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Effects of PAHs and dioxins on the earthworm *Eisenia andrei*: a multivariate approach for biomarker interpretation

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Abstract

In this study, a battery of biomarkers was utilised to evaluate the stress syndrome induced in the earthworm *Eisenia andrei* by exposure to environmentally realistic concentrations of benzo[*a*]pyrene (B[*a*]P) and 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) in OECD soil. The set of tests was then employed to assess the toxicity of field soils contaminated with organic xenobiotic compounds (such as PAHs, dioxins and PCBs). The results highlighted an impairment of immune and metabolic functions and genotoxic damage in worms exposed also to lower bioavailable concentrations of toxic chemicals. Multivariate analysis of biomarker data showed that all different contaminated soils had a detrimental effect on the earthworms. A separation between temporal and concentration factors was also evident for B[*a*]P and TCDD treatments; and field contaminated soils were further differentiated reflecting a diverse contamination. Multivariate analysis also demonstrated that lysosomal membrane stability can be considered a prognostic indicator for worm health status.

Capsule: Biomarkers were employed in *E. andrei* in laboratory and field studies. Multivariate analysis ranked the stress syndrome in worms. Lysosomal stability is prognostic for health status.

Keywords: earthworms; lysosomal membrane stability; biomarkers; multivariate analysis

1. Introduction

Biomarkers are sensitive tools for detecting exposure and adverse effects of toxic chemicals both on aquatic and terrestrial organisms (Moore et al., 2004; Peakall, 1994; Scott-Fordsmand and Weeks, 2000). The study of biological parameters at different level of functional complexity in diverse cells and tissues of the organisms and with different meaning (i.e. biomarkers of stress, exposure and genotoxicity) is useful to clarify the mechanisms of action of chemicals as well as to determine the level of pollutant-induced stress syndrome in animals exposed to environmental matrices, where a mixture of many different contaminants may be present (Asensio et al., 2013; Binelli et al., 2010; Cajaraville et al., 2000; Sforzini et al., 2011; Turja et al., 2014; van der Oost et al., 2003; Viarengo et al., 2007a, 2007b). However, this approach may result in an unclear correlation structure of the data; an aspect that could be more evident analyzing results obtained in field studies than from laboratory experiments, using known doses of a single substance. In addressing this problem, previous studies increasingly suggest that multivariate analysis techniques are a useful tool for interpreting multiple biomarker responses as they produce a two-dimensional pattern of the degree of similarity between different groups of data (Allen and Moore, 2004; Astley et al., 1999; Bernet et al., 2000; Brenner et al., 2014; Burgos et al., 2005; Galloway et al., 2004; Garmendia et al., 2011; Sanchez et al., 2007). The use of multivariate analyses to identify prognostic biomarkers, useful to provide better risk assessment at the early stages, is also of undoubted importance (Jenkins et al., 2011; Moore et al., 2006; Ortiz et al., 2011).

In the last decades, earthworms acquired a position of growing importance in terrestrial ecotoxicology. These animals have been shown to accumulate and respond to contaminants at various levels of complexity ranging from the whole animal to the most sensitive molecular/cellular changes (Caselli et al., 2006; Dimitrova et al., 2010; Hayashi et al., 2013; Santoyo et al., 2011; Sforzini et al., 2012; Spurgeon and Hopkin, 1999; Ville et al., 1995; Yang et al., 2012).

In this study, a battery of biomarkers was utilised to evaluate the alterations in the health status induced in the earthworm *Eisenia andrei* (Bouché, 1972) by exposure to environmentally relevant concentrations of benzo[*a*]pyrene (B[*a*]P) and 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) in OECD soil (OECD, 2004). These hazardous environmental chemicals, classified by the US EPA (Environmental Protection Agency) as priority pollutants, are among the frequently occurring soils contaminants (Martínez et al., 2006; USEPA, 2009; Wang et al., 2007). Stress responses at the various levels of biological organisation (including lysosomal membrane stability, lipofuscin and neutral lipid accumulation and tissue damage) were measured; the genotoxic effects caused by these substances on worms, in terms of both DNA and chromosomal damage, have previously been demonstrated (Sforzini et al., 2012). To verify the robustness of the selected multi-biomarkers in *E. andrei* to be used for the assessment of polluted natural soils, the set of tests was then applied in a field study conducted to evaluate the potential toxicity of soils contaminated by organic xenobiotic compounds (such as PAHs, dioxins, PCBs), as a consequence of different anthropogenic activities. Multiple biomarker responses in worms exposed to different chemical treatments and field contaminated soils were analysed by multivariate statistics **in order to identify any discernable similarities or dissimilarities in multidimensional biomarker response patterns.** An overarching objective was the use of predictive models of lysosomal and other biomarker reactions as both diagnostic and prognostic biomarkers for health status in the earthworms. For this purpose, multivariate analysis has been previously used to develop statistical models to study the role of lysosomal functions and responses to environmental variables, particularly chemical pollutants (Allen & Moore, 2004; Moore et al., 2006).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless otherwise indicated.

2.2. Animals

Earthworms were cultured essentially as described in the OECD guideline (OECD, 2004). Organisms were selected from a synchronised culture with an homogeneous age structure. Adult worms with clitellum of similar size and weight (of 400 to 500 mg) were utilised in the experiments.

2.3. Artificial soil test

The artificial soil tests were performed as described in the OECD guideline for the testing of chemicals (OECD, 2004). The tests were performed under controlled conditions for a period of 10 and 28 d. The selected B[*a*]P and TCDD concentrations were i) beginning with the lowest, the Italian law limit for residential areas, ii) for industrial areas, iii) five (B[*a*]P) and twenty (TCDD) times higher than the latter (for details see Sforzini et al., 2012). Vitality and reproduction were assessed at the end of the experiments.

2.4. Field soils

Five soils contaminated by organic xenobiotic compounds (i.e. PAHs, dioxins and PCBs) were collected from different areas in the Campania region (Italy): Site 1 (4536773/446660) is located close to the site of construction of an incinerator; Sites 2 and 3 (4538083/442802 and 4531906/424656, respectively) are industrial areas; Sites 4 and 5 (4540699/443848 and 4535670/440538, respectively) are areas subjected to uncontrolled waste fires. **The chemical analysis of these samples were performed by ISPRA (…) and are a part of a larger study published by Italian Ministry of the Environment (ISPRA, 2012).** As shown in Table 1, the

concentrations of the chemicals in the soils, in only a few cases, were higher than the limits for residential areas set by Italian law, but always lower than the limits for industrial areas (ISPRA, 2012). A control field soil was collected from a site with no detectable soil contamination (Reference site - 4569424/470553). Particle-size distribution, organic matter content and pH of soils from the different Sites were homogeneous (ISPRA, 2012).

Soil samples were collected from the top 0-5 cm layer at each site after removal of surface vegetation and litter. Each of these samples represented a composite of five subsamples from the center and four corners of a square sampling grid. Soil subsamples at each location were homogenized and transferred to clean containers. Soils were dried, sieved through a 2 mm mesh, moistened with deionized water and then, for each soil replicate, ten worms were kept in 500 g of soil placed in glass test containers. At least five replicates per soil were used. The test containers were maintained in a climatized chamber with a temperature of 20 ± 1 °C. The test was performed under controlled light-dark cycles (16 h light, 8 h dark) with illumination of 800 lx and for a period of 10 days. Vitality was assessed at the end of the incubation.

2.5. Biomarker tests

A battery of biomarkers was used to evaluate the harmful effects induced in worms by increasing concentrations of B[*a*]P, TCDD and field contaminated soils.

Coelomocytes used for the determination of lysosomal membrane stability (LMS) and MNi frequency were obtained by a non-invasive extrusion method i.e. ethanol extraction (Eyambe et al., 1991; Fugère et al., 1996; Sforzini et al., 2012). Briefly, earthworms were rinsed in saline (0.85 mg/ml NaCl at 4 °C) and the posterior portion was massaged to expel the gut contents of the terminal part of the intestine. Then, animals were placed in 4 ml of cold extrusion medium containing 5% ethanol, 2.5 mg/ml EDTA, 10 mg/ml of the mucolytic agent guaiacol glycerol ether (pH 7.3). After 3 min, the earthworms were removed and 2 ml of Hanks' balanced salt

solution (HBSS) (Sigma product H6648) were added. The cell suspension was centrifuged at 150 × g at 4 °C for 2 min to remove mucus, and then for 10 min to recover the cells. Chloragogenous tissue was used for the determination of LMS, lipofuscin and neutral lipid lysosomal content and lysosomal/cytoplasm volume ratio. At the end of the incubation, worms were washed and then kept on clean moist filter paper for 24 h to allow them to void their gut contents Pieces of five earthworms (4-5 mm length, posterior to the clitellum), were placed on an aluminium cryostat chuck and chilled in hexane at -70 °C as described previously (Sforzini et al., 2014).

LMS of amoeboid coelomocytes was evaluated by the neutral red retention time (NRRT) assay by fluorescence microscopy (Sforzini et al., 2011).

The determination of LMS in the cells of chloragogenous tissue was performed on cryostat tissue sections following essentially the method described by Moore (1988). This cytochemical assay is based on acid labilization characteristics of latent hydrolase β-*N*-acetylhexosaminidase (NAH) using naphthol AS-BI-N-acetyl-β-D glucosaminide as a substrate for NAH.

Lipofuscin and neutral lipid lysosomal content in the cells of the chloragogenous tissue as well as the lysosomal/cytoplasmic volume ratio (L/C) of this tissue were evaluated as described by Sforzini et al. (2011).

The micronucleus test in coelomocytes of worms exposed to field soils was performed following the methods described in Sforzini et al. (2012).

2.6. Univariate statistical analysis

At least five replicates per control and per treatment were analyzed. For the biomarker data obtained in coelomocytes, each replicate consists of cells from two earthworms pooled together; the two animals were collected from a separate replicate of soil, consisting of 10 earthworms incubated in 500 g of soil. For the biomarker data obtained in chloragogenous

tissue (cryostat sections), pieces of five earthworms, each one collected from a separate replicate soil, were analysed.

The non-parametric Mann-Whitney *U*-test was used to compare the data from treated earthworms with those of the controls ones.

2.7. Multivariate analysis

Biomarker data for earthworms exposed to B[*a*]P, dioxin and field soils from Campania Region were analysed using non-parametric multivariate analysis software, PRIMER v 6 (PRIMER-E Ltd., Plymouth, UK; Clarke, 1999). All data were log transformed [$\log_n(1+x)$] and standardised to the same scale. Principal component analysis (PCA), hierarchical cluster analysis and non-metric multi-dimensional scaling analysis (MDS), derived from Euclidean distance similarity matrices were used to visualise dissimilarities between sample groups. The results were further tested for significance using analysis of similarity (PRIMER v6 - ANOSIM), which is analogous to a univariate ANOVA and reflects on differences between treatment groups in contrast to differences among replicates within samples (the *R* statistic). Under the null hypothesis H_0 ("no difference between samples"), *R* = 0 and this was tested by a non-parametric permutations approach; there should be little or no effect on the average *R* value if the labels identifying which replicates belong to which samples are randomly rearranged.

The PRIMER v6 - BIO-ENV routine (Spearman's Rank Correlations, Rho) linking multivariate biomarker response patterns was used to identify "influential biomarkers" - small subsets of biomarkers capturing the full MDS biomarker response pattern.

Finally, in order to map integrated biomarker data onto "health status space" by using LMS; first principal components (PC1) for the biomarker data were derived using PRIMER v6 and then plotted against the LMS (as a measure of cellular well-being) values for each treatment/field sample (Allen and Moore, 2004; Moore et al., 2006).

3. Results

3.1. Biomarker responses

The assessment of the different biomarker responses in worms exposed for 10 and 28 d to increasing concentrations of B[*a*]P and TCDD in OECD soil demonstrated significant changes in treated worms (Fig. 1); at the concentrations used no effect on vitality and reproduction rate was found (data not shown).

LMS in coelomocytes of worms exposed for 10 d to both B[*a*]P and TCDD showed a significant decrease with respect to controls at the two higher concentrations; in particular, very strong effects were observed at 10 ppm B[*a*]P (-91% with respect to controls) and 0.1 and 2 ppb TCDD (-81% and -99% with respect to controls respectively) (Fig. 1A, B). In animals exposed for 28 d, significant changes were observed at all the concentrations of the two chemicals, with maximal effect at the highest one, i.e. 50 ppm B[*a*]P and 2 ppb TCDD (-70% and -88% with respect to controls respectively) (Fig. 1A, B).

The lysosomal responses in the cells of the chloragogenous tissue, in term of lipofuscin and neutral lipid accumulations, highlighted relevant variations in worms exposed for 28 d to the higher chemical concentrations (Fig. 1C, D and Fig. 1E, F respectively). In particular, a significant increase in lipofuscin content was observed in worm exposed to 0.1 ppb TCDD (+78% with respect to controls); the results of neutral lipid lysosomal content indicated a significant increase at 10 ppm and 50 ppm B[*a*]P (+53% and +36% with respect to controls respectively), with maximal effect at the highest dioxin concentration (+114% with respect to controls).

B[*a*]P and TCDD also caused an increase in the L/C (Fig. 1G, H). In particular, significant changes were observed in worms exposed for 10 d to 10 ppm and 50 ppm B[*a*]P and to all the dioxin concentrations; the alteration being particularly relevant at 0.1 and 2 ppb TCDD (+44% and +45% with respect to controls). After 28 d, the values measured were significant at all the doses of both

chemicals, with greater effects in animals exposed to 10 and 50 ppm B[*a*]P (+52% and +64% with respect to controls).

The results of biomarkers in worms exposed for 10 d to field soils collected in Campania, highlighted significant alterations at the contaminated sites (Sites 1-5) with respect to reference site (Fig. 2), without resulting in mortality (data not shown). It is important to point out that animals exposed to the reference site soil did not show changes with respect to laboratory controls (data not shown).

The results of LMS in the cells of the chloragogenous tissue indicated a significant reduction in worms exposed to soils collected from all the contaminated sites (Site 1-5), with maximal effect at Site 3 (Fig. 2A).

In the cells of the same tissue, significant changes were observed also in the other lysosomal biomarkers. In particular, the results of lysosomal accumulation of lipofuscin highlighted a significant increase in lipofuscin content in worms exposed to Sites 2, 4 and 5, with greater effect at Site 2 (+173% with respect to the Reference Site~~);~~). I~~i~~n animals exposed to Site 1, the value was significantly lower with respect to the Reference site (-78%) (Fig. 2B). Lysosomal accumulation of neutral lipids showed significant changes only in worms exposed to Sites 2-3, with a slight increase at Site 3 (+23%) and a marked reduction at Site 2 (-66%) with respect to the Reference site (Fig. 2C).

L/C in the chloragogenous tissue showed a significant increase in worms exposed to soils from all the contaminated sites (Sites 1-5); the effect was greater at Sites 3 and 4 (+44% and +45% with respect to Reference site respectively) (Fig. 2D).

The results of MN test in coelomocytes of worms exposed to soils from Sites 1-5 showed a significant increase of chromosomal damage with respect to the Reference site; the MNi frequency was higher at Sites 2 and 4 (7.5 ‰) (Fig. 2E).

3.2. Multivariate analysis of biomarker responses

Principal component analysis (PCA), multi-dimensional scaling (MDS) and hierarchical cluster analysis of the biomarker responses in worms exposed to B[*a*]P and TCDD (in addition to the results shown in this investigation, the data ~~of~~ for DNA damage and micronuclei induction obtained in a previous study -Sforzini et al., 2012), showed that **both chemicals had a detrimental effect on the earthworms. This effect is indicated by the ~~(delete): there is a~~ clear separation between the clusters ~~of~~ for the control groups and the treatment groups~~. Due to the biological meaning of these biomarkers of stress, the results could be interpreted as detrimental effects of both chemicals on the earthworms~~** (Fig. 3). Analysis of similarity shows that these clusters are significantly different (ANOSIM, R Statistic: B[*a*]P $R = 0.876$, $P < 0.001$; dioxin $R = 0.772$, $P < 0.001$). **The PCA analysis ~~was~~ also ~~suitable to~~ highlighted a separation between control worms and ~~animals~~ those exposed to the lower chemical concentration. In particular, at 0.1 ppm** B[*a*]P **after 28 d, the different distribution of the data seems to be due mainly to LMS and L/C ratio. In dioxin exposed animals (lower dose after 28 d) the main contributions (separation factors) seems to be MNi frequency and L/C ratio with a contribution of LMS.**

Multivariate analysis of the biomarker reactions from field soil samples collected in Campania also showed clear differences between the reference and contaminated samples (ANOSIM, $R = 0.815$, $P < 0.001$). Sites 1 and 5 (primarily PAHs) were clustered together and Site 2 (primarily dioxins and furans) was clearly distinct from all the other samples (Fig. 3)**; from this figure and the data reported in Fig. 2, it is evident that the separation of Site 2 is mainly due to the ~~huge~~ large difference in LF (positive) and LN (negative) from all the other treatments.**

Multiple regression analysis of the biomarker data indicated that some of the biological parameters are correlated (Fig. 4), although there is no consistent pattern of correlations across the various treatments. However, the BIO-ENV routine indicated that the lysosomal parameters were influential biomarkers in all three exposure studies. Combinations of lysosomal biomarkers showing

267 significant capture of the full MDS pattern are shown in Table 2. Experimental exposure of worms
268 to B[*a*]P and TCDD as well as to Campania soil samples resulted in LMS + lipofuscin (LF) + L/C
269 emerging as the strongest combinations of lysosomal biomarkers (Table 2).
270 By plotting LMS against the first principal component (PC1) of selected biomarker data (Fig. 5), we
271 effectively integrate the multi-biomarker results and the graph reflects the gradient of toxicity
272 between the samples. PC1 is a measure of the contaminant gradient with the left-hand side being the
273 most impacted and the right-hand side the least affected (Fig. 5). The correlation between LMS and
274 PC1 is highly significant for B[*a*]P and TCDD treatments. In the Campania data, the correlation is
275 also significant, but it is not as strong as in the experimental treatments in spiked soil (Fig.5).
276

277 ## 4. Discussion

278 Hydrophobic organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), dioxins and
279 furans (PCDD/Fs), and polychlorinated biphenyls (PCBs), are common contaminants in soils.
280 These toxic and genotoxic chemicals, which are generally strongly bound to soil particles, tend to
281 persist in the soil and can bioaccumulate, increasing the potential hazard for the ecosystem
282 (Baderna et al., 2013; Valentín et al., 2013).
283 In a previous study, we have demonstrated that the exposure of the earthworm *E. andrei* for 10 and
284 28 d to sublethal concentrations of B[*a*]P and TCDD has resulted in genotoxic effects (Sforzini et
285 al., 2012). In particular, even the lowest doses of the two chemicals utilised (representing the limit
286 for residential and industrial areas set by Italian low) induced in treated animals both DNA and
287 chromosomal damage. To further investigate the adverse effects caused by these chemicals on
288 worm health status, in this study the responses of well-established biomarkers of stress, namely
289 lysosomal membrane stability (LMS), the lysosomal lipofuscin content, the neutral lipid
290 accumulation in lysosomes (cell-level biomarkers); and the lysosomal/cytoplasmic volume ratio
291 (L/C, biomarker of tissue damage), were evaluated.

In B[*a*]P and TCDD-exposed worms, amoeboid coelomocytes (immunocompetent cells circulating in the coelomic fluid - Adamowicz, 2005; Cooper and Roch, 2003), showed a significant reduction of LMS; the effect was evident at all the different concentrations and at the shorter exposure time. **It is interesting that at the medium concentration used for B[*a*]P and TCDD (10 ppm and 0.1 ppb, respectively), the strongest effect was observed after 10 d of exposure with a partial recovery at 28 d. This effect is ~~of~~ difficult to interpret~~ation.; among t~~The possible explanations~~, there are~~include the biological responses of the animals~~, also~~ related to the turnover time of the coelomic cells in this ~~organism~~species of worm.** This type of subcellular pathological reaction represents an extremely sensitive general index of cellular condition, able to reveal the early adverse effects of pollutants in different animal models (Moore et al., 2012). The neutral red retention time (NRRT) assay has been successfully applied for the *in vivo* evaluation of LMS in fish hepatocytes, in the blood cells of a wide range of marine and freshwater invertebrates as well in earthworms coelomocytes (Binelli et al., 2009; Camus et al., 2000; Canesi et al., 2006; Guidi et al., 2010; Hauton et al., 1998; Lowe and Pipe, 1994; Moore et al., 2009; Sforzini et al., 2011; Weeks and Svendsen, 1996; Winzer et al., 2002).

Chloragogenous tissue surrounding the intestine, where most of the key metabolic processes occur (Prentø, 1987), also presented significant changes in treated animals. In particular, a significant lysosomal accumulation of lipofuscin (end-products of membrane lipid peroxidation - Terman and Brunk, 2004; Viarengo and Nott, 1993) and of neutral lipids (due to unbalanced fatty acid metabolism - Lüllman-Rauch, 1979) was induced in the chloragocytes of worms exposed for 28 d only to some concentrations. B[*a*]P and TCDD are known to provoke an increase of the cellular levels of reactive oxygen species (ROS) (Gelboin, 1980; Lin et al., 2007); and there is evidence that these chemicals can have an impact on lipid metabolism (Irigaray et al., 2006; Schiller et al., 1985). However, we need to consider that in the cells of the chloragogenous tissue of *E. andrei* exposed to different kinds of pollutants, accumulation of lipofuscin and neutral lipid ~~accumulation~~ in

lysosomes typically shows a bell-shaped trend (Gastaldi et al., 2007; Sforzini et al., 2011). This ~~could~~ can be partly explained by an enhanced rate of elimination of lipofuscin/neutral lipid-rich residual bodies into the coelomic fluid as well as by an augmented rate of turnover of chloragocytes, since several studies showed that chloragocyte depletion may occur in worms as a way of eliminating toxic chemicals (Cancio et al., 1995; Fischer and Molnár, 1992). Furthermore, when we also consider the results of the L/C, a biomarker reflecting the level of cellular autophagy (Lowe et al., 1981), there is a clear indication that an impairment of the physiology of the whole tissue was already occurring after 10 d at the lower doses of both chemicals.

Overall, the results obtained showed that environmentally realistic concentrations of B[*a*]P and TCDD in OECD soils, without affecting vitality and reproduction of earthworms, provoked significant alterations in the physiological status of the organisms. Relevant changes at cellular and tissue levels were observed in animals exposed, for only 10 d, to lower contaminant chemical concentrations.

To verify the robustness of the selected multi-biomarkers in *E. andrei* to be used for the assessment of polluted natural soils, the set of tests was then applied in a field study conducted to evaluate the potential toxicity of five soils contaminated by organic xenobiotic compounds (such as PAHs, dioxins, PCBs), as a consequence of different anthropogenic activities. Chemical analysis revealed that the concentrations of chemicals in soils were, in only a few cases, higher that the Italian law limits for residential areas.

Worms incubated for 10 d in the different contaminated soils showed sublethal stress and genotoxic effects. A significant decrease in LMS in the cells of the chloragogenous tissue (based on acid labilization characteristics of the latent hydrolase β-*N*-acetylhexosaminidase - Moore, 1988; Peeters-Joris, 2000) was observed at all sites; the alteration was particularly relevant in Site 3 (contaminated primarily with PCBs). Initial studies on the toxic effects of contaminants on lysosomes were originally carried out to determine LMS by using histochemical procedures applied

342 on frozen tissue sections of fish liver or mussel hepatopancreas (Köhler, 1991; Moore, 1976, 1990).
343 This methodology is currently used by many researchers in the laboratory as well as in field studies
344 (Banni et al., 2014; Broeg et al., 1999; Domouhtsidou and Dimitriadis, 2001; Franzellitti et al.,
345 2012; Köhler et al., 2002; Roméo et al., 2000; Shaw et al., 2011; Viarengo et al., 1987). Once the
346 tissues are frozen, it is possible to preserve them until the analysis, an aspect that could be important
347 when large-scale biomonitoring studies are performed.
348 PCDD in field soils (Sites 2, 4) at concentrations similar to those spiked in OECD soil induced
349 (differently from that observed in dioxin treatments) a significant increase of lipofuscin content; a
350 minimal, although significant, change was observed also in worms exposed to Site 5 (primarily
351 contaminated with PAHs). The results of lysosomal accumulation of neutral lipids indicated a
352 significant increase in neutral lipid content in Site 3 (primarily with PCBs).
353 The bioavailable contaminants present in the polluted soils also induced in the chloragogenous
354 tissue a significant increase of L/C; the higher tissue alteration being detected Sites 3 and 4
355 (primarily contaminated with PCBs and PCDD-PCDF, respectively).
356 Finally, all contaminated soils resulted genotoxic, as indicated by the MN test showing a significant
357 increase in MNi frequency in coelomocytes of exposed worms. The MN test has emerged as an
358 alternative approach to the classical techniques for assessing cytogenetic damage (Schmid, 1975).
359 The test procedure is technically easier and faster than the analysis of chromosomal aberrations
360 during metaphase. The MN test has been widely applied in human and other mammalian cell types,
361 amphibians, fish and molluscs (Bolognesi and Hayashi, 2011; Fenech, 2000); and recently this
362 method has also been developed on *E. andrei* coelomocytes (Sforzini et al., 2012).
363 Taken together, the results obtained in the field study confirm that LMS and L/C as well as MNi
364 frequency are sensitive biomarkers able to highlight the stress syndrome induced in worms by
365 exposure to bioavailable chemicals present in soils. The evaluation of lipofuscin and neutral lipid
366 lysosomal accumulation (biomarkers widely used in monitoring studies, easy to perform and not

expensive) is important in order to highlight additional relevant adverse effects on animal health. However, these parameters in worms have to be used together with LMS and L/C ratio (showing a clear response profile over a stress gradient) to avoid a possible misinterpretation when negative results are obtained.

Procedures capable of integrating and interpreting the biomarker responses within synthetic stress indices have been developed (Beliaeff and Burgeot, 2002; Dagnino et al., 2007; Moore et al., 2004; Narbonne et al., 1999; Sforzini et al., 2011; Viarengo et al., 2000). An addition useful tool for interpreting multiple biomarker responses is multivariate statistics as they produce a two-dimensional pattern of the degree of similarity between different groups of data (Allen and Moore, 2004; Astley et al., 1999; Bernet et al., 2000; Brenner et al., 2014; Burgos et al., 2005; Galloway et al., 2004; Garmendia et al., 2011; Sanchez et al., 2007).

Multivariate analysis indicated that the battery of biomarkers deployed in this study (lysosomal reactions and indicators of genotoxic damage) can effectively discriminate between experimental treatments of earthworms exposed to B[*a*]P~~,~~ and dioxin spiked ~~in~~ OECD soil and various types of ~~polluted~~ contaminated field soils (i.e. Campania samples). In particular, multivariate analysis of biomarker data showed that all of the different contaminated soils had a detrimental effect on the earthworms; control animals being clearly separated from the treated ones. Among these latter, worms exposed for 28 d to the highest B[*a*]P and TCDD concentration as well to soil from Site 2 (primarily dioxins and furans) were the most distant from the controls, these animals showing greater biological effects.

A separation between temporal and concentration factors were also evident for B[*a*]P and TCDD treatments; while, as for the Campania samples, Sites 1 and 5 (primarily PAHs) were clustered together and Site 2 (primarily dioxins and furans) was clearly distinct from all the other samples.

Many pollutants may exert toxicity and genotoxicity directly, as well as through oxidative stress. The resulting (oxidative) damage to membranes, proteins and DNA contribute to decreased protein

392 synthesis, cell injury and physiological dysfunction (Kirchin et al., 1992; Lowe et al., 2006; Moore
393 et al., 2006;Viarengo, 1989). LMS in blue mussels is directly correlated with total oxyradical
394 scavenging capacity (TOSC), polyribosome formation (translational efficiency in initiation of
395 protein synthesis); and inversely proportional to DNA damage (micronuclei formation), lipofuscin
396 (age/stress pigment, ceroid lipofuscin) formation, lysosomal swelling and autophagic accumulation
397 of lipid (Dailianis et al., 2003; Kalpaxis et al., 2004; Krishnakumar et al., 1994; Moore et al., 2006;
398 Regoli, 2000).
399 In this study, multiple regression analysis of the biomarker data indicated that in all three exposure
400 studies LMS is significantly correlated to L/C, and both these lysosomal parameters to MNi
401 induction.
402 Although lipofuscin content showed no consistent pattern of correlations across the various
403 treatments, the BIO-ENV routine indicated that experimental exposure of worms to B[*a*]P and
404 TCDD as well as to Campania soil samples resulted in LMS + lipofuscin (LF) + L/C emerging as
405 the most effective combination of lysosomal biomarkers (Table 2).
406 The recent developments in many research fields are leading to the discovery of prognostic
407 biomarkers that could be suitable as risk indicator of pathologies (Berghella et al., 2014; Jenkins et
408 al., 2011; Moore et al., 2006; Ortiz et al., 2011). Many biomarkers probably only exhibit a response
409 in a part of the "health status space" (Allen and Moore, 2004; Depledge et al., 1993; Moore et al.,
410 2006); where they will indicate that a reaction has taken place and may even indicate health status
411 within a narrow range, or what has induced the response, but they do not generally indicate the
412 health status of the whole range from healthy to irreversible damage (Köhler et al., 2002). In terms
413 of environmental prognostics, the first stage is to relate biomarker responses to health status of
414 individual organisms by mapping the said responses against an integrated "health status" indicator
415 (Allen and Moore, 2004; Köhler et al., 2002; Moore et al., 2004, 2006).

Lysosomes have attracted a great interest in the field of ecotoxicology as they are the target of a wide range of contaminants (Allison and Mallucci, 1964; Moore et al., 2009; Sforzini et al., 2014; Viarengo et al., 1985, 2007b) and they are present in all nucleated cells. Lysosomes contain numerous hydrolytic enzymes involved in diverse cellular processes including the degradation of cellular and extracellular macromolecules (Moore, 1976; Pipe, 1993). The evidence is steadily accumulating that LMS is a generic indicator of cellular health in eukaryotic cells, as is indicated by studies with protozoans, coelenterates, annelids, crustaceans, molluscs, fish and mammals (Lin et al., 2010; Moore et al., 2012; Sohaebuddin and Tang, 2013). This parameter is now considered a highly sensitive biomarker that allows to follow the evolution of the stress syndrome from its early phase to the development of pathological conditions (Moore, 1988; Moore et al., 2004). LMS has been used in the liver cells of the flatfish flounder (*Platichthys flesus*) to predict the degree of liver degeneration (from cell injury through to hepatocellular carcinoma) as a result of PAH and organochlorine exposure (Köhler et al., 2002); and lysosomal integrity in hepatopancreatic digestive cells of mussels is directly related to scope for growth; and also, in the digestive cells of oysters (*Crassostrea virginica*) to larval viability (Allen and Moore, 2004; Ringwood et al., 2004).

A useful method of integrating biomarker data into a "health status space" involves the use of Principal Components Analysis (PCA) to reduce the dimensionality of the problem to a simple two dimensional representation (Allen and Moore, 2004; Chatfield and Collins, 1980). PCA is commonly used as a cluster analysis tool and is designed to capture the variance in a dataset in terms of principle components. Hence, by plotting the first principal component (PC1) of the selected biomarker data against LMS, as an indicator of the gradient from health to pathology and disease, we effectively integrated the multi-biomarker data and the graph reflects the gradient of toxicity among the samples. **These results clearly ~~showed~~ indicate that LMS is prognostic ~~of~~ for the health status of the earthworms treated with benzo(a)pyrene and TCDD; and also in worms exposed to contaminated field samples from Campania.~~;~~ F~~f~~urther research on the**

second/third worm generation will clarify if this parameter is also prognostic of negative effects at population level.

5. Conclusion

Overall, the biomarkers utilised in this study were able to reveal in *E. andrei* the toxic and genotoxic effects of even low levels (close to the Italian legal limits for contaminants in residential areas) of bioavailable pollutants in OECD as well as in natural soils.

Multivariate statistical analysis proved to be a valuable additional tool for improving the interpretation of multi-biomarker results in exposed worms. In particular, multivariate statistics showed that the use of the selected parameters enabled us to distinguish between temporal and concentration factors of chemicals' exposure as well as between different contaminated soils.

Among the different parameters evaluated, diagnostic of a stress syndrome in the organisms, we have shown that LMS is a prognostic indicator for health of edaphic sentinel animals, such as earthworms. The approach described here will facilitate the validation, and further the new development of robust diagnostic and prognostic tools that can be used along with other chemical, ecotoxicological and ecological tools as indices of sustainability.

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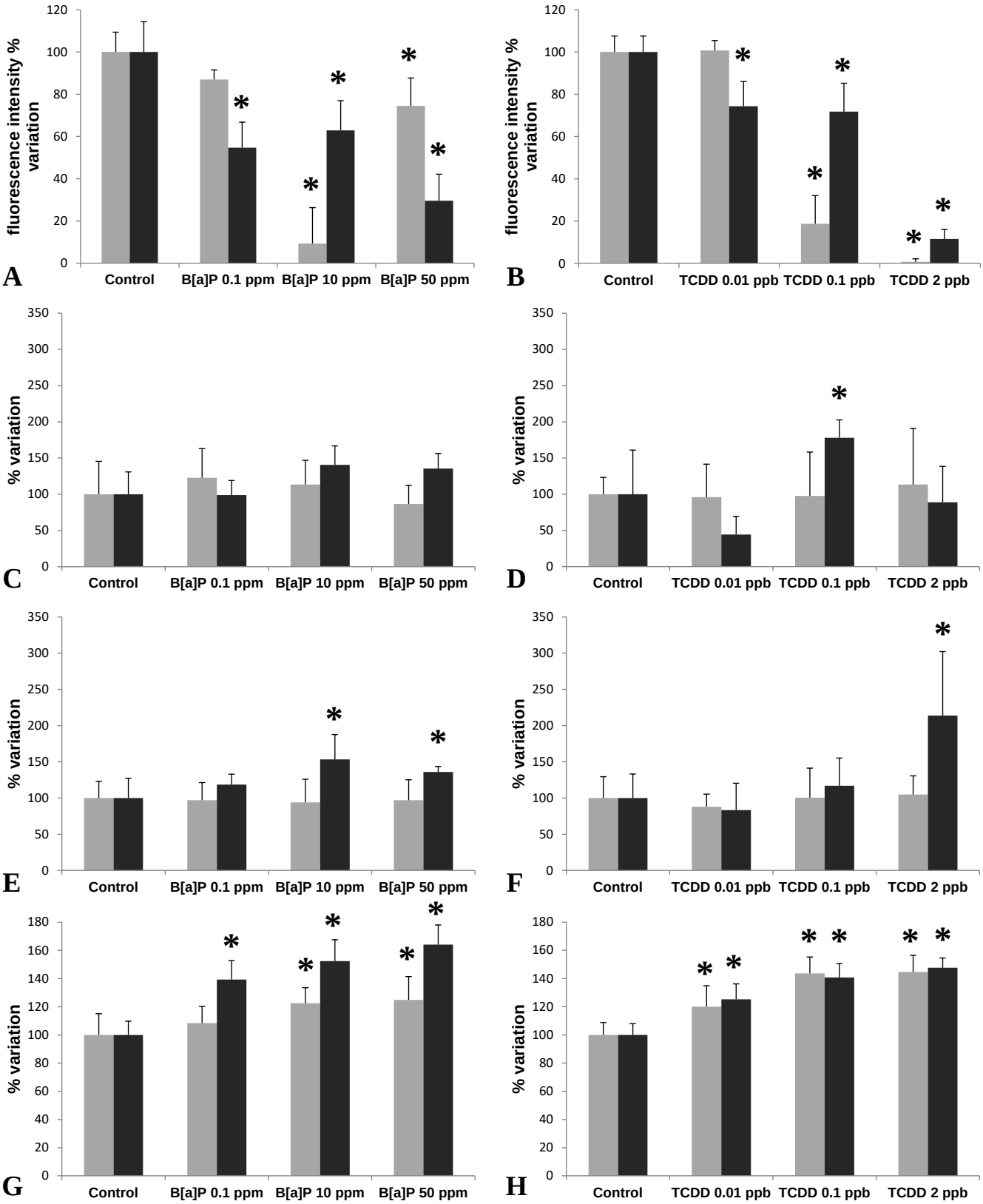
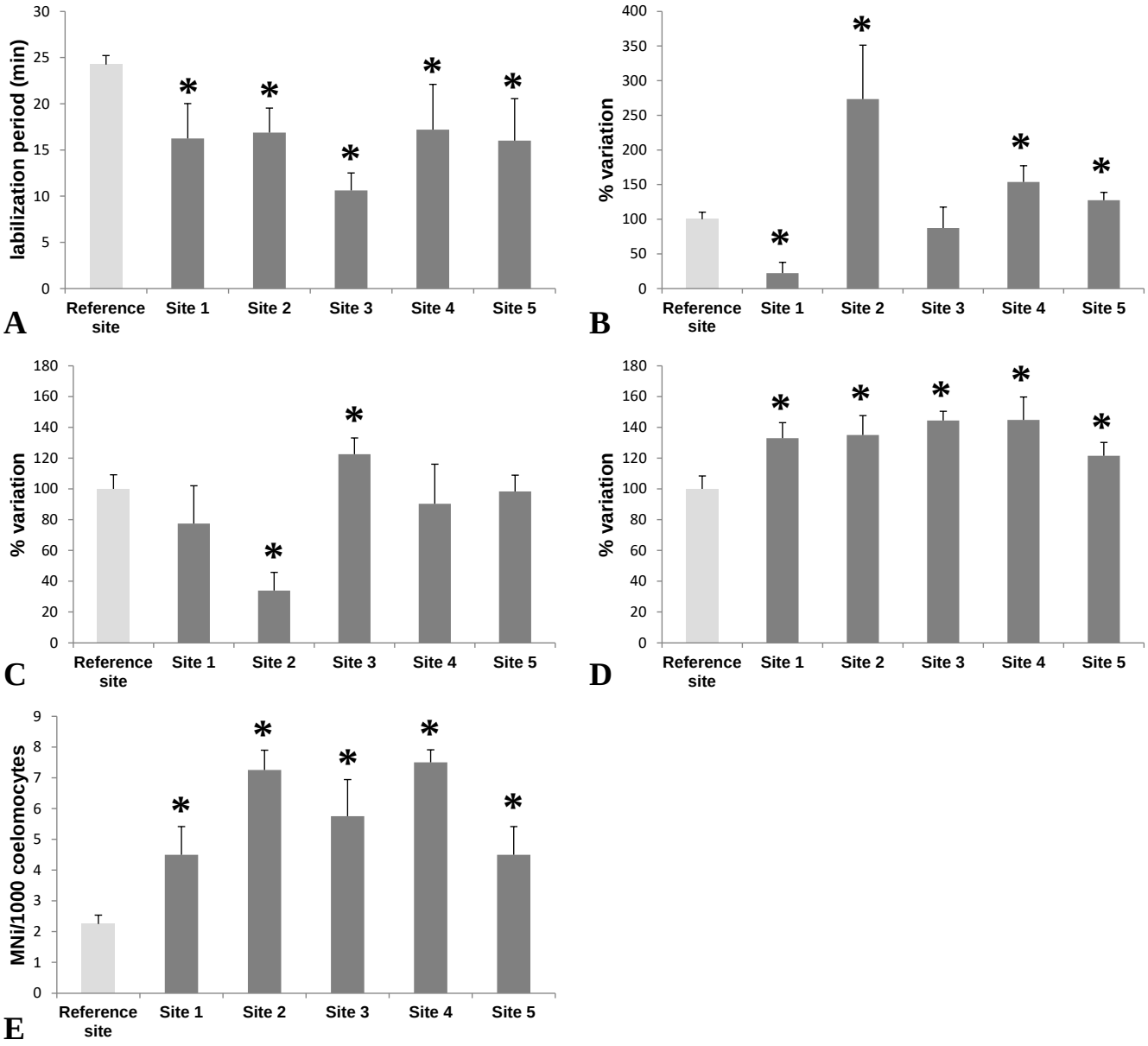
747

748 Fig. 1. Biomarker responses in *E. andrei* after exposure of worms for 10 d (grey columns) and 28 d
749 (black columns) to different concentrations of B[*a*]P and TCDD spiked in OECD soil. A, B)
750 Lysosomal membrane stability; C, D) lipofuscin content; E, F) neutral lipid accumulation; G, H)
751 lysosomal/cytoplasmic volume ratio. Data, expressed as percent change with respect to control
752 values, represent the mean ± SD of at least five replicates. * indicates statistically significant
753 differences ($p < 0.05$ Mann-Whitney *U*-test).
754
755 Fig. 2. Biomarker responses in *E. andrei* after exposure of worms for 10 d to soils collected from
756 different areas in Campania region (Italy). A) Lysosomal membrane stability in the cells of
757 chloragogenous tissue (based on latency of β-*N*-acetylhexosaminidase); B) lipofuscin content; C)
758 neutral lipid accumulation; D) lysosomal/cytoplasmic volume ratio; E) micronuclei frequency.
759 Data, expressed as percent change with respect to control values, represent the mean ± SD of at
760 least five replicates. * indicates statistically significant differences ($p < 0.05$ Mann-Whitney *U*-test).
761
762 Fig. 3. Principal Component Analysis (PCA) with superimposed Cluster Analysis showing
763 Resemblance Levels (distance), and vectors for the individual biomarkers.
764
765 Fig. 4. Regressions for B[*a*]P, TCDD and Campania data with correlation coefficients below each
766 set of plots. *Critical values for the Correlation coefficients are r = 0.381 for P = 0.05 (Campania),
767 and r = 0.349 for P = 0.05 (B[*a*]P and dioxin).
768
769 Fig. 5. Lysosomal membrane stability as an integrated indicator of health plotted against the first
770 principal component eigenvectors (PC 1) for all the remaining cellular biomarkers. The data has
771 been log transformed and normalised; and open triangles shown on the B[*a*]P plot are outliers not
772 included in the analysis.


A
fluorescence intensity % variation
120
100
80
60
40
20
0
Control
B[a]P 0.1 ppm
B[a]P 10 ppm
B[a]P 50 ppm
B
fluorescence intensity % variation
Control
TCDD 0.01 ppb
TCDD 0.1 ppb
TCDD 2 ppb
C
% variation
350
300
250
200
150
100
50
0
Control
B[a]P 0.1 ppm
B[a]P 10 ppm
B[a]P 50 ppm
D
% variation
Control
TCDD 0.01 ppb
TCDD 0.1 ppb
TCDD 2 ppb
E
% variation
Control
B[a]P 0.1 ppm
B[a]P 10 ppm
B[a]P 50 ppm
F
% variation
Control
TCDD 0.01 ppb
TCDD 0.1 ppb
TCDD 2 ppb
G
% variation
180
160
140
120
100
80
60
40
20
0
Control
B[a]P 0.1 ppm
B[a]P 10 ppm
B[a]P 50 ppm
H
% variation
Control
TCDD 0.01 ppb
TCDD 0.1 ppb
TCDD 2 ppb

773 Fig. 1.
774


labilization period (min)
Reference site
Site 1
Site 2
Site 3
Site 4
Site 5
A
% variation
B
% variation
C
% variation
D
MNi/1000 coelomocytes
E

775 Fig. 2.
776

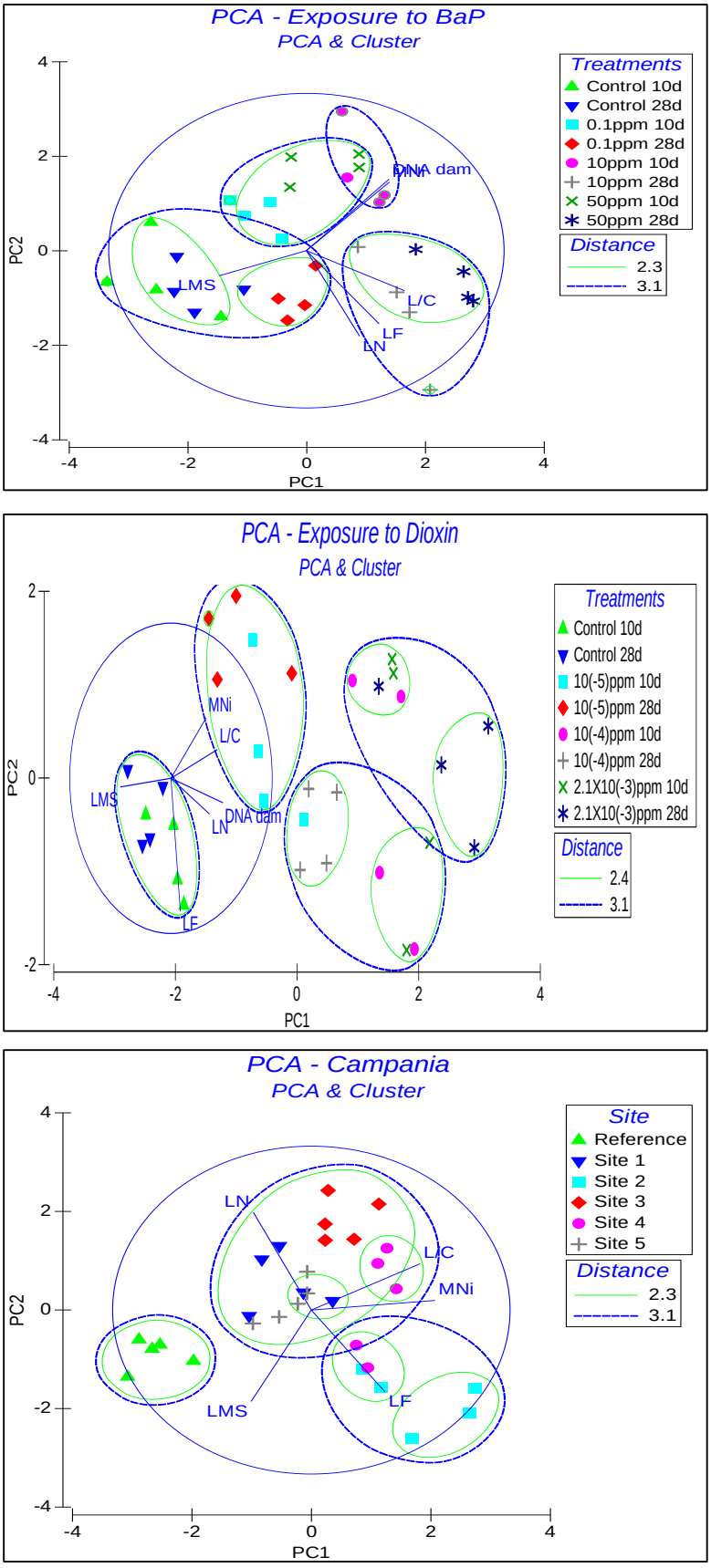
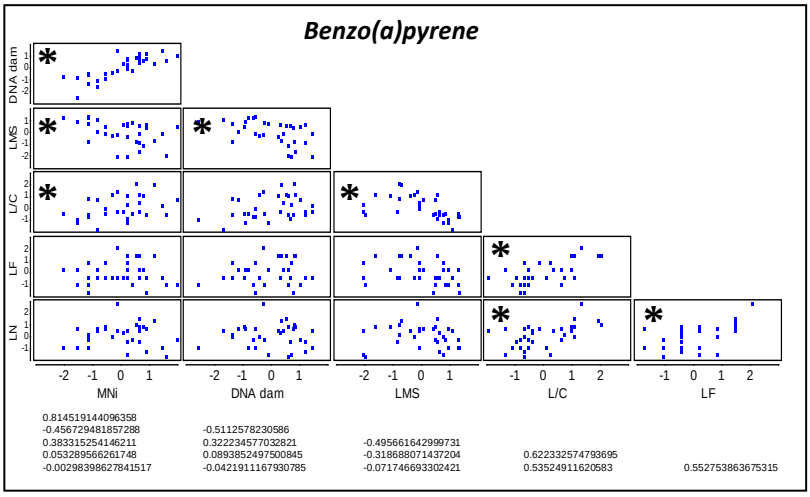
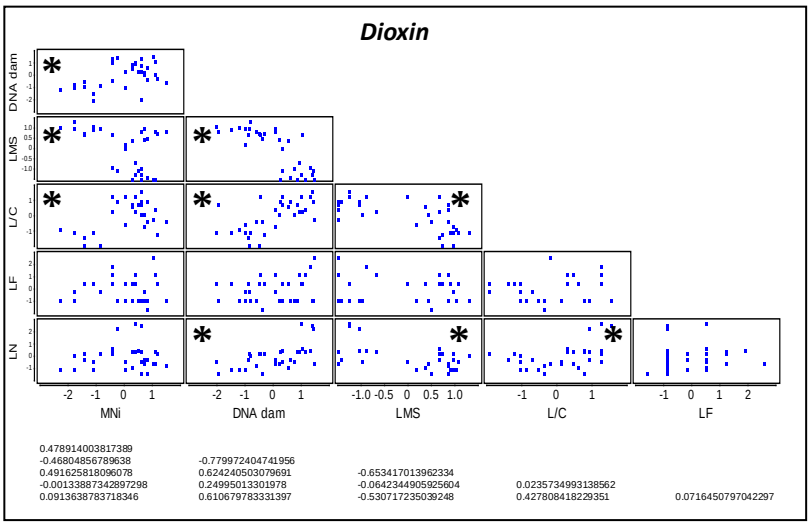
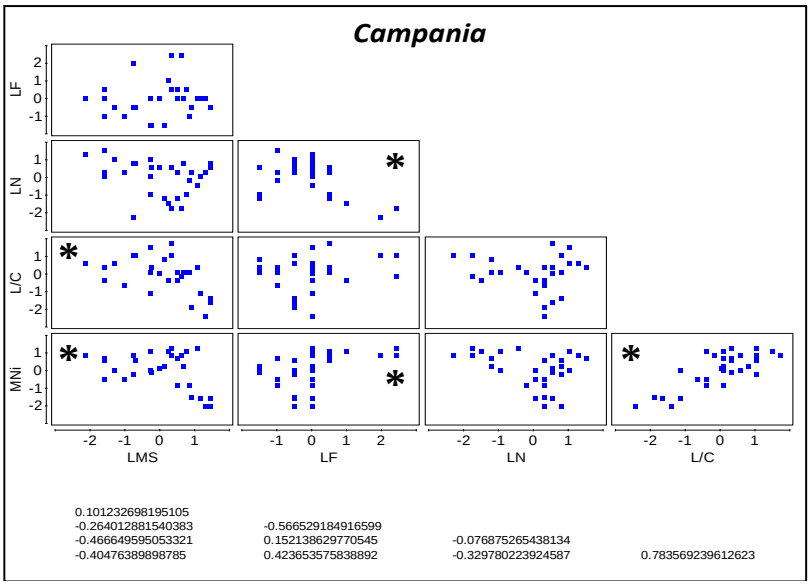

PCA - Exposure to BaP
PCA & Cluster
Treatments
Control 10d
Control 28d
0.1ppm 10d
0.1ppm 28d
10ppm 10d
10ppm 28d
50ppm 10d
50ppm 28d
Distance
2.3
3.1
DNA dam
LMS
L/C
LF
LN
PC1
PC2
PCA - Exposure to Dioxin
PCA & Cluster
Treatments
Control 10d
Control 28d
10(-5)ppm 10d
10(-5)ppm 28d
10(-4)ppm 10d
10(-4)ppm 28d
2.1X10(-3)ppm 10d
2.1X10(-3)ppm 28d
Distance
2.4
3.1
MNi
L/C
LMS
LN
DNA dam
LF
PC1
PC2
PCA - Campania
PCA & Cluster
Site
Reference
Site 1
Site 2
Site 3
Site 4
Site 5
Distance
2.3
3.1
LN
L/C
MNi
LF
LMS
PC1
PC2

Fig. 3.



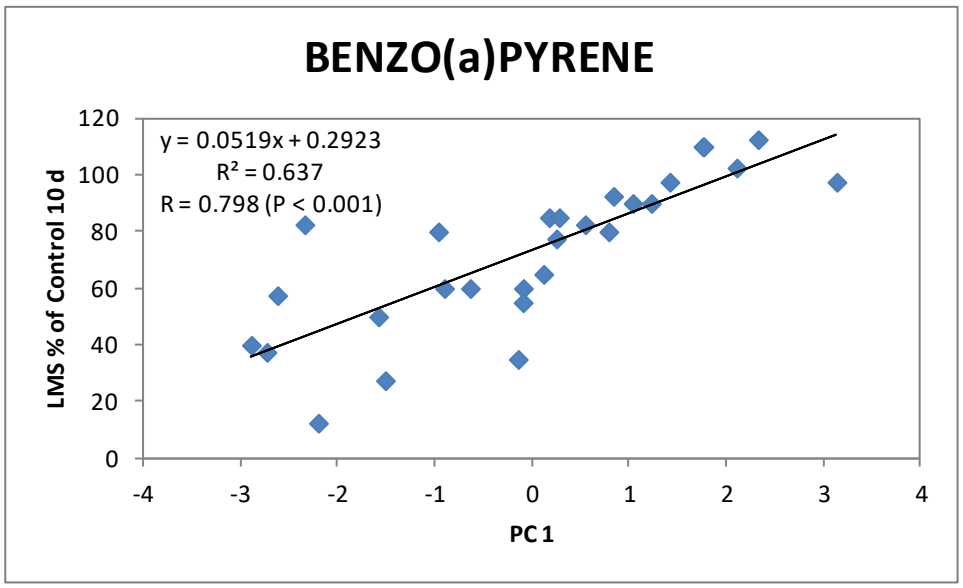


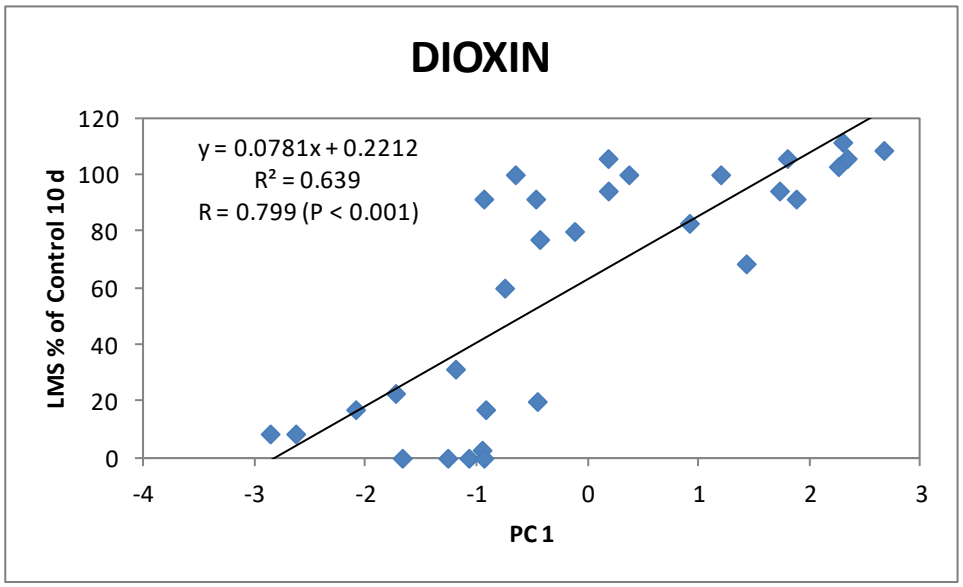


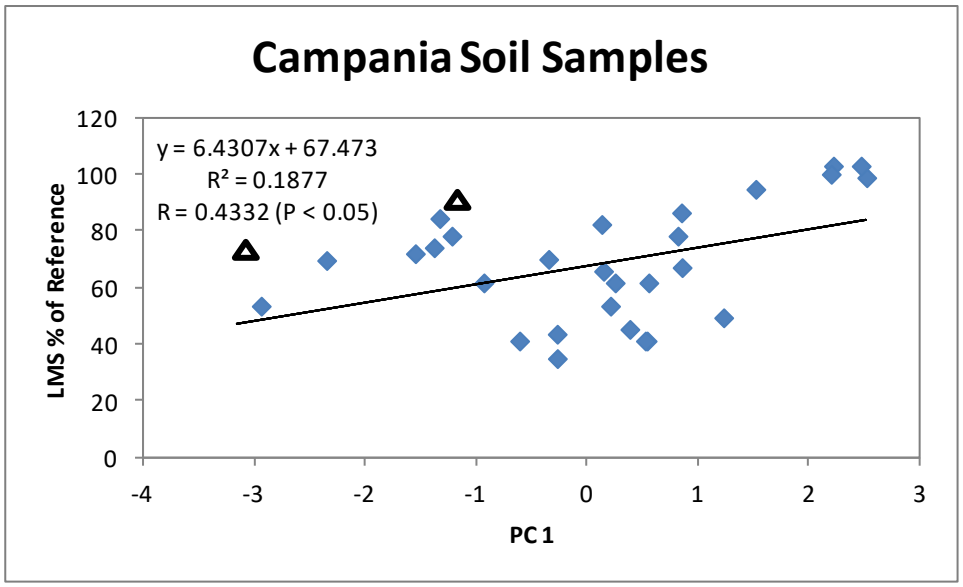
780

781 Fig. 4.

782


BENZO(a)PYRENE
y = 0.0519x + 0.2923
R² = 0.637
R = 0.798 (P < 0.001)
LMS % of Control 10 d
PC 1


DIOXIN
y = 0.0781x + 0.2212
R² = 0.639
R = 0.799 (P < 0.001)
LMS % of Control 10 d
PC 1


Campania Soil Samples
y = 6.4307x + 67.473
R² = 0.1877
R = 0.4332 (P < 0.05)
LMS % of Reference
PC 1

783

784 Fig. 5.
785