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Ability of the aquatic fern *Azolla* to remove chemical oxygen demand and polyphenols from olive mill wastewater

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RESUMEN

Eficacia del helecho de agua *Azolla* para reducir la demanda química de oxígeno y los polifenoles del alpechín.

La eficacia del helecho de agua *azolla* para eliminar polifenoles y reducir la demanda química de oxígeno (DQO) de los alpechines obtenidos en el proceso de obtención tradicional y continuo del aceite de oliva, fue investigado mediante ensayos de filtración. Cinco conos secuenciales de Imhoff y cinco columnas secuenciales se rellenaron de biomasa de *Azolla*. En ambos experimentos, el filtrado procedente de la quinta extracción mostró una disminución en el contenido de polifenoles de 7650 mg L^{-1} a 3610 mg L^{-1}en el alpechín obtenido mediante el sistema tradicional y de 3852 mg L^{-1} a 1351 mg L^{-1}en el alpechín del sistema continuo. La demanda química de oxígeno del alpechín del sistema tradicional disminuyó de 110200 mg L^{-1} a 52400 mg L^{-1} en y de 41600 mg L^{-1}a 2300 mg L^{-1}en el procedente del sistema continuo. Una proporción en peso 5:1 de alpechín: *Azolla* fue la óptima tanto para la reducción de los polifenoles como para la de la DQO. La eficiencia del tratamiento biológico con alfalfa se comparó con la obtenida con *Azolla*. Los resultados indicaron que el tratamiento con alfalfa no dio lugar a la reducción de los polifenoles ni de la DQO.

PALABRAS-CLAVE: Alpechín - Azolla - Demanda química de oxígeno - Polifenoles - Tratamiento biológico.

SUMMARY

Ability of the aquatic fern *Azolla* to remove chemical oxygen demand and polyphenols from olive mill wastewater.

We investigated the biofiltration ability of the aquatic fern *Azolla* to remove polyphenols and chemical oxygen demand (COD) from olive mill wastewater (OMWw) collected from the traditional (TS) and continuous (CS) extraction systems. *Azolla* biomass was packed into five sequential Imhoff cones and five sequential columns. In both experiments, the filtrates collected from the 5th biofilter showed a decrease in polyphenol contents: from 7650 mg l^{-1} to 3610 mg l^{-1} in TS OMWw and from 3852 mg l^{-1} to 1351 mg l^{-1} in CS OMWw. The COD contents decreased from 110200 mg L^{-1} to 52400 mg L^{-1} in TS OMWw and from 41600 mg L^{-1} to 2300 mg L^{-1} in CS OMWw. A 5:1 OMWw to *Azolla*-fresh-weight ratio was optimal for both polyphenol and COD removal.

The biofiltration ability of alfalfa was compared with that of *Azolla*, but the treatment with alfalfa did not result in the reduction of COD or polyphenols.

KEY-WORDS: Azolla - Biological treatment - Chemical oxygen demand - Olive mill wastewater - Polyphenols.

1. INTRODUCTION

The extraction and use of olive oil has been an integral part of Mediterranean culture for over 6000 years (Civantos, 1995; Tardàguila *et al*., 1996).

Olive oil is produced by two methods: the traditional system (TS) and the continuous system (CS). Highly pollutant wastewater is a by-product of both processing methods. Olive oil extraction involves an intensive consumption of water and produces large amounts of olive mill wastewater (OMWw), the average volume ranging from 0.5 to 1.5 m^3 per ton of processed olives (Monteoliva-Sanchez *et al*., 1996; Paredes *et al*., 1996).

The content of fresh organic matter in oil mill wastewater is an agricultural and environmental problem for olive oil-producing countries, since its effects on soil status and fertility, insect proliferation and groundwater contamination are more harmful than beneficial (Cox *et al*., 1996; Spandre and Dellomonaco, 1996).

OMWw contains high amounts of organic matter (30-200 kg COD m^{-3}), with a COD/BOD_5 ratio between 2.5 and 5, which is considered poorly degradable (Lopez, 1992). The organic compounds in OMWw (sugars, polyphenols, tannins, polyalcohols, pectins and lipids), in association with its high C/N ratio and low pH, compromise the biological degradation process of soils (Marques, 2000) and can cause eutrophication when the wastewater is collected in basins with low exchange rates (closed gulfs, lakes, etc.).

The toxicity of OMWw is also due to its high content of phenolic compounds in a wide range of molecular weights (MW), from low-MW substituted phenols to complex high-MW phenolic compounds (Montedoro *et al*., 1992). During olive oil production, large quantities of phenols are released along with the wastewater, according to their partition coefficient. Phenolics are derivatives of benzene (cyclic derivatives in the case of polyphenols) with one or more hydroxyl groups associated with their ring. The dark color of the water is caused by polyphenols (Pp) (Hamdi and Garcia, 1993) and depends on the type of olives processed and their ripening stage and on climatic conditions, as well as the technology used. However, despite their toxicity, polyphenols are considered the most interesting compounds in OMWw (Visioli *et al*., 1995), because of their potential use in the perfume and pharmaceutical industries.

A possible treatment of OMWw to recover valuable compounds like polyphenols would employ the aquatic fern *Azolla* as a biofilter. Dried *Azolla* biomass has already been used in the biosorption of a wide range of heavy metal concentrations from aqueous media (Sela and Tel-Or, 1988; Cohen-Shoel *et al*., 2002). Pectin is an important polysaccharide constituent of *Azolla* cell walls, made up of fragments of polygalacturonic acid chains that interact with Ca^{2+} and Mg^{2+} ions to form a three-dimensional polymer (Schols *et al*., 1989; Jauneau *et al*., 1997; Kamnev *et al*., 1998). Carlozzi *et al.* (1986) reported progress in the development of a culture technique suitable for growing *Azolla* spp. in mineral solutions without combined nitrogen or in the wastewater from a sugar-refinery.

Azolla biomass can be used as a biofertilizer or as a feed supplement for aquatic and terrestrial animals due to its protein, crude fiber and mineral contents (Lumpkin and Plunknet, 1980).

The aim of this study was to test the ability of *Azolla* (vegetable matrix) to reduce the phenol and organic matter contents in wastewaters from the two methods of oil production. This paper reports the results of laboratory experiments.

2. EXPERIMENTAL AND METHODS

OMWw Pre-treatment

Fresh OMWw were collected in January 2004 from two olive mills (TS, CS) near Florence (Italy). Table 1 shows the main characteristics of the two OMWw after centrifugation.

Azolla cultivation

The strain of *Azolla filiculoides* derives from the Botanical Institute of Naples, Italy. Since the 1980s, it has been preserved by the "Centro di Studio dei Microrganismi Autotrofi" of Florence, which is now part of the Institute of Ecosystem Study of the Italian CNR. During the summer, the fern was grown outdoors, first in four mini-ponds of 4.0 m^2 and later in a maxi-pond of 25 m^2, in the following nutrient medium: KCl, 0.149 g l^{-1}; $CaCl_2$.$2H_2O$, 0.294 g l^{-1}; KH_2PO_4, 0.054 g l^{-1}; $MgSO_4$ .7 H_2O, 0.197 g l^{-1}; 1 ml l^{-1} of Hughes' minor nutrients (Fe-EDTA). The Fe-EDTA consisted of: $EDTANa_2Fe$, 29.754 g + $FeSO_4$ $7H_2O$, 24.9g (total volume = 1.0 l). During the experiments, the optimal planting density of 50 g (d.w.) m^{-2} was maintained (Carlozzi *et al*., 1986).

Table 1
Chemical parameters of olive mill wastewater (OMWw) obtained by both the traditional (TS) and continuous (CS) processes.

Chemical parameters	OMWw from TS	OMWw from CS
pH	4.5	5.1
Colour	21.4 ± 0.9	15.7 ± 0.6
COD (mg L^{-1})	92000 ± 2200	44400 ± 800
Total Polyphenols (mg L^{-1})*	7360 ± 290	4367 ± 130

***** expressed in gallic acid

Biofiltration experiments

Before using *Azolla* as a biofilter, it was washed thoroughly with distilled water and left to drip dry.

Fresh *Azolla* biomass (200 g) was inserted and lightly packed into each of five sequential Imhoff cones and five sequential columns (7 cm diameter and 35 cm height). The OMWw volumes (1 L) were introduced and maintained in contact with the fern biomass for 30 min. The OMWw was then percolated slowly into the beaker and analyzed for the presence of COD (Chemical Oxygen Demand) and polyphenols.

Figures 1 and 2 show the five packed Imhoff cones (in series) and the five columns (in series) used as biofilters in the laboratory experiments.

Figure 1
Azolla biomass packed inside Imhoff cones.

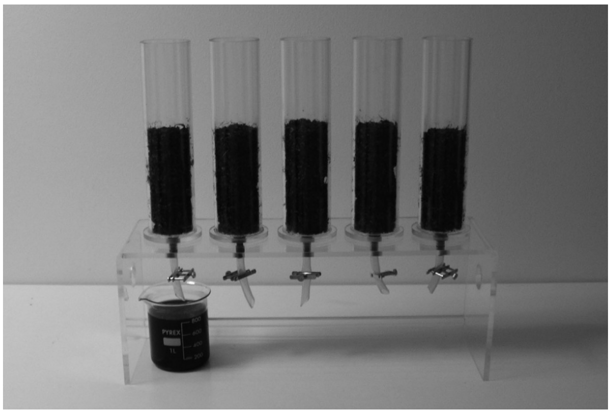

Figure 2
Azolla biomass packed inside columns.

Assay

Decoloration: OMWw samples were collected, centrifuged (20 min at 4000 rpm) and diluted 1:50 (v:v), and the absorbance was recorded at 395 nm (Yesilada *et al.*, 1997) with a Beckman DU 640 UV/Visible Spectrophotometer.

The COD concentration in the OMWw was determined with a HANNA C99 Multiparameter Bench Photometer for laboratories: 2 ml of sample were added to an oxidizing chromatic acid solution and digested for 2 h at 150°C. After cooling, the color of the sample varied from orange to green.

The Folin-Ciocalteau reagent was used to determine the total polyphenol (Pp) content; this reagent is a solution of complex polymer ions formed from phosphomolybdic and phosphotingstic heteropoly acids. It oxidizes phenolates, reducing the heteropoly acids to a Mo-W complex at alkaline pH, resulting in a blue color. The total content of polyphenols was estimated spectrophotometrically (Beckman DU 640) at 730 nm, with gallic acid as standard. The equation was: Pp (mgl^{-1}) = Abs/2.33855 D

D = dilution factor

3. RESULTS AND DISCUSSION

Data on the growth of the aquatic fern (*Azolla-Anabaena azollae* symbiosis) outdoors in the climatic conditions of Florence are reported in Table 2. The productivities achieved in the 25 m^2 pond were similar to those reported by Carlozzi *et al.* (1986) for a 100 m^2 pond. The highest yield (13.2 g dry weight (d.w.) m^{-2} d^{-1}) was achieved in July; the average productivity was 10.3 g (d.w.) m^{-2} d^{-1}.

Table 2
Yield and growth rate of Azolla filiculoides achieved outdoors in a maxi-pond of 25 m^2. The average planting density was 50 g (dry weight) m^{-2}.

Months	Productivity [g (d.w.) m^{-2} d^{-1}]	Growth rate (d^{-1})
July	13.2	0.235
August	10.1	0.183
September	7.7	0.144

Physico-chemical analyses of the two OMWw showed that they were dark acidic wastes with high levels of organic matter and polyphenols. The TS OMWw was darker than the CS OMWw, and the COD and polyphenol levels were respectively 107% and 68.5% higher in the TS OMWw. According to Ranalli (1992), phenolic compounds are the pigments responsible for the dark color of OMWw.

To determine the best OMWw/fresh-*Azolla* ratio (w/w) for the removal of organic compounds, four ratios were tested: 10:1, 5:1, 2:1 and 1:1.

The results for the Imhoff cones and columns were the same.

Figure 3 shows the *Azolla* adsorption of polyphenols at the four OMWw/fresh-*Azolla* ratios. Despite the different initial polyphenol concentrations in the two wastewater types (TS, CS), the proportional decrease in polyphenol content after *Azolla* treatment was the same. Figure 4 shows the polyphenol pull-down efficiency. The most effective OMWw/fresh-*Azolla* ratio was 5:1 in both OMWw types, and the highest values were 5 mg l^{-1} g(*Azolla*)$^{-1}$ in TS and 2.75 mg l^{-1} g(*Azolla*)$^{-1}$ in CS.

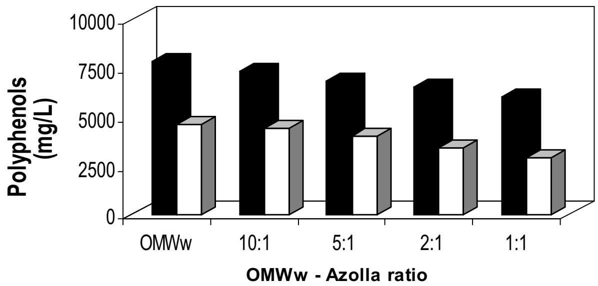

Figure 3
Polyphenol reduction in TS OMWw (black bars) and CS OMWw (white bars) as a function of the OMWw/fresh-*Azolla* ratio.

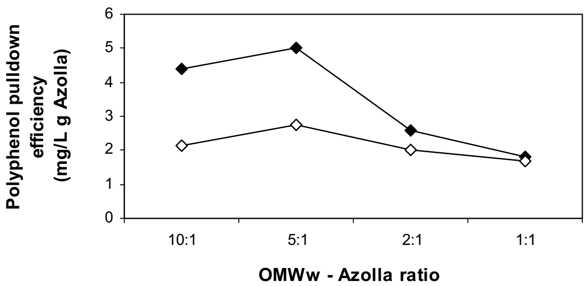

Figure 4
Polyphenol pull-down efficiency in TS OMWw (◆) and CS OMWw (◊) as a function of the OMWw/fresh-*Azolla* ratio.

The pattern of COD removal was similar (Fig. 5). The optimal OMWw/fresh-*Azolla* ratio was 5:1 (Fig. 6). This ratio was chosen for the experiments, since it always gave the highest COD pull-down efficiency in both types of OMWw (70 mg l^{-1} g(*Azolla*)$^{-1}$ in TS and 57 mg l^{-1} g(*Azolla*)$^{-1}$ in CS). A comparison of Figures 3 and 5 with Figures 4 and 6 respectively shows that the total organic matter content (polyphenols and COD) adsorbed by the *Azolla* biomass increased as the OMWw/*Azolla* ratio decreased, while the COD and polyphenols

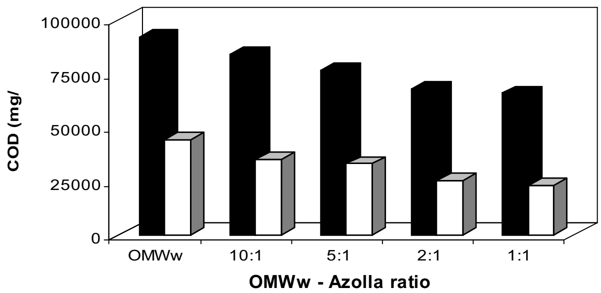

Figure 5
COD reduction in TS OMWw (black bars) and CS OMWw (white bars) as a function of the OMWw/fresh-*Azolla* ratio.

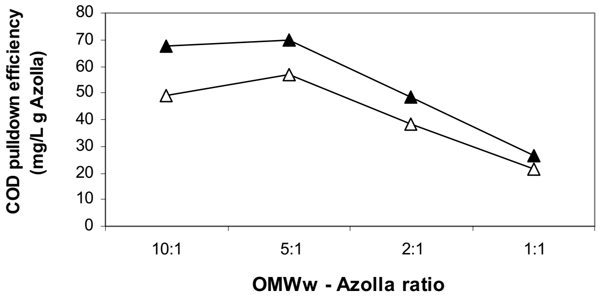

Figure 6
COD pull-down efficiency in TS OMWw (▲) and CS OMWw (Δ) as a function of the OMWw/fresh-*Azolla* ratio.

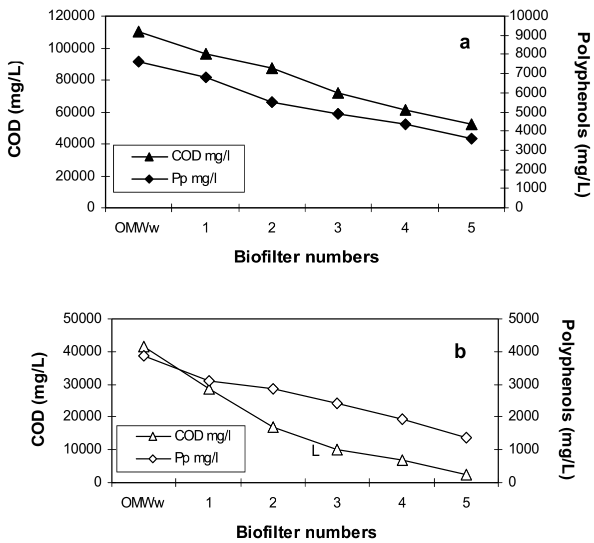

Figure 7
COD and polyphenol (Pp) removal in OMWw after five passages through fresh-*Azolla* biomass packed into Imhoff cones: traditional system (a); continuous system (b).

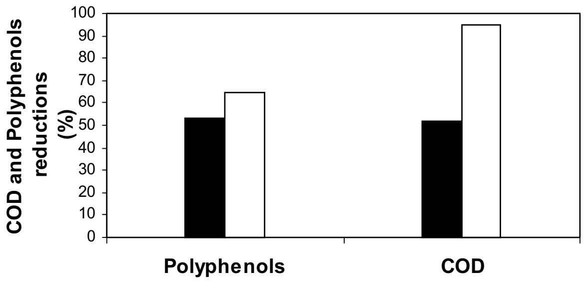

Figure 8
COD and polyphenol percentage decrease in TS OMWw (black bars) and CS OMWw (white bars).

adsorbed per unit of biomass dry weight decreased. This behavior was similar to that observed by Itho *et al*. (1975) in heavy metal removal by plant and microbial biomasses; the authors supposed that the dependence of adsorption on cell density was probably due to electrostatic interactions among binding sites, as more ions are adsorbed on the cell when cell distances are greater. Cohen-Shoel *et al*. (2002) reported a correlation between metal adsorption and the availability of carboxyl groups of galacturonic acid, the principal constituent of pectin. They used methylation, demethylation of carboxyl groups and pectinase treatment to demonstrate that the pectin of the *Azolla* cell wall is the major metal binding site. Likewise, lignin and cellulose, the major constituents of tree ferns, were mainly responsible for the adsorption of a basic dye by those plants, because of the availability of their polar functional groups (Newman, 1997; Ho *et al*., 2005). In our study, the adsorption efficiency of the 10:1 OMWw/fresh-*Azolla* ratio was similar to that of the 5:1 ratio. This was probably the result of the saturation of binding sites above a certain concentration of the organic compounds.

A single biofilter with the *Azolla* matrix was considered insufficient to achieve an effective pull-down of organic compounds (polyphenols and COD) in OMWw. Thus, we tested a new process using five Imhoff cones (biofilters) in series. The results of the sequential passages of the same OMWw are shown in Fig. 7a,b. The COD and polyphenol contents in the OMWw decreased progressively from the first to last cone. In the OMWw from the 5th cone, COD was reduced to 52400 mg l^{-1} in TS and to 2300 mg l^{-1} in CS. The polyphenols decreased to 3610 mg l^{-1} in TS and to 1351 mg l^{-1} in CS.

Figure 8 shows the percentage of decrease in polyphenols and COD in the TS and CS OMWw after passing through the series of five Imhoff cones. Polyphenols decreased by 53% in TS and by 65% in CS. COD decreased by 52% in TS and 95% in CS. The higher COD pull-down percentage in the CS OMWw was probably due to the complete saturation of the binding sites after treatment. In the TS OMWw, the initial COD concentration was probably higher than the number of available binding sites on the *Azolla* cell wall.

The color intensity of the TS and CS OMWw decreased by 45% and 52% respectively after *Azolla* treatment (data not shown). The remaining substrate color was mainly due to highly polymerized phenolic compounds, which are not readily absorbed by *Azolla.*

It is noteworthy that the organic matter adsorption capacity seems to depend on the type of organism used. Beccari *et al*. (2002) compared the COD removal obtained with activated sludge with that achieved with a co-culture of aerobic bacteria (Bertin *et al*., 2001) using the same oil mill effluent. The total COD pull-down percentages were 86.5% and 52.4% respectively.

Gardea-Torresdey *et al*. (1995a; 1995b) used fresh and silica-immobilized *Medicago sativa* (alfalfa) for heavy metal adsorption, and they successfully removed copper and nickel from aqueous solutions. They established that a pH of 5-6 was optimal for heavy metal binding to alfalfa tissues. Acidic pH was probably essential because it made the carboxyl groups of the alfalfa cell wall available for metal binding.

Results of the comparison of the biofiltration properties of alfalfa and *Azolla* for OMWw purification are shown in Fig. 9. OMWw was treated with alfalfa without changing the pH and the results were compared with those of *Azolla* removal of COD and polyphenols at the same pH (4.5 – 5.1). In these conditions, there was no reduction of either COD or polyphenols with the alfalfa treatment. This confirms the high affinity and binding capacity of both COD and polyphenols to *Azolla* biomass. Therefore, *Azolla* has great potential for bioremediation because it is easily obtained at low cost.

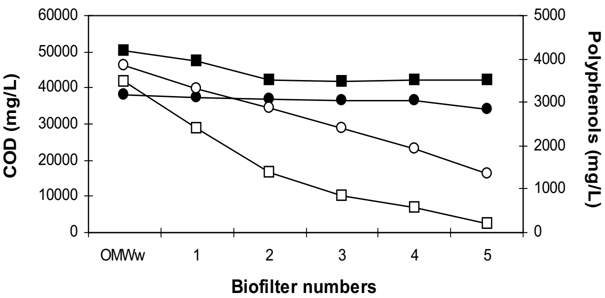

Figure 9
Comparison of COD (□) and polyphenol (○) abatement in OMWw after biofiltration with *Azolla* (open symbols) and alfalfa (closed symbols).

4. CONCLUSIONS

This preliminary study demonstrates the role of *Azolla* in reducing the organic load of polyphenols and COD in OMWw. This finding is enhanced by the negative results obtained with alfalfa.

Azolla production is easy and cheap because it can grow in a culture medium without nitrogen thanks to the N_2-fixing cyanobacteria *Anabaena azollae* living in cavities on the dorsal of the fern's leaves.

These aspects, together with the ability of *Azolla* biomass to remove both polyphenols and COD from OMWw, provide a new low-cost strategy for OMWw bioremediation and polyphenol recovery.

The treated wastewaters are not toxic and the effluent appears to be environmentally safe and suitable for agricultural irrigation, providing the soil with organic matter and potassium.

Research is in progress to investigate the mechanism by which *Azolla* biomass removes COD and polyphenols from OMWw.

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