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Monitoring the natural attenuation of a sewage sludge toxicity using the *Allium cepa* test

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ABSTRACT

Appropriate final disposal of sewage sludge (SS) generated by wastewater treatment plants (WWTP) has been considered a serious environmental problem, but also a viable alternative to be applied in agriculture, once SS is rich in organic matter and nutrients. However, SS can be a source of contamination of several toxic agents. Therefore, its use in agriculture requires special care to avoid possible damage to the environment and exposed organisms. Detoxification of toxic wastes can be performed using the monitored natural attenuation, which involves biological, physical and chemical processes that frequently occur in the environment. This study aimed to assess the feasibility of decontaminating SS after different periods of monitored natural attenuation. To this end, samples of SS and associations of soil/SS with proportions of 10, 25 and 50% SS were buried for 0, 2, 6 and 12 months in holes prepared in a place free of contamination. *Allium cepa* was used as an indicator to assess the efficiency of the natural attenuation process. According to chemical analysis, the SS samples presented a high concentration of *m*- and *p*-cresol, especially for samples analyzed after 0 or 2 months of natural attenuation. The microorganisms present in the SS belonged to 17 different genera of bacteria, which varied in the microbial composition among samples. Both, raw SS and aqueous SS extracts induced DNA damage in *A. cepa*, even when associated with soil. However, this effect was observed to decline during the attenuation period, although significant effects were detected for the highest tested concentration (100% SS) even at the end of this process. These results thus indicated the necessity of applying a stabilization process associating SS and soil for a period of at least 12 months and showed that the studied raw SS is not a viable material for use as a soil reconditioner, even after natural attenuation. *A. cepa* test proved to be a useful tool to assess the efficiency of SS detoxification process. Therefore, we suggest that the application of SS in agriculture should be approached with caution and that the SS must be previously submitted to methodologies that evaluate its toxic potential.

1. Introduction

Sewage sludge (SS) is a semi-solid residue resulting from the treatment of urban or industrial effluents. Although SS presents a quite variable composition, it is rich in organic matter, nitrogen and phosphorus and may contain other nutrients such as potassium, calcium and magnesium (Gray, 2010). Because SS contains a high concentration of organic matter and some basic elements required for plants, it can be characterized as a viable alternative for fertilizer in agricultural areas and/or as a reconditioner of the physical, chemical and biological properties of soil (Sánchez-Brunete et al., 2007).

The use of SS in agricultural activities can be characterized as a promising destination for this residue, both economically and environmentally, as the production of SS in urbanized areas continues to increase because of the constant growth of the amount of sewage produced by humans (Bright and Healey, 2003). However, SS may contain chemical contaminants such as metals and toxic organic compounds as well as pathogenic microorganisms (Holmstrup et al., 2001; Tsakou et al., 2001), which may result in soil degradation. Therefore, the use of SS in agriculture requires special care to avoid possible damage to the environment and to

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directly exposed organisms, including humans (Clarke and Smith, 2011).

The use of SS contaminated with metals, organic pollutants and pathogens in agricultural soils can affect functionality and edaphic biodiversity (Tas, 2010), which in the long term, can impact the entire associated food chain (Roig et al., 2012). Another problem is possible chemical lixiviation of SS-treated soils by pluvial waters, which can transfer this contamination to superficial and subterranean waters (Keller et al., 2002). These issues have stimulated worldwide efforts to develop new technologies to optimize the fate of SS (Tas, 2010).

Monitored natural attenuation is considered an advantageous remediation methodology for decreasing the toxicity of organic wastes because it is efficient, inexpensive and environmentally friendly (Makadia et al., 2011).

According to USEPA (1999a) the monitored natural attenuation can be defined as a process that occurs under favorable conditions in the environment, resulting in a decrease of the mass, toxicity, mobility, volume or concentration of contaminants as a consequence of a variety of physical, chemical or biological processes, such as biodegradation, dispersion, dilution, adsorption, volatilization, transformation and others.

The chemical analysis of environmental samples is extremely important for characterizing damage in contaminated areas. However, chemical analysis only estimates the concentrations of toxic substances in the environment and does not reflect the bioavailability of these chemicals to the associated biota (Moreira et al., 2008). Thus, it has become increasingly important to conduct bioassays that can establish the real impact of contaminants on the environment (Plaza et al., 2005). Bioassays allow the observation of isolated or combined effects of substances present in the environment and may therefore reveal the complexity of the associated biochemical and physiological processes (Roos et al., 2004).

The species *Allium cepa* has frequently been used to evaluate the effectiveness of environmental decontamination processes because it presents suitable biomarkers of cellular and genetic damages (Mazzeo et al., 2010, 2011; Miranda et al., 2013; Souza et al., 2013).

This study aimed to evaluate the effectiveness of monitored natural attenuation of domestic SS by means of bioassays using the test organism *A. cepa* to comparatively evaluate the cytotoxic, genotoxic and mutagenic potential of the SS before and after different periods of the attenuation process.

2. Materials and methods

2.1. Material and sample preparation

Samples of anaerobic SS, dewatered *via* centrifugation, were collected from a wastewater treatment plant (WWTP) that receives only effluents from domestic sewage, located in the municipality of Rio Claro, São Paulo, Brazil (latitude 22°24′39″S and longitude 47°33′39″W). This WWTP is responsible for treatment of 22% of the city's total wastewater, serving 36,000 inhabitants. The volume of treated sewage corresponds to 180,645 m^3 per month, generating a total of 113.53 t of anaerobic SS per month.

After sampling, the SS samples were mixed with a reference soil collected in the Experimental Garden of the University of São Paulo State—UNESP (Campus Rio Claro, São Paulo, Brazil) in proportions of 10, 25 and 50% SS (0.5 geometric progression from 100% SS). This soil was characterized based on its granulometry, organic matter content and electrical conductivity (Table 1).

2.2. Detoxification of SS through monitored natural attenuation

Samples of 100% SS and associations containing 10, 25 and 50% SS in soil were placed in individual plastic bags (42.5 × 65.0 cm) that were micro-perforated with openings that were 0.5 mm in diameter and 1 cm apart. Each bag contained 8 kg of each sample. The bags were buried in the external area of the Experimental Garden of UNESP in Rio Claro (São Paulo, Brazil) in holes with a maximum depth of 50 cm to allow interactions between the samples and the external environment, besides avoid significant temperature variations. The Experimental Garden is a reference site to conduct this kind of experiment, because it is considered free of contaminants, which avoids the contamination of the samples. Moreover, the Experimental Garden is also an isolated place, which prevents the dispersion of contaminants to other environments. The experiment was conducted in duplicate over periods of 2, 6 and 12 months, after which bioassays were carried out.

2.3. Production of aqueous extracts (solubilized)

Aqueous extracts of each fresh sample were obtained following the protocol proposed by ABNT NBR 10.006 (2004), using a suspension of 125 g of each sample (equivalent dry weight) in 500 mL of ultrapure water, followed by constant stirring at low speed for 5 min. For samples of 100% SS, twice that amount of water was added (1000 mL). After 7 days of decantation at 22 °C, the liquid phase of the sample/water mixture was collected and filtered through a 0.45 mm-pore-size membrane to obtain extracts containing water-soluble substances.

To obtain the equivalent dry weight, 3 independent aliquots of each sample (10 g) were dried at 105 °C and weighed until a stable dry weight was obtained (approximately 24 h). The average of each triplicate was considered as the equivalent dry weight of each sample and this value was used to prepare the aqueous extract.

2.4. Chemical analysis

After each period of monitored natural attenuation, chemical analysis of 100% SS solid samples was performed at the laboratory of the Analytical Technology Company (São Paulo—Brazil). The analyses were conducted according to the procedure described by USEPA SW-846 (1999b). The selection of the analyzed parameters were based on CONAMA resolution (National Environment Council) no. 375 (Ministério do Meio Ambiente, Conselho Nacional do Meio Ambiente, 2006), which determines the maximum limits of pollutants present in SS samples to allow their use in agricultural soils in Brazil.

The presence of volatile organic compounds (1,2,3-trichlorobenzene; 1,3,5-trichlorobenzene; 1,2,4-trichlorobenzene; 1,2-dichlorobenzene) and semivolatile organic compounds (1,3-dichlorobenzene; 1,4-dichlorobenzene; 1,2,3,4-tetrachlorobenzene; 1,2,4,5-tetrachlorobenzene; 1,2,3,5-tetrachlorobenzene; di-*N*-butyl phthalate; dimethyl phthalate; 2,4-dichlorophenol; 2,4,6-trichlorophenol; pentachlorophenol; benzo(*a*)anthracene; benzo(*a*)pyrene; benzo(*k*)fluoranthene; indeno(1,2,3-cd)pyrene; naphthalene; phenanthrene; hexachlorobenzene; *m*- and *p*-cresol; *o*-cresol; bis(2-ethylhexyl)phthalate) were quantified using gas chromatography-mass spectrometry (GC/MS), following the methods 8260B and 8270D (USEPA, 1999b), respectively.

The presence of organochlorine pesticides (heptachlor; aldrin; *g*-BHC; *a*-chlordane; *g*-chlordane; dieldrin; DDT; endrin; toxaphene; mirex) and inorganic compounds (phosphorus; potassium; sodium; sulfur; calcium; magnesium; arsenic; barium; cadmium; lead; copper; chrome; mercury; molybdenum; nickel; zinc) was analyzed using gas chromatography (GC), following the method 8081B (USEPA, 1999b) and inductively coupled

Table 1
Granulometric analysis and electrical conductivity of the reference soil.

Sample	Sand (g/kg)						Silt (g/kg)	Clay (g/kg)		Flocculation[h] (%)	Texture class	Electrical conductivity (μS/cm)
Soil	VC[a]	C[b]	I[c]	T[d]	VT[e]	TS[f]	79	w/disp.[g]	water	78	Clayey	146.7
		6	16	119	257	72		469	451	100		

[a] Very coarse.
[b] Coarse.
[c] Intermediate.
[d] Thin.
[e] Very thin.
[f] Total sand.
[g] With dispersant.
[h] Proportion of clay that is flocculated, reporting on the degree of aggregate stability.

plasma-atomic emission spectrometry (method 6010C—USEPA, 1999b), respectively.

Dioxins and furans were quantified using high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS) by the method 8290A (USEPA, 1999b).

The identification of the compounds detected in the raw 100% SS samples were also performed in the aqueous SS extract at the laboratory of the Global Analysis and Consultancy (São Carlos—Brazil) following the method proposed by Opeolu et al. (2010) using high performance liquid chromatography with diode array detection (HPLC-DAD).

2.5. Identification and quantification of microorganisms

To identify the microorganisms present in the SS and the soil, 1 g of each sample (100% SS or soil control) was added to 100 mL of sterile saline solution after each period of monitored natural attenuation. To assess the diversity of the microorganisms in the samples, 10 μL of this solution was seeded by depletion, using a laboratory loop, into Petri dishes containing blood agar, MacConkey agar, SS agar and Sabouraud agar. The plates were incubated at 35 °C, except for the Sabouraud agar plates, which were kept at room temperature. The plates were read every 24 h for 7 days. The various microorganisms found in the samples were isolated and initially identified using conventional biochemical tests. These microorganisms were subsequently identified using automated Vitek 2 Compact equipment (BioMerieux®, Inc, St Louis, MO, USA).

Microorganisms were quantified by scoring colony-forming units (CFUs).

2.6. Bioassays with A. cepa seeds

Seeds of *A. cepa* ($2n = 16$ chromosomes) from the same lot and variety (the "Baia Periforme" onion) were submitted to germination in individual Petri dishes containing: 1—SS/soil associations or 100% SS; or 2—aqueous extracts obtained from SS/soil associations or 100% SS, obtained both before and after the monitored natural attenuation process.

Controls were performed with ultrapure water (negative control), the aqueous extract of the reference soil, the reference soil itself (soil control) and 10 mg/L of methyl methane sulfonate (Sigma-Aldrich, CAS 66-27-3) (positive control). All tests were performed in duplicate.

After germination, onion roots *ca.* 2.0 cm in length were collected and fixed in a mixture of ethanol and acetic acid (3:1—v/v) for 6 to 18 h at room temperature. After this period, the roots were stored in freshly made fixation solution at 4 °C until use. The protocol described by Leme and Marin-Morales (2008) was followed to prepare slides of the meristematic and F1 root regions.

Genotoxic endpoints were estimated in meristematic cells of *A. cepa* that presented different types of chromosomal aberrations (*e.g.*, losses, bridges, delays, adherence) and nuclear abnormalities (*e.g.*, budding, binucleated cells, lobulated nuclei). Cytotoxicity was assessed through recording the changes in the mitotic index of these cells, and the mutagenic potential was assessed through recording the presence of micronuclei in meristematic and F1 cells and chromosomal breaks in meristematic cells.

These parameters were evaluated under a light microscope, and 10 slides per treatment were taken, and 500 cells were examined per slide.

The efficiency of the monitored natural attenuation was evaluated by comparing the results obtained in 5 individuals for each sample in each period tested to the negative control using the Mann–Whitney test ($p < 0.05$), as suggested by Caritá and Marin-Morales (2008), Mazzeo et al. (2011), Christofoletti et al. (2012), Miranda et al. (2013), Mazzeo and Marin-Morales (2015).

3. Results and discussion

3.1. Chemical analysis

Detailed results of the chemical analyses performed in the raw 100% SS during the different periods of natural attenuation as well as in the soil used to prepare the soil/SS associations can be found in the Supplementary data (Table S1).

A major concern regarding the application of SS to agricultural soils is related to the presence of high levels of metals in SS, which can negatively impact the soil. Once metals are added to the soil, it is very difficult to remove them due to their mobility and residual time, which leads to long-term interference with soil proprieties and, hence, with the quality of agricultural crops (Jiang and Fan, 2008). Moreover, unlike organic contaminants, most metals are not affected by microbial or chemical degradation (Park et al., 2011) and therefore require more specific technologies for their removal from the environment.

In this study, the metal concentrations recorded in the SS from the Rio Claro WWTP were below the limits established by CONAMA 375 (Ministério do Meio Ambiente, Conselho Nacional do Meio Ambiente, 2006—(Table S1)), which characterize this material as a potential fertilizer for agricultural soils.

The results of the chemical analysis indicated the presence of *m*- and *p*-cresol at concentrations that were much higher than those established by legislation (Ministério do Meio Ambiente, Conselho Nacional do Meio Ambiente, 2006) in both the initial SS and the SS degraded for 2 months (Table 2). These substances were also detected at high concentrations in the aqueous SS extracts at the 0- and 2-month sampling times (Table 2).

Cresols are substances belonging to a chemical group of phenolic compounds that are employed in several industrial processes (ATSDR, 2008; Sen et al., 2014). *m*-Cresol and *p*-cresol are used in the production of contact herbicides, pyrethroid insecticides, fragrances, antioxidants, flame-retardants, synthetic resins, disinfectants and explosives, among other products (OECD, 2003; ATSDR, 2008; Tallur et al., 2009). According to Arya et al. (2011),

Table 2
Quantification of *m*- and *p*-cresol in SS following different periods of monitored natural attenuation.

Parameter	Material	Unit	Initial sample	2-Month sample	6-Month sample	12-Month sample
m-, *p*-Cresol	Raw sludge	μg/kg	276,089.8	256,743.4	ND[a]	ND[a]
	Aqueous extract	mg/L	3.70	4.21	0.78 (81.47%)[b]	0.06 (98.58%)[b]

[a] Not detected.
[b] Percent decrease in *m*- and *p*-cresol concentrations.

these substances are also present in effluents from the textile, pulp and paper, coal conversion and fungicide industries. Furthermore, isomeric cresols are widely distributed in nature and naturally present in the oils of some plants (e.g., jasmine, cinnamon, mint, eucalyptus, conifers and camphor), petroleum, coal tar and geological materials from volcanic eruptions, in addition to often being produced by soil microorganisms as intermediary metabolites during biodegradation (IPCS, 1995). Thus, cresols reach the environment either through natural sources or *via* the combustion of coal and wood, vehicle exhaust, oil refineries and even cigarette smoke (ATSDR, 2008). Bright and Healey (2003) detected the presence of *m*- and *p*-cresol in SSs from five WWTPs in the metropolitan area of Vancouver (Canada) at concentrations ranging from 0.46 to 940 μg/g, with the highest concentration exceeding the limits allowed for the use of SS in soils. In a review, Harrison et al. (2006) also described studies identifying the presence of methylphenol in SS at concentrations up to 1160 mg/kg dry weight.

Evaluation of the natural attenuation process applied in this study demonstrated that after 6 months, *m*- and *p*-cresol were no longer present in the raw SS samples (at 6 and 12 months), at least at concentrations that were detectable by the methods used in our chemical analysis, indicating degradation of these compounds.

Dioxins (polychlorinated dibenzo-*p*-dioxins—PCDDs) and furans (polychlorinated dibenzofurans—PCDFs) were also detected in the studied samples (Table 3). According to Kulkarni et al. (2008) and Suzuki et al. (2011), the presence of dioxins and furans in the environment occurs mainly as a result of anthropogenic activities related to the incineration of solid domestic and hospital wastes; the combustion of diesel, wood and coal; industrial activities such as pulp bleaching; steel mills; the manufacture of pesticides and the incineration of halogenic material; and the action of microorganisms and photochemical reactions of certain substances. The presence of dioxins and furans in SS was also reported by Klimm et al. (1998).

The Brazilian government does not set limits for these substances in SS to be used in agricultural soil. However, Canadian legislation establishes thresholds for agricultural soils ranging from 17 to 50 ng TEQ/kg dry weight of SS and a limit for non-agricultural soils of up to 100 ng TEQ/kg dry weight of SS (Leblanc et al., 2008). In the present study, the chemical analyses showed an increase in the dioxin and furan concentration in the 100% SS along the natural attenuation period (Table 3).

Based on these results, it is possible to conclude that the increase of these substances may be related to the decrease in the total mass of SS during the natural attenuation process and the consequent mineralization of organic matter (loss of total organic carbon—Table S1) without the decrement of dioxins and furans, resulting in higher final concentrations of these substances. Another evidence for supporting this explanation is that the same increase of the metal levels was also observed. According to Matamoros et al. (2012), the dewatering and mineralization of organic matter of SS lead to a higher metal concentration in this matrix. In the present study, mineralization is more likely explanation because of the fact that the concentrations are expressed on a dry basis.

Another explanation is that the presence of these substances may be related to microbial degradation of compounds not identified in the chemical analysis, *e.g.* chlorinated biphenyls (PCBs), that

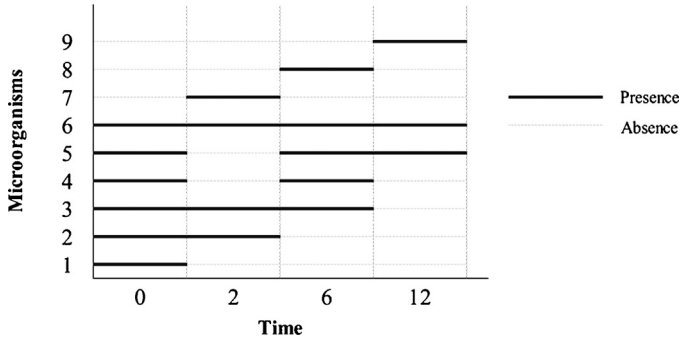

Fig. 1. *Microbiological succession observed during the monitored natural attenuation of SS.* (1) *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus* (coagulase-negative); (2) *Morganella morganii*, *Proteus mirabilis*; (3) *Enterobacter cloacae*; (4) *Pseudomonas aeruginosa*; (5) *Serratia marcescens*; (6) *Bacillus* sp.; (7) Gram-negative bacilli (non-fermenter), *Providencia rettgeri*; (8) *Citrobacter freundii*, *Ochrobactrum anthropi*; (9) *Achromobacter denitrificans*, *Burkholderia cepacia* complex, *Buttiauxella agrestis*, *Pseudomonas putida/fluorescens*.

can be transformed in dioxins and furans as observed in the study conducted by Weber et al. (2002).

Although there were increases in the amounts of dioxins and furans during the attenuation period, according to the indexes provided by Canadian legislation, the results are not alarming because they are below 17 ng I-TEQ/kg dry weight SS.

The chemical analysis performed on the SS samples that had been buried for 2 months revealed a new substance (bis(2-ethylhexyl)phthalate) that was not present in the analysis of the initial SS. This substance is a synthetic plasticizer that exhibits high stability, fluidity and low volatility and is widely added to plastics to make them more flexible, specifically those composed of polyvinyl chloride (PVC) (Carrara et al., 2011). Because this substance was not detected in the initial chemical analysis, we suggest that its presence in the SS sample attenuated for 2 months was resulted of the transfer of this compound from the plastic bag to the samples. However, this substance was not detected in the subsequent chemical analyses performed on the SS samples buried for 6 and 12 months (Table S1). Therefore, we conclude that the release of phthalate occurred during the first contact of the samples with the plastic material, which most likely occurred until the second month of the experiment. After this period, we believe that the phthalate had been depleted from the plastic bag and was degraded.

3.2. Microbiological characterization of SS

The results of the microbiological characterization of the SS after the tested attenuation periods and the reference soil are presented in Table 4.

Regarding the reference soil sample, a low diversity of microorganisms was recorded. For the SS samples, we observed a change in the microbial composition of the samples (Fig. 1) involving 17 different genera of bacteria along the different periods of natural attenuation. The presence of Gram-positive cocci (*Enterococcus faecalis* and *Staphylococcus* (coagulase-negative)) was exclusively observed in the initial sample. Enterobacteria (*Citrobacter freundii*, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Morganella morganii*, *Proteus mirabilis* and

Table 3
Results of the analysis of dioxins and furans in the reference soil and 100% SS after different periods of monitored natural attenuation, expressed as toxicity equivalents (TEQs), based on toxic equivalent factors (W-TEFs) for 2005 established by the World Health Organization (WHO).

Dioxins	Reference soil	Initial sample	2-Month sample	6-Month sample	12-Month sample	TEF
	W-TEQ (ng/kg)	W-TEQ (ng/kg)	W-TEQ (ng/kg)	W-TEQ (ng/kg)	W-TEQ (ng/kg)	W-TEF
2,3,7,8-TCDD	ND[a]	ND[a]	0.9423	ND[a]	0.42	1
1,2,3,7,8-PeCDD	0.2140	1.654	1.873	ND[a]	2.56	1
1,2,3,4,7,8-HxCDD	ND[a]	ND[a]	0.0619	ND[a]	0.499	0.1
1,2,3,6,7,8-HxCDD	0.03774	0.2513	0.4148	5.296	1.76	0.1
1,2,3,7,8,9-HxCDD	0.03071	ND[a]	0.1377	ND[a]	0.093	0.1
1,2,3,4,6,7,8-HpCDD	0.1199	0.3187	0.7052	1.678	1.86	0.01
OCDD	0.01778	0.06996	0.16302	0.19902	0.2289	0.0003
Total dioxin TEQs	0.42013	2.29396	4.29792	7.17302	7.4209	
Furans						
2,3,7,8-TCDF	0.02031	0.2105	0.2503	ND[a]	0.411	0.1
1,2,3,7,8-PeCDF	0.00659	ND[a]	0.06858	ND[a]	0.0804	0.03
2,3,4,7,8-PeCDF	0.10311	0.8442	0.7677	ND[a]	0.627	0.3
1,2,3,4,7,8-HxCDF	0.02150	0.2610	0.3212	ND[a]	0.293	0.1
1,2,3,6,7,8-HxCDF	0.01535	0.1461	0.2531	ND[a]	ND[a]	0.1
2,3,4,6,7,8-HxCDF	0.01476	0.1665	0.2353	ND[a]	0.304	0.1
1,2,3,7,8,9-HxCDF	0.01753	ND[a]	0.04516	ND[a]	0.440	0.1
1,2,3,4,6,7,8-HpCDF	0.01196	0.2147	0.3251	0.3874	0.0171	0.01
1,2,3,4,7,8,9-HpCDF	ND[a]	ND[a]	0.01193	ND[a]	0.491	0.01
OCDF	0.00087	0.01273	0.02562	0.018876	0.02097	0.0003
Total furan TEQs	0.21198	1.85573	2.30399	0.406276	2.68447	
General total TEQs	0.63211	4.14969	6.60191	7.579296	10.10537	

[a] Not detected.

Providencia rettgeri) predominated over the initial periods, remaining up to 6 months of natural attenuation. Non-fermenter Gram-negative bacilli (*Achromobacter denitrificans*, *Burkholderia cepacia* complex and *Pseudomonas fluorescens*/*putida*) and fungus were observed, predominantly, in samples from 12 months of attenuation. The only species presented in all of the analyzed samples was *Bacillus* sp. Petersen et al. (2003) also reported a microbial succession during the sewage sludge decomposition, as observed in the present study.

During the attenuation process, a decrease in the final counting of total bacteria was also observed for the final period (below 5×10^5). Thus, both the alteration in the groups of microbial community and the counting of total bacteria in SS can be used as indicators of each step of the natural attenuation process.

Several studies suggest that some of the microorganisms identified in the investigated SS may have the ability to degrade substances that were initially present in these samples. Among them, the bacteria *Pseudomonas* sp. (Saravanan et al., 2008; Ho et al., 2010); *Bacillus* sp. (Tallur et al., 2006; Tallur et al., 2009; Ho et al., 2010); *Serratia liquefaciens* (Sharma et al., 2002); *Serratia plymuthica* (Pradhan and Ingle, 2007); *Serratia marcescens* (Singh et al., 2007); *P. mirabilis* (Mohite et al., 2011) were cited in the literature as efficient species in the mineralization of phenolic derivatives, including cresol. According to our results, only the bacteria *Bacillus* sp., *E. cloacae*, *P. rettgeri*, *M. morganii*, *P. mirabilis* and non-fermenter Gram-negative bacilli can be inferred to be possible agents for the degradation of phenols present in the SS studied here, since *Pseudomonas aeruginosa* and *Serratia* sp., recognized

Table 4
Characterization of microorganisms isolated from SS after different periods of monitored natural attenuation.

Microorganisms	Reference soil	Initial sample	2-Month sample	6-Month sample	12-Month sample
Bacteria					
Achromobacter denitrificans[a]	–	–	–	–	2×10^4
Bacillus sp.[b]	4×10^5	10^7	4×10^5	10^5	5×10^5
Burkholderia cepacia complex[a]	–	–	–	–	3×10^5
Buttiauxella agrestis[a]	–	–	–	–	3×10^5
Citrobacter freundii[a]	–	–	–	10^7	–
Enterobacter cloacae[a]	–	8×10^6	4×10^5	10^7	–
Enterococcus faecalis[b]	–	5×10^6	–	–	–
Escherichia coli[a]	–	9×10^6	–	–	–
Klebsiella pneumoniae[a]	–	10^7	–	–	–
Morganella morganii[a]	–	10^7	10^5	–	–
Ochrobactrum anthropi[a]	–	–	–	10^4	–
Proteus mirabilis[a]	–	9×10^6	3×10^6	–	–
Providencia rettgeri[a]	–	–	3×10^5	–	–
Pseudomonas aeruginosa[a]	–	8×10^6	–	2.2×10^6	–
Pseudomonas putida/*fluorescens*[a]	–	–	–	–	10^4
Serratia marcescens[a]	–	10^7	–	10^7	10^4
Staphylococcus[b] (coagulase-negative)	–	10^6	–	–	–
Gram negative bacilli[a] (non-fermenter)	–	–	1×10^6	–	–
Fungus					
Penicillium sp.	5×10^4	–	–	–	10^6

[a] Gram negative.
[b] Gram positive.

Table 5
Alterations in cells of *A. cepa* exposed to various concentrations of raw SS after different periods of natural attenuation.

Evaluated parameters	MNA[a] (months)	Samples						
		Soil	NC[b]	PC[c]	10% SS	25% SS	50% SS	100% SS
Mitotic Index	0	42.17 ± 7.23	34.88 ± 13.40	36.03 ± 9.02	41.78 ± 8.61	43.04 ± 6.62	44.21 ± 12.93	40.78 ± 8.24
	2		34.11 ± 5.09	38.08 ± 5.05	37.50 ± 10.01	37.95 ± 5.30	35.24 ± 5.68	–
	6		46.17 ± 8.25	44.73 ± 5.09	39.93 ± 7.56	39.71 ± 7.58	42.39 ± 5.66	34.22 ± 6.51*
	12		45.87 ± 6.76	49.03 ± 9.88	42.84 ± 7.71	40.40 ± 10.36	48.08 ± 7.46	44.58 ± 12.92
Genotoxic alterations	0	5.36 ± 1.88	2.20 ± 1.29	8.78 ± 1.47*	6.58 ± 1.73*	10.13 ± 2.62*	14.44 ± 3.70*	16.82 ± 3.08*
	2		3.73 ± 1.51	10.35 ± 1.65*	9.37 ± 3.60*	12.88 ± 5.27*	11.69 ± 3.70*	–
	6		4.89 ± 2.28	14.30 ± 5.14*	5.99 ± 3.15	7.39 ± 2.10*	7.00 ± 1.85*	10.60 ± 4.13*
	12		2.07 ± 1.42	10.72 ± 2.84*	1.81 ± 0.84	2.30 ± 1.14	1.42 ± 1.65	3.38 ± 1.75
Mutagenic alterations	0	0.59 ± 0.69	0.48 ± 0.68	16.71 ± 6.22*	0.88 ± 0.56	1.28 ± 1.32	0.98 ± 0.80	1.28 ± 1.32
	2		0.00 ± 0.00	10.62 ± 6.12*	0.49 ± 0.51	2.27 ± 2.71*	1.65 ± 1.71*	–
	6		0.79 ± 0.62	6.67 ± 3.32*	0.69 ± 0.67	1.52 ± 1.19	1.00 ± 1.25	2.22 ± 1.55*
	12		020 ± 0.41	12.34 ± 4.45*	0.57 ± 0.49	0.28 ± 0.46	0.57 ± 0.49	0.38 ± 0.49
MN F1[d]	0	0.58 ± 0.68	0.38 ± 0.49	8.63 ± 6.80*	0.09 ± 0.29	0.29 ± 0.66	0.00 ± 0.00	0.19 ± 0.39
	2		0.78 ± 0.62	8.83 ± 5.44*	0.29 ± 0.65	0.30 ± 0.67	1.36 ± 0.51	–
	6		0.59 ± 0.68	8.09 ± 5.14*	0.39 ± 0.69	0.78 ± 0.77	1.27 ± 1.30	3.81 ± 4.73*
	12		0.39 ± 0.51	10.57 ± 4.28*	0.97 ± 1.22	0.20 ± 0.42	0.86 ± 2.09	0.58 ± 0.68

[a] Monitored natural attenuation.
[b] Negative control.
[c] Positive control.
[d] Micronuclei in F1 cells.
* Significantly different from the negative control ($p < 0.05$) according to the Mann–Whitney test.

as potential degradation agents for this type of waste, were not present at 2 months of natural attenuation and these compounds did not persist beyond this time (Fig. 1).

Species of the genus *Bacillus* (Juneson et al., 2001; Niazi et al., 2001; Azarova et al., 2003; Yuan et al., 2010) and *Pseudomonas* (Azarova et al., 2003) are recognized as bacteria with the potential to degrade phthalates. Our data also suggest that the genus *Bacillus* is effective in the degradation of bis(2-ethylhexyl)phthalate. However, we also observed the involvement of the bacteria *E. cloacae, P. rettgeri, M. morganii, P. mirabilis* and non-fermenter Gram-negative bacilli in the biodegradation of phthalates.

Even though several microorganisms have been described as degraders of dioxin and furan, including *Pseudomonas* sp. HH69 (Fortnagel et al., 1990), *Sphingomonas* sp. RW1 (Wilkes et al., 1996), *Burkholderia* JB1 (Parsons et al., 1998), *Terrabacter* sp. (Habe et al., 2002) and *Geobacillus* sp. UZO 3 (Suzuki et al., 2011), in the present study, under the conditions employed in our tests, none of the identified microorganisms appeared to be capable of degrading dioxins and furans, as there were increases in these compounds during the attenuation process.

3.3. *Evaluation of the effects of SS on the test organism* A. cepa

Tables 5 and 6 present results related to the effect of the studied SS on the test organism *A. cepa*.

The aqueous extract of the 100% SS at 0 and 2 months and the raw 100% SS at 2 months were considered highly toxic to the test organism, completely inhibiting seed germination, thus precluding the evaluation of the cytotoxic, genotoxic and mutagenic potential of these samples. Similar results were recorded by Walter et al. (2006) regarding the germination of cress seeds (*Lepidium sativum* L.) exposed to SS processed obtained following anaerobic

Table 6
Alterations in cells of *A. cepa* exposed to various concentrations of aqueous SS extracts (solubilized) after different periods of natural attenuation.

Evaluated parameters	MNA[a] (months)	Samples						
		Soil	NC[b]	PC[c]	10% SS	25% SS	50% SS	100% SS
Mitotic Index	0	30.58 ± 5.43	34.88 ± 13.40	36.03 ± 9.02	36.84 ± 13.04	35.44 ± 8.00	35.95 ± 7.11	–
	2		34.11 ± 5.09	38.08 ± 5.05	35.71 ± 7.59	36.61 ± 7.11	35.71 ± 13.12	–
	6		46.17 ± 8.25	44.73 ± 5.09	46.63 ± 9.85	47.78 ± 7.94	46.61 ± 8.17	48.31 ± 7.67
	12		45.87 ± 6.76	49.03 ± 9.88	48.86 ± 2.99	50.84 ± 7.38	51.05 ± 6.49	47.14 ± 5.48
Genotoxic alterations	0	3.02 ± 1.06	2.20 ± 1.29	8.78 ± 1.47*	4.34 ± 1.62*	8.00 ± 1.98*	7.41 ± 3.00*	–
	2		3.73 ± 1.51	10.35 ± 1.65*	5.69 ± 1.85*	9.59 ± 3.79*	10.49 ± 2.69*	–
	6		4.89 ± 2.28	14.30 ± 5.14*	4.95 ± 2.15	9.09 ± 2.58*	7.50 ± 2.54*	10.19 ± 3.32*
	12		2.07 ± 1.42	10.72 ± 2.84*	2.07 ± 0.85	1.34 ± 1.02	1.83 ± 1.33	4.38 ± 1.53*
Mutagenic alterations	0	0.55 ± 0.89	0.48 ± 0.68	16.71 ± 6.22*	0.76 ± 0.74	1.27 ± 1.61	1.45 ± 1.23	–
	2		0.00 ± 0.00	10.62 ± 6.12*	0.38 ± 0.67	2.67 ± 2.57*	1.42 ± 0.99*	–
	6		0.79 ± 0.62	6.67 ± 3.32*	0.69 ± 0.66	1.06 ± 1.13	0.49 ± 0.51	2.99 ± 2.73*
	12		0.20 ± 0.41	12.34 ± 4.45*	0.55 ± 0.64	0.28 ± 0.63	0.37 ± 0.63	1.13 ± 1.14*
MN F1[d]	0	0.47 ± 0.80	0.38 ± 0.49	8.63 ± 6.80*	0.00 ± 0.00	0.10 ± 0.30	3.09 ± 1.28*	–
	2		0.78 ± 0.62	8.83 ± 5.44*	1.56 ± 0.84	3.65 ± 3.68*	2.25 ± 1.21*	–
	6		0.59 ± 0.68	8.09 ± 5.14*	1.56 ± 1.24	1.67 ± 1.49	1.48 ± 1.07	3.53 ± 4.53*
	12		0.39 ± 0.51	10.57 ± 4.28*	1.17 ± 0.77	0.78 ± 0.78	0.58 ± 0.66	0.88 ± 0.72

[a] Monitored natural attenuation.
[b] Negative control.
[c] Positive control.
[d] Micronuclei in F1 cells.
* Significantly different from the negative control ($p < 0.05$) according to the Mann–Whitney test.

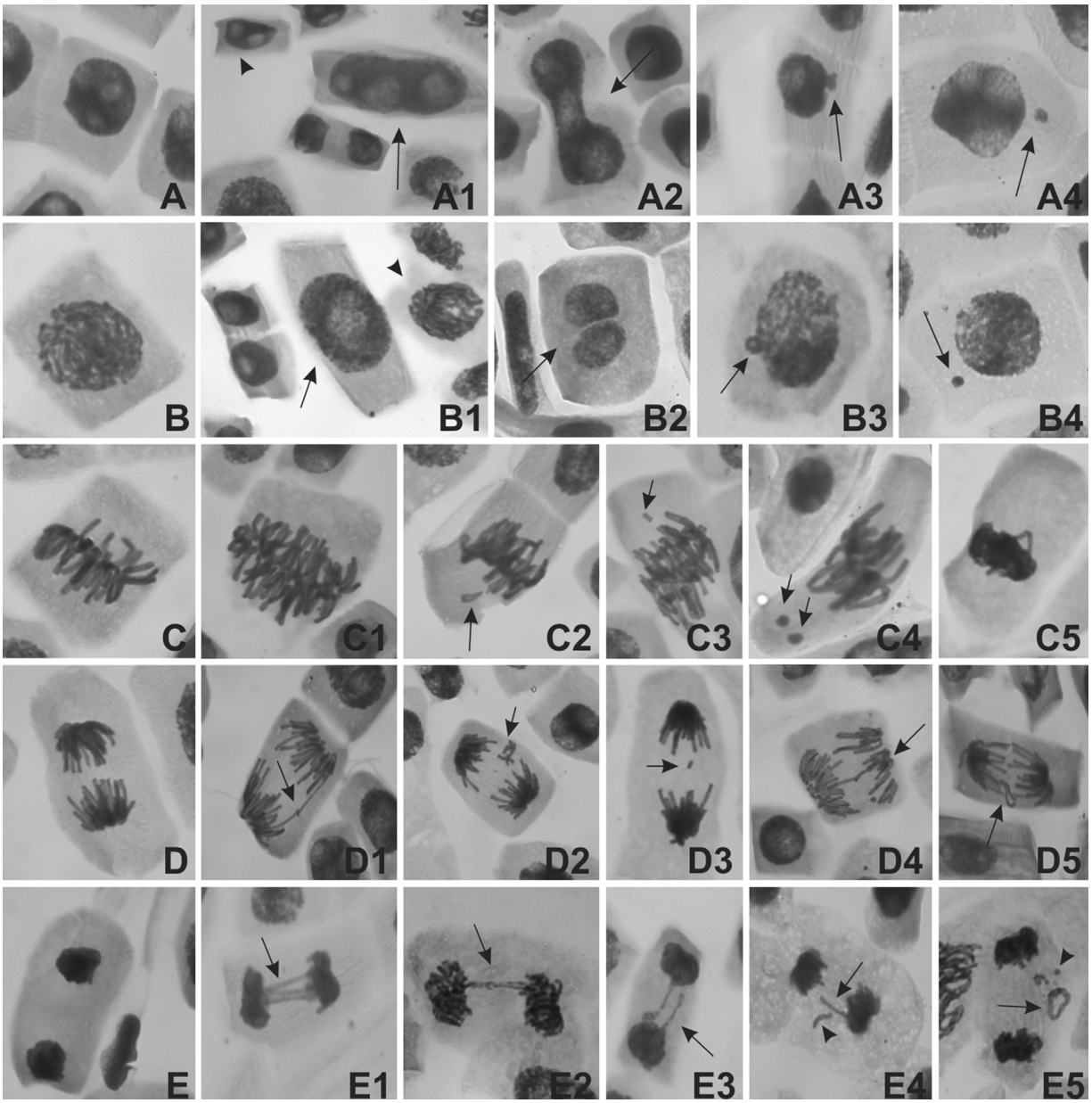

Fig. 2. *Meristematic cells of* A. cepa. (A) Normal interphase; (A1) Polyploid interphase (arrow), with the arrowhead indicating a cell of normal size. (A2) Nuclear bridge between two interphasic nuclei (arrow). (A3) Nuclear bud (arrow). (A4) Micronucleus (arrow). (B) Normal prophase. (B1) Polyploid prophase (arrow), with the arrowhead indicating a cell of normal size. (B2) Binucleated cell (arrow). (B3) Prophase with a nuclear bud (arrow). (B4) Prophase with a micronucleus (arrow). (C) Normal metaphase. (C1) Polyploid metaphase (arrow). (C2 and C3) Metaphase with chromosomal breakage (arrow). (C4) Metaphase with micronuclei (arrows). (C5) Metaphase with adherence. (D) Normal anaphase. (D1) Anaphase with a chromosomal bridge (arrow). (D2) Anaphase with chromosomal losses (arrow). (D3) Anaphase with chromosomal breakage (arrow). (D4) Anaphase with multipolarity (arrow). (D5) Anaphase with chromosomal loss (arrow). (E) Normal telophase. (E1) Telophase with chromosomal bridges (arrow). (E2) Telophase with a chromosomal bridge (arrow). (E3) Telophase with chromosomal delays (arrow). (E4) Telophase with chromosomal delay (arrow) and breakage (arrowhead). (E5) Telophase with chromosomal loss (arrow) and breakage (arrowhead).

digestion and thermal drying. In the present study, no inhibition of seed germination was observed for the other tested samples.

Evaluation of the cytotoxicity in seed germinated directly in SS after a period of 6 months indicated that at the 100% SS, mitotic cell division of *A. cepa* was inhibited (Table 5). However, after 12 months of natural attenuation, this effect was no longer observed. In the bioassays performed with aqueous extracts, no difference was observed in the mitotic index for the SS attenuated for 6 months (Table 6). These results indicate that the substances responsible for the observed cytotoxic effects are not transported by water because they were not present in the aqueous extract, and they may therefore have adhered to the particles of the SS itself and in this case, released during the decomposition of sludge. However, after 12 months of natural attenuation, these compounds were no longer present in the SS. These data show that the toxicity of the SS decreased during the period of natural attenuation of the SS samples, which confirms the efficiency of this process for the studied residue.

Concerning genotoxic effects, a significant frequency of cellular alterations was observed in association with SS concentrations of 10, 25 and 50% after periods of 0 and 2 months and for concentrations of 25, 50 and 100% of both raw SS and aqueous SS extracts after a period of 6 months (Fig. 2). However, the aqueous extracts induced a more severe genotoxic effect on the cells, as even after a period of 12 months, the 100% SS still induced significant genotoxic effects, indicating that substances with genotoxic potential

are bioavailable in SS even after 12 months of natural attenuation. Our results regarding the genotoxic potential of the SS indicated that 100% SS cannot be considered a candidate material for use as a soil reconditioner due to its persistent toxicity observed in this study. Thus, based on the obtained results, soil/SS mixtures are more suitable for use in agriculture.

Regarding the mutagenic effect observed in meristematic cells of *A. cepa*, significant results were recorded for concentrations 25% and 50% SS after a period of 2 months and for 100% SS after a period of 6 months in both types of tests (raw SS and aqueous SS extract). For the aqueous extract, 100% SS after a period of 2 months also induced a significant frequency of micronuclei (Fig. 2A4, B4 and C4). The significant results recorded for F1 cells indicated that the damage observed in meristematic cells was transferred to and fixed in cells of the F1 region. Thus, even at lower concentrations, the SS samples were capable of inducing damage to the genetic material of the test organism. These data support the findings for other toxicity parameters evaluated here, as 100% SS was difficult to detoxify compared with the soil/SS associations. Based on all of the results described above, it appears that a period of 12 months constitutes an excellent amount of time for the degradation of many mutagenic substances present in the studied SS.

Induction of genotoxic damage in *A. cepa* resulting from exposure to SS has also been reported by other authors, such as Srivastava et al. (2005), who observed a decrease in the mitotic index and a significant frequency of chromosomal aberrations induced by exposure to an aqueous extract of SS collected in a town in India. Aqueous extracts of SS from five WWTPs in the metropolitan area of São Paulo were also shown to induce genetic damage in *A. cepa* (Caritá, 2007). Furthermore, Christofoletti et al. (2012) observed a significant genotoxic effect in seeds of *A. cepa* exposed to raw biosolids from a municipality of São Paulo State (Brazil).

The genotoxic and mutagenic effects observed in the present study are at least partly related to the presence of toxic organic substances, such as cresol, as well as the synergistic effects of all of the substances present in the SS. According to ATSDR (2008), positive results obtained from *in vitro* tests performed in humans and animals suggest that cresols can react with DNA, thereby inducing genotoxicity. These compounds are also reported to be possible human carcinogens. Although the results of our chemical analyses indicate an absence of substances exceeding the limits established by legislation, significant genotoxic and mutagenic effects were observed for the species *A. cepa* after 6 and 12 months of attenuation, suggesting the presence of other substances that were not chemically identified in the SS because measurement of these substances is not required by legislation regarding the use of SS in agricultural environments. Another hypothesis related to the observed effects concerns the synergism of contaminants, which may be responsible for these results. The presence of dioxins and furans does not appear to be harmful to *A. cepa* because the concentrations of these substances in the samples increased over the examined periods of natural attenuation, while the detected cellular effects decreased.

However, because SS is a complex matrix, it is difficult to predict which substances are responsible for this effect, which reinforces the need to perform biological assays concomitantly with chemical analyses to better understand the potential risks of SS (Oleszczuk, 2008).

Although significant cytotoxic, genotoxic and mutagenic effects were observed in the present study, all of the assessed parameters indicated a reduction of the effects induced by the SS over the evaluated period of natural attenuation, particularly for the soil/SS associations. According to Wong et al. (1983), processes resulting in the stabilization of SS may decrease the toxicity of this waste. However, although the process of natural attenuation was effective in reducing the harmful effects of the soil/SS associations, the 100% SS did not appear suitable for agricultural applications even after 12 months of attenuation.

The results obtained in the assays performed with the raw SS and the aqueous SS extracts were very similar, which indicates that contaminants present in the SS responsible for the effects observed in *A. cepa* are bioavailable in the samples. An important point for consideration in the application of SS is that the presence of toxic substances in an aqueous extract indicates that these compounds can be transported by water and may thus be able to reach and contaminate deeper soil layers as well as groundwater and adjacent water resources. Although most studies report the presence of heavy metals in SS lixiviates (Mcbride et al., 1997; Keller et al., 2002; Bhogal et al., 2003), we emphasize that organic substances, such as cresol, may also be transported by water and should be considered major SS pollutants.

4. Conclusion

The chemical analyses revealed the presence of harmful compounds such as *m*- and *p*-cresol at high concentrations in the initial samples of SS (0 and 2 months). The results obtained in the *A. cepa* test demonstrated that the studied SS was potentially genotoxic and mutagenic to cells, even when present at low concentrations in association with soil. Thus, the application of SS in agricultural soils should not be implemented prior to evaluate its toxic potential because it may endanger exposed organisms and constitute a potential pollutant of both terrestrial and aquatic environments.

However, based on the reduction of the effects of the SS observed in the test organism *A. cepa* and on the absence of the toxic compounds observed in the chemical analysis, it can be concluded that monitored natural attenuation appears to be a very efficient and cost-effective method for the removal of toxic organic substances present in SS, such as *m*- and *p*-cresol. A period of 12 months of natural attenuation proved to be sufficient to decrease the toxicity of the soil/SS associations, proving to be a sustainable method to transform this waste in agricultural soils amendment. However, for the disposal of 100% SS (raw sludge), we recommend that the material undergo an attenuation period of more than 12 months to allow total elimination of its toxic effects.

Regarding the microbiological characterization of the SS, an alternation in microbial community was observed among enterobacteria and Gram-positive cocci during the initial periods and non-fermenter bacteria during the final period (12 months), serving as indicators of the attenuation process. The bacteria *Bacillus* sp., *E. cloacae*, *P. rettgeri*, *M. morganii*, *P. mirabilis* and a non-fermenter Gram-negative bacilli seem to be the main microorganisms involved in the degradation of toxic compounds present in the SS, such as *m*- and *p*-cresol and bis(2-ethylhexyl)phthalate, but they were not capable of degrading dioxins and furans.

The species *A. cepa* was shown to be an efficient test organism for detecting toxic effects induced by SS contaminants. The chromosomal aberrations and micronuclei assays performed in this organism were demonstrated to be very sensitive tools for detecting cellular damage induced by this type of residue.

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necessary to characterize the SS samples and prepare solubilized SS.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ecolind.2015.03.026.

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