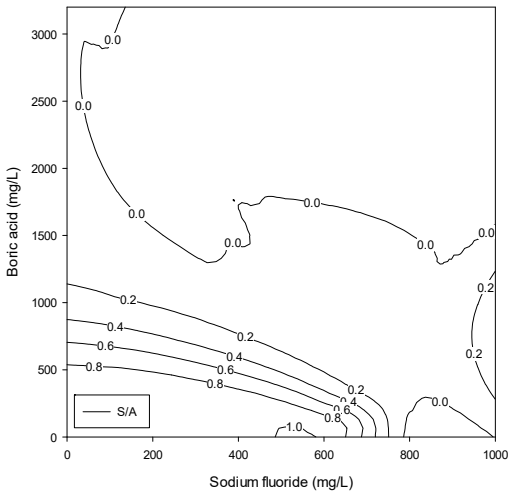
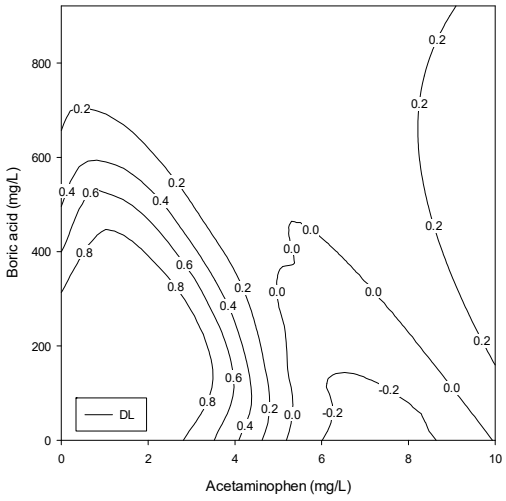


c)



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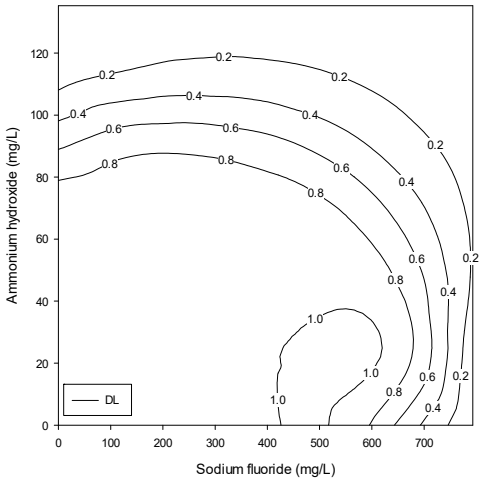

e) f)

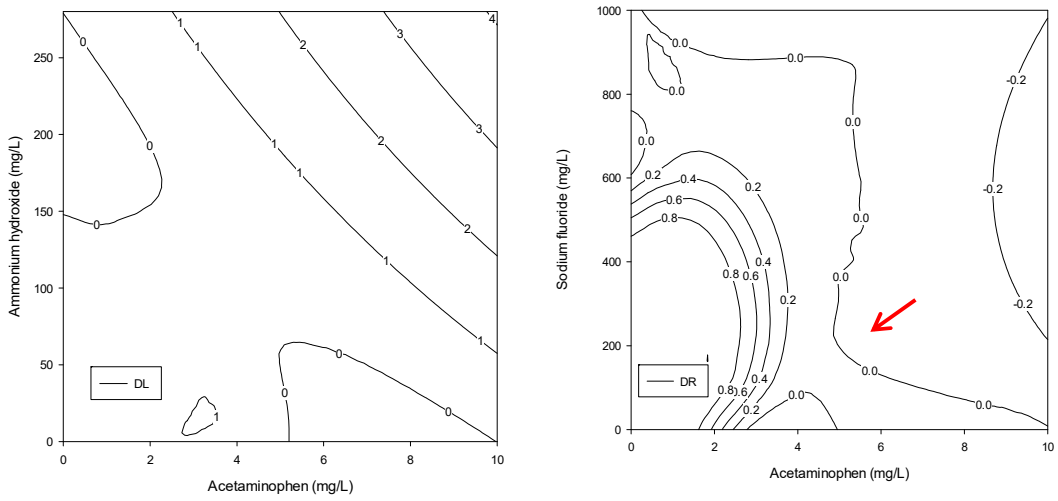

Figure 1. Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the immobilisation data of daphnids exposed to a) Boric acid with ammonium hydroxide b) Boric acid with sodium fluoride c) Boric acid with acetaminophen d) ammonium hydroxide with sodium fluoride e) ammonium hydroxide with acetaminophen f) sodium fluoride and acetaminophen. The red arrow indicates synergism.

Secondly, the toxicity patterns of the binary mixtures with the chemicals (acetaminophen, triclosan and PFOA), identified as priority chemicals within the WE-NEED consortium, were tested in Cremona and Bologna groundwater as well as ASTM standard culture medium using the daphnids immobilization standardized protocol [2]. In order to run the binary mixtures, the LC_{50} values of all three chemicals in all three waters were determined (**Table 3**).

Results from the single exposures of chemicals to the crustacean *D. magna*, show that PFOA is the least toxic compound while triclosan the most toxic when compared between compounds in all 3 media. When comparing between waters, there is no obvious pattern followed where toxicity is generally higher to one compared to the other. Bologna appears to be the medium that when daphnias are exposed to acetaminophen and triclosan causes the lowest toxicity (**Table 3**). Another interesting result comes from daphnias exposed to PFOS, where the Italian waters are significantly more toxic to the organism when compared to the culture standard water and the one proposed by international protocols.

Table 3. Summary of the LC_{50} values obtained from the 48h *D. magna* acute exposure to each compound in different media. LC_{50} values are expressed in mg/L with the 95% confidence intervals in brackets. n.d.- not defined

ASTM		
	Parameter	**LC_{50} value**
Acetaminophen	**Immobilization**	3.39 (3.04-3.76)
Triclosan		0.98 (0.70-1.69
PFOA		414.3 (374.9-453.6)
PFOS		21.2 (n.d.)*
Bologna		
	Parameter	**LC_{50} value**
Acetaminophen	**Immobilization**	5.7 (4.8-7)
Triclosan		2.23 (1.65-3.48)
PFOA		501.6 (442-582)
PFOS		6.25 (5.88-6.62)
Cremona		

	Parameter	LC_{50} value
Acetaminophen	**Immobilization**	3.11 (2.75-3.48)
Triclosan		0.95 (0.86-1.03)
PFOA		428.7 (363.7-507)
PFOS		4.51 (3.74-5.28)

*Yang et al (2019), Sci Total Environ

Based on the individual LC_{50} values of each chemical determined for each medium, a total of 9 binary mixtures were performed, 3 for each water.

An antagonistic pattern was identified for the binary mixture of acetaminophen with PFOA ($p(\chi2)<0.05$, R^2 =0.81, *SS*=91.41; **Table 4, a**) using ASTM as test medium. Such deviation to additivity can be observed through the isobologram (**Figure 2, a**). In Cremona, the same binary mixture elicited similarly antagonistic effect, but with the magnitude of such antagonism varying in a dose-level (DL) dependent manner ($p(\chi2)<0.05$, R^2 =0.71, *SS*=145.21; **Table 5, a**).

Regarding the binary mixture of acetaminophen and triclosan, a dose-ratio (DR) dependent deviation to CA model ($p(\chi2)<0.05$, R^2 =0.79, *SS*=93.58; **Table 4, b**) was obtained for both ASTM and Cremona synthetic water ($p(\chi2)<0.05$, R^2 =0.79, *SS*=45.10; **Table 5, b**). This means that considering the ratios of the chemicals within the mixture is essential to interpret the direction of the deviations to additivity. A positive a_{DR} parameter value parameter value and a negative value for b_{DR} denoted antagonism, except for those ratios where the toxicity was mainly caused by triclosan. For those ratios where the toxicity was led by triclosan, synergism was found. Otherwise, an antagonistic deviation pattern would occur. This pattern can be confirmed in **Figure 2, b and 3, b**.

Antagonism (a>0) was the best fitted deviation observed for the binary mixture of PFOA and triclosan in ASTM ($p(\chi2)<0.05$, R^2 =0.80, SS=121.87; **Table 4, c**). In Cremona synthetic water, a DR dependent deviation was registered ($p(\chi2)<0.05$, R^2 =0.80, SS=121.87; **Table 5, c**), consisting of antagonism, except for mixture ratios where triclosan is leading the toxicity of the mixture. This means that whenever the mixture toxicity is due to triclosan, it

assumes a synergistic pattern, but antagonism was the general rule when triclosan was not the main driver of mixtures effects.

Table 4. Summary of the mixture analysis of *Daphnia magna* (immobilization data - 48h) exposed to the mixture of **a)** acetaminophen and PFOA **b)** acetaminophen and triclosan and **c)** triclosan and PFOA in ASTM medium.

	a) acetaminophen and PFOA			
		Deviations from CA model		
	CA	S/**A**	DR	DL
R^2	0.73	0.81	0.81	0.81
SS	126.81	91.41	90.96	90.70
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	0.50	0.40
max	0.98	0.98	0.98	0.98
a	-	1.47	1.05	-0.02
b	-	-	0.77	41.80
	b) acetaminophen and triclosan			
		Deviations from CA model		
	CA	S/**A**	**DR**	DL
R^2	0.61	0.74	0.78	0.74
SS	170.82	114.57	93.58	116.35
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	**<0.05**	-
max	0.97	0.93	0.94	0.88
a	-	2.23	-0.58	-0.02
b	-	-	5.66	60.02
	c) triclosan and PFOA			
		Deviations from CA model		
	CA	S/**A**	DR	DL
R^2	0.50	0.69	0.69	0.97

SS	195.81	121.87	121.32	125.49
***p*(*F*-**	**<0.05**	-	-	-
test)	-	**<0.05**	>0.05	-
***p*(χ^2)**	0.97	0.94	0.94	0.96
max	-	2.00	1.79	3.15
a	-	-	0.67	0.16
b				

R^2 represents the coefficient of determination; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; *p*(χ^2) represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

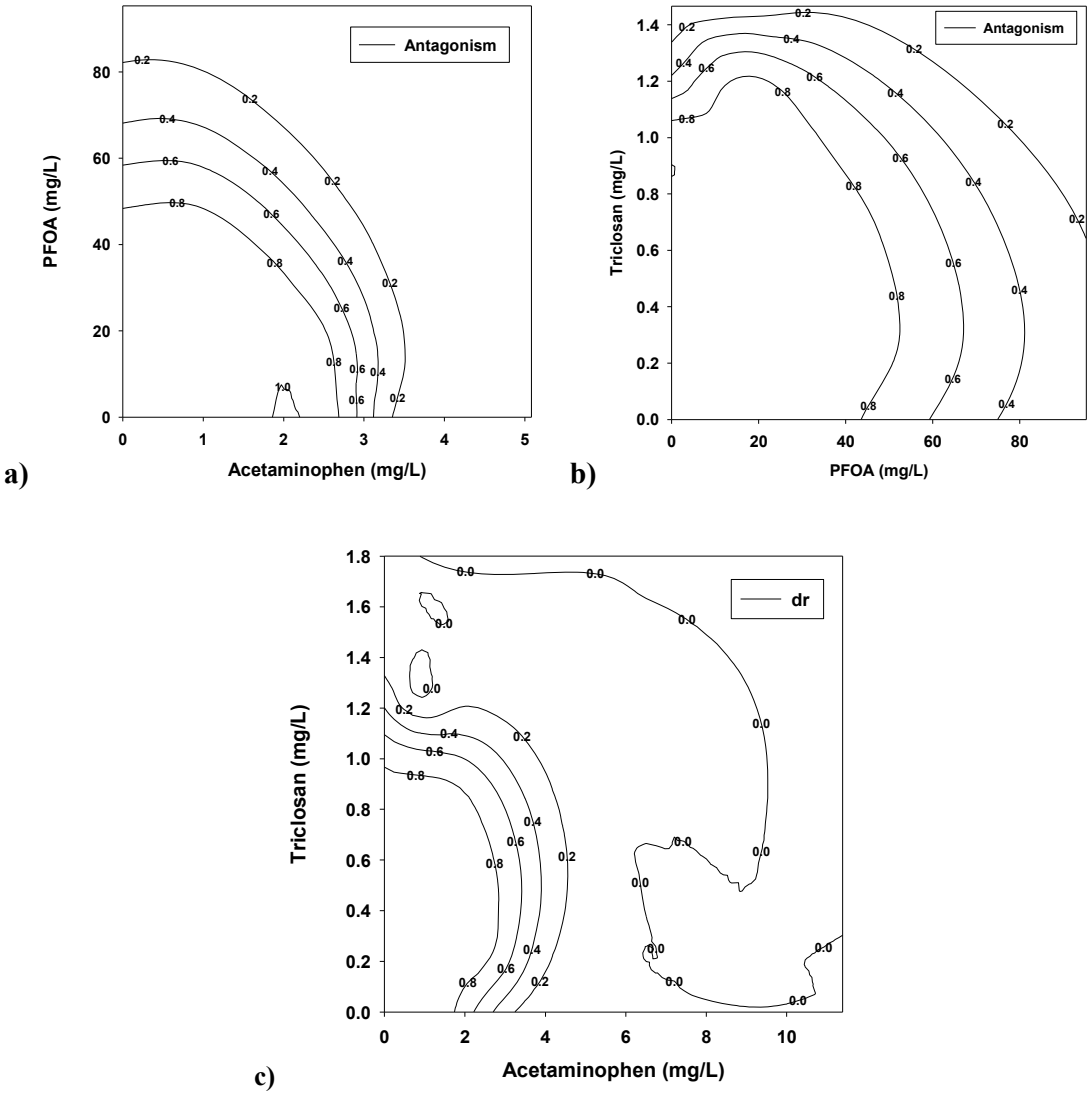

Figure 2. Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the immobilisation data of daphnids exposed to a) acetaminophen with PFOA b) triclosan with PFOA and c) triclosan with acetaminophen in the ASTM medium.

Table 5. Summary of the mixture analysis of *Daphnia magna* (immobilization data - 48h) exposed to the mixture of **a)** acetaminophen and PFOA **b)** acetaminophen and triclosan and **c)** triclosan and PFOA in Cremona water.

	a) acetaminophen and PFOA			
		Deviations from CA model		
	CA	S/**A**	DR	**DL**
R^2	0.59	0.70	(failed to converge)	0.71
SS	203.25	150.50		145.08
***p*(*F*-test)**	**<0.05**	-		-
$p(\chi^2)$	-	**<0.05**		**<0.05**
max	0.92	0.85		0.83
a	-	2.30		0.65
b	-	-		-1.46
	b) acetaminophen and triclosan			
		Deviations from CA model		
	CA	S/**A**	**DR**	DL
R^2	0.83	0.83	0.80	0.81
SS	60.38	59.29	45.10	89.72
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	0.30	**<0.05**	>0.05
max	0.98	0.98	0.98	0.98
a	-	0.37	3.53	0.099
b	-	-	-8.88	-5.02
	c) triclosan and PFOA			
		Deviations from CA model		
	CA	S/A	**DR**	DL
R^2	0.80	0.92	0.88	(failed to converge
SS	93.88	84.94	59.52	
***p*(*F*-test)**	**<0.05**	-	-	
$p(\chi^2)$	-	**<0.05**	**<0.05**	

max	0.93	0.92	0.89	
a	-	0.39	3.87	
b	-	-	-7.95	

R^2 represents the coefficient of determination; SS represents the sum of squared residuals; p(F-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, a and b represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

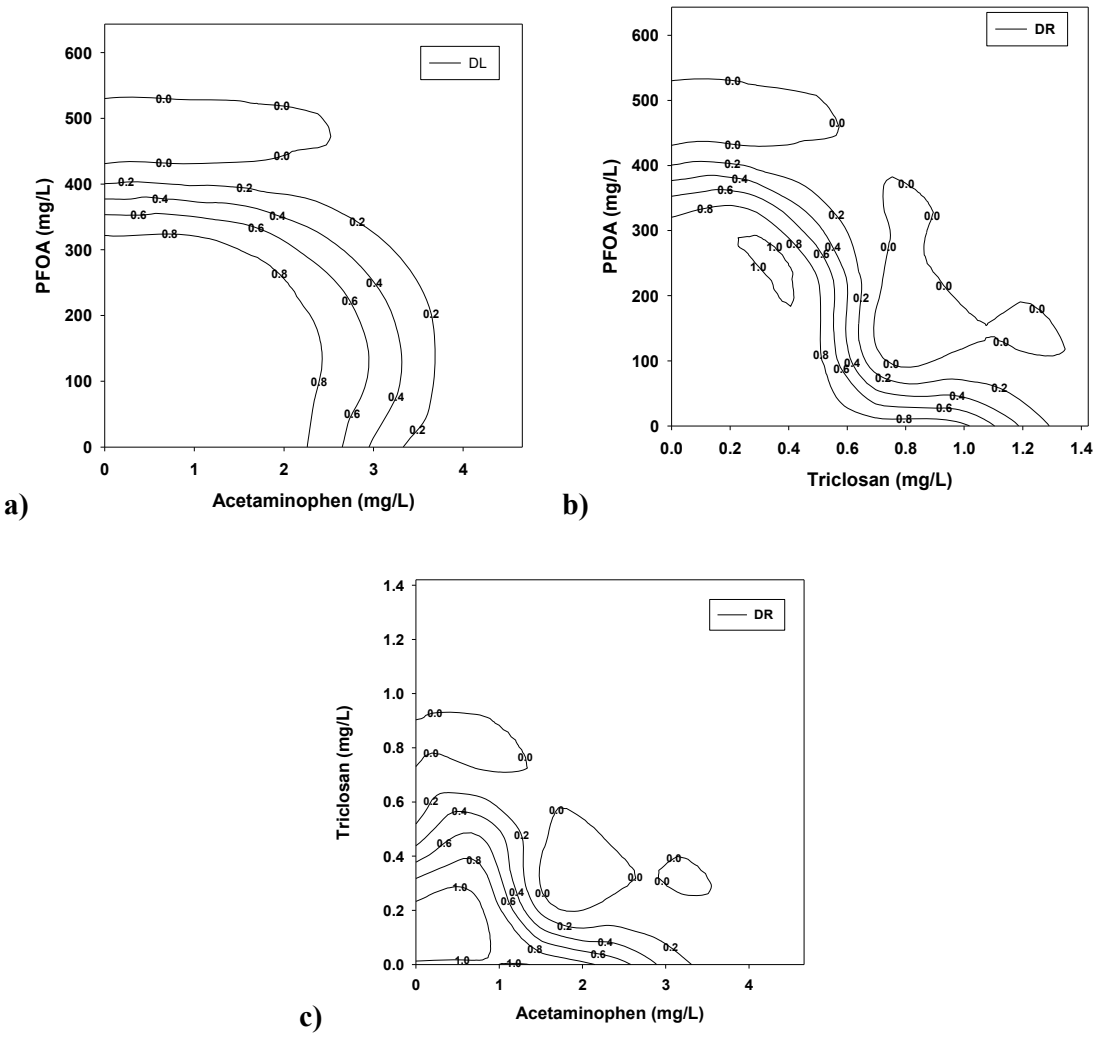

Figure 3. Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the immobilisation data of daphnids exposed to a) acetaminophen with PFOA b) triclosan with PFOA and c) triclosan with acetaminophen in the Cremona waters.

1.2.1 *Danio rerio*

Within the scope of WP4: Ecotoxicology, a total of 10 binary mixtures were performed with the standard model organism *Danio rer*io using Cremona and Bologna synthetic waters as well as Fish System Water (FSW) medium, a standard culture medium for comparison reasons. In order to run the binary mixtures, the LC_{50} values of all three chemicals in all three waters were determined for *D. rerio* (**Table 6**). Part of this work was presented in the SETAC Conference in May 2019 in Helsinki, Finland.

Table 6. Summary of the LC_{50} values obtained from the 96h *D. rerio* embryo toxicity exposure to each compound in different media. LC_{50} values are expressed in mg/L with the 95% confidence intervals in brackets. n.d.- not defined

ASTM		
	Parameter	**LC_{50} value**
Acetaminophen	**Immobilization**	1483.2 (n.d.)
Triclosan		0.42* (0.38-0.45)
PFOA		759** (643-875)
PFOS		3.04 (1.35-14.88)
Bologna		
	Parameter	**LC_{50} value**
Acetaminophen	**Immobilization**	634.6 (564.3-741.9)
Triclosan		0.73 (n.d.)

PFOA		377.9 (343.9-413.7)
PFOS		2.88 (2.02-4.47)
Cremona		
	Parameter	**LC_{50} value**
Acetaminophen	**Immobilization**	736.7 (594.3-928.9)
Triclosan		0.80 (n.d.)
PFOA		545.1 (483.7-611.7)
PFOS		6.34 (4.79-9.33)

*Oliveira et al (2009), Environ Sci Pollut Res

**Stengel et al (2018), Environ Sci Pollut Res

Following the determination of LC_{50} values for each chemical to the zebrafish, *D. rerio*, in each water tested, binary exposures were performed.

Starting with the Cremona water, data from the first binary mixture of acetaminophen and triclosan was well adjusted to the reference model, CA model, however continuing in the nested framework for assessing potential deviations, the best fitted deviation was the DR deviation, this is supported by the $p(\chi 2) < 0.05$, R^2 value= 0.85 and by the lowest sum of squared residuals (SS)= 47 comparing with all the others deviations in the CA model (**Table 7, a**). The parameter a_{DR} was positive, and b_{DR} was negative, denoting antagonism ($a>0$), except for mixture ratios where synergism was observed and caused mainly by one of the chemicals, in this case acetaminophen ($b<0$) (**Figure 4, a**).

Fitting the CA model to the data obtained from the triclosan and PFOS combination, the reference model showed a good fit to the dataset. After introducing parameter a to the

model, a significant fit was obtained showing deviations to CA. Parameter *a* is positive denoting clear antagonism (*a*>0) (**Figure 4, b**). By adding an additional parameter (parameter *b*) to the model, no significant improvement was obtained.

For the third mixture, acetaminophen and PFOS mixture results show a dose-ratio (DR) dependent deviation to CA model ($p(\chi 2)<0.05$, R^2 =0.89, *SS*=35.7; **Table 7, c**). As previously mentioned, the ratios of the chemicals within the mixture are essential to interpret the direction of the deviations to additivity. A positive a_{DR} parameter value parameter value and a negative value for b_{DR} denoted antagonism, except for those ratios where the toxicity was mainly caused by acetaminophen. For those ratios where the toxicity was led by triclosan, synergism was found. Otherwise, an antagonistic deviation pattern would occur. This pattern can be confirmed in **Figure 4, c**.

For the fourth and last mixture in the Cremona water, acetaminophen and PFOA results show an antagonistic deviation to CA model ($p(\chi 2)<0.05$, R^2 =0.84, *SS*=46.2; **Table 7, d**). This pattern can be confirmed in **Figure 4, d**. No further mixture approaches were performed with PFOA from this stage onward, as the concentrations were considered not relevant environmental-wise.

Table 7. Summary of the mixture analysis of *Danio rerio* (survival data - 96h) exposed to the mixture of **a)** acetaminophen and triclosan **b)** triclosan and PFOS **c)** acetaminophen and PFOS in the Cremona water.

	a) acetaminophen and triclosan			
		Deviations from CA model		
	CA	S/A	**DR**	DL
R^2	0.81	0.81	0.85	0.81
SS	58.8	58.1	47	58.11
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	>0.05	**<0.05**	>0.05
max	0.89	0.89	0.88	0.89
a	-	0.17	2.54	-0.11
b	-	-	-4.46	2.36
	b) triclosan and PFOS			
		Deviations from CA model		

	CA	**S/A**	DR	DL
R^2	0.83	0.98	0.89	0.89
SS	59.6	42.8	39.8	39.8
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	>0.05	>0.05
max	0.98	0.98	0.98	0.98
a	-	1.2	0.51	-0.03
b	-	-	1.84	30.5
	c) PFOS and acetaminophen			
		Deviations from CA model		
	CA	S/A	**DR**	DL
R^2	0.86	0.86	0.89	0.87
SS	43.7	43.7	35. 7	41.49
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	>0.05	**<0.05**	<0.05
max	0.88	0.88	0.86	0.87
a	-	0.04	1.06	-2.17
b	-	-	-2.94	1
	d) acetaminophen and PFOA			
		Deviations from CA model		
	CA	**S/A**	DR	DL
R^2	0.54	0.84	0.84	0.84
SS	136.6	46.2	46.2	45.9
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	**<0.05**	>0.05	>0.05
max	0.98	0.94	0.94	0.94
a	-	2.58	2.58	1.96
b	-	-	-0.002	-0.17

R^2 represents the coefficient of determination; SS represents the sum of squared residuals; p(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA

represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

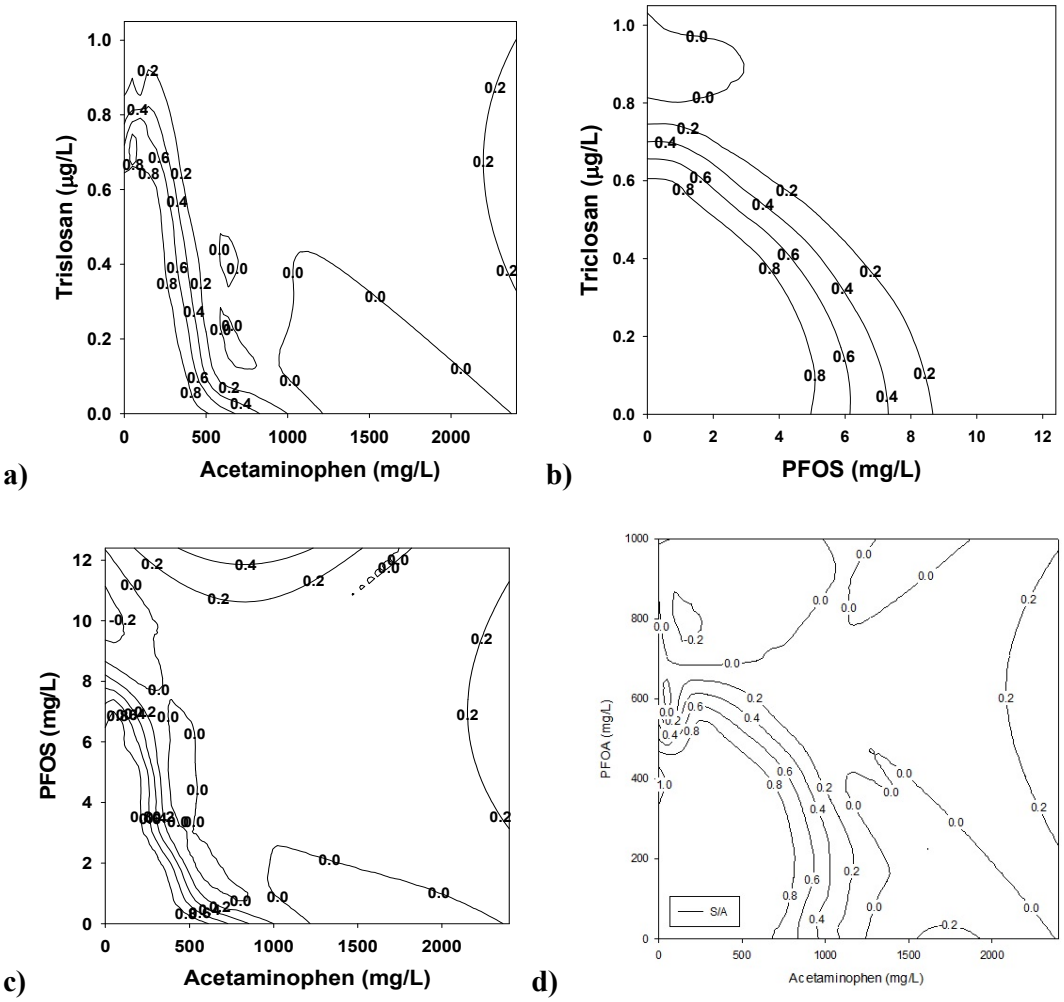

Figure 4. Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the survival data of zebrafish larvae exposed to a) acetaminophen with triclosan b) triclosan with PFOS, c) PFOS with acetaminophen and d) acetaminophen and PFOA in the Cremona water.

Lastly, the CA model was able to explain the data obtained from acetaminophen and PFOS exposure to *D. rerio* in the Bologna water ($p(\chi 2)<0.05$, R^2 =0.88, *SS*=41.9; **Table 8**). No significant deviations were observed when adding two more parameters to the model showing an additivity pattern (**Figure 8**).

The rest of the mixtures for the Bologna water (acetaminophen and triclosan, PFOS and triclosan) and FSW media (acetaminophen and PFOS, acetaminophen and triclosan, PFOS and triclosan) have been successfully conducted and completed up to this point but are still under

statistical analysis and therefore not able to present them at the moment. However, results will be published online in a peer-reviewed scientific paper and data after publication will be deposited to the appropriate depositary site chosen by WaterJPI consortium along with the rest of the results from the WE-NEED project.

Table 8. Summary of the mixture analysis of *Danio rerio* (survival data - 96h) exposed to the mixture of acetaminophen and PFOS in the Bologna water.

	acetaminophen and PFOS			
		Deviations from CA model		
	CA	S/A	DR	DL
R^2	0.88	0.88	0.89	0.88
SS	41.9	39.7	37.2	39.7
***p*(*F*-test)**	**<0.05**	-	-	-
$p(\chi^2)$	-	>0.05	>0.05	>0.05
max	0.93	0.93	0.93	0.93
a	-	-0.51	0.43	0.02
b	-	-	-2.26	29.28

R^2 represents the coefficient of determination; SS represents the sum of squared residuals; *p*(*F*-test) represents the result of the likelihood ratio test; $p(\chi^2)$ represents the outcome of the likelihood ratio test; max represents the control response, *a* and *b* represents the additional parameters of the function; CA represents the concentration addition model; S/A represents synergism/antagonism; DR represents the dose ratio dependence; DL represents the dose level dependence.

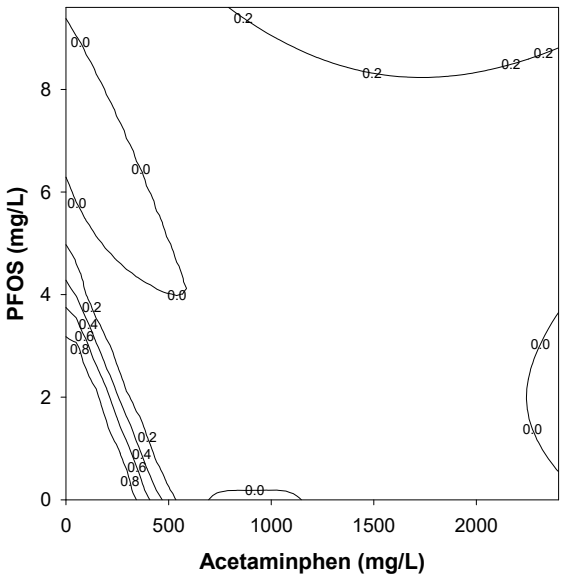

Figure 8. Binary mixture dose–response relationships (2D isobolic representation of the response surfaces) for the survival data of zebrafish larvae exposed to acetaminophen with PFOS in the Bologna water.

1.3 Complex mixtures approach in Cremona and Bologna groundwaters

Following a similar approach, as the one described in Section 1.2 Binary mixtures approach in Cremona and Bologna groundwaters, a total of 6 complex mixtures (three quaternary mixtures and three ternary mixtures) were performed in a step-by-step order using the model organism *Daphnia magna*.

The first three quaternary mixtures refer to four compounds found in the two groundwaters (boric acid, ammonium hydroxide and sodium fluoride) or in surface waters (acetaminophen). An equitoxic approach (fixed ratio design) was followed using the Toxic Unit (TU) concept where 1 TU equals to the LC_{50} of each compound. In **Table 9**, the experimental design can be seen. Results from the three quaternary mixtures when compared to the Concentration Addition model prediction show that, once again, the CA model is a conservative model (**Figure 9**). CA predicts a higher occurrence of a toxic effect in daphnids compared to the three waters, thus overestimating the effects of the four compounds when encountered in mixture. The least toxic water was the Cremona water, followed by the Bologna one and lastly, ASTM standard culture medium.

Table 9. Experimental design for acute toxicity experiments in *D. magna* testing four-component mixtures containing boric acid, ammonium hydroxide, sodium fluoride and

acetaminophen in ASTM standard culture medium, Cremona and Bologna groundwaters. Fixed-ratio design based on TU derived from ASTM data.

QUATERNARY MIXTURE	Boric acid (mg/L)	Ammonium hydroxide (mg/L)	Sodium fluoride (mg/L)	Acetaminophen (mg/L)	∑ TU
M1	5.51	0.70	4.13	0.02	0.03125
M2	11.02	1.41	8.26	0.04	0.0625
M3	22.04	2.82	16.52	0.08	0.125
M4	44.09	5.64	33.05	0.17	0.250
M5	88.17	11.27	66.09	0.33	0.5
M6	176.34	22.54	132.18	0.67	1
M7	264.52	33.82	198.27	1.00	1.5
M8	352.69	45.09	264.37	1.33	2
M9	705.37	90.18	528.73	2.66	4
M10	1410.75	180.35	1057.46	5.32	8

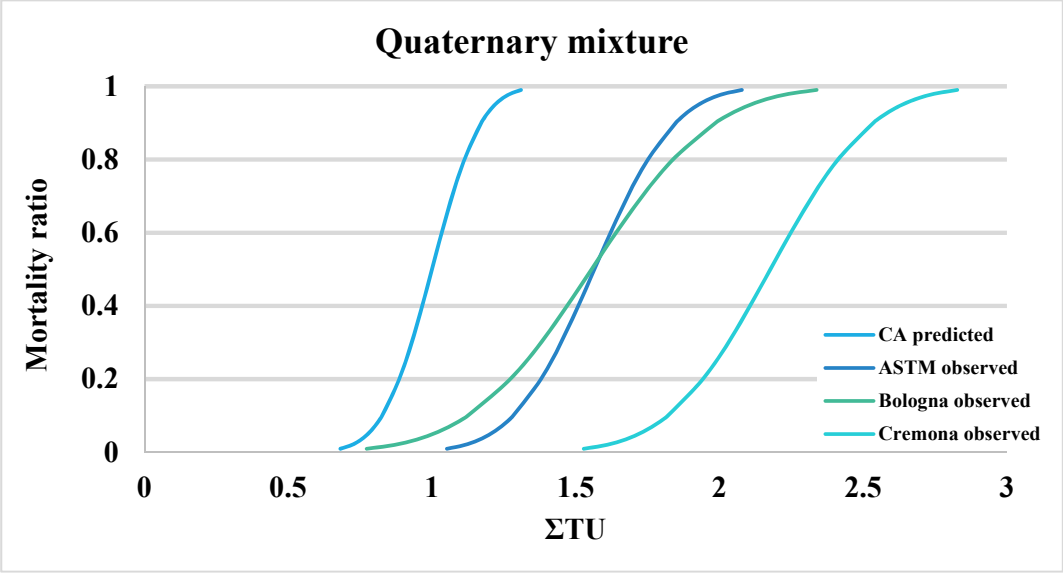

Figure 9. Quaternary mixture dose–response relationships showing the survival data of aphnids exposed to boric acid, ammonium hydroxide, sodium fluoride and acetaminophen in the ASTM culture medium, Cremona and Bologna groundwater.

Following the binary approach of the three identified as highly priority chemicals within the We-Need consortium (acetaminophen, triclosan nd PFOA) on *Daphnia magna*, two ternary mixtures were performed following the same concept as in the quaternary mixture described above. As observed above, the CA model appears to be more conservative in the ternary mixtures in both waters again and showing that CA predicts higher mortality of the daphnids compared to the one in the waters tested. Additionally, once again, the Bologna water promotes a higher toxicity (increased mortality) compared to the Cremona one. Such effect can be explained based on the differences of the groundwaters chemical composition.

Table 10. Experimental design for acute toxicity experiments in *D. magna* testing three-component mixtures containing acetaminophen, triclosan and PFOA in Cremona and Bologna groundwaters. Fixed-ratio design based on TU derived from ASTM data.

BOLOGNA MIXTURE	**Acetaminophen (mg/L)**	**Triclosan (mg/L)**	**PFOA (mg/L)**	**∑TU**
M1	0.04	0.02	3.39	0.02
M2	0.08	0.03	6.77	0.05
M3	0.15	0.07	13.57	0.09
M4	0.31	0.14	27.13	0.19
M5	0.62	0.28	54.26	0.38
M6	1.24	0.56	108.53	0.75
M7	1.85	0.84	162.79	1.13
M8	2.47	1.12	217.06	1.50
M9	4.94	2.23	434.12	3.00
M10	9.88	4.46	868.23	6.00
CREMONA MIXTURE	**Acetaminophen (mg/L)**	**Triclosan (mg/L)**	**PFOA (mg/L)**	**∑TU**
M1	0.02	0.01	3.34	0.02
M2	0.05	0.01	6.69	0.05
M3	0.10	0.03	13.40	0.09
M4	0.19	0.06	26.79	0.19
M5	0.39	0.12	53.59	0.38

M6	0.78	0.24	107.18	0.75
M7	1.16	0.36	160.77	1.13
M8	1.55	0.47	214.36	1.50
M9	3.10	0.95	428.71	3.00
M10	6.21	1.90	857.43	6.00

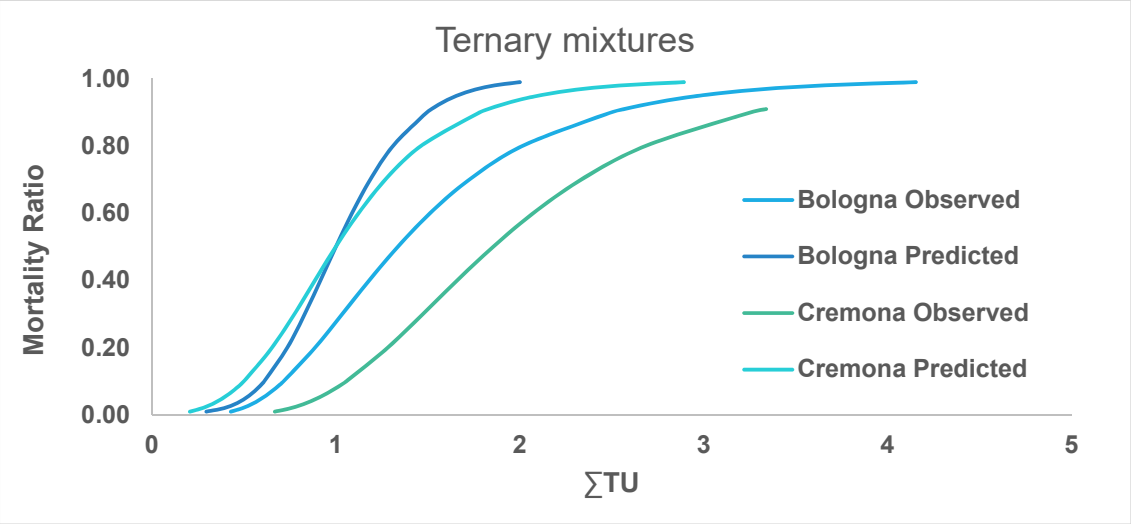

Figure 10. Ternary mixture dose–response relationships showing the survival data of daphnids exposed to acetaminophen, triclosan and PFOA in the Cremona and Bologna groundwater.

References

1. Jonker, M. J., Svendsen, C., Bedaux, J. J. M., Bongers, M. & Kammenga, J. E. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. *Environ Toxicol Chem* **24,** 2701–2713 (2005).
2. OECD. *OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems Test No. 202: Daphnia Sp. Acute Immobilisation Test*. (OECD Publishing, 2004). doi:http://dx.doi.org/10.1787/9789264069947-en

ANNEX 1

Table 1SD. Overview of mixture toxicity studies with *Pseukirchneriella subcapitata*, *Daphnia magna*, and *Danio rerio,* performed by Prof. Dr. Susana Loureiro's team where significant deviations to additivity were identified. $LC50_1$ and $LC50_2$, refer to the concentrations of chemicals 1 and 2 where 50% mortality occurs and values are expressed in mg/L. Reference model, denotes whether Concentration Addition (CA) or Independent Action (IA) were used as conceptual approach for testing additivity of the mixture. S/A denotes whether the deviations to additivity found in the toxicity of the mixture could be properly explained as synergism (Syn) or Antagonism (Ant) by extending the reference model with a single additional parameter $a_{S/A}$ (to note: if no values are shown, more complex deviations were required to properly explain the toxicity of the mixture).
DR/DL denotes whether deviations to additivity found required more complex dose-ratio (DR) or dose-level (DL) dependent deviation patterns, resulting from the extension of the reference model with two additional parameters (*a* and *b*).

Chemical mixture (Chem 1 + Chem 2)	***Species***	**$LC50_1$ (mg/L)**	**$LC50_2$ (mg/L)**	**Reference model**	**Deviation**					**medium**	**REF (DOI)**
					a **(S/A)**	**S/A**	**DR/DL**	***a***	***b***		
AgNP + ZnO NP	*Daphnia magna*	$12.49x10^{-3}$	3.74	CA			DL	-2.58	0.78	ASTM	10.1016/j.jhazmat.2016.07.068
AgNP + ZnO NP	*Daphnia magna*	$12.49x10^{-3}$	3.74	IA	-3.32	Syn				ASTM	10.1016/j.jhazmat.2016.07.068
$ZnCl_2$ + ZnO NP	*Daphnia magna*	1.74	4.75	CA	53.64	Ant				ASTM	10.1016/j.jhazmat.2016.07.068
$ZnCl_2$ + ZnO NP	*Daphnia magna*	1.74	4.75	IA						ASTM	10.1016/j.jhazmat.2016.07.068
AgNP + $AgNO_3$	*Daphnia magna*	$12.49x10^{-3}$	$15.23x10^{-3}$	CA			DR	-2.44	5.57	ASTM	10.1016/j.jhazmat.2016.07.068

AgNP + $AgNO_3$	*Daphnia magna*	$12.49x10^{-3}$	$15.23x10^{-3}$	IA	-21.92	Syn				ASTM	10.1016/j.jhazmat.2016.07.068
$ZnCl_2$ + $AgNO_3$	*Daphnia magna*	1.74	$15.23x10^{-3}$	CA			DL	2.04	10.01	ASTM	10.1016/j.jhazmat.2016.07.068
$ZnCl_2$ + $AgNO_3$	*Daphnia magna*	1.74	$15.23x10^{-3}$	IA			DL	34.12	2.17	ASTM	10.1016/j.jhazmat.2016.07.068
Imidacloprid + Chlorpyrifos	*Daphnia magna*	125.96	0.27	CA	4.56	Ant				ASTM	10.1002/etc.198
Imidacloprid + Chlorpyrifos	*Daphnia magna*	126.37	0.28	IA	22.06	Ant				ASTM	10.1002/etc.198
Imidacloprid + Thiacloprid	*Daphnia magna*	110.34	47.91	CA	-2.90	Syn				ASTM	10.1002/etc.198
Imidacloprid + Thiacloprid	*Daphnia magna*	109.32	47.36	IA	-8.17	Syn				ASTM	10.1002/etc.198
Ni + Chlorpyrifos	*Daphnia magna*	7.69	1.06	CA						ASTM	10.1002/etc.198
Ni + Chlorpyrifos	*Daphnia magna*	7.75	1.09	IA	-7.38	Syn				ASTM	10.1002/etc.198
Cd + Carbendazim	*Daphnia magna*	$79.05 x10^{-3}$	$156.7x10^{-3}$	IA			DR	-2.67	-13.93	ASTM	10.1016/j.aquatox.2008.05.012
Acetaminophen + Triclosan	*Danio rerio*	736.7	796.2	CA			DR	2.54	-4.46	Cremona	WE-NEED RESULTS
Acetaminophen + PFOS	*Danio rerio*	736.7	6.34	CA						Bologna	WE-NEED RESULTS
Acetaminophen + PFOS	*Danio rerio*	736.7	6.34	CA			DR	1.06	-2.94	Cremona	WE-NEED RESULTS
Acetaminophen + PFOA	*Daphnia magna*	2.92	323.8	CA			DL	0.66	-1.44	Cremona	WE-NEED RESULTS
Triclosan + PFOS	*Danio rerio*	792.2	6.34	CA	1.2	Ant				Cremona	WE-NEED RESULTS

Acetaminophen + PFOA	*Danio rerio*	801.8	544.9	CA	2.58	Ant				Cremona	WE-NEED RESULTS
Atrazine + Simazine	*Pseukirchneriella subcapitata*	196 $x10^{-3}$	252	CA			DR	0.18	-2.8	MBL	10.1007/s10646-011-0661-x
Atrazine + Terbuthylazine	*Pseukirchneriella subcapitata*	196 $x10^{-3}$	24 $x10^{-3}$	CA			DR	0.19	-4.81	MBL	10.1007/s10646-011-0661-x
Simazine + Terbuthylazine	*Pseukirchneriella subcapitata*	252 $x10^{-3}$	24 $x10^{-3}$	CA						MBL	10.1007/s10646-011-0661-x
Atrazine + Metalochlor	*Pseukirchneriella subcapitata*	196 $x10^{-3}$	98 $x10^{-3}$	IA			DR	5.59	-8.15	MBL	10.1007/s10646-011-0661-x
Simazine + Metalochlor	*Pseukirchneriella subcapitata*	252 $x10^{-3}$	98 $x10^{-3}$	IA			DR	-2.94	4.97	MBL	10.1007/s10646-011-0661-x
Terbuthylazine + Metalochlor	*Pseukirchneriella subcapitata*	24 $x10^{-3}$	98 $x10^{-3}$	IA			DR	-1.26	10.03	MBL	10.1007/s10646-011-0661-x
Ammonium perchlorate + Sodium perchlorate	*Daphnia magna*	396.21	3925	CA			DR	-4.37	9.10	ASTM	https://bit.ly/2x9aCnb
Triclosan + Carbendazim	*Daphnia magna*	856.8 $x10^{-3}$	87.6 $x10^{-3}$	IA			DL	0.017	-251.8	ASTM	10.1016/j.ecoenv.2015.02.022
Boric acid + Ammonium hydroxide	*Daphnia magna*	697.6	92	CA			DR	4.75	-5.65	ASTM	WE-NEED RESULTS
Boric acid + Sodium fluoride	*Daphnia magna*	697.6	540.2	CA	0.77	Ant				ASTM	WE-NEED RESULTS
Ammonium hydroxide + Sodium fluoride	*Daphnia magna*	92	540.2	CA			DL	0.62	-1.53	ASTM	WE-NEED RESULTS
Boric acid + Acetaminophen	*Daphnia magna*	697.6	3.39	CA			DL	6.48	0.36	ASTM	WE-NEED RESULTS
Ammonium hydroxide + Acetaminophen	*Daphnia magna*	92	3.39	CA			DL	-1.85	1.21	ASTM	WE-NEED RESULTS
Sodium fluoride + Acetaminophen	*Daphnia magna*	540.2	3.39	CA			DR	1.18	3.82	ASTM	WE-NEED RESULTS

ANNEX 2

Table 2SD. Toxicity data for PFOA, PFOS, Triclosan and Acetaminophen under the human risk assessment scope obtained after a bibliographic review.

Chemical	Species	Individual / organ / cell	Parameter	NOAEL	LOAEL	ED50	Ref (DOI)	Comments
Triclosan	*Homo sapiens*	Human keratinocyte HaCaT and hepatic L02 cells	DNA damage and chromosomal breakage	10 mM			10.1080/15287394.2019.1618758	
Triclosan	Salmonella	Ames test	Mutagenicity	0.1 mg/plate	10 mg/plate		10.1080/15287394.2019.1618758	
Acetaminophen	*Danio rerio*	Embryo development test	Cardiac edema	20 mg/L	25 mg/L		10.1371/journal.pone.0091874	
Acetaminophen	*Danio rerio*	Embryo development test	Red fluorescence intensity	2,5 mg/L	5 mg/L		10.1371/journal.pone.0091874	
Acetaminophen	*Danio rerio*	Embryo development test	Liver size	10 mg/L	20 mg/L		10.1371/journal.pone.0091874	
PFOA	CD-1 mice	Liver	Liver hepatomegaly	≈ 0.1 mg/kg	< 1 mg/kg		10.1093/toxsci/kfr076	
PFOA	Mice	Pregnant NMRI	Behaviour changes at age 4 months		0.49 $mg/kg_{bw}/d$		10.1289/ehp.11277	
PFOA	Rats	5 weeks-old, Male Fisher 344	Inhibition of gap-junctional intercellular communications in liver cells; increased liver weight		38 $mg/kg_{bw}/d$		doi.org/10.1289/ehp.11728	
PFOA	Japanese quail (*Coturnix coturnix japonica*)	3 week old, Male	Suppression of T-cell response		10 ppm		doi.org/10.1016/j.envpol.2013.03.063	

PFOA	Rats (Fischer 344)	6 weeks-old, Male (liver)	Increased liver weight and increased liver 8-hydroxydeoxyguanosine (8OH-dG)		24 $mg/kg_{bw}/d$	doi.org/10.1016/0304-3835(91)90063-N	
PFOA	*Macaca fasicularis* (cynomolgus monkeys)		Decreased serum T3 levels	0.03 mg/kg/d		doi.org/10.1093/toxsci/68.1.249	
PFOA	*Dugesia japonica*		Mortality	400 mg/L *	450 mg/L *	doi.org/10.1016/j.chemosphere.2007.08.032	(*) 96h, nominal concentrations
PFOA	Mouse	14 day feeding	Mortality (100%) at 3000 ppm; Decrease body weight and 1/5 females died at 300 ppm; Increase liver weights at P30 ppm. No histopathology.		30 ppm	doi.org/10.1016/0378-4274(87)90245-1	
PFOA	Mouse	21-day feeding	Increase liver weights at P3 ppm. No histopathology		1 ppm	doi.org/10.1016/0378-4274(87)90245-1	
APFO (ammonium perfluorooctan)	Mouse	28-day feeding	Mortality (100% or close to) at P300 ppm; Decrease body weights throughout study (100 ppm males and females); Decrease body Weight in week 4 (30 ppm females); Increase liver weights (30 and 100 ppm males and females); Panlobular diffuse hepatocellular hypertrophy accompanied by focal to multifocal cytoplasmic lipid vacuoles in 30 and 300 ppm animals.		30 ppm	doi.org/10.1080/15298668091425301	
APFO (ammonium perfluorooctan)	Rat	28-day feeding	Mortality (100%) at 10,000 and 30,000 ppm; Weight loss (males: P1000 ppm; females: 3000 ppm); Increase liver weights (males: P30 ppm; females: P1000 ppm). Liver effects: hepatocellular hypertrophy, hepatocyte degeneration and/or necrosis; Focal bile duct proliferation.		30 ppm	doi.org/10.1080/15298668091425301	
PFOA	Rat	28-day gavage	Decrease body weights (20 mg/kg/day); Increase relative liver, kidney and gonad weights (both doses); Hepatocyte hypertrophy with cytoplasmic vacuolation (both doses);		5 mg/kg/d	doi.org/10.1007/s00244-008-9194-6	

			Congestion and thickened epithelial walls in lung (both doses); Turbidness and tumefaction in kidney proximal convoluted tubular epithelium (20 mg/kg).			
APFO (ammonium perfluorooctan)	*Macaca fasicularis* (cynomolgus monkeys)	6-month gavage	Increase liver weights at all dose levels; Body weight loss at 30/20 mg/kg.		3 mg/kg/d	doi.org/10.1093/toxsci/69.1.244
PFOA	CD-1 mouse	Pre- and postnatal developmental	Decrease aternal weight gain (P20 mg/kg-day); Increase maternal liver weights (P1 mg/kg day); Increase full litter resorptions (P5 mg/kg-day); Increase prenatal fetal loss and decrease live fetal weights (<20 mg/kg-day); Increase reduced ossification and minor tail/ limb defects (P1 mg/kg); Increase neonatal mortality and survival (P10 mg/kg); Decrease fetal body weights (P3 mg/kg); Increase delayed eye opening (P5 mg/kg); Increase delay with either age at vaginal opening or time to first estrus (20 mg/kg); Increase age of preputial separation in males (P1 mg/kg).	3 mg/kg/day (other developmental effects)	1 mg/kg/d (reduced ossification)	doi.org/10.1093/toxsci/kfj105
PFOA	CD-1 mouse	Prenataldevelopmental	Histopathology of liver showed abundance of fine eosinophilic granules; Gene expression associated with fatty acid catabolism altered in fetal liver and lung; Gene expression associated with lipid transport, ketogenesis, glucose and lipoprotein metabolism, phospholipid and steroid metabolism, retinol metabolism, cholesterol and bile acid synthesis, proteosome activation, and inflammation altered in fetal liver;		1 mg/kg/d	doi.org/10.1016/j.tox.2007.06.095

PFOS	*Macaca fasicularis* (cynomolgus monkeys)	Oral (capsule)	Most sensitive endpoint: Increased TSH (males) Reduced T3 (males, females) Reduced HDL (f) (182 days)	0.03 $mg/kg_{bw}/d$	0.75 $mg/kg_{bw}/d$	10.2903/j.efsa.2008.653 10.1093/toxsci/68.1.249	
PFOS	Crl:CD® (SD)IGS BR VAF® rats		Reproductive outcome (F0)	1.6 mg/kg/d		10.1016/j.tox.2005.07.018	
PFOS	Rats	Chronic toxicity / carcinogenenicity. Oral diet	Most sensitive endpoint: Liver pathology: hepatocellular hypertrophy Neoplastic effects: hepatocellular adenomas (males/females); Thyroid follicular cell adenomas/carcinomas (females; 0.15 – 0.57 mg/kg_{bw}/ day).	0.02-0.06 (males) 0.04 * 0.07-0.2 (females) 0.14 * (mg/kg/d)	0.06-0.2 (males) 0.14 * 0.2-0.6 (females) 0.37 * (mg/kg/d)	Thomford, P. J. (2002). 104-week dietary chronic toxicity and carcinogenicity study with perfluorooctane sulfonic acid potassium salt (PFOS; T-6295) in rats. Final Report, 3M T-6295 (Covance Study No. 6329-183, vol. I–IX, 4068 pgs, 3M, St. Paul, MN.	(*) The mean daily exposure values as calculated as the average of the range of calculated exposure cited in the report. LD: Lactation day
PFOS	Rabbits	Developmental toxicity.Oral gavage	Most sensitive endpoint: GD7-20 Maternal: reduced body weight and food consumption Foetal: reduced birth weight and delayed ossification	Maternal: 0.1 Foetal: 1 (mg/kg/d)	Maternal: 1 Foetal: 2 (mg/kg/d)	Case, M.T., York, R.G., Christian, M.S. 2001. Rat and rabbit oral developmental toxicology studies with two perfluorinated compounds. Int J Toxicol. 20, 101-109.	
PFOS	Rats	Developmental toxicity.Oral gavage	Most sensitive endpoint: GD2-21 Maternal: reduced body weight Reduced serum T4 Newborns: Post natal death Reduced weight gain Delayed eye opening Sternal defects Reduced serum T4	n.a.	Maternal: 1 Foetal: 1 (mg/kg/d)	10.1093/toxsci/kfg122 10.1093/toxsci/kfg121	n.a. = not available

PFOS	Mice	Developmental toxicity.Oral gavage	Most sensitive endpoint: GD1-18 Maternal: increased liver weight and reduced serum triglycerides Foetus: Postnatal death Reduced foetal weight Delayed eye opening	Maternal: 1 Foetal: — (mg/kg/d)	Maternal: 5 Foetal: 1 (mg/kg/d)	10.1093/toxsci/kfg122 10.1093/toxsci/kfg121
PFOS	Rats	Developmental toxicity. Oral gavage Two generation study Rats (m/f) 35 rats /dose group	Most sensitive endpoint: F0 (males): from 42 days prior to mating, to the end of mating, F0 (females): from 42 days prior to mating to LD21 F1(males): from 22 days after birth to the end of mating, F1(females): from 22 days after birth to LD21 of F2 F0 (males/females) reduced body weight gain F1 reduced weight gain F2 reductions in mean pup body weight.	0.1 mg/kg$_{bw}$/d	0.4 0.1 (males) (lowest dose tested) 0.4 mg/kg$_{bw}$/d	Christian, M.S., Hoberman, A.M. and York, R.G. 1999. Combined Oral (gavage) Fertility, Developmental and Perinatal/Postnatal Reproduction Toxicity Study of PFOS in Rats. Argus Research Laboratories, Inc., Horsham, PA U.S EPA. Docket 8EHQ-0200-00374.
PFOS	Mouse, C57BL6	Neurotoxicity study (90 day)	Impaired learning and memory; increased apoptosis in hippocampal cells	0.43 mg/kg$_{bw}$/d	2.15 mg/kg$_{bw}$/d	10.1371/journal.pone.0054176
PFOS	Rat, Sprague Dawley	Developmental neurotoxicity	Developmental neurotoxicity	1.0 mg/kg$_{bw}$/d	>1 mg/kg$_{bw}$/d	10.1016/j.reprotox.2009.01.005
PFOS	Mouse, C57BL6	Immunotoxicity Study (60 day)	Reduced SRBC plaque forming cell response	0.008 mg/kg$_{bw}$/d	0.083 mg/kg$_{bw}$/d	10.1007/s00204-009-0424-0
triclosan	*Homo sapiens*	MCF-7 BOS human breast cancer cells	Cell viability	20 µL/mL		DOI 10.1002/jat.1736
triclosan	*Homo sapiens*	anoikis-resistant human lung cancer H460 cells	Cell viability and apoptosis	7,5 µM	10 µM	10.1371/journal.pone.0110851
acetaminophen	*Phalloceros harpagos* (fish)	Scototaxis test	Behaviour	8 mg/L		10.1007/s11356-018-2839-8

acetaminophen	*Oryzias latipes*	Acute fish toxicity test (OECD TG 203)	Organism lethality	>160 mg		10.1016/j.envint.2006.11.017
acetaminophen	*Homo sapiens*	human hepatocytes	Cell viability (liver chips at day 10)		2.4 mM	10.1007/s00204-019-02427-4
acetaminophen	*Homo sapiens*	human hepatocytes	Cell viability (liver spheroids at day 10)		1.7 mM	10.1007/s00204-019-02427-4
acetaminophen	*Homo sapiens*	human hepatocytes	Albumin release (endpoint for liver function, liver chips at day 10)		1.5 mM	10.1007/s00204-019-02427-4
acetaminophen	*Homo sapiens*	human hepatocytes	Albumin release (endpoint for liver function, liver chips at day 10)		2.4 mM	10.1007/s00204-019-02427-4