




Article

Evaluating Earthworms' Potential for Remediating Soils Contaminated with Olive Mill Waste Sediments

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Abstract: The olive-oil industry generates large amounts of residues that, in the past, were accumulated in evaporating ponds in many Mediterranean countries. Currently, these open-air ponds pose a serious environmental hazard because of toxic chemicals that concentrate in their sediments. Bioremediation of olive mill waste (OMW) sediments has emerged as a viable option for managing this environmentally problematic residue. Here, we postulate that inoculation of an OMW-soil mixture with earthworms may be a complementary bioremediation strategy to that using native microorganisms only. A laboratory study assessed the ecotoxicity of OMW-amended soils (10%, 20%, 40% and 80% *w/w*) combining earthworm biomarker responses and soil enzyme activities. The doses of 40% and 80% were toxic to earthworms, as evidenced by the high mortality rate, loss of body weight and signs of oxidative stress after 30 d of soil incubation. Conversely, doses $\leq 20\%$ *w/w* were compatible with earthworm activity, as indicated by the significant increase of soil enzyme activities. Total concentrations of phenolic compounds decreased by more than 70% respect to initial concentrations in 10% and 20% OMW treatments. These results suggest that OMW sediments intentionally mixed with soils in an up to 20% proportion is a workable bioremediation strategy, where earthworms can be inoculated to facilitate the OMW degradation.

Keywords: earthworm ecotoxicology; oxidative stress; soil enzymes; bioremediation; *Lumbricus terrestris*

1. Introduction

The olive-oil mill waste (OMW) is an environmentally problematic residue generated from the olive-oil processing industry, which may contaminate soil, surface water and groundwater [1,2]. OMW's toxicity is due to its chemical composition, which is highly variable depending on the olive variety, olive crop type, environmental conditions, time of olive storage prior to oil production and the olive oil extraction process. Essentially, OMW is an acidic, dark brown to black liquid emulsion composed of water (up to 92%); mineral nutrients (0.5–2.5%); and organic substances (4–15%), such as sugars, organic acids and especially phenolic compounds [3]. The OMW phenolic fraction is characterized by highly hydrophilic and persistent phenolic compounds (e.g., tyrosol and hydroxytyrosol) and polyphenols (e.g., flavonoids and secoiridoids) [4]. Because of the high phenolic content, OMW is phytotoxic and displays antimicrobial effects [5,6]. Recently, some studies have also shown that OMW is toxic to invertebrates and vertebrates [7,8].

Traditionally, disposal in evaporation ponds or direct discharge in soil were the two most common practices for managing OMW [2]. Although direct disposal of OMW is no longer allowed in Spain and other Mediterranean countries, many open-air shallow ponds still remain, constituting a serious threat for the environment [2,9]. Additionally, some studies suggest that irrigation of soil with olive mill wastewaters increases the organic matter content and nutrient availability [10,11], but its use in agriculture as a soil amendment is questionable. Undesirable side-effects, such as enhanced soil acidity, leaching of nutrients and phenolic-associated toxicity, may appear in OMW-amended agricultural soils [6]. For example, Kavvadias et al. [1] found that although direct application of untreated OMW increased soil electrical conductivity; organic matter content; total N and NH_4^+ ; and other macro and micronutrients, the residue also increased the concentration of total phenolic compounds, thereby compromising the soil quality.

Bioremediation is a common practice with which to manage OMW [12,13]. However, this technology has some limitations related to microbial adaptation to potential OMW toxicity, climatic conditions for microbial proliferation and the time taken to clean OMW-polluted soils, among others. Recently, some authors have proposed the use of soil-dwelling earthworms such as *Lumbricus terrestris* to facilitate microbial dispersion and proliferation in polluted soils, increase pollutant bioavailability and improve soil physicochemical properties for plant growth promotion [14,15]. With the purpose of exploring whether earthworms are able to reduce the potential toxicity of soils intentionally mixed with OMW sediments, we performed a laboratory study to investigate what OMW proportion in soil is suitable for an earthworm-assisted bioremediation. Therefore, earthworm survival, sublethal toxicity biomarkers and soil physicochemical and biochemical parameters were measured in OMW-contaminated soils to assess the viability of this potential bioremediation system. The aims of this study were: (i) to examine the avoidance response of earthworms to OMW-amended soils; (ii) to assess sublethal effects of earthworms incubated in OMW-amended soils, through the analysis of neurotoxicity and oxidative stress biomarkers; and (iii) to assess the impact of earthworms on soil enzyme activities, which were used as indicators of soil quality. Results in this study are expected to define a complementary remediation framework for reducing OMW toxicity using earthworms as stimulating biological agents.

2. Materials and Methods

2.1. Study Area, OMW Sediments, Soil and Earthworms

We used OMW sediments from abandoned OMW evaporation ponds located in Mora municipality ($39^\circ 40' 07.4''$ N $3^\circ 49' 40.2''$ W, Toledo, Spain), which occupy a total surface of 5 ha including eight evaporation ponds (2400 and 12,000 m^2) (Figure 1). Disposal of OMW at the study area started in 1982 and ended in 2007, and currently many of them contain concentrated OMW sediments. Samples of OMW sediments were collected for physicochemical characterization and measuring total water-soluble phenolic compounds (Table 1). The soil used (22% clay; 27% silt, 50% sand) had 1537 kg/m^3 of bulk density, with a plant available water storage capacity of 42 mm, alkaline reaction, low electrical conductivity and organic matter content (Table 1), being classified as Cambisol derived from carbonatation of parental granite [16]. Adult individuals of *L. terrestris* were obtained from a local supplier and maintained in temperature-controlled chambers (Radiber S.A., Barcelona, Spain) (15°C and permanent dark) following the recommendations by Lowe and Butt [17] until they were used in toxicity testing. The soil used in the earthworm culture was the same as that used in the toxicity testing with OMW sediments.

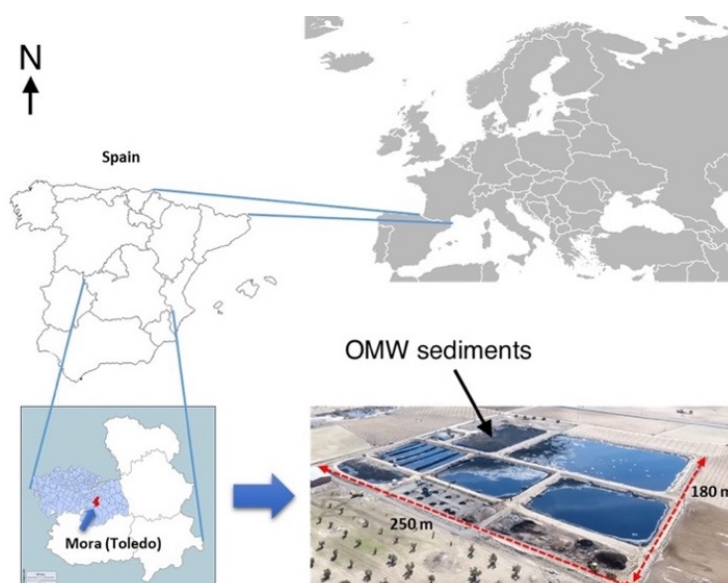


Figure 1. Study area at the locality of Mora (Toledo, Spain). Disposal of olive-mill waste (OMW) in open-air evaporation ponds.

Table 1. Selected physicochemical properties (mean \pm SD, n = 3) of OMW sediments and soil used in toxicity testing with *Lumbricus terrestris*.

| Parameter | OMW Sediments | Soil |
|---------------------------------------|-----------------|-------------------|
| pH | 9.38 \pm 0.02 | 8.76 \pm 0.06 |
| Electrical conductivity (dS/cm) | 1.40 \pm 0.06 | 0.08 \pm 0.01 |
| Organic matter (%) | 3.06 \pm 0.09 | 0.36 \pm 0.01 |
| Total organic carbon (%) | 3.08 \pm 0.05 | 0.27 \pm 0.02 |
| Total nitrogen (%) | 0.26 \pm 0.05 | 0.03 \pm 0.01 |
| Total soluble polyphenols (mg/kg) | 873 \pm 55 | 85 \pm 6.5 |
| CEC (meq/100 g OM) | 244 \pm 31 | 381 \pm 17 |
| Total P (g/kg) | 3.2 \pm 0.09 | 0.067 \pm 0.005 |
| K (g/kg) | 11.9 \pm 0.7 | 3.9 \pm 0.35 |
| Na (g/kg) | 1.9 \pm 0.01 | 0.6 \pm 0.05 |
| PO ₄ ⁻³ (mg/kg) | 32 \pm 0.6 | 8 \pm 0.4 |
| SO ₄ ⁻² (mg/kg) | 0.8 \pm 0.09 | 0.4 \pm 0.03 |

2.2. Experimental Design

The experimental design was identical to that in previously conducted research into biochar-spiked soils by Sanchez-Hernandez et al. [18]; therefore, it is only briefly described in the current paper. OMW toxicity was assessed by two successive bioassays with earthworms. The first one followed the ISO 2007 guidelines [19] for testing the avoidance behaviour response (ABR) of earthworms. The ABR assay consisted of quantifying the ability of earthworms to avoid OMW-amended soil in a two-chamber system (265 \times 162 \times 100 mm) holding the test soil in a compartment and the control (non-contaminated) soil in the opposite side. The doses of OMW sediment were: 0%, 10%, 20%, 40% and 80% OMW *w/w* dry mass (each treatment was run in triplicate). OMW sediment was mixed in soil using a spatula, and then moistened to 15% *v/w* with distilled water. Test boxes containing the OMW-amended and control soils were placed in an acclimatization chamber at 15 °C and continued dark for acclimatization (24 h). Next, five earthworms were released in each treatment and the containers were kept again for 48 h in the acclimatization chamber to allow earthworms to freely distribute between both soil sections. After this incubation time, individuals were counted in both compartments following the criteria by De Silva and van Gestel [20].

The second bioassay consisted of a microcosm study whereby earthworms were incubated in OMW-amended soils for 30 d. Treatments (0%, 10%, 20%, 40% and 80% *w/w* dry mass) were prepared in triplicate as described above, and 2.0 kg (wet mass) of each spiked soil was placed in a plastic container with a holed plastic lid for reducing water loss during incubation. Containers ($n = 15$) were placed in the thermostated chamber for 24 h for soil acclimatization, and five earthworms were released in each container after recording body weight. Individuals were maintained in Petri dishes for 24 h to allow gut voiding, and then body weight was recorded. Before adding the worms, we took one soil sample from each replicate, which served to measure the soil enzyme activity at $t = 0$ d. Containers were incubated for 30 d at 15 °C. During this period, earthworms received approx. 5 g of *Morus alba* leaves per container and week as food, which were put on soil surface. At the end of microcosm experiment, we recovered earthworms, and collected soil samples from the burrow walls and casts (excreta) from the soil surfaces. Soil and cast samples were frozen at -20 °C until measurement of enzyme activities. Earthworms were put again on placed in Petri dishes (15 °C for 24 h) to record post-incubation body weight. Animals were killed by freezing and dissected using a stereomicroscope, and muscle (body wall) and gastrointestinal tissues were collected and immediately frozen at -80 °C. Tissues were rinsed with distilled water to remove the rest of coelomic fluid, blood and other tissues. Gastrointestinal content was also collected for measuring digestive enzyme activities; the samples were frozen at -20 °C.

2.3. Sample Preparation for (Bio)Chemical Analysis

Earthworm tissues were homogenized in ice-cold buffer (pH = 7.4) made of 25 mM sucrose, 20 mM Tris-HCl buffer and 1 mM EDTA. The homogenates were centrifuged at $9000\times g$ for 20 min at 4 °C to obtain the post-mitochondrial fraction which was aliquoted and stored at -80 °C until analysis. Aqueous suspensions of soil and cast, and the luminal contents of the gastrointestinal tracts were used for enzyme activity measurements. The samples were prepared as described in Deng et al. [21], with minor modifications for the luminal content which consisted of mixing 0.2 g of sample with 4.0 mL of distilled water for 1 min using a vortex. Suspensions were used in the moment of preparation or maintained at 4–5 °C for a maximum period of 5 d.

2.4. Earthworm Biomarkers

Acetylcholinesterase activity (AChE, EC 3.1.1.7) was determined in a reaction mixture composed of 0.1 M Na phosphate buffer (pH = 8.0), 320 μ M (final concentration, f.c.) 5,5'-dithiobis-2-nitrobenzoic acid and 3 mM (f.c.) acetylthiocholine iodide as the enzyme substrate [22]. Kinetics (10 min) were read at 412 nm (22 °C), and the enzyme activity was expressed as nmol of product $\text{min}^{-1} \text{mg}^{-1}$ of total protein, using a calibration curve constructed with serial concentrations of reduced glutathione [23]. The glutathione-dependent antioxidant enzymes glutathione *S*-transferase (GST, EC 2.5.1.18) and glutathione reductase (GR, EC 1.6.4.2) were measured according to Habig et al. [24], and Ramos-Martínez et al. [25], respectively. The former activity was determined in a reaction mixture containing 0.1 M Na-phosphate buffer (pH 6.5), 2 mM CDNB, 5 mM reduced glutathione and the sample, and the reaction product read at 340 nm. The extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$ was used to express the specific enzyme activity. Glutathione reductase activity was measured in a reaction medium composed of 1 mM oxidized glutathione (GSSG), 60 μ M NADPH, 0.1 M Na-phosphate buffer (pH = 7.5) and the sample. The NADPH oxidation rate was monitored at 340 nm and the specific enzyme activity calculated using the extinction coefficient of $6.22 \text{ M}^{-1} \text{ cm}^{-1}$.

Determination of GSH and GSSG concentrations followed the colorimetric method by Rahman et al. [26]. Samples (100 μ L aliquots) were firstly deproteinized by adding 100 μ L of 0.6% sulfosalicylic acid. Proteins were then removed by centrifugation ($10.000\times g$ for 5 min at 4 °C). For GSH determination, 50 μ L of the acid supernatant was mixed with 125 μ L of 1:1 (v/v) DTNB and glutathione reductase (GR) mixture. Changes in absorbance were immediately monitored at 412 nm after adding 50 μ L of NADPH. For GSSG measurements, samples were incubated (1 h and 22 °C) with 2-vinylpyridine

to derivatize GSH, and afterwards, neutralized with triethanolamine for a 10-min incubation. Finally, these samples were treated with the same procedure as that for GSH determination.

Digestive alkaline phosphatase (EC 3.1.3.1) and β -glucosidase (EC 3.2.1.21) were measured in both the gastrointestinal epithelium and luminal content according to Nozaki et al. [27]. Lipase (EC 3.1.1.3) activity determination followed the method by Gupta et al. [28], whereas esterase activity (EC 3.1.1.1) was measured by the method of Thompson [29]. These enzyme assays were run with some slight modifications to adapt the protocols to microplate-scale format, which are described in detail in Sanchez-Hernandez et al. [30].

2.5. Soil and Cast Enzyme Activities

We measured the potential activity of soil carboxylesterase, β -glucosidase, alkaline phosphatase and dehydrogenase using microplate-scale format protocols, which are fully described elsewhere [31]. We selected these soil enzyme activities because of their known role in C (esterase and β -glucosidase) and P-cycling (alkaline phosphatase) [32], whereas dehydrogenase activity was used as a general index of soil microbial activity [33].

2.6. Physicochemical Analysis of OMW-Amended Soil

OMW samples were air-dried in oven equipped with forced aeration at 45 °C in order to avoid degradation of phenolic compounds. The samples were ground using a vibratory ball mill (Firscht Pulverisette Spartan, Fritsch GmbH, Idar-Oberstain, Germany) to obtain a dust particle size, and were dried to 105 °C for further measurements. The soil pH and electrical conductivity (EC) were measured in soil extracts (1:2.5 soil:water, w/v, ratio for pH and 1:5 ratio for EC) [34]. The organic content was determined gravimetrically after incineration of the dry sample at 550 °C for 4 h. Oxidizing organic carbon was measured by the Walkley and Black modified method [35]. Concentrations of P, Na and K were measured in acid extracts obtained by sample mineralization in HNO₃ using a MARS One (CEM, Buckingham, U.K.) microwave digester. Phosphorus was colorimetrically determined by the method of molybdate phosphoric acid [36], whereas Na and K were quantified by flame spectrophotometry (Jenway PF7P flame photometer, Staffordshire, U.K.). The total organic carbon and total nitrogen were determined by dry combustion at 1020 °C using an elemental analyzer (EuroVector Elemental Analyzer, Milano, Italy), whereas anion concentrations (Cl⁻, NO₃⁻ and SO₄⁻²) were measured in soil:water (1:10, w/v) extracts using an ionic liquid chromatograph (Dionex ICS 1000, ThermoFisher Scientific, Waltham, Massachusetts, U.S.A.). Water soluble phenols were determined in 1:20 soil:water extracts according to the Folin–Ciocalteu reaction; the absorbance of the product was measured at 765 nm, and compared to that of the gallic acid, which was used as the standard [37]. All determinations were performed in triplicate, and results are reported as mean values and standard deviations.

2.7. Data Analysis

Earthworm avoidance response to OMW-amended soils was quantified by using the following equation [20]:

$$NR = \left(\frac{C - T}{N} \right) \times 100 \quad (1)$$

where *NR* is the net avoidance response (%); *C* and *T* are the number of individuals found in the control and OMW-amended soils, respectively. *N* is the total number of earthworms in the test container. We used the one-way ANOVA test followed by Games–Howell post-hoc test to detect statistical differences between treatments.

Biomarker responses to OMW-amended soil exposure were explored via one-way ANOVA test with Welch’s homogeneity correction, followed by the Games–Howell post-hoc test. Because of the low number of replicates (*n* = 3), the impact of OMW sediment on soil (and cast) enzyme activities was assessed by the non-parametric Kruskal–Wallis test followed by the post-hoc Welch’s *t* test. We also

used the “Integrated Biomarker Response, version 2” (IBRv2) index by Sanchez et al. [38] to integrate the responses of all biomarkers into a single value, which was calculated using the equation:

$$IBRv2 = \sum_{i=1}^n |A_i| \quad (2)$$

where A_i is a deviation value for each biomarker with respect to the reference value from controls. The higher the IBRv2 value, the stronger is the biological stress induced by chemicals occurring in OMW sediments. We also plotted the A_i values in a star plot to identify inhibition (negative A_i or area in the sunray plot below the reference level) or induction response (positive A_i or area above the reference level) of each biomarker [38], and consequently to identify what biomarker had a stronger impact in the IBRv2 value.

3. Results and Discussion

3.1. Avoidance Behaviour Response Test

We found no individual dead after 48 h of incubation in the ABR test, and they randomly distributed between both sections, as suggested by the similar percentages of earthworms found in the control treatment ($53.3\% \pm 5.8\%$ in one section and $46.7\% \pm 5.8\%$ in the opposite section). However, the addition of OMW sediment to soil at the doses of 40% and 80% *w/w* caused a significant avoidance response ($F_{4,4.72} = 20.7$, $p = 0.003$) with respect to the control treatment (Figure 2A). The ABR test is a common screening test for assessing soil pollution [38], and it is generally accepted that avoidance response is judged as significant when more than 80% of earthworms are found in the compartment containing the control soil. This is a threshold value that defines the soil habitat limit, as suggested by Hund-Rinke et al. [39]. Many studies have used the ABR test to evaluate the potential toxicity of soils contaminated by metals [40], pesticides [41,42], cyanotoxins [43], veterinary pharmaceuticals [44], microplastics [45] or soil additives such as biochar [46,47]. Although the ABR test guidelines recommend the use of the epigeic earthworms *Eisenia fetida* or *E. andrei* [19], soil-dwelling earthworm species such as *L. terrestris* are now adopted in standardized methods such as the ABR test [48]. Moreover, some studies suggest that *L. terrestris* is more sensitive to organic pollutants such as pesticides than the common and recommended species *E. fetida* [49].

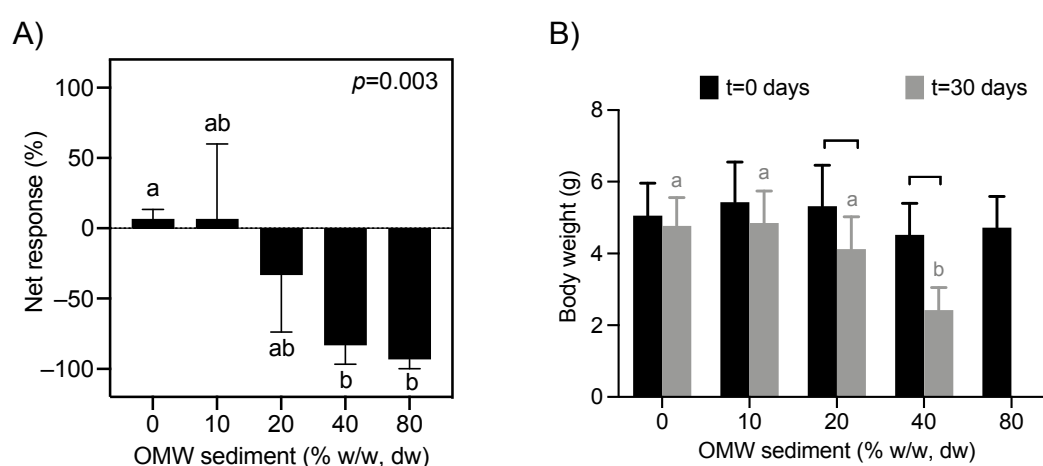


Figure 2. (A) Avoidance behaviour response of *Lumbricus terrestris* to olive mill waste (OMW)-amended soils. Statistical differences between treatments indicated by different letters ($p < 0.05$, Games–Howell post-hoc test). (B) Mean body weight of earthworms exposed for 30 d to OMW-amended soils. Different letters indicate significant differences between treatments within a sampling time ($p < 0.05$), whereas asterisks denote differences between sampling times ($p < 0.05$).

In this study, we used the ABR test as a screening assay to determine the amount of OMW sediment that, added to soil, does not result repellent to earthworms. Current results pinpoint to concentrations of OMW sediment $\leq 20\%$ *w/w* (dry mass) as viable to remediate OMW-amended soils using the earthworm *L. terrestris*.

3.2. Microcosm: Impact of OMW on Earthworm Biomarkers

All individuals ($n = 15$) from the 80% OMW treatment were found dead during the first two weeks of exposure. Furthermore, the dose of 40% OMW caused a 53.3% mortality after 30 d of incubation. The interaction between OMW treatment and time of exposure had a significant impact of earthworm body weight ($F_{3,104} = 4.2$, $p = 0.008$) (Figure 2B). While the earthworm weight was similar between treatments at the beginning of the microcosm trial ($F_{3,56} = 2.42$, $p = 0.076$), it decreased after 30 d of incubation ($F_{3,48} = 15.8$, $p < 0.001$) in the 20% and 40% treated groups ($p < 0.05$, Tukey post-hoc test).

We measured a set of biomarkers linked to oxidative stress, neurotoxicity and digestive disruption, using the body wall muscle and gastrointestinal tract as target tissues. We found no significant variation in muscle AChE activity ($F_{3,20} = 1.44$, $p = 0.26$), which is a common biochemical endpoint of neurotoxicity. However, exposure to OMW-amended soils caused signs of oxidative stress (Figure 3), which were evidenced by alteration of GSH ($F_{3,17} = 9.25$, $p < 0.001$) and GSSG ($F_{3,17.5} = 4.30$, $p = 0.019$) concentrations, and GST activity ($F_{3,18.4} = 6.73$, $p = 0.003$). The earthworm group exposed to 20% OMW-amended soils had the highest mean concentrations of muscle GSH (13.3 ± 4.3 nmol⁻¹ mg protein, mean \pm SD, $n = 15$) and GSSG (3.06 ± 1.12 nmol⁻¹ mg protein, $n = 15$), and the highest GST activity (310 ± 87 nmol⁻¹ min⁻¹ mg protein, $n = 15$) with respect to controls (GSH = 8.20 ± 1.35 nmol⁻¹ mg protein, GSSG = 1.93 ± 0.62 nmol⁻¹ mg protein and GST activity = 202 ± 38 nmol⁻¹ min⁻¹ mg protein; $n = 15$). Similar findings were found in the gastrointestinal tract, although the earthworms exposed to 40% OMW-amended soils had also the gastrointestinal GSH and GSSG concentrations significantly higher with respect to the control group ($p < 0.05$, Games–Howell post-hoc test) (Figure 3). These data suggest that exposure to OMW-derived toxic chemicals occurred via both the earthworm skin and the gut epithelium, which is in agreement with experimental data of organic contaminant uptake by earthworms [50].

In general, there was a dose-dependent response of digestive enzyme activities to OMW concentrations (Table 2). Lipase activity was the only enzyme with a significant inhibition (39% of controls) response in the gastrointestinal tissue of earthworms exposed to 40% OMW-amended soils ($p = 0.037$, Games–Howell post-hoc test). Additionally, there was a slight although significant increase of tissue phosphatase activities of earthworms exposed 10% and 20% OMW sediment. We also measured the activity of these enzymes in the gastrointestinal content, although the amount of sample was sufficient enough for all five enzymes (Table 2). At the dose of 20% OMW sediments, both alkaline phosphatase and β -glucosidase activities of the gastrointestinal content were significantly lower than those of the control group ($p < 0.05$, Games–Howell post-hoc test). This finding suggests toxic effects on gut symbionts because the mean activities of these two enzymes did not change (β -glucosidase), or even were higher (alkaline phosphatase), when the activities of gastrointestinal tissue are compared between 0% and 20% OMW treatments.

The IBRv2 index was used to assess the biomarker responses. The index clearly showed that the highest OMW concentration induced oxidative stress in earthworms, as evidenced by the high A_i scores for both GSH and GSSG (Figure 4). Moreover, the A_i score for the gastrointestinal GSSG concentration of 40% OMW treatment was higher than that of GSH, probably suggesting an oxidation of GSH by free radicals [26]. Glutathione S-transferase activity was induced in the muscle tissue of earthworms from all OMW treatments, and in the gastrointestinal tissue of the earthworms exposed to 10% and 20% OMW. It is well known that GST activity plays an important role in the metabolism of xenobiotics by conjugating glutathione with electrophilic metabolites [26,51].

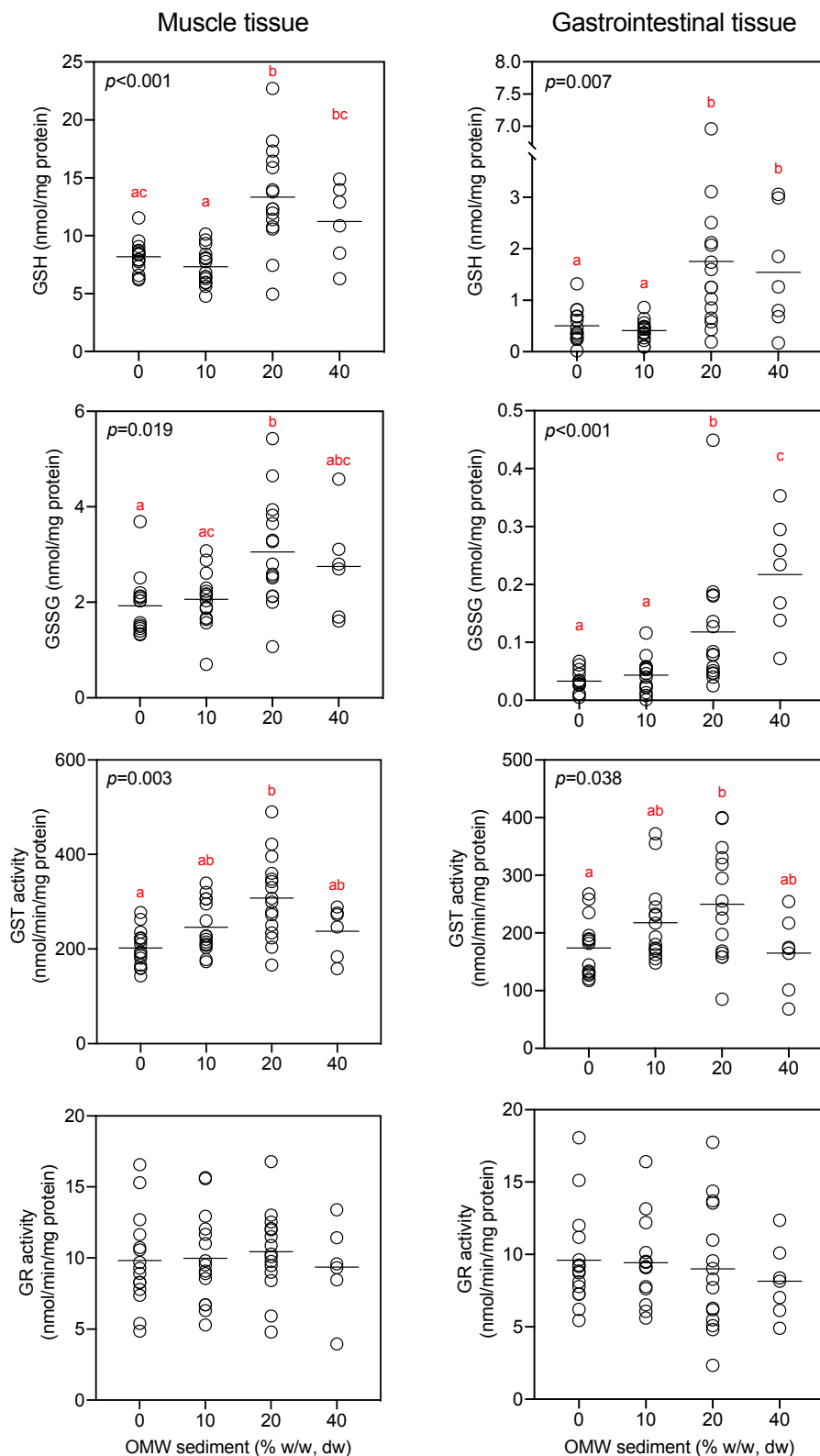


Figure 3. Response of glutathione-dependent antioxidant mechanisms in the body wall muscle and gastrointestinal tract of earthworms after 30 d of exposure to olive mill waste (OMW)-amended soils. Horizontal solid lines in the univariate scatterplots are the mean values. Different letters denote statistical differences ($p < 0.05$, Games–Howell post-hoc test). GSH = reduced glutathione, GSSG = oxidized glutathione, GST = glutathione S-transferase, GR = glutathione reductase.

Table 2. Digestive enzyme activities (mean ± SD, n = 15) in the gastrointestinal tissue and content of *Lumbricus terrestris* exposed to olive mill waste (OMW)-amended soils.

| Digestive Enzymes ¹ | OMW Dose (% w/w) | | | | p-Value ² |
|---------------------------------|------------------|----------------|----------------|----------------|----------------------|
| | 0 | 10 | 20 | 40 | |
| <i>Gastrointestinal tissue</i> | | | | | |
| Esterase | 74.2 ± 38.5 | 76.8 ± 36.4 | 92.3 ± 30.7 | 68.0 ± 46.7 | 0.407 |
| Lipase | 3.01 ± 0.85 a | 2.83 ± 0.80 ab | 2.56 ± 1.20 ab | 1.84 ± 0.80 b | 0.038 |
| Glucosidase | 5.76 ± 1.06 ab | 6.78 ± 1.78 a | 7.10 ± 3.45 ab | 4.90 ± 1.22 b | 0.039 |
| Acid phosphatase | 6.30 ± 1.16 a | 8.14 ± 1.87 b | 7.82 ± 4.15 ab | 7.26 ± 1.84 ab | 0.028 |
| Alkaline phosphatase | 6.87 ± 1.24 a | 8.58 ± 2.50 ab | 10.5 ± 5.64 b | 9.00 ± 4.67 ab | 0.034 |
| <i>Gastrointestinal content</i> | | | | | |
| Glucosidase | 1.09 ± 0.39 a | 0.85 ± 0.25 ab | 0.77 ± 0.25 b | – ³ | 0.049 |
| Acid phosphatase | 1.55 ± 0.70 | 1.65 ± 0.63 | 1.21 ± 0.47 | – | 0.086 |
| Alkaline phosphatase | 1.50 ± 0.46 a | 1.34 ± 0.51 ab | 1.01 ± 0.49 b | – | 0.029 |

¹ Enzyme activity expressed as nmol min⁻¹ mg⁻¹ total protein (tissue), and nmol h⁻¹ g⁻¹ content (gastrointestinal content). ² One-way ANOVA test (Welch’s homogeneity correction). Different letters denote within-effect differences for each enzyme’s activity (p < 0.05, Games–Howell post-hoc test). ³ Not measured (insufficient sample amount).

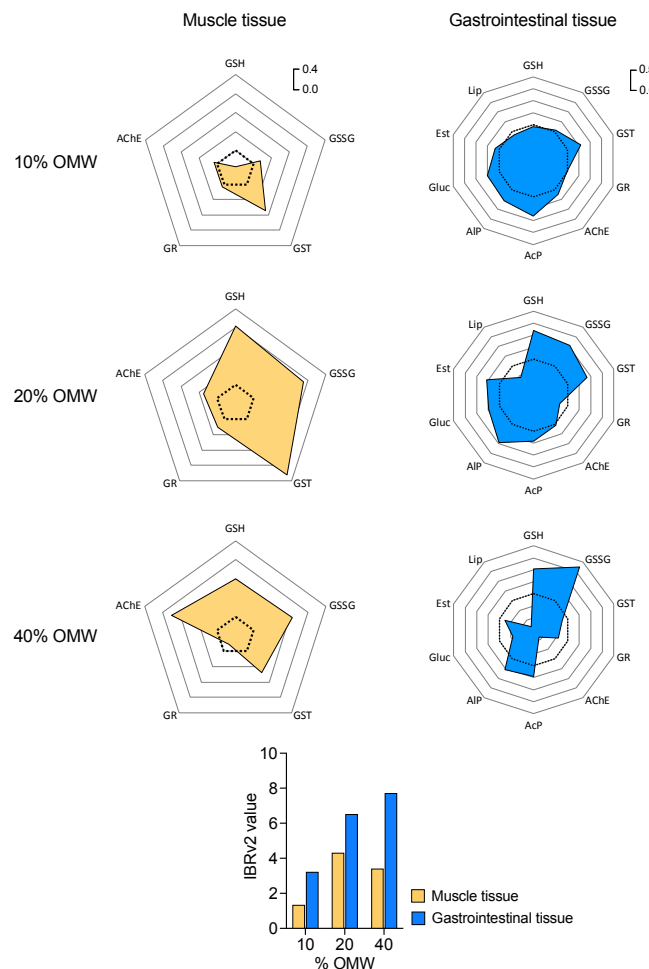


Figure 4. Sunray plots of the deviation index (A_i scores) of biomarker measured in body wall muscle and gastrointestinal tissue of *Lumbricus terrestris* exposed after 30 d of exposure to olive mill waste (OMW)-amended soils. Dotted lines in sunray plots indicate the reference values (see Materials and Methods for details). Bar plot shows the integrated biomarker response index (IBRv2) values calculated for each treatment and tissue. AChE = acetylcholinesterase, GSH = reduced glutathione, GSSG = oxidized glutathione, GST = glutathione S-transferase, GR = glutathione reductase, AcP = acid phosphatase, AIP = alkaline phosphatase, Gluc = β -glucosidase, Est = esterase.

Furthermore, GST activity also removes lipid peroxides, thus contributing to reduce cellular oxidative damage [52]. Its enhanced activity has been suggested as a biomarker of oxidative stress in earthworms, the body wall and foregut being the tissues expressing the highest levels of GST activity [53]. Therefore, the response of this antioxidant enzyme in our earthworms indicated oxidative stress induced by chemicals present in the OMW sediments.

Biomarker responses of control earthworms measured in this study were comparable to other studies that used the same species [18]. Therefore, we can assume that these are baseline values for these biomarkers, so they can be used as reference values in IBRv2 index calculations [38]. The IBRv2 index is commonly used to integrate the responses of multiple biomarkers measured in organisms inhabiting polluted sites compared to organisms from reference (unpolluted) sites [54,55]. In the present study, we found that IBRv2 values of OMW-exposed earthworms markedly increased from control earthworms, varying from 3.22 (10% OMW) to 7.71 (40% OMW) for the gastrointestinal tissue, and from 1.33 (10% OMW) to 3.40 (40% OMW) for the body wall muscle (Figure 4). This increase was dose-dependent in the case of the gastrointestinal tissue. The results suggest that the IBRv2 index assists in the data interpretation of multiple biomarkers, which have different responses to pollutants (inhibition or induction).

3.3. Microcosm: Impact of OMW on Soil Enzyme Activities

Time variation of soil enzyme activities in OMW-amended soils are shown in Figure 5. The OMW concentration had a dose-dependent response in the activity of carboxylesterase, alkaline phosphatase and dehydrogenase at $t = 0$ d ($p < 0.05$, Kruskal–Wallis test), which could be due to microbial proliferation during the incubation time of earthworm-free soils for acclimatization (24 h). However, the addition of earthworms only had a significant impact on β -glucosidase and dehydrogenase activities ($p < 0.05$, Welch's t test) of 10% OMW and control treatments after 30 d of soil incubation compared to initial values for these treatments ($t = 0$ d). This observation is consistent with data in the literature. It is well known that earthworms exert a stimulatory effect on soil microbial activity [56,57] and extracellular enzyme production [58]. Such an effect is caused by the intensive burrowing and feeding activities, which disperse microorganisms in soil [59], besides altering soil's physicochemical properties [60]. However, no significant increase in soil enzyme activities was observed in the 20% and 40% OMW treatments after 30 d of incubation. In addition, although casts displayed higher levels of enzyme activity than those of soils collected from burrow walls, they did not significantly vary between treatments (Figure 5, inset plots). The reason for not detecting a significant increase of enzyme activities in the 20% and 40% OMW-amended soils with respect to the other treatments is unclear. We postulate that, because these OMW doses were toxic to earthworms (Figures 2B and 4), the animals probably displayed depressed burrowing and feeding activities, which reduced their capacity to stimulate soil microorganisms and exoenzyme production.

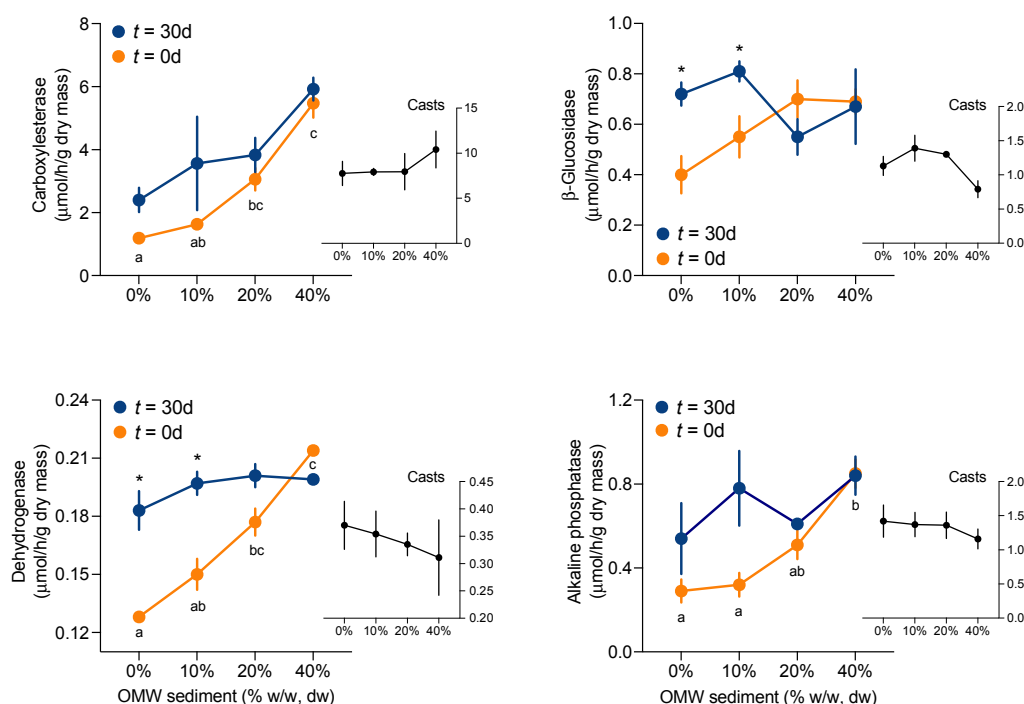


Figure 5. Mean (\pm SEM) enzyme activity in olive mill waste (OMW)-amended soils at $t = 0$ d and after 30 d of incubation. Different letters indicate significant differences between OMW treatments ($p < 0.05$, Welch's t post-hoc test), whereas asterisks denote differences between soils inoculated with earthworms and earthworm-free ($p < 0.05$). Inset plots show the mean (\pm SEM) enzyme activity in casts collected from the soil surface after 30 d of incubation.

3.4. Microcosm: Impact of Earthworms on Soil Physicochemical Variables

The addition of OMW sediments significantly increased the concentrations of Na, K, P, total organic carbon and nitrogen, total phenols and oxidized carbon; and the electrical conductivities and pH values of the spiked soils, in a dose-dependent manner (ANOVA test with Welch's homogeneity correction, $p < 0.05$) (Figure 6). The inoculation of OMW-amended soils with earthworms had a strong impact on total phenol concentrations, which significantly decreased in all treatments after 30 d of soil incubation ($p < 0.05$, Games–Howell post-hoc test). However, the percentage of total phenol decrease depended on their initial concentration; the more phenols were concentrated in soil, the lesser the degradation rate achieved (96%, 87.4%, 73.2% and 58.6% decreases for 0%, 10%, 20% and 40% w/w OMW sediments, respectively). The negative relationship may be attributed to the toxic effects of phenol compounds on earthworms, as showed by the biomarker and avoidance response outcomes, which would reduce their burrowing and feeding activities and thereby their potential remediation capacity. Likewise, TOC and total N and Na concentrations increased in the soils treated with the highest OMW concentration after 30 d incubation with respect to their initial values (Figure 6). Regular feeding of earthworms during the microcosm study could account for this finding because of exogenous organic matter input. In addition, earthworm-induced microbial mineralization of organic matter could be depressed because of toxic effects derived from high OMW concentrations on earthworms. Although these assumptions require further research, current data suggest that co-application of *L. terrestris* and leaf litter (as a food supply to earthworms) to OMW-amended soils improves soil quality in terms of enhanced exoenzyme activity as long as concentrations of OMW sediments do not exceed 20% (w/w).

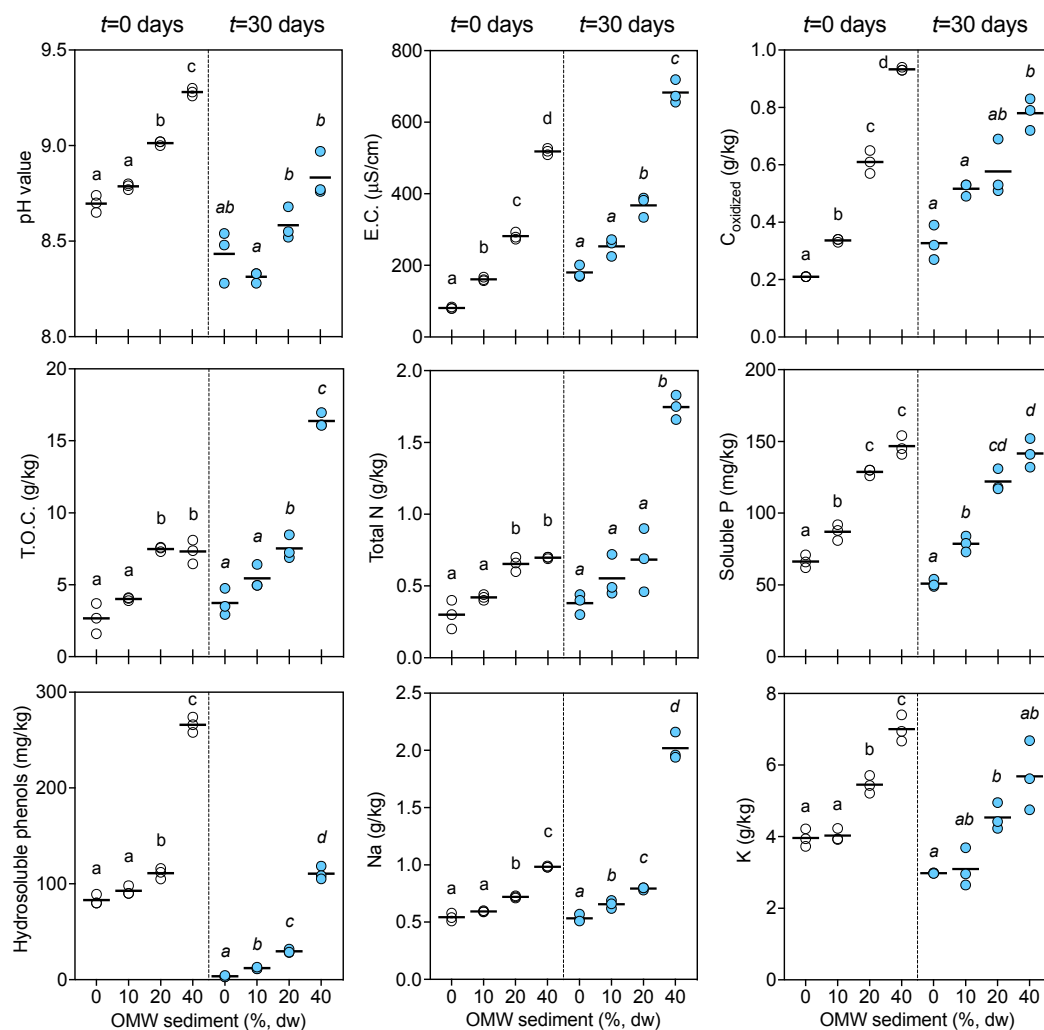


Figure 6. Variation of physicochemical parameters in olive mill waste (OMW)-amended soils during 30-d incubation in the presence of earthworms (*Lumbricus terrestris*). Different letters denote significant differences between treatments within a sampling time (normal fonts for $t = 0$ d, and cursive fonts for $t = 30$ d), ANOVA with Welch's homogeneity correction ($p < 0.05$). Horizontal solid lines in the univariate scatterplots are the mean values.

4. Conclusions

The findings of this study suggest that inoculation of OMW-amended soils with the earthworm *L. terrestris* facilitates the degradation of toxic phenolic compounds, and promotes soil quality through soil extracellular enzyme activities. The impact of OMW sediments at multiple levels of the drilosphere (i.e., the soil environment under the influence of earthworms, which include the burrow walls, gastrointestinal microenvironment, casts and middens), and the toxicity of the sediments to the earthworms (survival, growth and biomarker responses) indicated that a mixture proportion of OMW sediments in soil up to 20% *w/w* is suitable for managing this environmentally hazardous residue under a bioremediation scheme that includes the use of *L. terrestris*. Therefore, and pending of field validation, our study supports the idea that inoculation of contaminated or degraded soils with soil-dwelling earthworms is a potential bioremediation methodology (so-called vermiremediation), whereby these organisms act as biological vectors of microbial activity proliferation and exoenzyme production.

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J.C.S.-H.; visualization, J.C.S.-H.; writing—original draft, J.C.S.-H.; writing—review and editing, J.C.S.-H., R.M. and J.M. All authors have read and agreed to the published version of the manuscript.

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References

1. Kavvadias, V.; Doula, M.K.; Komnitsas, K.; Liakopoulou, N. Disposal of olive oil mill wastes in evaporation ponds: Effects on soil properties. *J. Hazard. Mater.* **2010**, *182*, 144–155. [[CrossRef](#)] [[PubMed](#)]
2. Kavvadias, V.; Elaiopoulos, K.; Theocharopoulos, S.; Soupios, P. Fate of Potential Contaminants Due to Disposal of Olive Mill Wastewaters in Unprotected Evaporation Ponds. *Bull. Environ. Contam. Toxicol.* **2017**, *98*, 323–330. [[CrossRef](#)] [[PubMed](#)]
3. Dermeche, S.; Nadour, M.; Larroche, C.; Moulti-Mati, F.; Michaud, P. Olive mill wastes: Biochemical characterizations and valorization strategies. *Proc. Biochem.* **2013**, *48*, 1532–1552. [[CrossRef](#)]
4. Senol, A.; Hasdemir, İ.M.; Hasdemir, B.; Kurdaş, İ. Adsorptive removal of biophenols from olive mill wastewaters (OMW) by activated carbon: Mass transfer, equilibrium and kinetic studies. *Asia-Pac. J. Chem. Eng.* **2017**, *12*, 128–146. [[CrossRef](#)]
5. Hentati, O.; Oliveira, V.; Sena, C.; Bouji, M.S.M.; Wali, A.; Ksibi, M. Soil contamination with olive mill wastes negatively affects microbial communities, invertebrates and plants. *Ecotoxicology* **2016**, *25*, 1500–1513. [[CrossRef](#)]
6. Enaime, G.; Baçaoui, A.; Yaacoubi, A.; Belaqziz, M.; Wichern, M.; Lübken, M. Phytotoxicity assessment of olive mill wastewater treated by different technologies: Effect on seed germination of maize and tomato. *Environ. Sci. Pollut. Res.* **2020**. [[CrossRef](#)] [[PubMed](#)]
7. Babić, S.; Malev, O.; Pflieger, M.; Lebedev, A.T.; Mazur, D.M.; Kužić, A.; Čož-Rakovac, R.; Trebše, P. Toxicity evaluation of olive oil mill wastewater and its polar fraction using multiple whole-organism bioassays. *Sci. Total Environ.* **2019**, *686*, 903–914. [[CrossRef](#)]
8. Smeti, E.; Kalogianni, E.; Karaouzas, I.; Laschou, S.; Tornés, E.; De Castro-Català, N.; Anastasopoulou, E.; Koutsodimou, M.; Andriopoulou, A.; Vardakas, L.; et al. Effects of olive mill wastewater discharge on benthic biota in Mediterranean streams. *Environ. Pollut.* **2019**, *254*, 113057. [[CrossRef](#)]
9. Aharonov-Nadborny, R.; Tschansky, L.; Raviv, M.; Graber, E.R. Mechanisms governing the leaching of soil metals as a result of disposal of olive mill wastewater on agricultural soils. *Sci. Total Environ.* **2018**, *630*, 1115–1123. [[CrossRef](#)]
10. Zema, D.A.; Esteban Lucas-Borja, M.; Andiloro, S.; Tamburino, V.; Zimbone, S.M. Short-term effects of olive mill wastewater application on the hydrological and physico-chemical properties of a loamy soil. *Agric. Water Manag.* **2019**, *221*, 312–321. [[CrossRef](#)]
11. Meftah, O.; Guergueb, Z.; Braham, M.; Sayadi, S.; Mekki, A. Long term effects of olive mill wastewaters application on soil properties and phenolic compounds migration under arid climate. *Agric. Water Manag.* **2019**, *212*, 119–125. [[CrossRef](#)]
12. Lee, Z.S.; Chin, S.Y.; Lim, J.W.; Witoon, T.; Cheng, C.K. Treatment technologies of palm oil mill effluent (POME) and olive mill wastewater (OMW): A brief review. *Environ. Technol. Innov.* **2019**, *15*, 100377. [[CrossRef](#)]
13. Martínez-Gallardo, M.R.; López, M.J.; Jurado, M.M.; Suárez-Estrella, F.; López-González, J.A.; Sáez, J.A.; Moral, R.; Moreno, J. Bioremediation of Olive Mill Wastewater sediments in evaporation ponds through in situ composting assisted by bioaugmentation. *Sci. Total Environ.* **2019**, 135537. [[CrossRef](#)] [[PubMed](#)]
14. Rodriguez-Campos, J.; Dendooven, L.; Alvarez-Bernal, D.; Contreras-Ramos, S.M. Potential of earthworms to accelerate removal of organic contaminants from soil: A review. *Appl. Soil Ecol.* **2014**, *79*, 10–25. [[CrossRef](#)]
15. Morillo, E.; Villaverde, J. Advanced technologies for the remediation of pesticide-contaminated soils. *Sci. Total Environ.* **2017**, *586*, 576–597. [[CrossRef](#)] [[PubMed](#)]
16. FAO. *World Reference Base for Soil Resources 2014*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2015.
17. Lowe, C.N.; Butt, K.R. Culture techniques for soil dwelling earthworms: A review. *Pedobiologia* **2005**, *49*, 401–413. [[CrossRef](#)]

18. Sanchez-Hernandez, J.C.; Ríos, J.M.; Attademo, A.M.; Malcevski, A.; Andrade Cares, X. Assessing biochar impact on earthworms: Implications for soil quality promotion. *J. Hazard. Mater.* **2019**, *366*, 582–591. [[CrossRef](#)]
19. ISO. *Soil Quality—Avoidance Test for Determining the Quality of Soils and Effects of Chemicals on Behaviour-Part 1: Test with Earthworms (Eisenia fetida and Eisenia andrei)*; ISO: Geneva, Switzerland, 2007.
20. De Silva, P.M.C.S.; van Gestel, C.A.M. Comparative sensitivity of *Eisenia andrei* and *Perionyx excavatus* in earthworm avoidance tests using two soil types in the tropics. *Chemosphere* **2009**, *77*, 1609–1613. [[CrossRef](#)]
21. Deng, S.; Popova, I.E.; Dick, L.; Dick, R. Bench scale and microplate format assay of soil enzyme activities using spectroscopic and fluorometric approaches. *Appl. Soil Ecol.* **2013**, *64*, 84–90. [[CrossRef](#)]
22. Wheelock, C.E.; Eder, K.J.; Werner, I.; Huang, H.; Jones, P.D.; Brammell, B.F.; Elskus, A.A.; Hammock, B.D. Individual variability in esterase activity and CYP1A levels in Chinook salmon (*Oncorhynchus tshawytscha*) exposed to esfenvalerate and chlorpyrifos. *Aquat. Toxicol.* **2005**, *74*, 172–192. [[CrossRef](#)]
23. Eyer, P.; Worek, F.; Kiderlen, D.; Sinko, G.; Stuglin, A.; Simeon-Rudolf, V.; Reiner, E. Molar absorption coefficients for the reduced Ellman reagent: Reassessment. *Anal. Biochem.* **2003**, *312*, 224–227. [[CrossRef](#)]
24. Habig, W.H.; Pabst, M.J.; Jakoby, W.B. Glutathione S-transferase: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **1974**, *249*, 7130–7139. [[PubMed](#)]
25. Ramos-Martinez, J.I.; Bartolomé, T.R.; Pernas, R.V. Purification and properties of glutathione reductase from hepatopancreas of *Mytilus edulis* L. *Comp. Biochem. Physiol.* **1983**, *75B*, 689–692. [[CrossRef](#)]
26. Rahman, I.; Kode, A.; Biswas, S.K. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat. Protoc.* **2007**, *1*, 3159–3165. [[CrossRef](#)] [[PubMed](#)]
27. Nozaki, M.; Ito, K.; Miura, C.; Miura, T. Examination of digestive enzyme distribution in gut tract and functions of intestinal caecum, in megascolecoid earthworms (Oligochaeta: Megascolecidae) in Japan. *Zool. Sci.* **2013**, *30*, 710–715. [[CrossRef](#)] [[PubMed](#)]
28. Gupta, N.; Rathi, P.; Gupta, R. Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Anal. Biochem.* **2002**, *311*, 98–99. [[CrossRef](#)]
29. Thompson, H.M. Esterases as markers of exposure to organophosphates and carbamates. *Ecotoxicology* **1999**, *8*, 369–384. [[CrossRef](#)]
30. Sanchez-Hernandez, J.C.; Ríos, J.M.; Attademo, A.M. Response of digestive enzymes and esterases of ecotoxicological concern in earthworms exposed to chlorpyrifos-treated soils. *Ecotoxicology* **2018**, *27*, 890–899. [[CrossRef](#)]
31. Sanchez-Hernandez, J.C.; Sandoval, M.; Pierart, A. Short-term response of soil enzyme activities in a chlorpyrifos-treated mesocosm: Use of enzyme-based indexes. *Ecol. Indic.* **2017**, *73*, 525–535. [[CrossRef](#)]
32. Lessard, I.; Sauvé, S.; Deschênes, L. Toxicity response of a new enzyme-based functional diversity methodology for Zn-contaminated field-collected soils. *Soil Biol. Biochem.* **2014**, *71*, 87–94. [[CrossRef](#)]
33. Shaw, L.J.; Burns, R.G. *Enzyme Activity Profiles and Soil Quality*; CABI: Oxford, UK, 2006; pp. 158–182.
34. Bustamante, M.A.; Paredes, C.; Moral, R.; Moreno-Caselles, J.; Pérez-Murcia, M.D.; Pérez-Espinosa, A.; Bernal, M.P. Co-composting of distillery and winery wastes with sewage sludge. *Water Sci. Technol.* **2007**, *56*, 187–192. [[CrossRef](#)] [[PubMed](#)]
35. Yeomans, J.C.; Bremner, J.M. A rapid and precise method for routine determination of organic carbon in soil. *Commun. Soil Sci. Plant Anal.* **1988**, *19*, 1467–1476. [[CrossRef](#)]
36. Kitson, R.E.; Mellon, M.G. Colorimetric determination of phosphorus as molybdivanadophosphoric acid. *Ind. Eng. Chem. Anal. Ed.* **1944**, *16*, 379–383. [[CrossRef](#)]
37. Beltrán, F.J.; García-Araya, J.F.; Álvarez, P.M. Wine Distillery Wastewater Degradation. 1. Oxidative Treatment Using Ozone and Its Effect on the Wastewater Biodegradability. *J. Agric. Food Chem.* **1999**, *47*, 3911–3918. [[CrossRef](#)]
38. Sanchez, W.; Burgeot, T.; Porcher, J.M. A novel Integrated Biomarker Response calculation based on reference deviation concept. *Environ. Sci. Pollut. Res. Int.* **2013**, *20*, 2721–2725. [[CrossRef](#)]
39. Hund-Rinke, K.; Achazi, R.; Römbke, J.; Warnecke, D. Avoidance test with *Eisenia fetida* as indicator for the habitat function of soils: Results of a laboratory comparison test. *J. Soils Sediments* **2003**, *3*, 7–12. [[CrossRef](#)]

40. Delgadillo, V.; Verdejo, J.; Mondaca, P.; Verdugo, G.; Gaete, H.; Hodson, M.E.; Neaman, A. Proposed modification to avoidance test with *Eisenia fetida* to assess metal toxicity in agricultural soils affected by mining activities. *Ecotoxicol. Environ. Saf.* **2017**, *140*, 230–234. [[CrossRef](#)]
41. De Sousa, A.P.; de Andréa, M.M. Earthworm (*Eisenia andrei*) avoidance of soils treated with cypermethrin. *Sensors (Basel)* **2011**, *11*, 11056–11063. [[CrossRef](#)]
42. Saggiaro, E.M.; do Espírito Santo, D.G.; Sales Júnior, S.F.; Hauser-Davis, R.A.; Correia, F.V. Lethal and sublethal effects of acetamiprid on *Eisenia andrei*: Behavior, reproduction, cytotoxicity and oxidative stress. *Ecotoxicol. Environ. Saf.* **2019**, *183*, 109572. [[CrossRef](#)]
43. Cao, Q.; Steinman, A.D.; Yao, L.; Xie, L. Toxicological and biochemical responses of the earthworm *Eisenia fetida* to cyanobacteria toxins. *Sci. Rep.* **2017**, *7*, 15954. [[CrossRef](#)]
44. Alves, P.R.L.; Bandeira, F.O.; Giraldo, M.; Presotto, R.; Segat, J.C.; Cardoso, E.J.B.N.; Baretta, D. Ecotoxicological assessment of Fluazuron: Effects on *Folsomia candida* and *Eisenia andrei*. *Environ. Sci. Pollut. Res. Int.* **2019**, *26*, 5842–5850. [[CrossRef](#)]
45. Prendergast-Miller, M.T.; Katsiamides, A.; Abbass, M.; Sturzenbaum, S.R.; Thorpe, K.L.; Hodson, M.E. Polyester-derived microfibre impacts on the soil-dwelling earthworm *Lumbricus terrestris*. *Environ. Pollut.* **2019**, *251*, 453–459. [[CrossRef](#)]
46. Elliston, T.; Oliver, I.W. Ecotoxicological assessments of biochar additions to soil employing earthworm species *Eisenia fetida* and *Lumbricus terrestris*. *Environ. Sci. Pollut. Res. Int.* **2019**. [[CrossRef](#)]
47. Li, D.; Hockaday, W.C.; Masiello, C.A.; Alvarez, P.J.J. Earthworm avoidance of biochar can be mitigated by wetting. *Soil Biol. Biochem.* **2011**, *43*, 1732–1737. [[CrossRef](#)]
48. Brami, C.; Glover, A.R.; Butt, K.R.; Lowe, C.N. Avoidance, biomass and survival response of soil dwelling (endogeic) earthworms to OECD artificial soil: Potential implications for earthworm ecotoxicology. *Ecotoxicology* **2017**, *26*, 576–579. [[CrossRef](#)]
49. Pelosi, C.; Toutous, L.; Chiron, F.; Dubs, F.; Hedde, M.; Muratet, A.; Ponge, J.-F.; Salmon, S.; Makowski, D. Reduction of pesticide use can increase earthworm populations in wheat crops in a European temperate region. *Agric. Ecosyst. Environ.* **2013**, *181*, 223–230. [[CrossRef](#)]
50. Jager, T.; Fleuren, R.H.L.J.; Hogendoorn, E.A.; de Korte, G. Elucidating the routes of exposure for organic chemicals in the earthworm, *Eisenia andrei* (Oligochaeta). *Environ. Sci. Technol.* **2003**, *37*, 3399–3404. [[CrossRef](#)]
51. Van der Oost, R.; Beyer, J.; Vermeulen, N.P.E. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol.* **2003**, *13*, 57–149. [[CrossRef](#)]
52. Hayes, J.D.; Flanagan, J.U.; Jowsey, I.R. Glutathione transferases. *Ann. Rev. Pharmacol. Toxicol.* **2005**, *45*, 51–88. [[CrossRef](#)] [[PubMed](#)]
53. LaCourse, E.J.; Hernandez-Viadel, M.; Jefferies, J.R.; Svendsen, C.; Spurgeon, D.J.; Barrett, J.; Morgan, A.J.; Kille, P.; Brophy, P.M. Glutathione transferase (GST) as a candidate molecular-based biomarker for soil toxin exposure in the earthworm *Lumbricus rubellus*. *Environ. Pollut.* **2009**, *157*, 2459–2469. [[CrossRef](#)] [[PubMed](#)]
54. Bonnail, E.; Buruaem, L.M.; Araujo, G.S.; Abessa, D.M.; DelValls, T.Á. Multiple biomarker responses in *Corbicula fluminea* exposed to copper in laboratory toxicity tests. *Arch. Environ. Contam. Toxicol.* **2016**, *71*, 278–285. [[CrossRef](#)] [[PubMed](#)]
55. Vieira, C.E.; Costa, P.G.; Lunardelli, B.; de Oliveira, L.F.; Cabrera, L.C.; Risso, W.E.; Primel, E.G.; Meletti, P.C.; Fillmann, G.; Martinez, C.B. Multiple biomarker responses in *Prochilodus lineatus* subjected to short-term in situ exposure to streams from agricultural areas in Southern Brazil. *Sci. Total Environ.* **2016**, *542*, 44–56. [[CrossRef](#)] [[PubMed](#)]
56. Dempsey, M.A.; Fisk, M.C.; Yavitt, J.B.; Fahey, T.J.; Balser, T.C. Exotic earthworms alter soil microbial community composition and function. *Soil Biol. Biochem.* **2013**, *67*, 263–270. [[CrossRef](#)]
57. Stromberger, M.E.; Keith, A.M.; Schmidt, O. Distinct microbial and faunal communities and translocated carbon in *Lumbricus terrestris* drilospheres. *Soil Biol. Biochem.* **2012**, *46*, 155–162. [[CrossRef](#)]
58. Hoang, D.T.T.; Razavi, B.S.; Kuzyakov, Y.; Blagodatskaya, E. Earthworm burrows: Kinetics and spatial distribution of enzymes of C-, N- and P-cycles. *Soil Biol. Biochem.* **2016**, *99*, 94–103. [[CrossRef](#)]

59. Yang, P.; van Elsas, J.D. Mechanisms and ecological implications of the movement of bacteria in soil. *Appl. Soil Ecol.* **2018**, *129*, 112–120. [[CrossRef](#)]
60. Blouin, M.; Hodson, M.E.; Delgado, E.A.; Baker, G.; Brussaard, L.; Butt, K.R.; Dai, J.; Dendooven, L.; Peres, G.; Tondoh, J.E.; et al. A review of earthworm impact on soil function and ecosystem services. *Eur. J. Soil Sci.* **2013**, *64*, 161–182. [[CrossRef](#)]



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