sustainability

MDPI

Article

The Comet Assay as a Sustainable Method for Evaluating the Genotoxicity Caused by the Soluble Fraction Derived from Sewage Sludge on Diverse Cell Types, Including Lymphocytes, Coelomocytes and *Allium cepa* L. Cells

Monica Adriana Costea, Cristina Adriana Rosan *, Vasile Laslo, Eliza Agud *, Cornelia Purcarea and Simona Ioana Vicas

Faculty of Environmental Protection, University of Oradea, 26 Gen. Magheru Street, 410048 Oradea, Romania; mcostea@uoradea.ro (M.A.C.); vasile.laslo@uoradea.ro (V.L.); cpurcarea@uoradea.ro (C.P.); svicas@uoradea.ro (S.I.V.)

* Correspondence: crosan@uoradea.ro (C.A.R.); eliza.agud@uoradea.ro (E.A.)

Abstract: With the expansion of urban areas, the amount of sludge produced by sewage treatment plants is increasing, raising big problems regarding the reintroduction of this sludge into nature in order to fully solve the wastewater problem. The application of sludge to agricultural surfaces or degraded land is a controversial solution since, despite the well-known benefits, sludge can, in certain cases, represent a real threat to both human health and the environment, with long-term harmful effects. The present study evaluates the potential genotoxicity of sludge using the Comet Test and three cellular bioindicators (lymphocytes, coelomocytes, and *Allium cepa* L.) for its quantification. To perform the tests, the soluble fraction of the sludge was used at concentrations of 25%, 50%, 75%, and 100%, as well as a negative control (H_2O) and a positive control (H_2O_2). The Comet test indicated an increase in DNA damage among cells exposed for 4 h in the following order: coelomocytes, lymphocytes, and *Allium cepa* L. cells. Our results indicate that *Allium cepa* L. nuclei are more sensitive, with genotoxic effects being evident at concentrations as low as 25%. In coelomocytes, we recorded nuclear damage starting at a concentration of 75%. These results indicate the necessity of using multiple genotoxicity tests, combined in a test battery, to achieve a greater level of relevance. The concentration of the soluble fraction of the sludge has an inverse relationship with the auxin content in leaves and roots, suggesting varying levels of stress. The results of this study can contribute to the creation of a genotoxic profile of sewage sludge, facilitating decisions related to reducing its negative impact.

Keywords: Comet assay; DNA damage; sewage sludge; lymphocytes; coelomocytes; *Allium cepa* L.; bioindicators

Citation: Costea, M.A.; Rosan, C.A.; Laslo, V.; Agud, E.; Purcarea, C.; Vicas, S.I. The Comet Assay as a Sustainable Method for Evaluating the Genotoxicity Caused by the Soluble Fraction Derived from Sewage Sludge on Diverse Cell Types, Including Lymphocytes, Coelomocytes and *Allium cepa* L. Cells. *Sustainability* **2024**, *16*, 457. https://doi.org/10.3390/su16010457

Academic Editor: Agostina Chiavola

Received: 8 December 2023
Revised: 26 December 2023
Accepted: 2 January 2024
Published: 4 January 2024

1. Introduction

The amount of wastewater produced by urban agglomerations and industrial activities is constantly increasing, at the same time increasing the amount of sludge resulting from the purification of these waters [1]. The properties of sludge depend on the concentration of pollutants in wastewater and the specific treatments applied to it [2,3]. Sludge contains compounds with high agricultural value (organic matter, nitrogen, phosphorus, potassium, and, to a lesser extent, calcium, sulfur, and magnesium) [4,5]. When applied on agricultural surfaces, it can partially substitute for classic fertilizers [4,6,7], and, at the same time, it can improve the physical characteristics of the soil (density, porosity, permeability, water retention capacity, etc.) as well as its chemical characteristics (pH and cation exchange) [8]. Worldwide, the use of sludge in agriculture is considered a way of reducing environmental pollution and contributing to the circular economy, specifically via using sludge as a

fertilizer and soil improver [9]. The amount of sewage sludge produced in Europe (EU27) in 2020 was estimated to be over 13 million tons of dry matter [10].

Even if the economic advantages of using sludge as fertilizer are obvious in the current energy context, the risks of its application should also be noted in the absence of proper management. These risks are related to the accumulation over time of potentially genotoxic compounds such as polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), organochlorine pesticides, and heavy metals [8,11,12], which can translocate into plants and then pass on throughout the food chain [13]. The presence of heavy metals in soil induces genotoxicity through different mechanisms: metal ions induce oxidative changes in free amino acids and proteins [14]; the binding of metal to the cell nucleus causes genotoxicity [15]; and metal-mediated oxygen induces genotoxicity in plants [16].

Some studies have reported on plant growth inhibition mediated by heavy-metal stress, especially in the early stages of plant growth [17,18], while others [19,20] have demonstrated favorable plant yield responses to sewage sludge application. A decrease in soil microbial biomass and enzymatic activities due to the application of sewage sludge was observed in [8,21]. In sewage sludge, Hg and Pb frequently appear, both being metals responsible for the generation of very toxic oxygen species, such as the superoxide radical (O^{2-}), the hydroxyl radical ($OH^{\bullet -}$), and hydrogen peroxide (H_2O_2) [18,22]. They act directly on membrane lipids and proteins, or indirectly, damaging the genome by degrading nitrogenous bases, generating single- and double-strand breaks, and inducing DNA–protein crosslinks [23]. The different types of DNA damage induced by heavy metals, through ROS activity, affect the stability of the genome by affecting replication and transcription [24–26].This fact leads to different physiological effects, including reduced protein synthesis and damage to the cell membrane and to proteins involved in photosynthesis that ultimately negatively affect plant growth and development.

The spread of mutagenic and genotoxic agents in aquatic ecosystems [27,28] has an adverse effect on aquatic animals, posing a significant risk to human health. The evaluation of genotoxic contamination in the aquatic environment can be conducted by using the Comet test and by using various aquatic organisms as biosensors [29]. The toxicity and bioavailability of metals in the aquatic environment are affected by various abiotic factors, including pH, water hardness, alkalinity, and the accumulation of humic substances. A significant correlation exists between these parameters and the accumulation of metals in the biota.

The heavy metals responsible for soil genotoxicity, when sewage sludge is applied for a long time, are arsenic (As), barium (Ba), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) [19,30,31]. The mobility of heavy metals in soil is conditioned by some of the latter's characteristics, such as texture and pH, and by the organic complexation of the metals [32]. Increased mobility of heavy metals is associated with their increased absorption by plants or with their leaching into underground water [33].

The concentration of heavy metals (including As, Pb, Cd, and Cr) in plants varies depending on the species [34]. In one study, treatment with sewage sludge did not significantly affect the uptake of heavy metals by *Loropetalum chinense* var. *rubrum* but significantly affected heavy metal uptake by *Dendranthema morifolium* and *Viola tricolor* [35]. Henning et al. (2001) [36] reported that *Zea mays* plants grown on a mixture of soil and sludge showed high levels of Pb, Cu, and Zn in their tissues. Another study revealed that heavy-metal accumulation occurs in plants grown in soil mixed with sewage sludge [37]. The reductions in the concentrations of Pb, Cd, Hg, and Zn in soil mixed with sludge after harvesting *Solanum lycopersicum* plants were 39.38%, 47.93%, 6.18%, and 49.89%, respectively [38]. According to Emamverdian et al. (2015) [39], lead is easily absorbed by plants through the roots but is translocated only in small amounts, while cadmium, although not essential for plants, is easily absorbed and accumulates easily in plant tissues.

Results have demonstrated that by interfering with essential metabolic processes like photosynthesis, heavy metals cause a decrease in endogenous auxin levels, which, in turn, slows down shoot and root growth processes [40,41]. Exogenous auxin supplementation mitigates growth inhibition by enhancing heavy metal tolerance, as demonstrated by Bücker-Neto [42]. Several investigations have provided evidence for the significant contribution of auxins in the production of enzymatic and non-enzymatic antioxidants to combat nitro-oxidative stress caused by metalloids or other abiotic factors. According to Zhang [43], auxins are a class of phytohormones that play important roles in the life cycle of plants and form part of a hormonal signaling network, and they affect how plants react to stressors.

In the European Union, the use of sewage sludge in agriculture is regulated by Directive 86/278/CEE [44].

Before application, the chemical composition of sewage sludge should be determined [4]. The complexity of the corresponding mixture greatly increases the costs of determinations. The determination of the genotoxic potential of sewage sludge through relatively simple and cheap tests such as the Comet assay can provide important information about the genotoxic potential of sludge.

The in vitro assessment of the genotoxic effects of different agents using human cells allows for a good extrapolation to the state of human health [45], and conducting the same procedure using plant cells allows a more accurate assessment of the health of the environment [46–48]. Earthworms are good environmental indicators, as they are present in the soil in large numbers and respond to a variety of environmental and ecological factors, including alterations in soil chemistry as well as in forestry and agricultural practices [49,50]. Verschaeve and Gilles (1995) [51] used, for the first time, coelomocytes obtained from earthworms of the species *Eisenia fetida* for the detection of genotoxic compounds in soil, and, later, Salagovic et al. (1996) [52] proposed the use of the Comet assay and *coelomocytes* to monitor pollution in terrestrial ecosystems. Earthworm coelomocytes represent a very suitable bioindicator for pollution monitoring because they are exposed to genotoxicity through dermal uptake/diffusion and the ingestion of soil pollutants. Three main subpopulations of coelomocyte have been proposed, namely, eleocytes, granulocytes, and amoebocytes, but this distinction is still under debate due to the different stages of cell functioning [53].

Several studies have shown the a negative impact of sewage sludge on soil organisms, particularly regarding the physiology, activity, and diversity of earthworms [54–57]. On the other hand, other studies have shown that there is a good correlation between the results of bioassays performed on plant and animal cells [58].

The comet assay was first proposed by Ostling and Johanson in 1984 [59]. They demonstrated that under neutral conditions, DNA fragments from nuclei migrate to an anode. This version of the comet test detects only DNA double-strand breaks, which are characteristic effects of radiation and radiomimetic agents [60]. This technique was later improved by Singh et al. [61], who found that alkaline conditions increased the sensitivity, specificity, and reproducibility of this method. This comet assay variant is widely employed and capable of identifying DNA single-strand breaks, alkali-labile sites, and cross-linked DNA within individual cells. This test is based on the ability of DNA breaks to relax supercoiling, allowing DNA loops to extend from the nuclear core (nucleoid) under an electric field to form a comet-like tail [62]. It is used in human-monitoring studies as a biomarker of exposure to agents that cause DNA damage [63] and in ecotoxicological studies for a variety of sentinel organisms [64].

Based on the above, in this study, the possible genotoxic potential of treated sludge from an urban wastewater treatment plant was investigated using cells of different origins, namely, from humans (lymphocytes), animals (coelomocyte), and plants (*Allim cepa* L.), in order to identify which of them are more sensitive and can be used as a bioindicator of environmental pollution. In addition, the effect of different sludge concentrations on the phytohormone auxin content was evaluated in barley leaves and roots.

2. Materials and Methods

2.1. Chemical Analysis of Sludge and Acquisition of the Soluble Fraction from Biosolids

The sludge was obtained from the Oradea sewage treatment plant (Romania), and the chemical analyses conducted on it were carried out in the WESSLING Hungary Kft laboratory accredited with the number NAH-1-1398, based on the accredited methods shown in Table S1.

The soluble fraction of the sludge (SFS) was obtained according to the recommendations of DIN procedure 38414-S4 (1984): 5 g of biosolid sample dried for 24 h at 105 °C was mixed with 50 mL of deionized water, and the mixture was shaken for 24 h at 25 °C (Orbital Shaker OS 20 Biosan, Riga, Latvia). The mixture was separated via centrifugation at 4500 rpm (EBA 20, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) for 30 min, and the suspension was then passed through a sterile 0.22 µm pore membrane filter (LABBOX, Labbox Labware, S.L Barcelona, Spain) to avoid microbiological contamination.

2.2. Isolation of Lymphocytes and Treatment with SFS

This task was performed according to the protocol described by Álvarez-Moya, C., and Reynoso-Silva, M., in 2015 [65], with minor modifications. A total of 100 µL heparinized blood + 400 µL RMPI 1640 (BIOWEST, Nuaillé, France) was gently mixed and deposited on 400 µL of Histopaque-1077 (SIGMA) without mixing the layers. This mixture was centrifuged for 20 min at 2000 rpm. At the interface between the 2 layers, a whitish layer of lymphocytes became visible. Using a micropipette tip (ISOLAB, Isolab Laborgeräte GmbH-Wertheim Deutschland, Germany), the layer of lymphocytes was collected and placed in a microcentrifuge tube with 500 µL of RMPI 1640 medium. After centrifugation for 10 min at 2000 rpm, the supernatant was removed, and the pellet was washed once more, after which it was taken up in 200 µL RMPI 1640. The lymphocytes (Figure 1a) were brought to a cell density of 2×10^5 (Bürker-Türk chamber, BRAND GMBH + CO. KG Wertheim, Germany) and exposed to the experimental variants (SFS 25%, 50%, 75%, and 100% and 200 µM of H_2O_2 (positive control) and H_2O (negative control) for 4 h.

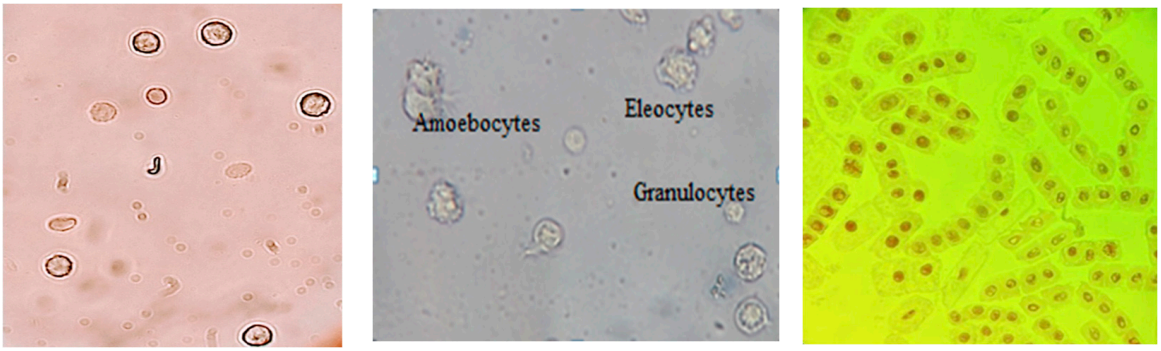
2.3. Isolation of Coelomocytes and Treatment with SFS

Earthworms were purchased from a company specializing in their culture for fishing (S.C. Maggot Fish S.R.L. Oradea, Romania). They belonged to the species *Eisenia fetida* (Annelida, Oligochaeta). To obtain coelomocytes (Figure 1b), we used a non-invasive method described by Eyambe GS et al. in 1991 [66]. Before extrusion, earthworms were washed with tap water at room temperature and placed on a paper towel overnight to allow them to empty their gut contents. Groups of 3 worms (approx. 15 g) were placed for 2 min in a Petri dish containing an extrusion medium (2 mL/individual) consisting of saline solution (95% *v*/*v*) and absolute ethanol (5% *v*/*v*) (S.C. CHIMREACTIV, S.R.L., Bucuresti, Romania), supplemented with 2.5 mg/mL of EDTA (Amresco Chemicals) and 10 mg/mL of the mucolytic agent guaiacol glycerol (1:1) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), adjusted to pH 7.3. The extruded cells were transferred to centrifuge tubes containing 6 mL of PBS (4 °C) (Roth), centrifuged at 8000 for 3 min, and washed again with 10 mL of PBS (4 °C). After washing, the cell density was determined using a Bürker-Türk counting chamber, and viability was determined via staining with trypan-blue 0.4% (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The cell density was 10^5 cells/mL. A total of 150 µL of cell suspension was exposed for 4 h to each of the experimental variants tested (SFS 25%, 50%, 75%, and 100% and 200 µM of H_2O_2 (positive control) and H_2O (negative control)) for 4 h.

2.4. Isolation of Allium cepa L. *nuclei and Treatment with SFS*

Bulbs of *Allium cepa* L. var. Alba de Buzău purchased from a local market, identified in the Vegetables Laboratory of the Faculty of Environmental Protection, and of approximately equal size were placed on the tops of 800 mL jars, with the root meristem submerged in water. They were kept in the dark at room temperature. After 5 days, the roots emerging

from the rhizogenic discs were about 4–5 cm long. The root discs together with the roots were detached from the bulb through a transverse section and then divided into 4 fragments (Figure 1c), which were immersed in the solutions with the experimental variants (SFS 25%, 50%, 75%, and 100% and 200 µM of H_2O_2 (positive control) and H_2O (negative control)) for 4 h. After exposure, the roots were washed and placed in a Petri dish with 400 mM of Tris-HCl buffer (AppliChem) at pH 7.5 (at an ice-cold temperature). The tips of roots measuring 1–1.5 cm long were fragmented with a fresh razor blade, and the isolated root nuclei were collected in 320 µL of 0.4 M Tris-HCl buffer (pH 7.5). This method ensures a low level of damage to the nuclei [67]. For all types of bioindicators, exposure to the different concentrations of SFS (soluble fraction of sludge) was performed for 4 h at 4 °C and in the dark.



(**a**) (**b**) (**c**)

Figure 1. The biological material used in the Comet assay: (**a**) lymphocytes; (**b**) coelomocytes; (**c**) cells from the root tips of *Allium cepa* L.

2.5. The Comet Assay

2.5.1. Slide Preparation

Slide preparation was performed according to the method reported by Singh (1996) [68] with minor modifications. Each slide was pre-coated with 1% normal-melting-point agarose (NMPA) (Sigma-Aldrich) and completely dried at room temperature. A total of 50 µL of 0.8% low-melting-point agarose (LMPA) (Thermo Fisher Scientific Inc., Waltham, MA, USA) kept at 37 °C was mixed with 50 µL of nuclear suspension and placed on top of the agarose layer, and a lamella was placed on top. The slides thus prepared were placed on ice for 10 min for solidification, after which the lamella was removed, and the slide was used in the next lysis step.

2.5.2. Lysis

The lymphocyte lysis solution contained 2.5 M of NaCl, 10 mM of Tris, and 100 mM of Na-EDTA plus 1% sodium lauryl sarcosinate (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) at pH 10.0. Immediately before use, 1% Triton X-100 (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) and 10% DMSO (dimethyl sulfoxide) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were added, with the role of capturing the radicals generated by hemoglobin iron. The lysis time was 1h, and lysis occurred at 40 °C and in the dark [69].

For lysis, slides with *Allium cepa* L. nuclei were immersed in freshly prepared, ice-cold lysis solution (1 M of NaCl and 30 mM of NaOH, 0.5% *w*/*v* SDS, pH 12.3) for 1 h at 40 °C in the dark [70].

Slides with coelomocytes were immersed in freshly prepared, ice-cold lysis solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, 10% DMSO, and 1% Triton X-100; pH 10.0) for 2 h [71].

2.5.3. Pre-Electrophoresis

The slides were placed in an electrophoresis unit filled with a freshly prepared alkaline buffer (300 mM of NaOH and 1 mM of Na EDTA) (Sigma-Aldrich) and placed at 40 °C. For lymphocytes, the despiralization time was 20 min; for coelomocytes, it was 20 min, and it was 30 min for *Allium cepa* L. nuclei [72].

2.5.4. Electrophoresis

A fresh, cold electrophoresis buffer (1 mM of Na_2EDTA and 300 mM of NaOH, pH 13) was prepared. Slides placed in an electrophoresis tank (SC PRECISA SRL, Sibiu, Romania) were exposed to 26 V (0.72 V/cm) at 300 mA for 25 min. After electrophoresis, the slides underwent three rinses using 400 mM of Tris (pH 7.5). They were then stained with 80 µL of ethidium bromide (20 µg/mL) (Amresco Inc., Cincinnati, OH, USA) for 10 min. Finally, the slides were washed with ice-cold water to eliminate the dye. All operations were carried out under red lights. Visualization was performed with a fluorescent microscope (BIOSYSTEMS Co., Ltd., Ulaanbaatar, Mongolia) equipped with an excitation filter of 515–560 nm, a barrier filter of 590 nm at a magnification of 200×, and a Microscope Digital Camera SP83. A minimum of 50 comets were analyzed per repetition using the software program CometScore2.00.38.

2.6. Determining 3-Indole Acetic Acid (IAA)

The *Hordeum vulgare* L. cv. Salamander seeds used originated from S.C. Totem SRL, Oradea, Romania, and were harvested in 2022. Following a washing procedure conducted using tap water, the items were disinfected by immersing them in a solution containing 0.15% mercuric chloride ($HgCl_2$) for 2 min. Subsequently, the seeds were rinsed 5 times in sterile distilled water.

Barley seedlings were obtained by germinating the seeds on an inert substrate moistened with solutions at the concentrations established in the experiment. After 14 days, root and leaf samples were collected in three repetitions, and the IAA content was determined. Data were analyzed in three independent replicates.

The procedure described by Goldschmidt et al. (1968) [73], with minor modifications, was used to obtain the auxin extract. Briefly, sterile plant tissue (160 mg) was cold-mortared with fine sand in 2 mL phosphate buffer (pH 6) and then centrifuged at 5000 rpm for 5 min. For the determination of IAA, 2.0 mL of Salkowski's reagent (1.0 mL of 0.5 M $FeCl_3$ (Fluka Chemie GmbH, Buchs, Switzerland) in 50 mL of 35% perchloric acid) was added to the supernatant (1.0 mL) (Alpha Chemika, Mumbai, India). Pink color development was measured after 1 h at 530 nm (Shimadzu UVmini 1240, Shimadzu Corporation Tokyo, Japan), and the results were expressed in µg/100 mg of fresh weight.

2.7. Statistical Analysis

All the experiments were carried out in triplicate, and the results indicate the mean ± standard deviation (SD). Statistical significance between treatment groups was evaluated using the one-way ANOVA test followed by Tukey's multiple comparison test, which were carried out using GraphPad Prism (version 8.01) software (GraphPad Software, Inc., La Jolla, CA, USA). A value of $p < 0.05$ was considered statistically significant. Different letters for each SFS treatment indicate statistically significant differences.

3. Results and Discussion

Certain sludges possess traits that define them as organic fertilizers owing to their significant concentrations of organic matter and essential macro-elements (such as nitrogen and phosphorus) that are essential for soil health and plant growth. However, an important barrier to using sludge as soil and plant fertilizer is its high concentration of heavy metals and other contaminants, which can be released into the soil and consequently negatively impact the development of cultivated plants. The physico-chemical parameters (pH and conductivity) and mineral content of sludge are presented in Table 1.

Table 1. The physico-chemical characteristics of sludge.

Elements Analyzed	u.m. *	Determined Values	Analysis Methods **
pH (25 °C)-SFS L/S:10/1	pH units	6	(1)
Conductivity (25 °C)-SFS L/S:10/1	µS/cm	1979	(2)
Arsenic (As)	mg/kg	1.18	(3)
Barium (Ba)	mg/kg	2.90	(3)
Cadmium (Cd)	mg/kg	<0.5	(3)
Chromium (Cr)	mg/kg	<0.5	(3)
Copper (Cu)	mg/kg	3.50	(3)
Mercury (Hg)	mg/kg	0.23	(4)
Molybdenum (Mo)	mg/kg	<1	(3)
Nickel (Ni)	mg/kg	<2	(3)
Lead (Pb)	mg/kg	<2	(3)
Selenium (Se)	mg/kg	<0.1	(5)
Antimony (Sb)	mg/kg	<0.1	(6)
Zinc (Zn)	mg/kg	18.9	(3)
Chloride	mg/kg	482	(7)
Fluoride	mg/kg	<100	(7)
Sulphate	mg/kg	109	(7)
Total dissolved solids (TDS)	mg/kg	16,560	(8)
Dissolved organic carbon (DOC)	mg/kg	620	(9)

* Unit of measurement. All the results are reported for dry matter. ** The methods are mentioned in Table S1.

The soluble fraction of the sludge was slightly acidic (pH = 6). The importance of sludge pH results from the fact that the solubility of heavy metals in a sludge sample is dependent on pH, which controls the bioavailability of metals that exist mostly in labile form. Concentrations of As, Ba, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Se, Sb, and Zn in the soluble fraction of the sludge were lower than the limited values for heavy metals defined in Order 95/2005 and European Directive 86/278/EEC. Mercury exhibited a slight excess of 0.23 mg/kg, surpassing the national standard of 0.2 mg/kg.

The present study exploits the sensitivity of the comet test, an indicator of exposure to genotoxic agents, with reference to DNA damage in the presence of chemical contaminants from the soluble phase of sludge from urban water treatment plants. Several systems have been developed to evaluate genotoxicity using a wide range of biosensors (bacteria, human, plant, or animal cells). Several studies addressing this topic concluded that the response of most bioindicators is not strictly linked to a specific contaminant but is associated with the complex mixture of contaminants present in treated sewage sludge [8,74–76].

The parameters of the comets obtained are presented in Table 2. The values for the comet tail length (µm), tail DNA (%), and olive tail moment (OTM) are described.

Table 2. Main parameters in comets obtained by exposing 3 cell types to the soluble phase of sewage sludge.

Cell Bioindicators	Tail Length (µm)	Tail DNA %	OTM * (µm)
Lymphocytes			
H_2O	2.34 ± 1.11 ^{c}	10.95 ± 6.36 ^{c}	0.41 ± 0.14 ^{c}
25	3.45 ± 1.38 ^{c}	16.42 ± 8.33 ^{c}	0.62 ± 0.11 ^{c}
50	37.96 ± 3.13 ^{b}	43.77 ± 3.71 ^{b}	5.18 ± 0.70 ^{b}
75	40.32 ± 4.01 ^{ab}	76.49 ± 7.81 ^{a}	6.91 ± 1.36 ^{b}
100	44.61 ± 0.54 ^{a}	80.72 ± 2.69 ^{a}	7.37 ± 1.33 ^{b}
H_2O_2	44.94 ± 2.08 ^{a}	82.92 ± 2.85 ^{a}	14.46 ± 0.82 ^{a}

Table 2. *Cont.*

Cell Bioindicators	Tail Length (µm)	Tail DNA %	OTM * (µm)
Coelomocytes			
H_2O	2.70 ± 0.39 ^{c}	7.8562 ± 0.88 ^{c}	0.2071 ± 0.04 ^{c}
25%	2.80 ± 0.22 ^{c}	8.25 ± 0.30 ^{c}	0.23 ± 0.02 ^{c}
50%	2.84 ± 0.15 ^{c}	8.27 ± 0.08 ^{c}	0.24 ± 0.01 ^{c}
75%	14.25 ± 1.48 ^{b}	16.80 ± 1.23 ^{b}	2.36 ± 0.25 ^{b}
100%	26.94 ± 4.53 ^{a}	18.01 ± 2.60 ^{ab}	5.22 ± 1.50 ^{a}
H_2O_2	30.08 ± 4.16 ^{a}	20.98 ± 3.143a	6.63 ± 1.51 ^{a}
Allium cepa L.			
H_2O	0.18 ± 0.15 ^{d}	7.75 ± 5.29 ^{c}	0.79 ± 0.15 ^{c}
25%	30.55 ± 5.91 ^{c}	72.34 ± 14.88 ^{b}	4.67 ± 0.68 ^{b}
50%	39.59 ± 3.87 ^{b}	75.02 ± 10.31 ^{b}	5.31 ± 0.27 ^{b}
75%	43.00 ± 3.34 ^{a}	77.91 ± 7.74 ^{b}	5.31 ± 0.55 ^{b}
100%	44.61 ± 6.35 ^{a}	90.39 ± 7.24 ^{a}	5.64 ± 0.41 ^{b}
H_2O_2	45.11 ± 1.65 ^{a}	98.39 ± 0.75 ^{a}	6.45 ± 0.42 ^{a}

The values are expressed as the mean ± SD of experiments conducted in triplicate (n = 3). Tukey's multiple comparison test was conducted to identify significant differences. Distinct lowercase letters denote a statistically significant difference ($p < 0.05$) in each column, for each cell type, and according to the treatments. Lymphocytes s, coelomocytes, and *Allium cepa* L. cells were treated with concentrations of 25, 50, 75, and 100% SFS; H_2O_2 was the positive control, and H_2O served as the negative control. * The olive tail moment (OTM) was used to assess DNA damage. The OTM is calculated as a product of two factors: the percentage of tail DNA (Tail DNA %) and the distance between the intensity centroids (centers of gravity) of the head and tail along the comet's x-axis.

The lymphocytes indicated the genotoxicity of the soluble phase of the biosolid with respect to the tail-length parameter, starting at a concentration of 50% (Figure 2). The degree of DNA damage, for the significance threshold ($p < 0.05$), at a concentration of 25% was not significant compared to that of the negative control ($p = 0.96863$) but was significantly lower compared to the level of damage recorded at the concentrations of 75%, 100%, and compared to the positive control. For the OTM descriptor, we recorded significant differences between the positive control and all other evaluated concentrations and an insignificant difference between the negative control and the 25% concentration. The *Allium cepa* L. nuclei exposed to the positive control (H_2O_2, 200 µM) showed a higher level of DNA damage, illustrated by increased comet length and OTM values, while the nuclei treated with the negative control (H_2O) were intact, round, and did not show signs of DNA fragmentation or significant increases in the length of the comet or OTM (Figure 2). The results showed that the nuclei used as bioassay, exposed to different concentrations of the soluble phase of the biosolid, reacted in a specific way depending on their origin. Nuclei in *Allium cepa* L., with regard to the significance threshold employed ($p < 0.05$), indicated genotoxicity starting at a concentration of 25% (Figure 2). Compared to the negative control, the differences recorded at this concentration are significant in terms of comet tail length (+30.41), the % of DNA in the tail (+64.59), and OMT (+3.88). The length of the comets increased with the increase in the concentrations of the soluble phase of the biosolid; however, the differences between the concentrations of 50%, 75%, and 100% were not significant, nor were they significant when compared to the positive control. The differences in terms of the amount of DNA in the tails of the comets were significantly higher in the positive control compared to the 25% ($p = 0.00063$), 50% ($p = 0.00288$), and 75% ($p = 0.01279$) variants and insignificant compared to the 100% variant ($p = 0.71014$). The values determined for the OTM parameter indicate significant differences between the positive control and the 25% ($p = 0.00005$), 50% ($p = 0.02366$), and 75% ($p = 0.02453$) variants and insignificant compared to the 100% concentration ($p = 0.19940$).

The higher sensitivity of plant cell nuclei to genotoxic agents can be associated with the higher DNA content (33 pg for onion diploid cells and 6 pg for human diploid cells). The results suggested [77,78] that compared to plant cells, animal cells may have an apparatus that is more efficient in repairing damaged DNA.

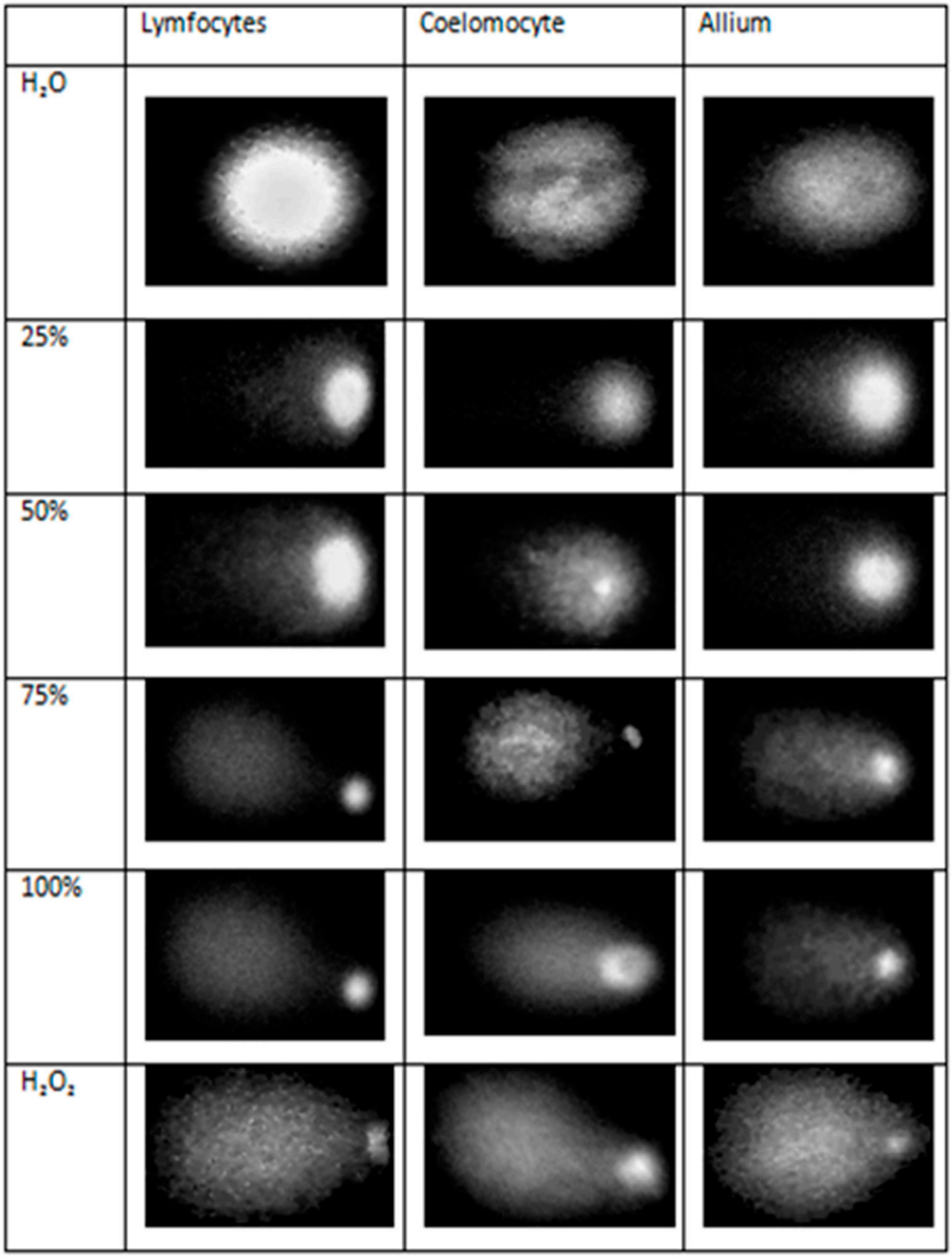


Figure 2. Morphology of DNA comets in relation to the tested bioindicator and SFS concentration. H_2O—negative control; 25%, 50%, 75%, and 100%—the concentrations of SFS with which the leucocytes, coelomocytes, and *Allium cepa* L cells were treated; H_2O_2—positive control.

The results of the Comet coelomocyte test showed that for all three analyzed parameters, the differences between the negative control and the concentrations of 25% and 50% were insignificant. This fact suggests a greater stability of the genetic material to the genotoxic factors present in the sewage sludge. Several studies have revealed mechanisms for reducing DNA damage in coelomocytes by eliminating damaged cells via apoptosis (programmed cell death), stopping them from becoming mutant cells [79] via DNA repair [80] or through the existence of distinct adaptive strategies (epigenetic or genetic) in response to exposure to a genotoxic environment [81]. The average length of the comet tails, % of DNA in the tail, and OTM determined in the three types of cells (leucocytes, coelomocytes, and *Allium cepa* L.) exposed to different concentrations of the soluble phase of the biosolid are shown in Figure 3a–c. The existence of a clear relationship between the

dose applied and the response obtained as well as the higher stability of coelomocytes with respect to the genotoxicity of the soluble phase were observed. It is important to mention that our genotoxicity study includes all coelomocytes in the population, without distinguishing between subpopulations.

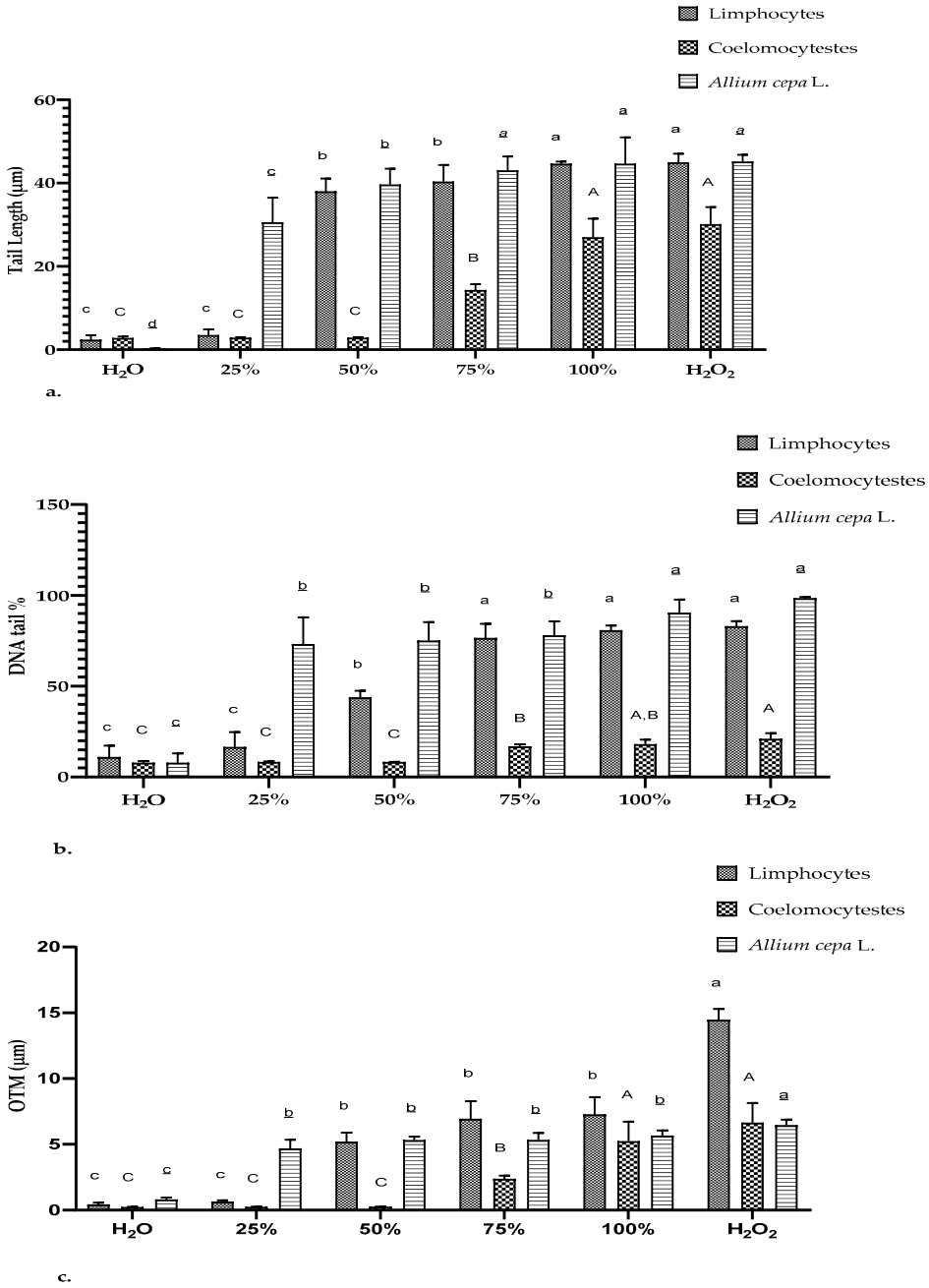


Figure 3. Evaluation of DNA damage using the Comet assay. (**a**) Mean tail length values. (**b**) Mean values of DNA tail %. (**c**) Olive tail moment mean values (OTM). The error bars depict the standard deviation of the mean values obtained from three repeated tests. Tukey's multiple comparison test was conducted to identify significant differences. Significant differences ($p < 0.05$) in the DNA tail of the experimental variants are indicated by different small letters. Significant differences in the experimental variants' DNA lengths are indicated by different capital letters. Significant differences in the OTM values between the experimental variants are indicated by different lowercase letters that are underlined. The experimental variants included a negative control using H_2O, and different concentrations (25%, 50%, 75%, and 100%) of SFS were used to treat leucocytes, coelomocytes, and *Allium cepa* L. cells. H_2O_2 was used as a positive control.

Heavy metals prevent the growth of roots, causing changes in water balance and in their absorption of nutrients [82] and thus affecting the growth of shoots and the accumulation of biomass in the above-ground parts of a plant. The stress induced by heavy metals in plants causes a decrease in endogenous levels of auxins affecting their growth and development [42]. For example, arsenic (As) was found to be able to alter the levels of three auxins (IAA, NAA, and IBA) in Brassica juncea [83]. Other studies demonstrated that short-term exposure of barley root tips to cadmium disrupted IAA homeostasis [84] or that it suppressed root elongation in Arabidopsis [85]. Upon entering the soil, heavy metal ions become involved in a competitive process with essential nutrient cations, namely, a competition to bind and be absorbed at the surface of the roots. After entering the plant cell, they exert their cytotoxic and genotoxic effects by disrupting the protein structure through attacking their thiol groups [18]. Stimulation of ROS production induced by heavy metals inflicts oxidative damage on cellular macromolecules and the photosynthetic apparatus, leading to a decrease in membrane stability and photosynthetic yield. Assimilative pigment production is also compromised, hormonal and nutritional imbalances are established, and the inhibition of DNA replication, gene expression, and cell division occurs [86]. To reduce and/or avoid heavy metal toxicity, plant roots use several strategies: avoidance of heavy metal uptake, the regulation of heavy metal toxicity through transporters, the activation of antioxidant mechanisms, the sequestration of heavy metals in vacuoles, and the synthesis and deposition of callose [87].

The effects of stress induced by pollutants in SFS on the auxin content in the roots and leaves of barley (*Hordeum vulgare* L. var. Salamandra) are shown in Figure 4. Compared to the control roots, treatment with 25% SFS reduced the content of endogenous IAA from 5.78 to 4.16 µg IAA equivalent/mL, and in the treatment with 100%, this content was reduced to 1.37 µg IAA equivalent/mL. In the case of leaves, the endogenous auxin decrease was from 3.36 µg IAA equivalent/mL for the control (H_2O) to 0.97 µg IAA equivalent/mL at the 100% concentration. Our observations are consistent with studies on sorghum [88,89], Pteris vittata and rice [43,90], and rice and Brassica juncea [91,92].

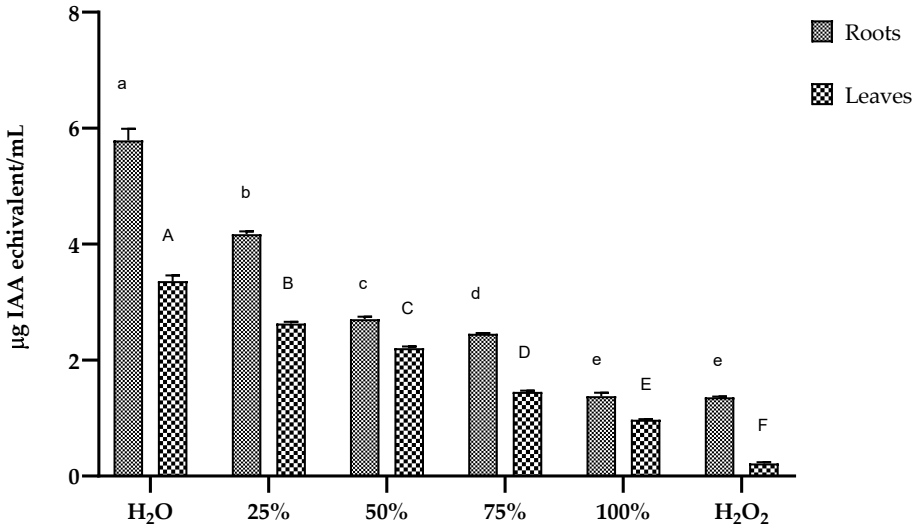


Figure 4. Effects of stress induced by heavy metals in SFS on auxin content in roots and leaves of barley (*Hordeum vulgare* L. var. Salamandra). The error bars depict the standard deviation of the mean values obtained from three repeated tests. Tukey's multiple comparison test was performed to determine significant differences ($p < 0.05$). The lowercase letters indicate significant differences between the root samples, while the uppercase letters denote statistical significance between the leaf samples based on the treatments applied. The treatments included a negative control, for which H_2O was used, and different concentrations (25%, 50%, 75%, and 100%) of SFS, while H_2O_2 was used as the positive control.

Studies on the genotoxicity of sewage sludge have shown different results (Table 3), probably due both to the different assay systems that were used and differences in the characteristics of the sludge due to the treatment technology applied [11,44,93]. Our results suggest the possibility of evaluating the sensitivity of particular tests, as integrated in a test battery, to accurately determine the genotoxic effects on relevant organisms.

Table 3. A summary of research confirming the incidence of genotoxic damage resulting from exposure to sewage sludge to different cell types.

Cell Types	Test/Assay	Findings	Reference
Allium cepa L.	Chromosomal aberrations, Micronucleus test	- Chromosomal aberration and micronucleus in root meristem cells; - A genotoxic effect was observed; - Inhibition of seed germination occurs when heavy metals are present in concentrations that are below the legal standards set in Brazil.	[37]
	Chromosomal aberrations, Micronucleus test	- Mitotic and chromosomal abnormalities (chromosome adherence chromosomal bridge, cell polyploidy, micronucleus); - Increase in phytotoxicity, cytotoxicity, genotoxicity, and/or mutagenicity.	[37]
	Chromosomal aberrations	- Chromosomal aberration in root meristem cells; - Genotoxic effects were observed for 10% sludge leachate that contains Cr, Cu, Ni, and Pb.	[94]
Lymphocytes	Comet assay	- There was no observed increase in the rate of DNA damage in peripheral blood leukocytes as a result of sewage sludge exposure; - There was not an increase in the levels of DNA damage.	[95]
Human Peripheral Blood	Comet assay	- Significant differences in the levels of lead (Pb) and cadmium (Cd) were noted in the blood of sewage workers; - Significantly increased levels of DNA damage were also detected in the groups that were exposed.	[96]
Lymphocytes	Comet assay	- No evidence was found indicating that channeling workers are more exposed to genotoxic substances compared to other workers; - No increase in DNA damage was observed among the sewage workers examined.	[97]
Lymphocytes	DNA diffusion assay, Micronucleus test, Comet assay	- Exposure to untreated waste leachate for a duration of 6 to 24 h resulted in significantly higher values of comet assay parameters in comparison to the other groups that were tested; - The lymphocytes examined were found to be cytogenotoxic when exposed to sludge leachate.	[98]
Eisenia fetida (coelomocytes)	Comet assay	- Earthworms exposed to the two highest concentrations (20 and 40%) showed a significant increase in DNA damage compared to the corresponding control group. Additionally, there was a decrease in the number of coelomocytes.	[80]

Table 3. *Cont.*

Cell Types	Test/Assay	Findings	Reference
Eisenia fetida	Comet assay	- Evaluating soil mutagenicity by measuring oxidative DNA damage caused by earthworms was used as a bio-monitoring technique; - This method is exclusively reliable for bio-accumulating metals, such as cadmium, but not for non-bio-accumulating metals, such as nickel.	[99]
Pheretima posthuma (earthworms)	Comet assay RAPD-PCR *	- As the concentration of sewage sludge increased, the amount of fragmentation in DNA also increased; - Sewage sludge induced DNA damage and inhibited the activities of esterase and phosphatase.	[100]
Eisenia fetida	Allium bioassay, Chromosomal aberration, Micronucleus, Mitotic index	- A decrease in chromosomal aberrations as well as an increase in root length and mitotic index in the final vermicompost were observed; - Earthworms have the ability to accumulate heavy metals, and the intestinal microflora and chloragocytic cells of earthworms have the ability to detoxify toxic substances.	[101]

* RAPD-PCR—Random Amplified Polymerase DNA Polymerase Chain Reaction.

However, there are other relevant aspects that need to be considered. Correa et al. [102] showed that the sewage sludge analyzed in their study presented genetic toxicity and mutagenicity in the context in which all physico-chemical parameters stay within the limits established by internal standards. The apparent absence of lymphocyte DNA damage in situations in which such damage should be obvious may be due to the selection of inadequate target cells Amresco [97]. The genotoxicity of sewage sludge applied on agricultural terrain may be diminished locally by the activity of earthworms, which can accumulate and detoxify heavy metals via their intestinal microflora and their chloragogen cells [101]. There is a certain difficulty in comparing results obtained via the Comet test [60] due to the lack of a standard procedure for the application of the test (processing duration, the comet parameters considered representative, sorting method, etc.).

4. Conclusions

The present study focuses on the impact of genotoxic agents found in sewage sludge on three trophic levels: human lymphocytes, plant cells, and coelomocytes. All the tested bioindicators demonstrated the existence of genotoxicity, with varying degrees of sensitivity. Our findings indicate that it is necessary to employ multiple tests, combined in a test battery, in order to properly evaluate genotoxic effects on relevant bioindicators. This approach is necessary for multiple significant reasons: (i) the diverse mixture of substances in sludge makes it challenging to identify a specific genotoxic factor within a broader context, such as a heavy metal; (ii) the acceptable levels of substances in sludge may not be relevant in terms of their genotoxic effects, as indicated in the existing literature and our own observations; (iii) the biosolid investigated herein (sewage sludge) contains a significant biotic component, and its metabolic processes are part of a complex network of interactions that can either enhance or diminish a specific effect. In addition, as the concentration of sludge increased, a decrease in the auxin's concentration was noted in both leaves and roots. Prior to utilizing sludge as a fertilizer and soil enhancer, it is necessary to develop an in-depth strategy that considers the sustainable management of soil's productive capacity. Before implementing a program of fertilization with sewage sludge, it is necessary to investigate the potential toxicity of treated sewage sludge. The results presented here can contribute to the compilation of a toxicological profile of sludge and to the completion of a database for the assessment of possible risks, aiding in making fully informed technological decisions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su16010457/s1. Table S1. The standards and methods used for the chemical analysis of sludge.

Author Contributions: Conceptualization, M.A.C. and V.L.; methodology, M.A.C.; validation, C.A.R., E.A. and M.A.C.; formal analysis, C.P. and V.L.; investigation, C.A.R. and E.A.; resources, V.L.; writing—original draft preparation, V.L.; writing—review and editing, C.P. and S.I.V.; supervision, V.L.; supervision, C.A.R. and E.A.; project administration, S.I.V. All authors have read and agreed to the published version of the manuscript.

Funding: The APC was funded by University of Oradea.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of UNIVERSITY OF ORADEA, Faculty of Medicine and Pharmacy (29 November 2023).

Informed Consent Statement: Informed consent was obtained from the subjects involved in the study.

Data Availability Statement: Data are contained within the article and supplementary materials.

Acknowledgments: The authors acknowledge S.C. Maggot Fish S.R.L., Oradea, Romania for donating the earthworms used in this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rusănescu, C.O.; Rusănescu, M.; Voicu, G.; Paraschiv, G.; Biriș, S.Ș.; Popescu, I.N. The Recovery of Vermicompost Sewage Sludge in Agriculture. *Agronomy* **2022**, *12*, 2653. [CrossRef]
2. Arthur Andersen Environment Risk Consulting Department. Disposal and Recycling Routes for Sewage Sludge. In *Part 3—Scientific and Technical Report*; Office for Official Publications of the European Communities: Luxembourg, 2001; pp. 151–2001.
3. Lima, M.; Mattos, C.; Vieira, P.; Almeida, L. Geração de Lodo de Esgoto e Seu Potencial Como Fonte de Matéria Orgânica Para a Agricultura. In *Manual de uso Agrícola e Disposição do Lodo de Esgoto Para o Estado do Espirito Santo*; INCAPER: Vitoria, Brazil, 2011.
4. Singh, R.P.; Agrawal, M. Potential Benefits and Risks of Land Application of Sewage Sludge. *Waste Manag.* **2008**, *28*, 347–358. [CrossRef] [PubMed]
5. Oleszczuk, P.; Malara, A.; Jośko, I.; Lesiuk, A. The Phytotoxicity Changes of Sewage Sludge-Amended Soils. *Water Air Soil. Pollut.* **2012**, *223*, 4937–4948. [CrossRef] [PubMed]
6. Yurievna Selivanovskaya, S.; Zinnatovna Latypova, V.; Aleksandrovna Artamonova, L. Use of Sewage Sludge Compost as the Restoration Agent on the Degraded Soil of Tatarstan. *J. Environ. Sci. Health Part. A* **2003**, *38*, 1549–1556. [CrossRef] [PubMed]
7. Nascimento, C.W.A.; Barros, D.A.S.; Melo, E.E.C.; Oliveira, A.B. Alterações Químicas Em Solos e Crescimento de Milho e Feijoeiro Após Aplicação de Lodo de Esgoto. *Rev. Bras. Ciênc. Solo* **2004**, *28*, 385–392. [CrossRef]
8. de Lourdes Marzo Solano, M.; de Lima, P.L.; Luvizutto, J.F.; Silva, P.R.; de Aragão Umbuzeiro, G.; de Camargo, J.L. In Vivo Genotoxicity Evaluation of a Treated Urban Sewage Sludge Sample. *Mutat. Res./Genet. Toxicol. Environ. Mutagen.* **2009**, *676*, 69–73. [CrossRef]
9. Sabbahi, S.; Ben Ayed, L.; Trad, M.; Berndtsson, R.; Karanis, P. Parasitological Assessment of Sewage Sludge Samples for Potential Agricultural Reuse in Tunisia. *Int. J. Environ. Res. Public. Health* **2022**, *19*, 1657. [CrossRef]
10. Collivignarelli, M.C.; Abbà, A.; Benigna, I. The Reuse of Biosolids on Agricultural Land: Critical Issues and Perspective. *Water Environ. Res.* **2020**, *92*, 11–25. [CrossRef]
11. Chenon, P.; Gauthier, L.; Loubières, P.; Séverac, A.; Delpoux, M. Evaluation of the Genotoxic and Teratogenic Potential of a Municipal Sludge and Sludge-Amended Soil Using the Amphibian *Xenopus laevis* and the Tobacco: *Nicotiana tabacum* L. var. *xanthi Dulieu. Sci. Total Environ.* **2003**, *301*, 139–150. [CrossRef]
12. Rank, J.; Nielsen, M.H. Genotoxicity Testing of Wastewater Sludge Using the *Allium cepa* Anaphase-Telophase Chromosome Aberration Assay. *Mutat. Res./Genet. Toxicol. Environ. Mutagen.* **1998**, *418*, 113–119. [CrossRef]
13. Grotto, D.; Carneiro, M.F.H.; Sauer, E.; Garcia, S.C.; De Melo, W.J.; Barbosa, F. Evaluation of Biochemical and Redox Parameters in Rats Fed with Corn Grown in Soil Amended with Urban Sewage Sludge. *Ecotoxicol. Environ. Saf.* **2013**, *95*, 188–194. [CrossRef] [PubMed]
14. Stadtman, E.R. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* **1993**, *62*, 797–821. [CrossRef]
15. Kasprzak, K.S. Possible Role of Oxidative Damage in Metal-Induced Carcinogenesis. *Cancer Investig.* **1995**, *13*, 411–430. [CrossRef] [PubMed]
16. Cunningham, R.P. DNA Repair: Caretakers of the Genome? *Curr. Biol.* **1997**, *7*, R576–R579. [CrossRef] [PubMed]
17. Li, W.-X.; Chen, T.-B.; Huang, Z.-C.; Lei, M.; Liao, X.-Y. Effect of Arsenic on Chloroplast Ultrastructure and Calcium Distribution in Arsenic Hyperaccumulator *Pteris vittata* L. *Chemosphere* **2006**, *62*, 803–809. [CrossRef] [PubMed]

18. Dutta, S.; Mitra, M.; Agarwal, P.; Mahapatra, K.; De, S.; Sett, U.; Roy, S. Oxidative and Genotoxic Damages in Plants in Response to Heavy Metal Stress and Maintenance of Genome Stability. *Plant Signal Behav.* **2018**, *13*, e1460048. [CrossRef] [PubMed]
19. Amin, A.W.; Sherif, F.K.; El-Atar, H.; Ez-Eldin, H. Residual Effect of Sewage Sludge on Soil and Several Yield Parameters of *Zea mays*. *Res. J. Environ. Toxicol.* **2009**, *3*, 86–93. [CrossRef]
20. Marzougui, N.; Ounalli, N.; Sabbahi, S.; Fezzani, T.; Abidi, F.; Jebari, S.; Melki, S.; Berndtsson, R.; Oueslati, W. How Can Sewage Sludge Use in Sustainable Tunisian Agriculture Be Increased? *Sustainability* **2022**, *14*, 13722. [CrossRef]
21. Kızılkaya, R.; Bayraklı, B. Effects of N-Enriched Sewage Sludge on Soil Enzyme Activities. *Appl. Soil. Ecol.* **2005**, *30*, 192–202. [CrossRef]
22. Gao, Y.; Guo, Y.-K.; Lin, S.-H.; Fang, Y.-Y.; Bai, J.-G. Hydrogen Peroxide Pretreatment Alters the Activity of Antioxidant Enzymes and Protects Chloroplast Ultrastructure in Heat-Stressed Cucumber Leaves. *Sci. Hortic.* **2010**, *126*, 20–26. [CrossRef]
23. Gjorgieva, D.; Kadifkova Panovska, T.; Ruskovska, T.; Bačeva, K.; Stafilov, T. Influence of Heavy Metal Stress on Antioxidant Status and DNA Damage in *Urtica Dioica*. *BioMed Res. Int.* **2013**, *2013*, 276417. [CrossRef] [PubMed]
24. Britt, A.B. Molecular Genetics of DNA Repair in Higher Plants. *Trends Plant Sci.* **1999**, *4*, 20–25. [CrossRef] [PubMed]
25. Lin, A.-J.; Zhang, X.-H.; Chen, M.-M.; Cao, Q. Oxidative Stress and DNA Damages Induced by Cadmium Accumulation. *J. Environ. Sci.* **2007**, *19*, 596–602. [CrossRef] [PubMed]
26. Siddiqui, S. DNA Damage in Cicer Plant Grown on Soil Polluted with Heavy Metals. *J. King Saud. Univ. Sci.* **2015**, *27*, 217–223. [CrossRef]
27. Das, A.; Parbat, D.; Shome, A.; Manna, U. Sustainable Biomimicked Oil/Water Wettability That Performs Under Severe Challenges. *ACS Sustain. Chem. Eng.* **2019**, *7*, 11350–11359. [CrossRef]
28. Das, A.; Naskar, S.; Dhar, M.; Manna, U. Rapid and Scalable Synthesis of a Vanillin-Based Organogelator and Its Durable Composite for a Comprehensive Remediation of Crude-Oil Spillages. *ACS Appl. Mater. Interfaces* **2021**, *13*, 46803–46812. [CrossRef]
29. Jiang, N.; Naz, S.; Ma, Y.; Ullah, Q.; Khan, M.Z.; Wang, J.; Lu, X.; Luosang, D.-Z.; Tabassum, S.; Chatha, A.M.M.; et al. An Overview of Comet Assay Application for Detecting DNA Damage in Aquatic Animals. *Agriculture* **2023**, *13*, 623. [CrossRef]
30. Amin, A.W. Evaluation of the Genotoxicity of Residual Repeated Applications of Sewage Sludge on M_2 Meiocytes of Zea Plants. *Res. J. Environ. Toxicol.* **2011**, *5*, 235–250. [CrossRef]
31. Gawdzik, J.; Gawdzik, B. Mobility of Heavy Metals in Municipal Sewage Sludge from Different Throughput Sewage Treatment Plants. *Pol. J. Environ. Stud.* **2012**, *21*, 1603–1611.
32. McBride, M.B. Toxic Metal Accumulation from Agricultural Use of Sludge: Are USEPA Regulations Protective? *J. Environ. Qual.* **1995**, *24*, 5–18. [CrossRef]
33. Udom, B.E.; Mbagwu, J.S.C.; Adesodun, J.K.; Agbim, N.N. Distributions of Zinc, Copper, Cadmium and Lead in a Tropical Ultisol after Long-Term Disposal of Sewage Sludge. *Environ. Int.* **2004**, *30*, 467–470. [CrossRef] [PubMed]
34. Mateuca, R.; Lombaert, N.; Aka, P.V.; Decordier, I.; Kirsch-Volders, M. Chromosomal Changes: Induction, Detection Methods and Applicability in Human Biomonitoring. *Biochimie* **2006**, *88*, 1515–1531. [CrossRef] [PubMed]
35. Dai, J.; Chen, L.; Zhao, J.; Ma, N. Characteristics of Sewage Sludge and Distribution of Heavy Metal in Plants with Amendment of Sewage Sludge. *J. Environ. Sci.* **2006**, *18*, 1094–1100. [CrossRef] [PubMed]
36. Henning, B.; Snyman, H.; Aveling, T. Plant-Soil Interactions of Sludge-Borne Heavy Metals and the Effect on Maize (*Zea mays* L.) Seedling Growth. *WSA* **2004**, *27*, 71–78. [CrossRef]
37. Corrêa Martins, M.N.; de Souza, V.V.; Souza, T. da S. Genotoxic and Mutagenic Effects of Sewage Sludge on Higher Plants. *Ecotoxicol. Environ. Saf.* **2016**, *124*, 489–496. [CrossRef] [PubMed]
38. Krishna, V.; Pandey, A.K.; Gupta, P.K. Heavy Metals in Sludge Produced from UASB Treatment Plant at Mirzapur, India. *Pollution* **2021**, *7*, 607–616. [CrossRef]
39. Emamverdian, A.; Ding, Y.; Mokhberdoran, F.; Xie, Y. Heavy Metal Stress and Some Mechanisms of Plant Defense Response. *Sci. World J.* **2015**, *2015*, 756120. [CrossRef]
40. Hąc-Wydro, K.; Sroka, A.; Jabłońska, K. The Impact of Auxins Used in Assisted Phytoextraction of Metals from the Contaminated Environment on the Alterations Caused by Lead(II) Ions in the Organization of Model Lipid Membranes. *Colloids Surf. B Biointerfaces* **2016**, *143*, 124–130. [CrossRef]
41. Rodriguez, E.; Santos, C.; Azevedo, R.; Moutinho-Pereira, J.; Correia, C.; Dias, M.C. Chromium (VI) Induces Toxicity at Different Photosynthetic Levels in Pea. *Plant Physiol. Biochem.* **2012**, *53*, 94–100. [CrossRef]
42. Bücker-Neto, L.; Paiva, A.L.S.; Machado, R.D.; Arenhart, R.A.; Margis-Pinheiro, M. Interactions between Plant Hormones and Heavy Metals Responses. *Genet. Mol. Biol.* **2017**, *40*, 373–386. [CrossRef]
43. Zhao, F.; Han, M.; Zhang, S.; Wang, K.; Zhang, C.; Liu, T.; Liu, W. Hydrogen Peroxide-Mediated Growth of the Root System Occurs via Auxin Signaling Modification and Variations in the Expression of Cell-Cycle Genes in Rice Seedlings Exposed to Cadmium Stress. *J. Integr. Plant Biol.* **2012**, *54*, 991–1006. [CrossRef] [PubMed]
44. Schowanek, D.; Carr, R.; David, H.; Douben, P.; Hall, J.; Kirchmann, H.; Patria, L.; Sequi, P.; Smith, S.; Webb, S. A Risk-Based Methodology for Deriving Quality Standards for Organic Contaminants in Sewage Sludge for Use in Agriculture—Conceptual Framework. *Regul. Toxicol. Pharmacol.* **2004**, *40*, 227–251. [CrossRef] [PubMed]

45. Azqueta, A.; Ladeira, C.; Giovannelli, L.; Boutet-Robinet, E.; Bonassi, S.; Neri, M.; Gajski, G.; Duthie, S.; Bo', C.D.; Riso, P.; et al. Application of the Comet Assay in Human Biomonitoring: An hCOMET Perspective. *Mutat. Res./Rev. Mutat. Res.* **2020**, *783*, 108288. [CrossRef] [PubMed]
46. Fomin, A.; Hafner, C. Evaluation of Genotoxicity of Emissions from Municipal Waste Incinerators with Tradescantia-Micronucleus Bioassay (Trad-MCN). *Mutat. Res.* **1998**, *414*, 139–148. [CrossRef] [PubMed]
47. Grant, W.F. Higher Plant Assays for the Detection of Genotoxicity in Air Polluted Environments. *Ecosyst. Health* **1998**, *4*, 210–229. [CrossRef]
48. Martins, M.; Costa, P.M. The Comet Assay in Environmental Risk Assessment of Marine Pollutants: Applications, Assets and Handicaps of Surveying Genotoxicity in Non-Model Organisms. *Mutagenesis* **2015**, *30*, 89–106. [CrossRef]
49. Tondoh, J.E.; Monin, L.M.; Tiho, S.; Csuzdi, C. Can Earthworms Be Used as Bio-Indicators of Land-Use Perturbations in Semi-Deciduous Forest? *Biol. Fertil. Soils* **2007**, *43*, 585–592. [CrossRef]
50. Peres, G.; Cluzeau, D.; Ferrand, C.; Peron, D. Earthworms Used as Indicators of Agricultural Managements. In *BIO-BIO Project. Biodiversity-Bioindication to Evaluate Soil Health*; Cenci, R.M., Sena, F., Eds.; 2006; Volume 22245, pp. 107–115; ISBN 92-79-02011-0. Available online: https://esdac.jrc.ec.europa.eu/ESDB_Archive/eusoils_docs/other/EUR22245.pdf (accessed on 7 December 2023).
51. Verschaeve, L.; Gilles, J. Single Cell Gel Electrophoresis Assay in the Earthworm for the Detection of Genotoxic Compounds in Soils. *Bull. Environ. Contam. Toxicol.* **1995**, *54*, 112–119. [CrossRef]
52. Salagovic, J.; Gilles, J.; Verschaeve, L.; Kalina, I. The Comet Assay for the Detection of Genotoxic Damage in the Earthworms: A Promising Tool for Assessing the Biological Hazards of Polluted Sites. *Folia Biol.* **1996**, *42*, 17–21.
53. Adamowicz, A. Morphology and Ultrastructure of the Earthworm *Dendrobaena veneta* (Lumbricidae) Coelomocytes. *Tissue Cell* **2005**, *37*, 125–133. [CrossRef]
54. Liu, W.; Li, P.J.; Qi, X.M.; Zhou, Q.X.; Zheng, L.; Sun, T.H.; Yang, Y.S. DNA Changes in Barley (*Hordeum vulgare*) Seedlings Induced by Cadmium Pollution Using RAPD Analysis. *Chemosphere* **2005**, *61*, 158–167. [CrossRef] [PubMed]
55. Lemtiri, A.; Liénard, A.; Alabi, T.; Brostaux, Y.; Cluzeau, D.; Francis, F.; Colinet, G. Earthworms *Eisenia fetida* Affect the Uptake of Heavy Metals by Plants *Vicia faba* and *Zea mays* in Metal-Contaminated Soils. *Appl. Soil. Ecol.* **2016**, *104*, 67–78. [CrossRef]
56. Karimi, F.; Rahimi, G.; Kolahchi, Z. Interaction Effects of Salinity, Sewage Sludge, and Earthworms on the Fractionations of Zn and Cu, and the Metals Uptake by the Earthworms in a Zn- and Cu-Contaminated Calcareous Soil. *Environ. Sci. Pollut. Res.* **2020**, *27*, 10565–10580. [CrossRef] [PubMed]
57. He, X.; Zhang, Y.; Shen, M.; Zeng, G.; Zhou, M.; Li, M. Effect of Vermicomposting on Concentration and Speciation of Heavy Metals in Sewage Sludge with Additive Materials. *Bioresour. Technol.* **2016**, *218*, 867–873. [CrossRef] [PubMed]
58. Poli, P.; Buschini, A.; Restivo, F.M.; Ficarelli, A.; Cassoni, F.; Ferrero, I.; Rossi, C. Comet Assay Application in Environmental Monitoring: DNA Damage in Human Leukocytes and Plant Cells in Comparison with Bacterial and Yeast Tests. *Mutagenesis* **1999**, *14*, 547–556. [CrossRef] [PubMed]
59. Ostling, O.; Johanson, K.J. Microelectrophoretic Study of Radiation-Induced DNA Damages in Individual Mammalian Cells. *Biochem. Biophys. Res. Commun.* **1984**, *123*, 291–298. [CrossRef]
60. Faust, F.; Kassie, F.; Knasmüller, S.; Boedecker, R.H.; Mann, M.; Mersch-Sundermann, V. The Use of the Alkaline Comet Assay with Lymphocytes in Human Biomonitoring Studies. *Mutat. Res./Rev. Mutat. Res.* **2004**, *566*, 209–229. [CrossRef]
61. Singh, N.P.; McCoy, M.T.; Tice, R.R.; Schneider, E.L. A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells. *Exp. Cell Res.* **1988**, *175*, 184–191. [CrossRef]
62. Azqueta, A.; Muruzabal, D.; Boutet-Robinet, E.; Milic, M.; Dusinska, M.; Brunborg, G.; Møller, P.; Collins, A.R. Technical Recommendations to Perform the Alkaline Standard and Enzyme-Modified Comet Assay in Human Biomonitoring Studies. *Mutat. Res./Genet. Toxicol. Environ. Mutagen.* **2019**, *843*, 24–32. [CrossRef]
63. Speit, G.; Vasquez, M.; Hartmann, A. The Comet Assay as an Indicator Test for Germ Cell Genotoxicity. *Mutat. Res./Rev. Mutat. Res.* **2009**, *681*, 3–12. [CrossRef]
64. Cordelli, E.; Bignami, M.; Pacchierotti, F. Comet Assay: A Versatile but Complex Tool in Genotoxicity Testing. *Toxicol. Res.* **2021**, *10*, 68–78. [CrossRef] [PubMed]
65. Alvarez-Moya, C. Use of Comet Assay in Human Lymphocytes as a Molecular Biomarker for Simultaneous Monitoring of Genetic Damage and Genotoxicity in Residents Who Lived Nearby the Santiago River, Mexico, in 2012. *Glob. J. Biotechnol. Biomater. Sci.* **2015**, *1*, 4–8. [CrossRef]
66. Eyambe, G.S.; Goven, A.J.; Fitzpatrick, L.C.; Venables, B.J.; Cooper, E.L. A Non-Invasive Technique for Sequential Collection of Earthworm (*Lumbricus Terrestris*) Leukocytes during Subchronic Immunotoxicity Studies. *Lab. Anim.* **1991**, *25*, 61–67. [CrossRef] [PubMed]
67. Gichner, T.; Mühlfeldová, Z. Induced DNA Damage Measured by the Comet Assay in 10 Weed Species. *Biol. Plant.* **2002**, *45*, 509–516. [CrossRef]
68. Singh, N.P. Microgel Electrophoresis of DNA from Individual Cells. In *Technologies for Detection of DNA Damage and Mutations*; Pfeifer, G.P., Ed.; Springer: Boston, MA, USA, 1996; pp. 3–24; ISBN 978-1-4899-0303-7.
69. Juchimiuk, J.; Gnys, A.; Maluszynska, J. DNA Damage Induced by Mutagens in Plant and Human Cell Nuclei in Acellular Comet Assay. *Folia Histochem. Cytobiol.* **2006**, *44*, 127–131.

70. Bandyopadhyay, A.; Mukherjee, A. Sensitivity of Allium and Nicotiana in Cellular and Acellular Comet Assays to Assess Differential Genotoxicity of Direct and Indirect Acting Mutagens. *Ecotoxicol. Environ. Saf.* **2011**, *74*, 860–865. [CrossRef] [PubMed]
71. Rajaguru, P.; Suba, S.; Palanivel, M.; Kalaiselvi, K. Genotoxicity of a Polluted River System Measured Using the Alkaline Comet Assay on Fish and Earthworm Tissues. *Environ. Mol. Mutagen.* **2003**, *41*, 85–91. [CrossRef] [PubMed]
72. Liu, W.; Zhu, L.-S.; Wang, J.; Wang, J.-H.; Xie, H.; Song, Y. Assessment of the Genotoxicity of Endosulfan in Earthworm and White Clover Plants Using the Comet Assay. *Arch. Environ. Contam. Toxicol.* **2009**, *56*, 742–746. [CrossRef]
73. Goldschmidt, E.E.; Goren, R.; Monselise, S.P. The IAA-Oxidase System of Citrus Roots. *Planta* **1966**, *72*, 213–222. [CrossRef]
74. Júnior, E.O.D.C.; Pereira, B.B.; Morelli, S. Monitoring Genotoxicity Potential in the Mumbuca Stream, Minas Gerais, Brazil. *J. Toxicol. Environ. Health Part. A* **2015**, *78*, 1277–1287. [CrossRef]
75. Fang, W.; Qi, G.; Wei, Y.; Kosson, D.S.; Van Der Sloot, H.A.; Liu, J. Leaching Characteristic of Toxic Trace Elements in Soils Amended by Sewage Sludge Compost: A Comparison of Field and Laboratory Investigations. *Environ. Pollut.* **2018**, *237*, 244–252. [CrossRef] [PubMed]
76. Siqueira, W.N.D.; França, E.J.D.; Pereira, D.R.; Lima, M.D.V.; Silva, H.A.M.F.; Araújo, H.D.A.D.; Sá, J.L.F.; Melo, A.M.M.D.A. Study of Genotoxic and Cytotoxic Effects after Acute and Chronic Exposures to Industrial Sewage Sludge on Biomphalaria Glabrata Hemocytes. *Chemosphere* **2020**, *249*, 126218. [CrossRef]
77. Navarrete, M.H.; Carrera, P.; de Miguel, M. Consuelo de la Torre A Fast Comet Assay Variant for Solid Tissue Cells. The Assessment of DNA Damage in Higher Plants. *Mutat. Res./Genet. Toxicol. Environ. Mutagen.* **1997**, *389*, 271–277. [CrossRef] [PubMed]
78. de Lima, R.; Oliveira Feitosa, L.; Rodrigues Maruyama, C.; Abreu Barga, M.; Yamawaki, P.C.; Vieira, I.J.; Teixeira, E.M.; Corrêa, A.C.; Caparelli Mattoso, L.H.; Fernandes Fraceto, L. Evaluation of the Genotoxicity of Cellulose Nanofibers. *Int. J. Nanomed.* **2012**, *7*, 3555. [CrossRef] [PubMed]
79. Evan, G.; Littlewood, T. A Matter of Life and Cell Death. *Science* **1998**, *281*, 1317–1322. [CrossRef] [PubMed]
80. Bonnard, M.; Devin, S.; Leyval, C.; Morel, J.-L.; Vasseur, P. The Influence of Thermal Desorption on Genotoxicity of Multipolluted Soil. *Ecotoxicol. Environ. Saf.* **2010**, *73*, 955–960. [CrossRef] [PubMed]
81. Kille, P.; Andre, J.; Anderson, C.; Ang, H.N.; Bruford, M.W.; Bundy, J.G.; Donnelly, R.; Hodson, M.E.; Juma, G.; Lahive, E.; et al. DNA Sequence Variation and Methylation in an Arsenic Tolerant Earthworm Population. *Soil. Biol. Biochem.* **2013**, *57*, 524–532. [CrossRef]
82. González-Moscoso, M.; Martínez-Villegas, N.; Cadenas-Pliego, G.; Juárez-Maldonado, A. Effect of Silicon Nanoparticles on Tomato Plants Exposed to Two Forms of Inorganic Arsenic. *Agronomy* **2022**, *12*, 2366. [CrossRef]
83. Srivastava, S.; Srivastava, A.K.; Suprasanna, P.; D'Souza, S.F. Identification and Profiling of Arsenic Stress-Induced microRNAs in *Brassica juncea*. *J. Exp. Bot.* **2013**, *64*, 303–315. [CrossRef]
84. Zelinová, V.; Alemayehu, A.; Bočová, B.; Huttová, J.; Tamás, L. Cadmium-Induced Reactive Oxygen Species Generation, Changes in Morphogenic Responses and Activity of Some Enzymes in Barley Root Tip Are Regulated by Auxin. *Biologia* **2015**, *70*, 356–364. [CrossRef]
85. Bennett, T.; Sieberer, T.; Willett, B.; Booker, J.; Luschnig, C.; Leyser, O. The Arabidopsis MAX Pathway Controls Shoot Branching by Regulating Auxin Transport. *Curr. Biol.* **2006**, *16*, 553–563. [CrossRef] [PubMed]
86. Singh, N.; Ma, L.Q.; Vu, J.C.; Raj, A. Effects of Arsenic on Nitrate Metabolism in Arsenic Hyperaccumulating and Non-Hyperaccumulating Ferns. *Environ. Pollut.* **2009**, *157*, 2300–2305. [CrossRef] [PubMed]
87. Riaz, M.; Kamran, M.; Rizwan, M.; Ali, S.; Wang, X. Foliar Application of Silica Sol Alleviates Boron Toxicity in Rice (*Oryza sativa*) Seedlings. *J. Hazard. Mater.* **2022**, *423*, 127175. [CrossRef] [PubMed]
88. Stobart, A.K.; Griffiths, W.T.; Ameen-Bukhari, I.; Sherwood, R.P. The Effect of Cd^{2+} on the Biosynthesis of Chlorophyll in Leaves of Barley. *Physiol. Plant.* **1985**, *63*, 293–298. [CrossRef]
89. Zhan, Y.; Zhang, C.; Zheng, Q.; Huang, Z.; Yu, C. Cadmium Stress Inhibits the Growth of Primary Roots by Interfering Auxin Homeostasis in Sorghum Bicolor Seedlings. *J. Plant Biol.* **2017**, *60*, 593–603. [CrossRef]
90. Balestri, M.; Ceccarini, A.; Forino, L.M.C.; Zelko, I.; Martinka, M.; Lux, A.; Ruffini Castiglione, M. Cadmium Uptake, Localization and Stress-Induced Morphogenic Response in the Fern *Pteris vittata*. *Planta* **2014**, *239*, 1055–1064. [CrossRef] [PubMed]
91. Shah, K.; Kumar, R.G.; Verma, S.; Dubey, R.S. Effect of Cadmium on Lipid Peroxidation, Superoxide Anion Generation and Activities of Antioxidant Enzymes in Growing Rice Seedlings. *Plant Sci.* **2001**, *161*, 1135–1144. [CrossRef]
92. Verma, K.; Shekhawat, G.S.; Sharma, A.; Mehta, S.K.; Sharma, V. Cadmium Induced Oxidative Stress and Changes in Soluble and Ionically Bound Cell Wall Peroxidase Activities in Roots of Seedling and 3–4 Leaf Stage Plants of *Brassica juncea* (L.) Czern. *Plant Cell Rep.* **2008**, *27*, 1261–1269. [CrossRef]
93. Gustavsson, L.; Engwall, M. Genotoxic Activity of Nitroarene-Contaminated Industrial Sludge Following Large-Scale Treatment in Aerated and Non-Aerated Sacs. *Sci. Total Environ.* **2006**, *367*, 694–703. [CrossRef]
94. Srivastava, R.; Kumar, D.; Gupta, S.K. Bioremediation of Municipal Sludge by Vermitechnology and Toxicity Assessment by *Allium cepa*. *Bioresour. Technol.* **2005**, *96*, 1867–1871. [CrossRef]
95. Silva, P.R.P.; Barbisan, L.F.; Dagli, M.L.Z.; Saldiva, P.H.N. Sewage Sludge Does Not Induce Genotoxicity and Carcinogenesis. *Genet. Mol. Biol.* **2012**, *35*, 657–663. [CrossRef]

96. Basu, R.; Talapatra, S.N.; Mukhopadhyay, A.; Roy Goswami, M.; Ray, S.S.; Chakrabarti, P.; Ram, S.S.; Sudarshan, M.; Chakraborty, A.; Dasgupta, A.; et al. Genotoxicity Study with Special Reference to Comet Test in the Blood Cells of Workers Exposed to Sewage Water. *Adv. Toxicol.* **2014**, *2014*, 251812. [CrossRef]
97. Friis, L.; Vaghef, H.; Edling, C.; Hellman, B. No Increased DNA Damage in Peripheral Lymphocytes of Sewage Workers as Evaluated by Alkaline Single Cell Gel Electrophoresis. *Occup. Environ. Med.* **1997**, *54*, 494–498. [CrossRef] [PubMed]
98. Gajski, G.; Oreščanin, V.; Garaj-Vrhovac, V. Cytogenotoxicity of Sewage Sludge Leachate before and after Calcium Oxide-Based Solidification in Human Lymphocytes. *Ecotoxicol. Environ. Saf.* **2011**, *74*, 1408–1415. [CrossRef] [PubMed]
99. Hirano, T.; Tamae, K. Earthworms and Soil Pollutants. *Sensors* **2011**, *11*, 11157–11167. [CrossRef] [PubMed]
100. Khalid, H.; Kashif Zahoor, M.; Riaz, D.; Arshad, M.; Yaqoob, R.; Ranian, K. Sewage Sludge-Induced Effect on Growth, Enzyme Inhibition, and Genotoxicity Can Be Ameliorated Using Wheat Straw and Biochar in *Pheretima posthuma* Earthworms. *Front. Environ. Sci.* **2022**, *10*, 888394. [CrossRef]
101. Bhat, S.A.; Singh, J.; Singh, K.; Vig, A.P. Genotoxicity Monitoring of Industrial Wastes Using Plant Bioassays and Management through Vermitechnology: A Review. *Agric. Nat. Resour.* **2017**, *51*, 325–337. [CrossRef]
102. Corrêa Martins, M.N.; Souza, V.V.D.; Silva Souza, T.D. Cytotoxic, Genotoxic and Mutagenic Effects of Sewage Sludge on *Allium cepa*. *Chemosphere* **2016**, *148*, 481–486. [CrossRef]