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ARTICLE

Original and Residual Phytotoxicity of Olive Mill Wastewater Revealed by Fractionations before and after Incubation with *Pleurotus ostreatus*

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Concentrations of dissolved organic carbon (DOC) and total phenols (TP), and the phytotoxicity to cress (*Lepidium sativum* L.) were determined for three molecular-sized fractions of olive mill wastewater (OMW), <1000, 1000−5000, and >5000 Da, before and after incubation with *Pleurotus ostreatus*. The <1000-Da fraction contained 82% of the total DOC and 48% of the TP, and was the most phytotoxic. Ethyl acetate separation of aqueous and solvent fractions showed that the aqueous fraction contained 93% of the total DOC, 83% of the TP, and was most phytotoxic, indicating low importance of monomeric phenols. Incubation of whole OMW and of the separate size fractions with *P. ostreatus* mycelia reduced TP by factors of 4.3−5.3, but exerted diverse impact on phytotoxicity; overall, *P. ostreatus* efficacy in organic load removal and OMW detoxification was limited. Additional size fractionation of the incubated fractions revealed that most residual phytotoxicity was associated with low-molecular weight (MW) compounds originated from the <1000 Da fraction and not with low-MW byproducts from the degradation of higher-MW fractions and that polymerized metabolites were nonphytotoxic. Total phenols should not be used as sole indicators of the successful remediation of OMW.

KEYWORDS: OMW (olive mill wastewater); fractionation; phytotoxicity; white rot fungi (*Pleurotus ostreatus*); polyphenols

INTRODUCTION

The recycling or disposal of olive mill wastes has become a major challenge in olive-oil-producing countries. Olive mill wastewater (OMW) contains high levels of chemical oxygen demand (COD) (40−220 g/L) and phenolic substances (0.5−10 g/L), is highly toxic to plants and to some microorganisms, and is produced in large quantities during the short olive harvest season October−December (*1*, *2*). The phytotoxicity associated with OMW constitutes one of the major difficulties regarding its remediation and disposal; therefore, it is of major importance to elucidate the origin of OMW phytotoxicity and the fate of its toxic substances during biological treatments.

OMW is a complex mixture of organic substances, such as sugars, fatty acids, and monomeric and high molecular-weight (MW) phenols (*3*). Several studies examined OMW by means of ethyl acetate extraction, which enabled monomeric phenols to be transferred into the organic ethyl acetate fraction to be isolated, identified, and quantified (*4*−*6*). Pure solutions of monomeric phenols (e.g., catechol and hydroxytyrosol) were found to be phytotoxic at concentrations similar to those found in OMW (*6*, *7*); therefore, monomeric phenols were considered as a major source of the phytotoxicity associated with OMW and dry olive residue (*7*, *8*). In contrast, Aliotta et al. (*9*) found that a synthetic solution that contained concentrations of monomeric phenols similar to those measured in OMW exhibited limited phytotoxicity as compared with that of the <300-Da fraction of OMW. Furthermore, examination of the phytotoxicity associated with the aqueous and ethyl acetate fractions, respectively, revealed higher phytotoxicity in the aqueous fraction (*10*). These results may suggest that the contribution of monomeric phenols to the overall phytotoxicity of OMW is relatively small.

Aerobic processes were previously investigated as viable biological treatments for OMW (*11*−*13*). Among various candidate microorganisms, white rot fungi were extensively considered because their ligninolytic system is capable of degrading phenolic compounds (*14*−*16*), and they were shown to substantially reduce the content of TP in OMW (*17*−*19*). Several studies investigated the fate of monomeric phenols during aerobic treatment; they included close examination of the fraction of ethyl acetate into which monomeric phenols were extracted, and HPLC analyses revealed a substantial decrease in the phenols extracted by ethyl

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acetate (6, 8, 20). However, despite substantial decreases in both total and monomeric phenols, the corresponding reductions in phytotoxicity varied, and questions arose regarding the nature and fates of phytotoxic substances in these degradation processes (16, 21). Two possible explanations were suggested: (1) that substances other than phenols made significant contributions to the overall phytotoxicity of OMW, substances that might not be degraded during the treatment process and therefore would contribute to the residual phytotoxicity (9, 20); (2) incubation of OMW with white rot fungi might form new toxic metabolites, which could contribute a significant part of the residual phytotoxicity (16, 21). It was shown that oxidation of phenolic compounds caused MW redistribution by parallel polymerization and depolymerization reactions (14, 22, 23).

To address such inconsistent findings regarding the relationship between phenolic compounds and phytotoxicity, the present study aimed to evaluate the relative contributions of various fractions to the overall phytotoxicity of OMW, before and after incubation with *P. ostreatus*. Fresh OMW was fractionated according to three molecular sizes, < 1000, 1000−5000, and > 5000 Da, and was separated into aqueous and solvent fractions with ethyl acetate. Concentrations of dissolved organic carbon (DOC) and total phenols (TP), and the phytotoxicity to cress (*Lepidium sativum* L.) were determined in all fractions. Additional fractionations were performed on each of the incubated fractions in order to assess the residual phytotoxicity contributed by metabolites formed through polymerization and degradation pathways.

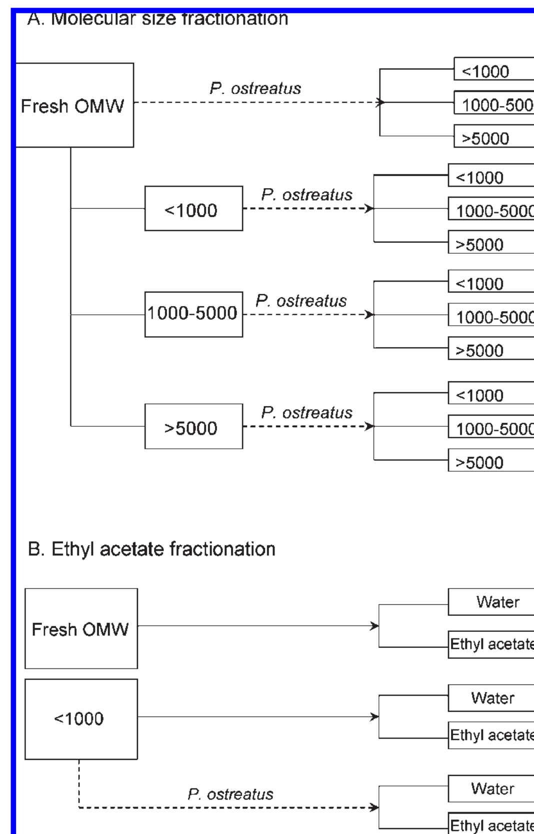

Figure 1. Fractionation scheme for OMW, before and after incubation with *Pleurotus ostreatus*.

MATERIALS AND METHODS

Olive Mill Wastewater (OMW). Fresh OMW generated from olives of cv. Souri by the three-phase extraction process was kindly provided by the Iksal Olive Mill (Nazareth, Israel); it was filtered through a 0.4-μm prewashed polycarbonate filter (Whatman, Brentford, UK) and stored at 4 °C. The filtered OMW had the following characteristics: pH 5.1 (Hanna electrode; HI 1332B), EC 14.8 dS/m (MC126, Mettler, Toledo), DOC 28.2 g/L (see Chemical Analyses), TP 4.8 g/L (see Chemical Analyses), and total Kjeldahl nitrogen 531 mg/L (using Nessler reagent). A stock filtered OMW was lyophilized (Christ, alpha 1−4, Germany) within 1 week after production and stored in a desiccator. For use in ongoing experiments, lyophilized OMW was redissolved in water to achieve the initial volume. DOC recovery after lyophilization and redissolution was 92%, and the phytotoxicity of this solution (see Phytotoxicity Bioassays) was similar to that of fresh OMW.

OMW Fractionations. The OMW was fractionated before and after incubation with *P. ostreatus*, according to the scheme illustrated in **Figure 1**.

MW-Based Fractionation. Fresh OMW was fractionated according to Raber and Kögel-Knabner (24), with slight modifications, using Cellu Sep H1 cellulose dialysis membrane tubes (Membrane Filtration Products, San Antonio, TX, USA) with nominal molecular-weight cutoffs (MWCOs) of 1000 and 5000 Da. The tubes were prewashed for 4 h with deionized water and then rinsed thoroughly with deionized water until the content of organic carbon in the membrane effluents was below the detection limit. The washing volumes were similar to those used in the actual fractionation procedure. A 75-mL aliquot of filtered OMW was poured into a prerinsed 1000-MWCO dialysis tube that was then placed in a beaker containing 1500 mL of distilled water at 10 °C, which was stirred continuously in the dark. The dialysate was renewed twice, each time after 24 h, and the total of 4500 mL was lyophilized down to a volume of 375 mL, in order to achieve a 5-fold dilution of the original volume of 75 mL. This lyophilized dialysate contained the < 1000-Da fraction. The contents of the dialysis tube were then transferred into a prerinsed 5000-MWCO dialysis tube and subjected to the same treatment. The dialysate of this step, which contained the 1000−5000-Da fraction, was also lyophilized down to 375 mL to achieve the same dilution factor. The remaining contents of the 5000-MWCO tube contained the > 5000-Da fraction, and this was also diluted 5-fold to achieve the same dilution factor as the two other fractions. All fractions were analyzed for DOC, TP, and phytotoxicity. Mass recovery was 76 and 102% for DOC and TP, respectively.

The same fractionation procedure was applied to the whole-OMW and to each molecular-sized fraction, after incubation with *P. ostreatus* (see Incubation with *Pleurotus ostreatus*). At the end of the incubation period, the contents of replicate vessels were combined, and 10-mL aliquots were taken and dialyzed as described above. The dialysate volumes in this case (200 mL) were lyophilized down to the original volume (10 mL). This additional fractionation yielded three molecular-sized fractions from each fraction (**Figure 1**), and all fractions were analyzed for DOC, TP, and phytotoxicity. Mass recoveries of DOC and TP were 76−121 and 40−144%, respectively. Since no correlation was observed between DOC and TP recoveries, low TP recoveries cannot be attributed to the loss of DOC during fractionation. The reason for the wider variability in TP recoveries obtained in the fractionation after incubation with *P. ostreatus* is unknown.

Fractionation by Ethyl Acetate. Ten milliliters of fresh OMW was sequentially extracted three times with 10 mL of ethyl acetate (1:1 v:v) using a separatory funnel. The three sequential extracts were combined and evaporated under vacuum (Zymark TurboVap, USA), and the solid residue was redissolved in 10 mL of water. Residues of ethyl acetate in the aqueous fraction and in the redissolved ethyl acetate fraction were allowed to evaporate in the hood for 1 day to eliminate the interference of ethyl acetate with the phytotoxicity bioassay.

The same extraction procedure was performed on the < 1000-Da fraction before (untreated) and after (treated) incubation with *P. ostreatus* (**Figure 1**). All fractions were analyzed for DOC, TP, and phytotoxicity. Mass recovery was 79−93 and 72−80% for DOC and TP, respectively.

Incubation with *Pleurotus ostreatus*. *P. ostreatus* (Florida f6 ATCC 58053) was maintained on Petri dishes containing 2% agar basidiomycete synthetic medium (BSM) as described by Ardon et al. (25), with the addition of 20% of filtered OMW. Preliminary experiments showed that acclimatization on agar with 20% OMW enhanced fungal growth in the subsequent liquid media that contained OMW (data not shown).

Incubation experiments were conducted on sterilized OMW solutions (121 °C, 20 min) of whole-OMW and of the three molecular-sized fractions, which were diluted 5-fold relative to their original DOC concentrations. Aliquots of 50 mL were transferred into four replicate 250-mL sterile Erlenmeyer flasks for each treatment, inoculated with *P. ostreatus* mycelium by inserting eight 6-mm-diameter agar plugs into each flask, without prior pH adjustment, and incubated on a rotary shaker at 100 rpm for 15 days at 28 °C. Vessels containing sterile, noninoculated OMW, also diluted 5-fold, served as controls. After incubation, the whole-OMW and each fraction were fractionated again into three molecular-sized fractions as described above. The pH, DOC, TP, and phytotoxicity were monitored during incubation.

In a separate experiment, the smallest-sized fraction (< 1000 Da) was incubated with *P. ostreatus* under the same conditions. This fraction was further fractionated with ethyl acetate before and after incubation. The DOC, TP, and phytotoxicity were measured in both fractions before and after incubation.

Chemical Analyses. Total phenols (TP) were analyzed after Box (*26*), with slight modifications, using 0.5 mL of Folin−Ciocalteau reagent (Merck) and 3 mL of 20% sodium bicarbonate in a total volume of 10 mL. Absorbance was measured at 725 nm (Cary 50 Bio, Varian) against a blank of deionized water and Folin reagent. Concentrations of TP were determined by comparison with a calibration curve obtained for caffeic acid (Merck). Analyses were performed on three subsamples except for the incubation experiments, in which one sample was withdrawn from each replicate vessel. Measurements of DOC were conducted with a TOC-5000 total organic carbon analyzer (Shimadzu, Japan) after acidification of samples to pH < 2 with 2 N HCl and subsequent purging. The presented DOC values are averages of two readings with standard deviation of < 2%. Samples were acidified before storage to minimize DOC biodegradation.

Phytotoxicity Bioassays. Four-milliliter aliquots of the sample were placed on GF/A glass microfiber filters (Whatman, Brentford, UK) in 90-mm glass Petri dishes. Ten seeds of cress (*Lepidium sativum* L.) were placed in each dish, of which two were used for each sample, except in the incubation experiments where one dish was used for each replicate vessel. Control dishes contained filters with 4 mL of deionized water. The bioassay was conducted over 48 h in darkness at 25 °C. Dishes from different treatments were wrapped separately in polyethylene bags to prevent desiccation and exchange of volatiles among treatments. The results were expressed as the phytotoxicity index:

$$\text{phytotoxicity index} = 1 - \frac{\text{root length in sample}}{\text{root length in control}}$$

The bioassays were conducted with 10-fold dilutions of the original fresh OMW.

Statistical Analyses. Data were subjected to ANOVA, and mean values were subjected to the Tukey−Kramer Honestly Significant Difference (HSD) test ($p \leq 0.05$) by means of JMP software.

RESULTS

Fractionation of Fresh OMW. The distributions of DOC, TP, and phytotoxicity after fractionation are presented in **Figure 2**. The < 1000-Da fraction contained 82% of the DOC (**Figure 2a**; left bar), and this fraction was also significantly more phytotoxic than the two other fractions (**Figure 2c**). The > 5000-Da fraction had 5.5 times less DOC than the < 1000-Da fraction and was significantly less phytotoxic, despite its similar TP concentration (**Figure 2b**). It is noteworthy that the > 5000-Da fraction contained about half of the TP but was the least phytotoxic. Finally, the 1000−5000-Da fraction exhibited phytotoxicity similar to that of the > 5000-Da fraction, despite containing very low DOC and TP concentrations.

Fractionation with ethyl acetate placed 93% of the DOC and 83% of the TP in the aqueous fraction (**Figure 2a**,**b**; right bars), and phytotoxicity was also significantly higher in the aqueous than in the ethyl acetate fraction (**Figure 2c**). The higher DOC

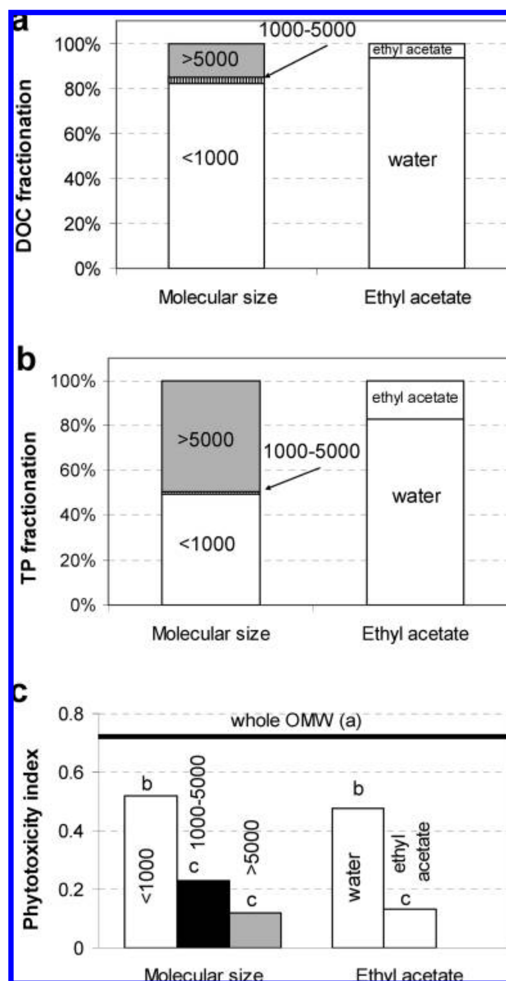

Figure 2. Fractionation of fresh (untreated) OMW: Distribution of DOC (**a**), TP (**b**), and phytotoxicity (**c**) among three molecular-sized fractions and between water and ethyl acetate fractions. DOC and TP are expressed as the percentage of each fraction. The phytotoxicity index is expressed as 1 − (root length in sample/root length in control). The toxicity of the whole OMW is indicated by a horizontal line. Letters represent significant differences between fractions (c) (α = 0.05).

concentration found in the aqueous fractions was to be expected because of the three-phase milling process, which eventually results in partitioning between the water (OMW) and the lipid phase (olive oil).

Incubation of OMW and Molecular-Sized Fractions with *P. ostreatus*. The dynamics of pH, DOC, TP, and phytotoxicity during the incubation period are presented in **Figure 3**. Control noninoculated vessels did not show consistent changes in pH, DOC, or phytotoxicity. Slight TP removal, by a factor of 1.2, was observed in control vessels, possibly as a result of some precipitation. All other inoculated treatments showed some increase in pH (**Figure 3a**), small removal of DOC (**Figure 3b**), substantial removal of TP (**Figure 3c**), and varying removal of phytotoxicity (**Figure 3d**). In all treatments TP concentrations were substantially reduced to less than 1 g/L, i.e., by factors of 4.3−5.3, mostly within the first 5 days of incubation. Although TP was substantially reduced, the DOC was only partly removed, with the greatest mass removal (31.3%) in the whole-OMW treatment. Phytotoxicity of the whole-OMW was partly but significantly reduced: the phytotoxicity index decreased from 0.73 to 0.53. The > 5000-Da fraction, which initially was the least phytotoxic, did not show any phytotoxicity after incubation. Phytotoxicity of the < 1000-Da and the

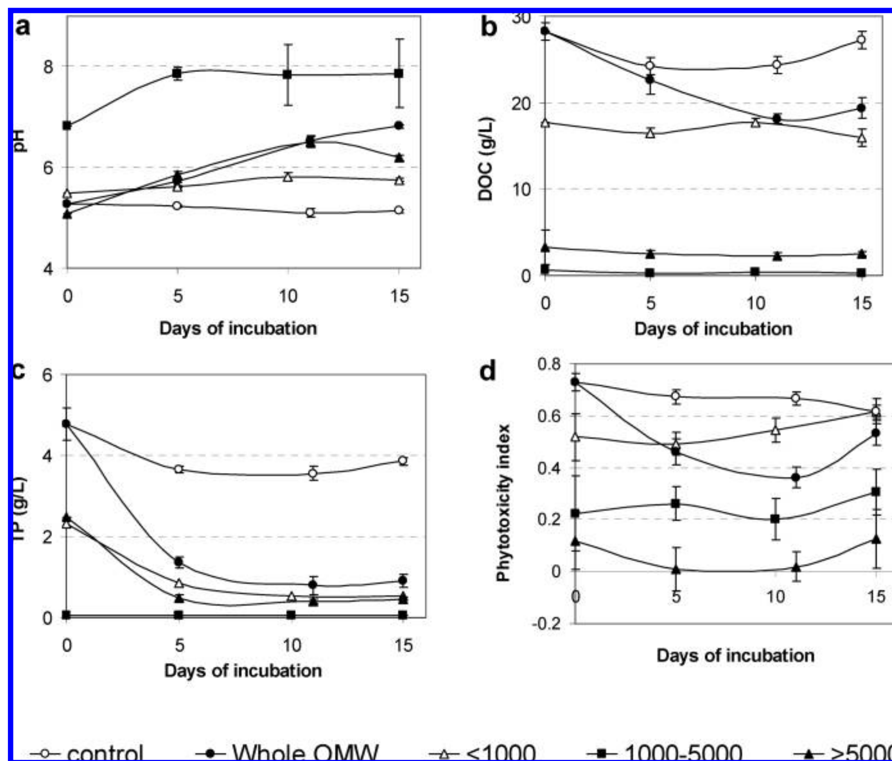

Figure 3. Dynamics of pH (**a**), DOC (**b**), TP (**c**), and phytotoxicity (**d**) of whole-OMW and of three molecular-sized fractions during incubation with *Pleurotus ostreatus*. The phytotoxicity index is expressed as 1 − (root length in sample/root length in control). Error bars represent the standard deviations of four replicate vessels (**a**, **b**, and **c**) and the standard errors of 40 seeds (cress; *Lepidium sativum* L.) (**d**).

1000−5000-Da fractions significantly increased with phytotoxicity index increases from 0.52 to 0.62 and from 0.22 to 0.30, respectively, for the <1000-Da and the 1000−5000-Da fractions. However, these trends were not consistent during the course of the incubation period.

Additional Fractionations after Incubation with *P. ostreatus*. The distributions of DOC, TP, and phytotoxicity in each of the incubated fractions are presented in **Figure 4**. The formation of polymerization metabolites was evidenced by the transition of some of the DOC and TP from smaller to larger molecular-sized fractions (**Figure 4a**,**b**). In parallel, the formation of degradation metabolites was evidenced by the transfer of DOC and TP from larger to smaller molecular-sized fractions.

Degradation metabolites which originated from larger-sized fractions showed very weak phytotoxicity, with phytotoxicity indexes ranging from 0.08 to 0.16. No evidence of phytotoxicity was found in >5000-Da metabolites that derived from all incubated size fractions; they even enhanced root growth. Notably, the <1000-Da fractions that originated from the whole-OMW and from the <1000-Da fraction were significantly the most phytotoxic.

The <1000-Da fraction was further fractionated with ethyl acetate before and after incubation with *P. ostreatus* (**Figure 5**). Before incubation (untreated), the distribution of DOC and TP between the aqueous and the solvent fractions was similar to that for whole-OMW, i.e., most of the DOC and TP were found in the aqueous fraction. After incubation (treated), DOC contents were slightly reduced in both the aqueous and the solvent fractions, but the TP contents were substantially reduced, by factors of 3.0 and 14.1 in the aqueous and the ethyl acetate fractions, respectively. Despite substantial removal of TP, the phytotoxicity of the aqueous fraction was not reduced and was even enhanced, whereas that of the solvent fraction was totally removed.

DISCUSSION

The relationship between DOC and TP contents, and the phytotoxicity of OMW was explored in two experimental systems: (1) fractions of fresh (untreated) OMW, obtained by molecular-size separation and by extraction with ethyl acetate; and (2) fractions of treated OMW, obtained after incubation with *P. ostreatus*. These two systems were examined in order to identify the fractions that carry most of the *original* phytotoxicity in OMW and the location of *residual* phytotoxicity after biological treatment. Investigation of these two systems also provided more insight into the potential of *P. ostreatus* to detoxify OMW.

Previous studies emphasized the roles of certain monomeric phenols in the toxicity of OMW (*4*, *7*, *27*). The focus on monomeric phenols was presumably related to the extensive use of ethyl acetate as an extraction solvent (*6*, *22*, *28*), although only limited phytotoxicity was found in a synthetic solution containing monomeric phenols at concentrations similar to those found in OMW (*9*). In a recent study (*29*), the phytotoxicity of OMW was measured in the aqueous (exhausted) and ethyl acetate fractions, after treatment with biotic and abiotic oxidative catalysts. Phytotoxicity was shown to be associated with monomeric phenols; yet, residual phytotoxicity was measured in the exhausted fractions as well as in treated samples containing negligible or non-detectable monomeric phenols.

The present study provided additional evidence for the limited contribution of monomeric phenols to the overall phytotoxicity of OMW. Only slight phytotoxicity was associated with the solvent fractions as compared with that in the aqueous fractions, although monomeric phenols were effectively extracted into ethyl acetate (ref *20* and Aviani, I., unpublished data). This was repeatedly observed for the fractions obtained from the whole-OMW and from the <1000-Da fraction, before and after incubation with *P. ostreatus*. Partial contribution of monomeric phenols to the overall phytotoxicity might suggest the

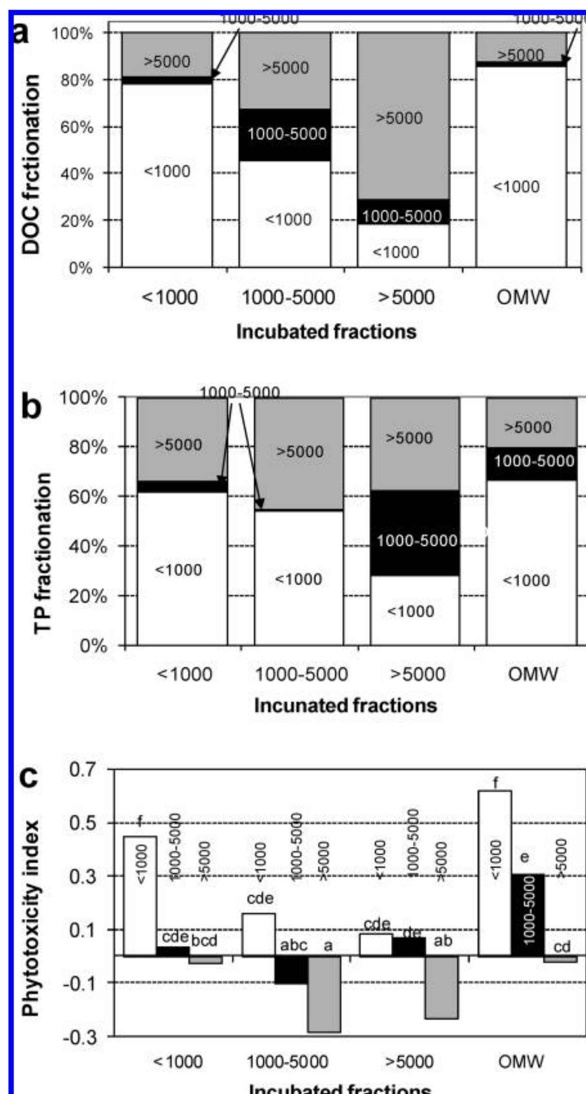

Figure 4. Additional fractionation of the incubated whole-OMW and of three molecular-sized fractions: Distributions of residual DOC (**a**), TP (**b**), and phytotoxicity (**c**). DOC and TP are expressed as the percentage of each incubated fraction. The phytotoxicity index is expressed as 1 − (root length in sample/root length in control). Letters represent significant differences between fractions (c) (α = 0.05).

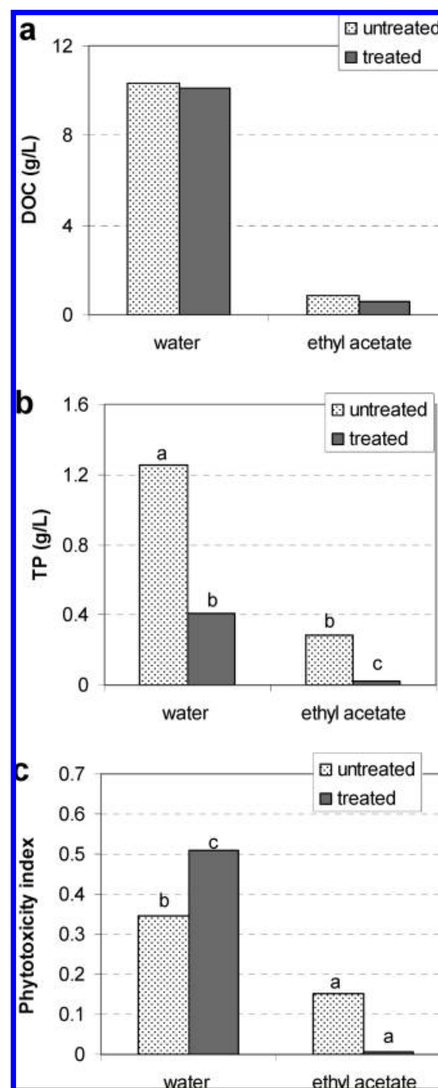

Figure 5. Fractionation of the untreated and treated <1000-Da fraction: Distribution of DOC (**a**), TP (**b**), and phytotoxicity (**c**) among ethyl acetate and water fractions before (untreated) and after (treated) incubation with *Pleurotus ostreatus*. The phytotoxicity index is expressed as 1 − (root length in sample/root length in control). Letters represent significant differences between fractions (c) (α = 0.05).

contribution of polyphenols of higher MW, but nevertheless smaller than 1000 Da (e.g., phenolic dimers and trimers which may not be extracted by ethyl acetate) or other nonphenolic organic compounds. A relatively small contribution of monomeric phenols to the overall phytotoxicity also could be related to the synergistic effects of several compounds, none of which makes a major contribution to the overall phytotoxicity.

These findings emphasize the significance of the < 1000-Da molecules that are not extractable by ethyl acetate as a primary source of phytotoxicity in OMW. This is consistent with several previous reports: The concentration of TP was shown to be higher by factors of 2.5 to 7 in the aqueous than in the ethyl acetate fraction obtained from OMW and dry olive mill residue (*10*, *20*, *30*); phytotoxicity was found to be the highest in the < 1000-Da fraction (*9*); and higher phytotoxicity was found in the aqueous than in the ethyl acetate fraction obtained from extracts of dry olive mill residue (*10*).

In the present study, incubation with *P. ostreatus* caused substantial TP reduction (by factors of 4.3 to 5.3) in the whole-OMW and the three molecular-sized fractions, despite relatively low DOC removal and diverse decreases in phytotoxicity. Similar results were obtained in previous studies in which aerobic treatments were effective in removing TP, but without corresponding reductions in phytotoxicity (*16*, *17*, *20*). In light of the findings of the present study, the > 5000-Da fraction can partially account for the inconsistent correspondence between removals of TP and of phytotoxicity. This fraction contained about half of the TP, but was least phytotoxic. The separate incubation of the > 5000-Da fraction with *P. ostreatus* caused a substantial decrease of TP by a factor of 5.3. However, since the initial phytotoxicity of this fraction was relatively low, the substantial TP decrease had a minor effect on the residual phytotoxicity.

Incubation of OMW with the fungus resulted in a redistribution of DOC and TP. Both degradation and polymerization metabolites were revealed by the additional MW fractionation of the *P. ostreatus*-incubated OMW fractions. Redistribution of TP could be facilitated through the following mechanisms: (1) oxidation of phenolics by laccase to quinonoids and phenoxy radicals which can then undergo nonenzymatic coupling and subsequent polymerization (*6*); (2) secretion of a reducing enzyme

that inhibits the nonenzymatic coupling of laccase oxidation products, as shown for *P. ostreatus by* Marzullo et al. (*31*). The results of the present study indicate the formation of non-phytotoxic polymerization products or slightly phytotoxic degradation metabolites. The majority of the phytotoxicity in treated OMW is related to low-MW substances which originated from the <1000-Da fraction and not from higher-MW fractions, which showed weak or no phytotoxicity, both before and after biological treatment. Treated high-MW fractions even enhanced root growth. The high-MW fraction in OMW, a metal polymeric organic fraction named Polymerin (*3*), was found to be only slightly phytotoxic. Polymerin was also detected in dry olive mill residue extract, and it elicited enhanced root activity after treatment with saprobic fungi (*8*).

In summary, water-soluble organic molecules that were smaller than 1000 Da and were not extractable by ethyl acetate constituted the most phytotoxic fraction in OMW, both fresh and after treatment with *P. ostreatus*; thus, we suggest that future studies focus on this fraction rather than on monomeric phenols, which seem to have a less important role in the overall phytotoxicity of OMW. *Pleurotus ostreatus* was of limited use for the removal of the organic load and of phytotoxicity from OMW; its degradation and polymerization metabolites appeared to make little or no contribution to the post-treatment residual phytotoxicity. The substantial decrease in TP was not accompanied by a corresponding removal of DOC and phytotoxicity, which shows that TP should not be used as the sole indicator of the success of OMW remediation. Phytotoxicity and microbial toxicity assays may provide better evaluation of OMW before and, especially, after treatment.

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