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Application of effect-based methods (EBMs) in a river basin: a preliminary study in Central Italy

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Abstract

Introduction. Effect-based methods (EBMs), i.e. *in vitro* and *in vivo* bioassays, represent innovative tools for the effect detection of environmental chemical pollutants on living organisms. The aim of this study was to evaluate the water quality of a river ecosystem implementing two *in vivo* bioassays on target freshwater animal species: the crustacean *Daphnia magna* and the small fish *Danio rerio*, also known as zebrafish.

Materials and methods. The methods applied in this study, i.e. the *Daphnia* sp. Acute Immobilisation assay and the Fish Embryo Acute Toxicity (FET) test, are commonly used in water quality research and their application in short-term ecotoxicity detection is suggested by recent European projects. Two sampling sites were chosen in the urban part of the Tiber River in Rome, while a third one was chosen as a reference site in the Farfa River, a tributary upstream of the city. The sites in the Tiber River are potentially affected by different pollution sources, including urban and industrial wastewater discharges, the pesticide release, livestock waste products, and waste dumps.

Results and discussion. The results of the study showed wide differences between the two applied bioassays. The FET test was generally more sensitive in detecting even low effects in all the water samples, but the strongest statistically results were observed with the *D. magna* Acute Immobilisation test. The results of this research confirm the effectiveness of EBMs in investigating and monitoring water chemical pollution, and stress the need for performing further studies, e.g. chemical analyses and other bioassays, to improve the knowledge of the health status of the Tiber River basin.

Conclusions. Further results will aim to support the local authorities in adopting measures to reduce and to eliminate the sources of chemical pollution in the study area.

Key words

- effect-based methods
- *Daphnia magna*
- zebrafish
- chemical pollution
- Tiber River

INTRODUCTION

The Water Framework Directive (WFD-2000/60/EC) commits the European Union (EU) Member States to ensure a good qualitative and quantitative status of all water bodies [1]. The chemical monitoring programmes of the WFD includes a list of different priority chemicals substances (Directive 2013/39/UE) and river specific pollutants that need to be constantly monitored in surface waters [2]. Despite the efforts of the EU to reduce the release of chemicals into the aquatic environment, new emerging pollutants and contaminant mixtures still make water pollution one of the main challenges across Europe [3].

The classical single-chemical risk assessment approach for the management of the chemical pollution in water bodies shows some limitations, as highlighted by recent European projects and monitoring networks [4, 5]. Analysing, detecting and quantifying all the substances in the aquatic environment is very challenging [3, 6]. Furthermore, the chemical pollutants present in the water bodies can combine themselves in mixtures whose effects may not be predictable on the only basis of chemical analyses. However, it is fundamental to investigate the effects of living organisms exposure to pollutants released in water ecosystems in order to understand the impact of chemical pollution on the aquatic biodiversity. It is also necessary to link the observed effects with the cost-effective management objectives. Therefore, in the context of the WFD, a specific activity was foreseen for the elaboration of a technical report [7] on aquatic effect-based tools (e.g. bioassays, biomarkers). As mentioned in the report of the WFD, these tools can be used as it follows:

- as screening tools to aid in the prioritisation of analysis of water bodies;

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- to establish early warning systems;
- to take into account the effects of chemical mixtures or chemicals that are not analysed (e.g. to support investigative monitoring when the reason why the decline of specific species is unknown);
- to provide additional support in water and sediment quality assessment.

In this WFD report, the adoption of apical short-term ecotoxicity bioassays, i.e. fish embryo toxicity and immobilisation of *Daphnia* sp., is recommended [4, 7]. In this research, the southern part of the Tiber River basin was chosen as a case study for a preliminary investigation of the chemical pollution effects in a water ecosystem particularly important from a human perspective. Indeed, the Tiber River is the third-longest river in Italy and the main watercourse in Rome. It rises in the Appennine Mountains in the Emilia-Romagna Region (Fumaiolo Mountain, 1407 m above s.l.) and it flows for 405 km to the southwest through Umbria and Lazio Regions towards the Tyrrhenian Sea. The river represents the largest basin in Central Italy with a drainage area of 17 375 km^2. The total human population living in this geographic area is approximately 4.7 million people. The plan of the Tiber River Basin Authority (TRBA) reports a large land agricultural use of the basin covering about 53% of the surface, while approximately 39% is forested and 5% is urbanised [8]. Thus, it can be assumed the presence of several chemical pollutants, also combined as contaminant mixtures, affecting its entire watercourse, even at very low concentrations. Environmentally hazardous concentrations of certain priority substances, e.g. heavy metals, nonylphenols, polycyclic aromatic hydrocarbons (PAHs), were found both in water and in sediments of the urban stretch of the Tiber River [9-11]. Chemical pollutants widespread into the watercourse represent an ecological risk for the river ecosystem and for the coastal area of the Tyrrhenian Sea, where the Tiber River flows. Previous studies found different organophosphate (OPPs) pesticides in the river and in its estuary, even if mostly in lower concentrations than the guideline values [12]. Moreover, other environmental pollutants were found in the last stretch of the river. For instance, high concentrations of pharmaceuticals, such as the hydroxy-metabolite of the mood-stabilising drug carbamazepine and the non-steroidal anti-inflammatory drug diclofenac, PAHs, pesticides, perfluorinated and polyfluorinated alkyl substances (PFAS), were detected [13]. The occurrence of antibiotics was also recorded [14]. The data of the local environmental agency obtained following the current legislation show that some priority substances, e.g. nickel, exceed the environmental quality standards (EQS) in the Tiber River [15]. Considering the high number of chemical pollutants that may be found in this river basin, and the resulting effects on living organisms, the use of EBMs appears to be a low-cost and sustainable opportunity for the water ecosystem management.

Aim of the study

The purpose of the present study was to apply two effect-based methods (EBMs), i.e. *Daphnia* sp. Acute Immobilisation assay and Fish Embryo Acute Toxicity (FET) test, with a view to investigating the effects of chemical substances in the last stretch of the Tiber River basin on two target freshwater model organisms: the crustacean *Daphnia magna* and the small fish *Danio rerio*, otherwise known as zebrafish. Each of these species can respond to the environmental pollution differently since they belong to separated trophic levels. Therefore, the implementation of bioassays on different organisms allows achieving a better overview of the effects of chemical pollution on water ecosystems. The bioassays used in this research represent common applied experimental procedures in water quality studies. Moreover, the use of these model organisms for environmental monitoring purposes is encouraged by recent European projects [4]. The general aim of this study was to support the effect-based evaluation of the quality status of the urban area of the Tiber River in the city of Rome and one tributary.

MATERIALS AND METHODS

Study area and sampling

The study area was located along the Tiber River basin in the Lazio Region, comprising two different sites along the main watercourse and one tributary *(Figure 1)*. The three selected sites were characterised by different types and levels of pollution, reflecting different anthropogenic pressures, potentially including drift of pesticides, urban and industrial wastewater discharges, waste dumps, and livestock waste products [15]. The southern part of the Tiber River reflects all the contamination sources affecting the river along its stream. Castel Giubileo (CG) is located in the North of Rome (upstream of the city): this site is likely affected by leaching of waste, agricultural and zootechnical discharges as well as the presence of small and medium factories. Mezzocammino (MC) is located in the South of Rome (downstream of the city), about twenty kilometers from the estuary: this site collects the urban discharges of the city of Rome and it is located right after a sewage treatment plant. Farfa River (FA) is a stream that flows into the Tiber River at around fifty kilometers before Rome: it was chosen as a reference site for its good ecological status according to the demands of the WFD [16, 17]. Two sampling campaigns were conducted in these sites: the first was performed during the summer of 2018, while the second was carried out during the summer of 2019. Water samples (2 L) were collected in each site between 0 and 20 cm from the surface and then they were quickly stored at +4 °C. Each sample was filtered at 0.45 µm in order to remove the suspended materials.

Instrumentation

The observations of the daphnids and the photographs of representative zebrafish individuals were performed using a Leica S8AP0 stereo microscope linked to a Basler acA 1300-60 gm camera. The images were acquired thanks to the software Media Recorder™ 4.0 provided by Noldus Information Technology.

Laboratory analyses

Analyses were carried out by the Laboratory of Ecotoxicology of the Unit of Ecosystem and Health of the Italian Institute of Health (Istituto Superiore di Sanità, ISS, Rome). The Laboratory works in quality according

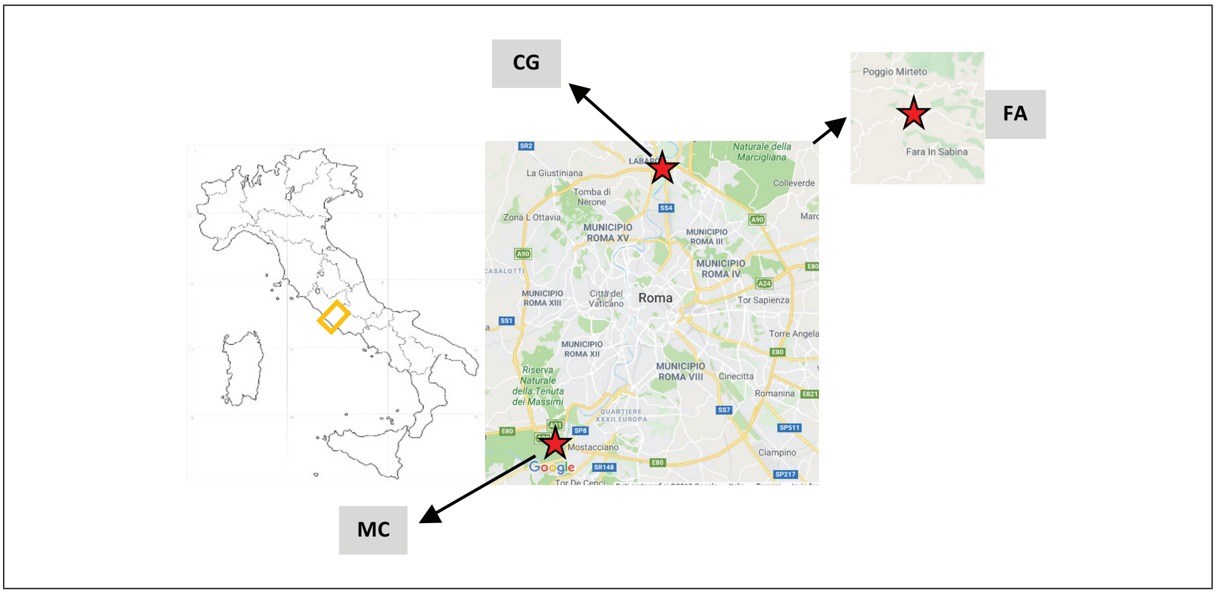

Figure 1
Map of the sampling sites in the study area.
CG: Castel Giubileo; FA: Farfa River; MC: Mezzocammino.

to the UNI CEI EN ISO/IEC 17025 standard [18] and participates in different national and European inter-laboratory comparisons.

Daphnia sp. Acute Immobilisation assay (OECD 202:2004)

The *Daphnia* sp. Acute Immobilisation assay is a screening method using freshwater crustacean daphnid species. In this study, the test was performed starting from the resistant forms 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 the OECD No. 202:2004 guideline [19]. The tests lasted 48 hours. All the physicochemical parameters were measured at the start of tests and after 48 hours. Six independent tests were performed, one for each sample. Three tests were carried out during 2018, while the other three during 2019. Each test was performed in three replicates. Twenty daphnids per sample (no longer than 24 hours after the hatching) were exposed in multi-well plates and they were incubated at 21 ± 1 °C in the dark. Each well contained five daphnids in 10 mL of the water sample. A control was performed exposing twenty daphnids to the test medium. At the end of the experiment, the number of immobilised individuals was recorded. Daphnids were considered immobilised if no directed movement was observed within 15 s after gentle stirring.

Fish Embryo Acute Toxicity (FET) test (OECD 236:2013)

Wild type zebrafish embryos were used to perform the analysis. The bioassay was conducted according to the OECD No. 236:2013 guideline [20]. The embryos were collected from the breeding groups at the Laboratory of Ecotoxicology of the ISS. Breeding fish were maintained in tanks with a loading capacity of 1-L 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. Three tests were carried out during 2018, while the other three during 2019. Each test was performed in two replicates. 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 the sample. A plate control and internal control were prepared with the test medium. Embryos were kept in dark conditions for four days at 26 ± 1 °C. The morphological observations of the embryos were made at 96 hours post fertilisation (hpf). Four apical observations were recorded as lethal endpoints indicating acute toxicity: coagulation of the embryo, non-detachment of the tail, lack of somite formation, and lack of heartbeat. Sublethal endpoints were also recorded in order to improve the 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.

Statistical analysis

The experimental data were analysed performing non-parametric tests. The Kruskal-Wallis test allowed the comparison among the sampling sites in the two different years (2018 and 2019) and the different time pattern showed by the Delta (values of 2019 – values of 2018). Moreover, the average and the standard deviation were calculated where possible. The statistical analysis was performed with the aid of the SAS® software. Please, see the *Supplementary materials* available on line for a detailed description of the statistical analysis and the related boxplots.

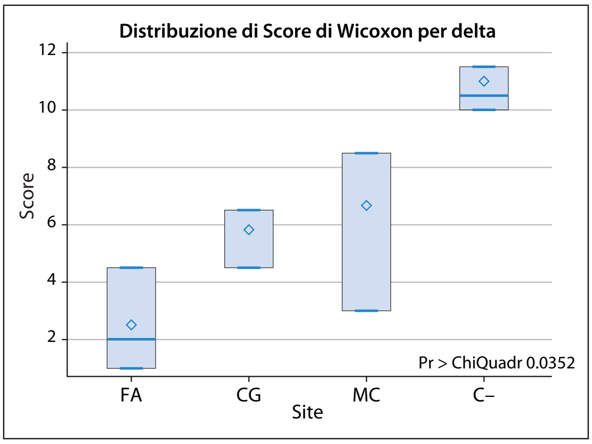

Figure 2
Wilcoxon Score Distribution showing the different time pattern (values of 2019 – values of 2018) indicated by the *delta*. Significance is expressed as Pr > Ch-square and it has be equal to ≤0.05. FA: Farfa River; CG: Castel Gandolfo; MC: Mezzocammino; C-: negative control.

RESULTS AND DISCUSSION

All the test results met the validity criteria in both the bioassays. Overall, the results showed toxic effects, i.e. lethal and sublethal effects, in the three sampling sites with differences between the applied bioassay and between the sites. However, the acute toxicity was generally weak, i.e. low mortality rate, for the organisms employed in this study. The *D. magna* Acute Immobilisation assay showed a significant difference between the results observed from the samples of 2018 and those of 2019 (*Figure 2*). Based on the average of the results, only the sample FA1 showed significant toxic effects (over 20% of the effect percentage) for the daphnids (*Table 1*). The results of the FET test did not show a significant difference between the samples of 2018 and those of 2019. However, this bioassay detected both lethal and sublethal effects. Indeed, the FET test is able to detect toxic effects even at low concentrations of chemical substances [5, 21]. Only the samples FA1 and MC1 showed significant toxicity (over 20% of the effect percentage) for zebrafish embryos (*Table 2*).

Surprisingly, most of the effects were observed in the organisms exposed to the tributary Farfa River. A possible explanation for the difference of the effects emerged in the Tiber River samples and the Farfa River samples could be related to the release of some pesticides into the tributary or to the use of some pharmaceuticals for veterinary purpose. Indeed, land exploitation for agricultural uses is carried out nearby the torrent, although this is limited to a restricted area. Other explanations could be sought in the presence of physical and chemical parameters in the samples that may have affected the development and survival of the tested organisms. Moreover, the low water flow rate of the Farfa River could amplify the effects of chemical pollutants because they could be more concentrated if compared with those released in the huge water flow of the Tiber River. Furthermore, other sources of pollution could be found in possible illegal wastes or in the presence of a dam upstream the site used for the abstraction of drinking water.

Daphnia *sp. Acute Immobilisation assay*

The results of *Daphnia* sp. Acute Immobilisation 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 (*Table 1*). The percentage of immobilised individuals recorded was closed 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 (*Table 1*). Samples that show values ranging between 20% and 50% are considered low toxic on the basis of the scale of toxicity used by Regional Environmental Protection Agency of Lazio – ARPAL. Statistical analysis revealed that the differences between the two campaigns are significant (*Figure 2*). However, considering the low amount of the data, these conclusions have to be treated very carefully and further analyses are needed. The results do not necessarily mean the absence of chemical pollution in the Tiber River since the *D. magna*

Table 1
Results of *Daphnia magna* Immobilisation Assay. The data represent the effect percentage calculated in each test for all the samples, including the negative control. Average and standard deviation are also reported. FA: Farfa River; CG: Castel Gandolfo; MC: Mezzocammino; C-: negative control.

Sampling	Site	Replicate 1 (%)	Replicate 2 (%)	Replicate 3 (%)	μ	σ
2018	FA1	30	15	35	26.7	10.4
	CG1	10	10	15	11.7	2.9
	MC 1	5	20	5	10	8.7
	C-	3,3	0	0	1.1	1.9
2019	FA2	5	5	0	3.3	2.9
	CG 2	0	0	0	0	0
	MC2	0	0	0	0	0
	C-	0	0	0	0	0

Table 2
Results of FET Test with *Danio rerio*. The data represent the effect percentage calculated in each test for all the samples, including the negative control. Average is also reported. Lethal and sublethal were calculated separately. NA (i.e. not applicable) means that the negative control was recorded only once for both lethal and sublethal effects. FA: Farfa River; CG: Castel Gandolfo; MC: Mezzocammino; C-: negative control.

Sampling		Site	Test 1 (%)	Test 2 (%)	µ
2018	*Lethal*	FA	15	45	30
		CG	5	5	5
		MC	25	30	27.5
		C-	0	0	0
	Sublethal	FA	0	9	4.5
		CG	0	11	5.5
		MC	13	0	6.5
		C-	NA	NA	
2019	*Lethal*	FA	5	10	7.5
		CG	15	10	12.5
		MC	5	5	5
		C-	0	0	0
	Sublethal	FA	11	6	8.5
		CG	0	6	3
		MC	5	11	8
		C-	NA	NA	

model could not be sensitive to the specific chemical substances and concentrations dissolved into the Tiber River. Furthermore, most of the chemical substances might have been stuck to the organic material that was removed during the sample filtering [22]. The chemical analysis could help in explaining the obtained results and revealing the chemicals in the samples and their concentrations.

FA1 and FA2 showed different effects, ranging from a low degree of toxicity to the absence of toxic effects on development, survival, and motility. The different results obtained with the Farfa River samples in the two sampling campaigns could be due to specific seasonal environmental perturbations as well as different levels of chemical discharge into the watercourses or single pollution phenomena. For instance, the available rainfall data show that the summer of 2018 was much rainier than the summer of 2019, especially in the area of the Farfa River [23]. However, it interesting to notice that a recently published study [24] indicated that the ecological status in a small investigated portion of this stream should be considered as *moderate* instead of *good*, according to the definition provided by the WFD obligations. These data refer to the period of 2018. However, the same study states that the ecological status of the Farfa River is *good* overall. The positive results recorded in the tested samples of the first campaign were weak and contrasting with those emerged in the samples from the second campaign. Therefore, more samplings are needed to confirm or reject these data.

Fish Embryo Acute Toxicity (FET) test

Lethal and sublethal endpoints on zebrafish embryos were observed and recorded in all the tests. The mortality rate was ≤10% in the plate control and internal control. Overall, the number of adverse effects was higher in the samples coming from the summer of 2018. This difference between the two sampling campaigns was due to MC and FA, while the organisms exposed to CG samples did not show any relevant difference between the two investigated periods (*Table 2*). 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. Indeed, only the embryo mortality percentage greater than 10% is considered significant according to the guideline. FA1 and MC1 registered levels of lethality that may indicate weak toxicity. CG showed a slight difference in lethality between CG 1 and CG2. Sublethal endpoints were also recorded in all the samples (*Table 2*). These observations could reveal the presence of chemicals or chemical mixtures that may not be assessed with the only record of lethal endpoints. Sublethal effects occurred as spine deformation, delay or absence in the hatching and general underdevelopment, and they were recorded in all the samples (*Figure 3*). Specifically, the application of the FET test has been useful in several scientific studies to detect the effects of chemical pollution on living organisms in surface water bodies, even for substances at very low exposure levels [16, 25]. Moreover, the detection of sublethal effects can be linked to some

Figure 3
Sublethal effects detected by the FET test. All the images were obtained at 96 hpf. A) represents a normal developing hatched embryo; B) shows the spine deformity; C) is a non-hatched individual.

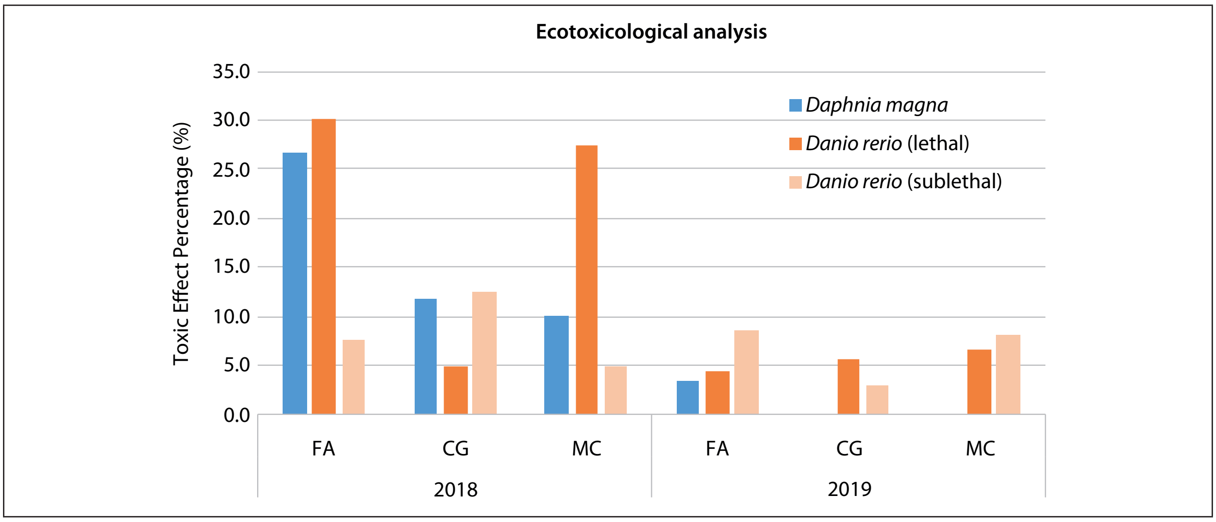

Figure 4
Results of the ecotoxicological analyses applied on three Tiber River basin samples (FA: Farfa, CG: Castel Giubileo, MC: Mezzocammino) in two sampling campaigns (2018-2019).

widespread classes of environmental pollutants and it allows the understanding of the main modes of action (MoAs) of these substances, e.g. DNA toxicity, neurotoxicity, developmental toxicity, cardio-circulatory toxicity [21]. The results of the test were congruent with those shown in *D. Magna* Acute Immobilisation assay, although very weak (*Figure 4*). The higher peaks of toxicity were registered in the organisms exposed to the Farfa River samples. The outcomes of the FET test also showed a certain level of toxicity in the site of MC as expected: this site is indeed located downstream to the city of Rome and it is affected by the pollution load typical for a big city, i.e. small enterprise discharges, waste pollution, personal care products, detergents, heavy metals. MC also receives the emissions of the urban wastewater treatment plant. Further studies integrating other ecotoxicological bioassays and chemical analyses are needed to improve our knowledge about the state of health of the study area.

CONCLUSIONS

Several activities have arisen along the banks of the Tiber River during the centuries, and this water ecosystem has been always relevant for its socio-economic activities and public health. Moreover, 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 [26, 27]. Therefore, in this context, every new result on the water quality of this river ecosystem should be carefully considered. The effects detected with the FET test can play an important role in future chemical screening of the Tiber River. The sublethal effects detected in all the sampling sites could be due to some classes of environmental pollutants left off the list of the chemical substances included in the European and Italian legislations [28]. Specifically, future studies should explain which sources could be responsible for the water contamination in the Farfa River, and the outcomes should be considered by the policymakers for monitoring and protecting this important naturalistic area. Integrating EBMs, e.g. FET test and *D. Magna* Acute Immobilisation assay, into surveillance, operational and investigative monitoring for water quality management is fundamental to evaluate the hazards of the whole chemical substances widespread in the water bodies. Furthermore, EBMs may reveal risks for natural communities working as environmental early warning systems, as already mentioned by past studies on the Tiber River [29].

It would be essential to combine the morphological observations detected in this study with the methods taking into account different MoAs, e.g. mutagenicity and neurotoxicity, in order to increase the knowledge on the underlined toxicity mechanism. These MoAs have been commonly revealed by bioassays that used samples collected in several European rivers [30]. A better comprehension of the chemical pollution levels in the Tiber River basin, including emerging substances, e.g. pharmaceuticals and pesticides not included in the legislation, would be fundamental to prevent risks for human health when there is a reuse of water for agricultural, aquaculture and drinking purposes.

In conclusion, the recommendation of this study is continuing to apply ecotoxicological bioassays in studying the Tiber River basin in support to the chemical analysis foreseen by the WFD. Future screening of these environmental sites could be essential for better explaining the results of this study, and to identify which substances are potentially responsible for these effects.

Authors contributions

The co-authors had together contributed to the completion of this article. Specifically, it follows their individual contribution. Conceptualisation: WC and IL; methodology: WC, IL, KDD, Ma.Co.; validation: IL, MC; investigation: WC, IL, KDD, MaCo; data curation: IL, WC; writing – original draft preparation: WC; writing – review and editing: MC, IL, WC; supervision: LM. All authors read and approved the final manuscript.

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Conflicts of interest statement

The authors declare no conflict of interest.

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