



## Original article

## Olive mill wastewater effects on the microbial communities as studied in the field of olive trees by analysis of fatty acid signatures

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## ABSTRACT

The aim of this work was to study the effects of spreading olive mill wastewater (OMW) on the soil surface of an olive grove on the soil microbial communities. Analyses of ester-linked fatty acid methyl esters (EL-FAME) were used to assess variations in the soil microbial community structure following land spreading of OMW. Our data provide evidence that agronomic application of OMW has important effects on soil microbial community. Bacteria were relatively more reduced by these treatments than fungi and actinomycetes as revealed by an increased index of fungal/bacterial FAME and actinomycetes/bacterial FAME. Specific FAME markers indicated a significant reduction in the Gram-positive bacteria. However, the relative proportion of the Gram-negative bacteria was not significantly different after agronomic application of OMW. The ratios of cyclopropyl/monoenoic precursors decreased and the total mono-unsaturated/total saturated fatty acids increased in the OMW amended soils, suggesting that the microbes inhabiting the control soil are more carbon limited than the OMW amended soils. The changes in the FAME pattern of the soil organisms possibly were related (i) to an altered substrate quantity, that is the availability of substrates after the treatments, (ii) the complex nature of OMW which also contains high molecular-mass recalcitrant polyphenols.

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## 1. Introduction

Mediterranean countries are responsible for more than 95% of world olive oil production. During the process of extraction of oil from olive, large amounts of dark liquid effluents called olive mill wastewaters (OMW) are generated. They are characterized by low pH, high organic load including pectin, polysaccharides, polyphenols and potassium contents [1,2]. Specifically, it was reported that the polyphenolic components of OMW are responsible for phytotoxic effects [3,4], and antibacterial activity [4,5]. Direct disposal of OMW to aquatic bodies results in environmental deterioration due to the large amount of organic loading discharged. Many physico-chemical and biological management strategies have been proposed for the treatment and valorisation of OMW. Physico-chemical treatments consist of the addition of chemical substances that promote the coagulation, precipitation or flocculation of the

organic matter of OMW [6,7]. These methods, however, are too expensive to find a wide application and unable to solve the problem completely because of the need to dispose of sludge or other by-products deriving from the process. One alternative and economical solution is the recycling of the OMW and their use as water for irrigation in agriculture. The OMW has also a high potassium concentration and notable levels of nitrogen, phosphorus, calcium, magnesium and iron, important factors in soil fertility [1]. Additionally the OMW potential herbicidal activity, and their ability to induce suppression against soil-borne plant pathogens are of extra value [8,9]. OMW applied as mulch, could favourably affect the soil water balance by reducing evapotranspiration [10].

The effects of the OMW on the physical and chemical characteristics of the soil are well documented [11–14]. Previous studies have addressed the impact on growth and yield of different types of cereal crops [11,15]. Several works have been devoted to the impact of OMW on soil microbial activity parameters, such as respiration, biomass, and hydrolytic activity [15–17], or more specifically on certain bacterial isolates [18,19]. However, knowledge about microbial community structure and its variants is very important in understanding nutrient fluxes and the effects of xenobiotica.

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Analysis of ester-linked fatty acid methyl ester (EL-FAMES) was used to assess variation in the soil microbial community structure. Fatty acid methyl ester (FAME) profiles of soils can be compared using multivariate statistical techniques to reveal differences in microbial communities [20,21]. EL-FAME analysis is relatively simple and fast, and its effectiveness for assessing community structure has been demonstrated [22].

This study was aimed at evaluating the influence of different application rates of OMW (amounts applied: 30, 60, 100 and 150 m<sup>3</sup>ha<sup>-1</sup>) on soil microbial community structure under natural field conditions in an olive grove.

## 2. Materials and methods

### 2.1. Olive mill wastewater origin

The OMW used in the present study were obtained from an olive oil production plant located in the city of Ouled Jaballah (Tunisia), which uses a discontinuous process for extraction of olive oil (The chemical characteristics of the OMW were: pH: 5.1; electrical conductivity (EC): 9.1 dS m<sup>-1</sup>; salinity: 6.37 g l<sup>-1</sup>; COD: 93 g l<sup>-1</sup>; N: 1340 mg l<sup>-1</sup>; P: 720 mg l<sup>-1</sup>; K: 6200 mg l<sup>-1</sup>; phenols: 8400 mg l<sup>-1</sup>; glucose 1200 mg l<sup>-1</sup>).

### 2.2. Field experiment and soil sampling

Olive trees spaced at 12 × 12 m<sup>2</sup> and growing in Ouled Jaballah (Tunisia, North latitude 35° 12', East longitude, 10° 59') were selected for the experiment. The climate of this region is typical Mediterranean, semiarid to arid, with an average rainfall of 200 mm year<sup>-1</sup> and an average annual temperature of 18–20 °C. Physico-chemical soil characteristics at the sites were analysed as follows: pH: 8.53; Sand: 78.1%; clay: 12.85%; Silt: 5.1%; electric conductivity (EC): 0.44 dS m<sup>-1</sup>; organic C: 0.37%; N: 0.042%; C/N: 8.81; Olsen P: 20.86 mg kg<sup>-1</sup>; exchangeable K: 0.43 (meq 100 g<sup>-1</sup>). The experiment included four levels of OMW application (T0: 0 m<sup>3</sup> ha<sup>-1</sup>, T1: 30 m<sup>3</sup> ha<sup>-1</sup>, T2: 60 m<sup>3</sup> ha<sup>-1</sup>, T3: 100 m<sup>3</sup> ha<sup>-1</sup> and T4: 150 m<sup>3</sup> ha<sup>-1</sup>). Three plots (576 m<sup>2</sup> each: 24 m × 24 m) were designed for each treatment. This amendment was realised in December 2004 in one application. All sampling events included the collection of soil samples from five random locations in each plot, from 0 to 20 cm after 50 days, 80 days and 240 days of the agronomic application of OMW. In the laboratory, soil subsamples were sieved (<2 mm) and used for fatty acid measurements.

### 2.3. Lipid extraction

Microbial community structure was characterized by the extraction and analysis of ester-linked fatty acid methyl esters (EL-FAMES) from soil, as described by Schutter and Dick, [22]. In brief, 3 g soils were extracted with 0.2 M KOH during a 37 °C, hour-long incubation with periodic mixing, followed by addition of 1.0 M acetic acid to neutralize the pH of the tube contents. EL-FAMES were partitioned into an organic phase by addition of hexane, which was removed from the aqueous phase after centrifugation at 480 × g for 10 min. The hexane layer was transferred to a clean tube, and the hexane was evaporated off, after which FAMES were resuspended in 0.5 ml of hexane–methyl tert-butyl ether (1:1) for analysis.

Individual FAMES were separated and quantified by gas chromatography (flame ionization detector) with an HP-5MS column (95% dimethyl–5% diphenyl polysiloxane, length 30 m × 0.25 mm). The temperature was programmed to increase from 170 to 270 °C at a rate of 5 °C per min. The temperature was increased to 270 °C for 2 min between samples in order to clean the column. Identification

of peaks was based on comparison of retention times to known standards (Supelco Bacterial Acid Methyl Esters #47080-U) and confirmed by gas chromatography mass spectrometry (GC–MS).

The fatty acids i15:0, a15:0, 15:0, 16:1ω9, 16:1ω7, i17:0, a17:0, 17:0, cy17:0, 18:1ω7 and cy19:0 were chosen to represent bacterial FAMES [21]. The FAME 10Me18:0 was used to indicate soil actinomycetes [23]. The branched, saturated FAMES i15:0, a15:0, i16:0, i17:0 and a17:0 were chosen to represent Gram-positive bacteria [24]. The fatty acids 16:1ω7, 18:1ω7, cy17:0 and cy19:0 were chosen to represent Gram-negative bacteria [24]. Soil FAME 18:1ω9 and 18:2ω6 were used as indicators of saprophytic fungi [25]. The ratio of fungal:bacterial FAME was used as an indicator of changes in the relative abundance of these two microbial groups [26]. The ratios between the bacterial fatty acids cy17:0 and cy19:0 and their metabolic precursors, 16:1ω7c and 18:1ω7c, and total mono-unsaturated (MUFA)/total saturated (SFA) have previously been used as indicators of nutritional stress in bacterial communities [27–29].

### 2.4. Statistical analysis

Results were statistically analysed using the SPSS statistical software Version 10.0 (SPSS Inc., Chicago). The significance of differences between means values was determined by tow-way analysis of variance. Indicator FAMES for soil fungi, bacteria, Gram-positive bacteria, Gram-negative bacteria and actinomycetes were evaluated with XLSTAT 2006 Version 2006.06 using principal components analysis (PCA) in order to compare the effects of OMW on the soil microbial community structure.

## 3. Results

The additions of OMW as well as time had a significant effect on the soil microbial community (Table 1,  $P < 0.05$ , two-way ANOVA). The relative abundance of bacterial FAMES, gram-positive FAMES, gram-negative FAMES, Fungal FAMES and fungal/bacteria ratio were significantly affected by the OMW doses and sampling time (Table 1). This indicated that agronomic application of OMW in each date responded distinctly from each other. These findings suggest that OMW had the potential to change bacterial populations in soil.

Agronomic application of OMW caused a significant decrease in the percentage of Gram-positive bacteria in all dates of sampling. This decrease was most significant in T4 amended soil (Table 2). The Gram+:Gram– ratio varied between 0.82 and 0.63 after 50 days and between 1.45 and 0.88 after 240 days in the control soil and the T4 amended soil respectively, and there was a tendency for this ratio to decrease with increasing doses of OMW. The ratio actinomycetes-to-bacteria FAMES increased in the OMW amended soil, suggesting that the actinomycete population increased with agronomic application of OMW. In comparison with the control soil, an increase in the percentage of fungal markers in the OMW amended soil was found (Table 2). In all dates of sampling, the markers of fungi increased when the doses of the OMW increased. This behaviour was reflected by a rapid increase of the fungal:bacteria ratio.

The effects of OMW on microbial stress indicators are shown in Fig. 1. The ratio of MUFA:SFA increased significantly ( $P < 0.001$ ) in all dates of sampling (Fig. 1A). This behaviour is due to the increase in the amount of MUFA (Fig. 2). This increase was the greatest in the plots with the highest OMW application rate. Lower cy17:0/16:1ω7c and cy19:0/18:1ω7c ratios were found in the amended soil than the control soil (Fig. 1B, C), indicating that microbial stress decreased after agronomic application of OMW.

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