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Attesting the efficiency of monitored natural attenuation in the detoxification of sewage sludge by means of genotoxic and mutagenic bioassays

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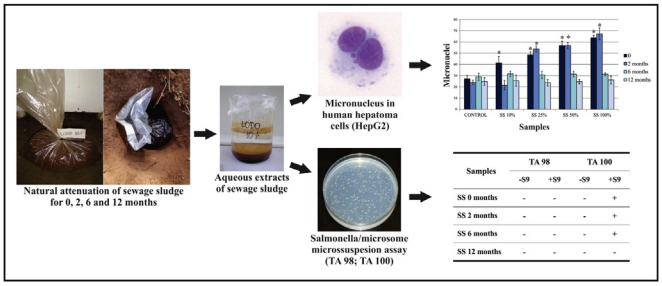
HIGHLIGHTS

- Genotoxicity/Mutagenicity of urban sewage sludge in HepG2 cells and *Salmonella* tests.
- Mutagenic effect of the studied sewage sludge observed in *Salmonella* strain TA 100/S9.
- Sludge/soil associations induced micronuclei in human hepatoma cell line.
- Natural attenuation detoxifies sludge and decreases potential risks to human health.

GRAPHICAL ABSTRACT



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ABSTRACT

A viable alternative to the use of sewage sludge (SS) would be using it as a reconditioner of agricultural soils, due to its high content of organic matter and nutrients. However, this solution may contaminate the soil, since SS may contain toxic substances. Monitored natural attenuation is a process that can be used in the decontamination of SS before its disposal into the environment. The effectiveness of the natural attenuation of a domestic SS was evaluated over 12 months by assays of *Salmonella*/microsome and micronucleus (MN) in human hepatoma cells (HepG2). Mutagenic activity was observed for the *Salmonella* strain TA 100, with S9, for the extracts from periods 0–6 months of natural attenuation. Genotoxic effects were observed in HepG2 cells, for 0 and 2 months, in almost all tested concentrations. Comparing obtained data by MN test to chemical analyses, it is possible to observe a coincidence between the induction of MN and the quantity of the m- and p-cresol, since these compounds were present in the initial SS and after 2 months of natural attenuation, decreasing their concentrations in samples from 6 to 12 months. The positive results obtained with *Salmonella*/microsome (from 6 months) suggest a combined action of other substances in SS. These results indicated that this SS, in the earlier periods tested, is potentially genotoxic and mutagenic and that its disposal can lead to severe environmental problems. Thus, the use of the studied SS as reconditioner requires pre-processing for over than 6 months of natural attenuation.

1. Introduction

With increasing urban development and the increase in the

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number of inhabitants, the world population is facing a serious environmental problem regarding the fate of solid waste generated by wastewater treatment plants (Pathak et al., 2009). Each year, worldwide, millions of tons of sewage sludge (SS) are produced (Aparicio et al., 2009) with the prospect of increased production.

Since SS contains high concentrations of organic matter, micronutrients and macronutrients, such as nitrogen and phosphorus, it has been considered as an excellent material for the reconditioning of agricultural soils (Çifci et al., 2013). However, SS may present a high variability of persistent and bioaccumulative toxic compounds, which, when deposited in the environment, may compromise the quality of soils and associated environment, such as atmosphere, subterraneous and superficial waters (Cincinelli et al., 2012). The exposure of plants and animals to these pollutants may compromise the trophic chain (Roig et al., 2012), as well as represent risks to the human health.

In an attempt to ensure the chemical and biological stability of SS and avoid harmful environmental consequences generated by its application on soil, several methods have been presented to decontaminate SS (Rathod et al., 2009). Among these methods, natural attenuation may decrease the concentration of some pollutants in the environment by means of natural processes, such as volatilization, transformation, dilution, dispersion (Megharaj et al., 2011), and mainly by biodegradation by the microorganisms present in the contaminated site. Thus, this process has been considered an advantageous method with low-cost implantation (Mills et al., 2003).

To better characterize the toxic substances present in SS, the association between chemical analysis and bioassays are strongly recommended (Farré and Barceló, 2003). Among the several bioassays available, the *Salmonella*/microsomal test has been widely used for the detection of the mutagenic potential of a large variety of chemicals, including complex environmental samples, due to its simplicity, fastness, efficiency and high reproducibility (Claxton et al., 2010).

The micronucleus test (MN test) has been extensively used to evaluate the genotoxic effects induced by SS (Tewari et al., 2005; Solano et al., 2009; Gajski et al., 2011) because it is a high sensitive and efficiency in the detection of biological effects of environmental pollutants. Moreover, the induction of MN is considered an indicative of cancer risk in humans (Bonassi et al., 2007).

Since the liver plays the main role in the detoxification of xenobiotics, human liver cells (HepG2) have been commonly used in environmental genotoxicity studies (Baderna et al., 2013), especially by closely resembling the characteristics of *in vivo* human cells (Van Der Water et al., 2011).

This study investigated the genotoxic effect of an urban SS, by means of the MN test, performed with human liver culture cells (HepG2), as well as its mutagenic potential by *Salmonella*/microsomal assay, after several periods of monitored natural attenuation. The effectiveness of this process was evaluated through the decreasing of the SS toxic activity over the periods of natural attenuation on the studied test-systems.

2. Materials and methods

2.1. Obtaining the samples

The studied SS was collected at a domestic sewage treatment plant, located at the municipality of Rio Claro (in the state of São Paulo, Brazil). After sampling, part of this SS was mixed with a clay soil control (146.7 μS/cm), in proportions of 10%, 25% and 50% (v/v). The soil was collected in the Experimental Garden of the University of São Paulo State—UNESP (Rio Claro, São Paulo State, Brazil), being considered as a reference soil (environmental control) due to its absence of toxicity and so, suitable to be used in this experiment.

All samples (10%, 25%, 50% and 100% SS) were then disposed, in duplicate, in plastic bags that were micro-perforated with 0.5 mm diameter holes 1 cm apart from each other, and buried in an uncontaminated site for periods of 0, 2, 6 and 12 months, in order to promote a natural attenuation of the samples, as described by Mazzeo et al. (2015).

The chemical characterization of the 100% SS, regarding the presence of chlorinated benzenes, phthalates, chlorinated and non-chlorinated phenols, polycyclic aromatic hydrocarbons, chlorinated dioxins and pesticides, after each period of natural attenuation, indicated that the studied samples contained the organic compounds shown in Table 1. A complete chemical characterization of the samples can be seen in Supplementary data.

2.2. Preparation of aqueous extract of SS (solubilized)

To obtain an aqueous extract of SS, 125 g of each sample (10%, 25% and 50% SS), regarding to their dry weight, were mixed in 500 mL of ultra pure water, which was followed by low-speed constant stirring for 5 min, according to ABNT NBR 10.006 (2004). Due to the high hygroscopic potential of the 100% SS, twice the volume of water (1000 mL) used for other samples (10%, 25% and 50% SS) was added to the same mass (125 g) of 100% SS, in order to obtain a similar supernatant liquid phase.

After 7 days of decantation, the supernatant was collected and filtered through a membrane of 0.45 μm porosity. With this procedure, aqueous extracts of each studied samples containing soluble substances, potentially bioavailable, were obtained. The presence of hydrophilic compounds with biological activity in these extracts was tested by bioassays of genotoxicity and mutagenicity.

Previous analyses using high performance liquid chromatography with diode array detection (HPLC-DAD) showed that the aqueous extracts related to the 100% SS samples presented a mixture of *m*- and *p*-cresol in concentrations of 3.70 mg/L, 4.21 mg/L, 0.78 mg/L and 0.06 mg/L for the periods of 0, 2, 6 and 12 months of natural attenuation, respectively (Mazzeo et al., 2015).

2.3. Salmonella/microsome microsuspension assay – Kado test

S. typhimurium strains TA98 and T100 were used in this study, with and without the metabolizing fraction, to detect frameshift mutations and substitution mutations at base pairs, respectively.

This test was based on the protocol described by Kado et al. (1983). The strains TA98 and TA100 were cultivated overnight in nutrient broth, in a shaker incubator, at 37 °C. Following this procedure, 100 μL of each strain (10^{10} cells/mL) were added to culture tubes containing 100 μL of phosphate buffer 0.1 M and the volume of the sample, corresponding to concentrations of 6.25, 12.5, 18.75, 25.00, 37.50 and 50.00 mg dry SS equivalent. These concentrations were obtained by diluting the 100% SS aqueous extract.

For the assays with S9 metabolic activation, the phosphate buffer was replaced by 100 mL of S9 mix [sterilized distilled water, phosphate buffer 0.2 M, NADP 0.1 M, glucose-6-phosphate 1.0 M, solutions of salts (KCl 1.65 M and $MgCl_2$ 0.4 M) and S9 fraction]. The tubes containing the mixture were pre-incubated at 37 °C for 90 min. Thus, their contents, already with the addition of 2 mL of surface agar, were poured in sterilized Petri dishes containing minimum solidified agar. The Petri dishes were incubated at 37 °C for 72 h. Each test was run in triplicate.

To perform the assay with metabolic activation, an exogenous metabolic activation system was used (S9 microsomal system, Moltox), composed of a homogenate of Sprague-Dawley rat liver cells, pretreated with polychlorinated biphenyl mixture (Araclor 1254).