This is the manuscript version of an article published in *Journal of environmental management* 300 (2021):113549.
Version of record is available at https://dx.doi.org/10.1016/j.jenvman.2021.113549

An integrated approach for chemical water quality assessment of an urban river stretch through Effect-Based Methods and emerging pollutants analysis with a focus on genotoxicity

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Abstract

The impact of emerging chemical pollutants, on both status and functionality of aquatic ecosystems is worldwide recognized as a relevant issue of concern that should be assessed and managed by researchers, policymakers, and all relevant stakeholders. In Europe, the Reach Regulation has registered more than 100.000 chemical substances daily released in the environment. Furthermore, the effects related to the mixture of substances present in aquatic ecosystems may not be predictable on the basis of chemical analyses alone. This evidence, coupled with the dramatic effects of climate changes on water resources through water scarcity and flooding, makes urgent the application of innovative, fast and reliable monitoring methods. In this context, Effect-Based Methods (EBMs) have been applied in the urban stretch of the Tiber

River (Central Italy) with the aim of understanding if detrimental pressures affect aquatic environmental health. In particular, different eco-genotoxicological assays have been used in order to detect genotoxic activity of chemicals present in the river, concurrently characterized by chemical analysis. Teratogenicity and embryo-toxicity have been studied in order to cover additional endpoints. The EBMs have highlighted the presence of diffuse chemical pollution and ecotoxicological effects in the three sampling stations, genotoxicological effects have been also detected through the use of different tests and organisms. The chemical analyses confirmed that in the aquatic ecosystems there is a diffuse presence, even at low concentrations, of emerging contaminants such as pharmaceuticals, not routinely monitored pesticides, personal care products, PFAS. The results of this study can help to identify an appropriate battery of EBMs for future studies and the application of more appropriate measures in order to monitor, mitigate or eliminate chemical contamination and remediate its adverse/detrimental effects on the ecosystem health.

1. Introduction

The European Regulation Reach (Registration, Evaluation, Authorisation, and Restriction of Chemicals) has registered until now more than 100.000 chemical substances, although the number of existing pollutants is possibly underestimated because it is not possible to analyse, detect and quantify all the substances found in the aquatic environment. Besides, the adverse biological effects from mixtures of substances present in the aquatic environment may not be predictable on the basis of chemical analyses alone. Therefore, in order to guarantee good water quality, it would be valuable to understand the potential adverse biological effects caused by the mixture of the chemicals present in the aquatic environment (including emerging pollutants, metabolites, and transformation products) and to link the observed effects with cost-effective management objectives (Brack et al., 2012; Altenburger et al., 2015). The European Water Framework Directive (WFD) has the aim to achieve a good chemical, ecological and quantitative status in all European waterbodies (European Union, 2000). The Chemical status assessment is based on a list of European priority substances (European Union, 2013), the concentrations must be in compliance with Environmental Quality Standard (EQS) that should protect the aquatic environment (different trophic levels) and human health (through the consumption of fishery products and drinking waters). However, this classical single-chemical risk assessment approach for the chemical pollution management of water bodies has some limitations (Brack et al., 2018, 2019): the prioritization, the derivation of EQSs and the monitoring programs for all these substances are challenging and the list covers only a limited number of substances. Among the current activities of the WFD the need to include Effect Based Methods (EBMs) in monitoring programs has been highlighted, EBMs are mainly bioassays in vivo and in vitro and biomarkers. EBMs are recommended as an integrated approach for monitoring water quality (Jia et al., 2019)) and EBMs using in vivo and in vitro assays have been applied as powerful tools for more than three decades (Escher et al., 2018; Altenburger et al., 2019). For instances, EBMs have been successfully applied to screen adverse effects in surface water, wastewater, recycled water and drinking water (Kuckelkorn et al., 2017; Tousova, 2017; Neale, 2017). These methods could fulfil different functions (Di Paolo et al., 2016; Wernersson et al., 2015), such as providing screening tools, as part of the pressure and impact assessment, and establishing early warning systems to prioritise

further studies in areas located far from known local pollutant sources and not considered at risk. Moreover, the effects from mixtures of pollutants could be quantified as well as outcomes of not routinely analysed chemicals. In this context genotoxicity assessment is a key aspect in the evaluation of surface water quality: De Souza and co-workers (De Souza et al., 2020) investigated acute toxicity and genotoxicity assays concluding that the latter can be considered as a reliable tool to identify the negative impacts of genotoxic substances present in wastewater. Moreover, the use of genotoxicity biomarkers may improve conventional physical-chemical methods of water quality assessment as suggested by a recent work (Francisco et al., 2019) in which genotoxic effects resulting from exposure to the water samples collected near the discharge of industrial and domestic waste waters were detected. Several EBMs allow the analysis of genotoxic effects which are the results of a damage to DNA and/or cellular structures involved in the maintenance of genome stability. The loss of genomic stability and the acquisition of genetic alterations are key steps in the process of carcinogenesis (Gray and Collins, 2000; Loeb et al., 2000). EBMs permit to evaluate the direct effects on organisms inferring the risks at individual level and provide information that can be interpreted at population level. Being DNA a common molecular target among living organisms, genotoxicity data provide extensive information useful in environmental risk evaluation. The impact of chemical pollutants on DNA integrity is a key aspect in the assessment of surface water quality (Francisco et al., 2019; De Souza Celente et al., 2020). DNA damage, as detected by the Comet (Single Cell gel Electrophoresis) assay, can be correlated to effects on life history traits such as growth, development, and reproduction of some organisms (Lourenco et al., 2010; Sukumaran et al., 2013; (Quinn et al., 2012); (Møller, 2018)) and may identify a potential risk for the fitness of natural populations. In general, the use of EBMs could be supportive also for the protection of human health (Mari´c et al., 2020). In fact, an association of specific cancers and exposure to disinfected water has emerged by epidemiological research, and several toxicity mechanisms for disinfecting by-products have been implicated, including genotoxicity which is of particular importance because of its link to mutagenicity and carcinogenicity (Lan et al., 2018). Furthermore, genotoxicity tests can be applied to evaluate the combined action of potentially hazardous compounds present in surface waters both as single compounds and complex mixtures (Da Silva, 2021), highlighting synergic, additive and antagonist effects at sub-lethal concentrations. Carvalho and co-authors, evaluating the single or mixed genotoxic effects of commonly used herbicides (glyphosate and imazethapyr) on tadpoles (Carvalho et al., 2019) showed an increase in genetic damage after the exposure to 5 and 10% of the LC50 96 h values, indicating a synergistic effect.

For these reasons, genotoxicity has been proposed recently as a priority effect to be addressed by risk assessment to chemical mixtures (Jeddi et al., 2021) towards a systematic use of effect biomarkers in population and occupational biomonitoring. Furthermore compounds “that possess carcinogenic, or mutagenic properties”, like polycyclic aromatic hydrocarbons (PAHs) and benzene, are indicated as main pollutants for European water bodies by the Annex VIII of the WFD. In addition, the Comet assay, an in vitro EBM detecting DNA damage at single-cell level, has been successfully applied as predictive biomarker introduced in model organisms exposed to assess sediments and surface waters (Guidi et al., 2017; Gajski et al., 2019).

In order to implement EBMs for research and policy uses, the aim of the present study conducted by a network of institutions was to select and apply methods feasible for the monitoring of surface water bodies.

The study area, comprising two different sampling sites along the main watercourse and the third one on a tributary, was located along the urban part of the Tiber River basin in the Latium Region in Italy. Tiber River has been also selected as a pilot river basin in the context of the WFD. Previous studies have found different organophosphate (OPPs) pesticides in the river and in its estuary, though often in lower concentrations than the guideline values (Montuori et al., 2016). Moreover, other environmental pollutants were found in the last stretch of the river; for instance, high concentrations of pharmaceuticals, such as the non-steroidal anti-inflammatory drug diclofenac, PAHs, pesticides, perfluorinated and polyfluorinated alkyl substances (PFAS), heavy metals were detected (Patrolecco et al., 2010; Sacca et al., 2019; Mancini et al., 2005). The occurrence of antibiotics was recorded (Grenni et al., 2018) as well as alkylphenols. Other studies have also detected mutagenic effects in the urban part of the river (Minissi and Lombi, 1997; Rizzoni et al., 1995). The data from the local environmental agency collected in compliance with the current legislation showed that some priority substances, e.g. nickel, exceed the environmental quality standards in the Tiber River (Lazio, 2018). Taking into consideration all these aspects, in the present study some genotoxicity EBMs have been selected and applied together with two acute in vivo toxicity bioassays largely used in monitoring programmes (Fish embryo toxicity test and Daphnia sp. Acute immobilization assay) and a teratogenicity assay. In a specific sampling campaign also targeted chemical analysis have been performed in order to add specific information to the study. Moreover, recommendations for including a battery of EBMs in future monitoring strategies at the legislative level suggested for preventive management measures are reported.

2. Materials and methods

2.1 Study area and sampling

The study area, located along the Tiber River basin in the Latium Region in central Italy, includes two different sites along the main watercourse and the third one on a tributary (see Fig. 1). The Tiber River basin has been selected as Pilot River basin in the context of the WFD common implementation strategy (Andreani et al., 2005). The Tiber River is the second largest river in Italy after the Po River. It originates on the slope of Monte Fumaiolo, a major summit of the Apennine Tosco-Emiliano, with a catchment area of 17,375 km^2. The water volume ranges from 60 $m^3\ s^{-1}$ to 3200 $m^3\ s^{-1}$, with a yearly average of 230 $m^3\ s^{-1}$. The river is 405 km long and runs through four administrative regions (Emilia-Romagna, Tuscany, Umbria e Latium). The Tiber lower stretch flows through the city of Rome and branches out into a delta entering the main channel being the Fiumara (Fiumicino) to the Tyrrhenian Sea.

The Tiber River has been classified, based on the principles established in the context of the WFD, as a “Very Large river” with a catchment area exceeding 10,000 km^2 and as an “R-L2” River type with water alkalinity exceeding 0.5 meq/L. The Tiber River is affected by numerous anthropogenic

activities, such as steel mills and paper mills, mainly located along Nera and Aniene tributaries that can deteriorate its water quality. The uses of the water resources are multiple: a significant use of surface water is linked by irrigation and the most important withdrawals are those of the upper Tiber valley and those of the final part of the river. Moreover, in the city of Rome, the river receives the wastewater of the treatment plants. The Tiber River Basin Authority (TRBA) reports that about 53% of the basin's surface is actually in agricultural use, while approximately 39% is forested and 5% is urbanized, in future possible drinking water uses are planned. The Tiber River basin was reported to undergo prolonged dry periods (drought events that affected the entire basin occurred in 1955, 1971, 1987, 1990, 1993, 2003 and 2007) and floods are also frequent.

The sampling strategy (Fig. 1) included 2 sites along the urban river stretch within Rome, in both the southern and the northern part of the city, and 1 sampling site located in the protected area of the tributary Farfa River (FA) where previous studies have been performed (Marcheggiani et al., 2019). Specifically, the upstream site Castel Giubileo (CG) is located in the northern part of Rome immediately before the city limit (this site is likely to be affected by leaching of waste, agricultural and zootechnical discharges as well as the presence of small and medium factories) while the downstream site Mezzocammino (MC) is located in the southern area of Rome, about 20 km from the estuary in the Tyrrhenian Sea and is located right after a sewage treatment plant. Lastly, the FA is located around 50 km from the city: it was chosen as a reference site for its good ecological status, determined following the WFD requirements. Three sampling campaigns were conducted in June/July 2018 (campaign 1), November/December 2018 (campaign 2), and June/July 2019 (campaign 3), in each site.

Water samples (10 L) were collected in each site between 0 and 30 cm from the water surface in inert containers. Water samples were then transported refrigerated to the laboratory, filtered in lab with 0.45 µm filters (in order to remove suspended material) and stored at 4 ◦C before testing. Only the water phase has been tested.

The codes of the sampling sites are the following: MC1, CG1, FA1 (first campaign), MC2, CG2, FA2 (second campaign), MC3, CG3, FA3 (third campaign).

3. Method applied

The following methods have been applied.

- Comet assay (Single Cell gel Electrophoresis) on: *Dreissena polymorpha*, *Daphnia magna*, *Danio rerio* embryos, *Hydra vulgaris*
- NRRT (neutral red retention time) assay on *Dreissena polymorpha*
- Cytome assay on *Dreissena polymorpha*
- SOD mRNA on *Hydra vulgaris*
- Daphnia sp. Acute Immobilization assay
- Fish Embryo Toxicity (FET) test on *Danio rerio*

- Teratogenicity assay on *Hydra vulgaris*
- Targeted Chemical analysis (only in the 3rd campaign)
- PFAS Chemical analysis (only in the 3rd campaign)

In supplementary materials there is a description of the exposure procedure for the different organisms used.

3.1 Comet assays

3.1.1. *Dreissena polymorpha*

The Comet assay was performed on haemocytes, from 10 specimens per tank at the end of the exposure, according to Singh et al. (1988) with slight modifications (Guidi et al., 2020). The amount of DNA damage was quantified as the percentage of DNA migrated into the comet tail (% tail DNA) (Kumaravel and Jha, 2006), using an image analyser (Kinetic imaging, Ltd., Komet, Version 5). At least 50 nuclei per slide and 2 slides per sample were scored, for a total of 100 nuclei and the mean calculated**.**

3.1.2. *Daphnia magna*

The Comet assay was carried out on 48 h-old organisms according to the methods described in Pellegri et al. (2014) and Pellegri et al. (2020), with minor revisions. The endpoint considered was the percentage of fluorescence intensity in the comet's tail (tail DNA %). The software IBM SPSS Statistics® v.25 was used for statistical analysis. Levene's test was first applied to evaluate variance homogeneity. When homoscedasticity was verified, a comparison was performed by ANOVA and Bonferroni's test as post hoc.

3.1.3 *Hydra vulgaris*

Hydras were mechanically disaggregated in 200 µl of PBS 1X and then centrifugated for 10 min at 2000 rpm 80 µl of PBS were added to the pellet and then 50 µl of this suspension were taken and mixed with 360 µl of Low Melting Agarose (LMA) 0.7% (previously dissolved and maintained at 37 ◦C). This suspension was then spread on two Normal Melting Agarose precoated slides and covered with a coverslip. The slides were then processed, dehydrated with 100% ethanol for 5 min and then air dried in the dark. Slides were stained with Ethidium Bromide (EtBr 10 µl/ml) and then images were acquired with an Axio Imager. M1 fluorescent microscopy (Carl Zeiss, Jena, Germany) equipped with a CCD camera. For each sample, 100 images were analysed using Comet Score software (Rex Hoover). Statistical analysis was performed by one-way ANOVA test with Dunnet post-test. *$p < 0.05$.

3.1.4 *Danio rerio*

Alkaline version (pH > 13) of Comet assay was performed on cell suspensions with viability >85% as described in Andreoli et al. (1997). The analysis was performed using a fluorescent microscope (Leica, Wetzlar, Germany) and DNA damage was quantified as a percentage of fluorescence intensity in the comet's tail (tail DNA%) by a dedicated image analysis system (IAS, 2000 Delta

Sistemi Italia), randomly scoring a total of 100 nucleoids for each experimental point. The statistical analysis was carried out using IBM SPSS Statistics® v.25 software and comparison was performed by ANOVA and LSD as post hoc test.

3.2. Neutral red retention time (NRRT) assay in *Dreissena polymorpha*

Lysosomal membrane destabilisation was assessed by the NRRT, according to Lowe et al. (1995), at the end of the exposure. Three specimens per tank were individually analysed. Zebra mussel hemolymph was incubated on a glass slide with a neutral red (NR) working solution. Granular hemocytes were examined at a light microscope at 15 min intervals, for a period up to 3 h, to evaluate the time at which 50% of cells had leaked to the cytoplasm the dye previously trapped by lysosomes.

3.3. Cytome assay in *Dreissena polymorpha*

The genotoxic effects were evaluated at chromosomal level by the micronuclei frequency assessment. The Micronucleus test (MN) was carried out on haemocytes, according to Scarpato et al. (1990) and Guidi et al. (2020). One thousand cells with well-preserved cytoplasm per specimen were scored (500 per slide) to determine the frequency of micronuclei according to the following criteria. Micronuclei were defined as round structures, smaller than one-third of the main nucleus diameter; micronuclei had to be on the same optical plan and non-refracted as the main nucleus but possess distinguishable boundaries from it (Fenech, 2007). The presence of cells with a morphologically altered nucleus (i.e. incomplete MN, lobed or multiple nuclei, nuclei connected by chromatin bridges in the same cell) was scored in parallel on the same slides and collectively reported. The frequency of the following nucleus abnormalities (NA) were assessed: nuclear blebs (BL), nuclear buds (NBUD), nuclear bridges (NPB), notched nuclei (NT), circular nuclei (CIR), lobed nuclei (LB), and anisochromatic nuclei (AN). Apoptotic cells (APO) were also evaluated. Ten specimens, two slides per specimen, 500 cells per slide were scored for each experimental group.

3.4. RNA extraction and cDNA synthesis on *Hydra vulgaris*

Hydra vulgaris specimens were maintained in Hydra medium as a control condition, or in the river water samples collected as indicated before, for 24 h. Total RNA from *H. vulgaris* was extracted by using TRIzol® Reagent (Life technologies Italia-Invitrogen, Monza, Italy). For each sample, 5 hydrae were used. 1 µg of total RNA was reverse transcribed to cDNA by using GoTaq 2 Step RT qPCR System Protocol (Promega Italia Srl, Milan, Italy). PCR product quantification was calculated by applying the SYBR-Green method. SYBR-Green Master Mix was from Promega. The SOD (Superoxide Dismutase) primer pairs were chosen as described in Malafoglia et al. (2016). Reactions were performed in a Promega detection system, by using the following temperatures: pre-incubation 95 ◦C, amplification at 95◦C-60◦C–72 ◦C for 45 cycles, melting at 95◦C-65◦C–97 ◦C, cooling at 37 ◦C. Data are calculated relative to the internal housekeeping gene (β-actin). The second derivative test, delta-delta Ct (2 ΔΔCT) method, choosing control samples to normalize data has been applied.

All data are expressed as the mean ± standard error of the mean (SEM) of n observations. Statistical analysis was performed by one-way ANOVA and subsequently by Bonferroni post-test. Differences are considered statistically significant at $p \leq 0.05$.

3.5. Daphnia sp. Acute Immobilization assay

This test has been performed by the Italian National Institute of Health (ISS) and the University of Parma (UNIPARMA) in 2 different laboratories. The Daphnia sp. Acute Immobilization assay is a screening method using freshwater crustacean daphnid species. In this study, the test was performed starting from the resisting stage of *D. magna* (i.e. ephippia) that were included in the kit named as Daphtoxkit® and developed by the Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE) at the University of Ghent, in Belgium. The experimental procedure application followed OECD No. 202:2004 guideline. Tests lasted 48 h according to the guideline. All the physicochemical parameters were measured at the start of tests and after 48 h. Six independent tests were performed, one for each sample.

Each test was performed in three replicates on undiluted samples. At the end of the experiment, the number of immobilized individuals was recorded. Daphnids were considered immobilized if no directed movement was observed within 15 s after gentle stirring.

3.6. Fish embryo toxicity (FET) test on *Danio rerio*

Wild type zebrafish (*Danio rerio*) embryos were used to perform the analysis. The bioassay was conducted according to the OECD No. 236:2013 guideline. The embryos were collected from the breeding groups at the laboratory of ISS. Breeding fish were maintained in tanks with a loading capacity of 2L water per fish at 26 ± 1 ◦C and with a fixed photoperiod of 12:12 (light: dark). Six independent tests were performed, one for each sample. Each test was performed in two replicates for a total of 40 embryos per sample. Zebrafish eggs were exposed in 24-well plates at a developmental stage ranging from 32 to 128 cells of segmentation. Each well contained one egg in 2 ml of sample. A negative control plate and internal negative control were prepared with the test medium. A positive control at a fixed concentration of 4 mg/L of 3,4-dichloroaniline was also performed. Embryos were kept in dark conditions for 4 day at 26 ± 1 ◦C. The morphological observations of the embryos were made at 96 h post fertilisation (hpf). Four apical observations indicating acute toxicity were recorded as lethal endpoints: coagulation of the embryo, non-detachment of the tail, lack of somite formation, and lack of heartbeat. Sub-lethal endpoints were also recorded for a more comprehensive evaluation of the sample toxicity with an enhanced level of detail. The investigated sublethal endpoints were spine deformation, hatching delay, general underdevelopment, absence of pigmentation, eye deformation, tail deformation, fin deformation, low heartbeat, head skeleton malformation, edema.

3.7. The *Hydra vulgaris* teratogenicity assay

To develop the experimental design based on the Hydra assay, we used specimens maintained in captivity at the Department of Sciences, University Roma Tre, within 30 × 30 × 15 cm glass tanks (oxygenated by water aerator) of a recirculating system filled with the Hydra medium (composed of $CaCl_2 \times 2H_20$ and $NaHCO_3$) at 16.5–20 ◦C with the 18/6 day/night photoperiod and fed *ad libitum* with nauplii of *Artemia salina* (Traversetti et al., 2017).

Before starting the assay, all specimens were cut with a scalpel under a stereoscope (Leica MZ 8) in two parts: hypostoma (mouthpart, head, and tentacles) and columna (remaining body portion). Columnae were immediately submerged for 96 h (the time range needed to regenerate a whole

specimen) in 20 ml circular wells of a multiwell plate (five specimens per well). Three wells contained the *Hydra* medium (representing the control condition – CTR), whereas the other wells contained water samples (not filtered) collected during the fieldwork. CTR and all treatments (i.e. field samples) were performed in triplicate and stored under the same laboratory conditions as the reared specimens. Observations were made at 24, 48, 72, and 96 h to note both the regeneration rate (RR), using the reference scale in (Spencer et al., 2016; Cera et al., 2020a, 2020b) and aberration frequency (AF, calculated as the ratio of the relative frequency of individuals with morphological aberrations to the total number of individuals). Then, RR and AF were used as descriptors of the double-entry Teratogenic Risk Index (TRI) matrix, as reported in Fig. 2. Crossing the values of RR (in lines) and AF (in columns) we may obtain a score associated with one of the five TRI judgment classes. Statistical analyses regard the application of the nonparametric analysis of covariance (ANCOVA) using RR values at each time point (24, 48, 72, 96 h) and time as covariates, and χ^2 using 2 × 2 contingency tables (with Yates correction) for AF at 96 h. In both cases, we test differences between treatments and control.

3.8. Target chemical analyses

Water samples collected in the 3rd Sampling campaign (Summer, 2019) were subjected to chemical analyses using liquid chromatography high resolution mass spectroscopy (LC-HRMS). A target screening of 504 organic micropollutants known to be used in Europe was conducted. The target list is covering a wide range of physico-chemical proprieties and compound classes including pesticides, pharmaceuticals and industrial compounds. An overview of the target list can be found in Supporting Information. Details information on the methods and QA/QC are given in Supporting Information. Furthermore, the toxic risk linked to the detected was compounds calculated by estimating the risk of extent of exceedance (EoE) using Equation (1):

Extent of Exceedance (EoE) = MEC / PNEC (1)

where MEC represents the measured environmental concentration detected from the chemical analyses and PNEC the lowest Predicted No Effect Concentration. All PNEC values used in this study were extracted from the NORMAN ECOTOX database (https://www.norman-network.com/nds/ecotox/). PNEC values were predicted using QSAR models for freshwater and in different environmental matrices (sediments, marine water, and biota).

3.9. PFAS

The analytical method used to analyse PFAS in aqueous environmental matrices was developed by the Unit “Human Exposure to Environmental Contaminants” of the Italian National Institute of Health (ISS), adapting a method validated by the Unit to analyse serum samples (Marra et al., 2020), and used in various national and international projects and in interlaboratory comparison exercises.

A detailed description is included in supplementary materials.

4. Results

4.1 Comet assays

4.1.1. *Dreissena polymorpha*

Taking together all the data from the three campaigns, a statistically significant difference among sites was only observed in the winter sampling, when the DNA primary damage level is lower ($p < 0.05$) in organisms exposed to water from the FA site (Fig. 3). Moreover, a seasonal variability indicating a loss of DNA integrity in organisms exposed to water samples collected during the summer season was observed, especially during the first sampling (Fig. 3).

Zebra mussels are exposed to water from Farfa (FA), Castel Giubileo (CG) and Mezzocammino (MC). Data are shown as mean ± SE. Different letters indicate different significance levels ($p < 0,05$). a, b (FA); a, b (CG); a, b (MC). # statistically significant difference ($p < 0.05$) within the same campaign.

4.1.2 *Daphnia magna*

Significant induction of DNA damage (Tail DNA%) on Daphnia magna organisms was recorded in the first sampling campaign (Fig. 4). In the first campaign the samples taken from the Farfa River (FA) and from the Tiber River in the Mezzocammino (MC) site induced genotoxicity on daphnids. The most significant genotoxic effects were detected in daphnids exposed to the sample drawn at the MC site ($p < 0.01$). During the second campaign, no genotoxic effects were recorded in the samples of the three sites, while in the third campaign (Fig. 4) only the sample derived from the MC site was shown to induce DNA damage ($p < 0.05$).

The samples collected in the three different campaigns on the site of Castel Giubileo (CG) did not cause genotoxic insults (Fig. 3). D. magna specimens exposed to water from FA (Farfa), CG (Castel Giubileo) e MC (Mezzocammino) Values are mean ± SE. *$p < 0.05$; **$p < 0.01$.

4.1.3 *Hydra vulgaris*

Among the samples of the 1st sampling, hydras treated with water taken from CG showed values significantly higher both than Control (Hydra Lab medium) and FA (Fig. 5). During the 2nd sampling, hydras treated with water taken from CG and MC showed values significantly higher than C. During the 3rd sampling, values obtained from hydras treated with water taken from MC were significantly higher than those of samples C. It should be noted that in the first and third sampling the DNA primary damage observed in Hydras grown in lab medium was comparable with that of FA. For the second sampling water from FA site has not been analysed due to few sample left. Lab: control samples treated only with hydra medium. Asterisks indicate significance compared to lab controls. Hashes indicate significance compared to FA. *, #$p < 0.05$. Farfa (FA), Castel Giubileo (CG) e Mezzocammino (MC).

4.1.4. *Danio rerio*

Zebrafish embryos were exposed for 48hrs to water samples from CG, MC, and FA sites and freshwater as a negative control. Comparable values of DNA damage were observed in cells from control and exposed embryos, being the Tail DNA (%) (mean ± SD) 0.52 ± 0.12 in controls; 0.62 ± 0.12 CG; 0.59 ± 0.16 MC and 0.84 ± 0.22 FA (Fig. 6). The experiment was repeated after 96 h of exposure to the water samples; no differences in DNA damage were observed in cells from controls and treated embryos (mean ± SD; 0.62 ± 0.11 controls, 0.61 ± 0.08 CG, 0.77 ± 0.20 MC). The exception was the site of FA showing a significantly higher amount of Tail DNA % compared to the negative controls (1.35 ± 0.01, $p = 0.01$ ANOVA). Zebrafish embryos treated at different stages of development showed a slight but significant higher value of Tail DNA % at 96hrs respect to 48hrs after exposure to FA water and to 4 Gy of ionizing radiations ($p = 0.05$, ANOVA). Four-fold and eight-fold increase in DNA damage was induced by ionizing radiation in cells from 48 to 96hrembryos, respectively.

The cells were exposed for 48hrs and 96hrs to water from FA (Farfa), CG (Castel Giubileo) and MC (Mezzocammino). Values are mean ± SD. *$p < 0.05$; **$p < 0.01$.

4.2. Lysosomal destabilisation. Neutral red retention time (NRRT) assay on Dreissena polymorpha

The lysosomal membrane stability of circulating haemocytes, assessed by the neutral red retention time, as the time necessary to obtain the 50% of cells displaying the dye released from the lysosomes to the cytoplasm was always very high, ranging from 81 to 180 min. No statistically significant differences were obtained in terms of lysosomal destabilisation among the sampling sites analysed during the first two campaigns. On the contrary, a membrane destabilisation effect was observed in specimens exposed to FA and MC site during the third sampling (81.67 ± 49.07 min; 93.33 ± 43.43 min, respectively). Moreover, a seasonal variability was detected indicating the highest ($p < 0.05$) level of lysosomal destabilisation in organisms exposed to waters sampled during the summer period in 2019.

4.3. Chromosomal damage. Micronuclei and nuclear abnormalities on *Dreissena polymorpha*

Taking into account the whole set of data, significant statistical differences were observed among sampling sites as organisms exposed to waters collected from CG showed an increase ($p < 0.05$) of MN and NA (BL, NBUD, BN, LB, NPB, NT, vacuolated nucleus and ring) compared to the organisms placed in the control tank (Fig. 7). Furthermore, within the two summer campaigns, a statistically significant difference due to the year of sampling was observed. Waters collected from MC and CG in July 2018 showed higher ($p < 0.05$) levels of micronuclei and nuclear abnormalities (limited to MC) respect to those collected in July 2019 (Fig. 8). As regards the third campaign (summer 2019), organisms exposed to the FA sample showed an increased ($p < 0.05$) apoptotic activity compared to the organisms exposed to water from MC and CG (Fig. 9).

Data are shown as mean ± SE and refer to the three campaigns taken together. * indicates significant differences ($p < 0.05$) respect to organisms exposed to the control water. FA (Farfa), Castel Giubileo (CG) and Mezzocammino (MC).

Data are shown as mean ± SE. * indicates significant differences ($p < 0.05$) between campaigns for each site.

Farfa (FA), Castel Giubileo (CG) and Mezzocammino (MC).

Data are shown as mean ± SE. * indicates significant differences ($p < 0.05$) between sites in each campaign.

Farfa (FA), Castel Giubileo (CG) and Mezzocammino (MC).

4.4. SOD mRNA expression in *Hydra vulgaris*

Hydra vulgaris specimens were maintained in different water samples for 24 h and then collected for molecular analysis by Real-Time PCR. mRNA expression levels were statistically analysed for each gene through three technical repeats from three biological replicates, n = 3 (3 triplicates for each gene in a single experiment, for a total of 3 experiments) with one-way ANOVA (and nonparametric) assay, followed by Bonferroni's Multiple Comparison Test. Data are expressed as mean fold change compared with control ±SEM. As shown in Fig. 10, SOD mRNA expression was strongly increased in specimens exposed to MC1, MC2, MC3 CG2, and CG3 water samples for 24 h. As a positive control, the polyps were treated with 10 µM H2O2 for 10 min. After 24 h exposure to MC2 and MC3 water, SOD mRNA levels increased significantly up to ~150 folds, compared to the control. These data indicate that Hydra vulgaris is able to strongly upregulate mRNA expression of SOD antioxidant enzyme after only 24 h exposition to polluted river water. For the second sampling water from FA site has not been analysed due to few sample left.

SOD mRNA transcription after exposure of samples to Hydra medium (Lab-1) or river water from different sites was quantitatively analysed by comparing it to actin expression, as a housekeeping gene. Hydra medium was used as a negative control whereas 10 µM H2O2 as the positive. *$p \leq 0.05$ vs LAB-1 and **$p \leq 0.0001$ vs Lab-1.

Farfa (FA), Castel Giubileo (CG) e Mezzocammino (MC).

4.5. *Daphnia* sp. Acute Immobilization assay

The results of the tests are similar in the two laboratories. The results of *Daphnia* sp. Acute Immobilization assay did not show any acute effect for the two Tiber River samples of MC and CG. The recorded percentage of mobility inhibition ranged between 0% and 20%, for both the campaigns. The recorded percentage of immobilized individuals was close to 0% as regards the second campaign (MC2 and CG2). However, the first sample of Farfa River (FA1) weakly affected the motility of daphnids showing an acute toxicity effect, ranging from 15% to 35%, while the second sample (FA2) was not toxic for the crustacean. Samples that show values ranging between 20% and 50% are considered lowly toxic on the basis of the scale of toxicity used by the Regional Environmental Protection Agency of Lazio – ARPAL. Overall the results of the three campaigns show no toxicity for Daphnia, only FA is weakly toxic in the first campaign. (Fig. 11). However, considering the low amount of data, these conclusions have to be treated very carefully and further analyses are needed.

4.6. Fish Embryo Toxicity (FET) test on *Danio rerio*

All validity criteria, such as a ≤10% mortality rate in the plate control and internal control and a ≥30% mortality percentage in the positive control were met at the end of the 96 h of exposure. Lethal and sub-lethal endpoints on zebrafish embryos were observed and recorded in every test (Fig. 12). Mortality was observed in all the tested samples for each site. However, a minimum number of lethal effects can be due to the mortality rate characteristic of this species but only an embryo mortality percentage greater than 10% is considered significant according to the guideline.

For sub-lethals also percentages less than 10% are considered. FA1 and MC1 showed levels of lethality that may indicate weak toxicity. CG showed a slight difference in lethality between CG1 and CG2 and CG3. Sub-lethal endpoints were also recorded in all the samples (Fig. 12). These observations could reveal the presence of chemicals or chemical mixtures that may not be assessed with the only record of lethal endpoints. Sub-lethal effects occurred as spine deformation, delay or absence in the hatching, and general underdevelopment, and they were recorded in all the samples. Overall the higher values of toxicity were registered in the organisms exposed to the FA1 and MC2 samples.

4.7. The *Hydra vulgaris* teratogenicity assay

As for RR, ANCOVA outputs on the trend comparisons between control and treatments showed significant differences in some cases. Specifically, CT (Lab medium) showed significant differences with all treatments for a different level of confidence (Table 1). As for AF, significant aberrations emerged in all treatments as well, with respect to CT. Once obtained both RR and AF, TRI was calculated for each site, ranging from I (no risk) to III (moderate risk). No site in IV (high risk) and V (very high risk) were observed.

For the second sampling water from FA site has not been analysed due to few sample left.

4.8. Target chemical analysis

Target chemical analyses highlighted the presence of 34 out of a list of 504 Organic Micropollutants (OMPs). The analysis has been performed during the 3rd sampling campaign. Among the 34 chemicals, 2 compounds were detected below the method detection limit (MDL). Six compounds could not be quantified due to poor calibration curves. OMPs concentrations ranged from 4.9 ng/L (metalaxyl, MC) up to 3.5 µg/L (di-n-butyl phosphate, CG). The highest number of compounds was detected in the site of MC (28 compounds), followed by CG (16) and FA (8). A wide variety of industrial chemicals, plasticizers, and flame retardants (i.e. tri(butoxyethyl)phosphate and di-n-butyl phosphate) was detected in all sites with concentration ranging from 1 up to 3 µg/L. A higher incidence of pharmaceutical products (10) and pesticides (7) was observed in the area of MC. In particular, the herbicide metazachlor (2.6 µg/L), the pharmaceuticals telmisartan (1.1 µg/L), and theophylline (747 ng/L) were detected at higher concentrations with respect to other OMPs. The area of CG was characterized by a lower number of pharmaceuticals (3) and pesticides (4) respect to MC. The herbicide metazachlor (1.2 µg/L) still showed a higher concentration in CG respect to other compounds. Finally, the only pharmaceutical detected in FA was the antibiotic erythromycin, while pesticides were not detected inthis area. An overview of the chemical analyses is given in Table 2. Five compounds

(telmisartan, caffeine, metazachlor, metolachlor, tri(butoxyethyl)phosphate) had calculated EoE higher than one in the area of MC and CG. Due to extremely low PNEC values, telmisartan showed a surprisingly high EoE of 2078 followed by metazachlor (133 in MC and 63 in CG) and the flame retardant tri(butoxyethyl)phosphate (22 in MC). An overview of the EoE in the different sites is given in Fig. 13.

4.9. PFAS chemical analysis

The results of the group of PFAS indicated that PFOA and PFOS have been detected at low levels in all 3 sampling sites (Table 3). For PFOA, the highest level detected has been at CG site, for PFOS at MC site. For short-chain PFAS the levels detected are lower than the EQS (environmental quality standard) of the national legislation (DGls 172/2015). The EQS for PFOS has been slightly exceeded in the MC site.

5. Discussion

A monitoring study of surface water quality of Tiber River basin was conducted in three sites selected upstream, downstream the city of Rome and in a protected rural area outside the city. The analysis was carried out applying an integrated approach of EBMs focused on genotoxicity. Chemical water quality and biological effects, such as acute toxicity and teratogenicity, were also considered.

The study confirmed that the application of EBMs in phylogenetically different model organisms is feasible and informative. The genotoxic effects were detected as DNA primary damage and chromosomal damage using Comet and Micronucleus assays, respectively.

Positive results were also obtained in *D. polymorpha*, a bivalve filter-feeding invertebrate, confirming that it is a suitable model in aquatic environmental investigation. *D. polymorpha* was found to be useful for screening the genotoxic effects of both classical and emerging pollutants due to their high filtration rate, adequate size for in vivo laboratory exposure and maintenance (Guidi et al., 2017, 2020).

The sensitivity of zebra mussel haemocytes to genotoxic compounds has been previously demonstrated through the detection of DNA strand breaks and DNA adducts (Chatel et al., 2015; Pavlica et al., 2001; Juhel et al., 2007). Moreover, the increase of micronuclei and nuclear abnormalities related with the level of contaminants in river water was reported in literature (De La Fontaine, 2000). Results from this model organism suggest a lower sensitivity of *D. polymorpha* heaemocytes respect to *H. vulgaris* cells. It is worth to note that in contrast to hydra cells, *D. polymorpha* heaemocytes are not directly in contact with environmental stressors, but are exposed to chemicals after their absorption, metabolisation, distribution, and excretion into the body, providing suggestions about the different mechanisms of cellular responses possibly related with different routes of exposure of Tiber River sample waters.

The Comet assay in *D. magna*, showed the ability to discriminate the presence of genotoxins among differently impacted sites, confirming data deriving from previous sampling campaigns on a

pilot basin located in the Parma district (Italy) (Suppa et al., 2020). The use of *D. magna* in a battery of EBMs, able to identify genotoxic pollutants in freshwaters, seems to be very interesting taking also into account the worldwide application of the daphnia toxicity assays in ecotoxicology (Brownik, 2017; Mattei et al., 2006).

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On the other hand, the analysis of DNA damage in *Danio rerio* embryos showed that this experimental model was less responsive compared to the other models in the experimental conditions applied. Also in this case, this result could be related to differences among phylogenetically different organisms in metabolism, detoxification processes, DNA repair capacity. The analysis of DNA damage in zebrafish embryos highlighted a different response between eggs (48 h) and early larval stages (96 h), showing an increased susceptibility to the adverse effects of environmental pollutants at later developmental stages (Kosmel et al., 2006). The issue of variability in sensitivity between developmental stages could be a confounding factor to be considered in hazard identification, although further studies are needed on this aspect.

The different responses, shown by the various organisms used, highlighted not only their different sensitivity to environmental DNA damaging compounds but also the importance of using a set of model organisms in monitoring environments contaminated by complex mixtures.

Overall, the results obtained with genotoxicity assays showed that the Comet assay can be a useful tool in biomonitoring of surface waters applying several experimental models in agreement with literature data (Guidi et al., 2017; Kolarevic et al.-, 2016a; Kolarevic et al.-, 2016b; Deutschmann et al., 2016; pellegri et al., 2020).

In relation to the results obtained with the acute toxicity and teratogenicitiy assays, it should be emphasized that the two model organisms, *H. vulgaris* and *D. rerio*, showed correspondence in the toxicity results for almost all water samples. In detail, the hydra Teratogenicity test detected toxicity in 8 out of 9 samples, while the FET test (lethal and sub-lethal endpoints) on *D. rerio* detected toxicity on all the samples. The FET test (OECD 236) is considered indeed a suitable model for the application of WFD monitoring programs (Cristiano et al., 2019). Moreover, the Hydra Teratogenicity test has been previously applied as an informative approach (Malafoglia et al., 2016). In the present study, the highest levels of teratogenicity have been detected in the MC and CG sites, while sub-lethal effects with zebrafish embryos have been detected in all the samples. The effects detected by the different assays for each sample are described in Fig. 14. As for the teratogenicity bioassays based on *H. vulgaris*, this study confirms that hydra is a suitable model to be exploited as rapid, economic, and target specific early warning systems for obtaining an evaluation on teratogenic risks for environmental health and human wellbeing (Cera et al., 2020a). Particularly, we exploited the *H. vulgaris* regeneration for a recently developed risk index especially tested for environmental monitoring of teratogenic effects in freshwaters (Traversetti et

al., 2017). The FET model gives information also on sub-lethal effects that can be linked to other relevant endpoints such as neurotoxicity or embryo-toxicity (Cristiano et al., 2020).

An unexpected result is the detection of acute effects in the FA site area. This effect could be maybe related to some substances detected in this area, like personal care products and veterinary pharmaceuticals.

Results discussed above as function of the test applied and the biological effects observed, were then elaborated considering the analyses grouped per sampling site. Based on the results obtained, it is difficult to rank the sampling sites, because the biological effects were not always consistent among the three locations (Fig. 14). In fact, at MC site toxic and genotoxic effects were detected by most of the assays applied (Tables 4 and 5).

The results indicate MC site as the most affected by environmental pressures possibly due to the location of this station downstream to the city of Rome. The site is affected by the pollution load typical of a big city, i.e. small plant discharges, waste pollution, personal care products, detergents and the emissions of the urban wastewater treatment plants.

The chemical analysis, conducted in the water samples of the third sampling campaign, detected thirty-four chemical compounds. All neuroactive compounds were classified as pharmaceuticals, and they involve different molecular targets. Five OMPs have been detected at levels above the PNEC, suggesting that rigorous monitoring measures need to be continuously applied. In particular, telmisartan and metazachlor showed EoE higher than 100 in MC, values which may highlight a potential risk for the local fauna. High concentrations of telmisartan were already reported in different European river basins (Gurke et al., 2015; Carmona et al., 2017; Diuzheva et al., 2018; Alygizakis et al., 2019). As examples, Gurke et al. (2015) reported a concentration of 1.3 µg/L in Elbe River (Germany), while Carmona et al. (2017) detected concentrations of telmisartan up to 950 ng/L in Turia River (Spain). In a study on the Danube River basin performed by Alygizakis et al. (2019), telmisartan exceeded the PNEC values in 83% of the samples. Furthermore, similar concentrations of metazachlor (1 and 3.3 µg/L) were also detected by other authors in Spain (Quintana et al., 2019).

It is difficult to directly link the effects highlighted by EBMs with the chemical substances detected in this study, but in some cases this association can be hypothesized based on the knowledge of their mode of action. Some molecules included in the European watch-list (European Commission. Commission Implementing Decision (EU) 2018/840) have been identified, such as erythromycin able to induce DNA damage in zebrafish erythrocytes after chronic exposure (Singh et al., 2014; Rocco et al., 2012).

High caffeine concentrations were detected in MC samples. Induction of DNA primary damage was found in Asian clam haemocytes after chronic exposure to 15 µg/L of caffeine (Aguirre-Martınez et al., 2013), likely related to the oxidative stress mechanism as reported by Martìn-Dìaz and co-Authors (2009) who detected lipid peroxidation induced by caffeine and carbamazepine in the mussel Elliptio complanata. On the other hand, no loss of DNA integrity was assessed in the freshwater teleost Prochilodus lineatus after chronic exposure to caffeine in the dose range of 0.3–30 µg/L (Santos-Silva et al., 2018). The non-steroidal pharmaceutical ketoprofen has been detected in the MC site. This compound is suspected to be mutagenic, being found to induce

chromosomal mutations (Kılıc and Tuylu, 2020) in human lymphocytes and acts on nucleic acid biosynthesis and RNA polymerase activity. The herbicide metazachlor, which acts on cells during mitosis, found at high concentration in MC and CG samples, has been reported to express its genotoxicity in the erythrocytes of the crucian carp exposed to the French Save River waters, both after basal flow and winter and spring floods, while induction of micronucleated cells was also found after the spring flood (Polard et al., 2011). Other contaminants detected are neuroactive (e.g dinoseb), cardiotoxic (e.g. Propamocarb-angiotensin receptor) or endocrine disrupting-chemicals (e.g. telmisartan, metholachlor), respiratory (ethofumosate) or photosynthesis (ranitidine) inhibitors.

Farfa is the site with the minor number of chemical contaminants, although some compounds have been also detected in this naturalistic site. This result indicates the presence of possible sources of pollution also in this area such as lauryl diethanolamide expected to be present in small urban areas. Pesticides such as terbuthylazine, a substance widely diffused in Italy and included in the national Italian decree (Dgls 172/2015) with a specific environmental quality standard (EQS) and metholachlor have been detected in CG site.

In relation to PFAS analysis, it should be remarked that the levels detected are far below the EQS of the Italian legislation and probably this group of compounds do not represent a current risk for the aquatic ecosystems of the monitored areas. However, even if at low levels, PFAS were detected in Farfa site where the anthropogenic pressures are minimal. Thus, the monitoring of the levels of these emerging contaminants, considering their relevant potential effects on environment and human health (EFSA European Food Safety Authority, 2020), should be followed up.

In this study different eco-toxicological effects have been detected and the results can contribute to improve the knowledge on the quality status of the Tiber River and help to identify the effects of mixtures of pollutants. Besides, the EBMs may detect the effects not only of a single contaminant but also of complex mixtures. A better comprehension of the effects (Ankley et al., 2010) induced by the mixture of compounds detected in the Tiber River basin, including emerging substances, is fundamental to prevent risks for human health when there is a reuse of water for agriculture, aquaculture and drinking purposes. It is worth to note that the Tiber River could be used in the future as a reservoir for drinking water in the city of Rome, especially considering the global climate change effects, e.g. water scarcity. Water scarcity can also increase the concentration and ecological effects of pollutants worsening the water quality. Tiber River is also affected by flooding that can give clogging of urban wastewater treatment plants, cross-contamination of rivers/soil/lands and increasing run-off, remobilization of chemically contaminated sediment. Therefore, in this context, every new result on the water quality of this river ecosystem should be carefully considered. The integration of a battery of EBMs in monitoring programmes of surface waterbodies (e.g. investigative in the WFD) such as the Tiber River is a key aspect in order also to identify the multiple sources of pollution (Fig. 15) and for the application of management measures.

In the figure are described the sources of pollution for surface waterbodies and the potential Application of EBMs.

6. Conclusions

This integrated study suggests that a battery of EBMs could have some advantages in the monitoring programmes of the WFD: the detection of effects indicates the possible presence of potential hazards (screening function), triggering further activities such as source identification, analysis of the pressure and impacts. EBMs can detect the adverse biological effects of mixtures of chemical pollutants in the ecosystems not disclosed by chemical analysis. Concerning the evaluation of Tiber River quality, this study has some weakness because of the limited number of sampling sites and frequency of monitoring, although it is possible to mention, considering the results of the study, that the Tiber River is affected by several sources of pollution as highlighted by the results of the EBMs. In all the sampling sites and during all the 3 sampling campaigns, genotoxic effects have been detected also in the site where the pressures are less intense.. At the same time teratogenicity, embryo-toxicity and neurotoxicity effects have also been detected. Acute effects have been rarely observed indicating that the contamination is not severe but is caused by multiple contaminants present at low concentrations. The detection of several contaminants indicates that the application of more stringent measures is needed.

These substances include pharmaceuticals, pesticides, plasticizers, antibiotics, and cosmetic products. The application of EBMs can be considered relevant also taking into account climate change effects on chemical contamination (e.g. flooding and water scarcity) and it is linked to human health aspects (e.g. genotoxicity). In conclusion, this study, performed by a network of institutions, could represent a strong support for the local authority in order to apply all the measures necessary to reduce or eliminate the release of chemical contaminants in the Tiber River considering the current and future uses of this precious ecosystem.

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Figure 1. Sampling sites of the study

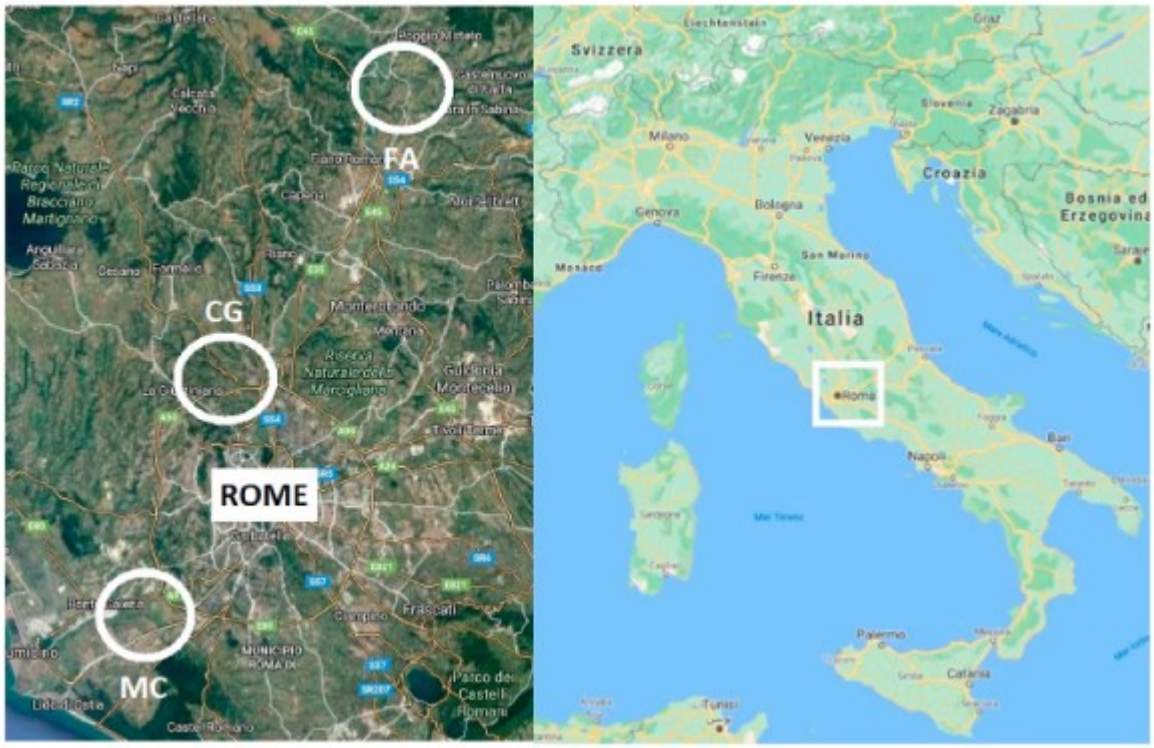

Figure 2. The Teratogenic Risk Index (TRI) double entry matrix

TRI matrix aberration frequency (AF)

regeneration rate (RR)	0.00-0.19	0.20-0.39	0.40-0.59	0.60-0.79	0.80-1.00
3.50-4.00	10	9	6	4	1
3.00-3.49	10	8	6	4	1
2.50-2.99	8	7	5	3	1
2.00-2.49	7	6	5	3	1
1.50-1.99	5	5	4	2	1
1.00-1.49	4	3	3	2	0
0.50-0.99	2	1	1	0	0
0.00-0.49	0				

score	class	risk level
10	I	none
9-7	II	low
6-4	III	moderate
3-2	IV	high
1	V	very high

(HI) HYDRA INDEX

Figure 3. Comet Assay in *D. polymorpha* haemocytes

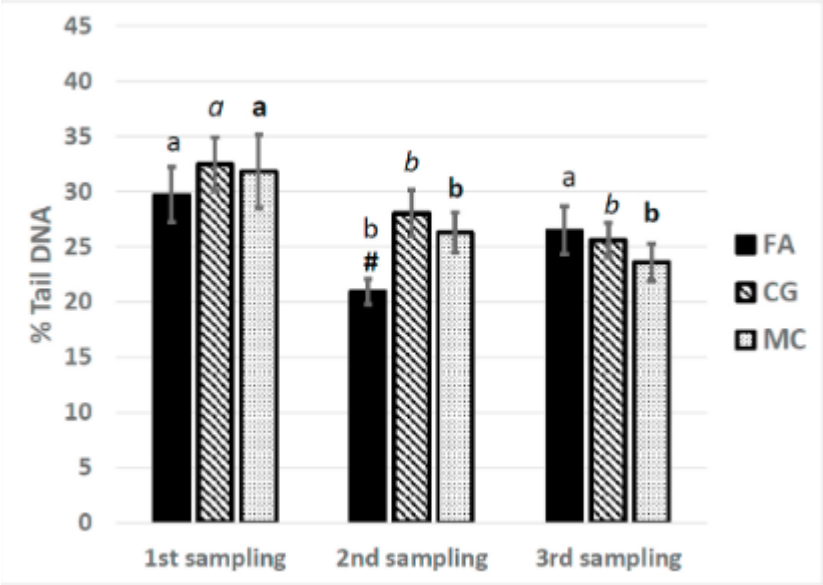

Figure 4. Comet assay in *D. magna*.

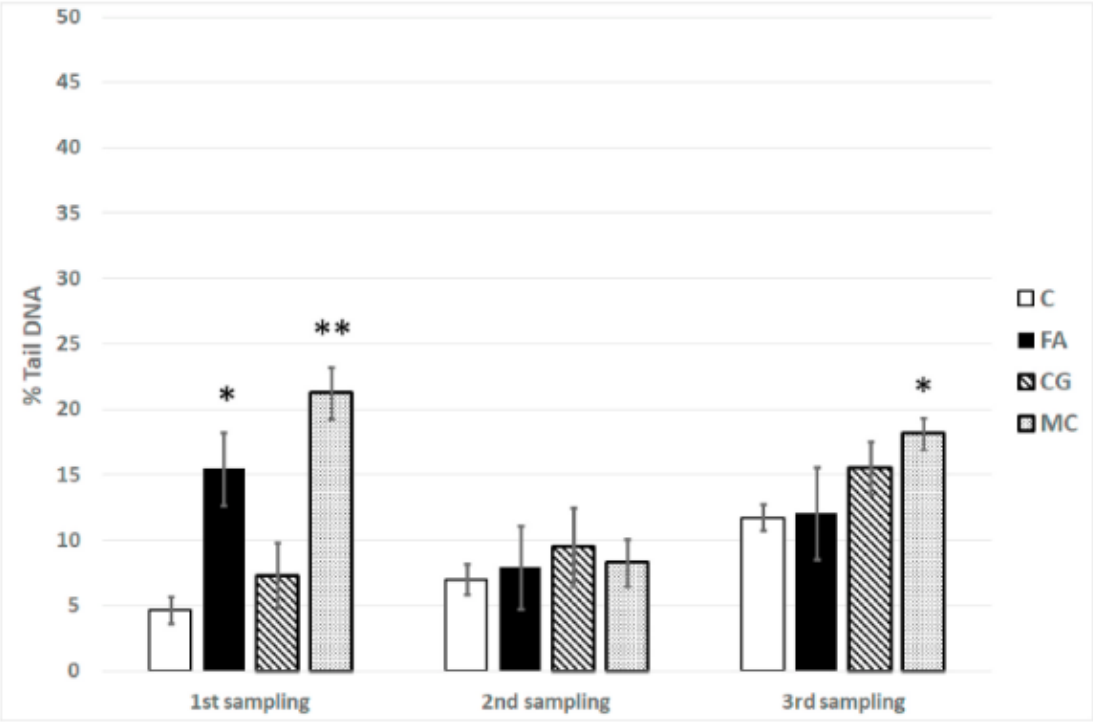

Figure 5. Comet assay in *H. vulgaris*

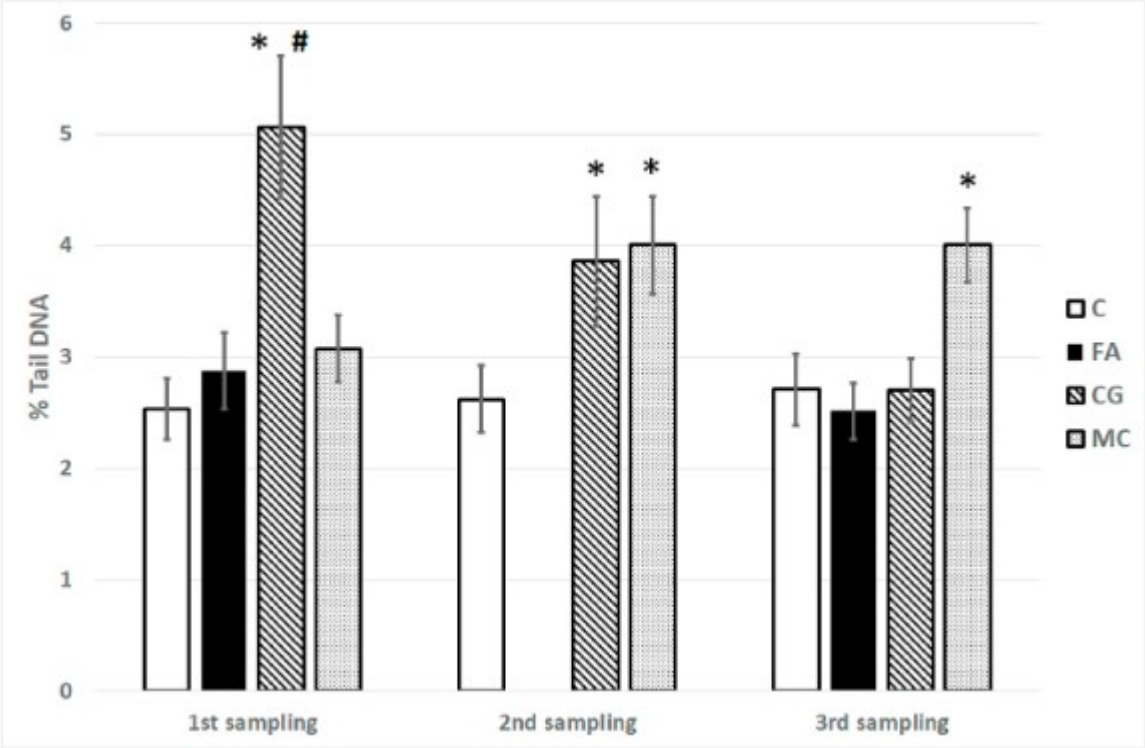

Figure 6. Comet assays observed in cells from zebrafish embryos

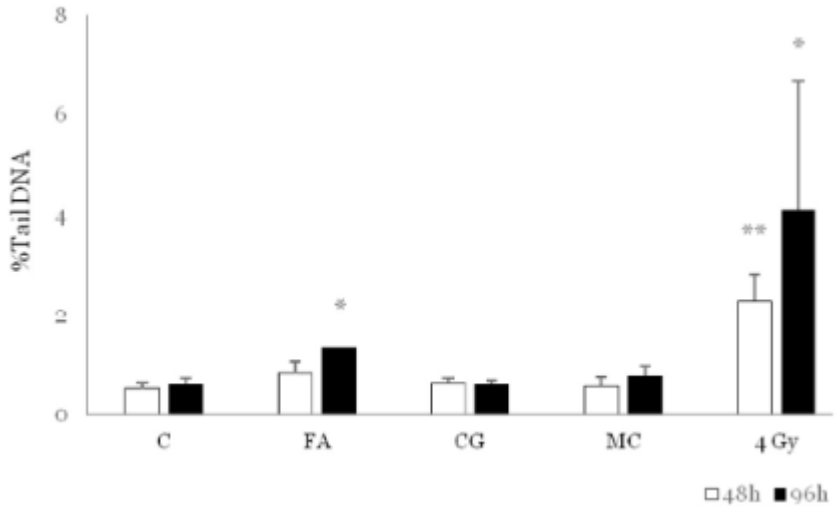

Figure 7. Micronucleated cell frequency (MN) and total nuclear abnormalities (NA) observed in *D. polymorpha* haemocytes

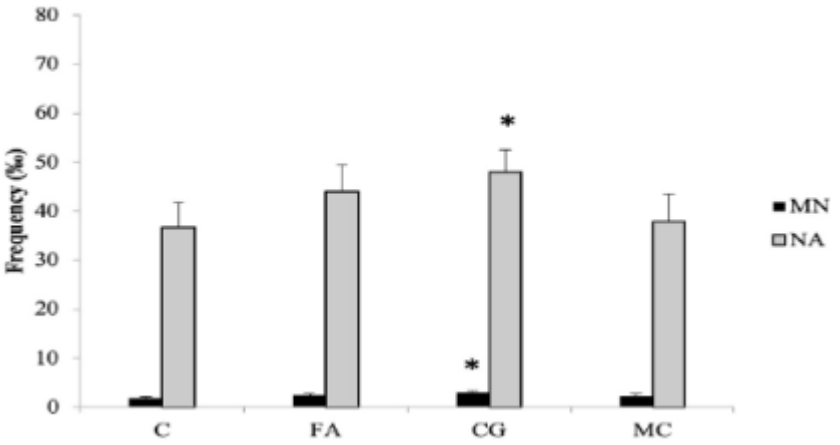

Figure 8. Comparison of the two summer campaigns. Micronucleated cell frequency (A), and total nuclear abnormalities (B) observed in *D. poymorpha* haemocytes

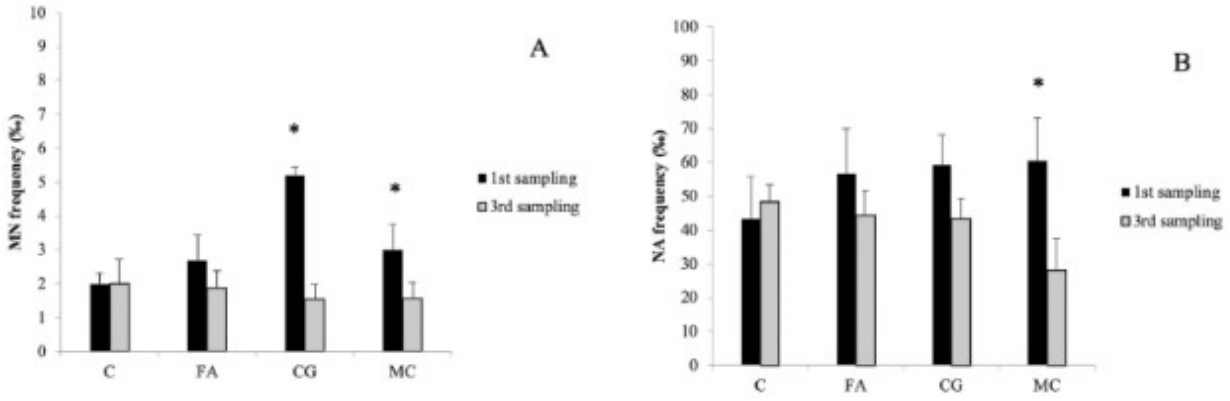

Figure 9. Apoptotic cells frequency evaluated in *D. poymorpha* haemocytes

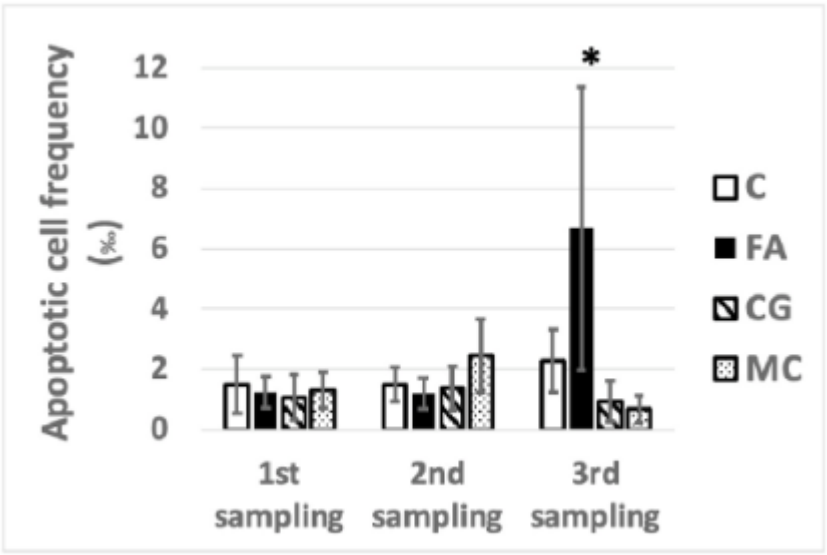

Figure 10. SOD mRNA transcription in *H. vulgaris*

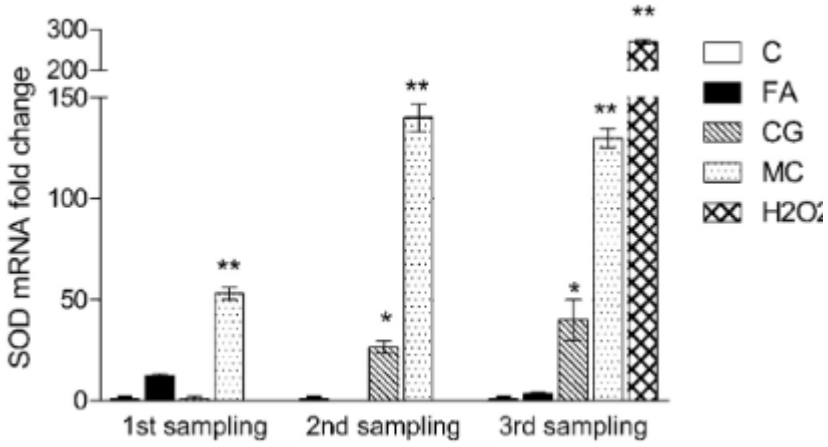

Figure 11. *D. magna* acute assay results expressed as means and the standard deviations of daphnid immobilization percentage

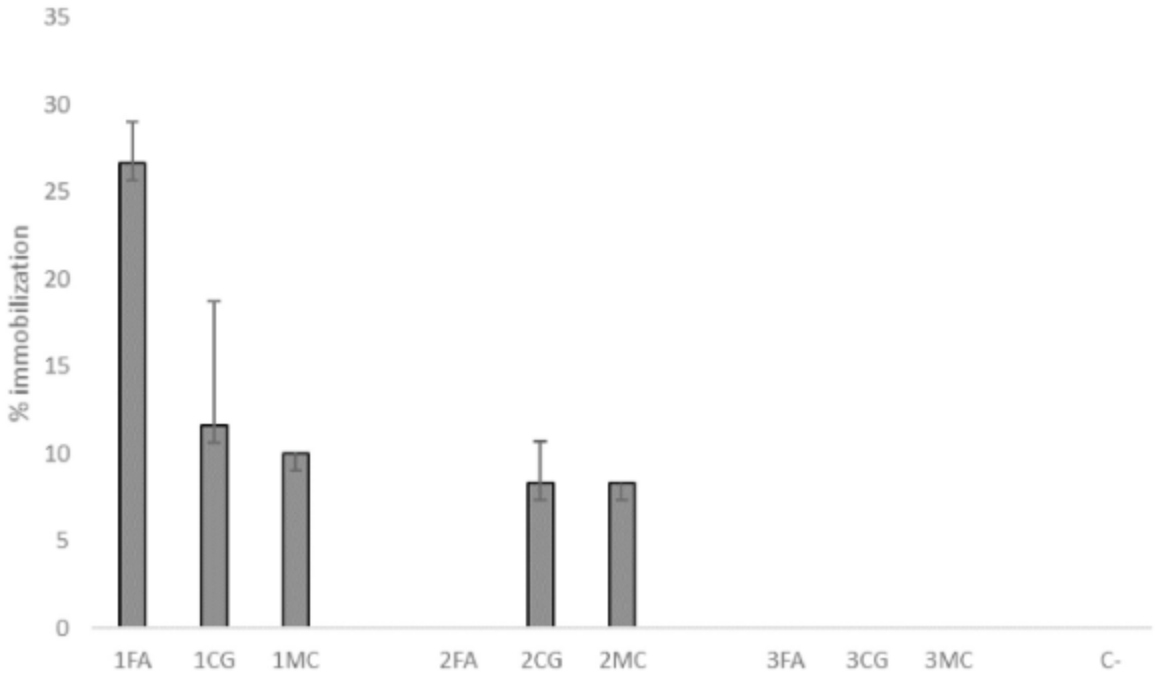

Figure 12. *Danio rerio* FET results; the straight line represents the limit for the acute toxicity (mortality)

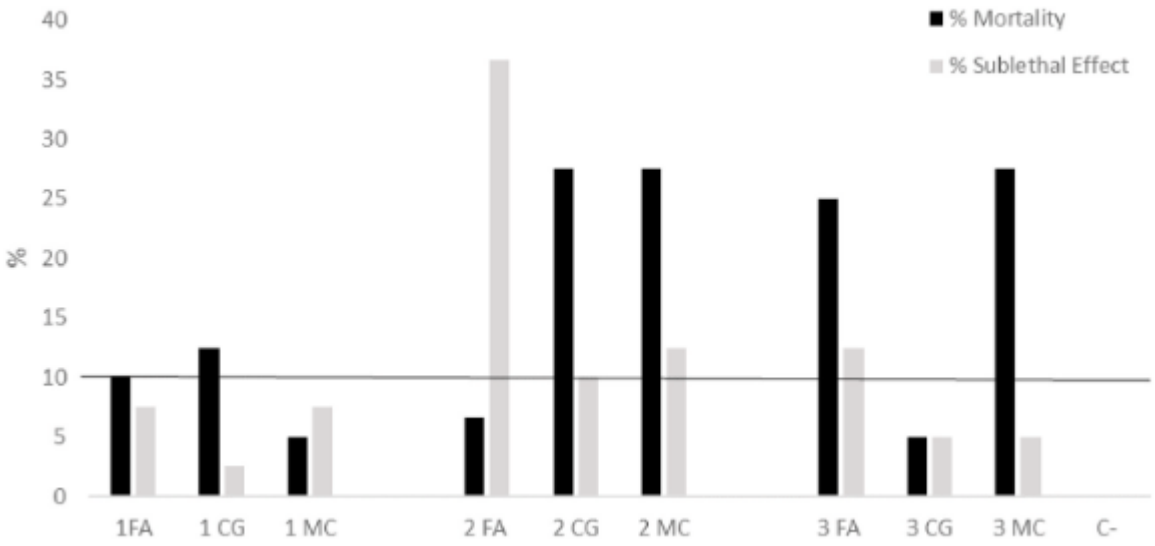

Figure 13. Calculated Extent of Exceedance (EoE) for Mezzocammino (MC) and Castel Giubileo (CG). Bars represent compounds with EoE values higher than the threshold of 1. TBeP (Tri(butoxyethyl)phosphate).

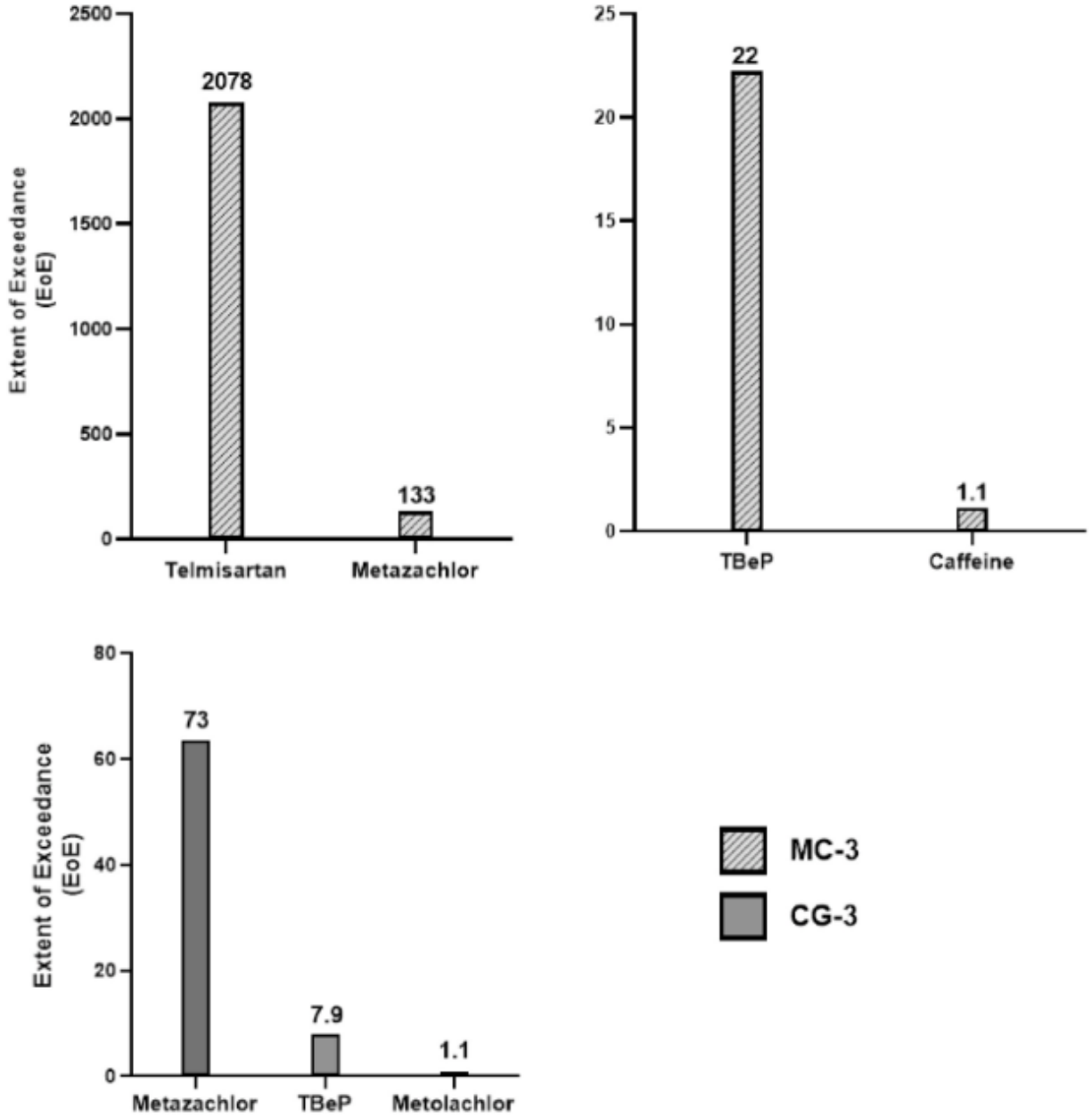

Figure 14. EBMs comparison. In this figure is described for each sample which EBMs have detected effects

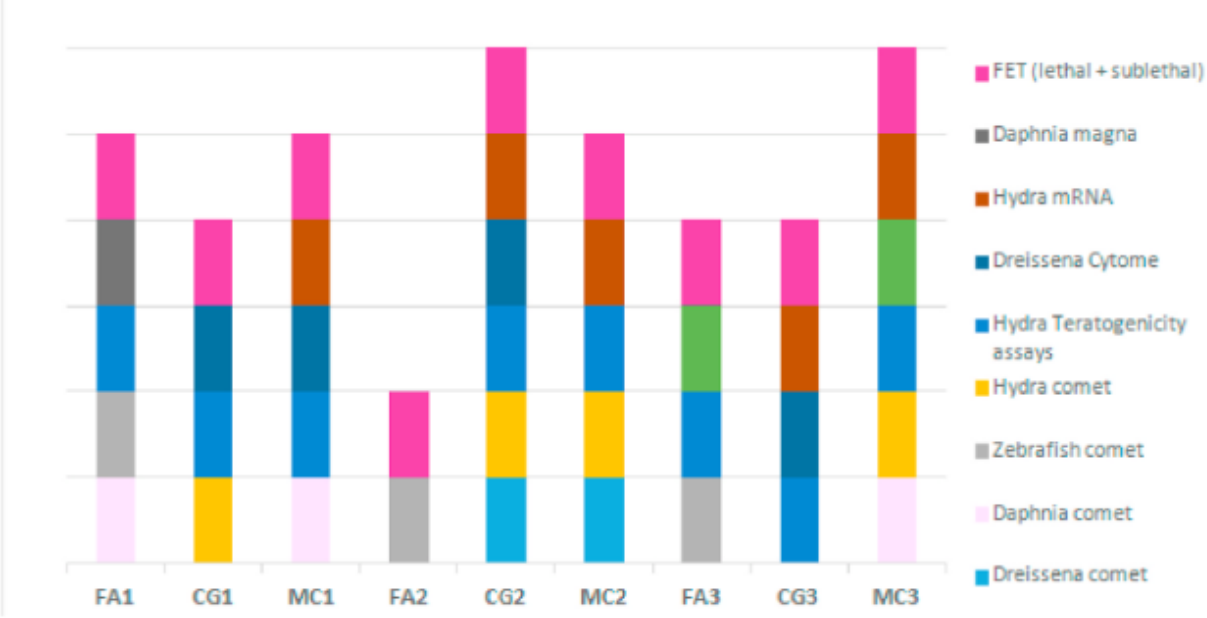

Figure 15. Application of Effect-Based methods and link to the pressures and sources

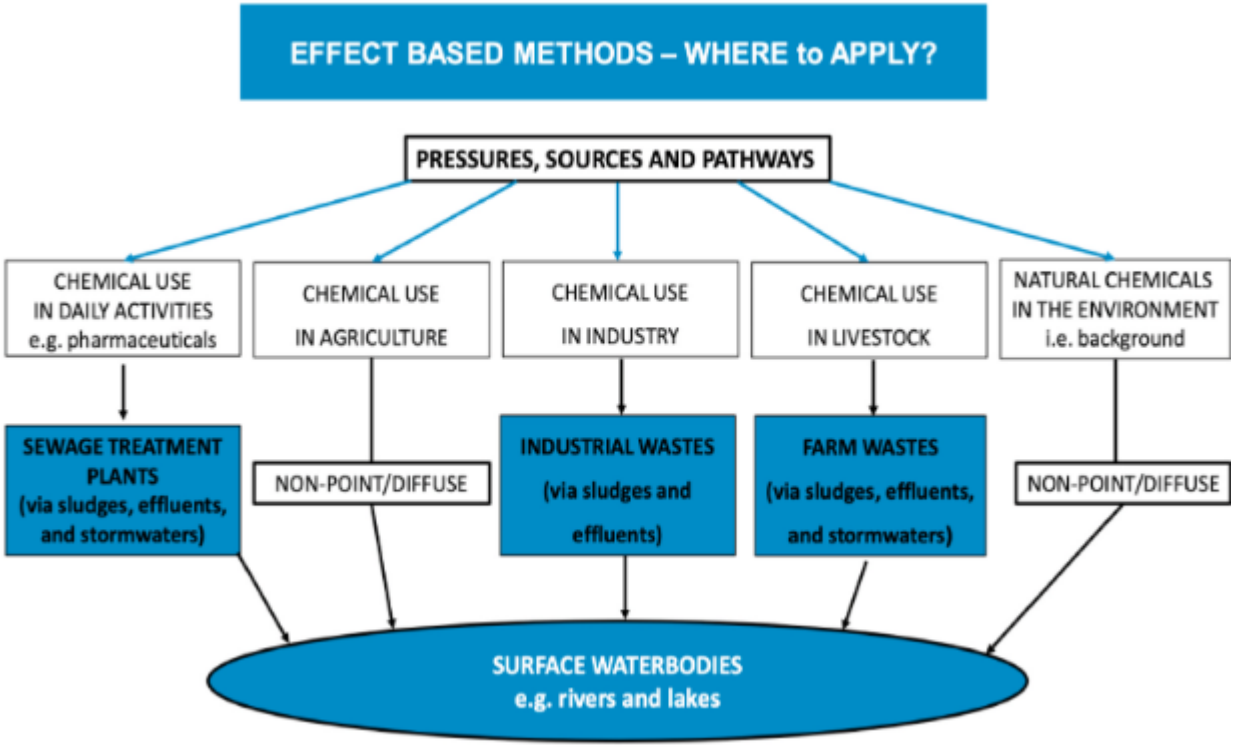

Table 1. The Teratogenic Risk Index (TRI) class (reported by Roman numbers) and judgement.

	TRI class	**TRI judgement**
CT1	I	no risk
FA1	II	low risk
CG1	II	low risk
MC1	II	low risk
CT2	I	no risk
CG2	II	low risk
MC2	III	moderate risk
CT3	I	no risk
FA3	II	low risk
CG3	III	moderate risk
MC3	III	moderate risk

Marks (in alphabetical order): CT=Control (treated only with Hydra medium) CG = Castel Giubileo; FA = Farfa River; MC = Mezzocammino. Arabian numbers associated to each mark represent the sampling sessions

Table 2. Concentrations of quantified contaminants and respective PNEC (predicted no effect concentrations).

Compounds	Concentration (µg/L)	PNEC (µg/L)
Amitriptyline	< MDL	0.1
Caffeine	1.3 (MC3)	1.2
Carbamazepine	0.03 (MC3) 0.007 (CG3)	0.05
Cotinine	0.1 (MC3)	10
DEET	0.2 (MC3) 0.01 (CG3)	88
Di-n-butyl phosphate	0.1 (FA3), 3.6 (CG3), 0.3 (MC3)	5.8
Dinoseb	0.04 (MC3)	0.4
*Erythromycin	(FA3), (MC3)-detected	0.5
Ethofumesate	0.08 (MC3)	3.1
Gabapentin-Lactam	0.3(MC3), 0.3 (CG3)	100
Kresoxim-methyl	0.05 (MC3)	0.1
Ketoprofen	0.04 (MC3)	2.1
Lauryl diethanolamide	0.02 (FA3) 0.04 (CG3)	0.95
Metalaxyl	0.005 (MC3)	20
Metazachlor	2.7 (MC3) 1.3 (CG3)	0.02
Metazachlor BH479-12	0.3 (MC3)	–
Metolachlor	0.02 (CG3)	0.2
N-Acetyl-4-aminoantipyrine	0.03 (MC3)	100
N-Butylbenzenesulfonamide	0.08 (MC3)	21
Propamocarb	0.06 (MC3) 0.06 (CG3)	710
Telmisartan	1.1 (MC3)	0.00055
Terbuthylazine	0.007 (CG3)	0.06
Theophyllin	0.7 (MC3)	15
Tri(butoxyethyl)phosphate	0.08 (FA3) 1.1 (CG3) 3.1 (MC3)	0.1
2-Aminobenzimidazole	0.1 (MC3) 0.1 (CG3)	2.3
5-Methyl-1H-benzotriazole	0.1 (MC3) 0.1 (CG3)	150
10,11-dihydroxycarbamazepine	0.085 MC3)	2.4

PNEC values were retrieved from the NORMAN Ecotoxicology database. MDL (Maximum Detection Limit). *Substance not quantified.

Table 3. PFAS results (ng/L)

	MC3	CG3	FA3	EQS
PFBA	ND	ND	ND	7000 ng/L
PFPeA	ND	ND	ND	3000 ng/L
L-PFBS	0.2	0.35	0.207	3000 ng/L
PFHxA	<0.47	<0.02	ND	1000 ng/L
PFHpA	<0.36	ND	ND	
PFHxS	0.15	0.16	0.040	
PFOA	2.58	9.92	8.60	100 ng/L
PFNA	0.43	ND	ND	
PFOS	0.75	0.29	0.036	0.65 ng/L
PFDA	<0.18	ND	ND	
PFUdA	<0.38	ND	ND	
PFHpS	0.05	0.05	0.041	
PFDoA	<0.4	ND	ND	

Table 4. Comparison of the results of genotoxicity assays.

Methods	FA1	CG1	MC1	FA2	CG2	MC2	FA3	CG3	MC3
Dreissena Comet	–	–	–	–	+	+	–	–	–
Daphnia Comet	+	–	+	–	–	–	–	–	+
Zebrafish *Embryo Comet	+	–	–	+	–	–	+	–	–
Hydra Comet	–	+	–	n.d.a	+	+	–	–	+
Dreissena NRRT	–		–	–	–	–	+	–	+
Dreissena Cytome	–	+	+	–	+	–	–	+	–
Hydra mRNA	–	–	+	n.d.a	+	+	–	+	+

For zebrafish (Comet assay) is not possible to differentiate the effects of the single sites, but for Farfa higher values have been remarked*. + presence of toxicity; – absence of toxicity; n.d.a. No data available

Table 5. Comparison of the results of other EBMs

Methods	FA1	CG1	MC1	FA2	CG2	MC2	FA3	CG3	MC3
Daphnia magna (Acute test)	+	–	–	–	–	–	–	–	–
FET lethal	+	–	+	–	+	+	–	–	–
FET sublethal	+	+	+	+	–	+	+	+	+
Hydra Teratogenicity assay	+	+	+	n.d.a	+	+	+	+	+

+ presence of toxicity; - absence of toxicity; n.d.a. No data available.