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Vicia faba Plant Suitability Assessment for Genotoxicity, Cytotoxicity, and Mutagenicity Testing of Pharmaceutical-Containing Wastewater

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Abstract: The article aimed to assess the *Vicia faba* plant's suitability in the micronucleus test for determining toxicity of wastewater containing diclofenac and sulfamethoxazole. Additionally, the study evaluated the activity of the antioxidant enzymes catalase and superoxide dismutase in plant leaves. The assessment of wastewater was performed on laboratory-constructed wetland models. Both influent and effluent samples were tested, and the study examined two methods of plant root exposure: hydroponic culture and soil culture. The analysis showed a decrease in the mitotic index (57% inhibition on average in hydroponic and 42% in soil culture for influent and 39% and 19%, respectively, for treated wastewater), indicating the toxicity of the wastewater. The inhibition of the cell division frequency was lower in soil culture, and the frequency of aberrations of chromosomes was also lower. However, there was no increase in micronuclei frequency. An upsurge in catalase activity was observed upon analyzing the wastewater, with a 67% increase in the influent and a 20% increase in the treated wastewater. Additionally, there was a notable boost in superoxide dismutase activity, primarily in hydroponic culture with raw wastewater, averaging 186%. The results showed genotoxic and cytotoxic effects, but there were no mutagenic effects. The *Vicia faba* assay is advantageous for its simplicity and rapid results; it offers representative assessment of genotoxicity through its broad range of detected effects.

Keywords: plants test; diclofenac; genotoxicity; sulfamethoxazole; wastewater reuse; constructed wetlands; oxidative stress

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1. Introduction

Water is a vital natural resource necessary for the survival of all living organisms and industrial operations. However, the world's freshwater resources are declining as a result of continuous population growth and increasing demand for water, particularly for land irrigation, which accounts for 80–90% of total freshwater use [1]. To address this issue, the use of treated wastewater for agricultural purposes is being proposed as a solution [2–4].

The wastewater treatment process involves primary, secondary, tertiary, and advanced treatment stages. Primary treated wastewater is not useful for irrigation, but secondary treated wastewater (activated sludge, membrane bioreactors, oxidation ditch, aerated basins, absorption biodegradation, or trickling filters) and tertiary treated wastewater (membrane filtration, adsorption, precipitation, chemical coagulation, or managed aquifer recharge) can be used for irrigation or groundwater recharge. Advanced treatment options such as advanced oxidation process, activated carbon, or reverse osmosis can even make wastewater suitable for drinking and surface/groundwater recharge [3,5].

Simply reducing or eliminating micropollutants during treatment does not always guarantee a decrease in wastewater toxicity. This is because the treatment process can

result in the formation of more harmful decomposition products that standard analytical methods may not detect [6,7]. Therefore, assessing the effectiveness of wastewater treatment based solely on physicochemical parameters is a significant oversimplification that can lead to uncontrolled environmental contamination. Thus, researching the influence of micropollutants from treated wastewater on the soil environment and plant growth is imperative due to these risks. On the other hand, acute toxicity tests, which are commonly used to assess toxicity, might be inadequate in reflecting the potential harm of the tested wastewater [8]. Consequently, researchers are exploring tests that can detect even minor changes, such as genetic alterations, that can have multi-generational impacts.

The utilization of wastewater to irrigate agricultural fields presents both advantages and disadvantages. The primary benefit is the conservation of drinking water resources. Moreover, soil nutrition, microbiological activity, and fertilization costs are all enhanced, and soil structure may also be improved. Nonetheless, drawbacks also exist, with the most critical being soil and food contamination. Insufficient wastewater treatment may also raise the number of soil pathogens [5].

Information regarding the impact of pharmaceuticals originating from wastewater on the soil environment is currently limited in the literature. Some authors suggest that the use of manure-originating fertilizers (MOFs) [9] or fertilizers derived from sewage sludge treatment [10] is associated with a low, but not insignificant, environmental risk. The authors emphasize the need to consider pharmaceutical contaminants when establishing quality guidelines, along with other pollutants. Other authors point out the risks associated with reduced crop yields due to exposure to pharmaceutical substances and the potential deterioration of crop quality from the uptake and transportation of contaminants to edible plant parts [11,12]. The authors also highlight the potential for antibiotic resistance to be transmitted to bacteria present on the surfaces of vegetables or fruits [13].

In fields subjected to extended irrigation using treated wastewater, there was an observed accumulation of specific pharmaceuticals including diclofenac, sulfamethoxazole, and trimethoprim [14]. The authors stressed the necessity for conducting comprehensive studies to obtain robust information regarding the safety of reusing wastewater for irrigation purposes. Among researchers, there is an agreement that only high-quality reclaimed water can be used for irrigation, as crops have the potential to bioaccumulate residual contaminants in their tissues, contributing to their entry into the food chain. However, a consistent methodology for developing a list of pollutants to be monitored in such cases is currently lacking [15].

Compared to other organisms, like bacteria or animal cells, plants present numerous advantages in ecotoxicological tests. One key benefit lies in the similarity between the chromosomal morphology of plants and mammals, resulting in comparable genetic responses to exposed hazardous agents. Additionally, plant-based testing is cost-effective and often does not necessitate sophisticated equipment [16].

Vicia faba, commonly known as broad bean, is a frequently used plant species in the field of ecotoxicology. It is employed for cytological, physiological, and radiobiological investigations as well as toxicity evaluations. One of the major benefits of using *V. faba* is its year-round availability, which greatly simplifies laboratory assessments. Moreover, breeding this plant is uncomplicated and cost-effective, and it does not require specialized equipment or sterile conditions. Additionally, these tests can be carried out in solid matrices such as soil and sediment, as well as liquid matrices including water and sewage [17].

In this study, two pharmaceutical compounds were investigated: diclofenac (DCF) and sulfamethoxazole (SMX). Diclofenac, a nonsteroidal anti-inflammatory drug (NSAID), possesses analgesic and antipyretic properties, often administered orally or as a skin ointment [18,19]. DCF functions by inhibiting cyclooxygenases (COX-1 and COX-2), the enzyme responsible for producing prostanoids, leading to the suppression of prostaglandins such as PGE2, PGD2, PGF2, prostacyclin (PGI2), and thromboxane (TX) A2. This reduction in PGE2 synthesis during inflammation is the primary mechanism for painkillers and anti-

inflammatory drugs, with DCF showing a higher COX-2 selectivity than most traditional NSAIDs [20].

In contrast, SMX is a broad-spectrum sulfonamide antibiotic effective against gram-positive/negative aerobic bacteria, protozoa, and certain fungi [21–23]. SMX operates by competitively inhibiting dihydropteroate production from two folic acid precursors: p-aminobenzoic acid (PABA) and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP). The enzyme dihydropteroyl synthetase (DHPS) catalyzes this reaction. The biological impact of SMX and other sulfonamides originates from competitive inhibition of DHPS, leading to the inhibition of bacterial growth and reproduction [23]. Sewage treatment plants release the highest pharmaceutical concentrations into the environment. Due to natural processes like biotransformation, sorption, or photolysis, the concentrations found in groundwater or freshwater are lower [24]. In surface water, the maximum DCF concentration is 0.0187 mg L^{-1}, and for SMX, it is 0.0119 mg L^{-1} [25]. These levels suggest that these compounds, as present in the natural environment, won't induce acute toxicity in model organisms. However, it is vital to acknowledge that pollutants coexist as mixtures, making their toxicity challenging to estimate and predict [26].

The aim of this study was to evaluate the effectiveness of the *Vicia faba* micronucleus test in identifying genotoxic, cytotoxic, and mutagenic effects in wastewater containing pharmaceuticals (diclofenac and sulfamethoxazole). The following effects were observed:

- chromosomal aberrations
- changes in the mitotic index,
- presence of micronuclei.

Additionally, the study investigated the catalase and superoxide dismutase enzyme activities in *V. faba* leaves to ascertain the potential occurrence of oxidative stress within the tested organisms.

2. Materials and Methods

2.1. Wastewater Characterization

2.1.1. Reagents

Pharmaceuticals: Two drugs were selected for the study: diclofenac (DCF; CAS: 15307-86-5) and sulfamethoxazole (SMX; CAS: 723-46-6). Both substances were purchased from Sigma-Aldrich (Taufkirchen, Germany)—purity above 99%. The properties of DCF and SMX are summarized in Table 1 at the base of [27].

Table 1. Properties of tested pharmaceuticals.

Tested Compound	Formula	CAS Number	Molar Mass, g mol^{-1}	pK_a	$logK_{OW}$, pH 8
DCF	$C_{14}H_{11}Cl_2NO_2$	15307-86-5	296.15	4.15	4.51
SMX	$C_{10}H_{11}N_3O_3S$	723-46-6	253.28	5.6–5.7	0.89

Synthetic wastewater: CH_3COONa, urea, KH_2PO_4, peptone, $FeSO_4{\cdot}7H_2O$, $MgSO_4{\cdot}7H_2O$, yeast extrace, skim milk powder, $KCr(SO_4)_2{\cdot}12H_2O$, $CuSO_4{\cdot}5H_2O$, $NiSO_4{\cdot}7H_2O$, $ZnCl_2$, $MnSO_4{\cdot}H_2O$, $PbCl_2$. All reagents were purchased from Avantor (Gliwice, Poland).

Soil substrate: kaolin clay (Surmin-Kaolin, Nowogrodziec, Poland), quartz sand (Biovita, Tenczynek, Poland), sphagnum peat (Biovita, Tenczynek, Poland), $CaCO_3$ (Avantor, Gliwice, Poland).

Micronucleus test: CH_3COOH (Sigma-Aldrich, Taufkirchen, Germany), C_2H_5OH (Avantor, Gliwice, Poland), HCl (Avantor, Gliwice, Poland).

Enzyme activity analysis: Coomassie Brilliant Blue G-250 (Merck, Darmstadt, Germany), bovine serum albumin (Merck, Darmstadt, Germany), $(NH_4)_2MoO_4$ (Sigma-Aldrich, Taufkirchen, Germany), Adrenaline reference standard (Merck, Darmstadt, Germany), H_2O_2 (Merck, Darmstadt, Germany), sodium phosphate buffer (pH 7.4) and carbonate buffer (pH 10.2) (Sigma-Aldrich, Taufkirchen, Germany).

2.1.2. Constructed Wetlands

A laboratory version of vertical-flow constructed wetlands (CWs) was made up of 12 columns (Figure 1). All of the columns were filled from the bottom with a layer of gravel (0.05 m), quartz sand (0.6 m), sand, and organic soil (0.05 m). All columns were planted with *Miscanthus giganteus*, commonly used in artificially constructed wetlands. Synthetic wastewater, following a modified protocol from Nopens et al. [28], was created. The details of the experimental setup and the recipe for the synthetic municipal wastewater have been provided by Drzymała et al. [29]. Each experimental column received wastewater injection, with the addition of DCF and SMX (2 mg L^{-1} of each) for testing purposes.

Figure 1. Constructed wetlands used in experiments.

A total of 12 test columns were utilized to investigate two technological parameters in the experiments, namely the frequency of wastewater dosing (either twice a week with a 2.5 L volume or five times a week with a 1 L volume) and the inclusion of a pharmaceutical mixture consisting of DCF and SMX at a 2 mg L^{-1} concentration (as indicated in Table 2). Eight sample sets were collected on a weekly basis, with all samples being subjected to triplicate analysis.

Table 2. The description of CW columns.

Columns Description		Frequency of Wastewater Dosing	Presence of DCF and SMX	Presence of *M. giganteus*
Rack 1	R1-CTRL	2 times a week in a volume of 2.5 L, HLR [1] = 80 L d^{-1} m^{-2}	−	+
	R1-PhC		+	
Rack 2	R2-CTRL	5 times a week in a volume of 1.0 L, HLR = 32 L d^{-1} m^{-2}	−	+
	R2-PhC		+	

Notes: [1] HLR—hydraulic loading rate; R1—rack 1; R2—rack 2; CTRL—control wastewater (without DCF and SMX); PhC—wastewater enriched with DCF and SMX.

To evaluate the effectiveness of wastewater treatment, changes in the following parameters were monitored: DCF and SMX concentrations—to determine the degree of removal of pharmaceuticals from wastewater; ammonium nitrogen and total organic carbon concentration—to assess the effectiveness of the purification process. The removal

efficiency (R) was calculated based on the influent and effluent concentrations according to (1):

$$R(\%) = \frac{C_{influent} - C_{effluent}}{C_{influent}} \times 100 \quad (1)$$

2.1.3. Chemical Analysis of Wastewater

The wastewater samples were filtered and then further analysis were performed. TOC was determined using a TOC-L analyser Shimadzu Corporation (Tokyo, Japan). The N-NH_4 concentration was measured with Merc KGaA (Darmstadt, Germany) test no. 1.00683.0001

Pharmaceutical concentrations were tracked using an HPLC system equipped with a C18 HypersilTM Gold column (250 mm × 4.6 mm, pore size: 5 µm) manufactured by Thermo Scientific, Poland. The mobile phase was a 40:60 mixture of acetonitrile and acetate buffer (pH 5.7) with a constant flow rate of 1.0 mL min^{-1}. The retention time for DCF was 8.4 ± 0.3 min, while for SMX, it was 6.4 ± 0.2 min. The limit of quantification was 0.2 mg L^{-1}. The experiments were conducted at four wavelengths: 220 nm, 240 nm, 268 nm, and 280 nm. Data analysis was carried out using Dionex Chromeleon™ v. 6.8 software [29].

2.2. Vicia faba Experiment

2.2.1. Germination of *Vicia faba* Seeds

The *V. faba* seeds of the Windsor White variety (Eden, Poland) were soaked in distilled water for 24 h and then placed on a moist layer of lignin to germinate in the darkness at a temperature of 20 ± 2 °C. After 3 days, seeds with a primary root length of 3 to 5 cm were selected for further cultivation. To stimulate the growth of secondary roots, roughly 5 mm of the primary root was removed and the seeds were allowed to grow secondary roots for an additional 3 days on a wet filter paper surface.

2.2.2. Micronucleus Assay

The ISO 29200 [30] protocol was followed to conduct the micronucleus test. A reduction in the mitotic index (MI) value and the presence of micronuclei (MN) or chromosomal aberrations (CA) in the test wastewater suggests its genotoxic, mutagenic, or cytotoxic potential. The study was conducted in two variations, hydroponic and soil cultures (with reference soil), in which plants were exposed to raw and treated sewage. The negative control was distilled water, while maleic hydrazide was used as the positive control (1.12 mg L^{-1} for hydroponic culture and 1.12 mg kg^{-1} for soil culture). Polystyrene pads were used to plant six seeds with secondary roots of around 10 mm in each of the 300 mL test containers for hydroponic culture.

To perform the soil culture experiment, the reference soil was prepared according to the OECD 222 [31] guidelines, consisting of 10% sphagnum peat, 20% kaolin clay, 0.3–1% calcium carbonate, and approximately 70% quartz sand. The total water-holding capacity (WHC) was determined and 250 g of soil was placed in each test container. The tested solutions were added to the soil, maintaining a 50% WHC, and six seeds with 10 mm long secondary roots were gently planted on the soil. The test containers were watered with the tested wastewater to maintain constant soil moisture (50% WHC), and the initial doses of pharmaceuticals in the soil were measured. For influent, the average initial doses were 0.52 mg DCF kg^{-1} and 0.72 mg SMX kg^{-1}, while for treated wastewater, they were 0.20 mg DCF kg^{-1} (with a range of 0.02–0.55 mg DCF kg^{-1}) and 0.08 mg SMX kg^{-1} (with a range of 0.01–0.31 mg SMX kg^{-1}). In the case of effluents, the range of doses varied depending on the removal efficiency of pharmaceuticals in CWs.

The test containers with the plants were kept under stable conditions, with a temperature of 20 ± 2 °C and a day/night cycle of 16 h light and 8 h darkness, for 48 h (for hydroponic culture) and 120 h (for soil culture).

In the hydroponic culture, roots were collected at 44, 46, and 48 h, while in the soil culture, roots were collected after 5 days (120 h). The roots were washed with distilled water, and then placed in Carnoy solution (a mixture of glacial acetic acid and 96% ethyl

alcohol in a 1:3 ratio) and stored at 4 °C for 24 h. After that, the roots were washed again with distilled water and preserved in 70% ethyl alcohol at 4 °C. For microscopic observation, the roots were rinsed with distilled water, treated with 1M HCl for 6 min at 60 °C, and then washed with distilled water for 3 min. Finally, root fragments were stained with 2% orcein for 1 min and viewed at a magnification of 1000× *g*.

One preparation was created for each plant, resulting in six preparations for each wastewater sample, corresponding to the six test plants. On each slide, 1000 cells were examined, and the following parameters were recorded:

- The Mitotic Index (MI) was the number of cells in the dividing process to the total number of observed cells;
- The Micronuclei Index (MN) was the number of cells with micronuclei to the total number of observed cells;
- The number of Chromosomal Aberrations (CA) to the total number of observed cells;

The decrease in the mitotic index (dMI, %) was calculated based on the MI for the negative control (K−) according to Equation (2):

$$MI(\%) = \frac{MI_{(K-)} - MI_{sample}}{MI_{(K-)}} \times 100; \quad (2)$$

Similar calculations were made to determine the respective increase or decrease in CA and MN.

2.3. Antioxidant Enzyme Activity

To assess the influence of the wastewater analyzed on the activity of antioxidant enzymes, the activity of catalase (CAT, EC 1.11.1.6) and superoxide dismutase (SOD, E.C. 1.15.1.1) was examined. After the roots were taken for the micronucleus experiment, the leaves of the plants were collected for enzymatic analysis. To prepare the enzyme homogenates, the Pro200 homogenizer (Pro Scientific Inc., Oxford, CT, USA) was used with the appropriate buffer (to determine CAT activity: 0.06 M sodium phosphate buffer, pH 7.4; for SOD activity: 0.05 M carbonate buffer, pH 10.2). The homogenates were then centrifuged (20 min, 4000 rpm, 4 °C) and frozen (−45 °C). The catalase [32] and superoxide dismutase [33] activity, as well as the protein concentration [34], were determined using an Evolution 220 spectrophotometer (Thermo Fisher Scientific, Warszawa, Poland).

The increase in the CAT/SOD activity (iCAT/iSOD) was calculated based on the CAT/SOD activity for the negative control (K−) according to Equation (3):

$$iCAT/iSOD(\%) = \frac{CAT/SOD_{sample} - CAT/SOD_{(K-)}}{CAT/SOD_{(K-)}} \times 100; \quad (3)$$

2.4. Statistical Analysis

All statistical analyses were performed with STATISTICA v. 13.3 software (StatSoft Inc., Kraków, Poland). Statistical tests were first performed with the Shapiro–Wilk test. The results were then analyzed using the Mann–Whitney U test (the differences were considered statistically significant if $p < 0.05$).

3. Results

The constructed wetland system successfully eliminated the tested drugs from the wastewater during the experiment. Rack 1 removed 69% of DCF while rack 2 removed 87%; for SMX: rack 1 removed 79% while rack 2 removed 98%. Differences observed in drug removal were attributed to variances in the sewage dosing system to the treatment plant. Rack 2, which received more frequent dosing of lower-load wastewater, exhibited higher removal efficiency. Our previous study [29] provides a detailed account of the results, which are summarized in Table 3 concerning the changes in wastewater characteristics following CW treatment.

Table 3. Removal efficiency of TOC, N-NH_4, DCF and SMX in CWs [29].

Types of Columns	Removal Efficiency, R %			
	TOC	N-NH_4	DCF	SMX
R1-CTRL	87.8 ± 3.9	19.9 ± 7.3		
R1-PhC	87.3 ± 1.9	18.0 ± 11.4	68.8 ± 8.2	79.1 ± 4.3
R2-CTRL	93.3 ± 1.4	45.8 ± 11.9		
R2-PhC	92.3 ± 1.1	58.9 ± 10.0	86.8 ± 9.7	98.0 ± 0.8

3.1. Genotoxicity Tests toward Vicia faba

The MI of *V. faba* root cells was affected by the wastewater that was examined, encompassing both influent and treated in CW. The results that were obtained are exhibited in Figure 2, and the outcomes of the statistical analyses are demonstrated in Table 4.

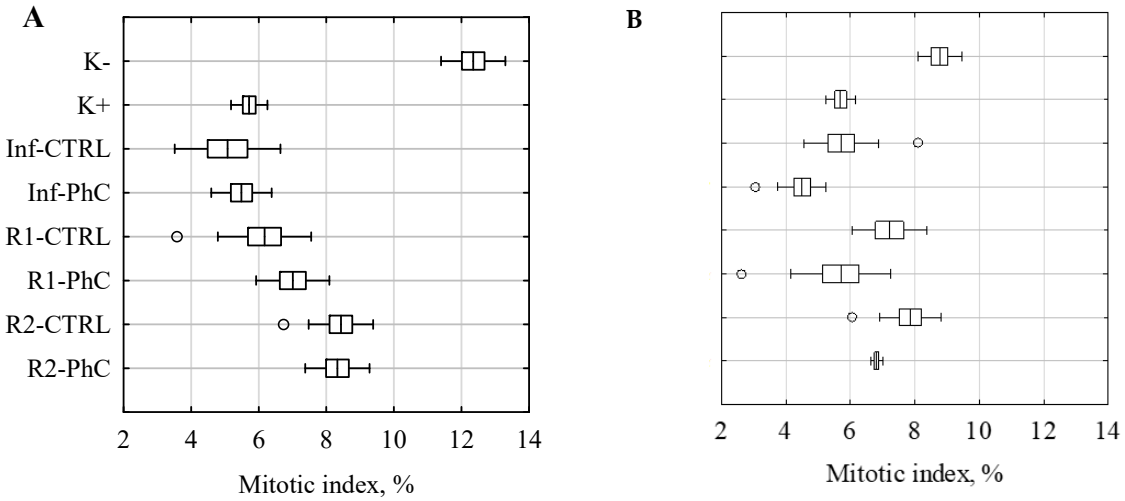

Figure 2. Effect of CWs wastewater on MI of *V. faba*; (**A**) hydroponic culture, (**B**) soil culture; K−/+—control negative/positive, Inf—influent, CTRL—control wastewater (without DCF and SMX); PhC—wastewater enriched with DCF and SMX; R1—rack 1; R2—rack 2.

In hydroponic culture, the tested wastewater had a more substantial impact on MI, resulting in a 57% decrease compared to a 42% decrease in soil culture. Similarly, effluent from CW exhibited a greater impact on hydroponic culture with a 39% average decrease in MI compared to only 19% in soil culture. The use of soil culture enabled the observation of the relationship between pharmaceutical-containing wastewater toxicity and control wastewater. Statistical analysis showed that the MI of *V. faba* root cells in effluents containing the tested drugs was significantly lower than those without pharmaceuticals (Mann–Whitney U test, $p < 0.05$).

In the soil experiment, a decrease in MI of only 14% was observed for control wastewater, whereas for treated wastewater containing DCF and SMX, the decrease in MI was almost 24%. Such correlations were not observed in hydroponic culture, as there were no significant differences in MI results between control effluents and effluents containing PhC (Mann–Whitney U test, $p < 0.05$). In hydroponic and soil cultures, a difference was observed in the proposed wastewater dosing systems, as determined by the Mann–Whitney U test ($p < 0.05$). In both experimental variations, wastewater from rack 1 (less frequent sewage dosing, higher load) demonstrated a greater increase in MI compared to the control conditions. Wastewater from rack 2 was characterized as less harmful to *V. faba* plants.

The statistical analysis of the results for MN did not reveal any influence of the examined wastewater (both influent and effluent) on this parameter (Mann–Whitney U test, $p < 0.05$). In both hydroponic and soil cultivation, only single micronuclei were observed,

which may have been a result of root contact with the tested sewage or other environmental or random factors. The results of the micronuclei analysis are presented in Table 5. No differences were observed in the incidence of MN when comparing hydroponic and soil cultivation or different sewage dosing systems, as determined by the Mann–Whitney U test ($p < 0.05$). The occurrence of statistically significant differences was limited to the positive control (maleic hydrazide at a concentration of 1.12 mg L^{-1} in hydroponic culture and 1.12 mg kg^{-1} in soil culture). In hydroponic culture, the frequency of MN was 0.77‰, while in soil culture, it was only 0.14‰.

Table 4. Impact of wastewater from CWs on the MI of *V. faba*: results and statistical analysis.

Samples	Hydroponic Culture		Soil Culture	
	MI, %	dMI, %	MI, %	dMI, %
Negative control (K−)	12.3 ± 1.0 ^{abd}	-	8.8 ± 0.7 ^{abd}	-
Positive control (K+)	5.7 ± 0.5 ^{ab}	53.7	5.7 ± 0.5 ^{ab}	35.0
Inf-CTRL	5.1 ± 1.6 ^{ac}	58.9	5.7 ± 1.2 ^{ac}	34.9
Inf-PhC	5.5 ± 0.9 ^{ac}	55.5	4.5 ± 0.7 ^{abc}	48.7
R1-CTRL	6.2 ± 1.4 ^{ae}	50.0	7.2 ± 1.2 ^{abcef}	17.8
R1-PhC	7.0 ± 1.1 ^{abcde}	43.2	5.7 ± 1.6 ^{acdef}	25.0
R2-CTRL	8.4 ± 1.0 ^{abce}	31.8	7.9 ± 0.9 ^{abcef}	10.4
R2-PhC	8.3 ± 1.0 ^{abcde}	32.5	6.8 ± 0.2 ^{abcdef}	22.2

Negative control (K−)—distilled water; Positive control (K+)—maleic hydrazide at concentration 1.12 mg L^{-1} in hydroponic culture and 1.12 mg kg^{-1} in soil culture; Statistical analysis according to the Mann–Whitney U test, $p < 0.05$: [a]—occurrence of statistically significant differences in relation to the negative control; [b]—occurrence of statistically significant differences in relation to the positive control; [c]—occurrence of statistically significant differences between toxicity levels of wastewater influent and effluent; [d]—occurrence of statistically significant differences between hydroponic and soil culture; [e]—statistically significant differences between toxicity of wastewater from racks with different frequencies of sewage dosing; [f]—statistically significant differences between toxicity of control wastewater and sewage containing pharmaceuticals.

Table 5. Impact of wastewater from CWs on the MN of *V. faba*: results and statistical analysis.

Samples	Hydroponic Culture	Soil Culture
	Micronuclei, ‰	
K−	0.00 ± 0.00 ^{ab}	0.00 ± 0.00 ^{ab}
K+	0.77 ± 0.29 ^{abd}	0.14 ± 0.07 ^{abd}
Inf-CTRL	0.01 ± 0.02 ^{b}	0.00 ± 0.00 ^{b}
Inf-PhC	0.01 ± 0.02 ^{b}	0.00 ± 0.01 ^{b}
R1-CTRL	0.00 ± 0.00 ^{b}	0.00 ± 0.01 ^{b}
R1-PhC	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}
R2-CTRL	0.00 ± 0.01 ^{b}	0.00 ± 0.00 ^{b}
R2-PhC	0.00 ± 0.00 ^{b}	0.00 ± 0.00 ^{b}

Statistical analysis according to the Mann–Whitney U test, $p < 0.05$: [a]—occurrence of statistically significant differences in relation to the negative control; [b]—occurrence of statistically significant differences in relation to the positive control; [d]—occurrence of statistically significant differences between hydroponic and soil culture.

The results of the chromosomal aberration (CA) analysis (Figure 3, Table 6) revealed more interesting correlations. No CA was observed under control conditions, where roots were placed in distilled water or when the soil was watered with distilled water. However, nearly all wastewater samples in the hydroponic culture (both influent and treated) had a negative effect in the form of chromosomal aberrations. For soil cultivation, CA effects were

only observed in the case of influents. While statistically significant differences between hydroponic and soil culture were observed for some results, this was likely due to the large standard deviations resulting from the infrequent occurrence of genetic material damage tests. Furthermore, no impact of the wastewater dosing system on the presence of CA was observed.

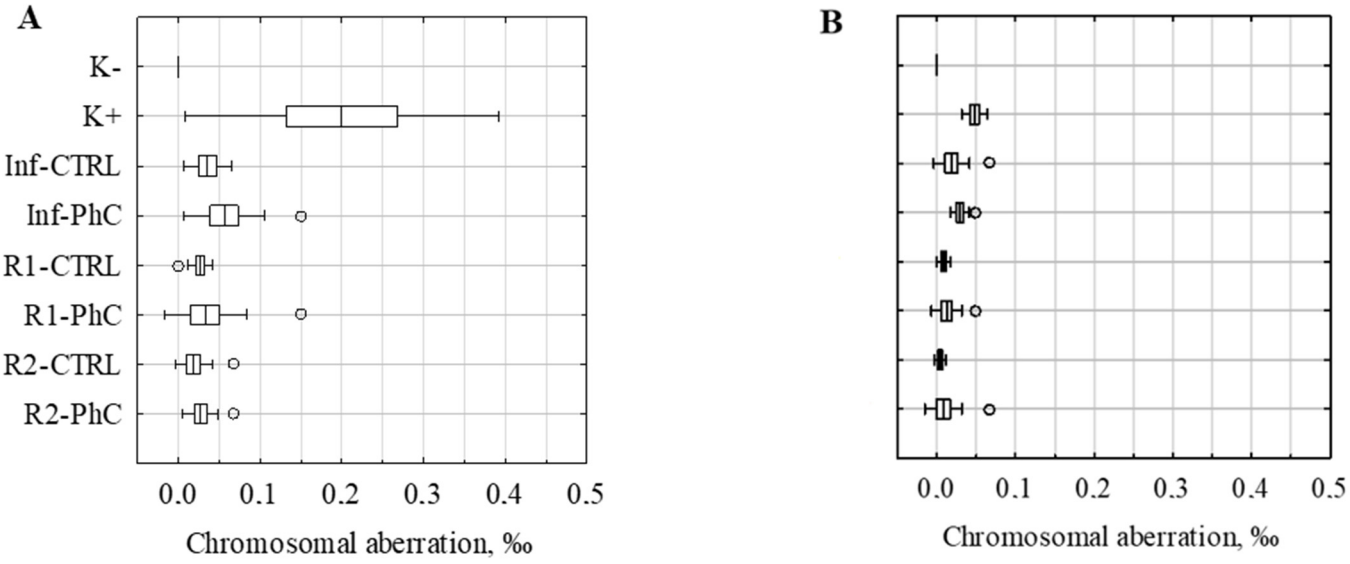

Figure 3. Effect of CWs wastewater on CA in *V. faba* cells; (**A**) hydroponic culture, (**B**) soil culture; K−/+—control negative/positive, Inf—influent, CTRL—control wastewater (without DCF and SMX); PhC—wastewater enriched with DCF and SMX; R1—rack 1; R2—rack 2.

Table 6. Impact of wastewater from CWs on the CA of *V. faba*: results and statistical analysis.

Samples	Hydroponic Culture	Soil Culture
	Chromosomal Aberrations, ‰	
K−	0.00 ± 0.00 ^{ab}	0.00 ± 0.00 ^{ab}
K+	0.20 ± 0.19 ^{abd}	0.05 ± 0.02 ^{abd}
Inf-CTRL	0.04 ± 0.03 ^{ab}	0.02 ± 0.02 ^{abc}
Inf-PhC	0.06 ± 0.05 ^{a}	0.03 ± 0.01 ^{abc}
R1-CTRL	0.03 ± 0.02 ^{abd}	0.01 ± 0.01 ^{bd}
R1-PhC	0.03 ± 0.05 ^{ab}	0.01 ± 0.02 ^{b}
R2-CTRL	0.02 ± 0.02 ^{ab}	0.00 ± 0.01 ^{b}
R2-PhC	0.03 ± 0.02 ^{abd}	0.01 ± 0.02 ^{bcd}

Note(s): Statistical analysis according to the Mann–Whitney U test, $p < 0.05$: ^{a}—occurrence of statistically significant differences in relation to the negative control; ^{b}—occurrence of statistically significant differences in relation to the positive control; ^{c}—occurrence of statistically significant differences between toxicity levels of wastewater influent and effluent; ^{d}—occurrence of statistically significant differences between hydroponic and soil culture.

3.2. Activity of Catalase and Superoxide Dismutase

Upon analyzing the results of CAT, it was observed that the tested wastewater caused an increase in enzyme activity compared to the control conditions, both in hydroponic and soil culture (Figure 4, Table 7). Raw sewage had the greatest impact on enzyme activity. In the case of raw wastewater tested in hydroponic culture, there was an average increase in CAT activity of 56%, while in soil culture, the increase was almost 78%. Although higher increases were observed for samples exposed to pharmaceutical-containing wastewater, statistical analysis did not confirm these observations (Mann–Whitney U test, $p < 0.05$).

Treated wastewater also caused an increase in CAT activity, but not at a statistically significant level (Mann–Whitney U test, $p < 0.05$). Additionally, CAT activity in soil culture was slightly lower, but there were no statistically significant differences. Moreover, no statistically significant differences were found during the analysis of sewage dosing systems (Mann–Whitney U test, $p < 0.05$). The absence of statistically significant differences, despite visible correlations, can be attributed to significant standard deviations resulting from a large scatter of the analyzed results.

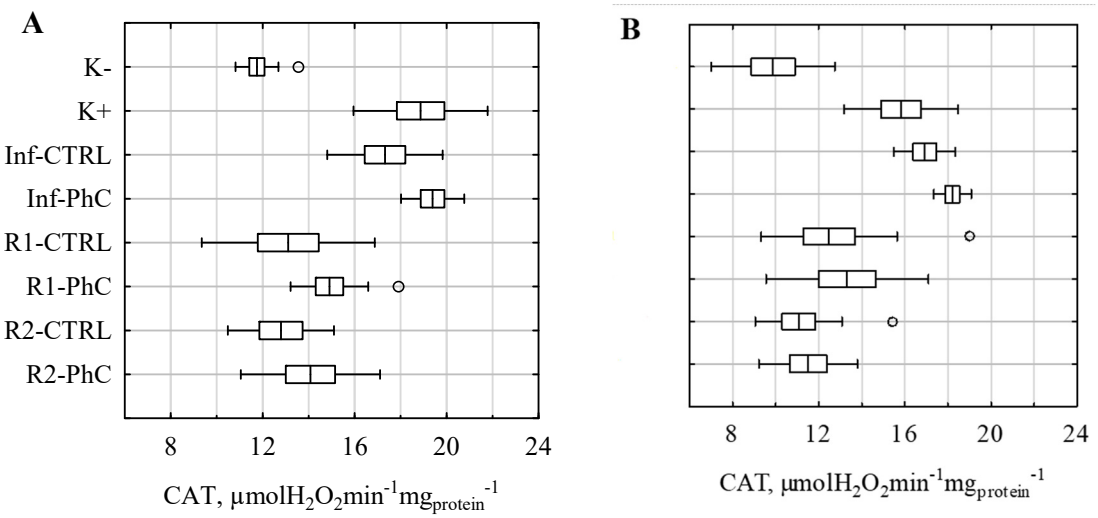

Figure 4. Effect of CWs wastewater on the CAT activity in *V. faba* cells; (**A**) hydroponic culture, (**B**) soil culture; K−/+—control negative/ positive, Inf—influent, CTRL—control wastewater (without DCF and SMX); PhC—wastewater enriched with DCF and SMX; R1—rack 1; R2—rack 2.

Table 7. Results and statistical analysis of the impact of wastewater from CWs on the CAT activity in *V. faba* cells.

Samples	Hydroponic Culture		Soil Culture	
	Catalase, $\mu mol\ H_2O_2\ min^{-1}mg_{\ Protein}{}^{-1}$	iCAT, %	Catalase, $\mu mol\ H_2O_2\ min^{-1}mg_{\ Protein}{}^{-1}$	iCAT, %
K−	$11.8 \pm 0.9^{\ ab}$	-	$9.9 \pm 2.9^{\ ab}$	-
K+	$18.9 \pm 2.9^{\ ab}$	60.5	$15.8 \pm 2.6^{\ ab}$	60.1
Inf-CTRL	$17.3 \pm 2.5^{\ ac}$	47.4	$16.9 \pm 1.4^{\ ac}$	71.3
Inf-PhC	$19.4 \pm 1.4^{\ ac}$	65.0	$18.2 \pm 0.9^{\ ac}$	84.3
R1-CTRL	$13.1 \pm 3.8^{\ bc}$	11.6	$12.5 \pm 3.2^{\ bc}$	26.3
R1-PhC	$14.9 \pm 1.7^{\ bc}$	26.8	$13.3 \pm 3.8^{\ bc}$	34.8
R2-CTRL	$12.8 \pm 2.3^{\ bc}$	8.9	$11.1 \pm 2.0^{\ bc}$	12.0
R2-PhC	$14.1 \pm 3.0^{\ bc}$	19.8	$11.5 \pm 2.3^{\ bc}$	16.5

Note(s): Statistical analysis according to the Mann–Whitney U test, $p < 0.05$: [a]—occurrence of statistically significant differences in relation to the negative control; [b]—occurrence of statistically significant differences in relation to the positive control; [c]—occurrence of statistically significant differences between toxicity levels of wastewater influent and effluent.

The results of the SOD activity analysis showed a clear impact of the tested wastewater on *V. faba* leaves (Figure 5, Table 8), similar to the CAT activity analysis. Influent caused a significant increase in SOD activity (average increase of 240% in hydroponic cultivation and 133% in soil cultivation). In hydroponic culture, effluents also caused a statistically significant increase in enzyme activity (48% on average). However, no such correlations

were observed in soil culture. While treated wastewater slightly increased SOD activity, the differences were not statistically significant (Mann–Whitney U test, $p < 0.05$).

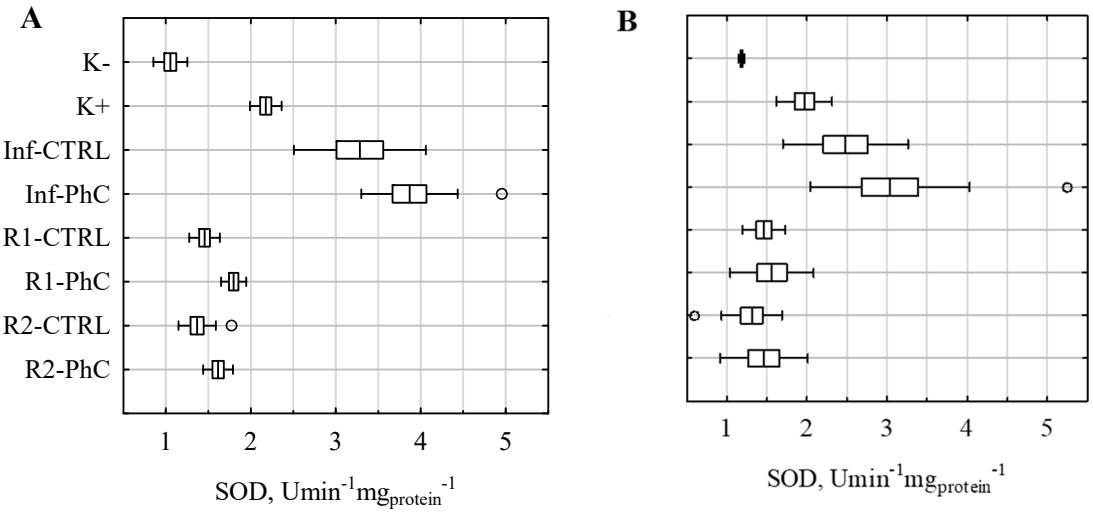

Figure 5. Effect of CW wastewater on the SOD activity in *V. faba* cells; (**A**) hydroponic culture, (**B**) soil culture; K−/+—control negative/positive, Inf—influent, CTRL—control wastewater (without DCF and SMX); PhC—wastewater enriched with DCF and SMX; R1—rack 1; R2—rack 2.

Table 8. Results and statistical analysis of the impact of wastewater from CWs on the SOD activity in *V. faba* cells.

Samples	Hydroponic Culture		Soil Culture	
	Superoxide Dismutase, $Umin^{-1}mg_{Protein}{}^{-1}$	**iSOD, %**	**Superoxide Dismutase, $Umin^{-1}mg_{Protein}{}^{-1}$**	**iSOD, %**
K−	1.1 ±0.2 ^{ab}	-	1.2 ± 0.0 ^{ab}	-
K+	2.2 ± 0.2 ^{ab}	106.6	2.0 ± 0.3 ^{ab}	66.2
Inf-CTRL	3.3 ± 0.8 ^{abcd}	211.8	2.5 ± 0.8 ^{acd}	109.6
Inf-PhC	3.9 ± 0.6 ^{abcd}	267.3	3.0 ± 1.0 ^{abcd}	156.5
R1-CTRL	1.5 ± 0.2 ^{abcf}	38.2	1.5 ± 0.3 ^{bc}	23.7
R1-PhC	1.8 ± 0.1 ^{abcef}	70.8	1.6 ± 0.5 ^{c}	32.1
R2-CTRL	1.4 ± 0.2 ^{abcf}	29.9	1.3 ± 0.4 ^{bc}	10.9
R2-PhC	1.6 ± 0.2 ^{abcef}	53.4	1.5 ± 0.5 ^{c}	23.6

Note(s): Statistical analysis according to the Mann–Whitney U test, $p < 0.05$: [a]—occurrence of statistically significant differences in relation to the negative control; [b]—occurrence of statistically significant differences in relation to the positive control; [c]—occurrence of statistically significant differences between toxicity levels of wastewater influent and effluent; [d]—occurrence of statistically significant differences between hydroponic and soil culture; [e]—statistically significant differences between toxicity of wastewater from racks with different frequencies of sewage dosing; [f]—statistically significant differences between toxicity of control wastewater and sewage containing pharmaceuticals.

Furthermore, the presence of pharmaceutical compounds (PhC) in wastewater was found to affect SOD activity in hydroponic culture, with effluents containing DCF and SMX causing a greater increase in enzyme activity. The average increase in SOD activity for PhC-containing effluent was 62% compared to 34% for control effluent (Mann–Whitney U test, $p < 0.05$). Significant differences were also observed between the two sewage dosing systems in hydroponic culture, with wastewater containing PhC from rack 2 showing better ecotoxicological properties than wastewater from rack 1 (Mann–Whitney U test, $p < 0.05$).

Moreover, significant differences were found between hydroponic and soil culture in terms of SOD activity (Mann–Whitney U test, $p < 0.05$), with a higher increase observed in hydroponic culture. However, in both variants of the experiment, a decrease in SOD activity was observed due to the wastewater treatment process. Influent caused a considerable increase in SOD activity, as mentioned earlier, with an average increase of 240% for hydroponic and 133% for soil culture. However, in the case of effluents, these values were only 48% and 23%, respectively.

4. Discussion

The chosen method for these studies, known as the micronucleus test, serves as a versatile tool for evaluating the genotoxic, cytotoxic, and mutagenic effects of both simple and complex mixtures and pure substances. This test yields significant insights into the substance's impact at a subcellular level. The occurrence of changes in the mitotic index of cells can be indicative of the substance's cytotoxic effects on the cells under examination. In addition, the presence of genetic material damage, such as micronuclei and chromosomal aberrations, can indicate mutagenic and genotoxic effects, respectively [35].

The findings indicate that both influent and treated wastewater samples have the potential to harm the natural environment. The study reveals a reduction in the mitotic index of *V. faba* root cells and an increase in the number of damages to genetic material, specifically chromosomal aberrations, while no noticeable increase in micronuclei was observed. Therefore, the tested wastewater can be classified as genotoxic and cytotoxic to the cells analyzed, while its mutagenic properties were not evident. Similarly, Zgórska and Borgulat [36] obtained comparable results while examining the toxic attributes of textile wastewater, where they noticed a rise in the number of instances of genetic damage, including micronuclei and chromosomal aberrations, and a decline in *V. faba* cells' mitotic index. On the other hand, Oubane et al. [37] observed a significant reduction in the genotoxicity of sewage from six wastewater treatment plants in Morocco. They concluded that using the *V. faba* micronucleus test was quite effective in evaluating wastewater genotoxicity, but most analyzed samples, even post-treatment, still exhibited a detrimental impact on the cells assessed. The researchers also concluded that direct contact between plants and wastewater, such as aquaculture, is a more promising approach for wastewater biomonitoring, as opposed to examining samples from soil culture. Mancini et al. [38] also found that the *V. faba* micronucleus test is an effective tool for assessing the genotoxicity of wastewater from medical devices and pharmaceutical manufacturers. They observed a decrease in the genotoxic properties of wastewater after undergoing the treatment process.

Although the analysis conducted did not reveal a rise in the number of micronuclei, there was a statistically significant increase in the number of chromosomal aberrations observed following exposure to sewage. Micronuclei are tiny extranuclear structures situated adjacent to the nucleus within the cytoplasm, detectable in root meristematic interphase cells in the subsequent cell cycle after exposure to mutagenic agents [39]. Therefore, the absence of micronuclei in this study could be attributed to inadequate exposure of the *V. faba* root cells to the wastewater samples under examination.

It is important to acknowledge that variances in experimental procedures can significantly impact the evaluation of a substance's toxicity. In this study, there were statistically significant differences between the results obtained from water and soil culture. A greater reduction in mitotic index was observed in water culture compared to soil culture, and a similar trend was also observed in the analysis of chromosomal aberrations.

In our previous study [26] the toxicity of DCF and SMX, as well as their combination, was evaluated using three model organisms: the bacterium *A. fischeri*, the crustacean *D. magna*, and the vascular plant *L. minor*. Notably, the binary mixture of DCF and SMX exhibited the highest level of toxicity, demonstrating a significant risk (high toxicity). DCF was categorized as a compound with moderate toxicity, showing a noteworthy environmental risk to aquatic organisms. In contrast, SMX was identified as a low-toxicity compound, indicating a lower environmental risk. We also proved that the plant *L. minor* was the most

sensitive to both SMX and the DCF-SMX combination (MIX). Drawing from our past experiences and the results of ecotoxicity tests involving DCF and SMX, we can conclude that the genotoxicity identified by the *V. faba* test was a result of the combination of pharmaceutical residues in the wastewater and the products resulting from their interactions.

A preliminary study examining parent active compounds (diclofenac and sulfamethoxazole) reached similar conclusions to the current work [40]. The variations in toxicity observed in water and soil culture could be attributed to the physicochemical properties of the substances being tested. The results indicated that DCF readily binds to soil particles, making it less available to test organisms (*V. faba*), while SMX does not accumulate in soil and thus shows higher toxicity [40]. Wastewater, also synthetic, such as those analyzed in this work, changes its properties depending on the conditions under which the treatment process takes place. Therefore, the properties of treated wastewater are a component of many parameters. The matter is much simpler if pure substances are introduced into the environment. Due to knowledge of the physicochemical properties of these pollutants, such as the water solubility, dissociation constant, or octanol-water partition coefficient, we are able to predict their properties after introducing them to various environments [41].

The selection of experimental conditions is important for determining the toxicity classification of a substance. For wastewater, it is essential to carefully consider the experimental conditions that closely resemble the actual use of treated wastewater. In cases where the effluent is intended for discharge into surface waters, hydroponic culture should be employed. However, when the effluent is used for irrigation or fertilization of agricultural fields, both soil culture (direct introduction of leachate into the soil) and aquaculture tests (leaching of contaminants from the soil with rainwater to surface water) should be conducted. This comprehensive approach is necessary to obtain a complete understanding of the potential environmental threat.

While there is a lack of literature data on using plants to assess the genotoxicity of pharmaceuticals, intriguing reports exist regarding the evaluation of genotoxicity in atrazine using *Allium cepa*, *Vicia faba*, and *Hordeum vulgare* [42–44]. Upon comparing the obtained results, it can be inferred that the sensitivity of the applied bioassays was similar, as researchers employed a comparable range of pesticide concentrations in their tests. Both a decline in the mitotic index and a concentration-dependent increase in the frequency of chromosomal aberrations were observed.

Thus, it cannot be definitively stated that *Vicia faba* surpasses other plants for genotoxicity testing. Nevertheless, in our view, the broad bean test holds several advantages as well as one disadvantage:

Advantages:

- Genomic Proximity: The presence of large chromosomes and proximity to cell division stages enables accurate genotoxicity assessment.
- Quantitative Data: The assay delivers quantitative data on the micronucleus frequency and chromosome abnormalities.
- Visual Assessment: Effects on root growth and chromosome structure are readily observable, simplifying result interpretation.
- Simplicity and Accessibility: The broad bean root tip assay is relatively straightforward, requiring no specialized equipment or extensive training. Additionally, the consistent tissue of the broad bean root tip simplifies the maintenance and preparation of microscope slides.
- Disadvantage:
- Time-consuming: The assay takes longer to produce results due to the specific cell cycle stages *Vicia faba* goes through.

The study conducted explored an important aspect of oxidative stress. In the natural environment, a balance between reactive oxidative species (ROS) and mechanisms that eliminate them is necessary. Any disturbance to this balance leads to oxidative stress [45]. The activity of enzymes responsible for maintaining balance in organisms can be used to assess oxidative stress. These enzymes include catalase, superoxide dismutase, glutathione

S-transferase, ascorbate peroxidase, and glutathione reductase [46]. This study focused on catalase and superoxide dismutase. The findings showed that both enzymes' activity increased when the plant was exposed to sewage samples compared to the control (distilled water). This indicated the activation of mechanisms to counteract oxidative stress. In a previous study by Drzymała and Kalka [40] on parent pharmaceuticals (diclofenac, sulfamethoxazole), increased activity of these enzymes was observed due to the drugs' effects. However, in this study, the control wastewater without pharmaceuticals also increased the enzyme activity, suggesting that the drugs were only one factor causing oxidative stress in *V. faba* leaf cells. Evaluating antioxidant enzyme activity is useful in detecting early effects of substances on model organisms that may not be evident in other tests (e.g., reproduction or mortality tests).

Our research revealed a significant increase in the activity of CAT and SOD in *V. faba* leaves exposed to the analyzed sewage, indicating the presence of oxidative stress in the cells. The testing of antioxidant enzyme activity is a commonly used tool for assessing the harmfulness of a substance at the subcellular level. For example, Fatima and Ahmad [47] studied eight antioxidant enzymes to assess the harmfulness of heavy metals in wastewater using the bioindicator *Allium cepa*. They found that testing the activity of antioxidant enzymes can be helpful in detecting heavy metal contamination. Enzyme activity studies can provide a broader perspective on the mechanisms of action of a mixture of different pollutants. Cai et al. [48] demonstrated the usefulness of a multilevel toxicity analysis of textile dye effluents against the algae *Scenedesmus obliquus*. They focused on algae growth, chlorophyll-A content, SOD activity, and cell membrane integrity. Zhang et al. [49] used *S. obliquus* to evaluate the process of wastewater detoxification in the treatment process, finding that SOD activity was a sensitive parameter for assessing wastewater toxicity.

Based on the test results, the tested wastewater was found to be genotoxic and cytotoxic to *V. faba*; however, mutagenic effects were not observed. The micronucleus test proved to be a highly sensitive tool, providing a wealth of information. When combined with antioxidant enzyme analysis, it offers valuable insights into the sewage system's functioning and detoxification process. Conducting such detailed research at the subcellular level enables the detection of even minor changes in the composition and properties of the analyzed wastewater.

In summary, the micronucleus assay offers a major advantage in that it can be easily conducted without the need for specialized laboratory equipment, is relatively inexpensive, and allows for delayed analysis of results. In addition, because the composition of real wastewater changes over time, periodic analysis is necessary. Despite these drawbacks, the micronucleus test provides valuable data for assessing the mutagenic, cytotoxic, and genotoxic potential of samples being tested, making it worthwhile to periodically conduct these tests to monitor the subcellular-level harmfulness of wastewater.

5. Conclusions

The findings from this study demonstrated the high effectiveness of the micronucleus test with *V. faba* for evaluating the genotoxic, cytotoxic, and mutagenic potential of wastewater-containing pharmaceuticals (namely diclofenac and sulfamethoxazole). After evaluating the pros and cons of this approach, it was suggested that subcellular-level tests be conducted periodically to detect changes in wastewater toxicity at an earlier stage, before they become apparent in standard acute toxicity tests. Additionally, assessing the antioxidant enzyme activity was found to be a valuable tool for monitoring the wastewater detoxification process and determining the degree of wastewater treatment.

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

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