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Bibliography data on Emergent Chemicals. Ecotoxicity tests with Daphnia magna and Danio rerio, using Emergent Chemical detected in Cremona and Bologna groundwater

D4.1 Report on EC toxicities from bibliography and laboratory experiments.

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1.1. Emerging Contaminants (Bibliography)

Groundwater contamination is one of the most important environmental threats resulting from anthropogenic activities. Besides constituting the primary source of drinking water, groundwater aquifers are vital for irrigation in agriculture, industry and for sustaining natural ecosystems functioning and health. Groundwater aquifers are thus limited resources, often overlooked and highly prone to deterioration. Groundwater Directive (2006/118/EC) was created to protect groundwater bodies from contamination by setting quality standards and establishing threshold limits for a set of priority pollutants. However, an extensive group of emerging contaminants (EC) whose effects are largely unknown, such as pharmaceuticals, personal care products, engineered nanomaterials or industrial chemicals, still have no regulatory status which has raised concerns over human and environmental health.

Under the scope of WP4: Ecotoxicology, the first approach towards such contaminants is to identify them and report their toxicity to the model organisms suggested in the current WP (*Daphnia magna* and *Danio rerio*) that will be further used for assessing EC toxicities and mixtures in groundwater samples in future works (reported in future deliverables). Further information concerning several EC effects (as suggested in WP3 including the ones identified in the two aquifers, Cremona and Bologna) in the two model organisms as found from bibliography can be seen in **Table 1**.

Table 1: Toxicity of Emerging Contaminants to *D. magna* and *D. rerio*. Units in mg/L. – not reported. hpf – hours post fertilization, dph – days post hatching

Emerging Contaminant	Species	Endpoint	Age	Temperature	Duration	EC_{50}	Author
Pharmaceuticals							
Acetaminophen	*Daphnia magna*	Immobility	<24h	20ºC	48h	30.1	(Y. Kim et al., 2007)
	Daphnia magna	Immobility	<24h	20ºC	24h	224	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	40	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	21d	5.32	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	21ºC	24h	53.87	(P. Kim et al., 2012)
	Daphnia magna	Immobility	<24h	21ºC	48h	11.85	(P. Kim et al., 2012)
	Daphnia magna	Reproduction (NOEC)	<24h	21ºC	21d	5.72	(P. Kim et al., 2012)
	Daphnia magna	Immobility	<24h	20ºC	48h	2.83	(de Oliveira et al., 2016)
	Daphnia magna	Reproduction (NOEC)	<24h	20ºC	21d	>1.25	(de Oliveira et al., 2016)
	Danio rerio	Mortality	2hpf	28.5ºC	24hpf	1530	(Selderslaghs et al., 2012)

	Danio rerio	Mortality	2hpf	28.5ºC	48hpf	1498	(Selderslaghs et al., 2012)
	Danio rerio	Mortality	2hpf	28.5ºC	72hpf	1188	(Selderslaghs et al., 2012)
	Danio rerio	Teratogenic Effects	2hpf	28.5ºC	72hpf	941	(Selderslaghs et al., 2012)
	Danio rerio	Mortality	2hpf	27ºC	7dph	0.1	(David and Pancharatna, 2009)
	Danio rerio	Hatching Success	2hpf	27ºC	5dph	0.1	(David and Pancharatna, 2009)
Amoxicillin	*Daphnia magna*	Immobility	<24h	20ºC	48h	>100	(Rizzo et al., 2009)
	Daphnia magna	Immobility	<24h	20ºC	48h	1060	(Ortiz de García et al., 2014)
	Danio rerio	Mortality	2hpf	27ºC	96hpf	>1125	(Oliveira et al., 2013)
	Danio rerio	Premature hatching/ Malformations	2hpf	27ºC	48hpf	>221	(Oliveira et al., 2013)
Azithromycin	*Daphnia magna*	Immobility	<24h	20ºC	48h	>10	(Harada et al., 2008)
Carbamazepine	*Daphnia magna*	Immobility	<24h	20ºC	48h	>100	(Y. Kim et al., 2007)
	Daphnia magna	Immobility	<24h	20ºC	48h	>10	(Harada et al., 2008)
	Daphnia magna	Immobility	<24h	20ºC	48h	>5	(Rizzo et al., 2009)
	Daphnia magna	Immobility	<24h	20ºC	48h	>100	(Cleuvers, 2003)
Clarithromycin	*Daphnia magna*	Immobility	<24h	20ºC	48h	38.65	(Ortiz de García et al., 2014)
	Daphnia magna	Immobility	<24h	20ºC	48h	>2	(Baumann et al., 2015)
	Daphnia magna	Reproduction (NOEC)	<24h	20ºC	21d	>2.1	(Baumann et al., 2015)
	Daphnia magna	Immobility	<24h	20ºC	48h	25.72	(Isidori et al., 2005)
	Daphnia magna	Immobility	<24h	20ºC	48h	>10	(Harada et al., 2008)
	Danio rerio	Mortality	2hpf	28ºC	48h	>2	(Baumann et al., 2015)
	Danio rerio	Mortality	Adults	23ºC	96h	>1000	(Isidori et al., 2005)
Diclofenac	*Daphnia magna*	Immobility	<24h	20ºC	48h	108	(Cleuvers, 2003)
	Daphnia magna	Immobility	<24h	20ºC	24h	82.3	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	18.1	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	21d	2	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	123.3	(de Oliveira et al., 2016)
	Daphnia magna	Reproduction (NOEC)	<24h	20ºC	21d	>72	(de Oliveira et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	39.9-44.7	(Haap et al., 2008)
	Danio rerio	Mortality	2hpf	-	96h	480	(Dietrich and Prietz, 1999)
	Danio rerio	Teratogenic Effects	2hpf	-	96h	941	(Dietrich and Prietz, 1999)
Erythromycin	*Daphnia magna*	Immobility	<24h	20ºC	48h	136.13	(Ortiz de García et al., 2014)

	Daphnia magna	Immobility	<24h	20ºC	48h	22.45	(Isidori et al., 2005)
	Danio rerio	Mortality	Adults	23ºC	96h	>1000	(Isidori et al., 2005)
Ibuprofen	*Daphnia magna*	Immobility	<24h	20ºC	48h	108	(Cleuvers, 2003)
	Daphnia magna	Immobility	<24h	20ºC	48h	>10	(Harada et al., 2008)
	Daphnia magna	Immobility	<24h	20ºC	48h	30.90	(Ortiz de García et al., 2014)
	Daphnia magna	Immobility	<24h	20ºC	24h	116	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	23.5	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	21d	3.97	(Du et al., 2016)
	Daphnia magna	Immobility	<24h	20ºC	48h	9.06	(Halling-Sørensen et al., 1998)
PCP							
Triclosan	*Daphnia magna*	Immobility	<24h	20ºC	48h	0.34	(Orvos et al., 2002)
	Daphnia magna	Immobility	<24h	20ºC	48h	0.48	(Ortiz de García et al., 2014)
	Daphnia magna	Immobility	<24h	20ºC	48h	0.26	(Harada et al., 2008)
	Danio rerio	Mortality	2hpf	27ºC	24hpf	1.57	(Oliveira et al., 2009)
	Danio rerio	Mortality	2hpf	27ºC	48hpf	0.52	(Oliveira et al., 2009)
	Danio rerio	Mortality	2hpf	27ºC	72hpf	0.43	(Oliveira et al., 2009)
	Danio rerio	Mortality	2hpf	27ºC	96hpf	0.42	(Oliveira et al., 2009)
Ethylparaben	*Daphnia magna*	Immobility	<24h	20ºC	48h	4.6	(Ortiz de García et al., 2014)
Propylparaben	*Daphnia magna*	Immobility	<24h	20ºC	48h	2.63	(Ortiz de García et al., 2014)
Methylparaben	*Daphnia magna*	Immobility	<24h	20ºC	48h	42.72	(Ortiz de García et al., 2014)
Industrial Chemicals							
Ammonium Hydroxide	*Daphnia magna*	Mortality	<24h	20ºC	24h	60	(Dowden and Bennett, 1965)
	Daphnia magna	Mortality	<24h	20ºC	48h	32	(Dowden and Bennett, 1965)
	Daphnia magna	Mortality	<24h	20ºC	96h	20	(Dowden and Bennett, 1965)
Boric Acid	*Daphnia magna*	Immobility	<24h	20ºC	48h	226	(Lewis and Valentine, 1981)
	Daphnia magna	Immobility (NOEC)	<24h	20ºC	48h	<200	(Lewis and Valentine, 1981)
	Daphnia magna	Mortality	<24h	20ºC	21d	53.2	(Lewis and Valentine, 1981)
	Daphnia magna	Reproduction	<24h	20ºC	21d	53	(Lewis and Valentine, 1981)
	Danio rerio	Teratogenic Effects	2hpf	28.5ºC	24hpf	3929	(Selderslaghs et al., 2012)
	Danio rerio	Mortality	2hpf	28.5ºC	48hpf	3906	(Selderslaghs et al., 2012)
	Danio rerio	Teratogenic Effects	2hpf	28.5ºC	48hpf	1561	(Selderslaghs et al., 2012)
	Danio rerio	Mortality	2hpf	28.5ºC	72hpf	2609	(Selderslaghs et al., 2012)
	Danio rerio	Teratogenic Effects	2hpf	28.5ºC	72hpf	1450	(Selderslaghs et al., 2012)

Sodium Fluoride	*Daphnia magna*	Immobility	<24h	20ºC	24h	454	(Dave, 1984)
	Daphnia magna	Immobility	<24h	20ºC	48h	216	(Dave, 1984)
	Daphnia magna	Reproduction (NOEC)	<24h	20ºC	21d	4.4	(Dave, 1984)
	Daphnia magna	Mortality	<24h	15ºC	24h	385	(Fieser et al., 1986)
	Daphnia magna	Mortality	<24h	20ºC	24h	279	(Fieser et al., 1986)
	Daphnia magna	Mortality	<24h	25ºC	24h	201	(Fieser et al., 1986)
Tetrachloroethylene (PCE)	*Daphnia magna*	Immobility	<24h	20ºC	48h	8.5	(Richter et al., 1983)
	Daphnia magna	Mortality	<24h	20ºC	48h	18	(Richter et al., 1983)
	Daphnia magna	Reproduction (NOEC)	<24h	20ºC	28d	0.25	(Richter et al., 1983)
	Daphnia magna	Reproduction (LOEC)	<24h	20ºC	28d	0.48	(Richter et al., 1983)
	Daphnia magna	Mortality	<24h	20ºC	24h	18	(LeBlanc, 1980)
	Daphnia magna	Mortality	<24h	20ºC	48h	18	(LeBlanc, 1980)
	Daphnia magna	Mortality (NOEC)	<24h	20ºC	48h	10	(LeBlanc, 1980)
ENM							
Ag NP	*Daphnia magna*	Immobility	<24h	20ºC	48h	0.01	(Ribeiro et al., 2014)
	Daphnia magna	Feeding Inhibition	<24h	20ºC	24h	0.01	(Ribeiro et al., 2014)
	Daphnia magna	Reproduction	<24h	20ºC	21d	0.001	(Ribeiro et al., 2014)
	Danio rerio	Mortality	2hpf	25ºC	96h	0.13	(Ribeiro et al., 2014)
TiO_2 NP	*Danio rerio*	Mortality	120d	23ºC	96h	124.5	(Xiong et al., 2011)
ZnO NP	*Daphnia magna*	Immobility	<24h	20ºC	48h	0.99	(Xiao et al., 2015)
	Daphnia magna	Immobility	<24h	20ºC	48h	1.32	(Lopes et al., 2014)
	Danio rerio	Mortality	120d	23ºC	96h	4.92	(Xiong et al., 2011)
	Danio rerio	Mortality	2hpf	25ºC	96h	27.21	(Azevedo et al., 2016)
Microplastics							
PE-1µm	*Daphnia magna*	Immobility	<24h	20ºC	48h	57.43	(Rehse et al., 2016)
PE-100µm	*Daphnia magna*	Immobility	<24h	20ºC	48h	No Observable Effect	(Rehse et al., 2016)

As can be seen from the table presented above regarding toxicities of those emerging contaminants, values vary significantly between studies. Such variation can be attributed to *e.g.* different medium characteristics, food type, temperature, test duration or organism clones.

1.2. Emerging Contaminants (laboratory experiments)

1.2.1 Research methodology, approach and results

Within the scope of WP4: Ecotoxicology, several laboratory standardized protocols were carried out to assess toxicity patterns for the following chemicals: sodium fluoride (NaF), boric acid (H_3BO_3) and ammonium hydroxide (NH_4OH), after being initially identified as the ones found and of some concern in the two groundwater circulation points from Italy, Cremona and Bologna aquifers. Acute toxicity tests with *Daphnia magna* were performed following the OECD 202 protocol (OECD, 2004), by exposing the daphnids to a range of concentrations of each chemical (5 replicates per concentration with 5 daphnids per replicate). Chronic toxicity tests were accomplished following the OECD 221 protocol (OECD, 2008) (10 replicates per concentration with 1 organism per replicate). Feeding inhibition tests with *Daphnia magna*, based on the experimental procedures proposed by McWilliam and Baird (2002) and Allen et al. (1995), were also performed (5 replicates per concentration with 5 daphnids per replicate). The second model species tested was *Danio rerio*. Herein, Fish Embryo Acute Toxicity tests (FET) were accomplished following the OECD 236 guideline (OECD, 2013) and the methodology described by Lammer, Carr et al. 2009, with some adaptations (30 replicates per concentration with 1 embryo per replicate). Each test was performed with the respective culture medium: American Society for Testing and Materials (ASTM) moderated-hard-water medium (ASTM, 1980) for *D. magna* and fish system water for *D. rerio*. These results will allow us to start the next step with valuable data on the toxicity patterns of each chemical, by adapting the same methodologies to the Cremona and Bologna groundwater types.

1.2.2 Boric acid (H_3BO_3)

Regarding boric acid, an acute toxicity test and a feeding inhibition test with *D. magna* were successfully completed. From the first test a 48h LC_{50} value of 705.73 mg/L was retrieved and a 24h EC_{50} of 573.22 mg/L was retrieved for the second. Figure 1 shows the feeding rates obtained for each concentration of exposure in the feeding inhibition test, where statistical significant differences were obtained for the last two concentrations tested.

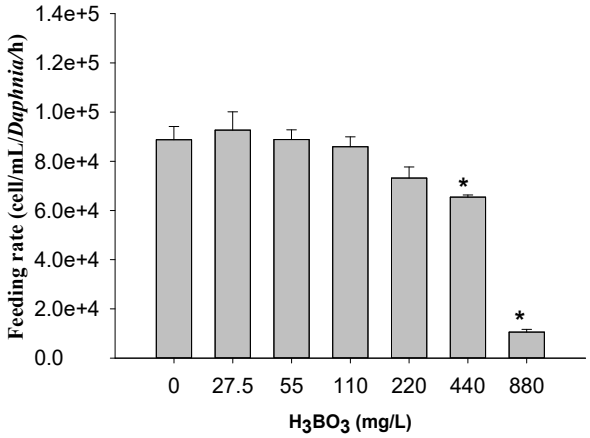


Figure 1. Feeding rates (cells/mL/daphnia/h) of *D. magna* exposed to boric acid for 24 h. Data are expressed as mean values with standard error. Asterisks denote statistically significant differences (Dunnet's method, $p < 0.05$).

The FET test with *D. rerio* was performed as well. A mortality of 100% was observed for the last two concentrations of exposure, as shown in Figure 2a), and a 96h LC_{50} value of 1617.63 mg/L retrieved. Hatching success was above 80% for all concentrations, except where mortality reached 100% (Figure 2b).

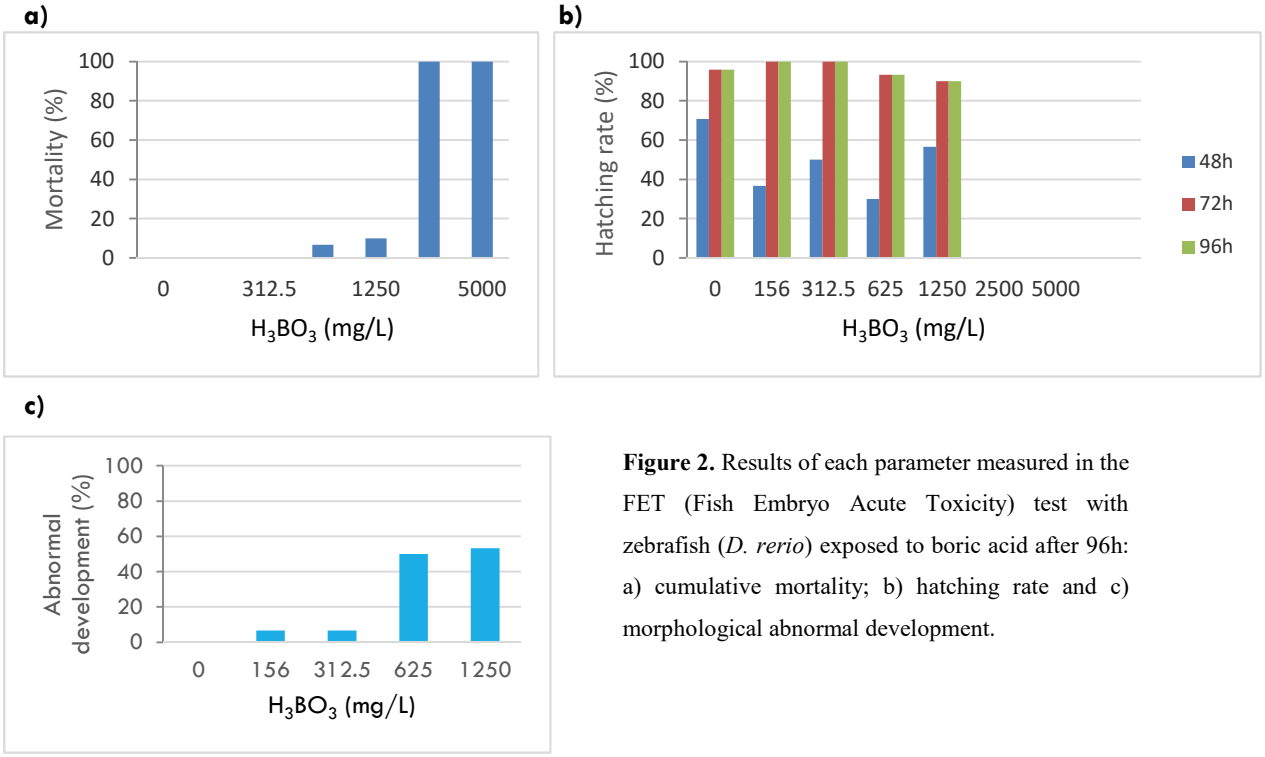


Figure 2. Results of each parameter measured in the FET (Fish Embryo Acute Toxicity) test with zebrafish (*D. rerio*) exposed to boric acid after 96h: a) cumulative mortality; b) hatching rate and c) morphological abnormal development.

Morphological malformations such as edema and malformation of the tail were observed in a higher percentage for the concentrations of 625 and 1250 mg/L of boric acid (Figure 2c and Figure 3).

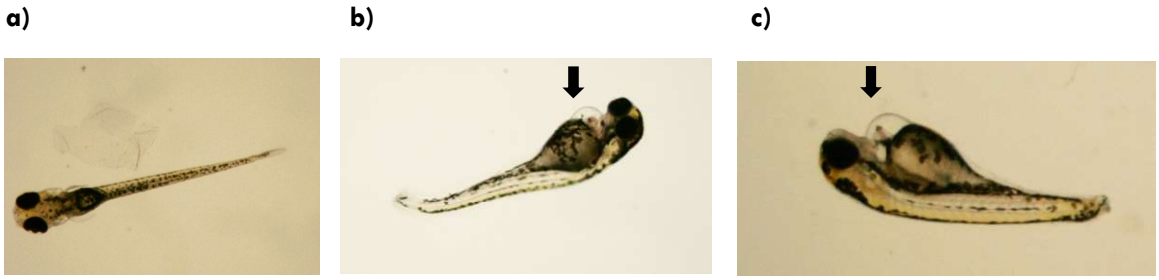


Figure 3. Morphological malformations observed in zebrafish (*D. rerio*) larvae exposed to boric acid after 96h: a) hatched control larvae with normal development; b) and c) larvae exposed to 625 mg/L of boric acid with edema (arrow) and tail malformation.

1.2.3 Sodium fluoride (NaF)

For the sodium fluoride (NaF), the acute toxicity test provided a 48h LC_{50} value of 528.73 mg/L, however, the feeding inhibition test was not concluded due to NaF reacting with the algae by creating precipitates. In this case, the green microalgae, *Raphidocelis subcapitata*, used as a food source for *D. magna* present in the test media (ASTM), seemed to react with NaF forming visible flocculates (Figure 4). In this way, food became unavailable for the daphnids making the purpose of this test impossible to fulfil.

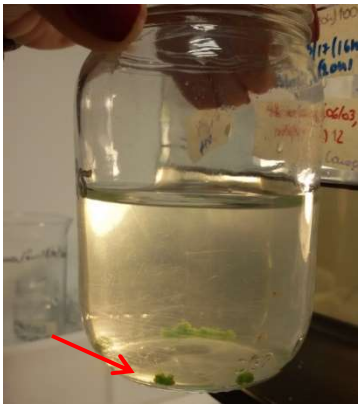


Figure 4. *Raphidocelis subcapitata* flocculation in the ASTM medium with NaF.

In the figure 5 below, are presented the results of the FET for NaF. The mortality observed was low in the 12.5 and 50 mg/L of NaF (Figure 5a). Additionally, hatching success was not affected, being higher than 90% in all the tested concentrations (Figure 5b). Although, in a low percentage (<20%), some abnormalities such as tail malformations and impaired equilibrium were found in the FET test (figure 5c and figure 6).

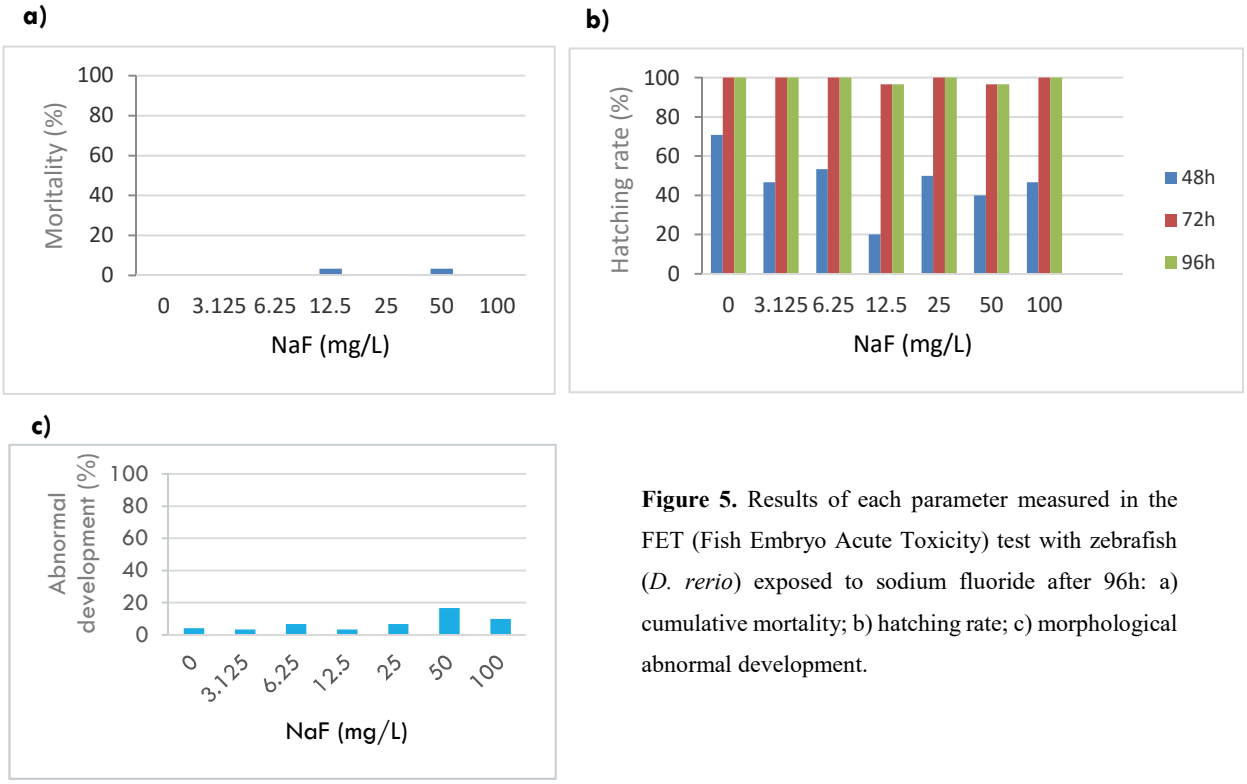
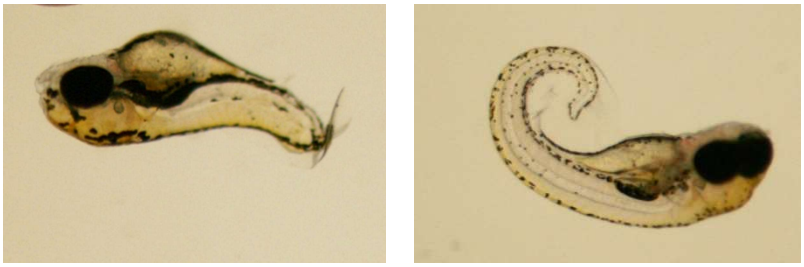


Figure 5. Results of each parameter measured in the FET (Fish Embryo Acute Toxicity) test with zebrafish (*D. rerio*) exposed to sodium fluoride after 96h: a) cumulative mortality; b) hatching rate; c) morphological abnormal development.



a) b)

ɔserved in zebrafish (*D. rerio*) larvae
ı: a) larvae exposed to 12.5 mg/L of NaF
o 100 mg/L of NaF with tail

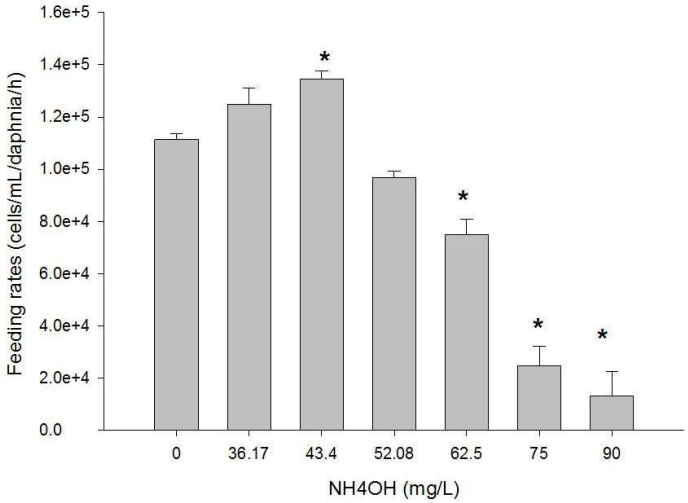


Figure 7. Feeding rates (cells/mL/daphnia/h) of D. magna exposed to ammonium hydroxide for 24 h. Data are expressed as mean values with standard error. Asterisks denote statistically significant differences (Dunnet's method, $p < 0.05$).

1.2.4 Ammonium hydroxide (NH_4OH)

For ammonium hydroxide all laboratory standardized protocols previously mentioned were successfully completed. While assessing the immobilization of daphnids, an LC_{50} value (48h) of 90.18 mg/L was observed. The feeding inhibition test followed a dose–response relationship (Figure 7), with an EC_{50} value of 64.93 mg/L (24h exposure).

In the reproduction toxicity test, after 21 days of exposure, the mean number of neonates per daphnia was similar in all the tested concentrations (Figure 8). Thus, no EC_{50} was retrieved.

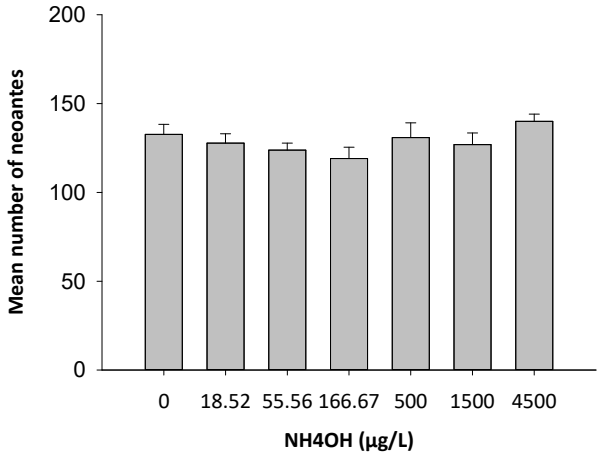


Figure 8. Reproduction effort during a 21 days exposure period of *D. magna* to ammonium hydroxide. Data are expressed as mean values with standard error.

As for the FET test with *D. rerio*, mortality was low (Figure 9a) and hatching success was high in all concentrations tested (>90%). Low percentages of abnormalities such as tail malformations and impaired equilibrium were observed.

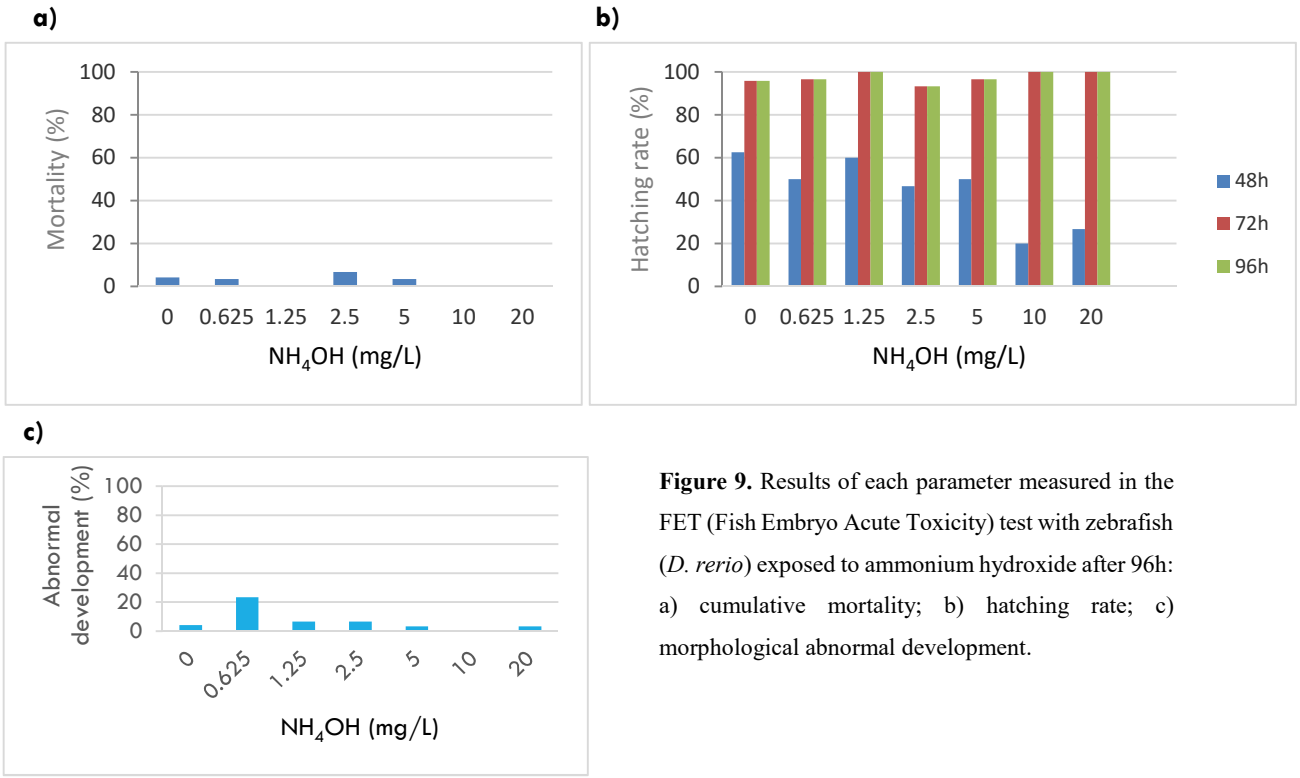


Figure 9. Results of each parameter measured in the FET (Fish Embryo Acute Toxicity) test with zebrafish (*D. rerio*) exposed to ammonium hydroxide after 96h: a) cumulative mortality; b) hatching rate; c) morphological abnormal development.

The above obtained results show that the chemicals at the measured concentrations encountered in the two groundwater points from Cremona and Bologna, will most likely have no ecological hazard effects. However, these chemicals normally appear as complex mixtures, comprising multiple compounds of different chemical classes and their metabolites. Understanding and predicting the toxicity and behavior of such mixtures is of paramount importance to adapt groundwater regulation to these new challenges and to develop sustainable management strategies. Future work will assess the toxicity of the compounds already

identified (WP3) using a synthetic water composition of the two Italian aquifers and their possible complex mixtures using conceptual models under realistic scenarios in order to be able to develop a risk assessment model for groundwater.

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