BIOPHYSICAL MEASUREMENTS OF LEAD IN SOME BIOINDICATOR PLANTS

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Abstract. Heavy metal (lead) concentrations in two marsh plants (*Phragmites australis* and *Cyperus rotundus*) and one free floating hydrophyte (*Eichhornia crassipes*) growing near metal smelters (lead pollutant area) were investigated. The shoots of the investigated plants were analyzed for lead content by flame atomic absorption spectrometry. The data indicated high lead levels due to the influence of urban and industrial effluents. This showed that the target plants could be considered as lead pollution biosensors. Scanning electron microscopy (SEM) and positron annihilation lifetimes (PAL) technique were used to ascertain the localization of lead in the living tissues. Cross-sections of plant organs were examined using SEM attached with an Energy Dispersed X-ray (EDX) unit for elemental analysis of the formed crystals that appeared abundant in the aerenchyma tissues of the polluted plants. PAL data reflected the correlation between the fraction of free volume hole and lead concentration; higher lead accumulation gives lower fraction of free volume hole. The results demonstrate the extreme sensitivity of the PAL technique towards structural changes occurring in polluted plants.

Key words: Lead, atomic absorption, positron, pollution, bioindicators.

INTRODUCTION

Lead (Pb), one of the heavy metal elements, has become the most important metal pollutant of the environment [26]. Its accumulation in the human body damages the central nervous system [5]. Plants are also poisoned by Pb [15] and Pb-contaminated soil causes sharp decrease in crop productivity. So, it raises a serious problem for agriculture.

Recently plants were used as biosensors of atmospheric pollutants and in aquatic environment [20]. Accumulators are capable of concentrating metals in the above-ground parts (in case of vascular plants) to an exceptional degree, from a low to a high substrate concentration. They have a general tendency to translocate most metals taken up from root to shoot [7].

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The mechanisms of metal tolerance can be classified into internal tolerance mechanisms in the symplasm and exclusion mechanisms in the apoplasm and at the plasma membrane [18]. The internal tolerance mechanisms immobilize, compartmentalize, or detoxify metals in the symplasm by using metal binding compounds [9]. In contrast the exclusion mechanisms prevent metals from entering or staying in the symplasm and coming in contact with sensitive intracellular sites. For example, Al tolerance is conferred by Al exclusion from the root tip, with an increased capacity to release organic acids such as citrate [19] and oxalate [31].

When positrons from a radioactive source are injected into a condensed medium, they lose their energy rapidly in collisions with atoms and molecules of the surrounding medium. Very soon (in the range of ~ps), they equilibrate with the surroundings. The thermalised positrons then annihilate with electrons leading to emission of two 511 keV photons. The mean implantation range varying from 10 to 1000 μm guarantees that positrons reach the bulk of the sample material [22]. Positron lifetime spectroscopy measures the time a positron spends in the sample between implantation and annihilation. The average lifetime of positrons is characteristic of each material and varies from 100 to 500 ps [22]. During the slow down process, a fraction of positrons combines with electrons to form positronium (Ps) atoms, which become trapped in atomic size holes. Ps can be formed in either a triplet state (ortho-positronium, o-Ps) or a singlet state (para-positronium, p-Ps) forming in a ratio of 3:1, respectively.

Using the positron as a probe for biological applications was attempted quite early [8, 10]. Two features of positron annihilation techniques make it specially attractive for the present study: the sensitivity of positron annihilation parameters to structural and conformational transformations occurring in the surrounding medium [13] and because it is a non destructive probe which enables measurements with great sensitivity without perturbing the studied samples [12]. Despite the significant problem that Pb toxicity poses for agriculture, Pb tolerance mechanisms of plants are to date not well understood [30]. Therefore, the aim of the present study is to clarify Pb tolerance mechanisms in some biosensors, namely two marsh plants (*Phragmites australis* and *Cyperus rotundus*) and one free floating hydrophyte (*Eichhornia Crassipes*). The sites of Pb accumulation in these plants were investigated via the application of microprobe techniques: positron annihilation and scanning electron microscope (SEM).

MATERIALS AND METHODS

The selected site is a Cairo district “Shobra”, which is one of the highly polluted multi-purpose areas. Lead smelters are distributed in many locations in it. In addition, the main water irrigation canal known as “Ismaelia canal” crosses the area. The lead concentrations (measured by Environmental Hazards and Mitigation Center – Cairo University) in air, soil and water were found to exceed the provincial soil cleanup guidelines for lead (500 μg/g) established for residential, parkland and agricultural levels [29].

Vegetative shoots of the plants were collected from their natural habitats, which were located 100 m from the lead smelters. *Phragmites* and *Cyperus* samples were collected from 1.5 m height plants, while *Eichhornia* samples were obtained from plants having about seven leaves per rosette. Samples were taken by transversal cuts of fresh stems, immediately after excision of the target plants. The investigated parts were taken from the second internode above the soil surface, stem at 5 cm above the soil surface and the lower third of the swollen leaf petiole for *Phragmites*, *Cyperus* and *Eichhornia*, respectively. *Cyperus* is a common helophytic plant in Egypt [6]; it was a co-dominant species with *Phragmites* in the selected polluted site. Ten samples were collected from ten individuals of each target plant. Sampling was carried out three times at intervals of one-month during summer. Control plants were collected 25 kilometers from the smelters from the non-polluted area in the Nile bank [lead concentration decreased linearly with the distance from smelters [4]].

Pb concentration was determined by a flame atomic absorption spectrophotometer [Perkin Elmer 2001]. The plant samples were washed, first with tap water and then deionized water, and dried at 80°C. The plant tissues were digested in 10 ml (1:1, analar concentration HCl:HNO3). The digest was slightly diluted and filtered through Whatman No. 42 ashless filter paper and then diluted to 25 ml with deionized water [3].

The freshly cut samples were preserved in fixative solution for 5 hours then cut by hand microtome. The sections were air dried and coated with carbon for SEM study.

SEM photographs were carried out for the samples, using SEM model Philips XL 30 attached with energy dispersed X-ray (EDX) unit, with accelerating voltage of 30 kV, magnification of 10× to 400,000× and resolution 3.5 nm. Plant sections were examined using SEM attached with an EDX unit for elemental analysis of the formed crystals, which appeared abundant in the polluted plants.

The positron source was prepared by depositing about 20μCi of aqueous 22NaCl on a thin kapton foil (7 μm thick). After drying, 22NaCl spots, it was covered with another similar foil glued together by epoxy glue and evacuated for a long time (more than 24 hours). Positron absorption by the kapton foil was about 10%, which contributes to the short lifetime component and was not separated in the analysis of the lifetime spectra. The PAL measurements were performed using a conventional fast-fast coincidence system. To calculate the time resolution of the system, the PAL spectrum of the kapton sample was measured. Kapton seems to be the only polymer with no positron yield; i.e., it has no long-lived component [24]. The resolution time was calculated using RESOLUTION program [17] and it was 240 ps (full width at half maximum, FWHM). The sample/positron source/sample sandwich was rubbed in Al foil in order to perform PAL measurements at room temperature (about 25°C).

The PAL spectrum for a molecular material is normally characterized by three lifetimes: τ_1, τ_2, and τ_3, and their intensities I_1, I_2, and I_3 (which indicate the number fractions of annihilation events for each mode). In the present work, the lifetime spectra were analyzed to finite term lifetimes using PATFIT program [17] without source correction.

RESULTS AND DISCUSSION

Table 1 presents Pb concentrations in the investigated plant samples as determined by a flame atomic absorption photometer. The recorded high Pb levels in the polluted plants reflect the influence of urban and industrial effluents and demonstrate the suitability of these plants as Pb pollution biosensors. This is in agreement with the results of other authors [16, 24, 27]. Keller *et al.* [16] showed that *Phragmites* are generally considered as filters, retaining allochthonous metals that can have toxic effects on biota. They are used efficiently for bioremediation of water and hydro-soils [1]. Schneider and Rubio [27] showed that *Eichhornia* is an excellent bio-absorbent for Pb. As shown in Fig. 1c, sclereids are observed arising from aerenchyma cells of control plant; they are smaller than the spindle crystals of polluted ones.

Table 1

Pb concentrations (ppm) in control and polluted plant samples

Plant	*Phragmites*	*Cyperus*
Control	225 ± 9.3	125 ± 5.75
Polluted	776 ± 38.6	331 ± 14.56

Numerous crystals of different forms are abundant in the aerenchyma tissues of the polluted plants as shown in Figure 1. For *Phragmites* samples quadrangular crystals are abundant in cortical parenchyma cells. For *Cyperus* and *Eichhornia* samples the crystals are of triangular and spindle shapes, respectively, and they are frequently met in cortical aerenchyma cells. A semi-quantitative elemental analysis for most of these crystals is presented in Table 2.

Mazen and El-Maghraby [21], using X-ray microanalysis, indicated the presence of three heavy metals (Cd, Pb and Sr) bound with Ca-oxalate crystals. Their results suggest a possible role of Ca-oxalate crystallization in toxic heavy metal deposition and thus tolerance by *Eichhornia*. Anderson *et al.* [4] showed that Pb was found in the parenchymatous cells of *Quercus nigra* and *Quercus velutina* trees (grown in lead smelting facilities in Alabama).

The different crystals shown in Figure 1 possibly play a protective role in regulating lead bioavailability [St-Cyr and Campbell [28] showed that iron plays a protective role in regulating zinc bioavailability in aquatic plants]. Also, it may be formed as a defense mechanism because it appears very frequently in plant samples collected from the polluted site.

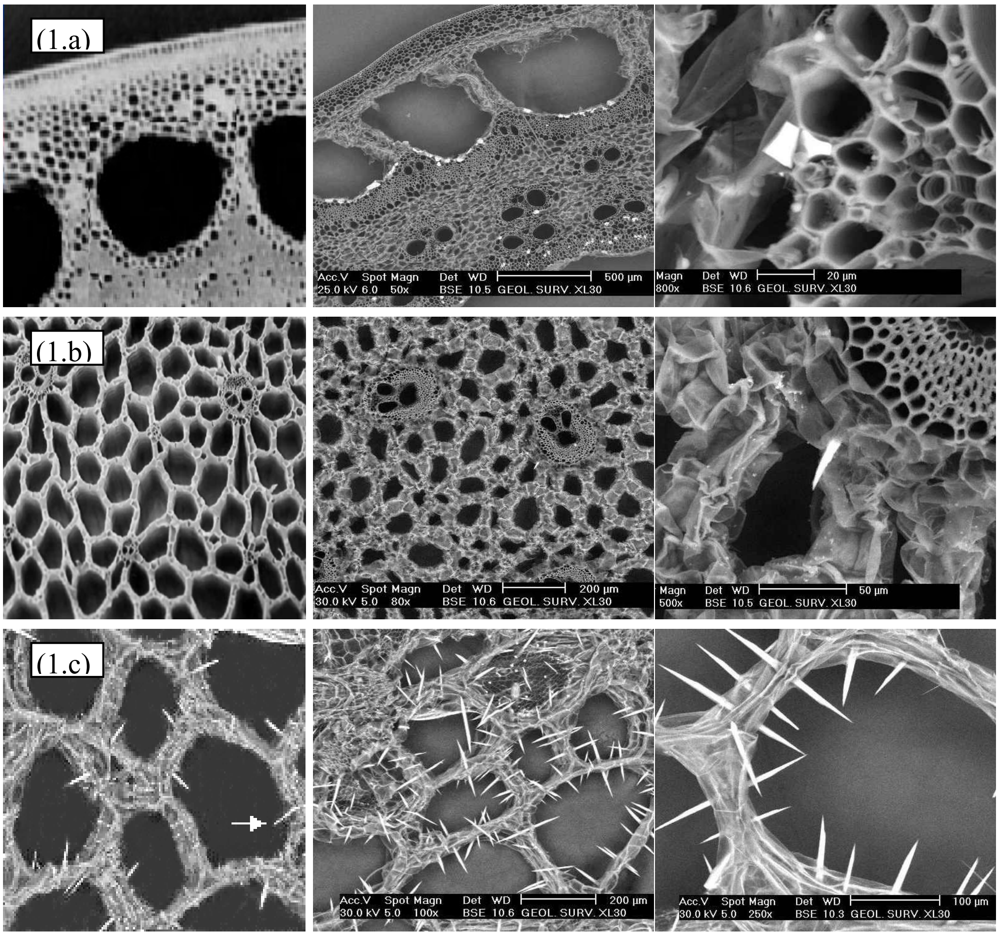

Fig. 1. Cortical parenchyma of control *Phragmites* stem (1.a), and cortical aerenchyma- of *Cyperus* stem (1.b) and *Eichhornia* petiole (1.c). Localization of lead-coated crystals is shown in the same corresponding parts of polluted plants. Arrow indicates the presence of sclereids in aerenchyma cells of *Eichhornia* petiole.

Figure 2 shows the PAL spectrum for nonpolluted *Cyperus* sample. Three lifetime components (τ_1, τ_2 and τ_3) were found to give the best variance ratio and the most reasonable standard deviations for the PAL spectra of the studied plant samples.

Table 3 presents PAL parameters for the studied samples. The short-lived component (τ_1 = 0.12–0.15 ns, I_1 = 40–49%) and the intermediate component (τ_2 = 0.37–0.46 ns, I_2 = 25–37%) are attributed mainly to p-Ps and direct annihilation of positrons, respectively.

Table 2

Elemental constituents of crystals for polluted plants estimated by semi-quantitative EDX analysis*

Phragmites	*Cyperus*	*Eichhornia*
1.2 Pb	2.06 Pb	3.2 Pb
97.53 Si	93 Ca	44.26 Cl
0.8 Al	4.94 K, Al and P	42.42 K
0.47 Cu	–	2.84 Ca
–	–	7.28 Fe, Cu, Al, P

*All concentrations are in %/wt.

Table 3

PAL parameters for control (C) and polluted (P) plants

Plant name		τ_3 (ns)	I_3 %	Free volume hole size $V_{Ps,h}$ (A^3)
Phragmites australis	C	1.350 ± 0.007	18.848 ± 0.238	42.105 ± 0.009
	P	1.265 ± 0.006	22.860 ± 0.260	35.969 ± 0.010
Cyperus rotundus	C	1.344 ± 0.008	20.457 ± 0.287	41.635 ± 0.011
	P	1.551 ± 0.009	17.967 ± 0.237	57.565 ± 0.013
Eichhornia crassipes	C	1.063 ± 0.005	26.106 ± 0.380	22.710 ± 0.008
	P	1.297 ± 0.006	22.938 ± 0.263	38.226 ± 0.007

Detailed analysis of these two components (τ_1 and τ_2) is difficult because of the possible formation of positron and positronium compounds that contribute to both of them. The long-lived component (τ_3 = 1.06–1.55 ns, I_3 = 18–26 %) is due to pick-off annihilation of the ortho-positronium (o-Ps) in free volume holes. It is expected to be sensitive to structural transformation occurring in plants, and it is considered as the mean value of the o-Ps lifetimes in the free volume holes of different sizes. I_3 reflects the number of free volume sites where pick-off annihilation occurs. Figure 2 shows the PAL spectrum for non polluted *Cyperus* sample.

As shown in Figure 3, for *Eichhornia* and *Cyperus* samples τ_3 are larger for polluted than for control ones. For *Phragmites* samples, τ_3 is shorter for polluted than for control sample. The variation of I_3 is suggestive for some changes taking place in the studied samples, i.e., the formation of numerous crystals of different forms in polluted plants is confirmed by SEM photographs. The decrease in o-Ps formation (smaller I_3) for polluted *Cyperus* and *Eichhornia* samples could be attributed to: Pb coated crystals in these plants acting as efficient traps for the precursor of Ps. Thus the deposited Pb act as efficient traps for free electrons generated in the spur, thus reducing efficiently the number of electrons available for the combination with the positron at the end of its track.

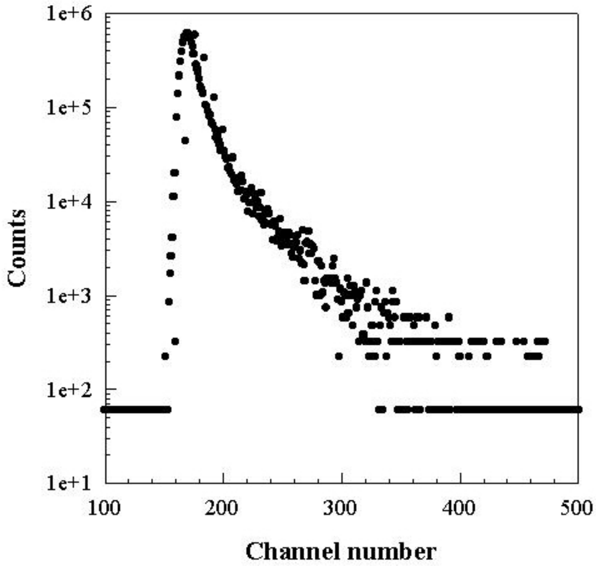

Fig. 2. PAL spectrum for non polluted *Cyperus* sample.

Figure 3 depicts the dependence of PAL parameters on Pb concentrations in the plant samples studied.

A simple kinetic model describing the competition between the positron and scavenger molecules for free electrons has been developed by Ache *et al*. [2]. Despite the increase in Pb concentrations in *Phragmites* samples in comparison with the other two species, o-Ps formation increased (larger I_3). This may be interpreted as follows: as shown in Figure 1, *Phragmites* have larger crystals than those for others. From the geometrical point of view, the formation of such big crystals gives a chance to form a free volume between them. So the o-Ps intensity is increased.

The average of the free volume hole size ($V_{Ps,h} = 4/3\ \pi R^3$) which probed by the o-Ps lifetime (τ_3) was calculated from the following relation between τ_3 and the average free volume hole radius "R":

$$\tau_3 = 0.5 \left\{ 1 - \frac{R}{R_o} + \frac{1}{2\pi} \sin\left(\frac{2\pi R}{R_o} \right) \right\}^{-1} \text{(ns)} \tag{1}$$

where $R_o = R + \Delta R$ and $\Delta R = 1.656$ Å is the thickness of the homogeneous electron layer in which the positron annihilates [23]. Figure 3 shows the variation of free volume hole size for the studied samples. As shown in figure 3, for polluted *Eichhornia* and *Cyperus* samples, the overall free volume is larger than that of control ones. This may be explained as follows: since the mass of P_s is small and the zero-point energy is large, as $E_o(\text{eV}) = 0.188 / R$ [11], a small difference in the free volume hole radius, *R*, can easily cause an energy difference larger than thermal energy (according to the spherical well model with infinite length). Owing to this particular quantum mechanical nature Ps can be preferentially transported to larger holes.

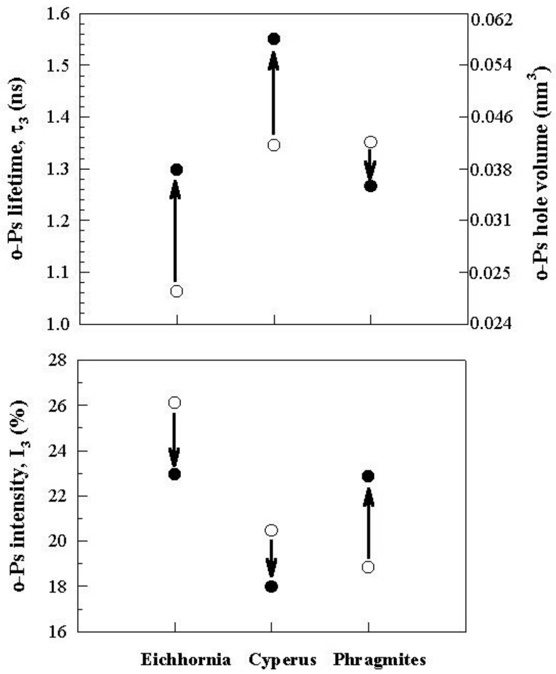

Fig. 3. Dependence of PAL parameters on Pb concentrations in the studied plants (●: control, o: polluted).

Thus it is quite probable that Ps is transported to larger holes generated as a result of formation of spendle-and triangular Pb crystals (Fig. 1). The behavior is opposite for polluted *Phragmites* samples, where the overall free volume is smaller than that of control ones, indicating a substantial (14.57%) constriction due to accumulation of large amount of Pb as revealed by atomic absorption data (Table 1) [776 ppm, a higher value in comparison with the other two species].

The probability of o-Ps is related to the fraction of free volume holes. For convenience, a relative fractional of the free volume hole is defined as [25]:

$$f_r = I_3\ V_{h,Ps}. \quad (2)$$

The ratios between the relative fraction of the free volume holes in polluted samples to that in control ones are 1.035, 1.215 and 1.479 for *Phragmites*, *Cyperus* and *Eichhornia*, respectively, corresponding to 3.45, 2.65 and 1.334 for lead concentrations. Thus, the increase in Pb concentrations results in the decrease in relative fraction ratio.

A structural behavior is evident in Figure 3 which seems to reflect changes in crystal shape, i.e., the Ps reactivity is affected by the form and size of the crystals, a structure effect which has previously been observed by Jean and Ache [14] and Jain [12].

CONCLUSION

The process of lead deposition is cooperative and brings about several structural transformations. Positron annihilation data is related to the overall molecular conformation. The change in crystal shape results in concurrent variation of positron annihilation parameters. So, the advantage of using positronium to probe the Pb deposition mechanisms in biosensors plants is very unique. Also, results showed that different plants vary in their capacity to tolerate increased lead concentrations in their shoots.

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