

Interspecies variation in DNA damage induced by pollution

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Abstract The choice of a suitable species to translate pollution signals into a quantitative monitor is a fundamental step in bio-monitoring plans. Here we present the results of three years of biomonitoring at a new coal power plant in central Italy using three different aquatic and terrestrial wildlife species in order to compare their reliability as sentinel organisms for genotoxicity. The comet assay was applied to the common land snail *Helix spp.*, the lagoon fish *Aphanius fasciatus*, and the green frog *Rana esculenta* sampled in the area potentially exposed to the impact of the power station. The tissue concentration of some expected pollutants (As, Cd, Ni, Pb, Cr) was analysed in parallel samples collected in the same sampling sites. The three species showed different values in the comet assay (Tail Intensity) and different accumulation profiles of heavy metals. *Aphanius fasciatus* showed an increasing genotoxic effect over time that paralleled the temporal increase of the heavy metals, especially arsenic, and the highest correlation between heavy metals and DNA damage. *Helix spp.* showed levels of damage inversely related to the distance from the source of pollution and in partial accordance with the total accumulation of trace elements. On the contrary, *Rana esculenta* showed a low capability to accumulate metals and had inconsistent results in the comet test. The fish appeared to be the most efficient and sensitive species in detecting chemical pollution. Overall, both the fish and the snail reflected a trend of increasing pollution in the area surrounding the power plant across time and space [Current Zoology 60 (2): 308–321, 2014].

Keywords Ecotoxicology, Genotoxicity, Wildlife, Coal power plants, Comet assay, Sentinel organism

Wildlife sentinels are regarded as those populations that can react to environmental contaminants before they impact human health and ecosystems without significant adverse effects (Stahl, 1997; Van der Schalie et al., 1999; Rabinowitz et al., 2005; Carere et al., 2010). A crucial issue is the selection of the species to monitor. This can be achieved by adopting a number of criteria: (i) a simple relationship between the source and tissue concentrations of the pollutant(s); (ii) its consistency across sampling sites and years; (iii) relative insensitivity to the pollutant(s); (iv) being abundant and easy to sample and to age; (v) a good knowledge about its ecology and physiology; (vi) being sedentary or having a limited home range (Martin and Coughrey, 1982; Philips and Segar, 1986; Philips and Rainbow, 1993; Beeby, 2001).

Many physical and chemicals contaminants can cause structural and functional changes in the molecular compounds of living cells. DNA is an important target of environmental stress due to persistent organic pollutants in both aquatic and terrestrial organisms (Frenzilli et al., 2001). The contaminants can create DNA lesions, including strand-breaks, modified bases, DNA-DNA and

DNA-protein crosslinks (Eastman et al., 1992). DNA strand-breaks (Shugart, 1990; Balpaeme et al., 1996) are a common modification that may be induced by a wide range of agents and mechanisms (Nacci and Nelson, 1992; Mitchelmore et al., 1998). Therefore, DNA damage has been proposed as a biomarker for assessing the genotoxic properties of environmental contaminants in biomonitoring studies (Everaats et al., 1998; Felder et al., 1998; Theodorakis and Shugart, 1998; Xu et al., 1999).

Among the various techniques used to assess the genotoxicity of environmental pollutants, the single cell gel electrophoresis (SCGE), or comet assay, has advantages that make it ideal for use in sentinel organisms (Mitchelmore and Chipman, 1998; Rojas et al., 1999). This technique can detect early signs of exposure to genotoxicants and show primary DNA damage as single or double strand breaks, alkali-labile sites and crosslinks in eukaryotic cells (*in vivo* and *in vitro*) in different tissues (Singh et al., 1988; Tice et al., 2000; Reinecke and Reinecke, 2004; Bonisoli-Alquati et al., 2010; Mosesso et al., 2012; Dallas et al., 2013). Many studies used the comet assay to determine DNA

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strand-breaks in aquatic organisms (Frenzilli et al., 2009; Lee and Steinert, 2003), while there are relatively few studies regarding the terrestrial environment (Table 1). The assay has been used under laboratory conditions, both *in vivo* and *in vitro*, for a variety of genotoxic agents and in organisms collected at polluted sites. In most studies, DNA damage has been linked to exposure to a wide spectrum of contaminants (polycyclic aromatic hydrocarbons, pesticides and heavy metals), standard genotoxicants (hydrogen peroxide, methyl methane sulfone, benzopyrene), cytotoxic compounds and physical agents like radioactivity and x-rays (for reviews see Lee and Steinert, 2003; Frenzilli et al., 2009; Vasseur and Bonnard, 2014; Bonisoli-Alquati, 2014).

In this study, the comet assay was used to assess DNA damage potentially caused by heavy metals, one

Table 1 Overview of aquatic and terrestrial species in which the comet assay was used

Organism	Reference
Aquatic invertebrates	
<i>Dreissena polymorpha</i>	Bolognesi et al., 2004
<i>Mytilus galloprovincialis</i>	Perez-Cadahia et al., 2004
	Laffon et al., 2006
<i>Mytilus edulis</i>	Hagger et al., 2005
	Rank et al., 2005
	Emmanouil et al., 2007
<i>Limnoperna fortunei</i>	Vilella et al., 2007; 2006
Aquatic vertebrates	
<i>Danio rerio</i>	Kosmehl et al., 2008
<i>Anguilla anguilla</i>	Regoli et al., 2003
<i>Cyprinus carpio</i>	Buschini et al., 2004
<i>Carassius auratus</i>	Rajaguru et al., 2003
<i>Octopus vulgaris</i>	Deguchi et al., 2007
<i>Rana clamitans</i>	Raimundo et al., 2010
<i>Rana pipens</i>	Ralph et al., 1996
<i>Xenopus laevis</i>	Ralph and Petras 1997
<i>Xenopus laevis</i>	Mouchet et al., 2006
<i>Bufo raddei</i>	Huang et al., 2007
<i>Tursiops truncatus</i>	Betti and Nigro, 1996
Terrestrial invertebrates	
<i>Eisenia foetida</i>	Reinecke and Reinecke, 2003
<i>Aporrectodea longa</i>	Rajaguru et al., 2003
	Martin et al., 2005
<i>Lumbricus rebus</i>	Button et al., 2010
<i>Helix spp.</i>	Angeletti et al., 2013
	Regoli et al., 2006; 2005
Terrestrial vertebrates	
<i>Hirundo rustica</i>	Bonisoli-Alquati et al., 2010
<i>Mus musculus</i>	Leon et al., 2007
<i>Rattus rattus</i>	Leon et al., 2007

of the most widespread form of environmental chemical contamination. Significant correlations have been found between DNA damage and the presence of heavy metals (Matsumoto et al., 2006; Hengstler et al., 2003; Cestari et al., 2004; Reinecke and Reinecke, 2004) in different sentinel organisms such as earthworms, mussels and fish. However, to our knowledge there are no analyses focused on comparing the response of different organisms to contaminants and simultaneously evaluating their relative resolving power

We aimed at evaluating three different wildlife sentinels representative of different habitats, both terrestrial and aquatic, in the area potentially exposed to the impact of a power station reconverted from oil to a coal-fired plant in 2010: the killifish, the common land snail and the green frog. The Mediterranean killifish *Aphanius fasciatus* (Valenciennes, 1821) is a cyprinodont living in coastal brackish waters, lagoons and salt marshes distributed over the central and Mediterranean coastal zones (Tortonese, 1986; Fischer et al., 1987; Cavarero et al., 2013). It is a well-known species in life history ecology and its entire life cycle is spent in these habitats with a low potential for dispersion (Maltagliati, 1998; Cavarero et al., 2013). The species is euricic by also being present in the extreme environmental conditions typical of these habitats, and it has already been evaluated as a potential sentinel to detect complex genotoxic mixtures in coastal lagoon ecosystems (Mosesso et al., 2012). In this study, samples collected in a significantly polluted site showed significant increases of DNA damage compared to samples of unpolluted sites.

The gastropods *Helix spp.* have the capability to accumulate different classes of chemicals and are considered suitable species for monitoring trace metals, agrochemicals, urban pollution and electromagnetic exposure (Berger and Dallinger, 1993; Gomot de Vaufléury and Pihan, 2000; Snyman et al., 2000; Beeby and Richmond, 2002, 2003; Viard et al., 2004; Regoli et al., 2005). Contact with a pollutant can occur through three routes: ingestion of soil and vegetation, contact with the soil and inhalation of air. Therefore, these species are potential sentinels of the bioavailability of contaminants in both soil and air. Due to the type of habitat, their low mobility, and their feeding behaviour, snails can provide information on the quality of the environment and are used in campaigns for environmental biomonitoring (Regoli et al., 2006; Leffa et al., 2010; Angeletti et al., 2013).

Rana esculenta complex (Linnaeus 1758) is formed

by the two parental species *Rana lessonae* and *Rana ridibundae* and by the hybrid *Rana esculenta* (Santucci et al., 1996; 2000). Amphibians may be useful for assessing the presence of toxic wastes in water and soil or sediment (Boone and Bridges, 2003). Most of the respiratory exchange occurs through the skin, so they can easily absorb environmental pollutants of their habitats. They have also a poor vagility, which makes them confined to the environment in which they live. Anurans were used to observe their sensitivity to pollution by heavy metals (Lefcort et al., 1998; Leontyeva et al., 1997), by nitrogenous substances (Oldham et al., 1997), and pesticides (Schuytema et al., 1991, 1993) and to monitor areas with intensive agriculture or heavy industrial activity (Ralph et al., 1997). In particular, the frogs of the *R. esculenta* complex have been used as biosensor (Kiseleva, 1997) or as bioindicators where high levels of DNA damage in erythrocytes of natural frog populations can be accounted by the presence of harmful compounds in waste dumping (Maselli et al., 2010).

We performed the primary DNA damage analysis through the comet assay and analysed the concentration of five heavy metals (As, Cd, Ni, Pb and Cr), as some of the expected pollutants emitted by the power station, in the tissues of these three species. The samples were collected in potentially contaminated sites and in control sites across two or three years.

1 Materials and Methods

1.1 Study area and sampling

Sampling was carried out in the area surrounding the ENEL power station “Torrevaldaliga Nord” (Civitavecchia, Central Italy) that was reconverted from an oil to a coal-fired plant in 2010. The area is potentially under different degrees of pollution due to the presence of different genotoxic compounds, which include heavy metals released by the new plant.

Different locations at different distance from the pollu-

tion source were selected for sampling across three years (2010–2012) for *A. fasciatus* (two locations), and two years (2011, 2012) for *Helix spp.* (five locations) and *R. esculenta* (three locations) (Table 2). Seven locations were selected with some of them coinciding across species (Fig. 1): a) Tarquinia Roccaccia Fontanil della Torre, a rural farmland of nonintensive agriculture; b) Natural Reserve “Saline of Tarquinia” (southern and northern ponds for *A. fasciatus*) located on the Tyrrhenian coast of central Italy was originally a site for salt work in which salt production was terminated in 1997. The basins are in connection only with the sea and are isolated from the inland by a canal which runs all around the area.; c), d) and e) three sites located in Civitavecchia in close proximity to the power station; f) Tolfa mountains.

Aphanius fasciatus were caught with appropriate nets. After capture, specimens were maintained alive in oxygenated recipient water and transported to the laboratory within 2–4 hours, until processed for experimental purposes. The snails were transported to the laboratory in a plastic box of the sort normally used for food and submitted for analysis on the same or the following day. *R. esculenta* individuals were captured by hand or with fishing nets in the evening hours. After capture, they were transported to the laboratory, housed in a plastic box and submitted for analysis the following day. Further details on the description of the sites and sampling procedure for *A. fasciatus* and *Helix spp.* are reported elsewhere (Angeletti et al., 2010; Angeletti et al., 2013; Mosesso et al., 2012).

1.2 Comet assay

The alkaline comet assay for *A. fasciatus* and *Helix spp.* was performed according to the procedures described for these species in Mosesso et al. (2012) and Angeletti et al. (2013). Briefly, the fish were sacrificed by a blow to the head and pericardial blood was collected with the aid of micropipettes, and in the snails, hemo-

Table 2 Sample size of each species for each year and sampling location.

	<i>Aphanius fasciatus</i>						<i>Helix spp.</i>				<i>Rana esculenta</i>			
	Heavy Metals			Comet Assay			Heavy Metals		Comet Assay		Heavy Metals		Comet Assay	
	2010	2011	2012	2010	2011	2012	2011	2012	2011	2012	2011	2012	2011	2012
a	-	-	-	-	-	-	10	10	9	6	10	10	5	5
b	40	20	20	10	15	16	10	10	12	6	-	-	-	-
c	-	-	-	-	-	-	10	10	5	6	-	-	-	-
d	-	-	-	-	-	-	10	10	5	6	-	-	-	-
e	-	-	-	-	-	-	10	10	5	6	10	10	8	5
f	-	-	-	-	-	-	-	-	-	-	10	10	5	5

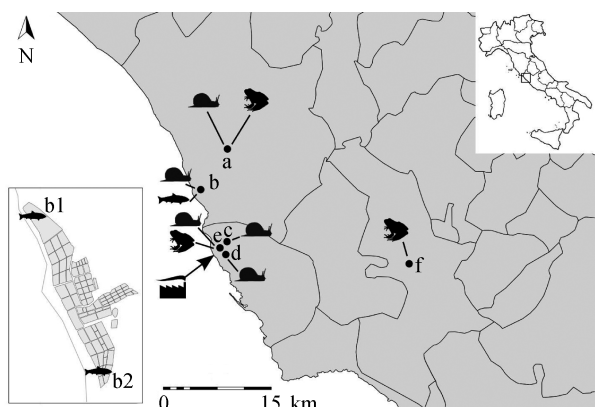


Fig. 1 Sampling sites for *A. fasciatus* (two locations), *Helix* spp (five locations) and *R. esculenta* (three locations). Distances (Km) of each site from the power plant are: (a) 20.2; (b) 9; (c) 1.48; (d) 1.24; (e) 0.27; (f) 13.9.

lymph was collected after a small hole was created on the shell at the hemocoele level (Regoli et al., 2006; Leffa et al., 2010). For both species, the times of unwinding of DNA and electrophoresis were standardized respectively at 10 min and 10 min. For *R. esculenta* complex, the test was carried out on the circulating blood taken with a hypodermic syringe, after the removal of the phalanx. About 1–2 μ l blood were collected from each individual and diluted with 10 μ l of PBS.

The alkaline comet assay was performed according to the method of Singh et al. (1988) and Mustafa et al. (2011) with some modifications necessary for the species *R. esculenta* concerning the times of unwinding DNA and electrophoresis that were standardized respectively to 30 min and 30 min. DNA damage was expressed as % Tail DNA (hereafter Tail Intensity, TI), which is considered the most reliable parameter (Kumaravel and Jha, 2006).

1.3 Heavy metals

In parallel to the primary DNA damage analysis, the chemical analysis of five heavy metals has been performed (As, Cd, Ni, Pb, and Cr). The samples (whole organism for *A. fasciatus*; soft tissue for *Helix* spp.; muscle tissue for *R. esculenta*) were thoroughly homogenized with suitable equipment after removing the parts not considered in the analysis. The samples were analysed by atomic absorption spectrophotometer. Briefly, the tissues were homogenized and 0.5 g were weighed with an analytical scale in 50 ml plastic centrifuge tube. Ten-milliliter hyperpure nitric acid (65%) was then added. Subsequently, the solution was boiled for 30 min under chemical hood. After cooling at ambient temperature, the acid solution was put in a 50 ml

flask and brought to volume with demineralized hyperpure water for trace analysis. Control trials were carried out with certified material and samples fortified with the target analytes were prepared. The mineral solution was finally analysed by atomic absorption spectrophotometry. The tissue concentrations are expressed as mg/kg fresh weight.

1.4 Data analysis

The data of both TI and heavy metal concentrations (hereafter HM) were successfully log-transformed to meet the assumptions of homoscedasticity for parametric analysis. We then ran two separate ANOVAs to test the effect of species (three levels) on the variables TI (comet assay) and total HM concentration. Due to the strong unbalance in the data, which made it impossible to run a single model including all factors, we ran three separate ANOVAs (one for each species) with year of sampling and sampling location as between-subject factors on these two variables. Fisher's PLSD post hoc was applied for multiple comparisons. Pearson correlation coefficient and linear regression with total HM concentration as the predictor variable were used to test the strength of association between HM and TI in each species and its sensitivity (regression slope). The differences between species were then analysed with a correlation difference analysis (Z-test, a test for comparing independent correlations based on correlation coefficients and sample sizes, using the Fisher r-to-z transformation, Cohen and Cohen, 1983). The correlational analysis was computed on the two parallel datasets nested per site and sampling year. The tests were two-tailed with an α -level = 0.05. The analyses were performed using Statview II (Abacus Concepts, CA, USA).

2 Results

2.1 Comet assay

The sample size for each species across years and sampling sites is reported in Table 2. A highly significant effect of species emerged for TI: *A. fasciatus* showed the highest values followed by *R. esculenta* and *Helix* spp. ($F_{2,130} = 27.8$, $P < 0.0001$; $0.0001 < P < 0.016$ in post hocs, Fig. 2A).

In *A. fasciatus*, TI differed across years, with lower values in 2011, while it always had higher values in the southern locations (year: $F_{2,35} = 5.2$, $P = 0.01$; post hocs 2011 vs 2012, $P = 0.0004$; sampling site: $F_{1,35} = 20.91$; $F < 0.0001$, Fig. 3A). The interaction between year and sampling site was borderline significant ($F_{2,35} = 2.7$; $P = 0.08$).

In *Helix spp.* TI differed across the different locations ($F_{4,56} = 3.5$, $P = 0.012$; a vs d and e; b vs d and e; c vs d and e, $0.004 < P < 0.04$ in post hocs Fig. 4A). The interaction between year and sampling site was not significant ($F_{4,56} = 0.66$; $P = 0.60$).

In *R. esculenta* TI differed between the two years with the highest values in 2012 ($F_{1,20} = 45.3$, $F < 0.0001$), and across sampling location ($F_{2,20} = 4.8$, $P = 0.02$; e vs f, $P = 0.007$; a vs f, $P = 0.04$ in post hocs, Fig. 5A). The interaction between year and sampling site was not significant ($F_{2,20} = 1.2$, $P = 0.30$).

2.2 Heavy metals

The descriptive statistics of the single pollutants for each species are shown in the Appendix (Tables A1, A2, A3). Here we report the statistical outcomes for the total HM concentration in the three species considered: *A. fasciatus* showed the highest values followed by *Helix spp.* and *R. esculenta* ($F_{2,237} = 21.9$ and $P < 0.0001$; $ps < 0.0006$ in post hocs Fig. 2B).

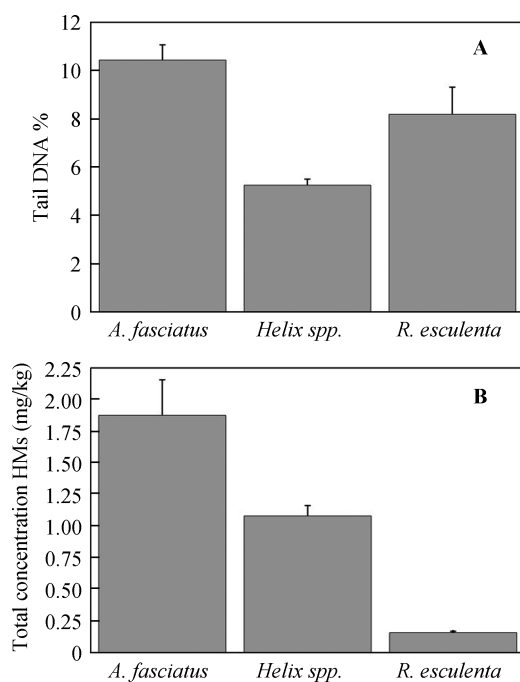


Fig. 2 (A) Tail Intensity in *A. fasciatus* ($n = 41$), *Helix spp.* ($n=66$) and *R. esculenta* ($n = 33$). Values are mean \pm standard error of % Tail DNA. Fisher's PLSD: *A. fasciatus* vs *Helix spp.* $P < 0.0001$; *A. fasciatus* vs *R. esculenta* $P = 0.0004$; *Helix spp.* vs *R. esculenta* $P = 0.016$. (B) Total concentration of heavy metals in *A. fasciatus* ($n = 80$), *Helix spp.* ($n = 100$) and *R. esculenta* ($n = 60$). Values are mean \pm standard error of total concentration (mg/Kg). Fisher's PLSD: *A. fasciatus* vs *Helix spp.* $P = 0.0006$; *A. fasciatus* vs *R. esculenta* $P < 0.0001$; *Helix spp.* vs *R. esculenta* $P = 0.0002$.

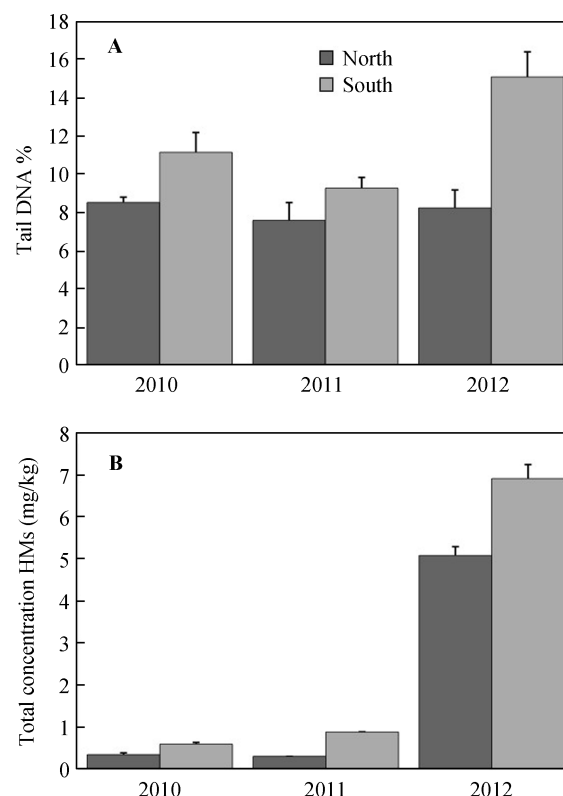


Fig. 3 (A) Tail Intensity across years and sampling sites for *A. fasciatus*. Values are mean \pm standard error of % Tail DNA. Fisher's PLSD: North vs South $P < 0.0001$; 2011 vs 2012 $P = 0.0004$. (B) Total concentration of heavy metals across years and sampling sites for *A. fasciatus*. Values are mean \pm standard error of total concentration (mg/Kg). Fisher's PLSD: North vs South $P < 0.0001$; 2010 vs 2012 $P < 0.0001$; 2011 vs 2012 $P < 0.0001$.

In *A. fasciatus* the total concentration of HM markedly differed between the two years of sampling and between the two locations with the highest values in 2012 ($F_{2,73} = 1106.1$, $P < 0.0001$; 2010 vs 2012 and 2011 vs 2012 $ps < 0.0001$ in post hocs) and in the southern locations ($F_{1,73} = 67.5$, $P < 0.0001$; $ps < 0.0001$ in post hocs, Fig. 3B). The interaction between site and year was also highly significant; the highest difference between northern and southern location was observed in 2012 ($F_{2,73} = 20.4$, $P < 0.0001$).

In *Helix spp.*, the total concentration of HM differed between the two years with the highest values in 2011 ($F_{1,90} = 68.04$, $P < 0.0001$; 2011 vs 2012, $P < 0.0001$ in post hoc) and across sampling sites ($F_{4,90} = 26.5$; $P < 0.0001$; a vs b, c, d, e; b vs c, d, e, $Ps < 0.0004$ in post hocs, Fig. 4B). The interaction between these two factors was also significant: the main contribution was given by sites c and d, in which HM concentrations were more than three times lower in 2012 compared to 2011 ($F_{4,90} = 25.8$, $P < 0.0001$).

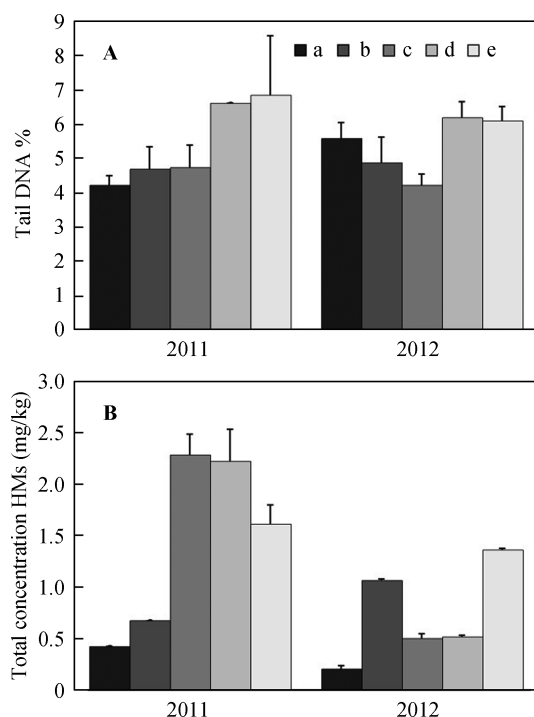


Fig. 4 (A) Tail Intensity across year and sampling site for *Helix spp.* Values are mean \pm standard error of % Tail DNA. Fisher's PLSD: a vs d $P = 0.015$; a vs e $P = 0.04$; b vs d $P = 0.0004$; b vs e $P = 0.013$; c vs d $P = 0.007$; c vs e $P = 0.02$. (B) Total concentration of heavy metals across years and sampling sites for *Helix spp.* Values are mean \pm standard error of total concentration (mg/Kg). Fisher's PLSD: a vs b $P = 0.0001$; a vs c, a vs d, a vs e, b vs e $P < 0.0001$; b vs c $P = 0.0002$; 2001 vs 2012 $P < 0.0001$.

In *R. esculenta* tissues no pollutant was detected in 2012 in two out of three sampling locations. Total concentration of HM was the highest in 2011 ($F_{1,54} = 2610.7$, $P < 0.0001$). There was also a significant effect of sampling site ($F_{2,54} = 12.3$; $P < 0.0001$; a vs e, f, $P \leq 0.0001$ in post hocs, Fig. 5B) and of the interaction between site and year ($F_{2,54} = 22.0$, $P < 0.0001$).

2.3 Association between genotoxicity and heavy metals

The correlation coefficients for the three species are presented in Table 3. *A. fasciatus* showed the highest coefficients, followed by *R. esculenta* and *Helix spp.* *R.*

esculenta showed an inverse correlation between HM concentration and TI. In the fish, the contribution to the significant positive correlation was given by As, Ni, and Cr, whereas in the snail the contribution to the (non significant) positive correlation was given by Ni and Cr (Figs. 6, 7). The regression analysis indicates a better sensitivity of the fish as suggested by the higher steepness of the lines. The species comparison in the coefficient, carried out considering total HM concentration, was as follows: *A. fasciatus* was almost significantly higher than *Helix spp.* ($z = 1.72$, $P = 0.08$) and not significantly different than *R. esculenta* ($z = -0.53$, $P < 0.29$); *Helix spp.* was significantly higher than *R. esculenta* ($z = -2.03$, $P = 0.04$).

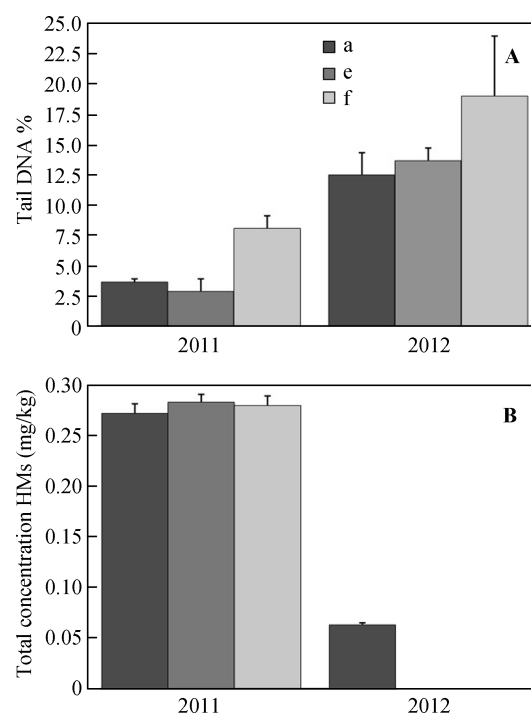


Fig. 5 (A) Tail Intensity across year and sampling site for *R. esculenta*. Values are mean \pm standard error of % Tail DNA. Fisher's PLSD: a vs f $P = 0.05$; e vs f $P = 0.007$; 2011 vs 2012 $P < 0.001$. (B) Total concentration of heavy metals across year and sampling site for *R. esculenta*. Values are mean \pm standard error of total concentration (mg/Kg). Fisher's PLSD: a vs e, a vs f $P = 0.0001$; 2011 vs 2012 $P < 0.001$.

Table 3 Correlation coefficients (Pearson) between primary DNA damage (T.I.) and tissue levels of heavy metals in the three species. The difference between species (Z-test) are reported in the text. * $P < 0.05$; ** $P < 0.01$.

	As	Cd	Ni	Pb	Cr	Tot
T.I. <i>Aphanius fasciatus</i>	**0.49	-0.14	**0.57	*-0.37	**0.68	**0.48
T.I. <i>Helix spp.</i>	-0.06	-0.04	*0.40	-0.05	0.16	0.18
T.I. <i>Rana esculenta</i>			-0.30		*-0.58	*-0.59

3 Discussion

In nature, the selection of suitable sentinel populations is inherently difficult (Hasspieler et al., 1995) and there have been relatively few studies providing inter-species comparisons. Here, we show that genotoxicity biomarker responses previously detected in the laboratory can be applied in native populations of the fish *A. fasciatus* and the snail *Helix spp.* These species meet most of the criteria enlisted in the Introduction for the choice of target suitable species for biomonitoring.

In *A. fasciatus* the profiles of DNA damage were consistent with those of heavy metal concentration. For both TI and HM the differences between *A. fasciatus* and the other two species were conspicuous with significantly higher values in the fish compared to the other two. The consistency clearly emerged when considering the trends over time and space with the highest values of HM in 2012 and in southern locations within each year, which parallels the TI values recorded in the corresponding samples. These results are supported by the positive correlations between TI and HM concentrations, which are the highest among the sampled species. The interaction between site and year, which was borderline significant for TI and highly significant for HMs, could indicate that the metals tend to especially accumulate in samples from the southern ponds, and this is followed by a parallel increase in DNA damage of slightly lower magnitude. These data indicate a good efficiency and sensitivity of *A. fasciatus* to genotoxic agents as previously suggested by both *in vitro* and *in vivo* assessments (Mosesso et al., 2012). The low values of TI recorded in our study (in 2010, 2011 and 2012 northern ponds) are in line, or slightly higher, with respect to the values found in previous studies in fish erythrocytes (Frenzilli et al., 2004; Mustafa et al., 2011). Moreover, we previously showed that this is not due to the presence of replicating cells in S-phase, but it is rather a species-specific feature (Mosesso et al., 2012). On the other hand, the TI values recorded in our study in the 2012 southern sample including a parallel increase of HM concentration, were similar to those found in specimens from Orbetello lagoon (about 40 km North, Tuscany, Italy), which is considered a significantly polluted site with high concentrations of As, Cd, Cu, Hg, Pb, and Zn of anthropogenic origin (e.g. up to 17 mg/kg of As and 245 mg/Kg of Pb, in sediments, Succi et al., 2008; Frontalini et al., 2010; Mosesso et al., 2012). Thus, the comet assay appears to have detected the increasing exposure

to genotoxic compounds. Notably, within each sampling year, *A. fasciatus* always showed higher TI and HM values in the southern area, which could be the consequence of the shorter distance of this site from the presumed source of pollution. Further, the connection with the sea of Tarquinia salterns is located north of the area and the water exchange is lower in the southern ponds, thus the persistence of pollutants may be favoured (Bellisario et al., 2013). Finally, HMs tend to be associated with the organic matter present in the thin fraction of the sediments (Soares et al., 1999), which progressively increases in the southern area (Bellisario et al., 2013). The increase of total HM concentration in fishes collected in 2012 was mainly due to arsenic, which is one of the pollutants expected from coal-fired power plants (Yudovich and Ketris, 2005; Goodarzi, 2006; Senior et al., 2006). Fish cell lines have a good sensitivity to the genotoxic effects of inorganic arsenicals, which are even higher than those expressed by mammalian cells in concomitant *in vitro* exposures (Raissudin and Jha, 2004).

In the snail *Helix spp.* there was no significant variation in DNA damage across two years, while total HM concentrations significantly decreased from 2011 to 2012 particularly in sites c and d. TI and HM concentrations tended to be higher near the power station. The correlation coefficients between TI and HMs were almost significantly lower than those found in *A. fasciatus*. Further, the lower steepness suggests a lower sensitivity (Figs. 6A and 7A). In Angeletti et al. (2013), TI values recorded in 2011 were significantly inversely correlated with the distance of sample locations from the power station. In 2012, this correlation was borderline significant ($P = 0.08$, unpublished results), despite the aforementioned decrease of HMs over time in sites c and d. This indicates that the higher DNA damage near the power station could be due to other pollutants beyond HMs. Indeed, terrestrial species, through their interaction with a diverse range of environmental compartments (e.g. soil, air, water and food) can be exposed to a complex array of contaminants (Tarazona and Vega, 2002; Langdon et al., 2003a) whose presence in the snail tissues were not considered here. Moreover, as demonstrated for earthworms, the bioavailability of genotoxic compounds can decrease as a consequence of soil ageing that strengthens pollutants sequestration (Vasseur and Bonnard, 2014). Lab and field studies have shown that, even if the comet assay has a sufficient sensitivity to detect DNA damage in fish, haemocytes of aquatic gastropods are often more sensitive to genoto-

xicants than fish erythrocytes (Jha, 2008; Osterauer et al., 2011). This seems to confirm that the different bioavailability of pollutants in aquatic versus terrestrial environments could be one main reason for the observed higher sensitivity of *A. fasciatus* with respect to *Helix* spp. Moreover, the higher bioavailability of metals in aquatic habitats could have enhanced the effect of other genotoxins, as it is known that metals can exert an indirect damage effect and inhibit the DNA repair systems (Dixon et al., 2002).

R. esculenta showed inconsistent results of difficult interpretation, which should be considered as preliminary. TI and HM concentration showed an opposite trend across years, with the first one significantly increasing and the second one significantly decreasing from 2011 to 2012. The differences of TI between sampling location seem to be consistent with the distance from the source of pollution, since the amount of DNA damage increases towards it, but this was not observed for HMs. In 2012, the farthest site showed the highest values of

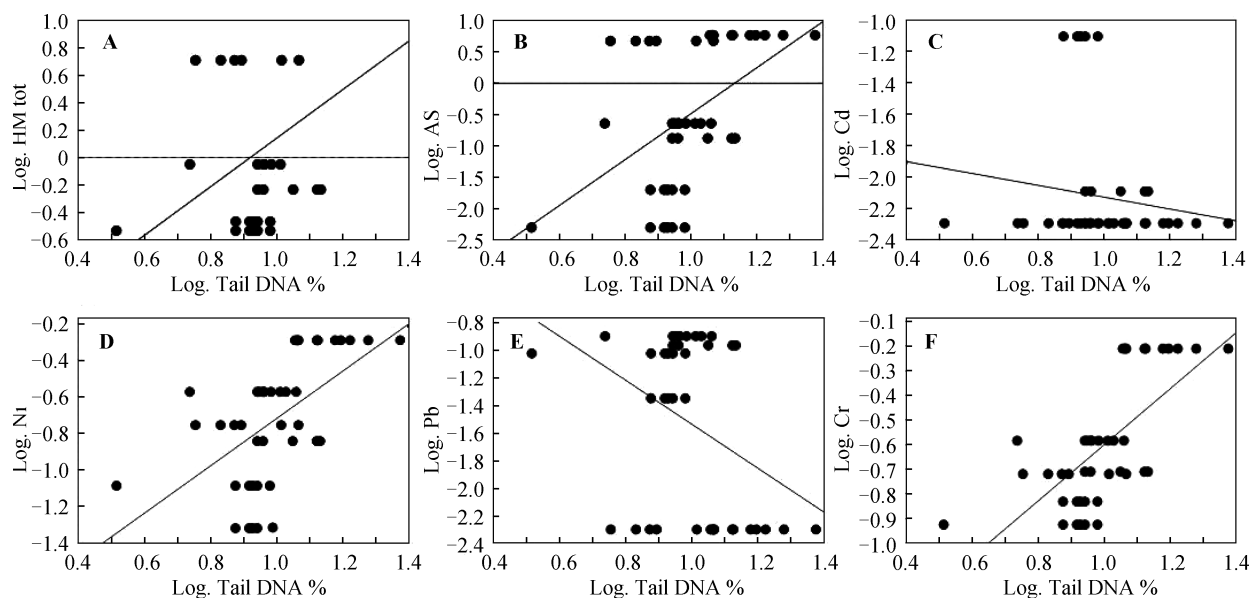


Fig. 6 Scatter plots of the total concentration (A) and the five heavy metal levels (B–F) in tissues of *A. fasciatus* versus % DNA damage (Tail Intensity). See Table 3 for correlation coefficients.

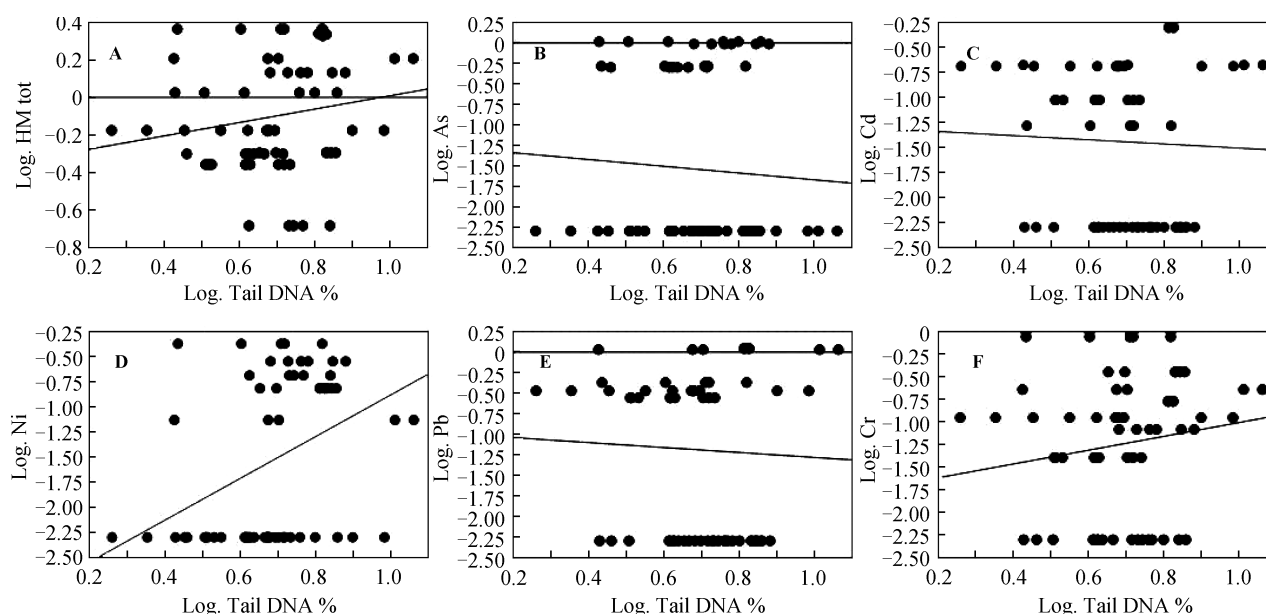


Fig. 7 Scatter plots of the total concentration (A) and the levels of the five heavy metals (B–F) in tissues of *Helix* spp. versus % DNA damage (Tail Intensity). See Table 3 for correlation coefficients.

total HMs. The differences between the sampling sites did not change for TI, which is different from what happens to HMs, whose concentrations in the sites e and f dropped to zero in 2012 and enhanced the gap between them and site a. Surprisingly, the correlation coefficients between the TI values and HM concentrations had a negative sign, indicating that higher DNA damage was associated with lower pollutant levels. The data obtained could be spurious or insufficient, may suffer the paucity of locations and individuals and could suggest that HM are not responsible for the genotoxic effect recorded in *R. esculenta*. Moreover, it is known that in amphibians, most trace elements tend to accumulate in tissues other than blood and muscle, such as kidney or liver (Loumbourdis, 1998; Vogiatzis and Loumbourdis, 1998; Loumbourdis et al., 2007), which were not analyzed here. Nevertheless, our preliminary tests for the comet assay on *R. esculenta*, already showed a limited sensitivity of its erythrocytes after *in vitro* exposure to genotoxic referent agents (e.g. hydrogen peroxide and x-rays; unpublished data), and it is known that the susceptibility of amphibians to pollutants, such as pesticides, varies across life stages (Greulich and Pflugmacher, 2003). Thus it is possible that part of the variability in the values registered here by the comet assay may be due to age or different seasonal conditions during sampling.

As regards possible mechanisms, some metals such as chromium (Zhirkovich et al., 1996; O'Brien et al., 2001; Matsumoto, 2006), cadmium (Hengstler et al., 2003), lead (Cestari et al., 2004), nickel, arsenic (Hirano and Tamae, 2010), and mercury can induce direct or indirect DNA damage, along with synergistic activity (Hengstler et al., 2003). Chromium can directly damage DNA producing DNA strand breaks, DNA-protein crosslinks and can cause cell to generate reactive oxygen species (De Flora et al., 1989; Klein et al., 1991). The damage could occur by inducing reactive oxygen species (ROS) that attack DNA generating oxidized bases (Bohr et al., 2002; Hirano and Tamae, 2010): for example, the effects of Ni can result from the generation of oxygen radicals in the interaction between metals and proteins (Reinecke and Reinecke, 2004). These radicals can subsequently interact with DNA, induce damage to its bases and cause DNA strand breaks (Kasprzak et al., 1992; Misra et al., 1993). Metals could also interact with the DNA repair process: Ni (Reinecke and Reinecke, 2004), Cr, Pb, As and Cd (Hirano and Tamae, 2010) can inhibit repair systems (Singh et al., 2009; Bolin et al., 2006).

Three years of biomonitoring the coal power plant allowed a comparative evaluation of three species as sentinel organisms. *A. fasciatus* clearly reflected the bioaccumulation of expected pollutants, such as arsenic, and the general increasing exposure to genotoxic compounds over time. *Helix spp.* reflected the geographical extent and the gradient of exposition to genotoxic compounds in the area surrounding the presumed source of pollution. Respect to the fish *Helix spp.* also have the advantage of ubiquity and the possibility to plan sampling campaigns in which individuals coming from not-polluted sites are artificially exposed according to a biomonitoring grid around the power plant. *R. esculenta* needs further testing, but the data collected so far seem to indicate that the species should be used in other ecological contexts to detect impacts that directly affect the hydrological sector (see Maselli et al., 2010).

It is still necessary to add complementary approaches: (i) a chemical characterization of environmental matrices of the sample sites to relate the genotoxicity and bioaccumulation to actual soil and water bioavailability in order to test the criterion of a simple relationship between source and tissue concentration of the pollutants; (ii) the use of the micronucleus test, as the simplest biomarker of effect to evaluate the occurrence of persistent DNA-damage (Bolognesi and Cirillo, 2014); (iii) an experimental approach using transplanted animals (in particular for *Helix spp.*), since organisms from already contaminated soils may develop resistance to the genotoxic effect of pollutants (Voua Otomo and Reinecke, 2010; Button et al., 2010; Button et al., 2012).

In sum, both the fish *Aphanius fasciatus* and the snail *Helix spp.* mirrored a trend of increasing pollution across time and space, which was evident by accumulation profiles being consistent with profiles of DNA damage. The fish appeared the most sensitive species in detecting heavy metal pollution. Conversely, the frog *Rana esculenta* showed a low capability to accumulate metals and puzzling results in the comet assay.

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References

- Angeletti D, Cimmaruta R, Nascetti G, 2010. Genetic diversity of the killifish *Aphanius fasciatus* paralleling the environmental changes of *Tarquinia salterns* habitat. *Genetica* 138: 1011–1021.
- Angeletti D, Sebbio C, Carere C, Cimmaruta R, Nascetti G et al., 2013. Terrestrial gastropods (*Helix spp*) as sentinels of primary DNA damage for biomonitoring purposes: A validation study. *Environ. Mol. Mutagen.* 54: 204–212.
- Balpaeme K, Delbeke K, Zhu L, Kirsch-Folders M, 1996. Cytogenetic studies of PCB77 on brown trout *Salmo trutta fario* using the micronucleus test and the alkaline comet assay. *Mutagenesis* 11:485–492.
- Beeby A, 2001. What do sentinels stand for? *Environ. Pollut.* 112: 285–298.
- Beeby A, Richmond L, 2002. Evaluating *Helix aspersa* as a sentinel for mapping metal pollution. *Ecol. Indic.* 1: 261–270.
- Beeby A, Richmond L, 2003. Do the soft tissues of *Helix aspersa* serve as a quantitative sentinel of predicted free lead concentrations in soil? *Appl. Soil Ecol.* 22: 159–165.
- Bellisario B, Carere C, Cerfolli F, Angeletti A, Nascetti G et al., 2013. Infaunal macrobenthic community in a manipulated hyperhaline ecosystem: A long-term study. *Aquat. Biosyst.* 9: 20.
- Berger B, Dallinger R, 1993. Terrestrial snail as quantitative indicators of environmental metal pollution. *Environ. Monit. Assess.* 25: 65–84.
- Betti C, Nigro M, 1996. The comet assay for the evaluation of genetic hazard of pollutants in cetaceans: Preliminary results on the genotoxic effects methyl-mercury on the bottle-nosed dolphin *Tursiops truncatus* lymphocytes *in vitro*. *Mar. Poll. Bull.* 32: 545–548.
- Bohr VA, Stevensner T, De Souza-Pinto NC, 2002. Mitochondrial DNA repair of oxidative damage in mammalian cells. *Gene.* 286: 127–134.
- Bolin CM, Basha R, Cox D, Zawia NH, Maloney B et al., 2006. Exposure to lead (Pb) and the developmental origin of oxidative DNA damage in the aging brain. *Faseb J.* 20: 788–790.
- Bolognesi C, Buschini A, Brenchi E, Carboni P, Furlini M et al., 2004. Comet and micronucleus assays in zebra mussel cells for genotoxicity assessment of surface drinking water treated with three different disinfectants. *Sci. Total Environ.* 333: 127–136.
- Bolognesi C, Cirillo S, 2014. Genotoxicity biomarkers in aquatic bioindicators. *Curr. Zool.* 60 (2): – .
- Bonisolì-Alquati A, Voris A, Mousseau TA, Möller AP, Saino N et al., 2010. DNA damage in barn swallows *Hirundo rustica* from the Chernobyl region detected by use of the comet assay. *Comp. Biochem. Physiol. Part C.* 151: 271–277.
- Bonisolì-Alquati A, 2014. Avian genetic ecotoxicology: DNA of the Canary in a coalmine. *Curr. Zool.* 60 (2): – .
- Boone MD, Bridges CM, 2003. Effects of pesticides on amphibian populations. In: Semlitsch RD ed. *Amphibian Conservation*. Washington: Smithsonian Institution, 152–167.
- Buschini A, Martino A, Gustavino B, Monfrinotti M, Poli P et al., 2004. Comet assay and micronucleus test in circulating erythrocytes of *Cyprinus carpio* specimens exposed *in situ* to lake waters treated with disinfectants for potabilization. *Mutat. Res.* 557: 119–129.
- Button M, Jenkin GRT, Bowman KJ, Harrington CF, Brewer TS et al., 2010. DNA damage in earthworms from highly contaminated soils: Assessing resistance to arsenic toxicity by use of the Comet assay. *Gen. Tox. En.* 696: 95–100.
- Button M, Koch I, Reimer KJ, 2012. Arsenic resistance and cycling in earthworms residing at a former gold mine in Canada. *Environ. Pollut.* 169: 74–80.
- Carere C, Costantini D, Sorace A, Santucci D, Alleva E, 2010. Bird populations as sentinels of endocrine disrupting chemicals. *Ann. Ist. Super. Sanità* 46: 81–88.
- Cavaro F, Daouti I, Leonardos I, Torricelli P, Malavasi S, 2013. Linking habitat structure to life history strategies: Insights from a Mediterranean killifish. *J. Sea Res.* 85: 205–213.
- Cestari MM, Lemos PMM, de Oliveira Ribeiro CA, Alves Costa JRM, Pelletier E et al., 2004. Genetic damage induced by trophic doses of lead in the neotropical fish *Hoplias malabaricus* (Cheraciformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations. *Genet. Mol. Biol.* 27: 270–272.
- Cohen J, Cohen P, 1983. *Applied Multiple Regression/Correlation Analysis for the Behavioral Sciences*. Hillsdale, NJ: Erlbaum.
- Dallas LJ, Cheung VV, Fisher AS, Jha AN, 2013. Relative sensitivity of two marine bivalves for detection of genotoxic and cytotoxic effects: A field assessment in the Tamar Estuary, South West England. *Environ. Monit. Assess.* 185: 3397–3412.
- Deguchi Y, Toyozumi T, Masuda S, Yasuhara A, Mohri S et al., 2007. Evaluation of mutagenic activities of leachates in landfill sites by micronucleus test and comet assay using goldfish. *Mutat. Res.* 627: 178–185.
- De Flora S, Camoirano A, Serra D, Bennicelli C, 1989. Genotoxicity and metabolism of chromium compounds. *Toxicol. Environ. Chem.* 19: 153–160.
- Dixon DR, Prusky AM, Dixon LRJ, Jha AN, 2002. Marine invertebrate eco-genotoxicology: A methodological overview. *Mutagenesis* 17: 495–507.
- Eastman A, Barry MA, 1992. The origin of DNA breaks: A consequence of DNA damage, DNA repair or apoptosis? *Cancer Invest.* 10: 229–240.
- Emmanouil C, Sheehan TM, Chipman JK, 2007. Macromolecule oxidation and DNA repair in mussel *Mytilus edulis* L. gill following exposure to Cd and Cr (VI). *Aquat. Toxicol.* 82: 27–35.
- Everaats JM, Den Besten PJ, Hillebrand MThJ, Hallbrook RS, Shugart LR, 1998. DNA strand break, cytochrome P-450 dependent monooxygenase system activity and levels of chlorinated biphenyl congeners in the pyloric caeca of the seastar *Aster rubens* from the North Sea. *Ecotoxicology* 7: 69–79.
- Felder DP, D'Surney SJ, Rodgers JH Jr, Deaidoff TL, 1998. A comprehensive environmental assessment of a receiving aquatic system near an unbleached kraft mill. *Ecotoxicology* 7: 313–324.
- Fischer W, Schneider M, Bauchot ML, 1987. Fiches FAO d'identification des espèces pour les besoins de la pêche. Méditerranée et Mer Noire (Zone de la pêche 37). Vertébrés, FAO CEE, Rome 2: 761–1529.
- Frenzilli G, Nigro M, Scarcelli V, Gorbi S, Regoli F, 2001. DNA integrity and total oxyradical scavenging capacity in the Mediterranean mussel *Mytilus galloprovincialis*: A field study in a highly eutrophicated coastal lagoon. *Aquat. Toxicol.* 53: 19–32.
- Frenzilli G, Scarcelli V, Barga ID, Nigro M, Forlin L et al., 2004. DNA damage in eelpout *Zoarces viviparus* from Goteborg harbor. *Mutat. Res.* 552: 187–195.

- Frenzilli G, Nigro M, Lyons BP, 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutat. Res.* 681: 80–92.
- Frontalini F, Coccioni R, Bucci C, 2010. Benthic foraminiferal assemblages and trace element contents from the lagoons of Orbetello and Lesina. *Environ. Monit. Assess.* 170: 245–260.
- Gomot de Vaufléury A, Pihan F. 2000. Growing snails as sentinels to evaluate terrestrial environment contamination by trace elements. *Chemosphere* 40: 275–284.
- Goodarzi F, 2006. Morphology and chemistry of fine particles emitted from a Canadian coal-fired power plant. *Fuel* 85: 273–280.
- Greulich K, Pflugmacher S, 2003. Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquat. Toxicol.* 65: 329–336.
- Hagger JA, Depledge MH, Galloway TS, 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. *Mar. Pollut. Bull.* 51: 811–816.
- Hasspieler BM, Alipour M, Ali FN, Haffner GD, Adeli K, 1995. Assessment of cyto- and genotoxicity of environmental samples. In Butterworth FM, Corkum LD, Guzmán-Rincón J ed. *Biomonitoring and Biomarkers as Indicators of Environmental Change: A Handbook* New York: Plenum Press, 149–168.
- Hengstler JG, Bolm-Audorff U, Faldum A, Janssen K, Reifemarth M et al., 2003. Occupational exposure to heavy metals: DNA damage induction and DNA repair inhibition prove coexposures to cadmium, cobalt, and lead as more dangerous than hitherto expected. *Carcinogenesis* 24: 63–73.
- Hirano T, Tamae K, 2010. Heavy metal induced oxidative DNA damage in earthworms: A review. *Appl. Environ. Soil Sci.* 2010
- Huang D, Zhang Y, Wang Y, Xie Z, Ji W, 2007. Assessment of the genotoxicity in toad *Bufo raddei* exposed to petrochemical contaminants in Lanzhou Region, China. *Mutat. Res.* 629: 81–88.
- Jha AN, 2008. Ecotoxicological application and significance of the comet assay. *Mutagenesis* 23: 207–221.
- Kasprzak KS, Diwan BA, Rice JM, Misra M, Eiggs CW et al., 1992. Nickel (II)-mediated oxidative DNA base damage in renal cells. *Chem. Res. Toxicol.* 5: 809–815.
- Kiseleva EI, 1997. Aspect of the chemical ecology and chemosensory guided behavioral reaction in anuran tadpole. In: Pensoft Publishers ed. *Advance in Amphibian Research in the Former Soviet Union*. Sofia 2: 95–101.
- Klein CB, Frenkel K, Costa M, 1991. The role of oxidative processes in metal carcinogenesis. *Chem. Res. Toxicol.* 4: 592–604.
- Kosmehl T, Hallare AV, Braunbeck T, Hollert H, 2008. DNA damage induced by genotoxicants in zebrafish *Danio rerio* embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutat. Res.* 650: 1–14.
- Kumaravel TS, Jha AN, 2006. Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutat. Res.* 605: 7–16.
- Laffon B, Rabade T, Pasaro E, Mendez J, 2006. Monitoring of the impact of Prestige oil spill on *Mytilus galloprovincialis* from Galician coast. *Environ. Int.* 32: 342–348.
- Langdon CJ, Pearce TG, Meharg AA, Semple KT, 2003a. Interactions between earthworms and arsenic in the soil environment: A review. *Environ. Pollut.* 124: 361–373.
- Lee RF, Steinert S, 2003. Use of the single gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. *Mutat. Res.* 544: 43–64.
- Lefcort H, Meguire RA, Wilson LH, Ettinger WF, 1998. Heavy metals alter the survival, growth metamorphosis and antipredatory behaviour of Columbia spotted frog *Rana luteiventris* tadpoles. *Arch. Environ. Contam. Toxicol.* 35: 447–456.
- Leffa DD, Damiani AP, da Silva J, Zocche JJ, dos Santos CE et al., 2010. Evaluation of the genotoxic potential of the mineral coal tailings through the *Helix aspersa* (Muller, 1774). *Arch. Environ. Contam. Toxicol.* 59: 614–621.
- León G, Perez LE, Linare JC, Hartmann A, Quintana M, 2007. Genotoxic effects in wild rodents (*Rattus rattus* and *Mus musculus*) in an open coal mining area. *Mut. Res.* 630: 42–49.
- Leontyeva OA, Semenov DV, Esenin AV, 1997. Brown frogs as bioindicator of heavy metal contamination: preliminary results on the common frog *Rana temporaria* in Moscow Province. In: Pensoft Publishers ed. *Advance in Amphibian Research in the Former Soviet Union*. Sofia 2: 125–131.
- Loumbourdis NS, 1998. Heavy metal concentration in the frog *Rana ridibunda* from a small river of Macedonia, Northern Greece. *Environ. Int.* 24: 427–431.
- Loumbourdis NS, Kostaropoulos I, Theodoropoulou B, Kalmanti D, 2007. Heavy metal accumulation and metallothionein concentration in the frog *Rana ridibunda* after exposure to chromium or a mixture of chromium and cadmium. *Environ. Pollut.* 145: 787–792.
- Maltagliati F, 1998. Does the Mediterranean killifish *Aphanius fasciatus* (Teleostei: Cyprinodontidae) fit the one-dimensional stepping-stone model of gene flow? *Environ. Biol. Fish.* 53: 385–392.
- Martin MH, Coughtrey PJ, 1982. *Biological Monitoring of Heavy Metal Pollution: Land and Air*. London: Applied Science Publishers.
- Martin FL, Pearce TG, Hewer A, Phillips DH, Semple KT, 2005. A biomarker model of sublethal genotoxicity (DNA single-strand breaks and adducts) using the sentinel organism *Aporrectodea longa* in spiked soil. *Environ. Pollut.* 138: 307–315.
- Maselli V, Polese G, Rippa D, Ligrone R, Rastogi RK et al., 2010. Frogs, sentinels of DNA damage include by pollution in Naples and the neighbouring Provinces. *Ecotox. Environ. Saf.* 73: 1525–1529.
- Matsumoto ST, Mantovani MS, Malagutti MIA, Dias AL, Fonseca IC et al., 2006. Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genet. Mol. Biol.* 29: 148–158.
- Mitchelmore CL, Chipman JK, 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutat. Res.* 399: 135–147.
- Mitchelmore CL, Birmelin C, Livingstone DR, Chipman JK, 1998. Detection of DNA strand breaks in isolated mussel *Mytilus edulis* L. digestive gland cells using the comet assay. *Ecotox. Environ. Saf.* 41: 51–58.
- Misra M, Olinski R, Dizdaroglu M, Kasprzak KS, 1993. Enhancement by L-histidine of nickel (II) induced DNA protein cross-links and oxidative DNA base damage in the rat kidney.

- Chem. Res. Toxicol. 6: 33–37.
- Mosesso P, Angeletti D, Pepe G, Pretti C, Nascetti G et al., 2012. The use of cyprinodont fish, *Aphanius fasciatus*, as a sentinel organism to detect complex genotoxic mixtures in the coastal lagoon ecosystem. *Mutat. Res.-Gen. Tox. Env.* 742: 31–36.
- Mouchet F, Gauthier L, Mailhes C, Jourdain MJ, Ferrier V et al., 2006. Biomonitoring of the genotoxic potential of aqueous extracts of soils and bottom ash resulting from municipal solid waste incineration, using the comet and micronucleus tests on amphibian *Xenopus laevis* larvae and bacterial assays (Mutatox and Ames tests). *Sci. Total Environ.* 355: 232–246.
- Mustafa SA, Al-Subiai SN, Davies SJ, Jha AN, 2011. Hypoxia-induced oxidative DNA damage links with higher level biological effects including specific growth rate in common carp *Cyprinus carpio* L. *Ecotoxicology* 20: 1455–1466.
- Nacci D, Nelson S 1992. Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves. *Mar. Environ. Res.* 33: 83–100.
- O'Brien T, Xu J, Patierno SR, 2001. Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. *Mol. Cell. Biochem.* 222: 173–182.
- Oldham RS, Latham DM, Hilton-Brown D, Towns M., Cooke AS et al., 1997. The effect of ammonium nitrate fertiliser on frog *Rana temporaria* survival. *Agr. Ecosyst. Environ.* 61: 69–74.
- Osterauer R, Faßbender C, Braunbeck T., Köhler HR, 2011. Genotoxicity of platinum in embryos of zebrafish *Danio rerio* and ramshorn snail *Marisa cornuarietis*. *Sci. Total Environ.* 409: 2114–2119.
- Perez-Cadahia B, Laffon B, Pasaro E, Mendez J, 2004. Evaluation of PAH bioaccumulation and DNA damage in mussels *Mytilus galloprovincialis* exposed to spilled Prestige crude oil, *Comp. Biochem. Physiol. C.* 138: 453–460.
- Phillips DJH, Rainbow PS, 1993. Cosmopolitan indicators of trace metals. *Mar. Pollut. Bull.* 26: 593–601.
- Phillips DJH, Segar DA, 1986. Use of bio-indicators in monitoring conservative contaminants: Programme design imperatives. *Mar. Pollut. Bull.* 17: 10–17.
- Raimundo J, Costa PM, Vale C, Costa MH, Moura I, 2010. DNA damage and metal accumulation in four tissues of feral *Octopus vulgaris* from two coastal areas in Portugal. *Ecotox. Environ. Safe.* 73: 1543–1547.
- Rabinowitz PM, Gordon Z, Holmes R, Taylor B, Wilcox M et al., 2005. Animals as sentinels of human environmental health hazards: An evidence-based analysis. *Ecohealth* 2: 26–37.
- Raissudin S, Jha AN, 2004. Relative sensitivity of fish and mammalian cells to sodium arsenate and arsenite as determined by alkaline single-cell electrophoresis and cytokinesis-block micronucleus assay. *Environ. Mol. Mutagen.* 44: 83–89.
- Ralph S, Petras M, 1997. Genotoxicity monitoring of small bodies of water using two species of tadpoles and the alkaline single cell gel (comet) assay. *Environ. Mol. Mutagen.* 29: 418–430.
- Ralph S, Petras M, Pandrangi R, Vrzoc M, 1996. Alkaline single cell gel (comet) assay and genotoxicity monitoring using two species of tadpoles. *Environ. Mol. Mutagen.* 28: 112–120.
- Rank J, Jensen K, Jespersen PH, 2005. Monitoring DNA damage in indigenous blue mussels *Mytilus edulis* sampled from coastal sites in Denmark. *Mutat. Res.* 585: 33–42.
- Rajaguru P, Suba S, Palanivel M, Kalaiselvi K, 2003. Genotoxicity of a polluted river system measured using the alkaline comet assay on fish and earthworm tissues. *Environ. Mol. Mutagen.* 41: 85–91.
- Regoli F, Gorbi S, Fattorini D, Tedesco S, Notti A et al., 2006. Use of the Land Snail *Helix aspersa* as sentinel organism for monitoring ecotoxicologic effects of urban pollution: An integrated approach. *Environ. Health Persp.* 114: 63–69.
- Regoli F, Gorbi S, Machella N, Tedesco S, Benedetti M et al., 2005. Prooxidant effects of extremely low frequency electromagnetic fields (ELFEM) in the land snail *Helix aspersa*. *Free Radic. Biol. Med.* 39: 1620–1628.
- Regoli F, Winston GW, Gorbi S, Frenzilli G, Nigro M et al., 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*: Toward development of a more holistic biomarker assessment. *Environ. Toxicol. Chem.* 22: 2120–2129.
- Reinecke SA, Reinecke AJ, 2004. The comet assay as a biomarker of heavy metal genotoxicity in earthworms. *Arch. Environ. Contam. Toxicol.* 46: 208–215.
- Rojas E, Lopez MC, Valverde M, 1999. Single cell gel electrophoresis: Methodology and application. *J. Chromatogr. B* 722: 225–254.
- Santucci F, Nascetti G, Bullini L, 1996. Hybrid zones between two genetically differentiated forms of the pond frog *Rana lessonae* in southern Italy. *J. Evolution. Biol.* 9: 429–450.
- Santucci F, Andreani P, Nascetti G, Bullini L, 2000. Genetic identification and geographic distribution of the water frogs *Rana lessonae* and *Rana esculenta* in Italy, Sicily and Corsica (amphibia, anura). *Mus. Reg. Sci. Nat. Torino* 359–367.
- Schuytema GS, Nebeker AV, Griffis WL, Wilson KL, 1991. Teratogenesis, toxicity, and bioconcentration in frogs exposed to dieldrin. *Arch. Environ. Contam. Toxicol.* 21: 332–350.
- Schuytema GS, Nebeker AV, Peterson JA, Griffis WL, 1993. Effects of pentachlorophenol - contaminated food organisms on toxicity and bioaccumulation in the frog *Xenopus laevis*. *Arch. Environ. Contam. Toxicol.* 24: 359–364.
- Senior CL, Lignell DO, Sarofim AF, Mehta A, 2006. Modeling arsenic partitioning in coal-fired power plants. *Combust. Flame* 147: 209–221.
- Shugart LR, 1990. Biological monitoring: Testing for genotoxicity. In: McCarthy JF, Shugart LR ed. *Biomarkers of Environmental Contamination*. Boca Raton: Lewis, 217–227.
- Singh NP, McCoy MT, Tice RR, Schneider EL, 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.* 175: 184–191.
- Singh KP, Kumari R, Pevey C, Jackson D, DuMond JW, 2009. Long duration exposure to cadmium leads to increased cell survival, decreased DNA repair capacity, and genomic instability in mouse testicular Leydig cells. *Canc. Lett.* 279: 84–92.
- Snyman RG, Reinecke SA, Reinecke AJ, 2000. Hemocytic lysosome response in the snail *Helix aspersa* after exposure to the fungicide copper oxychloride. *Arch. Environ. Contam. Toxicol.* 39: 480–485.
- Soares HMVM, Boaventura RAR, Machado AASC, Esteves da Silva JCG, 1999. Sediments as monitors of heavy metal contamination in the Ave river basin (Portugal): Multivariate analysis of data. *Environ. Pollut.* 105: 311–323.
- Stahl RG Jr, 1997. Can mammalian and non-mammalian sentinel species' data be used to evaluate the human health implica-

- tions of environmental contaminants. *Hum. Ecol. Risk Assess.* 3: 329–335.
- Succi MC, Romano E, Bergamin L, Carboni MG, 2008. I foraminiferi bentonici indicatori ambientali nella Laguna di Orbetello (Toscana, Italia), *Rendiconti online, Soc. Geol. It.* 2: 1–3.
- Tarazona JV, Vega MM, 2002. Hazard and risk assessment of chemicals for terrestrial ecosystems. *Toxicology* 181–182, 187–191.
- Theodorakis CW, Shugart LR, 1998. Genetic ecotoxicology II: the relationship between DNA strand breaks and genotype in mosquito fish exposed to radiation. *Ecotoxicology* 7: 227–236.
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A et al., 2000. Single cell gel/Comet Assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.* 35: 206–221.
- Tortonese E, 1986 Cyprinodontidae. In: Whitehead PJP, Bauchot ML, Hureau JC, Nielsen J, Tortonese E ed. *Fishes of the Northeastern Atlantic and the Mediterranean*. Paris: UNESCO, 1341–1345.
- Van der Schalie WH, Gardner HS Jr., Bantle JA, De Rosa CT, Finch RA et al., 1999. Animals as sentinels of human health hazards of environmental chemicals. *Environ. Health Perspect.* 107: 309–315.
- Vasseur P, Bonnard M, 2014. Ecogenotoxicology in earthworms: A review. *Curr. Zool.* 60 (2): – .
- Viard B, Pihan F, Promeyrat S, Pihan JC, 2004. Integrated assessment of heavy metal (Pb, Zn, Cd) highway pollution: Bioaccumulation in soil, Gramineae and land snails. *Chemosphere* 55: 1349–1359.
- Villela IV, de Oliveira IM, Silveira JC, Dias JF, Henriques JA et al., 2007. Assessment of environmental stress by the micronucleus and comet assays on *Limnoperna fortunei* exposed to Guaíba hydrographic region samples (Brazil) under laboratory conditions. *Mutat. Res.* 628: 76–86.
- Villela IV, de Oliveira IM, da Silva J, Henriques JAP, 2006. DNA damage and repair in haemolymph cells of golden mussel *Limnoperna fortunei* exposed to environmental contaminants. *Mutat. Res.* 605: 78–86.
- Vogiatis AK, Loumbourdis NS, 1998. Cadmium accumulation in liver and kidneys and hepatic metallothionein and glutathione levels in *Rana ridibunda*, after exposure to CdCl₂. *Arch. Environ. Contam. Toxicol.* 34: 64–68.
- Voua Otomo P, Reinecke SA, 2010. Increased cytotoxic and genotoxic tolerance of *Eisenia fetida* (Oligochaeta) to cadmium after long-term exposure. *Ecotoxicol.* 19: 362–368.
- Xu L, Zheng GH, Lam PKS, Richardson BJ, 1999. Relationship between tissue concentrations of polycyclic aromatic hydrocarbons and DNA adducts in green-lipped mussels *Perna viridis*. *Ecotoxicology* 8: 73–82.
- Yudovich YaE, Ketris MP, 2005. Arsenic in coal: A review. *Int. J. Coal Geol.* 61: 141–196.
- Zhitkovich A, Voitkun V, Costa M, 1996. Formation of the amino acid-DNA complexes by hexavalent and trivalent chromium *in vitro*: Importance of trivalent chromium and the phosphate group. *Biochemistry* 35: 7275–7282.

Appendix

Table A1 Concentration of the single heavy metals and total concentration (mg/Kg fresh weight) for *A. fasciatus* across years and sampling sites

Year	Site	As	Cd	Ni	Pb	Cr	Total
2010	b North	0.02 ± 0.002	0.08 ± 0.03	0.05 ± 0.02	0.04 ± 0.02	0.15 ± 0.07	0.34 ± 0.9
	b South	0.13 ± 0.016	0.008 ± 0.002	0.14 ± 0.08	0.10 ± 0.02	0.19 ± 0.08	0.58 ± 0.15
2011	b North	-	-	0.08 ± 0.01	0.09 ± 0.008	0.12 ± 0.002	0.29 ± 0.01
	b South	0.23 ± 0.01	-	0.26 ± 0.008	0.12 ± 0.004	0.26 ± 0.1	0.88 ± 0.02
2012	b North	4.73 ± 0.65	-	0.17 ± 0.02	-	0.19 ± 0.02	5.09 ± 0.64
	b South	5.77 ± 1.05	-	0.51 ± 0.11	-	0.62 ± 0.18	6.90 ± 1.08

Table A2 Concentration of single heavy metals and total concentration (mg/Kg fresh weight) for *Helix spp.* across years and sampling sites

Year	Site	As	Cd	Ni	Pb	Cr	Total
2011	a	-	0.09 ± 0.004	-	0.27 ± 0.05	0.04 ± 0.004	0.44 ± 0.04
	b	-	0.21 ± 0.01	-	0.34 ± 0.002	0.11 ± 0.007	0.66 ± 0.01
	c	0.52 ± 0.08	0.05 ± 0.001	0.42 ± 0.12	0.43 ± 0.28	0.88 ± 0.56	2.31 ± 0.65
	d	-	0.51 ± 0.23	0.15 ± 0.03	1.13 ± 0.94	0.17 ± 0.12	2.21 ± 1.00
	e	-	0.21 ± 0.04	0.07 ± 0.005	1.09 ± 0.54	0.23 ± 0.14	1.60 ± 0.61
2012	a	-	-	0.21 ± 0.07	-	-	0.21 ± 0.07
	b	1.05 ± 0.04	-	-	-	-	1.05 ± 0.04
	c	0.50 ± 0.13	-	-	-	-	0.50 ± 0.13
	d	-	-	0.15 ± 0.03	-	0.36 ± 0.04	0.51 ± 0.06
	e	0.99 ± 0.05	-	0.28 ± 0.02	-	0.08 ± 0.004	1.36 ± 0.05

Table A3 Concentration of single heavy metals and total concentration (mg/Kg fresh weight) for *R. esculenta*

Year	Site	As	Cd	Ni	Pb	Cr	Total
2011	a	-	-	0.12 ± 0.01	-	0.15 ± 0.01	0.27 ± 0.02
	e	-	-	0.08 ± 0.005	-	0.20 ± 0.03	0.28 ± 0.03
	f	-	-	0.06 ± 0.009	-	0.22 ± 0.02	0.28 ± 0.02
2012	a	-	-	-	-	0.06 ± 0.006	0.06 ± 0.006
	e	-	-	-	-	-	-
	f	-	-	-	-	-	-