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Toxicological and genotoxicological assessment of water extracts of sewage sludge and other biogenic wastes: A piece of the SLURP jigsaw puzzle

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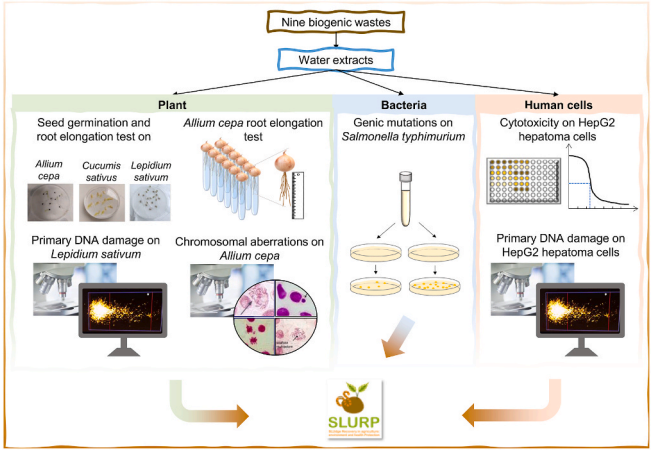
HIGHLIGHTS

- An integrated chemical and biological assessment was performed in the SLURP project.
- Water extracts of sewage sludge, manure, liming material, compost, digestate were tested.
- Application of biotests to assess the toxicity and genotoxicity of extracts.
- Toxicity and genotoxicity on plant and human cells, but not mutagenicity was found.

GRAPHICAL ABSTRACT



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ABSTRACT

Given the considerable quantities of biogenic matrices employed in agricultural applications, there is growing concern about the potential negative effects resulting from the presence of harmful contaminants. The project "SLURP - SLUdge Recovery in Agriculture: Environment and Health Protection" planned a multi-stage approach in which the application of a wide battery of bioassays was proposed as an effective tool to measure the direct interaction of matrices with the different components of the ecosystem, from the molecular to the whole

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Lepidium sativum
Salmonella typhimurium
HepG2 cells
Biotest

organism level. The aim of the present study, which is a part of the "SLURP" project, was to characterise the toxicological and genotoxicological properties of water extracts from biogenic wastes using several assays based on plant, bacterial and human cells. The aqueous extracts of four sewage sludges, a liming material, two manure slurries of swine and bovine origin, a digestate from bovine manure and a compost were chemically characterised for inorganic ions and heavy metals. Then the extracts were analysed using tests on *A.cepa, C.sativus*, *L.sativum*, *S. typhimurium* and human hepatoma cell line (HepG2) to assess toxicity (seed germination, root elongation, proliferation), mutagenicity and genotoxicity (primary DNA damage, chromosomal aberrations). The extracts exhibited chemical heterogeneity. Ammonia nitrogen, Ca^{2+}, Fe and Zn were the most abundant elements. Toxic effects were caused on *A.cepa* and *L.sativum* by all extracts, while there were non-toxic effects on human cells. Genotoxic effects on *A.cepa* and *L.sativum* were instead caused by almost all the extracts, at least at the highest dose tested, while only four samples from one sewage sludge, liming material, digestate, and compost, caused DNA damage on human cells. None of the extracts induced mutagenic effects in *S.typhimurium*. A comprehensive interpretation of these results can only be achieved through the integrated evaluation of all eco-toxicological and chemical data obtained throughout the entire project.

1. Introduction

As agricultural practices have developed, so has the need to fertilise the soil. Different types of matrices of biogenic origin are applied to agricultural land for this purpose, due to their valuable content. Many of these are readily accepted, such as manure or compost, while others cause more concern, such as digestate, biochar and sewage sludge. Particular attention is given to the latter, which is a "*mud-like residue resulting from wastewater treatment*" (European Commission, 2024). Around 45 million tons (dry solids) of sewage sludge are produced globally each year (Ferrentino et al., 2023). Europe accounts for approximately 8 million tons of this annual production (European Commission, 2023). Nearly half of the European production is spread on agricultural land, with a rise to 70–80% in France, Spain, and the United Kingdom (Vallet, 2018). Considering waste management priorities and the circular economy paradigms, material recovery is a top goal. Therefore, reusing sludge in agriculture is a potentially optimal solution as a fertiliser or soil improver due to its valuable properties. It contains approximately 30% of the organic carbon content of treated wastewater, around 20% of the influent nitrogen and up to 80% of the influent load of phosphorus (if phosphorus removal processes are in place), along with micronutrients such as metals, including nickel and copper (Mininni et al., 2015).

Despite the benefits mentioned above, it is important to pay close attention to the potential adverse effects of its use on ecosystems, crops, livestock, and human health. This is due to the presence of both known, such as organic pollutants, heavy metals, pathogenic microorganisms, and unknown contaminants (European Commission, 2024).

European Union (EU) legislation on fertilisers has recently been revised to extend its scope to fertilising products based on secondary raw materials, resulting in the publication of Regulation (EU) 2019/1009 (European Union, 2019), which includes sewage sludge, digestate and compost.

However, there is still much work to be done to clearly define the safety of a product before it can be applied to agricultural land. It is widely recognized that chemical characterisation alone is insufficient to achieve the goal of a definitive and robust description of the interactions between living organisms and a complex matrix of different origin, from medical devices (Laube, 2021) or cosmetics/food products (Severin et al., 2017) to environmental mixtures (Escher et al., 2020; European Commission, 2012).

Bioassays provide an effective approach, allowing the assessment of the direct interaction of a matrix with the different biotic components of an ecosystem. By this way, multitiered assays, including subcellular structures, cells, tissues and whole organisms, deliver a more realistic picture of the effects following the exposure to the organic residues like industrial wastes (Alias et al., 2021; Benassi et al., 2019) or wastewater (Bertanza et al., 2022; Pedrazzani et al., 2019).

In order to adhere to the toxicology principle of "battery", it is imperative to evaluate a variety of endpoints of both toxicity (e.g., immobilization, growth inhibition) and genotoxicity, as well as to consider multiple organisms from nearly three trophic levels (producers, primary, and secondary consumers) (e.g., point mutations, primary DNA damage, stable DNA damage). These precautions are essential for the purpose of reducing uncertainty and obtaining a more dependable assessment (Bierkens et al., 1998).

Moreover, the use of non-animal-based toxicity and genotoxicity assays has become increasingly important in characterising a variety of a wide range of environmental matrices, such as urban air (Feretti et al., 2019), drinking water (Alias et al., 2023a; Escher et al., 2021; Feretti et al., 2020), wastewater (Bertanza et al., 2021; Menghini et al., 2023), fertilised soil (Carraturo et al., 2024), sewage sludges and landfill leachates (Jabłońska-Trypuć, 2021; Jabłońska-Trypuc et al., 2019) due to the public and political rising concerns in the animal-based toxicity testing (Bos et al., 2020; SCHER et al., 2013). To address these issues, the multidisciplinary research project "SLURP - SLUdge Recovery in Agriculture: Environment and Health Protection" was conducted. The project employed a multi-stage approach, including numerous bioassays and broad-spectrum chemical analyses (Bertanza et al., 2024). The main novelty of this work lies in having compared different organic residues with the same integrated assessment methodology.

The research presented is a part of the SLURP project. Its aim was to characterise the toxicological and genotoxicological properties of water extracts from sewage sludge and other biogenic wastes using a battery of assays based on plant, bacteria and human cells.

2. Material and methods

All the protocols adopted in the SLURP project have been described in full detail (procedure, doses and statistical analysis) elsewhere (Bertanza et al., 2024). Therefore, each phase of this research will only be briefly outlined in the following paragraphs.

2.1. Samples collection and aqueous extracts production

Nine biogenic wastes were analysed belonging to four categories of products that can be potentially spread on agricultural land: sewage sludge, liming material, livestock manure, and soil improver.

In short, the following substrates were investigated:

- Sample S1: sewage sludge from a municipal (mainly domestic) wastewater treatment plant (WWTP).
- Sample S2: sewage sludge from a second municipal WWTP.
- Sample S3: sewage sludge from a facility that composts treated sludge by mixing it with biodegradable wastes.
- Sample S4: sewage sludge from a sludge treatment platform collecting sludge mostly from urban WWTPs and adding lime.
- Sample L: liming material derived from hydrolytic treatment (with lime and sulphuric acid) of sewage sludge collected by several WWTPs.

- Sample M1: swine manure slurry, from a swine farming facility.
- Sample M2: bovine manure slurry, from a bovine farming facility.
- Sample D: bovine manure digestate slurry derived from the anaerobic digestion of a mix of M2 and agricultural and food wastes.
- Sample C: compost from a composting plant that treats the organic fraction of municipal solid waste.

An aliquot of each solid, semi-solid or liquid substrate was mixed with distilled water at the 1:10 ratio (w/v) to obtain aqueous extracts with a nominal concentration of 100 g_{eq}/L, as described by Roig and colleagues (Roig et al., 2012).

2.2. Chemical characterization of aqueous extracts

The following parameters were measured to characterise aqueous extracts:

- Anions (Chloride, Nitrate, Sulphate), analysed through ion chromatography (US EPA, 1993).
- Metals (Calcium, Magnesium, Potassium, Sodium, Aluminium, Antimony, Arsenic, Beryllium, Boron, Cadmium, Copper, Iron, Lead, Manganese, Mercury, Nichel, Total Chromium, Vanadium, Zinc) determined by means of inductively coupled plasma mass spectrometer (UNI EN ISO 17294-2, 2016).
- Ammonia nitrogen, measured following the Italian Environmental Agency guidelines (APAT, 2003).

2.3. Plant-based tests

2.3.1. Allium cepa, Cucumis sativus, and Lepidium sativum seed germination and root elongation test

Seeds of *Allium cepa, Cucumis sativus* and *Lepidium sativum*, were placed on filter papers moistened with undiluted and diluted sample extracts at ratios of 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000, corresponding to concentrations of 100, 50, 10, 5, 1, 0.5 and 0.1 g_{eq}/L, respectively. After 72 h, the lengths of complete shoots (>1 mm) were measured, and the results were expressed as a percentage germination index (GI%). Negative controls were established using distilled water. For subsequent genotoxicity assessment in *L. sativum*, wherever feasible, the EC50, EC20, and EC10 values were calculated by probit regression with the confidence interval (CI) set at 95%.

2.3.2. Allium cepa bulb root elongation test

Twelve equal-sized commercial *A. cepa* bulbs were exposed for 72 h in the dark to extracts solutions undiluted and diluted at ratios of 1:2, 1:10, 1:20, 1:100, 1:200, and 1:1000, corresponding to concentrations of 100, 50, 10, 5, 1, 0.5 and 0.1 g_{eq}/L, respectively. The average root length (cm) was expressed as mean ± standard deviation (SD). The statistical analysis was performed by using Chi-square (χ^2) test, where $p < 0.05$ was considered significant. The EC50, EC20, and EC10 values were calculated by probit regression with the CI set at 95%. Macroscopic parameters such as changes in root consistency and color, presence of c-tumors, hooks, twisted roots, and flat/broken apices were also considered. A negative control was established using distilled water.

2.3.3. Lepidium sativum seedling single-cell gel electrophoresis (comet) test

L. sativum seeds were exposed to two sub-lethal doses of water extracts, corresponding to undiluted and 1:2 dilution or EC50 and ½EC50 (based on results of the toxicity test described in section 2.3.1). Negative and positive controls were performed using distilled water and methyl methanesulfonate (MMS, 10 mg/L), respectively. After 72 h, seedlings were collected in an ice-cooled dish. The tips were finely minced using a scalpel and then 500 μL of cold isolation buffer (200 mM Tris, 4 mM $MgCl_2{\cdot}6H_2O$, 0.5% Triton-X) was added. The nuclei were resuspended in low-melting agarose (LMA) to final concentration of 0.35% and distributed onto agarose pre-coated slides. Once solidified, the slides were immersed in cold buffer (1.5 mM Na_4EDTA, 30 mM NaOH, pH > 12.3) for 1 h of unwinding and 20 min of electrophoresis (0.8 V/cm), following Liman and colleagues (Liman et al., 2015) with some modification. The DNA was stained with 75 μL of GelRed Nucleic Acid Gel Stain (Biotinum) and fifty nuclei were examined under a fluorescence microscope (Olympus CX 41RF, Japan) for each slide (two slide per condition). The median value of the tail intensity (TI) values recorded per slide was used as the measure of the central value of the comet scores. The results per condition were expressed as the mean TI (±SD). The statistical analysis was performed by using ANOVA univariate and Dunnett's multiple comparison test, where $p < 0.05$ was considered significant.

2.3.4. Allium cepa bulb chromosomal aberrations test

Following pre-germination for 48 h in Rank's solution, the onion bulbs were exposed for 24 h to increasing doses of aqueous extract from each sample (EC50, EC20, and EC10), based on the results of the toxicity test (described in section 2.3.2). Positive and negative controls were performed using maleic hydrazide 10 mM (6-h exposure) and Rank's solution (72-h exposure), respectively. After exposure, the roots were cut, fixed in 1:3 acetic acid–ethanol for 24 h and stained with 2% acetic orcein. The root tips (3 mm long) were assessed for the mitotic index (MI) (5000 cells) and chromosomal aberration (CA) frequency (1000 cells in mitosis) (Cabaravdic, 2010). The statistical analysis was performed by using χ^2 test, where $p < 0.05$ was considered significant. The Mitotic Index Alteration (%MIA) was calculated to determine the percentage of cells undergoing mitosis in a specific concentration compared to the control sample (Tzima et al., 2022). The different types of aberrations were classified and divided into three main categories: fragments, rings, sticky chains, and bridges as direct DNA damage (DDD); laggards, binucleation, polar slips, multipoles, and c-mitosis as mitotic spindle defects (MSD), and buds as genic amplification (GA).

2.4. Salmonella/microsome mutagenicity (Ames) test

The TA98 and TA100 strains of *Salmonella typhimurium* were employed to detect frameshift and base-substitution mutations, respectively. The tests were conducted at doses of 10, 1, 0.1, 0.01 mg_{eq}/plate both with and without in vitro exogenous metabolic activation (±S9 mix). Negative controls consisted of distilled water. Strain-specific positive controls were employed: 2-nitrofluorene for the TA98-S9 strain (10 μg/plate); sodium azide for the TA100-S9 strain (10 μg/plate); and 2-aminofluorene for both strains with S9 (20 μg/plate).

The results were expressed using the mutagenicity ratio (MR). This was calculated by dividing the mean number of revertant colonies per plate for each sample by the mean number of revertant colonies per plate in the negative control, which represents the spontaneous mutation rate.

2.5. Human cell-based tests

2.5.1. Human hepatoma cell line HepG2 cytotoxicity (MTS) test

The cytotoxicity of HepG2 cells exposed to water extracts was determined with the MTS tetrazolium-reduction assay (Cell Titer 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega). HepG2 cells were seeded in 96-well plates at a density of 1×10^4 cells per well. The cells were then incubated in complete medium (DMEM high glucose, 10% foetal bovine serum, 2 mM L-glutamine and antibiotics) for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. Cells were treated with sample extracts diluted at concentrations of 50, 5, 0.5, 0.05 mg_{eq}/mL in serum-free medium and incubated for 3 h. After the incubation period, the treatment was discarded and replaced with 20% MTS in complete medium. The cells were then incubated again at 37 °C to allow for colour development, and after 3 h, the absorbance was recorded at 490 nm using a 96-well plate reader. The cell number was estimated from a calibration curve plotted from 0 to 1×10^4 cells per well (Fig. S1). The cell viability was evaluated by comparing the treated cells to the

unexposed cells. For subsequent genotoxicity assessment, wherever feasible, the EC50, EC20, and EC10 values were calculated by probit regression with the CI set at 95%.

2.5.2. Human hepatoma cell line HepG2 single-cell gel electrophoresis (comet) test

HepG2 cells were seeded in 6-well plates at a density of 2.5×10^5 cells/well and incubated in complete medium for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The culture medium was discarded and replaced with serum-free culture medium. Cells were exposed to increasing doses of aqueous extract from each sample for 3 h at 37 °C, based on the results of the toxicity test (described in section 2.5.1). Negative and positive controls were performed using distilled water and ethyl methanesulfonate (EMS, 2 mM), respectively. After treatment, cells were resuspended in LMA to final concentration of 0.6% and distributed onto agarose pre-coated slides. The slides were immersed in active lysis solution at 4 °C for 1 h. After that, slides were immersed in cold buffer (1 mM Na_4EDTA, 300 mM NaOH, pH > 13) for 20 min of unwinding and 30 min of electrophoresis (0.7 V/cm). The DNA was stained with 75 μL of GelRed Nucleic Acid Gel Stain (Biotinum) and seventy-five nuclei were examined under a fluorescence microscope (Olympus CX 41RF, Japan) for each slide (two slide per condition). The median value of the tail intensity (TI) values recorded per slide was used as the measure of the central value of the comet scores. The results per condition were expressed as the mean TI (±SD). The statistical analysis was performed by using ANOVA univariate and Dunnett's multiple comparison test, where $p < 0.05$ was considered significant.

3. Results

3.1. Chemical characterization

The aqueous extracts were chemically characterised for the presence of anions (NH_3–N, Cl^-, NO_3^-, SO_4^{2-}, PO_4^{3-}), cations (Ca^{2+}, Mg^{2+}, K^+, Na^+), and heavy metals (Al, Sb, As, Be, B, Cd, Cu, Fe, Pb, Mn, Hg, Ni, Cr, Va, Zn). Fig. 1 highlights the heterogeneity observed in the composition of the leachable fraction of the samples.

Samples S4, D, L and C were characterized by the highest nitrogen ammonia values (226.2, 198.4, 189.5, and 186.0 mg/L, respectively). Samples L and C, also, had the highest levels of calcium (648.9 and 252.3 mg/L, respectively), while samples D and C were found to contain the largest concentrations of iron (20.04 and 14.72 mg/L, respectively). Finally, extracts of samples C, M1, M2 and D exhibited the highest concentrations of zinc (1.48, 1.47, 1.21, and 0.99 mg/L, respectively). The extract of sample C is notable for its heterogeneous composition, which is the most diverse of all the samples. It exhibited the highest values of chloride (1381.74 mg/L) and phosphate (39.7 mg/L), as well as the highest concentrations of magnesium (101.5 mg/L), potassium (1653.6 mg/L), and heavy metals (aluminium [16.68 mg/L], zinc [1.48 mg/L], manganese and boron [0.86 mg/L], copper [0.46 mg/L]) in comparison to the other samples. Furthermore, sample C exhibited a minimal quantity of other elements, including nickel (0.11 mg/L), chromium (0.9 mg/L), arsenic (0.04 mg/L), vanadium (0.03 mg/L), and lead (0.02 mg/L), which contributed to its pronounced richness. The elements Sb, Be, Cd, and Hg are not displayed in Fig. 1 as they are below the limit of detection. The detailed data sets are shown in Table S1.

3.2. Toxicity and genotoxicity in plants

3.2.1. Effects of biogenic wastes on the seeds of Allium cepa, Cucumis sativus and Lepidium sativum

The germination and root elongation assays were conducted on seeds of *A. cepa* (monocot), *C. sativus*, and *L. sativum* (dicots), listed among the internationally recognized plant species for phytotoxicity evaluations (OECD, 2006). The results in Fig. 2 show the percentage of Germination Index (GI%). Detailed GI% values are provided in Table S2.

The germination of all three plant species was not affected by the three sludge samples (S1, S2, S3) and both the manure samples (M1 and M2), as indicated by the GI% values above 80% when undiluted. On the other hand, the other four samples (S4, L, D and C) caused a severe decrease in GI% in all plant species. More in detail, the phytotoxic effect varied among the three plants, with *C. sativus* being the least sensitive

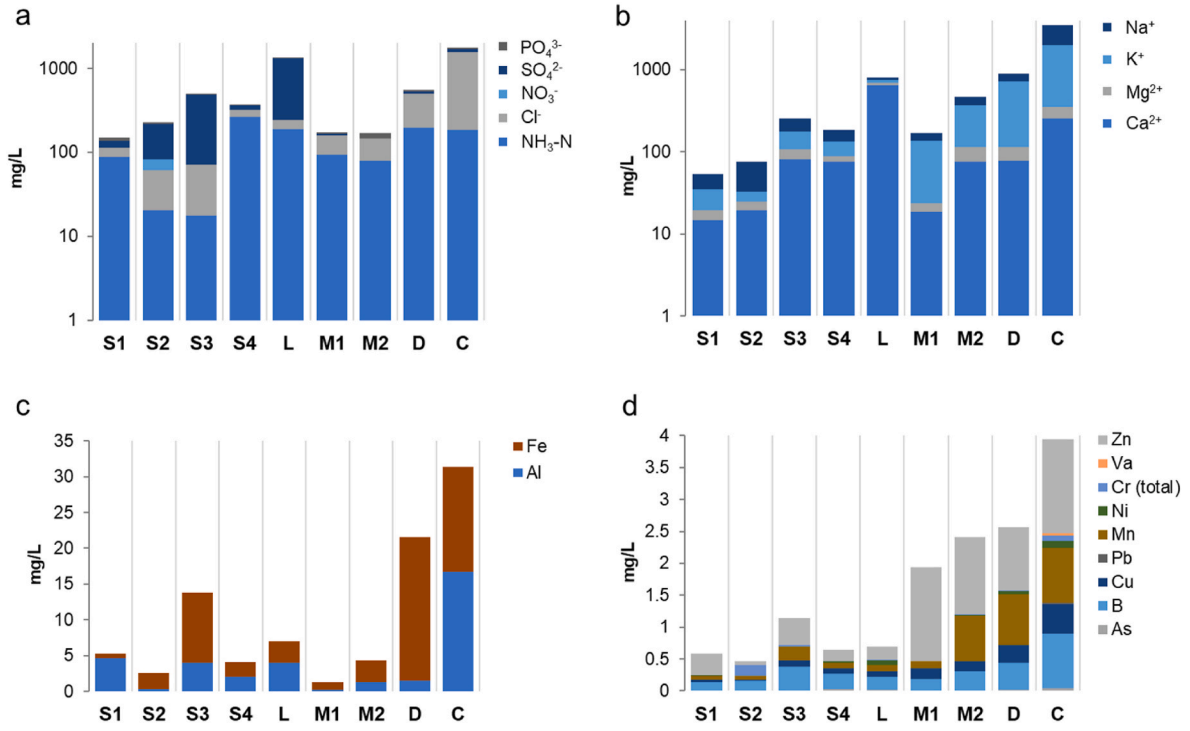

Fig. 1. Chemical characterisation of aqueous extracts of biogenic wastes. a) Anions b) Cations, c) Most abundant heavy metals (range 0.1–20 mg/L) and d) Less abundant heavy metals (range 0.01–4 mg/L). In panels a) and b), the concentrations are presented in logarithmic scale.

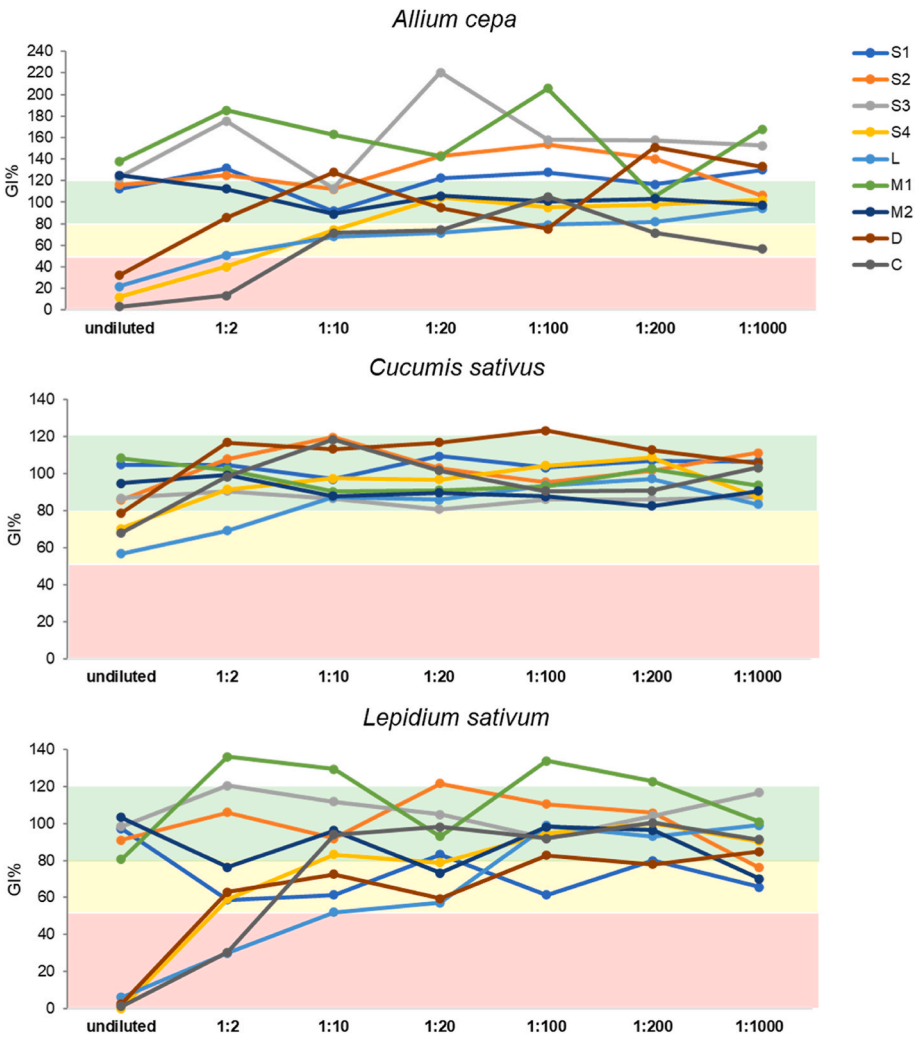

Fig. 2. The germination index percentage (GI%) was measured in *Allium cepa*, *Cucumis sativus*, and *Lepidium sativum* after treatment with aqueous extracts of biogenic wastes. The phytotoxicity range is highlighted by background colours according to GI% values: green for no phytotoxicity (GI%: 80-120%), yellow for moderate phytotoxicity (GI%: 50–80%), and red for strong phytotoxicity (GI%: <50%).

species. This was indicated by the majority of GI% values falling within the range of 80–120%. The most toxic samples (L, C, S4) caused a decrease in GI% of only between 50% and 80% at undiluted dose (and 1:2 dilution only for sample L), with a complete recovery (i.e., non-toxicity) along the serial dilutions. In the case of *A. cepa* seeds, samples S3, M1, and to a lesser extent, S2, did not exhibit phytotoxic effects and promoted germination and elongation of roots. This was demonstrated by the GI% values, which were well above 120% (Da Ros et al., 2018). In contrast, samples C, S4, L, and D caused a significant decrease in this parameter (<50%) when tested at an undiluted dose. Although sample D rapidly lost its toxicity when diluted by half, samples S4 and L required further dilution (1:20 to 1:100) to reduce and ultimately eliminate their toxic effects. In contrast, sample C was particularly effective in reducing germination and elongation performance, with the GI% well below 80% for all its dilutions except for 1:100.

Furthermore, the lowest GI% values were found in *L. sativum*. Also in this case, were samples C, S4, L, and D the cause of a comparable and severe decrease in this parameter (<10%) when tested at an undiluted dose. While sample S4 and D reduced their toxicity when diluted 1:2, samples L and C had to be further diluted (1:10 to 1:20) to obtain a decrease in the effects.

The sample S1 exhibits a distinctive effect on *L. sativum* in comparison to all other samples. It demonstrates no discernible influence from the dilution. Indeed, the GI% exhibits fluctuations within the range of 80-50% for all dilutions (from 1:2 to 1:1000).

Finally, the results of the test on *L. sativum* seeds were used to determine the sub-toxic doses for subsequent genotoxicity testing of DNA damage (refer to Table S3).

3.2.2. *Effects of biogenic wastes on the bulbs of Allium cepa*

The *A. cepa* bulbs root elongation test was conducted to evaluate the toxicity of the samples. Fig. 3 displays the lengths of *A. cepa* roots after 72 h of exposure to undiluted and diluted aqueous samples. Except for sample S2, all extracts inhibited root growth to varying degrees. Some samples, such as S3 and M1, only affected root growth at the undiluted dose, while others, such as S1, C, S4, and L, caused a significant reduction in root length even at a dilution of 1:20.

Moreover, the root length was significantly reduced by only samples S4 and L at a high dilution of 1:100.

In addition to the main endpoint, some morphological parameters of onion roots, such as colour, turgescence, root shapes were also considered (Table S4). All samples triggered the root shape of “hooks”, at least at the highest concentration (undiluted solutions). An exception was observed with samples S4 and L at higher concentrations (from undiluted to 1:2 or 1:20, respectively), as the roots were not evaluable. This was most likely due to the high toxicity of these samples. Less frequent, even if significant, were changes in the colour and the formation of c-tumors.

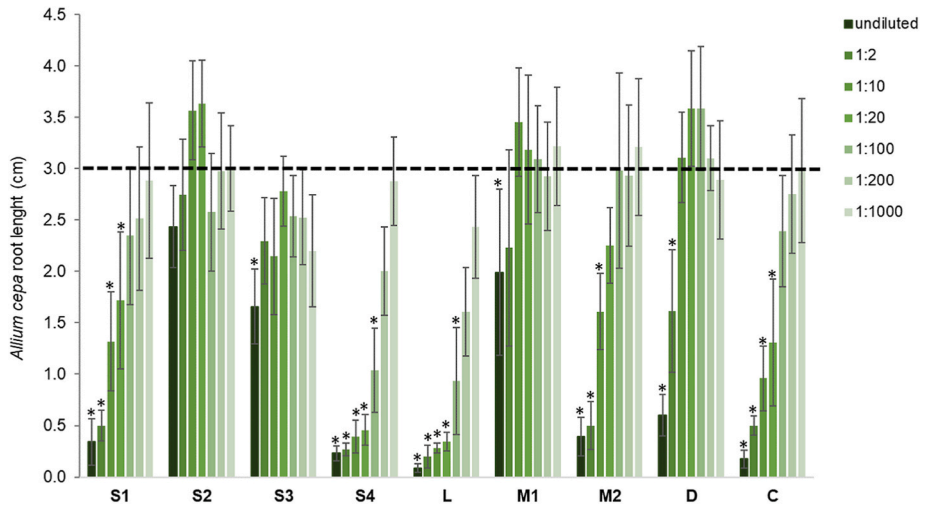

Fig. 3. Toxicity of aqueous extracts of biogenic wastes in *Allium cepa* bulbs, in terms of root elongation (mean ± SD). Dotted line represents the root length of negative control (mean value 3.03 ± 0.29 cm). Statistically significant versus negative control according to χ^2 test: *p < 0.05.

Finally, the elongation test on bulbs of *A. cepa* enabled the determination of sub-toxic doses (Table S5) for subsequent genotoxicity testing of chromosomal aberration assessment.

3.2.3. Genotoxicity on the seedlings of Lepidium sativum

The extent of damage to the DNA was evaluated in seedlings of *L. sativum* after exposure to sub-lethal doses of water extracts. Samples S1, S2, S3, M1 and M2 were tested without dilution and with a half dilution, whereas for samples S4, L, D, and C, the doses corresponded to the EC50 and half dilution of EC50. The results expressed as tail intensity (TI) are reported in Fig. 4. All samples exhibited a significant ability to increase the DNA damage, with the majority (S1, S2, S4, M2, D, and C) demonstrating a dose-response relationship.

The sole exception was represented by sample M1, which cause a damage that was not statistically significant.

3.2.4. Genotoxicity on the bulbs of Allium cepa

Genotoxicity was also evaluated in terms of chromosomal aberrations (CAs) on the bulbs of *A. cepa* exposed to sub-lethal doses of extracts (Fig. 5). The proliferation status of the cells did not appear to be affected by the samples tested, as shown by the percentage of the Mitotic Index Alteration (MIA%) (Tzima et al., 2022), which ranged from 72.4% of sample L at EC20 to 117.2% of sample C at EC20 (Fig. 5a).

Samples S1, S3, S4, L, M1, M2, D and C were observed to elicit an increase in the frequency of CAs, with some of them exhibiting a dose-response relationship (S1, S3, S4, M2). With respect to sample D, it was observed that an increase in chromosomal alterations occurred exclusively at the highest dose (14.0 ± 5.5 mean total CAs).

Samples S4 and L exhibited the greatest genotoxic potential, causing the highest number of CAs (23.2 ± 1.3 and 28.2 ± 5.6 mean total CAs, respectively) with the lowest EC50 (EC50 = 0.8 and 0.6 g_{eq}/L, respectively) among all the tested samples (see Table S5 for details). Finally, sample S2 did not cause any significant increase in CAs (Fig. 5b).

The different CAs were classified (fragments, rings, sticky chains, bridges, laggards, binucleation, polar slips, multipoles, c-mitosis, and buds) and grouped into three types: direct DNA damage (DDD), mitotic spindle defects (MSDs), and genic amplification (GA) (Fig. 5c). The mitotic spindle defects were strongly caused by sample S3 and D. The direct DNA damage was mainly caused by sample L at the highest dose (52.5%), followed by samples S1, S4 and C (40.0%, 37.3% and 31.2%, respectively). The detailed results of the CAs classification are presented in Table S6.

3.3. Mutagenicity of biogenic waste in bacteria

None of the samples induced a mutagenic effect on both

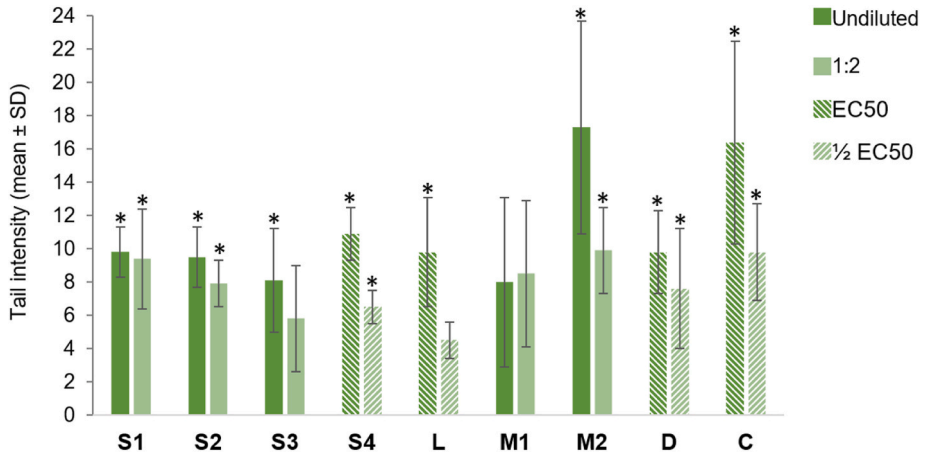

Fig. 4. DNA damage of aqueous extracts of biogenic wastes in *Lepidium sativum* seedlings expressed as tail intensity (mean ± SD). Fifty nuclei examined for each slide (100 nuclei per condition). Negative control: TI = 2.4 ± 1.0; Positive control: TI = 25.3 ± 0.9. Statistically significant versus negative control according to Dunnett's test: *p < 0.05.

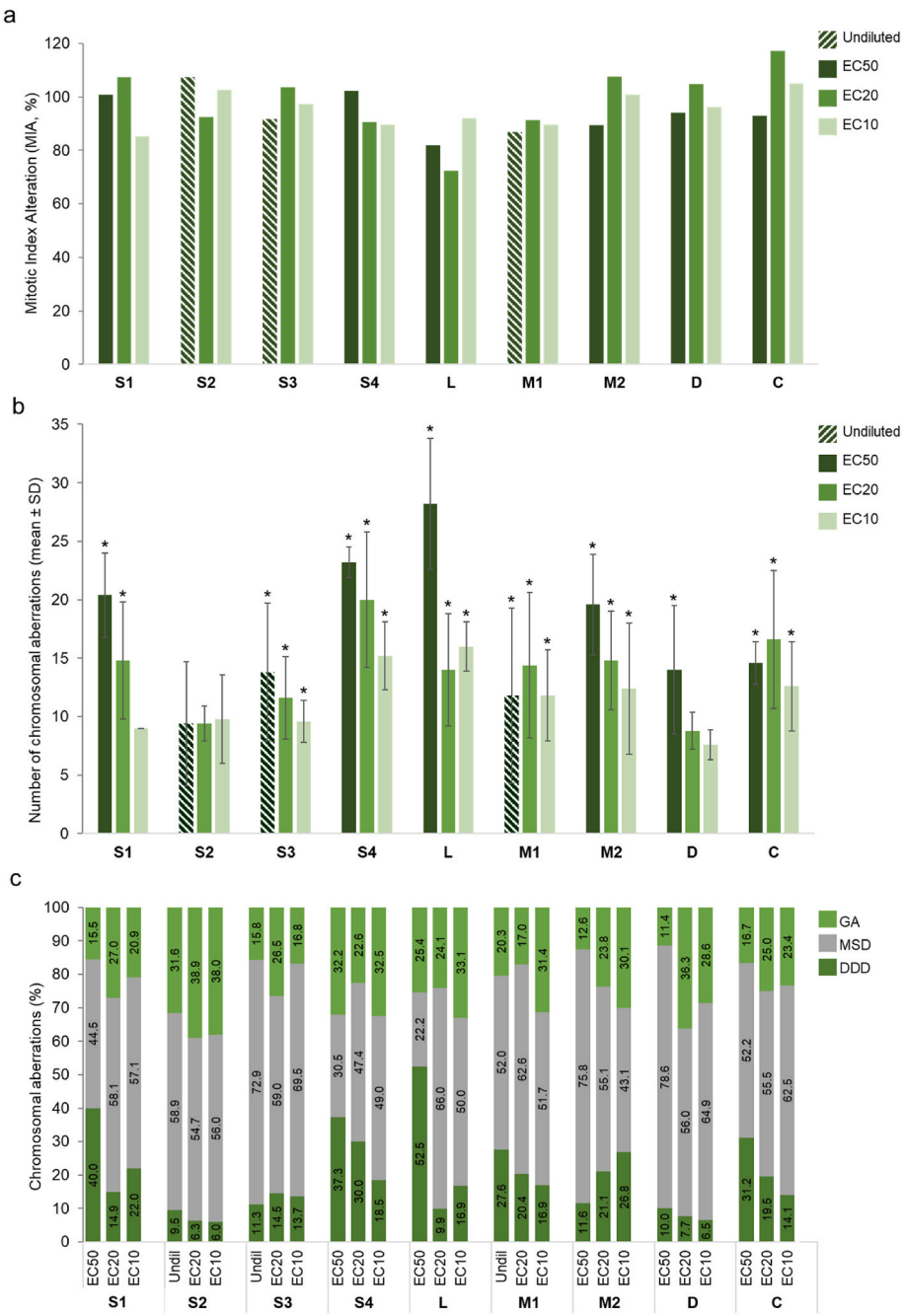

Fig. 5. Genotoxicity of aqueous extracts of biogenic wastes in *Allium cepa* bulbs. a) Percentage of the Mitotic Index Alteration (MIA%), b) Frequency of chromosomal aberrations (mean ± SD) and c) Distribution of the three categories of chromosomal aberrations: direct DNA damage (DDD), mitotic spindle defects (MSD), and genic amplification (GA). Statistically significant versus negative control according to χ^2 test: *$p < 0.05$.

S. typhimurium TA98 and TA100 strains tested with or without metabolic activation (±S9 mix). Results expressed as mean revertant number ± SD are outlined in Tables S7 and S9, while data expressed as mutagenicity ratio are presented in Tables S8 and S10.

3.4. Toxicity and genotoxicity of biogenic wastes on human cells

The toxicity of the aqueous extracts was evaluated using an MTS-based assay on the human hepatoma cell line HepG2. The results did not indicate any toxic effects of the samples (Table S11). Consequently, the samples were subjected to genotoxicity testing in terms of DNA damage on HepG2 cells at undiluted and half-diluted doses. The results of the comet assays are shown in Fig. 6. Samples S4, L, D, and C were found to significantly damage the DNA of cells at the highest dose, with two of them (L and C) causing an increase in the DNA damage even when diluted. The most adverse sample was C, which caused the highest impact on HepG2 cells, with $TI_{undil} = 33.9 \pm 5.7$ and $TI_{½dil} = 16.6 \pm 0.0$.

4. Discussion

The toxicological and genotoxicological properties of nine water extracts derived from sewage sludge and other biogenic wastes were characterised using a battery of assays based on plant, bacterial and human cell models. The findings revealed that all of the samples exhibited some degree of toxicity and/or genotoxicity in plant or human cells, while no evidence of mutagenicity was observed in bacterial

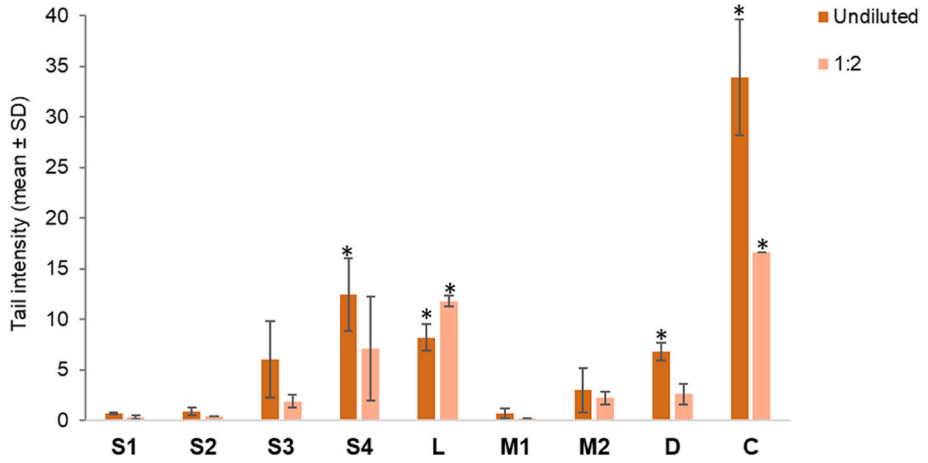

Fig. 6. DNA damage of aqueous extracts of biogenic wastes in human cell line HepG2 expressed as tail intensity (mean ± SD). Seventy-five nuclei examined for each slide (150 nuclei per condition). Negative control: TI = 0.7 ± 0.3; Positive control: TI = 22.5 ± 6.6. Statistically significant versus negative control according to Dunnett's test: *p < 0.05.

cultures.

Four samples were characterized by elevated levels of toxicity towards plant and demonstrated evidence of genotoxicity in both plant and human cells. These were the sewage sludge from a sludge treatment platform (sample S4), the liming material derived from hydrolytic treatments of sewage sludge (sample L), the bovine manure digestate mixed with agricultural and food waste (sample D), and the compost from the organic fraction of municipal solid waste (sample C). Among the multitude of compounds present in each sample, it is plausible to suggest that the phytotoxicity could be caused by the levels of ammonia nitrogen in the water extracts of these four samples (266.2, 189.5, 198.4, 186.0 mg/L, respectively). Indeed, ammonia toxicity has been shown to interfere with seed germination and development (Makaza and Khiari, 2023).

No other chemical parameter exhibits comparable trends among the four samples. However, if the data for samples D and C are considered separately, it is evident that these two samples contain the highest concentrations of multiple parameters (Cl^-, K^+, Na^+, B, Cu, Fe, Mn). A further consideration is warranted with regard to sample C, which is the richest of the samples in terms of both chemical quality and quantity. The phytotoxicity of compost from municipal wastes has been frequently associated to high concentrations of heavy metals, such as Cu, Ni, Zn, Pb (Siles-Castellano et al., 2020).

Nevertheless, it is of the utmost importance to approach chemical analyses with a critical eye. Despite the multitude of parameters that may be considered, it is evident that such analyses are inherently incomplete. This makes attributing an observed effect to a specific element a challenging, if not impossible, task. In light of these considerations, the necessity for a battery of biological assays becomes evident.

It is important to note that there are differences in the root growth response between monocots and dicots. Therefore, it is essential to consider test plants belonging to both botanical groups. Czerniawska-Kusza and colleagues observed different responses to sediment samples between monocot *Sorghum saccharatum* and dicots *Sinapis alba* and *L. sativum*, ranging from growth inhibition to growth stimulation. Of these, the monocot *S. saccharatum* exhibited the greatest sensitivity (Czerniawska-Kusza et al., 2006). Despite *C. sativus* being described as a sensitive organism, often the most sensitive in a battery (Visioli et al., 2014; Wang et al., 2001), it was surprisingly resilient when tested on these biogenic wastes. Differently, in this study, the dicot *L. sativum* emerged as the most sensitive plant of the battery. Its recognized high sensitivity to contamination, including heavy metals, petrochemicals and polycyclic aromatic hydrocarbons (Bożym et al., 2021) has made *L. sativum* an excellent plant model, frequently used either as a standalone or in conjunction with other plants or organisms, for the evaluation of the effects of biogenic waste, including sewage sludge (Mañas and De las Heras, 2018), digestates (Alias et al., 2022; Lencioni et al., 2016), and composts (Barral and Paradelo, 2011; Siles-Castellano et al., 2020). It is of note that these studies also ascribed phytotoxic properties to digestate and composts of an urban origin when tested on *L. sativum*. One noteworthy attribute of utilising *L. sativum* seeds is the potential to subsequently evaluate toxicity and genotoxicity through exposing the same organism to a chemical agent or a mixture. In the absence of toxicity, this approach could prove a more convenient and more rapid method of studying the ecotoxicological impact of a sample, as demonstrated in recent studies on bismuth (Passatore et al., 2022) and concrete leachates containing steel slags (Alias et al., 2023b).

Another important plant model used in this study is the monocot *A. cepa*, which offers a number of distinctive features, including the potential for two vegetative forms: seeds and bulbs. These forms exhibit varying degrees of sensitivity and response to contaminants as the result of distinct physiological states of the *A. cepa*. In addition, the dimension and the number of *A. cepa* chromosomes (2n = 16) allow the analysis of cytotoxicological (mitotic index) and genotoxicological parameters (chromosomal aberrations, micronuclei, DNA damage). All these characteristic have made onion a good model to determine the ecotoxicity and genotoxicity of a wide range of contaminants, as discussed elsewhere (Alias et al., 2023c, 2024b; Iqbal et al., 2019), including the impact of nitrogen fertilizers applied in agriculture (Arora et al., 2014; Verma et al., 2016).

Differently from the sentinel species, which are specific and representative of a particular habitat, the *A. cepa*, as well as the aforementioned *L. sativum*, are standardisable models that permit the investigation of a plethora of endpoints of toxicity and genotoxicity, even subsequently on the same organism. The results of these investigations should be regarded as indicators for the other organisms interrelated with the environment (i.e. bioindicators).

The four samples identified as the most toxic by the phytotoxicity assay (S4, L, D, C) and among the most genotoxic on both *L. sativum* and *A. cepa* bulbs were also found to be the sole samples capable of damaging the DNA of the human cells HepG2. This observation provides further evidence to support the idea that plant-based tests, demonstrating a concordance with animal systems, including humans (Tedesco and Laughinghouse IV, 2012), are a powerful tool for toxicological evaluations. This concordance between the outcomes of germination and elongation tests and those yielded by human cell-based genotoxicity assessments may indicate that the evaluation of phytotoxicity in aqueous extracts could serve as a rapid and cost-effective screening

instrument for comparing diverse biogenic materials in terms of their prospective use on agricultural lands. However, it is of utmost importance to exercise caution when designing a battery of bioassays to prevent any potential for underestimating the toxic or genotoxic capacity of a given sample. As evidenced by the results of the plant-based genotoxicity assays (comet assay on *L. sativum* and evaluation of chromosomal aberrations on *A. cepa*), numerous other samples were observed to interact with the genomic material, resulting in damage at varying degrees. A robust and reliable battery of bioassays should include a variety of tests targeting different endpoints. For these reasons, the plant-based toxicity and genotoxicity tests presented here can be considered an effective combination of assays for assessing the impact of biogenic-origin matrices in the form of aqueous extract. Moreover, since biotests can be applied to assess the behaviour of both pure chemicals and mixtures to target specific substances, in this case they were carried out for the evaluation of the effects of a mixture "*in toto*". This was a crucial aspect of the approach applied, as it enables the identification of biological effects associated with interactions between living organisms and undetectable substances, as well as the assessment of synergistic or antagonistic effects of molecules, toxicokinetic and toxicodynamic phenomena, independently of the chemical characterisation of a given sample. In addition, it is possible to estimate the effects of complex mixtures more realistically on the basis of the biological results than by chemical testing alone.

These considerations supported the decision to analyse the biogenic matrices of interest in the form of aqueous extracts, which are the most reliable representation of the solution that would be produced in the natural environment, as a result of water leaching of soil contaminants, in comparison to other extraction techniques. Furthermore, water extraction offers the additional benefit of enabling the same aqueous extract to be used for ecotoxicity assessment in a wide range of model organisms, including *Aliivibrio fischeri*, *Daphnia magna*, *Pseudokirchneriella subcapitata* and *Danio rerio* (Alias et al., 2024a; Della Torre et al., 2022). Additionally, it is in line with green chemistry principles (Ražić et al., 2023). The multi-tiered battery of bioassays proposed within this project included tests to be carried out on raw samples without pre-treatment, as well as on eluates and organic solvent extracts. The protocol we developed and followed within the SLURP project specifically included performing tests on aqueous extracts to simulate the actual bioavailability of various substances to organisms.

The selection of doses to be tested is a critical factor in the assessment of toxicity and genotoxicity. Standard practice involves treating test organisms with a wide range of doses, starting from the highest achievable concentration. In this study, a concentration of 100 g/L was used for the aqueous extracts. Conversely, it is crucial to assess the potential toxicity of a compound or mixture, as excessive toxicity can hinder the detection of genotoxic effects and may lead to erroneous data. Still, it should be noted that the various toxicological assays were performed according to the prescriptions of the respective methods. Consequently, certain dilutions can diverge from the actual conditions that occur during the application of biogenic waste in agriculture. This issue poses significant challenges in predicting the actual biological responses following exposure to complex matrices. The criticality of this problem becomes even more pronounced when attempting to compare the effects on different living organisms, measured using entirely different assays. To address this gap, within the SLURP project, as described in Bertanza et al. (2024) specific criteria were developed to translate test results in order to better simulate the real field application conditions.

5. Conclusions

This research provided comparative data on various matrices used in agriculture, focusing on the fraction of substances that can dissolve in soil water. The findings suggest that certain toxic and genotoxic effects may be linked to biogenic matrices generally considered posing low-risk (widely used in farming and, interestingly, generally seen as socially acceptable) such as compost and manure, whereas matrices often viewed as more hazardous, like sewage sludge (whose agricultural application is strictly regulated) showed limited interaction with the test organisms.

As part of the multilevel project "SLURP," the results of this study will be integrated with data from additional investigations, including endpoints from different organisms and extensive analyses of chemical, physical, microbiological properties and microplastics content (Bertanza et al., 2024; Magni et al., 2024).

This study demonstrated that the applied battery of bioassays is appropriate for evaluating the toxic behaviour of biogenic matrices, such as sludge, compost, and manure.

A reclassification of the toxicity of samples, based on their practical application rates in agricultural contexts, will be developed to achieve a more realistic assessment of their environmental impact.

This integrated approach aims to provide the most comprehensive evaluation of the suitability of these biogenic matrices for reuse in agriculture. By doing so, the study seeks to highlight key regulatory and operational considerations, offering valuable insights to guide future decision-making processes.

CRediT authorship contribution statement

Carlotta Alias: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Donatella Feretti:** Writing – review & editing, Validation, Supervision, Conceptualization. **Ilaria Zerbini:** Writing – review & editing, Visualization, Investigation, Formal analysis, Data curation. **Roberta Pedrazzani:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **Marta Domini:** Writing – review & editing, Project administration. **Giorgio Bertanza:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Alessandro Abbà:** Writing – review & editing. **Achille Amatucci:** Writing – review & editing. **Andrea Binelli:** Writing – review & editing. **Sara Castiglioni:** Writing – review & editing. **Catarina Cruzeiro:** Writing – review & editing. **Luca Del Giacco:** Writing – review & editing. **Camilla Della Torre:** Writing – review & editing. **Alberto Diana:** Writing – review & editing. **Marco Fossati:** Writing – review & editing. **Gianni Gilioli:** Writing – review & editing. **Stefano Magni:** Writing – review & editing. **Giovanna Mazzoleni:** Writing – review & editing. **Michele Menghini:** Writing – review & editing. **Silvia Schiarea:** Writing – review & editing. **Silvia Signorini:** Writing – review & editing. **Peter Schröder:** Writing – review & editing. **Anna Simonetto:** Writing – review & editing. **Nathalie Steimberg:** Writing – review & editing. **Vera Ventura:** Writing – review & editing. **Simona Vezzoli:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2025.144175.

Data availability

Data will be made available on request.

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