

1. Heavy Metal Bioaccumulation by the Important Food Plant, *Olea europaea* L., in an Ancient Metalliferous Polluted Area of Cyprus.
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## **ABSTRACT**

Aspects of the bioaccumulation of heavy metals are reviewed and possible evidence of homeostasis is highlighted. Examination and analysis of olive (*Olea europaea* L.) trees growing in close proximity to a copper dominated spoil tip dating from at least 2000 years BP, on the island of Cyprus, revealed both bioaccumulation and partitioning of copper, lead and zinc in various parts of the tree. A factor to quantify the degree of accumulation is illustrated and a possible seed protective mechanism suggested.

**KEY WORDS** Bioaccumulation, heavy metals, homeostasis, partitioning.

The town of Polis is located in northwest Cyprus; the ancient Limni copper workings are approximately 8 km to the east of the town. The control site was situated on the Akamas peninsular approximately 25 km west of Limni. The prevailing winds are from the south west and should not affect the deposition of spoil tip derived particulates on the control site. The spoil tips adjacent to the sample collection site at Limni are large covering an area of approximately 2 x 2 km and are often steep sided and prone to atmospheric, sheet and gully erosion (Pyatt and Birch, 1994) together with leaching during and following rainfall. Recently there has been a move to decrease the slope of the tip to reduce run off. Previous work by Pyatt (2001) investigated the plant accumulation of copper and lead by *Acacia retinoides* and *Eucalyptus torquata*, two tree species which had grown near this contaminated landscape for a long period. Wilson et al. (2005a) investigated bioaccumulation of copper, zinc, lead and cadmium by black spruce (*Picea mariana*) collected in the vicinity of an old copper mine in British Columbia, Canada and determined varying degrees of partitioning within the tree. Further research by Wilson and Pyatt on the sites of an early 20<sup>th</sup> century tungsten mine (2006) and an ancient copper mine (2007), both in the UK, highlighted bioaccumulation and partitioning in *Calluna vulgaris*.

The aims of the current research were to examine the commercially important plant species *O. europaea* (olives), currently growing in the close vicinity of the spoil tips, to determine copper, zinc, lead and cadmium concentration and the degree of partitioning.

## **METHODS AND MATERIALS.**

Soil and spoil samples were collected using a clean stainless steel trowel. Approximately 5mm of surface soil and detritus in 20cm x 20cm area was scraped off and discarded. Approximately 200g of soil or spoil was then collected to a maximum depth of 50mm and placed in polythene bags. Each sample was individually triple bagged to prevent transit damage and transported to the laboratory. The spoil and soil samples were air dried for seven days in a drying cabinet at 25<sup>0</sup>C and placed in an oven for 3 h at 60<sup>0</sup>C. After cooling the samples were passed through a 2mm sieve and ground for 5 min in a clean pestle and mortar before transfer to HDPE storage bottles.

Approximately 15g of olives were collected using clean stainless steel secateurs and the samples placed in a polythene bag; the samples were then triple bagged and transported to the laboratory. The olives were washed thoroughly with demineralised distilled water and dried for 24 h at 25<sup>0</sup>C in a positive pressure drying cabinet. They were then dried for 24 h at 70<sup>0</sup>C (Radofevic and Bashkin, 1999); after drying the flesh was separated from the stones using a clean stainless steel scalpel. Fleshy parts of olives were placed in a Waring Commercial Blender and blended at low speed for 2 min followed by 3 min at high speed, together with any oil which had exuded onto the storage dishes. Five seeds (stones) out of olives collected from Limni and the control site were placed in a clean 50 mL polypropylene centrifuge tube with 20 mL of Certified grade acetone and shaken for 30 min in an IKA model KS130 orbital shaker at 360 oscillations per min. This washing procedure was repeated, decanting away the acetone each time, stones were wiped with clean tissues and allowed to dry at room temperature overnight. The stones were prepared for analysis in the same manner as the flesh. The blended samples of flesh and stones were transferred into clean HDPE bottles.

Olive leaves were similarly collected using surgical examination gloves and placed in polythene bags. The samples were triple bagged for transport to the laboratory. When comparisons of heavy metal dispersion from a specific source are being carried out, leaf samples are taken from the same species of tree, at similar height and from the same aspect. Leaf samples were washed thoroughly with deionised distilled water and dried for 24 h at 25<sup>0</sup>C in a positive pressure drying cabinet. The leaves were then dried for 24 h at 70<sup>0</sup>C (Radojevic and Bashkin, 1999). The dried leaves were ground for 5 min in an agate pestle and mortar before transfer to HDPE storage bottles.

The immediate stems from which the leaves were detached were also collected, bagged and transported to the laboratory where they were processed in the same manner as olive flesh.

Samples of soil, foliage and fruits (olives) were collected from the control site on the Akamas peninsular on the same day from randomly selected trees of a similar height, vitality and from the same aspect.

1.0 g ± 0.05 g of prepared soil or spoil (0.5 ± 0.025 g vegetation) was weighed into a 50mL conical flask. 10 mL of aqua regia was added and the flask simmered for 90 minutes on a hotplate in a fume cupboard. The flask was removed, cooled and a further 4 mL of aqua regia were added. The flask was simmered for a further 30 minutes before cooling and filtering through Whatman 541 filter paper into a clean 50mL graduated flask. The 50 mL conical flask, filter paper and funnel were washed through thoroughly and the final volume made up with 0.5M HNO<sub>3</sub>. The extract was transferred to a HDPE bottle and stored in a refrigerator at 5<sup>0</sup>C until required for analysis (Wilson et al. 2005b) Ten replicates of each sample were extracted and analysed except olive stones from each site ( 4 replicates) and Limni olive stems (4 replicates).

Analysis was carried out using a Perkin Elmer Optima 2100 ICP-OES fitted with an AS-90 Plus autosampler. The instrument was calibrated using mixed Cu, Zn, Pb and Cd standards of 0.5 mgL<sup>-1</sup> and 1.0 mgL<sup>-1</sup> prepared from 1000mgL<sup>-1</sup> certified standards diluted with processed extractant which was also used as a blank during calibration. Three replicates of each sample were analysed by the instrument to obtain a mean metal concentration. Standard operating parameters of the instrument used are as shown in Table 1. Limits of detection of the instrument when used with this extraction matrix were Cu = 30 µg kg<sup>-1</sup>, Zn = 70µg kg<sup>-1</sup>, Pb = 0.08 mg kg<sup>-1</sup> and Cd = 80µg kg<sup>-1</sup>. Accuracy of the extraction and analytical processes for soil, using a standard reference material, CRM142R Light sandy soil are outlined in Table 2.

## **RESULTS AND DISCUSSION**

Table 3 illustrates the metal concentrations detected. The spoil sample collected from the Limni workings contained the highest concentration of copper but lead and zinc were not detected in 10 replicates of this sample. Similarly a relatively high concentration of copper was detected in the soil sample collected adjacent to the spoil tips but zinc and lead were not detected in 10 replicates of this sample. Soil from the control site however had a relatively high concentration of lead and lower concentrations of copper and zinc. Cadmium was not detected in any of the samples digested and analysed.

The samples of spoil collected from the Limni workings had a lower average concentration of copper than the samples examined by Raber (1984) and Pyatt (2001). Table 4 illustrates the metal concentrations recorded.

Metal concentrations in the spoil varies according to the site at which the trees were located, however it should be noted that both Raber and Pyatt expressed their copper and lead concentrations in terms of percentages and the current authors have adjusted these values to mg kg<sup>-1</sup> in order to facilitate comparisons. Table 3 demonstrates the variability in composition of samples collected in the same area and on different occasions. The results obtained for the current samples reflect the re-contouring of the spoil tip since 2001 and the recent addition of soil overburden; recontouring would alleviate problems caused by atmospheric erosion of such metalliferous spoil.

The soil sample at Limni was collected adjacent to the base of the olive trees and was found to contain a copper concentration of 193 mg kg<sup>-1</sup>. This sample site was situated approximately 35 m east of the spoil tip and would have accumulated there as a result of atmospheric transfer and leaching during rainstorms. The soil collected in the same manner from olive trees on the Akamas peninsular contained approximately one quarter of the concentration of copper when compared to Limni soil. The Akamas soil did however contain a high concentration of lead (345 mg kg<sup>-1</sup>), although this value does not exceed the soil guide line values for residential and allotment use (DEFRA, 2002).

Many workers have demonstrated phyto-accumulation of heavy metals (Boon et al. 1998. Malawska and Wilkomirski, 2001. Yukselen and Alpaslan, 2001. Walker et al. 2004. Wilson et al. 2005a). Lead concentrations in all the plant tissues examined were very similar and copper concentrations in the plant tissues were higher at the Limni site than those collected at the control site.

Investigations into the extent of partitioning within plants themselves have been much less apparent. Pyatt (2001) and Wilson et al. (2005a) demonstrated the varying concentrations of specified metals in different tissues of plant species and the metal concentrations values shown in Table 2 corroborate these findings, with copper and zinc concentrations in various plant tissues being higher where metal concentrations in soil are higher. Additionally, higher concentration in annual tissue i.e. florets and leaves in the apparently heavy metal tolerant plant *Calluna vulgaris* has been demonstrated at other abandoned heavy metal mine workings in the UK (Wilson and Pyatt, 2006,2007).

Partitioning of metal concentrations within the olive plants examined can be expressed as follows:-

Cu mg kg<sup>-1</sup> Limni Flesh>leaves> stem>stone

Cu mg kg<sup>-1</sup> Akamas Flesh>leaves>stem>stone

Zn mg kg<sup>-1</sup> Limni Stem>leaves>stone>flesh

Zn mg kg<sup>-1</sup> Akamas Leaves>stone>flesh>stem

Pb mg kg<sup>-1</sup> Limni Flesh>leaves>stem>stone

Pb mg kg<sup>-1</sup> Akamas Flesh >leaves>stem>stone

Higher accumulation of copper and lead is again apparent in annual tissue (flesh and leaves). There appears to be little or no accumulation of copper or lead in the stone (or seed) of the olive and the flesh of these fruits contain the highest concentration of these two metals. Thus such exposure to heavy metals (copper, lead and zinc) would have least effect on the seed and hence on its subsequent viability.

Maisto et al. (2004) used an accumulation factor, which they based upon the ratio between metal accumulated in *Quercus ilex* L. leaves and the concentration of metal in the soil surrounding the plant, as a method of comparing the accumulation of different metals. We have investigated the ratio between metal concentrations in soil and various plant tissues, but expressed this ratio as a percentage i.e.

$$A = \frac{C_{pt}}{C_s} \times 100$$

where A = accumulation factor %

C<sub>pt</sub> = metal concentration in plant tissue

C<sub>s</sub> = metal concentration in soil.

Figure 1 illustrates the accumulation factors (A) obtained when comparing the copper concentrations in various plant tissues collected at Limni and Akamas. The flesh of ripe olives collected at both sites had the highest relative accumulation

factor for copper thus a greater proportion of soil copper was accumulated by this tissue than other components examined and would therefore be the most useful as an indicator of copper contamination in soil. Zinc was not detected in Limni soil; therefore a similar comparison cannot be carried out. Lead concentrations were similar in tissue samples from both sites, therefore on the basis of this data; *O. europaea* does not accumulate significant concentrations of lead in any of the tissues examined. Evidence that annual tissue, in this case flesh of the fruit and leaves, accumulate higher concentrations of copper and lead. There is also an indication that the *O. europaea* exhibits a mechanism which appears to protect seeds from heavy metal accumulation.

Figure 1 also illustrates that all olive plant tissues collected from the control site, with the exception of seed, have higher accumulation factors than the same tissues collected near the spoil tips. It would appear that a higher proportion of the copper concentration in the soil on the Akamas peninsular is available for bioaccumulation.

Analysis of data recorded for the samples from Limni and the Akamas peninsular indicated that partitioning of the metals detected followed a similar sequence within the olive tree, with the flesh of the olive accumulating the highest concentration of copper and lead. Zinc concentrations varied within respective tissues depending on the collection site, but leaves accumulate more zinc than most other tissues. Seed samples collected at both sites failed to accumulate copper or lead but contained a small concentration ( $6.4 - 7.1 \text{ mg kg}^{-1}$ ) of zinc in *O. europaea* on Cyprus. Examination of the accumulation factors for copper at both sites shows that a higher proportion of the soil concentration is accumulated at the Akamas site, which may be a function of the speciation of the copper metal in the soils or a result of a form of homeostasis which is limiting the total concentration of copper in the various tissues of the plants.

There is also evidence that, due to agencies such as atmospheric turbulence, there has been opportunities over the last two millennia for widespread distribution of cations on the island of Cyprus. It is important to note that some species such as *O. europaea* have the ability to produce fruit which is considered suitable for human consumption despite the fact that they are growing in close proximity to a metalliferous spoil tip.

#### *Acknowledgements*

Higher Education Funding Council for England who provided financial assistance to Bob Wilson.

**Table 1.** Standard wavelength and survey range for analysing target metals on Perkin Elmer 2100 ICP-OES

Element	$\lambda$ nm	Survey Low nm	Survey Up nm	Peak Algorithm	Points per peak
Copper	327.393	327.244	327.542	Area	7
Zinc	206.2	206.108	206.192	Area	7
Lead	220.353	220.255	220.451	Area	7
Cadmium	228.802	228.669	228.905	Area	7

**Table 2.** Accuracy of extraction and analytical procedures when applied to standard reference material CRM 142R Light sandy soil.

CRM142R	Cu	Zn	Pb	Cd
Mean mg L <sup>-1</sup>	72.8	95.7	24.4	0.21
Rel SD %	1.13	2.32	6.4	8.9
Certified mg L <sup>-1</sup>	69.8	93.3	25.7	0.25
Accuracy %	95.7	97.7	94.9	84

**Table 3.** Metal concentrations in soil, spoil and vegetation samples collected in the vicinity of Limni workings and the control site.

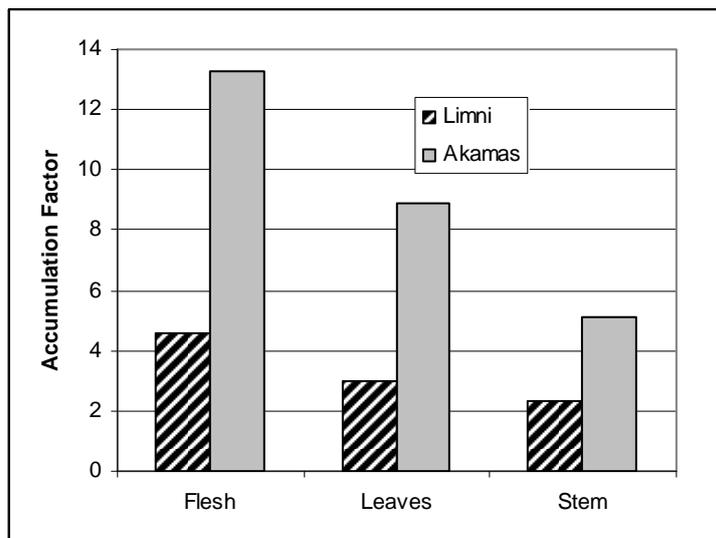
Sample Ref	Location Type	Mean Cu mg kg <sup>-1</sup>	RSD Cu %	Mean Zn mg kg <sup>-1</sup>	RSD Zn %	Mean Pb mg kg <sup>-1</sup>	RSD Pb %
25FBP01	Limni Spoil	674.0	2.78	<LOD		<LOD	
25FBP02	Limni Soil	193.0	5.8	<LOD		<LOD	
25FBP03	Limni olives Flesh	8.93	3.53	5.02	14.9	0.50	44.44
25FBP03A	Limni olives stone	<LOD		6.40		<LOD	
25FBP04A	Limni olives leaves	5.82	3.46	9.83	6.5	0.4	44.44
25FBP04B	Limni olives stem	4.43		15.3		0.21	
25FBP05	Akamas olives flesh	7.11	16.74	5.72	13.47	0.5	66.18
25FBP05A	Akamas olives stone	<LOD		7.15		<LOD	
25FBP06A	Akamas olives leaves	4.77	3.76	13.4	4.22	0.5	61.0
25FBP06B	Akamas olives stem	2.73	14.9	4.54	38.91	0.22	<LOQ*
25FBP07	Akamas soil	53.4	2.80	8.40	19.4	345.0	9.14

\* 6 replicate results fell below the LOQ for Pb of 0.21mg kg reported using 10 $\sigma$

**Table 4.** Metal concentrations in Cyprus spoils presented as mg kg<sup>-1</sup>

Metal	Source: Raber (1984)	Source: Pyatt (1999)	Limni current work
Cu	2000	3600	674
Zn	67	Not recorded	Not recorded
Pb	Not recorded	1200	Not recorded

**Figure 1.**



**Figure 1.** Partitioning of copper in olive trees at Limni and Akamas based upon accumulation factors.

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