



Particulate Matter Toxicity Evaluation Using Bioindicators and Comet Assay

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ABSTRACT

Atmospheric particulate matter (PM) characterizes the atmospheric air quality. PM particles can adsorb and include several toxic air pollutants of urban areas. The current study aimed to develop an experimental procedure to assess the toxicity of the pollutants on PM₁₀ by means of the comet assay on earthworms directly exposed to PM₁₀ collecting filters. A particular focus was the amount of polycyclic aromatic hydrocarbons (PAHs) in the filter, in spite of their very low concentration in PM, because of their strong mutagenic and carcinogenic effects. A quartz filter exposed to polluted air containing 24.9 mg/g of PM₁₀ and 14.1 µg/g of PAHs was characterized and mechanically reduced into a very fine powder by means of a planetary ball mill. This powder was combined with artificial soil samples allowing treatments at 15 µg/g of PM₁₀ (0.008 µg/g of PAHs), 22.5 µg/g of PM₁₀ (0.012 µg/g of PAHs), 30 µg/g of PM₁₀ (0.016 µg/g of PAHs). Earthworms were exposed to each treatment for seven days, including blank treatments with powdered clean quartz filter, such as phenanthrene (used as the standard), and an untreated soil. DNA damage was observed starting from 0.012 µg/g of PAHs in 22.5 µg/g of PM₁₀. No single PAH was detected or quantified in the bodies of the earthworms after microwave assisted solvent extraction (MASE) and GC-MS analysis. The results demonstrate that even a very low amount of PM₁₀ absorbed by the earthworms had a toxic effect on their immune systems, which could also have been caused by other xenobiotics included into the filter.

Keywords: PAHs; Earthworms; Biomarker; PM₁₀; Comet assay.

INTRODUCTION

Monitoring of atmospheric particulate matter (PM) is required in order to assess the air quality in urban areas. PM is a complex mixture of suspended solid and liquid particles with different physical, chemical and toxicological properties which originates from natural and anthropogenic sources (Stone *et al.*, 2011). According to the aerodynamic diameter, particles can be classified as PM₁₀ (< 10 µm diameter) and PM_{2.5} (< 2.5 µm). Respirable PM includes and adsorbs several organic and inorganic compounds; many of them are toxic and/or carcinogenic as the polycyclic aromatic hydrocarbons (PAHs) and heavy metals (Goyer 1986; Lighty *et al.*, 2000; Huggins *et al.*, 2004; Masih *et al.*, 2012). Although PAHs contribute minimally to PM mass (less than 0.1%), they contribute predominantly to the toxicity of PM₁₀ in urban areas (Pozzoli *et al.*, 2004). Most of PAHs and some of their degradation products are

known to have a high carcinogenic, mutagenic and allergenic potential, posing a threat to human health (Kameda *et al.*, 2005; Xue and Warshawsky 2005; Taioli *et al.*, 2007). Epidemiological studies demonstrated that people living in urban areas have a stronger risk of lung cancer than people living in rural areas due to higher levels of PAHs. Gasoline and diesel vehicle exhausts, tire wear debris, asphalt particles, re-suspended soils and power plants are considered PAH main sources in urban areas (Dyke *et al.*, 2003; Zielinska *et al.*, 2004). The highest concentrations of atmospheric PAHs were found in urban environments because of their extensive vehicular traffic and poor atmospheric dispersion (Miguel *et al.*, 1998; Amodio *et al.*, 2009). Italian and European legislations established an annual limit concentration for PM₁₀ (50 µg/m³) and a target value for BaP (1 ng/m³) in ambient air (Ministerial Decree n. 25/1994; Council Directive 1999/30/EC; Ministerial Decree n. 60/2002). Moreover Directive 2004/107/EC provided reference methods to assess PAH concentrations in atmosphere, evaluating their risk of carcinogenic on the basis of the quantitative ratios between BaP and other carcinogenic congeners. Toxicity of particulate matter on organisms can be evaluated by means of several *in vitro* assays. Skarek *et al.* (2007) assessed the genotoxic effects of organic air pollution present in gas

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phase and in the PM_{2.5}. Using a bacterial genotoxicity test (SOS chromotest) which measured the induction of SOS-repair system after damaging DNA on *Escherichia coli* PQ 37. Bonetta *et al.* (2009), applied a semipermeable membrane device (SPMD) to catch air pollutants in a coke plant. The SPMD extracts were used in the Ames test (mutagenic test) on *Salmonella typhimurium* TA98 and in the comet assay on human lymphoblastoid (Jurkat) cells. Finally, Vouitsis *et al.* (2009) collected PM emitted from light-duty vehicles on PTFE-coated fibre filters. Their extract toxicity was determined by means of Microtox® bioassay on the bioluminescence of *Vibrio fischeri*. However, the organic contaminants collected in the exposed filters were always Soxhlet extracted and, subsequently, applied in these previously cited bioassays.

Earthworms are affected by pollution and their metabolism can be modified in relation to environmental stress making them suitable as bio-indicators (Puglisi *et al.*, 2009; Bari *et al.*, 2010; Spagnuolo *et al.*, 2010). They ingest soil particles and absorb soil pollutants also through their integument. Several international protocols planned the use of earthworms as biomarkers to assess the acute toxicity or the sub-lethal effects of xenobiotics in natural or artificial soils but there are not applications of worms as biomarkers to evaluate air pollution impact (OECD, 1984; OPPTS, 1996). The comet assay directly assesses the breaks of DNA in a single cell acting as an effect biomarker (Fairbairn *et al.*, 1995; Lagadic *et al.*, 1997; Akcha *et al.*, 2004; Siu *et al.*, 2004). The current study was aimed at attempting to set experimentally a procedure evaluating the toxicity of air pollutants based on the application of comet assay applied on coelomocytes collected from earthworms exposed directly to PM₁₀ ground filters.

MATERIALS AND METHODS

Earthworms

Specimens of *Eisenia andrei* Bouché, 1972 (Annelida: Lumbricidae) were obtained by Compagnoni farm (Lecco, Italy). They were acclimated in the laboratory at room temperature, reared on compost soil and fed twice a week with a vegetable mixture for at least 1 month. The worms selected for this study were healthy and sexually mature adults (0.31 ± 0.05 g) according to OPPTS (1996) guidelines.

Artificial Soil Preparation

The artificial soil consisted of 68% silica, 20% kaolin clay, and 10% sphagnum peat moss (dry weight basis) (OPPTS 1996). Calcium carbonate (about 2%) was added to obtain pH equal to 7. The mixture was moistened with demineralised water up to 35% water content (by weight) and let stabilize overnight.

Sampling and Chemical Characterization of the Filters

PM₁₀ sample was collected in Taranto urban area (Apulia Region, South of Italy): the urban site was allocated according to Directive 2004/107/EC. Taranto (198,000 inhabitants) is a coastal city on the Mediterranean Sea; it is the chief city of the homonymous province and the third more densely-

populated city of the Southern Italy. The city, located in the middle of an area of 217 Km², is characterized by numerous maritime and military activities in the harbour area and high density of vehicular traffic in urban area. Sampling was performed by using a Graseby-Andersen (Smyrna, GA, USA) high volume sampler (model 1200) with a size selective inlet (SSI). The nominal flow was 70 m³/h, while the effective flow depended on the temperature and on the ratio between the pressure drop across the filter and the atmospheric pressure. PM₁₀ was collected on QM-A Whatman (Maidstone, Kent, UK) quartz filters (20 × 25 cm) for 24 hours. The filters were weighed, before and after the sampling, on a Genius Sartorius SE2-F analytical microbalance (Sartorius, Milan, Italy, sensitivity of 0.0001 mg) provided with an ionizer for electrostatic charges abatement in order to calculate PM₁₀ mass. Then, obtained filter was chemically characterized to determine PAH concentrations (Table 1). PAH determination was carried out extracting PM₁₀ samples by a microwave assisted solvent extraction (MASE) (Milestone s.r.l. model Ethos D, Sorisole (BG), Italy) and analyzing the extracted sample using an Agilent 6890 PLUS gas chromatograph (Agilent Technologies, Wilmington DE) interfaced to a mass selective spectrometer with an inert ion source (Agilent MS-5973 N) (Amodio *et al.*, 2007). Benzo[a]anthracene (BaA), benzo[b+j]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), indeno[1,2,3-cd]pyrene (IP), benzo[g,h,i]perylene (BgP) and dibenzo[a,h]anthracene (DbA) were detected according to Directive 2004/107/EC. Quantification was carried out based on the SIM acquisition mode using the signals corresponding to the molecular ions of PAHs: BaA (228), BbF (252), BkF (252), BaP (252), IP (276), DbA (278). Perylene-D12 (PrD, 264) was used as internal standard (I.S.). Part of exposed and clean filter was ground separately by means of a planetary ball mill (Retsch International) to obtain a very fine powder.

Filter Addition and Worm Exposure

The sampled ground filter was mixed to 10 g of artificial soil to set the following treatments: F1 - 6 mg of quartz filter (15 µg/g of PM₁₀ containing 0.008 µg/g of PAHs), F2 - 9 mg of quartz filter, (22.5 µg/g of PM₁₀ containing 0.012 µg/g of PAHs), F3 - 12 mg of quartz filter (30 µg/g of PM₁₀ containing 0.016 µg/g of PAHs) (Table 2). For each treatment, blank (FB) with powdered clean quartz filter (6, 9 and 12 mg) added to the artificial soil were set up. Moreover, a standard treatment was prepared with the addition of 0.015 µg/g of phenanthrene (PHE), a representative PAH, to the artificial soil following Northcott and Jones (Northcott and Jones, 2000). Each treatment was let to stabilize at 24°C in the dark for 14 days. Untreated sample (CTR) was set up using solely artificial soil. Each treatment was replicated simultaneously 5 times. Preliminary laboratory experiments showed that 10 g of artificial soil were suitable to support two earthworms; more specimens were stressed by scarce space and food. Consequently, two earthworms were exposed to each replicate for 7 days (10 earthworms in total for each treatment). The exposure time was chosen in accordance with previous preliminary

Table 1. Chemical characterization of quartz filter.

	Atmospheric concentration (ng/m ³)	Total mass on the filter (µg)	Concentration (µg/g of filter)
BaA	3.2	5.5	1.2
BbF	7.5	12.7	2.8
BkF	3.2	5.4	1.2
Bap	3.9	6.7	1.5
IP	5.0	8.5	1.9
BgP	3.7	6.3	1.4
DbA	1.8	3.0	0.7
PAHs	28.3	48.0	10.5

Table 2. Summary of the treatments; applied doses of xenobiotics.

Treatments	Quartz filter amount	PM ₁₀	PAHs	PHE	H ₂ O ₂
F1	6 mg	15 µg/g	0.008 µg/g		
FB1	6 mg	-	-		
F2	9 mg	22.5 µg/g	0.012 µg/g		
FB2	9 mg	-	-		
F3	12 mg	30 µg/g	0.016 µg/g		
FB3	12 mg	-	-		
PHE	-	-	-	0.015 µg/g	
CTR-	-	-	-		
CTR+	-	-	-		3%*

* Coelomocytes treated (with H₂O₂) in suspension.

experiments (data not shown) carried out with other contaminants and with Martin *et al.* (2005) that compared exposure time of 24 hours and 7 days. At least three worms for each treatment were subjected to the bioassay at the end of the exposure.

Comet Assay

Coelomocyte Collection

Exposed earthworms were rinsed with tap water and unfed for 24 h to empty their gut. Then, coelomocytes were extruded using ethanol and their viability was assessed by 0.4% Trypan blue exclusion dye (Vernile *et al.*, 2007). Coelomocytes samples showing at least 70% of viability were selected for the comet assay. Cell populations of each specimen were processed separately.

Positive Control Preparation

This assay was validated on coelomocytes extruded from earthworms reared on untreated samples (CTR) and incubated with 200 µM H₂O₂ for 30 min (CTR+).

Comet Assay Protocol

Comet assay protocol applied to coelomocytes of *E. andrei* was modified from Fourie *et al.* (2007). The comet assay kit and the pre-coated slides were supplied by Izkus Environment (Alessandria, Italy). Equal volumes of coelomocytes suspension and 1% low melting point agarose (LMPA) were mixed and applied to microscope slides pre-coated with 0.5% normal melting point agarose (Sigma-Aldrich). Obtained slides were kept at 4°C for 10 min before adding the third layer of 0.5% LMPA. Then, the slides were dipped into a cold lysis solution (2.5 M NaCl; 100 mM EDTA; 10 mM TRIZMA base; 1% Triton

X-100, with the final addition of 10% Dimethyl Sulfoxide, brought at pH 10 with 0.2 M NaOH) at 4°C in the dark, overnight. After washing the slides with cold distilled water to remove residues of the lysis solution, they were treated in a horizontal electrophoresis tank filled with fresh alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 25 min at room temperature to allow DNA unwinding and breakage at alkali labile sites. Electrophoresis was performed in the same buffer at room temperature, for 30 min at a constant voltage of 0.74 V/cm. After electrophoresis, slides were washed with a neutralizing buffer (0.4 M Tris, pH 7.5) and agarose micro-gel was stained with 300 µL of DNA dye FLUOplus (0.1 µL/mL) for 4 min. After removing the dye excess, slides were kept in a dark moist chamber until their observation at 200X magnification with a Nikon Eclipse E400 fluorescent microscope equipped with FITC filters (excitation 450–490, dichroic mirror 505, band pass 520). Images were recorded by a Nikon Coolpix 995 digital camera. For each comet, the tail length (distance between the head and the last DNA fragment) was recorded as index of the DNA damage and expressed in arbitrary units using the Comet Score™ freeware software (TriTekCorp). At least three treated earthworms were subjected to comet assay. Cells from each earthworm were used to prepare a single slide and at least 50 comets were screened for each slide (at least 150 comets were screened for each treatment). Collected data were expressed as mean values ± standard deviation. Mann-Whitney U-test was run for statistical analyses (Statistica software, Stat Soft®, Inc. Tulsa OK, USA).

Chemical Analysis of Earthworms

After the cell extrusion, the specimens were frozen at

–80°C and freeze-dried. Extraction of PAHs from earthworms was carried out using microwave-assisted solvent extraction (MASE) followed by filtration with 0.45 µm PTFE filters. For the quantification of the individual compounds, gas chromatography mass spectrometry (GC-MS) was applied in the same conditions of the filters (Amodio *et al.*, 2007).

RESULTS AND DISCUSSION

Comet Assay

Tail lengths of coelomocyte nuclei were always significantly more pronounced for earthworms exposed to soils spiked with polluted filters than those observed from exposure to blank filters (FB) and shorter than those measured for the positive control (CTR+) (Figs. 1.1, 1.3, 1.4, 2.2, 2.3, 2.4). The tail for F3 treatment (30 µg/g of PM₁₀ and 0.016 µg/g of PAHs) was a bit longer (50.95 ± 5.68 pixels) than that for F2 (22.5 µg/g of PM₁₀ and 0.012 µg/g of PAHs) resulting equal to 49.92 ± 1.76 pixels. However, both of them were significantly longer than 9.38 ± 0.64 pixels, observed in F1 (15 µg/g of PM₁₀ and 0.008 µg/g of PAHs) (F1 vs F2: $Z = 18.12$ $p < 0.001$; F1 versus F3: $Z = 6.37$ $p < 0.001$) showing, in part, a dose-dependent relationship. Specimens treated with a very low concentration of PHE (0.015 µg/g) provided a significantly longer tail (25.01 ± 0.83 pixels) than those observed in F1 ($Z = 12.87$ $p < 0.001$) and significantly shorter than those observed in F2 ($Z = -12.11$ $p < 0.001$) and F3 ($Z = -12.11$ $p < 0.001$) treatments (Figs. 1.2, 2). On the contrary, clean filter powder (FB) seemed to be largely harmless to the coelomocyte DNA because no differences were observed among three applied doses (tail lengths ranging from 2.90 ± 0.27 to 4.01 ± 0.49 pixels). However, as it is showed in Figs. 1.1, 1.2, 2.2, the nuclei of earthworms exposed to soil containing the clean filter showed a shorter tail length than that measured for worms kept in untreated artificial soil (CTR–: 17.87 ± 2.70 pixels) (CTR– vs. FB1: $Z = 9.24$ $p < 0.001$; CTR– vs. FB2: $Z = 8.36$ $p < 0.001$; CTR– vs. FB3: $Z = 7.97$ $p < 0.001$). The DNA damage detected on earthworms exposed in F1, F2 and F3 treatments did not seem to be bound to the quartz filters but to the presence of the mixture of pollutants caught on the filter which are more harmful than the phenanthrene one alone.

In this preliminary study, the percentage of DNA in the tail and the olive tail moment were not recorded along with the tail length. Usually, these latter parameters can avoid the underestimation of the toxic effects in case of high xenobiotic concentrations (McKelvey-Martin *et al.*, 1993; Duez *et al.*, 2004). In fact, the intensity of the tail increases after reaching its maximum extension from the head during its migration (Bowden *et al.*, 2003). This effect did not occur in the current experiments where the nuclei tail of earthworms exposed to the powder of the polluted filters could extend more, as demonstrated by the tails of coelomocytes treated with hydrogen peroxide (CTR+) in comparison with those obtained in F1, F2 and F3 treatments (Fig. 2). Applied concentrations of PM₁₀ were chosen arbitrarily, because of the lack of relative data in scientific literature on earthworms. Therefore, the current experiment

was started by testing different amounts of polluted filter (data not shown) in order to estimate the lowest dose able to produce a distinguishable tail of coelomocyte nuclei. This experimental approach, eliminating the pre-treatment of the filter, allowed reducing or avoiding losses of pollutants during their solvent extraction. In fact, the quartz filter was applied directly to the artificial soil just after being pulverized and without any chemical handling. This is quite useful when pollutants are collected in so small amount and applied solvent could be too selective in the xenobiotic extraction or not suitable in toxic evaluation. In addition, the sample can be directly assayed on a whole biological system, like live earthworms, it interacts with an integer immunological system and its effects are assessed by a very sensitive test. It is known that PM is deposited naturally on the soil surface by gravity or by adsorption on the water droplets (rain). Therefore, *Eisenia andrei*, such as other epigeic earthworms that burrows vertical tunnels reaching the soil surface, could come in contact with PM, even though in lowest concentrations, also in natural conditions.

Chemical Analysis on Earthworms

No PAHs were detected or quantified in the body of earthworms after solvent extraction: PAH concentrations were lower than the limit of detection (LOD) of the used analytical methodology (Table 3). However, it should be considered that in F3, the highest spiking treatment, the added amount of some PAHs was very close to the limit of the quantification estimated (LOQ): 1.4, 3.7, 1.4, 1.6, 2.5, 1.6 and 0.6 ng/g of soil for BaA, BbF, BkF, BaP, IP, BgP, and DbA, respectively. Therefore, we can say that the bio-concentration factor for each PAH in earthworms was below 1. This finding can be explained considering a partial sequestration of PAHs from the artificial soil, reducing the detectable amount inside their body, or degradation of all PAHs into other compounds. In order to confirm the finding obtained in this preliminary study, the analytical detection of adducts to DNA induced by PAHs in earthworms should be performed (Walsh *et al.*, 1997).

CONCLUSION

This preliminary study allowed investigating the impact of pollutants adsorbed on PM₁₀ collected on quartz filters by means of comet assay applied on earthworms exposed to contaminated soils. The negative toxic effect of the polluted filter compared to the blank one pointed out a clear relationship between these pollutants and the earthworms' immunological system. The high sensitivity of comet assay allowed to highlight this effect on DNA even at low PM₁₀ concentrations on which PAHs levels, not detected with conventional analytical techniques such as GC-MS, were adsorbed. Any bioaccumulation was estimate in the bodies of earthworms by applying MASE extraction and GC-MS analysis. This finding might be related to the very low concentration of pollutants in the spiked samples, to the sensitiveness of the analytical method and to the fate of PAHs in the earthworm bodies or in the artificial soil. Therefore, sampled filters added to the artificial soil and

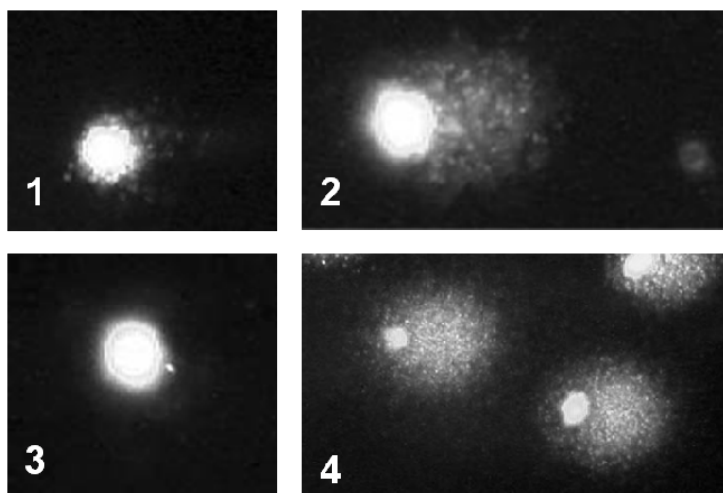


Fig. 1. Coelomocyte cells extruded from earthworms exposed to untreated artificial soil (1), to PHE spiked soil (2), to blank quartz filter FB2 (3) and to polluted quartz filter F2 (4).

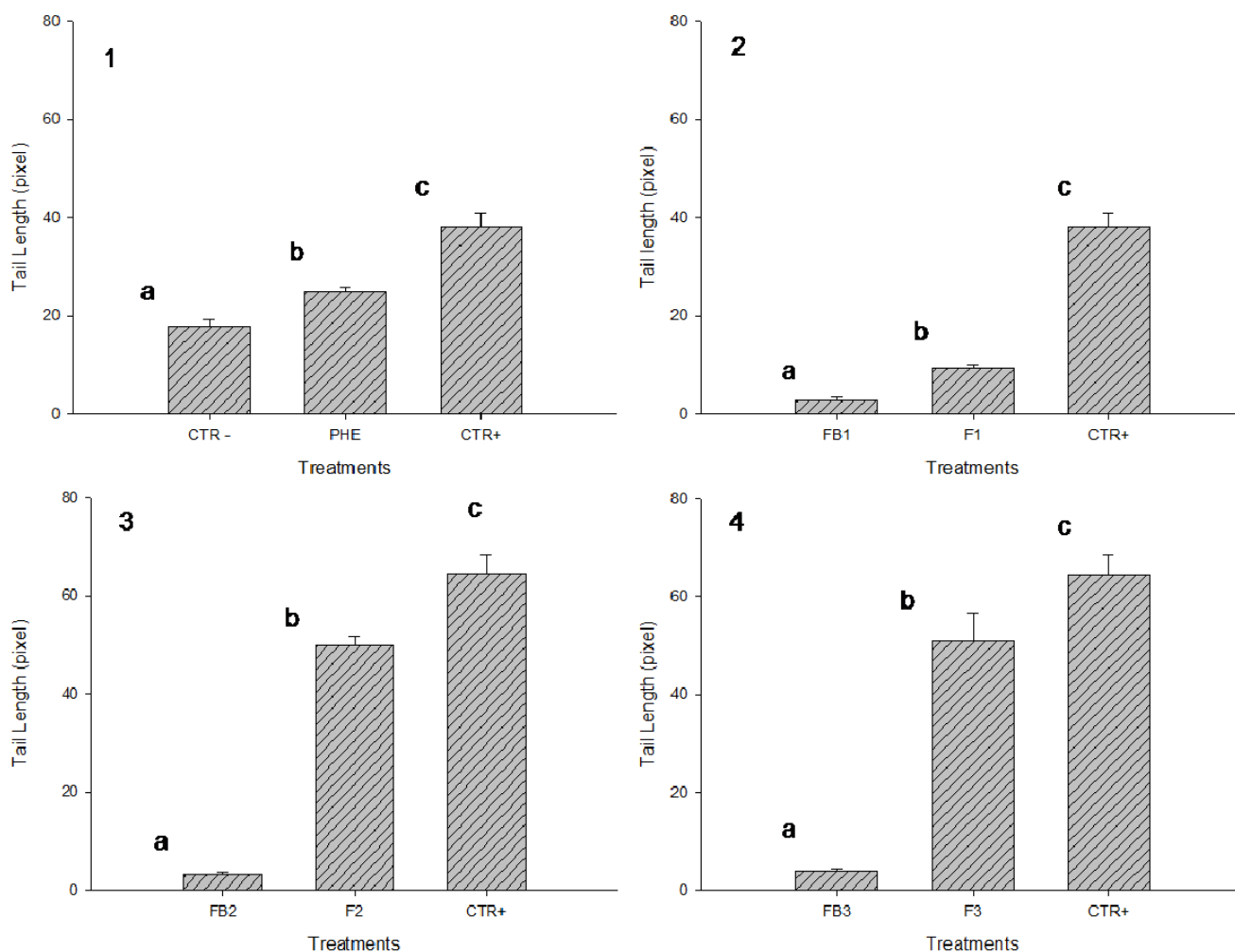


Fig. 2. Comet assay comparison of tail length of coelomocytes extruded from earthworms exposed to: 1). CTR-, untreated artificial soil; PHE, soil spiked with 0.015 $\mu\text{g/g}$ of PHE. 2). FB1, soil with 6 mg of clean quartz filter; F1, soil with 15 $\mu\text{g/g}$ of PM_{10} and 0.008 $\mu\text{g/g}$ of PAHs. 3). FB2, soil with 9 mg of clean quartz filter; F2, soil with 22.5 $\mu\text{g/g}$ of PM_{10} and 0.012 $\mu\text{g/g}$ of PAHs. 4). FB3, soil with 12 mg of clean quartz filter; F3, soil with 30 $\mu\text{g/g}$ of PM_{10} and 0.016 $\mu\text{g/g}$ of PAHs. For all cases: CTR+, coelomocytes treated with H_2O_2 . Means \pm SE were reported. Different letters indicate a significant difference among treatments at the $p < 0.001$ level (non-parametric Mann-Whitney U -test).

Table 3. Limit of detection (LOD) and limit of quantification (LOQ) for all investigated PAHs expressed as pg/mL. LOQ values were also calculated as ng of single PAHs on the fresh weight of earthworms (g).

PAHs	BaA	BbF	BkF	Bap	IP	BgP	DbA
LOD (pg/mL)	23	38	23	6	4	25	30
LOQ (pg/mL)	77	127	77	20	13	83	100
LOQ (ng/g)	0.82	1.35	0.82	0.21	0.14	0.88	1.06

earthworms seem to constitute a suitable model to assess in advance a mean risk of exposure to air pollution. Moreover, the use of not chemically handled sampled filters, eliminating the use of organic solvent, could provide a new methodological and multidisciplinary approach in ecotoxicological research, which should be standardized and encouraged in further studies.

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