

## Effects of single and combined olive mill wastewater and olive mill pomace on the growth, reproduction, and survival of two earthworm species (*Aporrectodea trapezoides*, *Eisenia fetida*)

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### ABSTRACT

The extraction processes of olive oil produce huge amounts of by-products, such as olive mill wastewater (OMWW) and olive mill pomace (OMP), these by-products have significant polluting properties related to their undesirable color and odor, high organic load, acidic pH, high phenolic compounds, high salinity, while their effect on soil organisms is unclear. The study into the effects of these by-products on soil bioindicators, such as earthworms should therefore be prioritized. Indeed, the purpose of this study was to evaluate the effect of environmentally realistic concentrations of olive mill wastewater and olive mill pomace on the growth, reproduction, and survival of the earthworms *Aporrectodea trapezoides*, and *Eisenia fetida*. Bioindicators were exposed under laboratory conditions to increasing doses (12.5%, 25%, 50%, 75%, and 100% w/w) of OMWW and OMP separately and in combination. The results showed a higher growth rate on *Eisenia fetida* when exposed to 12.5% OMP, with a neutral effect on reproduction and survival. Toxicological effects were found at higher doses, namely: there was no cocoon production, growth inhibition, and mortality; these effects were probably due to the higher level of phenolic compounds and higher salinity. In general OMP was found to have toxicity higher than OMWW, the combination of both by-products showed stronger effects on some earthworm endpoints. Comparative responses between the two species showed that *Aporrectodea trapezoides* proved to be more tolerant. Dilution and bioremediation of these by-products is recommended as a possible solution to reduce their toxicity.

### 1. Introduction

The olive oil industry is one of the most traditional agricultural industries and it is of fundamental economic importance for Mediterranean countries, from antiquity to the present. Algeria is the ninth largest olive oil producer country in the world with around 80,000 tons per year, equivalent to 4% of the world's production (IOC, 2018). There are different systems of oil extraction that are commonly used globally. The more traditional extraction process is the 3-phase separation system, due to the addition of large amounts of water used in the decanting process, a solid residue named Olive Mill Solid Waste (OMSW) or Olive Mill

Pomace (OMP) is generated together with a liquid effluent named Olive Mill Wastewater (OMWW). The two-phase system, which is more advanced, generates olive oil and the humid solid waste named Two-Phase Olive Mill Waste (TPOMW) (Vlyssides et al., 1998).

In this study, the focus is on the by-products of the 3-phase processing. The short time scale in which the olives are processed means that very high quantities of by-products are generated over a short period of time (1–3 months), thus increasing the problems related to their disposal. The OMP can be used to produce pomace oil and be devolved to the pomace mills, but it can also be used as a soil improver in crops or released in uncultivated fields. OMWWs can be released

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untreated in the fields or undergo decanting and purification processes. Most Mediterranean countries have their own legislation regarding the modality of the release of these effluents in agricultural systems, however, these by-products with their important toxicological potential, can represent a danger for ecosystems; in fact there are not as yet a sufficient number of research studies that have been conducted into their toxic effects, and their release tends to be difficult to control.

Olive Mill Wastes (OMWs) present different compositions related to their different origins, as they generally have a low pH, a high level of salinity, and a high amount of organic matter (Chatzistathis and Koutsos, 2017; Kavvadias et al., 2010) and their composition in water can range from 50 to 94%. The level of pollution is mainly related to their high values of biological oxygen demand as well as of chemical oxygen demand, together with their high content of phenolic compounds, potassium, phosphorous, and calcium (Chatzistathis and Koutsos, 2017; Lozano-García et al., 2011; Magdich et al., 2013). For the oil phase, there is only a residue of 1–2% of the total phenolic compounds of the olive fruit present, while around 53% is lost in the OMWW or in the solid olive residue (approximately 45%), due to the hydrophilic nature of phenolics and their high solubility in the water phase (Caporaso et al., 2018; McNamara et al., 2008; Rodis et al., 2002), moreover OMW may also contain pesticides that are used in olive cultivation.

To date, more than 50 phenolic compounds have been identified in OMWW (Preedy and Watson, 2010). Polyphenols are one of the main limiting factors for the toxicity of olive mill by-products because of their phytotoxic and antimicrobial effects. These by-products are considered to be one of the most polluting in the agrifood industry (Capasso et al., 1992; Cardinali et al., 2010; Preedy and Watson, 2010).

The toxic effects of OMW were investigated by several authors and focused on different plant species: *Trifolium repens*, *Triticum aestivum*, bacteria, and crustaceans, and indicate that olive mill waste has acute toxicity on *Daphnia pulex*, *Palaemonidae shrimp*, *Pelophylax ridibundus*, *Eisenia fetida* larvae, *Helianthus annuus*, *Chlorella vulgaris*, *Vibrio fischeri*, *Daphnia magna*, *Danio rerio* embryos and can also cause damage to the DNA's nucleotide and genomic structure (Inceli and Sengezer-Inceli, 2012; Rouvalis et al., 2013; Pavlidou et al., 2014; Campani et al., 2017; Aybeke, 2018; Babić et al., 2019).

The discharge of OMWW into different environmental compartments such as freshwater and soil on a yearly basis and over a relatively short period of time was found to negatively affect the soil properties by increasing the levels of soil salinity and the content of phenolic compounds, and electrical conductivity, and leads to a decrease in soil pH and fungal communities (Di Bene et al., 2013; Caruso et al., 2018; Zema et al., 2019; Ntougias et al., 2013). Piotrowska et al. (2011) observed that the application of OMWW immediately changes some biological properties of the soil. On the other hand, it must be mentioned that a couple of studies reported that olive mill wastewater can have positive effects on soil quality, suggesting they may be used as natural fertilizers after two-fold dilution to fertilize maize in calcareous soil or when used at a concentration of about 30 m<sup>3</sup>/ha/year (Belaqziz et al., 2016; Vella et al., 2016).

Earthworms are considered as the most fundamental actors in soil, representing a significant proportion of soil biomass (Diogène et al., 1997; Yasmin and D'Souza, 2010). They play an essential role in promoting soil bio-fertility with their waste products, being commonly considered as ecosystem engineers with their burrowing action (Spurgeon et al., 2003; Lavelle et al., 1997). They improve the activity of other beneficial soil organisms, and soil physical properties (Li and Cheng, 2006). These animals have been used as indicators to indicate soil health and quality (Edwards, 2004), they are also considered as sensitive bio-indicator organisms of soil pollution (Tejada and Masciandaro, 2011). Multiple tests were developed to investigate different toxicological endpoints, such as growth, reproduction, and the avoidance behavior of earthworms (OECD, 2004a, 2004b, 2004c).

The exposure of earthworms to the surrounding environment stressors of soil such as high salinity and high electrical conductivity

(EC), soil acidification, pesticides, heavy metals, organic pollutants, and phenolic compounds (Li et al., 2020; Wu et al., 2019; Babić et al., 2019), may result in significant damage at different biological levels, such as death, the inhibition of growth and reproduction, decreased hatching, DNA damage, the inhibition of AChE activity, (Hirano and Tamae, 2011; Maity et al., 2018a, 2018b; Wu et al., 2019; Babić et al., 2019; Campani et al., 2017). Earthworms are not just sensitive to the environmental stressors, because of their high strong interactions with soil, but also since they react quickly to either natural or anthropogenic pollutants in the environment (McGuirk et al., 2020).

Despite the wide use of OMW in agricultural land, very few studies have been conducted to date on the toxicity of these complex mixtures to earthworms, leaving many knowledge gaps that need to be addressed.

Hentati et al. (2016) reported the significant negative effects of dried OMW-amended soil on the reproduction of the earthworm *Eisenia fetida* and avoidance behavior. Fewer cocoons were produced at the higher doses of OMW; also the number of hatched cocoons was affected by OMW-amended soil. The authors point out the severe impact of OMW-amended soil on the functions of earthworms and the terrestrial ecosystem as well as the importance of establishing clear limits for the application of olive mill waste to land, and other aspects that should be considered apart from polyphenol contents. Campani et al. (2017) demonstrated that exposure to high concentrations (50%) of raw OMWW for 3 days caused high mortality on *E. fetida* while biochemical and cellular effects were found at lower concentrations. In a recent study, Chalkia et al. (2020) investigated the effects of the OMWW doses recommended by the Greek legislation for the fertilization of olive groves (80 m<sup>3</sup>/ha) on the survival and the growth of *Octodrilus complanatus* which is a very common species of earthworm found in olive groves and they maintained that the survival and the growth of earthworms were not affected by the tested doses.

The missing information on the toxicity of OMWs concerns in-depth studies conducted on the different types of OMWs, tested both individually and in combination, considering that in some countries they are also used in the fields simultaneously. It is also necessary to test different concentrations and different exposure times, to replicate the natural conditions in the best possible way, where the concentrations can vary and in some cases be higher than those allowed by law, given that it is practically impossible to thoroughly control their use. OMWs can decrease over time in terms of their concentration, but give rise to prolonged exposure. The time of exposure in the field may also vary depending on the type and characteristic of OMW used, that can be able to remain for either a short or a long period of time in the environment. Furthermore, different species can react differently to OMW exposure and another important point to consider is the need to explore the effects at different biological levels, from survival to the sublethal effects that can have an irreversible effect on the health of earthworms.

The current study has quite a complex experimental plan and was designed to investigate several aspects of the interactions between OMWs and earthworms and to contribute to addressing some of the knowledge gaps connected to this topic. The aim is to investigate the effects of olive mill wastewater, olive mill pomace and the combination of the two OMWs, by increasing doses, on several earthworm endpoints such as growth rate (weight gain or inhibition), reproduction (number of cocoons), and survival rate in two different species of adult earthworms: the endogeic *Aporrectodea trapezoides*, are widely dominant in Algerian soil, and the epigeic *Eisenia fetida*, a model soil invertebrate, is recommended as standard test species (OECD, 2004b). Tested doses go from 12.5% that for pomace is close to concentrations allowed by law in several Mediterranean countries and includes 25% and 50% concentrations that can potentially be reached in real situations. Regarding OMWW, 100% concentration correspond to 50m<sup>3</sup>/ha/year, the commonly allowed spreading doses. The study also aims to explore whether different exposure times result in different toxicological effects.

## 2. Materials and methods

### 2.1. Olive mill waste source

The study focused on the analysis of olive mill waste that has been collected from a modern olive oil mill, using a 3-phase cold-pressed system, named Al Hadja Yamina which is located in the municipality of Baghaï, Khenchela in the east of Algeria. The olive mill waste that is used comes from the Zabouch olive variety. This process generates two kinds of residue; a liquid phase (Olive mill wastewater-OMWW) and a solid phase (Olive mill pomace-OMP). The OMW samples were obtained in November 2019 and immediately stored at 4 °C until they were used.

### 2.2. Soil and earthworms sampling

Soil for the microcosm was collected from the topsoil (0–20 cm) of a natural apple grove (35° 29' 41" N, 6° 55' 27" E) that had not been treated with any kind of pesticide, in Khenchela. The soil sample was air-dried for 3 days, homogenized, sieved through a 2 mm mesh to remove stones, roots, and gravel prior to its use in the experiment. The main physico-chemical characteristics of the soil were: pH 7.24; EC 0.7 ds m<sup>-1</sup>; 61% sand, 22% clay, 17% silt; organic matter 2.92%; water holding capacity 38%. Adult earthworms with well-developed clitellum were randomly collected in November 2019 and they were sorted by hand and taken from two sites (35° 29' 41" N, 6° 55' 27" E) (35° 14' 59" N, 7° 02' 0" E) in Khenchela. Specimens of *Eisenia fetida* and *Aporrectodea trapezoides* were evenly selected so that they were the same size were, and they were identified by Prof. Kamel Eddine Bazri (The University of Constantine 01, Algeria), weighing between 0.350 g and 0.750 g for *E. fetida*, and between 0.700 g and 1.800 g for *A. trapezoides*. All earthworms were kept in Petri dishes on filter paper and moistened with distilled water for 3 h to allow them to expel their gut contents (OECD, 1984) and then acclimatized to laboratory conditions using soil for microcosms with ground hay added to provide food for the earthworms that were subsequently kept for two weeks before the tests started.

### 2.3. Physico-chemical analyses of olive mill wastewater samples

The physicochemical analysis of a fresh sample OMWW such as pH, electrical conductivity, and salinity was measured using a pH-meter, HANNA instruments, Hungary, a conductivity meter, HANNA instruments, Hungary, and a Multiparameter, Consort C535, Belgium, respectively (Rodier and Legube, 2009). Dry weight and moisture content were determined after the desiccation of 10 ml of an OMWW sample at 105 °C for 24 h as a (%) of the initial volume (Hamdi, 1991). The total suspended matter was evaluated as the difference between the residue weight of OMWW obtained by centrifugation of 15 ml of OMWW at 4000 t/min for 15 min, and its weight after drying overnight at 150 °C (Assas et al., 2000). The total nitrogen content has been determined in accordance with the Kjeldahl method (Greenberg et al., 1992) with minor modifications made. The results presented are the means of three replicates. The organic matter and mineral matter contents have been calculated by incinerating the OMWW sample at 550 °C in a muffle furnace "30–3000 °C" NABERTHERM, Germany, for 5 h (Helrich, 1990). The total sugar content was measured by using spectrophotometry UNICO, United States, at 488 nm in accordance with Dubois et al. (1956). The total phenolic content was estimated using the colorimetric method of Folin-Ciocalteu (Makkar et al., 1993). The biochemical oxygen demand (BOD<sub>5</sub>) was determined by a 5-day BOD test (Rodier et al., 1975).

### 2.4. Physico-chemical analyses of olive mill pomace samples

The raw olive mill pomace was dried in an oven at 105 °C and crushed in order to perform the physicochemical analysis. pH, electrical

conductivity and salinity were measured by using a pH-meter HANNA instruments, Hungary, a conductivity meter HANNA instruments, Hungary, and a Multiparameter Consort C535, Belgium, respectively, incorporating the international method at a solid: water ratio of 1:5 w/v (Mathieu et al., 2003). The moisture content was calculated by drying the OMP sample at 105 °C for 24 h (Fatianoff and Gouet, 1969). Organic matter was calculated through the calcination of the OMP sample on a muffle furnace at a 30–3000 °C NABERTHERM, Germany, and at 850 °C for 1 h (Aubert, 1978), assimilable phosphorus content was determined according to Olsen following the colorimetric method and spectrophotometry UNICO, United States, at 860 nm (Olsen, 1954). The total amount of nitrogen was measured using the Kjeldahl method (Mulvaney, 1982). Exchangeable potassium, sodium, and calcium were measured with a flame photometer (Pansu et al., 2003), the organic carbon content was determined in accordance with the Anne method through dichromate oxidation (Aubert, 1978). The polyphenol content was evaluated according to Folin–Ciocalteu spectrophotometric method (Singleton and Rossi, 1965).

### 2.5. Experimental design

#### 2.5.1. Treatment with olive mill wastewater and olive mill pomace

After the period of acclimatization, a homogeneous group of adult earthworms (n = 1080) were washed in tap water, dried on filter paper and weighed before being placed in microcosms. Tests were carried out using plastic containers that measured 16 cm × 14 cm × 18 cm (length × width × depth), ten worms with well-developed clitellum were placed in each container with 1000 g of soil. In the first treatment, earthworms were exposed to increasing concentrations of fresh olive mill wastewater (OMWW) T1 group: 12.5% w/w; T2 group: 25% w/w; T3 group: 50% w/w; T4 group: 75% w/w; and T5 group: 100% w/w. In the second experiment animals were exposed to olive mill pomace. The sample (OMP) was at first air-dried for 48 h to avoid enzymatic degradation, to concentrate bioactive compounds and to reduce the moisture content from 57.69% to approximately 15%, then applied with increasing concentrations to the T6 group: 12, 5% w/w; T7 group: 25% w/w; T8 group: 50% w/w; T9 group: 75% w/w; and the T10 group: 100% w/w. In the third experiment earthworms were exposed to a combination of OMWW and OMP at the same concentrations as below: T11 group: 12.5% w/w (6.25% OMWW + 6.25% OMP + 87.5% soil); T12 group: 25% w/w (12.5% OMWW + 12.5% OMP + 75% soil); T13 group: 50% w/w (25% OMWW + 25% OMP + 50% soil); T14 group: 75% w/w (25% OMWW + 50% OMP + 25% soil); T15 group: 75%\* w/w (50% OMWW + 25% OMP + 25% soil); T16 group: 100% w/w (50% OMWW + 50% OMP).

Untreated soil was used as negative control (T17 group) with three replicates for each treatment, and a positive control (T18 group) was also set, using the organophosphate dimethoate at a concentration of 0.6 mg/kg, obtained through suspension in distilled water that was mixed with soil (the dimethoate insecticide is widely used in agriculture and especially in the treatment of olive pests). Earthworms *E. fetida* and *A. trapezoides* were placed separately following the same approach. All microcosms were moistened with distilled water at about 35% of the dry weight, wrapped in gauze to keep the earthworms from escaping and limit water loss, which were then incubated in a growth chamber at 23 ± 2 °C under controlled light-dark cycles (16 h light, 8 h dark) with the illumination of 800 lx for 8 weeks.

OMW concentrations selected for treatments were based on realistic environmental use. In fact, the doses that are allowed by law in several Mediterranean countries are 50m<sup>3</sup>/ha/year for OMWW, corresponding to 100% in our experiment, and 50–80 tons/ha/years for solid oil mill by-products, including OMP, which is close to 12.5% in our experiment. Also, as already mentioned in some countries different OMW are spread simultaneously in the fields.

#### 2.5.2. Determination of survival, growth, and reproduction of earthworms

Alive adult worms were collected after being sorted by hand and

**Table 1**

Physicochemical characteristics of olive mill wastewater. The results are reported as mean  $\pm$  SD of 3 different measurements.

Parameter	Value
Color	Brown
pH 20 °C	4.84 $\pm$ 0.01
Electrical conductivity (mS cm <sup>-1</sup> )	9.7 $\pm$ 0.61
Salinity (25 °C) (g l <sup>-1</sup> )	7.4 $\pm$ 0
Dry weight (g l <sup>-1</sup> )	7.53 $\pm$ 0.03
Moisture content (%)	92.1 $\pm$ 0.96
Total suspended (g l <sup>-1</sup> )	9.422 $\pm$ 0.003
Total nitrogen (g l <sup>-1</sup> )	0.42 $\pm$ 0.02
Organic matter (%)	91 $\pm$ 1
Mineral matter (%)	3.3 $\pm$ 1.53
Total sugar (g l <sup>-1</sup> )	0.32 $\pm$ 0.04
Total phenolic content (g l <sup>-1</sup> )	2.31 $\pm$ 0.01
BOD (g l <sup>-1</sup> )	40.20 $\pm$ 0.01

separated from each microcosm, washed in distilled water, dried on filter paper, observed and counted, and weighed per group (10 organisms/container) weekly, and inspected for any morphological symptoms. Earthworms were considered to be dead when they did not respond to a gentle mechanical stimulus at the anterior end and if they were missing.

The growth proportion rate (%) of surviving earthworms was calculated as follows:

$$(W_t - W_0)/(W_0) \times 100\%$$

W<sub>t</sub> is the weight of the earthworm on the day they were checked, and W<sub>0</sub> the weight of the earthworm on day 0 (prior to the incubation). The survival rate (%) was the percentage of earthworm surviving over time T compared to the initial number of earthworms. To evaluate the reproduction of earthworms, at the eighth week the cocoons inside the microcosms were isolated, sorted by hand, washed, and counted (OECD, 2004b).

## 2.6. Statistical analysis

The values of the physicochemical analysis of olive mill wastewater and olive mill pomace were performed in triplicate and presented as means  $\pm$  SD using (XLSTAT 2014.5.03). The effect of OMWW and OMP on the growth, the reproduction, and the survival of the two species of earthworms were measured in triplicate and the data were analyzed using a two-way ANOVA according to the randomized factorial and the following additive model:  $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ijk}$ , with two levels for the species factor (i.e. *E. fetida* and *A. trapezoides*) and 18 levels for the treatment factor (i.e. T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, T16, T17, T18), with the statistical software XLSTAT 2014.5.03, testing the normality using the diagram Quantile-Quantile (D.QQ-plot). The homogeneous groups were analyzed using SAS 9.1 based on the *Least Significant difference* (LSD) of student at 5% significance level. Moreover, the linear regression was used to check the correlation between the growth and survival of the two earthworms species and the exposure time using XLSTAT 2014.5.03. The Principal component analysis (PCA) was performed for testing Multivariate difference between treatments and measured parameters, using Statistica 08 software.

## 3. Results and discussion

### 3.1. Physico-chemical analysis of olive mill wastewaters

The physicochemical characteristics of OMWW generally depend on the techniques used in the olive oil extraction process and other agronomic parameters. The compositions of olive mill wastewater used in this work are summarized in Table 1. The analysis indicated that the OMWW has a brown color caused by its high content of recalcitrant

**Table 2**

Physicochemical characteristics of olive mill pomace. Results are reported as mean  $\pm$  SD of 3 different measurements.

Parameter	Value
pH	4.7 $\pm$ 0.02
Electrical conductivity (mS cm <sup>-1</sup> )	14.8 $\pm$ 0.1
Moisture content (%)	57.69 $\pm$ 0.40
Organic matter (%)	90.27 $\pm$ 3.0
Assimilable phosphorus (mg g <sup>-1</sup> )	0.0253 $\pm$ 0.2
Total nitrogen (mg g <sup>-1</sup> )	0.5 $\pm$ 0.1
Na (mg g <sup>-1</sup> )	51.67 $\pm$ 1.53
Ca (mg g <sup>-1</sup> )	2.41 $\pm$ 0.19
K (mg g <sup>-1</sup> )	111.67 $\pm$ 2.09
Organic carbon content (%)	60.80 $\pm$ 0.59
Total polyphenol (mg g <sup>-1</sup> )	40 $\pm$ 0.3

compounds such as lignins and tannins (Paraskeva and Diamadopoulos, 2006) and an acidic pH (pH = 4.84  $\pm$  0.01), together with a high electrical conductivity (EC = 9.7  $\pm$  0.61 mS cm<sup>-1</sup>) when compared with the value cited by Malvis et al. (2019). A high salinity 7.4  $\pm$  0 g l<sup>-1</sup> can be explained by the high amount of salt that is added to conserve olives before extraction and in the mineral elements present in OMWW (Achat et al., 2008; Eroğlu et al., 2008; Bouknana et al., 2014). A high moisture content (92.1  $\pm$  0.96%) was found, while the OMWW total nitrogen content was very low (TN = 0.42  $\pm$  0.02 g l<sup>-1</sup>), and the results obtained are compatible with the values reported in other studies (Meftah et al., 2019; Babić et al., 2019). The content in the organic matter was high (MO = 91  $\pm$  1%), moreover a high biological oxygen demand (BOD = 40.20  $\pm$  0.01 g l<sup>-1</sup>) was found and the result obtained is in accordance with the value cited by Babić et al. (2019). Moreover, the physicochemical analysis revealed that the OMWW was highly enriched in phenolic content (2.31  $\pm$  0.01 g l<sup>-1</sup>) when compared to the previous studies (Bargougui et al., 2019; Magdich et al., 2016). A high level of phenolic content was also found, which is the main origin of OMWW toxicity (Mekki et al., 2013; Chaari et al., 2014). The characteristics of OMWW is very variable and depends on various factors such as the systems used for oil extraction, olive variety, the climatic conditions, the use of pesticides and fertilizers (Pardo et al., 2017; Magdich et al., 2016; Lanza et al., 2017; Al-Imoor et al., 2017).

### 3.2. Physico-chemical analysis of olive mill pomace

As mentioned above regarding OMWW, the physicochemical composition of olive mill pomace also varies according to the extraction process, culture conditions, olive species, the stage of maturation, the origin of the olives and the storage condition. The chemical characteristics of the olive oil pomace are reported in Table 2. The analysis showed that OMP has an acidic pH (4.7  $\pm$  0.02) when compared to previous studies where the pH level was between 6.2 and 5.4 (de la Fuente et al., 2011; Aviani et al., 2010; Medjahdi et al., 2014). The high electrical conductivity (14.8  $\pm$  0.1 mS cm<sup>-1</sup>), is in line with the values obtained by Medjahdi et al. (2014). The electrical conductivity is related to the concentration of dissolved substances and their nature, and it is an estimate of the degree of mineralization of olive pomace. The moisture contents of olive mill pomace varied considerably, depending on the oil extraction process, and the samples show a high value of moisture content of (57.69  $\pm$  0.40%), and a very high content of organic matter (90.27  $\pm$  3.0%) and organic carbon (60.80  $\pm$  0.59%). The results are close to those obtained by Ameziane et al. (2019). The content of polyphenols was approximately (40 mg g<sup>-1</sup>), which is significantly lower than the values cited by Bouknana et al. (2014), additionally polyphenols are considered to be one of the major pollutants of olive mill pomace. With regard to the major elements, OMP was particularly rich in potassium (111.67  $\pm$  2.09 mg g<sup>-1</sup>), which is a common characteristic in olive-mill waste and by-products, followed by sodium (51.67  $\pm$  1.53 mg g<sup>-1</sup>) and calcium (2.41  $\pm$  0.19 mg g<sup>-1</sup>). The high

**Table 3**

The values of a two-way ANOVA from a general linear model analysis of the species (S), treatments (T), and their interaction (S × T) on the growth, reproduction, and survival of the two earthworms (±SD n = 3).

Source of variation	Growth	Reproduction	Survival
Species (S)	2023.36***	9.48***	448.15 <sup>ns</sup>
Treatments (T)	9361.78***	31.46***	11722***
Interaction (S × T)	2497.87***	2.07***	44.23 <sup>ns</sup>

ns, Not significant.

\*\*\* Very high significance p < 0.001.

concentrations of these water-soluble salts are due to the high water content. However, the OMP samples were poor in phosphorus (0,0253 ± 0. 2 mg g<sup>-1</sup>) showing levels that are incompatible with the result cited by Albuquerque et al. (2004).

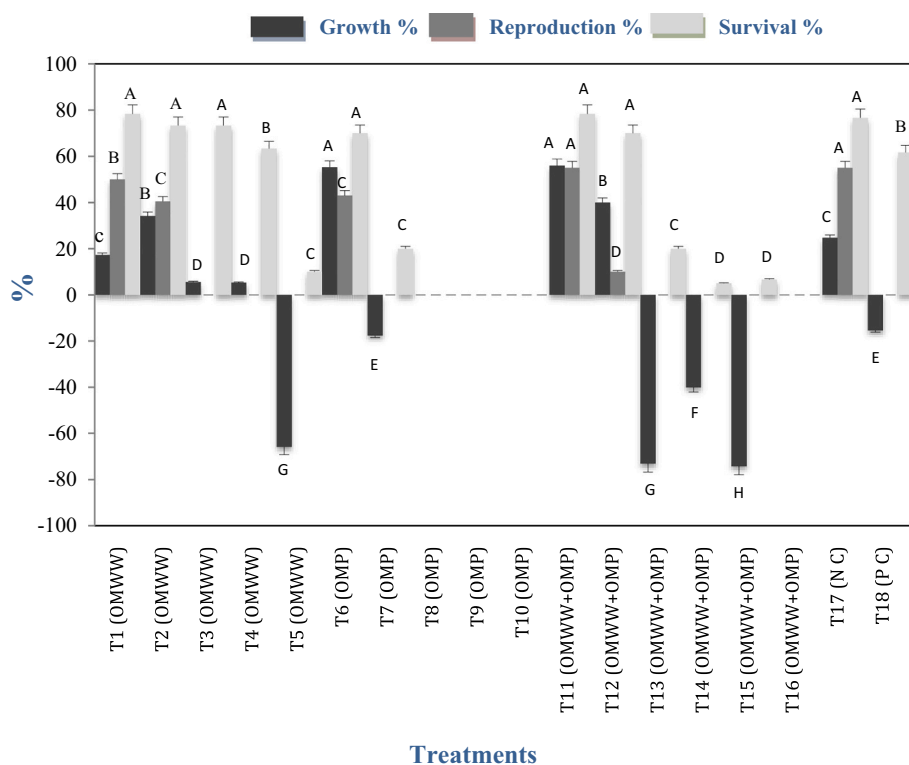
The olive mill waste is generally unexploited and released in the soils, becoming a potential environmental issue due to their negative effects on the soil properties, and soil organisms such as earthworms because of their high strong interaction with the soil. In this study, the initial data that was produced was on the effect of olive mill wastewater and olive mill pomace, and the combination of them on different endpoints such as growth, reproduction, and the survival of the two earthworm *E. fetida* and *A. trapezoides*, as reported below.

**3.3. The effect of olive mill wastewater and olive pomace and a combination of them on the earthworm growth**

The analysis of variance, which included both species, revealed the presence of highly significant differences between all the treatments (p < 0.001) for all the endpoints studied (growth, reproduction, survival) Table 3. Exposure to increasing concentrations of OMWW and OMP and the OMWW and OMP combination had highly significant effects on the growth and the reproduction of the two earthworm species. Fig. 1 shows that there is a clear difference between the various treatments for all the parameters that are measured. The results for the analysis of variance

indicated that all the factors have a very significant effect (treatments and species) on the growth of the earthworm (p < 0.001) Table 3 and indicates the presence of eight homogeneous groups Fig. 1. Groups A to D showed an increase in growth, the first (A) was characterized by high growth, and includes 12.5% OMP (T6) and 12.5% of the OMWW and OMP combination (T11), which were found to have a positive influence on *E. fetida* and *A. trapezoides* growth (55.2 ± 12.16 at 50.07 ± 12.48 respectively). The second group (B) including 25% of the OMWW and OMP combination (T12) and 25% OMWW (T2) also stimulate the growth of *E. fetida* and *A. trapezoides* (40.55 ± 10.02 and 34.12 ± 15.79% respectively). The fourth group (D) containing T3 and T4 were showing the lowest growth with less than 25%. Groups (E to H) showed a reduction in their growth, while the eighth group (H) which contains 75% of the OMWW and OMP combination (T15) presents the highest decrease in the weight of the two earthworm species (-74. 2 ± 14.37%). The rest of the treatments resulted in 100% mortality of the earthworm.

Chalkia et al. (2020) found that OMWW had an effect on the weight gain of earthworm, but this difference was not significant. A possible reason for the positive effect of olive mill pomace and olive mill wastewater found in the present study may be the wealth of nutrients revealed by the chemical analysis of OMP and OMWW. Edwards and Bohlen (1996), found that the growth of earthworm and their abundance was enhanced by the organic (manure, hay, green manure, crop residues, etc.) and inorganic fertilizers. Chen et al. (2017), revealed that *A. trapezoides* biomass could be increased by a low C/N ratio of organic residue. This may explain the reason why the findings demonstrate that the weight gain is present only at the low doses of OMP and OMWW, while with doses over 50% a decrease in the abundance and weight of the earthworm was recorded. These results are in agreement with the findings of Babić et al. (2019) where OMWW strongly inhibited the growth of algae *Chlorella vulgaris*, water fleas *Daphnia magna*, and zebra fish *Danio rerio* embryos. The decrease in the weight and abundance of earthworm may be due to the high level of salinity, the acidic pH, the presence of heavy metals, and also the content of polyphenols in our samples. Previous studies revealed that the biomass and size of earthworm populations in agricultural soil could be affected by soil salinity



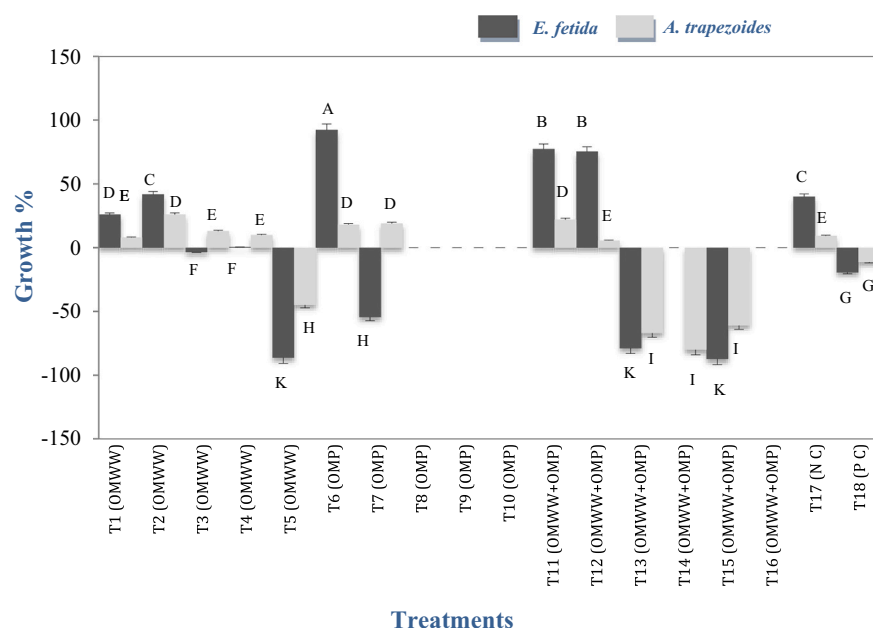
**Fig. 1.** Mean effect of treatments (% ± SD, n = 3) in measured parameters. T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; T13: 50% of the combination; T14: 75% of the combination; T15: 75%\*of the combination; T16: 100% of the combination; T17 (N C): negative control; T18 (P C): positive control, A, B, C, D, E, F, G, H: the homogeneous groups, G% Growth, R% Reproduction, S% Survival.

**Table 4**

The values of the mean effect of the two earthworm species (*A. trapezoides* and *E. fetida*) on growth, reproduction and, survival, the Least Significant difference (LSD<sub>5%</sub>); a, b the homogeneous group.

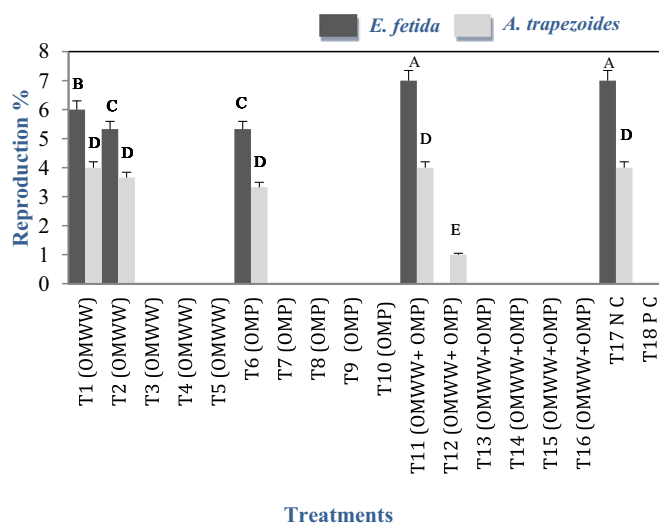
Parameters	Growth		Reproduction		Survival	
	<i>A. trapezoides</i>	<i>E. fetida</i>	<i>A. trapezoides</i>	<i>E. fetida</i>	<i>A. trapezoides</i>	<i>E. fetida</i>
Means	-7.30 <sup>b</sup>	1.36 <sup>a</sup>	1.11 <sup>b</sup>	1.70 <sup>a</sup>	51.30 <sup>a</sup>	47.22 <sup>a</sup>
LSD <sub>5%</sub>	2.76		2.66		75.75	

LSD<sub>5%</sub> Least Significant difference.



**Fig. 2.** Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters were T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; T13: 50% of the combination; T14: 75% of the combination; T15: 75%\*of the combination; T16: 100% of the combination; T17 (N C): negative control; T18 (P C): positive control; A, B, C,D, E, F, G, H, I, J, K,: the homogeneous groups.

(Ivask et al., 2012; Owojori and Reinecke, 2010; Jun et al., 2012). Heavy metals have the potential to inhibit growth even at low concentrations (Babić et al., 2019). The negative impact of the polyphenol content on earthworm growth was confirmed by Moço et al. (2010), which revealed that the high polyphenols content in cacao agro-forestry systems were the limiting factors to the growth and distribution of the soil invertebrates. Table 4 shows that *E. fetida* has a growth percentage that is significantly higher than *A. trapezoides* and in the treatments T1 (group A), T2 and T6 (group B), T11 and T12 of the combination (group C) and T17 (group D), these percentages are over 25% Fig. 2. These results might be because *E. fetida* is a compost earthworm that feeds only on organic matter in the top layer of soil (Campani et al., 2017). In addition, it has been shown that the physicochemical analysis of our samples indicate a high presence of organic matter content. In contrast, *A. trapezoides* presents a higher growth in comparison to *E. fetida* in T3 and T4 with  $13.71 \pm 1$  and  $10.04 \pm 2$ : 24% respectively for *A. trapezoides* and  $-2.57 \pm 1$ ;  $0.59 \pm 0.25\%$  respectively for *E. fetida*. T5, T7 and T13, T14, T15 of the combination and T18 treatments were found to be unfavorable for earthworm growth. The data indicates that *E. fetida* is more sensitive than *A. trapezoides* with weight reduction of up to  $-87.32 \pm 0\%$  regarding T15. *A. trapezoides* could keep growing to higher concentrations of OMWW 50% (T3) and 75% OMWW (T4), these concentrations presented a weight gain that was higher than *E. fetida*. This could be attributed to the endogeic geophagous nature of *A. trapezoides* that feeds on soil solution and mineral soil nutrient available (Lee, 1985; Zorn et al., 2008) and appears to be more tolerant than *E. fetida*, due to their size which is relative to a larger body surface area. In fact, smaller worms might absorb a greater proportion of chemicals through their skin than larger ones (Klaassen, 1991). In addition, T14 treatment shows a weight reduction of up to  $-80.21 \pm 0\%$



**Fig. 3.** Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters. Where T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; T13: 50% of the combination; T14: 75% of the combination; T15: 75%\* of the combination; T16: 100% of the combination; T17 (N C): negative control; T18 (P C): positive control; A, B, C, D,E: the homogeneous groups.

of *A. trapezoides*.

### 3.4. The effect of olive mill wastewater and olive pomace and a combination of them on the earthworm reproduction

According to the analysis of variance, the results indicated that all the factors have a very significant effect on the earthworm reproduction ( $p < 0.001$ ) Table 3. The 12.5% of OMWW and OMP combination and the negative control (group A) were characterized by the greatest reproduction ( $5.5 \pm 1.76\%$ ), then T1 (group B)  $5 \pm 1.26\%$ , followed by groups C (T2 and T6) and the combination D (T12) with reproduction at approximately 4.5% while the rest of the treatments indicate the total absence of reproduction for the two earthworm species Fig. 1. The interaction species  $\times$  treatment had a highly significant effect on *E. fetida* and *A. trapezoides* reproduction as shown in T1, T2, T6, and T11, T12 of the combination and T17 treatments. The D combination treatment *E. fetida* was found to have a percentage of reproduction that was higher than *A. trapezoides* Fig. 3 and Table 4. To our knowledge, little information has been published to date regarding the effects of OMWW and OMP on the reproduction of *A. trapezoides* and *E. fetida*. In our findings, the cocoons production of earthworm was not affected by the lower concentration of OMWW and OMP in the soil in 12.5% of the OMWW and OMP combination (T11), 12.5% OMWW (T1), 25% OMWW (T2), 12.5% OMP (T6) and, 25% of the OMWW and OMP combination (T12), when compared to negative control. Doses of 12.5% and 25% of OMP can be accepted as being close to the environmentally realistic concentrations (Campani et al., 2017). The lower concentration of the chemical components of OMWW and OMP in the soil might not negatively affect the reproduction of earthworm; this was partially confirmed by Jun et al. (2012) where it was shown that in the presence of low soil salinity *A. trapezoides* could keep reproducing. Taylor and Taylor (2014) demonstrated that the reproduction of earthworm could benefit from organic residues with a low C/N ratio. On the other hand, the negative effects of OMWW and OMP on the earthworm reproduction were proven with the rest of the treatment doses that clearly showed not to be a good habitat to support reproduction. These results are compatible with the results of Hentati et al. (2016) which indicated that OMW had a strong effect on *E. fetida* reproduction. This may be because of their high salinity levels, the high level of polyphenol, and the high content of heavy metals. Frouz et al. (2005), found that the complex interaction between soil parameters, polyphenols, moisture, phosphorus, and salinity inhibited *Enchytraeus crypticus* reproduction. According to Jun et al. (2012), *A. trapezoides* did not produce any cocoons in soil with a high salinity ( $5.26 \text{ dS m}^{-1}$  and  $7.35 \text{ dS m}^{-1}$ ). The findings can be related to those of some previous research papers (Jeziarska et al., 2009; Tu et al., 2017; Wu et al., 2014) which pointed out that the hatchability could be delayed and/or lower due to the presence of heavy metals (zinc, copper, manganese, and cadmium).

### 3.5. The effect of olive mill wastewater and olive pomace and a combination of them on the earthworm survival

The analysis of variance does not show there to be a significant effect of the following factors "species" and "interaction" (species  $\times$  treatment) on the earthworm survival, while the factor "treatment" was found to be highly significant ( $p < 0.001$ ) Table 3. According to the analysis of the mean results, the presence of four homogeneous groups was revealed Fig. 1. Group (A) was characterized by the highest survival rate, it includes (T1) 12.5% OMWW, (T2) 25% OMWW, (T3) 50% OMWW, (T6) 12.5% OMP, (T11) 12.5% of the OMWW and OMP combination, (T12) 25% of the OMWW and OMP combination and T17 with a survival rate of  $98.33 \pm 4.08$ ;  $93.33 \pm 5.16$ ;  $93.33 \pm 5.16$ ;  $90 \pm 0$ ;  $98.33 \pm 4.08$ ;  $90.0 \pm 12.64$  and  $96.66 \pm 5.16$  respectively, group (B) includes T4 ( $83.33 \pm 5.16$ ) and T18 ( $81.66\%$ ). Moreover, group D including 75% and 75%\* of the OMWW and OMP combination represents the lowest survival rates, while the rest of the treatments T8, T9,

**Table 5**

Most interesting regression equations describing the growth rate and survival rate over 8 weeks for species *A. trapezoides* and *E. fetida* presented in Fig. 4.

Treatment		Growth %	Survival %
T1 (12.5% OMWW)	<i>A. trapezoides</i>	$y = 0.113 \times 2 + 0.284x - 0.575$ $R^2 = 0.967$	No correlation
	<i>E. fetida</i>	$y = 3.710x - 5.010$ $R^2 = 0.922$	$y = -0.278x + 100.8$ $R^2 = 0.333$
T4 (75% OMWW)	<i>A. trapezoides</i>	$y = 1.747x - 1.930$ $R^2 = 0.886$	$y = -2.778x + 106.6$ $R^2 = 0.821$
	<i>E. fetida</i>	$y = -0.541x + 2.687$ $R^2 = 0.739$	$y = -2.421x + 106.3$ $R^2 = 0.741$
T5 (100% OMWW)	<i>A. trapezoides</i>	$y = 0.274 \times 2 - 2.156x - 12.77$ $R^2 = 0.042$	$y = 1.309 \times 2 - 24.88x + 132.7$ $R^2 = 0.955$
	<i>E. fetida</i>	$y = 2.564 \times 2 - 22.10x + 21.52$ $R^2 = 0.574$	$y = 1.607 \times 2 - 31.57x + 143.1$ $R^2 = 0.94$
T6 (12.5% OMP)	<i>A. trapezoides</i>	$y = -0.089 \times 2 + 2.995x - 3.876$ $R^2 = 0.914$	$y = -0.396 \times 2 + 2.220x + 97.62$ $R^2 = 0.974$
	<i>E. fetida</i>	$y = -0.451 \times 2 + 18.23x - 21.61$ $R^2 = 0.975$	$y = -0.396 \times 2 + 2.220x + 97.62$ $R^2 = 0.974$
T7 (25% OMP)	<i>A. trapezoides</i>	$y = 2.366x - 5.498$ $R^2 = 0.975$	$y = 2.182 \times 2 - 28.85x + 117.5$ $R^2 = 0.925$
	<i>E. fetida</i>	$y = 2.391 \times 2 - 17.40x - 16.04$ $R^2 = 0.289$	$y = -34.8\ln(x) + 77.05$ $R^2 = 0.743$
T8 (50% OMP)	<i>A. trapezoides</i>	No correlation	No correlation
	<i>E. fetida</i>	No correlation	No correlation
T12 (12.5% of the combination)	<i>A. trapezoides</i>	$y = 0.264 \times 2 + 0.913x - 1.304$ $R^2 = 0.986$	No correlation
	<i>E. fetida</i>	$y = 1.992 \times 2 - 9.476x + 5.719$ $R^2 = 0.987$	$y = 0.159 \times 2 - 1.908x + 102.0$ $R^2 = 0.825$
T15 (75%* of the combination)	<i>A. trapezoides</i>	$y = 4.913 \times 2 - 42.31x + 17.77$ $R^2 = 0.803$	$y = 2.579 \times 2 - 31.39x + 102.1$ $R^2 = 0.802$
	<i>E. fetida</i>	$y = 5.197 \times 2 - 44.89x + 18.52$ $R^2 = 0.595$	$y = 3.214 \times 2 - 38.13x + 111.3$ $R^2 = 0.733$
T17 (Negative control)	<i>A. trapezoides</i>	$y = 0.045 \times 2 + 0.955x - 0.305$ $R^2 = 0.948$	No correlation
	<i>E. fetida</i>	$y = 0.469 \times 2 + 1.476x - 1.290$ $R^2 = 0.987$	$y = -0.297 \times 2 + 1.925x + 97.67$ $R^2 = 0.900$

T10, T16 of the combination were found to be unfavorable to *E. fetida* and *A. trapezoides* survival, instead the two species show identical responses with very close mean values Table 4. The findings highlight that the lower concentration of OMWW and OMP together with the negative control did not indicate the mortality of the two earthworm species, and these results are confirmed by Campani et al. (2017), which found that exposure to 12.5%, and 25% of OMW and TPOMW did not show mortality in the earthworm *E. fetida*. Doses equal to or over 50% OMWW and OMP lead to the death of almost all earthworms, the most evident toxicological effects being associated with OMP. Our findings are in line with the results obtained by Campani et al. (2017), where exposure to 50% TPOMW showed the death of 90% of the earthworm after 72 h of exposure.

On the contrary, Hentati et al. (2016) and Chalkia et al. (2020) found there to be no mortality of earthworms *Eisenia fetida* and *Enchytraeus crypticus* maintained in soils amended with OMW at different dry weight ratios, additionally no lethal effect was found on the earthworm *Octodrilus complanatus* under the OMW application with a dose of up to 80

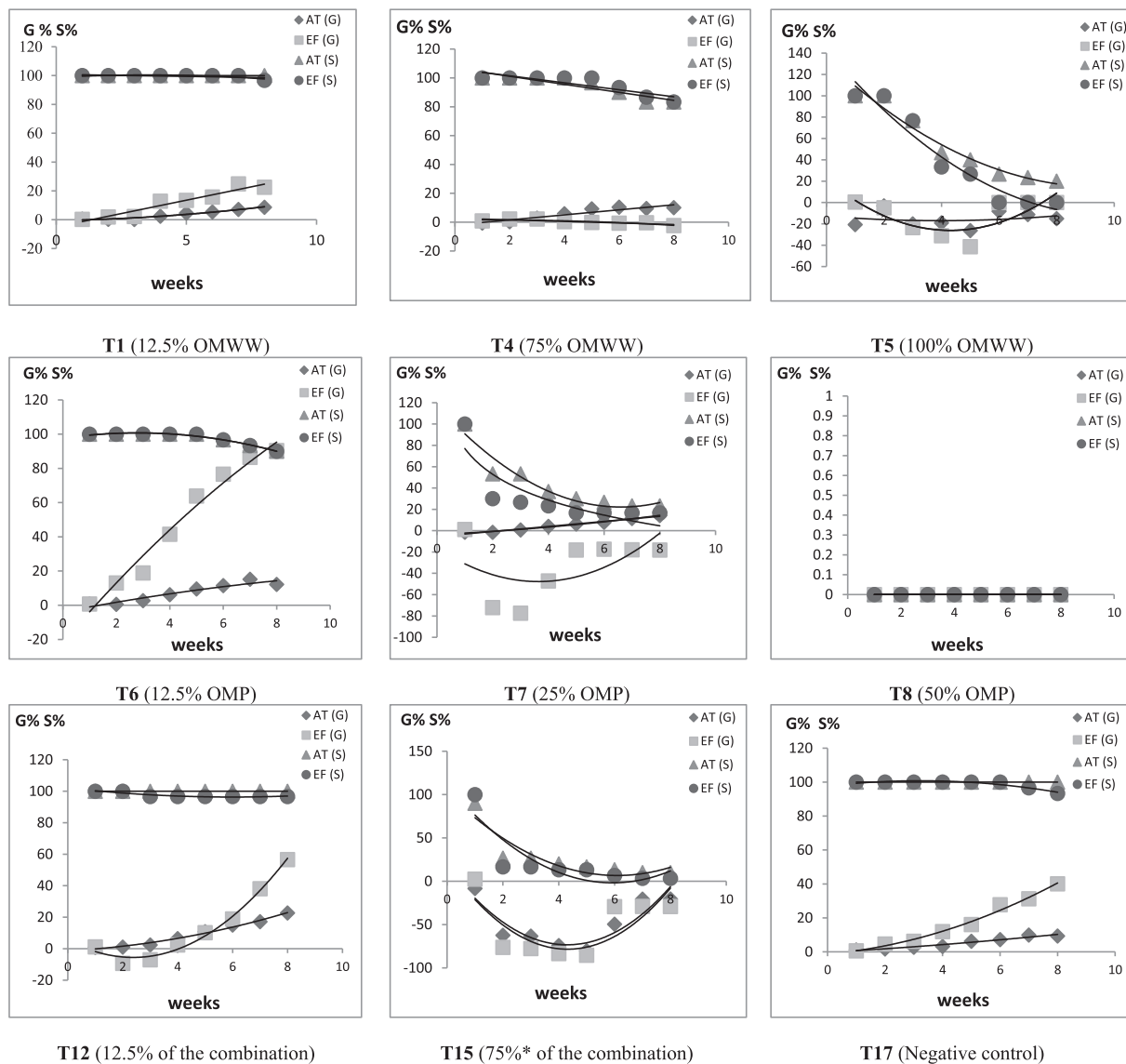


Fig. 4. Linear regression representing the most interesting results of the growth (G %) rate and survival rate (S %) over 8 weeks for species *A. trapezoides* (AT) and *E. fetida* (EF).

m<sup>3</sup>/ha. The negative effect found in our study and in the study of Campani et al. (2017) has a possible explanation that the tested treatments were mixed with the total volume of soil. In the study conducted by Chalkia et al. (2020), instead, the tested OMW were sprayed on the surface. While, Hentati et al. (2016) used soil samples from the olive mill evaporation ponds. In a related study Danellakis et al. (2011) investigated the impact of OMWW on the marine environment using *Mytilus galloprovincialis* as bioindicator organism and found the level of mortality to be at a high concentration of OMWW. The toxicological effect of OMWW is probably mainly due to their high polyphenol content. Campani et al. (2017), found that exposure to bioremediated TPOMW and OMWW had no effect on earthworm survival even at the highest concentration concluding that the reduction in toxicity of OMWW and TPOMW was a consequence of the bioremediation that reduced 90% of polyphenols and decreased acidity, while the salinity was increased.

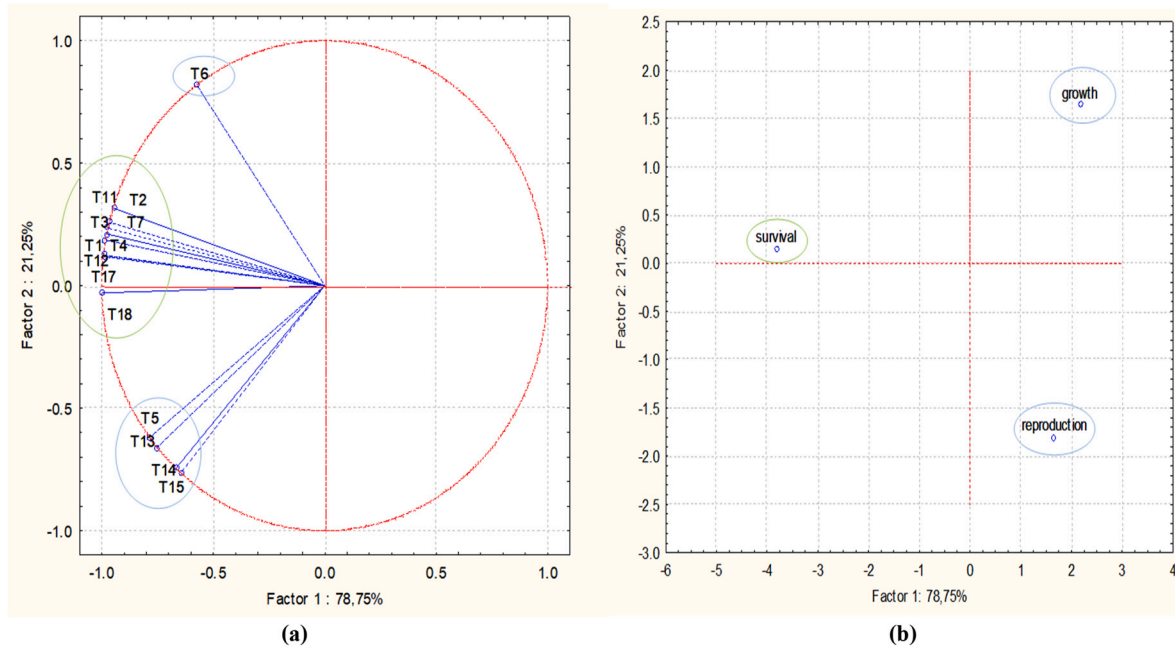
Although the principal responsible for OMWW toxicity is considered to be polyphenols (Sayadi et al., 2000), it is not advisable to base toxicity reduction only on the removal of phenolic compounds since, as we outlined, OMW have other components, including acidic pH, high salinity levels, together with heavy metals that can increase its toxicity (Bouknana et al., 2014). This is the case with olive mill pomace which

has quite low polyphenols content but a very high value of salinity (high electrical conductivity), acidic pH, and demonstrated a toxicological effect stronger than OMWW.

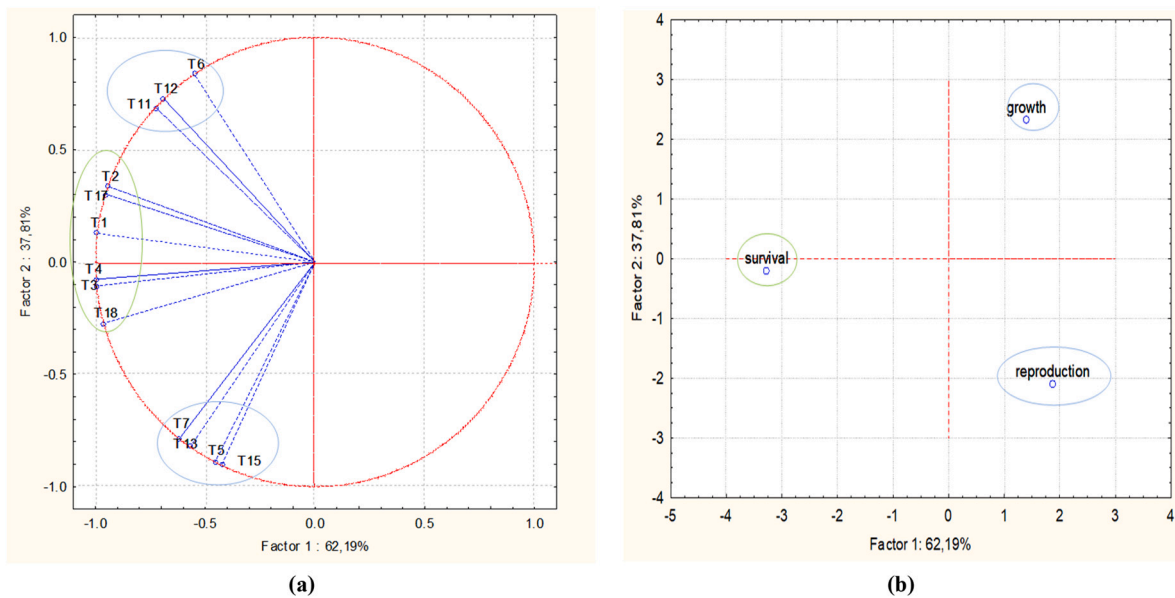
### 3.6. Relationship between growth and survival of earthworm and the exposure time

The linear regression analysis indicates a strong positive correlation between the growth and the period of exposure, meaning that growth increases when there is a rise in exposure time for both *A. trapezoides* and *E. fetida* in T1, T6, T12, a negative correlation was found in T15, T18, in these treatments; in fact, the growth of earthworm decrease when the number of weeks of exposure rises. Survival results show a negative relationship with the period of exposure in most of the treatments, T4, T5, T6, T7, and, T15 of the combination, then, and T18 indicating that the rate of survival decreases with the increasing period of exposure. Since T8, shows the mortality of earthworms no correlation was indicated. The regression equations are present in Table 5, and the most interesting results are shown in Fig. 4.

These results can only be compared with those of Chalkia et al. (2020), reporting an increase of body weight after 28 and 56 days of



**Fig. 5.** Principal component analysis of *Aporrectodea trapezoides* species.  
 a Correlation circles of different treatments of OMWW (T1 to T5) and OMP (T6 to T10) and the OMWW and OMP combination (T11 to T16) then T17 (N C); T18 (P C).  
 b Projection of experimental points according to growth, reproduction, and the survival of *Aporrectodea trapezoides*.



**Fig. 6.** Principal component analysis of *Eisenia fetida* species.  
 a Correlation circles of different treatments of OMWW (T1 to T5) and OMP (T6 to T10) and the OMWW and OMP combination (T11 to T16) then T17 (N C); T18 (P C).  
 b Projection of experimental points according to growth, reproduction, and the survival of *Eisenia fetida*.

exposure of *Octodrilus complanatus* to OMW. No other author has tested the variations in responses over time. However, they are important because if we refer to a natural situation, earthworm remain in the soil in contact with the OMW for long periods and it is important to see how and if the responses change over time.

**3.7. Multivariate analysis (PCA)**

The search for correlations was performed through principal

component analysis (PCA) separately for each species. In PCA correlation circle Fig. 5 for *A. trapezoides*, the first two principal components were F1 and F2, where F1 accounted for 78.75% and F2 for 21.25% of the information. T1, T2, T3, T4, T11, T12, T17, and T18 treatments were negatively correlated to axis 1. Moreover, T6 was positively correlated with axis 2 and opposed to T5, T13, T14, and T15 of the combination treatments.

The PCA projection of experimental points Fig. 5b indicates that only survival is negatively correlated with the first component of the PCA and

opposed to the other two variables: growth and reproduction. The score plot of PCA Fig. 5 indicates that T1, T2, T3, T4, and the T11, T12, T14 of the combination T17, and T18 treatments are responsible for *A. trapezoides* survival, while the T6 treatment is more beneficial to the growth than to the reproduction of *A. trapezoides* in contrast to T5, and T13, T14, and T15 of the combination treatments.

Regarding *E. fetida* Fig. 6, the first two principal components (axes 1 and 2) explain 62.19% and 37.81% information respectively. T1, T2, T3, T4, T17, and T18 treatments were negatively correlated with axis 1, while T6, T11, and T12 treatments are positively correlated with axis 2, and opposed to T5, T7, and T13, and T15 of the combination treatments Fig. 6a.

The projection of experimental points for *E. fetida* Fig. 6b shows that survival was negatively correlated and opposed to growth and reproduction. The PCA results indicate that T1, T2, T3, T4, T17, and T18 treatments resulted in the survival of *E. fetida*, while its growth was stimulated by T6, and T11, and T12 of the combination treatments than the reproduction in contrast to T5, T7, and T13, and T15 of the combination treatments.

### 3.8. Comparison of toxicity among the different by-products and between species

Based on the findings of our study, it is possible to compare the effects of the different types of OMWs tested individually and in combination. The treatment with olive mill pomace produces the most evident toxicological effects on all the earthworm endpoints (survival, reproduction, growth) of the two earthworm species (*E. fetida* and *A. trapezoides*) with the doses being over the amount that is allowed by law (12.5%), including the concentrations that can potentially be reached in a real situation (25% and 50%). Furthermore, the olive mill pomace and the combination were produced the most evident toxicological effects on the *E. fetida* endpoints, especially at the higher doses even the recommended. Moreover, *E. fetida* growth presents more sensitivity to the treatment with 100% of olive mill wastewater (corresponding to 50m<sup>3</sup>/ha/year) than the *A. trapezoides*.

The optimal soil electrical conductivity for earthworm is 0.5–1.0 dS m<sup>-1</sup> and the optimal soil pH for earthworms ranges from 6.0 (Kwak et al., 2019; OECD, 1984) to 7.0 (ASTM, 2004). The electrical conductivity 14.8 mS m<sup>-1</sup> that was found on olive mill pomace much higher than the ideal ranges, and the pH 4.7 was much more acidic than the ideal ranges, which might be the main cause of the highest toxicological effects of the olive mill pomace on the earthworm endpoints. Also, polyphenols might probably have played a role in the toxicological effects of OMP, although we found in our sample a quite low content (40 ± 0.3 mg g<sup>-1</sup>). Kwak et al. (2019) found *Eisenia andrei* to have 100% mortality and 100% abnormalities when soil has an electrical conductivity of 6.0 dS m<sup>-1</sup>. Wu et al. (2019) reported the effects of soil acid stress in earthworm *Eisenia fetida* with a significant inhibitory effects on the survival, growth, and reproduction of earthworm at a soil pH values of (3.0, 4.0, and 5.2). On the other hand, the negative effects of OMWW may be more directly related to the high levels of polyphenols.

The results highlighted also that the treatment with the combination of the olive mill wastes produced the most evident toxicological effect, on the growth of the two earthworm species, with a weight reduction up to -87.32 ± 0% after the highest dose treatment, and revealed a similar high toxicological effect with olive mill pomace at 100% dose on the survival of the two earthworm species. Additionally, the treatment with the combination of olive mill by-products produced a high toxicity on *E. fetida* reproduction than *A. trapezoides* at the doses higher than 12.5%. To our knowledge, this is the first study in which the effect of the OMWW and OMP combination on the earthworm endpoints was investigated, the main cause of this toxicological effect might be the combination of the high polyphenol content (present in OMWW) and the high electrical conductivity, and the acidic pH (present in OMP).

## 4. Conclusion

Based on the findings of this study, we recommend a significant dilution of olive mill wastes as a possible solution prior to their disposal in order to prevent risks to the soil invertebrates. In addition, our results confirm the importance of implementing remediation and bioremediation processes to reduce their environmental toxicity (e.g. by decreasing levels of polyphenols and salinity and by increasing pH) and facilitate the safe and environmental friendly use of these by-products. A biomarker approach is needed to investigate the potential effect of OMWW and OMP on earthworm biochemical and cellular parameters.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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