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Ecotoxicological and microbiological assessment of sewage sludge associated with sugarcane bagasse

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ABSTRACT

Sewage sludge (SS) obtained after sewage treatment process may contain several toxic substances. Bioremediation can decrease the toxicity of the sludge, mainly when it is associated with stimulant agents, such as sugarcane bagasse (B). Samples of pure SS (SSP); SS + B; SS + Soil; and SS + B + Soil were bioremediated for 1, 3, and 6 months (T1, T2, and T3, respectively). After each period, the cytotoxic, genotoxic, and mutagenic potentials of the solid samples and their respective aqueous extracts (aqueous eluate and percolate water) were evaluated by the *Allium cepa* test. A microbiological analysis of the samples was also performed after each period tested. All solid samples of SS + B (in T1, T2, and T3) and the solid sample of SSP (treatment T3) showed a significant decrease of cell division (cytotoxic effects). The aqueous eluate extracts of SS + B (T1 and T3) and SSP (T2 and T3) induced cytotoxic effect. The solid sample of SS + B (T2 and T3) and aqueous extracts of SSP (T1) were genotoxic, indicating a harmful effect of SS on *A. cepa*, even after 6 months of bioremediation. There was an alternation in the microbial community both in diversity and in abundance, with the predominance of non-fermenting gram-negative bacilli. The tested bioremediation periods were not sufficient for the complete detoxification of SS, and the use of B did not seem to contribute to the degradation of the pollutants to inert compounds. These data emphasize that a specific relationship should exist between the sludge characteristic and the biostimulating agent used to promote a more efficient bioremediation. These results suggest the necessity to study longer periods of biodegradation and the use of other decomposing agents for greater safety and sustainability for the agricultural use of this residue.

1. Introduction

Large amounts of sewage sludge (SS) are generated worldwide, creating an environmental contamination problem. The studies of Kelessidis and Stasinakis (2012) show that in European countries, such as Germany, Spain, France, and Italy, SS production is respectively 2.17; 1.121; 1.059, and 1.053 million tons of dry solids per year respectively. The studies of Pedroza et al. (2010) indicate the production of this residue in Brazil reaches around 150–220 thousand tons of dry matter/year. Wastes from wastewater treatment plants (WWTP) can be a global problem since the amount of waste generated is directly related to population density and water quality (Kliopova and Makarskienė, 2015) and, consequently, the socio-economic level of the country.

Some authors have noted that the composition of SS is related to the origin of the treated sewage, the type of treatment used by the WWTP, and the season of the year in which it was produced, so that this residue can present quite diverse characteristics (Bettiol and Camargo, 2006; Fytili and Zabaniotou, 2008; Silva et al., 2003; Singh and Agrawal, 2008). Rodríguez-Morgado et al. (2015) also highlighted that the use of different types of SS in the soil can generate distinct enzymatic activities due to the variation in the microbiological community present in each SS.

Due to the SS environmental risks, its growing world production and its rich composition of organic matter and nutrients (e.g., nitrogen, phosphorus, potassium, calcium, copper, cadmium, zinc, and manganese), some researchers have suggested the use of SS as an agricultural input, fertilizer or for reconditioning arable soil (Bovi et al., 2007; Singh and Agrawal, 2008; Mcgeehan, 2012; Gianico et al., 2013).

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Nevertheless, SS can also contain harmful substances for living organisms and the environment, such as copper, nickel, lead, cadmium, and mercury, in addition to organic compounds, such as phenols, benzenes, anthracenes, linear sulfonated alkylbenzenes, and pathogenic microorganisms (Almeida et al., 1998; Holmstrup et al., 2001; Lopes et al., 2005; Lourenço, 1997; Paraiba and Saito, 2005).

Bioremediation is a widely used approach to reduce the toxicity of organic waste by the use of living organisms that are able to metabolize, transform or decompose pollutants so that toxic substances are converted into less harmful products (Bamfoth and Sigleton, 2005; Makadia et al., 2011). An employed strategy to assist this technology is biostimulation, which involves the addition of stimulating agents to increase the native microbiota growth. The porosity and soil aeration can be improved by physical means or by the addition of decompacting materials, which allow the entrance of air into the system and the consequent aerobic conditions in the process (Vasudevan and Rajaram, 2001), favoring the biostimulation process. Sugarcane bagasse is a good soil decompacting agent because it improves the porosity of the substrate, in addition to being a good carbon source (due to its high content of carbohydrates) (Pandey et al., 2000).

Biological tests are essential to assess the success of the bioremediation process since they can estimate the possibility of the compounds present in the samples to interact with the genetic material of living organisms (Mazzeo et al., 2014), unlike the chemical analyses, which only measure the concentrations of the chemicals present in the environment. In this way, bioassays are essential for the bioavailability assessment of substances present in environmental samples (Moreira et al., 2008) and also for enabling the identification of additive, synergistic, and antagonistic effects of chemical compounds present in complex environmental samples (Pandard et al., 2006).

Plants are excellent genotoxicity bioindicators of soils, as this substrate is the growth medium for the great majority of them (White and Claxton, 2004). Among plants, *Allium cepa* has been considered an efficient test-organism for studies on the basic mechanisms of action of environmental contaminants (Fiskejö, 1985; Bushra Ateeq et al., 2002; Fernandes et al., 2009, 2007), as well as evaluation of the bioremediation process efficacy (Maila and Cloete, 2005; Mazzeo et al., 2015, 2010)

Bioremediation has potential to reduce the toxicity and improve the agronomical quality potential of SS, enabling its application as an agricultural soil conditioner. However, because of the specificity of the detoxification treatments for each SS type, it is now necessary to perform thorough evaluations of the steps involved in bioremediation. Thus, all obtained information in different assays developed with SS, regarding the applied techniques and the type of SS that was used, are important and must be disseminated because they can add relevant data to a better choice of technology to be adopted on the detoxification of different types of sludge produced by WWTPs.

Findings from such studies would likely contribute to the minimization of environmental impacts, which is badly needed. One strategy for the reduction of improper disposal of human waste is the identification of alternative that enable secure and sustainable implementation of SS into agricultural soils. Thus, this study aimed to evaluate the mutagenic, genotoxic, and cytotoxic effects of SS on the test-organism *A. cepa*, as well as estimate the possibility of detoxification of this waste by means of the biostimulation with soil and sugarcane bagasse.

2. Materials and methods

2.1. Material and preparation of the samples

The centrifuged anaerobic SS was collected from the WWTP of Jardim das Flores in the city of Rio Claro, São Paulo, Brazil (latitude 22°24'39"S and longitude 47°33'39"W), in 2011. This WWTP is installed in an area of 40,000 m^2 and only receives domestic effluent, generating 113.53 t of anaerobic SS per month.

For the preparation of the samples, clay soil was used, collected in the Experimental Garden of the University of São Paulo State - UNESP (Campus Rio Claro, São Paulo, Brazil). This soil was previously characterized by Mazzeo et al. (2015) and Christofoletti et al. (2013), regarding the presence of organic matter (ca. 20 g/Kg), electric conductivity (ca. 145 μS/cm), low concentrations of heavy metals, and absence of toxicity, being considered as a reference soil and, thus, adequate for this study. As a decompacting agent, dry and coarsely crushed sugarcane bagasse was used.

In a previous study, Mazzeo et al. (2015) evaluated the bioremediation process of SS mixed with soil in a ratio of 1: 1. Thus, in order to optimize the use of SS, an increase in the proportion of this residue in the tested mixtures was proposed. Thus, the mixtures were prepared in the following proportions: SS pure (SSP); SS mixed with sugarcane bagasse (SS + B; 3:1 [v/v]); SS mixed with soil (SS + S; 3:1 [v/v]); and SS mixed with sugarcane bagasse and soil (SS + B + S; 3:1:1 [v/v/v]).

2.2. Organization of the test

The mixtures were prepared in stainless steel vats (considered as an inert material), with dimensions of 24 cm wide, 20 cm high, and 30 cm long. These vats were prepared to be a suitable environment to estimate the effects of soil bioremediation as well as the potential for the toxic material of the mixture to percolate water during the process of bioremediation. Thus, the vats were assembled with a lower layer (bottom layer) of 2.7 kg of glass beads, to represent the porous layer of the soil and, only subsequently, the mixture under analysis was added. For extraction of the percolate water liquid samples, a hole was made in the bottom of the vats, to which a silicone hose was coupled. During the entire period of bioremediation, the vats were maintained in a covered place and under ambient temperature in the Experimental garden of São Paulo State University, Rio Claro, SP. Weekly, about 200 mL of water was sprayed in each of the vats, to maintain the humidity of the material in order to encourage the growth and maintenance of microorganisms. For each mixture studied, two vats were assembled (duplicate experiments). The periods chosen for evaluation of bioremediation were of 1 (T1), 3 (T2), and 6 months (T3) after the beginning of the experiment.

2.3. Acquisition of aqueous extracts

2.3.1. Aqueous eluate

The acquisition of the aqueous eluate was based on a Brazilian standard (ABNT NBR 10.006, 2004), adding (separately) 62.5 g of each sample (regarding its dry weight) in 250 mL of ultrapure water, followed by constant agitation for 5 min. Unlike the other samples, to obtain the SSP and SS + B aqueous extracts, we added 500 mL and 400 mL of ultrapure water, respectively. After 7 days of decantation at 22 °C, the liquid phase of each sample was collected and filtered through a 0.45-μm membrane.

The dry weight of each sample was obtained by weighing approximately 10 g of each sample in individual treated containers. The entire experiment was performed in triplicate. The drying was carried out at 105 °C for 24 h. Subsequently, a new weighing was performed, and the average of each triplicate was considered as equivalent to the dry weight of each sample.

2.3.2. The percolate water

The leachate was obtained by the collection of the liquid that flowed from each of the vats, due to the natural loss of water from the SS. The collection was carried out as described previously, through a hole in the bottom of the vats.

Table 1
Mitotic index of meristematic cells of *A. cepa* submitted to germination in different concentrations of SS, by three different periods.

Samples	Solid sample			Aqueous eluate			Percolate water	
	T1	T2	T3	T1	T2	T3	T1	T2
NC[a]	27.30 ± 9.48	18.08 ± 5.05	23.44 ± 4.84	18.46 ± 3.06	19.22 ± 3.81	18.59 ± 2.76	18.38 ± 2.49	21.30 ± 2.62
PC[b]	28.71 ± 5.56	18.25 ± 7.72	23.23 ± 6.39	14.26 ± 3.93	18.49 ± 2.38	21.42 ± 1.87	21.93 ± 3.56	27.28 ± 8.02
Soil	26.00 ± 5.84	18.04 ± 6.44	19.82 ± 5.09	16.70 ± 1.97	16.92 ± 1.65	17.19 ± 1.92	–	–
SSP	26.04 ± 7.19	14.70 ± 6.08	8.02 ± 4.20*	14.94 ± 2.48	8.88 ± 3.42*	13.45 ± 1.90*	13.90 ± 5.18	–
SS + B	15.46 ± 4.60*	7.83 ± 4.80*	12.71 ± 6.55*	13.01 ± 3.79*	15.54 ± 3.08	13.05 ± 3.09*	14.34 ± 3.04	18.11 ± 2.76
SS + S	19.60 ± 3.90	10.95 ± 5.25	18.19 ± 5.21	14.73 ± 2.40	17.55 ± 2.89	15.79 ± 2.99	–	15.71 ± 4.34
SS + B + S	16.33 ± 3.82	14.00 ± 3.15	18.33 ± 4.70	17.82 ± 2.96	18.69 ± 4.08	14.11 ± 1.65	22.35 ± 4.03	22.54 ± 5.25

SSP: SS pure; SS + B: SS mixed with sugarcane bagasse; SS + S: SS mixed with soil; SS + B + S: SS mixed with sugarcane bagasse and soil.
– Percolate water liquid was not obtained for this sample.
[a] Negative Control.
[b] Positive Control.
* Significantly different from the negative control ($p < 0.05$) according to the Kruskall Wallis test.

2.4. Identification and quantification of microorganisms

For the identification of microorganisms, 1 g of each sample (SSP; SS + B; SS + S and SS + B + S) were individually added to 100 mL of sterile saline solution after each period studied. For the assessment of the diversity of microorganisms in the samples, 10 µl of this solution was sown by the spread plate technique onto Petri dishes containing Blood agar, MacConkey agar, Sabouraud agar, and Cromagar UriSELECT 4 (Bio-Rad Laboratories, Marnes-La-Coquete, France). The plates were incubated at 35 °C, except the Sabouraud agar plates, which were kept at room temperature for 7 days. After this period, the plates were analyzed for the type and quantity of colonies found. The microorganisms were then isolated and initially identified by conventional biochemical tests, subsequently being identified using automation equipment (Vitek®2 Compact, BioMérieux®, Inc, St Louis, MO, USA). The quantification of microorganisms present in each sample was made by counting CFU (Colony Forming Units).

2.5. Bioassay with seeds of A. cepa

The germination of the seeds of *A. cepa* (2n = 16 chromosomes), variety "Baia Periforme", was performed in Petri dishes containing: a) Solid samples: SSP; SS + B; SS + S and SS + B + S; b) aqueous extracts obtained by digestion of the samples of SSP; SS + B; SS + S and SS + B + S; c) aqueous extracts obtained by percolation of liquid samples of SSP; SS + B; SS + S and SS + B + S, obtained for all three periods of bioremediation (T1, T2, and T3).

The control treatments were performed with ultrapure water (negative control), with the control soil and its aqueous eluate extraction (environmental controls) and in 10 mg/L methyl methanesulfonate (MMS, Sigma-Aldrich, CAS 66-27-3, as a positive control).

After germination, roots were collected and fixed in Carnoy (alcohol and acetic acid: 3;1 [v/v]) with approximately 2.0 cm in length for 6–8 h at ambient temperature. After this, the fixative was replaced, and the roots were stored at 4 °C. The protocol described by Leme et al. (2008) was followed for the confection of the slides of the meristematic region of roots.

For the analyses, the mitotic index (ratio between the number of cell divisions and the total number of cells) characterized the *endpoint* of cytotoxicity. For the *endpoint* of genotoxicity all of the chromosomal aberrations (loss, bridge, delay, chromosomal adhesion, polyploid metaphase and C-metaphases) and nuclear abnormalities that were found (bud, lobed nucleus and polyploid nucleus) were considered. The *endpoint* of mutagenicity was deemed as chromosomal breaks and the presence of MN. The analysis of all parameters was performed by counting of about 5000 cells per treatment; 10 slides per sample (approximately 500 cells each). The statistical analyses were made using the Kruskal Wallis test, the 0.05 significance level, using software BioEstat 5.0.

3. Results and discussion

3.1. Allium cepa

Bioassays with higher plants are important for monitoring the effects of SS when disposed of in soils since these organisms are direct targets of possible contaminants present in this material. The determination of the genotoxic potential of SS, through simple and low-cost testing, provides relevant information about the quality of the residue (Rank and Nielsen, 1998) and helps to estimate its potential for use in agricultural soils. Chemical analyses of complex mixtures are expensive and do not provide information on (eco-)toxicological effects or on the bioavailability of the contaminants (Martins et al., 2016; Semple et al., 2003). Because SS is a complex mixture of different chemical groups, bioassays are considered low-cost and efficient additions for the evaluation of the toxic potential of this residue, and complimentary to chemical analysis.

The results obtained in this study, regarding the cytotoxicity parameter, showed a decrease in cell division in all solid samples tested, over the periods studied, being statistically significant only for the SSP (at T3) and SS + B (at all times tested) samples (Table 1). The aqueous eluate of all samples presented mitotic index values lower than the negative control samples, but this was statistically significant only for the SS + B (at T1 and T3) and SSP (at T2 and T3) samples (Table 1).

The absence of cytotoxicity in the initial sample of SSP and a significant induction of this effect after 3 and 6 months of biostimulation indicated a possible bioavailability of cytotoxic substances. Previously inert organic substances might have been partially degraded by the microorganisms to more toxic metabolites than the substances initially present. An increase in the toxicity of SS after composting was also observed by Oleszczuk (2008), possibly related to the formation of toxic metabolites derived from organic compounds or by the increase in the concentration of heavy metals as a consequence of the decrease of the total mass of the SS. Phillips et al. (2000) monitored the bioremediation of three soils contaminated with creosotes and found that, despite a decrease of petroleum hydrocarbons in the soil, there was an increase in its toxicity, indicating that toxic intermediary metabolites may have formed during the process of biodegradation. According to Harrison et al. (2006), the degradation of organic compounds can result in products with either higher or lower toxicity than those initially present in the samples. In the present study, the process of bioremediation used for the SSP sample led to an increase in its cytotoxicity, indicating the need for monitoring of the bioremediation over time, since no toxic effect was observed in the initial sample. Thus, the direct application of raw sludge to soil can result in damage to the environment, even when this has already passed through a 6-months process of stabilization.

Table 2
Genotoxicity observed for meristematic cells of *A. cepa* submitted to germination at SS and their associations.

Samples	Solid sample			Aqueous eluate			Percolate water	
	T1	T2	T3	T1	T2	T3	T1	T2
NC[a]	0.10 ± 0.31	0.18 ± 0.38	0.54 ± 0.65	0.19 ± 0.39	0.48 ± 0.51	0.19 ± 0.40	0.09 ± 0.28	0.29 ± 0.47
PC[b]	4.64 ± 6.47*	4.74 ± 3.53*	5.45 ± 4.08*	2.25 ± 1.08*	3.35 ± 1.75*	3.54 ± 1.92*	3.12 ± 1.18*	2.45 ± 1.31*
Soil	0.09 ± 0.28	0.67 ± 0.46	0.94 ± 0.77	0.58 ± 0.68	0.86 ± 0.84	0.59 ± 0.51	–	–
SSP	0.66 ± 0.65	1.38 ± 1.06	1.59 ± 1.18	1.74 ± 1.29*	1.85 ± 1.51	0.50 ± 0.70	1.95 ± 0.91*	–
SS + B	0.49 ± 0.83	2.53 ± 2.48*	3.02 ± 1.91*	0.39 ± 0.67	0.68 ± 1.02	0.76 ± 0.87	0.89 ± 0.86	0.20 ± 0.42
SS + S	0.48 ± 0.69	0.48 ± 0.69	1.22 ± 1.33	0.20 ± 0.42	0.97 ± 0.63	0.49 ± 0.52	–	0.38 ± 0.50
SS + B + S	0.48 ± 0.68	1.07 ± 0.85	1.16 ± 0.62	0.58 ± 0.69	0.98 ± 0.65	0.58 ± 0.69	1.09 ± 1.59	0.20 ± 0.42

SSP: SS pure; SS + B: SS mixed with sugarcane bagasse; SS + S: SS mixed with soil; SS + B + S: SS mixed with sugarcane bagasse and soil.
– Percolate water liquid was not obtained for this sample.
[a] Negative Control.
[b] Positive Control.
* Significantly different from the negative control ($p < 0.05$) according to the Kruskall Wallis test.

Regarding the percolate water of SS and their mixtures, no statistically significant findings were detected (Table 1).

For the genotoxicity parameter for solid samples, statistically significant results were observed for the SS + B sample (at T2 and T3) (Table 2 and Fig. 1). Although the authors claim that the mixture of some vegetable agents, such as sugarcane bagasse, is important for the improvement of the bioremediation process (Infante et al., 2010; Molina-Barahona et al., 2004), in this study, we observed that the sugarcane bagasse seems not to assist the process of detoxification of the SS; a significant inhibition of cell division in the SS + B sample was observed at different periods of the study, as well as an increase in genotoxicity for two periods tested. According to Molina-Barahona et al. (2004), the plant residues improve bioremediation because they act as agents of volume favoring the oxygenation of the system in addition to being able to provide a bacterial biomass, which is important for the process of degradation of the pollutants. Additionally, the bagasse can continue to serve as a carbon source for microorganisms (Pandey et al., 2000). However, from the obtained results in this study it is possible to suggest that the use of sugarcane bagasse contributed to the generation of toxic metabolites, which were not efficiently eliminated during 6 months of bioremediation.

The addition of soil to SS seems to be important for the decrease of its cytotoxic and genotoxic effects since in these mixtures (SS + S and SS + B + S) there was no statistically significant effect on the meristematic cells of *A. cepa*. When SS is mixed with soil, a dilution of the chemical compounds present in SS occurs (Harrison et al., 2006), resulting in a less toxic mixture. Similar results were obtained by Mazzeo et al. (2015) when monitored the natural attenuation of an SS mixed with different proportions of soil. The authors observed that, in the majority of mixtures with soil, there was a decrease of toxicity for the test organism (*A. cepa*) when compared to the pure sludge.

With regard the genotoxicity of the aqueous extracts, for both aqueous eluate and percolate water samples, the results were statistically significant only for SSP (at T1) compared to the negative control (Table 2). Thus, the study of the different fractions of SS (solid part and the aqueous) allowed a complete observation of the chemical

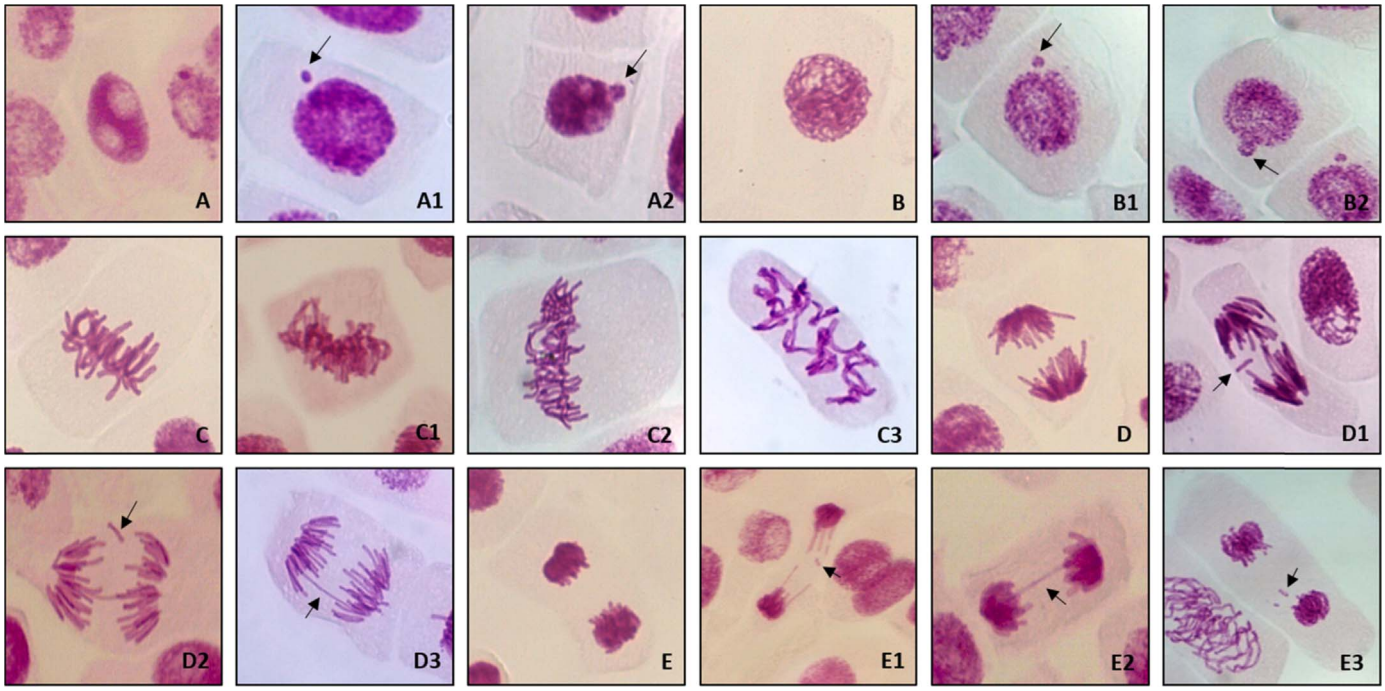

Fig. 1. Meristematic cells of *A. cepa*. (A) Normal interphase; (A1) Micronucleus (arrow), (A2) Nuclear bud (arrow), (B) Normal prophase, (B1)Prophase with micronucleus (arrow), (B2) Prophase with a nuclear bud (arrow), (C) Normal metaphase, (C1) Metaphase with chromosome adherence, (C2) Polyploid metaphase, (C3) C-Metaphase, (D) Normal anaphase, (D1 and D2) Anaphase with chromosomal loss (arrow), (D3) Anaphase with a bridge chromosomal, (E) Normal telophase, (E1) Telophase with chromosomal breakage (arrow), (E2) Telophase with chromosomal bridge, (E3) Telophase with chromosomal breakage (arrow).

characteristics of toxic substances present in this residue. According to Fernandez et al. (2009), the partition of samples of SS associated with the use of bioassays enables an initial screening of toxic compounds, which must be done before its agricultural use. The tests performed with the raw material contribute to a closer assessment than would occur when the sludge is prepared "*in natura*" in the environment. Another important assessment to be performed is the characterization of the toxicity of the aqueous extracts of the samples, to estimate the possibility of water-soluble substances present in SS being dispersed to the different physical media (i.e., to the soil and water). The results shown here demonstrated that, for the mitotic index, similar effects were induced by the aqueous extracts and solid samples, indicating that the substances with cytotoxic potentials were present in both fractions of SS. For genotoxicity, the SSP aqueous extracts had significant genotoxicity at T1, whereas the solid SSP sample was not genotoxic. This is because, in the initial period, substances with genotoxic effect were hydrosoluble, while in all other periods tested (T2 and T3), the substances soluble in water were no longer present in the SS. Some authors warn about the possibility of soils being contaminated by chemicals leaching from the SS, considering that there may be contamination of surface water and groundwater by percolation of exudates of SS, resulting from the action of rainwater (Keller et al., 2002). Therefore, analyses of aqueous extracts are important when considering the disposal of SS in soils. The association of these two analyses (solid and aqueous) allows a better understanding of the dispersion of contaminants in the environment, as demonstrated in this study.

For mutagenicity (Table 3), the results were not statistically significant for any of the tested samples, indicating that the studied sludge, pure or in association with sugarcane bagasse, soil, or both, was unable to induce chromosome breakage and micronuclei in the organism *A. cepa.*

3.2. Microbiological analysis

Bioremediation depends on the metabolic capacity of microorganisms in transforming the pollutants into less toxic or harmless compounds. Contaminated environments can be a source of inoculum for the bioremediation processes since the use of microorganisms adapted to the target pollutant favors the effectiveness and speed of the process (El Fantroussi and Agathos, 2005). Thus, by the proper characteristic of SS, the stimulation of the naturally present microorganisms in this material can assist in the processes of decontamination of this residue and contribute to its use in agriculture. SS has been used by other authors as a source of microorganisms for the degradation of various contaminants, such as contaminants in wastewater (e.g., pyrene, benzoate, and even natural estrogens) (Gupta and Thakur, 2015, 2016; Lee and Liu, 2002; Ma et al., 2013; Mountfort and Bryant, 1982).

The results of the microbiological characterization of the samples are presented in Table 4. An alternation in microbial composition was observed for samples during the periods of bioremediation tested, involving 16 genera of bacteria and 6 genera of fungi. Among the bacteria, gram-positive bacilli, enterobacteria, and non-fermenting gram-negative bacilli were detected, the latter being predominant at all evaluated periods, which indicates a preference of degradation by oxidative pathways. The predominance of bacteria in samples reflects the high tolerance of this microorganism to pollutants. According to Harms et al. (2011), the bacteria tolerate a greater variety of habitats, by the wide range of specific biochemical reactions that they promote. These organisms use the pollutants as substrates for growth, degrading contaminants and using them as an organic source, being effective in this process by presenting rapid growth. In addition, a great part of the pollutants is degraded, preferably by aerobic processes (Riser-Roberts, 1998).

The addition of sugarcane bagasse or soil into the sludge has contributed to an increase of the bacterial diversity in the initial phase, as can be observed for samples SS + B and SS + S. Wu et al. (2008), studying the bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs), observed that the sample that was biostimulated with a plant residue (ground corn cob) showed an increase in the number and abundance of microorganisms, promoting a change in the soil microbial community. However, in the present study, when these agents were combined (SS + B + S), at T1, there was a decrease of the bacterial diversity compared to the other samples (SSP, SS + B, SS + S). A decrease of this diversity was also observed during the tested periods of biostimulation (T2 and T3), with the exception of the SS + B + S sample. These results can be explained by interactions (competition and/or cooperation) between bacteria in their different growth substrates. According to Hibbing et al. (2010), the environment can favor the competition for space and resources, selecting the most adapted species, which can lead to a decrease in bacteria diversity.

The only species of bacteria present in all periods of the same sample was the *Alcaligenes faecalis* for the sample SS + B and *Pseudomonas* sp for the sample SS + B + S. The genus *Bacillus* was present in all samples tested at period T1, while the genus *Pseudomonas* was also present at period T2 of all samples tested. *Ochrobactrum anthropi* was identified in all assessed samples of periods T2 and T3. The family Bacillaceae was observed as the predominant bacteria in the initial sample of the soil in the study by Wu et al. (2008), which was expected as these species are widely spread in the soil. Thus, the genus *Bacillus* seems to be important for the beginning of the process of degradation, due to its resistance to the initial conditions of the SS. However, this genus was not the most abundant in the quantification of the microorganism of samples analyzed here. Other microorganisms also found in the studied samples in this study show characteristics that are already known to contribute to the degradation of some specific pollutants, for example, the genus *Pseudomonas*, which is widely

Table 3
Mutagenicity observed for meristematic cells of *A. cepa* submitted to germination in different concentrations of SS.

Samples	Solid sample			Aqueous eluate			Percolate water	
	T1	T2	T3	T1	T2	T3	T1	T2
NC[a]	0.09 ± 0.29	0.46 ± 0.65	0.10 ± 0.30	0.19 ± 0.40	0.38 ± 0.49	0.28 ± 0.45	0.28 ± 0.45	0.29 ± 0.47
PC[b]	16.61 ± 7.41*	14.34 ± 12.15*	21.85 ± 12.60*	6.34 ± 2.28*	13.57 ± 6.59*	24.32 ± 3.67*	8.31 ± 2.72*	10.53 ± 4.31*
Soil	0.27 ± 0.43	0.49 ± 0.50	0.19 ± 0.41	0.29 ± 0.47	0.58 ± 0.50	0.39 ± 0.50	–	–
SSP	0.82 ± 0.76	0.49 ± 0.70	0.10 ± 0.31	0.97 ± 1.03	1.47 ± 1.34	0.19 ± 0.41	0.39 ± 0.51	–
SS + B	0.99 ± 1.05	0.71 ± 0.52	0.19 ± 0.39	0.48 ± 1.21	0.58 ± 0.68	0.29 ± 0.46	0.79 ± 1.03	1.28 ± 0.83
SS + S	0.58 ± 0.96	0.28 ± 0.63	0.10 ± 0.31	0.30 ± 0.48	0.49 ± 0.52	0.29 ± 0.66	–	0.29 ± 0.65
SS + B + S	0.74 ± 0.85	0.48 ± 0.50	0.29 ± 0.66	0.39 ± 0.82	1.67 ± 1.33	0.19 ± 0.39	0.10 ± 0.31	0.58 ± 0.81

SSP: SS pure; SS + B: SS mixed with sugarcane bagasse; SS + S: SS mixed with soil; SS + B + S: SS mixed with sugarcane bagasse and soil.
– Percolate water liquid was not obtained for this sample.
[a] Negative Control.
[b] Positive Control.
* Significantly different from the negative control ($p < 0.05$) according to the Kruskall Wallis test.

Table 4
Identification and counting of microorganisms present in samples of SS and their associations.

	Microorganisms	SSP			SS + B			SS + S			SS + B + S		
		T1	T2	T3	T1	T2	T3	T1	T2	T3	T1	T2	T3
	Bacteria												
NF- GNB	*Achromobacter denitrificans*	–	2.5×10^6	2.6×10^4	–	–	2.0×10^2	–	5.0×10^6	–	–	3.0×10^6	–
	Acinetobacter baumannii	–	–	–	1×10^7	–	–	–	–	–	–	–	–
	Acinetobacter ursingii	–	5.0×10^6	–	–	3.0×10^6	–	–	–	–	–	–	–
	Alcaligenes faecalis	1.5×10^6	–	1.7×10^4	1×10^8	$> 10^7$	5.0×10^4	–	–	2.0×10^4	–	–	1.2×10^4
	Alcaligenes faecalis ssp	–	–	–	–	–	–	–	$> 10^7$	–	–	–	1.2×10^3
	BGN - unidentified	–	5.0×10^6	5.8×10^4	–	–	5.3×10^4	–	–	10^4	–	–	3.0×10^3
	Cupriavidus pauculus	–	5.0×10^6	3.7×10^4	–	–	–	–	5.0×10^6	–	–	–	–
	Ochrobactrum anthropi	–	5.0×10^6	2.0×10^3	–	$> 10^7$	3×10^3	–	$> 10^7$	4.2×10^3	–	5.0×10^6	10^4
	Pseudomonas aeruginosa 1[a]	1×10^7	1.0×10^6	–	1×10^6	–	–	4×10^5	–	–	–	–	–
	Pseudomonas aeruginosa 2[a]	5×10^6	–	–	6×10^5	–	–	4×10^6	–	–	–	–	–
	Pseudomonas aeruginosa 3[a]	4×10^6	–	–	–	–	–	4×10^6	–	–	–	–	–
	Pseudomonas aeruginosa 4[a]	–	–	–	–	–	–	5×10^6	–	–	–	–	–
	Pseudomonas putida	–	1.5×10^6	–	1×10^5	–	–	–	$> 10^7$	–	–	1.2×10^6	–
	Pseudomonas sp.	7×10^6	5.0×10^6	–	–	3.0×10^6	–	–	$> 10^7$	–	2×10^6	3.0×10^6	2.0×10^2
	Pseudomonas stutzeri	–	–	–	1×10^6	–	–	–	–	–	2×10^6	–	–
	Rhizobium radiobacter	–	–	–	–	–	–	–	–	–	–	–	10^4
	Sphingobacterium sp.	–	–	–	–	–	–	2×10^6	–	–	–	–	–
	Stenotrophomonas maltophilia	–	–	–	–	$> 10^7$	–	1×10^6	–	–	–	2.5×10^6	–
GPB	*Bacillus* sp.	1×10^5	–		1.3×10^6	–	–	1×10^5	–	–	1×10^6	–	–
	GPB unidentified	–	–	–	3×10^6	–	–	9×10^6	–	–	–	–	–
	Staphylococcus coagulase negativo	–	–	–	7×10^6	–	–	–	–	5.0×10^4	–	–	–
Enterobacteria	*Citrobacter freundii*	–	–	–	1×10^6	–	–	–	–	–	–	–	–
	Enterobacter aerogenes	–	–	–	–	–	–	4×10^6	–	–	1×10^7	–	–
	Serratia marcescens	–	–	–	–	–	–	–	–	–	–	2.5×10^6	–
	Fungi												
Filamentous	*Aspergillus niger*	–	–	–	–	–	–	–	–	–	–	–	6.0×10^2
	Aspergillus sp.	–	–	–	–	–	–	–	–	10^3	–	–	–
	Aspergillus sp. (brown)	–	–	–	–	–	–	–	–	–	–	–	8.0×10^3
	Aspergillus sp. (pink)	–	–	–	–	–	–	–	–	–	–	–	10^4
	Anemophilous fungus	–	–	–	–	–	–	–	–	2.0×10^4	–	–	–
	Penicillium sp.	–	–	2.2×10^5	–	–	$> 10^5$	–	–	10^5	–	–	–
	Penicillium sp. (green)	–	–	–	–	–	–	–	–	–	–	–	10^5
	Pseudallescheria sp.	–	–	1.4×10^4	–	–	–	–	–	–	–	–	–
	Scopulariopsis sp.	–	–	–	–	–	$> 10^5$	–	–	–	–	–	–
Yeast	*Candida parapsilosis*	–	–	–	–	–	10^4	–	–	–	–	–	–

-: Microorganisms were not detected in the sample; NF-GNB: non-fermenting gram negative bacilli; GPB: gram positive bacilli.

[a] *Pseudomonas aeruginosa 1,2,3* and *4* are strains with color, size and other characteristics that differentiate each other, although they belong to the same species.

studied in the degradation of PAHs and other recalcitrant organic pollutants, in addition to possessing biosorption properties of metals (Das et al., 2012; Ma et al., 2013; Quintelas et al., 2008). The species *O. anthropi* has also shown to be efficient at adsorbing heavy metals (such as chromium, copper, and cadmium) (Ozdemir et al., 2003). The genus *Alcaligenes* has been previously associated with the degradation of contaminated environments with phenol and crude oil (Essam et al., 2010; Lal and Khanna, 1996).

Regarding the quantitative analysis, a reduction in the number of microorganisms in the order from 10^6–10^7 to 10^3–10^4 was observed during the periods tested, possibly due to a reduction of the organic matter of the samples along those periods. In accordance with Boopathy (2000), a high content of organic matter favors a high diversity and a large number of microorganisms, whereas the availability of certain organic compounds can occasionally affect the activity of bacteria (Boopathy, 2000). Ivanov et al. (2004) observed a decrease in organic matter and microorganisms over time when monitoring the biodegradation of SS mixed with food residues. Other studies also show that a decrease in organic matter content during the biorremediation of SS led to a reduction in the microbial activity (Hernández et al., 2006) and in the number of microorganisms (Mazzeo et al., 2015). These data from the literature corroborates the results presented in this study, regarding the quantification of microorganisms.

Despite the bacterial diversity, certain toxicity was observed in some of the samples, as demonstrated by the *A. cepa* results, showing that biostimulation was not effective and possibly generated more toxic compounds than at the beginning of the study. This may be due to the presence of a low number of microorganisms with the capacity to mineralize the pollutants, or even, the microbial community present does not submit the appropriate metabolism for complete degradation of compounds in inert molecules (such as CO_2, H_2O, and CH_4) (El Fantroussi and Agathos, 2005).

Fungi were present only in the samples for the last period of biostimulation tested, and the genus *Penicillium* was the only fungi common in all assessed samples. A predominance of filamentous fungi was observed in relation to yeast. Among all the evaluated samples, the SS + B + S sample presented the greatest diversity of fungi.

Fungi are important during the degradation of organic compounds, being known to deteriorate or cause deterioration of a wide variety of materials (Singh et al., 2011). Six genera of fungi appeared only in the last period under analysis (6 months after the beginning of the experiment). According to Leitão (2009), fungi have slower growth compared to bacteria and usually need a substrate for co-metabolism; thus being considered less efficient in the degradation of some of the xenobiotics than the bacteria, but more efficient in the degradation of recalcitrant compounds (such as PAHs with four rings and more). The genus *Penicillium* sp, found in our study, is capable of degrading PAHs, pesticides,and organic compounds (e.g., fluorine and pyrene) (Cerniglia and Sutherland, 2010; Hofrichter et al., 1994; Leitão, 2009). In addition, the genus *Penicillium* sp has an important biosorbent role for metals, such as zinc and copper (Leitão, 2009). In this study, the genus *Penicillium* was found in all samples at T3, supporting the data of Leitão (2009), who proposed a delay in the colonization of samples by *Penicillium* because of its slower metabolism.

Changes in the microbial profiles of the tested samples might be related to competition for substrates and energy among microorganisms. According to Boopathy (2000), interactions between the existing microbiota affects bioremediation, either by competition, succession or predation. Microbial growth can also be prevented by factors such as temperature fluctuations, availability of water, pH, depletion of nutrients, and levels of toxic pollutants in environments (Gentry et al., 2004).

Thus, the identification of the microbiota present in the sample is interesting not only because of the possibility to select more efficient microorganisms for later use in the process of accelerating detoxification of SS, but also to determine whether the incorporation of new stimulating or dilution agents (sugarcane bagasse and soil) is favorable to bioremediation, by the introduction of new microorganisms to the system.

4. Conclusions

From the results obtained in this study, it is possible to conclude that the SS studied presented cytotoxic and genotoxic potential for the species *A. cepa*, even when associated with sugarcane bagasse. For microbiological data, a preference of degradation of SS by oxidative pathway was observed with a predominance of bacterial species. The periods of bioremediation tested and the addition of sugarcane bagasse seem not to have been sufficient for the attenuation of the toxic effects of SS because there was a bioavailability of toxic substances to the test organism *A. cepa* over the tested periods.

The set of methodologies used in this work was important for biological analysis of the effectiveness of detoxification of SS, as well as the study of new technologies of bioremediation. Thus, the present work emphasizes that, although the agricultural use of SS may seem to be a feasible alternative to its destination, this should not be carried out without their complete detoxification, since the SS can contain agents capable of inducing cell and genetic damages to the exposed organisms, both by direct contact (solid sample) and by through its aqueous eluate contaminants, proven by the evaluation of aqueous extracts (aqueous eluate and the percolate water).

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