Bull. Environ. Contam. Toxicol. (2006) 76:33–43

DOI: 10.1007/s00128-005-0886-6

Bulletin of Environmental Contamination and Toxicology

Aftermath of the Long-Term Application of Sludge and Water from a Sewage Treatment Plant to a Lemon Tree (*Citrus limon*) Plantation

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Received: 23 September 2005/Accepted: 7 November 2005

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 Allen 1997) have been investigated.

In Greece, the major problem of disposing sludge and water produced by Sewage Treatment Plants (STP) is under consideration for more than two decades. In most maritime cities where activities are limited to tourist services or agriculture, sewage treatment plants produce sludge and water characterized by the lack of heavy metals and toxic substances. The effect of the application of sewage sludge and irrigation with treated water from STP has, so far, been thoroughly investigated in annual (Tsakou et al. 2001a, Tsakou et al. 2001b, Tsakou et al. 2002) and perennial plants (Tsakou et al. 2003, Menti et al. 2005) with excellent results i.e. significant promotion of plant growth and productivity and the absence of heavy metals from the tissues of stems, leaves, roots and fruits.

Taking into account the exhortation included in the Council Directive 86/278/ECC (European Community 1986) for further research which would provide additional data for the effects of the use of STP products, we launched a project (Christodoulakis and Margaris 1996) which is currently at the final steps and, hopefully, gives well documented answers to all the questions about the effects of the long-term use of sludge and water in perennials from which parts (fruits, seeds, extracts etc) are also used as food for the humans.

The site of our experiments is round the STP on the island of Kos where hundreds of trees flourish since 1993 (Margaris et al. 1995), irrigated and fertilized with water and sludge from the installations. Considering this time span more than enough for any signs of bioaccumulation to appear, we thoroughly investigated heavy metal concentrations in all tissues of the lemon trees (*Olea europaea* L.). Leaves, fruits and seeds can, by no means, be considered sites of heavy metal accumulation. Wood tissues, even the oldest annual rings, deep in the trunks, did not host any toxic materials or traces of heavy metals (Menti et al. 2005).

In this paper, we present the results of our investigation on heavy metal accumulation for the lemon trees (*Citrus limon*). Lemons are fruits of high nutritional value (source of vitamin C) (Arias and Ramon-Laca 2005). They produce a broad spec-

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trum of secondary metabolites exhibiting desirable pharmacological properties (Del Rio et al 2003). As fruits they are consumed in large quantities in all Mediterranean countries.

MATERIALS AND METHODS

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 cultured there, more than 50 are lemon trees. Six lemon trees from this plantation were picked up in random.

Leaves and lemon fruits were collected in 2002 and 2003. Leaf primordia (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. 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).

Ripe lemons 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, 6·31 mega pixels 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 and presented in a previous paper (Menti et al. 2005).

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.

RESULTS AND DISCUSSION

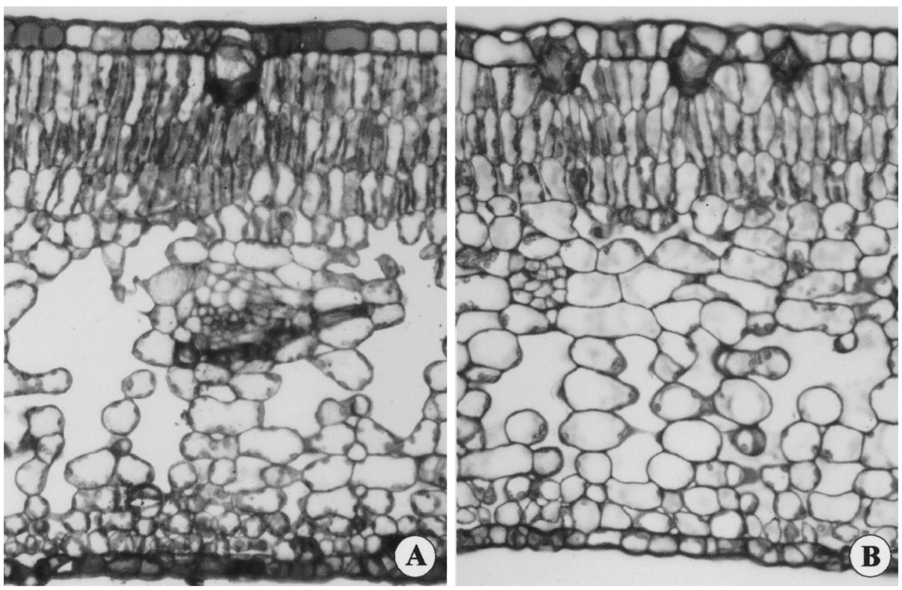
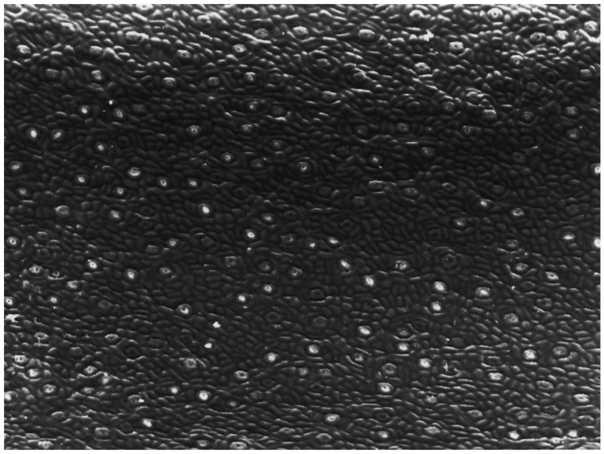


Figure 1. Cross-sections of leaves from naturally growing (A) and treated plants (B). Besides the elaborated conductive tissue observed in naturally growing plants and the lithocysts more common below the abaxial epidermal cells of the leaves of treated plants, no other differences can be observed among these two leaf types.

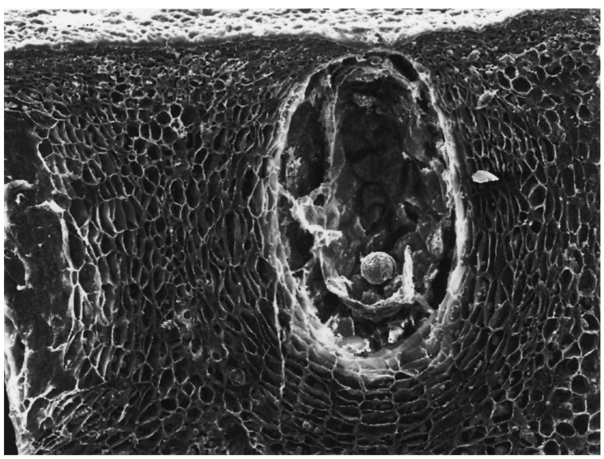
Leaves of naturally growing plants are somehow thicker, with more elaborated conductive tissue. Leaves from treated plants appear to develop far more lithocysts, between epidermal and palisade cells. Palisade parenchyma is equally developed in both leaf types occupying about 30% of the mesophyll while the spongy parenchyma seems looser in naturally growing plants. Accumulation of secondary metabolites, a response to environmental stress, was not observed. 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.

Energy Dispersive X-ray Microanalysis (EDX) performed on leaf tissue (epidermis and mesophyll) as well as within the pericarp of the lemons. 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).



Elmt	Spect. Type	Element %
Na K	ED	3.44
Mg K	ED	- 0.18*
Al K	ED	0.12
Ca K	ED	0.53
Cr K	ED	0.03*
Fe K	ED	0.00*
Co K	ED	- 0.09*
Ni K	ED	0.03*
Cu K	ED	0.36
Os K	ED	6.64
Hg K	ED	0.04
Pb K	ED	0.13

Figure 2. The adaxial (lower) surface of the leaf. A large number of stomata can be observed. The printout of the heavy metal microanalysis is given to the right of the picture (* = < 2 Sigma).



Elmt	Spect. Type	Element %
Na K	ED	1.50
Mg K	ED	- 0.03*
Al K	ED	- 0.06
Ca K	ED	0.42
Cr K	ED	0.08*
Fe K	ED	- 0.06*
Co K	ED	- 0.12*
Ni K	ED	0.12*
Cu K	ED	0.25
Os K	ED	12.64
Hg K	ED	0.01*
Pb K	ED	- 0.11*

Figure 3. Cross-sectioned pericarp of a lemon fruit. A large oil cavity is demonstrated. The printout of the heavy metal microanalysis, focused within the cavity and the oil droplet, is given to the right of the picture (* = < 2 Sigma).

The significantly high values in osmium concentration are 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. This is the reason for the higher concentration within the cells of the pericarp where lipids and terpenes are abundant, compared to osmium concentration recorded for the leaf surface.

Microanalysis data is encouraging for a long-term use of water and sludge yet, being quantitative, had to be checked further more. 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 there could be a direct comparison to any new data and the whole investigation would have a continuance. Inductively Coupled Plasma Atomic Emission Spectrometry, although challenging, remained a second choice.

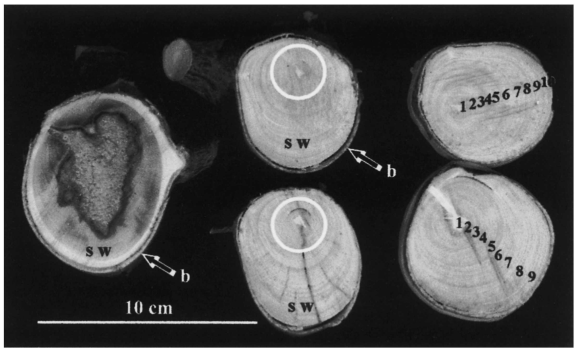


Figure 4. Slices from trunks of *Citrus limon* plants, nine or ten years of age. Numerals on the trunks (right) indicate annual rings. Heartwood is indicated within the circles. The externally located sapwood (sw) is covered by bark (b).

As presented in Table 1, young, fully expanded leaves and mature leaves detached from coetaneous lemon trees, growing in the same area, are analyzed. The mean values of the six specimens are compared to the mean values of the heavy metal analysis of the leaves from lemon trees growing outside the installations, on a field never exposed to sludge. The values of heavy metals detected with the "EDXRF QuanX Spectrace" Spectrometer are sprinkling and have no correlation to the leaf age. Heavy metals within the leaves were also investigated using Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), a method considered to be extremely precise. Data given in Table 2, confirm that heavy metal concentrations are very low, sometimes close to the resolution limit of the instrument and do not correlate to leaf age as well.

Values of heavy metals given in Table 3 (EDXRF) and Table 4 (ICP-AES) were detected either in recently sprouted branchlets collected in April (young) or in older branchlets collected in September (mature), from the same lemon trees. These stems are considered young organs. Heavy metal concentrations are very low, most of the times close to the resolution limits of the instruments.

Heartwood is a non-living tissue, the oldest on a plant. Sapwood is the younger, alive and functioning wood (Figure 4). The heartwood, sap wood and bark were carefully separated, grinded and undergone investigations with both methods. Data for heavy metal concentrations in heartwood and sapwood is presented in Table 5 and Table 6. This part of the investigation is crucial because it gives a piece of information on heavy metal dispersal and accumulation, within the plant body, versus time. If heavy metal concentrations in the heartwood were detected to be significantly higher than those in the sapwood then a scan along the annual rings would be necessary. In our case it seems that wood, regardless of it' s age, is free of heavy metals.

Table 7 and Table 8 present data for the heavy metal concentrations within the mesocarp, actually the edible part of the lemon fruit. Concentrations still remain close to the resolution limits of both instruments.

Table 1. Values of heavy metals detected within leaf tissues using "EDXRF Quan X Spectrace" Spectrometer (y= young leaf, m= mature leaf).

	specimen 1		**specimen 2**		**specimen 3**		**specimen 4**	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	1.15	ND	0.76	1.65	ND	2.78	ND	3.39
Cr mg/Kg	ND	3.65	ND	0.35	ND	1.54	ND	2.21
Cu mg/Kg	12.84	ND	21.65	20.54	13.25	8.07	20.14	3.72
Mn mg/Kg	15.28	10.82	14.79	9.99	23.11	14.81	16.39	16.15
Ni mg/Kg	5.82	13.1	2.08	4.71	8.64	ND	5.50	9.24
Pb mg/Kg	5.17	ND	ND	ND	ND	ND	ND	ND
Zn mg/Kg	17.02	16.92	19.84	25.16	20.86	11.77	18.05	21.43

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	ND	3.27	1.84	ND	**0.62**	**1.85**	*ND*	*ND*
Cr mg/Kg	ND	2.89	0.59	ND	**0.09**	**1.77**	*12.96*	*ND*
Cu mg/Kg	12.43	3.97	7.38	13.27	**14.61**	**8.26**	*6.35*	*10.03*
Mn mg/Kg	17.84	14.91	14.27	23.76	**16.95**	**15.07**	*35.48*	*11.13*
Ni mg/Kg	ND	6.05	ND	14.40	**3.67**	**7.92**	*8.82*	*2.53*
Pb mg/Kg	4.27	6.53	ND	ND	**1.57**	**1.09**	*ND*	*3.67*
Zn mg/Kg	8.40	8.13	21.53	23.86	**17.62**	**17.88**	*3.946*	*9.20*

Table 2. 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	y	m	y	m	y	m	y	m
Cd mg/Kg	0.30	ND	0.40	ND	0.50	ND	0.40	1.10
Cr mg/Kg	0.10	ND	1.10	ND	0.30	ND	0.60	ND
Cu mg/Kg	7.20	4.90	7.10	4.70	6.20	3.00	7.40	5.60
Mn mg/Kg	9.00	7.00	10.80	9.50	12.70	11.10	10.80	9.50
Ni mg/Kg	ND	2.60	ND	0.80	ND	1.60	ND	ND
Pb mg/Kg	5.90	1.20	ND	ND	ND	ND	ND	ND
Zn mg/Kg	20.10	16.20	25.00	14.00	18.80	14.50	46.50	13.70

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	0.4	ND	0.20	ND	**0.36**	**0.18**	*0.40*	*0.30*
Cr mg/Kg	ND	ND	0.90	ND	**0.50**	**ND**	*2.30*	*ND*
Cu mg/Kg	5.60	5.10	9.40	4.70	**7.15**	**4.66**	*10.20*	*2.20*
Mn mg/Kg	11.90	11.60	15.80	15.70	**11.83**	**10.73**	*49.90*	*8.40*
Ni mg/Kg	ND	1.10	ND	1.40	**ND**	**1.25**	*6.60*	*ND*
Pb mg/Kg	ND	ND	0.70	3.10	**1.10**	**0.72**	*ND*	*ND*
Zn mg/Kg	16.50	13.70	21.00	19.20	**24.65**	**15.22**	*18.70*	*14.50*

Table 3. 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	1.39	ND	ND	ND	ND	0.77	ND
Cr mg/Kg	2.0	ND	1.08	6.79	ND	1.05	ND	ND
Cu mg/Kg	2.21	15.2	6.90	5.69	1.5	ND	4.91	ND
Mn mg/Kg	3.45	5.18	4.24	1.41	5.67	3.96	7.74	2.81
Ni mg/Kg	ND	0.5	0.05	ND	1.04	ND	0.84	ND
Pb mg/Kg	ND	7.82	ND	ND	ND	ND	0.77	ND
Zn mg/Kg	14.68	20.73	17.79	16.23	14.68	12.16	7.91	7.81

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	2.63	3.97	ND	ND	**0.56**	**0.89**	*ND*	*1.66*
Cr mg/Kg	2.66	8.34	ND	1.94	**0.96**	**3.02**	*ND*	*3.17*
Cu mg/Kg	5.20	4.71	ND	2.96	**3.45**	**4.76**	*6.31*	*ND*
Mn mg/Kg	6.15	2.92	3.50	5.12	**5.12**	**3.57**	*1.66*	*5.28*
Ni mg/Kg	ND	ND	0.41	ND	**0.39**	**0.08**	*2.59*	*2.94*
Pb mg/Kg	ND	ND	3.96	3.30	**0.79**	**1.85**	*ND*	*0.18*
Zn mg/Kg	7.74	8.03	15.39	9.71	**13.03**	**12.44**	*6.87*	*17.75*

Table 4. 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	ND	ND	0.80	ND	ND	0.20	ND	ND
Cr mg/Kg	2.00	ND	2.10	0.30	0.70	ND	1.10	ND
Cu mg/Kg	2.90	9.50	3.80	8.90	8.90	8.10	5.40	11.00
Mn mg/Kg	3.90	3.10	2.60	3.40	4.90	3.40	5.60	4.50
Ni mg/Kg	ND	ND	ND	ND	3.30	ND	1.80	0.70
Pb mg/Kg	ND	ND	ND	ND	ND	ND	ND	ND
Zn mg/Kg	12.60	21.70	9.00	18.40	13.10	20.70	13.60	14.60

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m
Cd mg/Kg	ND	0.20	ND	ND	**0.13**	**0.07**	*0.20*	*0.10*
Cr mg/Kg	1.60	ND	ND	0.80	**1.25**	**0.18**	*ND*	*0.70*
Cu mg/Kg	3.50	10.50	6.10	8.70	**5.10**	**9.45**	*6.10*	*6.20*
Mn mg/Kg	3.00	4.00	5.97	2.90	**4.33**	**3.55**	*8.30*	*2.30*
Ni mg/Kg	0.70	ND	1.90	1.10	**1.28**	**0.30**	*2.30*	*ND*
Pb mg/Kg	ND	ND	ND	ND	**ND**	**ND**	*ND*	*ND*
Zn mg/Kg	ND	0.20	ND	ND	**8.05**	**12.6**	*0.20*	*0.10*

Table 5. Values of heavy metals detected within trunks of *Citrus limon* 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	ND	ND	1.77	ND	ND	ND	ND	ND
Cr mg/Kg	2.78	3.14	1.85	2.72	1.13	ND	4.96	2.76
Cu mg/Kg	1.98	2.67	3.11	2.34	4.21	1.97	10.54	17.5
Mn mg/Kg	7.89	8.56	8.23	10.86	0.79	7.65	2.32	8.33
Ni mg/Kg	ND	ND	9.24	10.66	ND	ND	14.87	1.48
Pb mg/Kg	ND	ND	0.59	ND	2.62	0.46	ND	ND
Zn mg/Kg	10.76	11.87	9.17	14.36	ND	5.17	2.76	11.11

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	ND	ND	ND	ND	**0.29**	**ND**	ND	3.96
Cr mg/Kg	3.23	5.76	4.16	5.02	**3.02**	**3.23**	3.66	3.42
Cu mg/Kg	0.98	1.67	3.34	2.98	**4.03**	**4.85**	4.27	3.81
Mn mg/Kg	6.78	5.76	9.12	10.43	**5.85**	**8.60**	3.05	5.55
Ni mg/Kg	ND	ND	1.13	0.78	**4.21**	**2.15**	5.89	4.46
Pb mg/Kg	ND	ND	ND	ND	**0.53**	**0.08**	1.29	ND
Zn mg/Kg	12.54	12.67	11.28	13.87	**7.75**	**11.51**	15.27	14.22

Table 6. Values of heavy metals detected within trunks of *Citrus limon* 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	ND	ND	0.20	0.10	ND	ND	ND	ND
Cr mg/Kg	1.99	2.30	ND	ND	0.50	0.50	ND	ND
Cu mg/Kg	4.10	5.20	ND	2.30	ND	ND	1.40	1.10
Mn mg/Kg	2.30	1.30	0.60	1.10	0.50	1.00	0.50	0.80
Ni mg/Kg	ND	0.40	ND	1.20	0.50	0.40	ND	0.20
Pb mg/Kg	ND	ND	ND	0.40	6.90	6.90	0.40	ND
Zn mg/Kg	13.10	9.90	3.70	11.30	2.50	15.50	2.60	10.60

	specimen 5		**specimen 6**		**mean values**		***untreated***	
metal	heart	sap	heart	sap	heart	sap	heart	sap
Cd mg/Kg	ND	ND	ND	ND	**0.03**	**0.02**	*ND*	*ND*
Cr mg/Kg	1.60	0.90	0.40	1.60	**0.75**	**0.88**	*ND*	*ND*
Cu mg/Kg	1.40	2.80	3.40	2.10	**1.72**	**2.25**	*13.10*	*9.20*
Mn mg/Kg	3.30	4.20	0.60	0.90	**1.30**	**1.55**	*0.80*	*0.70*
Ni mg/Kg	ND	ND	1.10	0.70	**0.27**	**0.48**	*0.20*	*ND*
Pb mg/Kg	0.40	ND	ND	ND	**1.28**	**1.22**	*ND*	*ND*
Zn mg/Kg	9.70	6.30	8.40	6.90	**6.66**	**10.08**	*16.30*	*8.00*

Table 7. Values of heavy metals detected within the mesocarp of *Citrus limon* fruits using "EDXRF Quan X Spectrace" Spectrometer.

	specimen 1		**specimen 2**		**specimen 3**		**specimen 4**		**specimen 5**		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m	y	m	y	m	y	m	y	m
Cd mg/Kg	0.97	1.48	1.48	ND	0.14	0.47	ND	ND	ND	ND	1.45	ND	**0.67**	**0.32**	*ND*	*ND*
Cr mg/Kg	1.42	ND	4.56	1.05	ND	ND	ND	ND	ND	ND	ND	ND	**1.76**	**0.17**	*5.17*	*1.82*
Cu mg/Kg	ND	5.45	9.04	10.65	ND	7.56	8.01	4.22	6.02	5.21	9.04	3.19	**5.35**	**6.05**	*2.71*	*2.04*
Mn mg/Kg	4.81	3.51	0.52	3.59	2.72	5.09	3.77	0.60	2.99	3.01	1.90	6.72	**2.78**	**3.75**	*3.99*	*4.79*
Ni mg/Kg	0.13	ND	ND	5.49	2.11	0.19	8.51	3.23	2.52	3.71	8.67	0.19	**3.66**	**2.14**	*ND*	*ND*
Pb mg/Kg	1.16	ND	2.86	ND	6.06	ND	ND	0.23	ND	ND	1.19	0.50	**1.88**	**0.12**	*ND*	*ND*
Zn mg/Kg	6.32	3.91	5.79	1.32	8.33	5.79	12.44	10.28	11.00	8.74	8.38	9.76	**8.71**	**5.01**	*6.99*	*4.99*

Table 8. Values of heavy metals detected within the mesocarp of *Citrus limon* fruits using Inductively Coupled Plasma Atomic Emission Spectrometry.

	specimen 1		**specimen 2**		**specimen 3**		**specimen 4**		**specimen 5**		**specimen 6**		**mean values**		***untreated***	
metal	y	m	y	m	y	m	y	m	y	m	y	m	y	m	y	m
Cd mg/Kg	1.00	0.10	1.20	ND	1.10	ND	1.20	ND	1.10	ND	1.30	ND	**1.15**	**0.02**	*ND*	*ND*
Cr mg/Kg	ND	0.10	ND	ND	ND	ND	ND	ND	ND	0.90	ND	0.30	**ND**	**0.22**	*0.60*	*ND*
Cu mg/Kg	5.80	6.20	8.90	3.70	6.80	6.80	7.80	7.10	8.90	6.50	9.30	5.80	**7.92**	**6.02**	*2.10*	*6.00*
Mn mg/Kg	1.90	1.90	2.00	1.90	2.40	1.70	3.00	1.40	2.00	2.30	3.00	3.70	**2.38**	**2.15**	*3.70*	*2.20*
Ni mg/Kg	0.50	0.20	ND	0.10	1.30	ND	ND	ND	ND	0.40	0.90	0.70	**0.45**	**0.23**	*ND*	*0.90*
Pb mg/Kg	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	**ND**	**ND**	*ND*	*ND*
Zn mg/Kg	9.87	7.40	7.90	4.70	7.30	8.20	6.90	5.70	7.80	6.70	5.90	7.20	**7.61**	**6.65**	*7.20*	*8.30*

Complicated statistical tests worked out (see "Materials and methods") do not appear in Tables 1 to 8 to avoid producing "bulky" and difficult-to-read images. Taking into account a) statistical analysis and b) the fact that very low and minor variations, as those recorded, are usually expected for natural specimens measured with different methods, in differently standardized instruments functioning at the limits of their resolution, we may conclude that heavy metals are hardly detectable within the oldest tissue of the lemon trees, the heart wood, about ten years of age in our experiment. The same is true for the leaf tissue, branchlets, trunks and mesocarp of *Citrus limon* (Tables 1-8) even after such a long-term culture in sludge amended soil. Both EDXRF and ICP analysis indicate that heavy metal content values in untreated and the mean values of treated samples are quite comparable for all metals examined. Concentration of Cd within the mature, edible mesocarp seems slightly increased in treated plants, a fact detected with both methods, yet the average concentrations of Pb and Cd in all samples investigated remain far lower than the strict limits (1 - 2 mg/kg of dry tissue in fruits) of the Directive 466/2001 of the European Committee.

Special attention should be devoted to Cu, Mn and Zn contents in *Citrus limon* trees since they appear increased compared to the other metals within all tissues studied. However, the Mn content appears significantly decreased compared to the corresponding values in *Olea europaea L.*, *Gossipium Hisrsitum* and *Linum usitatissimum* reported previously (Tsakou et al. 2001b, Tsakou et al. 2002, Menti et al. 2005). These concentrations increase in the order: trunks - branchlets - leaf. We may presume that Cu, Mn and Zn appear increased in the leaves because they keep an important metabolical role within them. Moreover, values of Cu, Mn and Zn in "young" samples usually appear higher or comparable to values detected within "mature" samples. The opposite trend is observed within the trunks, most probably because "mature" trunks, being metabolically inactive, retain some heavy metal traces.

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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