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

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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 content of polyphenols after five sequential Imhoff cones was 41600 mg L⁻¹ in TS OMWw and from 3852 mg L⁻¹ to 1351 mg L⁻¹ in the alpechin obtained through the traditional and continuous systems.

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 Ca²⁺ and Mg²⁺ 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 Micorganismi 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 m² and later in a maxi-pond of 25 m², in the following nutrient medium: KCl, 0.149 g l⁻¹; CaCl₂·2H₂O, 0.294 g l⁻¹; KH₂PO₄, 0.054 g l⁻¹; MgSO₄·7H₂O, 0.197 g l⁻¹; 1 ml l⁻¹ of Hughes’ minor nutrients (Fe-EDTA). The Fe-EDTA consisted of: EDTANa₂Fe, 29.754 g + FeSO₄·7H₂O, 24.9 g (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).

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.
**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 phosphotungstic 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 m² pond were similar to those reported by Carlozzi et al. (1986) for a 100 m² 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>
<thead>
<tr>
<th>Months</th>
<th>Productivity [g (d.w.) m⁻² d⁻¹]</th>
<th>Growth rate (d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>July</td>
<td>13.2</td>
<td>0.235</td>
</tr>
<tr>
<td>August</td>
<td>10.1</td>
<td>0.183</td>
</tr>
<tr>
<td>September</td>
<td>7.7</td>
<td>0.144</td>
</tr>
</tbody>
</table>

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

![Figure 4](image_url) 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...
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 2300 mg l⁻¹ in CS. The higher COD pull-down percentage in the TS 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.

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 N₂-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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