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Deliverable Number:	D4.2
Work package number:	WP4
Deliverable title	Report on the toxicity of samples from the two field sites
Type	Report
Dissemination Level	Public
Lead participant	UAVR
Contributing scientists and other personnel	Susana Loureiro, Rui Morgado, Maria Pavlaki, Ana Rita Silva, Sandra Gonçalves
Scheduled delivery date	20 October 2017
Actual / forecast delivery data	20 October 2017
Deliverable summary text: Ecotoxicity tests with Daphnia magna and Danio rerio, using Cremona and Bologna groundwaters	

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. This report follows the deliverable D4.1, 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, using standard protocols, of additional anthropogenic contaminants known to be relevant within the project case studies (Bologna and Cremona aquifers). After identifying and testing the toxicity of relevant ECs in *D. magna* and *D. rerio*, following well established protocols, the next steps include: 1) development and optimization of protocols for toxicity assessments using the synthetic groundwater that mimics Bologna and Cremona samples (provided by the Weizmann Institute of Science); 2) report the toxicity of selected ECs in *D. magna* and *D. rerio* using each of these synthetic groundwaters and further comparison to standard toxicity results.

As a first approach, toxicity tests using the synthetic groundwaters were performed for each selected ECs in order to compare the ecotoxicological patterns of each compound under several medium conditions (section 1.2 Ecotoxicity testing of selected ECs in synthetic groundwaters). Subsequently, we evaluated the performance of *D. magna* and *D. rerio* in the synthetic groundwater samples of Bologna and Cremona (with and without the ECs, according to concentrations measured in the field) (section 1.3 Testing of Bologna and Cremona synthetic groundwaters).

1.2. Ecotoxicity testing of selected ECs in synthetic groundwaters

For practical reasons, given the absence of sufficient groundwater model organisms for toxicity testing of groundwater settings, surface water organisms are used as surrogates. *D. magna* and *D. rerio* are among of the most widely used model organisms for ecotoxicity testing in aquatic ecosystems, including surface and groundwaters. Besides their convenience and tractability, the fact that standard protocols have been developed and these organisms' responses have been thoroughly described for many decades makes it possible to identify any causality, which is a critical requirement for supporting environmental risk assessments. Using these tests with conditions other than the standard, however, implies a previous assessment and validation of their responses.

After chemical analysis of the groundwater of the two aquifers (Bologna and Cremona), the **Weizmann Institute of Science** (**WP3 leader**) successfully recreated a recipe for synthetic groundwater that mimics the two field sites. **Table 1** shows the composition of the synthetic waters used to mimic Bologna and Cremona aquifers. The compounds at the top were considered as constituents of the waters whereas the compounds in the bottom part of the table (highlighted in red) were considered as contaminants of anthropogenic origin. Although it was detected in groundwaters, PCE could not be used since it was not possible to dissolve it in water nor ASTM/FSW, with or without solvents (it must be noted that the amount of solvent within the test system must not exceed 10%). As such, henceforth, PCE is not included in the synthetic groundwaters whenever these were tested along with the EC detected in the field.

Table 1. Water composition of Bologna and Cremona waters and emergent contaminants highlighted with the red line.

			Bologna Concentration		Cremona Concentration	
Substance	MW	Solubility	(mg/L)	mM	(mg/L)	mM
MgSO4	120	35.1g/100ml	138	1.15	46.0	0.38
NaHCO3	84	96 g/L	504	6	168.0	2.00
CaCl2	111	74.5 g/100 mL	77.7	0.7	25.9	0.23
Ca(NO3)2	164	1212 g/L	32.8	0.2	10.9	0.07
Ca(OH)2	74	1.73 g/L	259	3.5	86.3	1.17
Humic acid (sodium salt)			5		5	5
			ug/L			
tetrachloroethylene (PCE)			30		10	
NaF			75		25	
(NH4)OH			100		33.3	
H3BO3			800		266.7	

Before evaluating the performance of model organisms in a mixture (such as the one observed in the two aquifers), several toxicity testing protocols were carried to assess how the Bologna and Cremona waters affected the toxicity of the selected ECs individually: sodium fluoride (NaF), boric acid (H_3BO_3), ammonium hydroxide (NH_4OH) and acetaminophen.

Standard protocols for *D. magna* and *D. rerio* toxicity testing advise using standardized media like the American Society for Testing and Materials (ASTM) moderated-hard-water medium (ASTM, 1980) and fish water system (FWS), respectively. For validation purposes, these are media with which the performance in Bologna and Cremona groundwaters must be compared to. Acute and chronic toxicity tests with *D. magna* were adapted from the OECD 202 protocol (OECD, 2004) and OECD 221 protocol (OECD, 2008), respectively, and the FET experiments with *D. rerio* followed the OECD 236 guideline (OECD, 2013) and the methodology described by Lammer et al. (2009), with some adaptations. An additional control treatment with ASTM or FSW, for *D. magna* and *D. rerio* respectively, was always performed for validation purposes. The toxicity patterns resulting from these experiments with synthetic groundwaters were compared to those registered using the standard testing medium (previously reported in the deliverable **D4.1**).

i. Test species: *Daphnia magna*

Table 2 compares the LC_{50} values (i.e. concentration causing 50% effect on mortality) registered after exposing *D. magna* to boric acid, ammonium hydroxide, sodium fluoride and acetaminophen in different test mediums (ASTM, Bologna and Cremona) for 48h. LC_{50} values along with the confidence intervals were derived using the two-parameter probit model. The probit model is widely used in ecotoxicology and human toxicology to model quantal concentration-response data.

Overall, a pattern can be observed where the ECs when occur in the Cremona synthetic groundwater appear to cause higher toxicity to the crustacean, *D. magna*. However, even though such pattern can be acknowledged, statistically there are no significant differences between obtained LC_{50} values of most of the ECs for each water composition tested. The LC_{50} for boric acid was lower in both synthetic waters when comparing to ASTM, although the confidence intervals overlap. Regarding ammonium hydroxide, a significantly higher LC_{50} was found for Bologna water, when comparing to ASTM and Cremona. Such result could be explained due to the higher pH the Bologna water presents, thus keeping the $NH4^{+}$ ions concentration higher than in the Cremona waters. Additionally, the increased concentration of constituents to recreate the Bologna water as opposed to the Cremona, may result in a protective effect as they may react with ammonium hydroxide. A similar pattern was observed with sodium fluoride as the LC_{50} value was lower in Cremona than ASTM or Bologna. Finally, the LC_{50} values for acetaminophen varied considerably with the test medium, being highest for Bologna, followed by ASTM and lowest for Cremona. Such results indicate that the lower concentration of water constituents in the Cremona water are possibly responsible for the increased toxicity observed in most of the ECs tested.

Table 2. 48h-LC_{50} values (mg/L) for *D. magna* K6 clone exposed to selected ECs in ASTM, Cremona and Bologna waters. LC_{50} derived with probit model. Fiducial confidence intervals shown within brackets.

	ASTM	**CREMONA**	**BOLOGNA**
Boric acid	705.73 (479.49-792.14)	668.4 (632.52- 706.97)	686.5 (651.17- 724.95)
Ammonium hydroxide	99.31 (89.54-108.91)	111.2 (107.63- 114.95)	322.6 (293.01-359.54)

Sodium fluoride	649.4 (553.33-808.26)	508.06 (487.88- 529.77)	587.62 (538.22-646.05)
Acetaminophen	2.96 (2.6-4.08)	1.69 (1.55- 1.82)	3.26 (3.05-3.50)

At the time of writing the present report, chronic experiments with synthetic groundwaters and *D. magna* were still ongoing. The only selected EC to which chronic data is available is acetaminophen. **Figure 1a** shows the total number of juveniles produced by *D. magna* at the end of the experiment, for each treatment in Cremona water. **Figure 1b** shows the cumulative number of neonates produced throughout the experiment for each treatment, for each treatment in Cremona water. **Figure 1c** shows the total number of juveniles produced by *D. magna* at the end of the experiment, for each treatment in Bologna water. **Figure 1d** shows the cumulative number of neonates produced throughout the experiment for each treatment in Bologna water. Again, by comparing controls, it becomes clear that the reproductive output of *D. magna* was highest in ASTM and lowest in the Bologna synthetic groundwater. The toxicity patterns of acetaminophen to *D. magna* also seemed to be in line with those observed in the acute toxicity test. Whereas in Cremona groundwater the increase of acetaminophen concentrations generally led to reductions in total neonate production, such concentration-response relationship was not found in the Bologna water. Overall, results suggest that whilst the Cremona groundwater increases the toxicity of acetaminophen, the opposite seems to occur for Bologna. Such result can be possibly explained due to the low concentrations of water constituents in the Cremona water as opposed to the Bologna, thus increased availability of the pharmaceutical to interact with the target in the organism. It was not possible to calculate EC_{50} (concentration causing 50% of effect in the test organism, in this case, reduction by 50% of the reproduction output) values of acetaminophen in the two groundwaters. At the last concentration of acetaminophen in the Bologna water, it was observed a 90% mortality of adults after 21 days, while for both the Cremona and Bologna waters no concentration causing 50% of an effect compared to the control treatment was observed. Future work will be conducted using a wider range of concentrations in order to better pinpoint the concentration of acetaminophen that will induce 50% of reduction in the reproduction of *D. magna* for both waters.

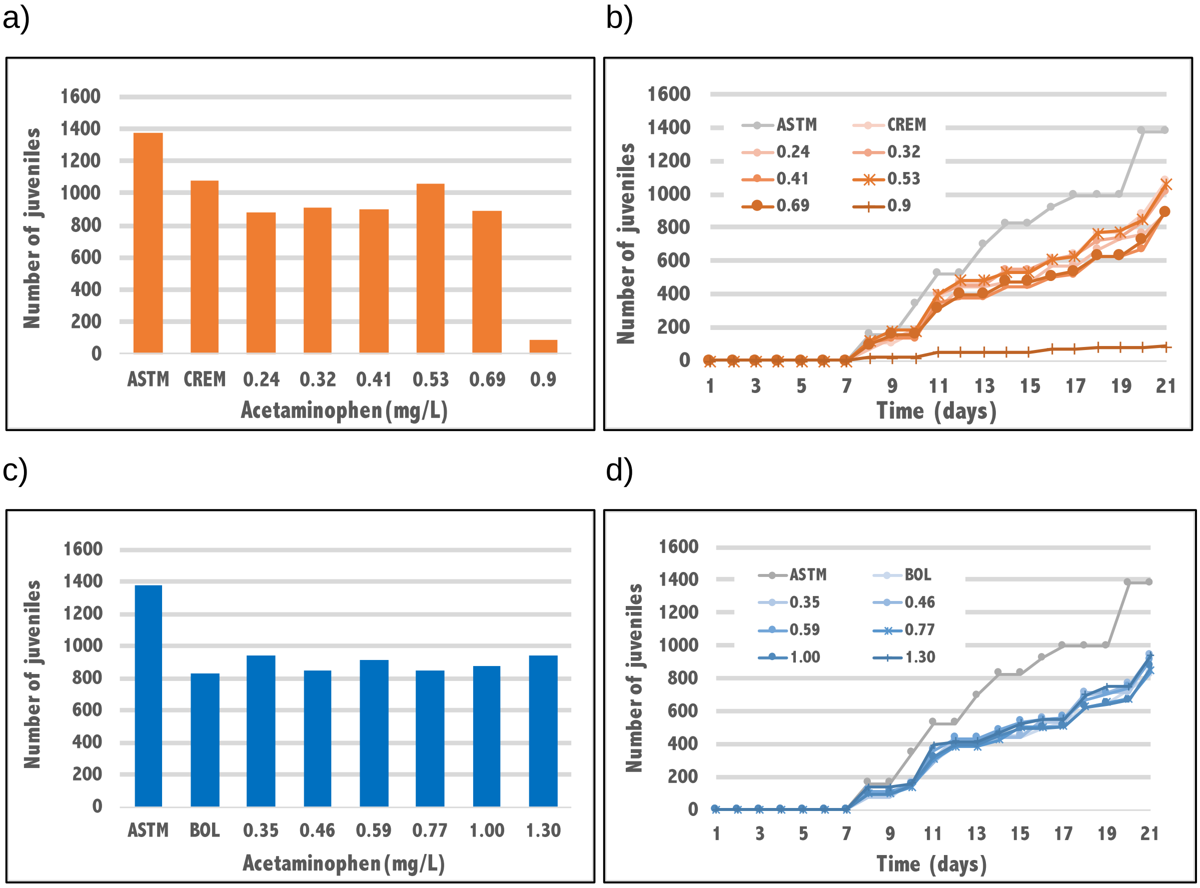

Figure 1. Reproduction output of *Daphnia magna* K6 after exposure to acetaminophen in synthetic groundwaters for 21 days. a) total number of neonates produced in Cremona water at day 21; b) total cumulative number of neonates produced throughout the experiment in Cremona water. c) total number of neonates produced in Bologna water at day 21; b) total cumulative number of neonates produced throughout the experiment in Bologna water.

ii. Test species: *Danio rerio*

Table 3 compares the LC_{50} values (i.e. concentration causing 50% effect on mortality) registered after exposing *D. rerio* to boric acid, ammonium hydroxide and sodium fluoride in different test mediums (FSW, Cremona and Bologna) for 96h. LC_{50} values along with the confidence intervals were derived using the two-parameter probit model.

The FET test with *D. rerio* generally showed similar toxicity results with both synthetic groundwaters, with the biggest differences being registered in boric acid. The 96h LC_{50} for boric acid was 401.5 mg/L for Bologna water and 991.2 mg/L for Cremona water (**Table 3**). Both values are considerably lower than 1617.63 mg/L which was the LC_{50} reported for FSW in deliverable **D4.1**. These results contrast with the acute test for *D. magna* in which the LC_{50} values for boric acid had been very close to each other. Hatching success was above 80% for all concentrations, except where full mortality occurred.

Table 3. 96h-LC_{50} values (mg/L) for *D. rerio* exposed to selected ECs in FSW, Cremona and Bologna waters. LC_{50} derived with probit model. Fiducial confidence intervals shown within brackets.

	FSW	**CREMONA**	**BOLOGNA**
Boric acid	1617 (1426-1876)	991.2 (863-1160.7)	401.5 (348.3-466.6)
Ammonium hydroxide	>20	94.2 (80.6-112.3)	122.2 (108.2-138.2)
Sodium fluoride	1009.1 (848.6-1169.5)	664.1 (580.7-746.4)	993.8 (876.6-1171-6)

The percentage of morphological abnormalities was higher at lower concentrations than reported for FSW medium in **D4.1**, particularly in the Bologna water. None were registered in FSW nor in any of the groundwater controls (without ECs). Abnormal morphological development includes low/no yolk sac absorption, tail and spine curvature malformations, inflated bladder and cardiac edema (**Figure 5**).

The 96h LC_{50} for ammonium hydroxide was 122.2 mg/L for Bologna water and 94.2 mg/L for Cremona water (**Table 3**). It was not feasible to compare values of both waters to FSW values for ammonium hydroxide.

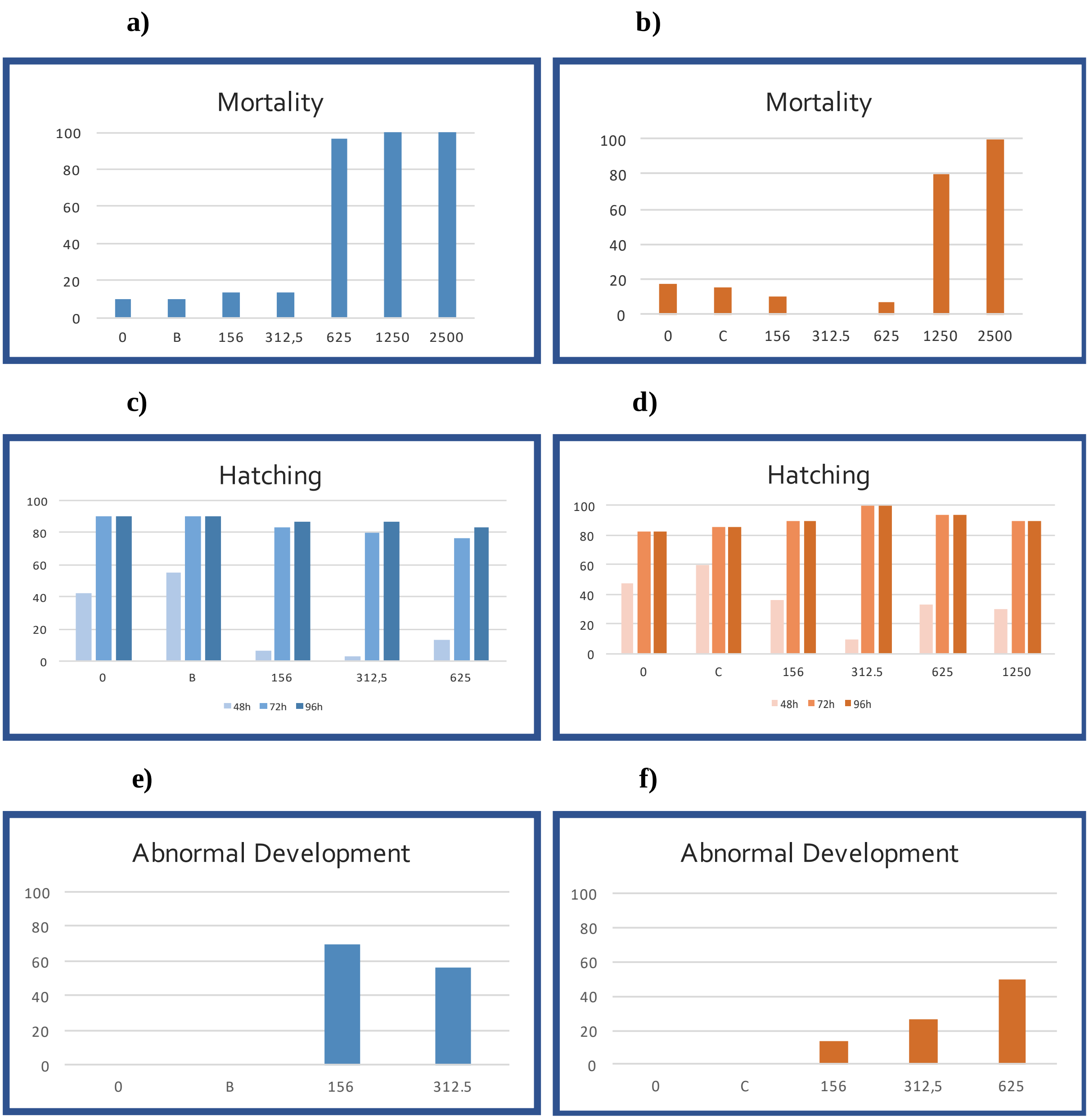

Figure 2. Performance of zebrafish (*D. rerio*) embryos exposed to boric acid in Bologna (blue panels) and Cremona (orange panel) groundwaters for 96h: top panels (a+b) show the cumulative mortality; middle panels (c+d) show the hatching rate; bottom panels (e+f) show the occurrence of morphological abnormal development. Treatments “0”, “B” and “C” represent the FSW control, the Bologna water control and the Cremona water control, respectively. Values are expressed as total percentages.

However, when comparing between the two water LC_{50} values, one can conclude that ammonium hydroxide’s toxicity towards zebrafish is higher in Cremona than in Bologna. Even though the confidence intervals appear to overlap, a pattern can be observed, as to the Cremona synthetic groundwater increases the toxicity of most of the selected ECs (ammonium hydroxide and sodium fluoride, see below) to zebrafish. Hatching success was above 80% for all concentrations, except the highest one (320 mg/L). The percentage of morphological abnormalities was higher at higher concentrations (~40% at 80 mg/L), particularly in the Cremona water, while for the Bologna, the two highest concentrations presented malformations at a lesser percentage (<20%). None were registered in FSW (used as control) nor in any of the groundwater controls (without ECs).

Figure 3. Performance of zebrafish (*D. rerio*) embryos exposed to ammonium hydroxide in Bologna (blue panels) and Cremona (orange panel) groundwaters for 96h: top panels (a+b) show the cumulative mortality; middle panels (c+d) show the hatching rate; bottom panels (e+f) show the occurrence of morphological abnormal development. Treatments “0”, “B” and “C” represent the FSW control, the Bologna water control and the Cremona water control, respectively. Values are expressed as total percentages.

The 96h LC_{50} for sodium fluoride was 664.1 mg/L for Cremona water and 993.8 mg/L for Bologna water (**Table 3**). Both values are considerably lower than 1009.1 mg/L which was the LC_{50} reported for FSW in deliverable **D4.1**. Additionally, when comparing between the two water LC_{50} values, one can conclude that sodium fluoride's toxicity towards zebrafish is higher in Cremona than in Bologna. As observed in ammonium hydroxide, a similar pattern is followed with sodium fluoride, as the Cremona synthetic groundwater increases the toxicity of the selected ECs to zebrafish. Mortality was above 80% in the two highest concentrations in the Cremona water while, below 80% for the Bologna one. Hatching success was above 80% for all concentrations of the Bologna water while in the highest concentration of sodium fluoride in the Cremona water was below 80%. The percentage of morphological abnormalities was higher at higher concentrations (>70% at 600 mg/L) (**Figure 5**). None were registered in FSW (used as control) nor in any of the groundwater controls (without ECs).

Figure 4. Performance of zebrafish (*D. rerio*) embryos exposed to sodium fluoride in Bologna (blue panels) and Cremona (orange panel) groundwaters for 96h: top panels (a+b) show the cumulative mortality; middle panels (c+d) show the hatching rate; bottom panels (e+f) show the occurrence of morphological abnormal development. Treatments “0”, “B” and “C” represent the FSW control, the Bologna water control and the Cremona water control, respectively. Values are expressed as total percentages.

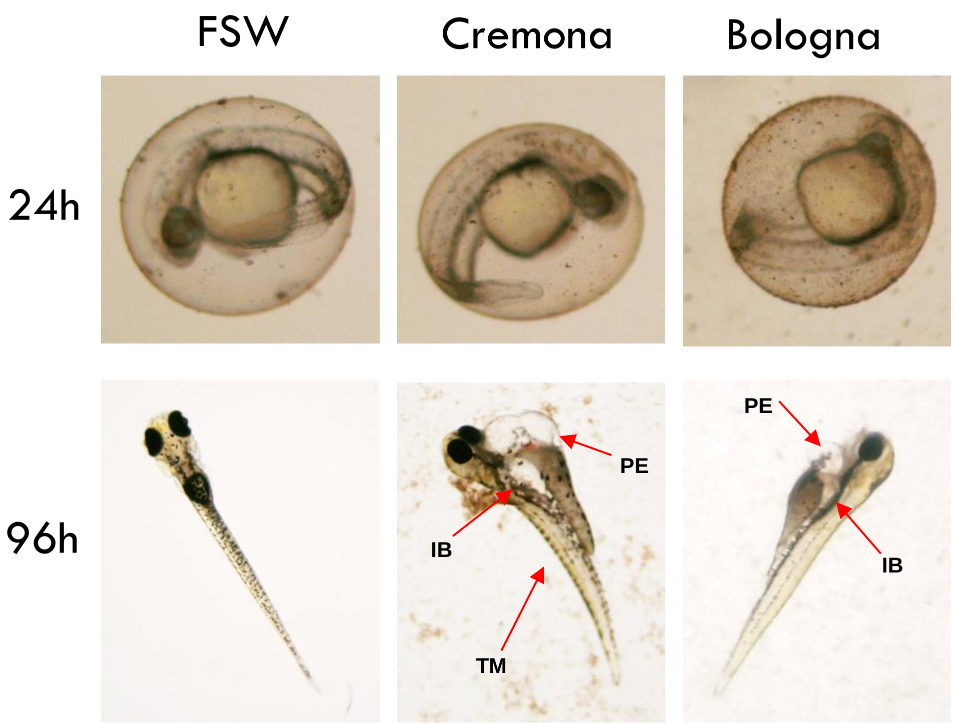

Figure 5. Morphological development and malformations observed in zebrafish (*D. rerio*) larvae exposed to sodium fluoride after 24h and 96h; PE – Pericardial Edema, IB – Inflated Bladder, TM – Tail Malformation (curvature).

Overall, a similar pattern can be observed for both organisms, *Daphnia magna* and *Danio rerio*, as the Cremona synthetic groundwater appears to increase the toxicity of most of the selected ECs tested individually.

1.3. Testing of Bologna and Cremona synthetic groundwaters

After proceeding with the toxicity tests for the selected ECs in the synthetic groundwaters from Bologna and Cremona aquifers, it is imperative to understand how these different mediums affect the performance of *D. magna* and *D. rerio*. As described in section **1.2**, standard protocols for *D. magna* and *D. rerio* toxicity testing were used. In the present section, the toxicity of the total composition of the two aquifers, Bologna and Cremona groundwater, were tested.

i. Test species: *Daphnia magna*

As a first approach towards the validation of synthetic groundwaters, an acute test with *D. magna* was performed whose treatments consisted of ASTM medium (as control) and the Bologna and Cremona synthetic groundwaters, with and without the ECs found in the field. The experiment followed the standard OECD 202 protocol (OECD, 2004) in terms of conditions and duration (48h), with slight adaptations when it comes to the four different groundwater compositions tested. The experimental design consisted of five replicates per treatment and five daphnids per replicate. After 24h and 48h, the immobilization of daphnids was recorded (including dead organisms). They were considered immobile if they were not capable of swimming after gentle agitation of the medium with the use of a pipette in the test-vial or mortality occurred.

Table 4 shows the results on survival/immobilization after 24h and 48h. All treatments showed to elicit no mortality/immobilization in daphnids within 48h.

Table 4. Survival (as mobility) results for *Daphnia magna* k6 exposed for 48h to ASTM, Cremona and Bologna synthetic groundwaters and Cremona and Bologna groundwaters with field detected ECs (boric acid, sodium fluoride and ammonium hydroxide).

Time	ASTM	CREMONA	CREMONA + ECs	BOLOGNA	BOLOGNA + ECs
24h	100%	100%	100%	100%	100%
48h	100%	100%	100%	100%	100%

Since none of the treatments caused any mortality to daphnids, they were used in a chronic *D. magna* experiment in order to evaluate the reproduction outcome. Again, the experiment followed the standard OECD 221 protocol (OECD, 1998) regarding the conditions and duration (21d), with adaptations to the groundwater compositions used. The experimental design consisted of ten replicates per treatment and one daphnid per replicate. Organisms were maintained in glass vials containing 50 mL of test media with seaweed extract and food (supplied as *Raphidocellis subcapitata*). The test media was renewed every other day and daphnids were fed daily. Every day throughout the experiment, neonates were counted and removed from the test system. After 21 days, adult daphnids were placed under a stereomicroscope and their body length measured, from the point immediately above the eyespot to the base of the dorsal spine.

Figure 6a shows the mean number of juveniles produced by *D. magna* at the end of the experiment, for each treatment. **Figure 6b** shows the cumulative number of neonates produced throughout the experiment for each treatment. Looking at the reproduction performance of *D. magna*, it was observed that the composition of the Bologna water induced the production of less juveniles than ASTM or Cremona, still fulfilling the validity criteria from the OECD guideline for controls (≥60 neonates; <20% adult mortality), but no differences were attained with the introduction of ECs between the Bologna and Bologna + ECS, and the Cremona and Cremona + ECs. **Figure 6c** shows the mean length of adult daphnids after 21 days of exposure to ASTM and four different groundwater compositions.

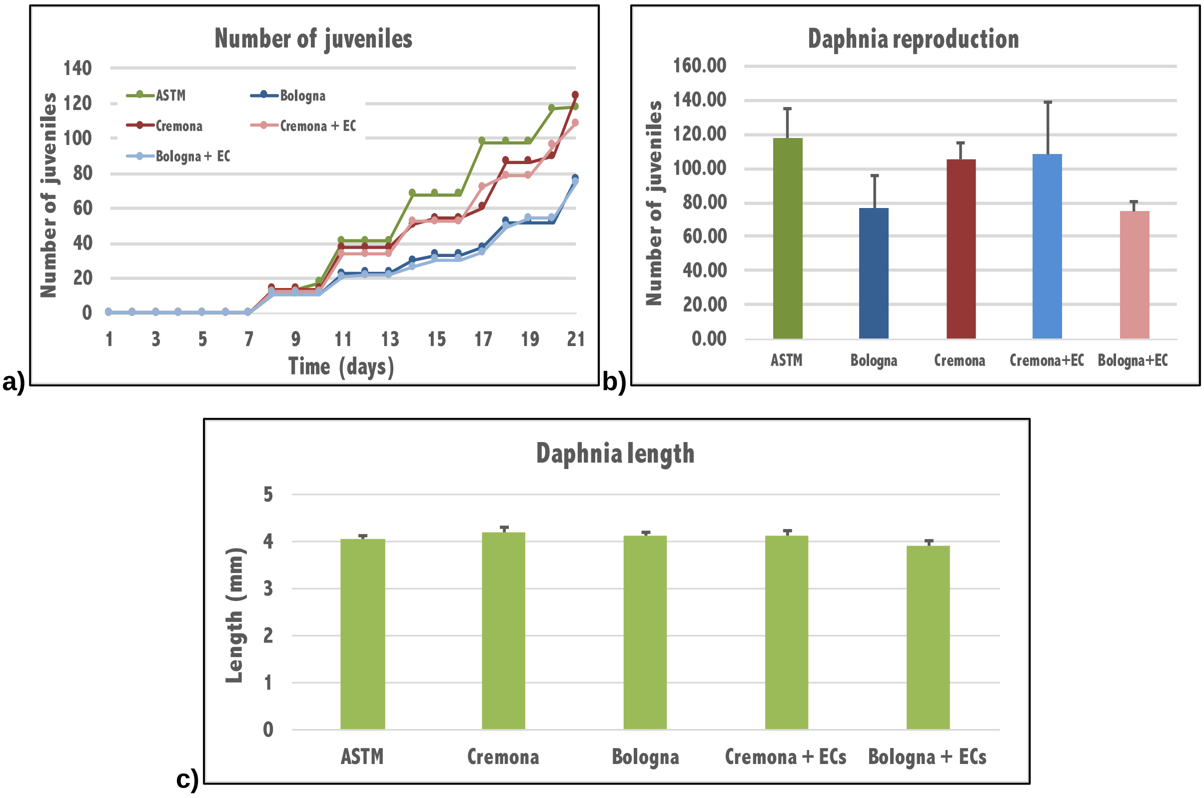

Figure 6. Reproduction of *Daphnia magna* k6 at the end of 21 days for each treatment: ASTM, Cremona and Bologna synthetic groundwaters and Cremona and Bologna groundwaters with field detected EC (Boric acid, sodium fluoride and Ammonium hydroxide): a) mean number of neonates produced at day 21; b) mean cumulative number of neonates produced throughout the experiment; c) mean length of adult daphnids at the end of the test.

ii. Test species: *Danio rerio*

A similar approach was taken for validating the use of synthetic groundwaters for fish embryo toxicity testing (FET) with *D. rerio*. This FET experiment followed the standard OECD 236 guideline (OECD, 2013) and the methodology described by Lammer et al. (2009), with adaptations. The rationale of comparing the organisms' performance in synthetic groundwater to that of standard medium was followed as previously mentioned in the *Daphnia* testing, with FSW (instead of ASTM) was the standard to compare to for fish. Test conditions and duration (96h) followed the standard protocol. The experimental design consisted of thirty replicates per treatment and one embryo per replicate. Every 24 h embryos were checked under the stereomicroscope and assessed for the following endpoints: survival, hatching and any occurring malformations (e.g. tail detachment, presence of edema, bladder malformation).

Figure 7a shows the cumulative mortality of zebrafish embryos for each treatment at the end of the experiment. **Figure 7b** shows the hatching rate for each treatment throughout the experiment. Results showed no difference in fish embryo survival between synthetic groundwaters, with or without the field detected ECs, and FSW. Additionally, there were also no differences in the hatching rate throughout the experiment. Hatching success was 100% for all treatments, except for Cremona with ECs but no significant differences were found. There were no morphological malformations in embryos resulting from this experiment.

a) b)

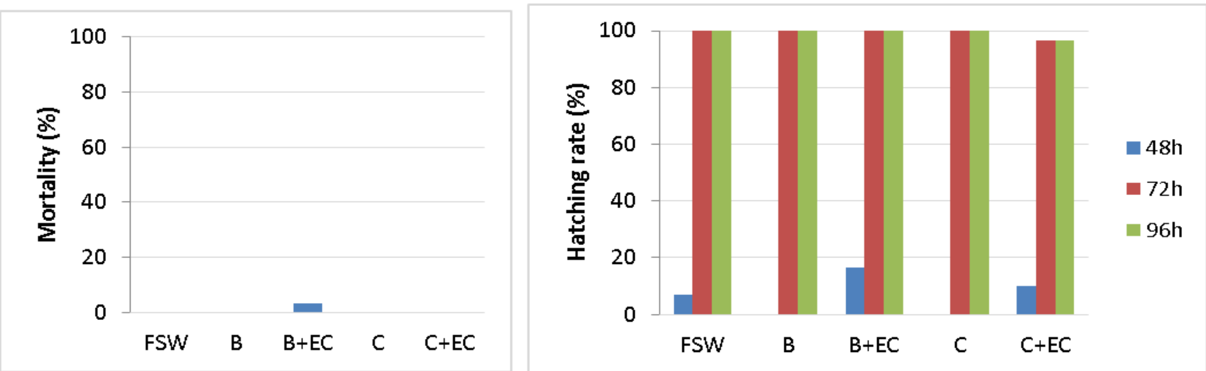

Figure 7. Results of each parameter measured in the FET (Fish Embryo Acute Toxicity) test with zebrafish (*D. rerio*) in fish water system (FSW), Cremona (C) and Bologna (B) waters and Cremona and Bologna waters with EC (C+EC and B+EC, respectively) after 96h: a) cumulative mortality; b) hatching rate.

As conclusion, none of the synthetic groundwaters affected the performance of *D. magna* and *D. rerio*. When non-standard media are being used for toxicity testing of additional compounds, adding a control (ASTM and/or FSW) suggested in standard protocols is advisable in order to confirm the fit of culture conditions.

The above obtained results show that the chemicals at the measured concentrations encountered in the two groundwater points from Cremona and Bologna have no ecological hazard effects. However, as mentioned in the **D4.1**, 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 genotoxicity and ecotoxicity of the compounds already identified (**Weizmann Institute of Sceince, WP3**) in addition to 3 more chemicals, usually encountered in surface waters, acetaminophen, azithromycin and PFAS (polyfluoroalkyl substances) individually and in mixtures. By using a synthetic water composition of the two Italian aquifers and their possible complex mixtures, it allows us to evaluate 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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