Evaluation of Genotoxic Potential of Waters from Two Italian Rivers in *Gammarus elvirae* (Amphipoda)

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ABSTRACT: The aim of the present work is to evaluate the genotoxic impact of contaminants along the whole course of Ninfa-Sisto and Amaseno (Latium, Italy) rivers. The authors performed the alkaline Comet assay to assess DNA damage in the freshwater amphipod *Gammarus elvirae*, exposed ex situ for 24 hours and 7 days to water collected at different sites. The assay, applied on haemocytes, provides a sensitive tool to reveal effects even at low concentrations of pollutants. The results indicate significant increase of DNA damage along the course of the two rivers, compared to the unpolluted upstream sites, even if the analytes do not exceed the permissible limits. Moreover, the results show that there is not a linear correlation between the concentration of analytes and DNA damage. Based on this study's results, it would be desirable to use Comet assay, on proposed test species, as an early warning method to detect genotoxic potential of waters. *Water Environ. Res.*, **87,** 2008 (2015).

KEYWORDS: Comet assay, DNA damage, water pollution, ex situ analyses/exposure, legal limits.

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Introduction

The investigation on the genotoxic potential of inland waters has become important in environmental monitoring because of the continuous production and release of contaminants into the aquatic environment (Rajaguru et al., 2002). In the present study, the authors proposed to evaluate the biological impact, as genotoxicity, of contaminants along two rivers in Latium (central Italy), Amaseno and Ninfa-Sisto. These two rivers are characterized by a good physicochemical water quality (Regional Agency of Environmental Protection of Lazio, ARPA-Lazio), as shown by chemical analysis conducted in previous monitoring programs (in 4 years, only 3 of 90 monitored analytes showing concentrations above the permissible limit).

The use of biological responses of sentinel organisms exposed to toxic substances (biological markers or biomarkers) may help to resolve the problems of causality, providing an early warning systems in cases where the chemical analysis reveal concentrations equal or below the limits imposed by national regulations (D.Lgs. 152/06) or below the detection limit of the analytical methods employed. Moreover, as often happens, the routine analysis does not provide a search of toxic contaminants, especially in the case of pesticides or their metabolites.

The genotoxic action of a xenobiotic substance occurs primarily at the biochemical and cellular level, as DNA damage and/or alteration of enzymatic activities. The authors investigate these biological response by means of exposures of different duration: a brief exposure (24 hours) to highlight the direct DNA damage and a longer exposure (7 days) to assess DNA damage mainly related with the reduced functionality of the repair systems.

Many genotoxicity assays, developed in the last few decades, allow assessment of DNA damage. Among these, the single-cell gel electrophoresis assay (Comet assay) has become one of the most popular methods to assess DNA damage (Rojas, 2009). The alkaline version of the Comet assay is capable to detect a wide variety of DNA damage, such as DNA single-strand breaks, double-strand breaks, oxidatively induced base damages, alkali–labile sites, and sites undergoing DNA repair (Alink et al., 2007; Frenzilli et al., 2009). The popularity of this test is the result of its sensitivity, relatively low costs, simplicity, time efficiency, and standardized scoring of the Comet assay by use of an automatic image-analysis software (Olive et al., 1993; Singh, 2000). Also shown in this work, the application of the Comet assay on sentinel organisms exposed to samples of water is effective, fast, and informative. In freshwater, the Comet assay has been used mainly in assessing the effects of pollution on fish DNA (Boettcher et al., 2010; Osman et al., 2012). The majority of freshwater genotoxicity studies using invertebrates focused on filter-feeding organisms such as bivalve (Frenzilli et al., 2009) like *Corbicula fluminea* (Rigonato et al., 2005), *Unio pictorum* (Stambuk et al., 2008), and *Limnoperna fortunei* (Villela et al., 2006). Other invertebrate species have also been used, such as planarians (Guecheva et al., 2001), snails (*Biomphalaria glabrata*) (Grazeffe et al., 2008), and chironomidae (*Chironomus riparius*) (Lee et al., 2008), although it was recently applied to aquatic invertebrates such as amphipod gammarids (Lacaze et al., 2010; Lacaze et al., 2011) to evaluate the genotoxicity of contaminants and their bioavailability in continental waters.

The gammarid genus *Gammarus* have been used in a vast number of ecotoxicological (Maltby et al., 2002) and toxicological studies (Gerhardt, 2011), confirming its sensitivity to a wide range of stressors (Barnard and Barnard, 1983). Moreover, the species of this genus are an important food source for macroinvertebrates, fishes, and amphibians (Macneil et al., 1997). They also play a major role in leaf litter breakdown and

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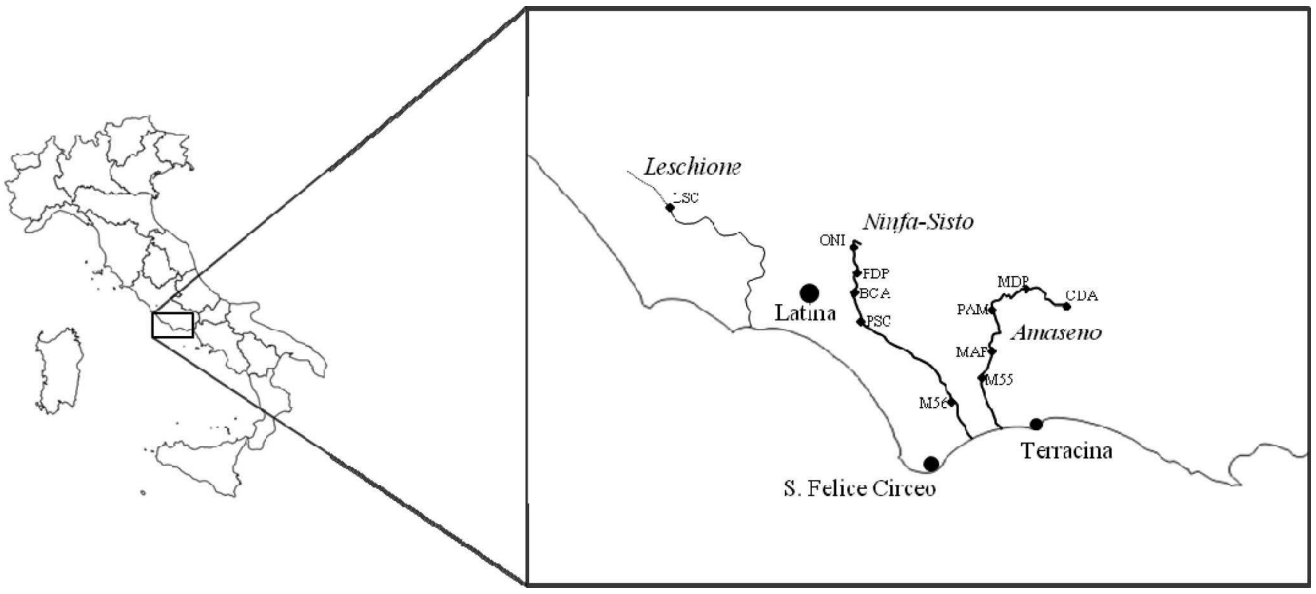

Figure 1—Map showing the sampling sites along the whole course of Amaseno and Ninfa-Sisto rivers.

thus in the entire food web (Forrow and Maltby, 2000). In the present study, the authors used *Gammarus elvirae*, a species endemic to the central Apennines (Iannilli and Ruffo, 2002), maintained in controlled laboratory conditions to reduce and homogenize baseline damage of DNA.

In most genotoxicity studies, using the Comet assay in aquatic species, the assay was performed on circulating cells such as haemocytes or erythrocytes as they play an important role in the transportation of toxic substances and in various defense mechanisms. In the present work, the authors used a genotoxicity biomarker by application of the Comet assay to haemocytes of *G. elvirae* exposed ex situ to the water sampled from different sites along the Amaseno and Ninfa-Sisto rivers. Ex situ toxicity evaluation allows exposing animals under more strictly controlled conditions, producing results more comparable with each other and then allowing using the method as early warning. The assessment of genotoxicity in aquatic organisms exposed to water under laboratory-controlled conditions can highlight the exposure to a mixture of potentially genotoxic pollutants (Avishai et al., 2003). The results obtained by the Comet assay have been related to the presence of environmental pollutants, trying to detect a causal relationship between toxic chemicals and genotoxic effects.

Methodology

Sampling Sites. Five sites were selected for each river (Figure 1). For the Amaseno River, the upstream site Capo D'Acqua (CDA; 13°17′52″ E, 41°27′53″N), was chosen as not polluted reference site because the analyses performed by ARPA Lazio (data not shown) and Regione Lazio (2007) showed a low level of contamination. Furthermore, it falls within the Site of Community Importance (SCI) named "Amaseno River upper course". The other sites, from the source to the mouth, are Madonna Del Ponte (MDP; 13°11′51″E, 41°25′51″N), Ponte Alle Mole (PAM; 13°12′07″E, 41°29′04″N), Mola dell'Abbadia Fossanova (MAF; 13°12′17″E, 41°27′16″N), and Migliara 55 (M55; 13°10′13″E, 41°21′43″N). For the Ninfa-Sisto River, the upstream site, Oasi di Ninfa (ONI; 12°57′19″E, 41°21′43″N) was selected as a not polluted reference site because the physicochemical properties of the water indicate that the area is relatively free of xenobiotics of antropogenic origin (ARPA Lazio, data not shown). Moreover this area was declared a Natural Monument by the Regione Lazio in 2000. The other sites along the river are Ponte Del Piegale (PDP; 12°57′20″E, 41°33′25″N), Borgata Carrara (BCA; 12°57′31″E, 41°32′35″N), Ponte Strada delle Congiunte (PSC; 12°57′30″E, 41°28′11″N), and Migliara 56 (M56; 13°07′37″E, 41°19′04″N). All these sites coincide with those chosen for official monitoring by the Regione Lazio and the Regional Agency for Environmental Protection of Latium (ARPA Lazio).

The authors also included a sampling site on the Leschione River (LSC; 12°40′54″E, 41°34′27″N) as a polluted reference site due to the high level of contaminant of its waters (Regione Lazio, 2007) (Figure 1).

Chemical Analyses of the Water. Physical parameters and pH were measured by pH meter (Hanna Instrument) and conductimeter (Eutech Instruments) during water sampling from different sites along the whole course of the two rivers.

Chemical analyses of the water of the Ninfa-Sisto and Amaseno rivers were carried out in April, June, and October/November 2011 by the National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). Total As, Ba, B, Cd, Cr, Cu, Fe, Mn, V, and Zn were measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES); chloride, fluoride, nitrates, nitrite, sulfate, and orthophosphate were measured by ion chromatography (IC); Hg was measured by automatic mercury analyzer (AMA) and Pb was measured by electrothermal atomic absorption spectroscopy (ET-AAS).

For computation of the abiotic index (AI) the authors considered the concentration of each analyte normalized to the respective Italian limit value (LV) for surface freshwater (D.Lgs 152/06). The sum of the normalized values was then divided by the total number of analytes considered for each sampling site. This calculation was repeated for each season:

$$AI_{season} = \frac{\sum_{i=1}^{n}(C_i/C_{LV_i})}{n} \quad (1)$$

where C_i = concentration of the single analyte, C_{LVi} = Italian LV for the ith analyte, and n = total number of analytes considered for each sampling site and for each season.

Sampling and Maintenance of Test Animals. The gammarids used for laboratory experiments were collected from the Amaseno spring Capo D'Acqua. Sexually mature *Gammarus* specimens were collected with a hand-held net (500 μm) in March 2011 and quickly brought to the laboratory where they were transferred to 10L aquarium tanks. They were kept for 15 days in the original site water, at constant temperature of 10 ± 1 °C, with a 16 hour/8 hour light/dark cycle and conductivity of 420 ± 14 μS; the water was changed weekly. During this period, the animals were fed with a common commercial food for freshwater crustaceans (JBL Novo Prawn) to ensure the right amount of nutrients.

Ex Situ Exposure Conditions. To carry out the genotoxicity test, the authors selected only precopula pairs. Three precopula pairs were exposed for 24 hours and three for 7 days to water samples collected from the different sites in April 2011. The specimens were placed in glass jars containing 500 mL of each water sample; during the exposure the same conditions of temperature and light/dark cycle described for the laboratory accommodation period were maintained. At the end of the exposure period, live specimens were sacrificed in order to proceed with the Comet assay.

Comet Assay. Haemolymph samples were collected from six individuals with an insulin syringe (30G needle) inserted between the cephalon and first mesosomite. The haemolymph of each individual was then placed in a 1.5-mL Eppendorf tube containing 100 μL of chilled phosphate buffered saline (PBS). The viability of the haemocytes was assessed by the Trypan-blue exclusion method. Only cell suspensions with viability >90% were used. The alkaline version of this technique, introduced by Singh et al. (1988) and recommended by international expert groups for genotoxicity testing, was chosen because it allows the evaluation of DNA damage including single- and double-strand breaks, DNA cross-links, alkali-labile sites, and incomplete repair sites (Singh et al., 1988). The authors followed the procedure as modified by Lacaze et al. (2010). Microscope slides were precoated with normal melting agarose in PBS (0.8%) and dried overnight. After collection of the cells, 20 μL of 1% low melting agarose in PBS (37 °C) were mixed with 20 μL cell suspension, added onto the coated slides, and finally covered with a coverslip. Slides were cooled for 5 minutes at 4 °C for solidification of the agarose. After removing the coverslip, slides were placed in a freshly prepared lysing solution at 4 °C (2.5 mol/L NaCl, 100 mmol/L Na2EDTA, 10 mmol/L Tris–HCl, 1% Triton X-100, and 10% DMSO, pH 10) in the dark for 1 hour; after cell lysis, slides were gently placed in a horizontal electrophoresis chamber filled with freshly prepared chilled buffer (300 mmol/L NaOH, 1 mmol/L EDTA, about pH 13). DNA was then allowed to unwind for 40 minutes. Electrophoresis was performed under 0.6 V/cm and 300 mA for 24 minutes. Lysis, DNA unwinding, and electrophoresis were performed at 4 °C. After the electrophoresis, the slides were washed in a neutralization buffer (0.4 mol/L Tris–HCl, pH 7.5). Finally, slides were dried for 15 minutes in absolute ethanol. After staining with 0.05 mmol/L ethidium bromide, the slides were covered with a cover glass and observed under an epifluorescence microscope (Zeiss Fluorescence Microscope System) equipped with a Zeiss camera (Axio Cam ICc 1). About 100 nuclei of each individual were captured at 40× magnification. To assess DNA damage, the authors considered the percentage of DNA in the tail (% DNA Tail) = 100 × (total tail intensity/total comet intensity), calculated by the software ©2006TriTekCorp CometScore, version 1.5. The authors performed the mean of six values of % DNA tail of six individuals. Comets showing an undistinguished head and a prominent tail were always excluded from the scoring. The authors considered one of the most commonly used parameters to monitor breaks in haemocytes DNA: percentage of DNA in the tail (% DNA tail) (Pereira et al. 2010), which is an objective parameter, less dependent on the technique; other authors consider this parameter as the most valid endpoint to quantify DNA strand-breakage in gammarids (Lacaze et al., 2010).

Statistical Analysis. The statistical analysis of both AI and Comet assay results was performed with the "PAST" software, version 1.93. Because the data were not normally distributed, the Kruskal–Wallis non-parametric test was used for the analysis. The parametric Pearson's correlation coefficient (r) was used to analyze correlations between the AI and Comet assay results.

Results and Discussion

The use of genotoxicity biomarkers is useful in detecting early and quantitatively measurable changes in molecular, biochemical, and cellular parameters such as the integrity of the genome (Shugart, 1990). The biomarkers of genotoxicity can highlight the occurrence of environmental disturbance before the effects are transferred to the higher levels of biological organization: population, community, and ecosystem (Clements, 2000).

For the correct interpretation of the results in monitoring studies, certain factors are considered critical, with the choice of a reference site being the most important. In the present study each chosen river has its spring as reference site. Besides the unpolluted reference sites, the authors considered another site on a third river, the heavily polluted Leschione (Regione Lazio, 2006, 2007), as a highly polluted site (polluted reference site).

The chemical profile is an important aspect in water quality monitoring and, in order to compare the different sites along a spatial gradient in the two rivers of interest containing different pollutants, the authors used the AI index. The index provides effective and synthetic information resulting from the contribution of each analyte, even if it was detected only in traces (Maes et al., 2005). The concentrations of analytes that have been used to calculate the AI values in each season are shown in Table 1. When an analyte was detected at a concentration below the detection limit of the method used, the authors considered the concentration to be half of the detection limit, as suggested by the APAT handbook (2003). In most cases the concentrations of the chemical species were below the legal limit (AI < 1 in Table 1). The only substances that exceeded the legal limit (D.Lgs 152/06) are nitrites at M56 in April, zinc at M55 in April, and orthophosphate at MDP and M56 in October/November. Cadmium and mercury were found always below the detection limit of the analytical methods used, so they were not included in AI calculation. The lowest values of AI are at the unpolluted reference site CDA in April and in June (0.063), the highest is 0.63 of the Leschione River. This study's analyses revealed no

Table 1—Values of analyzed chemical substances, obtained by normalization of concentrations with the limit values (D.lgs. 152/06). The AI values calculated in each month of 2011 and at each site along the Ninfa-Sisto and Amaseno rivers are also reported. Values exceeding the LV are in plain text; bold font indicates a value considered as half the concentration of the detection limit (APAT, 2003).

		Arsenic	Barium	Boron	Chlorides	Chrome	Copper	Fluorides	Iron	Lead
APRIL 2011	**ONI**	0.700	0.330	0.060	0.048	0.080	**0.100**	0.110	0.050	**0.010**
	PDP	**0.100**	0.650	0.030	0.055	**0.030**	0.300	0.120	0.030	**0.010**
	BCA	**0.100**	0.650	0.189	0.055	**0.030**	**0.100**	0.150	0.030	**0.010**
	PSC	0.300	0.670	0.020	0.065	**0.030**	**0.100**	0.120	0.440	**0.010**
	M56	**0.100**	0.710	0.005	0.265	**0.030**	0.750	0.340	0.150	0.062
	CDA	**0.100**	0.210	**0.001**	0.047	**0.030**	**0.100**	0.060	**0.010**	**0.010**
	MDP	**0.100**	0.230	**0.001**	0.050	**0.030**	**0.100**	0.070	0.020	**0.010**
	PAM	**0.100**	0.210	0.010	0.045	**0.030**	**0.100**	0.090	0.030	**0.010**
	MAF	**0.100**	0.250	0.060	0.050	**0.030**	**0.100**	0.050	0.040	**0.010**
	M55	**0.100**	0.290	0.030	0.050	**0.030**	0.185	0.160	0.060	**0.010**
JUNE 2011	**ONI**	0.650	0.500	0.064	0.049	0.080	0.150	0.115	0.050	**0.010**
	PDP	**0.100**	0.585	0.041	0.052	0.085	0.200	0.110	0.060	**0.010**
	BCA	**0.100**	0.535	0.116	0.051	0.055	0.150	0.120	0.050	**0.010**
	PSC	0.200	0.605	0.037	0.063	0.095	0.100	0.115	0.270	**0.010**
	M56	0.200	0.760	0.043	0.268	**0.030**	0.425	0.335	0.130	0.036
	CDA	**0.100**	0.210	**0.001**	0.047	**0.030**	**0.100**	0.060	**0.010**	**0.010**
	MDP	**0.100**	0.230	**0.001**	0.047	**0.030**	**0.100**	0.070	0.020	**0.010**
	PAM	**0.100**	0.290	**0.001**	0.046	**0.030**	**0.100**	0.080	**0.010**	**0.010**
	MAF	**0.100**	0.310	**0.001**	0.050	**0.030**	**0.100**	0.090	0.040	**0.010**
	M55	**0.100**	0.320	**0.001**	0.050	**0.030**	**0.100**	0.070	**0.010**	**0.010**
OCTOBER/NOVEMBER 2011	**ONI**	0.600	0.670	0.067	0.050	0.080	0.200	0.120	0.050	**0.010**
	PDP	**0.100**	0.520	0.052	0.048	0.140	**0.100**	0.100	0.090	**0.010**
	BCA	**0.100**	0.420	0.042	0.047	0.080	0.200	0.090	0.070	**0.010**
	PSC	**0.100**	0.540	0.054	0.060	0.160	**0.100**	0.110	0.100	**0.010**
	M56	0.300	0.810	0.081	0.270	**0.030**	**0.100**	0.330	0.110	**0.010**
	CDA	**0.100**	0.180	0.032	0.044	**0.030**	0.300	0.050	0.080	**0.010**
	MDP	**0.100**	0.240	0.027	0.050	**0.030**	0.200	0.050	0.140	**0.010**
	PAM	**0.100**	0.340	0.024	0.050	**0.030**	**0.100**	0.070	0.120	**0.010**
	MAF	**0.100**	0.370	0.053	0.075	**0.030**	0.350	0.070	0.130	**0.010**
	M55	**0.100**	0.430	0.058	0.270	**0.030**	0.450	0.100	0.160	0.020

significant differences between the AI values of the reference site and those of the other sites along each of the two rivers, except for site M55 in the Amaseno River in April ($p > 0.05$) (Figure 2). Hence, there is a homogeneous physicochemical quality of the water matrix from source to mouth, for both rivers. The downstream sites MAF, PSC, and M56 have high AI values, but are not considered contaminated because the concentration of analytes are lower than legal limits, and is not correct to indicate them as polluted sites. Moreover, only three analyte values out of 90 monitored in the last 4 years by the competent Italian institutions (ARPA Lazio, Regional Agency for Environmental Protection) for assessment and control of water quality, showing concentrations above the permissible limit, considering all sites, and all seasons (data not shown).

The detailed chemical analysis of waters along the Amaseno River revealed its good quality near the upstream site Capo D'Acqua (CDA), which the authors considered as a pristine reference (Regione Lazio, 2007, 2010); this site showed the lowest values of AI. On the contrary, at ONI, even if the authors considered this site as a reference, the AI value were not the lowest for the Ninfa-Sisto River because the arsenic and vanadium concentrations, although below the legal limit, were high in each month. These metals are generally found in volcanic hydrogeological structures such as the Albano Volcano, adjacent to the Lepini Mountains, at whose feet ONI is located. The hydraulic relationships between the two zones are not yet clear, but probably the presence of these metals could be linked with natural sources (Bono, 2005). Consequently, the authors considered ONI as unpolluted reference site because the chemicals are of natural origin and the physicochemical quality of the aqueous matrix of the reference site is not significantly different from that of the other test sites.

In the only sampling site of Leschione River (LSC), the AI value result in April is 0.63 (ARPA Lazio, data not shown). The LSC site was chosen as a polluted reference because it is the most polluted site in Latium, as highlighted in previous studies based on the LIM index (D.Lgs 152/99; Regione Lazio, 2007), which classify this site as bad quality and our AI value confirms this judgment.

The two rivers considered are characterized by a homogeneous physicochemical quality of the water. Table 2 shows that the three physical parameters considered are stable in the various sites monitored (low SD). The parameters did not exceed the respective permissible values, except for the temperature at the sites MAF and M56.

The aim of this work is to propose an integrative aspect on chemical analysis of water to understand the effects of the mixture of contaminants present in waters. In particular, the DNA damage, such as strand breaks, has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental biomonitoring studies (Frenzilli et al., 2004; Xu et al., 1999). The sentinel species that the authors chose, the

Table 1—(Extended)

Manganese	Nitrates	Nitrites	Sulphate	Vanadium	Zinc	O-phosphate	Al
0.012	0.212	**0.050**	0.003	0.160	0.010	**0.075**	**0.126**
0.038	0.096	**0.050**	0.020	0.040	0.034	**0.075**	**0.105**
0.018	0.124	**0.050**	0.025	0.080	0.072	**0.075**	**0.110**
0.260	0.148	**0.050**	0.032	0.100	0.006	0.400	**0.172**
0.022	0.272	*1.160*	0.120	0.140	0.014	0.550	**0.293**
0.004	0.268	**0.050**	0.025	**0.020**	**0.001**	**0.075**	**0.063**
0.142	0.220	**0.050**	0.033	**0.020**	**0.001**	**0.075**	**0.072**
0.044	0.396	**0.050**	0.033	**0.020**	**0.001**	**0.075**	**0.078**
0.160	0.136	**0.050**	0.033	0.060	0.004	**0.075**	**0.076**
0.160	0.252	**0.050**	0.035	0.060	*1.680*	**0.075**	**0.202**
0.015	**0.160**	0.050	0.015	0.170	0.008	**0.075**	**0.135**
0.040	**0.104**	0.050	0.023	0.060	0.024	**0.075**	**0.101**
0.030	**0.112**	0.050	0.026	0.090	0.052	**0.075**	**0.101**
0.413	0.128	0.325	0.034	0.110	0.007	0.238	**0.172**
0.042	0.236	0.605	0.137	0.170	0.011	0.993	**0.276**
0.004	0.268	**0.050**	0.025	**0.020**	**0.001**	**0.075**	**0.063**
0.142	0.220	0.120	0.029	**0.020**	**0.001**	**0.075**	**0.076**
0.090	0.148	**0.050**	0.028	**0.020**	**0.001**	**0.075**	**0.067**
0.024	0.760	**0.050**	0.029	**0.020**	**0.001**	**0.075**	**0.106**
0.026	0.140	0.120	0.029	**0.020**	**0.001**	0.225	**0.078**
0.018	0.108	**0.050**	0.027	0.180	0.006	**0.075**	**0.144**
0.042	0.112	**0.050**	0.025	0.080	0.014	**0.075**	**0.097**
0.042	0.100	**0.050**	0.027	0.100	0.032	**0.075**	**0.093**
0.566	0.108	0.600	0.036	0.120	0.008	**0.075**	**0.172**
0.062	0.200	**0.050**	0.153	0.200	0.008	*1.435*	**0.259**
0.024	0.236	**0.050**	0.025	0.100	0.010	**0.075**	**0.084**
0.152	0.236	**0.050**	0.028	0.120	0.008	*1.183*	**0.164**
0.164	0.188	**0.050**	0.028	0.100	0.010	**0.075**	**0.091**
0.124	0.180	0.400	0.041	0.120	0.008	**0.075**	**0.134**
0.158	0.208	0.600	0.062	0.140	0.008	**0.075**	**0.179**

freshwater gammarid *G. elvirae,* plays a key role in the recycling of organic matter and represents an important food source for vertebrates such as fishes and amphibians (Macneil et al., 1997). This species shows several advantages to be used in these in vivo tests, such as easy sampling and high adaptation to the laboratory conditions. In this work, the authors performed ex situ exposure of *G. elvirae* to water sampled at various sites along the rivers.

The specimens used for exposure were taken from CDA only in spring (April 2011) because the summer and winter water regimes, influenced by the climate, reduces the populations and has negative effects on individual viability (Villela et al., 2007). Consequently, it would be difficult to determine whether DNA damage is physiologically induced by stressful climatic conditions or by chemical contamination. However, it is possible to use gammarids bred and/or frozen water taken at other times/seasons.

In this study, the Comet assay was performed on haemocytes, somatic cells that can be easily collected from this gammarid species. Because this cell type has an important role in immune defense, phagocytosis, transport and excretion of toxic substances, and detoxification from xenobiotics, it is highly exposed to environmental agents (Iwanaga and Lee, 2005).

After exposure of *G. elvirae* for 24 hours and 7 days to water sampled at the five sites for each river, no one specimen was found dead. The comparative analysis of mean values of % DNA tail after exposure of *G. elvirae* to the water samples from the Amaseno River are shown in Figure 3 A. Madonna Del Ponte, closest to the source site, does not show a significant difference with the unpolluted reference site. However, there are significant differences between the unpolluted reference site and PAM and MAF for 7 days of exposure and M55 for both 24 hours and 7 days of exposure. Therefore, there is significant difference ($p < 0.05$) between 24 hours and 7 days of exposure to MAF water.

Figure 3B shows the Comet assay mean values after exposure of *G. elvirae* to water collected from the sites along the Ninfa-Sisto River.

For the sites closest to the source, PDP and BCA, the greatest DNA damage occur after 7 days of exposure significantly different from 24 hours of exposure ($p < 0.05$), whereas for the sites closest to the river mouth, PSC and M56, after 24 hours of exposure is greater and significant different from 7 days of exposure ($p < 0.05$). The % DNA tail values at PDP for 24 hours of exposure and at PSC and M56 for 7 days of exposure are similar to those at the unpolluted reference site ONI.

The greatest DNA damage was recorded in specimens exposed to water from MAF for 7 days and PSC for 24 hours; moreover, specimens exposed to PSC water shows % DNA tail values similar to them exposed to LSC values ($p = 0.65$). The % DNA tail values for specimens exposed to water from the unpolluted reference sites are always below 8, whereas the DNA

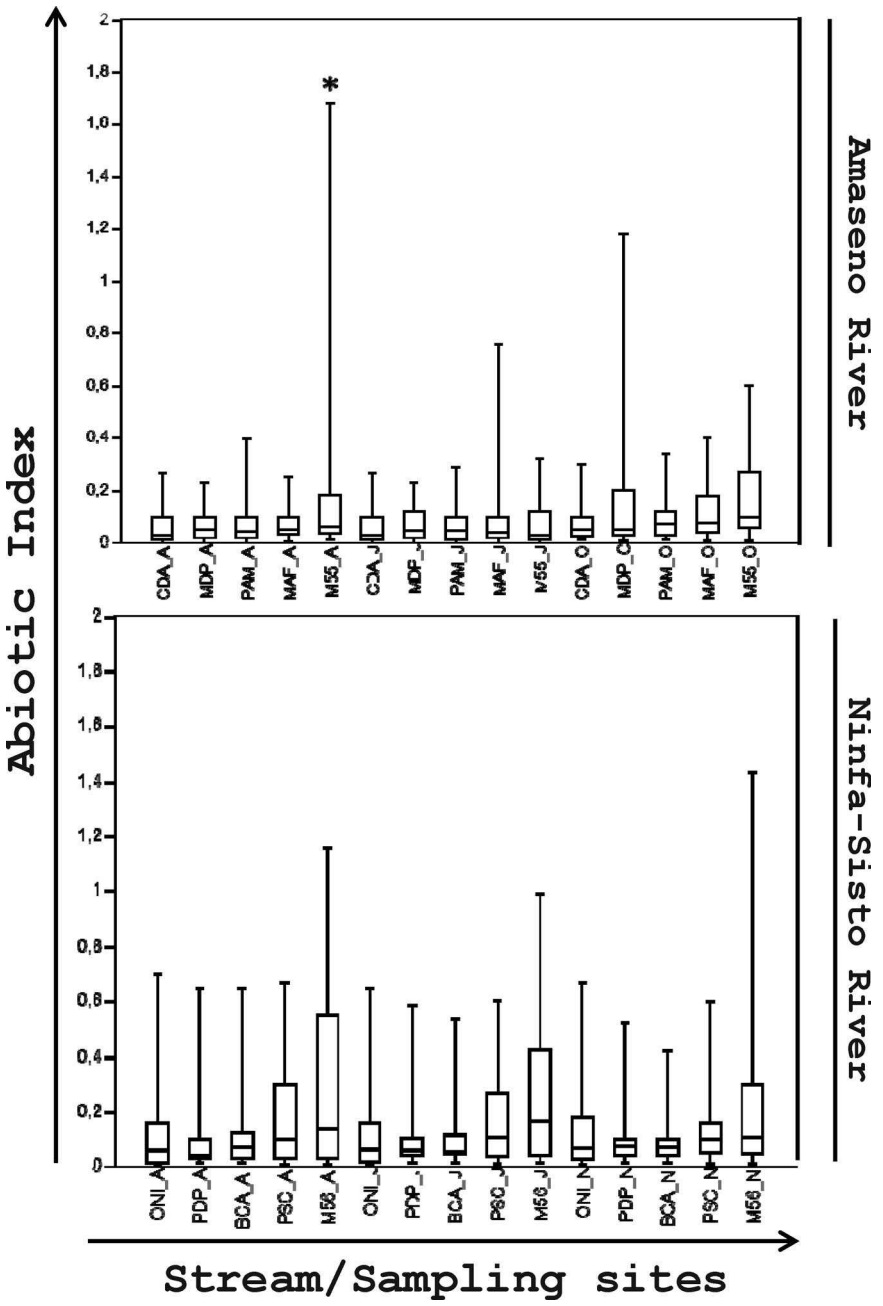

Figure 2—Boxplot of AI values for sampling sites along the Ninfa-Sisto and Amaseno rivers in each month considered in 2011, * significantly different from unpolluted reference site. For each site, the 25–75% quartiles are drawn using a box. The median is shown a horizontal line inside the box. The minimal and maximal values shown with short horizontal lines.

Table 2—Physical parameters of the water samples collected from different sites along the whole course of Amaseno and Ninfa-Sisto rivers.

Parameter (unit)	Sites (Mean ± SD)										
	CDA	MDP	PAM	MAF	M55	ONI	PDP	BCA	PSC	M56	Permissible limit
pH (unit)	7.5 ± 0.2	7.9 ± 0.4	8.1 ± 0.1	8.2 ± 0.2	8.1 ± 0.2	7.7 ± 0.1	8.1 ± 0.1	8.2 ± 0.1	8.2 ± 0.2	8.2 ± 0.3	6.5 – 8.5
Temperature (°C)	13.8 ± 0.4	16.4 ± 2.6	16.2 ± 3.1	19.5 ± 4.3	12.4 ± 2.7	13.9 ± 0.8	13.9 ± 1.7	15.4 ± 4.4	17.3 ± 6.1	19.2 ± 3.0	< 22
Conductivity (μs/cm)	410 ± 10	410 ± 20	410 ± 30	390 ± 20	420 ± 10	480 ± 30	460 ± 6.0	460 ± 4.0	490 ± 6.0	610 ± 20	< 1000

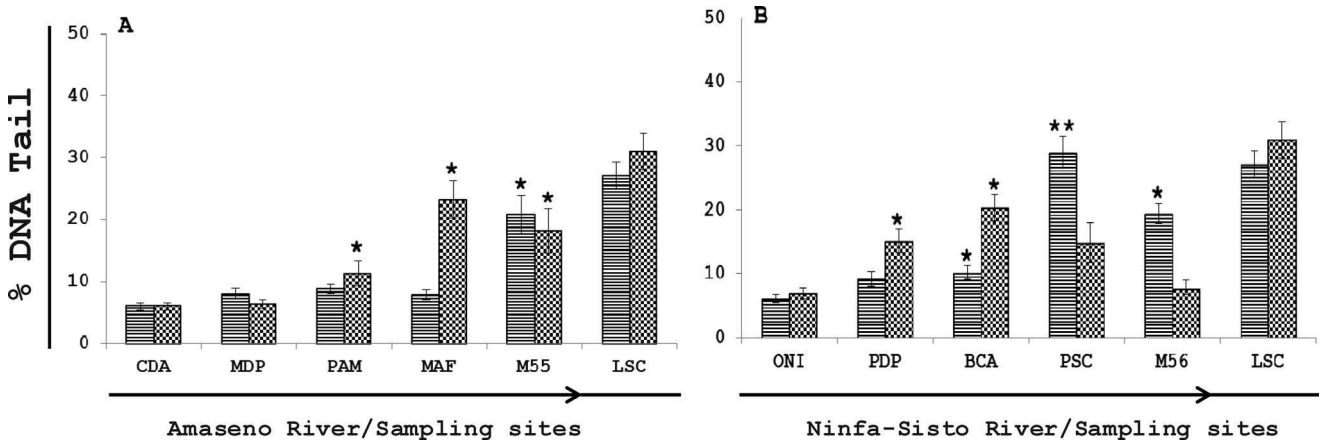

Figure 3—DNA damage. Mean ± Standard Error of the % DNA tail parameter of the Comet assay applied to *G. elvirae* haemocytes (▤ 24 hours and ▩ 7 days exposure; * significantly different from unpolluted reference site [$p < 0.05$]; ** significantly different from unpolluted reference site and not significantly different from polluted reference site [LSC]).

damage of specimens exposed to LSC water always shows values greater than 25.

The DNA damage in haemocytes showed low basal levels and low variability at the reference sites, providing detectable and reliable discrimination from the other sites. This study's results indicate that all waters collected from the sampling sites along the Ninfa-Sisto and Amaseno rivers have the potential to cause DNA damage in the species considered. In fact, the authors found significant differences among % DNA tail on the specimens exposed to the water from reference sites and from other sampling sites, even though the chemical analyses found concentrations of pollutants below the limits imposed by current legislation. It should be stressed that, in recent studies on genotoxicity potential conducted by in situ exposure (Osman et al., 2012; Villela, 2007), the genotoxic response was correlated to a specific xenobiotic or a set of xenobiotic substances but with concentrations higher than the legal limits.

It was not possible to detect a single xenobiotic agent capable of causing the observed DNA damage. However, it is known that synergistic effects due to the combination of several substances have a greater possibility of causing genotoxic insult (Osman et al., 2012). Water samples taken from specific sites are a solution of organic and inorganic substances that may have synergistic, additive, or antagonistic effects (Fent, 2003).

Therefore, the high levels of DNA damage observed in the *G. elvirae* haemocytes can be attributed to the combination of several contaminants arising mainly from the intense agricultural activities in the monitored area. Although the authors are unable to identify a specific cause–effect relationship, the chemical analysis of the water investigated shows that different mixtures of contaminants cause different genotoxic responses in exposed gammarids. The waters of MAF, like those of PDP and BCA on the Ninfa-Sisto River, probably have a higher potential to cause DNA damage in *G. elvirae* following long exposure (7 days) rather than acute (24 hours) exposure. The opposite pattern emerged at sites further downstream (PSC, M56) where the genotoxic response was higher at 24 hours than at 7 days ($p < 0.05$).

From the results obtained and the observations on land use, the authors can infer that the difference in genotoxic response at 24 hours and 7 days may be the result of the different crops grown in the two areas and thus to the different inputs of agriculture-related substances into the waters. Sites PSC and M56 are located in an area with intense agricultural activity: approximately 21 000 hectares are devoted to the alternate cultivation of vegetables and forage (http://censimentoagricoltura.istat.it; accessed 10 March 2014). The authors found high concentrations of nitrites, nitrates, sulphate, chlorides, orthophosphate, lead, iron and copper, and thus increased AI values, in the water. It is well known that lead can inhibit the activation of antioxidant enzymes and impairs cellular defenses, making the organism more susceptible to oxidative attack (Ercal et al., 2001) and then DNA damage. Furthermore, in a study conducted by the authors' research group, individuals of *G. elvirae* exposed to lead concentrations of the same order of magnitude as the legal limit (D.Lgs. 152/06) showed a significant genotoxic response already at 24 hours of exposure (Ronci, 2013).

The upstream sites of the Ninfa-Sisto River PDP, BCA, and site MAF of the Amaseno River are in an area dedicated mainly to cultivation of olives, vines and fruit trees (http://censimentoagricoltura.istat.it; accessed 10 March 2014). The authors found a detectable concentration of zinc, copper, and boron in the water. Zinc sulfate is also widely used as a mineral fertilizer for fruit trees (ISTAT, 2012). Exposure to copper can result in significant DNA damage, assessed by the Comet assay in *Crenicichla menezesi* (Egito et al., 2010). Moreover, in studies on *Gammarus locusta*, copper was shown to cause single DNA strand breakage (Costa et al., 2002). Before an induced DNA lesion is fixed or stabilized, several types of enzymatic processes, such as excision, may arrange for its repair and the final effect of the insult may be null. This could explain the greater damage found at 24 hours than at 7 days at the downstream sites of both rivers, where exposure to low doses allowed enzyme systems, not yet totally activated, to repair the DNA damage.

A recent study (Lacaze et al., 2011), on *Gammarus fossarum* exposed in caged upstream, in the vicinity and downstream a water resource recovery facility effluent in three rivers, shows that there are not significant differences in DNA damage on considered species haemocytes. Probably, the differences that emerged when compared to this study's findings are the result of

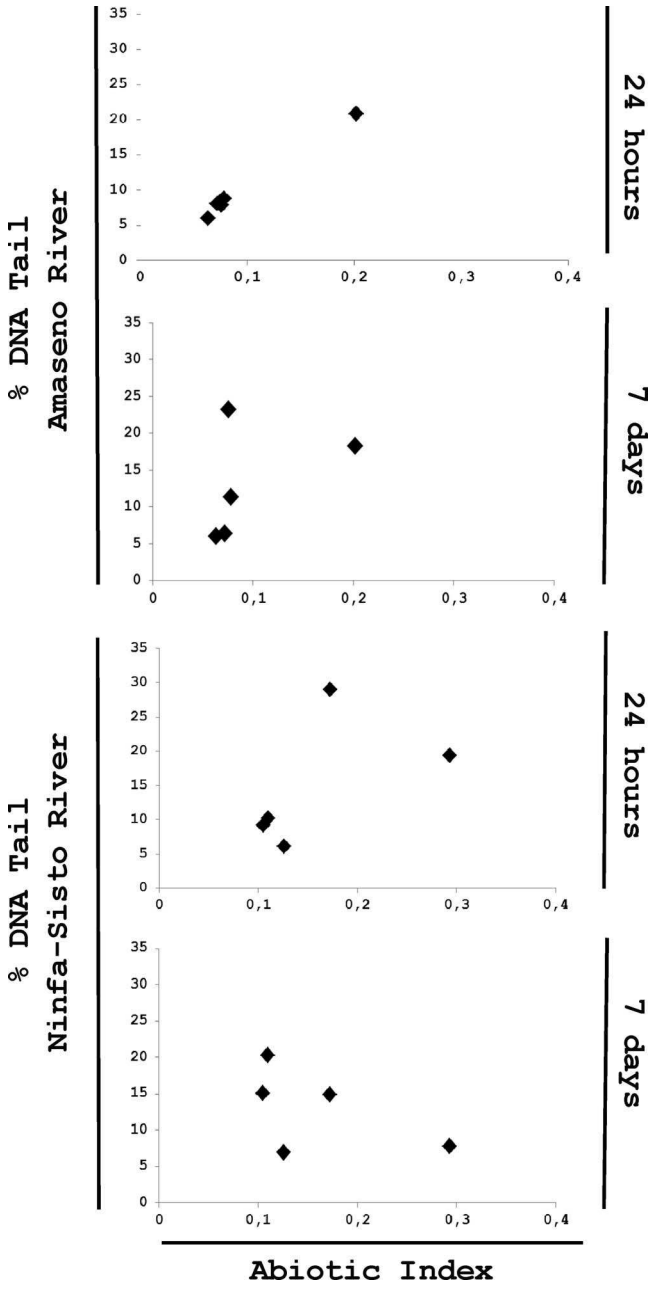

Figure 4—Correlations between AI and DNA damage (% DNA tail) in the haemocytes of *G. elvirae* in the Amaseno and Ninfa-Sisto rivers. The AI values refer to April 2011.

xenobiotics mixture linked with different contamination sources. However, it is to be noted that other variables must be taken into account to explain these differences. The most important is the type of exposure (caging versus ex situ) but also species and duration of exposure. To allow large-scale comparisons, further research would be necessary to investigate the relationship between the presence of mixture of pollutants in freshwaters and the genotoxic effects in gammarid species.

In the present study, the two considered variables (AI and DNA damage) are independent or in a nonlinear correlation (Figure 4). In fact, merging the AI values with DNA damage in *G. elvirae* no correlation between AI and DNA damage for the Ninfa-Sisto River was found (Figure 4). However there is linear correlation ($p < 0.05$) for 24 hours of exposure to water drawn from the Amaseno sites; but it disappears ($p > 0.05$) when M55 data (AI: 0.202; % DNA tail: 20.83) are removed.

Conclusion

To the authors' knowledge, the present study is the first attempt to detect genotoxicity potential of rivers in the freshwater invertebrate *G. elvirae*. The two considered rivers are characterized by a homogeneous physicochemical quality of the water. However, an apparent absence of significant contamination is contradicted by the molecular changes in the gammarid species considered. These changes could be attributed to synergic or additive effects of contaminants at low concentrations. Thus, this study demonstrates that evaluation of genotoxicity can reveal the effects of exposure to a mixture of pollutants in which, taken individually, none exceeds the limits of Italian legislation. This study's results clearly show that assessment of the toxicity of a single substance is not sufficient to determine its effects when it is present together with other substances. Indeed, although it is generally true that a substance can become toxic with increasing dose, this is not the case for a substance that is not genotoxic: it cannot become genotoxic at an increasing dose because genotoxicity implies the ability to interact, even if indirectly, with DNA.

The authors successfully applied the Comet assay to haemocytes of the gammarid *G. elvirae* exposed ex situ to water samples collected to whole course of two rivers. This species could become a sentinel species in ecotoxicological tools. Hence, this study has proven that the freshwater amphipod *G. elvirae* is a good candidate for genotoxicity assessments in freshwater ecosystems.

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