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Comparison between active and passive biomonitoring strategies for the assessment of genotoxicity and metal bioaccumulation in *Echinogammarus veneris* (Crustacea: Amphipoda)

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Abstract
The aim of this study was to investigate the relevance and the robustness of active and passive approaches used in freshwater biomonitoring with the ecologically relevant gammarid amphipod *Echinogammarus veneris*. To assess the contaminant bioavailability in two rivers of Latium (Central Italy), we measured the genotoxic potential in haemocytes by comet assay and metal bioaccumulation in tissues by analytical methods. We adopted an active strategy of exposure *in situ* and a passive method of sampling *in situ*. In the first case, the gammarids were exposed in cages in several sampling sites selected along two rivers, while in the sampling *in situ*, individuals were collected directly in the same sampling sites and then analyzed. The results indicate that the comet assay carried out on haemocytes from caged individuals proved to be a sensitive tool for freshwater genotoxicity monitoring. However, the sampling *in situ* is more appropriate for a realistic understanding of the presence of trace metal in *E. veneris*.

Keywords: *Gammaridae, comet assay, biomarker, in situ assessment, fresh water quality*

Introduction

Freshwater ecosystems receive large quantities of pollutants from various sources such as agricultural practices, industrial activities and domestic wastewaters. Therefore, aquatic samples are complex mixtures of organic and inorganic compounds that may interact to produce additive, synergistic or antagonist effects (Fent 2003; De Andrade et al. 2004). It has been demonstrated that the analysis of the biological response to subsequent exposures to complex mixtures of substances present in trace amounts, as in the case of freshwater ecosystems, is much more informative than just the quantitative profile of the individual components analyzed by chemical methods (Osman et al. 2012; Ronci et al. 2015). In addition, it is essential that the methods that lead to the results of these biological responses are well standardized and, therefore, comparable.

The use of biota to monitor levels and trends of chemical contamination in water (i.e., chemical biomonitoring) has been used in several monitoring programs in coastal and continental waters (Besse et al. 2012). Biota reflects the bio-accumulative and bioavailable fraction of contaminants in receiving waters, which are of direct eco-toxicological relevance. Finally, biota enables time-integrated measures over the exposure period, so it can be used to establish spatial and temporal trends of a bioavailable contamination (Rainbow 1995; Andral et al. 2004).

The response of biomarkers can be regarded as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (Van der Oost et al. 2003). There are currently two different strategies for chemical biomonitoring that can be adopted: passive and active. Passive approaches rely on indigenous organisms (Goldberg 1975),

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while active approaches rely on transplanted and caged individuals from a reference site (Andral et al. 2004).

The aim of this study is to investigate the relevance and the robustness of passive and active biomonitoring strategies, here called, respectively, sampling *in situ* and exposure *in situ*, to monitor trends of genotoxic potential (biomarker of effect) and metal bioaccumulation (biomarker of exposure) in the crustacean amphipod *Echinogammarus veneris* (Heller, 1865) from Amaseno and Ninfa-Sisto rivers (Central Italy), which flow through an area characterized by intensive agriculture (Regione Lazio 2004a).

We used the species *E. veneris* because it is widespread, easily identifiable and common in rivers and streams of the Peri-Mediterranean region. Moreover, it is ecologically relevant as it represents an important source of food for macroinvertebrates, fish, birds and amphibians; in addition, the species also plays a major role in the leaf litter breakdown processes (MacNeil et al. 1997). Finally, in a previous study we showed how *E. veneris* could be successfully employed to assess the actual genotoxic response to exposure of waters containing different chemicals (Ronci 2013).

The assessment of genotoxic potential in surface waters is one of the main tasks of environmental monitoring to control pollution (Raiaguru et al. 2003). The analysis of environmental genotoxicity provides early warning signals of adverse long-term effects of contamination (Rybakovas et al. 2009). DNA damage, such as strand breaks, has been proposed as a sensitive indicator of genotoxicity and an effective biomarker in environmental bio-monitoring studies (Xu et al. 1999; Frenzilli et al. 2004). The comet assay (Singh et al. 1988) is becoming an important tool for environmental biomonitoring (Cotelle & Ferard 1999; Valverde & Rojas 2009). Its advantages over other DNA damage quantification methods are related to its high sensitivity (Alink et al. 2007) and to the possibility to detect a variety of DNA damage, such as double- and single-strand breaks, alkali-labile sites and cross-linking (Lacaze et al. 2010, 2011). Haemocytes are easily obtainable and demand very little manipulation for preparation of slides, keeping the possibility of their damage to the minimum. They are closely exposed to environmental agents through their physiological roles in the transport of toxicants and in various defence mechanisms (Mersch et al. 1996). The demonstrated mutagenic and genotoxic effects of many metals (which can be released in waters from industrial and agricultural activities) such as arsenic, mercury, nickel and chromium (Anderson & Wild 1994) suggested the possibility to investigate the bioaccumulation of metals in the *E. veneris* specimens used for the sampling *in situ* and the exposure *in situ*. The study of relevant indicators of exposure is of great interest for environmental risk assessment (Besse et al. 2013), and one of the most important is bioaccumulation in the tissues of living organisms. Indeed, the bioaccumulation process integrates the organism's ability to regulate accumulated metals, the geochemical effects on metal uptake (McGeer et al. 2003; Luoma & Rainbow 2005) and the different ways, via water or via their diet, of accumulation. Metal determination in tissues is an important tool to monitor the effects of metals on biota because it reflects the fraction of metals bioavailable and potentially toxic for aquatic organisms (Campbell 1995; Meylan et al. 2004). In this study, the metal bioaccumulation analysis was assessed on *E. veneris* total tissues; in particular, we considered the following metals: aluminum (Al), arsenic (As), barium (Ba), boron (B), cadmium (Cd), chromium (Cr), iron (Fe), manganese (Mn), mercury (Hg), molybdenum (Mo), nickel (Ni), lead (Pb), zinc (Zn) and vanadium (V). The results were synthesized in the so-called individual mean (multi-metal) bioaccumulation index (IMBI; Maes et al. 2005), which corrects for the eventual lack of data and their homogeneity.

Materials and methods

Study area

Five sites were selected for each river (Figure 1). For the Amaseno River, the upstream site Capo D'Acqua (CDA) was chosen as non-polluted reference site because the analyses performed by ARPA Lazio (Regional Agency of Environmental Protection of Lazio, ARPA-Lazio; data not shown) and Regione Lazio (2007) showed a low level of contamination. Furthermore, it falls within the Site of Community Importance (SIC) named "Amaseno River upper course". The other sites, from source to mouth, are: Madonna Del Ponte (MDP), Ponte Alle Mole (PAM), Mola dell'Abbadia Fossanova (MAF) and Migliara 55 (M55). For the Ninfa-Sisto River the upstream site, Oasi di Ninfa (ONI), was selected as non-polluted reference site because the physical-chemical properties of the water indicate that the area is relatively free of xenobiotics (ARPA Lazio, data not shown). Moreover, this area was declared a Natural Monument by the Regione Lazio in 2000. Other sites along Ninfa-Sisto River are: Ponte Del Piegale (PDP), Borgata Carrara (BCA), Ponte Strada delle Congiunte (PSC) and Migliara 56 (M56). All these sites coincide with those chosen by the Regione Lazio and the Regional Agency

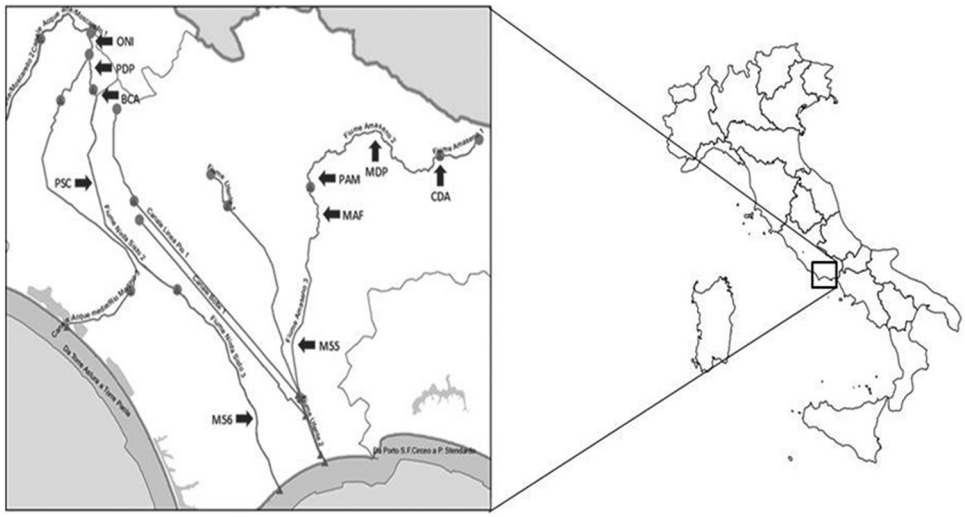

Figure 1. Map showing the sampling sites along the rivers. Amaseno River: Capo D'Acqua (CDA; 13°17′52″E, 41°27′53″N; reference site), Madonna Del Ponte (MDP; 13°11′51″E, 41°25′51″N), Ponte Alle Mole (PAM; 13°12′07″E, 41°29′04″N), Mola dell'Abbadia Fossanova (MAF; 13°12′17″E, 41°27′16″N) and Migliara 55 (M55; 13°10′13″E, 41°21′43″N). Ninfa-Sisto River: Oasi di Ninfa (ONI; 12°57′19″E, 41° 21′43″N; reference site), 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).

Table I. Physical parameters, number of gammarids collected during the "sampling *in situ*" and percentage of gammarids survivors after "exposure *in situ*", measured along the whole course of the Amaseno and Ninfa-Sisto rivers.

		January					May				
		CDA	MDP	PAM	MAF	M55	CDA	MDP	PAM	MAF	M55
Amaseno River	Water temperature (°C)	14.1	12.8	12.7	13.1	12.8	13.6	15.1	14.8	15.2	16.1
	Conducibility (μs/cm)	386	392	373	369	396	469	472	466	463	501
	pH	7.55	8.23	8.24	8.44	8.35	7.49	8.02	8.02	8.03	8.03
	Sampling *in situ* found (n)	20	0	20	0	0	20	0	20	20	0
	Exposure *in situ* survivors (%)	80	63	70	76	26	71	95	93	53	48
		January					May				
		ONI	PDP	BCA	PSC	M56	ONI	PDP	BCA	PSC	M56
Ninfa-Sisto River	Water temperature (°C)	12.7	12.5	12.3	12.3	12.8	14 .1	14.2	15.1	17.9	24.1
	Conducibility (μs/cm)	361	372	397	397	556	451	462	464	488	663
	pH	8.05	8.22	8.41	8.33	8.88	7.97	8.14	8.32	8.26	8.84
	Sampling *in situ* found (n)	20	20	20	0	0	20	16	20	0	0
	Exposure *in situ* survivors (%)	100	67	92	85	8	16	82	61	56	35

for Environmental Protection of Latium (ARPA Lazio) for official monitoring programs.

Experimental design

In sampling *in situ* experiments, we collected, where present (Table I), 10 pre-copula pairs (sexually mature) of *E. veneris* with a hand-held net (500 μm). We repeated the samplings in winter (January) and spring (May) 2013. After the sampling, the individuals were stored in plastic cans containing ambient fresh water, then stored in thermal containers and quickly brought to the laboratory. The following day, we proceeded with the genotoxicity assessment and bioaccumulation analysis (only in May). Table I shows the physical-chemical water parameters and the sites where the species was found in each month considered.

In exposure *in situ* experiments, amphipods were collected from CDA. Sexually mature *E. veneris* were collected using a hand-held net (500 μm). They were quickly brought to the laboratory where they were kept 15 days at 10 ± 1°C, using a 16/8 h light/dark cycle, continuously supplied with aerated

uncontaminated water. Adult gammarids with comparable body lengths were selected so that mature and similar age-ranked gammarids would be exposed. They were fed *ad libitum* on alder leaves (*Alnus glutinosa*) collected in a pristine site. For the exposure, 10 adult males and 10 adult females were caged in polypropylene cylinders (length, 10 cm, diameter, 5.5 cm) capped at their ends with net (mesh 1 mm) to guarantee free circulation of water and fed with the same alder leaves (*A. glutinosa*) as done for the individuals kept in laboratory. Three cylinders per site were placed. The cylinders were protected by a rigid, weighted plastic container. Caged individuals were exposed for 15 days at the sites previously described in the study area section in January 2013 and in May 2013. After 15 days of exposure, DNA damage and metal(loid) bioaccumulation (only in May) were measured. Table I shows the physical-chemical water parameters and the mortality rate after the exposure time.

Genotoxicity assessment

Haemolymph samples were collected from 12 individuals (limited to six individuals in M56 in exposure in situ, in January) with an insulin syringe (30G needle) inserted between the cephalon and first mesosomite. Haemocyte isolation was carried out to perform the comet assay as described in Lacaze et al. (2010) and in Ronci et al. (2015). A pool of four individuals (two females and two males) was required to get enough haemocytes for each replicate. The viability of the haemocytes was observed by the trypan blue exclusion method. Only cell suspensions with viability > 90% were used. We performed three replicates for each site. After the electrophoresis, we stained the slides with 50 µL ethidium bromide 30 µg/mL and observed them under a fluorescence microscope (Zeiss Fluorescence Microscope System). At least 60 nuclei per replicate slide were captured at 40X magnification. The comets were searched always following the same slide pathway. Images were analyzed with the software CometScore 1.5 by TriTekcorp™, in order to quantify the DNA damage. Tail moment (TM), defined as the product of the tail length and the fraction of total DNA in the tail, was chosen as a parameter of DNA damage.

Bioaccumulation analysis

In May, individuals of *E. veneris* were pooled (five individuals per sample) to obtain an average mass of 30 mg dry weight (about 150 mg wet weight). Three replicates of each pooled sample were subsequently analysed. Metal(loid)s (Al, As, Ba, B, Cd, Cr, Fe, Mn, Hg, Mo, Ni, Pb, Zn and V) were analysed by inductively coupled plasma optical emission spectroscopy (ICP-OES) after mineralization with nitric acid. The absolute values are expressed in mg/kg of sample. We calculated a relative bioaccumulation index by dividing (standardizing) the individual concentration of metal(loid) i (C_i) by the maximum observed concentration (C_{imax}) and averaging over all metal(loid)s of each site. Thus, the IMBI (Maes et al. 2005) was defined as:

$$\mathrm{IMBI} = \frac{\sum_{i=1}^{n} C_i / C_{imax}}{n} \quad (1)$$

with n the total number of metal(loid)s of each site, C_i the individual concentration of metal(loid) i, C_{imax} the maximal observed concentration of metal(loid) i and IMBI ranging from 0 to 1.

Statistical analysis

Statistical analyses were carried out with the software Past, version 1.93. For analysis of comet assay results and C_i/C_{imax} distributions, we performed a Kruskal-Wallis or one-way analysis of variance (ANOVA), depending on the normality test results. The parametric Pearson's correlation coefficient (r) was used to analyze correlations between TM and IMBI each versus percentage of survivors.

Results

Relevant physical-chemical parameters of water are given in Table I. In January the temperature is in a limited range (12.7–14.1°C) in both rivers; in May, in the Amaseno River the maximum temperature was found at site M56 and in both rivers the range is more extended (13.6–24.1°C). The highest conductivity values are at the downstream sites M55 and M56, while the lowest is in spring at ONI. Also, the pH values are lower in the reference sites and highest in those closest to the mouth of the rivers.

In terms of sampling *in situ*, only in two sites (one for Amaseno River in January), in addition to the reference ones, did we find sufficient specimens of *E. veneris* for the programmed assessment of genotoxicity and metal bioaccumulation. For the exposure *in situ*, after 15 days of caging, the gammarid percentage survival remained high (> 50%), except at the M55, M56 and ONI sites; in any case, sufficient individuals were found.

Figure 2 shows the level of DNA damage in the *E. veneris* haemocytes resulting from the sampling *in situ* experiment. In the 2 months, individuals at the upstream site CDA on Amaseno River exhibited a degree of DNA damage higher than the basal one

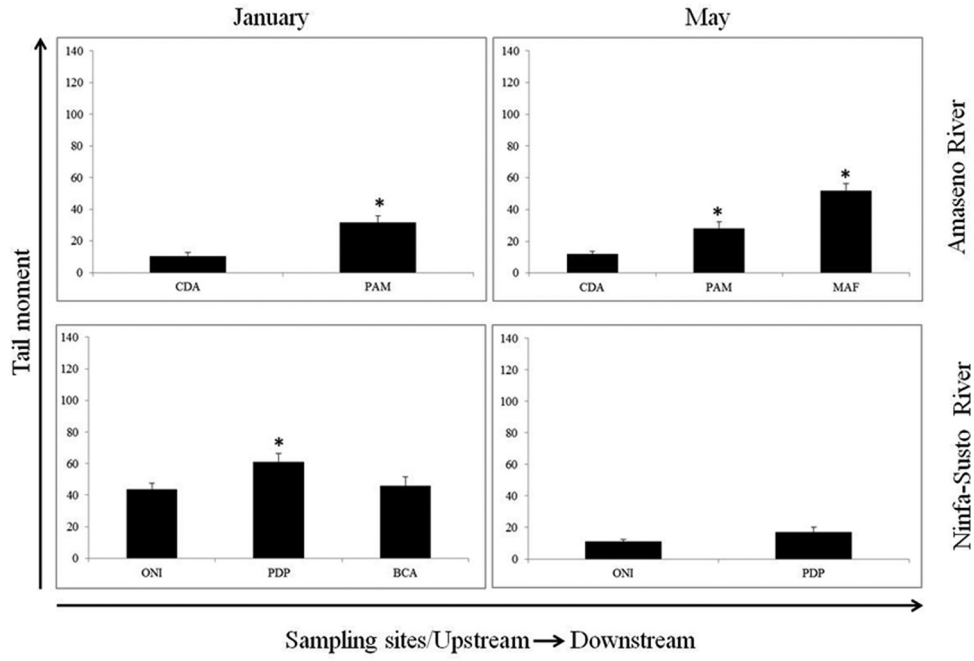

Figure 2. DNA damage in haemocytes from gammarids sampled for the sampling *in situ* strategy. Each block is the mean of tail moment values ± standard error (error bars). * Significantly different from reference site ($p < 0.05$).

found in previous experiments (data not shown) or in those individuals exposed *in situ* at the CDA site in the present study. However, values significantly higher were found in individuals collected from the sites PAM and MAF. In addition, an increase in the DNA damage from upstream to downstream along the river was also evident. In the Ninfa-Sisto River, ONI shows different and variable values of TM in the two considered seasons (January: 43; May: 11); the amount of DNA damage found at this site is not significantly different from that found in individuals collected at the other sites.

In the active approach, the completeness of the analysis relative to all of the sites defined should be highlighted. Figure 3 shows the level of DNA damage in the haemocytes of individuals considered for the exposure *in situ* experiment. In both months considered, individuals caged at the upstream sites, CDA and ONI, always exhibited low levels with low variability of DNA damage in the haemocytes. Our analyses unveiled a general pattern showing an increase of genotoxic damage from upstream to downstream in both rivers, with the lowest value in spring and the highest value in proximity of the river mouths, and with intermediate level for sites in between. The values of TM were found to be highest in the downstream site M55, both in January (125) and in May (54).

Metal bioaccumulation results, expressed in mg/kg of sample, are reported in Table II. We analyzed 14 different metal(loid)s (Al, As, Ba, B, Cd, Cr, Fe, Mn, Hg, Mo, Ni, Pb, Zn, V) for both exposure and sampling *in situ* monitoring of the two rivers in May. We found Hg under the detection limit (1 mg/kg). By contrast, Cd is present only in sampling *in situ* monitoring. Finally, Pb was not detectable in the tissues of the individuals caged along the Amaseno River. Also, Table II reports the IMBI values in the last column. In the sampling *in situ* approach, the lowest IMBI values were found in reference sites CDA and ONI, while in exposure *in situ* the distribution of the IMBI values does not differ significantly among sites. The significantly higher value of M55 compared to that of CDA ($p < 0.05$) is the only exception to this otherwise generalized pattern.

In order to emphasize the single contribution of each metal to the IMBI, we graphed the distributions of values of C_i/C_{imax} (Figure 4). A statistically significant upstream–downstream trend emerges along the Amaseno River associated with a low variance of the values considered in the sampling *in situ*. By contrast, after exposure *in situ* the obtained C_i/C_{imax} distributions do not increase from upstream to downstream and remain similar and with a wide variance along both rivers.

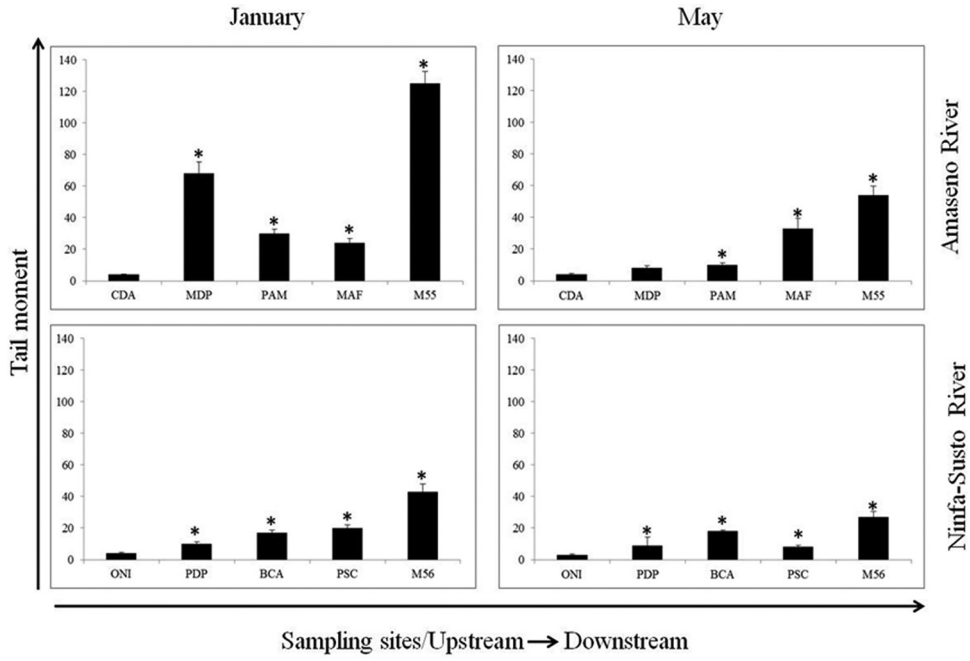

Figure 3. DNA damage in haemocytes from gammarids caged for the exposure *in situ* strategy. Each block is the mean tail moment values ± standard error (error bars). *Significantly different from unpolluted reference site ($p < 0.05$).

Table II. Concentration (C_i in mg/kg dry weight) values of each metal measured in gammarids tissues and respective IMBI values (last column) in each site of the Amaseno and Ninfa-Sisto rivers. The bold values are the C_{imax} considered. ND: not detected.

River	Sites														IMBI	
		Al	**As**	**Ba**	**B**	**Cd**	**Cr**	**Fe**	**Mn**	**Mo**	**Ni**	**Pb**	**Zn**	**V**		
Amaseno	**CDA**	25.2	0.6	0.6	15.6	**0.1**	1.4	1.8	57.6	0.3	0.7	1.3	14.6	1.9	0.41	**Sampling *in situ***
	PAM	**64.9**	0.4	0.9	**38.5**	**0.1**	3	25.6	85.3	**2.5**	3.1	4.6	**31.4**	**3.7**	0.78	
	MAF	46.3	**0.7**	**1.9**	16.9	**0.1**	**3.3**	**88.6**	**126.3**	1.4	**4.1**	**6.1**	25.4	2.9	0.86	
Ninfa-Sisto	**ONI**	12.4	**0.2**	0.1	2.5	**0.3**	**2.6**	0.8	0.1	ND	0.3	**0.9**	2.1	1.8	0.72	
	PDP	**12.5**	0.1	**0.5**	**8.2**	0.15	2.5	0.8	**23.5**	ND	**0.5**	0.8	**3.5**	**2**	0.90	
Amaseno	**CDA**	12	0.5	12.3	54.2	ND	2.6	253.1	102.5	4.1	**3.1**	ND	25.6	6.3	0.62	**Exposure *in situ***
	MDP	21.5	**0.7**	23.7	10.2	ND	2.4	125	28	**8.2**	1.3	ND	27.1	7.2	0.57	
	PAM	15.6	0.6	**24**	25.6	ND	**4.2**	254.2	106	2.5	2.2	ND	12.6	4.6	0.59	
	MAF	22.3	0.2	ND	38.2	ND	1.3	263.1	51	3.5	ND	ND	**41.3**	**8.4**	0.53	
	M55	**254**	0.5	13.5	**74.2**	ND	1.5	**284**	**236**	6.1	1	ND	28.6	5.4	0.74	
Ninfa-Sisto	**ONI**	22.3	0.4	**32.5**	36.5	ND	4.6	**264**	29	2.5	1.8	1.2	36.1	9.1	0.61	
	PDP	**51.3**	0.3	12.6	13.2	ND	5.6	198	36	2.8	3	1.5	**42.3**	**9.5**	0.61	
	BCA	45.6	0.5	24	18.4	ND	**8.1**	231	**84**	2.9	2.2	**4.2**	23.6	2.6	0.69	
	PSC	15.9	0.7	12	36.4	ND	4.6	144	56	3.6	**6.6**	2.2	28.4	4.6	0.61	
	M56	16.8	**0.8**	24.5	**84.6**	ND	5.2	111	76	**4.2**	1.5	2.3	27.3	6.5	0.68	

To assess whether the survival of *E. veneris* specimens was related to genotoxic potential or metal contamination of waters of exposure/sampling sites, we calculated the linear correlation between those parameters. No linear correlations between IMBI and percentage of survivors in both rivers and in both months considered were found. Instead, there is a single linear correlation between TM and percentage of survivors in January along the Amaseno River ($r = -0.9$; $p < 0.05$).

Discussion

The aim of this work was to evaluate the bioaccumulation of some metal(loid)s (biomarker of exposure) and the level of DNA damage (biomarker of effect) (Peakall & Shugart 1993) in the species *Echinogammarus veneris* in two rivers of Latium (Central Italy). To this end, we used two different biomonitoring strategies: (a) active, achieved by transplanting and caging individuals from a reference

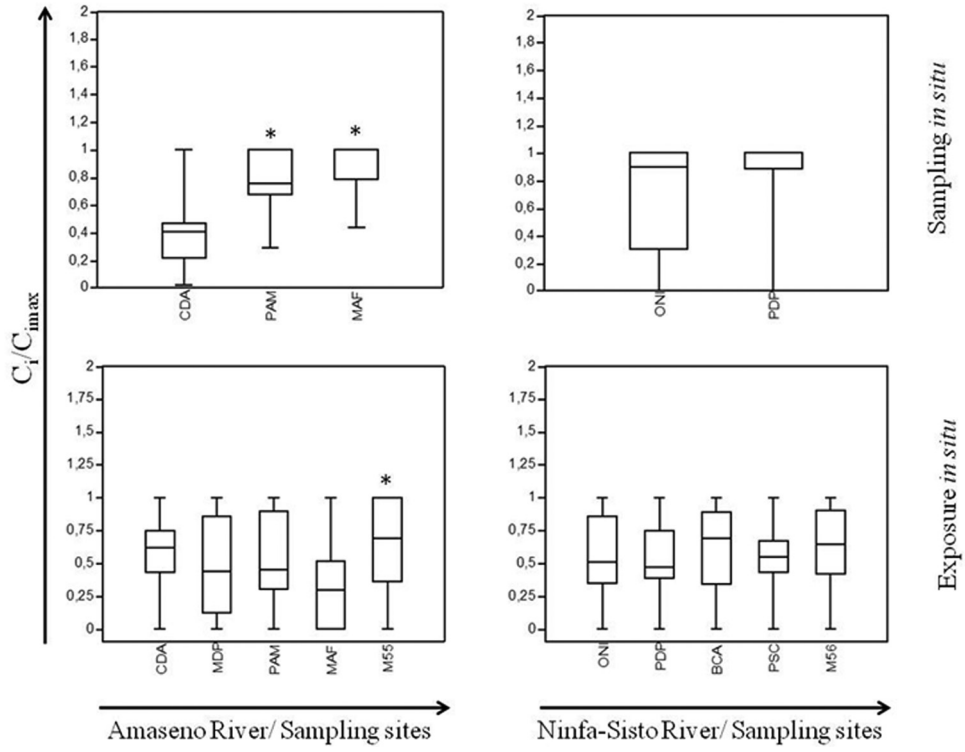

Figure 4. Boxplot of C_i/C_{imax} values (> 0 and ≤ 1) for sampling sites along the Ninfa-Sisto and Amaseno rivers. *Significantly different from unpolluted reference site. For each site, the 25–75% quartiles are drawn using a box. The median is shown as a horizontal line inside the box. The minimal and maximal values are shown with short horizontal lines.

site (Andral et al. 2004) into analysis sites; and (b) passive, relying on indigenous organisms (De Kock & Kramer 1994). The selected species offers good solutions because is found at high density, while its small size makes it possible to use easy-to-handle caging systems in active biomonitoring.

The passive approach, here called sampling *in situ*, was first introduced in 1976 with the aim of monitoring marine waters during the so-called "Mussel Watch" program (Goldberg 1975; Borja et al. 2008); in fresh water it was introduced in 1993 by the United States Geological Survey (USGS). Even if passive approaches have proved useful for monitoring contamination trends for metals and several organic contaminants, they are recognized as suffering from two major drawbacks: they depend on the effective presence of the native organism at the sampling sites, and on several factors such as variability in the exposure time, age and size of sampled organisms; all of these variables may hinder an accurate interpretation of the results (Besse et al. 2012).

Active approaches, here called exposure *in situ*, based on transplanted organisms, have been developed more recently with the aim of overcoming these limitations. They can minimize biological variability by using organisms collected from the same population, and they make it possible to control exposure time fully (Bervoets et al. 2005).

The results from the sampling *in situ* approach show that the species *E. veneris* was sampled in both rivers, but its presence was detected only at the upstream sites and never in downstream sites in both of the considered seasons (winter, spring). In rivers, the nature of the substrate and the water flow speed are important physical components that determine the distribution of macroinvertebrates in surface freshwater bodies (Siligardi et al. 2007). In particular, the decrease of riverbed slope in a flat area reduces the current speed in the potamal part of a river, creating conditions not conducive to the presence of our target species. These characteristics may also vary seasonally, explaining the finding of individuals in MAF in spring and not in winter (Amaseno River) and in BCA in winter and not in spring (Ninfa-Sisto River). Furthermore, the downstream sites of the two rivers have undergone severe human interventions such as earthworks, dams, concrete embankments, rectifications of the riverbed, and reduction and alteration of riparian vegetation and irrigation levies (Zerunian & Leone 1996; Mancini & Arcà 2000). Human pressure reduces the possibility of settlement by aquatic fauna,

decreases the self-purification capacity of the river and facilitates riverbank erosion, and the final result is significant biodiversity depletion in the downstream sites of the two rivers. From comet assay results, the DNA damage found in individuals from reference sites (CDA, ONI) is high and variable in the two seasons considered in the sampling *in situ* strategy: such a pattern does not allow discriminating effectively from those found at the other sites. Instead, the low variability in the distribution of the data relating to the metal(loid) bioaccumulation (Figure 4) suggests that sampling *in situ* is suitable for this analysis. In fact, the presence of the bioindicator organisms within the contaminated environments allows us to highlight even small amounts of accumulated toxic substances, since the bioaccumulation is a phenomenon influenced not only by a dose-response trend, but also by a time-response trend (Gobas et al. 1995; Shuhaimi-Othman & Pascoe 2007). The presence of Cd in the individuals collected in the sampling *in situ* only, although at a very low dose, supports this hypothesis.

Nevertheless, the issue remains of the availability of animals at all sites intended for the study. The absence of *E. veneris* in the downstream sites prompted the application of active biomonitoring (exposure *in situ*), which is based on the comparison of chemical and/or biological properties of samples that have been collected from one population and that have, after randomization and translocation, been exposed to different environmental conditions at monitoring sites (Wepener et al. 2005). This reduces the variability of results and thus increases the robustness and reproducibility of the method. Furthermore, the use of exposure *in situ* has proven in the past to be a winning strategy for the assessment of water quality (Gerhardt 2007), but it has rarely involved usage of the family Gammaridae (Lacaze et al. 2011) as proposed in the current study. Another advantage of this approach is the ability to control the effective time of exposure. The choice of the 15 days of exposure *in situ* along the two rivers follows what was demonstrated in previous studies which used amphipods species to evaluate DNA damage and tissue bioaccumulation after 7 or 15 days of exposure, in laboratory conditions or by "in cage" methods (Lacaze et al. 2011; Besse et al. 2013; Lebrun et al. 2015; Ronci et al. 2015). The use of animals kept in conditions of low impact for an appropriate period of time (acclimation in the laboratory) has given us the opportunity to start the exposure experiments with very low levels of basal stress. The physiological responses of the animals are thus more sensitive to genotoxic substances and with a wider range of response, such as to react to even low impacts due to substances very poorly represented in the aquatic mixture (Klobucar et al. 2003; Wepener et al. 2005). Our results show that the exposure *in situ* allows retrieving a full panel for the comet assay's results. In almost all sites, there is no correlation between DNA damage and survival rate: this implies that xenobiotics involved in the biological response do not cause the gammarids' death. Moreover, even when a linear relationship between amount of DNA damage and survival rate was found (the only case is in Amaseno River in January: $r = -0.9$; $p < 0.05$), nonetheless the number of surviving individuals was sufficient to complete the test. The survival of exposed animals is never correlated with IMBI values. It is evident that the mortality observed at some sites is due to the concomitant exposure to substances that taken alone would not meet the lethal dose (DL_{50}; xenobiotics present in the rivers in previous years occasionally exceeded the law limits; ARPA Lazio, data not shown). However, the synergistic effect leads to a mortality of 92% of the gammarids exposed (M56 in January). Nonetheless, according to Italian legislation (Legislative Decree 31/01), these waters could potentially be potable. Our results show that organisms' exposure to waters containing a mixture of toxic substances is still able to generate detectable DNA damage, allowing us, thus, to quantitatively evaluate the extent of the impact (Raiaguru et al. 2003; Wepener et al. 2005; Osman et al. 2012; Ronci et al. 2015). In fact, in both rivers, TM values of haemocytes were statistically significant when compared to the respective reference sites, showing how exposure to waters resulted in a genotoxic insult. In particular, the highest values of DNA damage recorded in M55, M56 and MDP could be related to the activities of intensive cultivation in the river surroundings (Mancini & Arcà 2000). The lowest values of DNA damage, observed in CDA and ONI, were due to a substantial absence of anthropogenic impacts in the two areas (Regione Lazio 2004a, 2004b). Regarding the bioaccumulation data obtained from exposure *in situ* (Figure 4), they show a flat trend and a very wide variability due to the different speed of uptake of the different metals and the exposure time (Mouneyrac et al. 2002; Santoro et al. 2009; Chojnacka 2010).

The information generated here will contribute to assessing the possibility of using *Echinogammarus veneris* as a sentinel organism for bio-monitoring

and to describe the temporal trends of contamination in the different environmental compartments of aquatic ecosystems, according to the Water Framework Directive (WFD 2000/60).

Conclusions

We have successfully used the crustacean gammarid *Echinogammarus veneris*, which plays a key role in freshwater ecosystems, to evaluate DNA damage in haemocytes and tissue bioaccumulation. These biomarkers allow us to estimate, to predict and to prevent events unacceptable in an ecological context. Also, tests such as comet assay and tissue bioaccumulation are easy to perform, economical and explanatory.

The comparison between the results obtained combining different methods (comet assay and bioaccumulation) and different strategies of freshwater monitoring (sampling *in situ* and exposure *in situ*) showed that the active biomonitoring system could be best suitable to study the effects (genotoxicity) of exposure to agents as toxic metals and other substances present in fresh waters. The passive biomonitoring could best be exploited to highlight phenomena that lead to bioaccumulation rather than being used as a biomarker of exposure.

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