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Heavy metal pathways and archives in biological tissue

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Abstract

Nuclear milli and micro probes at the Australian Nuclear Science and Technology Organisation (ANSTO) were used to determine lead accumulation in native Australian plants and animals. Three species of eucalypt plants (*Eucalyptus camaldulensis, E. globulus* and *E. lesouefii),* one species of salt bush *(Atriplex burbhanyana)* and one species of acacia *(Acacia saligna)* and an estuarine crocodiles (*Crocodylus porosus*) were investigated.

Experimentally grown plants were subjected to a nutrient solution with a pH of 5 and spiked with 200 µmol concentration of Pb. Lead concentrations in leaves of both *E. globulus* and *E. camaldulensis* showed an almost exponential decrease from the base of the main vein to the tip. Similarly Pb concentrations decreased from the main vein to secondary veins. Concentrations of essential elements such as K, Fe, Zn and Br in the main and secondary veins were constant within experimental uncertainty. In contrast, the concentrations of Pb in the leaf veins of *E. lesouefii* were much lower and evenly distributed. Stem and root material show that the highest concentration of Pb is found in roots and stem of *E. globulus* and *A burbhanyana* followed by *E. camaldulensis*. Some Pb was found in roots of *A. saligna* and only very low concentration in stem of the same plant. More detailed analysis of thin cross sectional samples of roots and stem showed that Pb is present in much higher concentration in epidermis and in relatively low concentration within the inner and outer cortex.

The osteoderms (dermal bones) of estuarine crocodiles, exposed to lead ammunition in food from the hunting activities of traditional Aboriginal owners, were sampled at two sites in Kakadu National Park, northern Australia. PIXE analyses showed enhanced, but relatively constant, ratios of Pb/Ca in the annual laminations. This was consistent with their history of long term exposure to elevated anthropogenic Pb sources and with the hypothesis that the osteoderm can be used as an archive of the crocodile's exposure to Pb during its life.

Results obtained by PIXE are compared with the results of ICP-ES and SIMS and found to be in a good agreement.

Key words: heavy metals, Pb, hyperaccumulation, PIXE.

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Introduction

Anomalies in elemental concentrations within biological structures are often used to assess levels of environmental pollution as well as the nutritional status of the ecosystem[1]. It is also well known that some plants hyperaccumulate heavy metals in their tissue and can therefore be successfully used as an affordable and effective remediation solution[2]. Phytoremediation needs to be rigorously assessed to determine its full potential. This includes understanding the concentration and distribution of heavy metals within plant structures (root, stem, leaf, etc.). Therefore, there is a growing demand for microanalytical techniques that can measure distribution of heavy metals within biological structures and thus evaluate the temporal variation of anthropogenic contamination in the environment[3].

Although conventional analytical techniques such as Atomic Absorption Spectroscopy (AAS) and Inductively Coupled Plasma spectrosopy (ICP) have good sensitivity and accuracy for heavy metal determination they lack positional sensitivity as they are in principle bulk techniques (ie sample have to be digested prior to analysis). Physical techniques such as electron and proton microprobes, as well as Secondary Ion Mass spectrometry (SIMS) techniques are being developed to measure the spatial distribution of heavy metals within biological structures (e.g. Sneider[4], Barnabas[5], Prinslo[6], Mesjasz[7], Bettiol[8], Twining[9]).

The principle aim of this study was to demonstrate the capabilities of nuclear milli and micro-probe facilities at the Australian Nuclear Science and Technology

Organisation (ANSTO) to determine the concentration and spatial distribution of Pb within several species of native Australian plants and the osteoderms of an estuarine crocodile.

Selected species of Australian flora were grown under well-controlled experimental conditions using nutrient solutions. The plants were then exposed to a Pb spiked nutrient solution for four weeks. The plants were then sampled and various structures of plants (roots, stem and leaves) were measured for their concentrations of heavy metals (including Pb) and other essential elements using PIXE, with the aim of determining the uptake and distribution of heavy metals in different plant species.

In a separate study, the distribution of Pb was measured in the osteoderms (dermal bones) of Pb-exposed Australian estuarine crocodiles (*Crocodylus porosus*) from the World Heritage-Listed Kakadu National Park (KNP), in the Alligator Rivers Region of Northern Australia. Long-term use of lead ammunition by the Aboriginal owners, who continue to hunt within KNP, poses a potential threat to resident crocodiles, due mainly to their consumption of fauna killed or crippled by Aboriginal hunters. In this study we have used osteoderms sampled from free-living crocodiles to identify populations with substantially enhanced Pb concentrations. The ANSTO milli-probe was used to irradiate the annual laminations of several osteoderm samples to indicate the historical pattern of enhanced exposure to Pb.

Materials and Methods (PIXE)

Plant tissues

Plant growth – Seeds of selected native Australian flora (*E. camaldulensis, E. globulus, E. lesouefii, A. saligna and A. burbhanyana*) were germinated and grown in trays of sterilised sand.

Constant growth conditions were maintained by watering once daily and feeding once per week with a nutrient solution (Thrive®; Yates and Co. Pty Ltd, New South Wales, Australia). When seedlings were approximately 150 mm in height, uniformly sized specimens were selected and placed in a hydroponic setup and treated for four weeks with a modified Hoaglands solution[10] according to the method outlined in Huang & Cunningham[11]. Then the plants were subjected for two weeks with a modified Hoaglands solution that was spiked with lead (200 μM as $Pb(NO_3)_2$). Subsequently, the plants were harvested, rinsed and root, stem and leaf tissues were sampled. Parts of the samples were oven-dried at 80°C and used to analyse bulk chemistry using conventional digestion and ICP-ES techniques. Some of the oven-dried leaf samples were used for preliminary microanalysis by broad-beam PIXE. Leaf tissue was sampled by punching approximately 13 mm circles along the main and secondary veins. A few leaves with straight main veins were left whole and irradiated without cutting. Other parts of the fresh samples (not oven-dried) were frozen in liquid nitrogen and dried in the vapour above liquid nitrogen (-80°C), in preparation for milli- and micro-beam PIXE analysis. After embedding the frozen tissues in Tissue-Tek (reference to be provided!!), 20 μm slices were cut using a cryo-microtome facility at Monash University. The slices were then sandwiched between formvar films and attached to metal sample holders designed for the PIXE sample compartments. The samples and holders were again freeze-dried and stored in dessicators at room temperature.

Crocodile tissues

Crocodiles were sampled at two sites in KNP where there has been a long history of hunting of wildlife with lead shot ammunition by the traditional Aboriginal owners. Total length of crocodiles in this study ranged from 1.6 to 4.99 m, representing an estimated age span of 4.9 to 40 years. Osteoderms were surgically removed from the pelvic region just forward of the hind leg. After cleaning, they were longitudinally sectioned along the major axis perpendicular to the horizontal plane. Cleaned and dried samples were mounted in a standard mix of Araldite and ground using SiC papers and distilled water, before being polished with diamond paste [9]

Sample Irradiation

Three modes of PIXE irradiation techniques were used in this study: broad beam (1-5 mm diameter), milli (50-200 μm) and micro-probe with a 2-5 μm beam diameter. A 3 MV Van de Graaff accelerator was used to provide a 2.6 MeV proton beam required for the broad beam and milli-probe irradiation. A semiconductor Si(Li) detector was used to acquire PIXE spectra with a typical chain of electronic components. A more detailed description of the set-up used in this study can be found in a previous paper[12]. To achieve satisfactory counting statistics samples were typically irradiated with the total charge of 3 to 12 μC. Simultaneously with PIXE spectra, Rutherford Backscattering Spectra (RBS) were collected to enable of the concentrations of major matrix elements (C, N and O) as well as the determination of the area density of plant thin sections.

The High Energy Heavy Ion Microprobe operating at the 10 MV Tandem accelerator of ANSTO's Physics Division was used to irradiate thin sections of stem and roots with 9MeV He ions. The beam was focused to an estimated spot size of 2-5 μm and scanned over the thin sections of root and stem samples.

Results

Plants

Broad beam PIXE and the proton milli-probe

2.6 MeV proton beam collimated to a 4 mm diameter was used to irradiate leaf samples. For each leaf, several points were analysed along the main vein and typically four points on the peripheral regions of the same leaf (2 from each side of the main vein). A typical PIXE spectrum of a leaf sample of *E. camaldulensis* is shown in Figure 1. The vertical scale is logarithmic to accommodate the three orders of magnitude difference in PIXE sensitivity and concentration difference between major low atomic number elements such as K and Ca and trace elements such as Zn and Pb. The concentrations of major elements as (Cl, K, Ca) and essential trace elements (Mn, Fe, Cu, Zn, Br and Pb) were determined in three different eucalypt species (*E. globules, E. camaldulensis* and *E. lesouefii).* Results are shown graphically for several elements in Figures 1 to 3. All concentrations are expressed in units of area density ($\mu g/cm^2$).

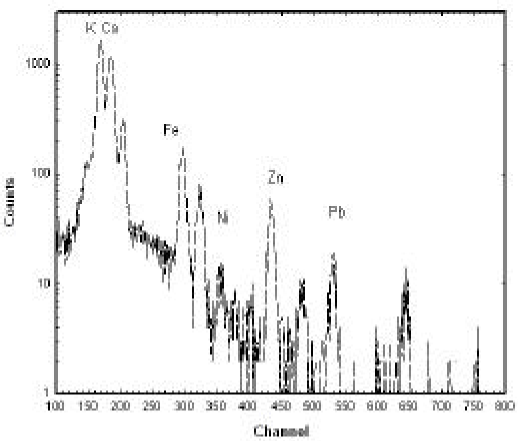

Figure 1 Typical PIXE spectrum of the leaf of *E. camaldulensis* plant. Please note relatively high concentration of Pb indicated by its L_α and L_β lines.

An example of the distribution of Pb, K, Fe and Zn in the main vein of a leaf of *E. camaldulensis* is shown in Figure 2. A very similar distribution of all elements was found in the main vein of a leaf of *E. globulus*. Both *E. globulus* and *E. camaldulensis* show elevated concentrations of Pb in the main vein of their leaves, with the highest values near the base of the leaf (2.1 $\mu g/cm^2$) and decreasing almost exponentially towards the tip (0.1 $\mu g/cm^2$), see Figure 2.

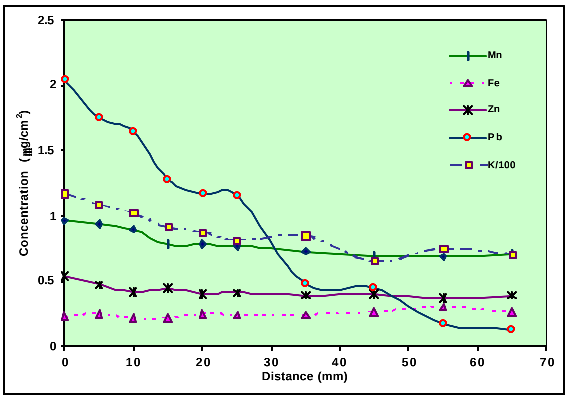

Figure 2 Concentration variation of Pb, K, Fe and Zn along the main vein of the leaf of *E. camaldulensis* plant. Concentrations are expressed in units of area density ($\mu g/cm^2$) with concentration of K scaled down 100 times to fit the vertical scale.

In both plant species, Pb concentration was in much lower concentrations in the secondary veins of their leaves (approximately 0.1 $\mu g/cm^2$). In contrast to the other eucalypt species, *E. lesouefii* leaves had much lower Pb concentrations (Figure 3; average Pb concentration of 0.05 $\mu g/cm^2$).

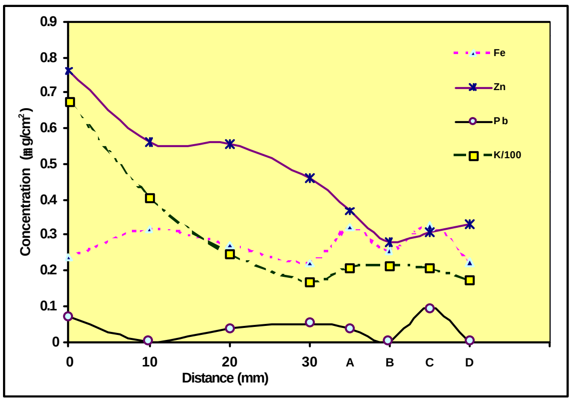

Figure 3 The distribution of Pb, K, Fe and Zn in the leaf of *E. lesouefii.* Please note that first 4 points are taken along the main vein and points A, B, C and D are from the peripheral areas of the leaf, two on each side of the main vein.

On the same figure, one can note that K and Zn concentrations decrease in the leaf vein from the stem to the tip of the leaf. This could be an apparent change in concentration due to the decrease in leaf thickness, as this leaf was relatively small and thinner than the range of incident protons (and therefore PIXE yield decreases with the thickness of the sample). To circumvent this problem, absolute concentrations will be calculated in our follow-up work by means of the corresponding RBS spectra.

Broad beam PIXE was used to irradiate all prepared transverse sections of roots and stems from four different plant species (*A. bunburyana, E. camaldulensis*, *E. globulus* and *A. saligna*). Results are summarized in Figure 4 for lead and cadmium. All concentrations are normalized to concentration of potassium (potassium was used as it showed the list concentration variation across all spectra). Clearly roots of *E. globulus* accumulate the highest concentration of Pb, with lesser values in roots and stem of *A. bunburyana* and stem of *E. globulus.* Due to relatively lower sensitivity of PIXE for the medium atomic number elements, Cd has been observed only in stem of *E. globulus* and *A. bunburyana.*

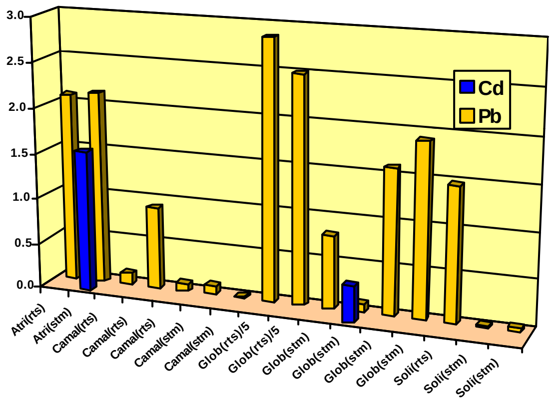

Figure 4 The summary of Pb and Cd analysis by the broad beam PIXE of transverse sections of roots and stem of *Artiplex bunburyana* (Atri), *E. Camaldulensis* (Camal), *E. Globulus* (Glob) and *A saligna* (Sali) species. All concentrations are normalized to concentration of potassium. Please note that the vertical scale of the Glob(rts) have been reduced 5 times to fit the scale. Clearly roots of *E. Globulus* accumulate the highest concentration of Pb, followed by roots and stem of *Artiplex bunburyana* and stem of *E. Globulus*.

ANSTO's proton milli-probe was used to determine concentration profile across the cross section of a *E. globulus* stem. The micrograph of the sample is shown in Figure 5 together with the measured concentration profiles of Pb, K and Ca. Lead is obviously concentrated in epidermis of the stem with only very low concentration in the inner and outer cortex.

Again, all concentrations are expressed in units of area density (μg/cm^2) and the diameter of the analysed stem sample is 5 mm. The beam size used in this experiment was 100 μm and it was moved across the sample initially with the step of 200 μm (left hand side of the figure) and latter 500 μm as it can clearly be seen from the burn marks caused by the exposure to the proton beam.

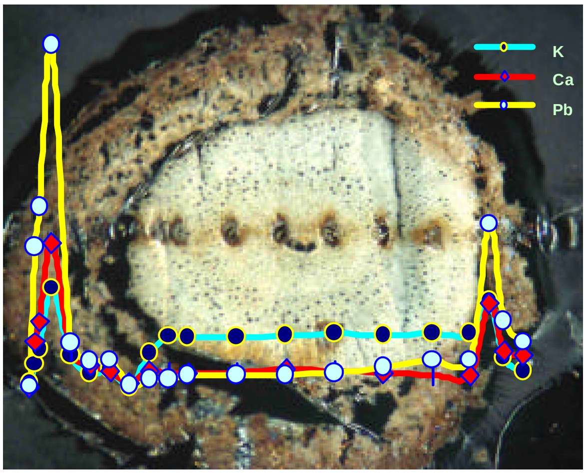

Figure 5 Thin transverse section of *E. globulus* stem with the corresponding concentration profiles of Pb, K and Ca. The overall diameter of the stem sample is 5 mm. All concentrations are expressed in units of area density (μg/cm^2). Please note the burn marks caused by the exposure to the proton beam.

Microprobe

To obtain element distributions on an even finer scale, ANSTO's heavy ion microprobe was used. The beam was focused to a 2-5 μm spot size and a thin transverse section of the root of *E. globulus* was irradiated with the scanning beam. The micrograph of the sample with the corresponding Pb distribution is shown in Figure 6. Again, Pb is found mainly in the epidermis of the root sample with very low concentrations in the cortex and vascular tissues.

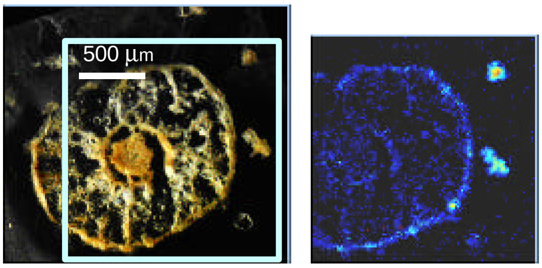

Figure 6 Micrograph of the thin transverse section of *E. globulus* and the corresponding Pb distribution in false color.

Crocodiles

Several samples of crocodile osteoderms were irradiated with the proton beam ranging in size from milli-probe to broad beam. The results for sample 13D are summarized in Figure 7. The Ca and Pb concentrations are relatively constant in the laminations deposited over the life time of the animal. In contrast, the Zn concentration appears to increase over the animal's life time, which is consistent with the pattern observed in their flesh [13].

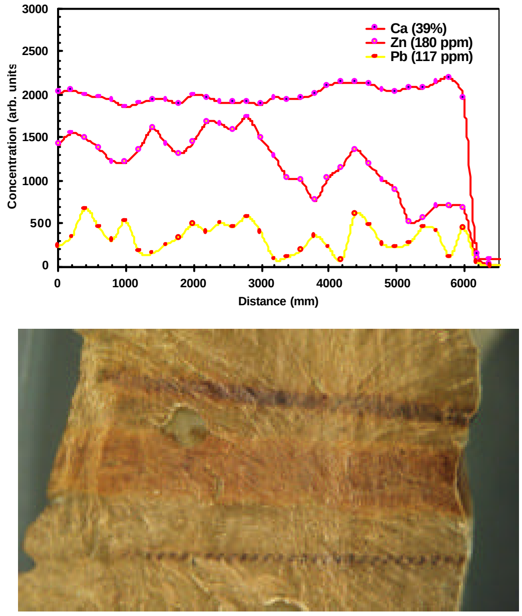

Figure 7 Micrograph of the 13D osteoderm with the corresponding concentration profiles of Ca, Zn and Pb. Dark brown area is the burn mark produced by the proton beam. Average concentrations in ppm are given in brackets.

Discussion

The capabilities of ANSTO's broad beam PIXE, as well as the PIXE milli and micro probe, were successfully applied in determining records of heavy metal pollution within the tissues of several plants and the osteoderms of a crocodile. Samples of leaves, stem and roots were analysed for variations in Pb concentration. Great variation in hyper accumulation of Pb between different plant species was observed. Our results show that leaves of *E. camaldulensis* and *E. globulus* accumulated the highest concentration of lead, while *E. lesouefii* accumulated hardly any lead in its leaves. On the other hand, the roots of *E. globulus* accumulated the highest concentration of Pb, followed by the roots and stem of *A. bunburyana* and the stem of *E.globulus.*

Also, a very prominent pattern was observed with regards to lead distribution: within a leaf, lead concentration decreases with the distance from the base of the leaf, as well as with the distance from the main vein. Within a root and stem the lead concentration is much higher in epidermal than in the vascular and cortex tissues. These results are in a good agreement with the results of ICP-MS.

PIXE analyses showed enhanced, but relatively constant, ratios of Pb/Ca in the annual laminations of the osteoderm from a Pb-exposed crocodile . This was consistent with its history of long term exposure to elevated anthropogenic Pb sources and consistent with the hypothesis that the osteoderm can be used as an archive of the crocodile's exposure to Pb during its life.

REFERENCES

1. (Hoffman et al., 2000)
2. DE Salt, RC Prince, IJ Pickering, I Raskin, Plant Physiol., 109, 1995 pp 1427-1433
3. Garbe-Schönberg et al., 1997
4. T Schneider, A Haag-Kerwer, M Maetz, M Niecke, B Povh, T Rausch and A Schubler, B158 (1999) 329-334
5. AD Barnabas, WJ Przybylowicz, J Mesjasz-Przybylowicz and CA Pineda, Nucl. Instr. Meth., B158 (1999) 323-328
6. MW Prinsloo, SA Reinecke, WJ Przybylowicz, J Mesjasz-Przybylowicz and AJ Reinecke, Nucl. Instr. Meth., B158 (1999) 317-322
7. J. Mesjasz-Przybylowicz, K Grodzinska, WJ Przybylowicz, B Godzik and G Szarek-Lukaszewska, Nucl. Instr. Meth., B158 (1999) 306-311
8. AA Bettiol, C Yang, GP Hawkes, ND Jamieson, KG Malmqvist and RW Day, Nucl. Instr. Meth., B158 (1999) 299-305
9. JR Twining, SJ Markich, KE Prince and RA Jeffree, Environ. Sci. Technol., 33 (1999) 4396-4400
10. reference for hoaglands solution
11. JW Huang and SD Cunningham, New Phytologist, 134, 1996, pp75-84
12. R. Siegele, D.D. Cohen and N. Dytlewski, Nucl. Instr. and Methods **B158** (1999) 31
13. RA Jeffree, SJ Markich and JR Twining, Arch. Environ. Contam. Toxicol., 40 (2001) 236-245.