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Aporrectodea caliginosa, a suitable earthworm species for field based genotoxicity assessment?

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Native populations of endogeic earthworm Aporrectodea caliginosa can be successfully applied in the genotoxicity field surveys using Comet assay.

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ABSTRACT

There is a growing interest for the application of biomakers to field-collected earthworms. Therefore we have evaluated the usability of native populations of endogeic, widely distributed earthworm *Aporrectodea caliginosa* in the assessment of soil genotoxicity using the Comet assay. Validation of the Comet assay on earthworm coelomocytes has been established using commercially available *Eisenia fetida* exposed to copper, cadmium, and pentachlorophenol, along with *A. caliginosa* exposed to copper in a filter paper contact test. Neutral red retention time (NRRT) assay was conducted on copper exposed and field-collected earthworms. Significant DNA and lysosomal damage was measured using Comet and NRRT assays in native populations of *A. caliginosa* sampled from the polluted soils in the urban area in comparison to the earthworms from the reference site. The results of this study confirm the employment of *A. caliginosa* as a suitable species for the *in situ* soil toxicity and genotoxicity field surveys.

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1. Introduction

In the recent past there has been an increasing concern about soil contamination due to the growing awareness of its importance for ecosystem structure and functioning. Earthworms account for the majority of animal biomass in the soil in a wide range of productive ecosystems and play an important role in improving the structure and fertility of the soil (Edwards and Bohlen, 1996; Paoletti, 1999; Bohlen, 2002). Through their intimate contact with the soil earthworms can be exposed to a wide range of contaminants and are therefore among the most relevant sentinel organisms for assessing the impact of anthropogenic stresses on the soil (Spurgeon et al., 2002).

Studies on several earthworm species (mostly *Eisenia fetida* sensu lato) have been performed for nearly 30 years and were focused mostly on survival endpoints in standardized toxicity tests (OECD, 1984). Recently, there has been a growing interest in studying earthworm biomarkers and validating their usefulness in the field conditions as an early warning of adverse ecological effects (Booth et al., 2005; Sanchez-Hernandez, 2006; Nahmani et al., 2007; Rodriguez-Castellanos and Sanchez-Hernandez, 2007). Additionally, using native earthworm populations for biomarker analysis integrates the bioavailability of pollutants, exposure pathways and temporal aspect of exposure (Spurgeon et al., 2002; Sanchez-Hernandez, 2006).

Having in mind the importance of DNA in maintenance of homeostasis and the transfer of information to offspring, assessment of DNA damage is highly relevant in the process of environmental risk assessment. So far there have been only a few studies which used earthworms (mainly *E. fetida*) for assessing the genotoxicity of field-contaminated soils (Table 1.). Moreover, to our knowledge, there have been only two studies which used native earthworm populations for genotoxicity assessment of polluted soils (Button et al., 2010; Espinosa-Reyes et al., 2010).

The Comet assay is a reliable, sensitive and relatively rapid technique for measuring DNA damage (single-, double-strand

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Table 1
Studies using earthworms in the genotoxicity assessment of field-contaminated soils.

Species	Biomarker of genotoxicity	Tissue	Exposure	Reference
Eisenia andrei	Comet assay	Coelomocytes	Soil collected after pesticide application	Piola et al., 2009
Eisenia sp. (native)	Comet assay	Coelomocytes	Contaminated soil from a industrial area	Espinosa-Reyes et al., 2010
Eisenia fetida	Comet assay	Coelomocytes	Contaminated soil sampled from a former coking plant	Bonnard et al., 2009, 2010
Eisenia fetida	Comet assay	Coelomocytes	Exposed to soil samples collected from the soya field	Casabé et al., 2007
Eisenia fetida	Comet assay	Coelomocytes	Contaminated soil sampled from farmland receiving wastewater irrigation	Qiao et al., 2007
Eisenia fetida	Comet assay	Coelomocytes	Contaminated soil sampled from idustrialized area	Xiao et al., 2006b
Eisenia fetida	Comet assay	Coelomocytes	PAH contaminated soil sample	Di Marzio et al., 2005
Eisenia fetida	Comet assay	Coelomocytes	Contaminated sediment from the river	Rajaguru et al., 2003
Eisenia fetida	Comet assay	coelomocytes	Contaminated soil from polluted area of a coke oven	Šalagovič et al., 1996
Eisenia fetida, *Lumbricus terrestris*	Comet assay	Coelomocytes	Contaminated soil	Verschaeve and Gilles, 1995
Eisenia fetida, *Lumbricus terrestris*	DNA adducts	Part of the body	PAH contaminated soil sampled at coal gasification plant	Walsh et al., 1995.; 1997
Lumbricus terrestris	DNA adducts	Part of the body	PAH contaminated soils sampled at old cooking plant	Van Schooten et al., 1995
Lumbricus rubellus, *Dendrodrilus rubidus*, *Lumbricus terrestris* (native)	Comet assay	Coelomocytes	Soils from a former mine site highly contaminated with arsenic	Button et al., 2010

breaks, alkali labile sites or DNA–DNA and DNA–protein cross-links) which has been used in a variety of aquatic and terrestrial organisms (Cotelle and Férard, 1999; Collins, 2004; Dhawan et al., 2009). The Comet assay applied to earthworms (mostly *E. fetida*) has been used in previous studies to investigate the genotoxicity of artificial or natural soils spiked with various chemicals (Zang et al., 2000; Reinecke and Reinecke, 2004; Martin et al., 2005; Zhu et al., 2006; Xiao et al., 2006a; Manerikar et al., 2008).

Taking the above into consideration, the aim of this study was to evaluate the applicability of *Aporrectodea caliginosa* for field assessment of soil genotoxicity. Contrary to the widely used epigeic *E. fetida* which is mostly found in top, organic rich soil, *A. caliginosa* is endogeic, abundant and widely distributed in both Northern and Southern hemisphere (Spurgeon et al., 2000; Booth and O'Halloran, 2001; Pižl et al., 2009). There is also a need for more studies on earthworm species other than *E. fetida* so that the large existing database on this earthworm could be applied to other, soil dwelling species (Nahmani et al., 2007).

In order to establish an appropriate protocol for the Comet assay on *A. caliginosa*, commercially available *E. fetida* were dermally exposed to two selected metals (Cd and Cu) and pesticide pentachlorophenol (PCP) under laboratory conditions. This approach also enabled the comparison of sensitivity of two eco-physiologically different earthworm species, the endogeic *A. caliginosa*, and the epigeic *E. fetida* in the Comet assay as well as in the neutral red retention time (NRRT) assay.

The NRRT assay is a commonly used nonspecific biomarker technique which measures lysosomal membrane stability. It is used here as an additional measure of toxic effect. The NRRT assay has proved to be reliable, dose-dependent and practical for use in aquatic (Lowe et al., 1992; Lowe and Pipe, 1994) and terrestrial systems (Weeks and Svendsen, 1996). It has become one of the most popular earthworm biomarkers since it provides a rapid and sensitive indication of a response to altered environmental conditions. Also it has already been linked to adverse effects on life cycle traits, e.g. survival, growth or reproduction (Sanchez-Hernandez, 2006).

Apart from industrial, mining and agriculture activities, vehicular traffic and its emissions is a major contributor to soil pollution. Elevated levels of heavy metals have been measured up to 200 m or more from the roads (Trombulak and Frissell, 2000) and genotoxicity of roadside soils has been reported by several authors (for review see Watanabe and Hirayama, 2001; White and Claxton, 2004). Both experimental studies and epidemiological evidence indicate that gasoline and diesel engine exhausts are mutagenic and carcinogenic to laboratory animals and possibly to humans (IARC, 1989). Therefore, the soils near heavy traffic roads were chosen for this research.

2. Material and methods

2.1. Collection and maintenance of earthworms

2.1.1. E. fetida

E. fetida earthworms were obtained from an earthworm farm "Eršek" (Donja Bistra, Croatia) and kept in the laboratory at 20 ± 2 °C in the dark, in glass containers filled with soil from the farm. Earthworms used in this research were adults with well-developed clitella (0.4–0.6 g of fresh weight).

2.1.2. A. caliginosa

Adult (clitellate) specimens of *A. caliginosa* (0.4–0.8 g of fresh weight) were collected from three sites characterized by a different degree of pollution. The reference site was located in the eastern Croatia, near the village Kozice (17°45′E, 45°40′W) in the vicinity of the town Podravska Slatina. The two polluted sites were situated in the city of Zagreb (15°58′E,45°46′W), near roads with heavy traffic. One of the sites was in the Dubrovnik Avenue, approx. 1 m from the road while the second site was in the Većeslav Holjevac Avenue approx. 25 m from the road (Fig. 1). Traffic intensity at the polluted sites has been approximated at 22,300 vehicles per day.

After sampling and taxonomic identification (Mršić, 1997; Mršić and Novak, 1995) earthworms were kept together with the soil from the sampling locations in glass containers in the laboratory in the dark at 20 ± 2 °C for no more than 24 h.

2.2. Laboratory exposure

Prior to the experiment, earthworms (*E. fetida* from the earthworm farm and *A. caliginosa* from the reference station Kozice) were incubated for one day on moist filter paper at room temperature to empty their gut content.

2.2.1. Filter paper contact tests

During the 24 h exposure the earthworms were placed in Petri dishes (12 cm in diameter) and exposed to three concentrations of $CuCl_2$ (0.25, 0.75, 2.25 μg Cu/cm^2), $CdCl_2$ (1.32, 6.6, 13.2 μg Cd/cm^2) and Na–PCP (0.05, 0.25, 0.45 μg PCP/cm^2), by adding 1 mL of prepared solution on the whole surface of the filter paper. Sublethal concentrations were selected using the range finding test where earthworms were exposed to the following concentrations of tested compounds: 0.01, 0.1, 1, and 10 μg/cm^2 of filter paper for a period of 24 h (OECD, 1984). Filter paper of control animals received only 1 mL of distilled water. Two earthworms per Petri dish were added. Four individuals were analysed for each concentration for the Comet assay and 4–10 for the NRRT experiments. After addition of earthworms, each dish was closed with a lid containing small ventilation holes. All exposures were conducted at 20 ± 2 °C in the dark.

2.3. Field-collected earthworms

Specimens of *A. caliginosa* were collected from the reference site (Kozice), and two polluted sites (Dubrovnik Av. and Holjevac Av.) (Fig. 1). They were immediately transported to the laboratory and incubated for one day on moist filter paper at room temperature to empty their gut content before analysed for the NRRT and Comet assay. From every site, five to eight earthworms were analysed for the Comet assay and five to ten earthworms for the NRRT assay.

2.4. Chemical analysis of soil

To evaluate the degree of pollution of soil samples we have measured the concentration of heavy metals (chromium, copper, nickel, lead, cadmium, zinc,

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