
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
BIOLOGIA CELULAR E MOLECULAR

AVALIAÇÃO DA TOXICIDADE DE RESÍDUOS INDUSTRIAIS E URBANOS APLICADOS NA AGRICULTURA

CINTYA APARECIDA CHRISTOFOLETTI

Tese apresentada ao Instituto de Biociências do Campus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas (Biologia Celular e Molecular).

Maio - 2013

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Orientadora: Prof^a Dr^a Carmem Silvia Fontanetti Christofolletti

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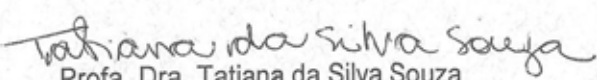
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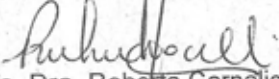
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Rio Claro, 27 de maio de 2013.


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*Para minha família,
que compartilha todos
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a caminhada,
as conquistas e realizações.
A eles, por tudo que têm me
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“O valor das coisas não está no tempo em que elas duram, mas na intensidade com que acontecem. Por isso, existem momentos inesquecíveis, coisas inexplicáveis e pessoas incomparáveis” (Fernando Pessoa).

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“Estou sempre alegre, essa é a melhor maneira de vencer os obstáculos da vida”.
Charles Chaplin

RESUMO

A degradação de solos provocada pelo mau gerenciamento das atividades humanas consiste em uma constante e crescente preocupação. O tratamento adequado e disposição final do grande volume de resíduos urbanos, industriais e agrícolas produzidos diariamente são um grande desafio à comunidade científica. Neste sentido, vem sendo pesquisadas inúmeras alternativas para o aproveitamento e destinação ambientalmente segura de tais resíduos, dentre as quais se destaca a aplicação destes como fertilizantes agrícolas. Tendo isso em vista, o presente trabalho teve por objetivo simular a dose de aplicação em cultura de cana-de-açúcar, de amostras de lodo de esgoto primário, biossólido e vinhaça de cana-de-açúcar, de acordo com a legislação brasileira vigente, e verificar seus potenciais tóxico, citotóxico, genotóxico e mutagênico, tanto das amostras brutas quanto de combinações destas com amostras de solo controle, antes e após o seu bioprocessamento por diplópodos. As amostras foram analisadas antes da exposição à diplópodos, designado por tempo 0 (t0 – momento da mistura) e após o bioprocessamento destas pelos animais (t30 – 30 dias após a exposição aos diplópodos). As análises químicas das amostras demonstraram diferentes concentrações de metais e macro/micronutrientes, evidenciando o bioprocessamento destes resíduos pelos animais. Para avaliar a toxicidade das diferentes combinações dos resíduos foram avaliadas a taxa de mortalidade e o comportamento dos indivíduos, e realizadas análises histológicas, histoquímicas e ultra-estruturais do intestino médio dos diplópodos, após 7, 30 e 90 dias de exposição. Os resultados obtidos nas avaliações histológica, histoquímica e ultra-estrutural, evidenciaram a toxicidade dos resíduos, observada por diferentes alterações como aumento nas taxas de renovação epitelial, espessamento do bordo em escova, acúmulo de grânulos citoplasmáticos nas células hepáticas dos animais e grupamentos de hemócitos por entre as células hepáticas, indicando um processo inflamatório severo, pois foram observadas ainda a presença de células hepáticas com núcleos heteropicnóticos e degradação citoplasmática. Adicionalmente, a toxicidade, citotoxicidade, genotoxicidade e mutagenicidade dos diferentes resíduos, antes e após o bioprocessamento, foram avaliadas por meio do sistema teste de *Allium cepa*. Embora somente algumas das amostras tenham se mostrado tóxicas ou citotóxicas, a genotoxicidade foi verificada para todas as amostras estudadas, por meio da quantificação das aberrações cromossômicas. A mutagenicidade, mensurada pela presença de quebras cromossômicas e micronúcleos nas células meristemáticas e micronúcleos nas células da região F₁, também foi verificada na maioria das amostras, provavelmente devido à ação de metais pesados. Contudo, os resultados apontaram para uma redução da genotoxicidade e

mutagenicidade após o bioprocessamento. Portanto, os resultados evidenciam que o bioprocessamento de amostras ambientais complexas pode ser uma alternativa antes da disposição destes na agricultura, uma vez que tais amostras apresentam perigo do ponto de vista ecotoxicológico, mesmo apresentando concentrações de contaminantes abaixo do exigido pela legislação. Logo, devem ser realizados novos estudos quanto à dosagem de aplicação destes, seus efeitos em outros organismos e em diferentes compartimentos, com o intuito de serem melhor empregados no condicionamento de solos agricultáveis e/ou como fertilizantes.

Palavras-chave: *Allium cepa*, *Rhinocricus padbergi*, histopatologia, bioprocessamento

ABSTRACT

The soil degradation caused by poor management of the human activities comprises a constant and growing concern. The proper treatment and final disposal of large volumes of industrial and agricultural wastes daily-produced are a big challenge to the scientific community. In this sense, several alternatives for the environmentally safe use and disposal of such waste have been studied, among which stands out the application as an agricultural fertilizer. Keeping this in view, the present study aimed to simulate the primary sewage sludge, biosolid and sugar-cane vinasse application rate in sugar-cane culture, according to the Brazilian legislation and checking their toxic, cytotoxic, genotoxic and mutagenic potential of both crude samples and combinations thereof with control soil samples, before and after its bioprocessing by diplopods. The samples were analyzed before exposure to diplopods, designated as time 0 (t_0 – time of mixture) and after being bioprocessed by these animals (t_{30} – 30 days after diplopod's exposure). The samples chemical analysis showed different concentrations of metals and macro/micronutrients, making evident the residues bioprocessing. Diplopods' behavior and mortality rate were evaluated to assess the toxicity of different residues combinations, as well as the histology, histochemistry and ultra-structure of diplopods midgut after 7, 30 and 90 days of exposure. The results obtained in the histological, histochemical and ultra-structural evaluations demonstrated the waste toxicity. It was observed through several changes, like increased epithelial renewal rates, thickening of the brush border, accumulation of cytoplasmic granules in the hepatic cells and hemocytes grouping among hepatic cells, indicating severe inflammation, seeing that they were still observed the presence hepatic cells with heteropycnotic nuclei and cytoplasmic degradation. Moreover, the toxicity, cytotoxicity, genotoxicity and mutagenicity of different residues before and after the bioprocessing were evaluated using the *Allium cepa* test system. Although only few samples have been shown to be toxic or cytotoxic, the genotoxicity was observed for all samples, by means of chromosomal aberrations quantification. The mutagenicity has been measured by the presence of chromosome breaks and micronuclei in meristematic cells and micronuclei in F_1 region cells. It was also found in most samples, probably due to the heavy metals action. However, the results indicated a reduction of genotoxicity and mutagenicity after bioprocessing. Therefore, these results highlight that the bioprocessing of the complex environmental samples can be an alternative before the agricultural disposal of this kind of waste, since these samples showed ecotoxicological hazard, even with contaminant concentrations below that required by law. Then, further studies regarding the application

rate, effects in other organisms and in different compartments should be performed, in order to be better employed in agricultural soils reconditioning and as fertilizers.

Key-words: *Allium cepa*, *Rhinocricus padbergi*, histopathology, bioprocessing

LISTA DE SÍMBOLOS E ABREVIATURAS

APMax	Valores orientadores para cenários de exposição agrícola - área de proteção máxima para solo (mg/kg) e água subterrânea no Estado de São Paulo, segundo a CETESB (195/2005-E)
As	Arsênio
B	Biossólido
Ba	Bário
Ca	Cálcio
Cd	Cádmio
CETESB	Companhia de Tecnologia de Saneamento Ambiental do Estado de São Paulo
cmol _c /dm ³	Centimol de carga por decímetro cúbico
CONAMA	Conselho Nacional do Meio Ambiente
CPM	Concentração máxima permitida no lodo de esgoto ou produto derivado, de acordo com o CONAMA (375/2006)
Cr	Cromo
CTC	Capacidade de troca catiônica
Cu	Cobre
DBO	Demanda bioquímica de oxigênio
DQO	Demanda química de oxigênio
EPA	Environmental Protection Agency
ETE	Estação de Tratamento de Esgoto
Fe	Ferro
FM	Fração de mineralização do nitrogênio
H+Al	Acidez pontecial
ha	hectare
Hg	Mercúrio
IAC	Índice de aberrações cromossômicas
ICASA	Instituto Campineiro de Solo e Adubo – Campinas/SP
IM	Índice mitótico
IMt	Índice de mutagenicidade
K	Potássio
ks	Concentração de potássio no solo, à profundidade de 0 a 0.80 m
kV	Quilovolt
kvi	Concentração de potássio na vinhaça, expressa em kg de K ₂ O /m ³
L	Lodo de esgoto primário
LQ	Limite de quantificação
Mg	Magnésio
Mn	Manganês
Mo	Molibdênio
MO	Matéria orgânica
N	Nitrogênio
Ndisp	Nitrogênio disponível
NKj	Nitrogênio Kjeldahl
NNH ₃	Nitrogênio amoniacal
NNO ₂	Nitrogênio - Nitrito
NNO ₃	Nitrogênio - Nitrato
P	Fósforo
Pb	Chumbo
S	Enxofre
SB	Solo+biossólido

SBV	Solo+biossólido+vinhaça
Se	Selênio
SL	Solo+lodo de esgoto primário
SLV	Solo+lodo primário+vinhaça
SM21	Standard Methods for the Examination of Water and Wastewater 21 th edition 2005
SV	Solo+vinhaça
T0	Tempo 0
T30	Após 30 dias de processamento das amostras pelos diplópodos
TASQA	TASQA – Serviços Analíticos Ltda – Paulínia/SP
TFSA	Terra fina seca ao ar
UNESP	Universidade Estadual Paulista
V	Vinhaça de cana-de-açúcar
V%	Saturação por bases
VI	Valor inconsistente
Zn	Zinco

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1. INTRODUÇÃO E RELEVÂNCIA DO TEMA

A contaminação do solo por resíduos complexos, como àqueles derivados da indústria sucroalcooleira e das estações de tratamento de esgoto, constitui um dos maiores desafios relacionado à qualidade do meio ambiente, principalmente no tocante ao volume gerado, tratamento adequado, disposição final e reutilização dos mesmos. Todavia, ambos os resíduos precisam ser adequadamente tratados e dispostos, com o intuito de evitar a contaminação ambiental. Considerando a destinação final destes, a vinhaça apresenta algumas alternativas de uso, destacando o seu potencial na fertilização dos solos. No entanto, muitos estudos comprovam que o seu uso pode provocar modificações nas propriedades físicas e químicas do solo, como: favorecer o aumento da disponibilidade de alguns elementos para as plantas e aumentar a capacidade de infiltração da água no solo, causando a lixiviação de íons e/ou elevação do escoamento superficial, com possível contaminação de águas superficiais e subterrâneas.

Já o lodo de esgoto apresenta outras alternativas, além do uso na recuperação de solos degradados, uso agrícola e florestal como fertilizantes, podem ser destinados para depósito em aterros sanitários, reuso industrial (fabricação de agregado leve, cimento, tijolos, entre outros, que deve ser realizado com cautela), incineração e conversão em óleo combustível. Para ser destinado à agricultura, o lodo de esgoto gerado pode ainda passar por processos com a finalidade de aumentar o teor de sólidos e diminuir o número de organismos patogênicos, gerando um resíduo denominado biossólido (HAYNES et al., 2009).

Entretanto, tanto a vinhaça quanto o lodo de esgoto, produzidos diariamente e em larga escala, podem apresentar alto poder poluente; a vinhaça pela acidez, alta quantidade de sais e alta DBO (WALISZEWSKI et al., 1997; ESPAÑA-GAMBOA et al., 2011) e o lodo, pela possibilidade de conter metais pesados, compostos orgânicos tóxicos e persistentes, bem como agentes causadores de doenças (ANDREOLI et al., 1998; BETTIOL; CAMARGO, 2006).

Neste sentido, têm sido empregadas inúmeras tecnologias que se baseiam na remoção ou redução, completa ou parcial, de poluentes ambientais por meio de organismos vivos. A biorremediação do lodo de esgoto é uma alternativa ambientalmente viável para a disposição segura desse resíduo em solos agrícolas, como para sua reutilização. Logo, encontram-se padronizados testes com anelídeos, uma vez que as minhocas conseguem reduzir a

concentração de metais presentes no lodo. Contudo, devido ao aumento das fontes de contaminação ambiental, vêm sendo desenvolvidos testes com outros organismos.

Assim sendo, diplópodos, organismos que desempenham papel semelhante ao das minhocas no solo, poderiam atuar na biorremediação de diferentes resíduos, tal qual a vinhaça da cana-de-açúcar, o lodo de esgoto, landfarming, dentre outros. Tais animais auxiliariam na produção de húmus a partir da matéria orgânica, podendo bioacumular e metabolizar metais presentes nestes resíduos.

A eficácia das tecnologias de bioprocessamento e ecotoxicidade tem sido comumente avaliada por meio de análises químicas. Entretanto, essas análises não fornecem informações sobre a ecotoxicidade dos locais avaliados após a biorremediação e/ou bioprocessamento dos diferentes resíduos pelos organismos. Logo, informações complementares, como as fornecidas pela avaliação de diferentes biomarcadores, nos mais diversos níveis de organização biológica têm sido cada vez mais requeridas.

Estudos de genotoxicidade e histopatologia têm se mostrado ferramentas importantes e eficientes na avaliação da qualidade ambiental. Os potenciais citotóxico, genotóxico e mutagênico de diferentes amostras ambientais têm sido mensurados pela indução de células ao processo de morte celular, alterações no índice mitótico, presença de aberrações cromossômicas e micronúcleos, em células de *Allium cepa*. Em contrapartida, a histologia, histoquímica e ultra-estrutura de órgãos responsáveis pela absorção de nutrientes e detoxicação, de diferentes animais, tem sido aplicadas na avaliação da toxicidade destas mesmas amostras.

Desse modo, sendo a vinhaça e o lodo de esgoto, ambos resíduos utilizados na agricultura, com estudos comprovando a toxicidade dos mesmos, o presente trabalho teve por objetivo geral avaliar os potenciais citotóxico, genotóxico e mutagênico desses resíduos em sementes de *Allium cepa*, antes e após o processamento destas amostras por diplópodos, assim como avaliar a possível toxicidade destes, no intestino médio destes invertebrados.

2. OBJETIVOS

O objetivo geral desse trabalho foi avaliar a toxicidade de amostras brutas de vinhaça de cana-de-açúcar, lodo de esgoto primário e biossólido, assim como da mistura de tais resíduos a amostras de solo controle, em dois períodos de exposição a diplópodos (t0 - momento da mistura dos resíduos e t30 - 30 dias após o início da exposição).

Os objetivos específicos foram:

- Avaliar a toxicidade, citotoxicidade, genotoxicidade e mutagenicidade da vinhaça, do lodo de esgoto primário e do biossólido, antes e após o processamento destes por diplópodos, por meio do teste de aberrações cromossômicas e micronúcleo, em células meristemáticas de *Allium cepa*, e do teste do micronúcleo em células da região F₁ deste mesmo organismo-teste, expostos diretamente às combinações dos resíduos ao solo, segundo norma P4.231 da CETESB, para a vinhaça e a resolução 375/2006, do CONAMA, para o lodo de esgoto e biossólido.
- Investigar a toxicidade dos resíduos vinhaça de cana-de-açúcar, lodo de esgoto primário e biossólido, por meio de estudos histológicos e histoquímicos no intestino médio de exemplares de *Rhinocricus padbergi* (Diplopoda) expostos diretamente às combinações de resíduos ao solo, segundo norma P4.231 da CETESB, para a vinhaça e a resolução 375/2006, do CONAMA, para o lodo de esgoto, verificando possíveis alterações morfológicas e/ou fisiológicas, quando comparados aos indivíduos expostos a um solo controle.
- Analisar a ultra-estrutura do intestino médio do diplópodo *R. padbergi*, por meio da microscopia eletrônica de transmissão, para detalhamento da constituição e organização das células deste órgão e para o entendimento da ação destes resíduos sobre as organelas/células, frente às alterações observadas na análise histológica.
- Verificar a viabilidade do uso de *R. padbergi* como bioindicador de solo e a análise morfológica de seu intestino médio como biomarcador, bem como sua eficácia no processamento destes resíduos.

3. REVISÃO DA LITERATURA

3.1 A problemática da contaminação de solos

O solo é a superfície inconsolidada que recobre as rochas e mantém a vida animal e vegetal da Terra. É constituído de camadas que diferem pela natureza física, química, mineralógica e biológica, que se desenvolve com o tempo sob a influência do clima e da própria atividade biológica (VIEIRA, 1975). É um sistema dinâmico e complexo, uma vez que serve como habitat para microrganismos, flora, fauna e humanos. Dentre outras funções, constitui uma fonte de alimento para diferentes grupos de organismos, atua como coletor de resíduos orgânicos e inorgânicos, seqüestrando compostos perigosos e atuando como manto protetor para as águas subterrâneas (SOUSA et al., 2008).

Durante as últimas décadas do século XX, houve a confirmação de que o solo não é um recurso inesgotável e, se utilizado inadequadamente ou mal administrado, tal sistema pode perder suas características em um curto período de tempo, com capacidades limitadas de regeneração (NORTCLIFF, 2002; SOUZA et al., 2013a). O uso inadequado do solo pode levar a necessidade de utilização de práticas que acabam gerando importantes fontes de poluição. O manejo não apropriado de defensivos agrícolas, águas de irrigação de baixa qualidade e a disposição indiscriminada de resíduos industriais ou domésticos podem provocar o acúmulo de substâncias que podem ser tóxicas às plantas e, ao entrar na cadeia alimentar, perigosos aos animais expostos e, conseqüentemente, ao homem. Em virtude dessas relações e da sua importância no ecossistema, o solo ocupa papel de destaque no controle da qualidade do ambiente (CAMARGO; DENARDIN, 2013).

Quando atingem o solo, seja proposital ou acidentalmente, os contaminantes sofrem a ação de fenômenos geoquímicos e biológicos, que os distribuem pela subsuperfície nas fases vaporizada, residual ou adsorvida, fase livre e fase dissolvida. A distribuição de tais fases depende das características físico-químicas e do tipo de solo (SILVA et al., 2004). Dessa forma, o comportamento dos contaminantes e, conseqüentemente, o seu potencial tóxico estão diretamente relacionadas à capacidade que o solo possui em mantê-los retidos em sua fase sólida, tornando-os indisponíveis para serem absorvidos pelas plantas, erodidos e/ou lixiviados (McBRIDE, 1995). Outros fatores, tais como a área superficial e as cargas elétricas das partículas do solo, podem determinar a ligação dos contaminantes a essa matriz.

De acordo com Lavonreti (1996) há vários processos de interação entre os diferentes contaminantes e o solo. Sucintamente, podemos citar estão as retenções por adsorção, absorção ou precipitações, a transformação biótica ou abiótica, o transporte por volatilização, lixiviação ou escoamento superficial. No entanto, a manutenção da qualidade do ecossistema terrestre está diretamente relacionada com o estudo das relações entre poluentes ambientais e organismos que desempenham papéis importantes no solo, tais como decomposição, fluxo de nutrientes e energia (SOCHOVÁ et al., 2006). Portanto, essas atividades podem ser afetadas devido à crescente contaminação do solo, decorrente das crescentes atividades industriais, agrícolas e urbanização.

A gestão dos resíduos no Brasil tem merecido destaque visto os impactos e/ou riscos ambientais representados por suas diversas fases, desde a coleta até o destino final dos mesmos. Dentre os impactos ambientais relacionados com o manejo e disposição inadequada desses resíduos, está a poluição química e biológica do solo, da água e do ar, com consequências na biodiversidade, na estrutura e funcionamento dos ecossistemas (BETTIOL; CAMARGO, 2006). Logo, inúmeras pesquisas vêm sendo realizadas no mundo todo, visando amenizar os efeitos da disposição dos mais diversos resíduos no solo. Dentre os processos mais utilizados, temos o físico, o químico e o biológico. De acordo com Sarkar et al. (2005), o método físico mais comum para o tratamento de solos contaminados é a disposição em *landfill* (disposição em aterro) e a incineração. No entanto, a incineração pode ser considerada uma fonte de poluição do ar. Os tratamentos químicos se baseiam na injeção direta de químicos oxidantes na matriz contaminada (RISER-ROBERTS, 1998). Já os processos biológicos surgiram na tentativa de transformar os contaminantes tóxicos em formas não-tóxicas, por meio de processos microbiológicos (RISER-ROBERTS, 1998); dentre eles temos a biorremediação.

A biorremediação pode ser definida como o uso de organismos vivos para remover poluentes ambientais do solo e da água (COLLIN, 2001; SARKAR et al., 2005). É frequentemente monitorada pelo acompanhamento dos contaminantes alvo. Entretanto, sua redução não indica, necessariamente, uma diminuição da toxicidade do solo. A degradação incompleta e a formação de metabólitos intermediários tóxicos podem resultar em um aumento da toxicidade do solo durante este processo (SOUSA et al., 2008).

Tecnologias de biorremediação são atrativas devido à enorme capacidade do solo reter contaminantes tóxicos e degradá-los por meio da atividade microbiana. Porém, informações inadequadas sobre a toxicidade do resíduo a ser tratado, o desconhecimento de seu comportamento pós-disposição no solo, assim como planejamento e manejo inadequados da

biorremediação, também têm contribuído seriamente para a contaminação do meio terrestre (WHITE; CLAXTON, 2004; SOUZA et al., 2011).

A compostagem é um sistema de biorremediação que leva à formação de produtos mineralizados, CO₂ e H₂O, e à estabilização da matéria orgânica, na forma de substâncias húmicas, contendo biomassa microbiana (CAI et al., 2007). Para acelerar esse processo, algumas modificações têm sido realizadas, como a técnica de vermicompostagem. Nesse processo, minhocas ingerem e digerem resíduos orgânicos presentes no resíduo com o auxílio da microflora aeróbia e anaeróbia presentes no intestino desses animais. O produto gerado é estável e homogêneo, além de, geralmente, apresentar redução considerável da concentração de contaminantes (GUPTA; GARG, 2008). Durante esse processo, nutrientes importantes, tais como N, P, K e Ca, presentes no resíduo, são convertidos em formas mais solúveis e disponíveis para as plantas (NDEGWA; THOMPSON, 2001). Além da redução de compostos orgânicos, a vermicompostagem é principalmente utilizada para reduzir a concentração de metais presentes no resíduo, uma vez que minhocas bioacumulam tais contaminantes (JAIN et al., 2004; SRIVASTAVA et al., 2005). Contudo, devido ao aumento das fontes de contaminação ambiental, testes com outros animais vêm sendo também desenvolvidos.

Está disponível na literatura, vasto número de diferentes bioensaios para a avaliação de solos. Os organismos utilizados como bioindicadores podem fornecer informações diretas, baratas e integradas da biodisponibilidade e da toxicidade de misturas complexas de contaminantes. Dentre os bioindicadores mais utilizados na avaliação de solos contaminados por diferentes resíduos estão os microrganismos, plantas e invertebrados (MAILA; CLOETE, 2005; SOUZA; FONTANETTI, 2011). A resposta obtida com os bioindicadores pode indicar a presença, os efeitos e, em alguns casos, o grau de contaminação de uma área. Desse modo, a indução de um efeito adverso significativo em um biomarcador pode ser considerada como um sinal de alerta da contaminação ambiental (WALKER et al., 1996 apud KLEMZ, 2002).

3.2 Diplópodos como bioindicadores de contaminação de solos

Dentre os organismos do sistema edáfico, os invertebrados têm sido considerados excelentes indicadores da qualidade de solos, uma vez que estes permanecem em contato direto com todos os contaminantes presentes no meio (TRIEBSKORN et al., 1991; HEIKENS et al., 2001). Não obstante, bioensaios com a fauna terrestre são relativamente simples de serem realizados, os taxa estão largamente distribuídos e o tamanho e movimentação de

muitas espécies resolve problemas associados com a heterogeneidade espacial da contaminação do solo (PATON et al., 2006).

Frente à praticidade e custos menos onerosos, a disposição final de resíduos potencialmente tóxicos no solo, tem sido, há tempos uma prática comum, que vem ocasionando alterações na comunidade de artrópodos (van STRAALLEN, 2004; FONTANETTI et al., 2011). Estas espécies podem apresentar alterações biológicas individuais (fisiológicas, morfológicas e comportamentais), as quais podem ser extrapoladas para estudos em campo a fim de analisar aspectos ecológicos, como dinâmica populacional e riqueza de diversidade nas áreas contaminadas. Portanto, a reunião de estudos biológicos, tanto laboratoriais quanto em campo, aliada às análises químicas dos contaminantes, fornece um panorama real dos efeitos que substâncias tóxicas podem causar neste ecossistema (SOUZA et al., 2013a).

Nesse sentido, têm sido utilizadas as mais diversas espécies de invertebrados terrestres como bioindicadores nas avaliações de ecotoxicologia terrestre, sobretudo nemátodos (SOCHOVÁ et al., 2006), minhocas (NATAL DA LUZ et al., 2004) e colêmbolas (EOM et al., 2007). Todavia, atualmente, devido ao aumento das fontes de contaminação, outras espécies com potencial bioindicador têm sido requeridas (LØKKE; VAN GESTEL, 1998; EOM et al., 2007).

A classe Diplopoda é constituída por artrópodos terrestres conhecidos popularmente, no Brasil, como piolhos-de-cobra, caramujis, gongôlos, emboás, dentre outros. Compreendem cerca de 80.000 espécies (HOFFMAN et al., 2002), embora as estimativas evidenciem que apenas 15% destas estejam descritas. Seus representantes estão distribuídos por todo o mundo, mas habitam preferencialmente os trópicos. Esses animais possuem hábito noturno, vivem em ambientes úmidos, sendo encontrados sob troncos e folhas caídas no solo; se alimentando de matéria orgânica, detritos, frutas e relativa quantidade de matéria mineral (SCHUBART, 1942; HOFFMAN et al., 2002; RUPPERT; BARNES, 2005).

A maioria dos Diplopoda é detritívora, podendo ocupar o nível trófico de decompositores (PETERSON; LUXTON, 1982) e colonizar diferentes camadas do solo. Participam da ciclagem e disposição de nutrientes presentes na matéria orgânica em decomposição, auxiliando na humificação do substrato, desempenhando, assim, importante papel na redução de material vegetal e formação da parte orgânica do solo (HOPKIN; READ, 1982; HOFFMAN et al., 1996). Pelas fezes, eles promovem a mineralização do solo, uma vez que secretam amônia e ácido úrico que, quando degradados, enriquecem o solo com nitratos (SCHUBART, 1942; GODOY; FONTANETTI, 2010). Desse modo, estimulam o

metabolismo microbiano, essencial para a ciclagem de nutrientes, como carbono, nitrogênio fósforo, além de promoverem a aeração ativa do solo (HOPKIN; READ, 1992; FONTANETTI et al., 2012).

Devido ao seu hábito alimentar, os diplópodos possuem ainda a habilidade de acumular metais pesados. Em diplópodos, a concentração corpórea de metais e as taxas de assimilação dos mesmos varia de espécie para espécie (HOPKIN et al., 1985; HOPKIN; READ, 1992). Estudos de bioacumulação de metais, realizados com diplópodos, tem sugerido que estes organismos são bons biondicadores da poluição de solos, logo úteis para a avaliação ecotoxicológica de solos (GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; PEREZ; FONTANETTI, 2011; SOUZA; FONTANETTI, 2010).

Hubert (1978; 1979) foi o primeiro pesquisador a identificar as estruturas onde os metais são armazenados pelos diplópodos. Geralmente, os grânulos contendo metais encontram-se associados a órgãos com funções digestória, de armazenamento e de excreção, como o intestino médio, células hepáticas, corpo gorduroso e túbulos de Malpighi. Logo, órgãos relacionados com a absorção e assimilação de nutrientes essenciais, tais como lipídios, carboidratos e proteínas, têm sido preferencialmente utilizados na avaliação de alterações tissulares e celulares decorrentes de contaminação ambiental (HOPKIN, 1989; PIGINO et al., 2005).

Em diplópodos, o intestino médio tem sido um dos órgãos alvo para estes estudos. Este órgão possui um importante papel nos processos de detoxicação e excreção de xenobióticos (HOPKIN et al., 1985; TRIEBSKORN et al., 1991; KÖHLER; TRIEBSKORN, 1998), pois funciona como uma barreira, impedindo que compostos tóxicos ou não essenciais, alcancem o restante do corpo (HOPKIN et al., 1985; TRIEBSKORN et al., 1991; KÖHLER; TRIEBSKORN, 1998; GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; 2011). Todavia, ao ingerirem solo ou alimentos altamente contaminados, compostos indesejáveis podem ultrapassar a barreira criada pelo epitélio, promovendo diversas alterações.

Foram estudados por Godoy e Fontanetti (2010), Perez e Fontanetti (2011), Nogarol e Fontanetti (2010; 2011) e Bozzatto e Fontanetti (2012) alterações tissulares no intestino médio do diplópodo *Rhinocricus padbergi*, decorrentes da sua exposição a solo contaminado com diferentes proporções de lodo de esgoto. Souza e Fontanetti (2011) também avaliaram as alterações tissulares no intestino médio de *R. padbergi*, após exposição a solo contaminado com landfarming, um resíduo oriundo das refinarias de petróleo. Merlini et al. (2012) avaliou a toxicidade do herbicida trifluralina, utilizando *R. padbergi* como biondicador, por meio de

alterações histopatológicas do intestino médio. As respostas mais significativas observadas nestes trabalhos foi aumento na taxa de renovação epitelial, aumento na liberação de vesículas de secreção para o lúmen, acúmulo de grânulos intracitoplasmáticos nas células hepáticas e aumento na ocorrência de hemócitos por entre as células hepáticas. As diversas alterações observadas pelos diferentes autores podem ser interpretadas como mecanismos de defesa desenvolvidos pelo animal, relacionado com processo de neutralização e eliminação de resíduos tóxicos. Por conseguinte, as alterações observadas indicam que esta resposta pode ser utilizada como um biomarcador de contaminação ambiental, não só para *R. padbergi*, mas também para outras espécies de diplópodos.

3.3 *Allium cepa* como organismo-teste

Os vegetais superiores apresentam características peculiares que os tornam excelentes modelos genéticos para avaliação de contaminantes ambientais (LEME; MARIN-MORALES, 2009); por esse motivo, têm sido utilizados frequentemente em estudos de monitoramento ambiental ou ainda no entendimento da ação de químicos sobre o material genético destes. Ainda assim, esse destaque não se deve, apenas, à sensibilidade na detecção de diferentes mutágenos nos mais distintos ambientes, como também a possibilidade de empregar diferentes células e órgãos como biomarcadores genéticos, capazes de detectar desde mutações pontuais até as aberrações cromossômicas (GRANT, 1994; LEME; MARIN-MORALES, 2009).

O uso de *A. cepa* como sistema teste foi originalmente introduzido por Levan, em 1938, quando este demonstrou que a colchicina poderia causar distúrbios no fuso mitótico, levando a uma poliploidização das células meristemáticas das raízes de *A. cepa*. Mais tarde, este mesmo autor mostrou que diferentes soluções de sais orgânicos induziam diversos tipos de aberrações cromossômicas nas células meristemáticas das raízes desse vegetal (LEVAN, 1945). Desde então, têm sido realizadas adaptações na metodologia do teste de *A. cepa* possibilitando uma avaliação e classificação mais abrangente de químicos, sendo eles misturas complexas, como o caso de grande parte das amostras ambientais (COTELLE et al., 1999; GROVER; KAUR, 1999; MATSUMOTO; MARIN-MORALES; 2004; GRISOLIA et al., 2005; EGITO et al., 2007; CARITÁ; MARIN-MORALES, 2008; LEME; MARIN-MORALES, 2008; SOUZA et al., 2009; 2013b; BIANCHI et al. 2011; CHRISTOFOLETTI et al., 2013) ou ainda de inúmeras substâncias (FISKEJÖ, 1985; RANK et al, 1993; GRANT, 1994; MA et al., 1995; KONUK et al., 2007; FERNANDES et al., 2007; 2009; YILDIZ et al. 2009; LIMAN et al., 2010; 2011; 2012; MAZZEO et al., 2011; ÖZKARA et al., 2011).

Apesar das diferenças estruturais e metabólicas, as plantas apresentam inúmeras características que as tornam excelentes organismos-teste. A cebola, por exemplo, tem o seu ciclo celular bem conhecido, grande número de células em divisão, rápido crescimento de raízes, tolerância às diversas condições de cultivo, disponibilidade, fácil manuseio e, por possuir cromossomos em número reduzido ($2n=16$) e de grande tamanho, pode ser utilizada nos estudos de avaliação de danos cromossômicos e/ou de distúrbios do ciclo de divisão celular, incluindo riscos de aneuploidia (FISKEJÖ, 1985; QUINZANI-JORDÃO, 1987; GRANT, 1994; EVSEEVA et al., 2003; EGITO et al., 2007; LEME; MARIN-MORALES, 2009).

As células meristemáticas de *A. cepa* constituem um material citogenético eficaz para analisar aberrações cromossômicas causadas pela poluição ambiental (KRISTEN, 1997), uma vez que podemos quantificar uma série de parâmetros morfológicos e citogenéticos, incluindo a morfologia e o crescimento da raiz, a determinação do índice mitótico, a indução de micronúcleos e de metáfases, anáfases e telófases aberrantes (GRANT, 1994; EVSEEVA et al., 2003; EGITO et al., 2007; LEME; MARIN-MORALES, 2009).

O teste de *A. cepa*, além de todas as vantagens mencionadas acima, tem mostrado alta sensibilidade e boa correlação, quando comparado com outros sistemas teste, principalmente com os de mamíferos. Segundo Grant (1982), de 148 químicos avaliados pelo teste de *Allium*, 76% apresentaram respostas positivas, o que levou o autor a sugerir a inclusão deste teste como rotineiro de triagem para determinação de danos cromossômicos induzidos por químicos. De acordo com CHAUHAN et al. (1999), o *A. cepa* constitui um dos melhores sistemas-teste já estabelecidos, aplicados rotineiramente na avaliação do potencial genotóxico de químicos no ambiente, devido à sua boa correlação com outros sistemas-teste.

Buscando corroborar a eficiência na detecção de diferentes substâncias com potenciais tóxico, citotóxico, genotóxico e mutagênico pelo organismo-teste *A. cepa* e firmar o seu uso como bioindicador, Leme e Marin-Morales (2009) publicaram uma revisão sobre a aplicação dos testes com *A. cepa* no monitoramento ambiental de diferentes classes de poluentes. Com base nas informações fornecidas nesta revisão, as autoras demonstraram que além de ser um ensaio rápido e sensível na detecção de xenobiontes, ele também permite a avaliação de mecanismos de ação dos agentes testados sobre o DNA dos organismos expostos. Logo, este ensaio proporciona um importante método de triagem de contaminação ambiental e os seus resultados podem ser utilizados como um alerta para outros organismos eventualmente expostos à contaminantes ambientais.

Rotineiramente, os testes com *A. cepa* têm sido realizados com diferentes amostras ambientais. De acordo com White e Claxton (2004), dentre os vegetais superiores, *A. cepa* tem sido a espécie mais utilizada para avaliação de solos contaminados.

Extratos aquosos de solos contaminados com resíduos industriais derivados do petróleo foram avaliados por Cotelle et al. (1999), pelo teste do micronúcleo em *A. cepa*, *Vicia faba* e *Tradescantia sp.* Pelos resultados obtidos, os autores afirmaram que as três espécies foram eficientes na avaliação da mutagenicidade das amostras de solo.

Grover e Kaur (1999), examinando os efeitos de amostras de lodo de esgoto sobre células meristemáticas de *A. cepa*, observaram que não houve diferença significativa entre o número de micronúcleos observados para as amostras de lodo e o controle negativo empregado.

Foram avaliados por Caritá (2007), por meio da indução de danos celulares e cromossômicos em células meristemáticas de *A. cepa*, diferentes solubilizados de lodo de esgoto, obtidos em cinco diferentes ETEs da região metropolitana de São Paulo. A autora observou uma elevada frequência de aberrações cromossômicas para todas as diluições testadas das amostras dos lodos utilizadas.

Souza et al. (2009) aplicaram os testes de aberrações cromossômicas e micronúcleos em *A. cepa*, com o intuito de verificar a eficácia da tecnologia de biorremediação de solo contaminado com resíduos de petróleo (*landfarming*) e a capacidade de biodegradação das amostras, após adição de casca de arroz. Estas mesmas autoras, em 2013, aplicaram os testes com *A. cepa* com o intuito de investigar a possível ação aneugênica/clastogênica de um solo de *landfarming* antes e após a adição da vinhaça de cana-de-açúcar. Os resultados obtidos indicaram que a vinhaça potencializou a clastogenicidade do *landfarming*, frente às inúmeras quebras cromossômicas observadas.

Estudos realizados por Maziviero (2011) também objetivaram avaliar os potenciais citotóxico, genotóxico e mutagênico de amostras de lodo de esgoto sobre o material genético de *A. cepa* e *Tradescantia*. O autor observou, para ambos os organismos empregados, que as amostras de lodo apresentaram-se genotóxicas e mutagênicas.

4. MATERIAL E MÉTODOS

4.1 Materiais biológicos

Para a realização dos bioensaios foram utilizados dois organismos-teste. Sementes de *A. cepa* (Liliaceae), da variedade Baia Piriforme, de mesma marca, lote e prazo de validade, a fim de evitar diferentes respostas às diversas etapas dos testes.

Também foram utilizados neste estudo espécimes adultos da espécie *R. padbergi* (Diplopoda), popularmente conhecido como piolho-de-cobra. No total foram utilizados 240 indivíduos com tamanho médio de 6 cm. Os espécimes foram coletados em amostras de solo controle, localizado dentro do Câmpus da Universidade Estadual Paulista (UNESP), Rio Claro-SP.

4.2 Caracterização da ETE Franca e seus resíduos

A ETE de Franca ocupa uma área de 20 hectares. A estação atende, aproximadamente, 80% da população da cidade de Franca, sendo os 20% restantes tratados em outras seis estações de tratamentos. O processo de tratamento de esgoto utilizado é o tratamento convencional de lodos ativados. Os esgotos afluentes à ETE de Franca são predominantemente domésticos, sendo desprezível a contribuição industrial (VANZO et al., 2000).

Em outubro de 1999, a estação recebeu o registro de Estabelecimento Produtor de Insumo Agrícola, pelo Ministério da Agricultura e do Abastecimento. O produto fabricado na estação é um biofertilizante, classificado como Condicionador de Solo. Sua denominação comercial é Sabesfértil (SP-09599 00001-0).

O lodo primário e o biofertilizante foram coletados nos anos de 2010 e 2011. Parte das amostras de tais resíduos foi levada imediatamente ao laboratório especializado para análises químicas e a outra parte, foi armazenada em caixas plásticas, envolvidas em sacos plásticos escuros e acondicionadas em câmara fria (4°C), até a liberação dos resultados para o início dos experimentos.

4.3 Amostras de vinhaça de cana-de-açúcar

A vinhaça foi coletada na Usina Santa Lucia, localizada na cidade de Araras-SP, nos anos de 2010 e 2011. Parte do resíduo foi submetido à análise química e o restante foi mantido em câmara fria (4°C), no Departamento de Bioquímica e Microbiologia da UNESP de Rio Claro, até o início dos experimentos.

4.4 Amostras de solo controle

O solo controle utilizado foi coletado no mesmo local onde foram coletados os diplópodos (dentro do Campus da UNESP de Rio Claro-SP). Para a montagem dos bioensaios, amostras de solo foram coletadas nos anos de 2010 e 2011, secas à temperatura ambiente, peneiradas em peneira com malha de 4 mm e submetidas à caracterização química e de fertilidade.

4.5 Caracterização química das amostras brutas e das combinações

Foram realizadas análises químicas, físico-químicas e do potencial agrônômico para as amostras de solo, referentes à macro e micronutrientes (N, Ca, Mg, P, K, S, Fe, Mn, Cu, Zn), relação carbono/nitrogênio (C/N), matéria orgânica, capacidade de troca catiônica (CTC) e saturação por bases realizado pelo Instituto Campineiro de Solo e Adubo (ICASA, Campinas-SP). Dados referentes aos metais (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se e Zn), às 16 substâncias orgânicas prioritárias pela Environmental Protection Agency (EPA) e do potencial agrônômico (condutividade elétrica, carbono orgânico, fósforo total, nitrogênio Kjeldahl, nitrogênio amoniacal, nitrogênio nitrato/nitrito, pH em água (1:10), potássio total, sódio total, enxofre total, cálcio total, magnésio total, umidade, sólidos voláteis e sólidos totais) foram mensurados pela laboratório TASQA, Paulínia-SP, para as amostras de solo, lodo primário e biossólido. Os parâmetros analisados seguiram os métodos SM21 (Standard Methods for the Examination of Water and Wastewater 21th Edition 2005) e da EPA (Environmental Protection Agency).

A vinhaça também foi analisada pelo laboratório TASQA, nos seguintes parâmetros: pH, resíduo não filtrável total, dureza, condutividade elétrica, nitrogênio nitrato, nitrogênio nitrito, nitrogênio amoniacal, nitrogênio Kjeldhal, sódio, cálcio, potássio, magnésio, sulfato, fosfato total, DBO (Demanda Bioquímica de Oxigênio) e DQO (Demanda Química de Oxigênio), bem como os metais (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se e Zn).

Esses mesmos parâmetros foram mensurados para as combinações dos resíduos antes e após o processamento pelos diplópodos.

4.6 Cálculos para a aplicação de lodo primário e biossólido, segundo a resolução CONAMA 375/2006

De acordo com esta resolução, a aplicação máxima anual de lodo de esgoto e produtos derivados em toneladas por hectare não deverá exceder o quociente entre a quantidade de nitrogênio recomendada para a cultura (em Kg/ha), segundo a recomendação agrônômica

oficial do estado de São Paulo, e o teor de nitrogênio disponível no lodo de esgoto ou produto derivado (N_{disp} em Kg/t), calculado de acordo com a fórmula:

$$\text{Taxa de aplicação (t/ha)} = \frac{\text{N recomendado (kg/ha)}}{\text{N}_{\text{disp}} \text{ (kg/t)}}$$

Para o cálculo do nitrogênio disponível (N_{disp}) no lodo de esgoto ou produto derivado, deverão ser utilizadas as seguintes frações de mineralização (FM), onde:

- Lodo de esgoto não digerido - 40% - Lodo de Franca - Lodo primário
- Lodo de esgoto digerido anaerobiamente - 20% - Lodo de Franca – Biossólido

O teor de N disponível (N_{disp}) do lodo de esgoto ou produto derivado é calculado pela equação:

$$\text{N}_{\text{disp}} = (\text{FM}/100) \times (\text{KKj}-\text{NNH}_3) + 0,5 \times (\text{NNH}_3) + (\text{NNO}_3 + \text{NNO}_2)$$

Sendo necessário para o cálculo do N_{disp}:

- Fração de mineralização do nitrogênio (FM) (%);
- Nitrogênio Kjeldahl (nitrogênio Kjeldahl = nitrogênio orgânico total + nitrogênio amoniacal (NK_j) (mg/kg);
- Nitrogênio amoniacal (NNH₃)(mg/kg);
- Nitrogênio Nitrato e Nitrito (NNO₃ + NNO₂) (mg/kg).

As concentrações utilizadas nestes cálculos devem ser em mg do parâmetro por kg de lodo de esgoto ou produto derivado em base seca.

4.7 Cálculos para a aplicação de vinhaça, segundo a norma P4.231, da CETESB

A dosagem máxima de vinhaça a ser aplicada é determinada pela equação:

$$\text{m}^3 \text{ de vinhaça/ha} = \frac{[(0,05 \times \text{CTC} - \text{ks}) \times 3744 + 185]}{\text{kvi}}$$

kvi

Sendo:

0,05 = 5% da CTC do solo.

CTC = Capacidade de Troca Catiônica, expressa em $\text{cmol}_c/\text{dm}^3$.

ks = concentração de potássio no solo, expresso em $\text{cmol}_c/\text{dm}^3$, à profundidade de 0 a 0,80 metros.

3744 = constante para transformar os resultados da análise de fertilidade, expressos em $\text{cmol}_c/\text{dm}^3$ ou $\text{meq}/100\text{cm}^3$, para kg de potássio em um volume de 1 (um) hectare por 0,80 metros de profundidade.

185 = massa, em kg, de K_2O extraído pela cultura por hectare, por corte.

kvi = concentração de potássio na vinhaça, expressa em kg de $\text{K}_2\text{O}/\text{m}^3$.

4.8 Preparação das amostras de solo e resíduos para montagem dos bioensaios com *R. padbergi*

Para a montagem dos bioensaios com *R. padbergi* foram utilizados seis terrários de vidro, de medidas 45 cm de comprimento, 25 cm de largura e 20 cm de altura, com capacidade de 22,5 l, contendo 5 kg de solo controle, cada. Para a aplicação do lodo primário e do bio sólido, seguiu-se a resolução CONAMA 375/2006 e para a aplicação da vinhaça, a norma P4.231 da CETESB. Após as misturas dos resíduos com o solo controle, 20 indivíduos da espécie *R. padbergi* foram adicionados em cada terrário. Com o intuito de avaliar o possível bioprocessamento destas amostras pelos diplópodos, análises químicas de cada terrário foram realizadas nos tempos 0 (t_0) e após 30 dias (t_{30}). Para avaliar a possível toxicidade, por meio de análises histológica, histoquímica e ultra-estrutural, os animais ficaram expostos à amostra de solo controle e às diferentes combinações de resíduos por períodos de 7, 30 e 90 dias.

Como os resultados obtidos pelas análises químicas das diferentes amostras variaram para as coletas realizadas, os cálculos e quantidades aplicadas em cada terrário estão apresentados nas seções 5.3 e 5.4. Entretanto, em ambos os bioensaios realizados, os animais foram expostos às seguintes combinações: SC (solo controle), SL (solo+lodo primário), SB

(solo+biossólido), SV (solo+vinhaça da cana de açúcar), SLV (solo+lodo primário+vinhaça) e SBV (solo+biossólido+vinhaça).

4.8.1 Dissecção dos animais

Após cada período de exposição, três animais de cada bioensaio foram anestesiados com clorofórmio e dissecados em solução fisiológica, a fim de se retirar o intestino médio, o qual teve porções fixadas em diferentes soluções, para que pudessem ser aplicadas diferentes técnicas. Após a fixação por 24 h, o material foi colocado em solução tampão fosfato de sódio pH=7,4, para manutenção sob refrigeração.

Os resultados observados nos indivíduos expostos ao lodo primário, ao biossólido, à vinhaça e suas combinações, por período de 7, 30 e 90 dias, foram comparados aqueles do grupo controle e às informações da literatura, com o intuito de detectar possíveis alterações nos animais expostos.

4.8.1.1 Histologia e histoquímica do intestino médio

Para aplicação das técnicas de histologia e histoquímica, o material foi desidratado em soluções de etanol a 70, 80, 90 e 95% e embebidos em resina (Leica historesin), por 24 h, sob refrigeração. Posteriormente, o material foi transferido para moldes plásticos contendo resina de inclusão. Após a polimerização, seções de 6 µm de espessura foram obtidos com auxílio do micrótomo LEICA RM 2245. Os cortes foram hidratados e recolhidos em lâminas.

Para análise histológica os cortes foram corados com hematoxilina e eosina (HE). Testes histoquímicos foram aplicados para a detecção de polissacarídeos neutros – técnica de PAS (JUNQUEIRA; JUNQUEIRA, 1983), proteínas totais – técnica azul de bromofenol (PEARSE, 1985), lipídios – técnica azul do Nilo (JUNQUEIRA; JUNQUEIRA, 1983) e cálcio, pela técnica de von Kossa (JUNQUEIRA; JUNQUEIRA, 1983).

4.8.1.2 Ultra-estrutura do intestino médio

Para análise ultra-estrutural, o material foi fixado em glutaraldeído a 2,5% em 0,1 M de tampão cacodilato de sódio, pH 7,2 a 4° C. Após a fixação, o material foi lavado neste mesmo tampão e pós-fixado em tetróxido de ósmio a 1%. Decorrida a pós-fixação, o material foi submetido ao acetato de uranila durante 8 h. A desidratação foi realizada em solução de acetona graduada e embebidos em uma mistura de resina epon araldite durante 12 horas a 4° C. Cortes finos obtidos em ultra-micrótomo foram contrastados com acetato de uranila e citrato de chumbo para observação e documentação com microscópio eletrônico de

transmissão ZEISS, EM 900, gentilmente cedido pelo Prof. Dr. Elliot W. Kitajima, da Escola Superior de Agricultura “Luiz de Queiroz” – ESALQ, Piracicaba-SP, operado em 80 kV.

4.9 Germinação das sementes de *A. cepa* nas amostras brutas dos resíduos, no tempo 0 (t0) e após processamento pelos diplópodos por 30 dias (t30).

As sementes de *A. cepa* foram submetidas à germinação em temperatura controlada 22°C, em placas de Petri, contendo primeiramente as amostras brutas de lodo primário (L), biossólido (B) e vinhaça (V).

Após as misturas das amostras nos terrários, 30 g de cada um foram coletados e dispostos em placas de Petri, resultando em: SL (solo controle+lodo primário), SB (solo controle+ biossólido), SV (solo controle+vinhaça), SLV (solo controle+lodo primário+vinhaça), SBV (solo controle+biossólido+vinhaça), sendo considerado o momento da mistura destes resíduos ao solo controle como tempo 0 (t0). O teste controle positivo foi realizado pela submissão das sementes a dois agentes, cujas concentrações são potencialmente citotóxicas e mutagênicas: o herbicida trifluralina, de ação aneugênica, na concentração de 0,019 ppm (FERNANDES et al., 2007) e o MMS (metil metanosulfonato), de ação clastogênica, na concentração de 4×10^{-4} M (RANK; NIELSEN, 1997). O controle negativo foi realizado com sementes submetidas à germinação em água ultrapura e o controle ambiental, por meio da germinação em solo controle.

Após 30 dias de exposição aos diplópodos (t30) foram retiradas amostras de cada terrário contendo as respectivas combinações, para nova exposição de sementes de cebola e análises químicas e físico-químicas.

4.9.1 Confecção das lâminas de *A. cepa*

Após germinarem e atingirem cerca de 2 cm de comprimento, as radículas foram coletadas e fixadas em Carnoy I (3 partes de etanol para 1 de ácido acético). Depois de fixadas, as radículas foram submetidas à reação de Feulgen (MELLO; VIDAL, 1978), com uma hidrólise ácida por 9 minutos. As pontas das radículas foram seccionadas, em lâmina, para extrair a região meristemática e região F₁. Com o intuito de intensificar a coloração e facilitar o espalhamento das células, foi adicionada ao material uma gota de carmim acético (2%). Todas as lâminas foram obtidas submetendo o material a um esmagamento suave entre lâmina e lamínula. As lamínulas foram extraídas em nitrogênio líquido e as lâminas montadas em Entellan. O material foi analisado em microscópio de luz, sob aumento de 400 x.

4.9.1.1 Teste de toxicidade, citotoxicidade, genotoxicidade e mutagenicidade em *A. cepa*

Para avaliar a toxicidade dos resíduos brutos e suas combinações, 100 sementes de *A. cepa* foram dispostas em placas de Petri contendo as amostras. Para tal avaliação, foi considerado o índice de germinação das sementes, de acordo com a fórmula:

$$\text{IG (índice de germinação)} = \frac{\text{n}^\circ \text{ total de sementes que germinaram} \times 100}{\text{Total de sementes expostas ao tratamento}}$$

Na avaliação dos potenciais citotóxico, genotóxico e mutagênico foram analisadas 5000 células para cada tratamento. O mesmo número de células foi analisado para os testes controles negativo e positivo.

A citotoxicidade foi verificada pela análise de alterações celulares morfológicas indicativas de morte celular e pela frequência do índice mitótico, obtido pela razão do número de células em divisão sobre o número total de células analisadas.

Por meio de análises citológicas nas diferentes fases de divisão celular (prófase, metáfase, anáfase e telófase), foram quantificados os diferentes tipos de aberrações, tais como C-metáfases, aderências cromossômicas, pontes cromossômicas, atrasos cromossômicos, poliploidia, brotos nucleares, dentre outras, para a avaliação da genotoxicidade das amostras, representado pelo índice de aberrações cromossômicas (IAC).

Para a análise da mutagenicidade (IMt) foi registrado a ocorrência de células portadoras de micronúcleos (MN) e quebras cromossômicas em células da região meristemática e micronúcleos (MN) em células da região F₁.

Os valores obtidos em todas as amostras foram comparados com os valores obtidos no controle negativo e no solo controle, por meio do teste estatístico de Mann-Whitney, com nível de significância de 0.05.

5. RESULTADOS

5.1 Caracterização química das amostras para correta aplicação dos resíduos

5.1.1 Caracterização química e análise de fertilidade do solo controle

Com o intuito de seguir a norma brasileira para aplicação da vinhaça e do lodo de esgoto na agricultura, se fizeram necessários dados referentes ao potencial agrônomo e à fertilidade do solo controle. Estão apresentados na tabela 1 os valores obtidos para o pH, matéria orgânica (MO), fósforo residual (P res), potássio (K), cálcio (Ca), magnésio (Mg), alumínio trocável (H+Al), soma de bases (S8), capacidade de troca catiônica (CTC), saturação por bases (V%) e as relações de Ca/Mg e Mg/K, para as duas amostras utilizadas.

Tabela 1 – Dados referentes à caracterização química e fertilidade do solo controle

Amostra	pH	MO	P res	K	Ca	Mg	H+Al	SB	CTC	V	Relações	
	CaCl ₂	g/dm ³	mg/dm ³			mmol _c /dm ³	TFSA			%	Ca/Mg	Mg/K
2010	6.20	18	3.0	0.8	2	1	88	3.9	91.9	4.2	2.0	1.25
2011	5.10	17	3.0	0.2	9	6	30	16.6	5.88	3.5	-	-

MO: matéria orgânica; **CTC:** capacidade de troca catiônica; **V:** saturação por bases; **SB:** soma de bases

5.1.2 Caracterização química das amostras brutas

Estão apresentados na tabela 2 os resultados obtidos pela análise físico-química e de metais para o solo controle e resíduos, assim como a comparação destes mesmos parâmetros, nas duas coletas realizadas. Por se tratar de amostras complexas, houve uma variação para todos os parâmetros analisados, comparando as duas coletas realizadas. Na segunda coleta de solo controle observaram-se valores diferentes para cobre, cromo, mercúrio, molibdênio, níquel e zinco.

Diferentemente das análises químicas do primeiro bioensaio, onde se detectou na amostra bruta de lodo primário, a presença dos compostos orgânicos fenantreno, fluoranteno e naftaleno; entretanto, tais compostos não foram encontrados na amostra do segundo bioensaio. Tal análise também foi realizada para as amostras brutas de biossólido e solo controle. No entanto, em nenhuma das amostras, para ambos os bioensaios, foi detectada a presença de compostos orgânicos (Tabela 3).

Tabela 2- Análise físico-química e de metais do solo controle e das amostras brutas de vinhaça, lodo primário e biossólido, coletadas em 2010 e 2011

Parâmetro	Amostras/2010						Amostras/2011					
	SC (mg/kg)	V (mg/L)	L (mg/L)	B (mg/kg)	SC (mg/kg)	V (mg/L)	L (mg/L)	B (mg/kg)	Método	APMax (mg/kg)	CMP (mg/kg)	
Arsênio	16.8	<LQ	14.5	<LQ	<LQ	<LQ	<LQ	<LQ	SM21 3120B	35	41	
Bário	5.91	0.41	11.0	158	<LQ	<LQ	7.73	222	SM21 3120B	300	1300	
Cádmio	<LQ	<LQ	<LQ	<LQ	<0.16	<LQ	<LQ	<0.66	SM21 3120B	3	39	
Cálcio Total	25.4	719	623	3939	42.8	671	432	5610	SM21 3120B	-	-	
Carbono Orgânico (g/kg)	12.6	NA	3939	279	32.3	NA	1345	590	SSSA Cap40	-	-	
Chumbo	49.3	<LQ	1.42	174	42.7	<LQ	<LQ	126	SM21 3120B	180	300	
Cobre	37.2	0.35	10.6	276	76.5	0.76	4.65	210	SM21 3120B	200	1500	
Conduktiv. Elétrica (µs/cm)	115	13530	2950	5389	97.9	15110	3700	1181	SM21 3120B	-	-	
Cromo	31.2	0.04	6.48	224	108	3.56	4.07	180	SM21 3120B	150	1000	
Enxofre Total	151	1219	373	11864	123	1681	6640	15475	SM21 3120B	-	-	
Fósforo Total	182	NA	707	17027	317	207	385	18156	SM21 3120B	-	-	
Magnésio Total	<LQ	237	75.4	358	<LQ	264	65.3	1132	SM21 3120B	-	-	
Mercurio	<LQ	0.0019	<LQ	1.08	0.065	<LQ	0.0016	<LQ	EPA 7470A	12	17	
Molibdênio	3.64	0.008	0.83	9.55	9.60	<LQ	<LQ	<0.44	SM21 3120B	50	50	
Níquel	13.0	0.03	1.80	82.3	24.2	<LQ	4.12	104	SM21 3120B	70	420	
Nitrato (mg/Kg)	4.40	1.30	1.20	6.79	8.14	1.49	1.09	8.63	SM21 4500-NO ₃ E	-	-	
Nitrito (mg/Kg)	0.06	0.008	0.02	1.39	<0.043	0.03	0.014	1.98	SM21 4500-NO ₂ B	-	-	
N Amoniacal (mg/kg)	31.8	NA	1467	167	49.6	NA	351	3969	SM21 4500-NH ₃ E	-	-	
N Kjeldahl (mg/Kg)	476	267	1597	21620	922	171	705	35740	SM21 4500-Norg B	-	-	
pH	6.20	3.9	6.55	8.01	5.1	4.37	6.72	6.8	EPA 4095 C	-	-	
Potássio Total	406	2056	107	2152	<LQ	3401	84.9	2513	SM21 3120B	-	-	
Selênio	<LQ	<LQ	<LQ	<LQ	52.1	<LQ	<LQ	<LQ	SM21 3120B	-	100	
Sódio Total	<LQ	50.2	41.8	<LQ	<LQ	114	71.5	597	SM21 3120B	-	-	
Sólidos Totais (g/g)	0.86	NA	55149	0.24	0.93	NA	42902	0.2146	SM21 2540B	-	-	
Sólidos Tot. Voláteis (g/g)	VI	NA	27978	VI	0.08	NA	25767	VI	SM21 2540B	-	-	
Teor de sólidos (g/g)	0.86	NA	NA	0.24	0.93	NA	NA	0.2146	SM21 2540B	-	-	
Umidade (g/g)	0.14	NA	NA	0.76	0.06	NA	0.9571	0.7854	SM21 2540B	-	-	
Zinco	23.2	1.66	40.5	825	96	<LQ	14.4	523	SM21 3120B	450	2800	

SC: solo controle; **V:** vinhaça; **L:** lodo primário; **B:** biossólido; **LQ:** Limite de quantificação; **NA:** não avaliado; **VI:** Valor inconsistente; **APMax:** valores orientadores para cenários de exposição Agrícola-Área de Proteção Máxima, para solo (mg/kg) e água subterrânea no Estado de São Paulo, segundo a CETESB (195/2005-E); **CMP:** concentração máxima permitida no lodo de esgoto ou produto derivado, de acordo com o CONAMA (375/2006).

Tabela 3 – Análise de hidrocarbonetos policíclicos aromáticos nas amostras brutas de solo controle, lodo primário e bio sólido, para as duas coletas realizadas

Parâmetro	Amostras/2010			Amostras/2011			Método	Concentração permitida no solo (mg/kg)	
	SC	L	B	SC	L	B		CB	CO
	Acenafteno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ		<LQ	EPA 8270 D
Acenaftileno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Antraceno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Benzo(a)antraceno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,025	0,025
Benzo(a)pireno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,052	0,052
Benzo(b)fluoranteno (mg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,38	-
Benzo(g,h,i)perileno (mg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,57	-
Benzo(k)fluoranteno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,38	0,38
Criseno (mg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	8,1	-
Dibenzo(a,h)antraceno (mg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,08	-
Fenantreno (µg/Kg)	<LQ	28,8	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	3,3	3,3
Fluoranteno (µg/Kg)	<LQ	16,8	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Fluoreno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Indeno(1,2,3-cd)pireno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,031	0,031
Naftaleno (µg/Kg)	<LQ	27,6	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,12	0,12
Pireno (µg/Kg)	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-

SC: solo controle; L: lodo primário; B: bio sólido; LQ: Limite de quantificação; CB: valores orientadores (prevenção) para solos no Estado de São Paulo, de acordo com a CETESB (195/2005-E); CO: concentração máxima permitida no solo, segundo o CONAMA (375/2006).

Foi detectada a presença de bário, cobre, cromo, mercúrio, molibdênio, níquel e zinco (Tabela 2), para as duas amostras de vinhaça utilizadas. Ambas as amostras coletadas apresentaram ainda baixo pH, altos valores de DBO e DQO, bem como valores de potássio para os anos de 2010 e 2011 (2056 mg/L e 3401 mg/L, respectivamente) (Tabela 4).

Tabela 4 – Características quali-quantitativas da vinhaça para as coletas de 2010 e 2011

Parâmetro	2010	2011	Método
Amônia (mg/L)	<LQ	<LQ	USEPA 440/5-85-001
Cálcio (mg/L)	719	671	SM21 3120 B
Condutividade Elétrica ($\mu\text{s}/\text{cm}$)	13530	15110	SM21 2510 B
DBO (mg/L)	5046	7941	SM21 5210 B
DQO (mg/L)	13380	25225	SM21 5220 D
Dureza (mg CaCO_3/L)	2493	276	SM21 2340 B
Fosfato total (mg/L)	1.30	NA	SM21 4500-P C
Magnésio (mg/L)	237	264	SM21 3120 B
Nitrato (mg/L)	1.30	1.49	SM21 4500- NO_3^- F
Nitrito (mg/L)	0.008	0.033	SM21 4500- NO_2^- B
pH	3.9	4.37	SM21 4500- H^+ B
Potássio (mg/L)	2056	3401	SM21 3120 B
Resíduo não-filtrável (mg/L)	2765	1800	SM21 2540 D
Sódio (mg/L)	50.2	114	SM21 3120 B
Sulfato (mg/L)	710	2993	SM21 4500- SO_4^{2-} E

LQ: limite de quantificação; NA: não avaliado

5.3 Cálculos para aplicação de lodo primário, biossólido e vinhaça

Após as análises químicas das amostras brutas, para a montagem dos bioensaios foi considerado o volume de solo a ser disposto em cada terrário, bem como sua densidade.

5.3.1 Primeiro bioensaio

Em relação às amostras de lodo primário e biossólido, a taxa de aplicação é dada de acordo com a quantidade de nitrogênio necessária para uma cultura e a quantidade de nitrogênio presente no lodo primário e no biossólido.

Assim sendo foram obtidos pelas fórmulas o resultado de 321,72 mL de lodo primário/terrário e 234,40 g de biossólido/terrário.

Seguem abaixo os dados obtidos pelas análises químicas e inseridos nas fórmulas para aplicação dos resíduos, para as duas coletas realizadas.

a) Lodo primário:

Dados obtidos nas análises químicas:

Nitrogênio Kj (NKj) = 1597 mg/L

Nitrito (NO₂) = 0.02 mg/L

Nitrato (NO₃) = 1.20 mg/L

Nitrogênio amoniacal (NNH₃) = 1467 mg de NH₃/L

Fração de mineralização (FM) = 40%

Quantidade de N recomendada para cana-de-açúcar: 30 kg/ha (PIRES; FERREIRA, 2008) ou 120 kg/ha de N, segundo Rajj et al. (1997). Na média, o ideal é 100 kg/ha.

Logo, aplicando os valores na fórmula:

$$N_{disp} = (FM/100) \times (KKj - NNH_3) + 0,5 \times (NNH_3) + (NNO_3 + NNO_2)$$

$$N_{disp} = (40/100) \times (1597 - 1467) + 0,5 \times (1467) + (1,20 + 0,02)$$

$$N_{disp} = (0,4) \times (130) + 733,5 + 1,22$$

$$N_{disp} = 52 + 733,7 + 1,22$$

$$N_{disp} = 786,72 \text{ mg/L ou } 0,78672 \text{ kg/m}^3$$

$$\text{Taxa de aplicação (t/ha)} = \frac{\text{N recomendado (kg/ha)}}{\text{N}_{disp} \text{ (kg/t)}} \left(\frac{\text{m}^3}{\text{ha}} \right) = \frac{\text{N rec (kg/ha)}}{\text{N}_{disp} \text{ (kg/m}^3)}$$

$$\text{Taxa de aplicação} = \frac{100 \text{ kg/ha}}{0,78672 \text{ kg/m}^3} = \mathbf{127,11 \text{ m}^3/\text{ha}}$$

Para transformar a quantidade a ser adicionada em uma amostra de terra ou substrato de t/ha (ou L/ha) para g/dm³ [ou uL(10⁻⁶L)/ dm³], deve se considerar que 1 ha (100 m x 100 m ou 1000 dm x 1000 dm) na camada de 0 a 20 cm (0 a 2 dm) de profundidade (devido ao método de análise de fertilidade do solo, conforme norma CONAMA 375):

Logo, 1 ha corresponde a 2x10⁶ dm³. Portanto,

$$\begin{array}{l} 127,11 \text{ m}^3 \text{ ----- } 2 \times 10^6 \text{ dm}^3 \\ X \text{ ----- } 1 \text{ dm}^3 \end{array}$$

$$2 \times 10^6 X = 127.11$$

$$X = \frac{127.11}{2 \times 10^6} = 63.555 \times 10^{-6} \text{ m}^3/\text{dm}^3 = 63.55 \times 10^{-3} \text{ L}/\text{dm}^3 = \mathbf{63.55 \text{ cm}^3/\text{dm}^3}$$

Para aplicação deste resíduo nos terrários, foi necessário calcular a área, o volume e a densidade dos 5Kg das amostras de solo.

- área do terrário: 1125 cm².

- volume: 22.5 L.

- densidade: 5.0625 dm³.

Logo:

$$63.55 \text{ cm}^3 \text{ ----- } 1 \text{ dm}^3$$

$$X \text{ ----- } 5.0625 \text{ dm}^3$$

$$X = 321.72 \text{ cm}^3/\text{dm}^3 = 0.32172 \text{ L de lodo primário/terrário ou } \mathbf{321.72 \text{ mL de lodo primário/terrário.}}$$

b) Biossólido:

Dados obtidos nas análises químicas:

Nitrogênio Kj (NKj) = 21620 mg/kg

Nitrito (NO₂) = 1.39 mg/kg

Nitrato (NO₃) = 6.79 mg/kg

Nitrogênio amoniacal (NNH₃) = 167 mg/kg

Fração de mineralização do biossólido = 20%

Quantidade de N recomendada para cana-de-açúcar: 30 kg/ha (PIRES; FERREIRA, 2008) ou 120 kg/ha de N, segundo Raij et al (1997). Na média, o ideal é 100 kg/ha.

Logo, aplicando os valores na fórmula:

$$N_{\text{disp}} = (FM/100) \times (KKj - NNH_3) + 0,5 X (NNH_3) + (NNO_3 + NNO_2)$$

$$N_{\text{disp}} = (20/100) \times (21620 - 167) + 0.5 \times (167) + (6.79 + 1.39)$$

$$N \text{ disp} = (0.2) \times (21453) + 83.5 + 8.18$$

$$N \text{ disp} = 4290.6 + 83.5 + 8.18$$

$$N \text{ disp} = \mathbf{4382.28 \text{ mg/kg ou } 4.38228 \text{ kg/t}}$$

$$\text{Taxa de aplicação (t/ha)} = \frac{N \text{ recomendado (kg/ha)} (m^3/ha)}{N \text{ disp (kg/t)}}$$

$$\text{Taxa de aplicação} = \frac{100 \text{ kg/ha}}{4.38228} = \mathbf{22.8 \text{ t/ha}}$$

Logo,

$$22.8 \text{ t} \text{ ----- } 1 \text{ ha} \text{ ----- } 2 \times 10^6 \text{ dm}^3$$

$$22.8 \times 10^6 \text{ g} \text{ ----- } 2 \times 10^6 \text{ dm}^3$$

$$X \text{ ----- } 1 \text{ dm}^3$$

$$\mathbf{X = 11.4 \text{ g/dm}^3 \text{ de base seca de bioossólido}}$$

Para a aplicação de bioossólido, também consideramos a área, o volume e a densidade dos terrários, chegando ao resultado de:

$$11.41 \text{ g de bioossólido} \text{ ----- } 1 \text{ dm}^3$$

$$Y \text{ ----- } 5.0625 \text{ dm}^3$$

$$Y = 11,41 \times 5,0625 = \mathbf{57.76 \text{ g de bioossólido seco}}$$

A resolução CONAMA 375/2006 exige que seja calculado o peso seco do bioossólido, para posterior aplicação. Assim sendo, o bioossólido úmido foi disposto em três placas de Petri, pesados e secos em estufa, à 65°C, por 48h. Com o resultado, obtivemos uma média (em g) de peso seco, em relação ao peso úmido (em g) do bioossólido. Logo, obtivemos como resultado:

$$14.25 \text{ g de bioossólido seco} \text{ ----- } 57.83 \text{ g de bioossólido úmido}$$

$$57.76 \text{ g de bioossólido seco} \text{ ----- } z \text{ g de bioossólido úmido}$$

$$14.25 z = 57.83 \times 57.76$$

$$z = 3340.26 / 14.25$$

$$z = \mathbf{234.40 \text{ g de bioossólido úmido/terrário}}$$

c) **Vinhaça:**

Dados do solo:

Valor de potássio (Ks): $0.08 \text{ cmol}_c/\text{dm}^3$

CTC: $9.19 \text{ cmol}_c/\text{dm}^3$

Dados da vinhaça:

Valor de potássio (Kvi): $2056 \text{ mg/L} = 2.056 \text{ kg/m}^3$.

O valor de potássio obtido nas análises químicas foi expresso apenas na quantidade do elemento potássio. Assim sendo, há uma relação que deve ser considerada para a vinhaça, onde 1 mg de K corresponde à 1.205 mg de K_2O .

1 mg de K ----- 1.205 mg de K_2O .

2056 mg de K ----- x

$$X = 1.205 \times 2056$$

$$X = 2477.48 \text{ mg/L ou } \mathbf{2.47748 \text{ kg/m}^3 \text{ de } \text{K}_2\text{O} = \text{Kvi}}$$

Aplicando os valores na fórmula:

$$\text{m}^3 \text{ de vinhaça/ha} = [(0,05 \times \text{CTC} - \text{ks}) \times 3744 + 185] / \text{kvi}$$

$$\text{m}^3 \text{vinhaça/ha} = \frac{[(0.05 \times 9.19 - 0.08) \times 3744 + 185]}{2.47748} = \frac{[(0.4595 - 0.08) \times 3744 + 185]}{2.47748}$$

$$\text{m}^3 \text{vinhaça/ha} = \frac{[(0.3795) \times 3744 + 185]}{2.47748} = \frac{[1420.848 + 185]}{2.47748} = \frac{1605.84}{2.47748}$$

$$\text{m}^3 \text{vinhaça/ha} = 648.177 \text{ m}^3/\text{ha} = 648177 \text{ dm}^3/\text{ha}$$

Para aplicação da vinhaça em 1 dm^3 :

648177 L ----- 1 ha ----- $8 \times 10^6 \text{ dm}^3$

X ----- 1 dm^3

$$8 \times 10^6 X = 648177$$

$$X = \frac{648177}{8 \times 10^6} = 0.0810 \text{ L} = \mathbf{81 \text{ cm}^3/\text{dm}^3}$$

Considerando novamente a área, o volume e a densidade dos terrários, temos:

$$1 \text{ dm}^3 \text{ ----- } 0.0810 \text{ L}$$

$$5.0625 \text{ dm}^3 \text{ ----- } y$$

$$Y = 0.0810 \times 5.0625 = 0.41 \text{ L ou } \mathbf{410 \text{ mL de vinhaça/terrário}}$$

5.3.2 Segundo bioensaio

a) Lodo primário:

Dados obtidos nas análises químicas:

Nitrogênio Kj (NKj) = 705 mg/L

Nitrito (NO₂) = 0.014 mg/L

Nitrato (NO₃) = 1.09 mg/L

Nitrogênio amoniacal (NNH₃) = 351 mg de NH₃/L

Fração de mineralização (FM) = 40%

Quantidade de N recomendada para cana-de-açúcar: 30 kg/ha (PIRES; FERREIRA, 2008) ou 120 kg/ha de N, segundo Raij et al. (1997). Na média, o ideal é 100 kg/ha.

Aplicando os valores na fórmula:

$$N_{\text{disp}} = (FM/100) \times (KKj - NNH_3) + 0,5 \times (NNH_3) + (NNO_3 + NNO_2)$$

$$N_{\text{disp}} = (40/100) \times (705 - 351) + 0.5 \times (351) + (1.09 + 0.014)$$

$$N_{\text{disp}} = (0.4) \times (354) + 175.5 + 1.104$$

$$N_{\text{disp}} = 141.6 + 175.5 + 1.104$$

$$N_{\text{disp}} = \mathbf{318.20 \text{ mg/L ou } 0.31820 \text{ kg/m}^3}$$

$$\text{Taxa de aplicação (t/ha)} = \frac{\text{N recomendado (kg/ha)}}{\text{N disp (kg/t)}} \left(\frac{\text{m}^3}{\text{ha}} \right) = \frac{\text{N rec (kg/ha)}}{\text{N disp (kg/m}^3\text{)}}$$

$$\text{Taxa de aplicação} = \frac{100 \text{ kg/ha}}{0.31820 \text{ kg/m}^3} = \mathbf{314.26 \text{ m}^3/\text{ha}}$$

Para transformar a quantidade a ser adicionada em uma amostra de terra ou substrato de t/ha (ou L/ha) para g/dm³ [ou uL(10⁻⁶L)/ dm³], deve se considerar que 1 ha (100 m x100 m ou 1000 dm x 1000 dm) na camada de 0 a 20 cm (0 a 2 dm) de profundidade (devido ao método de análise de fertilidade do solo, conforme norma CONAMA 375):

Logo, 1 ha corresponde a 2x10⁶ dm³. Portanto,

$$\begin{aligned} 314.26 \text{ m}^3 & \text{-----} 2 \times 10^6 \text{ dm}^3 \\ X & \text{-----} 1 \text{ dm}^3 \\ 2 \times 10^6 X & = 314.26 \\ X & = \frac{314.26}{2 \times 10^6} = 157.13 \times 10^{-6} \text{ m}^3/\text{dm}^3 = 157.13 \times 10^{-3} \text{ L}/\text{dm}^3 = \mathbf{157.13 \text{ cm}^3/\text{dm}^3} \end{aligned}$$

Para aplicação deste resíduo nos terrários, foi necessário calcular a área, o volume e a densidade dos 5Kg das amostras de solo.

- área do terrário: 1125 cm².
- volume: 22.5 L.
- densidade: 5.0625 dm³.

Logo:

$$\begin{aligned} 157.13 \text{ cm}^3 & \text{-----} 1 \text{ dm}^3 \\ X & \text{-----} 5.0625 \text{ dm}^3 \\ X & = 795.49 \text{ cm}^3/\text{dm}^3 = 0.79549 \text{ L de lodo primário/terrário ou } \mathbf{795.49 \text{ mL de lodo primário/terrário.}} \end{aligned}$$

b) Biossólido:

Dados obtidos nas análises químicas:

Nitrogênio Kj (NK_j) = 35740 mg/kg

Nitrito (NO₂) = 1.98 mg/kg

Nitrato (NO_3) = 8.63 mg/kg

Nitrogênio amoniacal (NNH_3) = 3969 mg/kg

Fração de mineralização do bio sólido = 20%

Quantidade de N recomendada para cana-de-açúcar: 30 kg/ha (PIRES; FERREIRA, 2008) ou 120 kg/ha de N, segundo Raij et al (1997). Na média, o ideal é 100 kg/ha.

Aplicando os valores na fórmula:

$$N_{\text{disp}} = (\text{FM}/100) \times (\text{KKj}-\text{NNH}_3) + 0,5 \times (\text{NNH}_3) + (\text{NNO}_3 + \text{NNO}_2)$$

$$N_{\text{disp}} = (20/100) \times (35740-3969) + 0.5 \times (3969) + (8.63+1.98)$$

$$N_{\text{disp}} = (0.2) \times (31771) + 1984.5 + 10.61$$

$$N_{\text{disp}} = 6354.2 + 1984.5 + 10.61$$

$$N_{\text{disp}} = \mathbf{8349.31 \text{ mg/kg ou } 8.34931 \text{ Kg/t}}$$

$$\text{Taxa de aplicação (t/ha)} = \frac{\text{N recomendado (kg/ha)} (\text{m}^3/\text{ha})}{N_{\text{disp}} (\text{kg/t})}$$

$$\text{Taxa de aplicação} = \frac{100 \text{ kg/ha}}{8.34931} = 11.977037 = \mathbf{11.98 \text{ t/ha}}$$

Logo,

$$11.98 \text{ t} \text{-----} 1 \text{ ha} \text{-----} 2 \times 10^6 \text{ dm}^3$$

$$11.98 \times 10^6 \text{ g} \text{-----} 2 \times 10^6 \text{ dm}^3$$

$$X \text{-----} 1 \text{ dm}^3$$

$$\mathbf{X = 5.99 \text{ g/dm}^3 \text{ de base seca de bio sólido.}}$$

Para a aplicação de bio sólido, também consideramos a área, o volume e a densidade dos terrários, chegando ao resultado de:

$$5.99 \text{ g de bio sólido} \text{-----} 1 \text{ dm}^3$$

$$Y \text{-----} 5.0625 \text{ dm}^3$$

$$Y = 6 \times 5.0625 = \mathbf{30.324 \text{ g de biossólido seco.}}$$

A resolução CONAMA 375/2006 exige que seja calculado o peso seco do biossólido, para posterior aplicação. Assim sendo, o biossólido úmido foi disposto em três placas de Petri, pesados e secos em estufa, à 65°C, por 48h. Com o resultado, obtivemos uma média (em g) de peso seco, em relação ao peso úmido (em g) do biossólido. Logo, obtivemos como resultado:

$$12.42 \text{ g de biossólido seco} \text{ ----- } 55.94 \text{ g de biossólido úmido}$$

$$30.324 \text{ g de biossólido seco} \text{ ----- } z \text{ g de biossólido úmido}$$

$$12.42 z = 55.94 \times 30.324$$

$$z = 1696.32 / 12.42$$

$$z = \mathbf{136.58 \text{ g de biossólido úmido/terrário}}$$

c) Vinhaça

Dados do solo:

Valor de potássio (Ks): 0.23 cmol_c/dm³

CTC: 5.88 cmol_c/dm³

Dados da vinhaça:

Valor de potássio (Kvi): 3401 mg/L = 3401 kg/m³.

O valor de potássio obtido nas análises químicas foi expresso apenas na quantidade de potássio. Assim sendo, há uma relação que deve ser considerada para a vinhaça, onde 1 mg de K corresponde à 1.205 mg de K₂O.

$$1 \text{ mg de K} \text{ ----- } 1.205 \text{ mg de K}_2\text{O.}$$

$$3401 \text{ mg de K} \text{ ----- } x$$

$$X = 1.205 \times 3401$$

$$X = 4098.205 \text{ mg/L ou } 4.098205 \text{ kg/m}^3 \text{ de K}_2\text{O} = \text{Kvi}$$

Aplicando os dados na fórmula:

$$\text{m}^3 \text{ de vinhaça/ha} = [(0,05 \times \text{CTC} - \text{ks}) \times 3744 + 185] / \text{kvi}$$

$$\text{m}^3\text{vinhaça/ha} = \frac{[(0.05 \times 5.88 - 0.23) \times 3744 + 185]}{4.098205} = \frac{[(0.294 - 0.23) \times 3744 + 185]}{4.09820}$$

$$\text{m}^3\text{vinhaça/ha} = \frac{[(0.064) \times 3744 + 185]}{4.098205} = \frac{[239.616 + 185]}{4.098205} = \frac{424.616}{4.098205} = \mathbf{103.610 \text{ m}^3/\text{ha}}$$

Para aplicação da vinhaça em 1 dm³:

$$103.610 \text{ m}^3 = 103610 \text{ dm}^3 = \text{L}$$

$$103610 \text{ L} \text{ ----- } 1 \text{ ha} \text{ ----- } 8 \times 10^6 \text{ dm}^3$$

$$X \text{ ----- } 1 \text{ dm}^3$$

$$8 \times 10^6 X = 103610$$

$$X = \frac{103610}{8 \times 10^6} = 12951.278 \times 10^{-6} = 0.012951278 \text{ L} = \mathbf{12 \text{ cm}^3/\text{dm}^3}$$

$$8 \times 10^6$$

Considerando novamente a área, o volume e a densidade dos terrários, temos:

$$1 \text{ dm}^3 \text{ ----- } 0.012951278 \text{ L}$$

$$5.0625 \text{ dm}^3 \text{ ----- } y$$

$$Y = 0.012951278 \times 5.0625 = 0.06556 \text{ L}$$

$$1 \text{ L} \text{ ----- } 1000 \text{ mL}$$

$$0.06556 \text{ L} \text{ ----- } z$$

$$Z = 0.06556 \times 1000 = \mathbf{65.56 \text{ mL/terrário}}$$

5.4 Preparação das amostras de solo e resíduos para montagem dos bioensaios com *R. padbergi* e *A. cepa*, após cálculos para aplicação

Após a caracterização química das amostras e cálculos para a aplicação dos resíduos e mensurações dos terrários, foram obtidos os seguintes valores:

Tabela 5 – Preparação das amostras de solo e resíduos para a montagem dos bioensaios, da coleta de 2010

Primeiro Bioensaio	
Amostras	
SC	5 Kg de solo controle + 20 diplópodos
SL	5 Kg de solo controle + 321.72 mL de lodo primário + 20 diplópodos
SB	5 Kg de solo controle + 136.58 g de biossólido + 20 diplópodos
SV	5 Kg de solo controle + 410 mL de vinhaça + 20 diplópodos
SLV	5 Kg de solo controle + 321.72 mL de lodo primário + 410 mL de vinhaça + 20 diplópodos
SBV	5 Kg de solo controle + 136.58 g de biossólido + 410 mL de vinhaça + 20 diplópodos

SC: solo controle; **SL:** solo+lodo primário; **SB:** solo+biossólido; **SV:** solo+vinhaça de cana-de-açúcar; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça

Tabela 6 – Preparação das amostras de solo e resíduos para a montagem dos bioensaios, da coleta de 2011

Segundo Bioensaio	
Amostras	
SC	5 Kg de solo controle + 20 diplópodos
SL	5 Kg de solo controle + 795.49 mL de lodo primário + 20 diplópodos
SB	5 Kg de solo controle + 234.4 g de biossólido + 20 diplópodos
SV	5 Kg de solo controle + 65.56 mL de vinhaça + 20 diplópodos
SLV	5 Kg de solo controle + 795.49 mL de lodo primário + 65.56 mL de vinhaça + 20 diplópodos
SBV	5 Kg de solo controle + 234.4 g de biossólido + 65.56 mL de vinhaça + 20 diplópodos

SC: solo controle; **SL:** solo+lodo primário; **SB:** solo+biossólido; **SV:** solo+vinhaça de cana-de-açúcar; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça

Após a mistura e disposição das amostras de resíduos com o solo controle, foram coletadas e dispostas em placas de Petri 30 g de cada terrário para a realização dos testes com o organismo teste *A. cepa*.

5.5 Comparação dos resultados obtidos pelo sistema-teste *A. cepa*

Durante o desenvolvimento deste trabalho, os dados obtidos nas avaliações dos potenciais tóxico, citotóxico, genotóxico e mutagênico das diferentes amostras pelo sistema-teste de *A. cepa* foram compilados com os resultados obtidos nas análises químicas e/ou na avaliação histopatológica do intestino médio de diplópodos que compuseram diferentes artigos. Portanto, a comparação dos resultados obtidos nas diferentes coletas está representada nas tabelas 6 e 7 e figuras 1 e 2.

Comparando os resultados, é possível observar que as amostras brutas de biossólido e lodo primário, SV, SLV e SBV, foram citotóxicas para o tempo 0 (t0), coleta de 2011. A genotoxicidade foi evidenciada para todas as amostras do tempo 0 (t0) de ambas coletas. Foi verificado efeito mutagênico, neste mesmo período, foi verificada para as amostras de SL, SV, SLV e SBV coletadas em 2010 e, SV, SLV e SBV da coleta de 2011.

Após 30 dias de exposição aos diplópodos (t30), nenhuma amostra apresentou citotoxicidade. Entretanto, ainda foi verificado efeito genotóxico para todas as amostras testadas, em ambas as coletas. As amostras de SB, SV, SLV e SBV, da coleta de 2010, não apresentaram efeitos mutagênicos.

Tabela 7 – Média e desvio padrão do índice mitótico (IM), do índice de aberrações cromossômicas (IAC) e do índice de mutagenicidade (IMt), em células meristemáticas de *Allium cepa*, após a exposição à água ultrapura (controle negativo), ao solo controle, ao MMS e à trifluralina (controles positivos), à amostra bruta de biossólido, lodo primário e às combinações de resíduos, no tempo 0 (t0), para os dois bioensaios realizados

Amostra	Coleta	Tratamento	IM	IAC	IMt
Controles	2010	CN	13.66±1.37	1.2±0.83	0.2±0.44
	2011	CN	10.68±1.62	2.8±2.04	1.4±1.14
	2010	SC	14.21±0.90	2±0.70	0.6±0.54
	2011	SC	12.02±1.15	4.6±0.54	2.0±2.0
	2010	MMS	11.53±0.88	8.6±3.04* ¹	25.6±8.5* ¹
	2011	MMS	12.23±0.82*	8.0±3.0* ¹	28.4±8.01* ¹
	2010	TRIF	9.47±0.54	51.4±8.79* ¹	12.2±3.42* ¹
	2011	TRIF	9.26±0.99 ¹	66.6±29.9* ¹	26±6.51* ¹
AMOSTRA BRUTA	2010	L	-	-	-
	2011	L	12.32±0.93*	20±3.39* ¹	4.2±1.48* ¹
	2010	B	15.07±0.56	2.15±1.12*	0.16±0.18
	2011	B	11.49±0.76*	19.4±3.97* ¹	3.2±1.3*
TEMPO 0 (T0)	2010	SL	13.26±1.19	18±2.44* ¹	1.2±0.44*
	2011	SL	11.53±2.02	19.2±4.43* ¹	1.4±0.54
	2010	SB	15.07±0.56	21±5.83* ¹	1.2±1.3
	2011	SB	12.45±1.33	22±8.33* ¹	2.2±1.33
	2010	SV	13.80±1.48	18.6±2.79* ¹	1.4±0.54*
	2011	SV	30.88±3.23* ¹	6.8±3.2*	3.2±1.09*
	2010	SLV	14.54±1.48	19±3.49* ¹	2±1.22*
	2011	SLV	12.38±0.73*	22.2±2.94* ¹	3.0±1.0*
	2010	SBV	15.01±1.26	21±5.04* ¹	2.2±0.44* ¹
	2011	SBV	12.6±1.44*	24.6±2.07* ¹	3.8±2.04*

SC: solo controle; **CN:** controle negativo; **MMS:** controle positivo; **TRIF:** controle positivo; **L:** amostra bruta de lodo de esgoto primário; **B:** amostra bruta de biossólido; e combinações; **SL:** solo+lodo primário; **SB:** solo+biossólido; **SV:** solo+vinhaça; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça.

* valores estatisticamente significativos, em relação ao controle negativo, pelo método estatístico de Mann-Whitney, com p<0.05.

¹ valores estatisticamente significativos, em relação ao solo controle, pelo método estatístico de Mann-Whitney, com p<0.01.

Tabela 8 – Média e desvio padrão do índice mitótico (IM), do índice de aberrações cromossômicas (IAC) e do índice de mutagenicidade (IMt), em células meristemáticas de *Allium cepa*, após a exposição à água ultrapura (controle negativo), ao solo controle, ao MMS e à trifluralina (controles positivos) e às combinações de resíduos, no tempo 30 (T30), para os dois bioensaios realizados

Amostra	Coleta	Tratamento	IM	IAC	IMt	
Controles	2010	CN	11.2±1.78	2±1	0.6±0.54	
	2011	CN	11.99±1.10	4.6±1.14	1.4±0.54	
	2010	SC	9.48±1.07	1.6±1.14	0	
	2011	SC	12.65±1.05	5.4±0.54	1.4±0.54	
	2010	MMS	5.73±1.15	14.2±2.94* ¹	24.8±7.29* ¹	
	2011	MMS	8.13±1.24	16.4±4.33* ¹	19.8±4.08* ¹	
	2010	TRIF	9.31±1.35	23.6±4.82* ¹	3.6±0.89* ¹	
	2011	TRIF	13.33±2.35	73.6±16.39* ¹	14.6±6.8* ¹	
	TEMPO 30 (T30)	2010	SL	13.33±1.04	10.2±1.48* ¹	1.2±0.83* ¹
		2011	SL	11.94±1.65	20.6±2.7* ¹	3.8±1.3* ¹
		2010	SB	15.03±0.79	10.6±3.33* ¹	0.4±0.54
2011		SB	11.84±1.69	20±2.34* ¹	3.2±1.3* ¹	
2010		SV	14.49±1.54	16±2* ¹	0.2±0.44	
2011		SV	12.75±1.93	27.8±6.68* ¹	2.4±0.54* ¹	
2010		SLV	11.49±1.59	12±5.24*	0.8±0.83	
2011		SLV	12.33±1.32	20.4±6.34* ¹	2.6±0.54* ¹	
2010		SBV	12.78±0.46	10.6±3.2* ¹	0.8±0.83	
2011		SBV	11.84±1.11	23±4.69* ¹	4.8±1.64* ¹	

SC: solo controle; **CN:** controle negativo; **MMS:** controle positivo; **TRIF:** controle positivo; e combinações: **SL:** solo+lodo primário; **SB:** solo+biossólido; **SV:** solo+vinhaça; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça.

* valores estatisticamente significativos, em relação ao controle negativo, pelo método estatístico de Mann-Whitney, com p<0.05.

¹ valores estatisticamente significativos, em relação ao solo controle, pelo método estatístico de Mann-Whitney, com p<0.01.

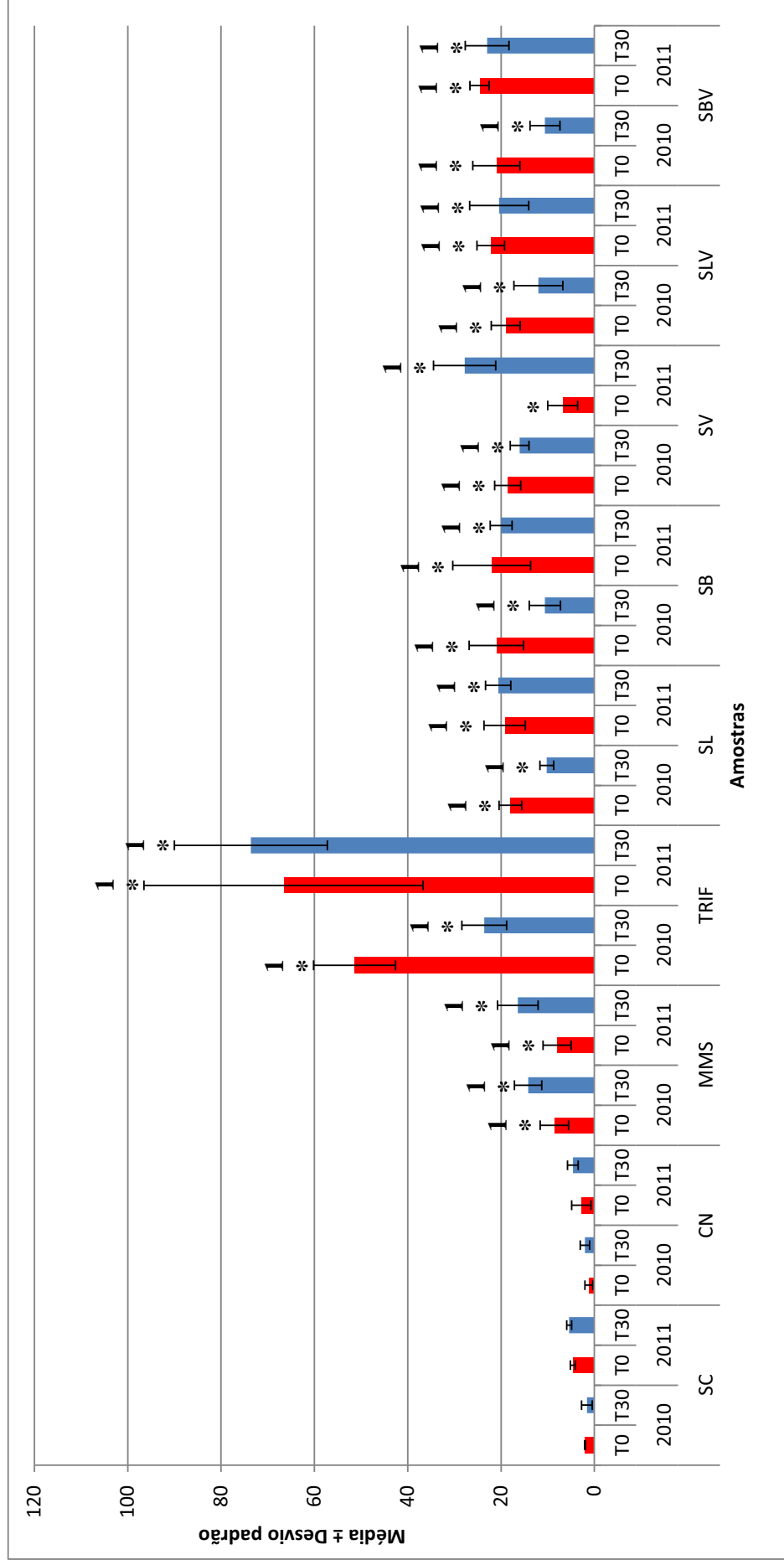


Figura 1 – Média e desvio padrão do índice de aberrações cromossômicas (IAC), indicativas de genotoxicidade, em células meristemáticas de *Allium cepa*, após a exposição à água ultrapura (controle negativo), ao solo controle, ao MMS e à trifluralina (controles positivos) e às combinações de solo controle, lodo primário, biossólido e vinhaça, no tempo 0 (t0) e após 30 dias (t30), para os dois bioensaios realizados

SC: solo controle; **CN:** controle negativo; **MMS:** controle positivo; **TRIF:** controle positivo; **SBV:** solo+biossólido+vinhaça. **SB:** solo+lodo primário; **SV:** solo+lodo primário+vinhaça; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça.

* valores estatisticamente significativos, em relação ao controle negativo, pelo método estatístico de Mann-Whitney, com $p < 0.05$.
 † valores estatisticamente significativos, em relação ao solo controle, pelo método estatístico de Mann-Whitney, com $p < 0.01$.

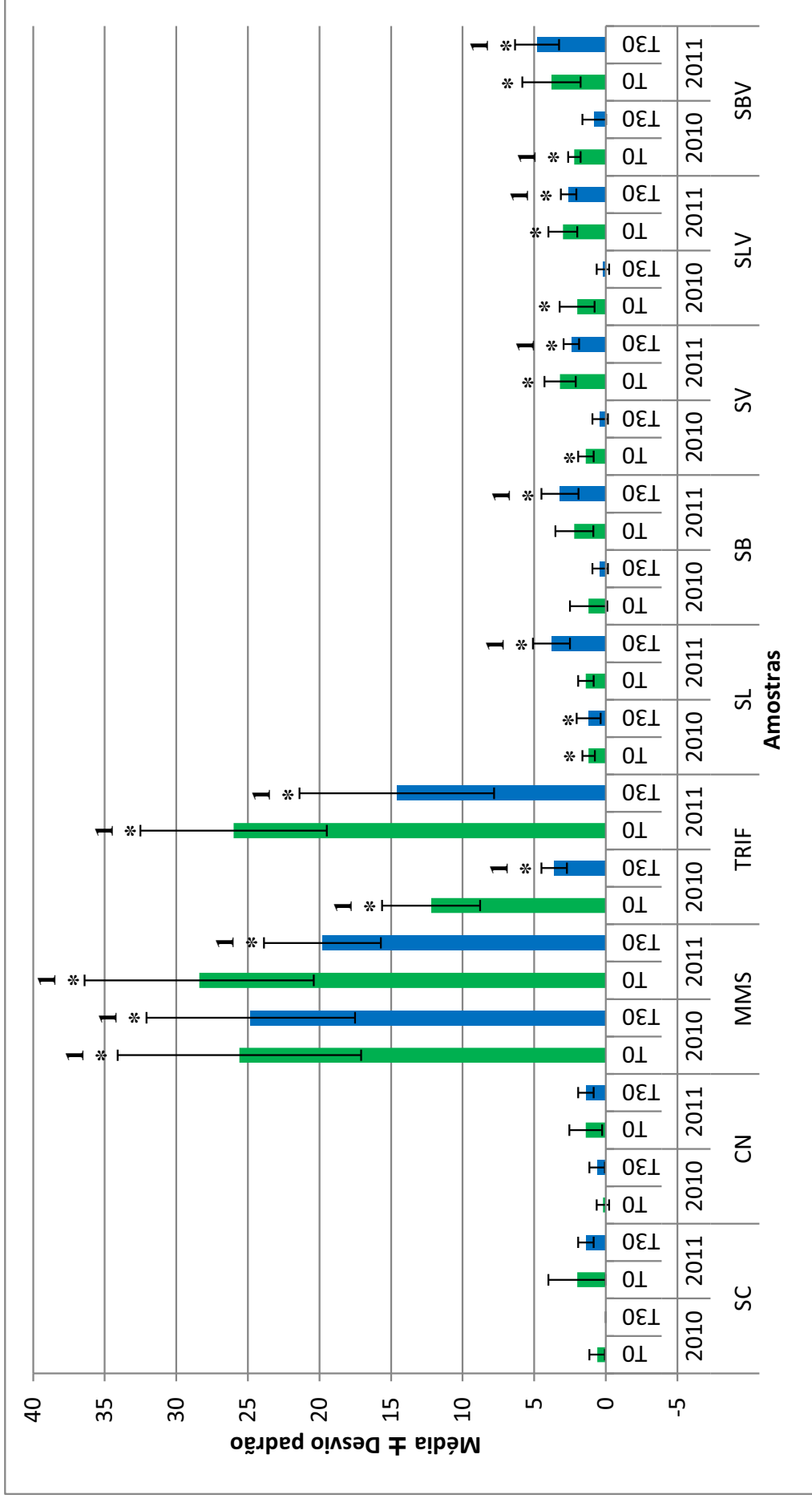


Figura 2 – Média e desvio padrão do índice de micronúcleos e quebras cromossômicas (IMt), indicativos de mutagenicidade, em células meristemáticas de *Allium cepa*, após a exposição à água ultrapura (controle negativo), ao solo controle, ao MMS e à trifluralina (controles positivos) e às combinações de solo controle, lodo primário, biossólido e vinhaça, no tempo 0 (t0) e após 30 dias (t30), para os dois bioensaios realizados

SC: solo controle; **CN:** controle negativo; **MMS:** controle positivo; **TRIF:** controle positivo; e combinações: **SL:** solo+lodo primário; **SB:** solo+biossólido; **SV:** solo+vinhaça; **SLV:** solo+lodo primário+vinhaça; **SBV:** solo+biossólido+vinhaça.

* valores estatisticamente significativos, em relação ao controle negativo, pelo método estatístico de Mann-Whitney, com $p < 0.05$.

† valores estatisticamente significativos, em relação ao solo controle, pelo método estatístico de Mann-Whitney, com $p < 0.01$.

Durante a realização desta tese foram produzidos seis artigos, dos quais dois já se encontram publicados, um já foi enviado para publicação e os outros estão em fase de envio para revistas especializadas.

ARTIGO 1. Biosolid soil application: toxicity tests under laboratory conditions

Cintya Ap. Christofolletti, Annelise Francisco, Carmem S. Fontanetti

Publicado na Applied and Environmental Soil Science

ARTIGO 2. Assessment of the genotoxicity of two agricultural residues after processing by diplopods using the *Allium cepa* assay

Cintya Ap. Christofolletti, Janaína Pedro-Escher, Carmem S. Fontanetti

Publicado na Water, Air and Soil Pollution

ARTIGO 3. Vinasse: environmental implications of its use and pollution potential

Cintya Ap. Christofolletti, Janaína Pedro-Escher, Jorge Evangelista Correia, Júlia Fernanda Urbano Marinho, Carmem S. Fontanetti

Submetido para publicação na Waste Management

ARTIGO 4. Diplopods as soil bioindicators toxicity after application of residues from sewage treatment plants and ethanol industry

Cintya Ap. Christofolletti, Annelise Francisco, Janaína Pedro-Escher, Renato Salaroli, Carmem S. Fontanetti

Em fase de submissão

ARTIGO 5. Sewage sludge: available treatments, main uses and effects on organisms

Cintya Ap. Christofolletti, Guilherme Thiago Maziviero, Carmem S. Fontanetti

Em fase de submissão

ARTIGO 6. Investigation of the genotoxicity of primary sewage sludge, the precursor of biosolids used in agriculture, with the *Allium cepa* system

Cintya Ap. Christofolletti, Janaína Pedro-Escher, Carmem S. Fontanetti

Em fase de submissão

Vinasse: environmental implications of its use and pollution potential

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Abstract

The inadequate and indiscriminate disposal of vinasse in soils and water bodies has received much attention since decades ago, due to environmental problems associated to this practice. Vinasse is the final by-product of the distillation of biomass, mainly for the production of ethanol, from sugar crops (beet and sugar cane), starch crops (corn, wheat, rice, and cassava), or cellulosic material (harvesting crop residues, sugar cane bagasse, and wood). Because of the large quantities of vinasse produced, alternative treatments and uses have been developed, such as recycling of vinasse in fermentation, fertirrigation, concentration by evaporation, and yeast and energy production. This review was aimed at examining the available data on the subject as a contribution to update the information on vinasse, from its characteristics and chemical composition to alternatives uses in Brazil, the effects on soil properties, seed germination, use as biostimulant, and environmental contaminant.

Keywords: agroindustrial residue, sugar cane, toxicity, genotoxicity.

1. Introduction

The management and disposal of agro-industrial residues have recently received attention because of the inadequate and indiscriminate discharge of many effluents in the environment. Given that each industrial effluent has properties and specific effects on the biota, assessments of their contribution to environmental contamination is essential (Srivastava and Sahai, 1987).

In this context, little is known about the agricultural vinasse residue. A by-product mainly of the sugar-ethanol industry, vinasse is usually an acidic compost (pH: 3.5-5), dark brown liquid, with a high organic content (COD: 50-150 gL⁻¹), and an unpleasant odor to humans (Espana-Gamboa et al., 2011; Waliszewski et al., 1997). According to Wilkie et al. (2000), the production of ethanol from sugar crops (beet, sugarcane, molasses, etc), starch crops (corn, wheat, rice, cassava, etc), and/or cellulosic material (sugar cane bagasse, harvesting and wood residues), produce a considerable volume of vinasse with high pollution potential. On

average, 10 to 15 liters of vinasse are produced per liter of ethanol, depending on the distillery equipment (Cortez et al., 1992).

In 1975, Brazil's government created the National Ethanol Program (PROÁLCOOL) during to the oil crisis as an alternative to petroleum products, in an attempt to supply domestic and international markets. Because of the expansion of the sugar-ethanol industry since 1975, refineries began to play an important role in environmental pollution. Brazil is currently the world's largest sugar cane producer (Demattê et al., 2004), with approximately 350 active refineries with capacity to produce 16 billion liters of ethanol per year (Junior et al., 2008; UNICA, 2007).

According to studies conducted in the 1980s, an average size refinery produced 10^6 L ethanol/year. Simultaneously with the increase in vinasse production, alternative uses have been proposed. In the past, considerable quantities of vinasse were discharged in water bodies, causing serious pollution problems (Demattê et al., 2004; Santos et al., 1981).

Researchers have then focused on finding adequate uses and treatments for vinasse. As a result some alternatives have been proposed, such as recycling of vinasse in fermentation, fertirrigation, concentration by evaporation, yeast production, energy production, and raw material for the production of livestock and poultry feed (Robertiello, 1982).

Currently, an environmental perspective has been emphasized, and in some cases, the application of vinasse has been debated due to its effects on the soil and ground waters (Gianchini and Ferraz, 2009; Silva et al., 2007). This review was aimed at examining the available data on the subject as a contribution to update the knowledge on vinasse from the characterization of its chemical composition to alternative uses in Brazil, effects on soil properties, seed germination, and its use as biostimulant and environmental contaminant.

2. Sugar cane crop: Characterization and chemical composition of vinasse

Brazil is the world's largest sugar cane producer, with nearly 5 million hectares of cultivated area (Basanta et al., 2003), followed by India, China, Thailand, Mexico, Kenya and Pakistan. According to the last census, since the 2011/2012 crop 492.70 million of tons of sugar cane were processed, producing 12,71 billion of liters of hydrated ethanol (CANAOSTE, 2012).

Despite the wealth that this industry generates, there are problems from the planting to the harvest of sugar cane (Alvarenga and Queiroz, 2009). Some negative environmental aspects in the cultivated area are: biodiversity reduction caused by deforestation and implementation of the sugarcane monoculture; soil and surface water contamination due to excessive

fertilization, mineral correctives, and application of chemicals, soil compaction caused by heavy machinery during planting, management and harvesting; silting of water bodies due to soil erosion in areas of crop renovation; release of ashes and greenhouse gases during the burning prior to harvesting (Alvarenga and Queiroz, 2009) and from vinasse along transportation ditches and after fertirrigation (Aguiar, 2011).

One of the most important and little discussed subjects regarding the negative impacts of sugar cane is vinasse, the byproduct of ethanol. Vinasse is produced during the distillation of ethanol following the fermentation of molasses (Figure 1). It is a residual liquid, also known in many regions of Brazil and other parts of the world as stillage, mosto, dunder or distillery pot ale (Camargo et al., 2009; Gianchini and Ferraz, 2009; Wilkie et al., 2000). It is produced in many countries as the by-product of ethanol from different feedstocks: sugar cane in South America, beet, wine, and fruits in Europe, and corn in North America (Gianchini and Ferraz, 2009). Thus vinasse may exhibit different properties. España-Gamboa et al. (2011) recently reviewed the different compositions of vinasse and available treatments. According to these authors, the characteristics of vinasse depend on the feedstock (biomass) used for the production of ethanol.

The chemical composition of sugar cane vinasse varies depending on the plant used for the production of ethanol and the distillation process. Thus, effluents from the distillation of molasses and sugar cane juice are different. The molasses mosto has higher concentrations of organic matter, potassium, calcium, and magnesium. Sugar cane mosto, on the other hand, have considerably lower concentrations of these elements – produced mainly in autonomous distilleries (Robertello, 1982).

In general, this effluent presents dark color and consists of basically water (93%) and organic solids and minerals (7%). It has high levels of organic matter, but is low in N and P. The main component of vinasse, whether from beet, sugarcane, or corn, is organic matter in the form of organic acids and cations such as K, Ca and Mg (Gianchini and Ferraz, 2009). Based on the data available from the literature and chemical analysis conducted by specialized laboratories, table 1 presents the main elements found in the different types of vinasse.

3. Alternatives for the use of sugar cane vinasse in Brazil

The main alternatives for the use of sugar cane vinasse (Figure 2) are discussed below. These alternatives are currently in different stages of technological development. According to Laime et al. (2011), aerobiosis, and recycling in fermentation and fertirrigation can be used in large scale, while vinasse combustion, yeast production, civil construction, livestock feed

production, anaerobic digestion, incineration, among others are in different stages of development and research. Table 2 summarizes the advantages and disadvantages of the alternatives discussed.

3.1. Fertirigation

According to Camargo et al. (2009), the first studies on the application of vinasse to the soil in Brazil started in the 1950s and were conducted by the Luiz de Queiroz College of Agriculture (ESALQ). The use as fertilizer in fertirigation became common in sugar cane refineries beginning in the 1980s (Corazza, 1996). Fertirigation consists of the infiltration of raw vinasse in the soil by irrigation of sugar cane crops (Camargo et al., 2009). When applied to the soil, in addition to irrigation, vinasse fertilizes the crop, simultaneously disposing of the effluent and lowering the costs with chemical fertilizers (Laime et al., 2011).

The use of vinasse in fertirigation is an alternative that focuses on the rational use of natural resources, preventing the discharge of vinasse in rivers, while fertilizing agricultural land (Gianchini and Ferraz, 2009).

Among the alternatives for the use of vinasse developed around the world, fertirigation is the most commonly used, as requires a low initial investment (tubes, pumps, trucks, and decantation tanks), low maintenance cost, fast application, does not require complex technologies, and increases crop yield (Camargo et al., 2009; Santana and Machado, 2008). This practice has totally or partially replaced the use of chemical fertilizers, mainly those containing phosphorus (Corazza, 1996).

According to Fronzalia (2007) and Junior et al. (2008), sugar cane occupies nearly three million of hectares in São Paulo State alone. Of these, 75 to 80% could be irrigated with vinasse. However, according to many authors, the direct application of vinasse in the soil can cause salinization, leaching of metals present in the soil to groundwater, changes in soil quality due to unbalance of nutrients, mainly manganese (Agrawal and Pandey, 1994), alkalinity reduction, crop losses (Kumar and Viswanathan, 1991), increase of phytotoxicity and unpleasant odor (Navarro et al., 2000; Santana and Machado, 2008). According to Santana and Machado (2008), fertirigation may be a palliative practice that provides a false impression of solving efficiently the problem of vinasse disposal.

On the other hand, certain environmental parameters need to be accounted for in fertirigation, such as soil type, distance from water bodies, soil field capacity (water retention) percentage of salts in the soil (Laime et al., 2011). Studies conducted by Coopersucar, one of the cooperatives of sugar cane producers in São Paulo State, and Penatti et al. (1999)

indicated that doses of 300 m³/ha of vinasse with potassium levels between 3 and 4 kg/m³ of vinasse, regardless of the type of soil, do not alter physical, chemical and biological properties of the soil.

3.2. Concentration by evaporation

This process is an alternative for the use of vinasse, since fertirrigation not always can dispose of total volume of vinasse produced. The product obtained in this process is used in the production of livestock feed and to improve the quality of vinasse as fertilizer. It can also be burned in special boilers generating energy or decreasing the water use in the facility, and the condensate removed by evaporation can be treated and reused in the process.

High energy demand is probably the main constraint of vinasse concentration. Some methods have been described in the literature for the treatment and concentration of vinasse (Gomes et al., 2011). However, according to Fitzgibbon et al. (1995) and Navarro et al. (2000), vinasse concentration and incineration are the only methods that can satisfactorily solve the pollution problem.

In the concentration process, water is removed from vinasse (without loss of solids), reducing its volume. The first vinasse concentration plants were installed in 1942 in Austria by the Austrian company Vogelbusch. This process can reduce the costs with transportation in tanker trucks, increasing radius of vinasse application, where fertirrigation in ducts is unfeasible (ANA, 2009). However, this process has problems associated with the fast incrustation of evaporators and spontaneous crystallization as the concentration of solids increases (Rodrigues, 2008).

In Brazil, only one plant concentrates vinasse. Installed as a demonstration facility more than 20 years ago, it processes 5% of the vinasse produced, concentrating it to 40% (ANA, 2009).

When not used as fertilizer, concentrated vinasse can be used in the production of livestock feed, due to its high levels of nutrients. The production of livestock feed from vinasse has also been studied in the 1980s (Laimé et al., 2011). The residue needs to have level of potassium reduced, and can be used as feed for cattle, pigs, and poultry. The feed produced does not interfere in the taste or odor of milk or dairy products, is well accepted by animals, and the conversion rate (weight gain in relation to feed consumption) is adequate. However, dosage limits should be observed (Corazza and Salles Filho, 2000).

According to Waliszewski et al. (1997), in some countries, dry vinasse is used to substitute molasses, mainly to feed ruminants. For these animals, the feed produced from

vinasse should not be over 10% of the daily feed; and under 2 to 3% for pigs, (Corazza and Salles Filho, 2000; Laime et al., 2011). However, the high levels of salts and low quantities of carbohydrates limit its use as poultry feed due to the low level of metabolizable energy (Waliszewski et al., 1997).

3.3. Energy production

An alternative that has increasingly being used in the ethanol industry is the anaerobic biodigestion of the organic load of vinasse. This process consists of the biodegradation of the organic load of vinasse to produce biogas and biodigested vinasse (Cortez et al., 2007).

The anaerobic process of biodigestion occurs in two stages, the acidogenic and methanogenic phases. In the acidogenic phase, the organic compounds of complex chains, such as lipids, carbohydrates and proteins are hydrolyzed until the formation of compounds with smaller carbon chains. These smaller-chain compounds are biologically oxidized and converted in organic acids, such as acetic acid (CH_3COOH) and propionic acid ($\text{CH}_3\text{-CH}_2\text{-CH}_2\text{-COOH}$) by facultative and obligate anaerobic bacteria. The reduction of the organic load of the effluent occurs in this phase (Cortez et al., 2007).

In the methanogenic phase, acids are converted into methane, carbon dioxide and organic acids, or carbon dioxide is reduced until the formation of methane by anaerobic microorganisms. This is the slowest phase of the process and controls the conversion rates. The maintenance of the adequate conditions to promote it is therefore essential (Cortez et al., 2007).

The biodigested vinasse is later used as fertilizer. Although it presents a reduced organic load, it maintains its original properties as fertilizer. On the other hand, biogas is mainly used to produce energy, due to its high methane content.

In the sugar-ethanol industry, biogas can be used to: operate gas turbines combined to an electric generator; substitute part of the fuels used in the agroindustry during the harvesting time; or use in boilers to generate vapors and to mill sugar cane (Cortez et al., 2007; Szymanski et al., 2010).

Anaerobic biodigestion has received more attention only after the development of high performance reactors, such as the UASB (Upflow Anaerobic Sludge Blanket), which is the most adapted to vinasse. In this type of system, the sludge at the bottom of the reactor adsorbs most of the organic matter, while gas is produced in the reaction compartment as bubbles during the anaerobic process, and removed to a separate compartment (Von Sperling, 2005).

This treatment in biodigesters and reactors has the advantage of producing biogas that can be used in the production of energy, in addition to have a low electric energy consumption, little production of biological sludge to be disposed and low polluting potential – mainly due to the reduction of the organic load of vinasse, since most of the BDO (biochemical demand of oxygen) is converted into biogas (Freire and Cortez, 2000). However, Cortez et al. (2007) describes the longer detention time compared to aerobic systems and production of corrosive gases with unpleasant odor as the main inconvenients of the anaerobic digestion.

Therefore, anaerobic biodigestion is an alternative of great economic as well as environmental interest in the treatment of vinasse, as the biogas produced, once purified, has calorific value similar to that of natural gas, with the advantage of being a renewable and easily available fuel (Szymanski et al., 2010).

4. Effects on the soil properties

According to many authors, the addition of vinasse on the soil can promote many beneficial or not effects on terrestrial ecosystems. When deposited in the soil, vinasse can improve fertility; but should not exceed the soil's ion retention capacity. Therefore, dosages should be determined based on the characteristics of each soil, due to its unbalanced quantities of mineral and organic elements, and leaching of ions may occur, especially nitrate and potassium (Silva et al., 2007). In general, its use can result in modifications in different soil properties.

The effects of the application of this residue on the soil depend on various factors, such as the quantity applied in the soil, soil type and chemical composition, relief, and crop type, and the economic conditions involved in the process.

Many studies on the disposal of vinasse in the soil have reported beneficial effects on crops and physico-chemical properties of the soils, as it increases moisture retention, porosity, potassium levels, and electric conductivity, in addition to biological activity. However, few studies have assessed the real polluting potential of vinasse in the soil and water table (Lyra et al., 2003; Tenório et al., 2000).

4.1. Physical properties

Many studies have examined the effects of vinasse on soil properties. Neves et al. (1983) reported that the addition of vinasse combined with organic matter can increase the physical conditions of the soil and the mobilization of nutrients, as a result of higher solubility

provided by the liquid residue. However successive applications in sandy soils can lead to an unbalance of bases and elements such as calcium, magnesium, potassium, and sodium, which are used to evaluate soil fertility (Silva et al., 2007).

Andrioli (1986) did not observed an effect of applications of 1.200 m³/ha of vinasse in total porosity, macroporosity, and microporosity of a latosol cultivated with sugar cane, and hypothesized that these results were probably due to a lack of increase in organic matter in the soil. Soil density was also altered as a result of the disposal of this residue, based on studies by Camargo et al. (1983).

On the other hand, Canellas et al. (2003) reported an increase in the level of organic matter, thus improving the physical conditions of the soil as a result of applications of vinasse throughout the years. Recent studies conducted by Zolin et al. (2011), in an exploratory study on the application of vinasse during years, observed an increase in the levels of organic carbon and potassium in the soil.

By promoting changes in the physical properties of the soil, vinasse can increase the infiltration capacity of the soil, contaminating the groundwater, reducing it, and increasing drainage with possible contamination of surface waters. In addition, the recharge mechanisms of water tables and aquifers are controlled mainly by rainfall. Thus, as it reaches the soil contaminated with vinasse, rainfall can infiltrate or drain superficially, polluting water bodies (Silva et al., 2007).

4.2. Biological and chemical properties

The addition of vinasse in the soil causes temporary changes in the population of microorganisms in the soil, resulting in many alterations in the biological and chemical processes, such as: decomposition of the organic matter, nitrification, denitrification, fixation of air N₂ and increase in pH. It indirectly facilitates the action of microorganisms in the agglutination of soil particles, improving its structure. Camargo (1954) observed an increase in the microbe population in soils treated with vinasse, with predominance of the fungi: *Neurospora* ssp, *Aspergillus* ssp, *Penicillium* ssp, *Mucor* ssp and *Streptomyces* ssp. In addition, Santos et al. (2009) reported that the addition of vinasse can significantly alter the population of fungi and bacteria in the irrigated soil, as well as actinomycetes and cellulolytic bacteria.

On the other hand, Tejada et al. (2007) reported that beet vinasse had a negative impact in the biological properties of the soil, interfering with microbe biomass, respiration, and enzymatic activities.

Glória and Orlando Filho (1983) described the effects of vinasse on the soil as: 1) increase in pH; b) increase in the availability of some ions and c) increase in the cation-exchange capacity. Leal et al. (1983) observed that the increase in soil pH after the application of vinasse may be associated with the development of the microbial population and the transformation of nitrogen during the denitrification process of nitrate (NO_3^-) into nitrite (NO_2^-).

4.3. Vinasse and effects on seed germination

The effects of vinasse on seed germination depend on its concentration and the crop (Pant and Adholeya, 2007). Algur and Kadioglu (1992) documented the toxic effects of this effluent in the growth, biomass, and primary productivity of *Pisum sativum* (pea) and *Helianthus annuus* (sunflower).

Ramana et al. (2002) evaluated the germination rates of tomato, onion, bell pepper, pumpkin, and cucumber seeds. The authors observed a decrease in the germination rate of seeds in five crops with the increase in the concentration of vinasse. Germination was inhibited in five of the crops examined in concentrations above 50%. However, vinasse at 10% had a positive effect in the germination of onion seeds.

Azania et al. (2004) applied vinasse in pots with arrowleaf sida (*Sida rhombifolia*), Suriname grass (*Brachiaria decumbens*) and sugar cane (variety RB72454) in greenhouse. The authors observed that vinasse had negative effects on the emergence and development of seeds of the Suriname grass and arrowleaf sida, but not on sugarcane, possibly due to the sensitivity of seeds to alcohol compounds present in the effluent.

Several studies have presented the influence of vinasse in the germination of seeds from different crops. Table 3 presents some studies on the effects of vinasse on different plant species.

5. Vinasse as source of nutrients for bioremediation

Bioremediation is one of the most viable options to remediate soils contaminated with organic and inorganic compounds considered harmful to the environment (Abioye, 2011). This process is defined as the use of microorganisms/plants to detoxify or remove xenobiotics from the environment. To promote the adequate activity of microorganisms, nutrients are often added to stimulate their activity. Thus, the use of vinasse has been evaluated as a source of nutrients for microorganisms in the decontamination of organic residues present in the soil (Crivelaro, 2005; Prata et al., 2001).

The organic matter from vinasse is an important source of soluble carbon, as glycerol, readily available to microorganisms (Prata et al., 2001). Because of these characteristics, these authors evaluated if vinasse could accelerate the degradation of the herbicide ametryn in the soil and obtained positive results.

According to Laime et al. (2011), vinasse promotes immediate soil acidification, favoring the development of fungi, which are the microorganisms responsible for the early stages of the decomposition process. As the pH of the soil increases, bacteria complete the final decomposition of vinasse. On the other hand, in the biodegradation of petroleum residues, nutrients need to be added to the process, among them N and P, S, Fe, Mg, Ca and Na. Pinto-Mariano et al. (2006) used vinasse in the bioremediation of soils contaminated with diesel oil. Using the Bartha respirometric assay, these authors observed an increase in the microbial population. However, they concluded that vinasse was not adequate to stimulate the bioremediation of contaminated soils, as the residue was biodegraded in the first 20 days of experiment, followed by a sharp decrease in CO₂ production.

Crivelaro et al. (2010) combined vinasse and petroleum sludge (produced by petroleum refineries) to accelerate the biodegradation of the latter. The authors also used the Bartha respirometric assay to assess the efficiency of the treatments consisted of soil and petroleum sludge, with soil moisture adjusted with and without vinasse for 121 days. Although the authors observed an increase in the microbial population in the treatments, vinasse was not adequate to increase the efficiency of biodegradation of petroleum sludge in the soil, as no differences were observed between CO₂ produced in the treatments with or without vinasse after the total consumption of vinasse. Thus the authors concluded that the use of vinasse as stimulant agent in biodegradation processes was inefficient under experimental conditions.

6. Vinasse as contaminant

6.1. Aquatic environments

Historically until the 1970s, growing volumes of vinasse were discharged into water bodies, mainly rivers located near sugar cane culture and ethanol refineries. Vinasse was considered highly toxic to animals, plants, microbes and microflora from freshwater and disturbed marine animals that came to the Brazilian coast to reproduce. Vinasse has a high pollution potential, approximately one hundred times more than household sewage, due to high organic matter content, causing a depletion of oxygen, low pH, high corrosivity, and high levels of biochemical demand of oxygen (BDO) (Freire and Cortez, 2000; Kannan and

Upreti, 2008). The consequences of the discharge of this effluent have been known for a long time. The organic load of vinasse causes the proliferation of microorganisms that deplete the oxygen dissolved in the water, kill aquatic animals and plants, and make contaminated water bodies more difficult to be used as sources of potable water. In addition, the discharge of vinasse in water bodies releases an unpleasant odor and contributes to disseminate endemic diseases such as malaria, amebiasis, and schistosomiasis (Laime et al., 2011) by absence of natural predators or transmissivity vectors.

Verma and Dalala (1976) investigated the capacity of survival of two fish species exposed to vinasse diluted in different temperatures and pHs. The authors observed that different concentrations killed 50% of fishes in the LC₅₀ assay within 96h. The authors recommended that raw vinasse should not be discharged in water bodies.

Gómez and Rodríguez (2000) reported the corrosive action of vinasse and its high potential of contamination of surface waters. More recent studies conducted by Gunkel et al. (2007), in the Ipojuca River, northeastern Brazil revealed that fertirrigation of sugarcane crops is one of the main sources of the contamination of the river, causing an increase in water temperature and acidification, increasing turbidity and oxygen depletion. The authors concluded that green methods of sugarcane cultivation need to be developed as well as technologies to reduce residues and water recycling processes in order to protect the water resources of the region.

The deleterious effects of vinasse were observed in studies conducted by Kumar and Gopal (2001). These authors reported that dilutions of sugarcane vinasse increased the production of mucus and reduced the quantity of proteins in different organs such as liver, brain, kidneys, and muscles in the fish *Channa punctatus*.

According to Piacente (2005), the infiltration of vinasse compromises groundwater potability, since it transfers high concentrations of ammonia, magnesium, aluminum, iron, manganese, chlorides, and organic matter to the water table. To confirm these results, Hassuda (1989) demonstrated that ground waters presented altered physico-chemical characteristics after the application of large quantities of vinasse, approximately 12.000 m³/ha⁻¹, for nine consecutive crops in sandy soils and temperate climate.

According to Ludovice (1997), in channels used for vinasse fertirrigation, the contamination of the water table can reach 91.7%, polluting the groundwater. In very sandy soils, the soil absorbs one meter of vinasse every four days. In more compact soils, on the other hand, absorption of vinasse may take twice as long (Piacente, 2005).

Botelho et al. (2012) investigated the toxicity of vinasse to cladocerans and fish before and after pH adjustment using an acute toxicity test. Linear and quadratic regression models were adjusted to demonstrate the concentration–response relationship between vinasse and the endpoints evaluated. The median lethal concentrations (LC50–48h) of vinasse before pH adjustment for *Ceriodaphnia dubia* and *Daphnia magna* obtained by the authors were 0.67% and 0.80% respectively, and the median lethal concentrations (LC50–96h) for *Danio rerio* was 2.62%. After pH adjustment, the values increased for all organisms, demonstrating a decrease in toxicity. This study reported marked toxicity for vinasse to aquatic organisms with toxicity reduction after pH adjustment.

6.2. Terrestrial environment

Pedrosa et al. (2005) assessed the toxicity of vinasse and concluded that exposed eggs of the nematoids *Meloidogyne javanica* and *M. incognita* exhibited reduced density and hatching rates. Yesilada (1999) demonstrated that the fecundity rate of eggs and the fertility of *Drosophila melanogaster* females were also reduced when exposed to high concentrations of vinasse.

Cruz et al. (2008) detected and mapped anomalies caused by the inadequate disposal of large quantities of vinasse in an old infiltration tank located in Ribeirão Preto, Brazil, using the electrical resistivity method. The authors observed that the contamination exceeded the limits of the tank and that the effect of vinasse can be characterized as low resistivity, a behavior comparable to that of leachate.

Waal et al. (2009) investigated the effect of vinasse on the sugar cane fields in the city of Patrocínio Paulista, São Paulo, Brazil, as an alternative to provide nutrients to the crop. Because of its relevance to environmental pollution in the area, with implications to human health, soil samples from six sites were collected and analyzed. The authors observed that contamination can occur in the study site due to soil granulation and chemical characteristics and high levels of potassium, organic matter, calcium, magnesium, nitrogen, phosphorus present in vinasse, indicating a possible contamination of surface and ground waters as a result of leaching of nitrogen compounds and ammonia.

7. Genotoxicity studies

Genotoxicity is the capacity of a given agent or substance to induce changes in the genetic material of exposed organisms. These alterations, when not adequately corrected, may result in cancers and hereditary diseases.

Studies conducted by Yesilada (1999) evaluated the genotoxic potential of vinasse and its effects on the fecundity and longevity of *Drosophila melanogaster*, as well as larva survival. The genotoxic effects were assessed with the somatic mutation and recombination test (SMART) using different concentrations of vinasse (0, 25, 50, 75 and 100%). Regarding fecundity, the author observed a decrease in egg production with the increase in the vinasse concentration, similar results were obtained for the average lifespan of flies. All concentrations of vinasse caused a decrease in larva survival rate. Based on the analysis of the genotoxic effects, the authors suggested that although there were small differences between the control and treatment groups, vinasse was not an effective genotoxic agent.

Srivastava and Jain (2010) evaluated the effect of raw vinasse, digested vinasse, and diluted digested vinasse (1:5 v/v) on the cytomorphology of 11 genotypes of sugar cane (*Saccharum* species hybrids). Raw vinasse and digested vinasse had an effect on the mitotic index and a wide spectrum of genotoxic effects, such as adherences and chromosome delays, C-metaphases, multipolarity, bi/multinucleated cells, and mutagenic effects such as chromosome breaks and micronuclei. According to these authors, these alterations were caused by the high concentrations of K, P, S, Fe, Mn, Zn and Cu and heavy metals such as Cd, Cr, Ni and Pb.

Souza et al. (2013) reported a significant increase in chromosome aberrations in *Allium cepa* seeds (onion) exposed to soil samples from a landfarming facility with and without sugar cane vinasse. According to the author, when vinasse was added, bridges, adherences and chromosome breaks were observed. After 33 days, landfarming soil with vinasse caused a prominent effect characterized by the presence of multiple chromosome breaks. According to authors, the results obtained indicated that vinasse potentialized the clastogenicity of landfarming soil.

In contrast, Christofolletti et al. (2013) followed Brazilian standards for application of sugar cane vinasse and biosolid samples in agriculture, and evaluated the genotoxicity of these residues after processing by a terrestrial invertebrate using the *A. cepa* assay. The authors observed that the sugar cane vinasse sample presented genotoxic effects to meristematic cells of *A. cepa*, even after processing of this residue by terrestrial invertebrate. In the end, the authors suggested that further studies are needed to adequately dispose of these residues in the environment.

8. Considerations

The low pH, electric conductivity, and chemical elements present in vinasse may cause changes in the chemical and physical-chemical properties of soils, rivers, and lakes with frequent discharges over a long period of time, and also have adverse effects on agricultural activities and biota in general. Thus, new studies and green methods need to be developed aiming at recycling and disposing sugar cane vinasse.

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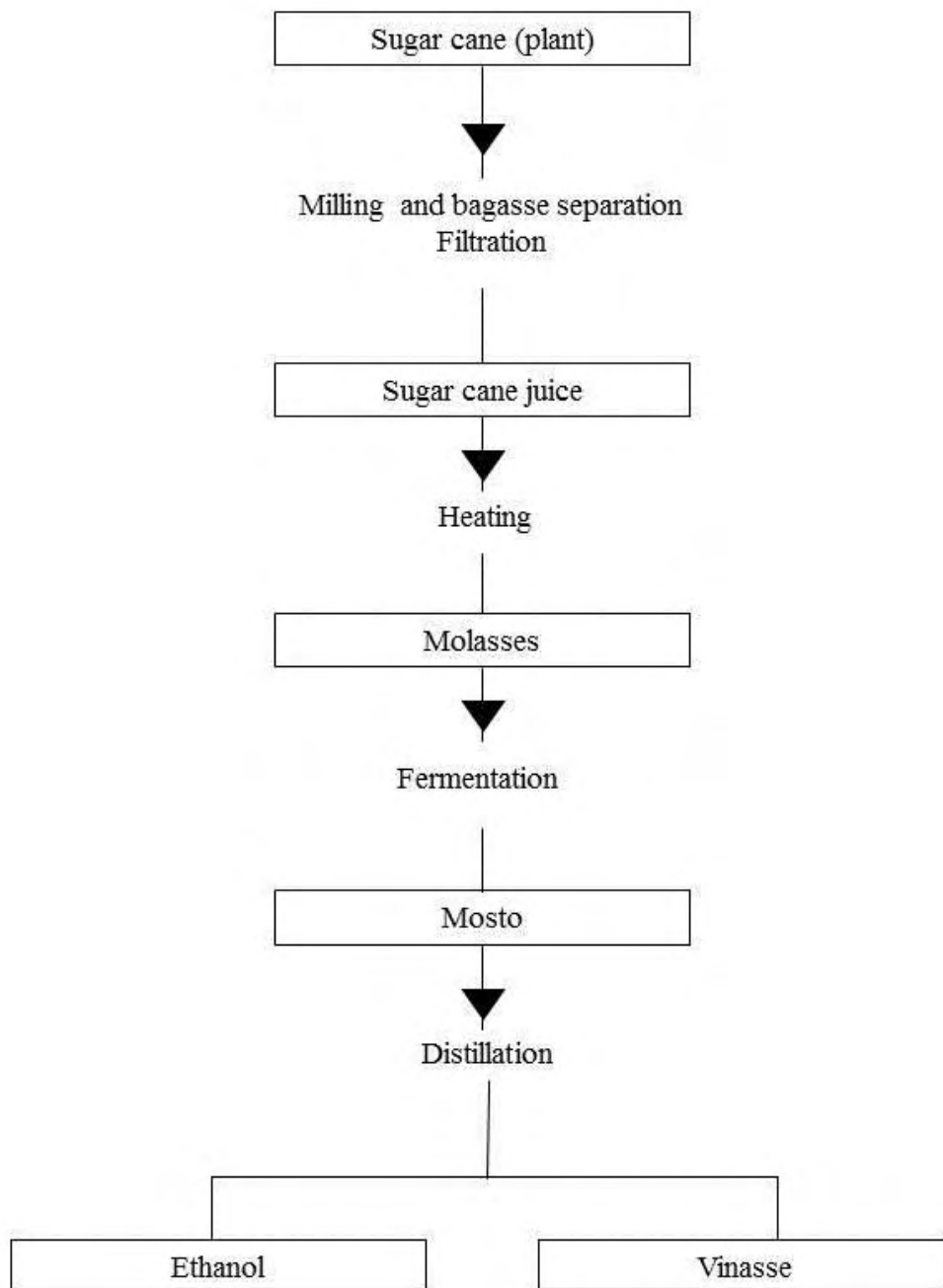


Figure 1: Flowchart of ethanol production process and underproduction of vinasse

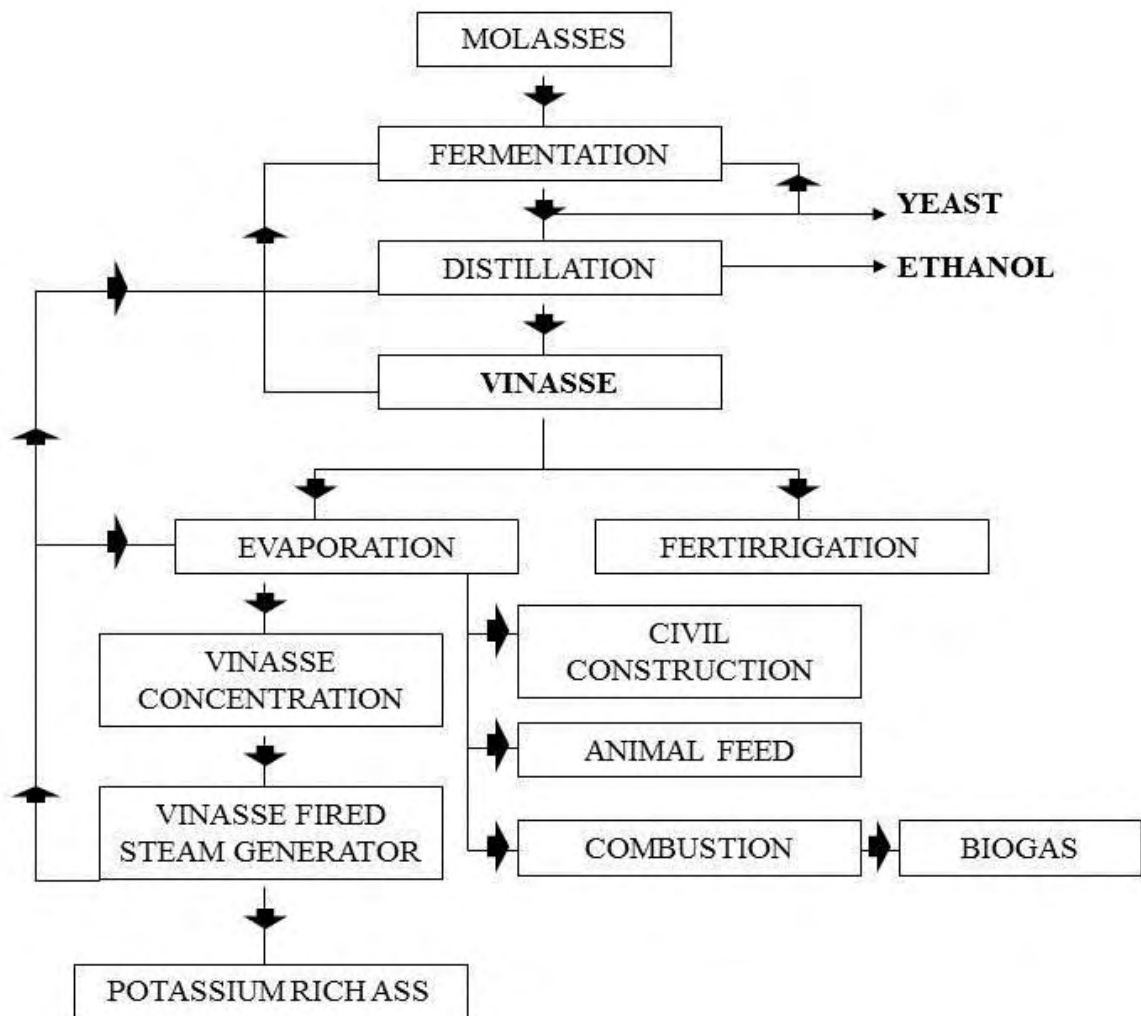


Figure 2: Flowchart of the main alternatives for the use/discharge of vinasse
Source: Adapted from Navarro et al. (2000)

Table 1 – Chemical composition of vinasses from different feedstocks

Parameters	Vinasse			
	Sugar cane	Grape (wine)	Beet	Sweet Sorghum
pH	3.9	2.9	5.1	4.5
BOD	5046	18900	78300	46
DQO	13380	NA	NA	NA
Potassium	2056	118-800	10.000-10.030	NA
Sodium	50.2	NA	3.79	NA
Sulfate	710	120	0.62	NA
Calcium	719	NA	0.71	NA
Magnesium	237	NA	1.23	NA
Total	190	83	91	1990
Phosphorus				
Hardness	2493	NA	NA	NA
As	NA	NA	NA	NA
Ba	0.41	NA	NA	NA
Cd	NA	0.05-0.08	<1*	NA
Cr	0.04	NA	NA	NA
Cu	0.35	0.2-3.26	2.1-5*	37
Hg	0.0019	NA	NA	NA
Mo	0.008	NA	NA	NA
Ni	0.03	NA	NA	NA
Pb	NA	0.55-1.34	<5*	NA
Se	NA	NA	NA	NA
Zn	1.66	NA	NA	NA

All values, except pH are expressed in mg.L⁻¹

* Unit is mg Kg⁻¹; **NA**: data not available

Source: adapted from Robertiello (1982) and España-Gamboa et al. (2012)

Table 2 – Applications of vinasse: advantages and disadvantages

Process/Final Use	Advantages	Disadvantages
Fertirigation	Inexpensive Easy to be implemented	Expensive transportation Unknown long-term effect
Animal Feed	Inexpensive Easy to be implemented	Little studied
Biodigestion/Biogas	Energy production BOD reduction Effluent used as fertilizer	Expensive High technology
Combustion in Boilers	Complete disposal Energy production Recovery of potassium in ashes	Little studied Small-scale tests
Protein production	Food No residues	Expensive Little studied

Source: Cortez et al., 1992

Table 3 – Effects of different effluents in plant species

		Observed effects		
Type of vinasse and concentration	Type of crop	Beneficial effects	Adverse effects	
Sugar cane vinasse in the concentrations of 1, 2.5, 5, 15, 30, 50, 75 and 100% (v/v).	<i>Phaseolus radiatus</i> L. (bean)	<ul style="list-style-type: none"> • Beneficial effects in treatment in the concentration of 5%; • Increase in carotenoids in the concentration of 30% in the first and second crops, 15 and 5% in the third and fourth crops, respectively. • Increase in soluble nitrogen and protein levels in the concentrations of 15 and 50%. 	<ul style="list-style-type: none"> • Decrease in % and germination speed with increase in concentration. 	Sahai et al. (1985)
Sugar cane vinasse in the concentrations of 1, 2.5, 5, 15, 30, 50, 75 and 100% (v/v).	<i>Cicer arietinum</i> L. (chickpea)	<ul style="list-style-type: none"> • Positive effect in all growth parameters at the concentration of 5%. 	<ul style="list-style-type: none"> • Delay in % and germination speed with increase of concentration; • Total inhibition of germination at the concentration of 100%. 	Srivastava and Sahai (1987)
Beet vinasse in the concentrations of 1, 2.5, 5, 10, 25, 50 and 100% (v/v).	<i>Pisum sativum</i> and <i>Helianthus annuus</i> (pea and sunflower)	<ul style="list-style-type: none"> • Increase in the length of the stem, leaf area, biomass, net productivity in pea and sunflower, at the concentration of 2.5%. 	<ul style="list-style-type: none"> • Decrease of these parameters with the increase in concentration. 	Algur and Kadioglu (1992)

<p>Beet vinasse Three mixtures of a concentrated depotassified</p>	<p><i>Zea mays</i> L., <i>Beta vulgaris</i> L. and <i>Helianthus annuus</i> L. (corn, beet, and sunflower)</p>	<ul style="list-style-type: none"> • No adverse effects were observed on the emergence of plants or phytotoxicity symptoms. 	<p>Madejón et al. (2001)</p>
<p>Distillery effluent 5, 10, 15, 20, 25, 50, 75 and 100%</p>	<p>Tomato, pepper, pumpkin, cucumber, and onion</p>	<ul style="list-style-type: none"> • Low concentrations of the effluent did not inhibit the germination of pepper, pumpkin, cucumber, and onion seeds; • Increase in germination rate of onion seeds at the concentration of 10%; 	<p>Ramana et al. (2002)</p>
<p>Vinasse, flegmass and fusel oil Concentrations 12.5, 25, 50 and 100% (v/v)</p>	<p><i>Sida rhombifolia</i>, <i>Brachiaria decumbens</i> and sugar cane (variety RB72454) (arrowleaf sida, Suriname grass, and sugar cane)</p>	<ul style="list-style-type: none"> • Negative effect on emergence and development of Suriname grass and arrowleaf sida seeds. 	<p>Azania et al. (2004)</p>
<p>Distillery effluent Concentrations of 1, 5, 10 and 25% in seeds Crop fertirrigation</p>	<p><i>Oryza sativa</i> and <i>Triticum aestivum</i> (rice and wheat)</p>	<ul style="list-style-type: none"> • Increase in germination rate of seeds at lower concentrations (1 and 5%). • Inhibition of seed germination at concentrations of 10 and 25%. 	<p>Ale et al. (2008)</p>

Pandey et al. (2008)	Distillery effluent 5, 10, 15, 20, 25, 50, 75 and 100%	<i>Zea mays</i> L. and <i>Oryza sativa</i> L. (corn and rice)	<ul style="list-style-type: none"> • Inhibition of seed germination and early growth of seedlings of rice and corn due to non-diluted effluent; • Significant reduction of the germination %, length of radicle and plumule, and fresh and dry weight; • Visible effects of toxicity on seedling leaves. 	Pandey et al. (2008)
Ramos et al. (2008)	Vinasse, flegmass and fusel oil Concentrations 12.5, 25, 50 and 100% (v/v)	Three varieties of sunflower, castor oil, and peanut	<ul style="list-style-type: none"> • Positive effect on castor oil, especially on variables associated to early developmental stages of seedlings; • Negative effects on emergence and early development of peanut plants and to a lesser extent sunflower plants, regardless of the variety examined; 	Ramos et al. (2008)
Doke et al. (2011)	Distillery effluent Concentrations of 1, 5, 10 and 25% in seeds Crop fertirrigation	<i>Vigna angularis</i> , <i>Vigna cylindrical</i> and <i>Sorghum</i> <i>ceruum</i> (azuki bean, cowpea, and sorghum)	<ul style="list-style-type: none"> • Decrease in germination rate of the three varieties of seeds tested with the increase of effluent concentration. 	Doke et al. (2011)

**Diplopods as soil bioindicators toxicity after application of residues from sewage
treatment plants and ethanol industry**

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Abstract

Large quantities of sewage sludge, biosolid, and vinasse are produced daily. These residues have the potential to degrade and contaminate the soil, but they can also be reused as agricultural fertilizers. Alternatives for the reuse of these residues has been the subject of intense investigation. Diplopods are among the many animals of the edaphic fauna that have been considered excellent bioindicators of soil contamination. In the present study, specimens of *Rhinocricus padbergi* were used as bioindicators of toxicity in samples of sewage sludge, biosolid, and vinasse. The exposure to these substances was evaluated at three different time periods, with samples collected in 2010 and 2011. The following parameters were used: behavioral analysis, mortality rate, and histological, histochemical, and ultrastructural analyses of the midgut of animals. The SB and SL samples (2010) were the most toxic, since induced high mortality of animals. The main tissue responses were: significant brush border thickening, induction of epithelial turnover, clustering of hemocytes between hepatic cells, accumulation of cytoplasmic granules in hepatic cells, as well as hepatic cells with heteropycnotic nuclei, cytoplasmic degradation, and high polysaccharide content. Alterations were observed at various levels among treatments with different samples and exposure times. The ultrastructural analysis revealed elongation of microvilli coated with a layer of an amorphous substance, resulting in a thicker brush border as observed in the histological analysis. This suggests that while revolving the contaminated soil, animals did not avoid the toxic compounds, assimilating them during digestion. After 30 days of exposure, animals showed signs of the presence of toxic components in their body, as suggested

by the accumulation of spherocrystals in principal cells and high absorption of substances, based on the elongation of microvilli. This alteration may be an attempt to prevent toxic substances from entering the organism through the gut. The results obtained in the chemical analysis and the behavioral responses observed in diplopods suggest that animals processed the residues, which contained compounds toxic to animals. Therefore, caution should be exercised in the disposal of these residues in agriculture.

Keywords: soil toxicity, histopathology, millipedes

1. Introduction

Sewage sludge is a byproduct of wastewater treatment, produced daily in large quantities in urban and industrial centers. It is characterized by high organic matter content as well as a myriad of xenobiotics in different quantities, depending on the source of sewage (LAMBAIS; CARMO, 2008). Sewage sludge treatment involves stabilization, and pathogen and volume reduction, resulting in a material termed biosolid, which is considered less harmful than raw sewage sludge (BERTELLI, 2007; ARTUSO et al., 2011).

Vinasse is a byproduct of the sugar cane industry. For each liter of ethanol produced, 10 to 14 liters of vinasse are generated (GRANATO; SILVA, 2002). Also, for each liter of cachaça (Brazilian liquor distilled from sugar cane) produced, 8 to 10 liters of vinasse are generated (OLIVEIRA et al., 2009). This residue is characterized by acid pH, conferring it corrosive properties, high organic matter content, biochemical demand of oxygen (BDO), and concentration of salts and metal ions.

Because of these properties, sugar cane vinasse and sewage sludge have a high pollution potential, but are also valuable fertilizers (VANZO et al., 2000; SILVA et al., 2007). Thus, their use in agriculture is promising, but can be very harmful to the environment. Studies on the environmental safety of large-scale land application of these effluents have been encouraged, since this practice can have negative effects on non-target organisms. Bioassays with higher plants, such as the *Allium cepa* test, have been used to evaluate the toxicity of vinasse and sewage sludge applied according to agricultural regulations (CHRISTOFOLETTI et al., 2012; 2013).

Many species of terrestrial invertebrates have also been used as bioindicators in terrestrial ecotoxicology, especially nematodes (SOCHOVÁ et al., 2006), earthworms (NATAL-DA-LUZ et al., 2004) and collembolans (EOM et al., 2007). However, due to an increase in sources of contamination, other species with bioindicator potential have been

necessary (LØKKE; Van GESTEL, 1998; EOM et al., 2007). Diplopods are important cosmopolitan soil macroinvertebrates capable of colonizing different soil layers, and act as decomposers (PETERSEN; LUXTON, 1982). Because of these traits and the responsiveness of their tissues as biomarkers (FONTANETTI et al., 2011), diplopods have been successfully used as bioindicators of soil quality (GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; 2011; PEREZ; FONTANETTI, 2011a; SOUZA; FONTANETTI, 2011; BOZZATTO; FONTANETTI, 2012; CHRISTOFOLETTI et al., 2012).

This study was aimed at accessing the toxicity of sewage sludge, biosolid, and sugar cane vinasse by behavioral analysis and histopathological alterations observed in the midgut of the diplopod *Rhinocricus padbergi* exposed to soil mixed with these effluents according to the Brazilian regulations for land application.

2. Materials and Methods

2.1 Soil

The soil sample used as control and for mixing with residues was collected at the site where diplopods were obtained at the São Paulo State University (UNESP), Rio Claro campus (22°24'36''S/47°33'36''W), São Paulo, Brazil. Two bioassays were set up using soil collected in 2010 and 2011 at a depth of 0.20 cm, previously dried at room temperature, sieved with 4 mm-screen sieve, and chemically characterized.

2.2 Sewage sludge and biosolid samples

Samples of primary sewage sludge and biosolid were collected in 2010 and 2011 in a municipal wastewater treatment plant in the state of São Paulo, Brazil. The plant is managed by the Basic Sanitation Company of the State of São Paulo (Companhia de Saneamento Básico do Estado de São Paulo - SABESP). Samples were collected and stored in plastic boxes wrapped in dark plastic bags and placed in cold room (4°C), until used in experiments.

2.3 Vinasse

This effluent was collected in a sugar cane processing plant, in the city of Araras, São Paulo, Brazil. After collection, the material was stored in cold room (4°C), at the Department of Biochemistry and Microbiology of UNESP – Rio Claro Campus, until being used in experiments.

2.4 Chemical characterization of samples

Chemical and physico-chemical characterization of all samples were carried out by the Institute of Campinas of Soil and Fertilizer (Instituto Campineiro de Solo e Adubo - ICASA), Campinas, São Paulo, Brazil and by the Laboratory TASQA, Paulínia, São Paulo, Brazil, according to the methodology described in Christofolletti et al. (2013).

2.5 Bioassays with *R. padbergi*

In the present study, adult individuals of *R. padbergi* averaging 6.0 cm in size were used in order to avoid interspecific differences associated with diplopod size and/or age. Specimens were collected on the Rio Claro Campus of the São Paulo State University, state of São Paulo, Brazil. After collection, animals were maintained in the laboratory for four weeks in a terrarium containing a mixture of soil, decomposing roots and tree branches from the collection site. The room temperature was set at $21 \pm 2^\circ\text{C}$ and 12 h light/12 h dark photoperiod.

Results related to mortality were expressed as the number of dead animals found in each bioassay, in relation to exposure time.

Six terraria measuring 20 cm in width x 25 cm in length and 45 cm in height were filled with 5 kg of soil from the site where diplopods were collected and mixed with primary sewage sludge, biosolid, vinasse, and combinations, according the Brazilian laws for land application. After chemical characterization of the samples and fertility analyses of the control soil samples, the following treatments were set up:

- 2010 Collection:

CS: 5 Kg of control soil + 20 diplopods

SL: 5 Kg of control soil + 321.72 mL of primary sludge + 20 diplopods

SB: 5 Kg of control soil + 136.58 g of biosolid + 20 diplopods

SV: 5 Kg of control soil + 410 mL of vinasse + 20 diplopods

SLV: 5 Kg of control soil + 321.72 mL of primary sludge + 410 mL of vinasse + 20 diplopods

SBV: 5 Kg of control soil + 136.58 g of biosolid + 410 mL of vinasse + 20 diplopods

- 2011Collection:

- CS:** 5 Kg of control soil + 20 diplopods
- SL:** 5 Kg of control soil + 795.49 mL of primary sludge + 20 diplopods
- SB:** 5 Kg of control soil + 234.4 g of biosolid + 20 diplopods
- SV:** 5 Kg of control soil + 65.56 mL of vinasse + 20 diplopods
- SLV:** 5 Kg of control soil + 795.49 mL of primary sludge + 65.56 mL of vinasse + 20 diplopods
- SBV:** 5 Kg of control soil + 234.4 g of biosolid + 65.56 mL of vinasse + 20 diplopods

2.5.1 Dissection of animals

Animals were exposed for 7, 30, and 90 days to examine the acute (7 days), subchronic (90 days) response and an intermediary period (30 days) between them. After each period of exposure, three animals of each treatment were anesthetized with chloroform and dissected in saline solution for insects. The midgut was removed and portions were fixed with Bouin, paraformaldehyde, and formol calcium for preparation of the different techniques. After fixation, the material was placed in sodium phosphate buffer pH 7.4 for 24 hours and stored in refrigerator.

2.6 Histology and histochemistry

For the preparation for histological and histochemical techniques, the material was dehydrated in a graded ethanol series (70, 80, 90 and 95%) and embedded in resin (Leica historesin) for 24 hours in the refrigerator. The material was later transferred to plastic molds filled with resin. After polymerization, 6- μ m sections were obtained with a LEICA RM 2245 microtome. Sections were hydrated and placed on slides.

For the histological analysis, slides were stained with hematoxylin and eosin (HE). Histochemical staining was performed to detect neutral polysaccharides - periodic acid-Schiff - PAS (Junqueira; Junqueira, 1983), total proteins - bromophenol blue (Pearse, 1985), lipids - Nile blue (Junqueira; Junqueira, 1983), and calcium - von Kossa (Junqueira; Junqueira, 1983). Slides were examined under light microscope.

2.6.1 Analysis of results

The histology of the midgut was qualitatively described and histopathological alterations were analyzed semi-quantitatively for each diplopod. The protocol followed was

based on the proposed by Bernet et al. (1999), by classifying cell pathology into three categories: (1) Minimal pathological importance, the lesion is easily reversible as exposure to irritants ends; (2) Moderate pathological importance, the lesion is reversible in most cases, when the stressor is neutralized; and (3) marked pathological importance, the lesion is generally irreversible, leading to partial or total loss of the organ function. In the present study, this classification was adapted to histopathology of the midgut of diplopods. Table 1 presents the classification used in this study. Scores from 0 to 6 were used to classify the histopathological alterations depending on the level or extent of the alteration: (0) unaltered; (2) small alteration; (4) moderate alteration; and (6) severe alteration. Intermediary scores were also used. Scores (a) were multiplied by the factor of importance of each alteration (w) to define an index of damage (I) per individual. Thus, the index of damage of the different alterations, for each individual was calculated as follows:

$$I(\text{individual}) = a(\text{score received}) \times w(\text{factor of importance})$$

To define the index of damage of each alteration for the control, as well as for each treatment, the sum of indices of damage was obtained for each individual and later the average and standard deviation were calculated.

The data obtained in the semi-quantitative histopathological evaluation were compared to the results obtained for the individuals exposed to the control soil sample with the Kruskal-Wallis test and $p < 0.05$.

2.6.2 Ultrastructure

For the ultrastructural analysis, the material was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4° C. The material was post-fixed in 1% osmium tetroxide in the same buffer and immersed in uranyl acetate for 8 h. The dehydration was performed with a graded acetone series and embedded in a mixture of epon araldite resin during 12 hours at 4° C. Thin sections were obtained with ultramicrotome and contrasted with uranyl acetate and lead citrate for the examination and documentation with a transmission electron microscope ZEISS, EM 900 operated at 80 kV, kindly provided by Dr. Elliot W. Kitajima, of the Luiz de Queiroz College of Agriculture - ESALQ, Piracicaba, São Paulo.

3. Results

3.1. Soil analysis

For the correct application of residues, data on the agronomic potential and on

the fertility of the control soil were necessary. The values obtained for pH, organic matter (OM), residual phosphorus (P res) potassium (K), calcium (Ca), magnesium (Mg), exchangeable aluminum (H+Al), sum of bases (SB), cation exchange capacity (CTC), and base saturation (V%) for the two soil collections varied and are presented in table 2.

3.1.1 Comparison and chemical characterization of the control soil and raw residue samples

The results obtained for the physico-chemical and metals analyses for the control soil and residues, as well as a comparison of these parameters for the two collections are presented in table 3. Because of their complexity, all parameters analyzed for the two soil collections varied. In the second soil collection, different results were obtained for copper, chromium, mercury, molybdenum, and nickel. Metals in samples of primary sludge and biosolid also had concentrations lower than those allowed in the 375/2006 resolution of CONAMA for disposal of sewage sludge and/or biosolid.

Unlike chemical analysis of the first bioassay, in which three organic compounds, phenanthrene, fluoranthene, and naphthalene, were detected in the sample of primary sewage sludge, these compounds were not detected in the chemical analysis of the sample used in the second bioassay. This analysis was also performed for samples of raw biosolid and control soil. However, no organic compounds were detected in these samples, in both bioassays performed.

3.1.2 Comparison of results obtained with the physico-chemical and metal analyses for the combinations of primary sludge, biosolid, and vinasse, before and after exposure to diplopods for both bioassays

The results obtained for the physico-chemical and metal analyses for the control soil and combinations of residues, before and after 30 days of exposure to diplopods, for both bioassays conducted are presented in table 4. Based on these analyses, alternation of accumulation and/or availability of metals was observed for all samples. The pH values varied slightly. Other elements, such as total phosphorus and total calcium varied (decrease or increase) for all samples.

3.2 Bioassays with *R. padbergi*

3.2.1 Behavioral analysis and mortality of diplopods exposed to sewage sludge, biosolid, vinasse, and combinations

The behavior of diplopods, such as the ability of bury themselves or remain on the surface, and the rate of mortality of these animals exposed to combinations of residues were observed since the beginning of experiments.

In both bioassays, the control group exhibited the behavior described for the species: animals were buried in the soil during the day, and were active at night. Also, based on the behavioral analysis, animals did not avoid the soil mixed with different residues until the 30th day of exposure. After this period, animals of from SS and SB treatments often did not buried themselves, and there were always some animals on the surface.

Regarding mortality rate, SSV and SBV of the first bioassay, and SB of the second bioassay were the most toxic, inducing the death of all individuals between 60 and 90 days of exposure (Table 5).

3.2.2 Histology, Histochemistry and Ultrastructure

3.2.2.1 Control group

The histological and histochemical patterns of the midgut of animals of the control group from both bioassays was typical for the species (FANTAZZINI et al., 2002), characterized by a pseudostratified epithelium with brush border, followed by a muscle layer and a layer of hepatic cell. Underneath hepatic cells, a discontinuous muscle layer is observed, and an external membrane encloses the entire structure (Figure 1A). Hepatic cells exhibit irregular morphology, spherical nuclei, and heterogeneous cytoplasm with granules of various aspects (arrows in figures 1A, 1B, and 1D). Between hepatic cells, some hemocytes, usually isolated, are observed (Figure 1B).

Neutral polysaccharides were detected in the brush border, which is 2.87 μm thick, in the basal membrane, and in the layer of hepatic cells (Figure 1B). Principal cells and hepatic cells were moderately stained for proteins (Figure 1C). Lipids were detected in the medial-apical region of the cytoplasm of principal cells of the epithelium and hepatic cells (Figure 1D). The von Kossa staining revealed the distribution of calcium in the principal cells of the epithelium as fine granules (Figure 1E).

The ultrastructural characteristics of the midgut of diplopods were in agreement with the pattern described for the species (CAMARGO-MATHIAS et al., 2004). The epithelium (Figure 2A) consists of principal or absorptive cells, regenerative cells, and secretory cells. The latter were seldom observed. Principal cells present a large number of microvilli in the

free surface. The cytoplasm of these cells is very electron-dense, with abundant mitochondria located mainly in the apical region (Figure 2B), and rough endoplasmic reticulum. Regenerative cells may be present at the base of the epithelium, between principal cells, and exhibit cytoplasm less electron dense than those of principal cells, with abundant mitochondria (Figure 2A, C). Epithelial cells exhibit many expansions in the basal region and are supported by a thick basal lamina (Figure 2C).

3.2.2.2 Group exposed to the different combinations of residues in the three time periods for both bioassays

The midgut of exposed animals from all treatments of both bioassays at 7 days exhibited an increase in epithelial turnover rate (Figure 3A,B).

After 30 days of exposure, a thickening of the brush border was observed (arrowhead in Figure 3C), measuring 4.87 μm , and an accumulation of cytoplasmic granules in hepatic cells (arrowheads in Figure 3D), for animals from SB, in both bioassays. Also, a high content of polysaccharides in hepatic cells (Figure 3E) was demonstrated by the PAS technique. Some cytoplasmic granules were stained for calcium (arrows in Figure 3F). The SV, SSV and SBV treatments of both bioassays still induced epithelial turnover in the midgut of animals. An increase in isolated hemocytes was also observed in samples of SS, SB, and SBV.

After 90 days of exposure, the SB treatment induced thickening of the brush border (arrowheads in Figures 3C, D). The increase was approximately 3 fold, measuring 9.13 μm , when compared to CS. Accumulation of cytoplasmic granules in hepatic cells of animals (arrows in Figure 3B) was observed in the first bioassay. Animals of the SV group exhibit an intense process of epithelial turnover and increase in the number of clusters of hemocytes (Figure 3E), as well as hepatic cells with heteropycnotic nucleus and cytoplasmic degradation (arrows in Figure 3B).

Regarding the histochemical pattern, no alterations were observed.

The averages and standard deviations of the sums of alterations observed for both bioassays are presented in table 6.

The ultrastructural analysis of SB samples demonstrated the elongation of principal cell microvilli (Figures 4A, B) covered by an amorphous substance, forming a continuous layer throughout the entire intestine (star in Figures 4A, B). Several spherocrystals were observed in epithelial cells, most of them arranged concentrically (Figures 4C, D).

4. Discussion

Studies on land application of sewage sludge and vinasse are mainly focused on the effects on soil fertility, since they provide nutrients and increase the dry mass production of several crops (BETTIOL et al., 1983; BETTIOL; GHINI, 2011), and the accumulation of heavy metals in soils treated with sewage sludge (McBRIDE, 1995; MARTINS et al., 2003) and vinasse (BRITO et al., 2007; CAMILOTTI et al., 2007). Few studies have focused on the toxic potential of these compounds on the different exposed organisms, such as plants and terrestrial invertebrates.

The chemical composition of these residues is very peculiar and complex, as it reflects the peculiarities of a given region. Thus, a chemical analysis is very important in order to know which elements are present in vinasse, sewage sludge, and in the soil, before disposal in the terrestrial environment. However the chemical analysis of the contaminant in geo-environmental samples only measures the concentration of the toxic element or substance, and does not reflect its real bioavailability (CESAR et al., 2008). For this, toxicity tests are performed, which are based on the analysis of adverse effects (lethal and sub-lethal) caused to bioindicators exposed to contaminants under controlled experiments in the laboratory (LANNON et al., 2003).

The evaluation of these responses in ecotoxicological studies have provided interesting results regarding the action of chemicals/contaminants in different organisms. Thus, several authors have recommend the use of a group of biomarkers in different levels of biological organization (Dittbrenner et al., 2011).

Based on the chemical analysis and the behavioral evaluation of diplopods, animals interacted with the substrate of all terraria and were able to make large quantities of organic carbon available in all treatments, including the control terrarium. However, according to Suthar (2010), some factors such as microclimatic variability (moisture level, temperature) and substrate depth, among others, may be responsible for the mineralization and/or assimilation of organic carbon. An increase in organic carbon and nitrate was observed in all treatments, corroborating the reported by Schubart (1942) and Godoy and Fontanetti (2010), that found that diplopods enrich the soil with nitrates. In addition, organic materials may form soluble or insoluble complexes with metals, directly affecting their availability (STEVENSON, 1982). This relationship can be observed in the levels of micronutrients and metals of all treatments. Compared to raw samples, levels were lower at t0 and higher after 30 days, demonstrating the bioprocessing of these samples after diplopods.

According to Cortet et al. (1999), bioaccumulation of metals by terrestrial invertebrates depends on the element, its concentration in the environment, and the physico-

chemical conditions of the soil. Nakamura and Taira (2005) and Nakamura et al. (2005) suggested that diplopods may provide information on the accumulation and regulatory mechanisms of harmful metals present in the environment.

Morphological alterations may be used in studies on the toxicity of specific chemical compounds and the monitoring of acute and chronic effects in impacted environments (MEYERS; HENDRICKS, 1985). Analysis involving the morphology and histology of tissues of invertebrates have been frequently used in the identification of different types of damage caused by harmful substances to individuals (KÖHLER; TRIEBSKORN, 1998; TRIEBSKORN et al., 1991; 1999; FONTANETTI et al., 2010). However, most studies on the histopathology of the midgut of diplopods exposed to different samples of sewage sludge, landfarming, and herbicides were focused on the qualitative alterations observed in this organ (CHRISTOFOLETTI et al., 2012; MERLINI et al. 2012; NOGAROL; FONTANETTI, 2010; 2011; PEREZ; FONTANETTI, 2011, SOUZA; FONTANETTI, 2011). According to Dittbrenner et al. (2011), it is crucial to combine a qualitative description of histological conditions and a semi-quantitative analysis in order to better classify the effects observed.

In the present study, the histological analysis revealed that the epithelium of diplopods was affected by the exposure to sewage sludge, biosolid, and vinasse after 7 days. According to Walker (1976), the epithelium is the first line of defense against the entry of potentially harmful substances that may be present in the gut lumen. Thus, the intense epithelial turnover observed in this study may be a physiological response to remove damaged cells or tissues, in order to maintain the structure of the organ, prevent changes in its functions by stressors, such as the contaminants present in the different residues examined.

Studies carried out by Nogarol and Fontanetti (2010), Perez and Fontanetti (2011a), Souza and Fontanetti (2011), and Merlini (2011) also observed an increase in the epithelial turnover rate in the gut of *R. padbergi*, as a response to acute exposure to samples of sewage sludge from different WTPs, an industrial soil contaminated with polycyclic aromatic hydrocarbons, and substrate contaminated with the herbicide trifluralin.

After 30 days, cytoplasmic granules were observed accumulating in hepatic cells as well as a thickening of the brush border in animals of the SB treatment, and increase in the epithelial turnover rate in animals of the SS, SB, and SBV treatments. After 90 days of exposure, these alterations became more severe. For the SB treatment, thickening of the brush border and accumulation of cytoplasmic granules in hepatic cells were observed. The SV treatment was the most harmful to animals, which exhibited an intense epithelial turnover,

with shedding of cells into the lumen, hepatic cells with heteropycnotic nuclei, degraded cytoplasm and clustered hemocytes.

The increase in cell turnover indicates tissue lesion, with possible replacement of cells in order to maintain the integrity and functionality of the tissue. The regeneration of epithelial constituents is a normal physiological process, due to the growth and division of regenerative cells (HOPKIN; READ, 1992). However, epithelial cells exposed to different contaminants are subjected to alterations that may lead to death and consequently their replacement, which may be an attempt of the animal's body to compensate the damage sustained after the ingestion of contaminated soil (SOUZA; FONTANETTI; 2011).

The layer of hepatic cells works actively in the detoxification of the organism when it is harmed by toxic substances (KÖHLER, 2002). This process was demonstrated with the increase in cytoplasmic granules in individuals exposed to different combinations of residues. The von Kossa staining revealed that part of the cytoplasmic granules in the hepatic cells consists of calcium. The formation of these granules, shown by the ultrastructural analysis of the epithelium, may be due to the absorption of metals and formation of spherocrystals in the attempt to maintain homeostasis and ion balance (FONTANETTI et al., 2006; PEREZ; FONTANETTI, 2011a), since these elements would be inert, avoiding compromising the entire organism.

Studies conducted by Godoy and Fontanetti (2010), Nogarol and Fontanetti (2010), and Perez and Fontanetti (2011a) exposed this same species of diplopod to substrate containing domestic and industrial sewage sludge and also observed an accumulation of granules in the hepatic cells of these animals.

The increase in hemocytes has also been reported as a frequent response in invertebrates exposed to different toxic agents. According to Perez and Fontanetti (2011b), in invertebrates, hemocytes play an important role in the recognition of foreign materials to the organism, and can mediate and perform cell defense. This response has also been observed in diplopods exposed to different samples of sewage sludge (GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; PEREZ; FONTANETTI; 2011), landfarming soil (SOUZA; FONTANETTI, 2011) and a herbicide (MERLINI et al., 2012).

In the present study, the increase in hemocytes was observed for SS, SB, and SBV after 30 days of exposure. After 90 days, large clusters of hemocytes were observed in SV. Thus, the occurrence of hemocytes indicates the presence of lesions in the intestine, possibly induced by different metals present in the samples examined, resulting in an inflammatory process. This may be considered a defense mechanism of the organism, which may act in the

destruction, dilution, isolation or sequester of the stressor, in the attempt to allow repair processes, such as the regeneration of the damaged tissue (ZWEIFACH et al., 1974). However, the inflammatory process was so severe that heteropycnotic nuclei were observed as well as cytoplasmic degradation of hepatic cells. Pycnotic chromatin, basophilic cytoplasm and loss of cell boundaries are among the main characteristics of cells undergoing death (ZAKERI; LOCKSHIN, 2002).

Elongation of microvilli of principal cells of animals exposed to SB were observed under electron microscope. The tip of epithelial cells of the midgut, usually exhibit microvilli, as they are involved in the digestion and absorption of nutrients, water, and secretion of liquids (TERRA et al., 1996). This alteration might have occurred due to contact with the contaminated soil by animals, which did not avoid the toxic substances present and assimilated them during digestion. Studies conducted with enteropathogenic bacteria, *Escherichia coli* demonstrated that the elongation and destruction of microvilli of the intestine are involved in the development of food allergies and diarrhea disturbances in animals and humans (PHILLIPS et al., 2000). However, after 30 days of exposure, animals showed signs of the presence of toxic compounds, given the accumulation of spherocrystals in principal cells and the thickening of the brush border. The latter may be an attempt of the animal's body to prevent the entry of toxic compounds through the intestine.

The examination of the midgut of diplopods exposed to different combinations of sewage sludge, biosolid, and vinasse with histological, histochemical and ultrastructural analyses provided important information on the effects of this exposure and which mechanisms may be used by animals in the attempt to detoxify the contaminants present in the different residues applied to the soil. After different periods of exposure, injuries became more severe in the midgut of these animals, and in the field, these animals would be chronically exposed to sludge, biosolid, and vinasse after land application.

5. Conclusion

Our findings demonstrate the efficacy of terrestrial invertebrates in bioprocessing complex environmental samples, as well as bioindicators in the evaluation of soil quality, also supporting histological, histochemistry and ultrastructural analysis of the midgut, as biomarkers of stress in these animals. The results obtained indicate the need for further studies on the biological effects of different residues to be disposed in the environment, in the different compartments of ecosystems, as well as in different levels of biological organization,

even when toxic agents are present in low concentrations, in order to safely use them in the sustainable conditioning of agricultural soils.

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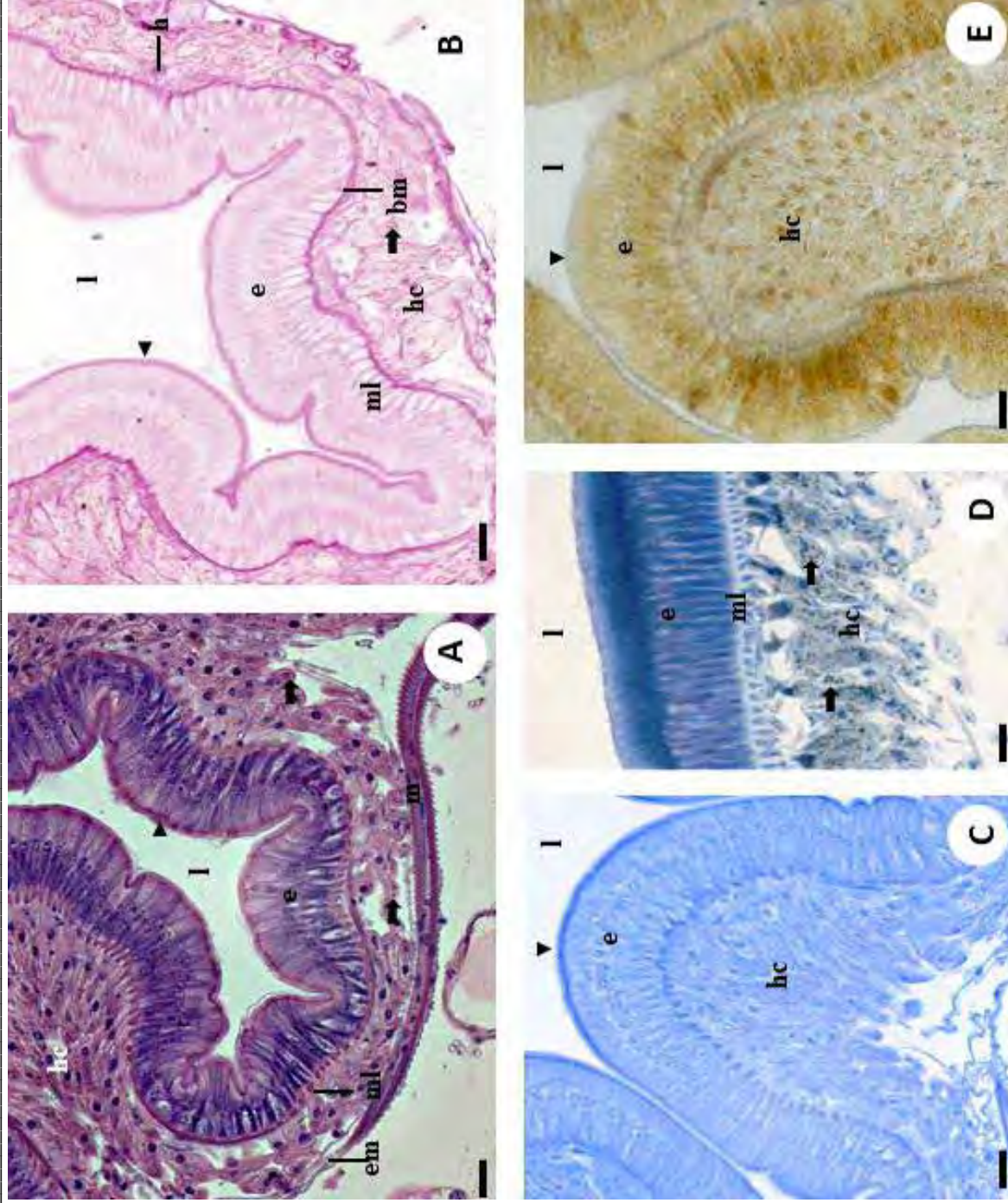


Figure 1. *R. padbergi* midgut exposed to control soil sample and subjected to HE (A), PAS (B), bromophenol blue (C), Nile blue (D) and von Kossa (E) techniques. **hc**= hepatic cells, **ml** = muscle layer, **e**= epithelium; **h**= hemocyte; **l**= lumen; **m**=muscle; **bm**= basal membrane, **em**= external membrane; **arrowhead**= brush border; **arrows**= cytoplasmic granules; bar= 20µm.

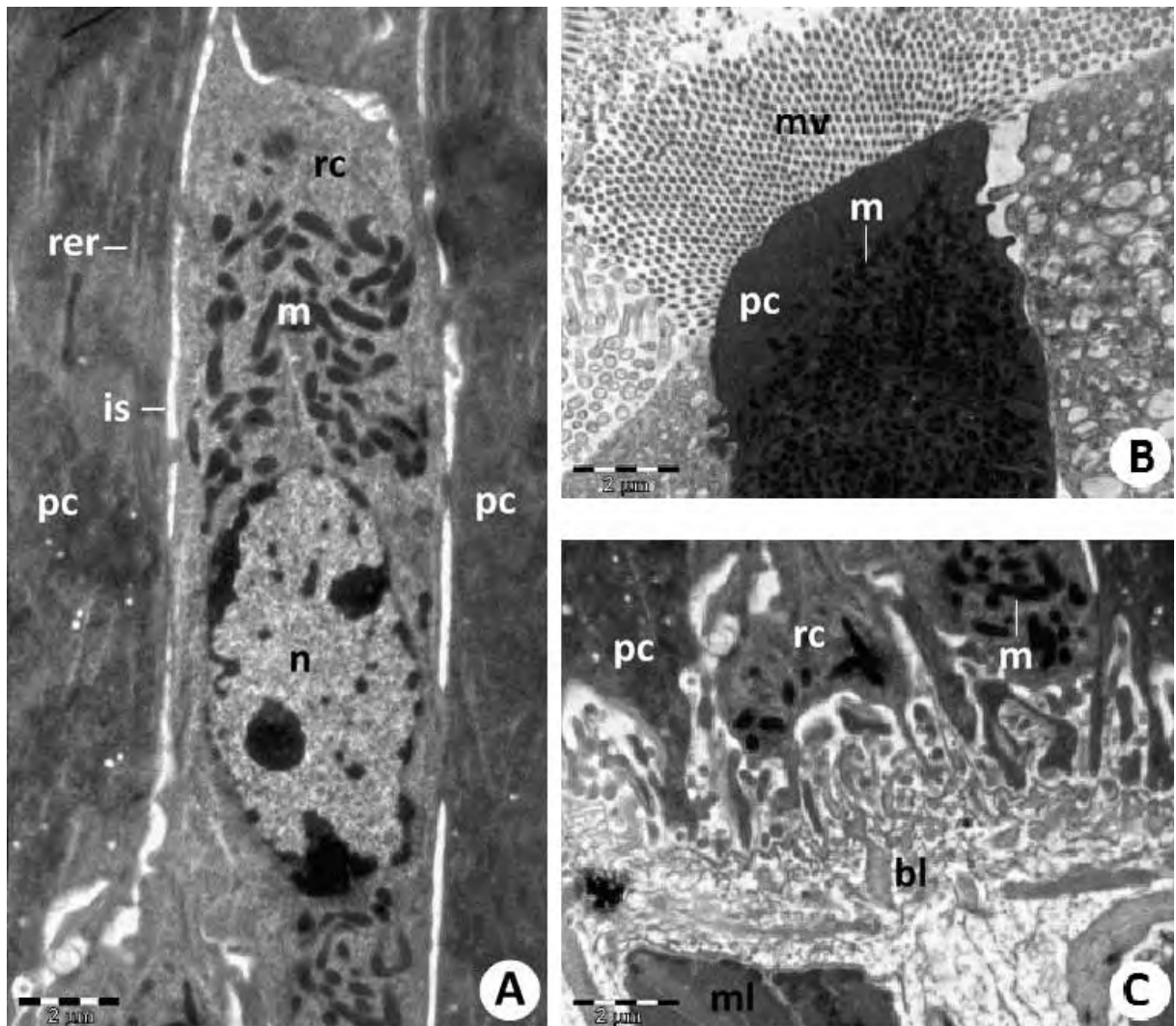


Figure 2. Ultrastructural analysis of *R. padbergi* midgut exposed to control soil sample (SC). **ml**= muscle layer; **pc**= principal cell; **rc**= generative cell; **is**= intercellular space; **bl**= basal lamina; **m**= mitochondria; **mv**= microvilli; **n**= nucleus; **rer**= rough endoplasmic reticulum.

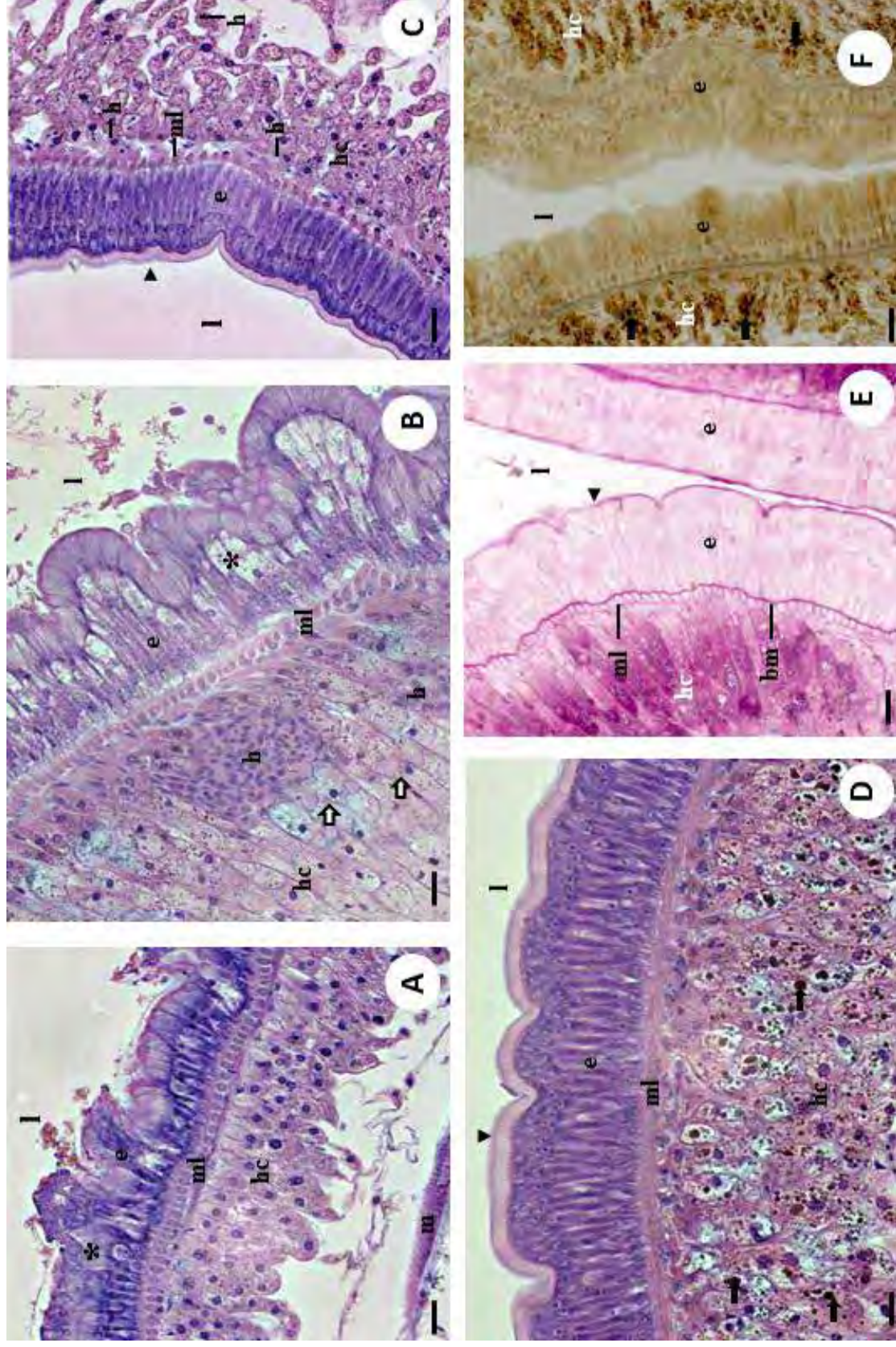


Figure 3. Major tissue alterations observed in the *R. padbergi* midgut texposed to different combinations of residues and subjected to HE (A, B, C, D), PAS (E) and von Kossa (F) techniques.
hc= hepatic cells; **ml**= muscle layer; **e**= epithelium; **m**= muscle; **h**= hemocyte; **l**= lumen; **bm**= basal membrane; **arrowhead**= brush border; **black arrows**= cytoplasmic granules; **white arrows**= heteropycnotic nuclei with cytoplasm degradation; *****= epithelial renewal; bar = 20µm.

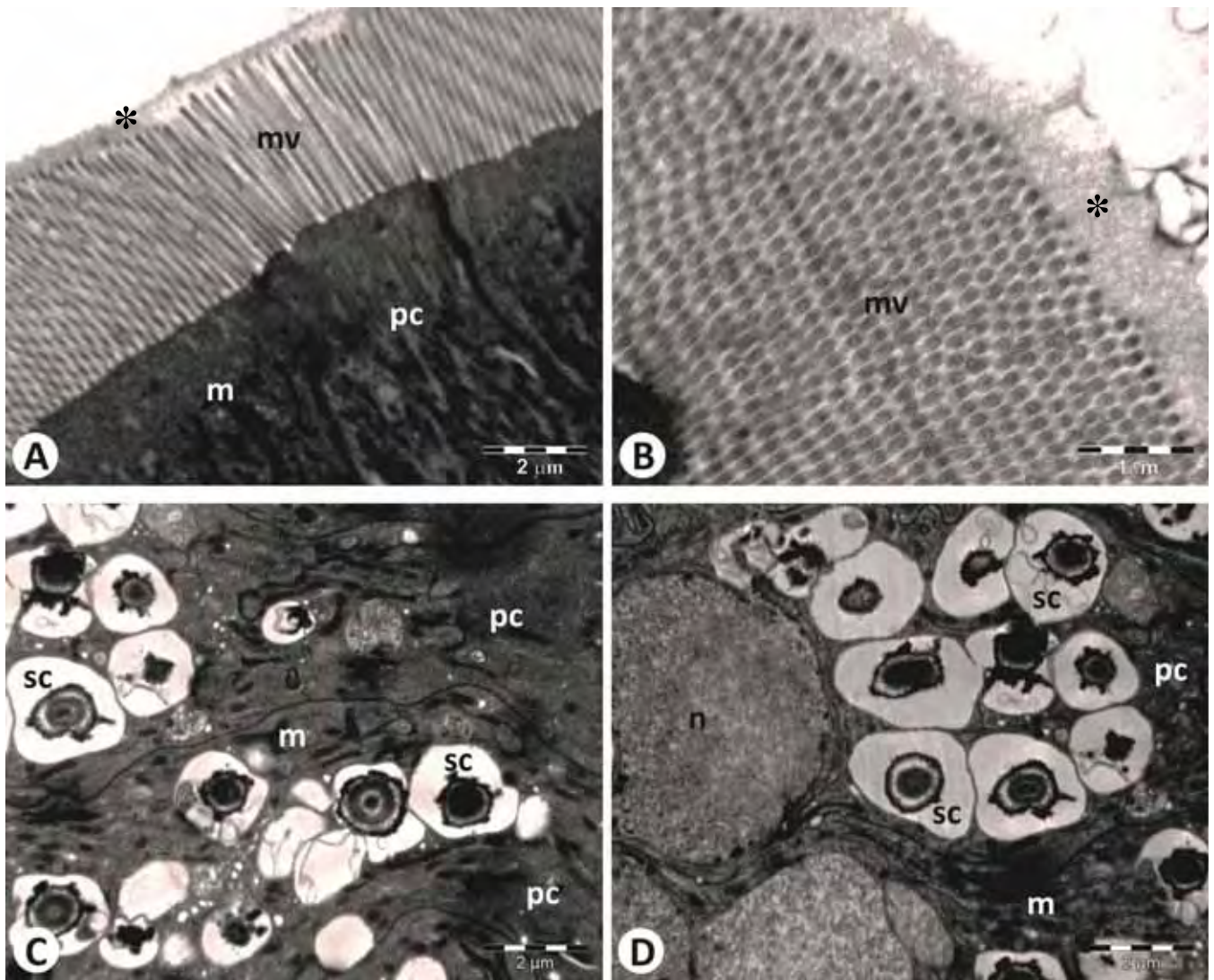


Figure 4. Major ultrastructural alterations observed in the *R. padbergi* midgut exposed to different combinations of residues. **pc**= principal cell; **sc**= esferocristal; **m**= mitochondria; **mv**= microvilli; **n**= nucleus; *****= amorphous substance.

Table 1 – Classification of the histopathological alterations in the midgut of diplopods based on a semi-quantitative evaluation

Region examined	Minimum pathological importance (1)	Moderate pathological importance(2)	Marked pathological importance (3)
Epithelium	Epithelial turnover	Thickening of the brush border	
Hepatic cells		Increase in the number of granules in hepatic cells	Heteropycnosis of the nucleus of hepatic cells
		Increase in the number of hemocytes between hepatic cells	Cytoplasmic degradation

Factor of importance: (1) Minimal pathological importance, damage is easily reversible when the exposure to the irritant ends; (2) Moderate pathological importance, damage is reversible in most cases, when the stressor is neutralized; and (3) Marked pathological importance, damage is usually irreversible, resulting in partial or total loss of the organ function.

Score: from 0 to 6 histopathological anomalies described, depending on the level and extension: (0) unaltered; (2) small change; (4) moderate change; and (6) severe change. In addition, intermediary scores may also be used.

Table 2 - Data on the fertility of the control soil for the two collections

Collection	pH	MO	P res	K	Ca	Mg	H+ Al	SB	CTC	V	Ratios	
	CaCl ₂	g/dm ³	mg/dm ³				mmol./dm ³ TFSA			%	Ca/ Mg	Mg/K
2010	6.20	18	3.0	0.8	2	1	88	3.9	91.9	4.2	2.0	1.25
2011	5.10	17	3.0	0.2	9	6	30	16.6	5.88	3.5	-	-

OM: organic matter; **CTC:** cation exchange capacity; **V:** base saturation; **SB:** sum of bases

Table 3- Physico-chemical and metal analysis of the control soil and raw samples of vinasse, primary sludge, and biosolid, for both collections

Parameter	2010 Collection						2011 Collection						Method	MaxPA (mg/kg)	MCA (mg/kg)
	CS (mg/kg)	V (mg/L)	L (mg/L)	B (mg/kg)	CS (mg/kg)	V (mg/L)	L (mg/L)	B (mg/kg)	CS (mg/kg)	V (mg/L)	L (mg/L)	B (mg/kg)			
Arsenic	16.8	<LQ	14.5	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	SM21 3120B	35	41
Barium	5.91	0.41	11.0	158	<LQ	158	<LQ	<LQ	<LQ	7.73	222	222	SM21 3120B	300	1300
Cadmium	<LQ	<LQ	<LQ	<LQ	<0.16	<LQ	<LQ	<LQ	<LQ	<LQ	<0.66	<0.66	SM21 3120B	3	39
Total Calcium	25.4	7.19	623	3939	42.8	623	3939	42.8	671	432	5610	5610	SM21 3120B	-	-
Organic Carbon (g/kg)	12.6	NA	3939	279	32.3	3939	279	32.3	NA	1345	590	590	SSSA Cap40	-	-
Lead	49.3	<LQ	1.42	174	42.7	174	174	42.7	<LQ	<LQ	126	126	SM21 3120B	180	300
Copper	37.2	0.35	10.6	276	76.5	276	276	76.5	0.76	4.65	210	210	SM21 3120B	200	1500
Electric conductivity (μ S/cm)	115	13530	2950	5389	97.9	2950	5389	97.9	15110	3700	1181	1181	SM21 3120B	-	-
Chromium	31.2	0.04	6.48	224	108	6.48	224	108	3.56	4.07	180	180	SM21 3120B	150	1000
Total sulfur	151	1219	373	11864	123	373	11864	123	1681	6640	15475	15475	SM21 3120B	-	-
Total Phosphorus	182	NA	707	17027	317	707	17027	317	207	385	18156	18156	SM21 3120B	-	-
Total Magnesium	<LQ	237	75.4	358	<LQ	75.4	358	<LQ	264	65.3	1132	1132	SM21 3120B	-	-
Mercury	<LQ	0.0019	<LQ	1.08	0.065	<LQ	1.08	0.065	<LQ	0.0016	<LQ	<LQ	EPA 7470A	12	17
Molybdenum	3.64	0.008	0.83	9.55	9.60	0.83	9.55	9.60	<LQ	<LQ	<0.44	<0.44	SM21 3120B	50	50
Nickel	13.0	0.03	1.80	82.3	24.2	1.80	82.3	24.2	<LQ	4.12	104	104	SM21 3120B	70	420
Nitrate (mg/Kg)	4.40	1.30	1.20	6.79	8.14	1.20	6.79	8.14	1.49	1.09	<8.63	<8.63	SM21 4500-NO ₃ E	-	-
Nitrite (mg/Kg)	0.06	0.008	0.02	1.39	<0.043	0.02	1.39	<0.043	0.03	0.014	1.98	1.98	SM21 4500-NO ₂ B	-	-
N Ammoniacal (mg/kg)	31.8	NA	1467	167	49.6	1467	167	49.6	NA	351	3969	3969	SM21 4500-NH ₃ E	-	-
N Kjeldahl (mg/Kg)	476	267	1597	21620	922	1597	21620	922	171	705	35740	35740	SM21 4500-Norg B	-	-
pH	6.20	3.9	6.55	8.01	5.1	6.55	8.01	5.1	4.37	6.72	6.8	6.8	EPA 4095 C	-	-
Total Potassium	406	2056	107	2152	<LQ	107	2152	<LQ	3401	84.9	2513	2513	SM21 3120B	-	-
Selenium	<LQ	<LQ	<LQ	<LQ	52.1	<LQ	<LQ	52.1	<LQ	<LQ	<LQ	<LQ	SM21 3120B	-	100
Total Sodium	<LQ	50.2	41.8	<LQ	<LQ	41.8	<LQ	<LQ	114	71.5	597	597	SM21 3120B	-	-
Total Solids (g/g)	0.86	NA	55149	0.24	0.93	55149	0.24	0.93	NA	42902	0.2146	0.2146	SM21 2540B	-	-
Total Volatile Solids (g/g)	VI	NA	27978	VI	0.08	27978	VI	0.08	NA	25767	VI	VI	SM21 2540B	-	-
Solid content (g/g)	0.86	NA	NA	0.24	0.93	NA	0.24	0.93	NA	NA	0.2146	0.2146	SM21 2540B	-	-
Moisture (g/g)	0.14	NA	NA	0.76	0.06	NA	0.76	0.06	NA	0.9571	0.7854	0.7854	SM21 2540B	-	-
Zinc	23.2	1.66	40.5	825	96	40.5	825	96	<LQ	14.4	523	523	SM21 3120B	450	2800

CS: control soil; V: vinasse; L: biosolid; B: biosolid; LQ: Limit of quantification; IV: Inconsistent value; MaxPA: values for agricultural exposure-Maximum Protection Area, for soil (mg/kg) and groundwater in the state of São Paulo, according to CETESB (195/2005-E); MCA: maximum concentration allowed in sewage sludge or derivatives, according to CONAMA (375/2006).

Table 4 – Comparison of results obtained for the physico-chemical and metal analysis of combinations of primary sewage, biosolid, and vinasse before and after exposure to diploids for the two bioassays

Parameter	Samples																																			
	CS						SL						SB						SV						SLV						SBV					
	2010		2011		2010		2011		2010		2011		2010		2011		2010		2011		2010		2011		2010		2011									
T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30	T0	T30									
Arsenic	16.8	<LQ	<LQ	<LQ	6.74	<LQ	3.49	<LQ	<LQ	5.79	<LQ	<LQ	<LQ	27.5	40.9	22.3	59.3	26.8	42.7	17.5	63.1	33.4	55.4	10.5	<LQ	<LQ	<LQ	2011								
Barium	5.91	11.3	<LQ	6.93	7.76	12.0	8.03	6.7	6.21	6.50	12.9	7.21	6.06	7.22	7.43	8.2	6.47	8.2	6.47	7.07	8.99	6.18	7.49	7.07	8.99	6.18	7.49	2011								
Cadmium	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	0.3	<0.18	<LQ	<LQ	<LQ	0.3	<0.18								
Total	25.4	170	42.8	2.43	24.5	242	49.2	<LQ	30.8	29.3	246	36.5	<LQ	41.7	96.5	35.6	<LQ	35.6	<LQ	26.7	97.3	37.6	3.04	26.7	97.3	37.6	3.04									
Calcium	12.6	91.8	32.3	47.9	33.3	128	34.3	42.1	35.2	28.8	81.7	27.5	40.9	22.3	59.3	26.8	42.7	17.5	63.1	33.4	55.4	10.5	<LQ	<LQ	<LQ	<LQ	<LQ	2011								
Organic Carbon	49.3	10.0	42.7	7.27	15.7	16.2	86.8	9.69	14.8	8.46	4.83	89.2	4.39	10.0	5.94	82.8	5.56	82.8	5.56	6.17	13.0	71.1	5.78	6.17	13.0	71.1	5.78									
Lead	37.2	68.4	76.5	43.6	62.3	75.7	54.1	41.6	67.8	58.2	70.2	64.2	49.5	40.7	51.6	59.1	52	59.1	52	38.1	47.1	56.9	48.9	38.1	47.1	56.9	48.9									
Copper	31.2	26.5	108	25.3	25.1	26.3	27.9	23.9	28.6	25.2	25.6	28.3	22.8	20.1	21.8	28.3	24	28.3	24	31.5	22.8	28.4	25.6	31.5	22.8	28.4	25.6									
Chromium	182	262	317	295	350	335	390	351	373	317	267	348	288	298	224	410	357	410	357	262	274	397	436	262	274	397	436									
Total Phosphorus	<LQ	<LQ	0.06	0.03	0.09	<LQ	0.17	0.04	0.10	0.20	<LQ	0.04	0.04	0.04	<LQ	0.04	0.04	0.04	0.04	0.04	0.04	0.19	0.05	0.04	0.04	0.19	0.05									
Mercury	3.64	<LQ	9.6	<0.12	<LQ	<LQ	1.54	<0.12	3.99	1.58	3.34	2.46	<0.13	1.70	<LQ	2.09	<0.12	2.09	<0.12	2.34	<LQ	2.24	<0.12	2.34	<LQ	2.24	<0.12									
Molybdenum	13.0	9.70	24.2	10.3	10.7	10.1	13.3	10.1	10.6	9.76	11.4	15	10.8	5.05	7.31	14.6	12.7	14.6	12.7	7.08	8.49	12.6	11.9	7.08	8.49	12.6	11.9									
Nickel	4.40	2.98	8.14	34.7	3.17	1.87	19.4	128	2.98	4.49	3.99	14.4	56.8	1.49	3.77	15.3	116	15.3	116	<LQ	2.70	22.5	132	<LQ	2.70	22.5	132									
Nitrate	6.20	5.85	5.1	6.1	5.22	6.15	5.85	6.8	6.80	4.49	3.99	5.55	5.7	6.02	5.74	5.4	5.3	5.4	5.3	4.46	5.84	5.5	6.3	4.46	5.84	5.5	6.3									
pH	<LQ	<LQ	52.1	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ								
Selenium	0.86	0.83	0.93	0.78	0.82	0.83	0.80	0.82	0.89	0.79	0.89	0.83	0.79	0.80	0.89	0.84	0.79	0.84	0.79	0.84	0.85	0.83	0.79	0.84	0.85	0.83	0.79									
Solid contents	23.2	44.2	96	47	40.5	49.3	55.7	43.2	41	37.3	42.2	57	45	30.8	35.2	57	49.9	57	49.9	31.3	37.4	54.7	61.4	31.3	37.4	54.7	61.4									
Zinc																																				

CS: control soil; **SL:** soil+sewage sludge; **SB:** soil+vinasse; **SV:** soil+sewage sludge+vinasse; **SLV:** soil+sewage sludge+vinasse; **SBV:** soil+biosolid+vinasse; **LQ:** Limit of quantification. Values for metals, calcium, total phosphorus, nitrate (mg/Kg), organic carbon (g/kg).

Table 5 – Number of dead diplopods, after exposure to the different combinations of residues

Treatment	N	Mortality											
		7 days		7 th to 21 st day		21 st to 60 th day		60 th to 90 th day					
		2010	2011	2010	2011	2010	2011	2010	2011	2010	2011	2011	
CS	11	0	0	2	3	3	2	3	2	3	2	3	2
SS	11	3	0	2	4	4	6	1	0	1	0	1	0
SB	11	1	3	3	0	2	8	4	All dead	4	All dead	4	All dead
SV	11	0	3	4	2	2	4	3	0	2	0	3	0
SSV	11	0	3	0	1	9	4	All dead	0	4	0	All dead	0
SBV	11	0	3	0	2	2	4	All dead	0	4	0	All dead	0

N: total number of diplopods per terrarium, excluded animals that were dissected for histological, histochemical, and ultrastructural analyses.

Table 6 – Mean and standard deviation of alterations observed in the midgut of *R. padbergi* exposed to different combinations of residues at 7, 30, and 90 days, for both bioassays.

YEAR	PERIOD	TREATMENT	ALTERATIONS					SUM OF ALTERATIONS
			ET	GR	HE	BB	PYC	
2010	7 DAYS	CS	1±0	-	-	-	-	1±0
		SL	1.6±0.57	-	-	-	-	1.6±0.57
		SB	2.6±0.57	-	-	-	-	2.6±0.57
		SV	2±0	-	-	-	-	2±0
		SLV	2±0	-	-	-	-	2±0
		SBV	4.33±0.57	-	-	-	-	4.33±0.57*
	30 DAYS	CS	1±0	0.66±0.57	-	-	-	2.33±1.15
		SL	1.66±0.57	-	2±0	-	-	3.66±0.57
		SB	2.33±0.57	6±3.46	2±0	5.33±3.05	-	15.66±2.88*
		SV	4±1	-	-	-	-	4±1
		SLV	5.66±1.57	-	-	-	-	5.66±1.52*
		SBV	3.66±0.57	-	6±2	-	-	9.66±2.08*
	90 DAYS	CS	2±0	1.33±1.15	-	-	-	3.33±1.15
		SL	5.5±0.7	0.66±1.15	-	-	-	6.5±0.7
		SB	-	5±1.41	-	6±2.82	-	11±1.41
		SV	6±0	-	8±2.82	-	13.5±2.12	39.5±3.53
		SLV	-	-	-	-	-	All dead
		SBV	-	-	-	-	-	All dead
2011	7 DIAS	CS	0.66±0.57	-	-	-	-	0.66±0.57
		SL	3.33±0.57	-	-	-	-	3.33±0.57*
		SB	5±1	-	-	-	-	5±1*
		SV	3.66±0.57	-	-	-	-	3.66±0.57*
		SLV	3±1	-	-	-	-	1±1
		SBV	3±1	-	4±2	-	-	7±2.64*
	30 DIAS	CS	1±0	1.33±1.15	-	-	-	2.33±1.15
		SL	2.33±0.57	-	2.66±2.3	-	-	5±1.7*
		SB	2.33±0.57	-	3.33±2.71	8.66±2.03	-	18.66±4.5*
		SV	3.99±1.14	-	-	-	-	4.33±0.57*
		SLV	4.33±0.57	-	-	-	-	4.66±0.57*
		SBV	3±0	-	3.33±2.30	-	-	7±2*
	90 DIAS	CS	2.33±0.57	2±0	-	-	-	4.33±0.57
		SL	-	-	-	-	-	-
		SB	-	-	-	-	-	All dead
		SV	4±0	-	8±2.82	-	12±4.24	37.5±3.53
		SLV	2.66±1	3±1.73	-	-	-	10±2.64*
		SBV	3±0.57	4±2	2±0	-	-	10.33±1.52*

CS: control soil; **SL:** soil+primary sludge; **SB:** soil+biosolid; **SV:** soil+vinasse; **SLV:** soil+primary sludge+vinasse; **SBV:** soil+biosolid+vinasse; Alterations: **ET:** epithelial turnover; **GR:** accumulation of cytoplasmic granules in hepatic cells; **HE:** increase in the number of isolated and/or groups of hemocytes; **BB:** thickening of the brush border; **PYC:** pycnotic nuclei.

Sewage sludge: available treatments, main uses and effects on organisms

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Abstract

Sewage sludge production has risen exponentially in Brazil and worldwide, due to population growth and increasingly restrictive requirements for effluent treatment. As a result, the management of sewage sludge from wastewater treatment plants (WTP) has become very complex and costly, that if not well conducted, can compromise the environmental and sanitary benefits expected from these systems. Thus, the adequate management of this residue is associated with the reduction of production, maximum increase in reuse and recycling, and promotion of environmentally healthy landfills and treatments. Seeking to examine the available data on the production and main uses of sewage sludge in Brazil and worldwide, this study presents a historical review, treatment technologies, advantages and disadvantages, and possible effects of sewage sludge use on organisms.

Keywords: solid waste management, sewage sludge reuse, recycling waste, biosolid

Introduction

In the biological processes of effluent treatment, part of the organic matter is absorbed and converted, making up the microbial biomass generally termed biological sludge, consisted of mainly biological solids (von SPERLING et al., 2001). Sewage sludge is a semi-solid residue from wastewater treatment plants (WTP) and/or water supply, with a predominantly organic composition, depending on the origin of the material (ANDRADE, 1999; BERTON; NOGUEIRA, 2010), the sewage treatment process (BETTIOL; CAMARGO, 2000), and seasonality (SINGH; AGRAWAL, 2008). In general, sewage sludge consists of approximately 40% to 60% of organic material, 4% of nitrogen, 2% of phosphorus and other macro and micronutrients, in addition to potentially toxic elements (BETTIOL; CAMARGO, 2006), and pathogenic organisms (ANDREOLI et al., 1998).

Sewage sludge management from wastewater treatment plants is very complex and costly. If not correctly conducted, it can compromise the environmental and sanitary benefits expected from these systems (LUDUVICE, 2001; PEDROZA et al., 2010). The importance of this practice has been acknowledged by the Brazilian Agenda 21, which includes the subject “Environmentally healthy management of solid residues and sewage related issues”, defining the following guidelines for sewage sludge management: reduction of production, maximum increase of reuse and recycling, and promotion of environmentally safe landfills and treatment (von SPERLING et al., 2001).

Therefore, the adequate final disposal of sewage sludge is a fundamental factor for the success of a sanitation system. Until recently, this activity was neglected in Brazil and because of that, inadequate alternatives for the final disposal were used, compromising the benefits of investments in sanitary sewage systems (von SPERLING et al., 2001). With the growth of this national sector along with technological advances of treatment systems, sewage sludge management has been the focus of research and new technologies aimed at finding economically feasible and environmentally safe alternatives for the production of biological sludge.

The present study was aimed at presenting a historical overview, treatment techniques, advantages, disadvantages, and possibles effects of the use of sewage sludge.

1. General overview of the production in some countries

In the USA in 2004, approximately 7.2 million of tons of sewage sludge were produced (LEBLANC et al., 2008). In Europe in 2005, the estimated production was 8-10 million of tons (IRANPOUR et al., 2004). According to Barneto et al. (2009), in this same year, the Spanish production of sewage sludge was 1,120,000 dry tons. According to Hossain et al. (2009), the production of sewage sludge in the United Kingdom was nearly 1 million of m³/year, 50 million of m³/ year in Germany, 4,2 million of m³/ year in Switzerland, and 170 thousand m³/ year in Singapore.

In Brazil, approximately 4 million of tons of sewage sludge are produced, and only 35% of the Brazilian municipalities use effluent treatment processes (BIOCICLO, 2012). According Andreoli (2002) *apud* Pedroza et al. (2010), the expansion of sewage collection has a potential to multiply the production of this residue in Brazil three to four fold.

A recent study conducted by the Technical Chamber of Sanitation of the Committee of the Water Basin of the Piracicaba, Capivari, and Jundiaí (PCJ) Rivers, São Paulo, Brazil, predicts a daily production of 630 to 760 tons of sewage sludge (20% of total solids) in 2020 for this region. Annually, this water basin could produce between 230,000 to 277,000 tons of sewage sludge (20% of total solids) (BIOCICLO, 2012). At a local scale, the estimated production of sewage sludge generated daily in this basin currently is 378 tons (20% of total solids).

Because of the large-scale production and the advances in the area as a result of incentive programs by the federal government, the production of sludge in Brazil, as well as worldwide, is a constant source of concern regarding environmental contamination (ANDREOLI et al., 1998).

There are several technically feasible alternatives for the treatment of sludge. The most common one involves the anaerobic digestion, which can be followed by final disposal in landfills, or surface disposal, ocean dumping, storage ponds, incineration or agricultural recycling (BETTIOL; CAMARGO, 2006; CAI et al., 2007; CAMARGO et al., 2008). The latter has received attention worldwide, from technical, economic, and environmental perspectives, as it recycles nutrients, promotes physical improvements in the soil, especially in its structure, and is a final solution for sewage disposal (ANDREOLI et al., 1998).

2. Main uses and applications

Because of the large production, mainly in large urban centers, researchers worldwide intensified their studies in the search of alternatives for the disposal of sewage sludge. As a result, sewage sludge has been used in the recovery of degraded areas, as fertilizer in different crops (ANDREOLI et al., 1998), or disposed in landfills, reused in the production of light aggregate, bricks and ceramic, and cement), incinerated, converted in combustible oil or dumped in the ocean (BETTIOL; CAMARGO, 2006; CAI et al., 2007).

In Brazil, there is a preference for the use of sludge in agriculture (although in early stages), due to the availability of land and relatively low costs. However, according to Camargo and Bettiol (2010), studies on the use of residues in agriculture developed until now in the country have been aimed at evaluating the agronomic efficiency of these products. Most studies have been conducted in greenhouses with little resemblance to field conditions. Studies on the contamination of the soil, water, air, plants, and organisms with heavy metals, persistent organic compounds, and pathogens still are in early stages to establish disposal guidelines for the safety of this practice.

On the other hand, some studies have demonstrated that sewage sludge, when incorporated to the soil, alters its physical properties, such as: soil density, aggregate size, water retention capacity (MELO; MARQUES, 2000); chemical properties, such as pH (SILVA et al., 2001), electric conductivity, and cation exchange capacity (OLIVEIRA et al., 2002), increase of phosphorus levels (SILVA et al., 2002), organic carbon (CAVALLARO et al., 1993) and nitrogen (BETTIOL; CAMARGO, 2006); and biological properties, usually increasing microbial activity (MELO et al., 2001).

The main constraint, however, when evaluating the possibilities of uses of sewage sludge in agricultural areas is the presence of metals and other persistent pollutants (NATAL DA LUZ et al., 2009), which can be toxic to plants (MARTINEZ; McBRIDE, 2000), microorganisms (KHAN; SCULLION, 2000), and soil invertebrates (SPURGEON; HOPKIN, 1999; GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; PEREZ FONTANETTI, 2011; FONTANETTI et al., 2012). This application can be limited by factors, such as presence of pathogenic organisms, toxic organic compounds, contamination of surface waters with nitrate, and transmission of heavy metals in the food chain. Of these factors, transference of metals and pathogenic organisms from the soil to crops and then to animals and humans, is probably the most harmful to health. As a result, many countries created guidelines for the use of sludge as soil amendment.

In Brazil, the application of sewage sludge or any other derivative in agriculture requires a detailed characterization of the agronomic potential of sludge, inorganic and organic substances that may be potentially toxic, presence of pathogens, as well as studies that evaluate its stability in the environment (BRASIL, 2006). The 375 Resolution of the Environmental National Council (Conselho Nacional do Meio Ambiente - CONAMA), of 29 August 2006, describes the maximum concentrations of inorganic compounds and pathogens in sewage sludge or its derivatives.

The sewage sludge produced may also undergo processes to increase the content of solids and decrease the number of pathogenic organisms, resulting in a residue termed biosolid, which is considered more innocuous than raw sewage sludge (HAYNES et al., 2009). Biosolid can be produced by sludge stabilization, reducing its volume with dry beds, sludge thickeners, press filters, press belts, vacuum filters, and centrifugation (BERTELLI, 2007). The term biosolid is used only when sewage sludge presents characteristics that allow its use in agriculture (ANDREOLI et al., 2006).

According to the definition adopted by USEPA (1995), biosolid is any organic product resulting from sewage treatment that can be beneficially used or recycled. Beneficial use implies the absence of environmental damage and of harm to animal and human health (USEPA, 1995).

Approximately 0.25 million of dry tons of sewage sludge are produced annually in Australia, and one third of biosolid is used in agriculture (MOLLOY et al., 2005). In Sidney, biosolid production reaches 190 thousands of tons/year (PEDROZA et al., 2010).

According to Tsutiya (2001), the USA and Europe produce approximately 13 million and 7 million of dry tons of biosolids per year, respectively, with final disposal in landfills, agricultural use, incineration, ocean dumping or in the reforestation and recovery of degraded areas.

In New Zealand, for example, the goal of residue management is to use 95% of biosolids for beneficial purposes, rather than disposal in landfills (FARIA; RODRIGUEZ, 2008).

In Brazil in 2004, the estimated production of biosolids (dry weight) is 202.530 t annually (LEMANSKI; SILVA, 2006). However, since sewage treatment practices are recent and still being developed, the agriculture use of biosolids is limited, occasionally in commercial scale and usually in experimental scales, in the states of São Paulo, Paraná and Distrito Federal (VANZO et al., 2002).

2.1 Physico-chemical aspects

The chemical characteristics of sludge depend on the sewage being treated, the facilities at the Wastewater Treatment Plant (WTP), sludge storage, treatment used to reduce pathogens, and seasonality (BERTON; NOGUEIRA, 2010).

From an agricultural perspective, sewage sludge contains significant quantities of nutrients essential for plant development. The decomposition of sewage sludge generates complexing agents that facilitate the solubilization of phosphates combined in the soil with iron and aluminum (CARVALHO; BARRAL, 1981), as well as nutrients in slow-release organic compounds. The improvements in physical and chemical characteristics of the soil result in an immediate increase in activity of the edaphic population (ANDREOLI et al., 1998).

The most abundant nutrients found are nitrogen and phosphorus, while calcium (Ca) and magnesium (Mg) are found in small quantities. When applied as the only source of nitrogen for plants, the quantities of micronutrients added are often enough to supply the nutritional needs of plants (von SPERLING et al., 2001).

The concentration of heavy metals in sludge is often significant. Among harmful elements are cadmium (Cd), copper (Cu), molybdenum (Mo), nickel (Ni), and zinc (Zn) (ALMEIDA et al., 1998). According to Rajj (1991), many of these metals are associated with environmental pollution or a natural occurrence in toxic levels for living beings. The ingestion of heavy metals may have toxic effects and promote the development of chronic or acute illnesses in animals and humans (GUPTA; GUPTA, 1998).

Metals may be present in the sludge and their availability can be affected by reactions such as adsorption, complexation, precipitation, oxidation, and reduction (FERREIRA; ANDREOLI, 1999). Absorption and/or accumulation of heavy metals by plants and the macro-pedofauna promotes the insertion of contaminants in the trophic chain, negatively affecting the health of ecosystems (CESAR et al., 2008). Therefore, knowledge on the fate of these elements in the soil is essential for the evaluation of the negative environmental impact caused by the use of sludge in agriculture, as the extent of this impact is directly associated with the ability of the soil to retain these metals (NOGUEIRA, 2008).

2.2 Microbiological Aspects

According to von Sperling (2001), the microorganisms found in sludge may be saprophytes, commensals, symbionts, or parasites. Only the latter are pathogenic and capable of causing illnesses to humans and animals. Among pathogenic organisms, five groups may

be present in the sludge: helminths, protozoa, fungi, viruses, and bacteria. Epidemiological studies have shown that bacteria, viruses, helminth eggs, and protozoan cysts are a risk to human and animal health. This risk is due mainly to the high incidence of parasitism in the population in different parts of the world; survival in the environment, and infectious dose (von SPERLING et al., 2001).

The origin of microbiological contamination of sludge is mainly due to fecal matter in the sewage, and therefore depends on the epidemiological characteristics of the local population and the effluents discharged in the sewer system (FERNANDES; SOUZA, 2001).

Among pathogens, streptococcus, *Salmonella* sp., *Shigella* sp., helminths (larvae and eggs), protozoa (cysts), and viruses (enterovirus and rotavirus) (ANDREOLI et al., 1998) are of special importance. It should be pointed out that bacteria are potential sources of epidemic diseases that need be monitored throughout the sewage treatment process.

Most enteric bacteria are transmitted by the fecal-oral route from contaminated water and foods. Inhaling of particles with pathogens is also possible. Therefore, the form of infection represents a higher risk for individuals directly handling sludge, such as workers of wastewater treatment plants, those involved in the transport and spreading of biosolids. However, Ottolenghi and Hamparian (1987) described that the land application of anaerobic digested sludge with bacteria may not increase the risk for farmers, as the survival of these pathogens in pastures is shorter (von SPERLING et al., 2001).

Among the pathogens most resistant to treatments are helminths, especially *Taenia solium*, whose eggs, if ingested with contaminated vegetables or polluted water, may cause neurocysticercosis, a common disease mainly in the state of Paraná, Brazil, that is the target of special programs by the State Secretariat of Health (ANDREOLI et al., 1998).

Data available in the literature have shown that the number of helminth eggs may be 10^3 a 10^4 or more in primary sludge, and for viruses, 10 a 10^6 /kg of dry matter (von SPERLING et al., 2001). According to the author, the survival of pathogens in the soil may vary depending on the ability of the microorganism, texture, and pH of the soil. In sandy soils, the survival of helminth eggs is shorter than in moist soils.

2.3 Agronomic aspects

Some crops are more suitable for the application of sludge, due to their ability to better use the content and slow release of nitrogen, such as grasses, as well as corn, wheat, sorghum, sugar cane among others. In addition to promptly responding to the sludge application as a result of directly absorbing nitrogen through the roots, the phytosanitary control and harvest

are mechanical, and the final product is processed, reducing to nearly zero the risk of any contamination (ANDREOLI et al., 1998).

The main crops recommended for sludge application are:

- Corn: studies demonstrate an increase in productivity of 49 to 77%.
- Other grasses: wheat, oat, and other grasses have good results with sludge. The application of sludge to sugar cane is also very promising, as it is a mechanized and industrialized crop, in addition to having high nitrogen requirements.
- Legumes: experiments with beans with bracinga have demonstrated that the production of beans doubled in comparison with areas without sludge. Legumes in general exhibit a less intense response than grasses, as they can fix air nitrogen by symbiosis, with phosphorus as the most important mineral for these crops.
- Fruits: during the transplant of seedlings into the hole, sludge can be mixed to the soil. For adult trees, sludge needs to be incorporated to the soil to prevent surface leaching. Good results have been obtained with apple and peach trees in the metropolitan region of Curitiba and with the productivity of citrus plants after sludge application.
- Pastures: the international literature recommends a period of quarantine of two months for the area where sludge was applied before grazing is allowed, avoiding the contamination of animals.
- Reforestation: topographic conditions of the terrain need to be taken into account, mainly regarding surface fertilization. There are studies on the use of sludge in the reforestation with bracinga in the metropolitan region of Curitiba, and the use of the product may include pine and eucalyptus trees. The use in reforestation areas is a good option, allowing the application of higher doses of sludge safely.
- Restoration of degraded land: the topographical condition of the terrain be taken into account, as the sludge will be applied to the bare soil, subjected to erosion. The physical and chemical properties of the sludge facilitate its use to restore degraded areas. Studies on mining areas reported an increase in phosphorus and magnesium levels, higher cation exchange capacity, and incipient trend to density reduction (ANDREOLI et al., 1998).

It should also be pointed out that information on the agricultural profile of the region where sludge will be applied, in addition to being important for the evaluation of the potential for the disposal of biosolids, is essential for planning this activity. The possibility of application of the product to many crops allow the scheduling of the distribution of biosolids

throughout the year, according to the requirement of each crop. Thus, the evaluation of the viability of the agricultural disposal of the residue must be in agreement with the demand for biosolids for each period of the year, according to the agricultural calendar of the region. In addition, it is recommendable that the application rate does not produce a nitrogen input higher than that needed for the development and production of the crop, to avoid risks of leachate (von SPERLING et al., 2001).

3. Available treatments (management and disposal)

In order to initiate the processes of final disposal, solid residues must be first classified to determine the most adequate procedure based on technological, economic and legal requirements. The Brazilian Association of Technical Standards (Associação Brasileira de Normas técnicas - ABNT) defined a set of guidelines to standardize the classification of solid residues. The NBR 10004 classifies the residues based on their potential risks to the environment and public health, indicating which residues must be managed and that require more strictly controlled disposal (CAVALCANTI, 2012).

Raw sewage sludge is rich in pathogenic organisms, putrescible, and quickly develop unpleasant odors. The process of stabilization or conditioning were developed to mineralize the biodegradable fraction of the organic mater present in the sludge, reducing the risks of putrefaction, as well as significantly decreasing the concentration of pathogens (von SPERLING et al., 2001; METCALF; EDDY, 2002; PEDROZA et al., 2010). The stabilization processes can be divided into:

- Biological stabilization: uses bacteria to promote the stabilization of the biodegradable fraction of the organic matter.
- Chemical stabilization: occurs by chemical oxidation of organic matter.
- Thermal stabilization: occurs from the action of heat on the volatile fraction in hermetically closed containers.

3.1. Chemical stabilization

The addition of chemical products to the sludge is aimed at promoting conditioning, consisted in the increase of the solid size by aggregating smaller particles, usually carried out with products such as coagulants and polymers (CAVALCANTI, 2012). Basically, in the chemical stabilization, products that can inhibit biological activities or oxidize organic matter

are added to sludge (FERNANDES; SOUZA, 2001). Processes of chemical stabilization are especially efficient to eliminate helminth eggs (CASSINI, 2003).

Liming is a stabilization method and chemical and thermal disinfection by the addition and mixture of large quantities of lime, to abruptly increase pH to slightly higher than 12, which often inactivates or destroys most of pathogens present in sludge and increases the temperature to approximately 60°C (ANDREOLI et al., 1998).

Some physical and chemical characteristics of sludge are altered by the addition of lime. Physically, a hard white cover may form on sludge when exposed to open air. Chemically, in addition to fixation of heavy metals, phosphorus insolubilization and nitrogen loss may occur, due to ammonia volatilization (FERNANDES; SOUZA, 2001).

3.2. Biological stabilization (composting and landfarming)

Composting is a biological treatment where an initial mixture of residues is subjected to the action of various groups of microorganisms. On the first days of the process, thermophilic microorganisms develop and maintain the temperature of the medium between 55-65°C for several days, destroying or reducing pathogenic microorganisms in the sludge (FERNANDES et al., 1996; ANDREOLI et al., 1998).

In the process of composting, sludge is mixed with a structuring residue rich in carbon, such as hay, wood bark or dust, organic part of urban trash, among others. This structuring residue, rich in carbon, unlike sludge, is poor in nitrogen, balancing the C/N ratio of the mixture that must be between 20 and 30 for the composting process to occur in favorable conditions. After the thermophilic phase, lasting approximately 30 days, the temperature gradually decreases to that of the environment and a maturation process takes place for approximately 60 days, characterized by the humification of the resulting material. The process generates a final product that is moist, black, with odor similar to that of mold, and easily manageable, termed compost (ANDREOLI et al., 1998).

Landfarming is an *ex situ* remediation technique, based on layering contaminated soil/and or residue up to 40 cm thick with agricultural machinery (BERGER, 2005; SOUZA et al., 2013).

In this system, an area receives large quantities of sludge for many years. The goal of this practice is to use the soil as treatment system. The soil becomes the support of biological activity, metal retention, site of sun exposure and biooxidation, degrading the organic matter (FERREIRA; ANDREOLI, 1999). Also according to these authors, the doses of sewage sludge application may vary between 60-70 t/year of dry matter for areas without

impermeabilization of the lower layer, and up to 300-600 t/year for areas with impermeabilization of the soil layer (60-80 cm in depth). This practice has been considered an inexpensive alternative and if correctly performed and monitored, it is innocuous to the environment and simple to be performed.

3.3. Thermal stabilization (Dewatering)

The solids in sludge come from the material originally present in effluents (suspended, dissolved, and colloidal solids), added to products for the reaction of additional chemical substances (coagulants, polymers, activated coal, and others) as well of non-reacted products. The goal of sludge dewatering is to decrease its volume by removing water and facilitating the costs of transportation and final disposal, in addition to eventually promoting conditions for recovering chemical products (reagents) and even specific pollutants (heavy metals) (CAVALCANTI, 2012).

Also according to Cavalcanti (2012), dewatering consists in removing water linked to sludge solids bonded by intermolecular forces of different types: interstitial, capillary, and absorbed.

The first phase is the separation of interstitial water by gravity thickening or mechanically. The resulting sludge is denser with solid content ranging from 2 to 8% that is still able to be pumped as it still is fluid.

The second phase is the separation of contact and capillary water. This separation requires significantly more energy, which are provided by mechanic dehydration equipment (centrifuges and presses). The result is solid cake transportable by conveyor belt with a solid content ranging between 15 and 50%. Finally, the third phase consists in the separation of absorption water, requiring higher quantities of energy per ton of water removed, only achieved by thermal power (evaporation). The result is a granulated solid or powder depending of the drying level, in which solid content can reach up to 95%.

Therefore, dewatering is a single operation that reduces the volume of sludge by decreasing its water content (GONÇALVES et al., 2001).

The process of sludge dewatering includes previous stages of thickening and/or conditioning, normally imposed by the equipment for thickening and drying, which need thicker sludge in order to reach higher efficiency in removing moisture (CAVALCANTI, 2012). Table 1 presents some the most common dewatering techniques in the management of sewage sludge, comparing costs of implementation and operation and the main advantages and disadvantages of each technique.

4. Final disposal

Currently in Brazil, landfills are the most common final disposal sites, followed by landfarming, ocean dumping, storage ponds, incineration or agricultural application after conditioning. The latter has been received much attention worldwide from a technical, economic, and environmental perspectives, as it recycles nutrients, improves the physical characteristics, especially soil structure and are a definitive solution for sludge disposal (ANDREOLI et al., 1998).

The costs of the alternatives of final disposal vary widely. Some factors such as the characteristic of the primary sludge, quantity of chemical products added for dehydration and conditioning, transportation to the final disposal site, machinery and labor used may directly interfere in the final costs. Table 2 presents some of the main alternatives of final disposal and their approximate costs.

5. Effects observed after exposure to sewage sludge on different test-organisms

In recent years, the concerns about soil pollution by metals due to the application of sewage sludge has increased, and are reflected in the growing number of studies on soil science in Brazil and worldwide, demonstrating that the application of this residue increases the levels of heavy metals in the soil (MARTINS et al., 2003; RANGEL et al., 2004; NOGUEIRA et al., 2007). Most of studies have reported an increase in metal levels based on the doses of sewage sludge application.

Increased levels of Cu in the soil have been observed by Pombo et al. (1989). Oliveira (2000) reported an increase in Cu and Cr levels in soils treated with sewage sludge, depending on the doses applied.

Although other studies have correlated the phytoavailability of these metals for many crops, few studies examined the genotoxic and mutagenic potential of sewage sludge.

Srivastava et al. (2005) evaluated the effects of leachate of city sewage sludge in India on meristematic cells of *A. cepa*. The chemical composition of raw sludge samples included the metals Cr, Cu, Ni and Pb. The concentrations evaluated were 2.5%, 5.0% and 10% of leachate diluted in distilled water. Regarding cytotoxic effects, the concentrations of 5.0% and 10% exhibited a significant decrease compared to the negative control. Regarding the mutagenic potential, the authors did not observed a significant mutagenic effect, based on the presence of micronucleus in cells. On the other hand, the concentration of 10% significantly

induced chromosome aberrations, demonstrating the genotoxic potential of this sludge. The authors attributed the effects observed to the combined action of heavy metals and other compounds, such as organic compounds, not characterized by the chemical analyses.

Caritá (2007) evaluated the potential of inducing damage to chromosomes of meristematic cells of *A. cepa* by the possible toxic agents present in diluted sewage sludge from WTPs from five metropolitan areas of the state of São paulo, Brazil. The author observed an increase in frequency of chromosome aberrations for all dilutions of sludge tested.

Studies conducted by Brossi (2008) evaluated the toxicity of four doses of sewage sludge applied according to the guideline P4.230 of CETESB, in one commercial area of eucalyptus. The objective was to test whether sewage sludge, when applied to the soil, had toxic effects on the soil-eucalyptus system. The toxicity was evaluated with tests using *Daphnia magna*, *Pseudokrichirella subcaptata*, *Lactuca sativa* and *Allium cepa*. The authors observed an increase in metal levels (Cr, Mn, Fe, Ni, Cu, Zn, Cd and Pb) in the soil, based on the doses as well as an increase of toxicity for *D. magna*, *P. subcaptata*, and genotoxicity and mutagenicity for *A. cepa*. The results demonstrated a correlation between the data obtained with the different test-organisms and the levels of potentially toxic elements in the soil.

Bioassays conducted by Maziviero (2011) were also aimed at evaluating the cytotoxic, genotoxic, and mutagenic potentials of sewage sludge and sludge dilutions on the genetic material of *A. cepa* and *Tradescantia*. This author observed that for both test-organisms, sewage sludge and sludge dilutions were genotoxic and mutagenic.

Assays with terrestrial organisms, such as colembola, earthworms, and diplopods were also conducted to assess the toxic potential of sewage sludge on these organisms.

Natal da Luz et al. (2009) conducted avoidance and reproduction tests using earthworms (*Eisenia andrei*) and collembolans (*Folsomia candida*) and evaluated the growth of the plants *Brassica rapa* and *Avena sativa*, in different concentrations of soil mixed with three sludges from Portugal (urban sludge, olive processing, and electroplating plants). The authors observed that sludge from electroplating plants was the most harmful to the test-organisms, due to the presence of chromium.

Bioassays with the diplopod *Rhinocricus padbergi* have been successfully conducted to evaluate the toxicity of sewage sludge from many Brazilian cities, by analyzing the histopathology of the midgut (GODOY; FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; 2011; PEREZ; FONTANETTI, 2011; BOZZATTO; FONTANETTI, 2012) and the fat body (SOUZA; FONTANETTI, 2012).

Samples of biosolid from a WTP from the state of São Paulo, evaluated by Christofolletti et al. (2012; 2013), also had a toxic potential to meristematic cells of *A. cepa* and were capable of inducing alterations in the midgut of the diplopod *R. padbergi*.

Regarding the presence of pharmaceutical products in sewage sludge, Miranda and Zemelman (2001) analyzed isolated bacteria from fish captured near urban sewage discharge areas, and observed a high frequency of bacteria resistant to the antibiotics ampicillin, streptomycin, tetracycline, and nitrofurantoin. The isolated bacteria belonged to Vibrionaceae and Enterobacteriaceae. These results suggest that fish living near urban sewage discharge areas might be responsible for transmitting antibiotic-resistant bacteria, and increasing the health risks of consumers of these fish. Woloszynek et al. (2000) also found ampicillin-resistant bacteria in a pond of primary sewage treatment.

Table 3 summarizes some of the main studies involving sewage sludge, biosolids, and different organisms.

6. Trends and perspectives (use as soil amendment, and the importance of qualitative monitoring with bioindicators)

Based on the available data on the use of sewage sludge as soil amendment, this practice may vary according to the edaphoclimatic conditions of a given region, type of crop, and its corresponding management. According to a report published by the PCJ Committees (2012), in Brazil since September 2011, only Class A sludge can be recycled as soil amendment, which invariably requires additional treatment of sludge from WTPs. In order to comply with the requirements of the Ministry of Agriculture, Livestock, and Supply it is possible to classify sewage sludge treated with adequate technology as an agricultural product. Considering that the Resolution 375/2006 of CONAMA describes the procedures aiming at mitigating risks, it should be pointed out that the treatment and elimination of risk factors at the Sewage Management Unit, is the most feasible.

At the national level, the new National Policies of Solid Residues, Law #12305/2010, in effect on 02 August 2014, defines that all Brazilian municipalities shall eradicate dumps and implement sanitary landfills for the disposal of solid residues. Only the part of the residue that cannot be reused, recycled, or other forms of treatment shall be disposed in landfills. Thus all and any alternative for the disposal of sludge that may use its beneficial potential are welcomed.

The future of sewage sludge in Brazil is moving toward the same path of countries such as Germany, France, and United States, where the use of sewage sludge as soil amendment is already a reality. As discussed in this review, there are several advantages and disadvantages to these practices. However, safety measures during management and treatment are equally essential for the success and consolidation of sewage sludge as soil amendment. As a result, biological tools combined with chemical analyses are efficient and necessary for a reliable qualitative monitoring of this practice in the laboratory as well as in the field and can provide a framework for the development of programs aimed at preserving different ecosystems.

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Table 1 - Main techniques for sewage sludge dewatering

Treatment System	Technique description	Efficiency (% SS)	Equipment cost / unit (R\$) ¹	Cost of operation / maintenance (R\$/ton) ²	Advantage	Disadvantage
Filter Press	Operate in batch, the slurry is pumped through the filter plates by filling and subsequently, the plates are subjected to pressures up to 170kPa by means of hydraulic pistons. The liquid is separated by means of filtration screens and led to the discharge channel clarified while the solids are retained in the pressing chambers between the walls of tissue. After pressure relief, the plates are separated and dewatered sludge is discharged by gravity or mechanical scraping its bottom	20-50%	400.000,00- 600.000,00	7,00-15,00	-Good efficiency of dehydration -Technology-diffused -Low polymer consumption	-Requires constant maintenance -Risk-clogging of filters -Requires skilled labor
Centrifuge	The solid / liquid separation in a centrifuge occurs under high rotation. The rotor consists of a cylindrical barrel and a conical-screw. The suspension is injected into the rotor reaching the distributor where it is subjected to centrifugal force. As the suspended solids are collected in the barrel wall, the screw dragging centrifuged particles carrying them to one end of the drum where they are discharged by gravity. The liquid phase is drained at the other end by gravity	18-35%	500.000,00- 600.000,00	7,00-15,00	-Good dewatering efficiency -High feed rate -Requires small area for installation	-High-energy consumption -Requires constant maintenance -Requires skilled labor
Rotary thickener	The previously flocculated sludge (ss> 0.6%) is inserted by gravity into the rotary drum inclined structure composed of a cylindrical-conical stainless steel where the screen is fixed. The suspension is thickened by means of rotation of the drum, leaving the system under gravity	4-10%	300.000,00- 400.000,00	5,00-7,00	-Low-power consumption -Requires little maintenance -Robust	-Low-rate power -Low-solids in the cake -Requires skilled labor
Geotextile tube	Injection of the suspension into the interior of a porous geotextile bag adopts the tubular shape when full. The filling operation repeated many times, it is only completed when the entire unit is loaded with solids. The suspension to be dewatered must be previously flocculated with chemicals in order to obtain flakes of a size to allow drainage. The cell settlement must be leveled properly as mild slope in the longitudinal direction of the tube	30-45%	200.000,00- 300.000,00	5,00-7,00	-Fácil operação -Boa taxa de desidratção -Baixo custo de energia	-Easy operation -Good rate of dehydration- -Low-cost energy
Drying bed	Built in brick, with square or rectangular shape, endowed fund composed of permeable sand and gravel of different particle size and collection device of the percolated liquid, usually exposed to the weather, to enjoy the sunshine, facilitating evaporation which causes rapid drying of the sludge	30-50%	50.000,00- 100.000,00	-	-Requires no addition of chemicals -Does not require electricity -Ease-operating	-Odors and unpleasant aspects -Attraction-vector -Requires large surface areas
Dryer thermal	t is a unit operation based on the evaporation of interstitial water previously dehydrated sludge by injection latent heat through auxiliary fuel. The drying is carried out in a single pass without recycling the product, regardless of water content in the material to be treated or finished product. A closed-circuit gas generated during the process prevents uncontrolled emissions	75-95%	800.000,00- 1.000.000,00	5-10,00	-High-efficiency dehydration -Can be self-sufficient -Sterilization-in residue	-High cost of implementation -Requires gas treatment -Operational Risk
Incinerator	The combustion process is carried out in plants with primary rotary kiln or static, post-combustion chamber system, gas treatment, sewage treatment devices and systems for emission monitoring. Can be used as fuel, the waste itself, natural gas, fuel oil or other. Waste solids, liquids or pastes are fed in balanced mixtures technically and incinerated in the furnace primary temperatures 800-1100° C with residence time longer than two seconds and then treated in cooling systems that remove and dry particulate matter, volatile and gases	75-95%	1.000.000,00- 8.000.000,00	5-10,00	-High-efficiency dehydration -Addition of sludge with high moisture content -Sterilization-in residue	-High cost of implementation -Requires gas treatment -Operational Risk

¹: Cost survey by market research (based on 2011/2012); ²: Cost on the Piracicaba, Capivari and Jundiá' basin (CBH-PCJ) (BIOCICLO, 2012).

Table 2 - Techniques disposal

Final Disposal System	Technique description	Approximate cost (R\$/ton)¹	Advantage	Disadvantage
Landfill	For these landfills should be directed only natural waste solid or pasty, being vetoed sending waste liquids or suspensions, and oily residues. Landfills consist of impermeable cells, equipped with gas collection system and leachate liquid under constant monitoring and restricted access. The solid wastes are disposed in stacks, compacted clay and covered with superimposed layers until full saturation area	40-250	-Layout-most used -Closer to the site -Well-control system	-Requires preliminary dehydration -Decreased life of landfill -Difficulty compression for pies below 20% solids
Coprocessor	It is one of the techniques of thermal destruction of solid waste, including some types of sludge and other waste from the sewage treatment systems, and may appear solid, pasty or as suspensions. The sludge is applied as a fuel substitute, the residue is verified to ensure energy gain of the clinker manufacturing process. The thermal destruction of waste in a cement kiln characterized reuse and disposal in a single operation in a rotary kiln firing at a temperature above 1700° C	*	-Economically-viable -Environmentally-sustainable -Aggregates-positive characteristics to the final product	-Not allowed to pies with chlorinated compounds -Not allowed for pies with metals -They must have specific function
Agricultural disposition	Consists of applying the previously stabilized sludge and / or sanitized the particular area under constant monitoring. For agricultural application, beyond culture, one should take into account soil characteristics, physico-chemical characterization of the sludge, especially with reference to heavy metals and nitrogen content	40-270	-Environmentally-sustainable -Reduces need for agricultural inputs -High content of nutrients	-Requires prior conditioning -It has restrictions on certain crops -Requires constant monitoring and tracking
Incineration	It is the process of thermal oxidation at high temperatures under controlled conditions to ensure that complex molecules are destroyed or cracked into simpler molecules. The incineration process is conducted in plants equipped with rotary kiln primary or static, post-combustion chamber system, gas treatment, sewage treatment devices and systems for emission monitoring. Can be used as fuel their own waste, natural gas, fuel oil or other. Waste solids, liquids or pastes are fed in balanced mixtures technically and incinerated in the furnace primary temperatures 800-1100° C	150-300	-Drastic reduction of the volume- -Sterilization / mineralization of waste -Allows agricultural application	-High Cost -Requires gas treatment -Requires large amount of energy

¹Source: Andreoli et al. (1998)

*: Cost may vary according to the application

Tabela 3. Biomarkers evaluated in different organisms after sewage sludge and biosolid exposure

Sewage sludge	Test organism	Biomarkers	References
Municipal sewage sludge from France Samples: test systems were performed either directly with sludge or sludge-amended soil samples (plant or with aqueous extracts)	<i>Xenopus laevis</i> (frog)	Micronucleus assay on the larvae Frog Embryo Teratogenesis Assay – <i>Xenopus</i> (FETAX)	Chenon et al. (2003)
Leachate prepared from municipal sludge in Lucknow city (India)	<i>Nicotiana tabacum</i> (tobacco)	Somatic mutation test	
	Swiss albino male mice (mammal)	Chromosomal aberrations, micronucleus test and comet assay with cells of bone marrow	Tewari et al. (2005)
Sewage samples from Franca and Barueri (São Paulo, Brazil)	<i>Daphnia similis</i> (microcrustacean)	Mobility	Jonsson and Maia (2007)
Latosols and chernosols amended with sewage sludge from Ilha do Governador Sewage Treatment Plant (Rio de Janeiro, Brazil)	<i>Eisenia andrei</i> (earthworm)	Avoidance, mortality and biomass	Cesar et al. (2008)
Stabilized anaerobic sewage sludge from Spain was applied once at three application rates: 30, 60 and 120t ha ⁻¹ wet wt. trying to cover agronomic requirements	<i>Triticum aestivum</i> (wheat), <i>Vicia sativa</i> (garden vetch) and <i>Brassica rapa</i> (turnip)	Seed germination, above-ground biomass production and shoot elongation	Carbonell et al. (2009)
	<i>Eisenia fetida</i> (earthworm)	Mortality and biomass	
Pelleted commercial diet containing 0, 5000, 10.000 and 50.000 ppm of a treated sludge sample (PCJ1-Brazil)	<i>Wistar</i> rats (mammals)	Bone marrow micronucleus test and comet assay	Solano et al. (2009)
Sludge effluent from Nigeria	<i>Allium cepa</i> (onion)	Root length and morphology Chromosomal aberrations	Ukaegbu and Odeigah (2009)

Substrate containing sewage sludge from a sewage treatment station (STS) of a city of São Paulo State (Brazil)	<i>Rhinocricus padbergi</i> (diplopod)	Midgut histopathology	Godoy and Fontanetti (2010)
Raw Sewage sludge and solubilized samples from Atibaia (São Paulo, Brazil).	<i>Allium cepa</i> (onion) <i>Tradescantia pallida</i>	Chromosomal aberrations and micronucleus test Micronucleus test in tetrads	Maziviero (2011)
Substrates containing sewage mud of a STS AT-1, located in the hydrographic region of the Alto Tietê Basin (São Paulo, Brazil).	<i>Rhinocricus padbergi</i> (diplopod)	Midgut histopathology	Nogairol and Fontanetti (2010, 2011)
Substrate containing sewage sludge from two STPs (SG and PCJ-2) located in two hydrographic regions of São Paulo State, Brazil.	<i>Rhinocricus padbergi</i> (diplopod)	Midgut histopathology	Perez and Fontanetti (2011)
Two sewage sludge samples were used; they were collected in two small towns in the countryside of São Paulo State. Both cities are part of the Piracicaba–Capivari–Jundiá basin (PCJ-1 and PCJ-3).	<i>Rhinocricus padbergi</i> (diplopod)	Midgut histopathology	Bozzatto and Fontanetti (2012)
Substrates containing sewage mud of a STS AT-1, located in the hydrographic region of the Alto Tietê Basin (São Paulo, Brazil).	<i>Rhinocricus padbergi</i> (diplopod)	Fat body histopathology	Souza and Fontanetti (2012)

Biosolid

Digest sewage sludge and derived composts. Each waste was tested mimicking a field application of 6 ton/ha or 12 ton/ha.	<i>Eisenia andrei</i> (earthworm) <i>Folsomia candida</i> (springtail) <i>Brassica rapa</i> (turnip) <i>Avena sativa</i> (oat)	Avoidance and reproduction assays Seed germination and plant growth	Moreira et al. (2008)
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Three types of municipal sewage sludge and one pig slurry from Spain. The samples were digested, composted and/or thermally dried.	<i>Brassica rapa</i> (turnip), <i>Lolium perenne</i> (perennial ryegrass) and <i>Trifolium pretense</i> (red clover)	Seed germination and shoot length	Ramírez et al. (2008)
Five biosolid samples from different sewage plants throughout Ireland	<i>Eisenia fetida</i> (earthworm) and <i>Folsomia candida</i> (springtail)	Mortality and reproduction	Artuso et al. (2011)
Sewage sludge digested samples from two sewage treatment plants in southeast part of Poland	<i>Lepidium sativum</i> (cress) <i>Sorghum saccharatum</i> (sorghum) and <i>Sinapis alba</i> (white mustard)	Phytotoxkit test (seed germination and root growth)	Oleszczuk and Hollert (2011)
	<i>Heterocypris incongruens</i> (crustacean)	Ostracodtoxkit test (mortality and growth inhibition).	
Biosolid sample from a domestic sewage plant throughout Brazil, used in agrilculture	<i>Allium cepa</i> (onion)	Chromosomal aberrations and micronucleus test	Christofoletti et al. 2012; 2013
	<i>Rhinocricus padbergi</i> (diplopod)	Midgut histopathology	

Investigation of the genotoxicity of primary sewage sludge, the precursor of biosolids used in agriculture, with the *Allium cepa* system

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Abstract

Soil contamination by residues from wastewater treatment plants (WTP) is one of the major challenges to the scientific community, due to the volume produced, their adequate treatment, final disposal and recycling. Several alternatives for the use of sewage sludge, such as fertilizer in crops, produced by WTPs have been examined. In order to evaluate the toxic, cytotoxic, genotoxic, and mutagenic potentials of primary sewage sludge from a WTP, precursor of a biosolid for agriculture, the chromosome aberration and the micronucleus tests using *Allium cepa* seeds (onion) were conducted with raw sewage sludge and a mixture of sewage sludge and control soil at time 0 (t0) and after 30 days (t30) of bioprocessing by millipedes. Sewage sludge samples were collected in 2010 and 2011 and chemically analyzed. The chemical analysis revealed that the 2010 sludge sample contained some heavy metals and organic compounds, such as phenanthrene, fluoranthene, and naphthalene, unlike the 2011 sludge sample, which did not contain these organic compounds. The 2010 sludge sample was toxic to onion seeds, completely inhibiting germination, while the 2011 sample had cytotoxic effects, inducing the proliferation of meristematic cells in comparison to the negative control. The chromosome aberration test demonstrated the genotoxicity of sewage sludge. Mutagenicity was observed for all treatments, except for the 2011 sludge sample at time 0. However, the latter had significant results for the micronucleus test in cells of F₁ region after 30 days of bioprocessing. The chemical analysis revealed a variation in the concentration of different metals after 30 days of exposure by millipedes, indicating bioprocessing. However, this did not reduce significantly the genotoxic and mutagenic effects of all samples of the two bioassays conducted. Based on our results, genotoxicity tests are important tools to evaluate environmental quality of sewage sludge samples and the disposal of residues should be conducted with caution.

Keywords: aneugenicity, clastogenicity, chromosome aberrations.

1. Introduction

The inadequate management of human activities have had major impacts on ecosystems, seriously compromising the quality and maintenance of environmental conditions. In the last decades, the final disposal of residues generated by wastewater treatment plants (WTP) has been a major concern. Sewage sludge is a predominantly organic semi-solid residue with variable levels of inorganic compounds from the treatment of domestic and industrial effluents (ANDRADE, 1999).

A feasible alternative for the disposal of sewage sludge is its use in agriculture, as soil fertilizer and/or conditioner, which can have some advantages from economic and environmental perspectives (LOPES, 2008). However, there is a concern regarding the high levels of heavy metals that may be added to the treated soils and consequently the harmful effects these elements may have on the biota and human health (GUPTA; GUPTA, 1998; CESAR et al., 2008).

In order to be use in agriculture, sewage sludge needs to undergo a process to increase the solid content and decrease the number of pathogenic organisms. This process involves sludge stabilization, reducing its volume with the use of drying beds, sludge thickeners, filter presses, belt presses, vacuum filters, and centrifugation (BERTELLI, 2007). After being processed, sewage sludge is termed biosolid (HAYNES et al., 2009).

The monitoring of sludge quality is a valuable tool to determine the conditions of applicability of this biosolid in agriculture. The 375/2006 resolution of the National Environmental Council (Conselho Nacional do Meio Ambiente – CONAMA) in effect in Brazil is currently being reviewed and defines procedures, criteria, and requirements for the development of projects, implementation, and generation of biological systems using liquid domestic sewage effluents in agricultural areas, aiming to fulfill environmental requirements. However, this resolution does not include studies on the genotoxic and mutagenic effects of sewage sludge. In other countries, adverse effects on biological systems, such as genotoxicity and carcinogenicity, have been correlated to heavy metals and organic pollutants in sewage sludge (PARKPAIN et al., 2000; SINGH; AGRAWAL, 2008).

Toxicity and genotoxicity tests could reduce the uncertainties on the quality of sewage sludge, and assist in the selection of those appropriate for agricultural use (ARAÚJO; MONTEIRO, 2005). On the other hand, tests with terrestrial invertebrates have been successfully used to evaluate soils contaminated with different residues (GODOY;

FONTANETTI, 2010; NOGAROL; FONTANETTI, 2010; 2011; SOUZA; FONTANETTI, 2011; MERLINI; FONTANETTI, 2012). Diplopods are important animals of the terrestrial fauna, occupy the trophic level of decomposers (PETERSEN; LUXTON, 1982), and can colonize different layers of the soil. These animals participate in the cycling and disposal of nutrients present in the decomposing organic matter, assisting in the humification of soil; and promoting mineralization with their feces, as they secrete ammonia and uric acid that when degraded, enrich the soil with nitrates (SCHUBART, 1942; GODOY; FONTANETTI, 2010). Thus, by processing the compounds present in the soil, they stimulate microbial metabolism, essential for the cycling of nutrients, such as carbon, nitrogen, and phosphorus, in addition to promoting active soil aeration (HOPKIN; READ, 1992).

The present study was aimed at evaluating the toxicity of sewage sludge from a WTP, the precursor of a biosolid registered as fertilizer, using *A. cepa* seeds exposed to raw sewage sludge, and combinations of sludge with control soil, according to Brazilian laws for agricultural use. The sewage sludge samples and combinations with control soil were also analyzed after processing by diplopods, representatives of the edaphic fauna.

2. Materials and Methods

2.1 Primary sewage sludge

Sewage sludge samples were collected in 2010 and 2011 in a wastewater treatment plant (WTP) of a city of São Paulo state, Brazil. The plant is managed by the Basic Sanitation Company of the State of São Paulo (Companhia de Saneamento Básico do Estado de São Paulo – SABESP). According to Vanzo et al. (2000), incoming sewage to this WTP is predominantly domestic, and industrial wastewater is negligible. The sewage treatment used in this plant is the conventional activated sludge process. The production of sludge is mainly carried out by the treatment systems used for the liquid phase. According to Pedroza et al. (2010), *a priori*, all processes of biological treatment generate sludge. Those that receive raw sewage in primary decanters generate primary sewage, composed of sedimentable solids of raw sewage. Primary sewage sludge may be mixed with the biological excess sludge from the aeration tank or from the sludge return line. After concentration in gravity thickeners, the sludge moves into digesters where stabilization by anaerobic digestion takes place, converting sludge into biosolid (VANZO et al., 2000).

Samples were collected and stored in plastic boxes, wrapped in dark plastic bags and maintained in a cold room (4°C), until used in experiments.

2.2 Control soil sample

The soil sample used as control and for mixing with residues was collected on the campus of the São Paulo State University - UNESP (22°24'36''S/ 47°33'36''W), city of Rio Claro, São Paulo, Brazil. For the two bioassays, soil was collected in 2010 and 2011 at a depth of 0-20 cm, dried at roomtemperature, sieved with a 4 mm-mesh, followed by chemical characterization and granulometric analysis.

2.3 Test system

Allium cepa seeds from the same lot and variety (Baia periforme) were used to examine the toxic, cytotoxic, genotoxic, and mutagenic potential of the sewage sludge samples and combinations of sludge and soil.

2.4 Chemical characterization of samples

Chemical, physico-chemical, and agronomic potential analysis of the control soil regarding macro and micronutrients (N, Ca, Mg, P, K, S, Fe, Mn, Cu, Zn), C/N ratio, organic matter, cation exchange capacity (CEC), and base-saturation percentage were performed by the Campinas Institute of Soil and Fertilizer (Instituto Campineiro de Solo e Adubo - ICASA), Campinas, São Paulo, Brazil. Metals (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se and Zn), the 16 priority organic compounds defined by the Environmental Protection Agency (EPA), and the agronomic potential (electric conductivity, organic carbon, total phosphorus, Kjeldahl nitrogen, ammoniacal nitrogen, nitrate/nitrite nitrogen, pH in water (1:10), total potassium, total sodium, total sulfur, total calcium, total magnesium, moisture, volatile solids, and total solids) in the soil and primary sludge samples were measured by the laboratory TASQA, Paulínia, São Paulo, Brazil. The parameters analyzed followed the Standard Methods for the Examination of Water and Wastewater 21th Edition 2005 (SM21) and EPA. These same parameters were measured for the combinations of soil and sludge in the beginning of the assay – time 0 (t0) and after 30 days (t30) of bioprocessing by diplopods.

In order to classify the type of soil used as control, a sample was sent to the Department of Soil Sciences of the Luiz de Queiroz School of Agriculture (Escola Superior de Agricultura “Luiz de Queiroz” - ESALQ/USP) for characterization based on granulometry.

2.5 Calculations for sewage sludge application

According to the resolution 375/2006 of CONAMA, the maximum annual application of sewage sludge and its derivatives in tons per hectare shall not exceed the ratio between

quantity of nitrogen recommended for the crop (in Kg/ha), following the official guidelines of the state of São Paulo, and the nitrogen content available in the sewage sludge or its derivatives (Navail em Kg/t), calculated as: $N \text{ recommended (kg/ha)} / N \text{ available (Kg/t)}$. To determine the nitrogen available (Navai) in the sewage sludge and/or biosolid, the mineralization fractions were calculated (MF). According to CONAMA, this fraction represents 40% of undigested sewage sludge.

2.6 Preparation of soil and residue samples for bioassay setup

Soil volume and density of each terrarium were calculated ($d= 5.0625 \text{ dm}^3$). According to CONAMA, the rate of sewage sludge application in agriculture shall be calculated according to the quantity of nitrogen needed for each culture and the quantity of nitrogen present in the sewage sludge or its derivatives. In this study, sugar cane was the crop examined. According to Raij et al. (1997), the quantity of nitrogen for this crop is 120 kg N/ha. However, the quantity used was 100 kg N/ha.

Four glass terraria measuring 45 cm in length, 25 cm in width, and 20 cm in height, with capacity for 22.5 L containing 5 Kg of soil each were used. After the physical-chemical analysis of soil and sludge, control soil (CS) and soil + sludge (SL) combinations were termed samples at time 0 (t0):

1. **CS (2010):** 5 Kg of control soil + 20 diplopods.
2. **CS (2011):** 5 Kg of control soil + 20 diplopods.
3. **SL (2010):** 5 Kg of control soil + 371 mL of primary sludge + 20 diplopods.
4. **SL (2011):** 5 Kg of control soil + 795.49 mL of primary sludge + 20 diplopods.

Terrestrial invertebrates were remained in the samples for 30 days (t30).

2.7 Germination of *A. cepa* seeds in raw sewage sludge at time 0 (t0) and after bioprocessing by diplopods for 30 days (t30).

Approximately 100 seeds of *A. cepa* were allowed to germinate at a controlled temperature of 22°C in Petri dishes. A sample from each terrarium was collected and place in Petri dishes for the germination of *A. cepa* seeds at time 0 (t0). The positive control test consisted of seeds exposed to two agents with potentially cytotoxic and mutagenic concentrations: trifluralin, an anaugenic herbicide at a concentration of 0.019 ppm (FERNANDES et al., 2007) and MMS (methyl methanesulfonate), a clastogenic agent at a concentration of 10 mg/mL (RANK; NIELSEN, 1997). The negative control consisted of seeds allowed to germinate in ultrapure water and the environmental control, in control soil.

After 30 days of bioprocessing by diplopods, a sample from each terrarium was collected, with the corresponding combinations, for further exposure of onion seeds and physico-chemical analysis. All treatments described above were conducted in duplicate.

2.7.1 Preparation of slides and evaluation of cytotoxic, genotoxic, and mutagenic effects on *A. cepa* seeds.

After roots reached approximated 2 cm in length followed seed germination, root tips were collected and fixed with Carnoy (3:1 ethanol: acetic acid). After fixation, root tips were stained with the Feulgen reaction (MELLO; VIDAL, 1978), with acid hydrolysis for 11 minutes. Root tips were sectioned on the slide to remove the meristem and F₁ region. A drop of acetic carmine (2%) was added to intensify staining and spread cells. All slides were obtained by lightly pressing the material between slide and coverslip. Coverslips were removed with liquid nitrogen and slides were mounted with Entellan. The material was analyzed under light microscope at magnification of 400x.

To evaluate the toxic, cytotoxic, genotoxic and mutagenic potential, 5000 cells were examined for each treatment at time 0 and after 30 days. The same number of cells was examined for the negative and positive controls.

Toxicity was assessed with the seed germination index. Citotoxicity was determined based on cell alterations indicating cell death and the mitotic index (MI) calculated as $MI = (\text{number of dividing cells} / \text{total number of observed cells}) \times 100$.

Genotoxicity was evaluated based on the quantification of cells with chromosome alterations (CAI), such as C-metaphase, chromosomal adherence, multipolar anaphase and telophase, chromosome bridge and loss.

Mutagenicity (MtI) was determined based on cells with micronuclei (MN), as well as chromosome breaks in meristematic cells and micronuclei in the F₁ region cells.

The results obtained for all treatments for the different times were compared with those obtained for the corresponding negative control and control soil with the Mann-Whitney test, with significance set at 0.05.

3. Results

3.1 Chemical and granulometric characterization of soil samples

For the correct application of sewage sludge, information on the agronomic potential and fertility of the control soil were needed. The values obtained for pH, organic matter

(OM), residual phosphorus (P_{res}), potassium (K), calcium (Ca), magnesium (Mg), exchangeable aluminum (H+Al), sum of bases (SB), cation exchange capacity (CEC), and base saturation (V%), for the two samples are presented in table 1. The results for pH, K, Ca, Mg, H+Al, SB and CTC varied. According to granulometric analysis, soil samples had a clayey texture.

3.1.1 Comparison and chemical characterization of the control soil and sewage sludge samples

The results of the physico-chemical analysis and metals for the control soil and for sewage sludge samples are presented in table 2. Because of their complexity, all parameters analyzed varied when comparing the samples from the two years. In the second control soil sample, different results were obtained for copper, chromium, mercury, molybdenum, and nickel.

Unlike the chemical analysis of the 2010 sludge sample, in which the presence of three organic compounds, phenanthrene, fluoranthene, and naphthalene, were found, these compounds were not detected by the chemical analysis in the second sample (table 3).

3.2 Analysis of the toxic, cytotoxic, genotoxic and mutagenic potential of sewage sludge

3.2.1 Toxicity

The sewage sludge sample collected in 2010 was toxic to the test-organism, as no seeds germinated. A comparison of germination indices of the samples collected in the two years is presented in figure 1.

3.2.2 Cytotoxicity

Cytotoxicity tests, based on the mitotic index and the presence of cells undergoing cell death, revealed that in addition to the positive controls, the second sewage sludge sample was significantly cytotoxic compared to the negative control. Table 4 presents the mitotic indices of cells examined for all samples evaluated.

3.2.3 Genotoxicity

The statistic analysis revealed a genotoxic effect for all treatments examined (Table 4), in samples collected in both years, as all exhibited significant indices ($p < 0.05$) of chromosome aberrations compared to those of the negative control and/or control soil, even after processing by diploids (t30).

The most commonly observed alterations in the present study were cells with nuclear buds (Figure 2A), metaphase with chromosomal adherence (Figures 2B), polyploid metaphase (Figure 2C), anaphase with chromosome bridge (Figure 2D). Cells with disform nucleus (polyploid) and hypertrophy (Figure 2E) were observed only in samples exposed to primary raw sludge sample collected in 2011. The frequencies of aberrations are presented in table 5.

3.2.4 Mutagenicity

Cells with chromosome breaks (Figure 2F) and micronuclei (Figure 2G) were quantified to evaluate the mutagenicity (IMt) of treatments. The statistic analyses revealed a significant mutagenic effect for all treatments examined, except for the combination of soil+sludge (SL) collected in 2011 at time 0 (Table 4).

Seeds exposed to MMS, trifluralin, and to the combination of soil+sludge after 30 days (t30) collected in 2011 exhibited significant indices of micronuclei in cells of F₁ region (Figure 2H), when compared to the negative and/or control soil (Table 6). Based on these results, the chromosome aberrations observed in meristematic cells of these treatments progressed into micronuclei in F₁ region cells.

4. Discussion

The levels of contaminants in sewage sludge can be measured by chemical analyses. However, these analyses are not frequently conducted, as they require an extensive knowledge of the class of pollutants that need to be analyzed. In addition, they are expensive, when considering all possible types of chemical contaminants that can be present; they provide little information regarding the bioavailability of pollutants and/or the products of their degradation (CROUAU et al., 2002), their toxicity to soil organisms, and the possible cumulative, synergistic or antagonistic effects among pollutants.

When an organism is injured, adversely affecting its physiology, the agent involved might be considered toxic. In the present study, the raw primary sludge sample collected in 2010 had a toxic effect in *A. cepa* seeds, as no seeds germinated. In this same sample, three polycyclic aromatic hydrocarbon (PAH) were detected. According to the literature, some of them, such as naphthalene, are phytotoxic and can interfere in the normal development of plants (ADAM; DUNCAN, 1999).

After 30 days of bioprocessing by diplopods, further germination tests with *A. cepa* seeds were performed with the same samples. However, no toxic effects were observed, as

seeds exposed to all samples had germination indices similar to those of the negative and environmental controls. Our results indicate that the toxicity of sewage sludge was minimized by the action of diplopods that by revolving the contaminated soil, and feeding (SCHUBART, 1942; GODOY; FONTANETTI, 2010), assimilated the toxicants.

Several studies have demonstrated that one of the major concerns and precautions regarding the use of sewage sludge in agriculture is the concentration of heavy metals in the sludge, which after successive applications can have negative impacts in its surroundings (MARTINS et al., 2003; RANGEL et al., 2004; NOGUEIRA et al., 2007). Once added to the soil, they are difficult to be removed, mainly due to their mobility and residual time, and can interfere in the properties of the soil and the quality of crops (JIANG; FAN, 2008). Unlike organic contaminants, most metals are not degraded by microbes or chemicals (PARK et al., 2010), and require more complex technologies to be removed from the environment, which often makes the use of sewage sludge unfeasible in agriculture.

According to Rajj (1991), many of these metals are associated to environmental pollution or naturally occur in toxic levels for living beings. The ingestion of heavy metals can have toxic effects and induce the development of chronic or acute illnesses in animals and humans (GUPTA; GUPTA, 1998).

Although the chemical analysis detected many heavy metals in concentrations below the allowed by Brazilian laws, the cytotoxic, genotoxic, mutagenic effects observed in our study may be due to the presence of heavy metals, or a synergistic effect of all compounds present, including organic substances and others not measured in the analysis.

The cytotoxic effect of a compound can be determined by the decrease and/or increase of the mitotic index of exposed cells (FERNANDES et al., 2007). Thus, mitotic indices lower than that of the negative control can indicate the presence of agents with a toxic action capable of compromising the growth and development of exposed organisms. On the contrary, mitotic indices above those observed in the controls induce cell division, leading to uncontrolled cell proliferation and the development of tumors (HOSHINA, 2002; CARITÁ; MARIN-MORALES, 2008; LEME; MARIN-MORALES, 2009). Thus, the mitotic index is an important indicator of the action of a substance, and can be used to evaluate environmental pollution levels (SMAKA-KINCL et al., 1996).

The raw sludge sample collected in 2011 induced a proliferation of exposed meristematic cells compared to those of the negative control. This may be due to the presence of organic matter, macro and micronutrients in the sludge sample, since the negative control consisted of ultrapure water.

According to the literature, all chromosome alterations observed may be due to the action of some metals. Heavy metals are potentially mutagenic and are strongly associated with environmental pollution. Studies conducted with plants and animals in the laboratory have demonstrated that many metals can inhibit the formation of mitotic fuse, resulting in an abnormal distribution of chromosomes and also disturb the mitotic apparatus (FISKEJÖ, 1983; 1988; De FLORA et al., 1994; MINISSI; LOMBI, 1997; VOUSINAS et al., 1997; CARDOSO et al., 2002).

Among the alterations associated with genotoxicity observed in the present study, chromosomal adherence is a common signal of the toxic influence on the chromosomes, which may characterize an irreversible damage to the cell (FISKEJÖ; LEVAN, 1993; MARCANO; DEL CAMPO, 1995; MARCANO et al., 1999). C-metaphases, chromosome loss, chromosome laggards, and polyploidy are events that can derive from problems in cytoplasmic microtubules (VIDAKOVIĆ-CIFREK et al., 2002; FERNANDES et al., 2007). Polyploid cells exhibit a large chromosome imbalance, due to the increase in chromosome content. Thus, chromosomes tend to be more condensed (FISKEJÖ, 1985; FISKEJÖ, LEVAN, 1993), promoting chromatid and chromosomal adherence (FERNANDES et al., 2007). The formation of chromosome bridges, chromatid and chromosome loss are phenomena that can occur as a consequence of adherence (MARCANO et al., 2004). In this case, chromosomes remain united and when separated, chromosomes can be broken or loss (MARCANO et al., 1999), as well as result in aneuploidy and polyploidy (FISKEJÖ, 1985; MATSUMOTO; MARIN-MORALES, 2004; MATSUMOTO et al., 2006).

Nuclear buds are structures similar to micronuclei, but that remain attached to the main nucleus by a nucleoplasmic connection (SALVADORI et al., 2003). According to Fernandes et al. (2007), this alteration may be a result of polyploidization in which the excess material is discharged from the cell.

Cells with micronuclei and chromosome breaks are considered endpoints of mutagenic action, since even with alterations in the genetic material, the viability of these cells is not visibly compromised and the alteration may persist in the cell cycle (CARITÁ, 2010).

Chromosome breaks show the direct action of an agent on the DNA, inducing fragmentation. After cell division, these fragments can result in micronucleus that will not be incorporated to the main nucleus (FENECH, 2002).

The induction of chromosome aberrations and micronuclei by raw and solubilized sewage sludge samples in meristematic cells of *A. cepa* and micronucleus in tetrads of *Tradescantia* have also been observed by Maziviero (2011). In this study, raw samples as well

as solubilized ones obtained from a predominantly domestic sewage sludge from a WTP in São Paulo, Brazil induced damage to the genetic material of both test-organisms.

Christofoletti et al. (2012, 2013) also observed several chromosome aberrations and micronuclei in *A. cepa* cells exposed to biosolid from the same WTP used in the present study and bioprocessed by diplopods. In these studies, the authors detected the presence of metals in the samples and observed that the bioprocessing of the biosolid by diplopods reduced the genotoxicity and mutagenicity of the sample.

Since all samples, including the control soil, had some metals, which according to the chemical analyses, were below and/or within the quantities allowed by law, we infer that the aberrations observed may be due to their action.

Our findings demonstrated significant frequencies of micronuclei in F₁ region cells only for the combination of soil+sludge (SS) at t30, when compared to the negative control and/or control soil. These micronuclei might be associated to the high frequency of chromosome aberrations and micronuclei in meristematic cells of *A. cepa* exposed to this treatment. Cells of F₁ region are derived from meristematic cells during mitotic divisions (MA; XU, 1986). Thus, micronuclei observed in cells of F₁ region might have resulted from damage not repaired or repaired incorrectly, induced in parental cells (RIBEIRO, 2003). Our findings on the chromosome aberration and micronucleus tests in *A. cepa* indicate that the sewage sludge samples tested had an aneugenic as well as clastogenic action, resulting in chromosome aberrations, chromosome breaks, and micronuclei. Also, the bioprocessing of primary sewage sludge by diplopods was not efficient to significantly reduce the genotoxicity and mutagenicity of samples, as demonstrated by tests with *A. cepa*.

5. Conclusion

In this study, the chemical analysis detected the presence of different metals and organic compounds, potentially toxic and in low concentrations. Although the concentrations of metals were below the limits determined by law, damage to the genetic material of onion was observed. Tests with *A. cepa* associated to chemical analyses are important tools to evaluate environmental quality of samples. In the present study, the bioprocessing of sewage sludge samples by diplopods was demonstrated by the variation in macro and micronutrients and metals over time of exposure. However, it was not efficient to significantly reduce the genotoxic and mutagenic effects of the two samples examined.

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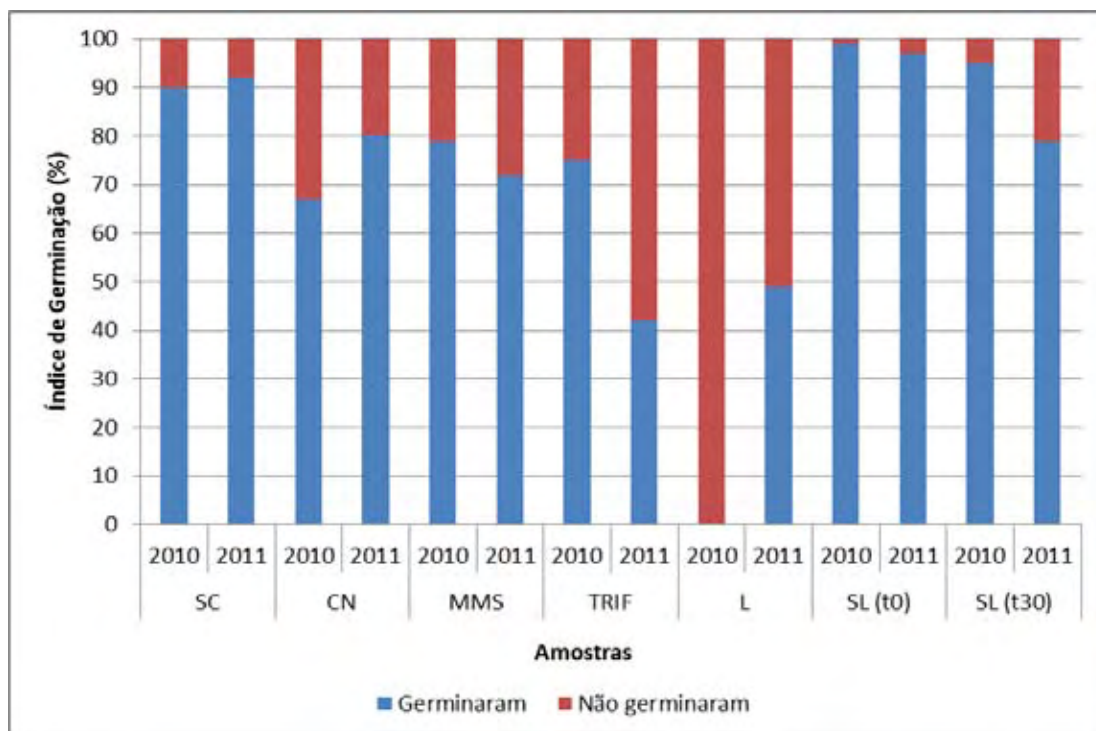


Figure 1 – Comparison of the germination indices of seeds (%) allowed to germinate in ultrapure water (negative control), control soil (environmental control), MMS and Trifluralin (positive controls), and raw sludge samples and combinations of residues, at time 0 (t0) and after 30 days (t30). **SC**: control soil; **CN**: (negative control; **MMS**: methyl methanesulfonate; **TRIF**: trifluralin; **L**: raw primary sewage sludge sample; **SL**: soil+sludge.

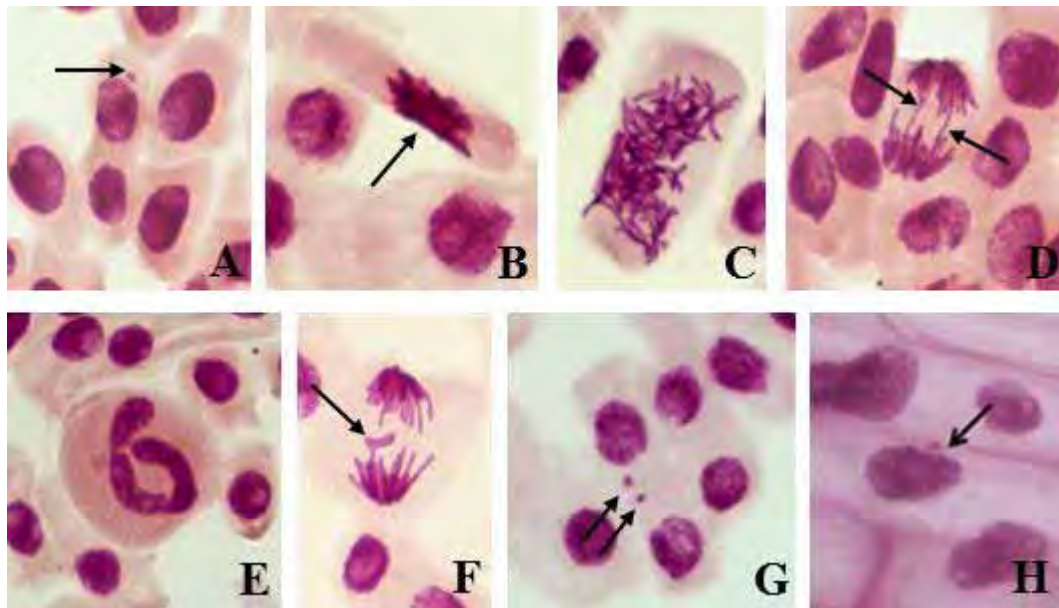


Figure 2 - Genotoxic and mutagenic alterations in *A. cepa* cells. **A.** Cell with nuclear bud (arrow); **B.** Metaphase with chromosomal adherence (arrow); **C.** Polyploid metaphase; **D.** Anaphase with chromosome bridge (arrow); **E.** Hypertrophied cell with disformed nucleus ; **F.** Anaphase with chromosome break (arrow); **G.** interphase cells with micronucleus (arrows); **H.** Cell of the F₁ region with micronucleus (arrow).

Table 1 – Results of the fertility of samples of control soil

Sample	pH	OM	P res	K	Ca	Mg	H+Al	SB	CEC	V	Ratios	
	CaCl ₂	g/dm ³	mg/dm ³				mmol/dm ³ TFSA			%	Ca/Mg	Mg/K
2010	6.20	18	3.0	0.8	2	1	88	3.9	91.9	4.2	2.0	1.25
2011	5.10	17	3.0	0.2	9	6	30	16.6	5.88	3.5	-	-

OM: organic matter; **CTC:** cation exchange capacity; **V:** base saturation; **SB:** sum of bases

Table 2- Physico-chemical and metals analysis of control soil samples and raw primary sewage sludge samples collected in 2010 and 2011

Parameter	2010 Collection			2011 Collection			Method	MaxPA (mg/kg)	MCA (mg/kg)
	CS (mg/kg)	L (mg/L)	CS (mg/kg)	L (mg/L)	CS (mg/kg)	L (mg/L)			
Arsenic	16.8	14.5	<LQ	<LQ	<LQ	<LQ	SM21 3120B	35	41
Barium	5.91	11.0	<LQ	<LQ	<LQ	7.73	SM21 3120B	300	1300
Cadmium	<LQ	<LQ	<0.16	<LQ	<LQ	<LQ	SM21 3120B	3	39
Total calcium	25.4	623	42.8	432	42.8	432	SM21 3120B	-	-
Organic carbon (g/kg)	12.6	3939	32.3	1345	32.3	1345	SSSA Cap40	-	-
Lead	49.3	1.42	42.7	<LQ	42.7	<LQ	SM21 3120B	180	300
Copper	37.2	10.6	76.5	4.65	76.5	4.65	SM21 3120B	200	1500
Electric conductivity (µs/cm)	115	2950	97.9	3700	97.9	3700	SM21 3120B	-	-
Cromium	31.2	6.48	108	4.07	108	4.07	SM21 3120B	150	1000
Total sulfur	151	373	123	6640	123	6640	SM21 3120B	-	-
Total phosphorus	182	707	317	385	317	385	SM21 3120B	-	-
Total magnesium	<LQ	75.4	<LQ	65.3	<LQ	65.3	SM21 3120B	-	-
Mercury	<LQ	<LQ	0.065	0.0016	0.065	0.0016	EPA 7470A	12	17
Molybdenum	3.64	0.83	9.60	<LQ	9.60	<LQ	SM21 3120B	50	50
Nickel	13.0	1.80	24.2	4.12	24.2	4.12	SM21 3120B	70	420
Nitrate (mg/Kg)	4.40	1.20	8.14	1.09	8.14	1.09	SM21 4500-NO ₃ E	-	-
Nitrite (mg/Kg)	0.06	0.02	<0.043	0.014	<0.043	0.014	SM21 4500-NO ₂ B	-	-
N Ammoniacal (mg/Kg)	31.8	1467	49.6	351	49.6	351	SM21 4500-NH ₃ E	-	-
N Kjeldahl (mg/Kg)	476	1597	922	705	922	705	SM21 4500-Norg B	-	-
pH	6.20	6.55	5.1	6.72	5.1	6.72	EPA 4095 C	-	-
Total Potassium	406	107	<LQ	84.9	<LQ	84.9	SM21 3120B	-	-
Selenium	<LQ	<LQ	52.1	<LQ	52.1	<LQ	SM21 3120B	-	100
Total Sodium	<LQ	41.8	<LQ	71.5	<LQ	71.5	SM21 3120B	-	-
Total Solids (g/g)	0.86	55149	0.93	42902	0.93	42902	SM21 2540B	-	-
Total Volatile Solids (g/g)	VI	27978	0.08	25767	0.08	25767	SM21 2540B	-	-
Solid content (g/g)	0.86	NE	0.93	NE	0.93	NE	SM21 2540B	-	-
Moisture (g/g)	0.14	NE	0.06	0.9571	0.14	0.9571	SM21 2540B	-	-
Zinc	23.2	40.5	96	14.4	96	14.4	SM21 3120B	450	2800

CS: control soil; L: primary sludge; LQ: limit of quantification; NE: data not evaluated; VI: Value inconsistent; MaxPA: values for agricultural exposure-Maximum Protection Area, for soil (mg/kg) and groundwater in the state of São Paulo, according to CETESB (195/2005-E); MCA: maximum concentration allowed in sewage sludge or derivatives, according to CONAMA (375/2006).

Table 3 – Analysis of polycyclic aromatic hydrocarbons in raw samples of control soil and primary sewage sludge collected in 2010 and 2011

Parameter	Samples/2010		Samples/2011		Method	Allowable concentration in soil (mg/kg)	
	CS	L	CS	L		CB	CO
	(µg/Kg)	(µg/Kg)	(µg/Kg)	(µg/Kg)			
Acenaphthene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Acenaphthylene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Anthracene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Benzo(a)anthracene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,025	0,025
Benzo(a)pyrene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,052	0,052
Benzo(b)fluoranthene (mg/Kg)	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,38	-
Benzo(g,h,i)perylene (mg/Kg)	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,57	-
Benzo(k) fluoranthene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,38	0,38
Chrysene (mg/Kg)	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	8,1	-
Dibenzo(a,h)anthracene (mg/Kg)	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,08	-
Phenanthrene	<LQ	28,8	<LQ	<LQ	EPA 8270 D	3,3	3,3
Fluoranthene	<LQ	16,8	<LQ	<LQ	EPA 8270 D	-	-
Fluorene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-
Indeno(1,2,3-cd)pyrene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	0,031	0,031
Naphthalene	<LQ	27,6	<LQ	<LQ	EPA 8270 D	0,12	0,12
Pyrene	<LQ	<LQ	<LQ	<LQ	EPA 8270 D	-	-

CS: control soil; **L:** raw primary sewage sludge; **LQ:** limit of quantification; **CB:** guiding values (prevention) for soils in São Paulo, according to Cetesb (195/2005-E); **CO:** maximum concentration in the soil according to CONAMA (375/2006).

Table 3 – Frequency (%) of the mitotic index (MI), chromosome aberration index (CAI) and mutagenicity index (MtI) in meristematic cells of *Allium cepa*, after exposure to ultrapure water (negative control), control soil, and MMS and trifluralin (positive controls), raw sludge, and combinations of control soil and sludge at time 0 (t0) and after 30 days (t30)

Samples	Collection	IM	IAC	IMt
NC	2010	13.66±1.37	1.2±0.44	0.2±0.44
	2011	10.68±1.62	2.8±2.04	1.4±1.14
CS	2010	14.21±0.90	2.0±0.27	0.6±0.54
	2011	12.02±1.15	4.6±0.54	2.0±2.0
MMS	2010	11.53±0.88* ¹	8.6±0.35* ¹	25.6±8.5* ¹
	2011	12.23±0.82*	8.0±3.0* ¹	28.4±8.01* ¹
TRIF	2010	9.47±0.54* ¹	51.4±8.79* ¹	12.2±3.42* ¹
	2011	9.26±0.99 ¹	66.6±29.9* ¹	26±6.51* ¹
L	2010	NE	NE	NE
	2011	12.32±0.93*	20±3.39* ¹	4.2±1.48* ¹
SL (t0)	2010	13.26±1.19	18±2.44* ¹	1.2±0.44*
	2011	11.53±2.02	19.2±4.43* ¹	1.4±0.54
SL (t30)	2010	13.3±1.04	10.2±1.48* ¹	1.2±0.83*
	2011	11.94±1.65	20.6±2.7* ¹	3.8±1.3* ¹

CS: control soil; **NC:** negative control; **MMS** and **TRIF:** positive controls; **L:** raw primary sewage sludge sample; **SL:** soil+sludge. **NE:** data not evaluated.

* statistically significant values compared to the negative control with the Mann-Whitney test, significance set at $p < 0.05$.

¹ statistically significant values compared to the control soil with the Mann-Whitney test, significance set at $p < 0.05$.

Table 4 – Frequency of chromosome aberrations observed in meristematic cells of *A. cepa*, after germination in ultrapure water (negative control), control soil, MMS, and trifluralin (positive controls), raw sludge, and combinations of control soil and primary sludge at time 0 (t0) and after 30 days (t30)

Samples	Collection	Nuclear bud	Adherence	Polyploidy	Chromosomal bridge
NC	2010	0±0	1.2±0.83	0±0	0±0
	2011	1.3±1.3	2±0.83	0.33±0.44	0±0
CS	2010	0.2±0.44	0.4±0.54	0.4±0.54	0±0
	2011	1.2±0.83	1.8±0.44	0±0	1.6±0.54
MMS	2010	3±1.22*	2.4±0.89 ¹	0±0	3.2±1.64
	2011	3±1.87*	1.8±0.48	0±0	2.6±1.94*
TRIF	2010	17.4±2.5* ¹	11.8±5.35* ¹	2.6±2.07	1.4±1.67
	2011	11.4±3.2* ¹	8.2±2.68* ¹	3.8±4.7* ¹	1.4±2.6
L	2010	NE	NE	NE	NE
	2011	3.2±0.83*	8.8±3.27* ¹	4±2.44* ¹	2.6±1.51*
SL (t0)	2010	3±1.22*	6.8±3.11*	3.6±1.3* ¹	3.2±1.6*
	2011	3.4±0.54*	7±1.73* ¹	5.6±3.12* ¹	3±1.41*
SL (t30)	2010	1.8±0.83*	4.8±0.83* ¹	0±0	3.2±1.3*
	2011	5.6±1.34* ¹	5.4±3.2* ¹	5.6±1.14* ¹	3±2.5*

CS: control soil; **NC:** negative control; **MMS** and **TRIF:** positive controls; **L:** raw primary sewage sludge sample; **SL:** soil+sludge. **NE:** data not evaluated.

* statistically significant values compared to the negative control with the Mann-Whitney test, significance set at p<0.05.

¹ statistically significant values compared to the control soil with the Mann-Whitney test, significance set at p<0.05.

Table 5 – Frequency of micronuclei in meristem (M) and in F₁ region cells of *A. cepa*, after germination in ultrapure water (negative control), control soil, MMS, and trifluralin (positive controls), raw sludge and to combinations of control soil and primary sludge, at time 0 (t0) and after 30 days (t30)

Samples	Collection	MN (M)	MN (F ₁)
NC	2010	0.2±0.44	0.2±0.44
	2011	1.4±1.14	0.8±0.44
CS	2010	0.6±0.54	0
	2011	1.8±1.78	0.8±0.8
MMS	2010	21.2±7.08* ¹	4.6±2.07* ¹
	2011	25.6±8.64* ¹	5.8±2.04* ¹
TRIF	2010	12.2±3.42* ¹	2.6±1.14* ¹
	2011	26±6.61* ¹	4.6±0.89* ¹
L	2010	NE	NE
	2011	2.6±1.51*	0
SL (t0)	2010	1.2±0.44*	0
	2011	1.4±0.54	1.2±1.09
SL (t30)	2010	1±0.7	0
	2011	3.4±1.14* ¹	1.6±0.54* ¹

CS: control soil; NC: negative control; MMS and TRIF: positive controls; L: raw primary sewage sludge sample; SL: soil+sludge. NE: data not evaluated.

* statistically significant values compared to the negative control with the Mann-Whitney test, significance set at p<0.05.

¹ statistically significant values compared to the control soil with the Mann-Whitney test, significance set at p<0.05.

6. Considerações Finais

Amostras ambientais complexas tais como lodo de esgoto primário, biossólido e vinhaça da cana-de-açúcar, precisam ser caracterizadas quimicamente para que se conheçam os diferentes elementos que as compõem. Para tanto, é necessário que se conheça sua origem (doméstica e/ou industrial), assim como o tratamento/processo empregado na sua obtenção.

Como as análises químicas determinam apenas as concentrações em que os elementos estão presentes em cada amostra, ensaios biológicos se tornam imprescindíveis para a avaliação da qualidade ambiental destas amostras, principalmente ao considerarmos que duas delas, biossólido e vinhaça, são frequentemente dispostas no solo e/ou em diferentes culturas.

Conforme abordado anteriormente, as amostras de biossólido e de vinhaça de cana-de-açúcar, têm sido frequentemente utilizadas na agricultura. Logo, o presente trabalho teve por objetivo simular a dose de aplicação destas em cultura de cana-de-açúcar, de acordo com a legislação brasileira vigente.

As análises químicas realizadas demonstraram que os diplópodos foram capazes de bioprocessar os elementos presentes nas amostras, visto as diferenças nas concentrações, principalmente de macro e micronutrientes e metais, após análise das amostras brutas, no momento da mistura destas amostras ao solo controle (designado por tempo 0) e após 30 dias de exposição aos diplópodos (t30). A aplicação de outros testes, tais como os respirométricos e a identificação dos microrganismos constituintes da flora intestinal destes animais, poderiam revelar se esses animais são capazes de atuar em processos de biorremediação, semelhantes ao processo de vermicompostagem, realizado pelas minhocas.

Os diplópodos representam grande importância ecológica. Embora o número de estudos com estes animais ainda seja escasso em comparação a outros invertebrados terrestres saprófagos, o seu uso como biondicador de contaminação de solos, por diferentes resíduos, têm sido demonstrado.

Diante disso, o presente trabalho objetivou investigar a sensibilidade de diplópodos como organismo-teste na avaliação de solos contaminados por lodo de esgoto primário, biossólido, vinhaça e pelas combinações destes resíduos. Tal espécie foi escolhida com base nos resultados obtidos na literatura, por ser uma espécie abundante, facilmente mantida em laboratório, com descrições de anatomia interna e externa bem estabelecidas, principalmente

do trato digestório. Logo, análises histológicas, histoquímicas e ultra-estruturais foram realizadas no intestino médio deste animal, uma vez que esta é a porção onde ocorre a digestão (assimilação) dos compostos. O intestino médio representa ainda importante papel nos processos de detoxicação e excreção de xenobióticos.

As respostas obtidas pelas análises realizadas no intestino médio dos animais expostos representam uma tentativa de defesa, com o intuito de neutralizar e/ou eliminar os resíduos tóxicos ingeridos.

Primeiramente foi observado um aumento nas taxas de renovação epitelial. Tal resposta fisiológica permite a remoção de tecidos alterados, visando a manutenção das funções do órgão em estudo. Posteriormente, observou-se um acúmulo de grânulos intracitoplasmáticos nas células hepáticas. As toxinas presentes nos diferentes resíduos são acumuladas em formas insolúveis nestes grânulos, para posterior excreção. Entretanto, como deve haver um limite para a absorção/assimilação de toxinas, quando este é atingido e não processado, outros danos histológicos e/ou histoquímicos podem ser induzidos. Também foi observado um espessamento do bordo em escova. Tal alteração provavelmente ocorreu com o intuito de impedir a absorção dos contaminantes presentes no solo, pelo trato digestório do animal.

Após 90 dias de exposição aos diferentes resíduos, uma grande quantidade de hemócitos foi observada por entre as células hepáticas, principalmente para a amostra de solo misturado à vinhaça da cana-de-açúcar. Tal alteração foi relacionada com o desencadeamento de processos inflamatórios, com o intuito de destruir as toxinas e reabsorver o tecido injuriado. No entanto, o processo inflamatório foi tão severo, que foram observadas ainda a presença de núcleos heteropicnóticos e degradação citoplasmática das células hepáticas, evidenciando, portanto, a citotoxicidade de tal resíduo às células hepáticas destes animais. A picnose da cromatina, a basofilia do citoplasma e perda dos limites celulares são características de células em processo de morte celular.

Portanto, observou-se um agravamento das injúrias ao intestino médio desses animais, de acordo com o aumento no tempo de exposição dos mesmos às diferentes combinações de resíduos. Logo, cabe ressaltar que em condições de campo, esses animais estariam expostos cronicamente ao lodo, bio sólido e vinhaça, dada a frequente disposição destes no solo. Nesse sentido, a ativação de processos visando a sobrevivência dos diplópodos, pode afetar outras funções vitais ou ainda, interferir negativamente na decomposição da matéria orgânica, prejudicando assim, o ecossistema como um todo.

Pelos resultados obtidos no uso de diplópodos como biondicadores da qualidade de solos e do seu intestino médio como biomarcador, conclui-se que a espécie *R. padbergi* é adequada para estudos ecotoxicológicos. Logo, ensaios realizados com essa espécie podem revelar o nível de contaminação dos solos e fornecer subsídios para a criação de programas voltados à preservação deste ecossistema.

Em contrapartida, foram avaliados os potenciais tóxico, citotóxico, genotóxico e mutagênico destas mesmas amostras por meio do teste de aberrações cromossômicas e micronúcleos em células meristemáticas e, micronúcleos em células da região F₁, em *Allium cepa* (cebola). Os efeitos dos resíduos associados, antes e após o bioprocessamento das amostras pelos diplópodos, também foram monitorados por meio de testes com *A. cepa*.

A toxicidade foi verificada por meio da inibição da germinação de sementes de cebola. As amostras brutas de lodo de esgoto e vinhaça, coletadas em 2010, e a de vinhaça coletada em 2011, foram tóxicas, pois inibiram a germinação das sementes.

A citotoxicidade foi mensurada apenas pelo aumento e/ou diminuição do índice mitótico das células meristemáticas do vegetal empregado, uma vez que não foram observadas células em processo de morte celular. Na coleta de 2010, apenas a amostra bruta de biossólido foi citotóxica. Em 2011, foram citotóxicas as amostras brutas de lodo de esgoto primário e biossólido e as amostras de SL, SLV e SBV, do tempo 0.

No decorrer deste estudo, brotos nucleares, aderências, pontes cromossômicas e poliploidia foram os principais tipos de aberrações cromossômicas observadas em *A. cepa*, evidenciando o potencial genotóxico das amostras. Tais alterações possivelmente são decorrentes da presença de diferentes metais nas amostras estudadas. Tais efeitos foram observados para as amostras brutas de biossólido (2010 e 2011) e lodo primário (2011) e para todas as combinações, dos tempos 0 e 30 dias, dos dois bioensaios realizados.

O potencial mutagênico foi evidenciado pela presença de quebras cromossômicas e micronúcleos em células meristemáticas e, células portadoras de micronúcleos na região F₁. Apresentaram tal efeito a amostra bruta de biossólido (2010 e 2011) e a de lodo de esgoto primário (2011). No tempo 0 (t₀) do primeiro bioensaio, apenas as amostras de SV e SBV; no segundo bioensaio, estas mesmas amostras continuaram apresentando potencial mutagênico, assim como a de SLV. Após 30 dias de exposição, para o primeiro bioensaio, nenhuma amostra apresentou tal efeito; no segundo, houve uma redução da mutagenicidade apenas para SV. Talvez tais resultados sejam decorrentes da presença de metais em maior concentração das amostras coletadas em 2011, o que pode ter dificultado o bioprocessamento de tais resíduos pelos diplópodos.

As análises realizadas mostraram que *A. cepa* constitui um excelente organismo teste para avaliação do potencial tóxico, citotóxico, genotóxico e mutagênico de misturas ambientais complexas. Pelos resultados obtidos, conclui-se também que o teste de aberrações cromossômicas e micronúcleos em células meristemáticas e micronúcleos em células da região F₁, aplicados a essa espécie vegetal pode ser rotineiramente aplicado por agências regulatórias, para avaliar a qualidade ambiental de diferentes amostras.

Portanto, os resultados do presente trabalho alertam que amostras ambientais complexas, como as aqui estudadas, com possível destinação agrícola, apresentam perigo do ponto de vista ecotoxicológico, mesmo apresentando concentrações de contaminantes abaixo dos padrões exigidos pela legislação brasileira. O bioprocessamento de tais amostras por diplópodos pode ser uma alternativa viável antes da disposição destes na agricultura; tal ação foi evidenciada pela redução dos efeitos genotóxicos e mutagênicos para as amostras de 2010, as quais apresentaram menores concentrações de metais, embora tais resultados não tenham se repetido nas análises de 2011. Logo, outros estudos quanto à dosagem de aplicação destes, seus efeitos em outros organismos e nos diferentes compartimentos do solo, devem ser realizados com o intuito de que estes possam ser melhor empregados no condicionamento sustentável de solos agricultáveis, subsidiando a tomada de decisão em medidas de controle ambiental.

7. CONCLUSÃO

A partir dos resultados apresentados, conclui-se que:

- A caracterização química das amostras, principalmente a de metais, foi importante para uma melhor compreensão das reações dos dois organismos-teste às diferentes amostras estudadas.
- As concentrações dos diferentes metais presentes nas diferentes amostras, mesmo possuindo valores abaixo do permitido pelo CONAMA 357/2006, para aplicação de lodo de esgoto e biossólido em solos agrícolas e, pela P2.341 da CETESB, para o uso da vinhaça, podem ter sido as responsáveis pelos danos induzidos aos dois organismos-teste.
- A espécie *A. cepa* foi eficiente na avaliação dos potenciais tóxico, citotóxico, genotóxico e mutagênico das amostras brutas de lodo de esgoto primário, biossólido, vinhaça de cana-de-açúcar e combinações, antes e após o bioprocessamento destas amostras pelos diplópodos.
- A espécie *R. padbergi* mostrou-se sensível para avaliar a qualidade de solos e pode ser utilizada no bioprocessamento de amostras ambientais complexas.
- As alterações observadas no intestino médio, evidenciadas pelas análises histológicas e ultra-estruturais, podem ser utilizadas como biomarcadores na avaliação de solos contaminados com misturas complexas, como o lodo de esgoto primário, biossólido e vinhaça da cana-de-açúcar.
- Outros estudos quanto à dosagem de aplicação destes, seus efeitos em outros organismos e nos diferentes compartimentos do solo, devem ser realizados com o intuito de que estes possam ser melhor empregados como fertilizantes, ou ainda, no condicionamento sustentável de solos agricultáveis, subsidiando a tomada de decisão em medidas de controle ambiental.
- O uso da vinhaça de cana-de-açúcar e de lodo de esgoto brutos na agricultura, como corretivos de solos degradados pode caracterizar um sério risco ao ambiente, pois os bioensaios com estas amostras brutas mostraram ser tóxicas para os organismos testes utilizados.

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ANEXOS

Research Article

Biosolid Soil Application: Toxicity Tests under Laboratory Conditions

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A large volume of generated sewage sludge makes its disposal a problem. The usage of sludge in agriculture is highlighted by a number of advantages. However, heavy metals and other toxic compounds may exercise harmful effects to soil organisms. This study evaluated the possible toxic effects of a biosolid sample, under laboratory conditions, for 30 days, using diplopods *Rhinocricus padbergi* and plants *Allium cepa* (onion) as test organisms. The data obtained demonstrated that the biosolid raw sample had genotoxic potential for *Allium cepa* root tip cells. In the diplopods exposed to biosolid sample, epithelium disorganization in the midgut and a reduction of the volume of the hepatic cells were observed after 7 days of exposure. After 30 days, the animals still showed a reduction of the volume of the hepatic cells, but in minor intensity. *Allium cepa* analysis showed genotoxicity, but this effect was reduced after 30 days of bioprocessing by diplopods. This study was important to know the effects as well as to determine how this waste could be applied concerning the soil living organisms and plants.

1. Introduction

In sewage treatment plants (STP), after the sewage had been treated, a sludge rich in organic matter and nutrients is generated as a waste, known as sewage sludge. The composition of this sludge is very variable since it depends on the source of the sewage treatment process and the seasonality [1]. Generally, the sewage sludge presents around 40% to 60% of organic matter, 4% nitrogen, 2% phosphorus, and other macro- and micronutrients, besides potentially toxic elements [2].

The generated sewage sludge still can go through processes in order to increase the solids and reduce the number of pathogenic organisms, generating a residue called biosolid, which is considered most innocuous than the sewage sludge itself [3]. Good quality fertilizers can be generated with the sludge stabilization, reducing its volume through the use of “sludge thickeners drying beds,” filter presses, band presser, vacuum filters, and centrifugation [4]. According to Lambais and Do Carmo [5], chemical composition of the sludge depends on the origin of the wastewater. This way, the material is variable, but generally it

is a compound rich in organic matter and essential nutrients for plants and microorganisms.

Currently, sewage treatment plants in different Brazilian cities are facing the problem of sludge disposal. The alternatives to the sewage sludge usual fate are landfill disposal, reuse in industry (light-weight aggregate production, bricks and ceramics manufacturing, and cement production), incineration, conversion into fuel oil, ocean disposal, recovery degraded soils, and agricultural use [2, 6].

In Brazil, there is a preference for the use of sludge in agriculture, since there is a considerable land availability and the costs would be relatively low. However, this practice is still incipient, so that the application is made without an adequate management [4]. According to Melo et al. [7], when incorporated into the soil, sewage sludge provides changes in physical properties such as density, aggregates size, and water retention capacity; on chemical properties such as pH, electrical conductivity, CEC, and increased levels of P and N and biological properties, usually by increasing soil microbial activity.

However, the main limitation observed during the evaluation of possible utilization of sewage sludge in agriculture

refers to the presence of metals and other persistent pollutants [8], which may be toxic to plants [9], microorganisms [10], and soil invertebrates [11]. Although other work related the phytoavailability of these metals to a variety of cultures, few studies relate the sewage sludge to genotoxic and mutagenic potential. The application of metal-rich biosolids in clay and sandy soils, compared with biosolids with low-metal concentration, causes a transient soil microbial community increase in mass and activity, with reduced carbon immobilization [5]. Many field studies, based on biosolids agronomic doses, reported soil biota stimulation, probably due to the addition of organic matter, which causes an increase in fertility. The application of this kind of compound, however, has shown inhibitory effect on soil invertebrates [12].

Thus, the aim of this study was to investigate the effects of a biosolid sample according to Brazilian standards for its application on soils, under laboratory conditions, using *Allium cepa* (plant) and *Rhinocricus padbergi* (terrestrial invertebrate) as test organisms.

2. Materials and Methods

2.1. *Rhinocricus padbergi*. Adult specimens of *R. padbergi* with a mean size of 5.0 cm were collected at the campus of the São Paulo State University (UNESP), Rio Claro, in order to avoid intraspecific differences related to either diplopod size or age. After collection, the specimens were maintained in the laboratory for 3 weeks for acclimation, in a terrarium containing soil, tubercles, and decomposing pieces of tree trunks from the capture area. The experimental temperature was $21 \pm 2^\circ\text{C}$ and the photoperiod was a 12 : 12-h light/dark cycle.

2.2. *Allium cepa*. All assays were carried out with only one kind of seeds of *A. cepa* (variety Baia Piriforme) to avoid different responses in the several stages of the process.

2.3. Control Soil. The control soil was obtained from the site where diplopods were collected at a depth of 0–20 cm, in the UNESP Campus of Rio Claro, SP. Soil samples were homogenized, dried at room temperature, and sieved with 4 mm mesh.

2.4. Biosolid Sample. The wastewater treatment plant where the biosolid was collected occupies an area of 20 hectares. The facility serves approximately 80% of the 318.785 inhabitants [13] of a city in São Paulo state where sewage is treated by conventional activated sludge process. In October 1999, the plant received the license of Producer of Agricultural Amendments, by the Ministry of Agriculture. The product produced in the facility is a biosolid classified as soil conditioner. The brand name is Sabesfértil (SP-09599 00001-0). Biosolid samples were collected and stored in plastic boxes wrapped with dark plastic bags and maintained in a cold room (4°C), until use.

2.5. Chemical Analysis of Samples. The concentration of trace elements (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se, and Zn) and the 16 priority organic compounds (PAHs) in the biosolid

and control soil was determined followed the Standard Methods for the Examination of Water and Wastewater 21th Edition 2005 (SM21) and USEPA. The characterization of samples was measured by TASQA Laboratory (Paulínia, São Paulo, Brazil). Analyses of trace elements were performed by inductively coupled plasma emission spectrometry (ICP-AES). The PAHs analyses were performed by atomic absorption spectrometry. Chemical and physicochemical analyses, as well as a characterization of control soil sample based on macro- and micronutrients (N, Ca, Mg, P, K, S, Fe, Mn, Cu, and Zn), C/N ratio, organic matter, cation exchange capacity (CEC), and base-saturation percentage, were carried out by the Instituto Campineiro de Análise de Solo e Adubo (ICASA), Campinas, São Paulo, Brazil.

2.6. Calculating Biosolid Quantities for Application

2.6.1. Application of Sewage Sludge. According to the law 375/2006 of the Environmental National Council (Conselho Nacional do Meio Ambiente, CONAMA) [14], the maximum annual application of sewage sludge and derivatives in tons per hectare shall not exceed the quotient between the quantity of nitrogen recommended for the crop (in kg/ha), following the official recommendation for São Paulo State and the nitrogen content available in the sewage sludge or derivatives (in kg/t), calculated as N recommended (kg/ha)/N available (kg/t).

To determine the nitrogen available in the sewage sludge and/or biosolid, mineralization fractions were calculated. According to CONAMA [14], this fraction represents 40% of undigested and 20% of digested sewage sludge.

2.6.2. Preparation of Soil and Residue Sample for the Bioassays with *R. padbergi*. Two glass terraria with capacity for 22.5 L were filled with 5 Kg of control soil each. After physicochemical analysis of soil samples and biosolid, the following bioassays were set up with control soil (CS) and soil + biosolid (SB):

- (1) CS: 5 Kg of control soil;
- (2) SB: 5 Kg of control soil + 234.4 g of biosolid.

Twenty specimens of *R. padbergi* were then placed in each terrarium, where they remained for 30 days to assess the toxicity of contaminants present in biosolid. The animals were monitored for 90 days. Six animals per bioassay were dissected for histological analyses, three animals on the seventh day, and three diplopods in 30th day of exposure.

2.6.3. Histology of the Midgut. The diplopods were anesthetized with chloroform, placed in Petri dishes containing isotonic salt solution, and dissected under the dissecting scope. The midgut was removed and fixed in paraformaldehyde 4%. Following that, the organ was dehydrated in increasing concentrations of ethanol (70%, 80%, 90%, and 95%), embedded in resin (Leica historesin), and kept in the refrigerator for 24 h. Later, the material was transferred to plastic moulds containing inclusion resin. After polymerization, $6\ \mu\text{m}$ slices were obtained with the help of a Leica

RM2245 microtome. For histological analyses, sections were stained with hematoxylin and eosin.

2.7. Germination of *A. cepa* Seeds in Residue Samples at Time 0 (t_0) and after Exposure to Diplopods after 30 Days (t_{30}). Approximately 100 seeds of *A. cepa* were allowed to germinate at 22°C in Petri dishes containing raw biosolid sample (B) and soil from each terrarium: CS and SB samples were collected and placed in Petri dishes for the germination of *A. cepa* seeds, at time 0 (t_0). Positive controls were made with the aneugenic herbicide trifluralin (TRIF) at the concentration of 0.019 ppm [15] and methyl methanesulfonate (MMS), a clastogenic agent at the concentration of 10 mg/mL [16]. Negative control (NC) consisted of seeds allowed to germinate in ultrapure water and the environmental control (CS) consisted of seeds allowed to germinate in the control soil.

After 30 days of exposure by diplopods, soil samples from each terrarium were collected for the tests with onion seeds.

2.7.1. Preparation of Slides of *A. cepa*. After germinating and reaching 2 cm in length, root tips were collected and fixed with Carnoy (3:1 ethanol/acetic acid). Samples were then stained with the Feulgen reaction [17], with acid hydrolysis for 11 minutes. Root tips were sectioned to remove the meristem and region F_1 . To intensify the staining and spread cells, one drop of 2% acetic carmine was added. All samples were lightly pressed between slide and coverslip. Coverslips were removed with liquid nitrogen and slides were mounted with Enthelan. The material was analyzed under light microscope, at magnification of 400x.

2.7.2. Evaluation of the Cytotoxic, Genotoxic, and Mutagenic Effects on Meristematic Cells of *A. cepa*. A total of 5000 cells were examined for each treatment at t_0 and t_{30} and for the negative and positive controls. Cytotoxicity was assessed based on morphological alterations indicating cell death, and the mitotic index (MI) calculated as $MI = (\text{number of dividing cells} / \text{total number of observed cells}) \times 100$. The cells in death process present a vacuolated cytoplasm, which is outcome of the cytoplasmic organelles digestion by lysosomal enzymes [18]. They can still present enhanced cytoplasmic volume, which can lead to a rupture of the plasmatic membrane exposing the cell content to the outer media [19].

Genotoxicity was evaluated based on the number of cells with chromosome aberration (CA). For the CA analyses, several aberrations within different cell divisions (metaphase, anaphase, and telophase) were considered such as C-metaphase, chromosomal adherence, multipolar anaphase and telophase, and chromosome bridge and loss [20]. The frequency of CA was calculated as $CAI = (\text{number of cells with chromosome aberrations} / \text{total number of observed cells}) \times 100$. Mutagenicity index (IMt) was determined based on the occurrence of cells with micronuclei (MN) and chromosome breaks, calculated as $IMt = (\text{total number of cells with MN and breaks} / \text{total number of observed cells}) \times 100$. The results obtained in all treatments at the different periods were compared with the negative control and soil control at

their corresponding times with the Mann-Whitney test, with significance set at 0.05.

2.7.3. Evaluation of Micronuclei in Cells of the F_1 Region of *A. cepa*. The damage to meristematic cells was assessed based on the number of micronuclei in cells of the F_1 region, which is composed by differentiated cells and is located about 1 mm above the meristematic region [21]. A total of 5000 cells were examined per treatment. The results obtained for all samples were compared with the negative control and soil control at their corresponding times with the Mann-Whitney test, with significance set at 0.05.

3. Results

3.1. Chemical Characterization of Samples. To follow a Brazilian standard for application of biosolids on soil, data regarding the agronomic potential and fertility of control soil became necessary. The values obtained are presented in Table 1. The results obtained by physical-chemical and trace elements analysis of control soil and biosolid sample are presented in Table 2. The concentrations of arsenic and copper found in the control soil were above the limits determined by CETESB-195/2005-E [22], but below the limits for intervention in agricultural areas. Levels of barium, lead, copper, chromium, molybdenum, nickel, and zinc found in the biosolid were high, but below the maximum level allowed by CONAMA (Table 2). None of the 16 PAHs, priority by EPA, were detected by analyses.

3.2. Histology of the Midgut of Diplopods. Animals from control soil presented the midgut (Figure 1(a)) as the histological pattern described for the species [23], being, therefore constituted by a pseudostratified epithelium with brush border (arrow head in Figure 1(a)). The epithelium showed principal cells with nuclei of round to oval morphology, located in middle apical region and regenerative cells in the basal portion; the epithelium is followed by a muscular layer and hepatic cells layer covered by an external membrane. The hepatic cells had an irregular morphology, spherical nucleus, and cytoplasm with cytoplasmic granules of varied content. Among the hepatic cells, some hemocytes were observed, generally isolated.

The group exposed for one week to SB showed epithelium disorganization (arrow in Figure 1(b)) with disruption in several places, indicating an epithelium renewal. The hepatic cells layer showed disorganization with volume reduction in some cells (Figure 1(c)).

After 30th day of exposure, animals from SB sample showed the minor disorganization of the hepatic cells layer when compared to the midgut of the animals exposed for 7 days (Figure 1(d)).

3.3. Cytotoxic, Genotoxic, and Mutagenic Effects on *A. cepa*. The mitotic index of cells examined for B, SB, TRIF, MMS, CS, and NC at 0 and after 30 days of exposure to diplopods is presented in Table 3. Seeds exposed to B and SB samples presented the highest mitotic index in both periods of exposure. However, no sample was statistically significant

TABLE 1: Fertility parameters of the control soil.

Sample	pH	g/dm ³	mg/dm ³	mmol/dm ³ TFSA					%	Ratio		
	CaCl ₂	OM	P res	K	Ca	Mg	H + Al	SB	CEC	V	Ca/Mg	Mg/K
Soil	6.20	18	3.0	0.8	2	1	88	3.9	91.9	4.2	2.0	1.25

OM: organic matter; CEC: cation-exchange capacity; V: base saturation.

TABLE 2: Physicochemical and metal analysis of the control soil and biosolid sample.

Parameter	Samples		Method	G (mg/kg)	MCA (mg/kg)
	CS (mg/kg)	B (mg/kg)			
Arsenic	16.8	<LQ	SM21 3120B	3.5	41
Barium	5.91	158	SM21 3120B	75	1300
Cadmium	<LQ	<LQ	SM21 3120B	<0.5	39
Total calcium	25.4	3939	SM21 3120B	—	—
Organic carbon (g/kg)	12.6	279	SSSA Cap40	—	—
Lead	49.3	174	SM21 3120B	17	300
Copper	37.2	276	SM21 3120B	35	1500
Electric conductivity (μ s/cm)	115	5389	SM21 3120B	—	—
Chromium	31.2	224	SM21 3120B	40	1000
Total sulfur	151	11864	SM21 3120B	—	—
Total phosphorus	182	17027	SM21 3120B	—	—
Total magnesium	<LQ	358	SM21 3120B	—	—
Mercury	<LQ	1.08	EPA 7470A	0.05	17
Molybdenum	3.64	9.55	SM21 3120B	<4	50
Nickel	13.0	82.3	SM21 3120B	13	420
Nitrate (mg/Kg)	4.40	6.79	SM21 4500-NO ⁻³ E	—	—
Nitrite (mg/Kg)	0.06	1.39	SM21 4500-NO ⁻² B	—	—
Ammoniacal nitrogen (mg/kg)	31.8	167	SM21 4500-NH ₃ E	—	—
Kjeldahl nitrogen (mg/Kg)	476	21620	SM21 4500-Norg B	—	—
pH	6.20	8.01	EPA 4095 C	—	—
Total potassium	406	2152	SM21 3120B	—	—
Selenium	<LQ	<LQ	SM21 3120B	0.25	100
Total sodium	<LQ	<LQ	SM21 3120B	—	—
Solid content	0.86	0.24	SM21 2540B	—	—
Moisture (g/g)	0.14	0.76	SM21 2540B	—	—
Zinc	23.2	825	SM21 3120B	60	2800

CS: control soil; B: biosolid; LQ: limits of quantification; IV: inconsistent value; G: guidelines of quality for soil (mg/kg) and groundwater in São Paulo State, according to CETESB (195/2005-E); MCA: maximum concentration allowed in sewage sludge or derivative product, according to CONAMA (375/2006).

TABLE 3: Mean and standard deviation of the mitotic (MI), the chromosome aberration (CA), and mutagenicity indexes (IMt) in meristematic cells of *Allium cepa* after exposure to control soil, negative and positive controls, and biosolid samples.

Samples	MI		ICA		IMt		
	t_0	t_{30}	t_0	t_{30}	t_0	t_{30}	
Controls	NC	13.66 \pm 1.37	11.2 \pm 1.78	1.2 \pm 0.83	2 \pm 1	0.2 \pm 0.44	0.6 \pm 0.54
	CS	14.21 \pm 0.90	9.48 \pm 1.07 ²	2 \pm 0.70	1.6 \pm 1.14	0.6 \pm 0.54	0
	MMS	11.53 \pm 0.88	5.73 \pm 1.15	8.6 \pm 3.04* ¹	14.2 \pm 2.94* ¹	25.6 \pm 8.5* ¹	24.8 \pm 7.29* ¹
	TRIF	9.47 \pm 0.54	9.31 \pm 1.35	51.4 \pm 8.79* ¹	23.6 \pm 4.82* ¹	12.2 \pm 3.42* ¹	3.6 \pm 0.89* ¹
Raw	B	15.36 \pm 0.99	NA	2.15 \pm 1.12	NA	0.16 \pm 0.18	NA
Combination	SB	15.07 \pm 0.56	15.03 \pm 0.79	21 \pm 5.83* ¹	10.6 \pm 3.33* ^{1,2}	1.2 \pm 1.3	0.4 \pm 0.54

NC: negative control; CS: control soil; MMS: positive control; TRIF: positive control; B: raw biosolid sample; SB: soil + biosolid.

t_0 : time of mixing and t_{30} : after 30 days of exposure to the diploids.

*Statistically significant values when compared to the negative control, by Mann-Whitney test, $P < 0.05$.

¹Statistically significant values when compared to the control soil, by Mann-Whitney test, $P < 0.01$.

²Statistically significant values when compared to the same treatments at 0 and 30 days.

NA: not available.

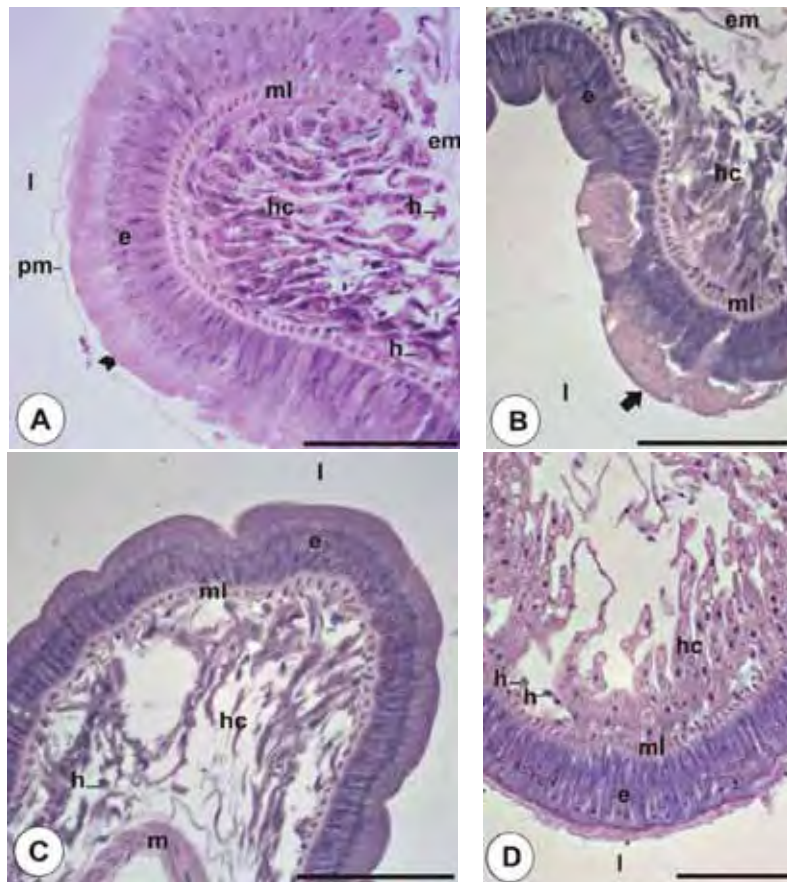


FIGURE 1: Micrographs of the *R. padbergi* midgut stained with HE. (A) Animals exposed for 7 days to the control soil; (B, C) animals exposed for 7 days to soil + biosolid; (D) animals exposed for 30 days to soil + biosolid. e: epithelium; em: external membrane; h: hemocyte; hc: hepatic cells; l: lumen; m: muscle fibers; ml: muscular layer; pm: peritrophic membrane; arrow head in (A) brush border; arrow in (B) epithelium renewal. Scale bars = 100 μm .

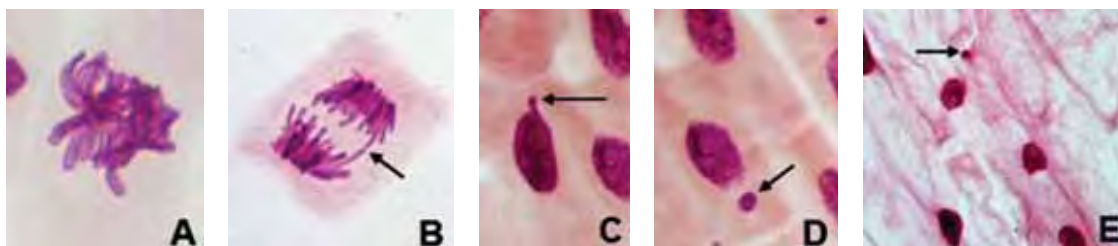


FIGURE 2: Chromosomal aberrations in *Allium cepa* exposed to raw biosolid (B) and control soil + biosolid (SB); (A) chromosomal adherence; (B) anaphase with polyploidy and chromosome bridge (arrow); (C) nuclear bud (arrow); (D) meristematic cell carrying micronuclei (arrow); (E) micronuclei in cells of the F_1 region (arrow).

when compared to the negative control. In comparison to the values obtained between the CS and B samples there was a significant difference. There were no cells in the cell death process for all samples.

B and SB samples induced genotoxic effects (Table 3), at t_0 and t_{30} ($P < 0.05$), characterized by chromosome aberrations, compared to the negative control and/or control soil. However, the genotoxic effect was significantly reduced after the bioprocessing by diplopods in the SB group. The most frequently observed alterations in the present study were cells

in metaphase with chromosomal adherence (Figure 2(a)), polyploidy (Figure 2(b)), anaphase with chromosomal bridge (Figure 2(b)), and nuclear bud (Figure 2(c)).

Meristematic cells with micronuclei (Figure 2(d)) and chromosome breaks were examined to assess the mutagenicity. Statistical analysis revealed no mutagenic effect of the samples examined at both times of exposure compared to the negative control and/or control soil (Table 3). Although micronuclei has been observed in meristematic and F_1 region (Figure 2(e)), the values were not statistically significant

TABLE 4: Mean and standard deviation for micronuclei in cells of the meristematic region (M) and F₁ of *Allium cepa* after exposure to control soil, negative and positive controls, and biosolid samples.

Samples		t_0		t_{30}	
		M	F ₁	M	F ₁
Controls	NC	0.2 ± 0.44	0.2 ± 0.44	0.6 ± 0.54	0.2 ± 0.44
	CS	0.6 ± 0.54	0	0	0.2 ± 0.44
	MMS	21.2 ± 7.08* ¹	4.6 ± 2.07* ¹	20.6 ± 5.12* ¹	4.6 ± 0.89* ¹
	TRIF	12.2 ± 3.42* ¹	2.6 ± 1.14*	3.6 ± 0.89* ¹	2.6 ± 0.89* ¹
Raw	B	0.14 ± 0.14	0.05 ± 0.04	NA	NA
Combination	SB	1 ± 1	0.6 ± 0.54	0.4 ± 0.54	0

NC: negative control; CS: control soil; MMS: positive control; TRIF: positive control; B: raw biosolid sample; SB: soil + biosolid.

t_0 : time of mixing and t_{30} : after 30 days of exposure to the diplopods.

*Statistically significant values when compared to the negative control, by Mann-Whitney test, $P < 0.05$.

¹Statistically significant values when compared to the control soil, by Mann-Whitney test, $P < 0.01$.

²Statistically significant values when compared to the same treatments at 0 and 30 days.

NA: not available.

(Table 4) and a decrease for these values was observed after 30 days of exposure to diplopods.

4. Discussion

Researches about sewage sludge disposal in soil are focused on its effects on soil fertility, plant development, and contamination by heavy metals and organic compounds [24]. However, few studies have been conducted to evaluate the toxic, genotoxic, and mutagenic potentials of the sewage sludge disposal on exposed plants and animals.

Chemical analysis of biosolid sample showed the presence of trace elements. According to the literature, these elements tend to induce genotoxic and/or mutagenic effects on plants [25, 26] and tend to concentrate in the terrestrial invertebrates tissues because their rate of absorption frequently surpasses their rate of elimination [27].

Several authors have used higher plants to diagnose and monitor the action of chemicals and environmental pollution. Among these, *Allium cepa* (onion) has been used in determination of cytotoxic, genotoxic, and mutagenic effects of substances [15, 28] and complex environmental samples [29–36].

In this study, *A. cepa* assays were carried out to assess abnormalities in dividing cells and to estimate the potential of B and SB samples to induct chromosome aberrations. The mitotic index of B was statistically significant compared to control soil. In our study, the mitosis stimulation observed in B and SB treatments may be due to the phosphorus and nitrogen presence and abundant elements in domestic sewage [37, 38].

Chromosomal aberrations are recognized as important consequences of the genotoxic environmental chemicals actions [39], to which many organisms, including humans, are exposed. Epidemiological studies have linked high chromosomal aberrations frequencies at significant cancer developing risk [40]. For this reason, numerous biological tests for chromosomal aberrations evaluation have been developed, in order to ensure the environmental quality [15, 31].

The most common aberrations observed in this study were metaphase with chromosomal adherence, polyploidy anaphase, and anaphase with chromosomal bridge and nuclear bud. According to the literature, all aberrations may be caused by the action of some trace elements/metals. Some metals are potentially genotoxic/mutagenic and are strongly related to environmental pollution. Several studies with plants have shown that the genotoxic effects of metals can cause changes in chromosome structure, chromosome number, and also the disturbances in the mitotic apparatus [25, 26]; they have the ability to inhibit mitotic spindle formation, leading to an abnormal chromosomes distribution and polyploidy [25, 41–43].

In this study was observed the presence of some elements such as barium, lead, copper, chromium, mercury, molybdenum, nickel, and zinc. Although their concentrations are within the standards established by CONAMA [14], according to De Godoy and Fontanetti [44], these standards do not take into account aspects such as the possible interaction of toxic metals and plants, as well as the lack of information regarding the influence of sludge on the soil fauna, including animals that promote humification, aeration, and enrichment of the same.

Like earthworms and collembola [8, 12, 45, 46], the diplopods are considered excellent test organisms for studying the organic amendment effects in the soil ecosystem due to their direct exposure and their sensibility to pollutants; therefore, the diplopods have been used as bioindicators of soil pollution and ecotoxicological assessment [27, 44, 47, 48].

The stimulation of soil biota revealed in some field studies using agronomic dosage of biosolids [49] is probably linked to the soil fertility enhancement, especially due to the contribution of the organic matter. However, in some laboratory investigations, the biosolid and sewage sludge application has caused inhibitory effects [50] and tissue damage on soil invertebrates [44, 47, 48].

Morphological changes can be employed in investigations of chemicals toxicity and in monitoring of acute and chronic effects, in impacted environments [51]. Analyses

involving morphology and histology of tissues in invertebrates have been widely used to identify different damage types caused by harmful substances to animals [52–55].

Preliminary histological analysis of the midgut of diplopods, exposed for 7 and 30 days to the SB sample, according to sewage sludge disposal for Brazilian law may help clarify the mechanisms used by animals in an attempt to detoxify the contaminants present in the biosolid when applied to the soil. It is inferred that the high rate of epithelium renewal of the midgut of these animals may be to maintain the integrity of the organ and an attempt by the body to compensate the damage suffered after the ingestion of contaminated soil [27]. Similar results were obtained by other authors in response to acute diplopods exposure to industrial soil contaminated with polycyclic aromatic hydrocarbons [27] and sewage sludge samples from different sewage treatment plants [47, 48].

Tissue changes related to defense and detoxification may be reversible, being present only as a result of an altered metabolic status of the organism [56]. In invertebrates, the intracellular accumulation of potentially toxic compounds in insoluble forms and in physiologically inactive ones is an efficient mechanism for detoxification of these elements [27, 57]. In diplopods, the midgut and the hepatic cells work actively in this process [47, 57].

The biotransformation of toxic compounds requires the consumption of energy reserves [27]. In the present study, after 7 days of exposure, the reduction of the volume of hepatic cells of the animals exposed to SB sample could be due to the large energy demand for detoxification of the toxic compounds found in the soil. However, after 30 days of exposure, a decrease in the aggression intensity in hepatic cells of the diplopods' midgut was observed, probably due to SB sample stabilization.

At the same time, there was a reduction of SB genotoxicity in onion seeds exposed to this sample. It is inferred that the genotoxicity reduction was obtained again due to SB sample stabilization for bioprocessing of this by diplopods and subsequent immobilization of trace elements. However, under field conditions, the animals would be chronically exposed to this residue, given the frequent application of this. In this sense, it is indispensable to develop studies that evaluate the subchronic exposure effects in an attempt to measure whether responses are sufficient to maintain the organ integrity and/or if will elapse in more severe cellular/tissue damage.

5. Conclusion

There is evidence that biosolids use will lead to increased soil fertility and trace elements levels; therefore, the environmental significance of such increases needs to be examined.

The results of this study reinforce the need for more research to evaluate the biological effects of waste to be discharged into the environment in different ecosystems compartments, as well as different levels of biological organization, even when toxic agents are present at low concentrations, since the sample studied in accordance with Brazilian standards for sewage sludge disposal has shown toxic and

genotoxic potential to onion seeds and terrestrial invertebrates.

In this sense, studies that evaluate the subchronic exposure effects in an attempt to measure how this provision can be harmful or not to the environment compartments and receptor organisms are necessary.

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Assessment of the Genotoxicity of Two Agricultural Residues After Processing by Diplopods Using the *Allium cepa* Assay

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Abstract Agroindustrial by-products and residues from treatment of sewage sludge have been recently recycled as soil amendments. This study was aimed at assessing toxic potential of biosolid, obtained from a sewage treatment plant (STP), vinasse, a by-product of the sugar cane industry, and a combination of both residues using *Allium cepa* assay. Bioprocessing of these samples by a terrestrial invertebrate (diplopod *Rhinocricus padbergi*) was also examined. Bioassay assembly followed standards of the Brazilian legislation for disposal of these residues. After adding residues, 20 diplopods were placed in each terrarium, where they remained for 30 days. Chemical analysis and the *A. cepa* assay were conducted before and after bioprocessing by diplopods. At the end of the bioassay, there was a decrease in arsenic and mercury. For the remaining metals, accumulation and/or bioavailability varied in all samples but suggested bioprocessing by animals. The *A. cepa* test revealed genotoxic effects characterized by different chromosome aberrations. Micronuclei and chromosome breaks on meristematic cells and F₁ cells with micronuclei were examined to assess mutagenicity of samples. After 30 days, the genotoxic effects were significantly reduced in the soil+biosolid and soil+

biosolid+vinasse groups as well as the mutagenic effects in the soil+biosolid+vinasse group. Similar to vermicomposting, bioprocessing of residues by diplopods can be a feasible alternative and used prior to application in crops to improve degraded soils and/or city dumps. Based on our findings, further studies are needed to adequately dispose of these residues in the environment.

Keywords Biosolid · Sugar cane vinasse · Diplopods · Genotoxicity · Mutagenicity

1 Introduction

Soil is a dynamic and complex system and a habitat for microorganisms, plants, animals, and humans. It is also a food source for different groups of organisms as well as a protective layer for groundwater (Sousa et al. 2008). Therefore, soil contamination by inadequate disposal of residues has become a concern due to environmental impacts and/or risks. As an important component of the soil, its fauna is involved in many aspects of organic matter decomposition, partial regulation of microbial activities, and nutrient cycles (Cortet et al. 1999). Soil disturbances caused by pollutants result in qualitative and quantitative changes in the fauna, affecting soil functions.

As decomposers, diplopods are important animals of the terrestrial fauna (Petersen and Luxton 1982) that can colonize different layers of the soil. They

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participate in recycling and release of nutrients in the decomposing organic matter, enhancing soil humification. Diplopods also promote mineralization, as they secrete ammonia and uric acid through feces, which when degraded, enriches the soil with nitrates (Schubart 1942; Godoy and Fontanetti 2010). Thus, they stimulate microbial metabolism, essential for the recycling of nutrients, such as carbon, nitrogen, and phosphorus, and promote active soil aeration (Hopkin and Read 1992).

Efficacy of invertebrate process has been evaluated through chemical analysis. However, they provide little information on toxicity of the sites after them (Plaza et al. 2005). In addition, determining which and how many compounds are needed to adequately assess imminent risk of a residue is a difficult task. Therefore, complementary information, such as those provided by biological assays, has been increasingly needed to aid in the decision-making process (Rank and Nielsen 1998).

Higher plants are suitable organisms to evaluate contaminated soils (Cotelle et al. 1999; Knasmüller et al. 1998; Souza et al. 2009, 2013). *Allium cepa* is among the most sensitive plant species to assess the genotoxicity of chemicals and environmental samples due to the size and quantity of its metacentric chromosomes (Ma et al. 1995). Incidence of anomalies during mitosis in the chromosomes of meristematic cells of *A. cepa* is an easy method to study mechanisms of several genotoxic and mutagenic compounds (Konuk et al. 2007; Leme and Marin-Morales 2008; Yıldız et al. 2009; Liman et al. 2010, 2011, 2012; Özkara et al. 2011) in different environments, such as aquatic (Bianchi et al. 2011; Caritá and Marin-Morales 2008) as well as terrestrial habitats, in the analysis of soils (Souza et al. 2009, 2013).

Use of biosolids, a residue obtained after treating, stabilizing, and drying sewage sludge produced by wastewater treatment plants (Marques et al. 2002) and vinasse, a by-product of the sugar cane industry, in crops as organic fertilizer and soil conditioner is an important alternative for disposal of these residues in addition to nutrient recycling. However, some environmental problems can arise when these residues are incorrectly used, like soil contamination by toxic heavy metals (Silveira et al. 2003) and/or soil salinization, leaching of metals present in the soil to groundwater, changes in soil quality due to unbalance of nutrients (Agrawal and Pandey 1994), alkalinity

reduction, and increase of phytotoxicity (Navarro et al. 2000).

Therefore, for greater safety in the use of a biosolid and sugar cane vinasse, this study aimed to determine whether there is any cytotoxic, genotoxic, and/or mutagenic potentials of these residues in soil using chromosome aberration test and micronucleus assay with *A. cepa* after bioprocessing for terrestrial invertebrates (diplopods).

2 Materials and Methods

2.1 Biosolid Sample

The biosolid sample was obtained in a sewage treatment plant (STP) of Basic Sanitation Company of the São Paulo State (Companhia de Saneamento Básico do Estado de São Paulo—SABESP). The plant covers an area of 20 ha and serves approximately 80 % of the 318,785 inhabitants (IBGE 2010) of a city in São Paulo state, Brazil, where sewage is treated by conventional activated sludge process. Sewage treated at the plant is predominantly domestic, while industrial wastewater is negligible (Vanzo et al. 2000). In October 1999, the plant received the license of Producer of Agricultural Amendments from the Ministry of Agriculture, Brazil. The product produced in the facility is a biosolid classified as soil conditioner. The brand name is Sabesfértil (SP-09599 00001–0). Biosolid samples were collected and stored in plastic boxes wrapped with dark plastic bags and maintained in a cold room (4 °C) until use.

2.2 Sugar Cane Vinasse Sample

Sugar cane vinasse samples were collected at a sugar cane processing facility in the city of Araras, São Paulo, Brazil and maintained in a cold room (4 °C) at the Department of Biochemistry and Microbiology of the São Paulo State University (UNESP) campus in Rio Claro City, São Paulo, Brazil to minimize bacterial degradation until the beginning of experiments.

2.3 Control Soil Sample

The control soil was obtained from the site where diplopods were collected at a depth of 0–20 cm in UNESP campus in Rio Claro City (22°24'36"S/47°33'

36°W), São Paulo, Brazil. Soil samples were homogenized, dried at room temperature, and sieved with 4-mm mesh and subjected to chemical characterization.

2.4 Chemical Characterization of Samples

Chemical and physicochemical analyses were carried out as well as a characterization of samples based on macro- and micronutrients (N, Ca, Mg, P, K, S, Fe, Mn, Cu, and Zn), C/N ratio, organic matter, cation exchange capacity (CEC), and base saturation percentage by the Campinas Institute of Soil and Fertilizer (Instituto Campineiro de Solo e Adubo—ICASA), Campinas, São Paulo, Brazil. Regarding metals (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se, and Zn), the 16 priority organic compounds were defined by the Environmental Protection Agency (EPA), and the characterization of control soil and biosolid samples was measured by TASQA Laboratory (Paulínia, São Paulo, Brazil). The metal analyses were performed by inductively coupled plasma emission spectrometry (ICP). The polycyclic aromatic hydrocarbons (PAH) analyses were performed by gas chromatography. The analyzed parameters followed the Standard Methods for the Examination of Water and Wastewater 21th Edition 2005 (SM21) and EPA 8270D, respectively.

For vinasse, the following parameters were measured by the TASQA Laboratory: pH, total nonfilterable residue, hardness, electric conductivity, nitrate nitrogen, nitrite nitrogen, ammoniacal nitrogen, Kjeldhal nitrogen, sodium, calcium, potassium, magnesium, sulfate, total phosphate, biochemical oxygen demand (BOD), chemical oxygen demand (COD), and metals (As, Ba, Cd, Cu, Cr, Hg, Mo, Ni, Pb, Se, and Zn). These parameters were measured for control and three experiments in the beginning of experiments—time 0 (t_0)—and after 30 days (t_{30}) of bioprocessing by diplopods.

2.5 Calculating Biosolid and Vinasse Quantities for Application

2.5.1 Application of Sewage Sludge (Biosolid)

According to law 375/2006 of the Environmental National Council (Conselho Nacional do Meio Ambiente—CONAMA), the maximum annual application of sewage sludge and derivatives, in tons per hectare, shall not exceed the quotient between the quantity of nitrogen recommended for the crop (in kilograms per hectare),

following the official recommendation for São Paulo State, and the nitrogen content available (N_{avai}) in the sewage sludge or derivatives (N_{avai} in kilograms per ton) calculated as: N recommended (kilograms per hectare)/N available (kilograms per ton).

To determine the nitrogen available (N_{avai}) in the sewage sludge and/or biosolid, mineralization fractions (MF) were calculated. According to the CONAMA, this fraction represents 40 % of undigested and 20 % of digested sewage sludge (biosolid).

2.5.2 Application of Sugar Cane Vinasse

The maximum dosage of vinasse used was determined according to the current legislation P4.231 of the Environmental Sanitation Technology Company (Companhia de Tecnologia de Saneamento Ambiental—CETESB).

2.5.3 Preparation of Soil and Residue Samples for the Bioassays with *Rhinocricus padbergi*

After the chemical analysis of raw samples, the soil volume to be disposed in each terrarium as well as their density ($d=5.0625 \text{ dm}^3$) for mounting the bioassays were considered. Regarding the biosolid sample, the application rate is given according to the amount of nitrogen needed for culture and the amount of nitrogen present in the sewage sludge or derivatives. The sugar cane crop was the culture considered in the present study, and the amount of nitrogen recommended is 120 kgN/ha, according to Raij et al. (1997). However, in this study, it was considered to be 100 kgN/ha.

Four glass terraria of 45 cm in length, 25 cm in width, and 20 cm of height, with capacity for 22.5 L, were filled with 5 kg of control soil each. After physicochemical analysis of soil samples and residues, data obtained were inserted into the equations present in both laws for calculating the amount to be applied. The following bioassays were set up with control soil (CS), soil+biosolid (SB), soil+sugar cane vinasse (SV), and soil+biosolid+sugar cane vinasse (SBV):

1. CS: 5 kg of control soil
2. SB: 5 kg of control soil+234.4 g of biosolid
3. SV: 5 kg of control soil+410 mL of sugar cane vinasse
4. SBV: 5 kg of control soil+234.4 g of biosolid+410 mL of sugar cane vinasse.

Twenty individuals of the diplopod species, *R. padbergi*, were then placed in each terrarium (t0), where they remained for 30 days (t30) to assess the bioprocessing of contaminants present in residues.

2.5.4 Germination of *A. cepa* Seeds in Residue Samples at Time 0 (t0) and After Bioprocessing by Diplopods after 30 Days (t30)

Approximately 100 seeds of *A. cepa* from the same lot and variety (Baia periforme) were used to examine the cytotoxic, genotoxic, and mutagenic potential of combinations of the biosolid and sugar cane vinasse samples with soil. Seeds of *A. cepa* were allowed to germinate at 22 °C in Petri dishes. Samples from each terrarium were collected and placed in Petri dishes for the germination of *A. cepa* seeds at time 0 (t0). The positive control group consisted of seeds exposed to two compounds at concentrations potentially cytotoxic and mutagenic: the aneugenic herbicide trifluralin at the concentration of 0.019 ppm (Fernandes et al. 2007) and methyl methanesulfonate (MMS), a clastogenic agent at the concentration of 4×10^{-4} M (Rank and Nielsen 1997; Caritá and Marin-Morales 2008). The negative control consisted of seeds allowed to germinate in ultrapure water. The environmental control consisted of seeds allowed to germinate in the control soil. After 30 days of bioprocessing by diplopods, samples from each terrarium were collected for the tests with onion seeds and physicochemical analyses.

2.5.5 Preparation of *A. cepa* Slides

After 5 days of seed exposure to samples, ultrapure water, trifluralin and MMS, root tips about 2.0 cm in length were collected and fixed in Carnoy's fixative 3:1 (ethanol:acetic acid, v/v), for 6–12 h. Afterwards, they were transferred to a new Carnoy's fixative and stored at 4 °C before utilization. The roots were hydrolyzed with 1 N HCl at 60 °C for 9 min and then treated with Schiff's reagent for 2 h (Mello and Vidal 1978). Root tips were sectioned to remove the meristem and region F₁. To intensify the staining and spread cells, one drop of 2 % acetic carmine was added and later covered with coverslips. Coverslips were removed with liquid nitrogen and slides were mounted with Entellan®. The material was analyzed under a light microscope at 400× magnification.

2.5.6 Evaluation of the Cytotoxic, Genotoxic, and Mutagenic Effects in Meristematic Cells of *A. cepa*

A total of 5,000 cells were examined for each treatment at t0 and t30 and for the negative and positive controls. Cytotoxicity was assessed based on morphological alterations indicating cell death, and the mitotic index (MI) was calculated as $MI = (\text{number of dividing cells} / \text{total number of observed cells}) \times 100$. Genotoxicity was evaluated based on the number of cells with chromosomal alterations (CAI), such as C-metaphase, chromosomal adherence, multipolar anaphase and telophase, chromosome bridge, and chromosome loss. The frequency of CAI was calculated as $CAI = (\text{number of cells with chromosome aberrations} / \text{total number of observed cells}) \times 100$. Mutagenicity (MTI) was determined based on the occurrence of cells with micronuclei (MN) and chromosome breaks, calculated as $MTI = (\text{total number of cells with MN and breaks} / \text{total number of observed cells}) \times 100$. The results obtained in all treatments at the different times were compared with the negative control and soil control at their corresponding times with the Mann–Whitney test, a test for nonparametric endpoints, with significance set at 0.05. The Bioestat 5.0 statistical program was used.

2.5.7 Evaluation of Micronuclei in Cells of the Region F₁ of *A. cepa*

The damage to meristematic cells was assessed based on the number of micronuclei in cells of the region F₁. A total of 5,000 cells were examined per treatment. The results obtained for all samples were compared with the negative control and soil control at their corresponding times with the Mann–Whitney test, with significance set at 0.05.

3 Results

3.1 Physicochemical Analysis and Metals

3.1.1 Soil Sample

The results of the control soil fertility are presented in Table 1. The control soil was classified as clay, slightly acidic (pH=6.20), with low levels of organic matter and heavy metals.

Table 1 Chemical characterization and clay content of the control soil

Sample	pH (CaCl ₂)	Organic matter (g/dm ³)	P (mg/dm ³)	K (mmol _e /dm ³)	Ca (mmol _e /dm ³)	Mg (mmol _e /dm ³)	H+Al (mmol _e /dm ³)	Sum of bases (SB) (mmol _e /dm ³)	Cation exchange capacity (CEC) (mmol _e /dm ³)	Base saturation (%)	Ratio		Clay (g/kg)
											Ca/Mg	Mg/K	
Soil	6.20	18	3.0	0.8	2	1	88	3.9	91.9	4.2	2.0	1.25	483

The results of the physicochemical analysis and metals of the control soil sample, raw vinasse, and biosolid samples are presented in Table 2. The

concentrations of arsenic and copper found in the control soil were above the limits determined by CETESB (195/2005-E) but below the limits for

Table 2 Physicochemical and metal analyses of the control soil and raw samples of vinasse and biosolid

Parameter	Samples			Method	G (mg/kg)	MCA (mg/kg)
	CS (mg/kg)	V (mg/L)	B (mg/kg)			
Arsenic	16.8	<LQ	<LQ	SM21 3120B	3.5	41
Barium	5.91	0.41	158	SM21 3120B	75	1,300
Cadmium	<LQ	<LQ	<LQ	SM21 3120B	<0.5	39
Total calcium	25.4	719	3,939	SM21 3120B	–	–
Organic carbon (g/kg)	12.6	–	279	SSSA Cap40	–	–
Lead	49.3	<LQ	174	SM21 3120B	17	300
Copper	37.2	0.35	276	SM21 3120B	35	1,500
Electric conductivity (μs/cm)	115	13,530	5,389	SM21 3120B	–	–
Chromium	31.2	0.04	224	SM21 3120B	40	1,000
Total sulfur	151	1,219	11,864	SM21 3120B	–	–
Total phosphorus	182	–	17,027	SM21 3120B	–	–
Total magnesium	<LQ	237	358	SM21 3120B	–	–
Mercury	<LQ	0.0019	1.08	EPA 7470A	0.05	17
Molybdenum	3.64	0.008	9.55	SM21 3120B	<4	50
Nickel	13.0	0.03	82.3	SM21 3120B	13	420
Nitrate	4.40	1.30	6.79	SM21 4500-NO ₃ ⁻ E	–	–
Nitrite	0.06	0.008	1.39	SM21 4500-NO ₂ ⁻ B	–	–
Ammoniacal nitrogen	31.8	–	167	SM21 4500-NH ₃ E	–	–
Kjeldahl nitrogen	476	267	21,620	SM21 4500-Norg B	–	–
pH	6.20	3.9	8.01	EPA 9045 C	–	–
Total potassium	406	2,056	2,152	SM21 3120B	–	–
Selenium	<LQ	<LQ	<LQ	SM21 3120B	0.25	100
Total sodium	<LQ	50.2	<LQ	SM21 3120B	–	–
Solid content	0.86	–	0.24	SM21 2540B	–	–
Moisture (g/g)	0.14	–	0.76	SM21 2540B	–	–
Zinc	23.2	1.66	825	SM21 3120B	60	2,800

CS control soil, V sugar cane vinasse, B biosolid, LQ limits of quantification, IV inconsistent value, SM21 Standard Methods for the Examination of Water and Wastewater 21th edition 2005, SSSA Soil Science Society of America—Methods of soil analysis—part 3—chemical methods, EPA 7470A Mercury by manual cold vapor technique, EPA 9045 C an electrometric procedure for measuring pH in soils and waste samples, G guidelines of quality for soil (mg/kg) and groundwater in São Paulo State according to CETESB (195/2005-E), MCA maximum concentration allowed in sewage sludge or derivative product according to CONAMA (375/2006)

intervention in agricultural areas. The levels of barium, lead, copper, chromium, molybdenum, nickel, and zinc found in the raw biosolid sample were high but below the maximum allowed by CONAMA (Table 2). The pH of samples also varied. Raw sugar cane vinasse sample contained barium, copper, chromium, mercury, molybdenum, nickel, and zinc, low pH, high DBO and DQO, and potassium. None of the 16 priority organic compounds listed by EPA were found in the control soil and raw biosolid samples (Table 3).

3.1.2 Samples at t_0 and t_{30}

After mixing residues, the results for the concentration of metals were below the maximum allowed in the soil with the application of sewage sludge and/or biosolid, according to CONAMA (375/2006). The pH of the control soil sample and the different residues varied between pH 4 and 6 (Table 4).

After 30 days of treatment, the concentration of metals remained below the maximum allowed (Table 4). However, the concentration of organic carbon increased in all samples. The pH of samples

ranged between 4 and 7. Figure 1 presents a comparison of metals and other parameters between t_0 and t_{30} .

3.1.3 Diplopods Behavior

Animals of control soil, soil+sugar cane vinasse, and soil+biosolid+sugar cane groups exhibited the same behavior during the 30 days of experiment. Some diplopods from soil+biosolid sample, however, were often on the surface instead of buried in the soil.

The highest mortality rate of diplopods during the first 7 days was observed in the soil+biosolid group, compared to those of the control soil and other groups (Fig. 2). After the seventh day, a high mortality rate was observed among animals from the soil+sugar cane vinasse treatment. From day 15th to 30th, the soil+biosolid and soil+sugar cane vinasse groups were the most toxic compared to the control soil and soil+biosolid+sugar cane vinasse (Fig. 2). After the 30th day, mortality rates increased for all treatments. Soil+sugar cane vinasse and soil+biosolid+sugar cane vinasse treatments were the most toxic (Fig. 2).

Table 3 Analysis of polycyclic aromatic hydrocarbons in raw samples of control soil and biosolid sample

Parameter	Samples		Method	Allowed concentration in the soil (mg/kg)	
	CS ($\mu\text{g}/\text{kg}$)	B ($\mu\text{g}/\text{kg}$)		CB	CO
Acenaphthene	<LQ	<LQ	EPA 8270 D	–	–
Acenaphthylene	<LQ	<LQ	EPA 8270 D	–	–
Anthracene	<LQ	<LQ	EPA 8270 D	–	–
Benzo(a)anthracene	<LQ	<LQ	EPA 8270 D	0.025	0.025
Benzo(a)pyrene	<LQ	<LQ	EPA 8270 D	0.052	0.052
Benzo(b)fluoranthene	<LQ	<LQ	EPA 8270 D	0.38	–
Benzo(g,h,i)perylene	<LQ	<LQ	EPA 8270 D	0.57	–
Benzo(k)fluoranthene	<LQ	<LQ	EPA 8270 D	0.38	0.38
Chrysene	<LQ	<LQ	EPA 8270 D	8.1	–
Dibenz(a,h)anthracene	<LQ	<LQ	EPA 8270 D	0.08	–
Phenanthrene	<LQ	<LQ	EPA 8270 D	3.3	3.3
Fluoranthene	<LQ	<LQ	EPA 8270 D	–	–
Fluorene	<LQ	<LQ	EPA 8270 D	–	–
Inden(1,2,3-cd)pyrene	<LQ	<LQ	EPA 8270 D	0.031	0.031
Naphthalene	<LQ	<LQ	EPA 8270 D	0.12	0.12
Pyrene	<LQ	<LQ	EPA 8270 D	–	–

CS control soil, B biosolid, LQ limits of quantification, EPA 8270 D method used to determine the concentration of semivolatile organic compounds in extracts prepared from many types of solid waste matrices and soils, CB guidelines (preventive) for soils of São Paulo State according to CETESB (195/2005-E), CO maximum concentration allowed in the soil according to CONAMA (375/2006)

Table 4 Comparison of the physicochemical and metal analyses for the control soil and combinations of residues at time 0 (t0) and after 30 days of bioprocessing by diplopods (t30)

Parameter	Samples								
	CS (mg/kg)		SB (mg/kg)		SV (mg/kg)		SBV (mg/kg)		MCA (mg/kg)
	t0	t30	t0	t30	t0	t30	t0	t30	
Arsenic	16.8	<LQ	3.49	<LQ	5.79	<LQ	10.5	<LQ	41
Barium	5.91	11.3	6.21	15.2	6.50	12.9	7.07	8.99	1,300
Cadmium	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	39
Total calcium	25.4	170	30.8	350	29.3	246	26.7	97.3	–
Organic carbon (g/kg)	12.6	91.8	35.2	118	28.8	81.7	17.5	63.1	–
Lead	49.3	10.0	14.8	16.6	8.46	4.83	6.17	13.0	300
Copper	37.2	68.4	67.8	93.6	58.2	70.2	38.1	47.1	1,500
Chromium	31.2	26.5	28.6	30.6	25.2	25.6	31.5	22.8	1,000
Total phosphorus	182	262	373	444	317	267	262	274	–
Mercury	<LQ	<LQ	0.10	<LQ	0.20	<LQ	0.04	<LQ	17
Molybdenum	3.64	<LQ	3.99	2.56	1.58	3.34	2.34	<LQ	50
Nickel	13.0	9.70	10.6	14.6	9.76	11.4	7.08	8.49	20
Nitrate	4.40	2.98	2.98	4.47	3.71	2.31	<LQ	2.70	–
pH	6.20	5.85	6.80	7	4.49	3.99	4.46	5.84	–
Selenium	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	<LQ	100
Solid content	0.86	0.83	0.89	0.82	0.79	0.89	0.84	0.85	–
Zinc	23.2	44.2	41	60.1	37.3	42.2	31.3	37.4	2,800

CS control soil, SB soil+biosolid, SV soil+sugar cane vinasse, SBV soil+biosolid+sugar cane vinasse, LQ limits of quantification, MCA maximum concentration allowed in sewage sludge or derivative according to CONAMA (375/2006)

3.2 Results with *A. cepa* Before and After Bioprocessing by Diplopods

3.2.1 Cytotoxicity

Table 5 presents a comparison among mitotic indices of cells from the negative and positive controls, control soil, and the treatments at the different times of exposure. At both times of exposure, mitotic indices of treatments were not significantly different from those of the negative control and/or control soil. Characteristics of cell death were not observed in any group at any time.

3.2.2 Genotoxicity

All treatments induced genotoxic effects (Table 5) at t0 and t30 ($p < 0.05$), characterized by chromosome aberrations, compared to the negative control and/or control soil (Fig. 3). However, the genotoxic effect was significantly reduced after the bioprocessing by

diplopods in the soil+biosolid and soil+biosolid+sugar cane vinasse groups, except for the soil+sugar cane vinasse group (Table 5, Fig. 3). The most frequently observed alterations in the present study were cells in metaphase with chromosome adherence (Fig. 4a and b), polyploid metaphases (Fig. 4c), polyploid anaphase (Fig. 4d), anaphase with chromosome bridges (Fig. 4e), anaphase with chromosome loss (Fig. 4f), and cells with nuclear buds (Fig. 4g).

3.2.3 Mutagenicity

Meristematic cells with micronuclei (Fig. 4h) and chromosome breaks (Fig. 4i) were examined to assess the mutagenicity. Statistical analysis revealed a mutagenic effect of the samples examined at both times of exposure compared to the negative control and/or control soil, except for the soil+biosolid group (Table 5, Fig. 5).

The micronucleus test with cells of the F₁ region (Fig. 4j) revealed that after 30 days, the mutagenicity

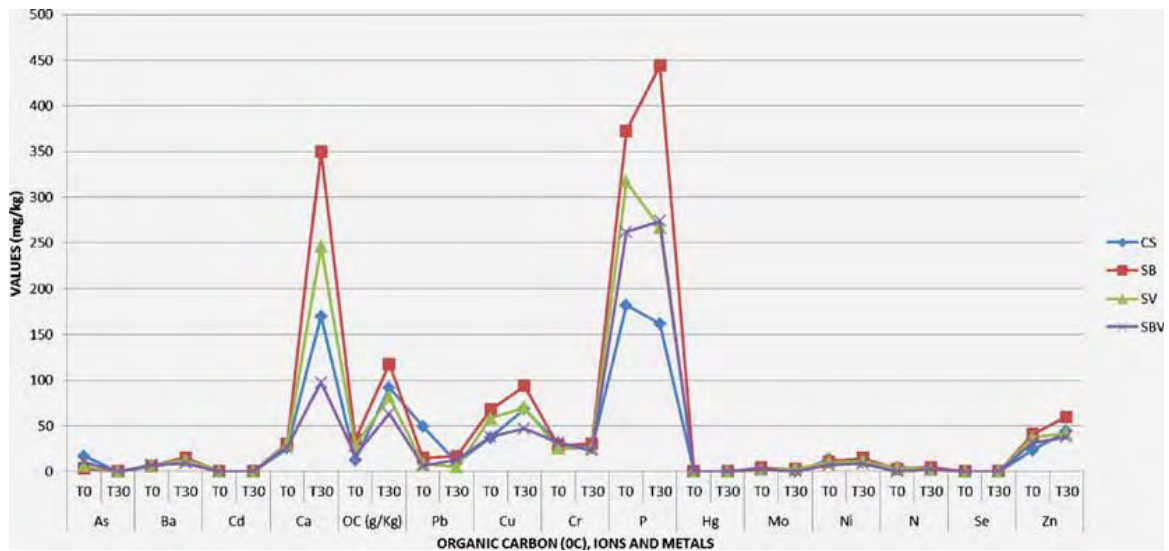


Fig. 1 Comparison of results obtained by chemical analyses, between time 0 (t0) and 30 days (t30), for soil and residue combination samples. CS control soil, SB soil+biosolid, SV soil+sugar cane vinasse, SBV soil+biosolid+sugar cane vinasse

of the soil+sugar cane vinasse and soil+biosolid+sugar cane vinasse groups significantly decreased (Table 6, Fig. 6), compared to the results obtained at t0, indicating the efficacy of diplopods in reducing the mutagenicity of these residues.

4 Discussion

The bioprocessing of biosolid and sugar cane vinasse by diplopods can be an important tool to reduce the

toxicity of agricultural residues. Our results with the *A. cepa* assay indicated that the genotoxicity and mutagenicity levels were reduced after 30 days of bioprocessing to diplopods.

Unlike vermicomposting in which earthworms reduce C/N ratio, by increasing the surface of exposure for microorganisms and making conditions more favorable for their activity and consequently to decomposition, Singh et al. (2011) observed that diplopods released large quantities of organic carbon in all groups, including the control group. According to

Fig. 2 Mortality rate for individuals of the *R. padbergi* exposed for 30 days to control soil and residue combination samples. CS control soil, SB soil+biosolid, SV soil+sugar cane vinasse, SBV soil+biosolid+sugar cane vinasse

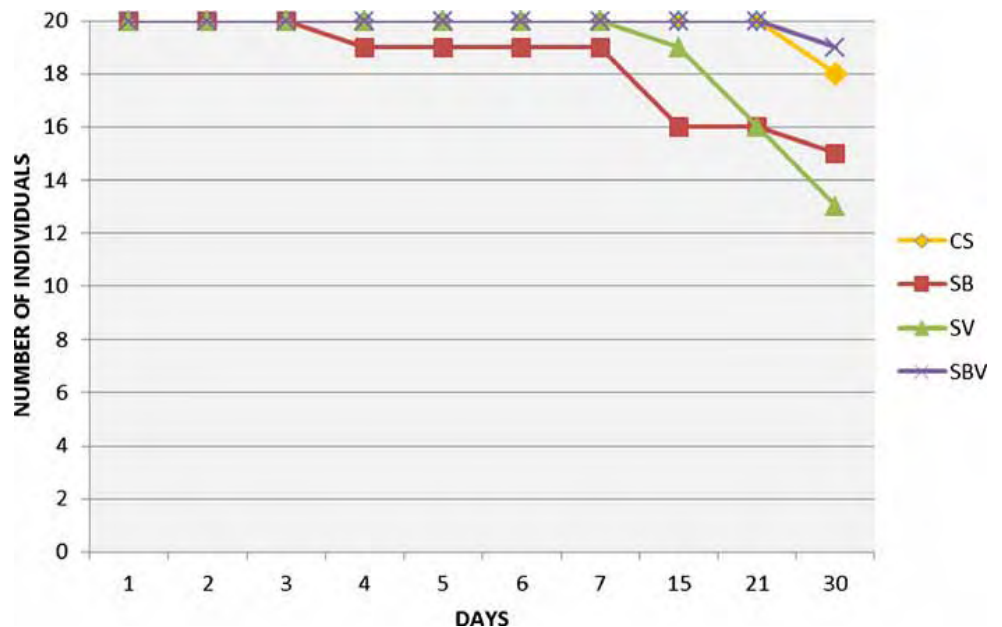


Table 5 Mean and standard deviation of the mitotic index (MI), the rate of chromosomal aberrations (CAI), and mutagenicity index (Mtl) in meristematic cells of *Allium cepa* after exposure

to ultrapure water (negative control), control soil, the MMS, and trifluralin (positive controls) and combinations of control soil, biosolid, and sugar cane vinasse at time 0 (t0) and 30 days (t30)

Treatment	Samples	Mitotic index (MI)		Chromosomal aberrations (CAI)		Mutagenicity index (Mtl)	
		Time 0 (t0)	30 days (t30)	Time 0 (t0)	30 days (t30)	Time 0 (t0)	30 days (t30)
Controls	NC	13.66±1.37	11.2±1.78	1.2±0.83	2±1	0.2±0.44	0.6±0.54
	CS	14.21±0.90	9.48±1.07 ^a	2±0.70	1.6±1.14	0.6±0.54	0
	MMS	11.53±0.88	5.73±1.15	8.6±3.04 ^{*,**}	14.2±2.94 ^{*,**}	25.6±8.5 ^{*,**}	24.8±7.29 ^{*,**}
	TRIF	9.47±0.54	9.31±1.35	51.4±8.79 ^{*,**}	23.6±4.82 ^{*,**}	12.2±3.42 ^{*,**}	3.6±0.89 ^{*,**}
Combinations	SB	15.07±0.56	15.03±0.79	21±5.83 ^{*,**}	10.6±3.33 ^{*,**} , ^a	1.2±1.3	0.4±0.54
	SV	13.80±1.48	14.49±1.54	18.6±2.79 ^{*,**}	16±2 ^{*,**}	1.4±0.54 [*]	0.2±0.44
	SBV	15.01±1.26	12.78±0.46 ^a	21±5.04 ^{*,**}	10.6±3.2 ^{*,**} , ^a	2.2±0.44 ^{*,**}	0.8±0.83 ^a

MI mitotic index, CAI rate of chromosomal aberrations, Mtl mutagenicity index, t0 time 0—time of mixing of the residues, t30 30 days after exposure to diplopods, CS control soil, NC negative control, MMS and TRIF positive controls and combinations, SB soil+biosolid, SV soil+sugar cane vinasse, SBV soil+biosolid+sugar cane vinasse

^a Values statistically significant when comparing the same treatments at 0 and 30 days

^{*} *p*<0.05 (statistical significance, compared to negative control by the Mann–Whitney test)

^{**} *p*<0.01 (statistical significance in relation to the control soil by the Mann–Whitney test)

Suthar (2010), some factors, such as microclimate variability (humidity and temperature) and substrate depth, can be responsible for the mineralization and/or assimilation of organic carbon. In addition, organic materials can form new soluble or insoluble complexes with metals, directly influencing their availability (Stevenson 1982).

The results of the chemical analysis also indicated that phosphorus was released in terraria in all treatments after 30 days. In earthworms, phosphorus is

released, in part, by alkaline phosphatases, which are produced in relatively large quantities. This essential enzyme is involved in the biogeochemical cycle of phosphorus in the soil (Le Bayon and Binet 2006; Suthar 2010). Another element released by diplopods was calcium. The same was observed by Suthar (2010) and Yadav and Garg (2009), which reported a significant increase in calcium after vermicomposting.

Sewage sludge and sugar cane vinasse have a high potential as soil amendments of degraded soils, as they

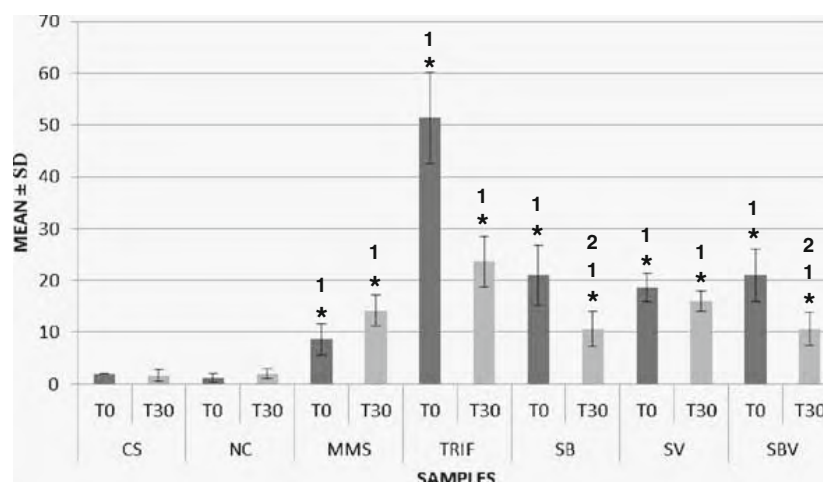


Fig. 3 Distribution of the genotoxicity index (mean±SD) for meristematic cells of *A. cepa* roots allowed to germinate in control soil (CS), ultrapure water (NC), MMS, and trifluraline (TRIF) for positive controls and soil+biosolid (SB), soil+sugar cane vinasse (SV),

and soil+biosolid+sugar cane vinasse (SBV) at t0 and t30 periods. *Significant statistic values in comparison to NC, with *p*<0.05; ¹significant statistic values in comparison to NC, with *p*<0.01; ²significant statistic values in comparison at t0 and t30 periods

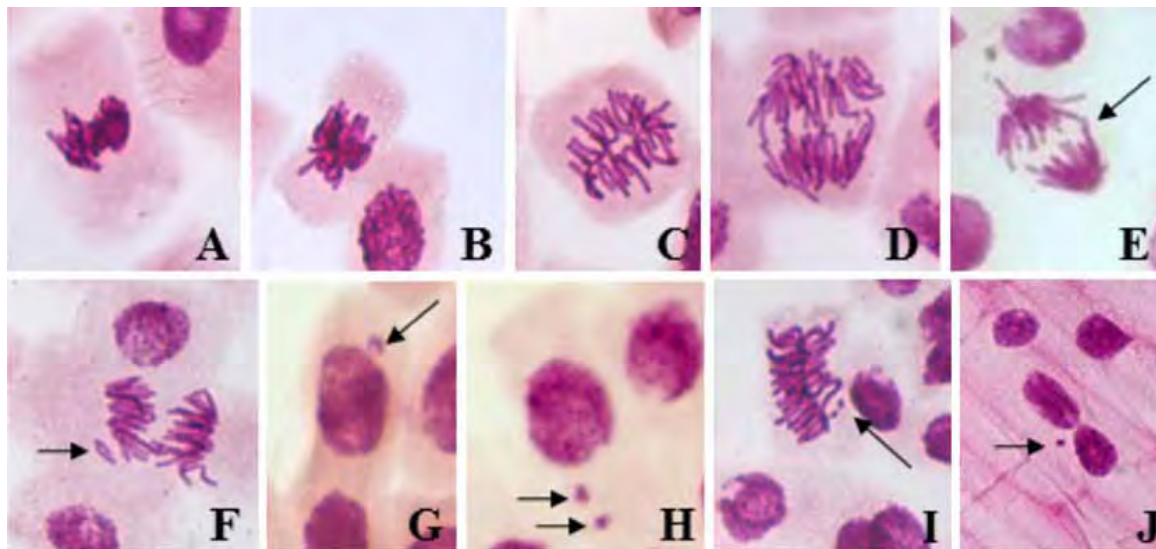


Fig. 4 Chromosomal aberrations, micronuclei, and chromosomal break found in *Allium cepa*. **a** and **b** Metaphase with chromosome adherence; **c** polyploid metaphase; **d** polyploid anaphase; **e** anaphase with chromosomal bridge (arrow); **f**

anaphase with chromosome loss (arrow); **g** nuclear bud (arrow); **h** meristematic cell in interphase carrying micronuclei (arrows); **i** metaphase with chromosomal break (arrow); **j** F₁ region cell carrying micronuclei (arrow)

contain increased levels of essential nutrients such as phosphorus and organic carbon. The interest in bioprocesses, such as vermicomposting, is due to the reduction of the volume of residues, making disposal and application easier (Singh et al. 2011), in addition to reduction of toxic pollutants and pathogenic microorganisms. According to Singh et al. (2011), during vermicomposting, earthworms absorb large quantities of heavy metals in the midgut. Shahmansouri et al. (2005) reported that the concentration of heavy metals in the substrate decreased with time. Saxena et al. (1998) observed that *Eisenia fetida* accumulated

heavy metals in higher concentrations during this process. Cortet et al. (Cortet et al. 1999) suggested that the bioaccumulation of metals by terrestrial invertebrates depends on the element, its concentration in the environment, and the physicochemical conditions of the soil.

Köhler et al (1995) and Fontanetti et al. (2006) demonstrated that diplopods can metabolize and/or accumulate many types of metals, especially in the midgut and the fat body. The accumulation of minerals as spherocrystals is an important mechanism to maintain homeostasis of these animals. This has been

Fig. 5 Distribution of the mutagenicity index (mean ± SD) for meristematic cells of *A. cepa* roots allowed to germinate in control soil (CS), ultrapure water (NC), MMS, and trifluraline (TRIF) for positive controls and soil+biosolid (SB), soil+sugar cane vinasse (SV), and soil+biosolid+sugar cane vinasse (SBV) at t0 and t30 periods. *Significant statistic values in comparison to NC, with $p < 0.05$; ¹significant statistic values in comparison to NC, with $p < 0.01$; ²significant statistic values in comparison at t0 and t30 periods

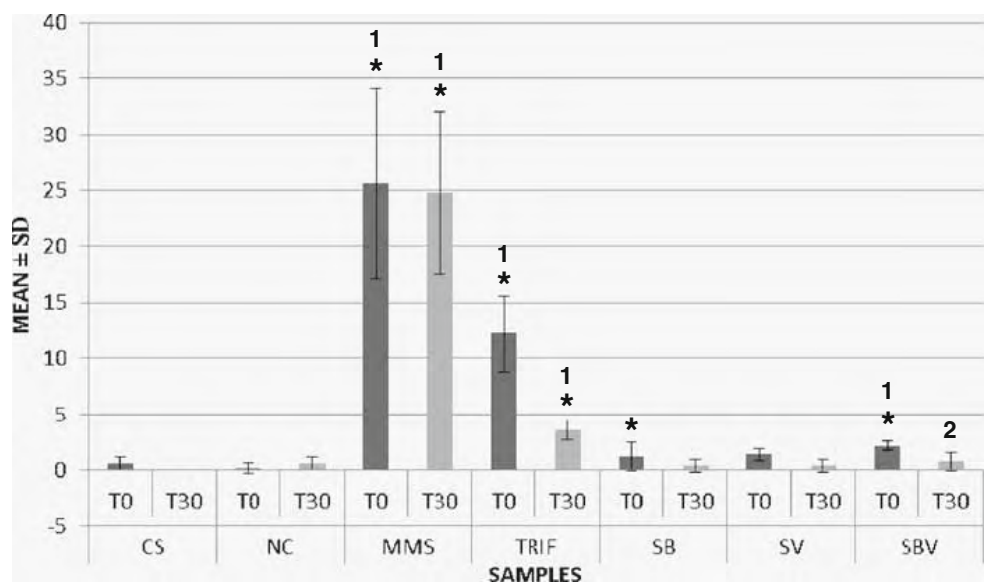


Table 6 Mean and standard deviation for micronuclei in cells of the meristematic (M) and F₁ regions of *Allium cepa* after exposure to ultrapure water (negative control), control soil,

MMS, and trifluralin (positive controls) and combinations of control soil, biosolid, and sugar cane vinasse at time 0 (t0) and 30 days (t30)

Treatment	Samples	Time 0 (t0)		30 days (t30)	
		Meristematic region (M)	F ₁ region	Meristematic region (M)	F ₁ region
Controls	NC	0.2±0.44	0.2±0.44	0.6±0.54	0.2±0.44
	CS	0.6±0.54	0	0	0.2±0.44
	MMS	21.2±7.08 ^{*,**}	4.6±2.07 ^{*,**}	20.6±5.12 ^{*,**}	4.6±0.89 ^{*,**}
	TRIF	12.2±3.42 ^{*,**}	2.6±1.14 [*]	3.6±0.89 ^{*,**}	2.6±0.89 ^{*,**}
Combinations	SB	1±1	0.6±0.54	0.4±0.54	0
	SV	1.2±0.44 [*]	0.4±0.54	0.4±0.54 ^a	0.6±0.54
	SBV	1.8±0.44 [*]	0.4±0.54	0.8±0.83 ^a	0

t0 time 0—time of mixing of the residues, t30 30 days after exposure to diplopods, CS control soil, NC negative control, MMS and TRIF positive controls and combinations, SB soil+biosolid, SV soil+sugar cane vinasse, SBV soil+biosolid+sugar cane vinasse

^a Values statistically significant when comparing the same treatments at 0 and 30 days

^{*} p<0.05 (statistical significance, compared to negative control by the Mann–Whitney test)

^{**} p<0.01 (statistical significance in relation to the control soil by the Mann–Whitney test)

observed in many invertebrates and in different organs, and is associated with ion balance, which involves recycling, storage, and excretion of minerals (Fontanetti et al. 2006).

In this study, it was observed that some metals, such as arsenic and mercury, probably accumulated in diplopods, in all groups, including the control soil.

However, other metals, such as barium, copper, molybdenum, nickel, and zinc, were found in much higher concentrations at t0. Since increased levels of metals occurred in the soil+sugar cane vinasse or sugar cane vinasse combined with biosolid groups, sugar cane vinasse may have influenced the results obtained due to its acidic pH. Souza et al. (2013)

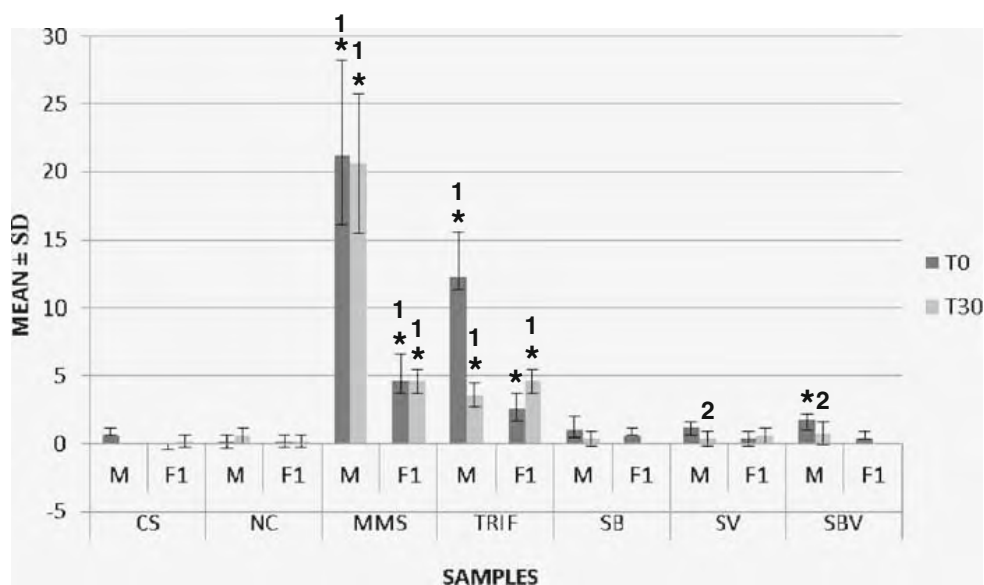


Fig. 6 Distribution of the micronuclei index (mean±SD) in meristematic cells (M) and F₁ region of *A. cepa* roots allowed to germinate in control soil (CS), ultrapure water (NC), MMS, and trifluraline (TRIF) for positive controls and soil+biosolid (SB), soil+sugar cane vinasse (SV), and soil+biosolid+sugar cane

vinasse (SBV) at t0 and t30 periods. *Significant statistic values in comparison to NC, with p<0.05; ¹significant statistic values in comparison to NC, with p<0.01; ²significant statistic values in comparison at t0 and t30 periods

evaluated the toxicity and mutagenicity of the soil from a landfarming area and observed a decrease in pH in soils associated with sugar cane vinasse.

According to Fernandes and Silva (1999), low pH values may be associated to the availability of metals in the soil. The pH and the quantity of organic matter in the soil influence the availability of macro- and micronutrients as well as of metals. The quantity of organic matter in the soil may minimize the mobility and the biological effects of metals by forming insoluble organometal compounds (McBride 1995). The pH affects the activity of the microorganisms responsible for the decomposition of the organic matter and many chemical transformations in the soil (Caldwell 2000). Andréa et al. (1997) reported that low pH values resulted in lower decomposition rates by chemical processes alone and affected the selection of microorganisms that can be decomposers, as they increase recalcitrance of compounds. Therefore, it is possible that the estimated time for biodegradation by the test organism as well as the microbial community was not enough to stabilize and decrease the concentration of metals.

Biosolid and sugar cane vinasse, residues used in agriculture, induced genotoxic effects at both times of exposure. However, genotoxicity was significant only for the treatment soil+sugar cane vinasse after 30 days. According to Kannan and Upreti (2008), toxicity of vinasse might be due to the large quantities of organic and inorganic chemicals and very acidic pH or the effect of ion exchange, during which dissolution of metals is limited, as it is a nonselective process and metals and elements have higher concentrations in the soil, such as calcium (Laterrel et al. 1978).

Souza et al. (2013) reported a significant increase in chromosome aberrations in *A. cepa* (onion) seeds exposed to soil samples from a landfarming facility with and without sugar cane vinasse. According to the authors, when the sugar cane vinasse was added, bridges, adherences, and chromosome breaks were observed. After 33 days, landfarming soil with sugar cane vinasse caused a prominent effect characterized by the presence of multiple chromosome breaks. The results obtained indicated that sugar cane vinasse potentialized clastogenicity of landfarming soil, probably due the release of metals that were previously adsorbed into the organic matter.

Chromosome aberrations are frequently induced by aneugenic agents, such as metals, present in both

residues. Heavy metals are potentially mutagenic and are closely associated with environmental pollution. Several studies conducted with plants have shown that the genotoxic effect of metals can induce alterations in chromosome structure, and number and disturbances in the mitotic apparatus (Fiskejö 1983; Minissi and Lombi 1997). Although present in concentrations below those permitted by CETESB and CONAMA in both experimental times, these elements had genotoxic and mutagenic potential at t0 in the soil+sugar cane vinasse and soil+biosolid+sugar cane vinasse groups. After bioprocessing by diplopods, these elements may not be available for plants, given the reduction of the genotoxic and mutagenic effects, as demonstrated by the decrease in chromosome aberrations and micronuclei in meristematic cells and F₁ cells. The F₁ region analyzed in *A. cepa* is characterized by cells resulting from the cellular differentiation of meristematic cells (Ma and Xu 1986). Lesions induced in the cells of the meristematic region, when not corrected and/or incorrect, may remain as micronuclei in the F₁ region. Thus, this test confirms the mutagenic effects of the different substances and/or chemicals in *A. cepa*. Based on the genotoxic/mutagenic effects shown by chromosomal aberration test and the mutagenicity for the observation of micronuclei in the meristematic and F₁ regions, the cell repair mechanism was able to reverse the damage caused by the metals during the cell process. Therefore, the damage induced in meristematic cells did not progress into micronuclei in F₁ cells.

Like vermicomposting, bioprocessing of agroindustrial and/or agricultural residues by diplopods can be a feasible alternative to be used before application in crops, degraded soils, and/or disposal in city dumps. Nevertheless, further studies are needed on the adequate disposal of these residues in the environment.

5 Conclusion

Management of residues is one of the major global challenges for the scientific community regarding environmental safety, especially their adequate treatment, final disposal, and recycling. Like vermicomposting, bioprocessing of residues by other terrestrial invertebrates, such as diplopods, can be a viable technology for the management of residues, as they aid in reduction of toxicity and can improve in nutrient recycling and

degraded soils. These technologies can also reduce the use of inorganic fertilizers.

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