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Environmental Effects Caused by Olive Mill Wastewaters: Toxicity Comparison of Low-Molecular-Weight Phenol Components

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Olive oil mill wastewaters (OMWs) show significant polluting properties due to their content of organic substances, and because of their high toxicity toward several biological systems. Wastewaters' toxicity has been attributed to their phenolic constituents. A chemical study of wastewaters from a Ligurian oil mill characterized phenolic products such as 1,2-dihydroxybenzene (catechol), derivatives of benzoic acid, phenylacetic acid, phenylethanol, and cinnamic acid. The OMWs were fractioned by ultrafiltration and reverse osmosis techniques and tested for toxicity on aquatic organisms from different trophic levels: the alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum*); the rotifer *Brachionus calyciflorus*; and two crustaceans, the cladoceran *Daphnia magna* and the anostracan *Thamnocephalus platyurus*. The fraction most toxic to the test organisms was that from reverse osmosis containing compounds of low molecular weight (<350 Da), and this was especially due to the presence of catechol and hydroxytyrosol, the most abundant components of the fraction.

KEYWORDS: ***Olea europaea*; olive oil mill wastewater; phenolic components; toxicity**

INTRODUCTION

The disposal of olive oil mill wastewaters (OMWs) is one of the main environmental problems in the Mediterranean area, where the greatest quantities of olive oil are produced with a large volume of wastewaters within only a few months (from November to February). The high polluting activity of OMWs is linked with their high content of organic molecules, especially polyphenolic mixtures (1−10 g/L) with different molecular weights (*1*), as well as their acidity and high concentrations of potassium, magnesium, and phosphate salts (*2*−*4*). Besides aromatic compounds, OMWs contain other organic molecules, including nitrogen compounds, sugars, organic acids, and pectins (*5*, *6*), that increase their organic load (COD = 80−200 g/L; BOD = 50−100 g/L). Furthermore, the physicochemical characteristics of OMWs are rather variable, depending on climatic conditions, olive cultivars, degree of fruit maturation, storage time, and extraction procedure.

Because of these characteristics, the disposal of OMWs in urban sewage treatment plants is not practicable. Several pretreatment techniques have been worked out to reduce the impact of OMWs on municipal plants and on the receiving water bodies by using microrganisms and chemical or physicochemical methods (*7*−*11*). However, at present, Italian regulations allow the spreading of OMWs on agricultural soil, subject to certain limitations (*12*), because of the expense of new technologies for pretreatments and the difficulty of conventional treatment methods. This practice causes extensive pollution of the soil and even transfers harmful compounds into other media, such as groundwaters and surface waters. Recently, Aliotta et al. (*13*) reported the phytotoxicity of polyphenols from OMWs on seed germination, and Yesilada and Sam (*14*) reported their toxic effects on the soil bacterium *Pseudomonas aeruginosa*, but only a few studies have reported the toxic potential of this matrix on the typical organisms of the freshwater food chain (*15*−*17*). In this study, small-scale toxicity tests, incorporated into a multitrophic battery of organisms (producers and consumers of carbon), were used to measure the effects that may occur if OMWs reach surface waters.

The aim of this investigation was to determine the toxicity of the entire OMW matrix and its several fractions obtained by microfiltration (MF), ultrafiltrations on different cutoff membranes (UF1 and UF2), nanofiltration (NF), and reverse osmosis (RO) processes on the indicated biota of receiving environments. Furthermore, for the highest toxic potential of the reverse osmosis fraction, the toxic effects of individual low-molecular-weight polyphenols isolated from this fraction were also evaluated.

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MATERIALS AND METHODS

Chemicals. 4-Hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid), 3,4-dihydroxycinnamic acid (caffeic acid), 4-hydroxycinnamic acid (*p*-coumaric acid), 4-hydroxy-3-methoxycinnamic acid (ferulic acid), 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid), 4-hydroxy-3-methoxybenzoic acid (vanillic acid), and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) were obtained commercially (Aldrich Chemical Co.).

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded at 500 MHz for ^{1}H and 125 MHz for ^{13}C on a Varian 500 Fourier transform NMR spectrometer. Electronic impact mass spectra (EI-MS) were obtained with a HP 6890 spectrometer equipped with an MS 5973 N detector. Infrared spectra were determined on a Perkin-Elmer 1740 FT-IR spectrometer. UV/vis spectra were recorded in ethanol on a Perkin-Elmer Lambda 7 spectrophotometer. The analytical HPLC apparatus consisted of a System Gold 127 pump (Beckman), a System Gold 166 UV detector (Beckman), and a Chromatopac C-R6A recorder (Shimadzu). The column was a 250 mm × 4.6 mm i.d., 5 μm Hibar LiChrosorb RP-18 (Merck). The preparative HPLC apparatus consisted of a Shimadzu LC-10AD pump, a Shimadzu RID-10A refractive index detector, and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed using a 250-mm × 10-mm-i.d., 10-μm Luna RP-8 column (Phenomenex). Analytical TLC was performed on Kieselgel 60 F_{254} or RP-18 F_{254} plates with 0.2 mm layer thickness (Merck). Spots were visualized by UV light or by spraying with H_2SO_4/AcOH/H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Kieselgel 60 F_{254} plates with 0.5 or 1 mm film thickness (Merck). Flash column chromatography (FCC) was performed on Kieselgel 60, 230−400 mesh (Merck), at medium pressure. Column chromatography (CC) was performed on Kieselgel 60, 70−240 mesh (Merck), or on Sephadex LH-20 (Pharmacia).

Chemical Parameters of OMWs. Chemical oxygen demand (COD) was determined by the open reflux method (*18*). Biochemical oxygen demand (BOD_5) was determined by a 5-day BOD test (*19*). Phenols were determined spectrophotometrically (Helios α, Unicam, Cambridge, UK) with 4-aminoanti-pyrine by sample distillation (*20*).

Isolation of Low-Molecular-Weight Phenols from OMWs. Olive oil mill wastewaters were collected from a mill located in Liguria, Italy. The sludge-free olive oil mill wastewaters were supplied by Prof. Canepa (Università di Genova), who separated them into five fractions, on the basis of the molecular weight of the components, by microfiltration (MF), ultrafiltrations on different cutoff membranes (UF1 and UF2), nanofiltration (NF), and reverse osmosis (RO) processes (*10*). Three types of polysulfone membranes (cutoffs 8000, 20 000, and 200 000) were used at 20 °C and 300 kPa to obtain the ultrafiltrated and nanofiltered fractions. The RO fraction was obtained from an apparatus containing cellulose acetate and cellulose nitrate membranes at 5870 kPa.

The phenol components of the RO fraction have been previously isolated and characterized by Della Greca et al. (*6*). An aliquot (250 mL) of the fraction obtained from reverse osmosis, containing phenols with molecular weight <350, was extracted in a separator funnel using ethyl acetate. The organic layer was dried with Na_2SO_4 and concentrated under vacuum, yielding 400 mg of residual material. The extract was chromatographed on Sephadex LH-20 under low pressure, eluting with different mixtures of MeOH/H_2O. Chromatographic processes led to pure catechol (**1**, 50 mg), 4-hydroxybenzoic acid (**2**, 3 mg), 3,4-dihydroxybenzoic acid (protocatechuic acid, **3**, 3 mg), 4-hydroxyphenylethanol (tyrosol, **9**, 40 mg), 3,4-dihydroxyphenylethanol (hydroxytyrosol, **10**, 35 mg), 3,4-dihydroxyphenylethyleneglycol (**11,** 20 mg), 4-hydroxycinnamic acid (*p*-coumaric acid, **12**, 5 mg), 4-hydroxy-3-methoxycinnamic acid (ferulic acid, **14**, 3 mg), and 4-hydroxy-3,5-dimethoxycinnamic acid (sinapic acid, **15**, 1 mg).

4-Hydroxy-3-methoxybenzoic acid (vanillic acid, **4**), 4-hydroxy-3,5-dimethoxybenzoic acid (syringic acid, **5**), 4-hydroxyphenylacetic acid (**6**), 3,4-dihydroxyphenylacetic acid (**7**), 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid, **8**), and 3,4-dihydroxycinnamic acid (caffeic acid, **13**) were detected directly in the OMWs with a HPLC method using a RP-18 column. The column was equilibrated with a 9:1 (v/v) mixture of A (H_2O containing 1% CH_3COOH) and B (MeOH containing 1% CH_3COOH) and was used with the following program: isocratic run for 25 min, followed by an increase of B up to 60% in 30 min and a decrease to 10% in 5 min. The identification of phenolic compounds was performed by comparison with authentic standards.

Toxicity Testing. The acute toxicity of fresh sludge-free OMWs and their fractions was determined on the crustacean anostracan *Thamnocephalus platyurus* and on the rotifer *Brachionus calyciflorus*.

All the compounds isolated from RO were tested for their toxicity on the same organisms and on the crustacean cladoceran *Daphnia magna*. All the test organisms were provided in cryptobiotic stages by Creasel (Deinze, Belgium). A chronic toxicity test on the green alga *Pseudokirchneriella subcapitata* (formerly known as *Selenastrum capricornutum* and *Raphidocelis subcapitata*) was added to the acute bioassays using microalgae immobilized on an alginate matrix, also supplied by Creasel. All the tests were performed in compliance with international standard organizations (U.S. Environmental Protection Agency, Organization for European Cooperation and Development, European Economic Community, American Society for Testing and Materials) (*21*−*26*).

For bioassays, chemicals isolated in small quantities from OMWs were purchased. A range-finding test was performed to determine the tolerance range of organisms to sludge-free OMWs, their fractions, and pure chemicals before definitive tests were carried out to determine the 50% threshold effect.

All bioassays were conducted under static conditions, with no renewal of the test solution, measuring dissolved oxygen and pH in each sample both at the start and at the end of testing.

Briefly, juveniles (age 0−2 h) of the rotifer *Brachionus calyciflorus*, hatched from cysts, were exposed to the test sample in reconstituted freshwater (moderately hard EPA medium). Tests were run in 36-well plates with five organisms in each well, containing 0.3 mL of test solution at five concentrations, in six replicates per concentration and a negative control. Organisms were exposed to the toxicants for 24 h at 25 °C. The test endpoint was mortality (LC_{50}).

The bioassay with the anostracan crustacean *Thamnocephalus platyurus* was conducted in a similar manner, using second and third instars fairy shrimp larvae. Tests were performed in 24-well plates with 10 larvae in each well, containing 1.0 mL of test solution at five concentrations, in three replicates per concentration and a negative control. Organisms were exposed to the toxicants for 24 h at 25 °C. The test endpoint was mortality (LC_{50}).

The procedure for the test with *Daphnia magna* Straus was similar, with differences as follows. Glass beakers were used instead of the multiwell plates, and the test volume was 10 mL. The reconstituted freshwater, aerated before use, was the ISO hard medium (hardness 250 mg/L expressed as $CaCO_3$). Five organisms (age less than 24 h) were added to each container of test solution in four replicates for each of five concentrations and the negative control. Juveniles hatched from ephippia were exposed to the samples for 24 h at 20 °C, and the test endpoint was immobilization (EC_{50}). An organism was considered to be immobilized if it was not able to swim after gentle agitation of the liquid in 15 s of observation, even if it still moved its antennae (*21*).

The algal growth inhibition test was run for 72 h, using the OECD reconstituted freshwater (*25*) as dilution water for the test solutions. The alga, de-immobilized from the beads of alginate, was inoculated (1×10^4 cells/mL) in 25 mL of test solution, prepared in three replicates for each of five concentrations and control. The cell density was measured at time 0 and every 24 h for 3 days by an electronic particle dual-threshold counter (Coulter Counter Z2, 100 μm capillary), and from these data the algal growth inhibition was calculated by integrating the mean values from $t_0 = 20$ to $t_{72\,h}$ (area under the curve) (*25*, *26*).

Data Analysis. Raw data for all bioassays, except for the algal test, were analyzed using Toxcalc (*27*). The L(E)C_{50} endpoints and 95% confidence intervals were calculated using probit or trimmed Spearman−Karber method, as appropriate. The algal growth inhibition, estimated by integrating the mean values from time 0 to 72 h (area under the curve), was reported against log-transformed data of concentrations (μmol/L) to calculate the median inhibition (IC_{50}, in

Table 1. Toxic Units (TU) of OMW Fractions on *T. platyurus* (Crustacean) and *B. calyciflorus* (Rotifer)[a]

fraction	*B. calyciflorus*	*T. platyurus*
sludge-free OMW (SF)	106 (92–112)	78 (59–94)
microfiltrate MW > 120 000 Da (MF)	21 (17–26)	21 (17–26)
ultrafiltrate MW 120 000–20 000 Da (UF1)	18 (14–22)	10 (7–14)
ultrafiltrate MW 20 000–1000 Da (UF2)	17 (14–21)	9 (8–11)
nanofiltrate MW 1000–350 Da (NF)	17 (14–20)	29 (25–34)
reverse osmosis MW < 350 Da (RO)	52 (35–79)	37 (29–46)

[a] 95% confidence interval in parentheses.

μmol/L) for the pure chemicals, and this value was transformed into toxic units (TU) with the formula $1/IC_{50} \times 100$ for the OMW fractions (*28*).

RESULTS AND DISCUSSION

The fresh OMWs were collected from a mill in Liguria, Italy, and separated into five fractions on the basis of the molecular weight by ultrafiltration and reverse osmosis techniques. The MF fraction was constituted by compounds over 120 000 Da; the UF1 fraction by compounds ranging from 120 000 to 20 000 Da, the UF2 fraction by compounds ranging from 20 000 to 1000 Da, the NF fraction by compounds ranging from 1000 to 350 Da, and the RO fraction by molecules <350 Da. The sludge-free OMW (SF) was characterized by pH = 4.2, COD = 150 g/L, BOD = 80 g/L, and total phenols = 1.2 g/L. The SF was immediately tested for its toxic potential to avoid a loss of simple phenolic compounds due to the phenomenon of polymerization (*29*). It was tested on the rotifer *B. calyciflorus* and the crustacean anostracan *T. platyurus* because of their high sensitivity and rapidity of response (time from hatching to scoring of results <48 h). The results are reported in **Table 1** in toxic units (TU) with their confidence limits ($p < 0.05$). The SF showed high toxicity values for both organisms. In the literature, toxic effects of OMWs were also found for amphibian tadpoles, a bactericidal activity for Gram-positive and -negative bacteria, and a genotoxic effect for *Drosophila melanogaster* (*15*, *30*). The TUs obtained in this sample represented the response of interactions between additive, synergistic, or antagonistic effects, and therefore they were not the sum of TU values of all the other fractions, as previously reported (*31*). Among the fractions, RO was the most toxic, with an impact on total toxicity of about 50%, although the results for *T. platyurus* did not show a significant difference from TUs of the nanofiltered fraction. Rotifers were more sensitive than crustaceans for both SF and RO. This latter fraction contained phenols with molecular weight less than 350 Da, and it was found to be inhibitory also for seed germination and algal growth (*13*, *17*).

The separation of this fraction by chromatographic methods allowed us to obtain 15 phenols as the major components of RO. All the phenols were identified by ^{1}H and ^{13}C NMR analyses as catechol (**1**), four benzoic acids [4-hydroxybenzoic acid (**2**), protocatechuic acid (**3**), vanillic acid (**4**), and syringic acid (**5**)], three phenylacetic acids [4-hydroxyphenylacetic acid (**6**), 3,4-dihydroxyphenylacetic acid (**7**), and 4-hydroxy-3-methoxybenzoic acid (**8**)], three phenylethanols [tyrosol (**9**), hydroxytyrosol (**10**), and 3,4-dihydroxyphenylethylene glycol (**11**)], and four cinnamic acids [*p*-coumaric acid (**12**), caffeic acid (**13**), ferulic acid (**14**), and sinapic acid (**15**)] and are shown in **Figure 1**.

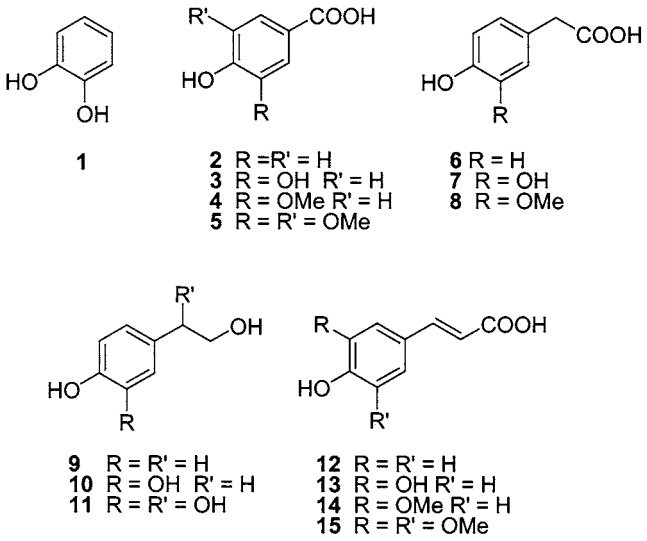

Figure 1. Phenolic compounds isolated from RO fraction.

Table 2. Median Effective Concentration Expressed as EC_{50} (in μM) for Cladocerans, LC_{50} for Rotifers and Anostracans, and IC_{50} for Algae of Phenols from RO Fraction[a]

	organisms			
compd	*D. magna*	*T. platyurus*	*B. calyciflorus*	*P. subcapitata*
1	10 (8–14)	8 (7–10)	17 (13–21)	34 (22–55)
2	446 (340–585)	983 (961–1005)	225 (179–283)	256 (221–311)
3	413 (381–446)	589 (563–616)	385 (301–492)	344 (320–391)
4	386 (359–416)	431 (358–465)	1 (0.7–2)	255 (216–300)
5	177 (116–270)	97 (80–118)	141 (119–167)	214 (164–307)
6	391 (299–511)	689 (559–737)	273 (239–300)	486 (463–511)
7	331 (245–446)	390 (331–442)	136 (120–153)	33 (28–40)
8	268 (214–336)	299 (253–346)	407 (290–572)	440 (416–465)
9	861 (678–1091)	296 (265–331)	47 (32–67)	210 (123–603)
10	11 (9–15)	4 (3–5)	9 (8–10)	120 (66–296)
11	208 (145–297)	65 (54–81)	144 (121–170)	137 (101–188)
12	290 (205–411)	591 (486–719)	108 (84–142)	225 (184–242)
13	326 (274–388)	626 (554–710)	359 (272–473)	120 (106–136)
14	249 (165–377)	300 (228–325)	247 (212–286)	413 (375–432)
15	208 (170–254)	628 (459–860)	398 (362–437)	254 (235–274)

[a] 95% confidence interval in parentheses.

All individual compounds were tested for their toxicity on the green alga *P. subcapitata*, the rotifer *B. calyciflorus*, the crustacean cladoceran *D. magna*, and the anostracan *T. platyurus*, and the results are summarized in **Table 2**. For all test organisms, the most toxic compound was catechol (**1**) with IC_{50}'s ranging from 8 μM for *T. platyurus* to 34 μM for *P. subcapitata*. Hydroxytyrosol (**10**) was almost as toxic as **1**, except on *P. subcapitata* (IC_{50} = 120 μM). Data for catechol

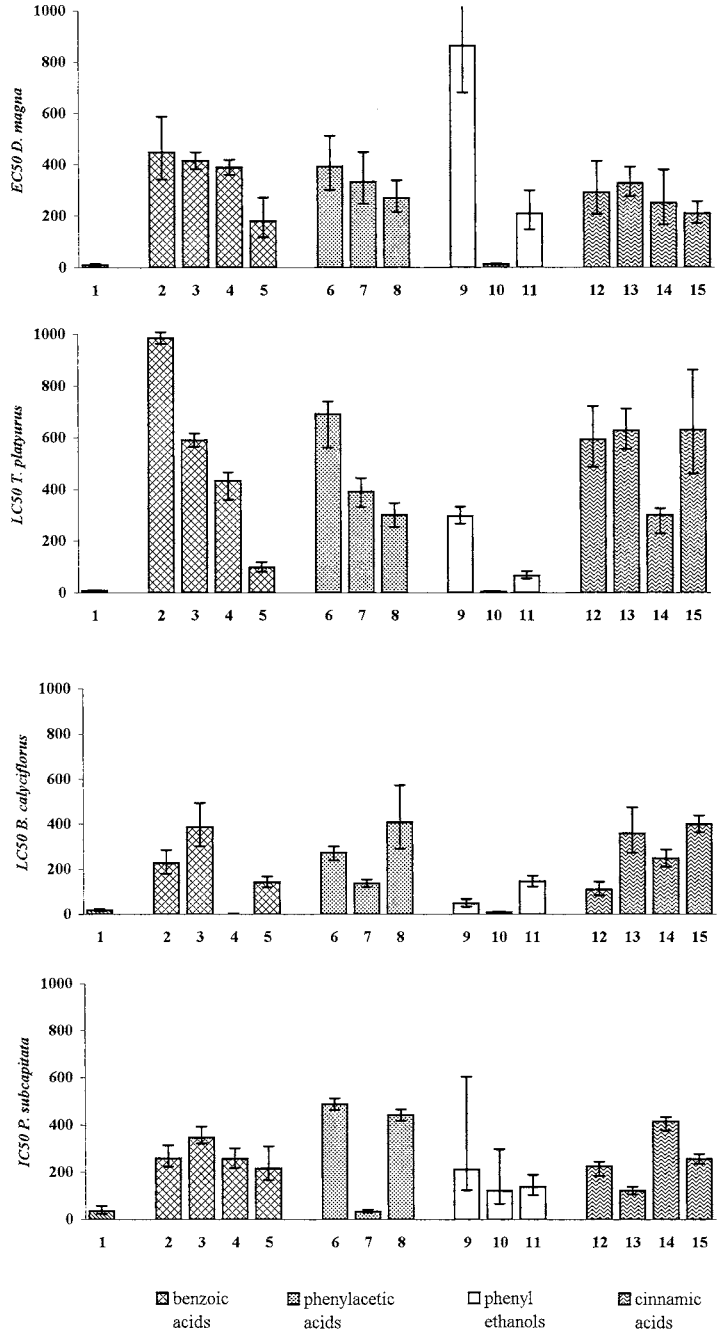

Figure 2. Toxicity (in μM) of phenolic compounds from RO fraction.

agreed with previous investigations that showed a strong phytotoxicity of this compound on seed germination of *Raphanus sativus* and *Triticum durum* (*13*), *Lycopersicon esculentum* and *Cucurbita pepo* (*31*), and on the alga *Ankistrodesmus braunii* (*17*), and a deterrent activity on oviposition of the olive fly *Bactrocera oleae* (*32*). These data assume a particular value if it is considered that catechol is the most abundant chemical in the RO fraction. High variability existed among species, so it was difficult to compare relative toxicity for the different species unless the differences in toxicity were very great. An example was vanillic acid (**4**), which was found to be the most active in this investigation, but only for rotifers ($LC_{50} = 1\ \mu M$). For this reason, an analysis closely related to structure−activity among the compounds was chosen. The identified chemicals were grouped in five compound classes: catechol (**1**), benzoic acids (**2**−**5**), phenylacetic acids (**6**−**8**), phenylethanols (**9**−**11**), and cinnamic acids (**12**−**15**). As shown in **Figure 2**, the toxicity of benzoic acids increased when methoxy groups were present in the structure, as did the toxicity of phenylacetic acids for both crustaceans. In contrast, for the phenylacetic acids, the less evolved organisms, such as rotifers and algae, were especially affected by the presence of a further hydroxyl group, as in **7**. The number of hydroxyl groups in the aromatic ring and the side chain influenced the toxicity of phenylethanols. Compound **10** showed the highest toxicity for this class of compounds, as previously indicated. No structure−activity relationship was found for the cinnamic acids, which did not seem to be affected by the presence of a hydroxyl group, except for *P. subcapitata* (**13**).

As the results showed, all of these compounds had a strong toxic potential on different trophic levels in the aquatic system. The sensitivity comparison of algal, rotifer, and crustacean bioassays confirmed the need for a battery of organisms in aquatic toxicity testing because no one organism was uniquely

sensitive to the chemicals tested. The results obtained indicated that the great RO toxicity was mainly due to the presence of catechol and hydroxytyrosol, two of the most abundant phenols in RO, which are largely present in all the OMWs cited in the literature.

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NOTE ADDED AFTER ASAP

This article was released ASAP on 1/4/2003 before final corrections were made. In two instances, μM was incorrectly used as mM. The correct version was posted on 1/10/03.

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