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Valorization of Lignocellulosic Wastes – Evaluation of Its Toxicity When Used in Adsorption Systems

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***Abstract*—**The agriculture lignocellulosic by-products are receiving increased attention, namely in the search for filter materials that retain contaminants from water. These by-products, specifically almond and hazelnut shells are abundant in Portugal once almond and hazelnuts production is a local important activity. Hazelnut and almond shells have as main constituents lignin, cellulose and hemicelluloses, water soluble extractives and tannins. Along the adsorption of heavy metals from contaminated waters, water soluble compounds can leach from shells and have a negative impact in the environment. Usually, the chemical characterization of treated water by itself may not show environmental impact caused by the discharges when parameters obey to legal quality standards for water. Only biological systems can detect the toxic effects of the water constituents. Therefore, the evaluation of toxicity by biological tests is very important when deciding the suitability for safe water discharge or for irrigation applications.

The main purpose of the present work was to assess the potential impacts of waters after been treated for heavy metal removal by hazelnut and almond shells adsorption systems, with short term acute toxicity tests.

To conduct the study, water at pH 6 with 25 mg.L^{-1} of lead, was treated with 10 g of shell per litre of wastewater, for 24 hours. This procedure was followed for each bark. Afterwards the water was collected for toxicological assays; namely bacterial resistance, seed germination, *Lemna minor* L. test and plant grow. The effect in isolated bacteria strains was determined by disc diffusion method and the germination index of seed was evaluated using lettuce, with temperature and humidity germination control for 7 days. For aquatic higher organism, Lemnas were used with 4 days contact time with shell solutions, in controlled light and temperature. For terrestrial higher plants, biomass production was evaluated after 14 days of tomato germination had occurred in soil, with controlled humidity, light and temperature.

Toxicity tests of water treated with shells revealed in some extent effects in the tested organisms, with the test assays showing a close behaviour as the control, leading to the conclusion that its further utilization may not be considered to create a serious risk to the environment.

***Keywords*—**Acute toxicity tests, adsorption, lignocellulosic wastes, risk assessment.

I. Introduction

SEVERAL industrial wastewaters have as main pollutant agents heavy metals, as result of its activities. Examples are the metal plating industries, mining activities, smelting, or even battery manufactures and recyclers. One common wastewater treatment is the utilization of several materials to adsorb heavy metals. Bhatnagara and Sillanpa [1] made an important review that emphasise the importance in this field of materials like agriculture lignocellulosic by-products. This is the case of hazelnut and almond shells [2], [3] or *Eichhornia crassipes* [4] and vegetables such as *Bradyrhizobium* [5]. Bulut and Tez established that the thermodynamic process for metals adsorption by hazelnut and almond shells is spontaneous because the value of free energy (ΔG) evaluated for the process was negative [2]. On the other hand, Mehrasbi et al. also verified that adsorbent materials of low cost can successfully remove 9 mg Pb^{2+} and 7 mg Cd^{2+} per gram of adsorbent [3].

In Portugal, almond shells (AS) and hazelnut shells (HS) are abundant as by-products of the almond and hazelnut production. The production of almond is an important activity, with Portugal occupying the 10th position worldwide. Annually, nearly 36 530 ha yields nearly 14 000 tones of almond. On the other hand, hazelnut production occupies an area of 600 ha, with a yield of approximately 1 ton/ha. Worldwide, Portugal is the 16th producer of hazelnut.

Although the legislation defines levels of parameters such as BOD5, COD, oil and grease, sulphates, nitrates and metals, including complete lead, for wastewater quality control, increase attention is being given to ecotoxicological tests, complementing the treated wastewaters chemical characterization. The toxicological analysis is the study of adverse effects caused by the interaction of chemicals, living organisms and/or biological systems, keeping in mind that aquatic and terrestrial plants are essential components of a healthy ecosystem. The toxicity of effluents has been an extremely useful tool to indicate toxic effects of water discharges on the environment [6]. Several organisms are used as targets, like luminescent bacteria, *Lemna gibba* L., seeds (for germination or growing assays), among others. Some controversy yet arises from these tests results. For instance, while certain authors argue that Lemnas are sensitive to toxicity, others claim that they are tolerant to environmental toxicity [7]. In tests conducted with Lemnas exposed to Cu and Ni, it was found that they not only have capacity for metals accumulations, but also have higher accumulation potential for Cu than for Ni [7]. Seed germination, being the first step in the life of a plant, and one of the most sensitive physiological process, is affected by hormonal interaction with environmental factors (abiotic and biotic) and the presence of metals in excess. This is the feature used in toxicity tests. The study of the influence of different concentrations of heavy metals (Cu, Mn, Ni, Zn) on the germination of *Atriplex halimus* and *Salicornia ramosissima*, showed that all metals

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tested affect the final germination percentage of *A. halimus* and only the Ni reduces the germination of *S. ramosissima* [8].

Keeping this in mind, and having as intent the need of discharge the water treated for lead removal by HS and AS, the main objective of this work was to evaluate the potential impacts of this effluent with short term acute toxicity tests, specifically assess whether the residual lead and the compounds leached from sorbent surface are toxic to bacteria, duckweed and seeds. Different tests were chosen in order to highlight the effect in organism with different complexity and that are characteristic from water or soils.

II. Materials and Methods

A. Adsorption Experiments

The adsorption of lead on HS and AS was studied by the batch technique. A known weight (1.5 g) of HS and AS (40-60 mesh) was equilibrated with 150 mL of 25 mg Pb^{2+}/L solution (pH 6) in a stopper Pyrex glass flask at a fixed temperature in a thermostatic shaking water bath. Blank assays were performed with the same volume of de-ionized water for each adsorbent. Lead, calcium, magnesium and potassium concentrations were evaluated by atomic absorption spectroscopy. pH and conductivity were measured by specific electrodes.

B. Acute Toxicity Tests

The behavior of isolated strains in the presence of water treated by adsorption with HS and AS was determined by disc diffusion method [9]. Four bacterial strains were isolated from a sample of surface water. After isolation, stock cultures of each microorganism were maintained at 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.

Duckweed, floating and flowering plants of the family *Lemnaceae*, are fast growing plants and adapt easily to various aquatic conditions. *Lemna gibba* L. was the test organism used. The test protocol has as reference the 8211method from Standard Methods for Water and Wastewater Examinations [10]. Duckweed growth was measured after four days of exposure to different test solution. Nine *Lemna* fronds were gently placed in 100 mL Erlenmeyer containing 25 ml of leach 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). A control (with nutrient medium) was necessary to compare the results and to demonstrate the effect of shells leachable content and residual metal content on duckweed growth. The plant growth index was calculated as follows [11].

$$Growing\ index = \frac{Biomass\ (t=4\ days)}{Biomass\ (t=0)} \quad (1)$$

Seeds of lettuce (*Lactuca sativa*) were prepared for the evaluation of leach from the adsorption systems effects in germinations. Twenty five plant seeds with good conditions and equivalent sizes were spread on each sterilized Petri dish, with sterilized Wathman paper disk and then irrigated with 5 mL of the different leach solutions. The same procedure was followed with sterile water as control. 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 2.

$$GI = \frac{\overline{GS_s} * \overline{RL_s}}{\overline{GS_c} * \overline{RL_c}} * 100 \quad (2)$$

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

The plant growth experiments were conducted in 200 cm^3 plastic vessels with about 180 cm^3 of a mixture of sand, perlite and peat, in the same proportion, per pot [12]. The prepared soil was sieved through 5 mm mesh screens to remove larger fractions. Ten seeds of *Lycopersicum esculentum mill* were then embedded just beneath the surface. After the seeds were planted and watered with different leach solutions, the pots were placed in a set 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 14th day after germination. After this period, the seedlings were counted, collected and dried in an oven at 60 ° C for 24 hours to evaluate the growing index, *GRI* (%) (3).

$$GRI = \frac{DW_s}{DW_c} * 100 \quad (3)$$

with DW representing the dry weight (g) of samples – s and control - c.

III. Results and Discussion

The chemical analysis of HS and AS showed that the majority constituents are lignin, cellulose and hemicelluloses but an important fraction regards water soluble extractives and tannins [13]. The AS has higher content of water soluble extractives, cellulose, proteins and tannins, while HS has higher content of lignin. The leachable components of shells, water soluble extractives and tannins, represent 3.0% and 8.8% in the HS, respectively, and 5.7% and 18.3% in AS.

The chemical characterization of the water treated by adsorption is represented in Table I. It is noticeable that HS have higher efficiency in the lead removal than AS, with nearly 100% removal verified by HS against 85% for the latest.

TABLE I
CHEMICAL COMPOSITION OF THE LEACH

	pH	Conductivity (µS/cm)	Pb (mg/L)	Ca (mg/L)	Mg (mg/L)	K (mg/L)
HS (with Pb)	5.57	67.10	< QL	2.12	0.32	21.11
AS (with Pb)	5.13	405.5	3.81	28.6	4.42	128.7
HS (blank)	5.82	61.75	< DL	1.98	0.18	19.79
AS (blank)	5.13	376.0	< DL	25.4	4.23	155.9

QL – quantification limit; DL – detection limit

The adsorption is commonly explained by the lignin, tannins or other phenolic compounds of the materials content. The structure of the phenolic groups of those compounds may be responsible for the Pb^{2+} ion exchange with the proton. The lead attaches to two adjacent hydroxyl groups and two oxyl groups that may donate two pairs of electrons to the metal ion, and release two hydrogen ions into solution. The pH decrease in the HS adsorption experiment with Pb^{2+}, comparing with the blank assay, may support this conclusion. With the AS, the decrease was the same but lower adsorption was verified and higher content of ions were leached by the almond surface. AS leach has higher values of calcium, magnesium and potassium and therefore conductivity. The extractable tannins were also more evident in AS assays once the water solutions had a stronger brown color comparing with HS water solutions that were colorless. Nevertheless, is well known that soil amendments intent to strengthening the soil nutrient in organic matter and ions like Ca, Mg, K among others, which shows the importance of this type of water in agricultural soils.

Acute toxicity tests were done in order to evaluate the effect of residual Pb^{2+} and the shells extractable in different organisms, and evaluate the safety of the treated water discharge. Four tests were chosen in order to verify the consequences in organisms with different resistances, sizes and characteristic of different environments, water and soils. In Table II is possible to verify the effect on bacteria dead in presence of the solutions under evaluation. The test consists of the inoculation of the bacterial strain in Petri dishes with a solid medium. Above the culture, are placed the discs previously immersed in solutions under study. After incubation is made the measurement of the so-called "zone of inhibition", or the area around the disk where there was no bacterial growth. The inhibitory zone diameter, or the diameter of the inhibition circle is measured and is proportional to the inhibitory effect.

TABLE II
INHIBITORY ZONE DIAMETER OF ISOLATED STRAINS

Strain	Inhibitory Zone Diameter (mm)				
	HS (with Pb)	AS (with Pb)	HS (blank)	AS (blank)	Control
1	6	-	7	10	-
2	-	-	8	12	-
3	8	-	14	10	-
4	18	15	12	9	-

The most obvious conclusion to take out from the data shown in Table II is that the residual Pb^{2+} is not an inhibition factor of bacterial growth, once stronger effects were verified in blank assays. Further evaluations are necessary to identify the differences between assays with and without Pb^{2+}. It was expected that, at least, the different bacteria response should be identical in solutions of leach from the same shells. The controls revealed no inhibition and Strain 4 evidenced the most sensible behavior in the presence of possible toxic compounds. Generally, some inhibition action on microorganisms is a consequence of essential functional groups blocking or modifying the active conformation of biological molecules by external toxic compounds.

The *Lemna* test is performed using frond-count increase as the test end point. Any visible protruding frond was counted. The results are presented in Table III.

TABLE III
GROWTH RESPONSE OF THE DUCKWEED *LEMNA GIBBA* L. TO LEACH SOLUTIONS

	HS (with Pb)	AS (with Pb)	HS (blank)	AS (blank)	Control
Frond Increase/Vessel	9	6	7	2	8
Growing Index	2.4	1.7	1.7	1.2	1.9
Visual Inspection	Chlorosis	Necrosis	Chlorosis	Necrosis	Healthy

The fronds quality was evaluated, comparing test assays with controls, by visual inspection observing the duckweed plants under a magnifying glass (2x) for symptoms like chlorosis (loss of pigment), necrosis (localized dead tissue), colony breakup, root destruction, loss of buoyancy and gibbosity (humpback or swelling). The Lemna fronds in the controls were healthy and had an uniform green color but were the smallest of all the assays. In all the solutions it was verified the development of Lemnas fronds, with growing index above 1. It was also verified a development of roots in all the assays. Both tests with AS and HS showed an effect on Lemnas development, although with HS solutions the growing index was higher than the control. The loss of pigmentation in the Lemnas of the HS assays, with a slightly chlorosis, and an evidence of 1 dead frond in the AS assays, show some effect of the compounds leached from the shells in the plant development. Thus, some evidence is taken that the organic compounds may induce the plant growth but with some shortcomings.

In the germination tests, the germination index, described by equation 2, is evaluated having in account the number of seeds germinated but also the size of its roots. Although the number of germinated seeds in the control was higher, the roots were smaller. Therefore, the %GI in the test assays was above 100%.

TABLE IV
RESULTS OF THE GERMINATION TOXICITY TEST

	HS (with Pb)	AS (with Pb)	HS (blank)	AS (blank)	Control
GI (%)	188	115	213	164	-
Lettuce Germinated Seeds (%)	50	45	54	53	64
$\overline{RL}$ (mm)	44	29	45	36	24

Again, is evident that AS leach is more toxic than HS and some evidence shows that residual Pb^{2+} may have effect in the seeds development. While for HS with and without Pb^{2+} the GI percentage of germinated seeds and average root length were similar, in AS solutions, the results were different. In the solution with residual Pb^{2+} the parameters were lower than in the blank solutions.

Data from Table V, regarding the behavior of growing tomato tests, show, once more, that HS solutions are less toxic to seeds than AS solutions. This behavior is similar in all the performed tests. The effect of high concentration of soluble ions may be the most important problem affecting the organisms. Additional information is that even in the control assays the development of living species was not 100%.

TABLE V
RESULTS OF THE *LYCOPERSICUM ESCULENTUM Mill* GROWTH TOXICITY TEST

	HS (with Pb)	AS (with Pb)	HS (blank)	AS (blank)	Control
GRI (%)	74.8	37.9	64.3	42.8	-
Tomato Germinated Seeds (%)	55	30	45	40	70

Is worthwhile to further strengthen that the HS assays, with and without Pb^{2+} had similar results meaning that probably the main extractable from the HS are key factor in the seeds development and not the residual Pb^{2+} in the solution.

Taken account all the tests, it is possible to strength the importance of the toxicity tests once solutions with low pollutant profile have effect in living organisms. The most reproducible test was found to be with Lemnas, with very close results between replicates. An interest suggestion arising from the results is that although some negative effect is felt by the living organism, at the same time, higher development rate is suggested in assays, namely larger fronds in Lemnas and longer roots in the germinated seeds than in the control. These observations probably result as effect of the organic compounds leached from the shells surface.

In fact, several plants may be used as additives of some organic material to soil as an alternative for diseases control. Is the case of kudzu (*Pueraria lobata*), velvetbean or mucuna (*Mucuna deeringiana*) and pine bark (*Pinus* spp.) that were used in an attempt to improve soybean plant growth and to reduce the disease induced by *Rhizoctonia solani* [14]. Although further evaluations are needed to understand the extent of the effects in the organisms and, if valuable, what are the components of the leached solutions that have an important role, we may conclude that not only the leach may not be considered highly toxic to the environment, as it still, and according to other authors, the extractable components from barks, may have an immediate role in growing and on pathogen and consequently diseases suppression.

IV. CONCLUSION

According to the Pb^{+2} adsorption procedures and consequent toxicity tests is possible to conclude that:

1. HS has higher efficiency than AS for the metal removal from contaminated water;
2. The adsorption process using AS involves higher leaching extent of compounds from the shell surface, namely ions like Ca, Mg or K, but also tannins with an effective brown color;
3. The water treated by AS apparently has some effect in the tested organisms development and more pronounced than the HS leach;
4. Albeit the influence of the leach compounds in the initial germination inhibition of the tested organisms, some evidences were found leading to the conclusion that the organic compounds may enhance it grow.

Toxicity tests of water treated with shells revealed in some extent effects in the tested organisms. Although more research is needed in order to consolidate the results, deepen the effects of leached compounds in the test organisms and possible inhibition of pathogenic, is possible to expect that further utilization of hazelnut and almond shells as adsorption materials may not be considered to create a serious risk to the environment.

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