Ecotoxicological Safety of Wastewater Treated with Lignocellulosic Adsorbents

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Abstract**—**Portugal is an important wine and olive oil producer, activities which generate a high quantity of residues commonly called grape stalks and olive cake, respectively. In this work grape stalks and olive cake were used as lignocellulosic adsorbents for wastewater containing lead treatment. To attain a better knowledge of the factors that could influence the quality of the treated wastewater, a chemical characterization of the materials used in the treatment was done. To access the ecotoxicological safety of the treated wastewater, several tests were performed.

The results of the toxicity test show that the samples leachate has a mild effect on the living models tested. The tests performed in lemna and bacteria were the most sensible to toxicity effects of the samples. The results obtained in this work evidenced the importance of use of simple and fast toxicity tests to predict impacts in the environment.

Keywords**—**Chemical composition, ecotoxicological, lignocellulosic residues, safety.

I. Introduction

ONE of the most important activities of Portuguese agriculture, is wine production which occupies the 10th place worldwide, with an occupied area of occupation of approximately 239 951 ha, with a wine production of about 7 hL annually [1]. The production of olive is also an important activity in Portuguese agriculture. Olive production currently occupies the 8th position worldwide, with about 60 273 ha and a production of 42 182 ton/year [2]. The agricultural lignocellulosic by-products are receiving increased attention, particularly by its environmental applications. Due to the high cost of activated carbon [3] and of the common hydroxides used in heavy metal recovery from wastewater, a lot of research has been recently done in order to find an economically viable lignocellulosic material that can adsorb the main heavy metals from wastewater. Usually the main concerns in relation to safety considerations is the concentration of heavy metals however there are some evidences that this kind of adsorbents may leachate some organic compounds to the treated wastewater. Some of these compounds, mainly phenolic compounds, have been shown to have some toxic activities to environmental organisms. On the other hand publications covering lignocellulosic adsorption are mainly focused on the adsorption kinetics and adsorption capacity [4]-[7] and forget fundamental chemistry aspects of leachates in treated wastewater. The aim of this work was to study the best conditions to attain the maximum removal of lead from a wastewater using lignocellulosic residues (GS and OC) and provide new strategies for understanding the quality and safety of wastewater obtained after the adsorption which may contribute to increase the list of its potential uses.

The influence of chemical composition of the material on the possible contamination of treated wastewater and its security in several uses will be discussed.

Chemical analysis may not be sufficient to assess the ecological safety of complex mixtures of organic and inorganic compounds originated from leachate of solid material. Whole-effluent toxicity tests have been referred to as extremely useful approach to identify toxicity impacts in the environment. It is also important to apply batteries of assays to evaluate toxic effects in organisms representing diverse taxonomic and trophic levels [8].

In this work we access toxicity of these treated effluents in bacteria and in aquatic and terrestrial plants in order to evaluate its safety.

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II. Experimental

A. Sample Preparation

GS and OC were used in the tests. The samples were grounded and sieved into three fractions > 40 Mesh, 40-60 Mesh and < 60 Mesh. The 40-60 Mesh fraction was selected for the tests.

B. Chemical Composition Determination

The OC was characterised regarding the ash content, extractives (in acetone, dichloromethane and in hot water), proteins, tannins, cellulose, lignin and hemicelluloses. The ashes content was determined by calcinations of the material at 525ºC, according to the standard procedure Tappi T 211 om-93[9].

The extractives content in acetone and dichloromethane were determined by Soxhlet extraction according to the Tappi T 204 om-88 [10]. The determination of extractives in hot water was carried out with a solution of ammonium citrate (10 g/L) for 1 hour under reflux (liquid-to-solid ratio 100). The proteins content in extractives-free shells, after extraction with acetone, was determined by treatment with 1% pepsin solution in 0.1 M HCl at 37ºC for 16 hours [11]. The tannins content was assessed in extractives- and proteins-free shells by reflux with 0.3% NaOH solution (liquid-to-solid ratio 100) under nitrogen atmosphere for 1 hour. The extracted material was filtered, washed with hot water until neutral reaction of filtrate and dried at 60ºC to a constant weight. The content of tannins was assessed by the difference in weights of initial and extracted materials. The alkaline extract was precipitated by adding 3M H_2SO_4 until pH 3. After 24 hours, precipitated tannins were centrifuged and washed with water to pH 5. Finally, the tannins fraction was freeze dried. The cellulose was determined by 4 consecutive treatments with HNO_3: EtOH mixture (1:4, v/v) under reflux for 1h each according to the Kürschner and Höffer method [12]. The lignin content in olive cake free of extractives, proteins and tannins was determined by Klason method with 72% H_2SO_4 [10].

The results of GS were already published [13].

C. Adsorption Tests

The best operating conditions to attain the maximum removal of lead (Pb^{2+}) from a wastewater were determined before by testing the lead adsorption of 100 mg of residues (GS and OC) for 24 hours [14]. The best operating conditions were obtained at pH=6. Olive cake and grape stalks showed a removal percentage of 86% and 100%, respectively.

The lignocellulosic material (1.5grams)was placed in erlenmeyer to which 150 mL solution of 25 mg Pb^{2+}/L or 150 mL of distilled water at pH=6 were added. The erlenmeyers were then placed in a shaker bath for 24 hours. The obtained solutions were filtered and the Pb^{2+} concentration was determined by atomic absorption spectroscopy flame.

D. Chemical Characterization of the Samples

Since some chemical parameters are known to affect the toxicity in living systems, while other may act like nutrients, the leachate samples were characterized in terms of its chemical composition. Electrochemical device is used to measure the pH by potentiometry and conductivity by condutimetry. We also chose to determine the Pb content to evaluate if the presence of some residual metal could *contribute* to toxicity. Also the Ca, Mg and K were determined duo to its abundance in solid by-product, its ability to leachate and its potential influence in the toxicity test results. The concentration of Pb, Ca, Mg and K were determined using atomic absorption spectrometry by flame atomization.

E. Toxicity Tests

Germination assays were performed using seeds of lettuce (*Lactuca sativa*). Twenty five plant seeds were spread on each sterilized Petri dish, containing sterilised Wathman paper disk and then irrigated with 5 mL of the different leachate solutions. The control was made with sterile water, using the same procedure. Each assay was done in duplicate. The Petri dishes were incubated at 27ºC for 7 days, after which the germinated seeds were counted and root length measured. The germination index (GI, %) was evaluated by (1):

$$GI = \frac{\overline{GS}(s)\times\overline{RL}(s)}{\overline{GS}(c)\times\overline{RL}(c)}\times 100 \quad (1)$$

where $\overline{GS}$ represents de average number of germinated seeds and $\overline{RL}$ the average root length (mm) of seeds in samples (s) and the control (s) tests.

The behavior of the bacterial strains, isolated from surface water to leachate water from adsorption with HS and AS was determined by disc diffusion method [15]. After its isolation stock cultures of each one of the microorganisms were maintained at temperature of 4ºC in PCA medium (Merck Cat. No. 1.05463.0500).The target microorganisms were grown in nutrient broth medium (Merck Cat. No. 1.07882.0500) until reaching 1.0 of optical density. Nutrient agar plates were prepared by pouring sterile nutrient agar into Petri dishes, previous sterilized. After the solidification of the medium, plates were incubated for 24 hours at 25°C. The isolated strains were inoculated, scattering in solid medium in a Petri dish. Filter paper discs (5 mm) were dipped in each test solutions and, without excess of water, were placed in the individual Petri dishes. Plates were incubated at 30ºC for 18 hours. Control assays were performed with sterile water as test solution. The tests were evaluated by the inhibition diameter in each disc. Each sample was tested in duplicate and the halo of the inhibition zones measured.

The influence of samples in grow of *Lemna gibba* L. (Duckweed) was determined according to standard methods for water and wastewater [16]. Nine Lemna fronds were gently placed in 100 mL Erlenmeyer containing 25 mL of leachate solution added with the nutrient medium. The flasks were placed at 25°C in the presence of light (continuous cool white fluorescent lighting with a 100 W lamp) during four days. Control treatment (with nutrient medium) was made All the test were performed in triplicate.

In plant growth experiments, 200 cm^3 plastic vessels were filled with 180 cm^3 of a mixture with equal parts of sand, perlite and peat [17]. The prepared soil was sieved through 5 mm mesh screens to remove larger fractions. Ten seeds of *Lycopersicum esculentum* Mill were buried just near the surface. After the seeds were planted and watered with different leachate solutions, the pots were placed in a cycle with a 14-h light/10-h dark, a day/night temperature of 25/20ºC and a relative humidity of 70%.The pots were watered daily with 10mL of each solution from the day following its planting until the day that equaled 14 days after germination. After this period, the seedlings were counted, collected and placed in a box, which was placed in an oven at 60°C for 24 hours to evaluate the growing index, GRI (%) according to (2).

$$GRI = \frac{DW(s)}{DW(c)}\times 100 \quad (2)$$

with DW representing the dry weight (g) of sample(s) and control (c).

III. Results and Discussion

A. Chemical Composition of the Materials

Aiming a better understanding of the results obtained the general chemical composition of the GS and OC have been done. Table I presents the GS and OC chemical composition.

TABLE I
CHEMICAL COMPOSITION OF GS AND OC (% DRY MATERIAL)

Parameters		Content (%)	
		GS [13]	OC
Ashes		7.00	3.44
	K	0.90	7.61
	Ca	0.15	3.61
	Mg	0.02	0.15
	Na	<0.01	0.01
	Zn	0.01	0.01
Extractives	Acetone	3.30	4.85
	Water-soluble	23.70	19.85
Cellulose Kürscher and Höffer		30.30	26.69
Proteins		6.10	18.22
Tannins		15.90	17.14
Lignin		17.40	24.09
Hemicelluloses		21.00	5.57

The chemical characterization showed that GS and OC are lignocellulosic materials, in which cellulose is the major component (with around 30 and 27% for GS and OC, respectively). Regarding other components in GS, this value is followed by insolvent hemicelluloses in hot water (with around 24%) and lignin (17.4%). It's important to highlight the high presence of tannins (15.9%) and extractives in water (23.7%). To OC the second major component is lignin (24%) followed by insolvent hemicelluloses in hot water (with around 20%) and tannis (24%). The chemical analysis of the ash cations made to these residues shows that OC had high contents of Ca^{2+} and K^{+} and GS of K^{+}.

B. Chemical Composition of the Leachate Samples

The chemical characterization of the water treated by adsorption is represented in Table II.

In accordance with the chemical composition of the materials the leachate samples present conductivity values ranging from 100 to 300 µS/cm and potassium as the main cation present. As a result from the treatment only OC samples showed minor concentration of Pb while in the others the values were below detection limit. The pH values were between 5.05 and 6.35.

TABLE II
CHEMICAL COMPOSITION OF LEACHATE OF GS AND OC

	pH	Cond (µS/cm)	Pb (mg/L)	Ca (mg/L)	Mg (mg/L)	K (mg/L)
OC (with Pb)	5.24	295.5	3.09	13.89	2.07	88.80
GS (with Pb)	5.96	222.5	< DL	11.81	2.29	35.30
OC (blank)	5.05	302.0	< DL	13.24	2.01	100.75
GS (blank)	6.35	103.1	< DL	9.01	1.67	31.59

DL – detection limit

C. Toxicity Tests

Although the conductivity values present, the germination process doesn't seem to be affected by the samples while we could notice some stimulation on grow of the roots compared with controls (Table III). It is necessary to make more studies to assess if this stimulation on growing could be attributed to the presence of a stimulating compound or simply a higher concentration of nutrients in the leachate.

TABLE III
RESULTS OF THE GERMINATION TEST

	OC (with Pb)	GS (with Pb)	OC (blank)	GS (blank)	Control
GI (%)	114	203	142	273	-
Lettuce Germinated (%)	32	59	42	57	48
$\overline{RL}$ (mm)	3.9	3.9	3.8	5.35	2.4

A different behavior was observed in the grow test where the leachate samples seemed to inhibited the germination slightly while no stimulation was seen on growing (Table IV). The fact that a different species of plant was used for the test may be one possible explanation for the differences observed from the germination test.

TABLE IV
RESULTS OF THE GROWTH TEST

	OC (with Pb)	GS (with Pb)	OC (blank)	GS (blank)	Control
GRI (%)	40.8	34.1	51.1	73.2	-
Tomato germinated seeds (%)	50.0	50.0	65.0	60.0	70.0

The Lemna toxicity test showed that some leachate samples have the ability to generate some disturbances on its growing process (Table V). We could observe, in general, less grow, especially with the OC samples. Visual inspection evidenced that lemnas treated with OC (with Pb) and GS (blank) showed necrosis and less pigmentation, respectively, which is characteristic of some form of toxicity in the extracts. It could also be observed some increasing on dimensions of leaves, probably as a result of higher concentration of nutrients in comparison with the controls.

TABLE V
RESULTS OF THE LEMNA TOXICITY TEST

	OC (with Pb)	GS (with Pb)	OC (blank)	GS (blank)	Control
Frond increase/vessel	16.5	15.0	12.0	16.0	17.0
Visual inspection	Necrosis	-	-	Big leaves; less pigmentation	Small leaves

The bacteria diffusion test seemed to be the most sensible of all the assays performed in this work (Table VI). All the strains showed some inhibition on growing. The Bact1 was the most resistant while the Bact4 evidenced the most sensible behavior. OC sample was the only one to show effects on all the strains.

TABLE VI
RESULTS OF THE BACTERIA DIFFUSION TEST

Strain	Inhibitory zone diameter (mm)				
	OC (with Pb)	GS (with Pb)	OC (blank)	GS (blank	Control
1	10	-	-	-	-
2	4	10	9	13	-
3	8	8	12	7	-
4	12	10	10	10	-

Despite the application of a treatment process to remove Pb, and the differences observed between the toxicity tests, it was noticed that all the samples showed some mild effects on the four living models. These results suggest the presence of some compounds in the leachates, originated from the material, capable to exert some disturbance in the environment. Further investigation is necessary to identify these compounds and comprehend the mechanisms responsible for its biological activities.

In routine work it is frequently difficult to perform a complete chemical analysis sufficient to predict the ecological safety due to the complexity of this kind of effluents. The use of simple and fast toxicity tests using whole effluent, like those that were used in this work, may be a useful and low cost strategy to identify toxicity effects, implement better treatment processes and predict impacts in the environment.

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