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Diversity of soil fungi exposed to fresh and stored Olive Mill Wastewater

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Abstract

Olive Mill Wastewater (OMW) management is one of the most challenging environmental problems in Mediterranean countries. Its recycling in soil is an alternative of valorization mean of interest. The effect of OMW on growth of soil fungi, a principal element of biodegradability in soil, was investigated in this study. In a field trial, OMW application to soil at 8 l/m² and 16 l/m² caused an increase in abundances of soil fungi during the 6 months following the spreading. In a microcosm essay, growth of soil fungi was better in fresh OMW than in stored OMW becoming after storage more antimicrobial and phytotoxic. In fresh OMW sterilized then inoculated by soil microflora, survivors of soil fungi were constituted mainly by yeasts which showed an increase of abundances from 5.09 10^4 CFU/ml to 5.02 10^8 CFU/ml after 15 days of incubation at 20°C. In stored sterilized then inoculated OMW, yeasts showed a fast reduction then a survival at low levels. Soil moulds were a sensitize group to OMW even fresh or stored. This group presented a fast reduction of abundances then disappearance. It could be concluded that OMW spreading in high amounts would conflict soil fungi's homeostasis and that spreading of OMW which was stored for a prolonged period should be avoided.

Key words: *Olive Mill Wastewater, fungi, soil, Olive Mill Wastewater storage*

1. Introduction

The effluent of olive oil production units called olive mill wastewater (OMW) is blackish wastewater characterized by a low pH (3.5 to 5.5), a high load of organic matter (COD of 45 to 220 g O_2/l) and phenolic compounds (0.5 to 24 g/l) (Paraskeva et Diamadopoulos, 2006). OMW valorization is a major problem for Mediterranean countries producing nearly 95% of the world olive oil production (Tomati et al., 2001). In these countries, OMW is generally stored in ponds or directly discharged in sewers or rivers what lead to environmental and socioeconomic problems. In Morocco, OMW discharge is causing a deterioration of surface and groundwater quality (Boukhoubza et al., 2008). Consequently, the cost of water potabilization increases and the risk of formation of carcinogenic chlorophenols occurs during chloration of waters contaminated by phenols. On the other hand, OMW released into sewers will cause dysfunction of wastewater treatment stations located downstream.

Although many physicochemical and biological treatments were suggested for OMW, their practical application is generally limited by the high cost of the treatment because of the high OMW production estimated to 30 000 m^3/year (Casa et al., 2003). OMW recycling in soil is a way of valorization that seems promising because it is low cost in comparison with the other possibilities (Cabrera et al., 1996) and because it respects the natural OMW destiny in nature. Indeed, ripe olives constituted to nearly 50% by olive vegetable water fall naturally in the soil and are biodegraded on it. Because OMW natural destiny is soil, OMW recycling in soils need to have a particular interest on studies interested to OMW treatment. Moreover, along with its acidity and phenols load, OMW is rich of fertilizing

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elements such as organic matter, N, P, K and Mg (Casa et al., 2003; Rinaldi et al., 2003). OMW doesn't contain high loads of heavy metals nor pathogenic microorganisms.

Studies interested to OMW spreading to soils have well focused on the impact of spreading on physicochemical properties of soil (Di Giovacchino et al., 2001; Gamba et al., 2005; Mekki et al., 2006 ; Sierra et al., 2007 ; Jarboui et al., 2008). These studies revealed that spreading is beneficial to physicochemical characteristics of soil when applied doses are moderate and suitable with soil characteristics. When spreading is made at high doses, negative aspects such as immobilization of soil nitrogen have been noticed (Sierra et al., 2007).

However, less interest was given to understand the microbial aspects of OMW spreading to soil (Mekki et al., 2006) while microorganisms are a key element in biodegradability processes in soil. Studies interested to the microbial aspect of OMW spreading were generally limited to determination of soil respiration (production of CO_2) and at less extent the abundances of microbial groups. These studies showed that OMW spreading at reasonable doses in controlled conditions leads to an enhancement in soil microflora abundances (Mekki et al., 2006) and soil respiration (Kotsou et al., 2004; Gamba et al., 2005). However, spreading high amounts could cause a decrease in soil microorganisms' abundances and soil respiration (Mechri et al., 2007). OMW antimicrobial activity was mainly linked to OMW high amount of phenolic compounds which can vary from 0.5 g/l to 24 g/l depending on culture conditions in orchards, degree of ripeness of olive fruit, climatic conditions, storage conditions and olive oil extraction process (Ramos-Cormenzana et al., 1997; Casa et al., 2003 ; D'Annibale et al., 2004).

The aim of this work is to study the growth of soil fungi in OMW in presence and in absence of soil inert fraction. Growth will be tested in fresh and stored OMW since the effect of OMW storage before spreading is a factor generally neglected on OMW spreading experiments (Mechri et al., 2007). In this work we will check if the increase of soil microflora abundances observed in field experiments is caused actually by OMW. On the other side, we are looking for detecting the most sensitive fungi to OMW and to follow their growth in OMW. This study would highlight the protective function of the inert fraction of soil to microorganisms while OMW application to soil.

2. Material and Methods

2.1. OMW origin and characterization

In this study, fresh and stored OMW were used. Fresh OMW was just produced from the mill. Stored OMW was produced the previous year and was stored in closed tanks of 30 liters of capacity during 1 year at 4°C. Fresh and stored OMW were procured from the same olive mill press located in Fez-Dokkarat-Morocco.

Fresh and stored OMW were characterized for pH, COD (Chemical oxygen demand), Total phenols and phytotoxicity toward maize seeds germination, before and after their sterilization. COD was determined by a COD meter HACH according to the standard micromethod (Rodier, 1996). Total phenols were quantified by means of Folin-Ciocalteu colorimetric method (Box, 1983). For total phenols, the absorbance was determined at λ=750 nm. Toxicity of OMW toward maize (*Zea mays*) seeds germination was determined according to Casa et al. (2003). Maize seeds were disinfected (rinsing by NaOH 1%), washed, dried, then distributed in sterilized and papered plates as 64 seeds per plate of 9 cm of diameter. OMW was added to plates as 10 mm. Distilled water was added to control plates. Plates were then incubated in darkness at 4°C during 48 hours (vernalization) then at 20°C during 6 days. At the end of the 8 days of incubation, germinated seeds were counted in the different plates. Seeds are considered germinated when the embryo perforates the seed's epidermis. Each essay was done in quadruplicate.

2.2. Study site and sampling

The study area consisted in a field of Saïs valley-Morocco, a site containing 42% of Moroccan mills. The weather typical Mediterranean, semiarid to arid, with an average rainfall of 450 mm $year^{-1}$ and an average annual temperature of 18–20 °C. The field was divided to three plots of 5 m². Plots C, P_1, and P_2 were amended in April 16th with 0, 8, and 16 l/m² of Fresh untreated OMW, respectively. The soil is a lime constituted by 51.16± 5.88 % of sand, 32.56± 3.92 % of silt and 16.25± 1.96 % of clay. Soil characteristics are presented on table 1.

Table 1. Soil characteristics

Parameter	Value
pH (25°C)	7.82±0.04
Conductivity (25°C) (ms/cm)	0.21±0.01
Humification degree (%)	10.68 ±2.96
Viable microbiota (CFU/g of dry soil)	4.05 (10^5) ±7.77
Yeasts (CFU/g)	0.04 (10^4) ±0.008
Moulds (CFU/g)	0.09 (10^4) ±0.03

Since one month from the OMW spreading, soil samples were collected from four random locations in each plot in the top 10 cm of soil layer which is the most relevant to microflora activity (Mekki et al., 2006). For each plot, microbial abundances were followed monthly for a period of 6 months since spreading. All soil samples, taken from each plot were mixed, air-dried, sieved with a mesh size of 2 mm and stored at 4°C until use.

2.3. Microbial abundances determination

The determination of abundances of soil total viable microbiota (bacteria, archae, and fungi), yeasts and moulds was done by indirect enumeration of CFU (colony forming units) in solid mediums. 5 g of soil was suspended in 100 ml of physiologic solution (NaCl 0.85 %) added by 100 µl of Tween 20 (polyoxyethylenesorbitan monolaurate) then agitated during 30 min. Tween 20 would allow dissociation of microflora from soil particles. 10-fold dilutions in sterile physiologic solution (NaCl 0.85 %) were prepared and used to inoculate specific media plates, three plates for each dilution. Viable microbiota abundances were determined in TSA medium (Tryptone-Soja-Agar) (Biokar Diagnostics, France), yeasts in YPG medium (Yeast extract- peptone- glucose) added by ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml) and moulds in Malt extract agar (Biokar Diagnostics, France). Inoculated plates were incubated at 28°C. After 1, 3 and 7 days, abundances of bacteria, yeasts and moulds were determined, respectively.

2.4. Growth of soil microflora in fresh and stored OMW, in microcosms

For all growth essays, OMW was used without any dilution. OMW was first filtered many times through glass wool in order to avoid preferential orientations within microcosms. Microcosms were sterilized glass pyrex Erlenmeyers of 250 ml of capacity. OMW was sterilized by autoclaving (110 °C during 20 min) for constitution of microcosms where OMW is intended to be sterile. OMW (sterile or not sterile) was distributed in erlenmeyers at a rate of 100 ml each. Microcosms were prepared in duplicate.

To prepare inoculum of soil microflora, 50 ml of sterile nutritive broth (10 g tryptone, 5 g meat extract, 5 g NaCl in 1000 ml of distilled water) were inoculated by 2 g of soil then incubated 16 hours at 28 °C under moderate agitation (150 rpm). After incubation, the culture was aseptically centrifuged at 4500 g during 15 min in sterile 15 ml tubes full to 10 ml of their volume. The supernatant was eliminated and the precipitate was suspended in sterile physiologic solution (NaCl 0.85 %) then the suspension was centrifuged. This washing was repeated three times and after the last centrifugation, the base was suspended in 5 ml of sterile physiologic solution, and the inoculum was so constituted. The average abundances of viable microbiota, yeasts and moulds in the inoculum are respectively 1.5 10^7 CFU/ml, 9.8 10^5 CFU/ml and 5.6 10^5 CFU/ml. 2 ml of the inoculum were added to each microcosm. Microcosms prepared for different essays were as follow:

- Fresh OMW not sterilized and not inoculated by soil microflora;
- Fresh OMW not sterilized and inoculated by soil microflora;
- Fresh OMW sterilized and inoculated by soil microflora;
- Stored OMW not sterilized and not inoculated by soil microflora;
- Stored OMW not sterilized and inoculated by soil microflora;
- Stored OMW sterilized and inoculated by soil microflora.

Microcosms were incubated in darkness at 20 °C. Microbial abundances of total microbiota, yeasts and moulds were followed-up for a period of 15 days and were determined by indirect enumeration of CFU as previously cited. Abundances were determined in the days: d_0 (just after inoculation), d_1, d_4, d_6, d_9, d_{12} and d_{15}. For this, 1 ml sampled from each microcosm and was used to prepare 10-fold dilutions in a 0.85% NaCl solution to inoculate plates of suitable media. Plates were then incubated at 28 °C.

2.5. Statistical analyses

Statistical analyses were conducted using prism pad, version 4 software. Student test was used to compare means ((P<0.05). ANOVA one way allowed testing significance of the difference between two growth profiles. The microbial abundances in growth essays underwent a logarithmic transformation (log 10) before their presentation on graphs. The evolution of abundances of the studied microbial groups was compared to the Monod theory: $\mu=\mu_{max}S/K_s+S$ (µ: growth rate; µmax: maximum growth rate; S: limiting substrate concentration; Ks: concentration of substrate for which the growth rate is half-maximum.

3. Results

3.1. Effect of OMW spreading on abundances of soil microflora

Microbial counts of total microbiota, yeasts and moulds were followed during 6 months since OMW spreading. OMW spreading at 8 l/m² and 16 l/m² caused an increase in abundances of the studied groups (Figure 1). The load of increase in abundances depended on the microbial group and on the dose spread. For viable microbiota which gives information about the totality of soil microflora, when the dose spread was 8 l/m² (P_1) the abundances increased progressively since the spreading and reached a maximum value of 8.76 10^6 CFU/ g of soil after three months from spreading while the control had a value of 4.01 10^5 CFU/ g of soil (Figure 1a).The same tendency was obtained for yeasts and moulds and the maximum of abundances was obtained after three months for yeasts and after four months for moulds (Figure 1b and Figure 1c). When the dose spread was 16 l/m², abundances of viable microbiota, yeasts and moulds started increasing lately in comparison with the lower dose 8 l/m² (Figure 1a).

The increase in abundances of the studied microbial groups was followed by a decrease what should be due consumption of OMW organic matter. The profile of growth follows the Monod theory: $\mu=\mu_{max}S/K_s+S$ (µ: growth rate; µmax: maximum growth rate; S: limiting substrate concentration (OMW organic matter); Ks: concentration of substrate for which the growth rate is half-maximum. OMW organic matter is mainly constituted by carbohydrates, aliphatics, phenolic compounds and fats (El Hajjouji et al., 2008).

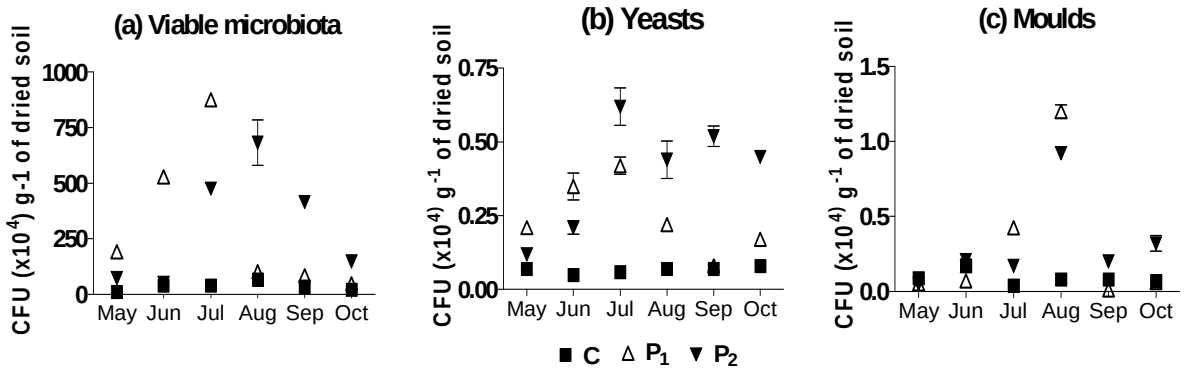

Figure 1. Effect of OMW spreading to soil at 8 l/m² (P_1) and 16 l/m² (P_2) on abundances of soil viable microbiota (a), yeasts (b) and moulds (c)

3.2. Effect of storage on OMW characteristics

OMW storage for 1 year at 4°C caused a significant decrease (p<0.05) of 16.38% of phenolic compounds and 15.31% of COD (Table 2). OMW stored 1 year at 4°C became 100% toxic to maize seeds germination in comparison with fresh OMW which was toxic for only 38.05% (Table 2). OMW pH was not significantly affected by storage.

Table 2. Effect of storage for 1 year at 4°C on OMW characteristics. Letters (a,b) indicate statistical difference at 0.05 level.

	Before storage	After storage
pH	4.70 ± 0.11a	3.98 ± 0.55a
Total phenols (g/l)	18.92 ± 0.98a	15.82 ± 1.21b
COD (gO2/l)	130.6 ± 2.46a	110.68 ± 9.44b
Phytotoxicity (% of germination)	61.95 ± 5.43a	0.00 ± 0.00b

3.3. Effect of sterilization on OMW characteristics

OMW sterilization had as a main consequence a significant decrease of COD and phenolic compounds for fresh OMW (Table 3A). For stored OMW, we only obtained a decrease in phenolic compounds without decrease of COD (Table 3B). Sterilization didn't have any significant effect on OMW pH and toxicity toward maize seeds germination. As shown in Table 3A and Table 3B, sterilized OMW was characterized by a low pH unusual to soil microflora (soil pH= 7.82) and by a high load of phenolic compounds and organic matter.

Table 3. Effect of sterilization on characteristics of fresh (A) and stored (B) OMW. Letters (a,b) indicate statistical difference at 0.05 level.

(A)

	Before sterilization	After sterilization
pH	4.64 ± 0.05a	4.74 ± 0.07a
Total phenols (g/l)	17.49 ± 0.13a	12.45 ± 0.09b
COD (gO_2/l)	124.42 ± 1.95a	105.06 ± 0.33b
Phytotoxicity (% of germination)	59.35 ± 6.67a	57.00 ± 3.51a

(B)

	Before sterilization	After sterilization
pH	3.98 ± 0.55a	4.28 ± 0.02a
Total phenols (g/l)	15.82 ± 1.21a	10.49 ± 0.18b
COD (gO_2/l)	110.68 ± 9.44a	108.75 ± 5.86a
Phytotoxicity (% of germination)	0.00 ± 0.00a	0.00 ± 0.00a

3.4. Growth of soil microflora in OMW, in microcosms

Raw OMW, fresh or stored, had its own indigenous microflora (Figure 2a, Figure 3a). Since its incubation at 20°C, fresh OMW grew rich out of yeasts (Figure 2a). Indeed, after one day of incubation, abundances of OMW yeasts significantly increased from 2.04 10^6 CFU/ml to 2.95 10^7 CFU/ml. No increase of abundances was obtained for moulds.

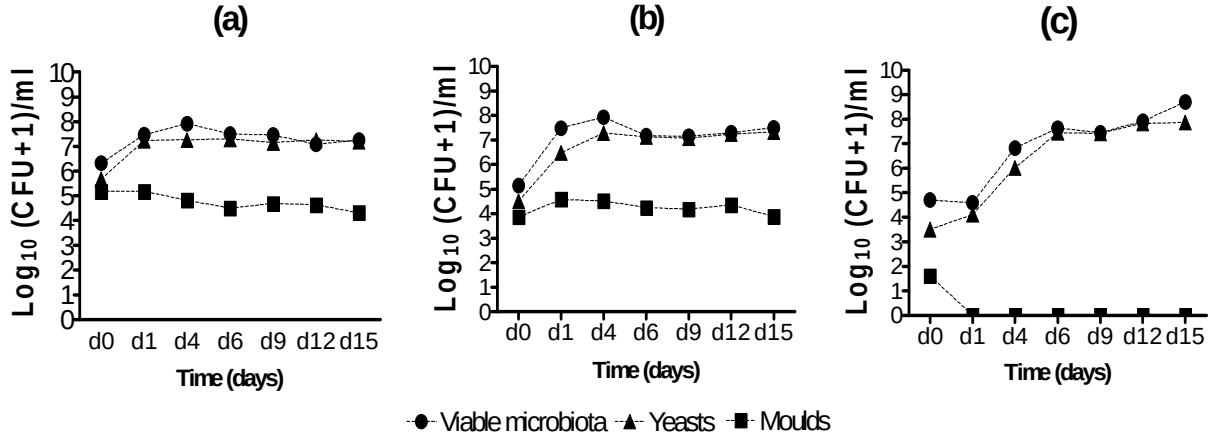

Figure 2. Growth of soil viable microbiota, yeasts and moulds in fresh OMW, in microcosms; (a): fresh OMW not sterilized and not inoculated by soil microflora, (b): fresh OMW not sterilized and inoculated by soil microflora, (c): fresh OMW sterilized and inoculated by soil microflora.

Raw sterilized OMW, fresh or stored, was a biotope where groups of soil microflora could grow (Figure 2c, Figure 3c). In fresh sterilized and inoculated OMW (Figure 2c), soil yeasts survived and showed an important and fast increase of abundances. The profile of growth of soil yeasts in OMW showed a phase of latency of nearly one day, a phase of exponential growth which lasted nearly 6 days during which the growth rate reached a maximum ($\mu=\mu_{max}$) and a stationary phase during which there is compensation between multiplication and mortality of microorganisms ($\mu\approx0$). In stored OMW, growth of soil yeasts showed a profile of fast reduction then survival at low level.

Soil Moulds were a sensitive group to both fresh and stored OMW (Figure 2c and Figure 3c). Indeed, just after inoculation of fresh sterilized OMW with soil microflora, soil moulds abundances were lower than 10 CFU/ml. Soil moulds disappearance was faster when inoculated OMW is the stored one and disappearance happened immediately after contact between inoculum and OMW (Figure 3c).

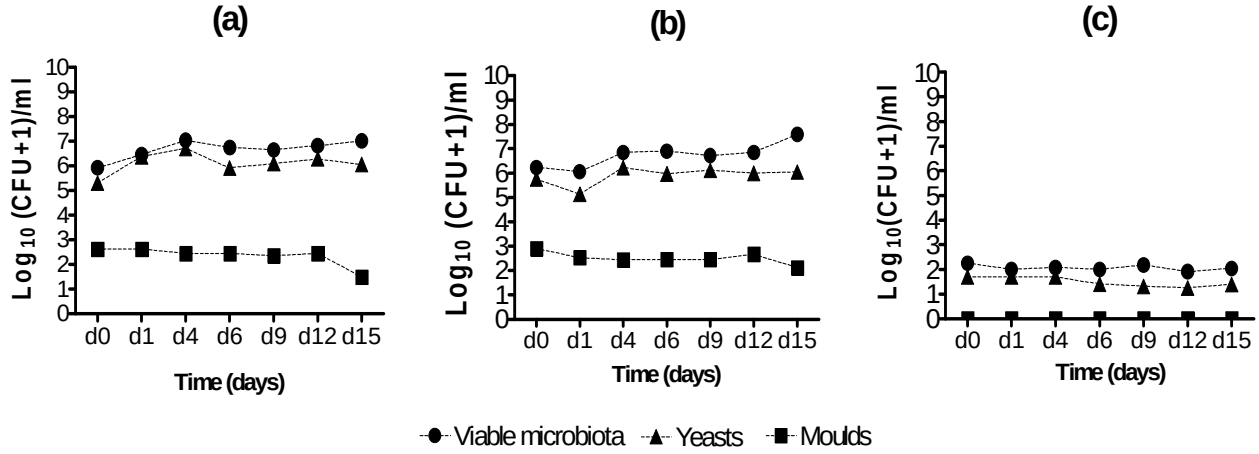

Figure 3. Growth of soil viable microbiota, yeasts and moulds in OMW stored 1 year at 4°C, in microcosms; (a): stored OMW not sterilized and not inoculated by soil microflora, (b): stored OMW not sterilized and inoculated by soil microflora, (c): stored OMW sterilized and inoculated by soil microflora.

4. Conclusions

OMW spreading to field at doses 8 l/m² and 16 l/m² caused an increase in abundances of total microbiota, yeasts and moulds what agree with previous findings (Kotsou et al., 2004; Gamba et al., 2005; Mekki et al., 2006). The increase in abundances started after a phase of latency which was longer when the spread dose was higher. The latency phase could be a phase of adaptation of microorganisms to the new substrate which is OMW applied to soil (Tomati and Galli, 1992) or could be a consequence of unavailability of OMW organic matter to microflora under adsorption or reaction with soil (Mekki et al., 2006). Some compounds in OMW especially phenols and organic acids may inhibit the soil microorganisms especially in the high doses and neutralize the favorable influence of OMW high nutrient content (Ramos-Cormenzana et al., 1997; Mekki et al., 2006 ; Sierra et al., 2007; Capasso et al., 1995). The increase of abundances was temporary and followed the Monod Law what was also reported by Kotsou et al. (2004) showing that the microbial abundances in soil receiving OMW increase to reach a top then start decreasing.

OMW storage at 4°C during 12 months caused a reduction of OMW phenols and organic matter what would be mainly assured by OMW indigenous psychrophilous microorganisms (Vavilin et al., 2000). A reduction of the load of phenols and COD of OMW after storage was reported. OMW storage under field conditions for 3 months has as result a reduction of 10% of COD and 20.3% of phenolic compounds (Saadi et al., 2007). According to previous studies, a reduction of OMW load of organic matter and phenolic compounds is linked to a reduction of OMW toxicity (Casa et al., 2003; D'Annibale et al., 2004). This was not in agreement with our findings showing that the toxicity of stored OMW increased. A prolonged storage of OMW is favorable for the transformation of oxidation stat of phenolic compounds what could increase their toxicity (Field et Lettinga, 1989). However, OMW storage under field conditions for less than four months was considered as a pretreatment (Borja et al., 1995 ; Marrara et al., 2002). According to our results, a prolonged storage of OMW at low temperatures should be defective to its interest as fertilizer.

OMW sterilization caused a significant diminution of COD and phenolic compounds for fresh OMW (Table 3A) and only a reduction of phenolic compounds without COD for stored OMW. This result should be explained by the fact that for stored OMW, available organic matter that could be easily oxidized during sterilization was consumed during storage, so that we did not obtain a reduction of COD after sterilization. This result should also show that the COD measurement is not relevant for phenolic compounds because even that we obtained a decrease on their load the COD was not reduced.

Raw OMW, fresh or stored, had its indigenous microflora having enzymatic devices allowing these microorganisms to grow in OMW as an only source of carbon (El Hajjouji et al., 2008). After incubation of fresh OMW at 20°C, abundances of indigenous yeasts increased but not those of moulds what showed that OMW would constitute a more adequate biotope for survival of OMW yeasts than moulds. Soil yeasts showed an exponential growth profile in sterilized OMW. This result show that soil yeasts are r-selected species involving first in metabolization of OMW available organic matter (Kotsou et al., 2004). Yeasts are efficient in biodegradation of labile organic matter of OMW constituted mainly by sugars, urea and aliphatic acids (El Hajjouji et al., 2008). It was demonstrated that yeasts isolated from different habitats are able to grow and multiply in OMW (Lanciotti et al., 2004 ; BenSassi et al., 2007). In stored OMW, growth of soil yeasts showed a profile of fast reduction then survival at low level which is one of the typical growth profiles in stressful media (Inamori et al., 1992).

Soil Moulds were a sensitive group to both fresh and stored OMW. OMW antimicrobial activity toward moulds was previously announced. Tardioli et al. (1997) showed that the soil common moulds *Scopulariopsis brevicaulis* and *Cladosporium cladosporioides* are unable to grow in OMW for a dilution higher than 50%. Rubia et al. (2008) demonstrated that the OMW phenolic compound tyrosol is highly inhibiting the development of moulds mycelium. On the other hand, OMW acidity and its high load of antioxidant phenolic compounds and fats should be inhibitor to germination of moulds spores. A reduction in moulds abundances after spreading a high dose of OMW was reported (Mekki et al., 2006a), however, a disappearance of moulds from soil amended by OMW was never reported, as we know. OMW spreading at high amounts should be avoided in practices of OMW spreading to protect soil moulds' homeostasis since this group set up a paramount role in lignin degradation in soil (Evelyn et al., 2005). OMW toxicity toward soil moulds would lead us to note that during an OMW spreading to soil, the increase in moulds' abundances (Tardioli et al., 1997 ; Mekki et al., 2006 ; Mechri et al., 2007 ; Figure 1) would correspond to an increase in abundances of OMW's moulds added to soil. Increases of moulds abundances during OMW spreading would also be due to multiplication of soil moulds if it's admitted that the inert fraction of soil play a protective part to its microorganisms. Indeed, it is reported that sediment's granules are a protective microbiotope for microorganisms against physical and biological aggressions of the medium (Davies et al., 1995) and that survival of a microbial population is better in presence of a support allowing fixing microorganisms (Maunoir et al., 1990). Also, it is demonstrated that moulds are more performing when they are cultivated in immobilized conditions rather than in free culture (Kim and Shoda, 1999).

Evolution of abundances of soil microbial groups following inoculation of fresh, not sterilized OMW (Figure 2b) did not show a profile which is plurality of profiles of abundances obtained in not sterilized not inoculated OMW (Figure 2a) and sterilized inoculated OMW (Figure 2c). This result would be explained by intervention of other biological phenomena such as competition between OMW microorganisms and soil microorganisms in favor to OMW microorganisms since competition is considered as a primordial factor controlling growth of microorganisms in a biotope (Brandi et al., 1996).

From this study, we can conclude that OMW has an indigenous population of fungi where yeasts are more abundant than moulds. Soil yeasts are able to grow and multiply in fresh not diluted OMW without addition of nutrients or treatment except sterilization. Soil moulds are a sensitive group to OMW antimicrobial activity. The antimicrobial activity of OMW and its toxicity toward maize seeds germination is highly important when OMW is stored 1 year at 4°C. From this study, we can recommend that OMW spreading to soil should be an interesting way of its valorization, since after spreading we obtained an increase in abundances of total microbiota, yeasts and moulds recycling the OMW. However, spreading high doses or a long time stored OMW is not recommended since it could threaten soil fungi homeostasis.

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