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The Effects of Olive Mill Wastewater on Soil Microbial Populations

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Abstract

Olive mill wastewater (OMW) is a common pollutant in Jordan due to the large number of olive mills and the importance of the olive oil industry in the country. In this study, the effects of OMW and fertilizer on soil microbial populations were examined by counting the number of microbial colonies on each plate after respective treatments with water, OMW, and fertilizer. Colonies were identified based on macroscopic and microscopic examination as well as a range of biochemical tests. After treatment with OMW, a significant increase was exhibited in the *Bacillus* (*p*-value of 0.011 in clay) and *Yeast* (*p*-values of 0.001 in clay and 0.037 in sand) populations. In contrast, *Staphylococcus*, *Streptomyces (p*-values of 0.034 in clay and 0.016 in sand) and Mold (*p*-value of 0.013 in sand) exhibited population decreases. Our results showed that OMW significantly affects natural soil microbial populations, which is an important finding as most of the OMW in Jordan is disposed of in a way that exposes it to the soil. This study illustrated that OMW has a potential to be recycled and utilized as an antibacterial agent. Further studies should be conducted using molecular PCR analysis in order to accurately determine the species of each studied microorganism.

Keywords: olive mill wastewater; soil microbes; fertilizer; Jordan.

1. Introduction

Olive and olive oil production is of particular importance to the economic sectors of Jordan in particular and Mediterranean countries as a whole (Rusan *et al*., 2015). However, the olive oil industry generates a large amount of toxic drainage known as olive mill wastewater (OMW), the latter of which is characterized by an acidic pH as well as a high content of organic compounds and polyphenols (Ribeiro *et al*., 2018). The disposal of untreated OMW poses a number of threats to both environmental and public health, and its management is a source of considerable problems for most of the Mediterranean region (Ioannou-Ttofa *et al*., 2017). OMW has phytotoxic and inhibitory effects on plant growth, acts as an anti-bacterial agent, and contains compounds that are toxic to non-bacterial microorganisms, all of which result in an altered state of soil microbial diversity (Mekki *et al*., 2013; Ntougias *et al*., 2013; Rusan *et al*., 2016). Therefore, OMW can neither be disposed of directly into the environment nor into the sewage systems, and several different OMW management, treatment, and valorization strategies have been proposed (Souilem *et al*., 2017).

Jordan's eastern Mediterranean climate makes it particularly suited for the cultivation of olive trees, which hold great cultural, religious, and economic importance for the Jordanian people (Al Ganideh and Good, 2016). In fact, Jordan is a major exporter of olives and olive oil, and olive trees cover 73% of the agricultural land occupied by fruit trees (El Hanandeh and Gharaibeh, 2016). As a result, nearly 180,000 families are supported by the farming of 20 million olive trees, and the annual income from olive oil production is approximately 100 million Jordanian dinars (*The Jordan Times*, 2015). More than 70% of the Jordanian olive oil processing industry utilizes three-phase oil mills (86 out of a total of 118 oil mills as of 2011), while the remaining 30% consists of two-phase oil mills (20%) and traditional press mills (10%) (Qdais and Alshraideh, 2016). Three-phase oil mills produce large amounts of OMW, a pulp-like substance called olive cake, and a substantial amount of wastewater from washing the olives prior to extraction (Dourou *et al*., 2016).

Several studies have been conducted concerning OMW itself as well as its management and disposal in Jordan and abroad. OMW can be treated in jet-loop (JACTO) reactors in order to reduce its chemical oxygen demand (COD) and total phenol content by 85% and 80%, respectively (Ribeiro *et al*., 2018; Khoufi *et al*., 2015). Furthermore, the simple act of diluting OMW with water was reported to eliminate its phytotoxic effects on plant growth (Rusan and Malkawi, 2016). In addition, biodegradation of OMW by various types of thermophilic bacteria is another potential treatment strategy that is under investigation (Al-Qodah *et al*., 2015). Moreover, volcanic tuff treated with nitric acid was found to reduce the COD and total phenol content of OMW by 14% and 21%, respectively (Azzam, 2018). Likewise, natural Jordanian clay that was subject to calcination and acid treatment reduced the COD of OMW by up to 50% (Azzam *et al*., 2015). Lastly, the use of OMW as a fertilizer was found to enhance plant growth, but such growth was lower than that obtained by

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conventional fertilizer and potable water (Rusan *et al*., 2015; Barbera *et al*., 2013).

Since OMW has been investigated in the context of plant growth and fertilization, it is important to also consider how OMW application might affect the microbial diversity of the soil it is being applied to. It has previously been found that olive washing conditions and the resulting OMW can affect the growth of certain fungal species in Jordan's environment (Massadeh *et al*., 2010; Al-Ameiri *et al*., 2015). Therefore, the main aim of this study is to investigate the impact of raw OMW on various soil microbiota obtained from local sources, specifically in comparison with fertilizer and water treatments.

2. Materials and methods

2.1. Field and soil sampling

Two types of soil samples were used in this study: clay and sand. Soil samples were collected from an on-campus site at Jordan University of Science and Technology, while raw OMW was collected from local olive mills in Irbid, Jordan. A greenhouse experiment was conducted to evaluate the effects of three treatments (tap water (W), tap water and fertilizer (W+F), and raw OMW) on the microbiota populations of clay and sand.

A total of 18 pots were filled with 5 kg of air-dried silty clay loam (n=9) or 5 kg of air-dried sandy loam (n=9), so that each treatment was replicated three times in a randomized complete block design. Three corn seeds were planted in each pot, after which the soil moisture was brought up to the field capacity water content. The amount of water required to bring the sand to field capacity was 900 cm^3, while the amount required for the clay was 1,500 cm^3. Afterwards, all the pots were watered periodically to keep the soil moisture level at field capacity. Irrigation solution was added three times per week depending on the losses of soil moisture by evapotranspiration, the latter of which was determined by the periodic weighing of the pots. Pretreatment characteristics of the soil and irrigation solutions are summarized in **Tables 1** and **2**, respectively. After 8 weeks of growth, the plants were harvested, and representative soil samples were taken from each pot after thoroughly mixing the soil.

Table 1. Characteristics of soil samples.

Parameters	**Units**	**Sand**	**Clay**
pH*	-	7.8	8.18
Electrical conductivity*	dS/m	1.2	0.61
Calcium carbonate ($CaCO_3$)	%	27.1	15.3
Cation exchange capacity (CEC)	cmol/kg	24.1	34.3
Organic matter	%	1.01	0.72
Texture Class	-	Sandy loam	Silty clay loam

*of 1:1 soil:water suspension

Table 2. Characteristics of irrigation water and olive mill wastewater (OMW).

Parameters	Units	Water	Raw OMW	Treated OMW
pH	-	7.8	4.7	6.2
Electrical conductivity	dS/m	0.6	7.6	5.1
Total suspended solids (TSS)	mg/l	10	1236	-
Total polyphenol content	mg/l	0.98	1666	700

2.2. Treatment of soil

Soil samples were sieved and dried, and 1g of each sample was mixed with 99 mL of sterile distilled water and placed in a reciprocal shaker to be shaken for three hours at 120 rpm. Each sample was properly diluted, and 0.1 mL was inoculated on nutrient agar (for general bacteria, *Bacillus* spp, and *Staphylococcus* spp), oatmeal agar (for *Streptomyces* spp), malt extract agar (for yeast), and potato dextrose agar (for molds).

2.3. Culturing of microorganisms

Inoculated plates were placed in an incubator at 37ºC for incubation. The general bacterial, *Bacillus* and *Staphylococcus* colonies were counted after an incubation period of 24 hours, while the *Streptomyces*, yeast and mold plates required 4 days of incubation before their colonies were counted. There were two types of colonies on the nutrient agar plate, and they were separated into two groups depending on their morphology (**Table 3**).

Table 3. Colony morphology, microscopic examination and biochemical tests.

Biochemical test	Type 1 (*Staphylococcus*)	Type 2 (*Bacillus*)
Colony morphology	White, round	Large, flat, white, smooth, non-pigment producer
Gram staining	Gram-positive	Gram-positive
Shape of cell	Cocci	Long rod
Arrangement of cells	In clusters	In chains
Endospore staining	Non-spore former	-
Catalase	-ve	-
Benzidine	-ve	-
Nitrate red	-ve	-
Motility	-ve	-

2.4. Microbial parameters

In this study, the main parameters were the colony counts, macroscopic and microscopic examination, and a series of biochemical tests.

2.4.1. Colony counts

The main parameter used for observing the effect of each treatment on soil microbial populations was the colony count, which was carried out via a Quebec colony counter. The colony count is a basic microbiological technique that attempts to quantify the amount of bacterial growth in terms of number of colonies. Therefore, this technique is useful in the present study as it provides a

quantitative means of measuring the effects of the various treatments on each bacterial population.

2.4.2. Macroscopic and microscopic examination

As there were two types of colonies on the nutrient agar plates, further examination was necessary to identify each genus. Macroscopically, it was found that Type 1 (*Staphylococcus*) microorganisms had a white and round appearance, while Type 2 (*Bacillus*) microorganisms had a large, flat, white and smooth surface with no pigment production. Upon microscopic examination, Type 1 (*Staphylococcus*) microorganisms had a cocci shape and were arranged in clusters, while Type 2 (*Bacillus*) microorganisms appeared as long rods that were linked together to form chains.

2.4.3. Biochemical tests

Several biochemical tests were employed in order to further ascertain the identities of the microorganisms. The first biochemical test was the basic Gram stain procedure in order to determine whether the cell was Gram-positive or Gram-negative. Afterwards, a series of biochemical tests were applied to the Group 1 (*Staphylococcus*) microorganisms in order to fully confirm their identity. These included the catalase, benzidine, nitrate red, and motility tests.

2.4.4. Statistical analysis

The data was analyzed by one-way ANOVA using IBM SPSS. Levene's test was first applied in order to test homogeneity of variance, and the post-hoc tests consisted of Tukey's test (for equal variances) and Games-Howell test (for unequal variances). All statistical analyses were conducted using SPSS statistical package 19.0 (SPSS Corp., USA).

3. Results

The application of OMW resulted in an increase in the general bacterial count compared with the application of fertilizer. *Bacillus* populations were the highest after OMW application and increased substantially after treatment with OMW in both clay and sand. The lower population was observed in sand. Like *Bacillus*, yeast populations were highest after OMW application in both sand and clay. However, yeast populations were much higher in clay compared to sand. On the other hand, the *Streptomyces*, *Staphylococcus*, and mold populations were the lowest after OMW application, as they decreased substantially after treatment in both clay and sand. The lower populations were observed in sand (**Figure 1**).

Table 4 shows statistical comparisons between the different types of treatments. There was a significant effect of the treatments on *Bacillus* populations in clay [F (2, 6) = 10.388, p = 0.011]. The Tukey post hoc test indicates that the mean for the OMW treatment (M = 20.3 x 10^5, SD = 75.2 x 10^5) differs significantly from the mean for no treatment (M = 8 x 10^5, SD = 2 x 10^5). Moreover, the treatments also had a significant effect on *Streptomyces* populations in clay [F (2, 6) = 6.288, p = 0.034]. The Tukey post hoc test indicates that the mean for the OMW treatment (M = 36.7 x 10^5, SD = 15.2 x 10^5) differs significantly from the mean for the fertilizer treatment (M = 11.6 x 10^6, SD = 2.1 x 10^6). A significant result was also observed for *Streptomyces* populations in sand [F (2, 6) = 8.988, p = 0.016].

There was a significant effect of the treatments on mold populations in sand [F (2, 6) = 9.653, p = 0.013]. The Tukey post hoc test indicates that the mean for the OMW treatment (M = 10 x 10^3, SD = 2 x 10^3) differs significantly from the mean for the fertilizer treatment (M = 50 x 10^3, SD = 17.3 x 10^3) and the no treatment (M = 40 x 10^3, SD = 10 x 10^3) groups. There was a significant effect of the treatments on yeast populations in clay [F (2, 6) = 34.543, p = 0.001]. The Tukey post hoc test indicates that the mean for the OMW treatment (M = 24 x 10^4, SD = 5.57 x 10^4) differs significantly from the mean for the fertilizer treatment (M = 50 x 10^3, SD = 17.3 x 10^3) and the no treatment (M = 30 x 10^3, SD = 10 x 10^3) groups. For yeast populations in sand, a significant effect was also shown [F (2, 6) = 6.000, p = 0.037]. The Tukey post hoc test did not indicate which groups showed the significant difference.

Table 4: Statistical comparisons between microbiota in the two types of soil.

	Type of soil	Mean Square	Sum of Squares	F	*P-value*
Bacillus	Clay	1.754 x 10^{12}	3.509 x 10^{12}	10.388	**0.011 ***
	Sand	4.878 x 10^{11}	9.756 x 10^{11}	2.851	0.135
Staphylococcus	Clay	2.413 x 10^{12}	4.827 x 10^{12}	3.899	0.082
	Sand	6.544 x 10^{11}	1.309 x 10^{11}	4.007	0.078
Streptomyces	Clay	5.782 x 10^{13}	1.156 x 10^{14}	6.288	**0.034 ***
	Sand	4.219 x 10^{13}	8.439 x 10^{13}	8.988	**0.016 ***
Mold	Clay	25.2 x 10^7	50.5 x 10^7	1.857	0.236
	Sand	13 x 10^8	26 x 10^8	9.653	**0.013 ***
Yeast	Clay	40.3 x 10^9	80.6 x 10^9	34.543	**0.001 ***
	Sand	16 x 10^8	32 x 10^8	6.000	**0.037 ***

***significant at p<0.05 at 2 df

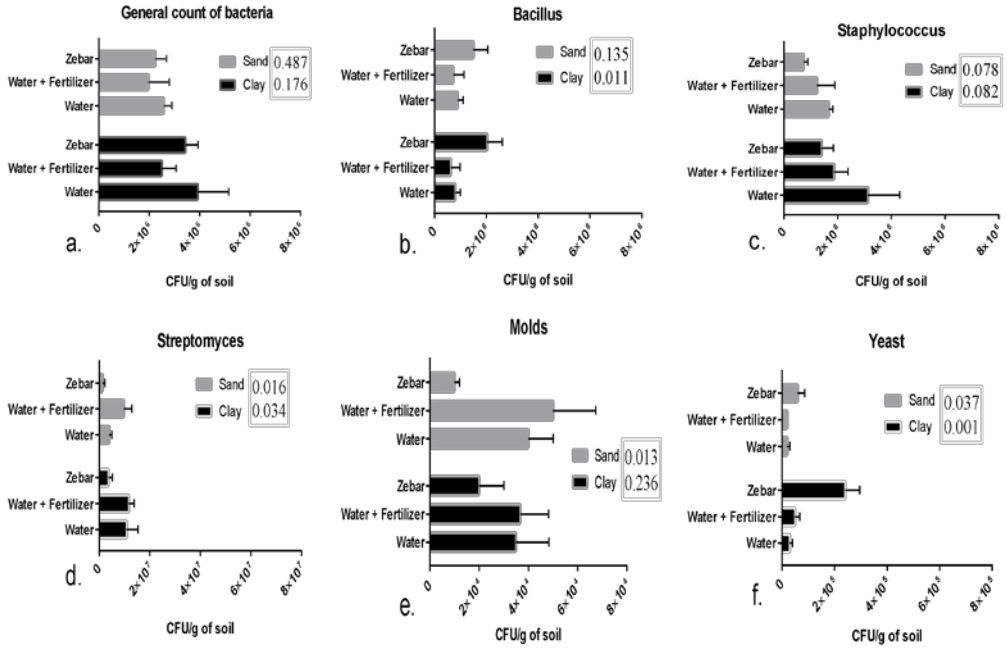

Figure 1: Graphical representation of colony counts (values in the legend represent the p-values): **a**. Applying raw OMW (zebar) resulted in an increase in the general count of bacteria in both sand and clay **b**. *Bacillus* populations exhibited increases in both sand and clay after application with OMW, with p-values of 0.135 and 0.011, respectively **c**. while *Staphylococcus* populations decreased to almost half after OMW application, their p-values in sand (0.078) and clay (0.082) were not significant **d.** *Streptomyces* populations decreased substantially after OMW treatment, with p-values of 0.016 in sand and 0.034 in clay that were statistically significant **e.** Mold populations were also reduced after being treated with OMW, with p-values of 0.013 in sand and 0.236 in clay **f.** Yeast populations (like *Bacillus*) increased significantly after application of OMW, and the p-values in sand 0.037 and in clay 0.001 reflected this significance.

4. Discussion

The olive oil industry is predominant in Jordan and other Mediterranean countries, making the safe disposal of any waste and harmful by-products of the utmost environmental importance. As a consequence of the OMW's high phenolic composition, it has a significant antimicrobial effect on several types of native soil microorganisms. In Jordan, oil mills are prohibited from the direct disposal of OMW, and they cannot discharge the OMW into municipal wastewater treatment systems (Rusan *et al*., 2015). Instead, raw OMW is disposed of at specified dumping sites and their surrounding areas (Rusan and Malkawi, 2016). Therefore, the aim of the present study was to investigate the effects of raw OMW on native soil microbiota sourced from local sites.

The fact that OMW is generally detrimental to the microbial populations of soil was corroborated by the results obtained in this study, which showed that general bacterial populations decreased after exposure to raw OMW (Ntougias *et al*., 2013). Moreover, the application of OMW resulted in a major reduction of *Staphylococcus* and *Streptomyces* populations in both clay and sand, which agreed with previously published results (Tafesh *et al*., 2011). In contrast, the *Bacillus* population flourished in the presence of OMW, which could be explained by the fact that *Streptomyces* produces toxic substances that diminish *Bacillus* growth (de Lima Procópio *et al*., 2012). Correspondingly, one study reported that OMW sustained indigenous populations of *Bacillus*, while another found that OMW was conducive to *Bacillus* growth by protecting it from UV radiation (Yangui *et al*., 2008; Jallouli *et al*., 2014).

In terms of molds, our findings showed that the application of OMW led to an overall decrease in mold populations. Similarly, the application of OMW to fruit infected with gray mold as well as plum tree orchards led to a significant decrease in fungal formation (Saadi *et al*., 2007; Vagelas *et al*., 2009). Several types of molds, namely *Alternaria*, *Colletotrichum*, *Sclerotium*, and *Rosellinia*, species were strongly inhibited by the application of OMW (Cibelli *et al*., 2017). In contrast, the spreading of OMW to a field of olive trees led to an almost 5-fold increase in arbuscular mycorrhizal fungi, which caused the fungal-bacterial ratio to increase from 0.23 to 1.11 (Mechri *et al*., 2008). Concerning yeast, our findings show that yeast populations grew substantially when treated with OMW, with increases of 700% and 200% in clay and sand, respectively. Few studies about the effects of OMW on yeast were reported. However, the role of various yeast strains in the biodegradation of OMW phenols has been previously confirmed (Jarboui *et al*., 2012; Bevilacqua *et al*., 2013).

There are several limitations of the present study. Firstly, the populations of microorganisms were not measured before treatment, a step that is required to fully understand the anti-microbial effects of OMW. Secondly, representative soil sampling after plant harvest may have missed root-associated bacteria, i.e., rhizobacteria, which might also be affected by OMW.

In the present study, Jordanian OMW is suggested to unequally impact the growth of a variety of microorganisms depending on the type of soil. While *Bacillus* spp and yeast flourished under OMW treatment, *Staphylococcus* spp, *Streptomyces* spp, mold, and the general bacteria all exhibited decreases in colony counts. More research is needed to elucidate this difference in bacterial response, and future studies could further analyze OMW's antimicrobial effects in order to utilize it as a possible disinfectant. In addition, molecular PCR analysis could also be employed in order to determine the exact species of each microorganism that was studied.

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