



Sewage sludge does not induce genotoxicity and carcinogenesis

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Abstract

Through a series of experiments, the genotoxic/mutagenic and carcinogenic potential of sewage sludge was assessed. Male Wistar rats were randomly assigned to four groups: Group 1 - negative control; Group 2 - liver carcinogenesis initiated by diethylnitrosamine (DEN; 200 mg/kg i.p.); Group 3 and G4- liver carcinogenesis initiated by DEN and fed 10,000 ppm or 50,000 ppm of sewage sludge. The animals were submitted to a 70% partial hepatectomy at the 3rd week. Livers were processed for routine histological analysis and immunohistochemistry, in order to detect glutathione S-transferase positive altered hepatocyte foci (GST-P⁺ AHF). Peripheral blood samples for the comet assay were obtained from the periorbital plexus immediately prior to sacrificing. Polychromatic erythrocytes (PCEs) were analyzed in femoral bone-marrow smears, and the frequencies of those micronucleated (MNPCEs) registered. There was no sewage-sludge-induced increase in frequency of either DNA damage in peripheral blood leucocytes, or MNPCEs in the femoral bone marrow. Also, there was no increase in the levels of DNA damage, in the frequency of MNPCEs, and in the development of GST-P AHF when compared with the respective control group.

Key words: comet assay, micronucleus, sewage genotoxicity.

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Introduction

Sewage sludge disposal requires due attention, through the risk of toxic-substance content (Ghanbari *et al.*, 2012). Although numerous studies have been dedicated to evaluating the biodegradation potential of waste by chemical or biochemical means, little has been done to investigate the genotoxic/mutagenic and carcinogenic hazards involved (Anne *et al.*, 2011). Since sewage sludge is rich in nutrients, its use for farmland can be highly beneficial. However, the potential impact of this usage on the environment, and on animal and human health has not been adequately evaluated (Tollefson, 2008). In order to protect public health against the adverse effects of metals, pathogens and organic contaminants, and in response to human exposure, sewage sludge use and disposal is now regulated

in many countries. The European Union (EU) Directive 86/278/EEC of June, 1986, established, limits for the total amounts of several heavy metals for land sludge application (European Union, 2000). In 2000, the EU published the third draft of a future sludge directive, fixing even more restricted concentration-limits. In the United States, oceanic dumping of sewage sludge was prohibited in 1992, whereas the amendment for agricultural sludge usage was regulated by 40 CFR Part 503 (USEPA, 1995). In Brazil, the National Environmental Council (CONAMA, 2006), issued a legal norm regulating the agricultural use of sewage sludge. These regulations define acceptable management practices and provide specific limits for selected chemicals and pathogens related to sewage-sludge land application. Nevertheless, with the increase in the release of new complex substances in the environment, it is becoming very difficult to check their toxicity through chemical analysis of target compounds alone. Meanwhile, the scientific community

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continues the search for suitable methods to ensure safe application (Mielli *et al.*, 2009).

The Ito model has proved to be a consistent bioassay for detecting potential hepato-, as well as certain other non-liver, carcinogens (Ito *et al.*, 2003). The endpoint of this 8-week rat-liver assay is the development of altered foci of hepatocytes (AHF), expressing the placental form of enzyme glutathione S-transferase (GST-P⁺ AHF), and considered plausible as early indicators of liver carcinogenesis (Ito *et al.*, 2003). AHFs, described as surrogate hepatocellular preneoplastic lesions, supposedly lead to hepatocellular adenomas and carcinomas (Bannasch and Zerban, 1992; Hasegawa and Ito, 1994). Recently, various types of AHF, with similar morphological and biochemical changes in the cellular phenotype, were identified in chronic human liver disorders, as associated with hepatocellular carcinoma (Su and Bannasch, 2003; Libbrecht *et al.*, 2005).

Among the short-term mutagenicity/genotoxicity assays, the bone marrow micronucleus (MN) and the peripheral blood comet assay are widely used for identifying noxious chemicals (Burlinson *et al.*, 2007; Zeiger, 2010). The difference is basically in variations in the type of DNA alterations detected by the two forms: the MN test detects irreparable lesions that manifest as chromosome aberrations and/or aneugenic effects, whereas the comet assay detects primary DNA lesions. In contrast to the classical MN test, the comet assay is technically simple and fast in detecting genotoxicity in *in vitro* and *in vivo* studies alike (Tice *et al.*, 1991). The version of the alkaline comet assay permits detecting DNA strand breaks, alkaline labile sites, and transient repair sites (Tice *et al.*, 1991).

In this study, a series of experiments were undertaken to assess the mutagenic/genotoxic and carcinogenic potential of sewage sludge through medium-term liver carcinogenesis assay in male Wistar rats. Bone marrow and blood samples were herein used to perform MN tests and comet assays, respectively, as these are employed as target organs in mutagenicity/genotoxicity tests (MacGregor *et al.*, 1987; Nadin *et al.*, 2001; Fukumasu *et al.*, 2006), even though assays involving the liver depend on *in situ* two-step collagenase perfusion techniques for isolating viable hepatocytes (Eckl and Raffelsberger, 1997).

Material and Methods

The present study was approved by the Ethics Committee in Research at the University of São Paulo Medical School (FMUSP), protocol 021/06.

Experimental design

Male Wistar rats were obtained from the “Multidisciplinary Center for Biological Research” (CEMIB) at the State University of Campinas (UNICAMP). The animals were kept in propylene cages measuring 41x34x16 cm (five animals per cage), with autoclaved pine-wood bedding that was changed three times a week. During the experiment,

the rats remained in an environmentally-controlled facility in Botucatu, UNESP Medical School, under the following conditions: room temperature of 22 °C, 55% relative humidity, and 12 h of alternating light/dark cycles, with continuous air exhaustion. They were fed with pelleted chow (Biobase, Bio-tec, Bases Química Ltda., Colombo, PR), and received filtered water *ad libitum*. After a two-week acclimatization period, they were randomly assigned to groups composed of five animals per cage. Body weight and food consumption were registered twice a week throughout the experimental period. Euthanasia was by CO₂ asphyxiation.

Experimental diet mixed with sludge

In natura sewage sludge (SS), obtained through successive samplings, was provided by the Sao Paulo State Environmental Protection Agency (CETESB). These samples, collected from a specific wastewater treatment plant, were kept at -20 °C until mixing with commercial Nuvilab-CR1 (Nuvital/PR) powder chow at 10,000 or 50,000 mg/kg (ppm). The resultant powered mix was transformed into pellets with an industrial mixer (CAF-model M60, Brazil). Ventilation-drying of the pellets was at room temperature for 24 h. Various concentrated diets (Figure 1) were stored in plastic bags, identified, and then freezer-stored at -20 °C for a maximum of 30 days. Selection of the 50,000 mg/kg maximum-dose-level in our feeding study was according to the observed absence of toxicity in previous rat prechronic and chronic studies (Solano *et al.*, 2009; Luvizutto *et al.*, 2010).

Altered Foci of Hepatocytes (AHF) assay

The AHF assay protocol was according to Ito *et al.* (2003) (Figure 1). At the start of the experiment, the initia-

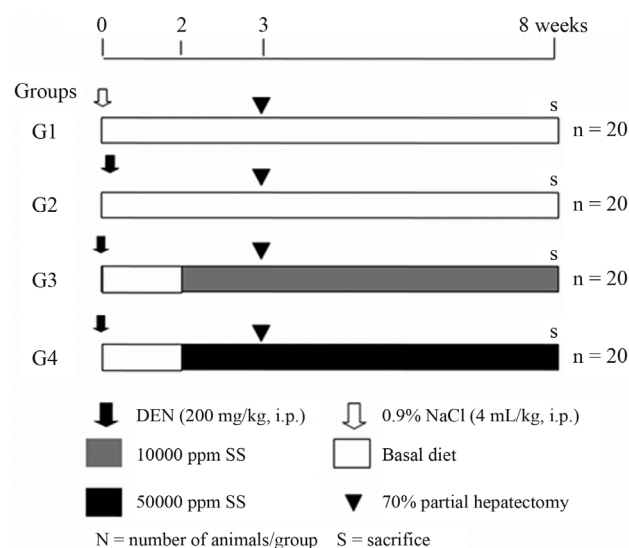


Figure 1 - Experimental design of the rat liver medium-term bioassay. DEN = diethylnitrosamine (200 mg/kg/day, i.p., single dose at the start); SS = basal six-week diet containing either 10,000 ppm or 50,000 ppm of sewage sludge, starting at the 2nd week.

tion of liver carcinogenesis was by single intraperitoneal (ip) injection of 200 mg/kg/body weight of diethylnitrosamine (DEN, CAS 7756; Sigma-Aldrich Co., St. Louis MO, USA). Three groups were thus initiated. The first, an untreated control group, received only 0.9% NaCl (DEN vehicle), whereas the other two received experimental diets containing SS from the 2nd week until the end of the experiment. At the 3rd experimental week, all the animals underwent 70% partial hepatectomy to stimulate regenerative proliferation of hepatocytes. After euthanasia, the liver was removed, blotted dry and weighed. Samples from each lobe were fixed in 10% buffered formalin for 48 h.

Liver histology, immunohistochemistry and foci image analysis

After fixation and paraffin embedding, the liver was sliced into 3-4 μm thick sections and stained by hematoxylin and eosin (HE). Immunohistochemical analysis with the avidine-biotin complex (ABC) method was to detect AHFs (Barbisan *et al.*, 2003), expressing the placental form of the glutathione S-transferase (GST-P) enzyme. Briefly, slides were sequentially treated with 3% H₂O₂ in PBS for 10 min, polyclonal antibody rabbit anti-rat GSTP (Medical and Biological Laboratories Co. Tokyo, Japan) (1:1000 dilution) overnight, biotinylated goat anti-rabbit IgG (1:200 dilution) for 60 min and avidin-biotin-peroxidase solution (1:50 dilution) for 45 min (Elite ABC kit, Vector Laboratory, Burlingame, CA, EUA). Chromogen color development was done with 3-3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., MO, USA), as the substrate, in order to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris hematoxylin, prior to quantitative evaluation in a KS300 image analysis system (CARL ZEISS, Germany) connected to a Nikon optical microscope (AXIOPHOT-X, Japan). The number and area of GST-P positive foci larger or equal to 0.15 mm² in diameter were calculated in each section (Barbisan *et al.*, 2003). After measuring the area of each histological section, the number of GST-P⁺ foci per cm² and of accumulated foci per area (mm²/cm²) was statistically analyzed to check for differences among groups.

Comet assay

The protocol was according to Nadin *et al.* (2001) and Fukumasu *et al.* (2006). The animals treated with MNU received 1 mL per 100 g of body weight of a solution of MNU diluted in 0.9% NaCl at a concentration of 50 mg/kg, administered intraperitoneally in a single dose. Samples were collected 3 h after MNU treatment. Blood samples from all the animals were collected from the periorbital vein with a microcapillary tube. After collection, the material was transferred to an identified microtube, prior to mixing with low melting point agarose (0.5%). An aliquot from each tube was aspirated onto an individual preparation of smears on slides precoated with

normal melting-point agarose (1.5%). For proper cell adhesion, the slides were first left in the refrigerator for 5 min. This was followed by cell lysis, storage in the refrigerator for 24 h, and membrane rupture and DNA release with detergents (n-lauryl sarcosinato 1%), high concentrations of salts (100 mM EDTA, 2.5 M NaCl, 10 mM Tris, pH 10), Dimethylsulfoxide (10%) and Triton X-100 (1%). The slides were then placed in a horizontal electrophoresis apparatus containing an alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na₂EDTA, pH > 13) at 4 °C, for a 20 min unwinding time. Using the same buffer, electrophoresis was performed at 25 V/cm and 300 mA for 20 min. For slide staining, 32 mL of solution A (50gNa₂CO₃ q.sp. 1000 mL double-distilled H₂O), and 68 mL of solution B (0.2 g NH₂NO₃, 0.2 g of AgNO₃, 1 g of tungstosilicic acid, 500 μL of formaldehyde, 1000 mL of double distilled H₂O) were stirred in a bucket for 1 min. After staining, the slides were left for 5 min in a bath solution finisher (1 mL of acetic acid q.s.p 100 mL of distilled water), and then in a double distilled H₂O bath for 1 min. Evaluation of the extent of DNA damage in nucleoids was made using an microscope (200X magnification) connected to the Image Pro-Plus ®, version 4.5 (Media Cybernetics) analysis system.

Micronucleus test

The micronucleus test was done according to a previously established protocol (MacGregor *et al.*, 1987). The animals treated with MNU received 1 mL per 100 g of body weight of a diluted solution of MNU in 0.9% NaCl at a concentration of 50 mg/kg, administered intraperitoneally in a single dose. Sample collection was 24 h later. Briefly, the contents of femoral bone marrow obtained from each animal immediately after euthanasia, were homogenized with 3 mL of fetal-calf serum (Gibco), and centrifuged for 5 min to obtain a homogeneous cell suspension. Drops of this suspension were then smeared onto slides, two per animal. After 24 h of drying at room temperature, the slides were stained by Leishmann (eosin-methylene blue, Merck, Germany). The frequency of micronucleated polychromatic erythrocytes (MNPCEs) was obtained by analyzing 1000 polychromatic erythrocytes (PCEs) per animal. Analysis was by light transmission microscopy (1000X).

Statistical analysis

Statistical analysis was with Jandel Sigma Stat software (Jandel Corporation, San Rafael, CA, USA). Data for body weight and body-weight gain, relative liver weight, amount of food and sewage-sludge consumption, and area of GST-P positive AHF were analyzed with ANOVA or Kruskal-Wallis tests. Comet and micronuclei data were analyzed with Kruskal-Wallis followed by Dunn tests for multiple comparison (comet) and X² (micronucleus). The level of significance was set at 5%.

Results

No significant differences in body weight were detected, except for groups 2, 3 and 4, which exhibited a significant reduction in body weight with respect to group 1 (Figure 2). DEN-initiated groups presented a certain difference in mean body weights when compared to the untreated negative control group, possibly caused by the impact of DEN-treatment and partial hepatectomy. Although no other histological alterations were apparent, all the DEN-treated animals developed altered foci of hepatocytes (Figure 3, Table 1), thereby indicating the effective initiation of liver carcinogenesis. These putative preneoplastic lesions, comprising clear or eosinophilic cell phenotypes (Figure 3A), also expressed the GST-P enzyme (Figure 3B). However, no significant differences were found in GST-P⁺AHF values (number and area) in sewage sludge exposed groups when compared to their respective controls (Table 1). Furthermore, there were no alterations in relative incidences of these types of AHF through treatment with higher concentrations of sewage sludge. The findings from *in vivo* comet assaying and micronucleus tests appear in Figure 4 and Table 2. Significant differences were only observed in groups that were treated with MNU as positive control. No increases in either the frequency of DNA damage in peripheral blood leucocytes or in that of MNPCEs in the femoral bone marrow were induced by sewage sludge.

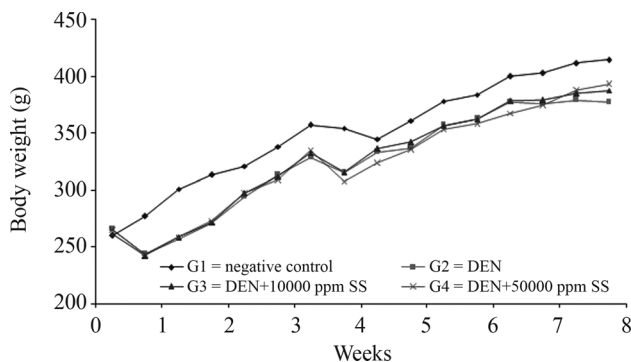


Figure 2 - Evolution of mean body weight (g) of the various groups during the experiment. DEN = diethylnitrosamine (200 mg/kg/day, i.p., single dose at the start); SS = Basal six-week diet containing either 10,000 ppm or 50,000 ppm of sewage sludge, starting at the 2nd week.

Table 1 - Numbers and areas of hepatic preneoplastic GST-P-positive liver foci.

Groups/Treatment	Relative liver weight (%)	GST-P analysis	
		Number (foci/cm ²)	Area (mm ² /cm ²)
0.9% NaCl/Negative Control (n = 19)	3.87 ± 0.49	0	0
DEN/Positive Control (n = 14)	3.96 ± 0.52	6.02 ± 3.20	0.20 ± 2.12
DEN + SS (10.000ppm) (n = 14)	3.86 ± 0.40	2.58 ± 2.13	0.12 ± 0.08
DEN + SS (50.000ppm) (n = 14)	4.23 ± 0.62	5.79 ± 2.72	0.29 ± 0.21

Values are mean ± SD). DEN = diethylnitrosamine (200 mg/kg/day, i.p., single dose at the start); SS = Basal six-week diet containing either 10,000 ppm or 50,000 ppm of sewage sludge, starting at the 2nd week. No statistical difference was observed among DEN-initiated groups when using Kruskal-Wallis.

Results from the chemical analysis of samples of sewage from CETESB treated wastewater appear in Table 3. Final values proved to be within the limits decreed by the Resolution of the Conselho Nacional do Meio Ambiente (CONOMA).

Discussion

The results indicated that the rat medium-term liver carcinogenicity bioassay protocol employed was appropri-

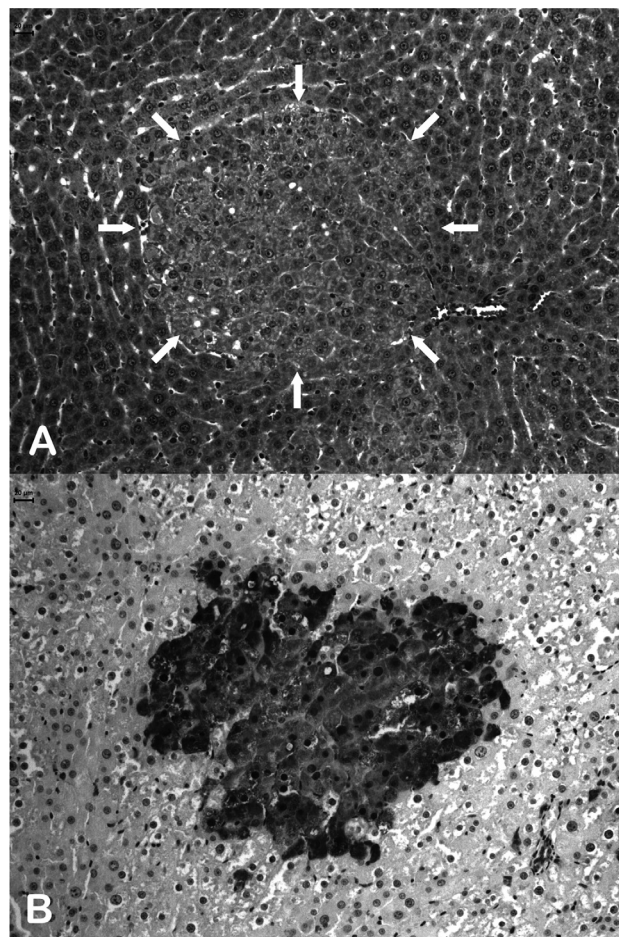


Figure 3 - Diethylnitrosamine-initiated rat liver (x400). A) Eosinophilic cell foci identified by hematoxylin and eosin staining (arrows); B) GST-P-positive foci identified by immunohistochemical staining.

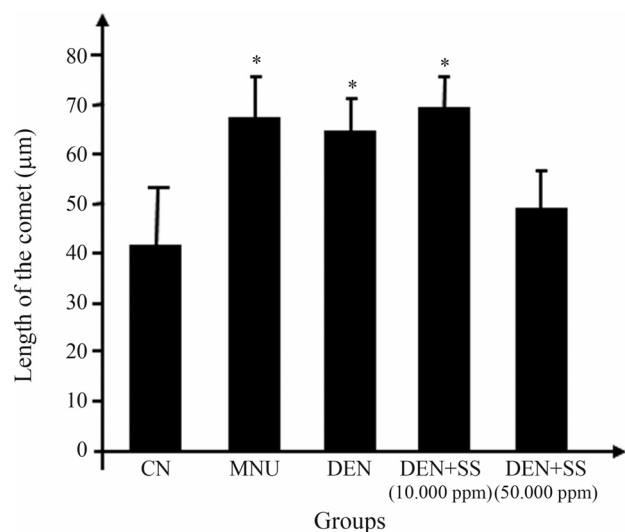


Figure 4 - Measurements of total DNA damage in lymphocytes from rat peripheral blood (values are mean \pm SD). DEN = diethylnitrosamine (200 mg/kg/day, i.p., single dose at the start); SS = Basal six-week diet containing either 10,000 ppm or 50,000 ppm of sewage sludge, starting at the 2nd week. Asterisks indicate a statistical difference from CN group ($p < 0.05$), by Kruskal-Wallis ANOVA followed by the Dunn Test for multiple comparisons.

ate for detecting the inherent carcinogenic risk to the liver from waste-water sludge. Even at high doses (50,000 mg/kg), sludge did not lead to the development of putative preneoplastic lesions in the liver of male Wistar rats initiated by DEN, and as registered by GST-P⁺ AHF development. Furthermore, no significant differences were found as regards GST-P⁺ foci values (number and area) in exposed groups, when compared to their respective controls (Table 3). The putative preneoplastic lesions generated, characterized by clear or eosinophilic cell phenotypes (Figure 3A), also expressed the GST-P enzyme (Figure 3B). Medium-term liver bioassaying, developed in combination with partial hepatectomy, has become well-established for the quick detection of carcinogenic activity in chemicals, as a means of estimating the number and area of preneoplastic S-transferase glutathione, a placental form (GST-P)-positive foci induced by hepatocarcinogens as an endpoint marker (Shirai, 1997; Ito *et al.*, 2003). Compared to the standard long-term carcinogenesis bioassay, such al-

Table 3 - Pontual Chemical values of sewage sludge from WWTP PCJ-1 and the second CONAMA Resolution n^o 375/2006.

Metals	Pontual values (mg/kg)	CONAMA (Resolution n. 375)
Arsenic	< 2	41
Barium	573	1300
Cadmium	5.41	39
Lead	102	300
Copper	317	1500
Chrome	106	1000
Mercury	< 0.1	17
Molybdenum	< 15	50
Nickel	51.7	420
Selenium	4.2	100
Zinc	1095	2800
Aluminum	10240	-
Antimony	187	-
Boron	31.9	-
Cyanide	< 3	-
Cobalt	< 4	-
Tin	< 75	-
Iron	16160	-
Fluoride	5.89	-
Silver	< 1	-
Sulfate	9798.04	-
Sodium	274	-
Vanadium	50.9	-
Total calcium	16310	-
Total phosphorus	6000	-
Total magnesium	1622	-
Total potassium	528	-

ternative assays are quicker, less expensive and may provide additional information on the mode of action of the tested chemicals (Ito *et al.*, 2003). In a previous study in Brazil, from 2004 to 2006, sludge samples collected from the same treatment plant, viz., PCJ1, were tested for genotoxicity using short-term assays. Three out of the five samples tested by the Salmonella microsome assay provided

Table 2 - Frequency of micronuclei (MN) in rat bone marrow.

Groups	MN Frequency	PCEMNs N ^o (%)	Ratio PCE/NCE
0.9% NaCl/negative control (n = 19)	190000	33 (0.33)*	1.22*
MNU/positive control (n = 10)	10000	270 (2.7)	1.07
DEN (n = 16)	160000	21 (0.21)*	1.43*
DEN+ SS (10.000 ppm) (n = 20)	20000	63 (0.63)*	1.63*
DEN + SS (50.000 ppm) (n = 18)	180000	42 (0.42)*	1.44*

DEN = diethylnitrosamine (200 mg/kg/day, i.p., single dose at the start); SS = Basal six-week diet containing either 10,000 ppm or 50,000 ppm of sewage sludge, starting at the 2nd week. * Difference from MNU group ($p > 0.05$) using the Chi-square test.

positive results (1.1 to 2.8 revertants per mg/dry weight). These values are considered relatively low when compared to normal mutagenicity in soil samples (White and Claxton, 2004). Positive results were also obtained with the *Tradescantia* micronucleus test (Mielli *et al.*, 2009). In a previous study, we reported on systemic oral toxicity and genotoxicity in male and female Wistar rats, as evaluated by the comet assay in peripheral blood, and by the bone marrow micronuclei assay (Solano *et al.*, 2009). Furthermore, and in the same laboratory, systemic oral toxicity of treated sewage-sludge samples obtained from a specific wastewater treatment plant had been previously evaluated in male and female Wistar rats (Luvizutto *et al.*, 2010). Findings from the present study also indicated that a 6-weeks treatment with sewage sludge was not genotoxic/mutagenic in male Wistar rats initiated for chemical hepatocarcinogenesis.

According to a chemical analysis undertaken by the Environmental Company of Sao Paulo State (CETESB, 2005), four of the chemically characterized samples from PCJ1 used in this study, with results expressed in mg/kg, were of this compound. The samples collected on 02/04/2007 presented no values above the limits established by the National Environmental Council Resolution 375 (CONAMA/BRASIL, 2006). Furthermore, the relative incidence of these AHFs was not altered by treatment with higher concentrations of sludge. The absence of sewage-sludge carcinogenic potential, as noted in this study, is in line with previous findings from the same laboratory, testifying that sewage-sludge samples collected at the very same specific wastewater treatment plant were not toxic or genotoxic/mutagenic in rodents (Solano *et al.*, 2009; Luvizutto *et al.*, 2010). However, the lower DNA damage level observed in the DEN plus 50000 ppm SS-treated group, in relation to the respective control group (DEN), could indicate a potential DNA cross-linking effect, whence a possible theme for further studies.

Our current results obtained through comet and micronucleus assays following exposure to any of the sludge concentrations tested did not indicate increased levels of DNA damage or frequency of MNPCEs, when compared to non-exposed concurrent positive controls. However, as a precaution before implementing a large-scale program of sewage-sludge use, it is a necessary to investigate beforehand the potential toxicity of the sewage sludge. The present results could contribute to compiling a toxicological profile of specific urban sewage-sludge, as well as providing a base for risk analysis of eventual human exposure, thus aiding in technical decisions for legislation and disposal of that produced in urban areas.

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Internet Resources

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