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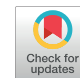
Evaluation of the potential agricultural use of biostimulated sewage sludge using mammalian cell culture assays



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HIGHLIGHTS

- Biostimulation with rice hulls applied for detoxification of domestic sewage sludge.
- Sewage sludge induced micronucleus and nuclear abnormalities in HepG2 cells.
- Sewage sludge induced genotoxic effects before and after bioremediation process.
- Sewage sludge mixed with rice hulls and soil was not genotoxic.
- Biostimulation of sludge by rice hulls is effective to eliminate sludge genotoxicity.

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ABSTRACT

Among the bioremediation processes, biostimulation is an effective methodology for the decontamination of organic waste by the addition of agents that stimulate the indigenous microbiota development. Rice hull is a biostimulating agent that promotes the aeration of edaphic systems and stimulates the aerobiotic activity of soil microorganisms. The present study aimed to evaluate the efficacy of the bioremediation and biostimulation processes in reducing the toxicity of sewage sludge (SS) and to evaluate its possible application in agriculture using cytotoxic and genotoxic assays in human hepatoma cells (HepG2). SS of domestic origin was tested as both the pure product (PSS) and mixed with soil (S) and with a stimulating agent, such as rice hull (RH), in different proportions (SS + S and SS + S + RH); we also examined different remediation periods (3 months - T1 and 6 months - T2). For the PSS sample, a significant induction of micronucleus (MN) in T2 was observed with nuclear buds in all of the periods assessed, and we observed the presence of more than one alteration per cell (MN and nuclear bud) in T1 and T2. The PSS sample caused genotoxic effects in the HepG2 cells even after being bioremediated. For the samples containing soil and/or rice hull, no toxic effects were observed in the test system used. Therefore, the addition of SS to agricultural soils should be conducted with caution, and it is important that the SS undergoes a remediation process, such as bioremediation and biostimulation treatments.

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Abbreviations: RH, rice hull; SS, sewage sludge; MN, micronucleus; HepG2, human hepatoma cells line; S, soil; PSS, pure sewage sludge; SS + S, sewage sludge with soil; SS + S + RH, sewage sludge with soil and rice hulls; T0, before the bioremediation; T1, three months of bioremediation; T2, six months of bioremediation; MEM, minimum essential medium; PBS, phosphate-buffered saline; MTT test, thiazolyl blue tetrazolium bromide test; DMSO, dimethyl sulfoxide; NC, negative control; PC, positive control; CBPI, cytokinesis-block proliferation index.

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1. Introduction

Humans produce countless residues that are deposited daily into the environment. Sewage sludge (SS) is a semi-solid residue with a worldwide production of millions of tons per year (Aparicio et al., 2009). Despite its varied composition, this residue is mainly composed of organic matter and macronutrients (nitrogen, phosphorus, potassium and magnesium) (Gray, 2010), which makes it an interesting material to be used for the reconditioning of agricultural soil (Çifci et al., 2013).

Due to its increasing production, the application of SS in agricultural activities is an alternative use (Bright and Healey, 2003) and is a viable option for recycling this residue, which is rich in organic matter, instead of discarding it. However, this residue may contain a large amount of persistent and bioaccumulative toxic compounds, which could contaminate and impair the quality of the soil where the residue is disposed, as well as interfere in other environmental matrices, such as the atmosphere and surrounding hydric resources (Cincinelli et al., 2012).

Considering the benefits that SS can confer to agriculture, as well as the possible toxicity to the soil to which it is added, efforts have been directed towards the development of new technologies to minimize toxicity and facilitate use in the reconditioning of agricultural soil (Tas, 2010).

Bioremediation is one of the detoxification methods for organic residues that uses organisms that are capable of modifying or decomposing pollutants (Tyagi et al., 2011). The degradation of organic compounds is directly related to the quantity of oxygen available in the environment, and therefore, improvements in the aerobic conditions of the soil could promote greater efficacy during the bioremediation process (Harmsen, 1991; Mazzeo et al., 2014; Molina-Barahona et al., 2004; Perez-Armendáriz et al., 2004). Increased aeration of a given system can be achieved by the addition of stimulating agents to the substrate (Souza et al., 2009; Vasudevan and Rajaram, 2001), such as vegetable residues. Rice hull is an agroindustrial residue that consists mainly of cellulose, hemicellulose and lignin (Fernandes et al., 2015) and that can act as an effective stimulating agent to contribute to the aerobic conditions of the bioremediation processes. In addition to the compounds present in the rice hull that are incorporated into the soil during application, rice hull also contains microbial strains (mainly filamentous fungi) that metabolize contaminants, thereby aiding in the bioremediation process of the organic compounds (Hamdi et al., 2007).

Some authors have warned that SS that is intended for soil application should be treated to improve the stability of the organic matter and prevent effects that alter the soil properties and cause environmental damage (Nafez et al., 2015; Sommaggio et al., 2018). Rathod et al. (2009) reported that many methods have been proposed to promote the detoxification of SS and to ensure its chemical and biological stability.

Biostimulation technology, which consists of the addition of stimulating agents, such as oxygen, and other nutrients is an interesting means for stimulating the growth of autochthonous microorganisms (Mazzeo et al., 2014), constituting a possible alternative to SS decontamination.

The development of bioassays to determine the impact of contaminants on the environment is of extreme importance for environmental monitoring studies (Plaza et al., 2005). Micronuclei (MN) are indicators of chromosomal damage and genomic instability (Lajmanovich et al., 2014) and can be evaluated in different cell types (Fenech, 2000). The MN test is a validated and standardized method for use in toxicology studies (Vral et al., 2011) and has been employed in the assessment of SS toxicity in several studies (Bonomo et al., 2016; Gajski et al., 2011; Mazzeo et al., 2016; Solano et al., 2009).

Assays with cultured mammalian cells have been widely used in research studies on the genotoxic and mutagenic potential of environmental pollutants (Cardozo et al., 2006; Mazzeo et al., 2010), since these cells correlate with the effects observed in humans (Brusick, 1987). HepG2 cells are considered an effective test system for evaluating the cytotoxic and genotoxic potential of contaminants (Leme et al., 2011; Liu et al., 2012). This cell line also presents metabolizing enzymes that are important for the activation and detoxification of toxic compounds within the organism (Knasmüller et al., 2004; Uhl et al., 2000) and has been extensively used for the screening of mutagens due to its efficient metabolic response (Chiang et al., 2011). The toxicity evaluation of the attenuated SS, carried out by Mazzeo et al. (2016), showed that HepG2 cells are sensitive, which verified the genotoxic potential of SS.

In this context, the present study aimed to evaluate whether the bioremediation process, using rice hull as a stimulating agent, contributes to the detoxification of SS, facilitating its application for agricultural soil fertilizers. This assessment was conducted using cytotoxic and genotoxic assays with HepG2 cells, which is considered an efficient test system for monitoring the environmental quality.

2. Material and methods

2.1. Collection of samples

SS of domestic origin was collected in 2014 from the Wastewater Treatment Plant Praia Azul, located in the city of Americana, São Paulo, Brazil (latitude 22° 44′ 21" S and longitude 47° 19′ 53" W). After sampling, the SS was immediately used to prepare the samples. To improve the oxygenation of the system, rice hull was obtained from a rice processing plant in the city of Rio Claro, SP and was used as a decompacting agent. The soil (S) used in the preparation of the mixtures was a clayey soil with no toxicity (Mazzeo et al., 2015), which was collected from the Experimental Garden of UNESP in Rio Claro, SP.

For the bioremediation and biostimulation study, three different samples containing SS were prepared: PSS (pure SS); SS + S (SS mixed with soil at a ratio of 3:1v/v); and SS + S + RH (SS mixed with soil and rice hull at a ratio of 3:1:1 v/v/v). After preparation, the samples were placed into stainless-steel vats (14 L volume) to be submitted to bioremediation periods for 3 (T1) and 6 (T2) months. During the bioremediation period, the vats were maintained in a covered place at ambient temperature at the Experimental Garden of São Paulo State University, Rio Claro, SP. For each mixture studied, three vats were assembled for triplicate experiments.

2.2. Preparation of the aqueous extracts (solubilized)

Solubilized extracts were obtained first by determining the dry weight of each sample. Approximately 10 g of each sample was weighed in an individual tare container, followed by oven drying at 105 °C for 24 h. This assay was performed in triplicate. Dried samples were then weighed, and the mean value of triplicate samples was calculated.

Solubilized extracts (liquid phase) were obtained using a method based on the norm Brazilian Association of Technical Standards NBR 10.006 (ABNT NBR 10006, 2004). A quantity of each sample (62.5 g; relative to its dry weight) was added to 250 mL of distilled water. However, more water was added to the system to obtain a sufficient liquid phase for solubilization, since there was substantial retention of water in the sludge. Thus, the final volume of water for all of the samples was adjusted to 600 mL. After

constant agitation for 5 min, followed by 7 days of decanting at 22 °C, the liquid phase of each sample was collected and filtered through membranes with 0.45-μm porosity. The resulting aqueous extracts from each sample were then filtered using 0.22-μm porosity syringe filters to prevent microbiological contamination of the HepG2 cell line.

2.3. Assays with HepG2 cell culture

2.3.1. Cell lineage

The HepG2 cells (*Human derived hepatoma cells*) used in this study were obtained from the Rio de Janeiro Cell Bank (Brazil). The cultures, which were maintained in 25 cm^2 disposable flasks, were cultivated in 5 mL of MEM medium supplemented with 10% fetal bovine serum and 0.1% antibiotic-antimycotic solution (10,000 U.I./mL penicillin and 10 mg/mL streptomycin) in a CO_2 incubator (5%) at 37 °C. Under these conditions, the cell cycle of this lineage was approximately 24 h.

2.3.2. MTT test

The MTT test (Thiazolyl blue tetrazolium bromide – CAS 298-93-1, Sigma) was performed to determine the non-cytotoxic concentrations of the samples for HepG2 cells treatment and was carried out according to the protocol of Mosmann (1983) with some modifications. Cells (2.34×10^4) were seeded into each of the 96 wells of a microtiter plate and incubated for a stabilization period of 24 h in a CO_2 incubator (5%) at 37 °C. The cells were then treated with different concentrations of the solubilized extracts of PSS, SS + S and SS + S + RH from different bioremediation periods (T1 and T2), as well as from before the bioremediation process (T0) for 24 h. The negative control (NC) was carried out using culture medium only, and the positive control (PC) used Triton X-100 at 1%. The medium containing the different treatments was then removed from each well, and 150 μL of the MTT solution (5 mg/mL) was added. The plates were then incubated for 4 h at 37 °C, and then, the MTT solution was discarded, and 100 μL of dimethyl sulphoxide (DMSO) was added to each well. This procedure was performed in triplicate in independent experiments.

The plates were read using a spectrophotometer with a microplate reader (Multiskan FC Apparatus – Thermo Scientific) at 540 nm. The concentrations that induced viability lower than 80% were considered cytotoxic. Thus, based on results of the MTT test, non-cytotoxic concentrations of the solubilized SS were determined for subsequent use in the MN test.

2.3.3. MN test with cytokinesis block

The MN test was performed using the protocol described by Natarajan and Darroudi (1991) with some modifications. The concentration of the solubilized extract used in this assay was of 50 μL/mL of medium for each sample (PSS; SS + S; and SS + S + RH of T0, T1 and T2); this corresponded to the highest non-cytotoxic concentration determined in the MTT test (SS dry matter at 4.95 mg/mL).

Approximately 1×10^6 cells were cultured in 25 cm^2 flasks for a complete cell cycle (24 h). Subsequently, treatments with the solubilized extract that were prepared as previously described were carried out in individual flasks and in triplicate. The NC was performed using PBS (50 μL/mL), and the PC was performed using methylmethane sulphonate (1×10^{-2} M; 50 μL in 5 mL). After treatment, the cells were washed twice with PBS, and 5 mL of complete culture medium containing 3 μg/mL of cytochalasin B was added. The flasks were incubated at 37 °C for 28 h (Chequer et al., 2009; Mazzeo et al., 2016; Tsuboy et al., 2007). The cells were then harvested and fixed using Carnoy (methanol: acetic acid, 3:1 v/v). For the preparation of the slides, a few drops of the cell culture were dropped onto clean slides containing a water film. After drying, the slides were stained with 5% Giemsa for 10 min.

The analysis was performed under a light microscope (1000 × magnification). Approximately 1000 binucleated cells with intact cytoplasmic membrane, nuclear envelope, and nuclei with similar non-overlapping sizes were counted. Two slides for each replicate were analyzed, totaling approximately 6000 cells for each treatment and controls. For the assessment of the genotoxic potential of the SS, the results for the treated cells were compared with the NC using the non-parametric Kruskal–Wallis test ($p < 0.05$).

In this assay, MNs, nuclear buds, nucleoplasmic bridges and alterations that appeared together were counted, such as binucleated cells that contained bridge and bud (P + B), MN and bud (MN + B), MN and bridge (MN + P) and those that presented other types of association of alterations, such as bridge, bud and micronucleus (MN + B + P) in the same binucleated cell. Furthermore, by scoring 1000 cells as mononucleate, binucleate or multinucleate, the cytokinesis-block proliferation index (CBPI) was determined using the following formula: $CBPI = [M1 + (2 \times M2) + (3 \times M3)]/N$, where M1, M2 and M3 represent the number of cells with one, two and multi nuclei, respectively, and N is the total number of cells scored (OECD, 2012). The results of this assay were compared with the NC using a parametric one-way ANOVA ($p < 0.05$).

3. Results and discussion

Due to the agricultural reuse of SS, there is a need to monitor this residue in order to understand its potential risk to the environment. SS can contain a complex mixture of toxic compounds and can impair the organisms that are exposed when it is deposited into the surrounding soil (Engwall and Hjelm, 2000). According to Alvarenga et al. (2007), a strategy to evaluate the harmful risks that this residue can cause in exposed organisms would be the use of different bioassays, which can measure the bioavailability of the contaminants present in the SS. Chemical analyses, besides being costly and laborious, cannot predict the combined toxic effects of substances present in a sample. Thus, assessments using biological systems are indispensable since they can offer a prognosis of the toxicity of complex mixtures (Tewari et al., 2005).

Since human hepatoma cells (HepG2) are an effective test system for evaluating the genotoxic potential of contaminants (Leme et al., 2011), several studies have been carried out with mammalian cell culture to investigate the toxic effects of environmental pollutants (Leme et al., 2011; Matsumoto et al., 2005; Mazzeo et al., 2010, 2013).

The MTT test was performed with 5 different concentrations of the solubilized extracts of PSS, SS + S and SS + S + RH, for all of the bioremediation periods studied (T0, T1 and T2). According to this test, none of the concentrations tested demonstrated cytotoxicity (5, 10, 15, 25 and 50 μL of sample per mL of culture medium); the viability values were 100% for all of the samples. Therefore, the highest concentration of each solubilized extract (50 μL/mL) was used in the MN test. Thus, it was ensured that none of the concentrations tested were considered cytotoxic for the HepG2 cells.

Another method for assessing cytotoxicity is the cytokinesis-blocked proliferation index (CBPI). This index is used to indicate the average number of cell cycles after the treatment with cytochalasin B (OECD, 2012; Surrallés et al., 1995). The results presented in Table 1 show a statistically significant increase in CBPI for SSP samples at T0 and T1 compared to the negative control. These results suggest that PSS presented proliferative action even after 3 months of bioremediation (T1), since a significant increase on cell division occurred in relation to the negative control.

For the MN test, the presence of one, two, three or more MNs in

a single binucleated cell (Table 1) were evaluated. According to Fenech (2007), these alterations are biomarkers of chromosomal breaks and/or losses. From the results shown in Table 1, it was possible to observe a higher quantity of binucleated cells carrying only one MN per cell than compared with two or three MN. However, this value was not statistically significant for any of the treatments that were performed.

The total number of micronucleated cells for the PSS sample was 38.09, 39.12 and 40.17 for the periods T0, T1 and T2, respectively; these values are higher than those obtained with other treatments. For SS + S, the results were 24.46 (T0), 21.5 (T1) and 29.76 (T2), and SS + S + RH presented values of 22.72 (T0), 20.80 (T1) and 26.75 (T2), respectively. The results were statistically significant compared to the negative control (24.16) for the PSS-T2 sample (after six months of bioremediation). These results suggest that after six months of the bioremediation process, the aqueous extract of the PSS sample had potential to induce MNs in the HepG2 cells, indicating that the SS studied may contain substances capable of inducing chromosomal breaks or losses.

Mazzeo et al. (2016) used HepG2 cells to investigate the mutagenic potential of aqueous extracts of SS that underwent periods of natural attenuation. The authors reported a significant mutagenic effect, relative to the amount of MNs, in the initial periods of the study (0 and 2 months of bioremediation). In a study performed with SS leachate, Gajski et al. (2011) observed a significant increase in the MN frequency during *in vitro* tests that were performed with human lymphocytes. The authors concluded that the SS sample tested could cause adverse effects in the exposed human populations. Tewari et al. (2005) also reported a significant increase in the MN frequency in rat erythrocytes that were exposed to SS leachate. Hopke et al. (1982) observed that extracts obtained from SS of domestic origin were also able to induce point mutations in germ cells of *Zea mays* and a clastogenic effect in *Tradescantia paludosa*. These studies corroborate the MN data that were obtained in this study, in which the solubilized extract of PSS also induced clastogenic effects in human cells maintained in culture.

In the analysis of nuclear buds, the presence of one or two buds was found in HepG2 binucleated cells, as shown in Table 1. The results showed a high quantity of single nuclear buds in binucleated cells for the PSS samples during all of the remediation periods that were assessed, and the results were statistically significant in relation to the NC. Statistically significant results were also observed for the sum of binucleated cells with 1 and 2 nuclear buds for the PSS samples for T0, T1 and T2. According to Fenech (2006) and Fernandes et al. (2009), nuclear buds originate from a cell mechanism of amplified and/or excess DNA removal, and they are a biomarker of amplified genes or altered gene dosage. The significant increase of nuclear buds in the cells exposed to the raw sludge indicates that the SS analyzed can also induce genotoxic damage in the test system used in this study, through the elimination of amplified genetic material in addition to chromosomal breaks and losses.

Rank and Nielsen (1998) also evaluated the genotoxic effects of SS in meristematic cells of *Allium cepa*. In their study, the observation of significant genotoxic effects caused by the sludge that was studied led the authors to conclude that these effects could be due to the presence of potentially toxic metals in the SS. However, in the present study, we cannot confirm that the observed effects are related exclusively to the presence of metals because SS is a complex mixture composed of several substances. It is difficult to attribute the toxicological effects of SS that were observed to a specific compound since, according to Oleszczuk (2008), countless chemical reactions can occur between the substances that are present in this residue.

Regarding the presence of nucleoplasmic bridges, none of the samples induced statistically significant changes in the occurrence of this alteration. Therefore, in this study, MNs appear to have an aneugenic origin instead of a clastogenic one, since the results for the presence of chromosomal bridges were not statistically significant. The bridges are important markers of DNA damage, being related to the formation of MN due to strand breaks in DNA (Fenech, 2006).

According to Fenech (2007), the presence of micronuclei, buds and nucleoplasmic bridges are indicative of chromosome damage or genetic instability. The assessment of DNA damage provides important information about the effects of toxic substances, since mutations represent crucial events in carcinogenic processes (Fenech, 2008).

Table 1
Genotoxic alterations and Cytokinesis-Block Proliferation Index (CBPI) in binucleated HepG2 cells submitted to the treatments with the solubilized extracts of SS and its mixtures with soil and rice hull, during different bioremediation periods.

Alterations	NC	PC	T0			T1			T2		
			PSS	SS + S	SS + S + RH	PSS	SS + S	SS + S + RH	PSS	SS + S	SS + S + RH
Cytotoxicity assessment											
CBPI	1.57 ± 0.04	1.48 ± 0.02*	1.64 ± 0.03*	1.60 ± 0.0	1.59 ± 0.02	1.64 ± 0.03*	1.55 ± 0.06	1.58 ± 0.06	1.59 ± 0.01	1.58 ± 0.03	1.55 ± 0.02
Presence of MN											
1 MN	24.16 ± 3.38	65.87 ± 3.71 *	38.09 ± 3.15	24.46 ± 3.74	22.72 ± 3.12	39.12 ± 4.02	21.50 ± 2.42	20.80 ± 2.08	40.17 ± 4.36	29.76 ± 3.76	26.75 ± 3.15
2 MN	3.8 ± 1.47	8.98 ± 2.75	4.99 ± 2.83	3.49 ± 1.64	2.32 ± 1.75	4.81 ± 1.74	2.83 ± 1.17	3.33 ± 1.86	5.81 ± 3.42	4.82 ± 2.04	3.49 ± 1.87
3 MN	0.17 ± 0.41	2.33 ± 1.85	0.33 ± 0.52	0 ± 0	0.17 ± 0.41	0 ± 0	0 ± 0	0.17 ± 0.41	0.99 ± 1.25	0.66 ± 0.81	0.17 ± 0.41
Total MN	28.16 ± 4.22	77.18 ± 5.31*	43.42 ± 3.58	27.96 ± 4.43	25.21 ± 3.05	43.93 ± 4.97	24.33 ± 2.57	24.29 ± 2.46	46.98 ± 4.48*	35.24 ± 3.98	30.41 ± 2.42
Presence of nuclear abnormalities											
1 bud	13.33 ± 4.08	35.92 ± 6.38*	27.12 ± 3.73*	21.96 ± 3.73	19.23 ± 2.27	27.04 ± 3.68*	18.50 ± 3.39	18.47 ± 2.90	31.03 ± 6.11*	25.11 ± 2.77	18.46 ± 3.07
2 buds	0 ± 0	0 ± 0	0 ± 0	0.17 ± 0.41	0.33 ± 0.52	1.16 ± 0.98	0.33 ± 0.52	0.33 ± 0.51	2.32 ± 1.84	1.49 ± 1.64	1.00 ± 0.63
Total buds	13.33 ± 4.08	35.92 ± 6.83*	27.12 ± 3.07*	22.13 ± 3.64	19.56 ± 2.10	28.20 ± 4.30*	18.83 ± 3.31	18.80 ± 2.65	33.34 ± 7.32*	26.61 ± 2.30	19.46 ± 2.94
Bridge	10.50 ± 2.42	28.28 ± 5.78*	16.97 ± 2.83	15.31 ± 3.73	13.61 ± 2.59	19.40 ± 5.66	11.00 ± 2.28	10.98 ± 3.83	14.12 ± 4.72	13.96 ± 5.19	13.80 ± 3.93
Presence of multiple alterations											
P + B	1.33 ± 0.82	0.33 ± 0.81	1.00 ± 2.45	0.50 ± 0.55	1.00 ± 0.89	1.83 ± 1.94	0.50 ± 0.84	0 ± 0	2.82 ± 1.33	0.83 ± 1.58	1.49 ± 1.04
MN + B	2.33 ± 1.37	8.48 ± 4.97*	4.82 ± 1.59	3.50 ± 1.98	4.48 ± 1.76	5.97 ± 1.26*	4.17 ± 1.47	2.50 ± 1.64	8.63 ± 3.77*	5.65 ± 1.93	3.99 ± 1.88
MN + P	0.67 ± 1.21	4.49 ± 1.52*	1.33 ± 1.51	1.00 ± 1.09	0.99 ± 0.61	0.83 ± 0.98	1.17 ± 1.60	1.00 ± 1.55	0.33 ± 0.82	1.66 ± 1.02	0.33 ± 0.51
MN + B + P	0 ± 0	0.17 ± 0.41	0 ± 0	0 ± 0	0 ± 0	0.17 ± 0.41	0 ± 0	0 ± 0	0.17 ± 0.41	0.16 ± 0.40	0.33 ± 0.51
Total of multiple aberrations	4.33 ± 1.37	13.48 ± 5.75*	7.32 ± 3.01	4.99 ± 2.76	6.46 ± 1.97	8.80 ± 2.16	5.83 ± 2.23	3.49 ± 1.23	11.95 ± 3.94*	8.30 ± 4.29	6.14 ± 2.81

*value statistically significant in relation to the NC ($p < 0.05$). NC: negative control; PC: positive control; PSS: solubilized of the sewage sludge without any mixture; SS + S: sewage solubilized mixed with soil; SS + S + RH: sewage solubilized mixed with soil and rice hull. T0: initial samples (before the start of the bioremediation); T1: samples after 3 months of bioremediation; T2: samples after 6 months of bioremediation. MN: micronucleus; P: bridge; B: bud. CBPI: Cytokinesis-Block Proliferation Index.

With regard to multiple alterations, or more than one abnormality occurring in one binucleated cell, a statistically significant increase in cells bearing MN and bud (Table 1) was observed for the PSS sample (T1 and T2), which corroborates the most common abnormalities found when analyzed separately. Therefore, it is possible to infer that the SS studied may contain substances capable of inducing the formation of nuclear buds by several pathways, such as loss and elimination of the surplus genetic material.

Aqueous extracts of the mixtures of SS with soil and rice hull did not cause statistically significant results for any of the periods tested, showing the importance of a pretreatment for this residue before its disposal in the environment. Roig et al. (2012) analyzed different types of SS in toxicity assays with plants and bacteria, and found that sludge that did not receive pre-treatment presented the highest toxicity indices, while the SS that was submitted to composting was considered less toxic. In a natural attenuation study with SS, in which SS was mixed with different proportions of soil, the authors observed that, after a certain period of bioremediation, the genotoxicity in HepG2 cells decreased significantly in relation to the initial period of the analysis. This result demonstrated the efficiency of the process that was used and adequacy of the test system for use as an indicator of DNA damage (Mazzeo et al., 2016).

Although the PSS underwent a bioremediation process, in which the microorganisms that were naturally present in the SS acted in the process, the periods assessed (3 and 6 months) were insufficient to diminish the toxicity of the aqueous extract of PSS. Studies carried out by Donnelly et al. (1990), using the Ames test, found that for mutagenic substances in the SS, which persisted for long periods in the soil, a period of approximately two years was necessary to decrease the mutagenic activity.

The Water and Sewage Management Department of Americana (São Paulo, Brazil), who provided the SS samples, classified the SS used in this study as IIA Class – non-inert residue by Brazilian Association of Technical Standards (ABNT NBR 10004, 2004). The characteristics of this class of waste include biodegradability, combustibility or solubility in water. Based on these data, it can be postulated that the organic compounds in the SS that were investigated underwent biodegradation, producing hydrosoluble substances with higher toxicity than the compounds that were originally present in the raw sludge. According to Harrison et al. (2006), the degradation of organic compounds can produce other compounds with higher or lower toxicity than the original substance. This finding supports the results that were observed for the PSS sample, since there was no reduction in toxicity, even after the remediation process. Thus, it is important to emphasize that the SS disposal in soils, without prior treatment, may be harmful to the environment.

When the SS was mixed with soil and rice hull, a decrease in the genotoxicity of this residue was observed. This decrease is probably due to an optimization of the bioremediation process, which may have resulted from the incorporation of new microorganisms into the system, in addition to an improvement in the aeration of the sample as a result of the rice hull.

In the present study that was performed with HepG2 cells, samples of solubilized SS + S + RH, in general, presented the lowest indices of alterations. These results show that rice hull was an important agent for the detoxification of the SS and for the consequent reduction in the genotoxic potential of the PSS samples. The use of vegetal agents favors bioremediation by optimizing the aerobiosis of the system, besides increasing the microbial biomass present in the process, thus leading to an improvement in the degradation of pollutants (Molina-Barahona et al., 2004). In a study conducted by Souza et al. (2009), the use of rice hull improved the biodegradation process, decreasing the clastogenic potential of contaminated samples that were submitted to a landfarming treatment.

Conducting previous treatment of the SS directly influences some of its properties, such as its toxicity (Roig et al., 2012). The genotoxicity of an aqueous extract of a composted SS was evaluated in *Vicia faba*, and a decrease in the genotoxic potential of this sludge was observed when compared to the non-composted SS, demonstrating that composting was essential for the reduction of the SS toxicity (El Fels et al., 2015). Kapanen et al. (2013) assessed the efficiency of the composting of domestic SS samples mixed with rice hull and turf. The authors conducted chemical analyses and found a reduction in the acute toxicity and genotoxicity of the composted sample. Based on results of the study, the authors concluded that the ecotoxicity assessment provides important information about the fate of these materials. They suggested that bioassays provide a useful tool for evaluating the quality of SS or its composted product, indicating the possible risks of these products for agricultural applications.

This study reinforces the need to use bioremediation techniques to decontaminate SS and demonstrates the relevance of using biomarkers, such as cellular and DNA damage in HepG2 cells, as efficient indicators for diagnosing the toxic potential of this residue.

4. Conclusions

HepG2 cells were sensitive to the contaminants present in the SS, characterizing an appropriate cell line for toxicological studies against this residue.

The bioremediation periods for the SS samples without mixtures (PSS) were not effective in reducing the toxicity of the sludge that was investigated in this study, indicating that SS should not be disposed of into the environment without pre-treatment.

When the SS was mixed with soil and rice hull, no significant genotoxic effects were identified for these samples (SS + S and SS + S + RH) at any bioremediation periods studied, showing that the mixtures were important for decreasing the toxicity of the sludge. These results indicate that the biostimulation can be a potential and interesting method to detoxify SS, supporting its application as an agricultural fertilizer.

This study demonstrated the importance of monitoring SS over time using biological assays, as well as the need to search for new methodologies for the remediation of SS.

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