Bull. Environ. Contam. Toxicol. (2005) 75:57–66

DOI: 10.1007/s00128-005-0718-8

Bulletin of Environmental Contamination and Toxicology

Aftermath of the Long-Term Application of Sludge and Water from a Sewage Treatment Plant to an Olive Tree (*Olea europaea* L.) Plantation

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Received: 9 November 2004/Accepted: 1 April 2005

The construction of Sewage Treatment Plants (STP) in many maritime cities of Greece led to an imperative need for the manipulation of the disposal problem for sludge and wastewater. Sewage of these cities, where the activities are limited to tourist services or agriculture, is characterized by the lack of heavy metals and toxic substances and the utilization of the STP products in various ways seems rather friendly to the environment. Microbial loads of the water are eliminated by chlorination or ozone application prior to disposal yet the sludge retains the high microbial load and, although rich in inorganic nutrients, has to be treated carefully.

The effect of sewage sludge application to agricultural soils in industrial countries (Raven and Loeppert 1997; Towers and Horne 1997) and that of the treated domestic and industrial effluents on plants (Hooda et al. 1997; Logan et al. 1997; Palacios et al. 1999; Samaras and Kallianou 2000, Weir and Alllen 1997) have been investigated.

Taking into account Council Directive 86/278/ECC (European Community 1986) for further research, we launched a project in order to investigate the effects of sludge and water application on crop plants of great economical importance (Margaris et al. 1995). Interesting results on plant growth and heavy metal accumulation were obtained for corn (Christodoulakis and Margaris 1996), cotton (Tsakou et al. 2001a, Tsakou et al. 2001b), flax (Tsakou et al. 2002) and poplar (Tsakou et al. 2003). All four species, among which only poplar is a perennial, exhibited tremendous growth promotion and fruit production when cultured on sludge amended agricultural soil irrigated with STP water. Concentrations of heavy metals in plant tissues (leaves, stems, roots, fruits and seeds) were far bellow the lower limits set up by the E.U. 's directives.

The final step of our investigation is of crucial importance since it is supposed to answer the questions on the effects of the long-term use of sludge and water in perennials from which parts (fruits, seeds, extracts etc) are used as food for the humans. The site round the sewage treatment installations on the island of Kos was the scene of a major environmental restoration including landscaping and planting - in 1993 - more than two thousand young individuals of plant species cultured in Mediterranean areas (Margaris et al. 1995). Olive (*Olea europaea* L.), peach (*Prunus persica*), almond (*Prunus dulcis*), pear (*Pyrus communis*), lemon (*Citrus limon*) and many other trees flourish round the installations for more than ten years

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irrigated and fertilized with water and sludge from the STP. Isn't this time span enough for any signs of bioaccumulation of heavy metals and toxic substances to appear? Are the leaves, the fruits or the seeds, although temporary plant organs, sites of heavy metal accumulation? Do the wood tissues of the deepest annual rings in the trunk - the oldest tissues of a plant - hide any secret? Are they hosting toxic materials or traces of lead?

MATERIALS AND METHODS

Six species of commercial and nutritional interest were selected. Among them is the Olive tree. Olives and olive oil are major constituents of the mediterranean nutrition, so frequently praised for their beneficial effects on human health. In this paper we present the results of our investigation on *Olea europaea* L.

This project was worked out at the Sewage Treatment Plant (STP) of the Municipality of Kos (an island of the southeastern Aegean Sea, Greece). Among the 2000 fruiting trees of the plantation more than 250 are olive trees. Six olive trees from this plantation were picked up in random.

Leaves and fruit were collected from the trees in 2002 and 2003. Very young leaves (leaf primordial - 20 April 2002), fully unfolded leaves (40 days later, on the 1st of June 2002) and mature leaves (95 days later, on the 5th of September 2002) were detached from branches of the current vegetative period. Small pieces of the branches were also collected for analysis. Leaves from each of the three groups were cut into small pieces, separately, and fixed in phosphate buffered 3% glutaraldehyde (pH 6· 8) at 0 °C for 2 hours (Sabatini et al., 1963). Some of the pieces from each group were dehydrated in a graded ethanol series, critical point dried, coated either with carbon or with gold or palladium and viewed with a JEOL JSM-6500F Scanning Electron Microscope. The Energy Dispersive X-ray Microanalysis (EDX) was executed on carbon-coated specimens with the JEOL JSM-6500F using the Oxford Link^{TM} ISIS^{TM} 300 microanalysis system through the Oxford SEMQuant^{TM} software (statistics and error correction). The accelerating Voltage was 20KV, the beam current 0,5 nA, the beam diameter 2μm and the live time 50 seconds.

A part of the tissue was post fixed in 1% osmium tetroxide in phosphate buffer (Ledbetter & Porter, 1963), dehydrated in a graded ethanol series and embedded in Durcupan ACM (Fluka, Steinheim, Switzerland).

Mature olives were also collected and prepared, using the same methods, for transmission and scanning electron microscopy and microanalysis.

All leaf and fruit semi-thin sections were viewed with a Zeiss Axioplan optical microscope. For the observation of the uranyl acetate-, lead citrate- double stained ultra thin sections (Reynolds, 1963); a Philips 300 Transmission Electron Microscope was used.

Original light micrographs were recorded digitally using a Nikon D100, 6· 1 megapixels camera. SEM images were digitally recorded while TEM micrographs were shot on Agfa TechPan B&W negative film.

Physicochemical properties of the local soil used as a basis for the growing substrate, were investigated (Table 1).

Table 1. Chemical profiles of: a) the soil used in the experiment (surface and 15 cm deep), b) not amended soil of the area, c) sewage sludge and d) the maximum values indicated in the Directive 201/688 of the European Community.

Element	Soil (surface) (mean value of 15 samples)	Soil (depth 20cm) (mean value of 15 samples)	Soil not amended (mean value of 15 samples)	Sewage sludge	Directive 2001/688 /EC (*)
K %	1.329	1323	1.053	0.969	
Ca %	18.605	17.717	19.433	8.047	
Ti %	0.233	0.205	0.176	0.156	
Fe %	2.058	1.912	1.709	0.930	
V mg/Kg	47.943	60.040	39.833	ND	
Cr mg/Kg	267.714	244.082	296.283	153.797	100
Mn mg/Kg	533.226	503.697	348.703	215.397	
Co mg/Kg	ND	1.536	ND	15.234	
Ni mg/Kg	146.373	141.765	134.374	104.515	50
Cu mg/Kg	41.383	46.743	7.162	267456	100
Zn mg/Kg	151.507	136.626	38.106	1108.791	300
As mg/Kg	11.741	12.847	2.823	32.937	10
Pb mg/Kg	43.593	42.037	51.092	168.488	100
Hg mg/Kg	5.815	5.939	8.012	2.426	1
Ag mg/Kg	3.019	3.300	ND	36.318	
Cd mg/Kg	2.831	2.115	1.185	0.670	1
Sb mg/Kg	4.709	1.945	ND	ND	
Ba mg/Kg	705.429	675.121	502.983	400.954	
SiO_2 diff	77.778	78.654	77.486	89.647	

Soil substrate, sewage sludge and wastewater as well as wood tissues, were analyzed for heavy metals and other elements by means of "EDXRF QuanX Spectrace" Spectrometer.

For crosschecking the heavy metal accumulations within the plant tissues, Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) was also used. Leaves and twigs were dried at 60 °C for 5 days, grinded, digested with HNO_3 65% in a microwave apparatus (MARS 5 CEM, USA) and filtered through Whatman 41 (20 - 25μm) filters. The extract was injected, with argon plasma, in an Iris Advantage AP/EWR-Duo Option (THERMO JARREL ASH, USA).

Analysis of variance was performed with the SPSS software. Heavy metal concentrations were compared using the Mann-Whitney U, Wilcoxon and Kruskall-Wallis tests.

* Directive 2001/688/EC defines the criteria for dispensation of the ecological label to soil amenders and culture media.

RESULTS AND DISCUSSION

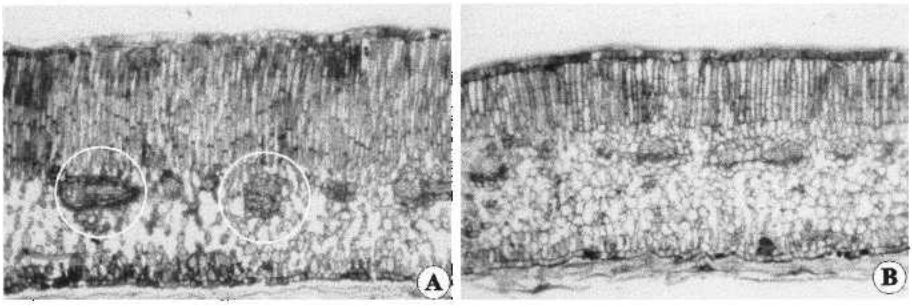
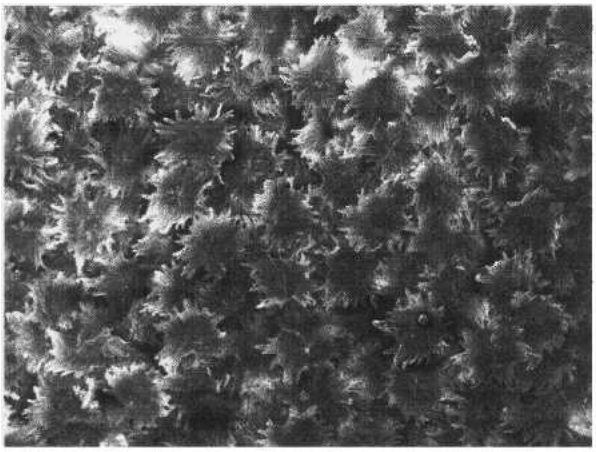


Figure 1. Cross sections of leaves from naturally growing (A) and treated plants (B). Naturally growing plants produce thicker, more xeromorphic leaves with developed palisade parenchyma and more elaborated conductive tissue (circles).

Leaves from both treated and naturally growing olive trees were primarily observed for structural differences at the light microscope level. Leaves from naturally growing plants were thicker, more xeromorphic, with well-developed palisade parenchyma - an important xeromorphic character - occupying more than 50% of the mesophyll, developed conductive tissue protected in bundle sheaths and large amount of secondary metabolites accumulated within the mesophyll cells. On the contrary, treated plants, although developing under the same stressing conditions of the Mediterranean climate, produce thinner leaves possessing less developed palisade parenchyma and less elaborated conductive tissue. Accumulation of secondary metabolites, a response to environmental stress, also seems inferior. The differences between the two leaf types are probably due to the rich in nutrients soil and the irrigation that treated plants regularly receive with STP water.

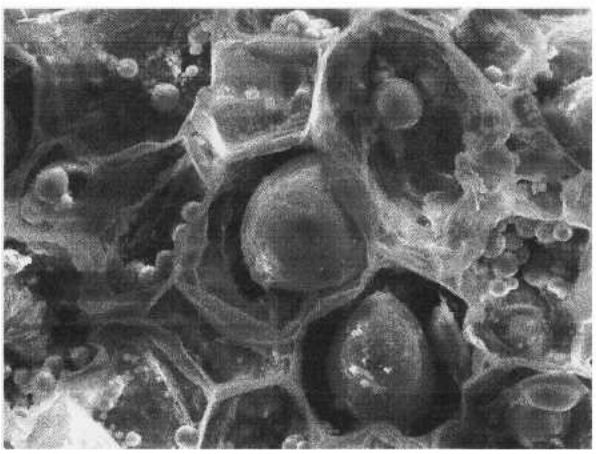
The next step was to perform Energy Dispersive X-ray Microanalysis (EDX) on leaf tissue (epidermis, mesophyll and hair cover of the adaxial surface) as well as within the mesocarp of the olives. The results indicated that heavy metals were either absent (negative values) or present in absolutely inconsiderable quantities, as the analysis printouts indicate (Figures 2 and 3).

A careful comparison of the printouts from the leaf tissue and the olive mesocarp indicates a significant difference in osmium concentration being elevated about four times in the olive mesocarp tissue. This is due to the use of osmium tetroxide for tissue fixation. Osmium atoms bind to form cross-links with the erected double bonds of the unsaturated lipids. Therefore, oil accumulation within the cells is to be blamed for the elevated values of osmium concentration in olives compared to those in the leaf tissue. Actually the huge peak of osmium in the graph was thought to be obstructive for other elements to appear. This was the reason for a new fixation of olives using only glutaraldehyde. Although the solvent used for dehydration (ethanol) removed much of the oil content, microanalysis indicated that in the position or in the vicinity of the missing peak of osmium, no other metals could be detected.



Elmt	*Spect. Type*	*Element %*
Na K	ED	2.58
Mg K	ED	- 0.04*
Al K	ED	0.06*
Ca K	ED	0.30
Cr K	ED	- 0.01*
Fe K	ED	0.09*
Co K	ED	0.09*
Ni K	ED	0.07*
Cu K	ED	0.26
Os K	ED	7.33
Hg K	ED	- 0.06*
Pb K	ED	- 0.14*

Figure 2. The lower surface of the leaf where a large number of scale-like protective hairs can be observed. This surface can withhold various airborne particles. The printout of the heavy metal microanalysis is also given to the right of the picture (* = < 2 Sigma).



Elmt	*Spect. Type*	*Element %*
Na K	ED	0.57
Mg K	ED	- 0.05*
Al K	ED	- 0.04*
Ca K	ED	0.08*
Cr K	ED	- 0.12*
Fe K	ED	0.02*
Co K	ED	- 0.06*
Ni K	ED	0.16*
Cu K	ED	0.72
Os K	ED	28.68
Hg K	ED	- 0.51*
Pb K	ED	- 0.21*

Figure 3. The cells from the mesocarp of an olive. Various droplets - large and smaller - are oil. The printout of the heavy metal microanalysis, focused within the cell and the oil droplets, is also given to the right of the picture (* = < 2 Sigma).

The above data is encouraging for a long-term use of water and sludge, yet microanalysis is a quantitative method and the results had to be checked further more so the "EDXRF QuanX Spectrace" Spectrometer was our primary option because all our investigations on heavy metal accumulation were so far conducted with this method (Tsakou et al. 2001a, Tsakou et al. 2001b, Tsakou et al. 2002, Tsakou et al. 2003). Eventually any new data could directly be compared and the whole investigation would have a continuance. Inductively Coupled Plasma Atomic Emission Spectrometry, although challenging, was our second choice.

Table 2. Values of heavy metals detected within leaf tissues using "EDXRF Quan X Spectrace" Spectrometer (p = leaf primordium, y = young leaf, m = mature leaf).

	specimen 1			**specimen 2**			**specimen 3**			**specimen 4**		
metal	p	y	m	p	y	m	p	y	m	p	y	m
Cd mg/Kg	ND	ND	ND	ND	0.4	2.7	ND	ND	ND	ND	ND	2.8
Cr mg/Kg	4.5	2.9	0.1	ND	2.8	6.9	4.7	ND	6.1	3.1	9.7	ND
Cu mg/Kg	2.1	9.9	5.7	6.4	13.5	6.1	9.7	ND	5.4	6.3	2.4	7.7
Mn mg/Kg	21.6	44.1	41.3	92.3	47.4	54.7	62.4	53.0	58.7	50.0	79.6	87.6
Ni mg/Kg	10.9	ND	ND	7.3	6.9	2.3	2.2	4.0	ND	2.5	3.7	3.7
Pb mg/Kg	ND	1.9	4.3	6.6	ND	ND	ND	ND	ND	2.7	ND	1.7
Zn mg/Kg	25.0	36.1	17.7	24.0	15.1	18.4	30.7	14.0	12.3	17.3	20.2	13.1

	specimen 5			**specimen 6**			**mean values**			***untreated***		
metal	p	y	m	p	y	m	p	y	m	p	y	m
Cd mg/Kg	ND	ND	ND	27.7	ND	ND	**0.4**	**0.1**	**0.8**	ND	ND	ND
Cr mg/Kg	ND	ND	ND	5.2	ND	2.3	**2.9**	**1.6**	**2.6**	2.4	ND	0.8
Cu mg/Kg	6.0	10.1	7.8	6.1	1.3	5.8	**6.1**	**6.2**	**6.4**	9.8	4.6	4.7
Mn mg/Kg	79.7	94.0	53.2	56.7	53.3	49.0	**60.4**	**61.9**	**57.4**	19.9	37.0	56.3
Ni mg/Kg	2.0	8.2	2.4	5.7	4.5	4.9	**5.1**	**4.6**	**2.2**	ND	3.5	4.3
Pb mg/Kg	ND	ND	ND	ND	ND	ND	**1.5**	**0.2**	**1.0**	ND	ND	10.1
Zn mg/Kg	30.4	23.1	8.8	17.1	14.7	13.0	**24.1**	**20.5**	**13.8**	14.8	22.1	12.6

Table 3. Values of heavy metals detected within leaf tissues using Inductively Coupled Plasma Atomic Emission Spectrometry (p = leaf primordium, y = young leaf, m = mature leaf).

	specimen 1			**specimen 2**			**specimen 3**			**specimen 4**		
metal	p	y	m	p	y	m	p	y	m	p	y	m
Cd mg/Kg	0.8	ND	0.1	0.3	ND	ND	1.0	0.4	0.3	0.4	ND	ND
Cr mg/Kg	1.4	0.8	0.2	2.3	1.5	1.4	1.0	1.1	2.5	1.3	1.1	2.1
Cu mg/Kg	4.2	4.7	5.9	3.1	6.6	4.0	4.9	3.0	5.9	4.6	5.0	4.2
Mn mg/Kg	27.5	28.7	51.7	99.1	31.4	56.1	72.1	34.5	51.3	52.2	61.0	80.2
Ni mg/Kg	31.3	4.6	11.0	10.9	4.4	1.9	4.9	3.0	6.3	5.9	6.9	7.8
Pb mg/Kg	ND	0.4	ND	ND	2.4	ND	ND	ND	ND	ND	ND	ND
Zn mg/Kg	25.2	25.4	2.2	22.3	18.0	1.5	28.2	19.4	1.8	19.4	18.3	1.4

	specimen 5			**specimen 6**			**mean values**			***untreated***		
metal	p	y	m	p	y	m	p	y	m	p	y	m
Cd mg/Kg	0.1	0.3	ND	0.4	0.2	ND	**0.5**	**ND**	**ND**	*0.2*	*ND*	*ND*
Cr mg/Kg	2.0	1.5	1.9	2.1	1.3	1.2	**1.7**	**1.2**	**1.5**	*ND*	*2.1*	*1.3*
Cu mg/Kg	3.8	3.9	3.0	1.3	4.9	4.9	**3.6**	**4.7**	**13.5**	*2.9*	*3.2*	*3.2*
Mn mg/Kg	87.1	60.8	46.0	52.9	33.0	46.7	**66.9**	**41.6**	**55.3**	*18.4*	*29.5*	*48.6*
Ni mg/Kg	8.3	5.1	5.7	4.1	2.8	7.6	**10.9**	**4.5**	**6.7**	*1.4*	*1.2*	*3.6*
Pb mg/Kg	ND	ND	ND	0.3	ND	ND	**ND**	**0.5**	**ND**	*ND*	*1.8*	*ND*
Zn mg/Kg	27.1	21.6	1.2	18.9	67.9	1.4	**23.3**	**28.4**	**1.6**	*12.9*	*22.0*	*15.4*

Table 4. Values of heavy metals detected within branchlet tissues using "EDXRF Quan X Spectrace" Spectrometer (y = young branch, m = mature branch).

	specimen 1		specimen 2		specimen 3		specimen 4	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	ND	ND	ND	ND	ND	ND	1.9	1.7
Cr mg/Kg	ND	ND	4.4	0.3	1.1	ND	4.1	ND
Cu mg/Kg	11.9	8.2	11.9	6.9	6.9	3.3	8.1	6.3
Mn mg/Kg	29.8	21.4	99.5	17.5	67.7	44.5	111.5	53.5
Ni mg/Kg	5.8	3.1	3.7	ND	3.4	ND	6.5	2.9
Pb mg/Kg	1.9	5.1	ND	ND	ND	ND	ND	2.7
Zn mg/Kg	14.6	15.0	15.1	6.3	18.9	6.4	19.6	8.5

	specimen 5		specimen 6		mean values		*untreated*	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	ND	ND	1.5	1.5	**0.6**	**0.5**	*ND*	*ND*
Cr mg/Kg	0.4	2.7	1.7	4.3	**1.9**	**1.2**	*ND*	*0.7*
Cu mg/Kg	4.8	2.4	13.0	1.2	**9.4**	**4.7**	*10.3*	*8.3*
Mn mg/Kg	98.8	96.0	55.5	38.6	**84.9**	**45.2**	*21.5*	*30.4*
Ni mg/Kg	6.2	ND	1.5	7.3	**4.5**	**2.2**	*2.7*	*2.5*
Pb mg/Kg	ND	ND	5.4	ND	**1.2**	**1.3**	*ND*	*3.4*
Zn mg/Kg	20.9	10.3	23.2	11.8	**18.7**	**9.7**	*15.9*	*14.2*

Table 5. Values of heavy metals detected within branchlet tissues using Inductively Coupled Plasma Atomic Emission Spectrometry (y = young branch, m = mature branch).

	specimen 1		specimen 2		specimen 3		specimen 4	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	0.1	0.2	0.3	0.4	0.4	0.4	0.2	0.1
Cr mg/Kg	1.8	0.8	3.3	0.7	2.9	0.1	1.2	0.9
Cu mg/Kg	1.9	8.0	3.9	9.8	2.8	5.2	9.8	2.9
Mn mg/Kg	17.6	14.9	109.0	10.6	50.0	28.1	29.2	37.8
Ni mg/Kg	2.0	1.8	6.2	7.4	4.2	8.5	0.7	2.3
Pb mg/Kg	ND	4.2	ND	ND	0.8	ND	1.9	2.2
Zn mg/Kg	14.9	16.7	15.6	29.2	17.5	20.1	21.4	16.5

	specimen 5		specimen 6		mean values		*untreated*	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	0.1	0.1	0.6	0.1	**0.3**	**0.2**	*0.5*	*0.2*
Cr mg/Kg	1.7	ND	2.7	0.5	**2.3**	**0.5**	*1.5*	*0.4*
Cu mg/Kg	2.8	3.4	4.9	2.7	**4.4**	**5.3**	*2.9*	*8.1*
Mn mg/Kg	95.6	85.1	46.0	26.9	**57.9**	**33.9**	*14.6*	*24.8*
Ni mg/Kg	1.2	1.3	6.8	0.5	**3.5**	**3.6**	*0.9*	*0.8*
Pb mg/Kg	3.8	ND	2.0	ND	**1.4**	**1.1**	*ND*	*1.6*
Zn mg/Kg	14.2	20.9	13.2	14.5	**16.1**	**19.6**	*17.0*	*22.0*

Table 6. Values of heavy metals detected within trunks of *Olea europaea* plants using "EDXRF Quan X Spectrace" Spectrometer.

	specimen 1		**specimen 2**		**specimen 3**		**specimen 4**	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	0.8	0.2	ND	0.6	ND	ND	ND	ND
Cr mg/Kg	ND	ND	0.1	ND	1.3	1.1	1.7	4.9
Cu mg/Kg	0.2	ND	7.3	10.9	3.6	0.1	4.3	7.2
Mn mg/Kg	1.6	2.3	2.0	2.7	1.0	ND	2.1	4.1
Ni mg/Kg	0.9	2.1	3.6	ND	1.9	3.4	2.5	ND
Pb mg/Kg	ND	0.7	ND	ND	ND	ND	ND	ND
Zn mg/Kg	ND	ND	5.9	8.7	2.9	8.7	2.4	3.2

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	ND	2.6	ND	0.3	**0.1**	**0.6**	*ND*	*ND*
Cr mg/Kg	0.5	ND	0.6	ND	**0.7**	**1.8**	*ND*	*ND*
Cu mg/Kg	2.9	4.7	0.3	6.2	**3.1**	**4.8**	*3.3*	*8.0*
Mn mg/Kg	3.2	4.8	1.9	3.1	**1.7**	**2.8**	*3.2*	*8.3*
Ni mg/Kg	0.1	ND	ND	1.3	**1.5**	**1.1**	*ND*	*ND*
Pb mg/Kg	ND	ND	ND	ND	**ND**	**0.1**	*ND*	*ND*
Zn mg/Kg	ND	ND	ND	2.0	**2.3**	**3.8**	*2.6*	*8.9*

Table 7. Values of heavy metals detected within trunks of *Olea europaea* plants using Inductively Coupled Plasma Atomic Emission Spectrometry.

	specimen 1		**specimen 2**		**specimen 3**		**specimen 4**	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	0.1	ND	ND	0.3	ND	ND	0.1	0.2
Cr mg/Kg	ND	ND	ND	ND	ND	ND	ND	ND
Cu mg/Kg	ND	ND	ND	ND	ND	ND	ND	0.7
Mn mg/Kg	0.8	1.4	1.1	3.0	0.9	3.0	1.2	3.3
Ni mg/Kg	ND	ND	ND	ND	ND	ND	ND	ND
Pb mg/Kg	ND	ND	ND	ND	ND	ND	ND	ND
Zn mg/Kg	1.1	4.9	49.6	4.0	4.6	2.9	3.0	72.2

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	ND	3.9	ND	3.9	**ND**	**1.4**	*0.4*	*0.1*
Cr mg/Kg	ND	ND	ND	ND	**ND**	**ND**	*ND*	*ND*
Cu mg/Kg	ND	5.0	ND	6.3	**ND**	**2.0**	*ND*	*0.6*
Mn mg/Kg	1.1	1.6	ND	1.1	**0.8**	**2.2**	*0.7*	*3.0*
Ni mg/Kg	ND	ND	ND	ND	**ND**	**ND**	*ND*	*ND*
Pb mg/Kg	ND	0.3	ND	1.3	**ND**	**0.3**	*0.3*	*ND*
Zn mg/Kg	5.9	9.0	ND	0.1	**10.7**	**15.5**	*2.1*	*4.5*

differently standardized instruments functioning at the limits of their resolution, we may conclude, primarily, that data obtained with both methods seem to be in agreement.

Analyzing these data from treated plants and comparing to those from naturally growing olive trees we can point out that for Pb and Zn no accumulation was detected, even after the long-term culture, in soil amended with sludge. The concentration of Cd seems increased in treated plants, a fact detected with both methods yet the concentrations of Pb and Cd remain far lower than the strict limits (0,001- 0,002 g/kg of dry tissue in fruits) of the Directive 466/2001 of the European Committee. For Cr, Cu, Mn and Ni a minor increase is detected with ICP-AES while records obtained with XRF seem to be within the values recorded for the natural plants.

Acknowledgments. We thank the General Secretariat of Research and Technology (Ministry of Development) and FAMAR S.A. for their financial assistance to this investigation through project 01ED29. We also thank the Municipality of Kos – Greece for providing the STP facilities and travel expenses.

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