

Phytotoxicity Tests Applied on Sewage Sludge Resulted from Urban Wastewater Treatment Plants

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Four types of urban sewage sludge were tested to estimate their effects on higher plants (*Lepidium sativum* - LES, *Sinapis alba* - SIA and *Sorgum saccharatum* - SOS) highlighting their reusable potential. Seeds germination and roots growth inhibition were used as parameters to measure the phytotoxic sensitivities. The tested samples were collected from three Romanian Waste Water Treatment Plants (WWTPs). The chemical investigations of samples revealed metal concentration values within accepted limits except Arsenic, Manganese, Calcium, Iron and Aluminium. The plants expressed different phytotoxic sensitivities related to the contamination level. The seeds germination was inhibited between 20 to 100% for LES and SIA and between 25 and 75% for SOS. In case of roots growth, all the tested plants were significant inhibited (28 to 100%). The results indicated that all the samples were toxic for the tested plants, especially for the roots growth. The toxic effects of sludge could be reduced by adding more than 50% unpolluted soil that would allow to be reuse in agriculture as fertilizer. The study has proved that the phytotoxic microbiotests could be used as routine toxicity screening tests to provide rapid and relevant informations for sludge reuse and their environmental risk.

Keywords: phytotoxicity, plants, soil, sludge, wastewater, metals

The management of the sludge sewage obtained during wastewater treatment processes represents an environmental issue. The increase of sludge production triggers the need to find solutions for its disposal or reuse. Many studies showed that the reuse of sewage sludge in agriculture has been a method of disposal and a path to use its organic matter and nutrients content [1-4]. Sewage sludge samples represent a substantial source of nitrogen, phosphorus, organic matter as well as a deposit of various pollutants and bacteria. The use of sewage sludge as soil fertilizer could (1) increase the biomass production on nutrient-poor soils, (2) reduce the energy consumption used for the disposal / storage of sewage sludge, and (3) recycle the content of nutrients [4, 5].

However, the non-biodegradable pollutants and their metabolites from sludge could induce toxic effects for plants, animals and humans. For these reasons, the sludge intended to be used in agriculture as fertilizer, needs to be treated and strictly monitored for the toxic pollutants (especially for metallic elements). Pathogenic bacteria and parasites (e.g. *E.coli*, *Salmonella*, *Tenia*, and *Giardia*) represent another hazard to human health. The number of these organisms could be considerably diminish after sludge treatment. Moreover, if the sludge is directly spread on the field the organism's adaption fails under the new environmental conditions (climate, organic matter, pH, and presence of other toxic substances or other competitive microorganisms of soil) [6].

The Sewage Sludge Directive 278/1986/EEC promotes the use of sewage sludge in agriculture and control its use to prevent harmful effects on soil, vegetation, animals and humans [7]. The Directive mentioned above is presently under revision concerning the impact of emerging contaminants (metallic elements, polychlorinated biphenyls, polychlorinated dibenzodioxins, polycyclic aromatic hydrocarbons, brominated flame retardants, ingredients of personal care products, pharmaceuticals and some industrial chemicals) on the terrestrial and aquatic

biota when the sludge is used in agriculture. Currently, terrestrial toxicity tests are based on terrestrial plants, according to Environmental Protection Agency (EPA), Organisation for Economic Co-operation and Development (OECD), Food and Drug Administration (FDA) or International Organization for Standardization (ISO) methodologies, and involve monitoring of acute and chronic effects on the following species: i) dicotyledons (*Sinapis alba*, *Lepidium sativum*, *Brassica alba*, *Lactuca sativa*, *Phaseolus aureus*, *Lycopersicon esculentum*, *Cucumis sativum*, *Daucus carota*) and ii) monocotyledons (*Zea mays*, *Oryza sativa*, *Hordeum vulgare*, *Allium cepa*, *Sorgum saccharatum*) as well as other cereal species [8- 11].

The present study, compared to the previously mentioned studies, is based on chemical characterization and phytotoxic assessments for different urban sewage sludge samples aiming to estimate their toxic effects on three higher species of plants: *Lepidium sativum* (LES 290310), *Sinapis alba* (SIA 051011) and *Sorgum saccharatum* (SOS 140611).

Experimental part

Sludge samples

The sewage sludge samples (about 1 kg) were collected in duplicates from the drying beads of three urban Wastewater Treatment Plants (WWTPs) from Romania (Calarasi, Pascani and Timisoara). The collected samples were stored in glass containers and immediately transported to laboratory. The samples were air-dried, crushed and sieved (2 mm mesh size) to obtain representative samples (fig. 1).

Table 1 presents the sample sites names, samples symbols and the percentages of sludge concentrations used in phytotoxicity tests. The samples tested were either as such (100%) or mixed with artificial soil in a proportion of 50%, hydrated with distilled water or wastewater collected from the associated WWTP. The chemical and

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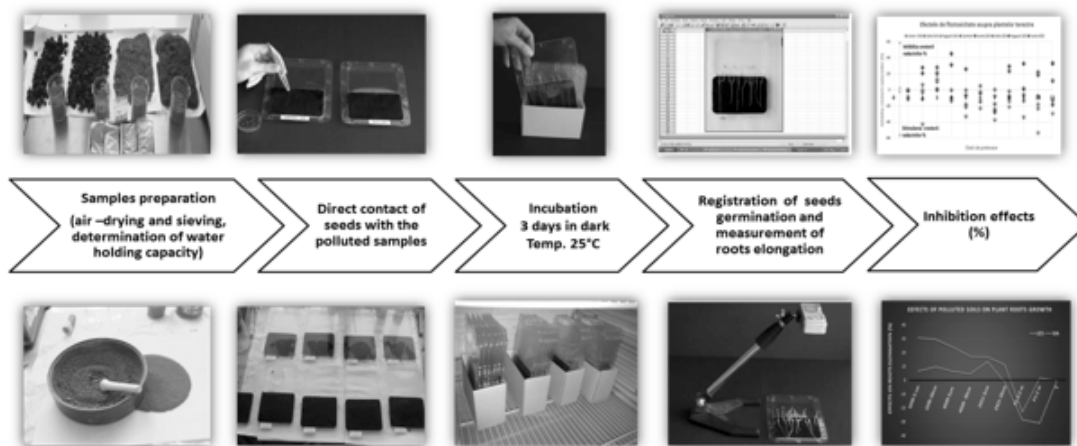


Fig. 1 Steps of phytotoxicity test (updated after Phytotoxkit protocol)

Sample sites of sludge	Symbol	Sludge conc. 100%	Sludge conc. 50%	Type of moisture
Calarasi WWTP	CAL-SS	x	x	d.w.
		x	-	w.w.
Pascani WWTP	PAS-SS	x	x	d.w.
		x	-	w.w.
Timisoara WWTP deposit from 2013	TIM-SS 2013	x	x	d.w.
Timisoara WWTP deposit from 2014	TIM-SS 2014	x	x	d.w.
Control (reference soil)	REF.SOIL	x	-	d.w.

Table 1
SAMPLE: COLLECTION SITES,
SYMBOLS, PERCENTAGE OF
DILUTION

Note: w.w. – wastewater; d.w. - dilution water;

bacteriological features of the wastewater used for hydration were analyzed.

Their quality for irrigation purposes fit in M2 class according to standard STAS 9450/1988 - water for crop irrigation, which could be used for all soils and plants crops, other than very permeable soil and plants intended for human and animal consumption.

Phytotoxicity tests

The toxicological effects of sludge samples were assessed using a Phytotoxkit microbiotest (MicroBioTest Inc. Belgium) which follows the ISO 11269-1:2012 methodology. The seeds of SOS, LES and SIA were grown on the contaminated sludges and after three days the seeds germination and the roots growth inhibition were analyzed compared to the control (sludges untreated seeds). The toxicity test followed the Phytotoxkit microbiotest protocol (fig. 1). The analyses and the roots length measurements were performed using ImageTool Programme for Windows. The assays were carried out in three replicates for each plant. The percentages of seed germination inhibition (SGI) and root growth inhibition (RGI) were calculated with formula: $SGI / RGI = A-B/A \times 100$ where: A is the average of seeds germination or roots length in the control; B is the average of seeds germination and roots length in the samples). Control tests were conducted using standard OECD soil (85% sand, 10% kaoline and 5% peat) and boric acid (250 mg/kg control soil) as reference test according to Phytotoxkit Standard Operational Procedure, 2012.

Results and discussions

Sludge characteristics

The sludge samples were chemical and bacteriological investigated (table 2) and their values were within the accepted limits of Directive 278/1986/EEC (Annex 2B) [7], Romanian Governmental Decision no. 344/2004 [12] and 40 CFR EPA 503 [13]. The dry matter content of dewatered sludge samples were between 15.69 and 28.73 %. The pH values were higher than 8 pH units for PAS-SS and TIM-SS, and 7.33 pH units for CAL-SS.

The sewage sludge metals were the most studied parameters because of their bioaccumulation potential and biomagnification in trophic chain [14].

All international norms limit the metal concentrations in the sewage sludge (table 3) which allowed the sewage sludge to be used as fertilizer in Europe [7], USA [13], England [15] and Romania [12].

Those limits depend on the metal soil loadings and environmental quality standards of each region.

The metals concentrations detected in the sewage sludge were below the regulations set limits and belonged within the class *a* limit of the African Classification System [16] or in category *EQ* (*Exceptional Quality*) bio solids according to EPA regulation [13]. Some metals, such as: Mn (detected in TIM-SS samples both in 2013 and 2014), As (in TIM-SS sample during 2013), Fe, Al and Ca (in all tested samples) showed high concentrations. Fe, Al and Ca were present in all tested samples as results of coagulant agents used in wastewater treatment processes. The experimental results regarding chemical characterization of the sludge were in accordance with 2011 Romanian Sewage Sludge National Management [17], excepting for Ni (in all samples) and for As (TIM-SS).

The metal concentrations were below 10 mg/kg d.m. for Cd, Co, Mo, Se; in the range 15 to 67 mg/kg d.m. for Cr, Ni, Pb; in the range 45 to 2100 mg/kg d.m. for Zn, Cu, Mn, Ti; and more than 20 000 mg/kg d.m. for Al, Ca and Fe (table 2). Bacteria tests revealed that all studied matrices corresponding to EPA 503 class *b* [13] and African Classification System [16], considering the fecal coliforms loading (> 1000 MPN/g.d.w.).

Phytotoxicity tests

The results of phytotoxicity evaluation of sludge samples, respectively seeds germination and roots elongation of LES, SIA and SOS are presented in table 4. The WWTPs sewage sludge showed a high effect on seeds germination and roots growth. The phytotoxic effects depend on contamination level of the sewage sludge.

Physical and chemical indicators	UM	Methods	CAL-SS	PAS-SS	TIM-SS 2013	TIM-SS 2014
pH	pH units	SR EN 15933/2013	7.33	8.10	8.5	8.3
Dry matter	%	SR EN 15935/2013	26.90	15.69	28.73	16.39
Arsenic (As)	mg/kg d.m.		5.02	1.36	15.3	8.61
Cadmium (Cd)	mg/kg d.m.		1.83	0.64	3.20	1.64
Chromium (Cr)	mg/kg d.m.		42.6	14.5	60.2	28.9
Cobalt (Co)	mg/kg d.m.		4.69	2.86	8.17	4.19
Cooper (Cu)	mg/kg d.m.		156	56.2	282	188
Nickel (Ni)	mg/kg d.m.		27.7	33.1	33.1	24.6
Lead (Pb)	mg/kg d.m.		66.7	18.1	71.8	59.6
Manganese (Mn)	mg/kg d.m.	SR EN	239	206	2113	2054
Titanium (Ti)	mg/kg d.m.	ISO11885/2009	153	43.8	222	136
Vanadium (V)	mg/kg d.m.	SR EN 16174/2012	17.8	5.41	22.9	11.4
Molybdenum (Mo)	mg/kg d.m.		4.92	4.43	3.39	3.71
Zinc (Zn)	mg/kg d.m.		829	260	815	557
Selenium (Se)	mg/kg d.m.		4.27	<0.1	35.5	16.1
Aluminum (Al)	mg/kg d.m.		37200	21500	37900	29550
Calcium (Ca)	mg/kg d.m.		47700	107500	23700	23650
Iron (Fe)	mg/kg d.m.		32500	20950	39650	27100
Total Coliforms	MPN/g d.m.	ICIM chap.IV2	34x10 ⁴	8x10 ⁵	8x10 ³	15x10 ⁶
Fecal Coliforms	MPN/g d.m.	EPA1680/2006	129x10 ³	1x10 ⁵	8x10 ³	15x10 ⁶
Enterococcus	MPN/g d.m.	ICIM chap. IV2	1293	3x10 ⁴	84	2x10 ⁶
<i>Escheriachia coli</i>	MPN/g d.m.	EPA1680/2006	2x10 ³	8x10 ⁵	8x10 ³	79x10 ⁵
<i>Salmonella</i>	-	EPA1682/2006	absent	absent	absent	absent

Note: d.m. – dry matter

Heavy metals	Directive 278/1986/EEC – Europe	40 CFR EPA 503 – USA	SI 1263/1989 (880/1990) - England	Governmental Decision no. 344/2004 - Romania
Cd	10=40	85; 39	1	10
Cu	1000 -1750	4300; 1500	25	500
Ni	300-400	420; 420	10	100
Pb	750 -1200	840; 300	25	300
Zn	2500-4000	7500; 2800	50	2000
Hg	16-25	57	0.1	5
Cr	1000	3000; 1200	25	500
As	-	75;41	-	10
Mo	-	75	-	-
Se	-	36	-	-
Co	-	-	-	50

Table 3
ADMISSIBLE LIMITS OF METALLIC ELEMENTS CONCENTRATIONS (IN mg/kg D.M.) IN THE SEWAGE SLUDGE FOR AGRICULTURE USE

Sample	LES		SIA		SOS	
	GS (10)	RG (10)	GS (10)	RG (10)	GS (4)	RG (4)
CAL-SS-dw	10	10.24±1.41	10	8.52±2.71	2	0
CAL-SS50%-dw	10	12.99±3.77	10	12.68±12.6	4	0
CAL-SS-ww	7	0	9	0	4	0
PAS-SS-dw	0	0	0	0	1	0
PAS-SS50%-dw	4	0	5	0	3	0
PAS-SS-ww	0	0	0	0	2	0
TIM SS-dw 2013	10	56.11±8.56	10	48.16±23.03	4	63.65±18.79
TIM SS50%-dw 2013	10	54.83±5.15	10	72.15±20.51	3	60.58±18.07
TIM SS-dw 2014	8	20.76±11.58	10	35.52±13.64	4	0
TIM SS50%-dw 2014	8	21.41±15.80	10	57.27±13.65	4	47.20±6.82
Control	10	66.88±20.68	10	80.94±9.82	4	76.45±23.43

Note: GS (10) (4) – average of 10 (LES and SIA) or 4 (SOS) seeds germination (number); RG (10) (4) – average of (LES and SIA) or 4 (SOS) roots length (mm)

Table 4
SEEDS GERMINATION - GS AND ROOTS GROWTH - RG

Seeds germination

The sludge from WWTP Pascani caused a significant inhibition of seeds germination (PAS-SS-dw > PAS-SS-ww > PAS-SS50%-dw) for all studied plants: SIA (50 to100%), LES (60 to100%) and SOS (25 to75%) (fig. 2 C, D and fig. 3).

In case of the sludge samples from Calarasi and Timisoara WWTPs, the seeds germination was lower or equal with 50% (fig. 2 A, B, E, F). The toxic effects of sludge

can be reduced if the samples are mixed more than 50% with unpolluted soil (in the study it was used artificial soil).

Other studies showed that a pH value above the normal range (6-7 pH units) caused the inhibition of seeds germination [18]. The fact was also presented in this work where a pH higher than 8 units inhibit germinating capacity of samples PAS-SS. In case of TIM-SS samples a different response was detected. Even if the pH values were over

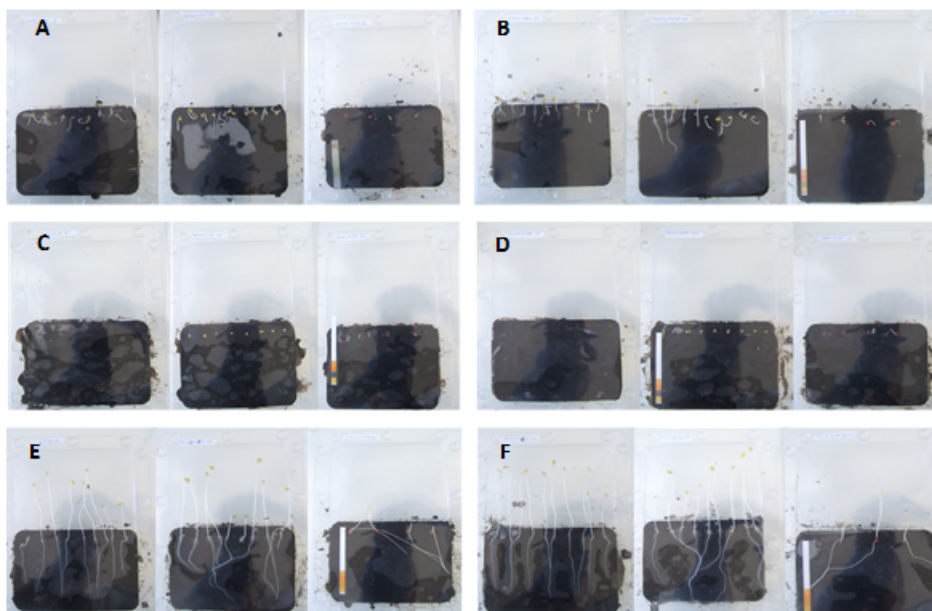


Fig. 2 Illustrations of phytotoxicity tests on LES / SIA / SOS; A - CAL-SS, 100%, d.w.; B - CAL-SS, 50%, d.w.; C - PAS-SS, 100%, d.w.; D - PAS-SS, 100%, w.w.; E - TIM-SS 2013, 100%, d.w.; F - TIM-SS 2013, 50%, d.w.

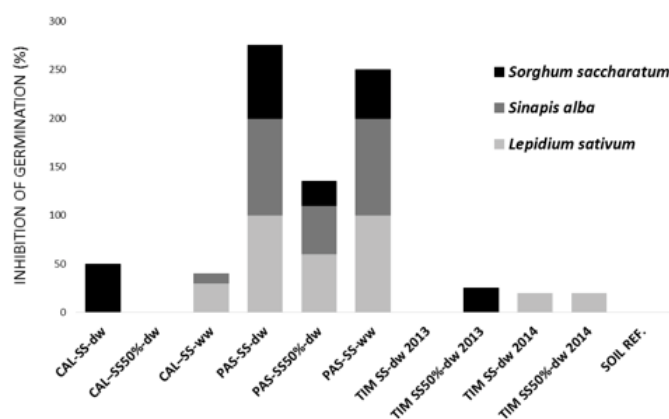


Fig. 3 Inhibition of seeds germination (%)

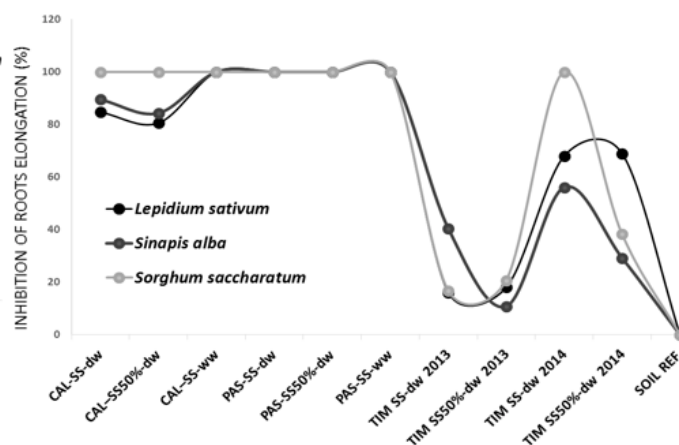


Fig. 4 Inhibition of roots growth (%)

8.3 units, the seeds germination process was situated in the range 80 to 100%.

Another study revealed that the high concentrations of Al could cause phytotoxic effects at high values of pH [19]. The presence of Mn and Fe and lack of Ca, Mg, P and K could influence the toxicity of Al. This metal interact with lipid membranes and causes stiffness of the membrane and impede seeds germination. Also Al could have effects on antioxidant enzymatic system and lipid peroxidation with free radical formation [18].

The results of seeds germination showed that SOS seeds could be considered the more sensitive compared with LES and SIA in Calarasi, Pascani and Timisoara samples (fig. 2).

No significant improvements of seeds germination were observed in case of TIM-SS50%-dw (fig. 2F) and CAL-SS50%-dw (fig. 2B) diluted samples. In case of PAS-SS50%-dw the seeds germination was increased with 40% for LES and 50% for SIA and SOS. When it was used wastewater for sample hydration, the seeds germination decrease with 30% for LES and 10% for SIA in case of CAL-SS-ww. In case of PAS-SS-ww the harmful effects of germination were unchanged (table 4).

Roots growth

After three days of incubation, the plants roots growth inhibition (%) of the sludge samples tested in 100% concentration were significant (37 to 100%). Inhibition of roots elongation occurs severe (88% to 100%) for the sludge samples collected from Calarasi and Pascani, for all three

types of plants studied (table 4 and fig. 4). A highest sensitivity of SOS plants growth it was observed in case of CAL-SS samples. No other differences between the plants species in case of PAS-SS samples were observed. In case of TIM-SS samples, insignificant changes of plants sensitivities was highlighted, keeping the same trend of roots growth inhibition (fig. 2 E, F and fig. 4). Other studies of sewage sludge with approximately 24% dry matter and metal contamination indicated the same phytotoxic effects on roots growth of SIA (70% to 100%) [20]. It seems that our results showed toxic effects both on seeds germination capacity and roots length more than 10% indicated a toxicity degree of 2 and 3, respectively *Toxic* (inhibition between 10 and 50%) or *Strongly toxic* (inhibition more than 50%).

Some studies revealed that certain metals are essential for plants growth (Cu, Mn, V, Mo, Zn, Co, Fe and Mg) and other are toxic (As, Cd, Pb and Cu). Some essential metals such as Cu and Zn introduced in soil through sludge in high concentration, showed phytotoxic effects on crop plants [21]. This hypothesis was confirmed in our study through the presence of these metals in all tested sludge samples (156 to 188 mg/Kg d.m for Cu and 260 to 829 mg/Kg d.m for Zn) that could induce the phytotoxic effects on tested plants.

Another cause of observed sludge toxic effects on roots growth could be due to the presence of high concentration of Cu > Cd > Fe = Zn > Pb. All these metals, excepting the Fe, can accumulate in roots [22]. All the tested samples

showed high concentrations of Fe, Cu, Cd, Zn and Pb, were recorded values of dozens or hundreds mg / kg, but within accepted limits.

Moreover the antagonistic activity of Fe-Zn and Fe-Mn (increased concentrations of these metals were detected in all samples) induced the roots growth inhibition [23]. A high concentrations of Fe could cause inhibition of Mn or Zn absorption and vice versa. Also, Ca and Al had antagonistic actions leading to metabolic disturbances in processes involving Mg or phosphates. Al inhibited the Ca absorption necessary for metabolic processes in plants, especially for the root growth that depends on Ca transportation [24].

Ni toxicity could be increased in the presence of Co, Cr, Zn, Mn, and Mo [23]. All these metals were identified in the tested samples, but their concentrations were under the limits.

The roots elongation could be affected also by pH value higher than the normal range (6-7 pH unities) [18] as it was the case of PAS-SS and TIM-SS samples. Mn, Al [19] and Fe were reported to induce a phytotoxic effect at a pH more than 6 [18].

Mn induced phytotoxicity at concentrations of 150 mg / kg to 5000 mg / kg [25]. In all the samples, Mn concentrations were situated in mentioned range.

The use of plants sensitivities as indicators of chemical contamination became an intensive research practice in environmental risk assessments studies and contaminated site remediation [26-28]. The phytotoxicity tests performed with the three different plants (SIA, SOS and LES) could give a species dependent response of sludge contamination for routine toxicity screening studies.

Conclusions

The phytotoxicity assessment (inhibition of seeds germination and roots elongation) of four different sludge was revealed to evaluate their potential as soil fertilizers. The experimental tests performed on three different plants provided relevant and correlated informations about sludge contamination using their responses. The plants had different phytotoxic sensitivities according to sludge site collection, level of contamination and sludge concentration. The seeds germination showed inhibitions about 20 to 100% for LES and SIA and 25 to 75% for SOS. The roots growth inhibition (%) of the sludge samples were significant (28 to 100%) for all plants. The results indicated that all samples were toxic or strongly toxic for the tested plants, especially concerning the roots growth. The study had proved that the phytotoxic microbiotests could be used as routine toxicity screening tests to provide rapid and relevant informations for sludge reuse and their environmental risk assessment.

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