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In Applied Biotechnology

Branch: Biological science

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Presented By: Nawal MEKERSI

THEME

Study the effect of by-products from olive oil extraction on the earthworm activity

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<u>In front of the jury composed of:</u>

President:	**Mr. Abdelhamid KHABTANE**	Senior Lecture class A, University of Khenchela
Supervisor:	**Mrs. Kenza KADI**	Professor. University of Khenchela
Examiner:	**Mrs. Sofia HAMLI**	Senior Lecture class A, University of Khenchela
Examiner:	**Mr. Smail CHAFAA**	Professor. University of Batna 2
Examiner:	**Mr. Rachid ROUABHI**	Professor. University of Tebessa
Guest:	**Mrs. Dalila ADDAD**	Senior Lecture class A, University of Khenchela

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I dedicate this work to my family. I would not be where I am today without your endless love and support.

Thank you for believing in me.

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ملخص عام

تهدف الدراسة الحالية إلى التحقيق في تاثير مرجين (OMWW) وثفل (OMP) ومزيجهما على النمو والتكاثر والبقاء على قيد الحياة لنوعين من دودة الأرض *Aporrectodea trapezoides* و *Eisenia fetida*. علاوة على ذلك، تأثيرها على الخصائص الفيزيائية والكيميائية للتربة مثل (الأس الهيدروجيني، التوصيل الكهربائي (EC)، المادة العضوية (OM) ، الكربون العضوي (OC) ، نيتروجين العضوي (TN)، الفوسفور المتاح P، محتوى البوليفينول (PP) ، بالإضافة إلى ذلك ، تم استخدام التحليل الطيفي FTIR لتحديد البوليفينول في التربة, تهدف الدراسة الحالية كذلك إلى تحديد تراكم المعادن الثقيلة من OMWW و OMP في التربة والتراكم البيولوجي في نوعين مختلفين من ديدان الأرض باستخدام خمس جرعات متزايدة من OMWW و OMP وتتراوح على النحو التالي: 12.5٪ ، 25٪ ، 50٪ ، 75٪ و 100٪ وزن / وزن. كشفت النتائج أن مخلفات مطاحن الزيتون لها تأثيرات كبيرة على دودة الأرض (البقاء، والنمو، والتكاثر) ، وأظهرت تعزيزًا للنمو بتركيزات منخفضة من OMP ، بينما تم العثور على نفوق ديدان الأرض وتثبيط التكاثر بجرعات عالية من OMP والمزيج ، تم العثور على ان *Eisenia fetida* أكثر حساسية للتعرض لمخلفات مطاحن الزيتون من *Aporrectodea trapezoides*. بالإضافة إلى OMWW و OMP لهما أيضًا تأثيرات كبيرة على الخصائص الفيزيائية والكيميائية للتربة، وانخفاض درجة حموضة التربة، وزيادة في EC للتربة، ومحتوى OM للتربة، وكذلك محتوى OC للتربة. أظهر تحليل FTIR زيادة في محتوى البوليفينول في التربة تحت المعاملة مع OMWW وOMP، وفي الوقت نفسه، لم تظهر التربة TN و P التربة تغيرًا كبيرًا في ظل المعالجات المطبقة خاصةً تحت الجرعات المنخفضة. إلى جانب ذلك، تم العثور على OMWW و OMP تحتوي على كميات كبيرة من المعادن الثقيلة، مما تسبب في تراكم ملحوظ للمعادن في التربة مثل (الكادميوم (Cd) والنحاس (Cu) والكروم (Cr) والزنك (Zn) والحديد (Fe)) ، والتي تسبب مخاطر بيئية خطيرة. بالإضافة إلى تراكم المعادن في التربة، وجد أن نفايات مطاحن الزيتون تسبب تراكمًا خطيرًا للمعادن الثقيلة في أنسجة دودة الأرض. أظهرت النتائج أن *Aporrectodea trapezoides* لديها القدرة على تراكم المعادن الثقيلة في أنسجتها أكثر من *Eisenia fetida*.

الكلمات المفتاحية: مياه الصرف الصحي لمعاصر الزيتون، ثفل الزيتون، دودة الأرض، التربة، المعادن الثقيلة.

General abstract

The current study aims to investigate the effects of olive mill wastewater (OMWW) and olive mill pomace (OMP) and their combination on the growth, reproduction, survival of the two earthworm species *Aporrectodea trapezoides*, and *Eisenia fetida*. Furthermore, their effects on the soil physicochemical characteristics such as (pH, Electrical conductivity (EC), Organic matter (OM), Organic carbon (OC), Total nitrogen (TN), Assimilable phosphorus (P), Phenolic compounds (PP), in addition, FTIR spectroscopy is used to determine the soil polyphenols. Moreover, the current study aims to determine the accumulation of heavy metals from OMWW and OMP in the soil and the bioaccumulation in the two different earthworm species using five increasing doses of OMWW and OMP ranging as follows: 12.5%, 25%, 50%, 75%, and 100% w/w. Findings revealed that olive mill wastes have significant effects on the earthworm endpoints (survival, growth, and reproduction), and showed growth enhancement at lower concentrations of OMP, while mortality of earthworms and reproduction inhibition are found at high doses of OMP and the combination, *Eisenia fetida* were found more sensitive to exposing to olive mill wastes than *Aporrectodea trapezoides.* As well as OMWW and OMP have also significant effects on soil physicochemical properties, decrease in soil pH, increase in soil EC, soil OM content, as well as soil OC content. FTIR analysis was showed raise in soil polyphenols content under treatment with OMWW and OMP, meanwhile, soil TN and soil P were not shown a significant change under the treatments applied especially under lower doses. Besides, OMWW and OMP were found contain significant amounts of heavy metals, as a consequence causing a remarkable accumulation of metals in the soil such as (Cadmium (Cd), Chromium (Cu), Copper (Cr), Zinc (Zn), and Iron (Fe), which cause crucial environmental hazards. In addition to the soil accumulation of metals, olive mill wastes were found to cause serious bioaccumulation of heavy metals in earthworm tissue. Results revealed that *Aporrectodea trapezoides* has the ability to accumulate heavy metals in their tissue more than *Eisenia fetida*.

Keywords: Olive mill wastewater, Olive mill pomace, Earthworm, Soil, Heavy metals.

Résumé général

L'étude actuelle vise à étudier les effets de margines et de grignons et leur combinaison sur la croissance, la reproduction, la survie des deux espèces des lombriciens *Aporrectodea trapezoides,* et *Eisenia fetida*. Par ailleurs, leurs effets sur les caractéristiques physicochimiques du sol telles que (pH, Conductivité électrique (CE), Matière organique (MO), Carbon organique (CO), Azote totale (NT), Phosphore assimilable (P), Polyphénols totale (PP), en addition, la spectroscopie FTIR est utilisée pour déterminer les polyphénols du sol. La présente étude vise à déterminer l'accumulation des métaux lourds provenant de margines et de grignons dans le sol et la bioaccumulation dans les deux différentes espèces des lombrics en utilisant cinq doses croissantes margines et grignons comme suit : 12,5%, 25%, 50%, 75%, et 100% v/v. Les résultats ont révélé que les sous-produits de l’extraction de l’huile d’olive ont des effets significatifs sur les paramètres des lombriciens (survie, croissance et reproduction), et ont montré une augmentation de la croissance à des concentrations plus faibles de grignons, tandis que la mortalité des lombriciens et l'inhibition de la reproduction sont trouvées à des doses élevées de grignons et de la combinaison, *Eisenia fetida* s'est avéré plus sensible à l'exposition aux déchets de l’extraction d’huile d’olive que *Aporrectodea trapezoides.* De même que les margines et les grignons ont également des effets significatifs sur les propriétés physico-chimiques du sol, la diminution du pH du sol, l'augmentation de la CE du sol, la teneur en MO du sol, ainsi que la teneur en CO du sol. L'analyse par FTIR a montré une augmentation de la teneur en polyphénols du sol sous le traitement des margines et de grignons, tandis que, le NT du sol et le P du sol n'ont pas montré un changement significatif sous les traitements appliqués en particulier sous les doses les plus faibles. En effet, on a constaté que les margines et les grignons contiennent des quantités importantes des métaux lourds, ce qui entraîne une accumulation remarquable des métaux dans le sol tels que le Cadmium (Cd), le Chrome (Cu), le Cuivre (Cr), le Zinc (Zn) et le Fer (Fe), ce qui constitue un danger crucial pour l’environnement. En plus de l'accumulation des métaux dans le sol, on a constaté que les déchets de l’extraction de l’huile d’olive provoquaient une bioaccumulation importante des métaux lourds dans les tissus des lombriciens. Les résultats ont révélé que *Aporrectodea trapezoides* a la capacité d'accumuler les métaux lourds dans leurs tissus plus que *Eisenia fetida.*

Mots-clés : Margines, Grignons, Lombrics, Sol, Métaux lourds.

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50% OMP, T9: 75% OMP, T10: 100% OMP, T11: 12.5% Combination, T12: 25% Combination, T13: 50% Combination, T14: 75% Combination, T15: 100% Combination, Control: untreated soil.

List of Abbreviations

AAS	Atomic absorption spectrophotometry
ANOVA	Analysis of variance
AT	*Aporrectodea trapezoides*
Bdl	Below detection limit
BOD$_5$	Biological oxygen demande
C/N	Carbon/Nitrogen
Ca	Calcuim
Cd	Cadmium
Cr	Chromium
Cu	Copper
CuSO$_4$	Copper sulfate
DM	Dry matters
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EF	*Eisenia fetida*
Fe	Iron
FTIR	Fourier transform infrared spectrometer
H$_2$SO$_4$	Sulfuric acid
IOC	International Olive Council
K	Potassium
K$_2$SO$_4$	Potassium Sulfate
Kbr	Potassium bromide
LSD	Least Significant Difference
M^3	Cubic meter
MC	Moisture content
Na	Sodium
NaOH	Sodium hydroxide
Ni	Nickel
Ns	Not significant
OC	Organic carbon
OH	Hydroxyl group
OM	Oragnic matter
OMP	Olive mill pomace
OMW	Olive mill waste
OMWW	Olive mill wastewater
P	Assimilable phosphorus
Pb	Plomb
PCA	Principal component analysis
pH	Potentiel hydrogène
PP	Phenolic compounds
ppm	Parts per million

PVC	Polyvinyl chloride
q/ha	quintals per hectare
SD	Standard deviation
T	Time
TN	Total nitrogen
UV	Ultraviolet
w/w	weight / weight
Ze	Zinc

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Chapter II: Effects of olive mill wastewater and olive mill pomace single and in combination on Soil physicochemical properties and soil polyphenols using FTIR spectroscopy

General Introduction

General Introduction

The olive tree (*Olea europaea L.*) is a Mediterranean species that has been planted for table olives and olive oil since ancient times. The cultivation of olives is extremely important in Algeria. This puts Algeria in 9th place in the world rankings, after Spain, Greece, Italy, Turkey, Morocco, Syria, Tunisia and Portugal **(IOC, 2018).**

Algerian olive production is mainly concentrated in the northern regions of the country, with most orchards (80%) located in hilly areas. Algerian olive oil production has increased compared to the previous year, reaching about 80 thousand tons **(Mendil etal., 2009).** The agriculture departments of the future Wilaya have estimated yields for oleaginous olives at 7 to 25 q/ha. The latter is an interesting threshold with 25, 22 and 21 q/ha respectively, especially in the wilayas of Skikda, Tizi Ouazou and Jijel. The wilaya of Bejaia alone covers 51 874 hectares and produces olive oil on 123 316 hectares. The olive tree also helps in erosion control, in the improvement of agricultural lands and in the settlement of hilly areas **(Aggoun-arhab., 2016).**

Olive oil is produced by harvesting the fruit of the olive tree in November, crushing it and then kneading it to increase the amount of oil obtained **(Klen and Vodopivec, 2012).** Their extraction processes involve two fundamental methods for extracting oil. Namely, the centrifugation process and traditional pressing. For centuries, traditional pressing has been used. The majority of olive mills, though, have used three-phase and two-phase centrifugation processes in recent decades **(Roig et al., 2006; Christoforou et al., 2016).** The quantity of oil generated by two and three-phase centrifugation is similar, but the amount of oil produced and the composition of solid and liquid residual fractions are significantly different **(Arvanitoyiannis and Kassaveti, 2008; Christoforou et al., 2016).**

At the end of the process, the three-phase centrifugation system produces three fractions: a solid residue fraction called olive husk or olive pomace (Three-Phase Olive Mill Waste – 3POMW), as well as oil and wastewater liquid fractions. The two-phase process, on the other hand, generates two phases at the end: the oil fraction and a solid/water mixture (Two-Phase Olive Mill Waste – 2POMW) residue that may be extracted and dried later **(Niaounakis and Halvadakis, 2006; Christoforou et al., 2016).**

The wastewater (OMWW) is the liquid phase of the olive mill byproducts, and they are composed of the water content of the olives as well as the milling waters (olives and equipment washing waters and the waters of dilution process in the three phases centrifugal systems). OMWW is characterized by an acidic pH, undesirable color and odor, and, the inclusion of organic compounds such as organic acids, lipids, alcohols, and polyphenols, which transform it into phytotoxic material with potentially negative impact o the environment **(Kavdir & Killi, 2008).** If not properly managed, it also presents a severe environmental hazard. OMWW, on the other hand, includes valuable resources such as a high organic matter content and some nutrients, particularly potassium, that might be used to improve physic-chemical and biological qualities, as well as soil fertility and production, so completing the residue-resource cycle **(Roig et al., 2006; Toscano et al., 2012).**

Moreover, according on the extraction procedure utilized, olive pomace is composed of fruit matter (olive skins, flesh, seeds, and stone fragments), as well as different amounts of vegetation and process water that includes the water-soluble compounds of the fruits **(Toscano et al., 2012).** The direct discharge of olive mill pomace to the land is not allowed because of the pomace's phytotoxic and antibacterial properties (e.g., polyphenols), and elevated salt concentration, their acidic pH (around 4-5) **(Gigliotti et al. 2012).** which might create major environmental concerns **(García-Ruiz et al 2012).** However, olive mill pomace is used as a soil organic amendment due to its high organic content as well as potassium, nitrogen, and phosphorous Besides, the agronomic use of the pomaces allows to increase soil organic content **(Regni et al., 2017).**

The focus of this research is on the by-products of three-phase processing. Because the olives are processed on a short time scale, large amounts of by-products are created in a short period of time (1–3 months), exacerbating the challenges associated with their disposal.

Apart from the aforementioned agronomic effects, their phytotoxic and antibacterial activities are due to the polyphenols present in olive mill by-products, and it's one of the keys limiting factors for the toxicity of olive mill by-products. These by-products are among the most polluting in the agricultural business. **(Capasso et al., 1992; Cardinali et al., 2010; Preedy and Watson, 2010).** Several authors studied the toxic effects of olive mill waste on various plant species, including *Trifolium repens*, *Triticum aestivum*, bacteria, and crustaceans, and found that it has acute toxicity on *Daphnia pulex*, *Palaemonidae shrimp*, *Pelophylax ridibundus*, *Eisenia fetida* larvae, *Helianthus annuus*, *Chlorella vulgaris*, *Vibrio fischeri*, *Daphnia magna*, *Danio rerio* embryos, can also harm the nucleotide and genomic structure of

DNA **(Inceli and Sengezer-Inceli, 2012; Rouvalis et al., 2013; Pavlidou et al., 2014; Campani et al., 2017; Aybeke, 2018; Babi´c et al., 2019).**

Besides the aforementioned toxic effects, the use of OMWW and pomace on soils may have significant environmental advantages. Previous research has suggested that OMWW could be used as a low-cost soil conditioner and fertilizer **(Ayoub et al., 2014; Barbera et al., 2014),** and represents a supplementary resource of water and nutrients for the irrigation and fertilization of Mediterranean olive groves, chronically affected by water and organic matter scarcity **(Ayoub et al., 2014; Chaari et al., 2015).** Because of their high amounts of nutrients essential for plant growth and development, such as (K, N, and P) and organic matter, as well as their high concentration of macro and microelements, olive mill pomace (OMP) soil application may have a favorable impact on soil productivity **(Lozano-García et al.,2011; Ameziane et al.,2019).**

Furthermore, olive mill wastes include a high level of heavy metals (Fe, Cr, Pb, Cu, Ni, Cd, Zn, Mn) **(Paredes et al., 2005).** Heavy metals are a class of toxic elements that severely limit the usefulness of wastewater and its agricultural applications **(Chaoua et al.,2019).** Heavy metal contamination of agricultural soils and crops has been recognized as a major environmental danger because of their non-biodegradable nature and extended biological half-life, as well as their potential accumulation in various body organs. Individuals who consume contaminated crops and/or vegetables, as well as soil fauna and bacteria, are affected by high heavy metal concentrations in agricultural soils **(Lenart-Boron and Wolny-Koladka, 2015; Bhargava et al., 2017; Midhat et al., 2019).**

Earthworms account for a larger amount of terrestrial invertebrate biomass (>80%), and they play a significant role in soil structure and nutrient content. As a result, they might be useful bioindicators of soil chemical pollution in terrestrial ecosystems, providing early warning of soil degradation **(Culy et al., 1995).** This is critical for the health of natural habitats, and it is becoming increasingly relevant in the context of human health and the health of other terrestrial animals that prey on earthworms **(Sivakumar et al., 2015a).** They also increase soil quality, nutrient absorption, plant development, and yield through their feeding, burrowing, and casting activities **(Sivakumar et al., 2015b).** Earthworms have a close relationship with the soil, as they consume enormous amounts of it and have minimal exterior barriers to the soil solution. Earthworms have been utilized widely in ecotoxicological soil research for these and other reasons **(OECD 1984).**

The habitat of the major varieties of earthworms found in the soil ecosystem may be categorized as *Epigeic* earthworms, these compost worms don't create permanent burrows underground and spend the most of their time on the top of the soil. They are phytophagous worms with excellent biodegradation abilities. Consider the following scenario: *Lumbricus rubellus, L.castaneus*, *Eisenia fetida* While the *Anecic* earthworms Worms in this group create vertical tunnels into the earth, but their principal food supply is decomposing debris on the surface of the soil, making them geophytophagous. Ex: *Lumbricus terrestris*. **(Shefali et al., 2018).** In addition, *Endogeic* earthworms, they feed on soil organic matter and dead roots, as well as enormous amounts of dirt, on the subsoil surfaces. As a result, they are classified as geophagus. Ex: *Aporrectodea trapezoides* **(Parihar et al., 2019).**

High salinity and high electrical conductivity soil acidification, pesticides, heavy metals, organic contaminants, and phenolic compounds are among stressors that earthworms are exposed to in their habitat, may cause considerable harm at several biological levels, such as mortality, growth and reproduction inhibition heavy metals accumulation in their tissue **(Li et al., 2010; Hirano and Tamae, 2011; Maity et al., 2018a, 2018b; Wu et al., 2019; Babi´c et al., 2019; Campani et al., 2017).**

To investigate the effects of the olive mill by-products single and in combination on the earthworms and on the soil as well as to contribute to closing some of the knowledge gaps associated with this subject. The aims of this study were to investigate the effects of olive mill wastewater and olive mill pomace and their combination on several earthworm endpoints such as growth rate (weight gain or inhibition), reproduction (number of cocoons), and survival, of two earthworm species *Aporrectodea trapezoides* and *Eisenia fetida*. As well as their effects on soil properties including (pH, EC, OM OC, TN, P, PP). Moreover, this study aims to determine the heavy metals present in olive mill waste, determine the concentration of Cd, Cr, Cu, Fe, and Zn in soil, and determine the bioaccumulation of Cd, Cr, Cu, Fe, and Zn in two different earthworm groups. The conducting experiment was carried out using increasing concentrations of OMWW and OMP and the combination ranged from 12.5% to 100% w/w.

Chapter I:

Effect of Olive mill wastewater and Olive mill pomace and their combination on earthworm growth, reproduction and, survival

Materiels & Methods

1. Materials & methods

The experimental part of the current study was carried out at the pedagogical laboratory complex of the Abbes Laghrour University El Hamma Khenchela.

1.1. Olive mill waste source

The study focused on the analysis of olive mill waste that has been collected from a modern olive oil mill, usinga3-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.

1.2 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^{-1}; 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 Figure 01 and **(Figure 02) (Table 01).** 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.

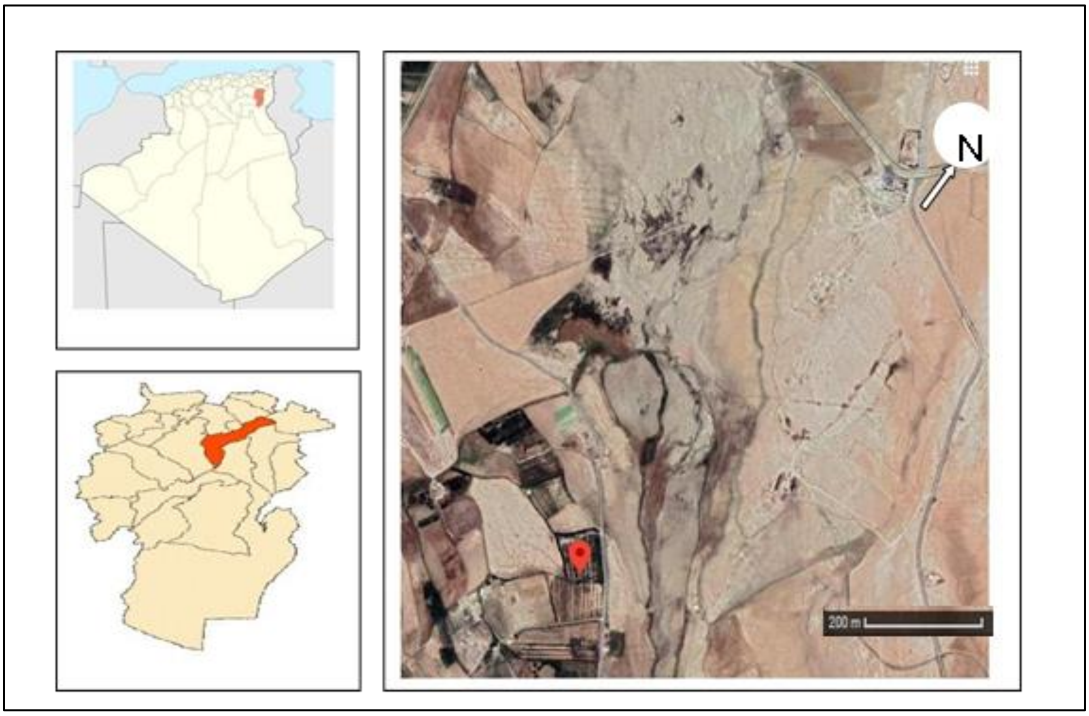


Figure 01: Collection site of *Aperroctodea trapezoides* douar Tmagra commune of N'sigha Wilaya of Khenchela.

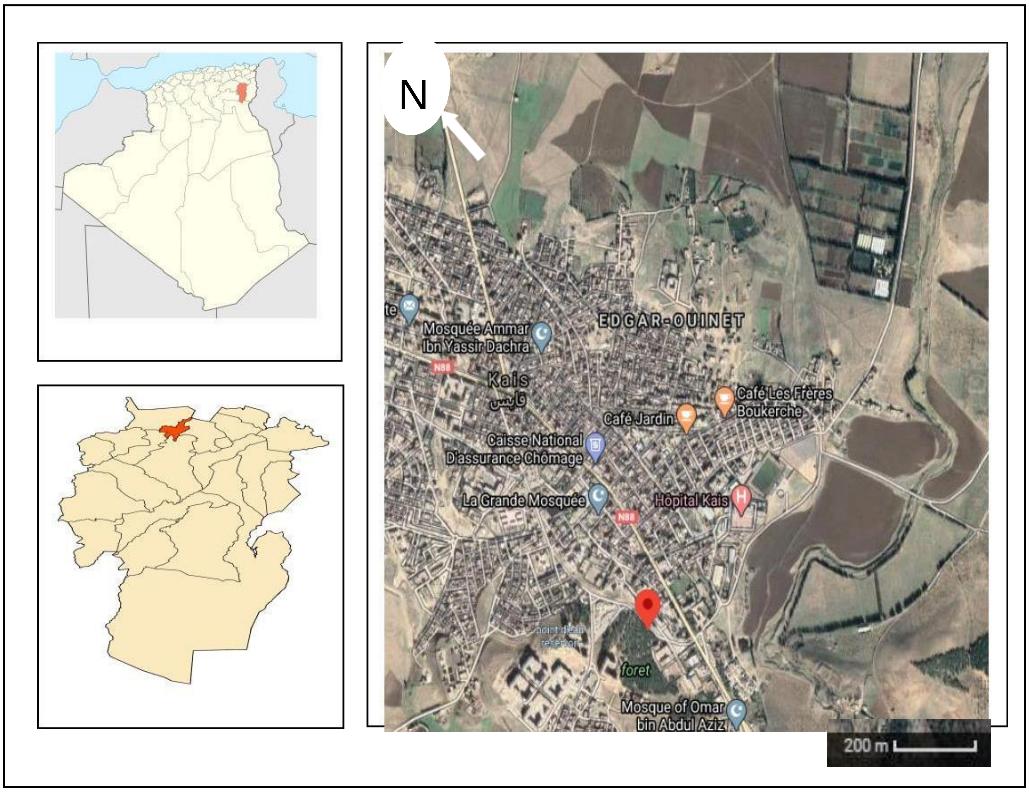


Figure 02: Collection site of *Eisenia fitida* commune of Kais Wilaya of Khenchela.

Table 01 : Characteristics of selected earthworm species.

Characteristics	*A. trapézoides*	*E. fetida*
Kingdom	Animalia	Animalia
Branch	Annelida	Annelida
Class	Clitellata	Clitellata
Order	Haplotaxida	Haplotaxida
Family	Lumbricidae	Lumbricina
Genre	*Apporoctodea*	*Eisenia*
Ecological categories	Endo -anecic	Epigee
Color	Brownish on the upper side and pale on the bottom	red green or tiger green
Size	Length 80 to 140 mm, diameter 3.5 to 8 mm	Length of 4 to 5 mm

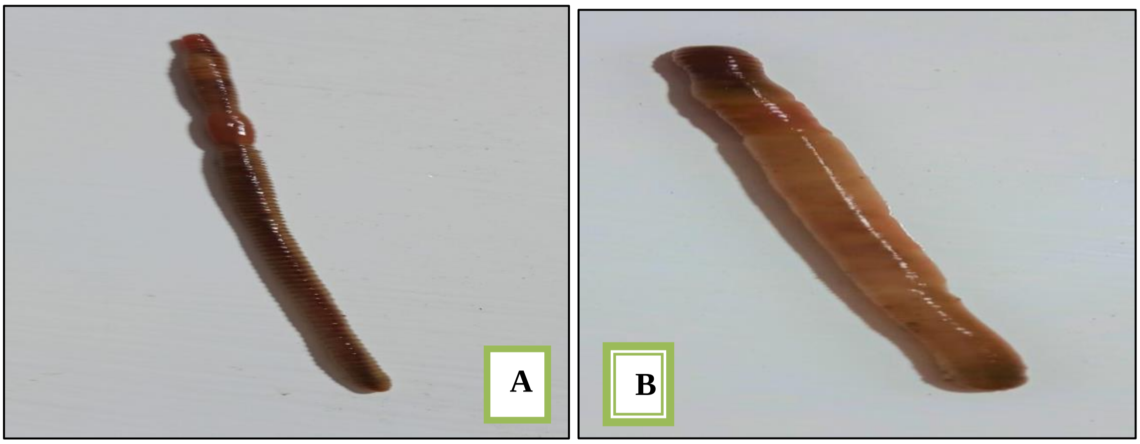


Figure 03: The two earthworm species used in this study A: *Eisenia fetida* and B: *Aporrectodea trapezoides.*

1.3. Physico-chemical analyses of olive mill wastewater samples (OMWW)

1.3.1. Determination of pH, Electrical conductivity (EC), Salinity of OMWW

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).**

1.3.2. Determination of dry weight of OMWW

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 percentage of the initial volume **(Hamdi, 1991).** The following formulae are used to determine the measurements:

DM ($g\ l^{-1}$): ((M-M0)/ V) × 100

M (%): ((M1-M) / (M1-M0)) ×100

M0: mass of the empty crucible in g

M1: mass of crucible and olive mill wastewater before drying in g.

M: mass of crucible and olive mill wastewater after drying in g.

V: volume of the test sample in ml.

1.3.3. Determination of total suspended of OMWW

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).**

1.3.4. Determination of total nitrogen in OMWW

The total nitrogen content has been determined in accordance with the Kjeldahl method **(Greenberg *et al*., 1992)**. 5 mL of olive mill effluent with a catalyst ($CuSO_4$+K_2SO_4) and some selenium in matras. As an antifoam, 10 mL H_2SO_4 and 10 mL 30°C hydrogen peroxide (H_2O_2). As an anti-shock measure, place a few glass beads. Heat at 100°C for a few minutes. Then raise the mineralization temperature to 400°C until a distinct green color shows, continue heating for 30 minutes. In an automated distillation equipment, soda (32c/o) and distilled water. The distilled ammonia was trapped in an erienmeyer containing 20 mL 4c/o boric acid.

The nitrogen rate was calculated according to the following formula:

$$\boxed{\%NTK\ (g) = (V1\times 0.014\times 100\times N)/V}$$

N: The normality of the sulfuric acid solution.

Vo : volume of the sample in ml (5ml).

V1 : volume in ml of the sulfuric acid solution used for the titration.

1.3.5. Determination of organic matter (OM) content in OMWW

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).**

1.3.6. Determination of total sugar content in OMWW

The total sugar content was measured by using spectrophotometry UNICO, United States, at 488 nm in accordance with **Dubois *et al*. (1956).**

1.3.7. Determination of total phenolic content in OMWW

The Folin ciocalteu colorimetric method is used to determine polyphenols **(Makkar *et al*., 1993).** The Folin-Ciocalteu reagent is a combination of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolibdic acid ($H_3PMo_{12}O_{40}$) that is reduced by phenols to a mixture of tungsten and molybdenum blue oxides (W_8O_{23}) (Mo_8O_{23}**).** This blue color has an absorption maximum at 760 nm, which is related to the amount of phenolic chemicals present in the medium.

1.3.8. Determination of biochemical oxygen demand (BOD_5) of OMWW

The biochemical oxygen demand (BOD_5) was determined by a 5-day BOD using OxiTop IS 6 BOD meter **(Rodier *et al*., 1975).** A quantity of 2.5 mL of purified and centrifuged olive mill effluent is obtained and diluted 100 times with distilled water. To begin, each sample's pH is adjusted to a range of 6.5 to 7.5 by adding NaOH, and then each sample is placed in a BOD_5-meter vial. The caps are then filled with 250 mg O_2/l, which corresponds to the quantity introduced (250 ml).

The BOD_5 values are expressed as follows:

$$\boxed{\mathbf{BOD_5}\ (\text{mg } O_2/l) = \text{Readings} * \text{Dilution factor}}$$

1.4. Physico-chemical analyses of olive mill pomace samples (OMP)

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

1.4.1. Determination of pH, Electrical conductivity (EC), salinity of OMP

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).**

1.4.2. Determination of moisture content in OMP

The moisture content was calculated by drying the OMP sample at 105 ◦C for 24 h **(Fatianoff and Gouet, 1969).** The sample then calculating it using the following formula:

$$\boxed{\text{MC}= \frac{\text{fresh weight} - \text{dry weight}}{\text{fresh weight}} \times 100}$$

1.4.3. Determination of organic matter content in OMP

It was determined by calcining the samples for one hour at 850°C in a muffle furnace NABERTHERM, Germany, and then calculating it using the conventional approach. Assume: P1: Weight of the empty capsule; P3: Weight of the capsule, Plus sample after 24 hours in the oven; P4: Weight of the capsule + sample after calcinations **(Aubert, 1978).**

$$\boxed{MO = \frac{P3 - P4}{P3 - P1} \times 100}$$

1.4.4. Determination of Assimilable Phosphorus in OMP

It was determined using Olsen's technique **(Olsen *et al.*, 1954),** which was based on the concept of the blue reagent and included measuring optical densities using a spectrophotometer at a wavelength of 860nm.

a) Sample preparation:

- 5 g of pulverized sample + 100 ml of 0.5M sodium bicarbonate "$NaHCO_3$," shaking for 30 minutes, filtering on filter paper

• 5 mL filtrate + 5 mL ammonium molybdate "$(NH_4)\ _6MO_7O_{24}\ 4H_2O$", shaken, dilute to 22 mL with distilled water

• Dissolve 1 mL of the diluted tin chloride solution "$SnCl_2\ 2H_2O$" in 1 mL of water.

• Fill the previous solution with distilled water until it reaches 25 mL, then shake for 10 minutes to allow the color to develop (sky blue).

• At a wavelength of 860 nm, provide the colorimeter measurement.

b) Phosphorus solution (standard) preparation for the standard range:

- Dissolve 0.439 g potassium dihydrogen phosphate "KH_2PO_4" in 500 ml distilled water while stirring, then top up with distilled water to create the volume 1 l. This solution has 100 milligrams of P per liter.
- Fill a 1liter bottle halfway with distilled water and 20 ml of the latter solution. The standard range was made from this stock solution, which includes 2 g/ml: take from 1 ml to 6 ml, which is comparable to 2 g to 12 g of P/ml in 25.
- Combine 5 mL $NaHCO_3$ and 5 mL $(NH_4)\ 6MO_7O_{24}\ 4H_2O$ in a shaker and dilute to 22 mL with distilled water.
- Pour 1 mL of the diluted $SnCl_2\ 2H_2O$ solution into the test tube.
- Using pure water, top up the solutions to 25ml and shake.
- Take note of the colorimeter measurement at 860nm.

1.4.5. Determination of total nitrogen content in OMP

The total amount of nitrogen was measured using the Kjeldahl method **(Mulvaney, 1982).**

- 2 g sample + 5 g copper sulfate "$CuSO_4$" in a Kjeldahl flask
- 10 g potassium sulfate (K_2SO_4) + 20 ml sulphuric acid (H_2SO_4) (98 %). Put the matras on the attack ramp placed under the host.
- Heat softly at first, then gradually raise the heat intensity until the matras contents come to a boil.

The transition of the samples to a greenish tint indicates the end of mineralization.

- Allow the solutions to cool.
- Collect supernatant in distillation flasks with 80 mL distilled water 80 mL pure water + 80 mL sodium hydroxide solution "NaOH" at a concentration of 33 percent

- Ammonia and water vapors are gradually heated and drawn into a cooling column, where they condense.
- Ammonia is measured in an Erlenmeyer flask using sulfuric acid and a pH indicator called "methyl red." A pH indicator is "Methyl red." Consider X to be the amount of H_2SO_4 required to neutralize the ammonia. To do so, add a few drops of methyl red to the ammonia solution, then titrate it with sulfuric acid at 0.1N using a burette until the color changes pink, then record the volume of sulfuric acid supplied.
- Calculations:

Since 1 mL of 0.1N sulfuric acid contains 0.2 mg of nitrogen, the total weight of nitrogen in the sample is determined using the formula: X (volume of H_2SO_4 used for neutralization) x0.2x2 (amount of sample).

1.4.6. Determination of the Exchangeable elements « Na, Ca, K » of OMP

The flame spectrophotometer was used to determine them using the traditional way. spectrophotometer for flames **(Pansu and Gautheyrou, 2003).**

Before continuing to the flame spectrophotometer reading, we produced aqueous extracts for each sample.

1.4.7. Determination of organic carbon content of OMP

According to the concept of colorimetry by diphenylamine by titration, it was calculated using Anne approach **(Aubert, 1978).**

- Fill flasks with 1 g of each sample, 10 ml of 8% potassium dichromate "$K2Cr_2O_7$," attach the flask to the condenser, and turn on the water faucet.
- Pour 15 mL of pure sulfuric acid (H_2SO_4) into the container.
- Using a Bunsen burner, heat the solution until it boils, then turn it off after 5 minutes.
- Allow time for it to cool.
- Pour the contents of the flask into a volumetric flask and top up with distilled water to create up to 100 ml.
- Add 200 mL distilled water to 20 mL of the contents.
- Combine 1.5 g sodium fluoride "NaF" with 3–4 drops diphenylamine.
- Shake the beaker, which is topped with a graded burette.
- Shake and use Mohr's solution at 0.2N to calculate the amount of dichromate present.

- V is the number of ml of Mohr's solution that was utilized.

Perform the same processes without the samples (control test), and multiply V' by the quantity of mL of Mohr's solution used in the control.

Calculations:

$$\boxed{\% C = (V' - V) \times 0.3}$$

1.4.8. Determination of polyphenol content in OMP

The polyphenol content was evaluated according to Folin-Ciocalteu spectrophotometric method at 460nm as follows: **(Singleton and Rossi, 1965).**

- The solvant used is acetone / water in proportions of 70/30 ml.
- 2 g of plant powder from each échantillon was extracted with 50 ml of solvent and agitated for 2 hours at room temperature.
- After filtering the extract, the residue was taken again with the same volume of solvent. The contents of the three filtrations obtained were combined in the same volume of solvent to yield the crude extract (aqueous).
- From a linear calibration curve (y=ax) constructed with gallic acid at different concentrations: (0.1-0.2-0.3-0.4-0.5) mg/ml under the same circumstances as the sample, the quantities of total polyphenols were reported as mg of gallic acid equivalent per gram of extract.
- To 500 l of Folin-ciocalteu diluted (1/10 dissolved in distilled water), 100 l of acetone extract was added. The mixture was vortexed and kept at room temperature for 5 minutes in the dark, after which 1.5 ml of 2 percent sodium carbonate "Na_2CO_3" was added with agitation, and the solutions were incubated for 1 hour at room temperature and in complete darkness.
- Finally, take a spectrophotometer reading at 460nm wavelength.

1.5. Experimental design

1.5.1. 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^3/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 (**Figure 04).**

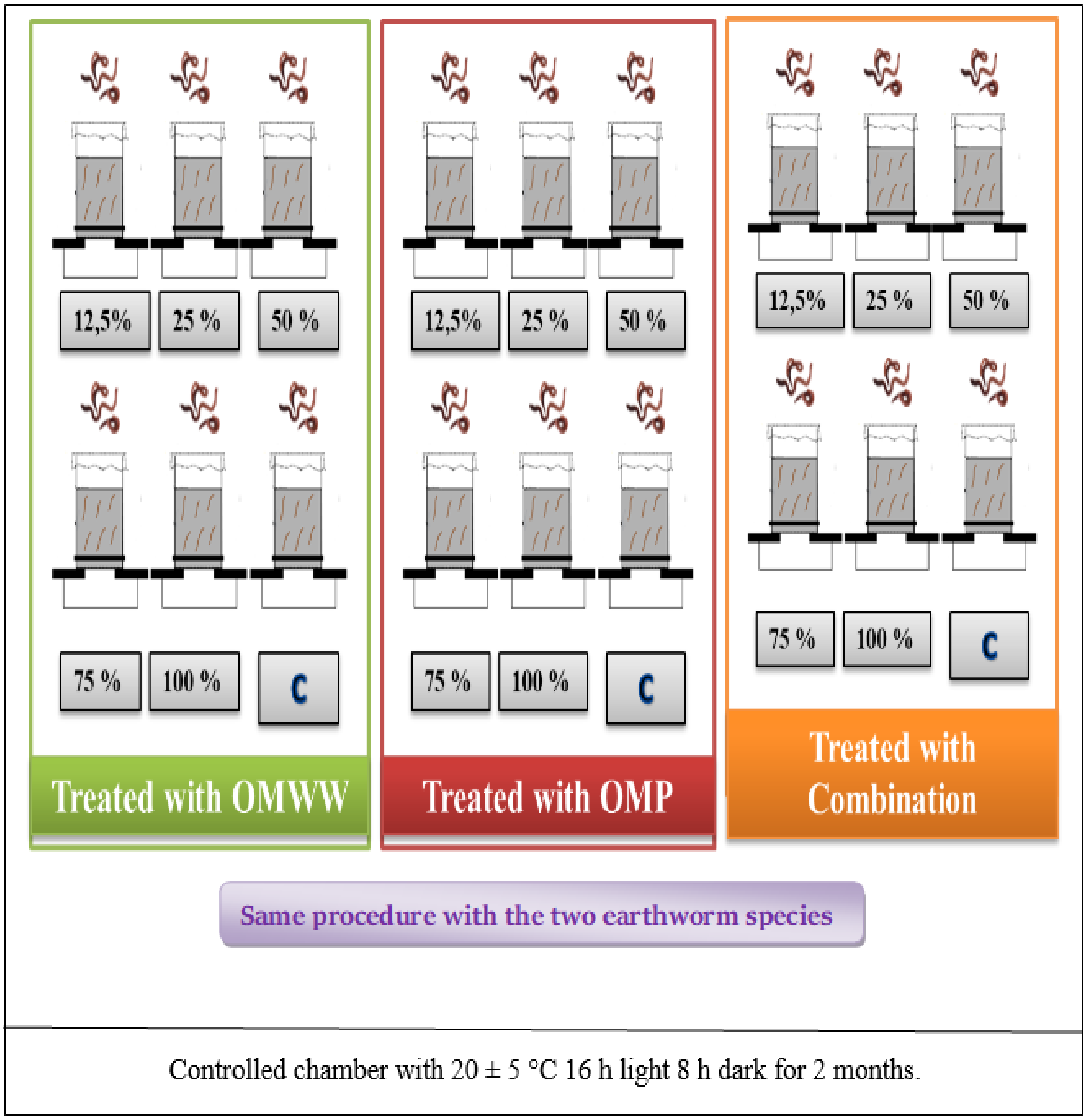


Figure 04: The presentation of the experimental design.

1.5.2. Determination of survival, growth, and reproduction of earthworms

Alive adult worms were collected after being sorted by hand and 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:

$$\boxed{\frac{(\mathrm{Wt}-\mathrm{W0})}{(\mathrm{W0})} \times 100\%}$$

Wt is the weight of the earthworm on the day they were checked, and W0 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) (Figure 05).**

Figure 05: The determination of survival, growth and reproduction of earthworms.

1.6. Statistical analysis

The values of the physicochemical analysis of olive mill wastewater and olive mill pomace were performed in triplicate and presented as means ± 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: Yijk =

$\mu + \alpha i + \beta j + (\alpha\beta)ij + eijk$, 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.

Results & Discussion

2. Results and Discussion

2.1. 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 02).**

The analysis indicated that the OMWW has a brown color caused by its high content of recalcitrant compounds such as lignins and tannins **(Paraskeva and Diamadopoulos, 2006)** and an acidic pH (pH = 4.84 ± 0.01), together with a high electrical conductivity (EC = 9.7 ± 0.61 Ms. cm^{-1}) when compared with the value cited by **Malvis *et al.* (2019).** A high salinity 7.4 ± 0 g. l^{-1} 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 **(Achak *et al.*, 2008; Ero˘glu *et al.*, 2008; Bouknana *et al.*, 2014).** A high moisture content (92.1 ± 0.96%) was found **(Figure 06),** while the OMWW total nitrogen content was very low (TN = 0.42 ± 0.02 g. l^{-1}), and the results obtained are compatible with the values reported in other studies **(Meftah *et al.*, 2019; Babi´c *et al.*, 2019).**

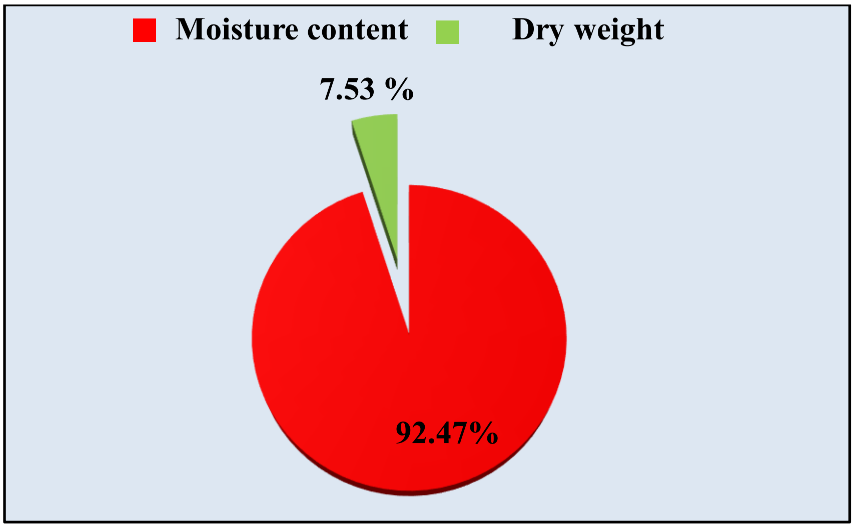


Figure 06: The moisture and dry matter contents of the studied olive mill wastewater samples.

The content in the organic matter was high (MO = 91 ± 1%) **(Figure 07),** moreover a high biological oxygen demand (BOD = 40. 20 ± 0. 01 g. l^{-1}) was found and the result obtained is in accordance with the value cited by **Babi´c *et al.* (2019).**

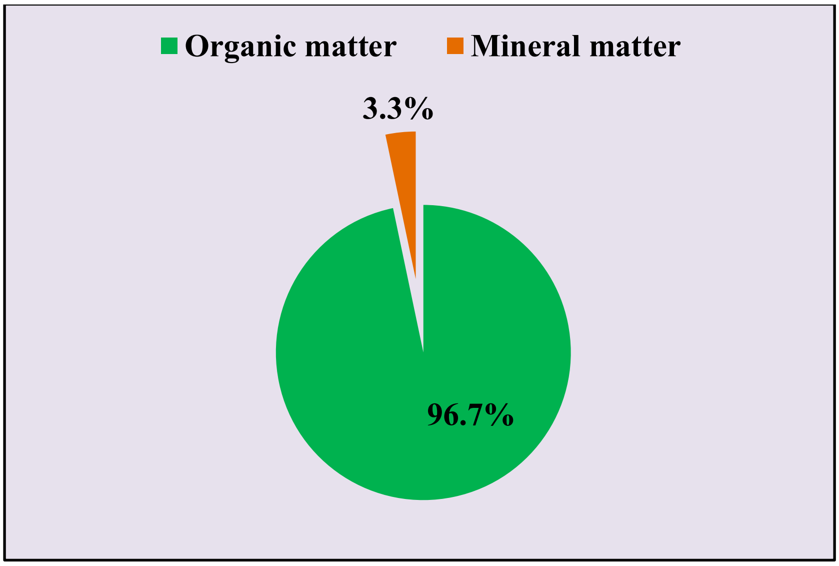


Figure 07: The organic and mineral matter contents of the studied olive mill wastewater samples.

Moreover, the physicochemical analysis revealed that the OMWW was highly enriched in phenolic content (2. 31 ± 0. 01 g l^{-1}) 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).**

The main physico-chemical results of the studied olive mill wastewater are recorded in the table below:

Table 02: Physicochemical characteristics of olive mill wastewater. The results are reported as mean ± SD of 3 different measurements.

Parameter	Value
Color	Brown
pH 20 C°	4.84 ± 0.01
Electrical conductivity (mS. cm^{-1})	9.7 ± 0.61
Salinity (25 C°) g. l^{-1}	7.4 ± 0
Dry weight (%)	7.53 ± 0.03
Moisture content (%)	92.47 ± 0.96
Total suspended (g. l^{-1})	9.422 ± 0.003
Total nitrogen (g. l^{-1})	0.42 ± 0.02
Organic matter (%)	96.7 ± 1
Mineral matter (%)	3.3 ± 1.53
Total sugar (mg. ml^{-1})	0.32 ± 0.04
Total phenolic content (g. l^{-1})	2.31 ± 0.01
BOD (g. l^{-1})	40.20 ± 0.01

2.2. 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 03).**

The main physico-chemical results of the studied olive mill pomace are recorded in the table below:

Table 03: Physicochemical characteristics of olive mill pomace. Results are reported as mean ± SD of 3 different measurements.

Parameter	Value
pH	4. 7 ± 0.02
Electrical conductivity (mS. cm^{-1})	14.8 ± 0.1
Moisture content (%)	57.69 ± 0.40
Organic matter (%)	90.27 ± 3.0
Assimilable phosphorus (µg. g^{-1})	25.3 ± 0.2
Total nitrogen (mg. g^{-1})	0.5 ± 0.1
Na (mg. g^{-1}) **Ca (mg. g^{-1})** **K (mg. g^{-1})**	51.67 ± 1.53 2.41 ± 0.19 111.67 ± 2.09
Organic carbon content (%)	60.80 ± 0.59
Total polyphenol (mg. g^{-1})	40 ± 0.3

The analysis showed that OMP has an acidic pH (4.7 ± 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 ± 0.1 mS. cm^{-1}), 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 ± 0.40%) **(Figure 08),** and a very high content of organic matter (90.27 ± 3. 0%) and organic carbon (60.80 ± 0.59%). The results are close to those obtained by **Ameziane *et al.* (2019).**

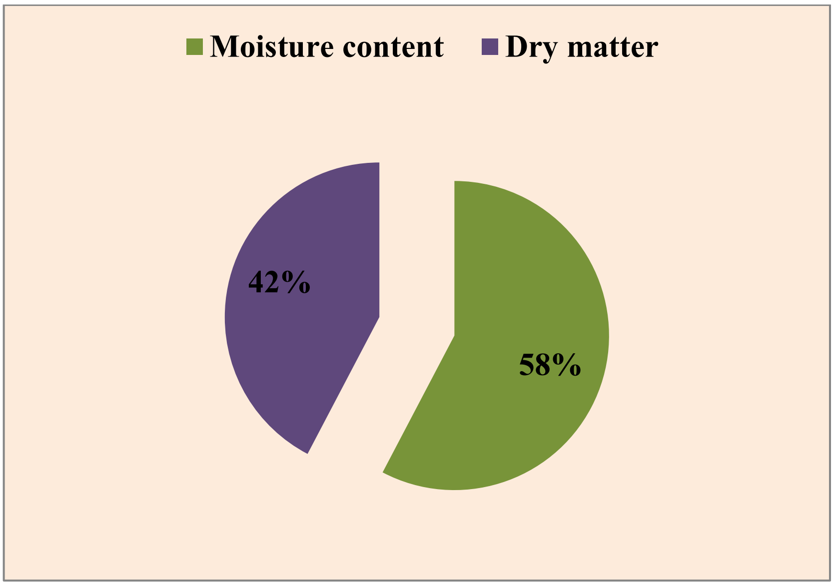


Figure 08: The moisture and dry matter contents of the studied olive mill pomace samples.

The content of polyphenols was approximately (40 mg. g^{-1}), 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 ± 2. 09 mg. g^{-1}), which is a common characteristic in olive-mill waste and by-products, followed by sodium (51. 67 ± 1.53 mg. g^{-1}) and calcium (2. 41 ± 0. 19 mg. g^{-1}).

The high 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^{-1}) showing levels that are incompatible with the result cited by **Alburquerque *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.

2.3. 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 04).**

Table 04: The effects of variables "species" and "treatments" and their interactions on earthworm survival, growth, and reproduction were tested using a two-way ANOVA.

Source of variation	DF	Growth	Reproduction	Survival
Species effect (S)	1	2023.36***	9.48***	448.15^{ns}
Treatment effect (T)	17	9361.78***	31.46***	11722***
Interaction effect (S ×T)	17	2497.87***	2.07***	44.23^{ns}
Error	72	51.73	0.15	120.37
ns, Not significant; *: highly significant P < 0.001**				

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. **(Figure 09)** 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 04)** and indicates the presence of eight homogeneous groups **(Figure 09)** 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 et 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.

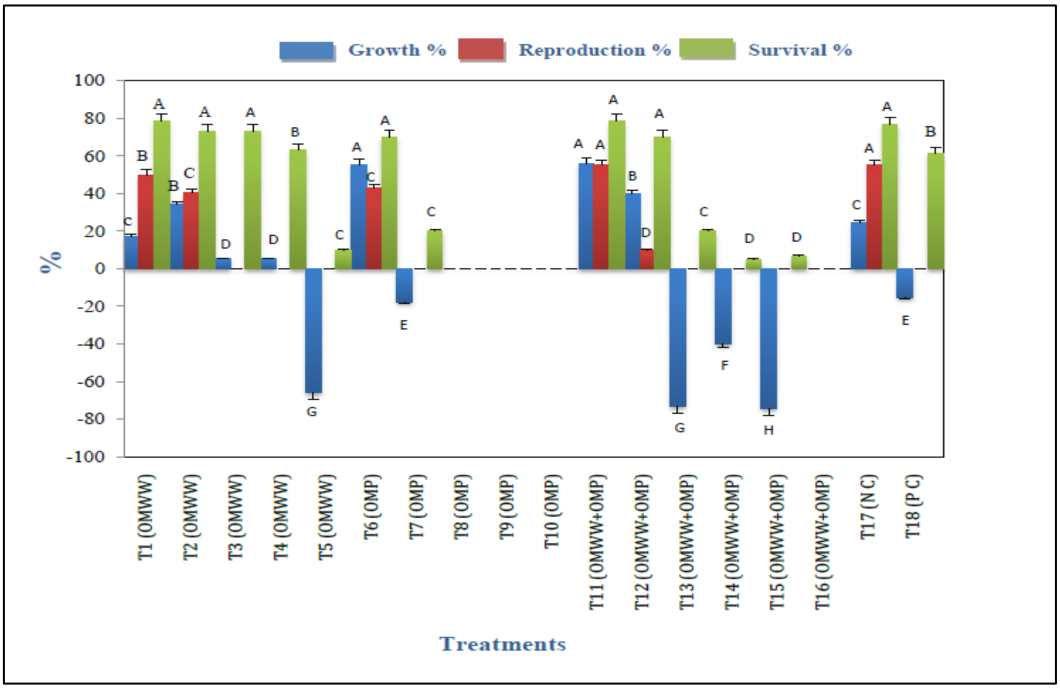


Figure 09: Mean effect of treatments (% ± SD, n = 3) in measured parameters. T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; T13: 50% of the combination; 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, and homogeneous groups, G% Growth, R% Reproduction, S% Survival.

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 **Babi´c *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 **(Ivask *et al.*, 2012; Owojori and Reinecke, 2010; Jun *et al.*, 2012).**

Heavy metals have the potential to inhibit growth even at low concentrations **(Babi´c *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 05)** 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% **(Figure 10).**

Table 05: The Least Significant Difference (LSD5 percent) between the two earthworm species (*A. trapezoides and E. fetida*) on growth, reproduction, and survival; a, b the homogenous group.

Parameters	Growth		Reproduction		Survival	
Species	AT	EF	AT	EF	AT	EF
Means	-7.30^b	1.36^a	1.11^b	1.70^a	51.30^a	47.22^a
LSD$_{5\%}$	2.76		2.66		75.75	
LSD$_{5\%}$ *Least Significant difference*						

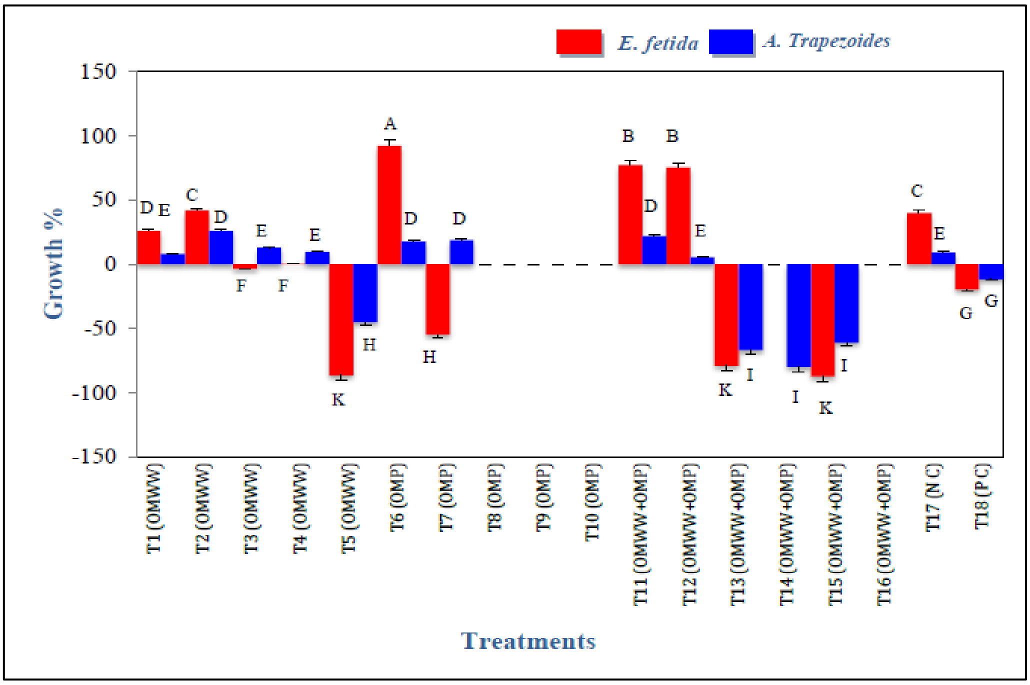


Figure 10: Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters were T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; 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; and homogeneous groups.

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 indicates 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 ± 1 and 10.04 ± 2: 24% respectively for *A. trapezoides* and - 2.57 ± 1; 0.59 ± 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 ± 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 ± 0% of *A. trapezoides*.

2.4. 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 04).** The 12.5% of OMWW and OMP combination and the negative control (group A) were characterized by the greatest reproduction (5.5 ± 1.76%), then T1 (group B) 5 ± 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 **(Figure 09).**

The interaction species × 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* **(Figure 11)** and **(Table 05).** 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).**

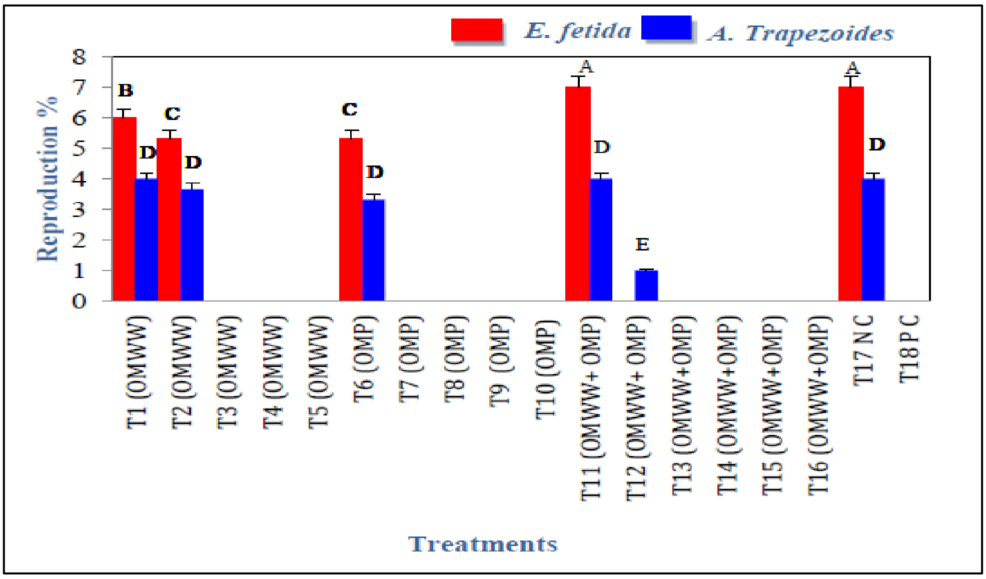


Figure 11: Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters. Where T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; 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; and homogeneous groups.

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 dS m- 1 and 7.35 dS m- 1). The findings can be related to those of some previous research papers **(Jezierska *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).

2.5. 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 × treatment) on the earthworm survival, while the factor “treatment” was found to be highly significant ($p < 0.001$) **(Table 04).** According to the analysis of the mean results, the presence of four homogeneous groups was revealed **(Figure 09).** 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 ± 4.08; 93.33 ± 5.16; 93.33 ± 5.16; 90 ± 0; 98.33 ± 4.08; 90.0 ± 12.64 and 96.66 ± 5.16 respectively, group (B) includes T4 (83.33 ± 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, 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 05).**

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.

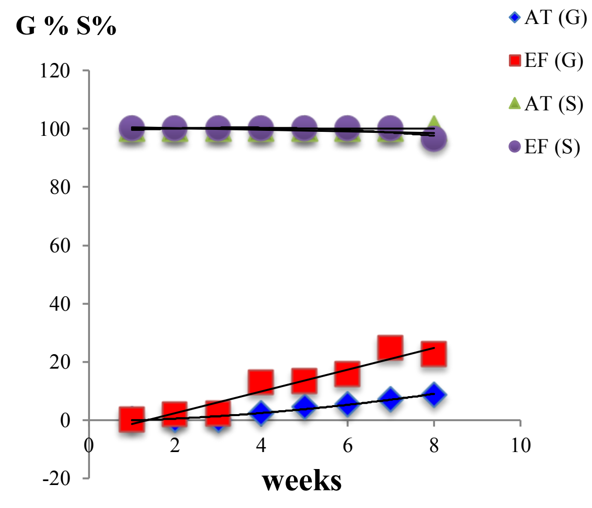
On the contrary, **Hentati *et al.* (2016)** and **Chalkia *et al.* (2020)** found there to be no mortality of earthworms *Eisenia fetida* and *Enchytraeus crypticus* maintained in soils amended with OMW at different dry weight ratios, additionally no lethal effect was found on the earthworm *Octodrilus complanatus* under the OMW application with a dose of up to 80 m^3/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.

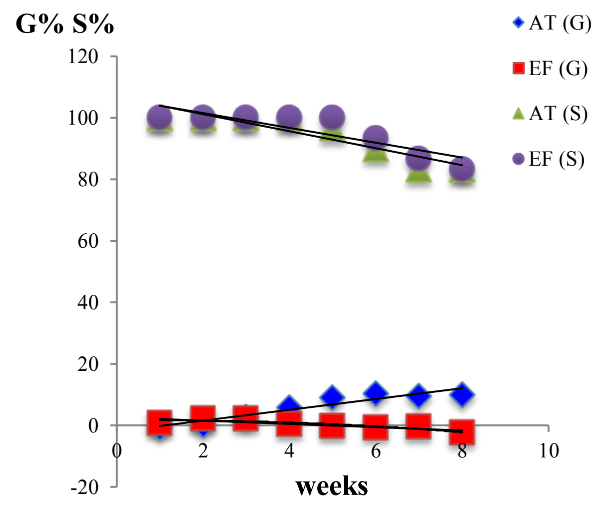
Although the principal responsible for OMWW toxicity is considered to be polyphenols **(Sayadi *et al.*, 2000),** it is not advisable to base toxicity reduction only on the removal of phenolic compounds since, as we outlined, OMW have other components, including acidic pH, high salinity levels, together 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.

2.6. Relationship between growth and survival of earthworm and the exposure time

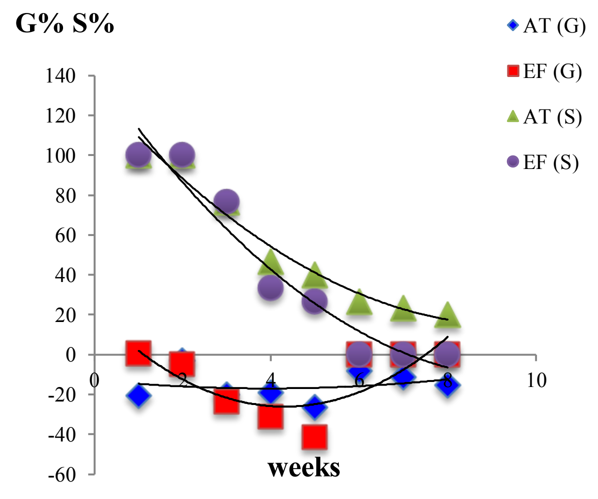
The linear regression analysis indicates a strong positive correlation between the growth and the period of exposure, meaning that growth increases when there is a rise in exposure time for both *A. trapezoides* and *E. fetida* in T1, T6, T12, a negative correlation was found in T15, T18, in these treatments; in fact, the growth of earthworm decreases 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 06)** and the most interesting results are shown in **(Figure 12).**



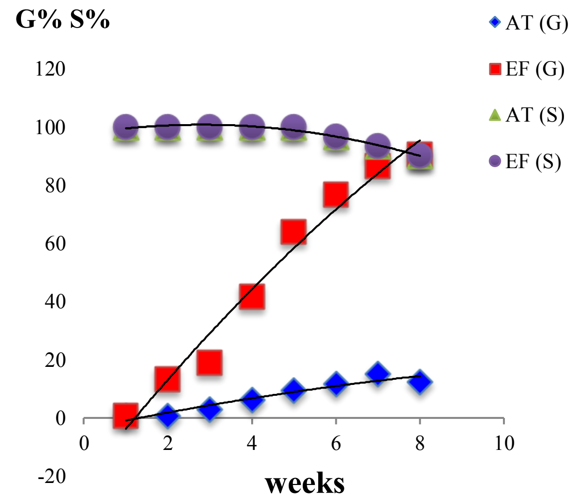
12.5% OMWW



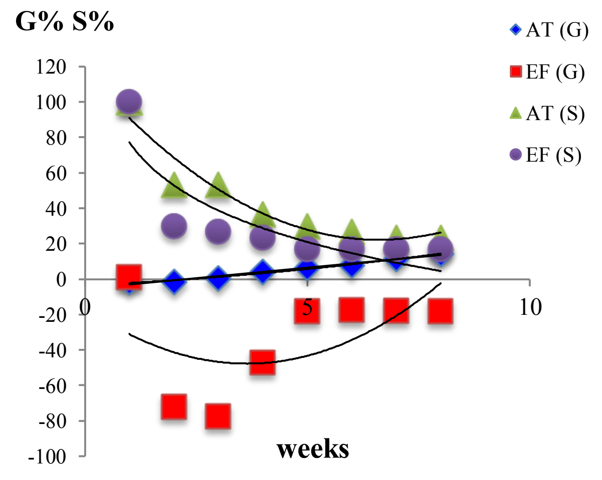
75% OMWW



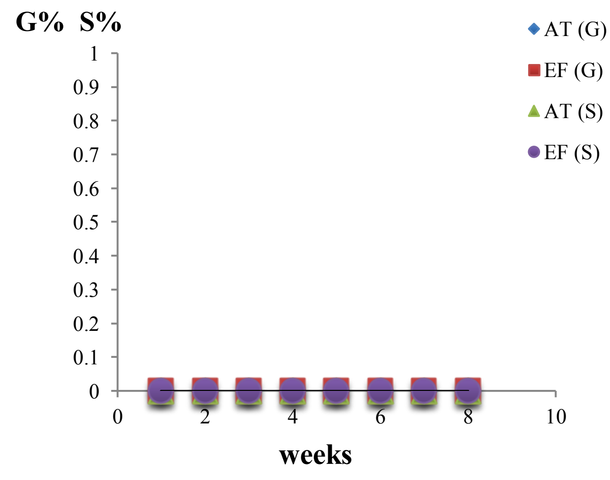
100% OMWW



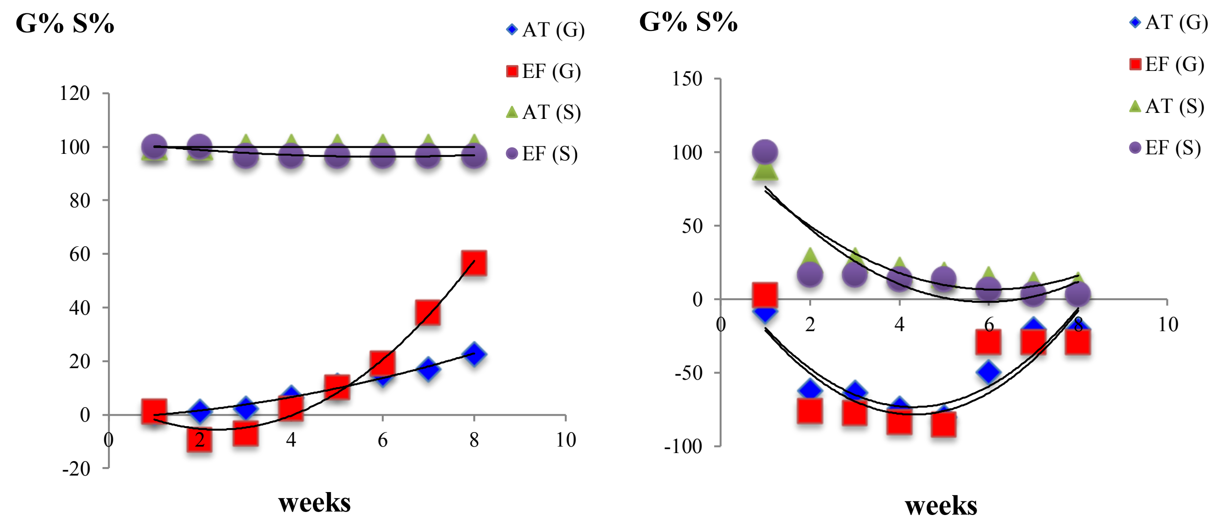
12.5% OMP



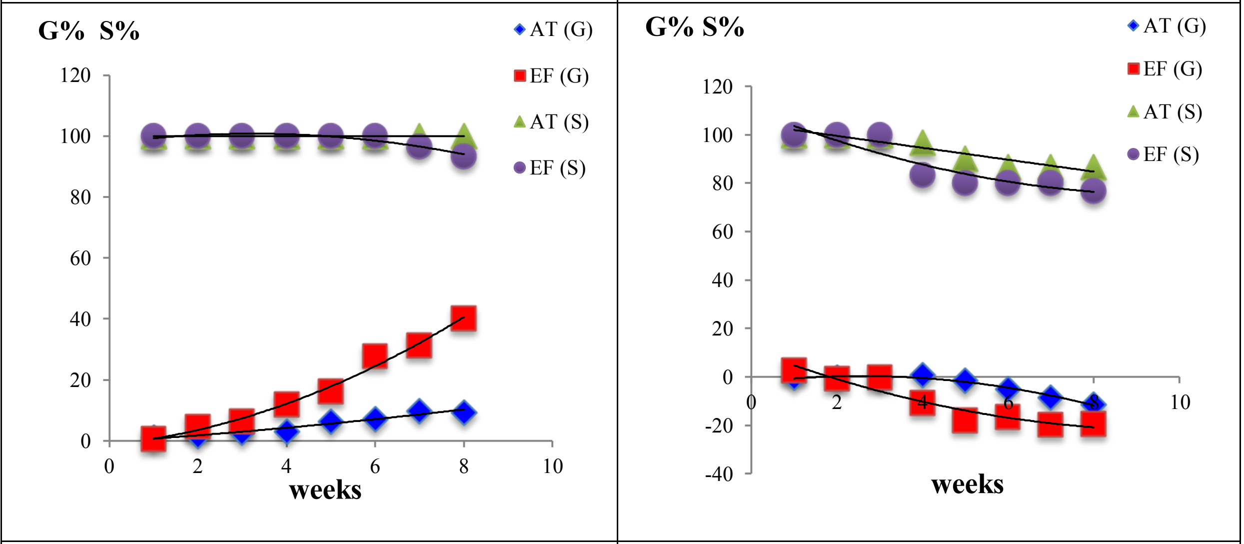
25% OMP



50% OMP



12.5% of the combination **75%* of the combination**



Negative control **Positive control**

Figure 12: 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).

Table 06: Most interesting regression equations describing the growth rate and survival rate over 8 weeks for species *A. trapezoides* and *E. fetida* presented in Figure 12.

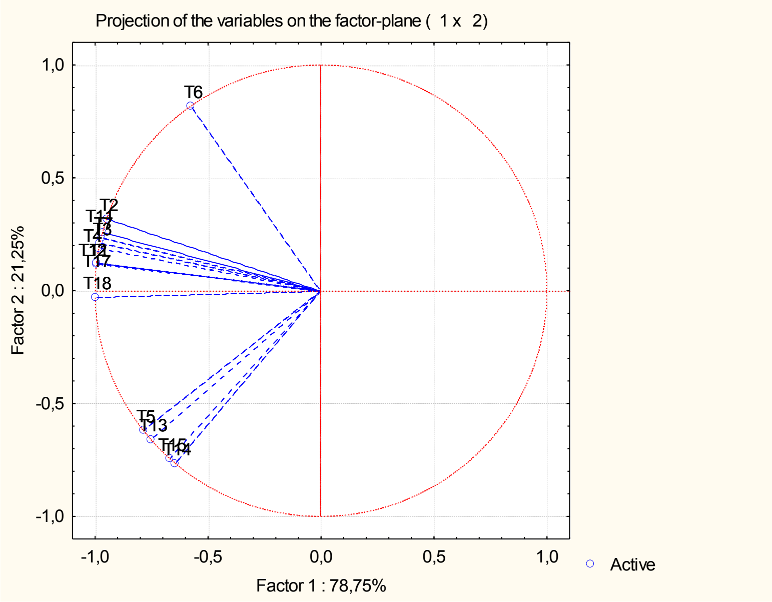
Treatments		Growth %	Survival %
T1 (12.5% OMWW)	*A. trapezoides*	$y = 0.113x2 + 0.284x - 0.575$ $R^2 = 0.967$	No correlation
	E. fetida	$y = 3.710x - 5.010$ $R^2 = 0.922$	$y = -0.278x + 100.8$ $R^2 = 0.333$
T4 (75% OMWW)	*A. trapezoides*	$y = 1.747x - 1.930$ $R^2 = 0.886$	$y = -2.778x + 106.6$ $R^2 = 0.821$
	E. fetida	$y = -0.541x + 2.687$ $R^2 = 0.739$	$y = -2.421x + 106.3$ $R^2 = 0.741$
T5 (100% OMWW)	*A.trapezoides*	$y = 0,274x2 - 2,156x - 12,77$ $R^2 = 0,42$	$y = 1,309x2 - 24,88x + 132,7$ $R^2 = 0,955$
	E. fetida	$y = 2,564x2 - 22,10x + 21,52$ $R^2 = 0,574$	$y = 1,607x2 - 31,57x + 143,1$ $R^2 = 0,94$
T6 (12.5% OMP)	*A.trapezoides*	$y = -0,089x2 + 2,995x - 3,876$ $R^2 = 0,914$	$y = -0,396x2 + 2,220x + 97,62$ $R^2 = 0,974$
	E. fetida	$y = -0,451x2 + 18,23x - 21,61$ $R^2 = 0,975$	$y = -0,396x2 + 2,220x + 97,62$ $R^2 = 0,974$
T7 (25% OMP)	*A.trapezoides*	$y = 2,366x - 5,498$ $R^2 = 0,975$	$y = 2,182x2 - 28,85x + 117,5$ $R^2 = 0,925$
	E. fetida	$y = 2,391x2 - 17,40x - 16,04$ $R^2 = 0,289$	$y = -34,8\ln(x) + 77,05$ $R^2 = 0,743$
T8 (50% OMP)	*A.trapezoids*	No correlation	No correlation
	E. fetida	No correlation	No correlation
T12 (12.5% of the combination)	*A.trapezoides*	$y = 0,264x2 + 0,913x - 1,304$ $R^2 = 0,986$	No correlation

	E. fetida	y = 1,992x2 - 9,476x + 5,719 $R^2 = 0,987$	y = 0,159x2 - 1,908x + 102,0 $R^2 = 0,825$
T15 (75%* of the combination)	*A.trapezoides*	y = 4,913x2 - 42,31x + 17,77 $R^2 = 0,803$	y = 2,579x2 - 31,39x + 102,1 $R^2 = 0,802$
	E. fetida	y = 5,197x2 - 44,89x + 18,52 $R^2 = 0,595$	y = 3,214x2 - 38,13x + 111,3 $R^2 = 0,733$
T17 (Negative control)	*A.trapezoides*	y = 0,045x2 + 0,955x - 0,305 $R^2 = 0,948$	No correlation
	E. fetida	y = 0,469x2 + 1,476x - 1,290 $R^2 = 0,987$	y = -0,297x2 + 1,925x + 97,67 $R^2 = 0,900$
T18 (Positive control)	*A. trapezoides*	y = -0,410x2 + 0,990x $R^2 = 0,947$	y = -2E-14x2 - 2,461x + 104,4 $R^2 = 0,880$
	E. fetida	y = 0,338x2 - 6,694x + 11,10 $R^2 = 0,911$	y = 0,357x2 - 7,102x + 110,3 $R^2 = 0,850$

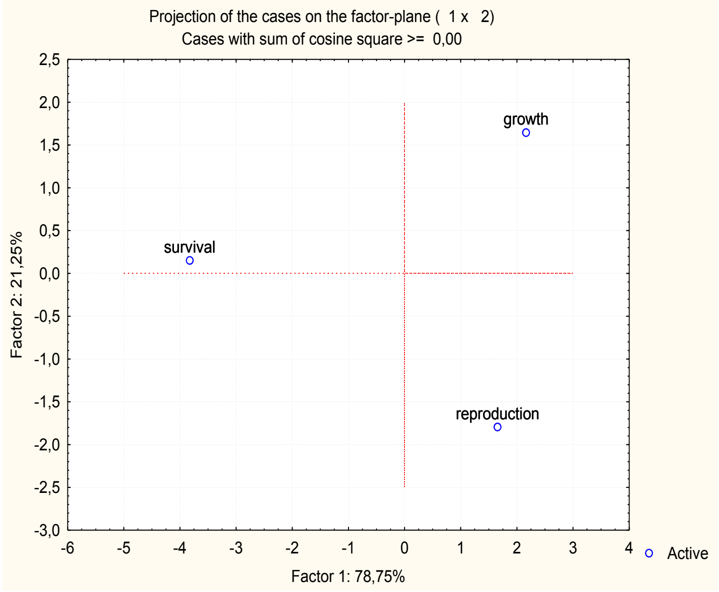
These results can only be compared with those of **Chalkia *et al.* (2020),** reporting an increase of body weight after 28 and 56 days of exposure of *Octodrilus complanatus* to OMW. No other author has tested the variations in responses over time. However, they are important because if we refer to 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.

2.7. Multivariate analysis (PCA)

The search for correlations was performed through principal component analysis (PCA) separately for each species. In PCA correlation circle **(Figure 13)** 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 **(Figure 13b)** 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 **(Figure 13)** 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.



(a)



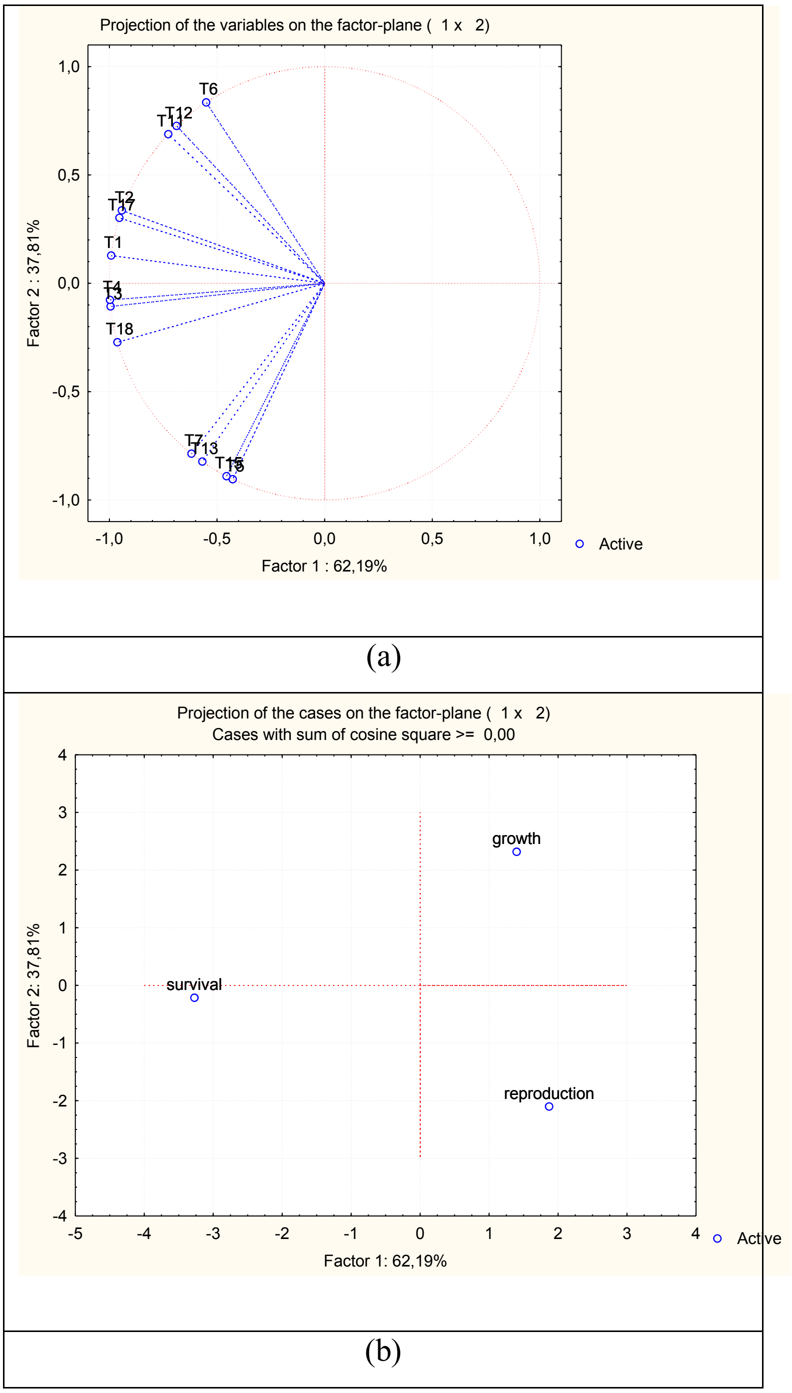
(b)

Figure 13: 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.*

Regarding *E. fetida* **(Figure 14)** 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 **(Figure 14a).** The projection of experimental points for *E. fetida* **(Figure 14)** 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.



(a)

(b)

Figure 14: 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*.

2.8. 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 50 m^3/ha/year) than the *A. trapezoides*. The optimal soil electrical conductivity for earthworm is 0.5–1.0 dS. m^{-1} 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^{-1} 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^{-1}). **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^{-1}. **Wu *et al.* (2019)** reported the effects of soil acid stress in earthworm *Eisenia fetida* with a significant inhibitory effect 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).

Chapter II :

Effects of olive mill wastewater and olive mill pomace single and in combination on Soil physicochemical properties and soil polyphenols using FTIR spectroscopy

Materiels & Methods

1. Materials and methods

The practical part of the current study was occurred at the laboratory of Biotechnology Research Center (C.R.B.T) Constantine. FTIR analysis was carried out at Structures, Properties and InterAtomic Interactions Laboratory (LASPI2A), Faculty of Science and Technology, University of Abbes Laghrour, Khenchela.

1.4. Soils physicochemical analyses

The soils in the containers were dried at room temperature at the end of the experiment, then crushed and sieved at 2 mm. The data was then analyzed, with each step being repeated three times.

For soil characterization, use the following formula:

1.4.1. Measurement of soil pH

The pH of the aqueous extract was determined using the ratio (1:2.5 soil: water suspension) **(Mohawesh et al., 2014).** The measurement was taken with a HANNA model HI 2209 pH meter.

1.4.2. Measurement of soil electrical conductivity (EC)

The electrical conductivity (EC) of the soil was determined using a (1:5 soil:water suspension) method **(ISO 11265, 1994).** The reading was taken with a HANNA model EC 215 conductivity meter.

1.4.3. Measurement of soil organic carbon content (OC)

The Walkley–Black method was used to determine organic carbon (OC) **(Walkley and Black, 1934).** In a 500 mL Erlenmeyer flask, the soil was crushed up to pass through a 0.5 mm mesh sieve. The amount of soil utilized in the analysis was computed based on preliminary data on the soil's C concentration and ranged from 0.1 to 0.5 g. To achieve good mixing of the soil with the reagents, ten milliliters of 0.167 potassium dichromate (K2Cr2O7) and 20 milliliters of concentrated sulfuric acid (H2SO4) were added to the soil while stirring it. 200 mL distilled water, 10 mL concentrated H3PO4, and 1 mL 0.16 percent diphenylamine.

1.4.4. Measurement of soil organic matter content (OM)

Total organic carbon was multiplied by 1.724 to get the organic matter (OM) **(Dabin, 1970).**

1.4.5. Measurement of soil total nitrogen content (TN)

The Kjeldahl Method was used to determine total soil nitrogen (TN) **(Kandeler et al., 1995).**

- **Mineralization**

The solid samples were manually crushed to a 2mm size, 1g of soil was placed in a matras, 25ml of concentrated sulfuric acid was added, and the mixture was allowed to sit for 30 minutes before adding the catalyst (5g K2SO4+0.5g CuSO4) and boiling at 400°C until the samples turned white. Mineralization is collected in 100ml flasks, and the size of the mineralization is modified with distilled water.

In the same way, the blank is made.

- **Solutions preparation:**
- put 96g of NaOH and add 300 ml of distilled water.
- 4g of boric acid, and complete with water to 100ml.
- **Procedure**
- Place 25mL of the sample and a few drops of phenolphthalein in the distillation flasks.
- 10ml boric acid and a few drops of Tashiro indicator in a 250ml Erlenmeyer.
- A control is prepared under the same conditions.
- **Titration**
  - **Solution preparation**

0.56l of sulfuric acid in 500 ml of distilled water

- **Procedure**

The surplus is titrated with a sulfuric acid solution until it turns purple after shaking.

1.4.6. Measurement of soil assimilable phosphorus (P)

The assimilable phosphorus was measured using the OLSEN NF ISO 11263 international standard **(Buol et al., 2011).**

- **Procedure**
- Weigh 1 g of soil and add a quantity of activated carbon.
- Add 20 ml of the extraction solution.
- Shake mechanically for 30 minutes.
- Filter with a watman paper until a clear solution is obtained.
- Take 10 ml of the filtrate and add 5 ml of sulfuric acid in a 50 ml flask.
- Let stand overnight or shake for one hour.
- Add 10 ml of distilled water and 5 ml of Ammonium Molybdet.
- **In 20 ml vials:**

- 1 mL phosphate standard solution (1,2, 3, 4, and 5 ppm) + 4 ml extraction solution + 2 mL H_2SO_4 + 30 minutes of shaking.
- Add 6 ml of water and 2 ml of ammonium molybdate and 0.1 ml of stannous chloride solution.
- Allow for a 10-minute rest period.
- Proceed to a 660 nm spectrophotometric reading (wavelength determined after scanning).

➢ **Calculation:**

The mass concentrations C in ppm (mg. l^{-1}) and the mass of phosphorus in mg. g^{-1} of soil determined using UV-VIS are as follows:

$$\boxed{m = \frac{C \times 20}{10}}$$

1.4.7. Measurement of soil phenolic compounds content (PP)

The phenolic compounds were evaluated using the Folin–Ciocalteu technique with acid galic as a reference and an absorbance dose of 725 nm (Singleton et al., 1999). The reading was taken with an Agilent Technologies model Cary 60 UV-Vis Malaysia Spectrophotocolorimetry.

1.5. Fourier transform infrared spectroscopy

➢ **Principle**

Fourier transform infrared spectrometer (FTIR) is one of the instruments based on infrared spectroscopy. It is the most modern type and preferred over the other dispersive spectrometers. It is because of its high precision, accuracy, speed, enhanced sensitivity, ease of operation, and sample non destructiveness.

The fundamental of infrared spectroscopic technology is on atomic vibrations of a molecule that only absorbs specific frequencies and energies of infrared radiation. The molecules could be detected and classified by FTIR because different molecules will have different infrared spectrum. A block diagram of FTIR working process is shown in **(Figure 16).**

The FTIR spectrometer essentially uses an interferometer to measure the energy that is being transmitted to the sample. The infrared radiation emitted from the black body reaches the interferometer where the spectral encoding of signals happens. The resultant interferogram signal is transmitted through or bounces from the sample surface, where specific energy wavelengths are absorbed. The beam eventually passes through the detector and further passed on to processing computer for Fourier transformation of energy signals **(Undavalli *et al*., 2021).**

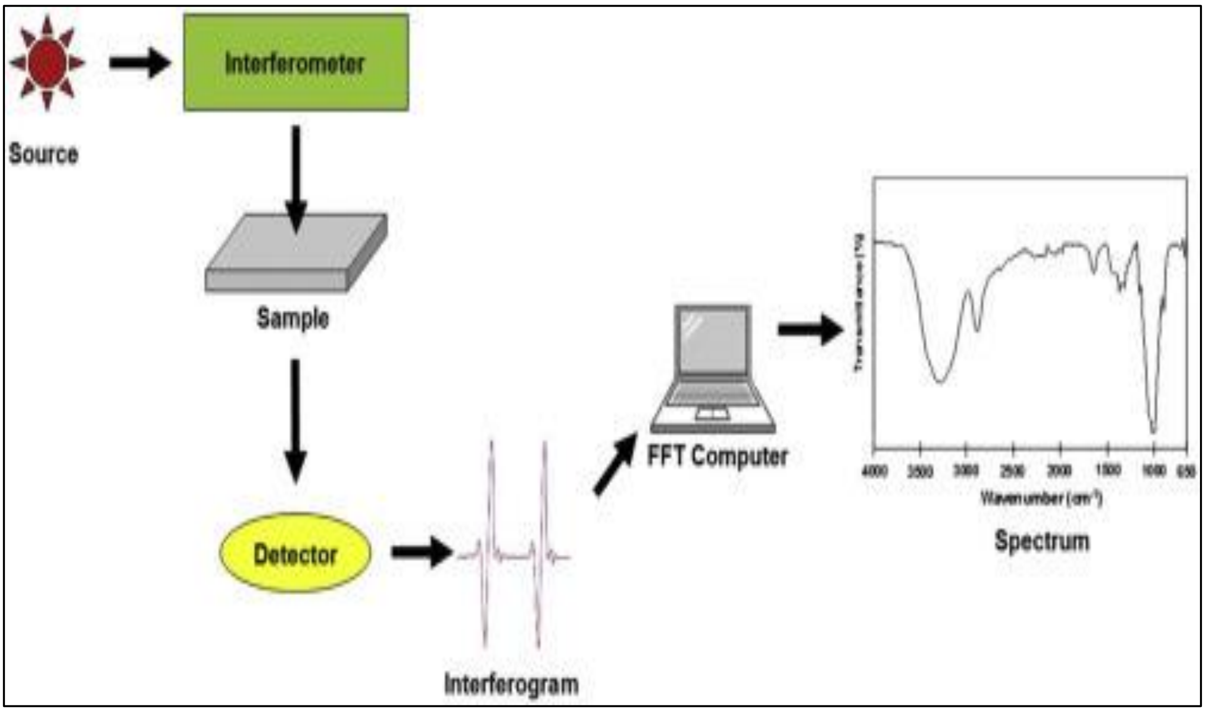


Figure 15: FTIR processing. *FTIR*, Fourier transform infrared.

1.5.1. FTIR analysis

Thermo Scientific Nicolet iS50 spectrometer was used to perform the FTIR analysis. The most popular way is using a KBr pressed disc. An agate mortar was used to smash the samples using dry potassium bromide powder. Following that, the samples were compressed into a clear disc. From 500 to 4000 cm^{-1}, all spectra were recorded.

1.6. Statistical analysis

The findings of the physicochemical properties of the soil after treatment with OMWW, OMP, and a combination were measured in triplicate and given as means SD. The influence of the various treatments (T1, T2, T3, T4, T5, T6, T7, T8, T9, T10, T11, T12, T13, T14, T15, and the control) on the soil parameters was evaluated using a one-way ANOVA (XLSTAT 2014.5.03).

The treatment effect is studied using the figure Quantile-Quantile (D. QQ-plot). The Least Significant Difference (LSD) of students at a 5% significance level was utilized to assess the homogeneous groups using SAS 9.1. The degree of relationship between the different properties of the soil treated was determined using correlation analysis (pH, EC, OM, OC, TN, P, PP). The correlation matrix is determined as a first step in the principal component analysis.

Principal component analysis (PCA) was utilized to assess the multivariate difference between treatments and physicochemical characteristics of the soil parameters using Statistica 08 software, and box plot diagrams were created using Origin Pro software (version 9.0).

Results & Discussion

2. Results and discussion

The agronomic use of OMWW and OMP on agricultural fields in large concentrations appeared to be a possible environmental threat. Although they have a negative influence on soil characteristics, they do have a good impact on soil production. The influence of olive OMWW and OMP, as well as their combination, on soil characteristics and phenolic compounds was investigated in this study. There is currently no data on this subject to our knowledge.

ANOVA stands for analysis of variance (Table 07). The treatment with OMWW and OMP, both alone and in combination, had a very significant impact on soil physicochemical parameters such as pH, EC, OM, OC, and PP, with the exception of P and TN, which exhibited a significant difference (p0.001).

Table 07: The values of one-way ANOVA from a general linear model analysis of the effects of OMWW and OMP and combination on the soil properties.

Source of variation	DF	pH	EC	OM	OC	TN	P	PP
Treatments	15	1.8469534***	0.78658348***	2.98763783***	0.98632758***	0.66547892*	0.65643983*	0.86383455***
Error	32	0.38473899	0.987832024	0.873752902	0.46723902	0.47638928	0.64762382	0.35783445
*** Highly significante $p<0.001$, * significant, ns Not significant.								

The following table summarizes the major physicochemical results of OMWW and OMP impacts on soil physicochemical characteristics (pH, EC, OM, OC, TN, P, PP):

Table 08: Physicochemical characteristics of soil treated with OMWW and OMP alone and in combination (mean values of three replications SD of each specimen). When it comes to homogenous groupings,

Treatments	pH	EC	OM	OC	TN	P	PP
T1	5.01±0.02c	3.83±0.04a	1.14±0.05a	4.24±0.04a	4.21±0.06a	0.05±0.04b	5.44±0.03c
T2	4.99±0.01c	1.95±0.02a	1.18±0.08a	4.26±0.08a	4.30±0.06a	0.07±0.02b	5.64±0.04c
T3	3.76±0.15c	2.11±0.01b	1.47±0.41b	4.34±0.41a	4.46±0.10a	0.09±0.01b	5.89±0.07c
T4	4.64±0.01b	2.32±0.02b	1.80±0.12b	4.62±0.05a	4.55±0.11a	4.02±0.05a	3.01±0.08e
T5	7.55±0.02b	2.51±0.03d	2.38±0.03c	4.96±0.05b	4.76±0.20b	4.07±0.02a	3.32±0.04f
T6	8.66±0.03b	2.26±0.23b	2.54±0.09c	4.05±0.33b	4.28±0.08a	4.06±0.03b	4.74±0.03c
T7	2.46±0.01b	2.69±0.01d	3.06±0.22d	4.35±0.10b	4.42±0.12a	4.09±0.04b	4.99±0.11d
T8	3.37±0.02b	2.78±0.02d	3.54±0.06d	4.63±0.10b	4.57±0.04b	4.10±0.02a	4.11±0.10b
T9	5.35±0.01b	3.00±0.03d	4.09±0.05e	4.95±0.05c	4.65±0.08b	4.07±0.13a	5.41±0.05e
T10	6.70±0.02a	3.21±0.01c	4.49±0.04e	4.18±0.04c	4.73±0.06b	4.06±0.13a	6.03±0.05e
T11	7.73±0.02c	3.94±0.02a	4.97±0.13b	3.70±0.14a	4.23±0.03a	4.05±0.03b	2.69±0.08b
T12	8.64±0.03b	3.02±0.03b	2.28±0.10c	3.90±0.07b	4.41±0.08a	4.06±0.03b	2.84±0.05b
T13	8.38±0.02b	3.35±0.02b	2.63±0.06c	3.10±0.10b	4.55±0.06a	4.09±0.03b	4.12±0.13b
T14	4.94±0.03b	3.70±0.06d	3.48±0.17d	5.95±0.07c	4.62±0.07b	4.01±0.02a	3.58±0.04d
T15	5.76±0.01b	1.03±0.01c	4.29±0.06e	5.06±0.05c	4.69±0.13b	4.04±0.01a	3.74±0.09d
Control	7.24±0.02c	2.70±0.03a	4.92±0.20a	5.11±0.08a	4.17±0.09a	4.04±0.03b	2.23±0.10a
LSD	0.3545	0.3252	0.4535	0.4523	0.5245	0.52356	0.3532

SD stands for standard deviation (P 0.05). EC (mS. cm1); OM (g. kg -1 DM); OC (g. kg -1 DM); TN (g. kg -1 DM); P: Assimilable phosphorus (g. kg -1 DM); PP: Phenolic compounds (mg. kg -1 DM).

2.1. Effect of OMWW, OMP and the combination on soil pH

A comparison of average pH revealed the presence of three groups, the first of which (control) had a neutral pH (7.240.02). (Table 08).

While the pH evolution of soil treated with various dosages of (OMWWW, OMP, and the combination) has shown a significantly substantial drop in soil pH (Figure 17). However, following the OMWW treatment, which caused a little reduction, the soil pH stayed around neutral, ranging from 7.010.02 to 6.550.02, including The suggested dose of OMWW was 50m3 ha^{-1} year^{-1}, with a modest drop in soil pH.

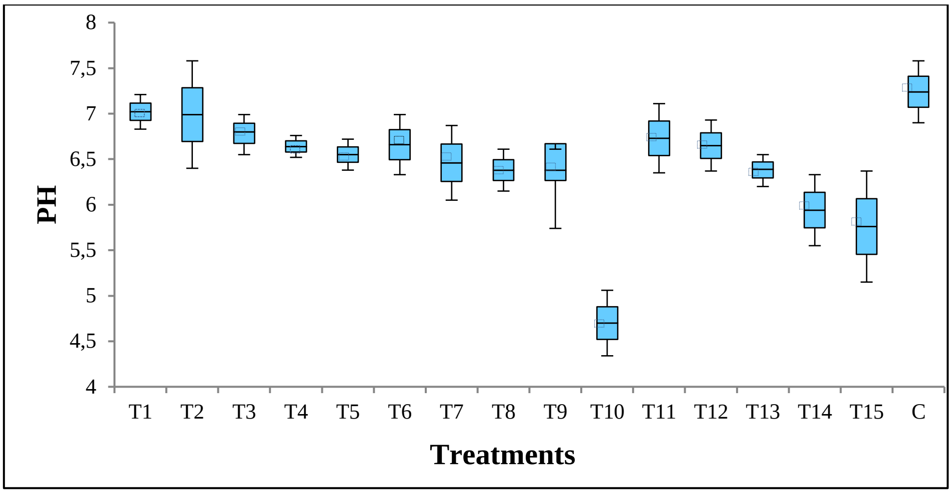


Figure 16: Boxplots of the effects of OMWW and OMP and the Combination on the soil pH with p values showing the significant effects revealed by ANOVA.

Furthermore, with both the treatment olive mill pomace and the combination, a significant decrease in soil pH values has been detected, ranging from (6.730.02 to 4.700.02) with the 100 percent OMP treatment compared to the control, and the treatment dose of 50–80 tons-1 ha-1 year-1 for the OMP has induced a pH of 6.370.02. The small change in soil pH following OMWW treatment, even at large dosages of 100% OMWW, is most likely due to the soil's high buffer capacity, which has neutralized the OMWW acidity; the presence of organic acids is mostly responsible.

These findings were supported by Meftah et al. (2019), who reported that despite the acidic OMWW (4.46), there was a minor change in soil pH at the depth (S50). Indeed, the carbonate in the soil converted to bicarbonate, neutralizing the OMWW acidic pH. The acidic influence of olive mill pomace on soil pH up to (4.700.02) at 100% dosages, due to the presence of organic acids (phenolic acids, fatty acids...) in the OMP (Aviani et al., 2010; de la Fuente et al., 2011).

Most research suggest a modest drop in soil pH following amendment with OMP (Ameziane et al., 2019; Nasini et al., 2013; Ferarra et al., 2012), which contradicts our findings. Otherwise, the combined treatment resulted in a decrease in soil pH (ranging from 6.730.02 to 5.760.01), owing to the acidic nature of the OMWW and OMP present together in the soil.

The soil pH is reduced as a result of the combination of different dosages. According to earlier studies (Lopez-Pineiro et al., 2011; Magdich et al., 2013), soil pH drops following OMWs spraying. This drop might be explained by organic acids and polyphenols found in olive mill wastes, as well as the ripening degree and post-harvest state of the storage (Barbera et al., 2013).

2.2. Effect of OMWW, OMP and the combination on soil EC

A study of average EC revealed the existence of four groups, with the control having the lowest soil EC (0.700.04 mS cm1) (Table 08). When compared to the control, the soil EC rose considerably following the treatment of (OMWW, OMP, and the combination). The largest rise was seen with both the 100 percent OMP and the 100 percent combination treatment (2.030.03 mS.. cm1 and 2.210.01 mS.. cm1, respectively). Furthermore, the OMWW treatment resulted in an increase in soil EC (from 0.830.04 mS cm1 to 1.510.03 mS cm1). While the indicated dosages for OMWW and OMP increased soil EC significantly (1.110.01 mS. cm1 for OMWW, 1.780.02 mS. cm1 for OMP) (Figure 18).

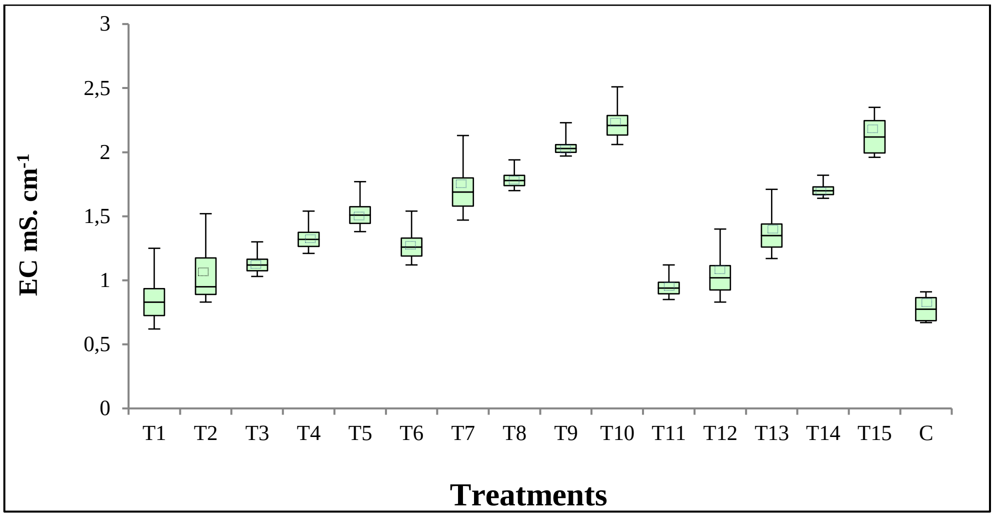


Figure 17: Boxplots of the effects of OMWW and OMP and the Combination on the soil Electrical Conductivity (EC mS. cm^{-1}) with p values showing the significant effects revealed by ANOVA.

In this context, our findings corroborate those of Di Bene et al. (2013), Magdich et al. (2016), and Meftah et al. (2019), who reported that treatment with OMWW boosted soil EC considerably. The high concentration of salts found on the OMWW was mostly responsible for the rise in soil EC (Barbera et al., 2013).

Furthermore, the effect of OMP on soil EC is dose dependent; indeed, the higher the doses of OMP used, the higher the soil EC, owing to the high content of soluble salts, particularly potassium salt, present in the olive mill pomace. Our findings are consistent with those of Ameziane et al. (2019), who found that the EC of the soil changes depending on the amount of olive mill pomace applied. Furthermore, since the soil electrical conductivity indicated the content of salts in the soil, the combination treatment showed a significant increase ranging from (0.940.02 mS. cm1 to 2.030.03 mS. cm1), the high effect of the combination treatment was due to the high content of salts present in the OMWW.

2.3. Effect of OMWW, OMP and the combination on soil OM

A study of the average OM revealed five distinct groupings. The analysis of soil organic matter revealed that the first control had a low organic matter content (1.920.20 g. kg -1 DM) (Table 08). Because of the quantity of organic material in this trash, treatment with OMWW and OMP alone and in combination resulted in a large increase in soil organic matter. Furthermore, in the treatment with OMP and the combination, the increase in soil organic matter was quite noticeable, ranging from (3.540.09 to 5.490.04 g. kg -1 DM) and (2.970.13 to 5.290.06 g. kg -1 DM) correspondingly (Figure 19).

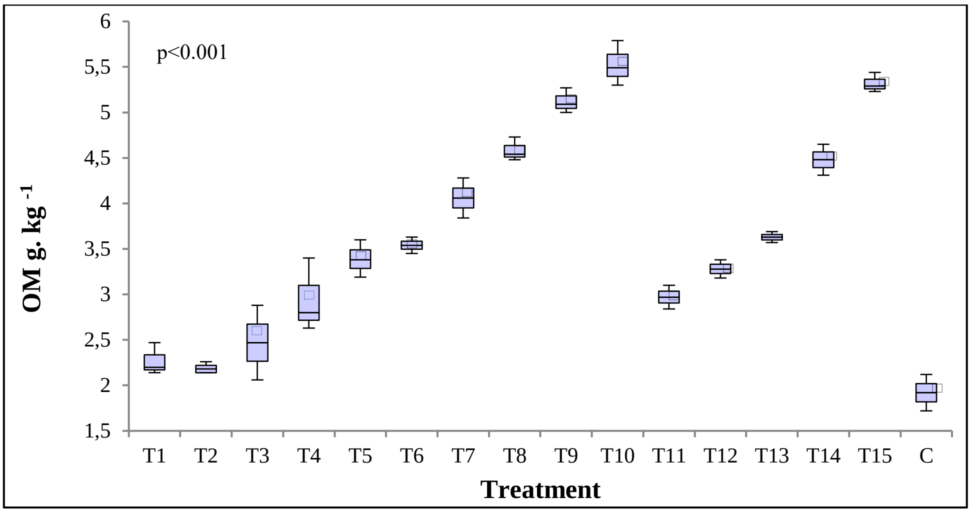


Figure 18: Boxplots of the effects of OMWW and OMP and the Combination on the soil Organic matter (OM g. kg $^{-1}$) with p values showing the significant effects revealed by ANOVA.

While the treatment with OMWW caused a slight modification when compared to the increase caused by OMP and the combination, this increase ranged from (2.140.05 to 3.380.03 g. kg -1 DM), and the recommended doses caused an increase in soil OM, this increase was not significant when compared to the high doses. Furthermore, increases in soil organic matter are proportional to the treatment dosages applied to the soil. The large amount of organic matter in OMWW and OMP is the major explanation for this increase; consequently, a high level of organic matter in the soil promotes water retention and soil stability by building colloidal complexes with clay, and these complexes are formed of

While the treatment with OMWW caused a slight modification when compared to the increase caused by OMP and the combination, this increase ranged from (2.140.05 to 3.380.03 g. kg -1 DM), and the recommended doses caused an increase in soil OM, this increase was not significant when compared to the high doses. Furthermore, increases in soil organic matter are proportional to the treatment dosages applied to the soil. The large amount of organic matter in OMWW and OMP is the major explanation for this increase; consequently, a high level of organic matter in the soil promotes water retention and soil stability by building colloidal complexes with clay, and these complexes are formed of

2.4. Effect of OMWW, OMP and the combination on soil organic carbon (OC)

The contribution of olive mill waste treatment to soil organic matter will result in an increase in soil organic carbon content (Figure 20). The organic carbon value of the various soil samples matched the value of soil organic matter perfectly.

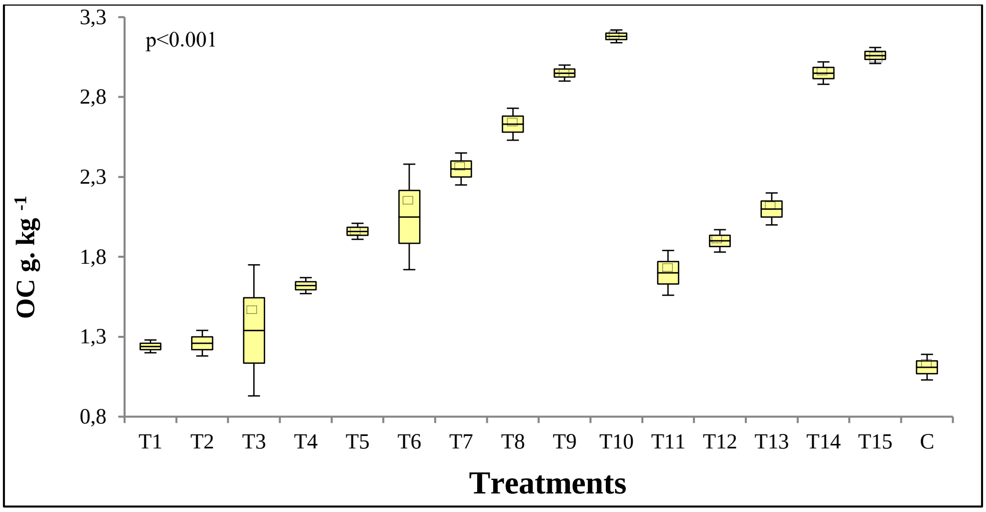


Figure 19: Boxplots of the effects of OMWW and OMP and the Combination on the soil Organic carbon (OC g. kg $^{-1}$) with p values showing the significant effects revealed by ANOVA.

In fact, the analysis of variance revealed a highly significant increase in soil organic carbon depending on the treatment applied compared to the control, ranging from (1.240.04 to 1.960.05 g. kg -1 DM) with OMWW, to (2.050.33 to 3.180.04 g. kg -1 DM) with OMP, and to (1.700.14 to 3.060.05 g. kg -1 DM) with the combination treatment (Table 08).

All prior study on the effect of OMWW on soil organic matter has shown that these effluents have a favorable influence on soil fertilization in this situation. (Kavvadias et al., 2015; Vella et al., 2016; Zema et al., 2019, Meftah et al., 2019, Mbarek et al., 2020) Furthermore, Ameziane et al. (2019) revealed that adding olive mill pomace to soil boosted soil organic carbon considerably depending on the dosages supplied. Furthermore, Garca-Ruiz et al. (2012) and Aranda et al. (2015) discovered that using olive mill pomace as a co-compost for a long time boosted soil organic carbon.

2.5. Effect of OMWW, OMP and the combination on soil total nitrogen (TN)

The presence of two groups was shown by comparing the average TN. The use of olive mill wastewater and olive mill pomace, as well as their combination, resulted in a little change in soil total nitrogen (Figure 21). The treatments had a substantial influence on the total nitrogen in the soil, according to the analysis of variance. Indeed, the control soil was first determined to be deficient in total nitrogen, with a mean value of 0.170.09 g. kg-1 DM.

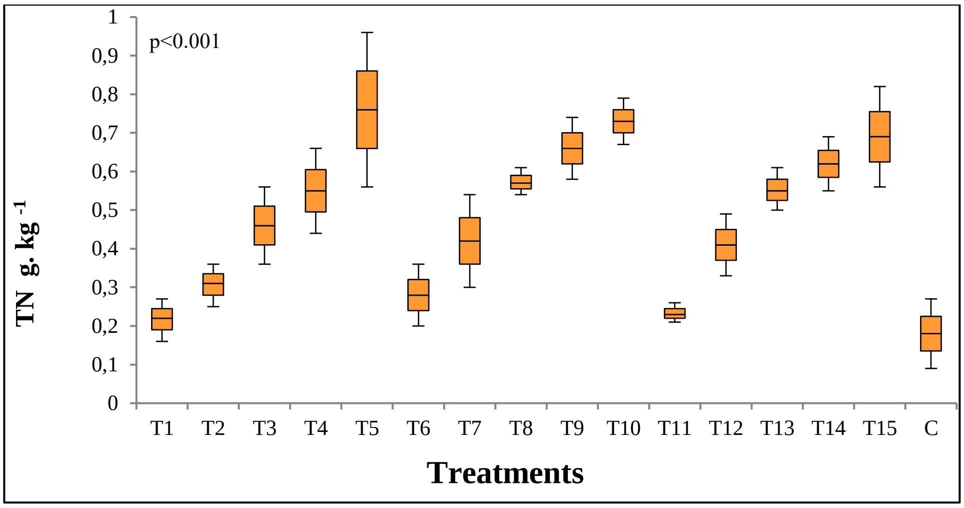


Figure 20: Boxplots of the effects of OMWW and OMP and the Combination on the soil Total nitrogen (TN g. kg $^{-1}$) with p values showing the significant effects revealed by ANOVA.

The applied treatments resulted in a slight increase in soil total nitrogen ranging from (0.210.06 to 0.760.20 g. kg-1 DM) with OMWW and (0.280.08 to 0.730.06 g. kg-1 DM) with OMP, including the doses allowed by law; additionally, the combination resulted in a slight increase ranging from (0.230.03 to 0.690.13 g. kg-1 DM) compared to the control (Table 08). This is most likely owing to the element's dynamics. All prior studies have found that treatment with OMWW and OMP increases soil total nitrogen, which boosts plant yield. Nitrogen is also the most important ingredient in fertilization. With a few exceptions, such as Piotrowska et al. (2011), Lanza et al. (2017), and Zema et al. (2019), few studies revealed declines or no significant changes in the number of people who smoke.

2.6. Effect of OMWW, OMP and the combination on soil Assimilable phosphorus (P)

The presence of two groups was shown by comparing the average P. In terms of soil phosphorus content, the analysis of variance found no significant effect for low doses, including doses of 50m3 ha-1 year-1 for OMWW and 50–80 tons-1 ha-1 year-1 for OMP of all the treatments applied (12.5 percent, 25%, and 50%) ranged from (0.050.04 to 0.090.01 g. kg -1 DM) with almost all the treatments (Table 08). (Figure 22).

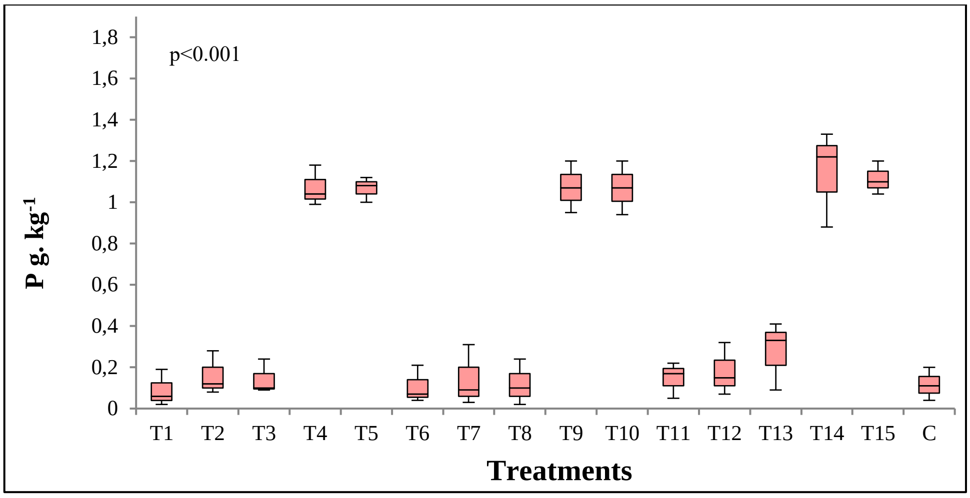


Figure 21: Boxplots of the effects of OMWW and OMP and the Combination on the soil Assimilable phosphorus (P g. kg $^{-1}$) with p values showing the significant effects revealed by ANOVA.

These numbers indicate that the soil is deficient in phosphorus. Chartzoulakis et al. (2010) reported no improvement in soil phosphorus concentration after three years of treatment with raw OMW. Magdich et al. (2013), on the other hand, reported no significant effect on soil phosphorus when OMWW was applied at three levels (50, 100 and, 200 m3 year^{-1}) this is In addition, Dakhli, et al. (2021) discovered that OMWW had no effect on soil assimilable phosphorus, most likely due to the element's immobilization produced by the soil humic feature. Ameziane et al. (2019) discovered that amending with olive mill pomace did not significantly increase soil phosphorus, owing to the OMP's low phosphorus level. In contrast, the treated soil with high doses of OMWW and OMP (75 and 100 percent) and the combination showed a considerable increase in soil assimilable phosphorus from (0.040.03 g. kg -1 DM) to (1.070.06 g. kg -1 DM).

Our findings are consistent with those of Kavvadiasa et al. (2010), who discovered an increase in accessible phosphorus in the soil following treatment with untreated OMWW. Furthermore, after 5 days of distributing 80 m3 ha-1 OMWW, Di Bene et al. (2013) observed increases in soil total and accessible phosphorus. Furthermore, according to a recent study by Zema et al. (2019), the OMWW increases soil phosphorus, improving soil fertility, and reducing the need for artificial fertilizers, which has clear economic and environmental advantages.

2.7. Effect of OMWW, OMP and the combination on soil phenolic compounds (PP)

Phenolic compounds in olive mill wastes are one of the most important limiting factors for their toxicity; their phytotoxic and antibacterial properties may cause pollution of groundwater and soil. These wastes are among of the most polluting in the agrifoods industry (Mekersi et al., 2021). Six groups were discovered when the average PP was compared. The treatment of OMWW and OMP, as well as their combination, significantly enhanced the soil phenolic compounds content when compared to the control, and this increase was proportionate to the dosages of olive mill wastes given to the soil, according to the analysis of variance.

The results showed an increase in soil phenolic compounds under treatment with OMWW ranging from (5.440.03 to 9.320.04 mg. kg -1 DM), as well as an increase in soil polyphenols ranging from (5.740.03 to 8.030.05 mg. kg -1 DM) and the combination treatment indicated results ranging from (4.690.08 to 6.740.09 mg. kg -1 DM) together with the allowed doses which induced an augmentation in soil polyphenols of (Figure 23).

These findings have been in accordance with previous study demonstrating that the application of OMWW has an important increase on the soil phenolic compound (Kavvadias et al., 2010; Magdich et al., 2012; Belaqziz et al., 2016; Vella et al., 2016; Zema et al., 2019).

Phenolic compounds, together with other organic compounds, play an important part in the creation of soil. Due to the phytotoxicity of polyphenols metabolites, their presence in high quantities might have an impact on soil functioning (Belaqziz et al., 2016).

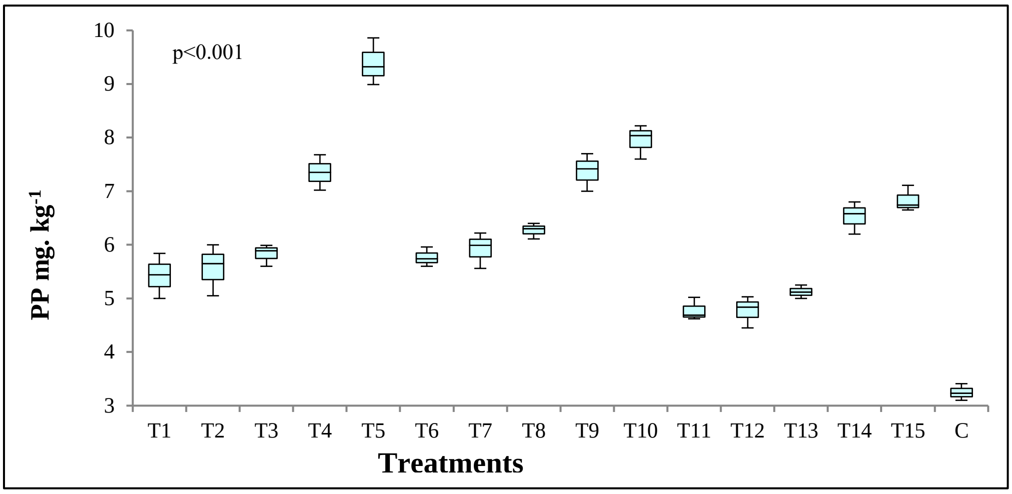


Figure 22: Boxplots of the effects of OMWW and OMP and the Combination on the soil phenolic compounds (PP mg. kg $^{-1}$) with p values showing the significant effects revealed by ANOVA.

2.8. FTIR analysis of OMWW and OMP

The FTIR analysis is regarded as one of the most effective qualitative analyses that monitored to determine the functional groups of organic waste such as the OMWW and OMP, where the FTIR analysis offers a unique signature of the chemical or biochemical substance present (torsions of chemical bonds, bending, and stretching) through the molecular vibrations of the chemical or biological compounds contained in the sample (Lupoi et al., 2015; Zghari et al., 2017). (Figure 24). The FTIR spectra of the OMWW and OMP samples are shown.

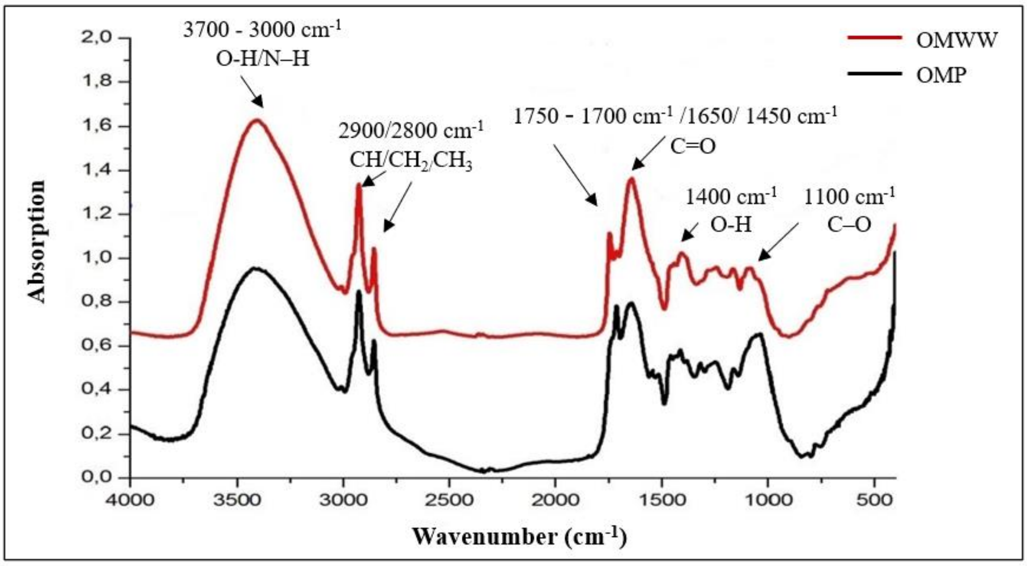


Figure 23: FTIR spectra peaks of olive mill wastewater and olive mill pomace from 4000 - 500 (cm^{-1}).

The stretching vibration O-H observed of alcohols, phenolic groups, carboxylic groups, and the amide N–H functions hydrogen vibration was concentrated between 3700 cm-1 and 3000 cm-1 among OWWW and OMP. Several prior investigations have linked the phenolic chemicals to an intensive, broadband absorption that occurred between 3,000 and 3800 cm1 in OMWW and OMP (Droussi et al., 2009a; Gursoy-Haksevenler and Arslan-Alaton, 2015; Gursoy-Haksevenler and Arslan-Alaton, 2016; Zghari et al., 2017; Jaouadi, 2021).

The two peaks at 2900 cm-1 and 2800 cm-1 indicating long-chain lipids comprise long-chain aliphatic methylene molecules with CH, CH2, and CH3 groups (Gursoy-Haksevenler and Arslan-Alaton, 2016; Zaier et al., 2017; El Hassani et al., 2020; Jaouadi, 2021). The stretching vibration C=O of the functional groups Ketones, aldehydes, and carboxylic acid groups, as well as ester, might explain the sequential peaks detected around the absorption bands of 1750-1700 cm-1, 1650 cm-1, and 1450 cm-1. Based on prior research that discovered the same bands, this trait exposes the OMW's acidic nature (Rubio-Senent et al., 2015; Zghari et al., 2017; Bekiaris., 2020; Jaouadi, 2021). Due to the aromatic ring C=C stretching of the quinone, the band of 1650 cm-1 denotes aromatic compounds.

Furthermore, at 1100 cm-1, the absorbances show C–O stretching, which corresponds to the structure of polysaccharides cellulose, confirming the existence of glucidic chemicals.

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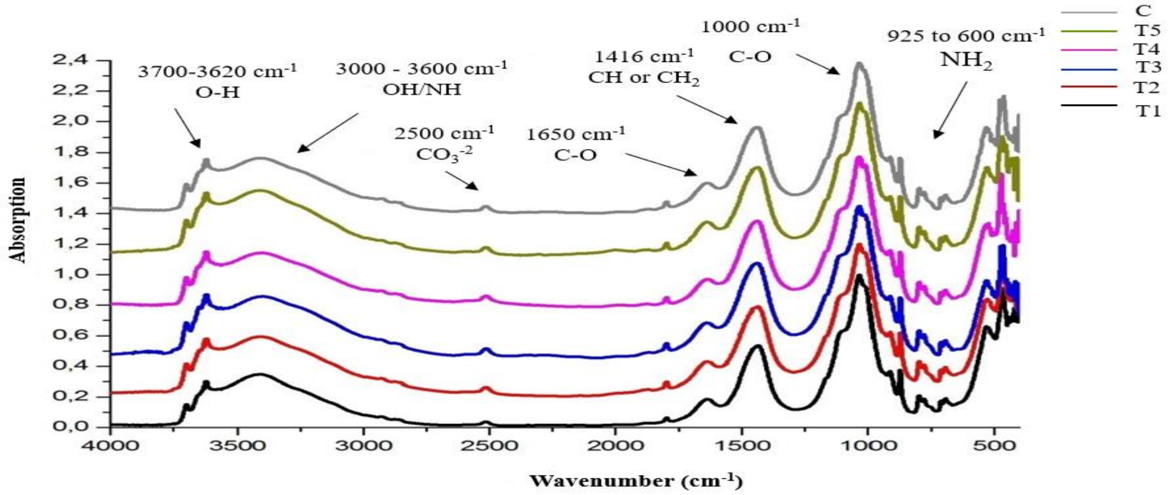
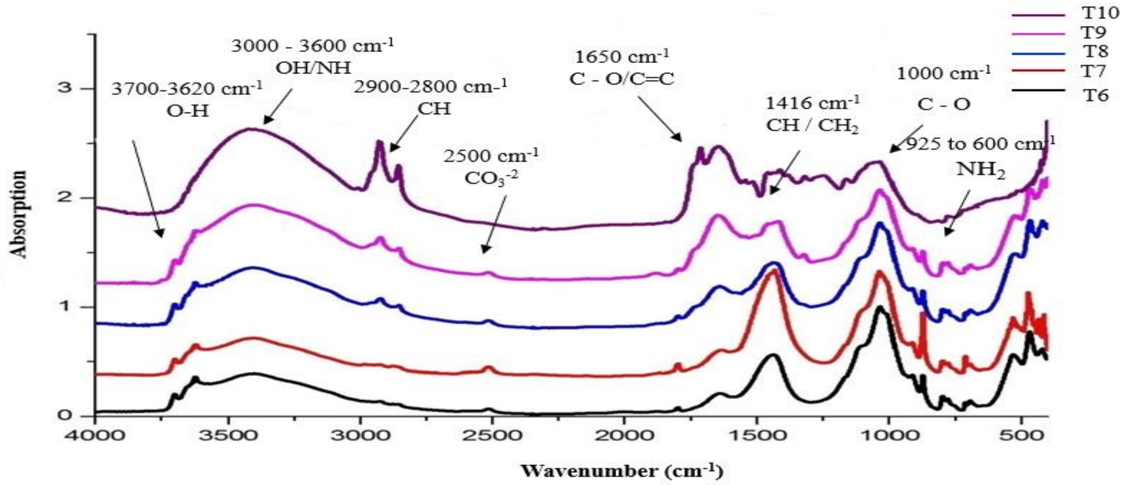
2.9. A comparative analysis by FTIR of the effects of OMWW and OMP and the combination on the soil

The FTIR spectra on average are presented in (Figure 25). For the soil treated with olive mill effluent, olive mill pomace, and the combination, as well as the varied dosages. Under the treatment with olive mill pomace and the combination, the spectral waves were mainly identical in form, while the treatment with OMWW exhibited virtually similar peaks between the different dosages utilized. With the vibration mode, a number of peaks can be distinguished corresponding to the functional groups of various components; the bands at around 3700-3620 cm-1 were present in almost all of the treatments, as well as the control, with the exception of the T10 and T15 treatments, which corresponded to the O-H stretching of clay minerals (Xing et al., 2019).

Their absence in the 100 percent OMP and 100 percent combination treatments is mostly owing to the high level of OMWs in these treatments and low soil content. Except for the untreated soil, all treatments had a broad absorption peak ranging from roughly 3600 cm-1 to 3000 cm-1. This huge peak is attributed to the OH stretching and or NH stretching of the carboxyl, alcohols, amine, and amide, hydroxyl groups, and phenolic chemicals (El Hassani et al., 2020; Xu et al., 2020; Xing et al., 2019). The phenolic chemicals discovered in the soil by FTIR analysis, particularly at high concentrations of OMWW, OMP, and the combination, are mostly due to the treatments.

These findings correlate with Monetta et al. (2012), who found that adding OMWs to the soil induces a large rise in soil phenolic compounds. Furthermore, Dakhli, et al. (2021) revealed that applying OMWs to soil results in a considerable rise in soil phenolic compounds. Both peaks at 2900-2800 cm-1, which are present with treatments T8, T9, T10, T14, and T15, correspond to the CH stretching of aliphatic methyl and methylene groups (El Hassani et al., 2020), which is likely due to the high organic matter content in the soil under the treatment with OMP and the combination, whereas the absence of the aliphatic bands in the rest of treatments is likely due to the degradation of the organic matter. The 2500 cm-1 peak is credited to application of the OMP and the combination, with the high doses T10, T15 renders the soil not calcareous.





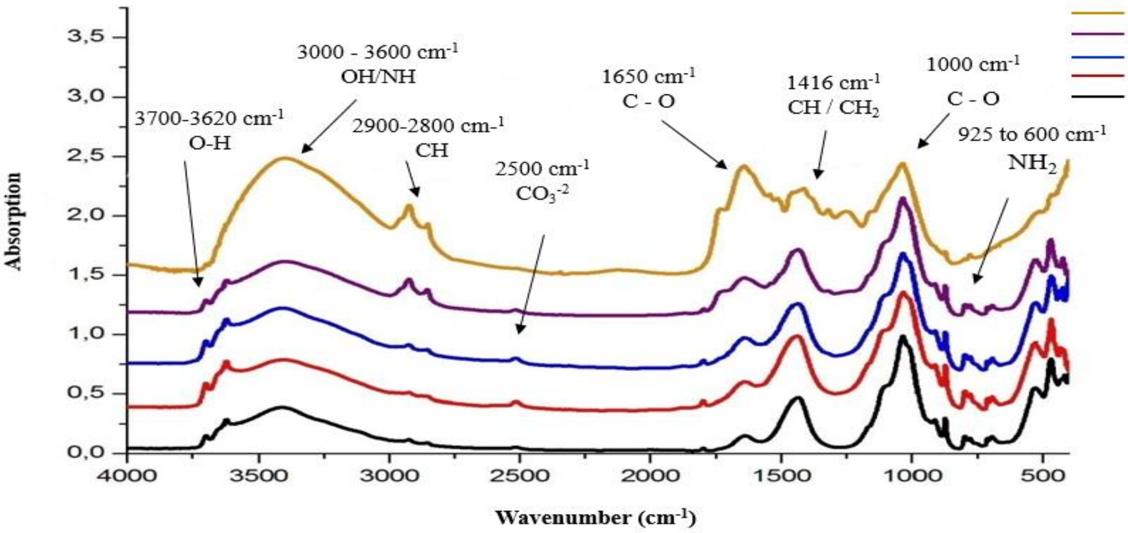


Figure 24: FTIR spectra peaks of the soil treated with OMWW and OMP and the combination from 4000 - 500 (cm^{-1}). T1: 12.5% OMWW, T2: 25% OMWW, T3: 50% OMWW, T4: 75% OMWW, T5 : 100% OMWW, T6 : 12.5% OMP, T7 : 25% OMP, T8 : 50% OMP, T9 : 75% OMP, T10 : 100% OMP, T11: 12.5% Combination, T12 : 25% Combination, T13 : 50% Combination, T14 : 75% Combination, T15 : 100% Combination, C : Control.

Furthermore, the band found at 1650 cm-1 in practically all treatments is linked to C-O stretching and aromatic C=C groups of amides (Calderón et al., 2013). Furthermore, the CH or CH2 bending vibrations of Methyls were likely reflected in the shoulder band at 1416 cm-1 (Xing et al., 2019). Furthermore, the peaks at 1000 cm-1 that appear in all treatments indicate Si-O stretching and C-O bending of Silicates and soil polysaccharides (Xing et al., 2016). The Al-OH stretching and NH2 vibration of Kaolinite Primary amine and Iron oxides, respectively, were ascribed to the consecutive bands from 925 to 600 cm-1 (Soriano-Disla et al., 2014; Xing et al., 2019).

2.10. Correlation analysis

The study of correlations between distinct pairs of qualities is done to determine which traits evolve in the same way and which traits evolve in opposite directions. In the first situation, a rise in one feature inevitably leads to an increase in the other, whereas in the second case, a reduction in one trait always leads to negative changes in the other. With EC, OM, OC, TN, P, and PP, the correlations between soil pH and the measured variables are strongly negative. While the correlations between EC and OM are considerably positive, the correlations between OC, TN, P, and PP are strongly negative (Table 09).

Table 09: Correlation matrix (Pearson rank correlation) among the physicochemical parameters of the treated soil with OMWW and OMP and the combination with significance probability.

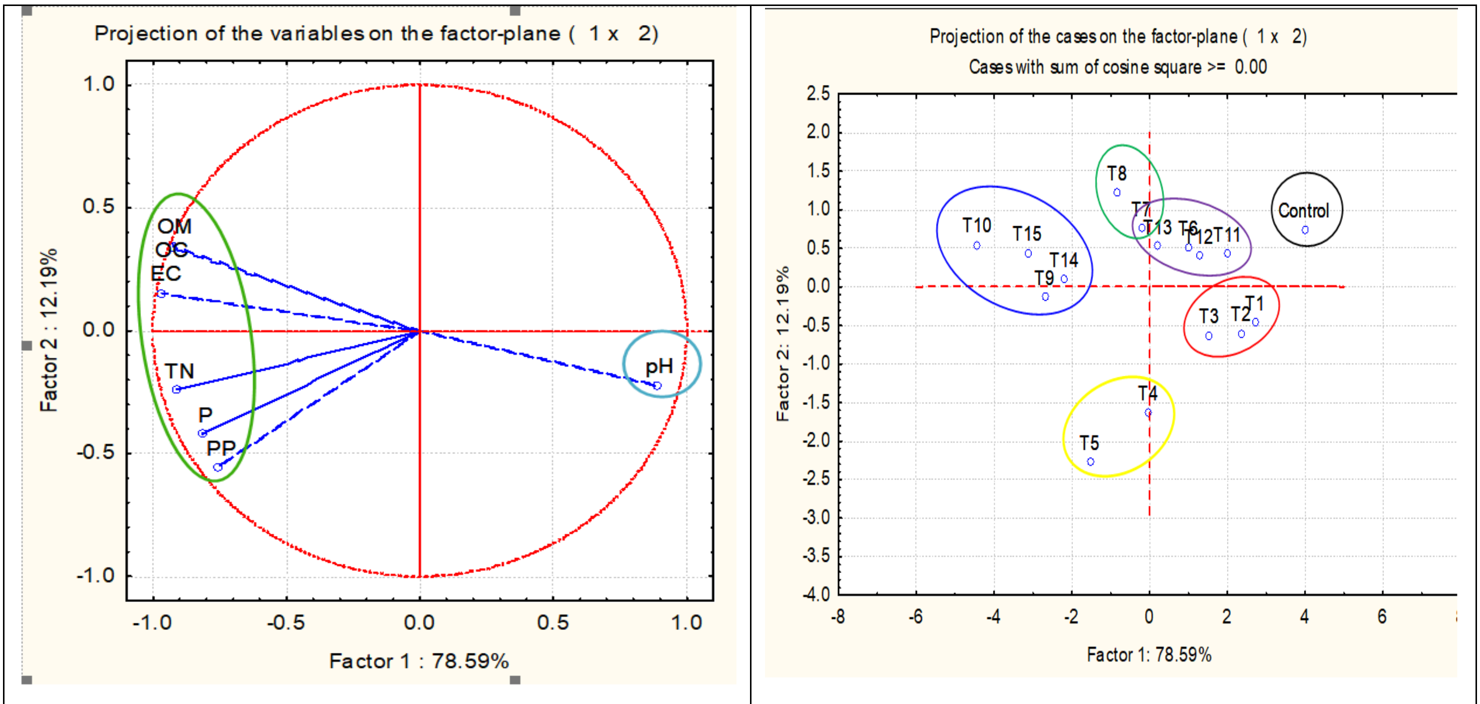
	pH	EC	OM	OC	TN	P	PP
pH	1.000	<0.0001	<0.0001	<0.0001	0.0013	0.0095	0.0254
EC	-0.845	1.000	<0.0001	<0.0001	<0.0001	0.0023	0.0060
OM	-0.855	0.957	1.000	<0.0001	0.0007	0.0136	0.0383
OC	-0.851	0.943	0.992	1.000	0.0008	0.0099	0.0419
TN	-0.730	0.847	0.754	0.749	1.000	0.0001	0.0004
P	-0.626	0.704	0.602	0.623	0.813	1.000	0.0011
PP	-0.556	0.654	0.522	0.513	0.778	0.739	1.000

EC: Electrical conductivity, OM: Organic matter, OC: Organic carbon, TN: Total nitrogen, P: Assimilable phosphorus, PP: Phenolic compounds.

2.11. Multivariate analysis (PCA)

A principal component analysis was utilized to examine the evolution of the several parameters under research (pH, EC, OM, OC, TN, P, and PP). The presence of two groups was shown by the PCA (Figure 26a). The first group included pH, which had positive correlations with axis 1 and explained 78.59 percent of the data, whereas axis 2 explained just 12.19 percent. OM, OC, EC, TN, P, and PP were also included in the second group, which had a negative connection with axis 1.

First, OM, OC, EC, and TN, P, PP were all altered in the same direction due to the considerable influence of the therapy administered. While the pH changes in an individual group owing to the high content of EC, OM, OC, and PP in the soil generated by the treatment, in other words, the high concentration of all these parameters in the soil increases soil acidity. Some of these findings match those of Chaari et al. (2015) and Zema et al. (2019). Those pertaining to OM, TN, P, and PP, in particular.



(a) **(b)**

Figure 25: Principal component analysis applied to soil properties treated with OMWW and OMP and the combination. a Correlation circles of the physico-chemical parameters of the soil pH, EC (electrical conductivity), OM (organic matter), OC (organic carbon), TN (total nitrogen), P (assimilable phosphorus), PP (phenolic compounds). b Projection of experimental points according to the treatment applied 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% Combination, T12: 25% Combination, T13: 50% Combination, T14: 75% Combination, T15: 100% Combination, Control: untreated soil.

The PCA projection for experimental points, in particular (Figure 26b). The treatments T8, T9, T10, T14, and T15 were shown to be negatively linked with axis 1, indicating that large dosages of OMP, as well as the combination and recommended dose of OMP, had a substantial impact on soil parameters. Except for T1, T2, T3, T4, T6, T7, T11, T12, T13, and the control treatment, all of the treatments were positively associated with the two-axis (1 and 2), with the exception of T1, T2, T3, T4, and the allowed dose of OMWW, which were negatively associated with axis 2. These treatments had an effect on soil properties, albeit a minor one. Furthermore, the T5 linked with the 100 percent OMWW therapy was adversely related to axis 1.

General Conclusion & Perspectives

General conclusion and Perspectives

Olive mill wastewater and olive mill pomace are generally unexploited and released in nature, especially on the soil are constitute an important problem because of the possible consequences and their negative effects on soil properties and soil organisms such as the earthworms.

Earthworms are the most important actors in soil, and few data are available regarding the effects of the interaction of OMWW and OMP on the growth, cocoon production, and survival of the earthworms. Data obtained within this study showed that OMWW and OMP separately and in combination had significant effects on the life-history traits of these soil invertebrates.

In this context, we have focused on determining the potential effects of different doses of OMWW and OMP and their combination on the growth, reproduction, survival of earthworms, soil properties, and heavy metals from olive mill wastes accumulation on the soil, and the earthworm tissue.

We can generate the following conclusions based on the results obtained within this study:

- At the lower doses, we found a positive effect on the growth, this is probably due to their high organic matter, and no negative effect on the reproduction and survival.
- Toxicological effects were found at higher doses. The main cause of this toxicity is probably the high level of polyphenols, heavy metals, salinity; the highest toxicological effects were produced by OMP.

In order to demonstrate its safety and make its application more efficient, the current research has focused on determining the potential consequences of various concentrations of OMWW and OMP including the recommended doses, further has focused on the combination effects on the most fundamental soil characteristics. Based on the findings of this study, we may conclude that:

- The treatment of the soil with various concentrations of OMWW, OMP, and the combination has had a significant effect on the soil physicochemical properties. A remarkable increase in the concentrations of several mineral elements has been observed, as well as a significant richness in the soil organic matter, especially at high doses.

  - Comparative effects of olive mill waste, the results showed mostly similar effects of the treatment with OMWW and OMP and the combination on the soil properties, except for the OMP and the combination which significantly improved the soil fertility compared to OMWW.
  - Highlights the benefits of exploiting FTIR to characterize variations on soil characteristics and soil phenolic compounds after treatments with olive mill waste, especially for the soil phenolic compounds, this qualitative analysis might be obtained quickly and inexpensively.
  - OMWW and OMP could be regarded as helpful and inexpensive amendments and fertilizers. Even with the promising findings obtained, longer investigations in various soil conditions are still needed to determine the short and long-term impact of OMWW and OMP and simultaneous treatments on soil characteristics.

- Olive mill wastewater and olive mill pomace have non-negligible amounts of heavy metals ranged as follow: Fe> Zn> Cu> Cd> Cr for OMWW, and Fe> Zn> Cu> Cr and below the detection limit for Cd for OMP.
- The application of olive mill waste on soil caused significant accumulations of heavy metals, in particular, under the treatment with a combination, showed significant accumulation of Cr, Fe, Cu, and Zn, and for Cd under the treatment with OMWW.
- Results showed also that earthworm tissue has a high tendency to bioaccumulate metals and the concentration of accumulation is proportional to the concentration of metals in the soil
- The bioaccumulation factor of *A. trapezoides* was ranged as follow: Cr> Cu> Fe> Cd> Zn, while for *E. fetida* was Cd> Cr> Fe> Zn> Cu. Endogeic earthworm *A. trapezoides* was revealed to be uptake metals in their tissue more than the epigeic earthworm *E. fetida*, especially for Cu.

Finally, we can conclude that olive mill wastes have a significant effect on the accumulation of heavy metals on soil and the earthworm tissue.

Perspectives:

- We suggest significant dilutions of olive mill wastes as a prospective approach for preventing dangers to soil invertebrates and soil properties prior to disposal.
- Bioremediation of these by-products is also recommended to lower their environmental toxicity and promote their safe and environmentally friendly utilization.
- Olive mill waste can be considered an inexpensive fertilizer.
- The possible impact of OMWW and OMP on earthworm biochemical and cellular parameters requires a biomarker approach.

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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*)

Nawal Mekersi [a], Kenza Kadi [a], Silvia Casini [b,*], Dalila Addad [c], Kamel Eddine Bazri [d], Salah Eddine Marref [e], Sabrina Lekmine [f], Asma Amari [g]

[a] *Laboratory of Biotechnology, Water, Environment and Health, University of Abbes Laghrour, Khenchela, Algeria*
[b] *Department of Physical, Earth and Environmental Sciences, University of Siena, via Mattioli, 4, 53100 Siena, Italy*
[c] *Department of Ecology, SNV Faculty, Abbes Laghrour University, Khenchela, Algeria*
[d] *Laboratory of Ecology, Department of Plant Biology and Ecology, University Constantine 1, Algeria*
[e] *Laboratory of Biotechnology of Bioactive Molecules and Cellular Physiopathology (LBMBPC), University of Batna 2, 05000 Batna, Algeria*
[f] *Laboratory of Bioactive Molecules and Applications, Larbi Tébessi University, Tebessa 12. 000, Algeria*
[g] *Laboratory of Plant Biology and Environnement, Faculty of Sciences, University Badji Mokhtar-Annaba, BP12, 23000 Annaba, Algeria*

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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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* Corresponding author.
E-mail addresses: mekersinawal@gmail.com (Nawal Mekersi), silvia.casini@unisi.it (S. Casini).

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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^3/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 bioindicator 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^3/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^3/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, usinga3-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^{-1}; 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-Ciocalteau (Makkar et al., 1993). The biochemical oxygen demand (BOD_5) 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 $50m^3$/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 ± SD of 3 different measurements.

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

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

Wt is the weight of the earthworm on the day they were checked, and W_0 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 ± 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 compounds such as lignins and tannins (Paraskeva and Diamadopoulos, 2006) and an acidic pH (pH = 4.84 ± 0.01), together with a high electrical conductivity (EC = 9.7 ± 0.61 mS cm^{-1}) when compared with the value cited by Malvis et al. (2019). A high salinity 7.4 ± 0 g l^{-1} 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 (Achak et al., 2008; Eroğlu et al., 2008; Bouknana et al., 2014). A high moisture content (92.1 ± 0.96%) was found, while the OMWW total nitrogen content was very low (TN = 0.42 ± 0.02 g l^{-1}), 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 ± 1%), moreover a high biological oxygen demand (BOD = 40. 20 ± 0. 01 g l^{-1}) was found and the result obtained is in accordance with the value cited by Babić et al. (2019). Moreover, the physico-chemical analysis revealed that the OMWW was highly enriched in phenolic content (2. 31 ± 0. 01 g l^{-1}) 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).

Table 2
Physicochemical characteristics of olive mill pomace. Results are reported as mean ± SD of 3 different measurements.

Parameter	Value
pH	4. 7 ± 0.02
Electrical conductivity (mS cm^{-1})	14.8 ± 0.1
Moisture content (%)	57.69 ± 0.40
Organic matter (%)	90.27 ± 3.0
Assimilable phosphorus (mg g^{-1})	0,0253 ± 0.2
Total nitrogen (mg g^{-1})	0.5 ± 0.1
Na (mg g^{-1})	51.67 ± 1.53
Ca (mg g^{-1})	2.41 ± 0.19
K (mg g^{-1})	111.67 ± 2.09
Organic carbon content (%)	60.80 ± 0.59
Total polyphenol (mg g^{-1})	40 ± 0.3

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 ± 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 ± 0.1 mS cm^{-1}), 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 ± 0.40%), and a very high content of organic matter (90.27 ± 3. 0%) and organic carbon (60.80 ± 0.59%). The results are close to those obtained by Ameziane et al. (2019). The content of polyphenols was approximately (40 mg g^{-1}), 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 ± 2. 09 mg g^{-1}), which is a common characteristic in olive-mill waste and by-products, followed by sodium (51. 67 ± 1.53 mg g^{-1}) and calcium (2. 41 ± 0. 19 mg g^{-1}). 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^{ns}
Treatments (T)	9361.78***	31.46***	11722***
Interaction (S × T)	2497.87***	2.07***	44.23^{ns}

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^{-1}) showing levels that are incompatible with the result cited by Alburquerque 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 et 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

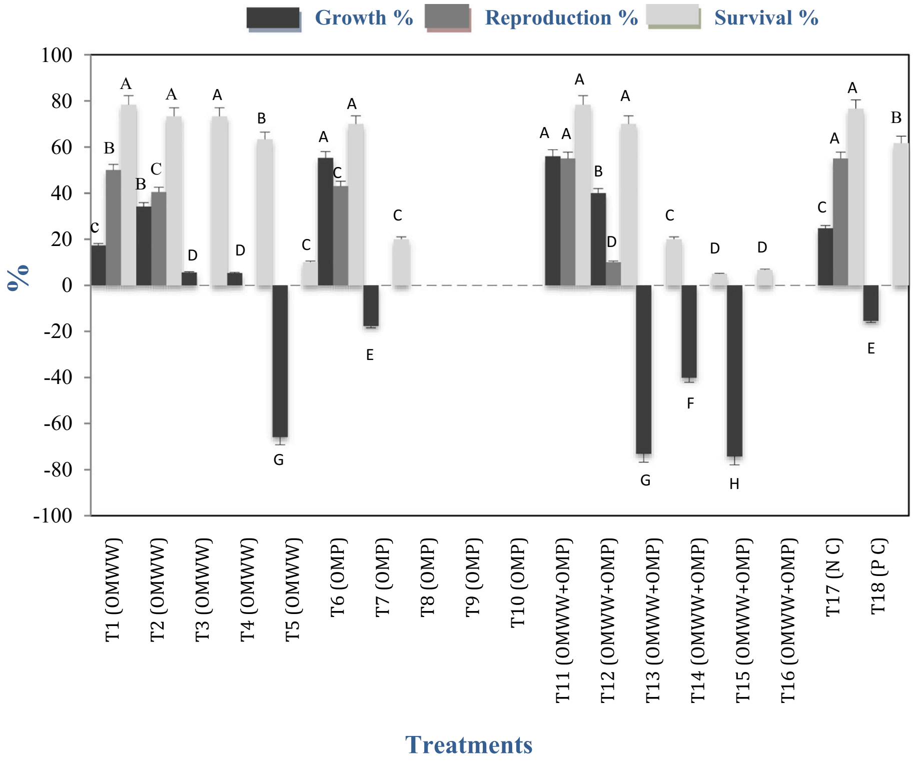


Fig. 1. Mean effect of treatments (% ± SD, n = 3) in measured parameters. T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; T13: 50% of the combination; 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 ($LSD_{5\%}$); a, b the homogeneous group.

Parameters	Growth		Reproduction		Survival	
Species	*A. trapezoides*	*E. fetida*	*A. trapezoides*	*E. fetida*	*A. trapezoides*	*E. fetida*
Means	-7.30^b	1.36^a	1.11^b	1.70^a	51.30^a	47.22^a
$LSD_{5\%}$	2.76		2.66		75.75	

$LSD_{5\%}$ *Least Significant difference.*

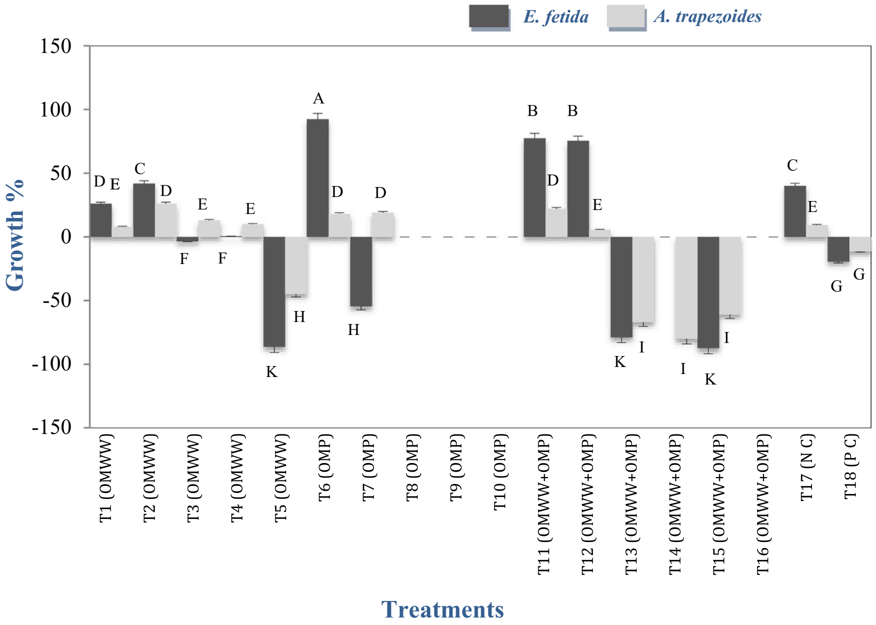


Fig. 2. Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters were T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; 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 ± 1 and 10.04 ± 2: 24% respectively for *A. trapezoides* and −2.57 ± 1; 0.59 ± 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 ± 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 ± 0%

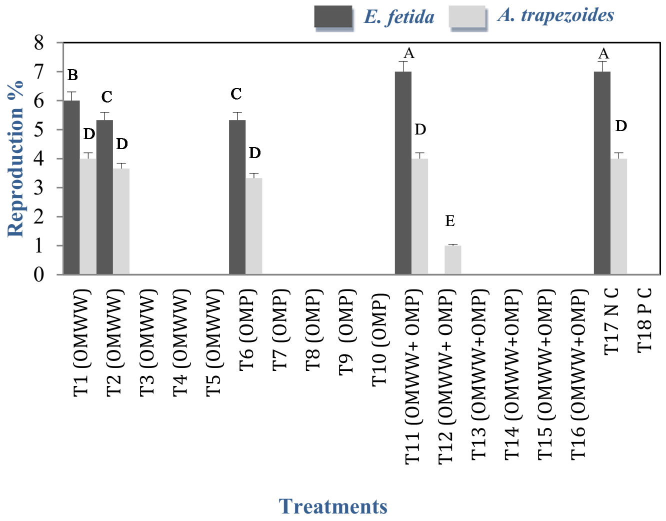


Fig. 3. Mean effect of interaction species × treatment (% ± SD, n = 3) on the measured parameters. Where T1: 12.5% OMWW; T2: 25% OMWW; T3: 50% OMWW; T4: 75% OMWW; T5: 100% OMWW; T6: 12.5% OMP; T7: 25% OMP; T8: 50% OMP; T9:75% OMP; T10: 100% OMP; T11: 12.5% of the combination; T12: 25% of the combination; 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 ± 1.76%), then T1 (group B) 5 ± 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 × 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 dS m^{-1} and 7.35 dS m^{-1}). The findings can be related to those of some previous research papers (Jezierska 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).

Table 5

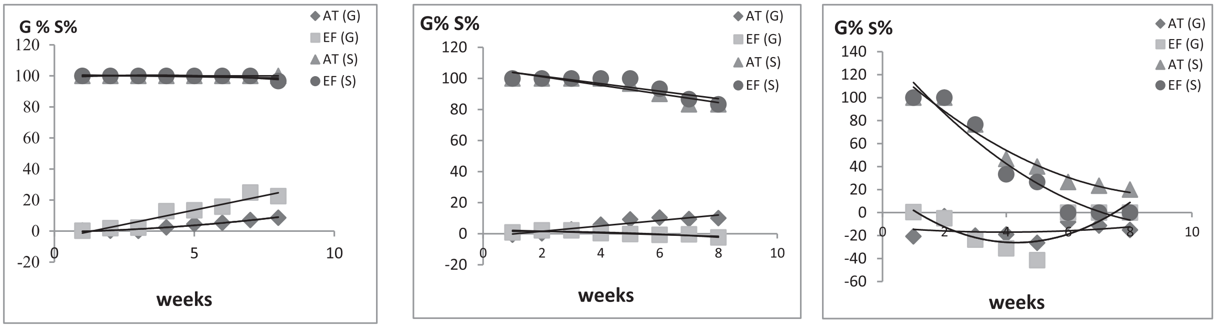
Most interesting regression equations describing the growth rate and survival rate over 8 weeks for species *A. trapezoides* and *E. fetida* presented in Fig. 4.

Treatment		Growth %	Survival %
T1 (12.5% OMWW)	*A. trapezoides*	$y = 0.113 \times 2 + 0.284x - 0.575$ $R^2 = 0.967$	No correlation
	E. fetida	$y = 3.710x - 5.010$ $R^2 = 0.922$	$y = -0.278x + 100.8$ $R^2 = 0.333$
T4 (75% OMWW)	*A. trapezoides*	$y = 1.747x - 1.930$ $R^2 = 0.886$	$y = -2.778x + 106.6$ $R^2 = 0.821$
	E. fetida	$y = -0.541x + 2.687$ $R^2 = 0.739$	$y = -2.421x + 106.3$ $R^2 = 0.741$
T5 (100% OMWW)	*A. trapezoides*	$y = 0,274 \times 2 - 2,156x - 12,77$ $R^2 = 0,042$	$y = 1,309 \times 2 - 24,88x + 132,7$ $R^2 = 0,955$
	E. fetida	$y = 2,564 \times 2 - 22,10x + 21,52$ $R^2 = 0,574$	$y = 1,607 \times 2 - 31,57x + 143,1$ $R^2 = 0,94$
T6 (12.5% OMP)	*A. trapezoides*	$y = -0,089 \times 2 + 2,995x - 3,876$ $R^2 = 0,914$	$y = -0,396 \times 2 + 2,220x + 97,62$ $R^2 = 0,974$
	E. fetida	$y = -0,451 \times 2 + 18,23x - 21,61$ $R^2 = 0,975$	$y = -0,396 \times 2 + 2,220x + 97,62$ $R^2 = 0,974$
T7 (25% OMP)	*A. trapezoides*	$y = 2,366x - 5,498$ $R^2 = 0,975$	$y = 2,182 \times 2 - 28,85x + 117,5$ $R^2 = 0,925$
	E. fetida	$y = 2,391 \times 2 - 17,40x - 16,04$ $R^2 = 0,289$	$y = -34,8\ln(x) + 77,05$ $R^2 = 0,743$
T8 (50% OMP)	*A. trapezoides*	No correlation	No correlation
	E. fetida	No correlation	No correlation
T12 (12.5% of the combination)	*A. trapezoides*	$y = 0,264 \times 2 + 0,913x - 1,304$ $R^2 = 0,986$	No correlation
	E. fetida	$y = 1,992 \times 2 - 9,476x + 5,719$ $R^2 = 0,987$	$y = 0,159 \times 2 - 1,908x + 102,0$ $R^2 = 0,825$
T15 (75%* of the combination)	*A. trapezoides*	$y = 4,913 \times 2 - 42,31\times + 17,77$ $R^2 = 0,803$	$y = 2,579 \times 2 - 31,39\times + 102,1$ $R^2 = 0,802$
	E. fetida	$y = 5,197 \times 2 - 44,89x + 18,52$ $R^2 = 0,595$	$y = 3,214 \times 2 - 38,13x + 111,3$ $R^2 = 0,733$
T17 (Negative control)	*A. trapezoides*	$y = 0,045 \times 2 + 0,955x - 0,305$ $R^2 = 0,948$	No correlation
	E. fetida	$y = 0,469 \times 2 + 1,476x - 1,290$ $R^2 = 0,987$	$y = -0,297 \times 2 + 1,925x + 97,67$ $R^2 = 0,900$

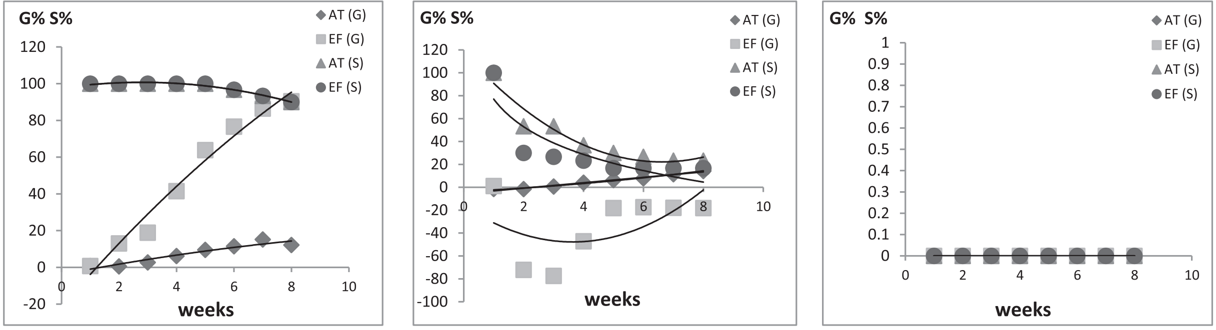
3.5. *The effect of olive mill wastewater and olive pomace and a combination of them on the earthworm survival*

The analysis of variance does not show there to be a significant effect of the following factors "species" and "interaction" (species × 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 ± 4.08; 93.33 ± 5.16; 93.33 ± 5.16; 90 ± 0; 98.33 ± 4.08; 90.0 ± 12.64 and 96.66 ± 5.16 respectively, group (B) includes T4 (83.33 ± 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, 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.

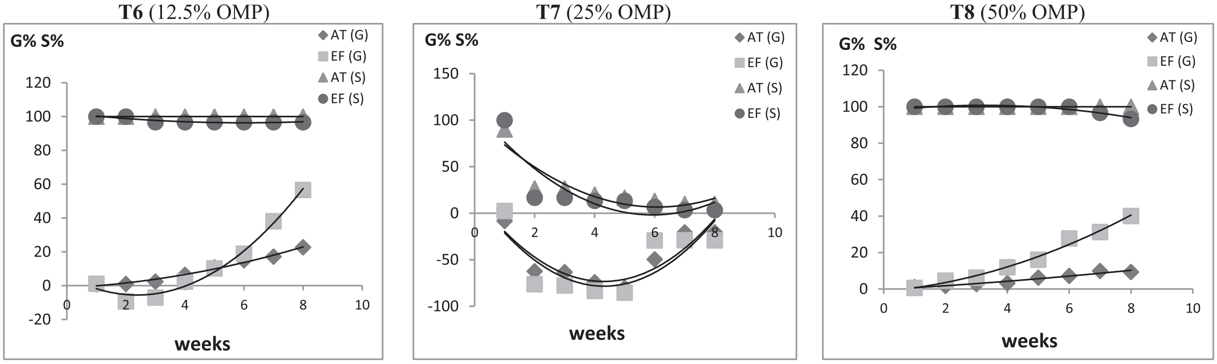
On the contrary, Hentati et al. (2016) and Chalkia et al. (2020) found there to be no mortality of earthworms *Eisenia fetida* and *Enchytraeus crypticus* maintained in soils amended with OMW at different dry weight ratios, additionally no lethal effect was found on the earthworm *Octodrilus complanatus*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



T1 (12.5% OMWW) **T4** (75% OMWW) **T5** (100% OMWW)



T6 (12.5% OMP) **T7** (25% OMP) **T8** (50% OMP)



T12 (12.5% of the combination) **T15** (75%* of the combination) **T17** (Negative control)

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).

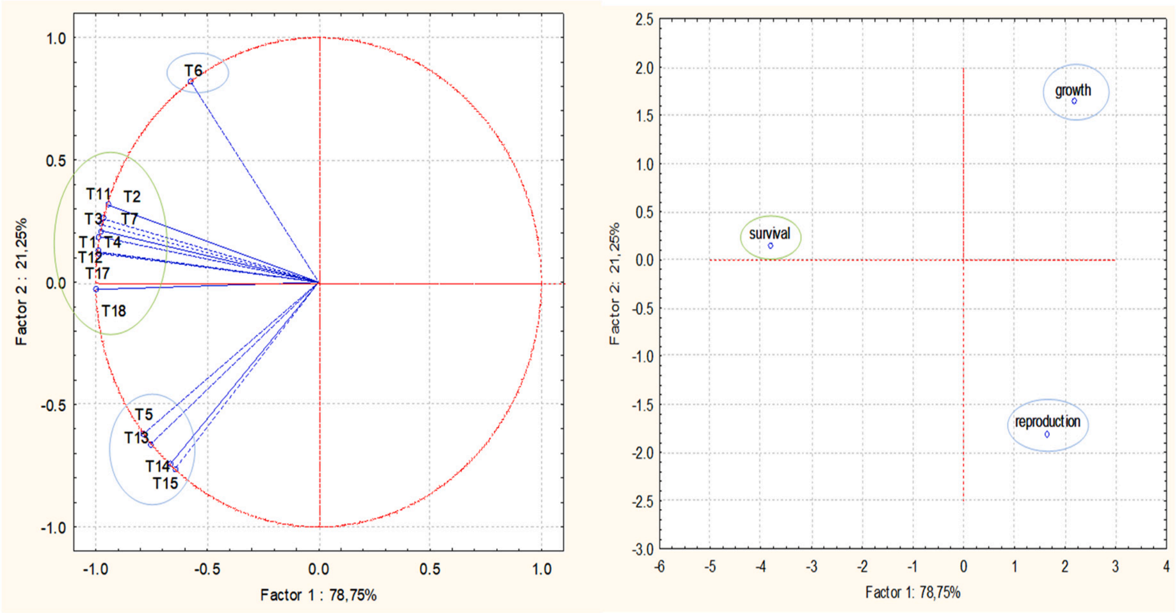
m^3/ha. The negative effect found in our study and in the study of Campani et al. (2017) has a possible explanation that the tested treatments were mixed with the total volume of soil. In the study conducted by Chalkia et al. (2020), instead, the tested OMW were sprayed on the surface. While, Hentati 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

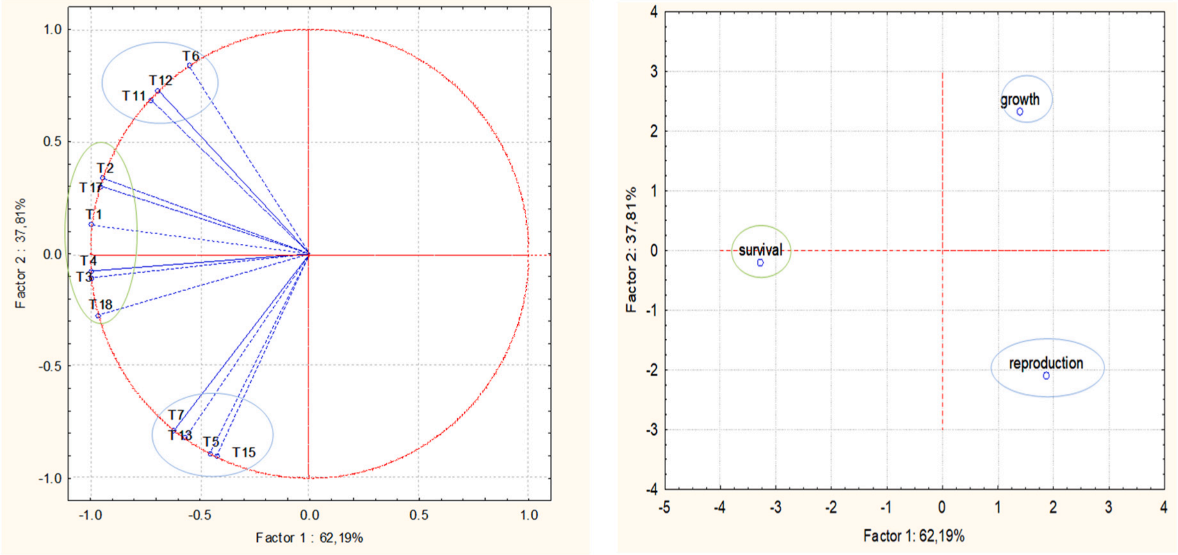
The linear regression analysis indicates a strong positive correlation between the growth and the period of exposure, meaning that growth increases when there is a rise in exposure time for both *A. trapezoides* and *E. fetida* in T1, T6, T12, a negative correlation was found in T15, T18, in these treatments; 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



(a) **(b)**

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*.



(a) **(b)**

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^3/ha/year) than the *A. trapezoides*.

The optimal soil electrical conductivity for earthworm is 0.5–1.0 dS m^{-1} 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^{-1} 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^{-1}). 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^{-1}. 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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