Water Air Soil Pollut (2023) 234:159
https://doi.org/10.1007/s11270-023-06102-4

Fish-Kills in the Urban Stretch of the Tiber River After a Flash-Storm: Investigative Monitoring with Effect-Based Methods, Targeted Chemical Analyses, and Fish Assemblage Examinations

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Received: 9 June 2022 / Accepted: 15 January 2023 / Published online: 24 February 2023

Abstract In 2020 and 2021, fish-kills events occurred in the Tiber river in the city of Rome. These events, which caused the death of thousands of fish of different species (e.g., *Barbus* spp., *Cyprinus carpio*, *Squalius* spp.), were preceded the days before by severe flash-storms. Heavy rains in urban areas in recent years are linked to climate change and fish-kills events. With the aim to investigate the causes of these events, effect-based methods (EBMs) and targeted chemical analyses have been performed on a specific site of river Tiber in the center of the city. Additionally, examination and classification of the floating dead fishes have been performed. The chemical analysis performed on several groups of contaminants showed the presence of some pharmaceuticals, insecticides, and PFAS at up to ng/L. Results with the Fish Embryo Toxicity (FET) test confirmed lethal and sub-lethal effects, while acute effects were not detected with the *Daphnia magna* acute test. The fish-kills events probably can be generated by several factors as a consequence of an increasing human anthropization of the area with the contribution of different stressors together with chemical releases and emissions. This study shows that in multiple stressor scenarios characterized by heavy rainfall, droughts, and strong anthropogenic pressures, the application of EBMs, chemical analysis, and fish assemblage examinations can represent a useful support in the investigation of the causes of extensive fish-kills events.

Keywords Effect-based methods · Water pollution · Fish-kills · Climate changes

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11270-023-06102-4.

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1 Introduction

Global warming affects the hydrological cycle which increases the intensity of extreme events such as heavy rainfalls. Flash-storms represent strong rain events happening in a short time period. They are an important key factor in the impact of contaminants on ecosystems and are becoming increasingly frequent because they are linked to climate change (Mancini et al., 2017; Tabari, 2020). Heavy rains and consequent flooding may represent the cause of industrial accidents (Krausmann et al., 2011) or may result in the redistribution of polluted sediments (Hilscherová et al., 2007; EEA,

2016) and in run-off phenomena, for example, of tire rubber antioxidants (Tian et al., 2021). The sudden release and mobilization of a large number of chemicals in water bodies may pose a risk to the health of the environment overall, including biodiversity and humans. The European Water Framework Directive (WFD) aims to achieve a good status of water quality in all European aquatic ecosystems, both in terms of chemical and ecological parameters (European Union, 2000). In this framework, the chemical status assessment is based on a list of priority substances defined by the European Union (European Union, 2013), whose concentrations need to meet the Environmental Quality Standards (EQSs) established to protect aquatic ecosystems and human health. However, this assessment approach shows some limitations, since every single water body can be simultaneously affected by the discharge of thousands of different pollutants which can interact with each other resulting in effects hard to predict (Brack et al., 2018).

Since it may be difficult to detect environmental toxicity through chemical analysis alone (Brack et al., 2019), it is important to apply both chemical analyses and effect-based methods (EBMs) which include in vivo and in vitro bioassays. EBMs are effective and low-cost tools to detect toxicity in water (Kuckelkorn et al., 2018; Neale et al., 2017; Wernersson et al., 2015). There is, consequently, the need to apply holistic methods able to detect such effects, on aquatic ecosystems and play a role also as a screening and early warning system.

Over the years 2020 and 2021, the city of Rome and the surrounding area were subjected to massive flash-storms that involved in particular the Tiber river. Such events were followed by the deaths of thousands of fish belonging to different species. With the aim of investigating the causes of the fish mortality, the water quality of the urban part of the Tiber river in the hours following two specific fish-kills events has been assessed through an integrated approach taking into account the application of EBMs as well as targeted chemical analysis of several contaminants typical of urban areas and run-off such as PFAS, pharmaceuticals, and personal care products; some of these contaminants have been selected also because they are included in the list of priority (e.g., PFOS, simazine) substances and in the EU watch-list (e.g., diclofenac, fipronil) of the WFD that is particularly relevant for the detection and knowledge of emerging compounds. Furthermore, examinations and classification of the dead floating fish have been carried out. The final results have been also discussed taking into account the analysis performed by ARPA Lazio (Environmental Protection Agency of Lazio Region) about physico-chemical parameters analyzed after the events.

The main goals of the study were to assess the possible effects on the aquatic ecosystems and to provide useful information in order to understand the causes of the fish-kills.

2 Materials and Methods

2.1 Study Area and Sampling Site

The Tiber river rises from "Monte Fumaiolo," in the Apennine Tosco-Emiliano, and has a water flow with a yearly average of 230 $m^3 \cdot s^{-1}$. The river is 405 km long, it runs through four regions (Emilia-Romagna, Umbria, Tuscany, and Latium), and its last stretch flows through the city of Rome and to the Tyrrhenian Sea. The Tiber river has a catchment area that exceeds 10,000 km^2, and its water is used mainly for irrigation, recreational fishing, and hydroelectric production although future uses as drinking water sources are under evaluation. The river is affected by numerous anthropogenic activities that may deteriorate its water quality: agricultural, urban (including sewage treatment plants), and industrial (mainly small enterprises).

Two water sampling campaigns were carried out in May 2020 and August 2021 at the same sites of the river, shortly after two extreme weather events that took place in the city of Rome. Such events followed prolonged dry periods (Fig. 1) and resulted in floods and in the enlargement of the river. The site where the water sampling collection has been performed is located under Ponte Duca d'Aosta (Fig. 2) in the city of Rome. It is important to remark that the sampling site is included in the stretch where the fish-kills were observed. This site is also located downstream of the inlet of the Aniene river (the second river of the city). Ten liters of water samples (TB20-year 2020 and TB21-year 2021) were collected between 0 and 30 cm in depth in inert containers and were then filtered at 0.45 μm and stored for 1 month at 4 °C in dark conditions at the Laboratory of Ecosystems and Health unit of the Italian Institute of Health (ISS). In

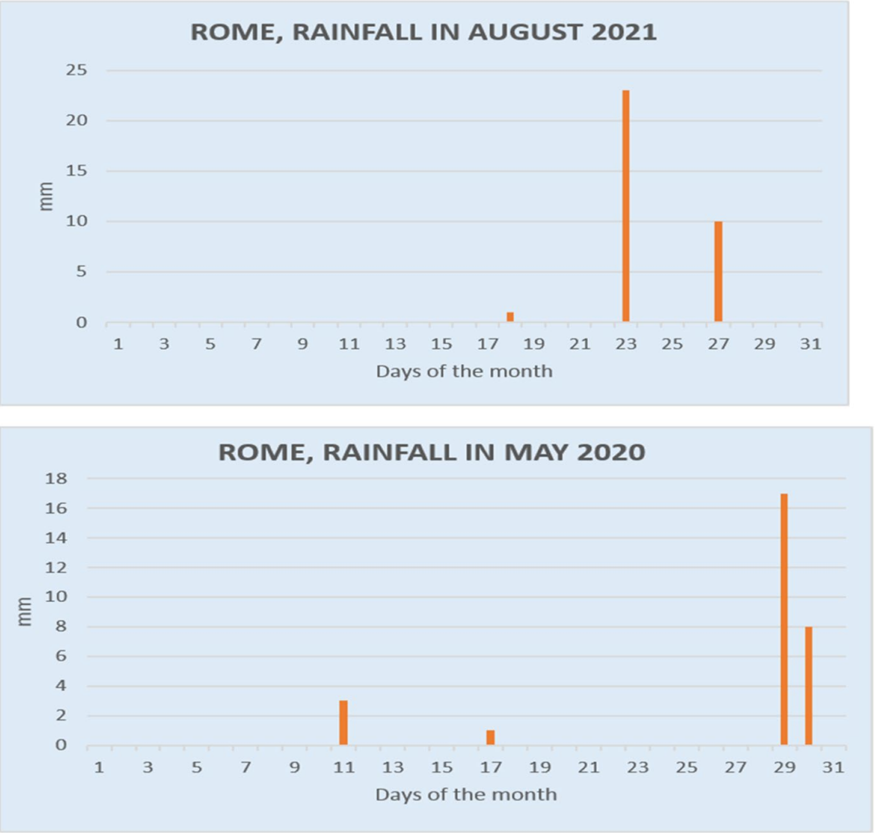


Fig. 1 Rome rainfall in May 2020 and August 2021

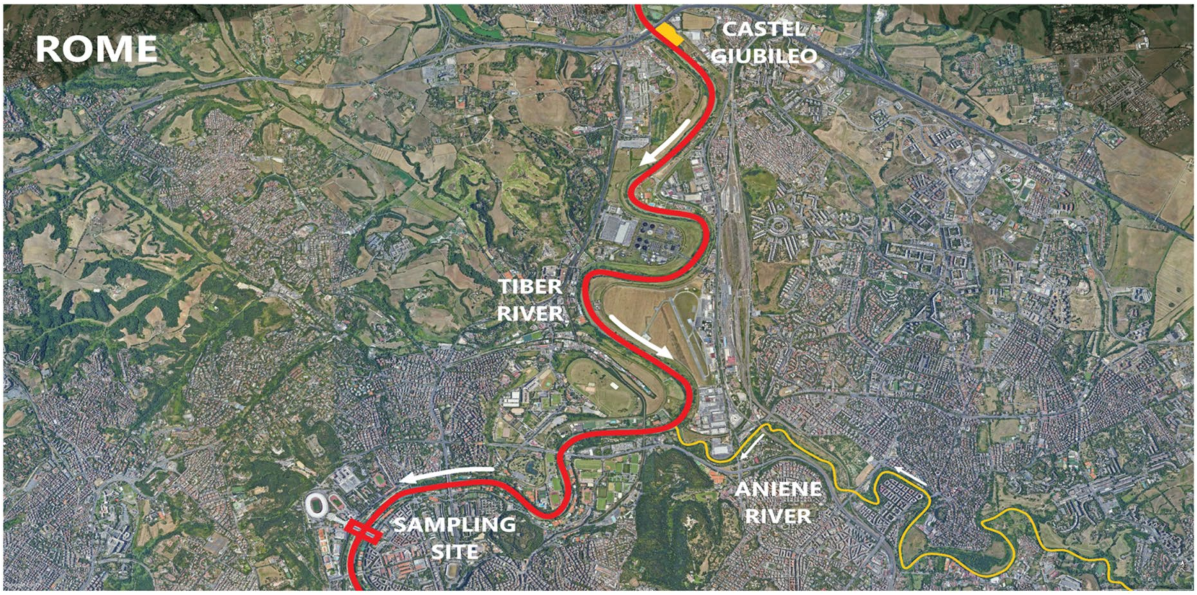


Fig. 2 Water sampling area. Coordinates water sampling site: Ponte Duca D'Aosta (41° 55′ 52.53″ N and 12° 27′ 38.16″ E)

the same stretch ARPA Lazio (Lazio Environmental Protection Agency) has detected physico-chemical parameters (ARPA Lazio., 2021), see Supporting Informations (SI).

2.2 Species Assemblage Assessment

In order to assess the fish taxa of local assemblages affected by the two fish-kills events, we firstly checked for dead floating fish on the riverine surface. The collection of fish carcasses along the urban stretch of the river has been conducted on May 30–31, 2020, and on August 25–26, 2021, respectively. These "*in situ*" activities were made in different sites, in particular, they have been made in the stretches where there was a massive accumulation of fish carcasses (e.g., where aquatic macrophytes were present) downstream to the water sampling site; we collected the available fish at the sites.

During the fish mortality event at the end of May 2020, the field examinations were primarily focused on the area between Umberto I and Sant'Angelo Bridge, about 8 km downstream of the Aniene river inlet and 5 km downstream of the water sampling site. The examinations in August 2021 focused on the same area of 2020 and at the same time, they were also extended to the upstream stretch of the Aniene river inlet. Thus close attention was given to two different river stretches in 2021:

1) downstream of the dam of Castel Giubileo (see Fig. 2), about 8 km upstream of the inlet of the main tributary Aniene river (coordinates: 41° 59′ 17″ N 12° 29′ 52″ E)
2) Sant'Angelo Bridge (same as of 2020), about 8 km downstream of the inlet of Aniene river (coordinates: 41° 54′ 06.49″ N and 12° 27′ 59.22″ E)

In all cases, macroscopic examinations of both floating carcasses of dead fish or stunned fish swimming at the water's surface have been carried out. A netted fishing basket with a handle of 3 m in length was used to collect the fish carcasses and facilitate the observations of the species. To describe and facilitate the analysis of the numerous impacts that these phenomena can have on different fish species, from the riverine fish assemblage of the Tiber river, an ecological/functional guild approach (Austen et al., 1994) has been applied. The guild/functional approach has also been used to develop many multi-metric fish-based indices: such metrics can be used for assessing the ecological status of water ecosystems (i.e., Karr et al., 1986; Noble & Cowx, 2002; Oberdorff & Hughes, 1992). According to this approach, the fish species were grouped depending on their preference for quality of water, habitat, breeding requirements, etc., (Breine et al., 2005), Noble & Cowx, 2002; Tancioni et al., 2006). The taxonomic-ecological guild classification applied (see supporting informations) includes trophic, habitat, reproductive, and tolerance guilds (in particular, tolerances to the reduction of oxygen saturation in the water and relative hypoxia status). The classification of teleosts fish and cyclostomes species is based on samples between 2007 and 2021 in the urban part of the river.

2.3 EBMs and Chemical Analyses

2.3.1 Fish Embryo Acute Toxicity (FET) Test with zebrafish (Danio rerio)

The Fish Embryo Acute Toxicity (FET) test was conducted according to the OECD 236 (OECD, 2013). The embryos were collected from wild-type zebrafish breeding groups at the Unit Ecosystems and Health of the ISS. Breeding groups were maintained at 26 ± 1 °C in tanks with a loading capacity of 2 L water per fish, with a fixed photoperiod of 12:12 (light:dark) with males and females at a ratio of 2:1. Fertilized eggs were exposed in 24-well plates at a developmental stage ranging from 32 to 128 cells of segmentation. In each well, a single egg was exposed to 2 mL of the whole sample. A negative control plate and internal negative controls (4 wells in every plate) were prepared with ISO standard freshwater (ISO—7346–3.). Embryos were kept in controlled conditions of temperature and light in a thermostatic chamber for 4 days at 26 ± 1 °C. The morphological observations of the embryos were made at 96 hours post-fertilization (hpf). At the end of the test, four lethal endpoints indicating acute toxicity were recorded: coagulation of the embryo, non-detachment of the tail, lack of somite formation, and lack of heartbeat.

Furthermore, the following sublethal endpoints were recorded: depigmentation, cardiac edema, and spine deformation, i.e., scoliosis and lordosis. We also analyzed body length and eye size with Danioscope software to determine possible deviations from normal development caused by chemical substances;

these sublethal endpoints were investigated since they can reveal potential toxicity due to several chemical compounds (Dang et al., 2021; Krzykwa et al., 2019).

Each test was performed in three replicates for a total of 60 embryos per sample. A positive control at a concentration of 4 mg/L of 3,4-dichloroaniline (3,4 DCA) was also performed.

Statistical analysis was performed: the effect percentage was calculated normalizing the sample values with the control, applying the following formula:

$$(\%)\mathrm{Effect} = \frac{X_{\mathrm{Control}} - X_{\mathrm{Exposed}}}{X_{\mathrm{Control}}}$$

X_{Control} represents the mean value of the single parameter (body length and eye size) calculated on the control, and X_{Sample}, the mean value on the samples separately. Furthermore, the statistical analysis was performed using GraphPad Prism version 7.03 for Windows (GraphPad Software, La Jolla, California, USA). Kruskal–Wallis test (ANOVA-on-ranks) was used in combination with Dunn's test for analysis of effects on body length and eye size.

2.3.2 *Daphnia* spp. Acute Immobilization Assay

The *Daphnia* spp. acute immobilization assay is a method to measure acute toxicity in freshwater that uses crustacean daphnid species. The test is performed starting from the resting stage of *D. magna* (i.e., ephippia) that are included in the Daphtoxkit® kit developed by the Laboratory for Environmental Toxicology and Aquatic Ecology (LETAE) at the University of Ghent, in Belgium. The experimental procedure follows the OECD 202 guideline (OECD, 2004).Tests lasted 48 h according to the guideline, and each test was performed in three replicates on undiluted samples; a total of 60 daphnids were exposed to each sample and control. At the end of the procedure, the number of immobilized individuals was recorded. The individuals that show no directed movements within 15 s after gentle stirring have been considered immobilized. The results are expressed as the mean of the three replicates with relative standard deviation. This test has been performed with the aim to integrate another trophic level for the possible detection of effects of chemical contaminants.

2.3.3 Targeted Screening Chemical Analyses

Water samples were analyzed by liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) with a target list of 505 compounds. In order to obtain a comprehensive overview of the chemical pollution of the area, our target list included a wide range of compound classes (i.e., pesticides, pharmaceuticals, industrial compounds, plastic additives) that are expected to be present in European (EU) freshwater environments. PFAS were added on the list because of their ubiquitous diffuse presence also in urban environments and relevance for the European and worldwide aquatic environment and their potential effects. A similar list was applied in previous studies for the chemical characterization of environmental samples and to assess potential chemical risks for aquatic species (Carere et al, 2021; Kandie et al., 2020; Beckers et al., 2018). Additional information on the instrument settings, data analysis, sample preparation, and targeted list is given in Supporting Informations (SI) and in the excel file attached.

Chemical concentrations were compared with experimental acute effect concentrations (LC and LOEL) for fish species (all life stages) retrieved from the United States Environmental Protection Agency's (USEPA) ECOTOX database (https://cfpub.epa.gov/ecotox/, updated to 14 September 2022). In the case of multiple values, we calculated the 5$^{\mathrm{th}}$ percentile of all measured acute values. Missing experimental values were filled with predicted values estimated with the software ECOSAR (v 2.2).

Water samples were also analyzed for nine perfluorocarboxylic acids (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUdA, and PFDoA) and three perfluorosulfonates (PFBS, PFHxS, and PFOS). The results, for some PFAS, have been compared with acute effect concentrations for fish species retrieved from literature studies.

The extraction methodology was adapted from Organtini et al. (Organtini et al., 2020), see Supporting Informations (SI).

3 Results

3.1 Species Assemblage Assessment (2020 and 2021)

In 2020, the examination of 116 specimens of floating fish carcasses along the banks of the Tiber river between Umberto I and Sant'Angelo bridges, was carried out. The data concerning the taxa assemblage and the relative observed occurrence (more than 65% of dead fish), can be summarized as follows (see also SI): barbel (*Barbus* spp.) was frequently observed (17.24%) and represented by specimens of different size classes (sub-adults and adults), thinlip mullet (*Chelon ramada*), mainly adult-sized, was detected at a frequency of 16.38%, and several specimens of chub (*Squalius* spp.) (16.38%), and common carp (*Cyprinus carpio*) (15.52%) were also observed. The other taxa observed were common bream or white bream (*Abramis brama*, *Blicca bjoerkna*) (12.93%), roach (*Rutilus rutilus*) (12.07%), wells catfish (*Silurus glanis*) (2.59%), tench (*Tinca tinca*) (2.59%), rudd (*Scardinius* spp.) (1.72%), pikeperch (*Sander lucioperca*) (0.86%), seabass (*Dicentrarchus labrax*) (0.86%), and eel (*Anguilla anguilla*) (0.86%).

In 2021, a total number of 112 carcasses were examined (see SI) between Umberto I and Sant'Angelo bridges. The taxa assemblages most affected by the phenomenon were again: barbels (*Barbus* spp.) (16.96%), thinlip mullet (*C. ramada*) (16.96%), chub (*Squalius* spp.) (16.07%), and common carp (*C. carpio*) (15.18%). The other taxa observed were common bream (*A. brama*) (9.82%), white bream, (*B. bjoerkna*) (2.70%), roach (*R. rutilus*) (8.93%), tench (*T. tinca*) (6.25%), rudd (*Scardinius* spp.) (2.68%), wells catfish (*S. glanis*) (1.78%), pikeperch (*S.lucioperca*) (1.78%), and sea bass (*D. labrax*) (0.89%). On the contrary, the observations made in the upstream section from the inlet of the tributary Aniene, below the barrier of Castel Giubileo, did not yield any carcasses of dead fish. There were no macroscopic injuries detected, and some fish showed an "open mouth" as a possible sign of hypoxia/anoxia (see pictures in SI).

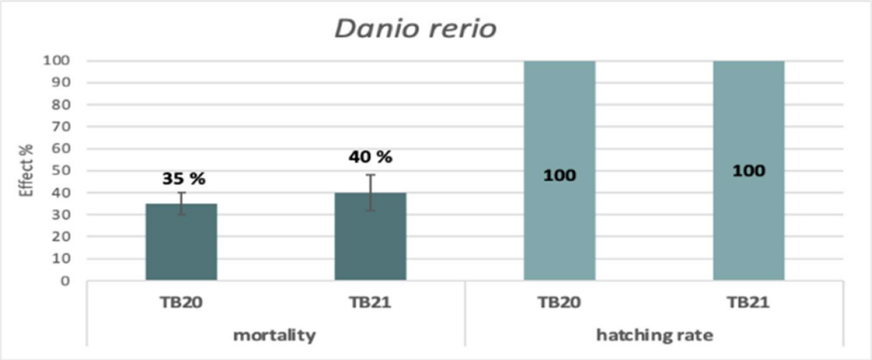
3.2 Fish Embryo Acute Toxicity (FET) Test with zebrafish (D. rerio)

The results highlight the presence of acute toxicity in both samples analyzed. Specifically, TB20 shows average mortality of 35%, while TB21 shows an average mortality of 40% (Fig. 3). No effects of hatching delay were recorded, and all the surviving larvae hatched within the time required by FET test conditions. All validity criteria required by the guideline, such as a $\leq$10% mortality rate in the control plate and in the internal controls and a mortality percentage $\geq$30% in the positive control, have been met at the end of the exposure.

3.2.1 Sublethal Endpoints (FET)

Several sublethal endpoints have been observed: depigmentation, spine deformation (scoliosis and lordosis), cardiac edema, body length, and eye-size variation. Depigmentation, spine deformation, and cardiac edema have been found in TB20; spine deformation and cardiac edema in TB21 (Fig. 4). The most recurring effects in TB20 were depigmentation and spine deformity (45%), while in TB21, the most commonly recorded sub-lethal effect was spine deformation, with a value of 75% (all the deformations were attributable to lordosis). In Fig. 5 it is possible to see normal embryos.

Fig. 3 Results of the Fish Embryo Acute Toxicity (FET) test—OECD 236: mortality and hatching rate expressed as mean +/− SD



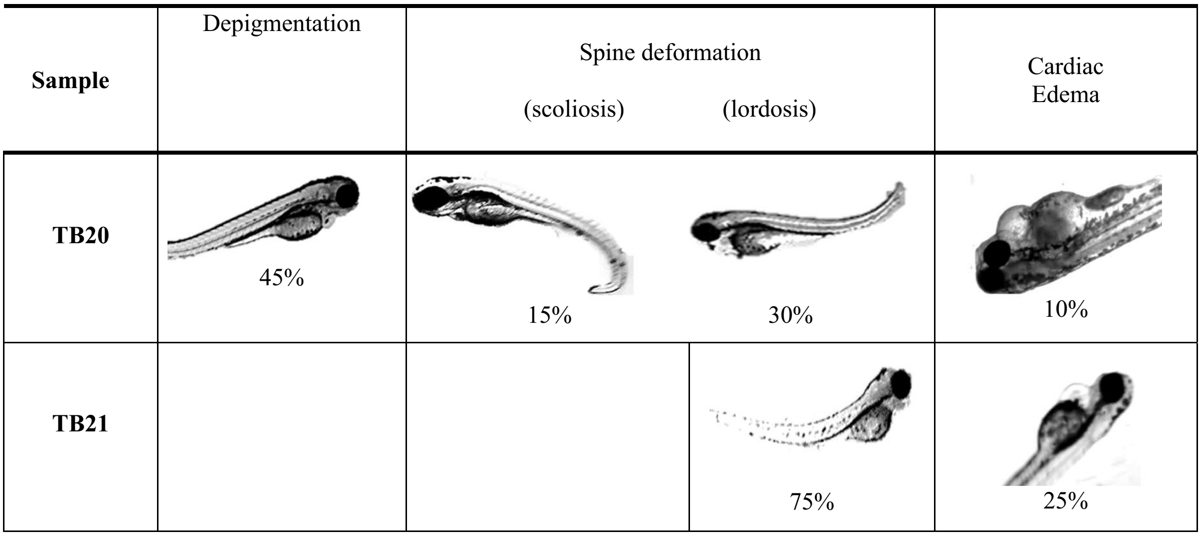


Fig. 4 Sublethal effects recorded in TB20 and TB21

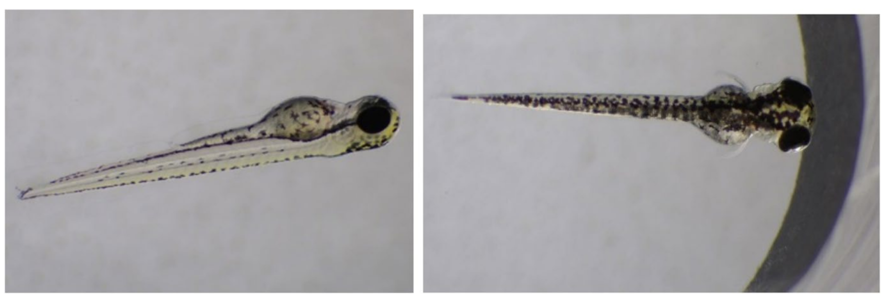


Fig. 5 zebrafish embryo normal development: lateral view (left), dorsal view (right)

3.2.2 Body Length and Eye Size (FET)

The surviving individuals were analyzed with Danioscope software for the measurement of two morphological parameters: body length and eye size (Table 1, Fig. 6).

Both samples showed a toxic effect that is reflected in the length of the total body of the larvae and the total surface of the eyes, which appeared statistically significantly smaller in size (Table 1, Fig. 6). Particularly, zebrafish larvae exposed to TB21 showed the main effects.

The effect percentages of the two samples related to the control were 7.2% (TB20) and 11.9% (TB21) for body length; 13.4 (TB20) and 25.3 (TB 21) for eye size.

3.3 *Daphnia* sp. Acute Immobilization Assay

The results of the Daphnia sp. acute immobilization assay revealed no acute toxicity. The number of immobilized daphnids is similar in both samples, with a percentage of mobility inhibition that ranges between 5 and 15% (Table 2).

According to the main scale of toxicity for *D. magna*, proposed by the Regional Environmental Protection Agency of Lazio Region (ARPAL), values exceeding 20% of effect are considered toxic. Overall,

Table 1 Variations of body length and eye size in the TB20 and TB21 calculated with the Danioscope software. *St:* standard error

Sample	Body length (μm)		Eye size (μm^2)	
	Mean	*St*	*Mean*	*St*
Control	3276	57.3	51172.7	2339.4
TB21	2887	116.6	38220.3	2069.3
TB20	3040.7	60.0	44330.2	1593.1

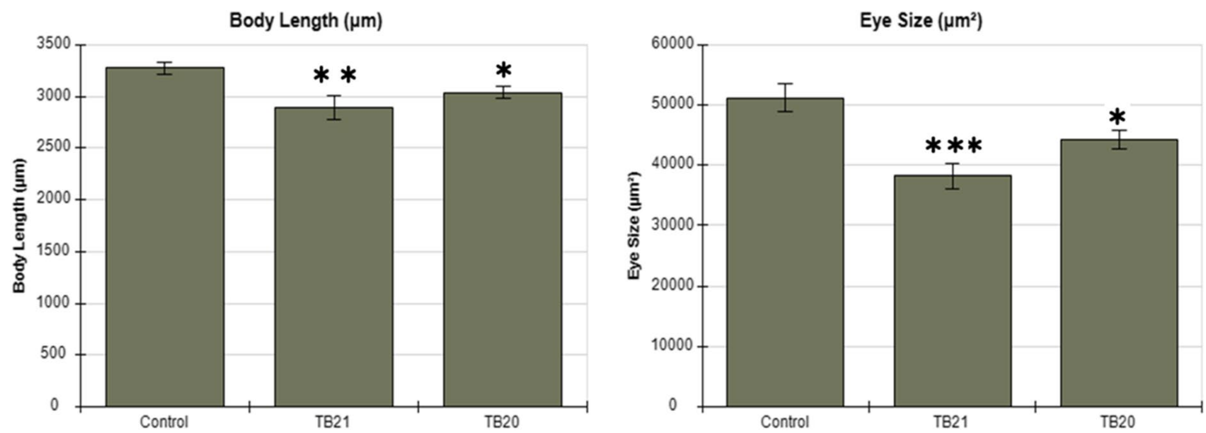


Fig. 6 Body length and eye size (mean ± SE.; $n=35$ per group) in zebrafish larvae exposed to river samples TB20 and TB21 (ANOVA-on-ranks, Dunn's test). The symbol * indicates a significant difference between tested samples and negative control (*$p<0.01$, **$p<0.001$, ***$p<0.0001$)

Table 2 Immobilization percentages in the three replicates for *Daphnia magna* exposed to the rivers samples TB20 e TB21

Replicate	Control immobilization %	TB20 immobilization %	TB21 immobilization %
1	0	5	5
2	0	5	10
3	0	10	10
Mean	0	6.7	8.3
SD	0	2.9	2.9

the test results show no relevant acute toxicity on the crustacean for both samples, with toxicity values of 6.7% in TB20 and 8.3% in TB21, (Fig. 7, Table 3).

3.4 Targeted Chemical Analysis

The targeted chemical analysis confirmed the presence of 12 pharmaceuticals and metabolites. The herbicides terbuthylazine and metolachlor (8.6 and 6.1 ng/L, respectively) and the biocide DEET (up to 37.7 ng/L) have been also detected. The plastic additive Bisphenol S at a concentration of 108 ng/L has also been detected in the sample TB21. All detected concentrations were several orders of magnitude below the experimental and predicted acute effect threshold for fish species. It is important to notice that just seven experimental values have been retrieved for 16 detected chemicals and that the predicted effect values can have an uncertainty range of 10–1000. Table 3 gives an overview of the results of the chemical analysis and effect concentrations.

The PFAS have been detected at low concentrations widely below the effect concentrations based on acute assays (Table 4). PFHpS has not been detected. The concentration levels of TB20 and TB21 were comparable. The effect acute concentrations for fish were retrieved from literature studies.

4 Discussion

EBMs and targeted chemical analysis were carried out in the Tiber river in Rome in correspondence with two significant weather events that caused the death of thousands of fish in 2020 and 2021.

The targeted chemical analysis has shown low concentrations of contaminants in both years. Some of these compounds can potentially have different effects on fish, but the concentrations detected are too low to hypothesize a key role in the fish-kills events. Among the pharmaceuticals, carbamazepine has been detected, an antidepressant widely found in aquatic environments; this substance can give several effects on zebrafish embryos (Santos et al., 2018) and also on *Cyprinus carpio* (Gasca-Pérez et al., 2019), a species that has been found in the river in this campaign; one of the main modes of action of this contaminant is neurotoxicity. The antibacterial sulfapyridine has also been already detected in previous studies in the Tiber river even at higher concentrations (Grenni et al., 2018); terbutylazine, an insecticide, has been detected at concentrations in compliance with the EQS defined

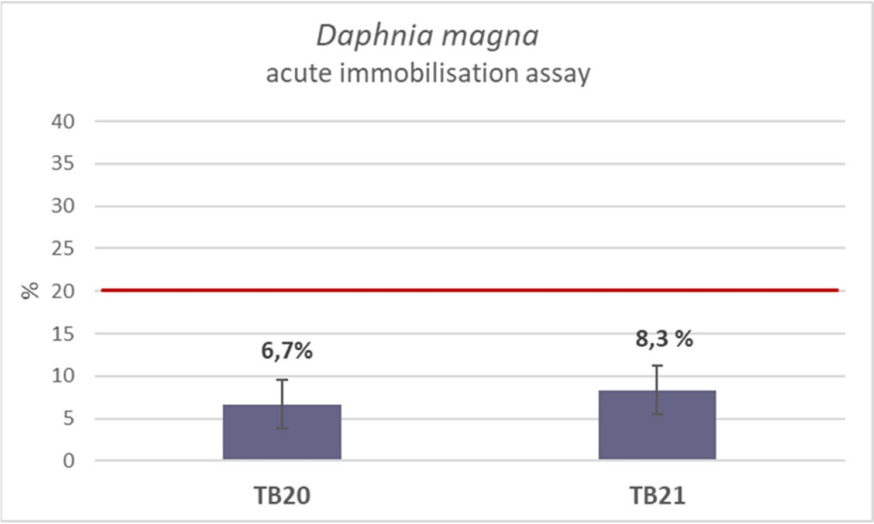


Fig. 7 Results of *Daphnia* sp. acute immobilization assay expressed as mean ± SD of the immobilized daphnids percentage. The values under the red line are not considered toxic (ARPAL)

by the Italian legislation (Dgls 172/2015); also, this substance is widely present in the Italian aquatic environments due to his large use. In relation to the fish's sensitivity, it is evident (see Tables 3 and 4) that the effect levels in fish are on average several orders of magnitude higher than the levels of the chemical contaminants detected. The PFAS chemical analyses showed the presence of several congeners, although at low concentrations in comparison to the effect acute data for fish. The levels detected for this class of compounds are an indicator of the chemical anthropogenic pressure of the area. PFAS are ubiquitous contaminants, and the concentrations we found are similar to those previously detected in other projects carried out in the same river. It is important to mention that PFAS have several chronic effects on fish also at low concentrations. Some of the chemical contaminants detected in this study were also found in a previous study (Table 5) conducted in the urban part of the Tiber river (Carere et al., 2021), although the sampling sites were located in different areas of the river. The levels of the antidepressant carbamazepine and the PFAS were comparable with those found in the previous campaign, while the levels of the insecticide anti mosquito DEET (10–200 ng/L) and of the anticonvulsant Gabapentin-Lactam were higher in the previous campaign (300 ng/L). An interesting variation is that in this study in the 2021 campaign, the plastic additive Bisphenol S has been found.

It is also relevant to highlight that during the events, the Regional Environmental Agency ARPA Lazio (ARPA Lazio, 2021) has carried out monitoring campaigns. In the context of the chemical analysis carried out in 2020, the pesticide cypermethrin (0.014 μg/L) and clothianidin (0.75 μg/L), a neonicotinoid substance have been detected. In 2021, the same contaminants were found at lower levels (ARPA Lazio, 2021). The effect fish acute concentrations for these substances are 104.2 mg/L for clothianidin and 0.00151 mg/L for cypermethrin (Pesticide Properties Database (herts.ac.uk).

The levels of macrodescriptors (e.g., BOD_5, COD) detected by ARPA Lazio immediately after the events are in the rangeof the normal values for the Tiber river (see SI). Furthermore, the levels of heavy metals (lead, nickel, chromium, arsenic) did not increase in comparison to previous campaigns carried out by ARPAL.

The test with *D. magna* did not show relevant acute toxicity in both samples, in line with other analyses performed in the urban part of the river (Cristiano et al., 2020). The samples analyzed with the FET test show mortality values up to 40%, with several sublethal effects that occurred along with the exposure: spine deformation (scoliosis or lordosis), which has been the most recurring effect, low pigmentation, variations of body length and eye size, and heart edema.

Spine deformations and the other morphological malformations detected could be associated with several classes of contaminants (Von Hellfeld et al., 2020, Galus et al., 2013, Massei et al., 2019) such as pharmaceuticals (ibuprofen) also in mixtures,

Table 3 Results of the targeted chemical analysis. *MDL*, maximum detection limit; *N.d.*, not detectable

Compound	CAS	Concentration (ng/L)		Effect concentration source	Effect concentrations for fish species (mg/L)
		TB 20	TB21		
Pharmaceuticals and metabolites					
2-Aminobenzimidazole	934–32-7	11	n.d	Predicted (ECOSAR v2.2)	1120
2-Hydroxycarbamazepine	68,011–66-5	3.9	4.5	Predicted (ECOSAR v2.2)	692
Acetyl-sulfamethoxazole	21,312–10-7	n.d	14.7	Predicted (ECOSAR v2.2)	1250
Candesartan	139,481–59-7	n.d n.d	24	Predicted (ECOSAR v2.2)	11
Carbamazepine	298–46-4	47	34.3	Experimental (ECOSAR)	0.86
Cetirizine	83,881–51-0	< MDL	n.d	Predicted (ECOSAR v2.2)	38,700
Citalopram	59,729–33-8	< MDL	n.d	Predicted (ECOSAR v2.2)	4.5
Gabapentin-Lactam	64,744–50-9	34	34	Predicted (ECOSAR v2.2)	150
Lamotrigine	84,057–84-1	36	n.d	Predicted (ECOSAR v2.2)	126
Sulfamethoxazole	723–46-6	46	16.5	Experimental (ECOSAR)	572
Sulfapyridine	144–83-2	26	n.d	Predicted (ECOSAR v2.2)	4310
Trimethoprim	738–70-5	n.d	17.4	Experimental (ECOSAR)	100
Pesticides and biocides					
Terbuthylazine	5915–41-3	8.6	n.d	Experimental (ECOSAR)	2.4
DEET	134–62-3	27	37.7	Experimental (ECOSAR)	72
Metolachlor	51,218–45-2	6.1	n.d	Experimental (ECOSAR)	1.7
Plastic additives					
Bisphenol S	80–09-1	n.d	108	Experimental (ECOSAR)	100

pesticides, PAH, and heavy metals; also, contaminants detected in this study (e.g., carbamazepine) can give the same effects. It is also important to mention that the EBMs results are consistent with those recorded in previous sampling campaigns (Table 5) in the urban stretch of the river (Carere et al., 2021), in which water samples were collected also under different weather conditions.

The differences reported in the results between the acute effects of the *Daphnia magna* test and the FET test, similarly to previous campaigns, can be probably explained by the different sensitivity of the species involved to the chemical contaminants that are present in this stretch of the river (Cristiano et al., 2020).

The high mortality of riverine fish concerned almost all the species and the different ecological guilds (e.g., habitat, tolerance vs intolerance, reproductivity, autochthonous vs allochthonous) that live in this stretch of the river.

The fish-kills events might be caused by several abiotic and biotic factors. Godinho et al., (2019) recently

Table 4 PFAS results (ng/L) of samples TB20 and TB21

	TB20	TB21	Effect concentrations for fish (mg/L)
PFBA	N.D	1.35	> 3000[3]
PFPeA	0.25	1.44	31.8[4]
PFBS	0.65	0.43	1938[5]
PFHxA	3.35	2.66	> 99.2[4]
PFHpA	1.30	0.79	-
PFHxS	0.09	0.09	-
PFOA	3.16	4.59	365.02[2]
PFNA	1.00	0.75	84[6]
PFOS	0.60	0.37	6.6[1]
PFDA	0.57	0.62	5[3]
PFUdA	0.63	0.33	-
PFDoA	0.15	0.05	-

[1]RIVM, 2010

[2]Yang et al., 2014

[3]Ulhaq et al., 2013

[4]Hoke et ale., 2012

[5]ECHA support document: https://echa.europa.eu/documents/10162/891ab33d-d263-cc4b-0f2d-d84cfb7f424a

[6]Zheng et al., 2011

Table 5 Substance concentrations and FET in the different sampling campaigns of the urban part of Tiber river

Compound	TB20	TB21	CG*	MC*
(ng/L)				
Carbamazepine	47	34.3	7	30
2-Aminobenzimidazole	11	n.d	10	10
Gabapentin-Lactam	34	34	300	300
DEET	27	37.7	10	200
Metolachlor	6.1	n.d	20	200
PFOA	3.16	4.59	9.92	2.58
PFOS	0.60	0.37	0.29	0.75
FET (lethal)	+	+	+	+
FET (sublethal)	+	+	+	+

CG, Castel Giubileo; *MC*, Mezzo Cammino; *previous study; + = effects detected

investigated factors related to fish-kills events in 67 Mediterranean reservoirs near the Iberian Peninsula, and they also reported a change in the likelihood of fish-kills event with the foreseen climate change. The authors outlined that such events occurred more often in shallow reservoirs with particular conditions such as lower oxygen content, larger surface area, and higher chlorophyll levels. Other factors related to mortality, such as toxic spills, were not taken into consideration by the authors, but their role cannot be excluded.

In the Pacific Northwest, coho salmon (*Oncorhynchus kisutch*) inhabiting the USA, acute mortality is annually related to polluted rainfall exposure when adult individuals migrate to urban areas for reproduction. Through the application of effect-directed analysis, some researchers (Tian et al., 2021) recently identified a tire rubber antioxidant, a highly toxic quinone transformation product of N-(1,3-dimethylbutyl)-N′-phenyl-p-phenylenediamine (6PPD) as the main cause.

The concentration of chemical contaminants detected in this study is not high enough to suggest a key role for them in the fish-kills events, although it was not possible to analyze all chemical contaminants that could derive from a run-off event. On the other hand, on the basis of the results obtained with the EBM (fish embryo test), it was possible to hypothesize a role for some chemical contaminants or some specific mixtures in the fish-kills events, and there are several examples in literature where chemical analysis showed low levels of contaminants, while EBMs detected effects on fish (Babić et al., 2017). The results from the examinations on the fish species affected in both events suggest the involvement of the inlet water that from the Aniene river flows directly in the Tiber river a few kilometers up to the sampling site. According to the technical report published by the Regional Environmental Agency (ARPA Lazio, 2021), this hypothesis is also supported by the fact that no dead fish was found upstream of the Aniene river inlet. Also, other water additions (i.e., drainage channels, run-off from paved urban surfaces, other untreated waters bypassing sewage plants) may have contributed to the injection of several other stressors in the river.

In addition, taking into account the analysis of the dead species, their ecological characteristics, and the proposed classification in ecological guilds, further considerations can be done: in particular, the discovery of many species classified as very tolerant (i.e., *S. glanis*) and relatively tolerant, in both the benthic area (i.e., *C. carpio*, *T. tinca*, *Barbus* spp.) and in the water-column (i.e., *C. ramada*, *Squalus* spp.), may suggest that a state of acute hypoxia and anoxia involved both the deepest layers of the waters (5–6 m of depth) and the entire water column (see also photos in SI), although at the same time, it is important to consider that the levels of macrodescriptors detected (see data in SI) are in a normal range for the river.

What can be assumed is that the fish-kills events can be caused by several factors, all generated by anthropogenic stress: the impact of the massive input of organic materials, the possible effects caused by specific chemical contaminants, and the combined action of biotic and abiotic factors may have contributed (Capoccioni et al., 2020, Tancioni et al., 2016). The strong rainfall, following a prolonged period of drought, may have resulted in localized floods in small tributaries with the leaching of run off from the main water passages in the urban stretches of the Aniene-Tiber rivers; these floods mobilized the sediments which had previously accumulated at the bottom of the tributary watercourses (additionally, the sediments had been enriched by a substantial amount of organic matter as well as chemical run-off from paved urban surfaces).

5 Conclusions

Fish-kills are events considered as a major cause of concern for ecosystem health and water quality. The search for the causes needs to apply all scientific tools and methods available. This study has been carried out with the aim to understand the reasons of these events and to detect the level of chemical pollution after the flash-storms. It focuses on the evaluation of chemical pollution in the areas where several floating fish carcasses were found. However, it is difficult, on the basis of the results obtained with chemical analysis, to attribute a clear role to the chemical contaminants that were found, while the analysis with EBMs has given potential additional informations and confirm the need to apply an integrated approach for this type of evaluation. A very recent report of the environmental Italian ONG Legambiente (Legambiente, 2021) mentioned that the city of Rome is the area in Italy where most of the extreme weather events occur. Furthermore, the growing anthropization and the alteration of natural flows in many lotic systems worldwide, in particular within the Mediterranean area, can increase the river vulnerability in the presence of heavy storms in global warming scenario.

In this context, an integrated approach that takes into account chemical, ecotoxicological and ecological parameters can help the decision makers to put in place adaptation measures aimed at mitigating the effects of these extreme events.

These events happen quickly, and their effects tend to be difficult to be detected after few hours; a station with a continuous automatic monitoring system should be deployed at least for the macrodescriptors, while for EBMs, a trend monitoring system with the use of fish biomarkers could be adopted; other chemical substances could be also included in the monitoring campaigns (e.g., 6PPD). The measures must be aimed at treating and reducing the releases, losses, and emissions of pollutants and organic materials from drainage channels and run-off. In this scenario, all the interventions aimed at slowing down the flow (i.e., artificial flood expanding areas) and the treatment of drainage (i.e., with constructed phytoremediation areas) can be supportive. In conclusion, it is necessary to think about the identification of new strategies for sustainable management of the water cycle (e.g., first-rain water) in urban areas, in order to safeguard the resilient capability of the aquatic ecosystems.

Author Contribution MC, LM, and LT contributed to the study conception and design. Material preparation, data collection, and analysis for EBM were performed by IL, WC, MC, and KD. Analysis and data interpretation for PFAS by ED, AI. Analysis and data interpretation for other target chemical analysis by RM. Collection, analysis and data interpretation for fish by LT. The first draft of the manuscript was written by MC, LM, and LT, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval The research has involved animals (fishes) in compliance with the ethical standard foreseen. All data and materials support their published claims and comply with field standards.

Competing Interests The authors declare no competing interests.

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