Environmental Science and Pollution Research (2022) 29:24956–24967
https://doi.org/10.1007/s11356-021-17593-1

RESEARCH ARTICLE

Effects of olive mill waste (OMW) contaminated soil on biochemical biomarkers and reproduction of *Dendrobaena veneta*

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Received: 20 April 2021 / Accepted: 14 November 2021 / Published online: 27 November 2021

Abstract

Olive oil industry is economically important in Mediterranean countries. Disposal of olive mill waste (OMW) presents an environmental concern in those countries due to its high salinity and its high level of polyphenols. In order to reuse OMW, those properties have to change either through the filtration process and addition of adsorbents or by composting. One of the most important organisms in composting of organic wastes is earthworms. However, data on the effects of OMW on earthworms are scarce. The main aim of our study was to investigate whether OMW contaminated soil (OMW CS) causes adverse effects on molecular and organism level in epigeic earthworm *Dendrobaena veneta* and on microbiological activity. Changes of measured biochemical biomarkers (AChE, CAT, GST, lipids, MDA) varied depending on the quantity of added OMW CS and the exposure duration. Oxidative stress occurred after 7 days of exposure, while in most cases enzyme activity recovered after 28 days. At the highest ratio of contaminated soil (50%), reproduction was completely inhibited. The second aim was to investigate the impact of earthworms on phenol degradation and microbial activity, indicating an important role in the bioremediation of contaminated soils. Our results show that above a certain quantity an OMW CS has an adverse effect on earthworms, while the impact of earthworms on soil microbial activity was positive but transient. Yet, as the results also imply that earthworms have an impact on phenol degradation, they can be used to help remediation of OMW CS and its subsequent usage in agriculture. However, the quantity of OMW CS that can be safely added should be determined first.

Keywords Earthworm · Olive mill waste contaminated soil · Phenol degradation · Biomarkers · Reproduction · Microbiological activity · *Dendrobaena veneta*

Introduction

Mediterranean countries are characterized by an important olive oil industry, which reaches 98% of the world's olive oil production (Galliou et al. 2018). This important economic activity leads to the generation of a significant amount of liquid waste during the harvest season, known as olive mill waste water (OMW) (Kapellakis et al. 2006). Due to the widespread disposal of these effluents in evaporation ponds, their number has increased over time throughout the Mediterranean region (Jarboui et al. 2010; Komnitsas and Zaharaki 2016). The management and disposal of this waste represent a crucial environmental issue in Mediterranean countries (Chouchene et al. 2010).

OMW is composed of 83–94% water, 4–16% organic compounds and 0.4–2.5% mineral salts (Davies et al. 2004). The organic fraction contains 2–15% phenolic components such as caffeic acid, tyrosol and tannins (Hachicha et al.

Responsible Editor: Chris Lowe

Highlights
- Responses of biomarkers were correlated with exposure time and OMW CS ratio.
- The highest applied dose of OMW CS (50%) prevented reproduction of *D. veneta*.
- A change in soil microbial activity indicated a transient stimulatory effect by earthworms.
- Earthworms stimulated a degradation of phenolic compounds.

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2009), with concentrations ranging from 0.8 to 24 g/l (Asses et al. 2009). This concentration depends on the processing system used for olive oil production (Sassi et al. 2006). Polyphenols are potentially hazardous to the environment and human health and represent the most recalcitrant fraction of OMW (Chtourou et al. 2004; Mekki et al. 2007). The presence of these recalcitrant organic compounds is one of the major problems in the management of these wastes, which are often disposed into the environment (Saadi et al. 2007). Soluble phenolic compounds and other pollutants from OMW, such as heavy metals and pesticides, could leach to deeper soil layers as long as olive wastes remain on the soil surface (Kavvadias et al. 2010). This significantly increases the risk of soil degradation (Doula et al. 2017) and the likelihood of impacts on soil fauna. Several authors studied the effects of OMW on soil microbial activity, suggesting a beneficial effect on soil microbes (Gamba et al. 2005; Saadi et al. 2007), although OMW is considered toxic (Mekki et al. 2008). These results could be explained by the removal of toxicity by other soil organisms such as fungi (Mekki et al. 2012). In view of this, a joint assessment of the effects of OMW on soil microbes and soil fauna is desirable. Therefore, it is important to investigate the effects of OMW and OMW contaminated soil (OMW CS) on non-target soil organisms such as earthworms, potworms or springtails. Earthworms have been used to assess the toxicity of metal-contaminated soils (García-Carmona et al. 2017) and for organic contamination (Rivier et al. 2019). Except their role in toxicity assessment, earthworms may have the potential for bioremediation. Because of their biological, chemical and physical actions, earthworms can be used directly in bioremediation strategies to promote biodegradation of organic contaminants. However, there has been only scarce research conducted on the effects of OMW on different soil organisms such as earthworms (Hentati et al. 2016; Campani et al. 2017; Chalkia et al., 2020., Sáez et al. 2020), arthropods (Karaouzas 2011) and collembolans (Kurtz et al. 2015). These authors demonstrated that OMW contamination affects the avoidance behavior of earthworms (*Eisenia fetida*), springtails (*Folsomia candida*) and the reproduction of enchytraeids (*Enchytraeus crypticus*). OMW toxicity depends on soil type, pH, carbonate content, nutrient content and organic carbon. Doula et al. (2017) confirmed that high carbonate content protects soils from the acidity of OMW as carbonates permanently react with acids and neutralize them while emitting gaseous CO_2. The degradation efficiency of phenol is improved by the presence of carbonate ions as carbonate radical is a selective oxidant in the degradation reacting with organic compounds. These properties are of great importance for the fate of OMW in the soil and thus for soil organisms (Hentati et al. 2016). The direct feedback of those organisms exposed to different types of contamination could be measured using a set of biomarkers. Biomarkers have been regarded as an efficient tool to investigate and evaluate the influence of pollutants, providing sensitive early-warning signs of pollution's risk to the organisms (Lam and Gray 2001). Antioxidative enzymes such as catalase (CAT), glutathione-S-transferase (GST) with other biochemical biomarkers such as acetylcholinesterase (AChE) activity, malondialdehyde (MDA), protein and lipid content are important bioindicators that represent a reliable tool in revealing the sublethal effect of the chemicals in soil ecosystems.

A few studies investigated the impact of OMW by evaluating the effects on different biomarkers, and most of them used marine organisms such as mussels as test organism. Danellakis et al. (2011) demonstrated the ability of OMW to induce oxidative stress, neurotoxic, genotoxic and cytotoxic effects in *Mytilus galloprovincialis* tissues by evaluating a set of biomarkers and the comet assay. Furthermore, the disposal of OMW into the marine environment could provoke pre-pathological alterations in marine organisms (Danellakis et al. 2011). Other research has focused on soil organisms such as earthworms (Campani et al. 2017). Namely, the toxicological effects of two different types of OMW on the earthworm *Eisenia fetida* were investigated using biomarkers of neurotoxicity (AChE), oxidative stress (LPO, CAT) and genotoxicity (comet assay). Raw OMW caused earthworm mortality at high doses and measurable biochemical and cellular effects at lower doses. They attribute this to the high acidity and polyphenols in OMW.

In order to create the necessary knowledge for the regular use of OMW in agriculture using vermicomposting, it is necessary to study the effects of OMW contamination on earthworms. Thus, the first aim of the present study was to assess the impact of OMW CS using a set of biomarkers at biochemical and population level. The second aim was to investigate the effects of earthworms on phenol degradation and soil microbial activity, which could indicate their important role in bioremediation of contaminated soil. The results obtained could be used in conjunction with the microbial activity data for the risk assessment of OMW CS.

Materials and methods

Test organisms

Earthworms (*Dendrobaena veneta* Rosa 1886) were obtained from a laboratory culture at the Department of Biology at J.J. Strossmayer University in Osijek, Croatia. *Dendrobaena veneta* is an epigeic species commonly used for vermicomposting. Therefore, *D. veneta* has a great potential to mitigate the adverse effects of soils contaminated with olive mill waste.

Adult earthworms with a well-developed clitellum weighting between 400 and 900 mg were used. Earthworms were removed from the synchronized culture and placed on a damp filter paper for 24 h in the dark to void their gut content prior to each exposure.

Test soils

OMW contaminated soil (OMW CS) samples were collected from the olive mill evaporation pond (OMW discharge pond) of Agareb, located 20 km from the city of Sfax, Tunisia (34°43′ N to 10°26′ E). OMW CS consisted of 55% sand, 16% silt and 29% clay. Initial phenol concentration was 375.28 mg/kg. Soil pH was 8.1 ± 0.2.

The artificial soil (AS) was prepared according to the protocol of the OECD (2004) with 10% sphagnum peat, 20% kaolin clay and approximately 70% air-dried quartz sand. The soil pH was adjusted to 6 ± 0.5 by addition of $CaCO_3$.

Experiment setup

Due to the lack of information on the impact of OMW CS on earthworms, a preliminary test was made to estimate sublethal concentrations.

Briefly, organisms were exposed to a mixture of AS and OMW CS sampled from the evaporation basin. Used mixture of OMW CS/AS was (MX1:10/90, MX2:25/75, MX3:50/50, MX4:75/25 and MX5:100/0). Control was run in parallel containing AS only. As the mortality occurred after exposure to MX4 (75% of OMW CS) and MX5 (100% of OMW CS), those ratios were omitted from further experiments. According to the results of the preliminary test, three mixtures MX1:10/90, MX2:25/75, MX3:50/3 were used in the reproduction test, marked as R2:10, R3:25 and R4:50% OMW CS; a new ratio R1:5% was also added. The OMW CS was added at the top of the dry AS and then mixed with a spatula for approximately 5 min, ensuring that the soil is well homogenized.

Reproduction test

In this experiment; four ratios were used (R1:5, R2:10, R3:25 and R4:50% of OMW CS), and three exposure periods (7, 28 and 56 days) were assessed. Control (R0) was run in parallel containing AS only.

Reproduction test was assessed following the Organization for Economic Cooperation and Development guideline (OECD 2004) including 28-day exposure of adult animals followed by another 28-day incubation of cocoons to enable the assessment of juvenile production.

Before adding earthworms, distilled water was added until 50% of WHC and all mixtures were left overnight to equilibrate. After that, adult earthworms were introduced. Vessels were covered with perforated lids for aeration and placed in a climate chamber with a stable temperature of 20 ± 1 °C, moisture of 40 ± 2% and 12:12 light:dark cycle. All treatments were performed in duplicate.

Containers were monitored weekly for maintaining soil moisture content and food required conditions. After 7 days of exposure, three earthworms were removed from each vessel and placed on sterilized Petri dishes to empty their guts. Earthworms were weighted, homogenized and prepared for further analyses. Same procedure was repeated on the 28th day. Additionally, the cocoons were counted and carefully placed back into the soil for the next 28 days. After that period, the number of juveniles was determined by placing the containers in a water bath at 60 ± 5 °C for 1 h, forcing juveniles to emerge to the surface, where they were counted. The number of unhatched cocoons was counted by hand sorting of soil.

Test vessels without earthworms were used as a control. Total phenol concentration was determined in all vessels and for each exposure period (0, 7, 28 and 56 days). FDA (fluorescein diacetate hydrolysis) and BFA (biofilm forming ability) were determined in R3 (25% OMW CS) for each exposure period (0, 7, 28 and 56 days).

Sample preparation and measurements

Earthworms were homogenized (1:5 w:v) in sodium phosphate buffer (0.1 M, pH 7.4) under ice-cold conditions using an OMNI TH-220 homogenizer. Homogenates were centrifuged at 9000 × g and 4 °C for 30 min to yield the post-mitochondrial fraction (S9). The resulting supernatant was used for further analysis.

AChE activity was detected with the method of Ellman et al. (1961). Kinetics was recorded at 412 nm. The reaction mixture contained sodium phosphate buffer (0.1 M, pH 7.2), DTNB (0.03 mM), AcSChl (3.6 mM) and sample post-mitochondrial fraction (S9). The enzymatic activity was expressed as nmol of acetylthiocholine hydrolyzed per min per mg of protein, and for calculations the absorption coefficient of 13.6 × 10 3 M^{-1} cm^{-1} was used. CAT activity was determined as described by Claiborne (1985). The enzyme activity was calculated from the decrease in absorption at 240 nm in 30 s, following the degradation of hydrogen peroxide by CAT present in the sample. The reaction medium consisted of sodium phosphate buffer (0.1 M, pH 7.2), hydrogen peroxide (9.3 mM) and sample (S9). An absorption coefficient of 42.6 M^{-1} cm^{-1} was used, and the activity was expressed as µmol of degraded H_2O_2 per min per mg of protein. The activity of glutathione S-transferase was measured spectrophotometrically at 340 nm according to the method of Habig et al. (1974). The reaction medium included CDNB (0.78 mM), GSH (4.9 mM) and sample (S9). According to Gagné (2014), lipid peroxidation was

determined by measuring the formation of thiobarbituric acid reactive substance (TBARS). TBARS were detected fluorometrically at 535-nm excitation and 635-nm emission in a microplate. The amount of MDA was calculated with calibration curve using tetramethoxypropane. The reaction mixture is composed of TCA (0.22 M), TBA (46.5 mM), $FeSO_4$ (1.85 mM) and sample (S9). Lipid content per earthworm was detected in the homogenate using the method of Frings et al. (1972). The absorbance was measured at 540 nm, and the amount of lipids was calculated based on the calibration curve with Triton X-100 as standard. Protein content per earthworm was detected with the method of Lowry et al. (1951). A calibration curve using bovine serum albumin as a standard was made for the calculation of the amount of proteins based on the absorbance measured at 730 nm. All activities, proteins, lipids and MDA content were expressed as relative to a corresponding control.

Concentration of phenolic compounds was determined in water extract of tested soil. Five grams of soil was mixed with 25 ml of distilled water for 20 h, filtered and centrifuged for 10 min at 5000 g. Phenol concentration was measured colorimetrically using Folin-Ciocalteu reagent. The absorbance was determined at 725 nm. Results were expressed in mg/kg of gallic acid (Box 1981).

The biofilm forming ability (BFA) of the microbial community was determined by the slightly modified method of Golby et al. (2012). Briefly, 302.5 mg ± 2.5 mg of soil samples were collected into the sterile plastic centrifuge tubes (2 ml) and mixed with 500 µl of sterile distilled water. Mixture was vortexed for 5 s at 2990 rpm. In each well of 48-well plate (Sarstedt), 100 µl of medium consisting of: 2 × M63 medium, supplemented with magnesium sulphate heptahydrate (493 µg/ml), glucose (4 mg/ml) and 1:500 dilution of the nutrient broth (Biolife) was added. After medium, 100 µl of soil slurry was added in three wells per sample using wide orifice pipette tips. The plates were incubated for 24 h at 25 °C. The samples were discarded by inverting the plates and washed three times by submerging into the container with the distilled water. Excess water was dipped, and samples were stained with crystal violet solution (0.1%) for 15 min at room temperature. The excessive stain was washed three times by submerging into the container with distilled water, and plates were left to air dry. The stain was dissolved in 1 ml of 96% ethanol, while absorbance was measured at 588 nm using a Shimadzu UV–Vis-1601 spectrophotometer.

Total microbial activity, i.e. non-specific activity of protease, lipase and esterase, was estimated by spectrophotometer measuring fluorescein diacetate (FDA) hydrolysis (Adam and Duncan 2001). The soil slurry left in the centrifuge tubes was mixed with 800 µl of 75 mM phosphate buffer pH 7.6 and 40 µl of 250 µl/ml FDA solution in acetone. The samples were vortexed after the FDA solution was added to start the reaction. The tubes were then placed on an orbital shaker (GFL 3005, 200 rpm) inside the incubator (30 °C) for 30 min. The reaction was stopped by addition of chloroform/methanol (2:1 v/v). The tubes were centrifuged at 16,500 g for 5 min, and the supernatants were measured immediately at 490 nm on Shimadzu UV–Vis spectrophotometer. FDA and BFA were determined only in R3 treatment as it was selected based on the results of the preliminary test. Namely, because of the lack of reproduction at 50% OMW CS in the preliminary test, the next lower concentration was chosen for the analysis of soil microbial activity.

Data analysis

All data was analyzed in R version 3.4.0 and RStudio (R Studio team 2016). Data were tested for normality using Shapiro–Wilk test and homogeneity of variance with Bartlett test. When no significant differences from normality were detected, all data were analyzed using analysis of variance (ANOVA). One-way ANOVA followed with a post hoc Dunnett's test was used to analyze differences in enzyme activity between concentrations and control. Data that were not normally distributed were analyzed using the Kruskal–Wallis test followed by Gao post hoc test. Differences between the same concentrations after different exposure periods were analyzed using paired *t* test.

Results

After 7 and 28 days of exposure to the mixture of AS and OMW CS, significant changes in activities of measured biomarkers were observed. All applied ratios were sublethal, and no mortality was recorded during the experiments. Moreover, there were no visible morphological changes in the exposed earthworms.

Significant loss of weight was observed after exposure to OMW CS (Fig. 1). After 7 days, a significant decrease of earthworm weight (over 40%) was detected at R4 (50% of OMW CS), and after 28 days for more than 40% in R3 (25% of OMW CS) and R4 (50% of OMW CS) treatments.

Exposure to OMW CS significantly affected AChE activity (Fig. 2A). Namely, after 7 days of exposure, there was a significant activity increase in R1 ($p = 0.0063$), R2 ($p = 0.0031$), R3 ($p = 0.0041$) treatments compared to control. After 28 days of exposure, smaller quantities of OMW CS did not affect AChE activity, while the highest ratio (50% of OMW CS) caused a significant induction of AChE activity (20%).

There was no significant change in CAT activity after 7 and 28 days of exposure (Fig. 2B). A significant inhibition of GST activity was observed after exposure to the highest ratio of OMW CS (Fig. 2C). After 7 days, GST activity decreased significantly (> 40%) at R4 ($p = 0.0061$). Furthermore, after

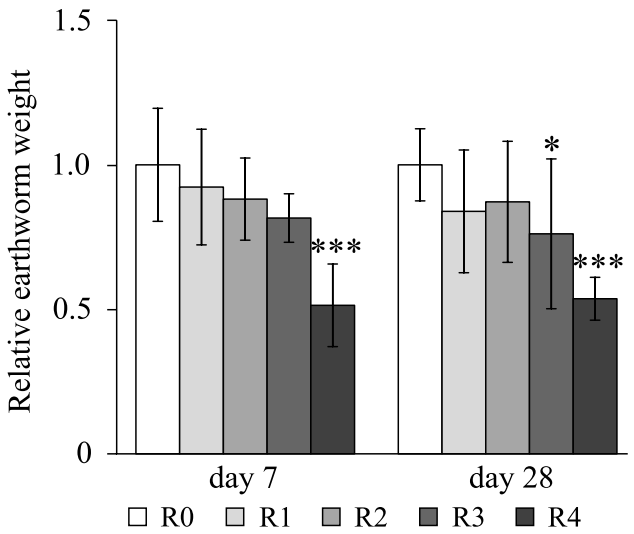

Fig. 1 Relative earthworm weight of *Dendrobaena veneta* exposed for seven and 28 days to different OMW CS ratios (R0 (0%), R1 (5%), R2 (10%), R3 (25%) and R5 (50%)). Significant differences within same exposure time obtained with Dunnett's post hoc test compared to control are labeled with * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$)

28 days of exposure, inhibition in dose–response manner was observed. Strong inhibition of GST activity was recorded at R3 (> 59%) and R4 (> 70%).

No significant differences were detected in protein content after 7 and 28 days of exposure (Fig. 3A); also, there was no observed change in lipid content after 7 days of exposure to OMW CS (Fig. 3B). Significant change in dose–response manner was obtained after 28 days (R2 ($p=0.0204$), R3 ($p=0.0117$) and R4 ($p<0.001$)). The higher decrease of lipid content was observed at R4 (50% of OMW CS) after 28 days of exposure (51% compared to control). There were no significant differences in the MDA content (Fig. 3C) or indication that significant lipid peroxidation occurred.

OMW CS also affected *D. veneta* reproduction (Table 1). Furthermore, there was a slight, but not significant, increase in cocoon number at lower ratio and a decrease at higher ratio of OMW CS. Number of cocoons was highest at R2, and no changes in cocoon hatchability were observed. The number of juveniles per cocoon decreased in a dose–response manner. Number of cocoons was highest at R2, and no changes in cocoon hatchability were observed. The number of juveniles per cocoon decreased in a dose–response manner.

Changes in BFA and FDA activity during exposure time were observed between treatments with and without earthworms (Fig. 4A, B). Furthermore, BFA activity was the highest on day 0, while changes of FDA activity occurred only at day seven.

Initial phenol concentrations were 19.39 ± 0.44 mg/kg (R0), 33.26 ± 0.68 mg/kg (R1), 45.07 ± 1.62 mg/kg (R2), 90.59 ± 1.11 mg/kg (R3) and 165.46 ± 2.04 mg/kg (R4). There were significant differences in total phenol content between all tested ratios and within tested ratios considering sampling time. Expected increase of total phenol content with increased OMW CS ratio was measured. Moreover, the expected decrease in total phenol content with time was observed. Furthermore, differences between treatments without and with earthworms were observed (Fig. 5). Significant difference was observed at R1 (5% of OMW CS) on day seven and 28 considering treatment without and with earthworms. Namely, total phenol content was significantly decreased in treatments with earthworms. Same pattern was observed at R2 (10% of OMW CS), while at R3 (25% of OMW CS) a significant decrease was observed only on day 28. At the highest ratio of OMW CS, there was a significant decrease on day seven, 28 and 56.

Discussion

Data presented in this study show that exposure to OMW CS caused measurable variation in biomarkers of *D. veneta* earthworm. However, *D. veneta* showed potential in accelerated remediation of soils significantly polluted with OMW probably through selective stimulation of specific microbial communities of phenol degraders of the OMW polluted soil.

At the end of the experiment, the earthworms were active, and no mortality was recorded, although earthworms lost weight. A significant decrease of earthworm weight was detected at higher ratio of OMW CS. Sanchez-Hernandez et al. (2020) also recorded a loss of body weight after 30 days of earthworm incubation in soils amended with 40% and 80% OMW. One possible reason for the decrease of earthworm weights could be a lower feeding rate. Another explanation could be earthworm activity. Increase of AChE activity indicates that earthworms were more active, consuming more energy, consequently resulting in weight loss. An opposite effect was observed by Chalkia et al. (2020) after exposure of earthworm *Octodrilus complanatus* to olive mill waste water. In their results, the trends of body weight change under the influence of OMW indicate that there was no adverse effect of OMW on the body weight gain of earthworms. Besides different olive byproducts were tested (wastewater vs. contaminated soil), the contrast could result from adaptation of O. complanatus to olive phenols, as earthworms from that research were collected in an olive grove. Additionally, different application was applied; in our research the OMW CS was mixed with the whole substrate and in Chalkia et al. (2020) OM waste water was sprayed on top.

Our results indicate that exposure to OMW CS has a negative influence on the lipid content. The same relationship was observed by Holmstrup et al. (2011) for earthworms living in sites polluted with heavy metals. At the highest

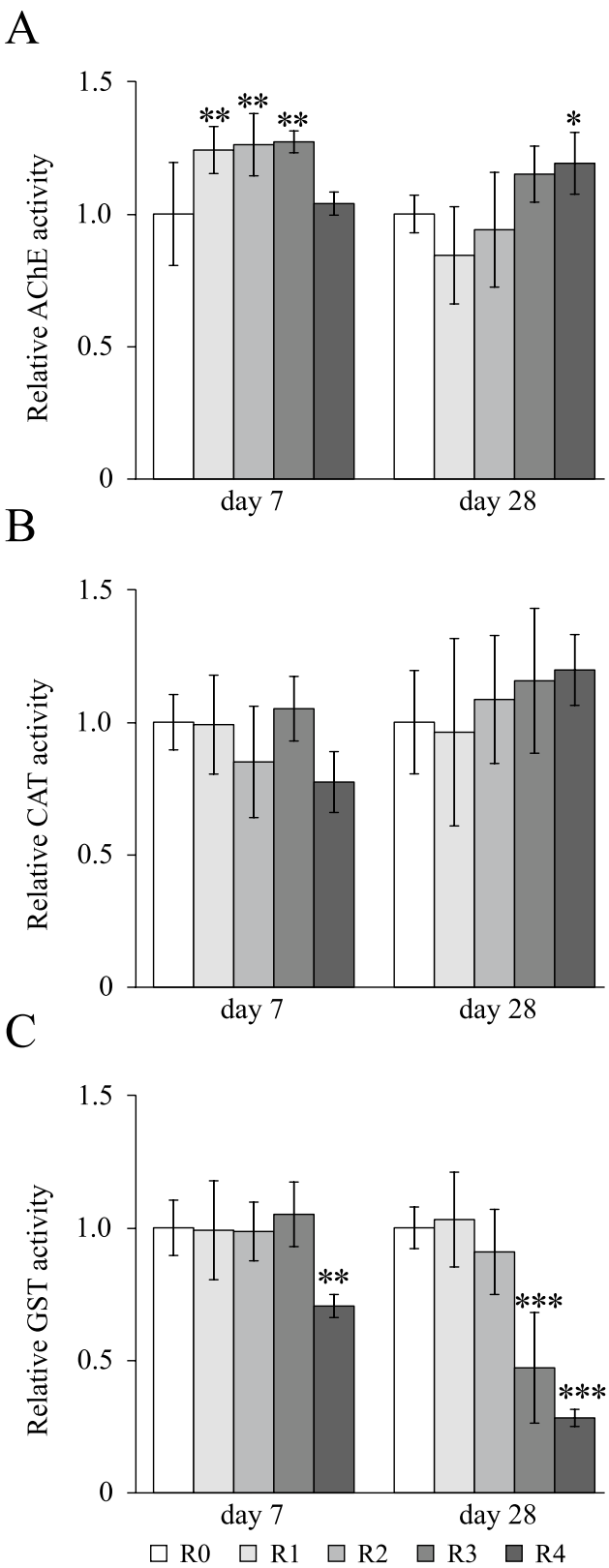

Fig. 2 Activities of acetylcholinesterase (AChE) (**A**), catalase (CAT) (**B**) and glutathione S-transferase (GST) (**C**) measured in *Dendrobaena veneta* exposed for 7 and 28 days to different OMW CS ratios (R0 (0%), R1 (5%), R2 (10%), R3 (25%) and R5 (50%)) and expressed as relative to a corresponding control. Significant differences within same exposure time obtained with Dunnett's post hoc test compared to control are labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

ratio of OMW CS, decrease was more than 50% compared to control. We assumed that these results are in correlation with AChE activity, where we observed induced activity at these ratios, and weight loss. Earthworms were more active, consume more energy and did not renew it enough, consequently leading to reduced energy reserves, i.e. lipid content.

A change of AChE activity is a widely used biomarker for detecting neurotoxic effects. After 7 days of exposure, dose–response curve for AChE activity was an inverted U-shape characteristic for hormesis (Fig. 2A). Hormesis characterizes the dose–response continuum as stimulatory at low doses and inhibitory at high doses. Campani et al. (2017) also found that low doses of raw olive mill waste water (12.5% w:v) induce AChE activity in earthworm *E. fetida*. The induction of AChE activity was correlated to an apoptosis inducing substance exposure (Zhang et al. 2002) and tissue inflammation (Gambardella et al. 2014) as an act to mitigate inflammatory response and restore homeostasis. Another plausible reason for AChE activity induction could be a higher physical activity in order to avoid stress inducing substrate. This assumption would be interesting to explore in an avoidance test, such as Djerdj et al. (2020).

Catalase activity did not show significant alterations in the treated groups compared to control. Campani et al. (2017) also did not observe significant changes in CAT activity after exposure of *E. fetida* to different concentrations of raw two-phase OMW.

GST is one of the most important phase II metabolizing enzymes in organisms. GST is responsible for the detoxification of xenobiotic and electrophile compounds by catalyzing the conjugation of the thiol group of reduced glutathione (GSH) (Singhal et al. 2015). The depletion of GST has been described as a sign of its consumption due to the activation of detoxification mechanisms (Hackenberger et al. 2018). Our study shows a strong GST activity inhibition of more than 70% at the highest ratio of OMW CS. According to our results, the decrease in activity could indicate a non-specific response to the chemicals present in OMW. This may be due to the low level of glutathione, which reflected the absence of additional synthesis after the consumption of glutathione in the early stage of detoxification. According to our results of GST activity, phase II biotransformation pathway was not induced by organic chemical constituents of OMW.

Lipid peroxidation is a reaction that occurs in organisms under oxidative stress by pollutants. Lipid peroxidation (LPO) was measured as thiobarbituric acid reactive substances (TBARS) and expressed as MDA equivalents. MDA is a major LPO metabolite, and it is considered a

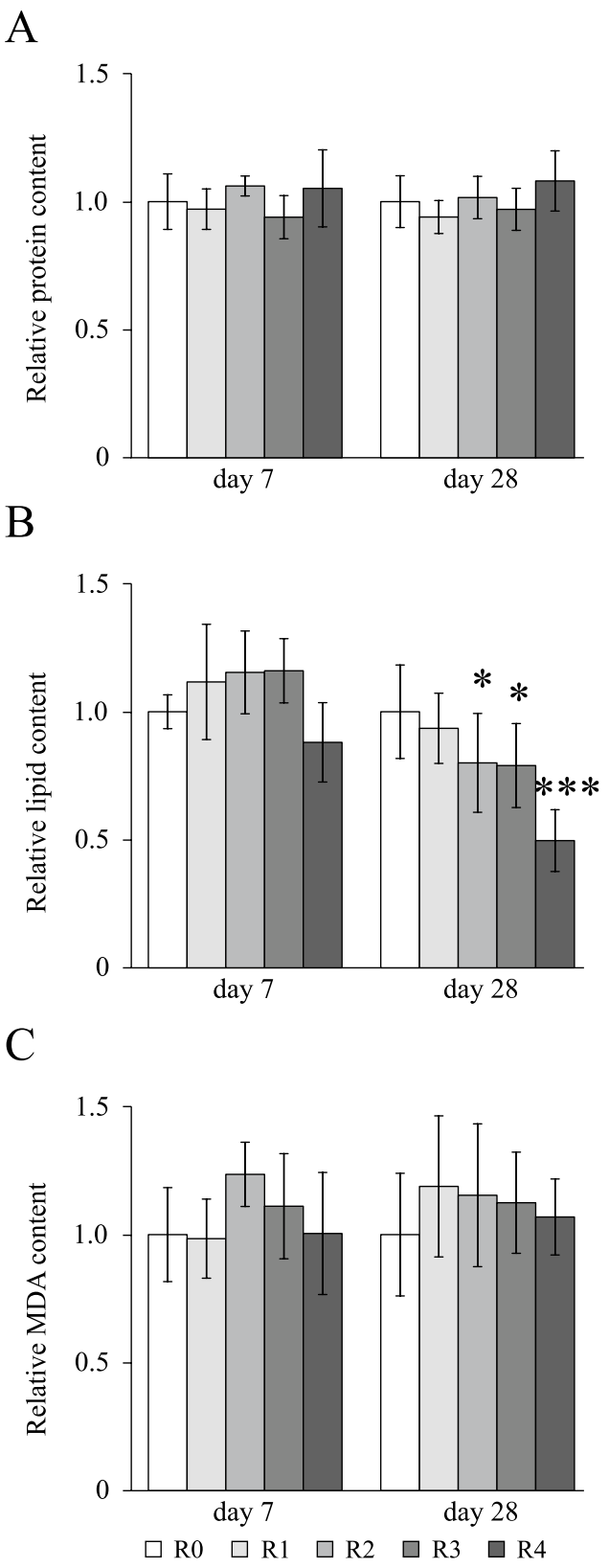

Fig. 3 Protein (**A**), lipid (**B**) and MDA (**C**) content measured in *Dendrobaena veneta* exposed for 7 and 28 days to different OMW CS ratios (R0 (0%), R1 (5%), R2 (10%), R3 (25%) and R5 (50%)) expressed relative to a corresponding control. Significant differences within same exposure time obtained with Dunnett's post hoc test compared to control are labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

reliable indicator of oxidative damage of cellular membranes (Tavazzi et al. 2000). Our results show that lipid peroxidation does not occur in earthworms after exposure to investigated mixtures of AS and OMW CS, probably as any existing peroxidant radicals were scavenged by antioxidant enzymes (e.g. superoxide dismutase (SOD) and peroxidase (POD)), which alleviate oxidative stress (Schmitt et al. 2007). It can be attributed to lower concentrations of polyphenols and, possibly, heavy metals in our mixtures when compared to unmixed substrates investigated in Mkhinini et al. (2019) that caused a significant induction of MDA indicating LPO.

The adverse effect of higher amount of OMW CS was also observed on the reproduction level. At the highest ratio of OMW CS (50%), there was no reproduction recorded at all. The same deleterious effect was observed in a study by Hentati et al. (2016) after exposure of *E. fetida* to OMW from Tunisia. The number of juveniles gradually decreased as the relative amount of dried OMW increased. Number of cocoons was not affected, but a negative effect on hatchability was detected. Similar trend was observed during exposure of OMW polar fraction to zebrafish embryos where the results have shown increased mortality, higher abnormality rate and decreased hatching (Babić et al. 2019). At R2 (10% OMW CS), the number of cocoons per earthworm was the highest (3.08 ± 0.35) which could indicate a hormetic effect as responses to stressors are often biphasic, i.e. a reaction towards stress as an increase of reproductive rate (Santadino et al. 2014). The enhanced reproduction rate in earthworms was already observed after exposure to different pesticides at low concentrations (Suthar 2014; Hackenberger et al. 2018).

High phenol content in OMW represents a major reason for its toxicity. It is well known that phenol content in high doses induces oxidative stress in living organisms (Charan et al. 2015; Varadarajan and Philip 2016; Duan et al. 2018). Martins et al. (2008) investigated the acute toxicity of OMW in rat liver mitochondrial bioenergetics and concluded that the inhibitory action of OMW on the phosphorylation efficiency of mitochondria could be a result of the incorporation of organic compounds present in OMW. To reduce the toxicity of OMW CS, different bioremediation techniques can be applied. Bioconversion of wastes from the olive oil industry by vermicomposting using different species of earthworms can be one. Earthworms form a major part of the soil decomposer community and are able to modify soil structure and nutrient cycling (Butenschoen et al. 2009; Natal-da-luz et al. 2012).

Table 1 Reproduction output of *Dendrobaena veneta* after 56 days of exposure to different OMW CS ratios (R0 (0%), R1 (5%), R2 (10%), R3 (25%) and R5 (50%))

Treatment	Number of cocoons	Cocoons per earthworm	Unhatched cocoons	Number of juveniles	Juveniles per cocoon
R0	13 ± 2.83	2 ± 0.47	6.5 ± 2.12	14 ± 8.48	2.09 ± 1.0
R1	16 ± 9.89	2.6 ± 1.65	3.5 ± 0.7	32 ± 8.18	1.97 ± 1.37
R2	18.5 ± 2.12	3.08 ± 0.35	2 ± 0	26 ± 11	1.54 ± 0.49
R3	7 ± 9.89	1.16 ± 1.06	2.5 ± 3.5	6 ± 4	0.94 ± 0.66
R4	0	0	0	0	0

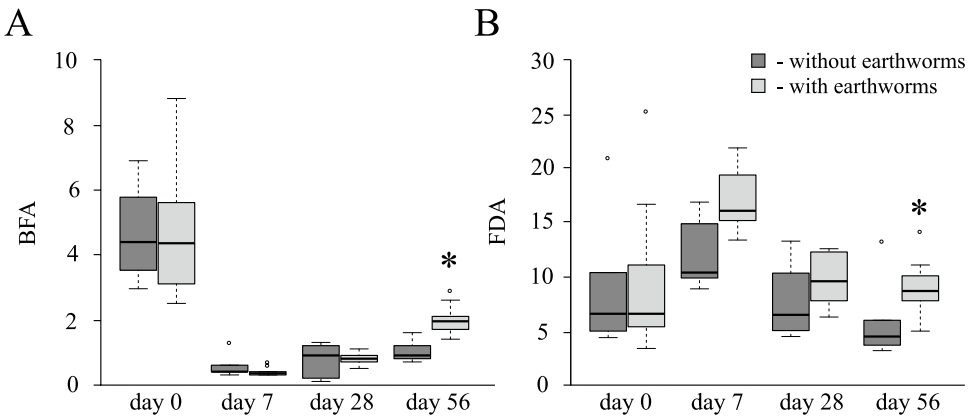

Fig. 4 Impact of earthworms on biofilm formation ability (BFA) (**A**) and fluorescein diacetate activity (FDA) (**B**) expressed as an absorption per g of soil at different exposure periods to R3 (25% of OMW CS). Significant differences between treatments with and without earthworms obtained with paired *t* test and were labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$)

Furthermore, earthworms can promote the degradation of many compounds in soil, such as polycyclic aromatic hydrocarbons (Natal-da-luz et al. 2012; Lin et al. 2018). According to that, earthworms may have the potential to degrade single cyclic molecules such as phenolic compounds with simpler structures than the complex substances mentioned above. Our results show that there is a significant decrease in a soil phenol concentration in the presence of earthworms after only 7 days in R1 (5% of OMW CS) and R2 (10% of OMW CS), after 28 days in all treatments and after 56 days only in R4 (50% of OMW CS). It could be assumed that on day 28 at lower doses (R1–R3), the quantity of phenols in the substrate has already been consumed by decomposers, and since the concentration of phenols was already low and earthworms were removed, the degradation rate slowed down and no significant difference between treatments could be observed at day 56 (Fig. 5). Furthermore, after day 28 no adult earthworms were present in soil, only cocoons (and juveniles by the end) that were not capable of having a significant impact on the phenol degradation. Only in the highest ratio (R4) where the concentration of phenols was still high at 28th day, the degradation rate between treatments stayed significantly different. Interestingly, microbial activity was only transiently increased at day seven, and later while by the end of the investigation, the difference between treatments with and without earthworms disappeared. On the other hand, BFA was significantly increased at the beginning of the investigation when contaminated soil was mixed with the artificial soil and wetted. This resulted in BFA stimulation as noted for some natural soils (Palijan et al., submitted). Also, increased biofilm formation at the beginning of the experiment could be explained by the effects of antimicrobial OMW constituents which could have caused stress for microbes (Piotrowska et al. 2006) present in the artificial soil and in that way induced biofilm formation (Sultana et al. 2019). Later, at the end of experiment, BFA stimulation was related to earthworms. Relationship of this microbial characteristic with earthworms and their influence on the phenol degradation should be investigated in the future. Microbial activity measured as FDA hydrolysis was constantly higher in treatments with earthworms. After 56 days, significantly more active microbial community was established in the R3 treatment with earthworms compared to treatment without earthworms. This microbial community was not responsible for phenol degradation in R3 as there was no difference in phenol concentration between treatments with and without earthworms. Such results suggest that specific community of phenol degraders of the OMW polluted soil was related to the earthworms (Toyota and Kimura 2000; Vivas et al. 2009) but not to the activity of the whole microbial community. These are important results which suggest the potential of earthworm *D. veneta* in accelerated remediation of soils significantly polluted with OMW. Bi et al. (2016) reported how earthworm species differ in their capacity to degrade phenolic compounds (PCs). Namely, anecic earthworm (*M. guillemi*)

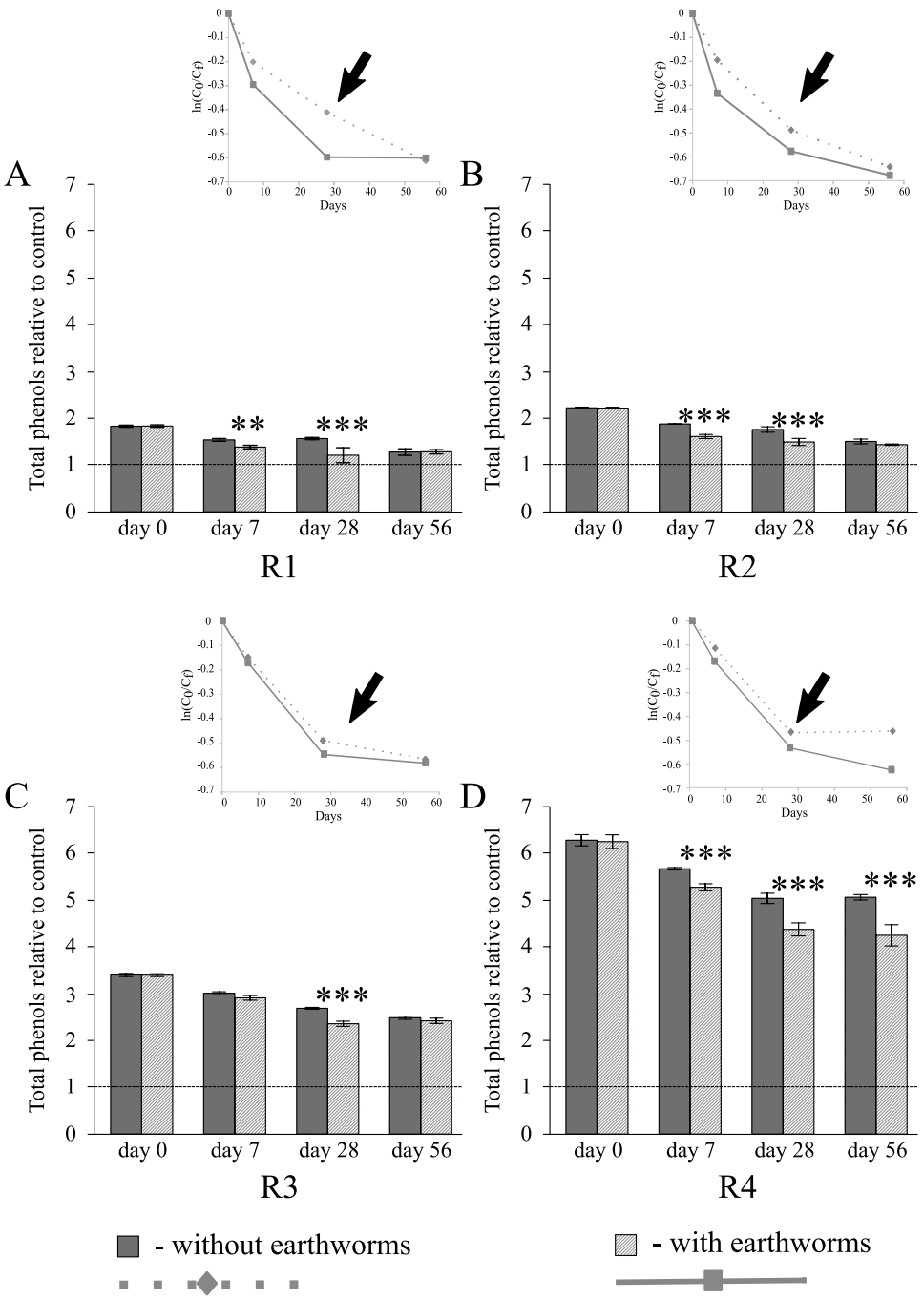

Fig. 5 Total phenol content measured in soil at day zero, seven, 28 and 56 with and without earthworms. Significant differences between treatments without and with earthworms were obtained with paired *t* test and were labeled with * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). The phenol degradation rate is presented as $\ln(C_0/C_f)$ (C_0 (initial concentration), C_f (phenol concentration at measuring point). The arrow shows Day 28 when adult earthworms where removed

was more successful in the degradation of phenols than the epigeic one (*E. fetida*), but both species have accelerated the degradation of PCs. Nevertheless, our results suggest that the highest level (50%) prevented reproduction of *D. veneta* suggesting that bioremediation organisms should be checked for their suitability for long-term exposure to OMW. It is also important to determine which percentage to add to make the earthworms the most effective in bioconversion of waste. A study by Melgar et al. (2009) demonstrated that bioconversion of waste is possible using a vermicomposting process and that the present vermicompost can be used in conventional and organic agriculture.

Conclusions

The usage of earthworms for in situ bioremediation of OMW through vermicomposting first requires predicting possible toxic effects. Knowledge provided by this study can contribute to better alternatives for the management of OMW

disposal. We conclude that the biomarker response was related to the ratio of OMW CS and the exposure duration. Furthermore, high amounts of OMW may have a negative effect on the reproduction. Nevertheless, earthworms can decrease phenol concentrations in olive waste and present a good tool for mitigating the negative effect of OMW. Future investigations should be focused more on how to reduce toxic effects on earthworms by strikethrough different additives or through some other pretreatments. Also, more research needs to be conducted on different soil organisms to assess the toxicity of OMW and possible usage of processed waste as agriculture fertilizer.

Acknowledgements This research was conducted in the framework of the DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) financed by the Croatian Science Foundation (HrZZ) (contract number: IP-09-2014-4459). We are grateful to the members of the Subdepartment of Quantitative Ecology who supported our work.

Author contribution All authors contributed to the study conception and design. Resource acquisition, funding and visualization were performed by ST, AK and BKH. The investigation and formal analysis were performed by ST, DKH, MK, NS and GP. The first draft of the manuscript was written by ST, MK and NS, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This research was financially supported through DEFENSoil project (Diverse Effects of Environmentally Relevant Metal-based Nanoparticle and Pesticide Mixtures on Soil Fauna: A Novel Issue for Risk Assessment) financed by the Croatian Science Foundation (HrZZ) (contract number: IP-09–2014-4459).

Availability of data and material Data, associated metadata and calculation tools are available from the corresponding author.

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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