Bull. Environ. Contam. Toxicol. (2001) 67:352–359

DOI: 10.1007/s00128-001-0132-9

Bulletin of Environmental Contamination and Toxicology

Phytotoxicity of Low-Molecular-Weight Phenols from Olive Mill Waste Waters

M. DellaGreca,[1] P. Monaco,[1] G. Pinto,[2] A. Pollio,[2] L. Previtera,[1] F. Temussi[1]

[1] Department of Organic Chemistry and Biochemistry, University Federico II, Complesso Universitario Momtesantangelo, Via Cinthia, I-80126 Napoli, Italy
[2] Department of Biologia Vegetale, University Federico II, Via Foria 223, I-80139 Napoli, Italy

Received: 19 December 2000/Accepted: 1 June 2001

The olive (*Olea europaea*) culture represents one of the most important crops in the Mediterranean region, and is widely diffused not only in European countries such as Italy, Spain and Greece, but also in other Mediterranean countries as Tunisia and Morocco. The manufacturing process of olive oil usually yields two waste residues: the exhausted husks and the olive mill waste-waters (OMW). The total production of this latter effluent is more than 30 million m^3 per year (Fiestas Ros de Ursinos 1981). The high levels of BOD and COD, until concentrations of 100 and 220 kg/m^3, respectively (Della Monica et al. 1978), make OMW one of the major agent of pollution in Mediterranean region. The waste of processing of 500 m^3 olives/year has a polluted load equivalent to the urban sewage of a 7000 people town (Boari and Mancini 1987).

The organic fraction of OMW includes sugars, tannins, polyphenols, mucilages, polyalcohols, pectins and lipids (Balice et al. 1982). The large concentration of highly toxic phenolics (Fedorak and Hrudei 1984; Borja et al. 1992) limits the biological treatment of this effluent (Hamdi et al. 1992). In force of the actual Italian regulations it is allowed to shed OMW in the soil, but several studies have evidenced that this practice may be cause of environmental pollution. Paredes et al. (1986) have reported microbiological and physico-chemical soil changes after pollution with OMW, and Rodriguez et al. (1988) have proved the strong antibacterial activity of phenolics present in OMW, testing the effects of several extracts on the soil bacterium *Bacillus megaterium*. OMW have also phytotoxic activity and Capasso et al. (1992) have studied the effects of the main phenolic components of OMW on the seed germination of tomato and vegetable marrow.

While all these studies have been useful to evaluate the land pollution, no data is available to assess the potential contamination of both ground and superficial waters by OMW. Algae occupy a unique position among the primary producers as they are an important link in the food chain and are essential to the economy of freshwater ecosystems. These organisms have proved to be exceptionally suitable in analysing water quality (Trainor 1984). Algae are particularly sensitive to phenols. It has been shown that tannins extracted from various fruits (Hussein Ayoub and Yankou 1985), as well as polyphenols released from barley straw decomposition (Ridge and Pillinger 1996), are toxic to several algal species.

Correspondence to: P. Monaco

Moreover, phenylpropanoids isolated from aquatic and wetland plants are active against several strains of microalgae (Della Greca et al. 1999). On this basis we have employed the green alga *Ankistrodesmus braunii* CCAP 202.7a, one of the assay organisms recommended for the standard algal assay procedure developed for aquatic systems (Pipe and Shubert 1984), to assess the toxicity of OMW and their phenolic components.

MATERIALS AND METHODS

Sephadex® LH-20 (Pharmacia Biotech) was used for column chromatography. Hibar LiChrosorb RP-18 (5 μm, 250 × 4 mm i.d) (Merk) column was used for HPLC analysis: conditions: 1.0 mL/min, absorbance 260 nm; A (H_2O-AcOH 99:1) B (MeOH-AcOH 99:1); mobile phase: A –B 9:1 for 25 min, then linear gradients to A – B 2:3 in 30 min. NMR experiments were performed at 400 MHz for ^{1}H and 100 MHz for ^{13}C on a Bruker AM400 spectrometer.

Algal growth experiments were carried out in quadruplicate and repeated two times. For each experiment four replicates of a control, without toxic substances, were also prepared. To test statistical significance of results, one way ANOVA was performed at $\alpha = 0.05$. For each experiment a comparison among means was performed using Student-Newman-Keuls test (SNK), at $\alpha = 0.05$. The SPSS statistical package was used.

CCAP 202.7a *Ankistrodesmus braunii* was cultivated in Bold Basal Medium (BBM) (Nichols 1973) in 1 L Erlenmeyer flasks placed on a shaking apparatus at 20 ± 1°C, under continuous illumination, with a total irradiance of 100 μE m^2s^{-1} provided by a daylight fluorescent lamp (Philips TLD 30w/55). OMW were fractionated by ultrafiltration using membranes with different molecular weight cut-off, and reverse osmosis (Canepa 1987). The following fraction were obtained: UF1 (M*r* above 20,000 dalton), UF2 (M*r* between 20,000 and 1000 dalton), UF3 (M*r* between 1000 and 350 dalton) and RO (M*r* less than 350 dalton). For the growth tests, the OMW fractions were diluted by adding sterile BBM to restore the initial concentrations: UF1 (1:14), UF2 (1:16), UF3 (1:13) RO (1:20). These solutions were aseptically transferred in test tubes (6 mL each) and algal inocula corresponding to a final concentration of 1×10^5 cells/mL were added. The inhibition was monitored daily for four days by measuring the absorbance at 550 nm with a colorimeter Baush & Lomb (Spectronic 20) or by counting the cell numbers with a Burker blood-counting chamber. When the cultures were coloured only the second method was used.

For the determination of EC_{50} five dilutions (1:1.5, 1:2, 1:3, 1:4 and 1:5) of the RO fraction were tested. To evidence the relationship between toxicity and algal biomass, the RO fraction was added to test tubes to have final algal concentrations from 0.5 to 5×10^5 cells/mL. For the experiments of acute shocks, measures of photosynthesis and respiration on samples prepared as described for chronic shocks were performed in a 1 mL glass cell equipped with a Clark oxygen

electrode (Hansatech Instruments Ltd.), thermostated at 20 ± 1°C with a heather bath. Saturating light intensity was provided by light from an incandescent lamp (Philips Comptalux E44 100 w) filtered through a 2% $CuSO_4$ solution.

In the experiments of resilience, algae were grown for 1-4 days on BBM containing the reverse osmosis fraction. The algae were then centrifuged at 4000 rpm for 15 min and washed in phosphate buffer at pH 6.5. The algal pellet was resuspended in BBM and the growth was followed daily by measuring the absorbance of the cultures at 550 nm. When it was present, the lag-phase of growth was evaluated according to Pinto and Taddei (1988).

Reverse osmosis fraction (300 mL) was extracted with EtOAc and the crude extract (300 mg) was chromatographed on Sephadex LH-20 eluting with MeOH - H_2O mixtures. MeOH - H_2O (3:2) gave fractions A - D, while MeOH - H_2O (7:3) gave fractions E and F.Fraction A was chromatographed on preparative TLC [$CHCl_3$ - MeOH (47:3)] to give *3,4-dihydroxy-phenylacetic acid* (**9**) (4 mg) which had MS: *m/z* 168 $[M]^+$. ^{1}H-NMR spectral data (CD_3OD): δ 6.48 (2H, *m*, H-2 and H-6), 6.42 (1H, *d*, *J* = 8.4 Hz, H-5), 3.64 (2H, *s*, H-7). Preparative TLC [$CHCl_3$ - MeOH (19:1)] of fraction B gave: **5** (40 mg), **6** (35 mg), **7** (4 mg) and **8** (5 mg). *Tyrosol* (**5**) had MS: *m/z* 138 $[M]^+$. ^{1}H-NMR: δ 7.02 (2H, *d*, *J* = 8.3 Hz, H-2 and H-6), 6.69 (2H, *d*, *J* = 8.3 Hz, H-3 and H-4), 3.68 (1H, *t*, *J* = 7.0 Hz, H-8), 2.71 (1H, *t*, *J* = 7.0 Hz, H-7). *2-(3,4-Dihydroxy)-phenylethanol* (**6**) had MS: *m/z* 154 $[M]^+$. ^{1}H-NMR spectral data ($CDCl_3$): δ 6.68 (1H, *d*, *J* = 8.0 Hz, H-5), 6.64 (1H, *d*, *J* = 1.9 Hz, H-2), 6.52 (1H, *dd*, *J* = 1.9 and 8.0 Hz, H-6), 3.67 (1H, *t*, *J* = 7.2 Hz, H-8) 2.66 (1H, *t*, *J* = 7.2 Hz, H-7). *2-(4Hydroxy-3-methoxy)-phenylethanol* (**7**) had MS: *m/z* 168 $[M]^+$.^{1}H-NMR spectral data (CD_3OD): δ 6.79 (1H, *d*, *J* = 1.9 Hz, H-2), 6.70 (1H, *d*, *J* = 8.9 Hz, H-5), 6.64 (1H, *dd*, *J* = 1.9 and 8.0 Hz, H-6), 3.83 (3H, *s*, OMe), 3.71 (2H, *t*, *J* = 7.2 Hz, H-8), 2.73 (2H, *t*, *J* = 7.2 Hz, H-7). *2-Hydroxy-2-(3,4-dihydroxy)-phenylethanol* (**8**) had MS: *m/z* 170 $[M]^+$ ^{1}H-NMR spectral data ($CDCl_3$): δ 6.81 (1H, *d*, *J* = 1.6 Hz, H-2), 6.70 (1H, *d*, *J* = 7.0 Hz, H-5), 6.65 (1H, *dd*, *J* = 1.6 and 7.0 Hz, H-6), 4.53 (1H, *t*, *J* = 6.2 Hz, H-7), 3.56 (2H, *d*, *J* = 6.2 Hz, H-8). Preparative TLC [$CHCl_3$-MeOH (19:1)] of fraction C gave *catechol* (**1**) (83 mg) which had MS: *m/z* 110 $[M]^+$. ^{1}H-NMR spectral data ($CDCl_3$): δ 6.69 (4H, *m*, H-3, H-4, H-5 and H-6). Fraction D gave by preparative TLC [$CHCl_3$-MeOH (93:7)] **4** (10 mg), and dimers **13** (12 mg) and **14** (10 mg). *Vanillic acid* (**4**) had MS: *m/z* 168 $[M]^+$. ^{1}H-NMR spectral data (CD_3OD): δ 7.54 (2H, *m*, H-2 and H-6), 6.83 (1H, *d*, *J* = 8.4 Hz, H-5), 3.87 (3H, *s*, OMe). Dimer **13** had MS: *m/z* 306 $[M]^+$. ^{1}H-NMR spectral data (CD_3OD): δ 6.73 (1H, *d*, *J* = 2.0 Hz, H-2), 6.72 (1H, *d*, *J* = 8.5 Hz, H-5), 6.65 (1H, *d*, *J* = 8.3 Hz, H-5'), 6.61 (1H, *d*, *J* = 2.1 Hz, H-2'), 6.59 (1H, *dd*, *J* = 2.0 and 8.5 Hz, H-6), 6.48 (1H, *dd*, *J* = 2.1 and 8.3 Hz, H-6'), 4.21 (1H, *dd*, *J* = 8.4 and 9.2 Hz, H-7), 3.50 (2H, *m*, H-8), 3.51 (2H, *m*, H-8'), 2.72 (2H, *m*, H-7'); ^{13}C-NMR spectral data (CD_3OD): 146.6 (C-3), 146.4 (C-4), 132.5 (C-1), 120.0 (C-6), 116.5 (C-5), 115.3 (C-2), 84.7 (C-7), 68.2 (C-8), 146.4 (C-3'), 144.9 (C-4'), 132.2 (C-1'), 121.5 (C-6'), 117.0 (C-2'), 116.5 (C-5'), 71.6 (C-8'), 37.0 (C-7'). Dimer (**14**) had MS: *m/z* 290 $[M]^+$.^{1}H-NMR

spectral data (CD_3OD): δ 6.98 (2H, *d*, *J* = 8.2 Hz, H-3' and H-5'), 6.71 (1H, *d*, *J* = 1.8 Hz, H-2), 6.70 (1H, *d*, *J* = 8.0 Hz, H-6), 4.21 (1H, *dd*, *J* = 8.4 and 9.2 Hz, H-7), 3.55 (2H, *m*, H-8), 3.45 (2H, *m*, H-8'), 2.74 (2H, *m*, H-7'); ^{13}C-NMR spectral data (CD_3OD): 157.0 (C-4'), 131.4 (C-1'), 131.1 (C-2' and C-6'), 116.5 (C-5'), 116.3 (C-3'), 71.6 (C-8'), 36.8 (C-7'), 146.6 (C-3), 146.4 (C-4), 132.5 (C-1), 120.0 (C-6), 116.5 (C-5), 115.3 (C-2), 84.7 (C-7), 68.2 (C-8). Preparative TLC [$CHCl_3$-MeOH (19:1)] of fraction E gave compounds **2** (3 mg), (**3**) (7 mg), **11** (3 mg) and **12** (2 mg). *4-Hydroxybenzoic acid* (**2**) had MS: *m/z* 138 $[M]^+$. ^{1}H-NMR spectral data (400 MHz, $CDCl_3$): δ 7.87 (2H, *d*, *J* = 7.0 Hz, H-2 and H-6), 6.79 (2H, *d*, *J* = 7.0 Hz, H-3 and H-5). *Protocatechuic acid* (**3**) had MS: *m/z* 154 $[M]^+$.^{1}H-NMR spectral data (400 MHz, CD_3OD): δ 7.45 (H, *d*, *J* = 2.1 Hz, H-2), 7.45 (1H, d*d*, *J* = 2.1 and 8.0 Hz, H-6), 6.73 (1H, *d*, *J* = 8.0 Hz, H-5). *Ferulic acid* (**11**) had MS: *m/z* 194 $[M]^+$. ^{1}H-NMR spectral data (400 MHz, $CDCl_3$): δ 7.60 (1H, d, *J* = 16.0 Hz, H-7), 7.18 (1H, *d*, *J* = 2.0 Hz, H-2), 7.04 (1H, *dd*, *J* = 2.0 and 8.2 Hz, H-6), 6.81 (1H, *d*, *J* = 8.2 Hz, H-5), 6.31 (1H, *d*, *J* = 16.0 Hz, H-8). *Sinapic acid* (**12**) had MS: *m/z* 224 $[M]^+$. ^{1}H-NMR spectral data (400 MHz, $CDCl_3$): δ 7.58 (1H, *d*, *J* = 16.0 Hz, H-8), 6.88 (2H, *s*, H-2 and H-6), 6.34 (1H, *d*, *J* = 16.0 Hz, H-7), 3.88 (6H, *s*, OMe). Preparative TLC [$CHCl_3$-MeOH (47:3)] of fraction F gave *coumaric acid* (**10**) (9 mg) which had MS: *m/z* 164 $[M]^+$. ^{1}H-NMR spectral data (400 MHz, $CDCl_3$): δ 7.59 (1H, *d*, *J* = 16.0 Hz, H-7), 7.43 (2H, *d*, *J* = 7.0 Hz, H-2 and H-6), 6.80 (2H, *d*, *J* = 7.0 Hz, H-3 and H-5), 6.28 (1H, *d*, *J* = 16.0 Hz, H-8).

The phenolic compounds were dissolved in acetone and each solution (20 μl) was added to the test tubes containing 6 mL of inoculated medium (algal concentration = 1×10^5 cells/mL), to give final concentrations between 10^{-3} and 10^{-4} M. In the experiments of chronic toxicity the total exposure to toxic agents was 4 days, and the conditions of incubation were the same previously described for the maintenance of algal cultures.

In the experiments of synergism the RO fraction (50 mL) was extracted with EtOAc (2 × 25 mL). The organic layer was evaporated in vacuo. The residue was diluted in BBM (500 mL) and tested against *A. braunii*. Algae were added to have a cell density ranging from 0.5 to 5 × 105 cells/mL. To evaluate the combined effects of phenols, a mixture of the seventeen compounds isolated from the RO fraction, was dissolved in BBM (PM). The final concentration of each phenol was that found in RO. Algae were added to have the above reported initial inocula.

RESULTS AND DISCUSSION

The OMW fractions, diluted with Bold Basal medium (BBM) to restore the initial concentration of their components, were tested on *A. braunii* cultures inoculated with 1.0×10^5 cells/mL, by measuring the growth inhibition after 4 days. The fractions UF1 (M*r* >20,000) and UF2 (M*r* 20,000-1000) did not inhibit algal growth or had a small stimulating effect. On the contrary, the nanofiltered fraction

(UF3), M*r* between 1000 and 350 dalton, and the reverse osmosis fraction (RO), M*r* less than 350 dalton, were toxic to algal cultures. The RO fraction was the most active fraction and inhibited completely the growth of *A. braunii*. Experiments with different dilutions of the RO fraction showed that 50% inhibition still occurred at 1:2 dilution, and that it was inactive at 1:5 dilution.

The effect of algal biomass on acute and chronic toxicity of RO fraction was evaluated by measuring respectively the photosynthesis and the growth of algal cultures, with inocula from 0.5 to 5 $\times 10^5$ cells/mL. All cultures, independently of the cell concentration, showed about 60% inhibition of photosynthesis, measured in terms of oxygen produced and consumed in the light, after 1h exposition. The inhibition of growth, measured as number of cells respect to the control, was monitored daily, and after 4 days ranged from 90% to 100% in the cultures with initial inocula 0.5 $\times$ 10^5 and 1 $\times$ 10^5 cells/mL. The inhibition diminished to 77% at 2.5 $\times 10^5$ cells/mL and at 5 x10^5 cells/mL, only 50% inhibition was observed.

Cells from all the cultures, after 4 days exposition, showed reduced dimensions, along with a pale-green colour, instead of the bright green of control cells; moreover, several cells presented irregular shape. To ascertain if irreversible damages occurred to cells, experiments of resilience were carried out. Algal cultures, with 1.0 $\times$ 10^5 cells/mL inoculum, were exposed to RO fraction and, after centrifugation, re-suspended in BBM. The algae from cultures kept 4 days with the RO fraction were irreversibly damaged, while the cultures kept 2 or 3 days resumed the growth respectively after two and five days. No lag-phase was observed after one day of exposition to RO fraction. To assay the toxicity of the individual phenolic constituents, the RO fraction was extracted with EtOAc and the extract was chromatographed on Sephadex LH-20. Subsequent CC, TLC and HPLC separations afforded fourteen compounds **1** – **14**, identified on the basis of their spectroscopic features. Besides catechol (**1**), three C_6-C_1, five C_6-C_2, three C_6-C_3 and two dimeric C_6-C_2 components were isolated. The C_6-C_1 components were identified as 4-hydroxybenzoic (**2**), protocatechuic (**3**) and vanillic (**4**) acids. The structures of tyrosol (**5**), 2-(3,4-dihydroxy)phenylethanol (**6**), 2-(4-hydroxy-3-methoxy)phenylethanol (**7**), 2-(3,4-dihydroxy)phenyl-1,2-ethandiol (**8**) and 3,4-dihydroxyphenylacetic (**9**) acid were attributed to the C_6-C_2 components. The C_6-C_3 compounds were identified as *p*-coumaric (**10**), ferulic (**11**) and sinapic (**12**) acids while structures **13** and **14** were attributed to the dimers. The fourteen compounds were utilised as standards in the quantitative and qualitative HPLC analysis of the RO fraction. The analysis evidenced the presence of three further components in the chromatogram. On the basis of the data reported in a previous paper on the *Olea europaea* fruit components (Bianchi & Pozzi, 1994) and by using commercial samples, they were identified as 4-hydroxy-phenylacetic (**15**), 4-hydroxy-3,5-dimethoxybenzoic (**16**) and caffeic (**17**) acids. Compounds **1-17** were assayed with *A. braunii* by measuring the inhibition at five different concentrations, ranging from 1 $\times$ 10^{-5} M to 1 $\times 10^{-3}$ M, using 1 $\times$ 10^5 cells/mL

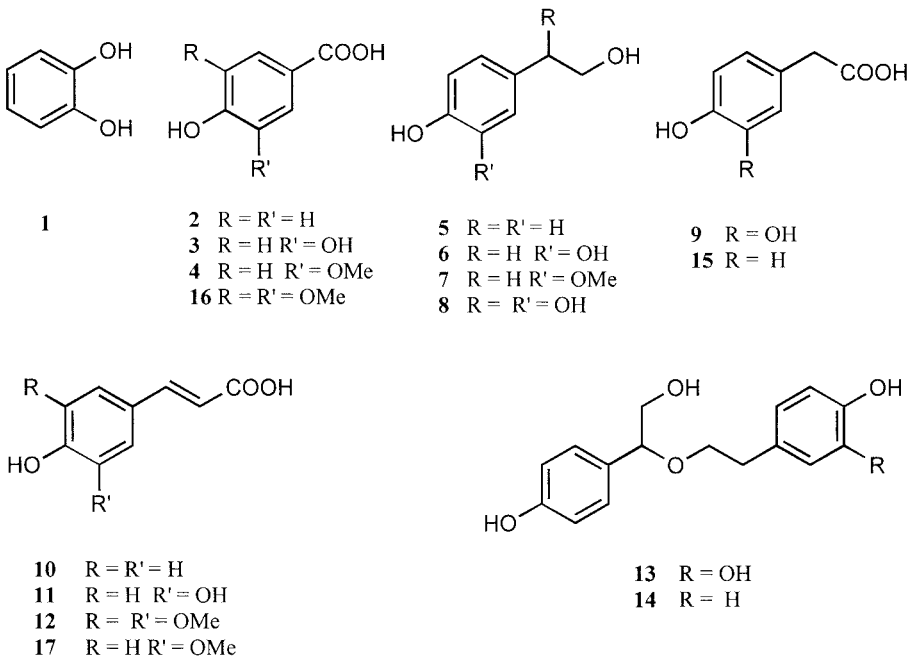

Figure 1. Phenolic components of the reverse osmosis (RO) fraction of olive mil waste-waters (OMW).

algal inoculum (Table 1). Compounds **3 - 8** and **12 - 15** did not inhibit *A. braunii* in the range of tested concentrations. Compounds **2**, **9**, **10** and **16** had a slight activity at 10^{-3} M concentration and only compounds **1**, **11** and **17** were significantly active at the same concentration. In each column values followed by different letters are statistically significant; Student-Newman-Keuls test; P=0.05.

Table 1. Inhibition of active phenolic compounds on *A.braunii*. expressed as percent of inhibition with respect to the control.

	1	2	9	10	11	16	17
10^{-4}	8b	0a	0a	0a	6b	0a	5b
5×10^{-4}	39c	0a	5b	7b	15c	0a	13c
10^{-3}	97d	14b	18c	22c	50d	15b	52d
control	0a	0a	0a	0a	0a	0a	0a

Catechol (**1**) was the most active and caused 97% inhibition, while ferulic acid (**11**) and caffeic acid (**17**) caused about 50% inhibition. The concentration of catechol (**1**), the most abundant and active component, is about 10^{-4} M in the RO

fraction, while the concentrations of remaining components are considerably lower, so that none of the phenolic constituents of RO fraction may account for the toxicity of the whole fraction. To exclude the occurrence in the RO fraction of highly inhibitory compounds besides those extracted with EtOAc, RO fraction was extracted with EtOAc and both the organic and the aqueous layers were tested on *A. braunii*. The EtOAc extract, tested at the same concentrations previously used, gave responses identical to those observed with the entire RO fraction, whereas the aqueous extract was inactive. To evidence a possible synergistic action of phenolic constituents, a mixture of all seventeen compounds (PM), in the same ratio found in RO fraction, was assayed on *A. braunii*. The mixture was dissolved in BBM to give the same concentration as present in the RO fraction; algae were added at concentrations from 0.5×10^5 to 5×10^5 cells/mL.

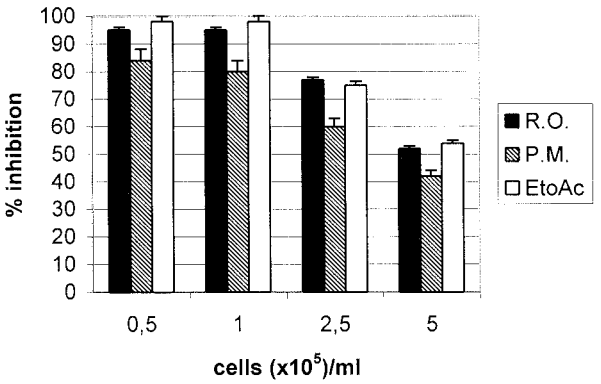

Figure 2. Percent of inhibition of *A. braunii* vs RO fraction mixture and EtOAc extract. Error bars represent the standard deviation.

The inhibition rates, measured after 4 days, closely resembled those obtained with the RO fraction (Figure 2), thus suggesting that the inhibitory effects observed on the growth were mainly due to the synergistic action of phenols. In conclusion, the assays on *A. braunii* are indicative that OMW may also affect water ecosystems. The damage is depending on algal concentration and by exposure time. The main pollutants of OMW are the phenolic components, and synergistic effects may enhance their toxicity.

REFERENCES

Balice V, Boari G, Cera O, Abbaticchio P (1982) Indagine analitica sulle acque di vegetazione. Inquinamento 7: 49-53

Bianchi G, Pozzi N (1994) 3,4-Dihydroxyphenylglycol, a major C_6-C_2 phenolic in *Olea europaea* fruits. Phytochemistry 35: 1335-37

Boari G, Mancini IM (1987) Trattamento delle acque di vegetazione. Ingegneria Sanitaria 12: 167-172

Borja R, Martin A, Maestro R, Alba J, Fiestas J.A (1992) Enhancement of the anaerobic digestion of olive mill wastewaters by removal of phenolic inhibitors. Process Biochem 27: 231-237

Canepa P (1987) Trattamento delle acque di vegetazione mediante processi a membrana. Inquinamento 5: 73-78

Capasso R, Cristinzio G, Evidente A, Scognamiglio F (1992) Isolation, spectroscopy and selective phytotoxic effects of polyphenols from vegetable waste waters. Phytochemistry 31: 4125-4128

Della Greca M, Fiorentino A, Monaco P, Previtera L, Pinto G, Pollio A (1999) Release of Potential Allelochemical from Aquatic Plants. In: Macías FM, Galindo JCG, Molinillo JMG, Cutler HG (eds) Recent Advances in Allelopathy – A Science for the Future, vol. I Servicio de publicaciones – Universidad de Cadíz, Cadíz, p 514

Della Monica M, Potenz D, Righetti E, Volpicelli M (1978) Effetto inquinante delle acque reflue della lavorazione delle olive su terreno agrario 10: 81-87

Fedorak PM, Hrudei SE (1984) The effects of som alkylphenolics on batch anaerobic methanogenesis. Water Res 18: 361-367

Fiestas Ros de Ursinos JA (1981) Différentes utilisations des margines. In: Proc Séminaire International sur la Valorisation des sous Produits de l'Olivier. Organisation des Nations Unies pour l'alimentation et agriculture. Tunisie, p 110

Hamdi M, Garcia JL, Ellouz R (1992) Integrated biological process for olive mill wastewater treatment. Bioprocess Eng 8: 79-84

Hussein Ayoub SM, Yankou LK (1985) Algicidal properties of tannins. Fitoterapia 56: 227-229

Nichols HW (1973) Growth media - freshwater In: Stein JR (ed) Handbook of Phycological Methods, Cambridge University Press, Cambridge, p 7

Paredes MJ, Monteoliva-Sanchez M, Moreno E, Perez J, Ramos-Cormenzana A, Martinez J (1986) Effect of waste-water from olive oil extraction plants on the bacterial population of soil. Chemosphere 15: 659-664

Pinto G, Taddei R (1988) Evaluation of toxic effects of heavy metals on unicellular algae. V - Analysis of the inhibition manifesting itself with an increased lag phase. Boll Soc Naturalisti in Napoli XCV:303-316

Pipe AE, Shubert LE (1984) The use of algae as indicators of soil fertility. In: Shubert LE (ed.) Algae as Ecological Indicators. Academic Press, London, p. 213

Ridge I, Pillinger JM (1996) Towards understanding the nature of antialgal inhibitors from barley straw. Hydrobiologia 340: 301-305

Rodriguez M.M, Perez J, Ramos-Cormenzana A, Martinez J (1988) Effect of extracts obtained from olive oil mill waste waters on *Bacillus megaterium* ATCC 33085. J Appl Bacteriol 64: 219-226

Trainor FR (1984) Indicator algal assays: laboratory and field approaces. In: Shubert LE (ed) Algae as Ecological Indicators. Academic Press, London, p 3