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Response of Maize (*Zea mays* L.) to Soil Contamination with Diclofenac, Ibuprofen and Ampicillin and Mixtures of These Drugs

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Abstract: Diclofenac (DIC) and ibuprofen (IBU) are popular non-steroidal anti-inflammatory drugs (NSAIDs), while ampicillin (AMP) is a relatively common antibiotic for treating bacterial infections. All of these drugs are only slightly retained in the human body, and therefore, their presence is found in the environment. In the present study, an attempt was made to determine the effects of diclofenac, ibuprofen and ampicillin on the growth and development of early stages of maize. The drugs were used both separately and in binary mixtures and a ternary mixture. The study found that NSAIDs exhibited the greatest phytotoxicity. Both diclofenac and ibuprofen, applied at the highest doses, reduced the fresh weight yield of maize seedlings relative to the control. Ampicillin, on the other hand, showed no adverse effect on the growth and development of maize seedlings. Analyzing the effect of selected drugs on changes in the content of photosynthetic pigments, it should be noted that they led to a systematic decrease in the content of chlorophylls and carotenoids in maize seedlings. Small changes in the values of the basic parameters of chlorophyll fluorescence may indicate the possibility of stress in maize seedlings.

Keywords: maize; phytotoxicity; NSAIDs; ampicillin; photosynthetic pigments; chlorophyll fluorescence

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1. Introduction

According to the United Nations definition, sustainable development is "development that meets the needs of the present without compromising the ability of future generations to meet their own needs." To achieve it, we must harmonize three key elements: economic growth, social inclusion and environmental protection. In the era of striving for sustainable development of human civilization, the aim is to ensure the well-being of the present generations while guaranteeing the ability to meet the basic needs of future generations. Such an approach to socio-economic development must also take into account the care of natural balance and environmental quality. One of the primary factors having a major impact on improving the quality and prolongation of human life are substances known as Pharmaceuticals and Personal Care Products (PPCPs). These include personal care substances, active ingredients in cosmetics, as well as all kinds of pharmaceutical products used in human treatment. As a result, there is a tremendous increase in drug production and the progressive development of the pharmaceutical industry, which, in turn, translates into increased consumption of drugs by the public. Several reasons contribute to the observed general trends indicating increasing demand for pharmaceuticals, such as aging populations, ubiquitous advertising of drugs on television and the Internet, and the ability to purchase many medications without the need for prescriptions issued by doctors. Another reason for the increased consumption of medicines is also the constant progress in the development of medical research, which is bringing new pharmaceuticals and drugs to the market for diseases that were previously considered incurable. Currently, more than

4000 active pharmaceutical substances are available for use in animal and human treatment in Europe alone [1–5].

Realizing, as it were, the second main premise related to sustainable human development, concerning the need to maintain the quality of the environment for future generations, scientists around the world are drawing attention to the fact that increased global consumption of drugs inevitably brings with it increased excretion. None of the available drugs is absorbed 100% by the human body; a large proportion, either in unchanged form or as metabolites, ends up as feces in the sewage system. At the same time, it should also be noted here that the metabolites show similar toxicity to the active pharmaceutical substance very often. Wastewater containing drugs and their metabolites, in turn, goes to treatment plants, and it is now known that most classical treatment plants cannot cope with the complete removal of drugs, and the process reaches a maximum of about 90%. Therefore, there are always some quantities of drugs in the treated wastewater, which, in turn, causes these substances to enter watercourses, rivers or groundwater and underground waters. Current available analytical equipment has made it possible to determine drug concentrations in water within the range of ng and μg per liter of water [6–10]. Another anthropogenic source of environmental contamination by pharmaceuticals is the irresponsible handling of expired medicines, which are dumped directly in landfills or thrown into domestic sewage systems. In addition, groundwater can be "fed" with various types of drugs due to leaching from decomposing cadavers [11,12].

A very important source of contamination of water and soil environments are the so-called veterinary drugs commonly used in animal husbandry. Although the European Union banned the use of antibiotics as growth-stimulating substances in animals in 2006, many countries continue to use these practices in poultry, cattle and pig farming, as well as in aquaculture. In this way, drug residues end up directly in water and soil, and an additional source of pharmaceuticals is slurry and manure from treated animals or those to which these agents are administered for non-therapeutic purposes [13–15]. Treated sewage, which is increasingly used to irrigate crops, may be an important source of medicines for soil environments. The extent of this phenomenon can be proven by the fact that in some countries of the Middle East, as much as 50% of treated sewage is used to irrigate agricultural fields. The use of sewage sludge as organic fertilizers is also important as a factor influencing the increase of drugs in the soil environment [4,5,7,16–18].

As previously written, the concentrations of pharmaceuticals found in the aquatic or soil environment are in the order of ng/L and μg/L, which should not pose a significant ecotoxicological threat. There have even been reports in the literature that indicate there is no threat to the environment from treated sewage used for irrigation [18,19]. However, we should be aware that there is never one drug in nature, only their mixtures, and the impact of which on individual elements of the environment we cannot predict. This applies to both synergism and antagonism in their mutual interactions. Moreover, drugs are permanent pollutants, and their constant supply from many sources causes their concentrations in the environment to be higher and the long-term effects unknown. Therefore, over the last twenty years, a huge number of publications have appeared proving the harmful impact of drug residues on many elements of the environment, mainly water [1,3,4,9,13,17,20–23].

The contamination of aquatic environments with pharmaceuticals, which is undeniable for the moment, must, at the same time, cause these substances to enter the soil. Soil composition, soil water and air conditions, physicochemical properties such as pH, sorption properties or even the content of organic fractions determine that drugs can accumulate in the soil. This results in a situation where drugs, in turn, end up in crops, where they are then metabolized, which is why plants are often called "green livers." However, a large supply of pharmaceuticals in the soil can prevent plants from completely metabolizing them, which will affect the yield and quality of these plants. In addition, plant food then becomes an additional source of drugs for animals and humans. This raises legitimate concerns, especially if the human body is exposed to additional amounts of antibiotics, which can compound the already observed phenomenon of antibiotic resistance or the

disruption of normal human hormonal balance due to excessive consumption of hormonal drugs. Excessive consumption of non-steroidal anti-inflammatory drugs [1,5,15,16,23–27] is also not without its impact on the normal condition of both plant and animal organisms and humans.

For these reasons, in the work presented here, we undertook the task of determining the effects of two drugs classified as NSAIDs—diclofenac and ibuprofen, as well as the antibiotic ampicillin—on the growth and development of maize (*Zea mays* L.) seedlings. In the studies conducted, we were more interested in the effects of mixed binary and trinary on this plant than in the individual effects of each of these drugs. The choice of IBU and DIC from the group of NSAIDs was based on the fact that these are the most popular drugs. Ibuprofen as an aspirin alternative was introduced in 1969. Today, it is used in huge quantities in the form of tablets, ointments, etc. IBU is excreted from the human body in 15% unchanged form, the remaining amounts being two isomers designated COOH-IBU and OH-IBU. Ibuprofen is now considered a persistent environmental pollutant, as it is 90% eliminated in wastewater treatment plants [6,19,28,29]. Diclofenac is considered the most effective therapeutic agent with analgesic and anti-inflammatory effects, having been introduced in 1979 and now consumed in the thousands annually. Diclofenac's removal from wastewater is lower than ibuprofen's, with a maximum of 70%; hence, it is commonly detected in water and soil samples. At the same time, a number of studies have proven that DIC exhibits relatively high toxicity to aquatic organisms, which is why it was already placed on the list of substances subject to monitoring by the European Commission in 2013 [3,7,9,11,22,30,31]. Ampicillin, on the other hand, is a semi-synthetic β-lactam with a broad spectrum of activity. The antibiotic was introduced in 1961 and is still frequently used to treat humans and animals, and in some countries, it is also used as a muscle growth promoter for livestock. Ampicillin is excreted about 70% unchanged by the kidneys 12 h after ingestion, so the unchanged form of this antibiotic and its metabolites are often detected in wastewater [13,25,29]. The choice of maize as a research object was prompted by the relatively high sensitivity of this plant to pharmaceutical contamination of soils due, among other things, to the fact that it conducts C4-type photosynthesis [32]. In addition, the fact that this plant ranks first in cereal production and that about 850 million tons of maize grain are produced annually worldwide was not insignificant for the selection of this plant for the study.

2. Materials and Methods

2.1. Chemicals

The non-steroidal anti-inflammatory drugs diclofenac (DIC), 2-[(2,6-Dichlorophenyl) amino] benzeneacetic acid sodium salt (≥98% purity) and ibuprofen (IBU), (±)-2-(4-isobutylphenyl)propanoic acid (≥98% purity) and the antibiotic ampicillin (AMP) D-(−)-α-aminobenzylpenicillin sodium salts (anhydrous, 96.0–102.0%) used in the study were purchased from Sigma-Aldrich Chemical Co. (Poznan, Poland).

2.2. Maize Seedling Growth Test

The phytotoxicity test of the tested drugs was carried out in a vegetation hall under strictly defined and controlled conditions, concerning the temperature of 20 ± 2 °C, illumination at 170 µmol m^{-2} s^{-1} in a 16 h day/8 h night system, and substrate humidity equal to 70% ppw, as described in OECD/OCDE 208/2006 [33]. Three drugs, DIC, IBU and AMP, were used for the test, and their effects on the growth and development of maize (*Zea mays* L.) seedlings were determined separately and as binary mixtures (DIC + AMP−1:1, IBU + AMP−1:1, DIC + AMP−1:1) and a ternary mixture (DIC + IBU + AMP−1:1:1). Both drugs and their mixtures were introduced into the soil, which was clayey sand, at concentrations of 0 (control), 1, 10, 100 and 1000 mg kg^{-1} of soil dry weight (DW). In each pot containing 250 g of soil with medicines and their mixtures, 10 seeds of common maize of the Rywal variety, which came from the seed station—Breeding and Production Plant in Nieznanice, Silesia Province, Poland, were sown. Fourteen days after sowing the

maize seeds, digital photos of the seedlings were taken, documenting the external appearance of the plants, and chlorophyll fluorescence was measured. In order to determine the phytotoxicity of the tested drugs and their mixtures, the length of the seedlings and their roots were also measured [34], the fresh weight yield of the plants was determined and, using the collected material, the dry matter content [35] and the level of all photosynthetic pigments [36] were determined. In addition, seed germination potential (GP) and seed germination capacity (GR) were also determined according to the method described by Liu et al. [37].

$$GP = \frac{\text{Number of germinated seeds on the } 3^{rd} \text{ day}}{Seeds\ number\ for\ the\ test} \times 100\%$$

$$GR = \frac{\text{Number of germinated seeds on the } 7^{th} \text{ day}}{Seeds\ number\ for\ the\ test} \times 100\%$$

All determinations were performed in a minimum of 4 replicates.

2.3. Photosynthetic Pigments Content Measurement

In order to determine the content of photosynthetic pigments, 200 mg of fresh plant material was sampled and crushed with 80% acetone. The contents were centrifuged, absorbance was measured in the filtrate at 470, 647 and 664 nm, and calculations were made using the relevant formulas given by Oren et al. [36].

a = 11.78 (ext. 664)–2.29 (ext. 647)

b = 20.05 (ext. 647)–4.77 (ext. 664)

c = 1000 (ext. 470)–3.27a-104b

Chl a = 25a/DW (mg)

Chl b = 25b/DW (mg)

Car = 25c/229DW (mg)

2.4. Chlorophyll Fluorescence Measurement

Chlorophyll fluorescence was measured using an OS1p-type chlorophyll fluorescence meter (GEOMOR TECHNIK, Poland). As part of the measurement carried out, after prior adaptation in the dark, the magnitude of the initial fluorescence (F_0), the maximum fluorescence (F_m) and variable fluorescence (F_v), the maximum quantum yield of PSII photochemistry (F_v/F_m) and its more sensitive form F_v/F_0 were determined.

2.5. Statistical Analysis

Statistical analysis of the results obtained was performed using STATISTICA 13.3. Based on a one-way ANOVA analysis of variance followed by Tukey's post hoc test, the significance of differences was determined in the form of determining homogeneous groups. Significant differences were found at $p < 0.05$. Analyses of all biomarkers of phytotoxicity were performed a minimum of four times ($n = 4$), and the results are presented in tables as arithmetic mean ± standard deviation.

3. Results and Discussion

3.1. Phytotoxicity Test Results

Analyzing the results obtained from the tests carried out on the germination potential (GP) and germination rate (GR) of maize grain grown on soil contaminated with DIC, IBU and AMP and mixtures of these drugs, it should be noted that there were no significant differences between the control and the test objects (Table 1).

Table 1. Effect of drugs on the germination potential (GP) and germination rate (GR) of maize. Data are means ± SD from four independent experiments. Values denoted by the same letters in the columns do not differ statistically at $p < 0.05$.

Concentration of Drugs [mg·kg^{-1} of Soil DW]	DIC	IBU	AMP	DIC + AMP	IBU + AMP	DIC + IBU	DIC + IBU + AMP
				GP [%]			
0	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a
1	86.70 ± 15.30a	96.70 ± 5.80a	90.00 ± 0.00a	90.00 ± 10.00a	100.00 ± 0.00a	96.70 ± 5.80a	96.70 ± 5.80a
10	86.70 ± 15.30a	90.00 ± 0.00a	96.70 ± 5.80a	96.70 ± 5.80a	100.00 ± 0.00a	100.00 ± 0.00a	86.70 ± 5.80a
100	96.70 ± 5.80a	90.00 ± 10.00a	90.00 ± 0.00a	100.00 ± 0.00a	96.70 ± 5.80a	80.00 ± 0.00a	96.70 ± 5.80a
1000	70.00 ± 10.00a	86.70 ± 15.30a	100.00 ± 0.00a	100.00 ± 0.00a	86.70 ± 15.30a	90.00 ± 10.00a	86.70 ± 5.80a
				GR [%]			
0	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a	86.70 ± 15.30a
1	86.70 ± 15.30a	96.70 ± 5.80a	90.00 ± 0.00a	90.00 ± 10.00a	100.00 ± 0.00a	96.70 ± 5.80a	96.70 ± 5.80a
10	86.70 ± 15.30a	90.00 ± 0.00a	96.70 ± 5.80a	96.70 ± 5.80a	100.00 ± 0.00a	100.00 ± 0.00a	86.70 ± 5.80a
100	96.70 ± 5.80a	90.00 ± 10.00a	90.00 ± 0.00a	100.00 ± 0.00a	96.70 ± 5.80a	80.00 ± 0.00a	96.70 ± 5.80a
1000	70.00 ± 10.00a	86.70 ± 15.30a	100.00 ± 0.00a	100.00 ± 0.00a	86.70 ± 15.30a	90.00 ± 10.00a	86.70 ± 5.80a

In the available literature, one can find scientific papers in which the authors also indicate that there is no effect of drugs on seed germination strength. Ziółkowska et al. [38], analyzing the effect of diclofenac on selected phytotoxicity parameters, observed no effect of these NSAIDs on the germination strength of lupin, peas and lentils. Similar conclusions were reached by Bellino et al. [39], who, conducting a study to determine the effect of four antibiotics (cloramphenicol, spiramycin, spectinomycin and vancomycin) on tomato seed germination, found a complete lack of effect of the drugs on this parameter of phytotoxicity. The same observations were made by Li et al. [15], who studied the effects of oxytetracycline and enrofloxacine on wheat grain germination strength. The lack of effect of five antibiotics (enrofloxacin, kanamycin, oxytetracycline, penicillin and tylosin) on the germination rate of sunflower, maize, soybean, sorghum and wheat seeds was also found in their study by Eluk et al. [13]. Some authors suggest that this may be related to the fact that seed coats protect seeds from the penetration of contaminants present in the soil; however, this protection is effective until such coats begin to break [40]. However, there are also works that have obtained results quite different from those cited above. Analyzing the effects of five antibiotics (ciprofloxacin, levofloxacin, ofloxacin, amoxicillin and ampicillin) on the germination and growth and development of rice seedlings, we found that all drugs caused a marked decrease in the germination power of the grain of this cereal [27]. In our earlier work [41,42] on the effects of DIC and IBU on the growth and development of spring barley seedlings, we found that the drugs used had an inhibitory effect on the germination potential (GP) of the grain and the magnitude of such an effect depended on the concentration used. Zezulka et al. [32], on the other hand, proved that the direction of the effect of drugs, as well as other contaminants, on plants is always dependent on the plant species, its physiological state, leaf area size, transpiration intensity, root morphology and nutrient availability. The same rules also govern the observed degree of effect of pharmaceuticals on plant seed germination. In their study, the authors found that paracetamol and diclofenac had no effect on the germination of lettuce, onion, pea and tomato seeds, while they reduced the germination strength of maize grain.

The plant organ that first comes into direct contact with contaminants present in the soil is the root. Therefore, phytotoxicity studies always determine the effect of contaminants, including drugs, not only on changes in root length but also on root morphology. Root responses to contaminants present in the growth environment are considered the primary measure of the phytotoxicity effect in environmental studies [19]. Analyzing the literature data on the effect of drugs on the growth and appearance of roots, it should be noted that most of the authors of these works proved that such an effect is toxic. Studies already cited earlier in this work on the determination of phytotoxicity of a number of non-steroidal anti-inflammatory drugs clearly indicate that they lead to a pronounced reduction in the length of roots of wheat, lupin, peas and lentils, among others [1,38,40]. In contrast, in a

study by Pawłowska et al. [41,42], the greatest inhibition of spring barley root growth was found when high concentrations of diclofenac, ibuprofen, naproxen and ketoprofen were introduced into the medium on which the cereal was grown. A decrease in root length was also found in studies treating the effects of antibiotics on the growth and development of wheat, sunflower, maize, soybeans and sorghum [13,15,39]. Schmidt and Redshaw [4], on the other hand, reported that the first reaction of plants to the presence of drugs in the growing environment can sometimes be root elongation. Such a reaction of radish and lettuce was observed in their study on NSAIDs. An increase in the length of the main root of peas, by as much as 30%, was observed by Svobodníková et al. [43] after naproxen was used in their study. However, the overall root mass was reduced due to a marked decrease in the number of lateral roots. In addition, these authors found unfavorable morphological changes in pea roots that were in contact with naproxen. However, in the scientific literature, we can also find papers [31] in which no effect of drugs was observed on the length of roots of onions, lettuce, peas and tomatoes grown on a DIC-supplemented medium. With such varied responses of plant roots to the presence of xenobiotics in the medium, the authors depend not only on the plant species but also on the chemical structure of the drug, which determines its physicochemical properties [14].

The results of our own study, which determined the effects of DIC, IBU and AMP, applied both separately and in binary mixtures and one ternary mixture, clearly show that all drugs led inevitably to a reduction in maize root length (Figure 1, Table 2). The magnitude of the observed decrease in length depended on the type of drug and mixture used, as well as the amount of drug introduced into the soil. The most toxic appeared to be both NSAIDs, reducing root length starting from a concentration of 10 mg kg^{-1} of soil DW, and at the highest concentration (1000 mg kg^{-1} d.m. of soil), the observed reduction in maize root length compared to the control was as high as 80% (DIC) and 85% (IBU). At the same concentration of AMP, maize root reduction was less than 20%. High concentrations of NSAIDs not only led to dwarfing of the main root, but the number of lateral roots also decreased significantly. Introducing binary mixtures DIC + AMP, IBU + AMP and DIC + IBU and a ternary mixture DIC + IBU + AMP into the soil, we did not observe any synergistic interactions leading to increased phytotoxic effects, which are often reported in the literature [7,21,26]. In the case of our study, all mixtures in which AMP was present showed lower toxicity to maize.

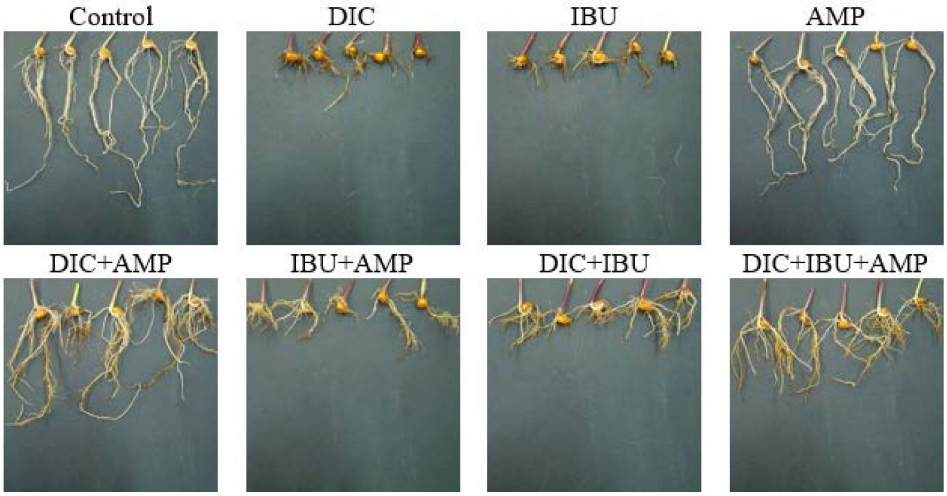

Figure 1. Digital photographs of maize roots without the addition of drugs (control) and after the introduction of DIC, IBU and AMP and their mixtures into the soil at a concentration of 1000 mg·kg^{-1} of soil DW.

Table 2. Effects of DIC, IBU and AMP and their mixtures on fresh and dry weight yield and length of aboveground parts and roots of maize seedlings. Data are means ± SD from four independent experiments. Values denoted by the same letters in the columns do not differ statistically at $p < 0.05$.

Concentration of Drugs [mg·kg^{-1} of Soil DW]	DIC	IBU	AMP	DIC + AMP	IBU + AMP	DIC + IBU	DIC + IBU + AMP
			Fresh weight [g pot^{-1}]				
0	6.531 ± 0.190a	6.531 ± 0.190a	6.531 ± 0.190a	6.531 ± 0.190b	6.531 ± 0.190a	6.531 ± 0.190ab	6.531 ± 0.190a
1	6.596 ± 0.173a	6.613 ± 0.330a	6.098 ± 0.080a	6.370 ± 0.182b	7.392 ± 0.308a	6.713 ± 0.686a	6.130 ± 0.552a
10	6.580 ± 0.404a	6.278 ± 0.081a	6.432 ± 0.454a	7.556 ± 0.530a	7.423 ± 0.487a	6.704 ± 0.079a	5.910 ± 0.024a
100	6.170 ± 0.669a	5.478 ± 0.318b	6.670 ± 0.073a	6.605 ± 0.262ab	6.445 ± 0.029a	5.634 ± 0.485b	5.867 ± 0.427a
1000	1.960 ± 0.025b	1.846 ± 0.016c	6.409 ± 0.212a	5.305 ± 0.510c	2.991 ± 0.575b	3.042 ± 0.101c	4.174 ± 0.386b
			Dry weight [g g^{-1} FW]				
0	0.0919 ± 0.0015b	0.0919 ± 0.0015b	0.0919 ± 0.0015a	0.0919 ± 0.0015b	0.0919 ± 0.0015b	0.0919 ± 0.0015b	0.0919 ± 0.0015b
1	0.0901 ± 0.0013b	0.0928 ± 0.0026b	0.0916 ± 0.0008a	0.0852 ± 0.0033b	0.0875 ± 0.0013bc	0.0870 ± 0.0027b	0.0928 ± 0.0013ab
10	0.0880 ± 0.0025b	0.0910 ± 0.0006b	0.0899 ± 0.0011a	0.0865 ± 0.0033b	0.0878 ± 0.0017bc	0.0896 ± 0.0034b	0.0902 ± 0.0016b
100	0.0895 ± 0.0027b	0.0908 ± 0.0029b	0.0895 ± 0.0027a	0.0923 ± 0.0034b	0.0865 ± 0.0021c	0.0876 ± 0.0022b	0.0917 ± 0.0014b
1000	0.1403 ± 0.0075a	0.1291 ± 0.0030a	0.0932 ± 0.0022a	0.1005 ± 0.0019a	0.1147 ± 0.0028a	0.1108 ± 0.0007a	0.0967 ± 0.0020a
			Shoot length [cm]				
0	24.07 ± 2.05a	24.07 ± 2.05a	24.07 ± 2.05a	24.07 ± 2.05a	24.07 ± 2.05a	24.07 ± 2.05a	24.07 ± 2.05a
1	22.80 ± 1.77a	22.45 ± 1.34ab	22.65 ± 1.51ab	22.35 ± 1.47ab	22.65 ± 1.45a	23.85 ± 1.45a	22.60 ± 2.16ab
10	22.95 ± 1.99a	23.35 ± 1.31ab	21.35 ± 1.56b	23.90 ± 1.31a	23.35 ± 1.08a	23.20 ± 1.86a	21.75 ± 1.74bc
100	22.20 ± 1.25a	21.65 ± 1.18b	22.75 ± 1.60ab	22.55 ± 1.42a	23.40 ± 1.15a	23.20 ± 1.27a	21.40 ± 1.02bc
1000	6.57 ± 1.81b	9.00 ± 1.94c	22.55 ± 1.07ab	20.35 ± 1.38b	14.90 ± 1.15b	14.30 ± 1.03b	19.75 ± 1.32c
			Root length [cm]				
0	18.25 ± 3.00a	18.25 ± 3.00a	18.25 ± 3.00ab	18.25 ± 3.00a	18.25 ± 3.00a	18.25 ± 3.00a	18.25 ± 3.00a
1	18.20 ± 1.78a	17.77 ± 1.16ab	18.19 ± 1.18ab	18.20 ± 1.49a	18.84 ± 1.71a	17.88 ± 1.74a	18.93 ± 1.70a
10	16.21 ± 1.60b	16.27 ± 1.64b	19.15 ± 1.20a	19.56 ± 1.64a	17.40 ± 1.58a	17.82 ± 1.31a	18.43 ± 1.60a
100	16.07 ± 1.50b	13.38 ± 1.73c	16.98 ± 1.63bc	17.64 ± 1.44a	17.31 ± 1.54a	15.36 ± 1.59b	16.93 ± 1.16a
1000	3.70 ± 0.74c	2.90 ± 1.09d	15.13 ± 1.57c	11.14 ± 1.80b	4.62 ± 1.21b	4.61 ± 1.20c	8.26 ± 1.06b

The reduction and decrease in the overall weight of plant roots pose a real threat to the growth and further development of their aboveground parts. The result can be reduced plant productivity, which, in turn, can lead to a threat to sustainability by disrupting the security associated with available food for humans and livestock [4]. In our study, the reduction in the length of the main roots and the number of lateral roots, as expected, led to a marked shortening of the length of maize seedlings and, thus, also a reduction in fresh weight yield (Figure 2, Table 2). As was the case with maize roots, DIC and IBU showed the most inhibitory effect. NSAIDs, introduced into the soil at the highest concentration (1000 mg kg^{-1} of soil DW), led to a decrease in the length of the aboveground parts of maize seedlings by about 73% (DIC) and 63% (IBU) with respect to the control objects. This translated into a reduction in maize seedling fresh weight yield of about 70% for both drugs. No synergism was found in the potentiation of the toxic effects of either the binary mixtures or the ternary mixture; on the contrary, all the mixtures used in the study showed significantly less phytotoxicity to maize. Moreover, it should be noted that ampicillin showed no toxicity to the growth of maize seedlings.

Confirmation of the results obtained can be found in the scientific literature, where the prevailing view is that drugs have an inhibitory effect on plant growth and, consequently, also reduce the amount of biomass. Such conclusions were reached by Sharma et al. [19], Wieczerzak et al. [7], Pawłowska et al. [41], Ziółkowska et al. [38], Drzymała et al. [21], Majewska et al. [2], among others, analyzing the effect of DIC on the growth of wheat, peanut, sorghum, lupin, pea, lentil, watercress and algae *Chlamydomonas reinhardtii*. Of the other NSAIDs, toxic effects on the growth and development of wheat and spring barley were found following the introduction of naproxen, ibuprofen, ketoprofen and paracetamol into the soil [40–42]. Similar effects for both terrestrial and aquatic plants (wheat, rice, watercress, green algae) were found by analyzing the effects of many antibiotics, including ampicillin [15,26,27]. Some authors [7,21,26] also report that very large increases in toxicity to plants are observed for various mixtures not only of drugs alone but also mixtures of drugs with other xenobiotics found in the environment.

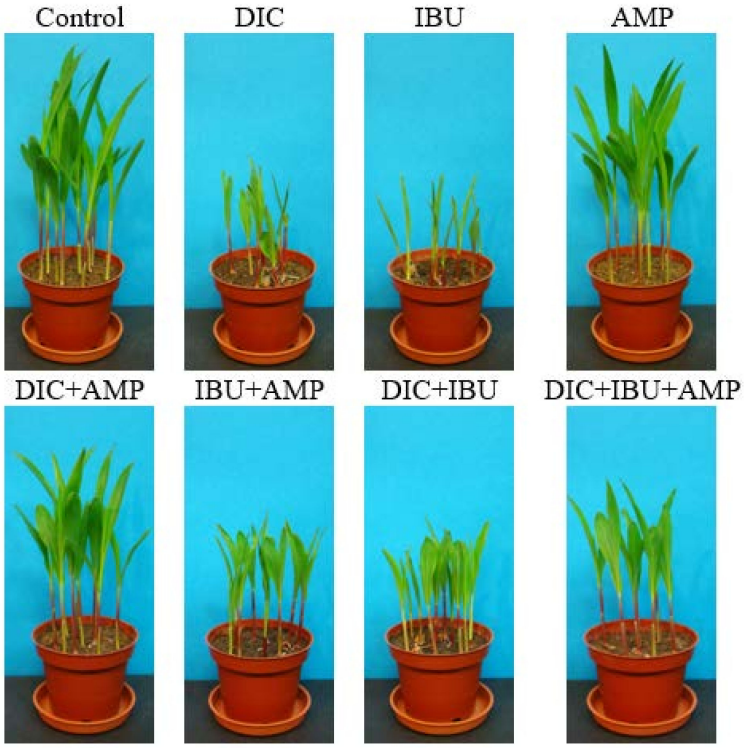

Figure 2. Digital photographs of maize seedlings without drug treatment (control) and after introduction of DIC, IBU and AMP and their mixtures into the soil at a concentration of 1000 mg·kg^{-1} of soil DW.

An indicator of the phytotoxicity of contaminants present in the environment of plant growth and development is the changes in the level of dry weight [4,19,41,42]. Therefore, in the presented work, we also analyzed changes in the dry weight yield content of maize seedlings grown in soil contaminated with DIC, IBU and AMP and mixtures of these drugs. A statistically significant increase in dry weight content was observed only after applying the highest concentrations of the drugs and their mixtures, which was most likely related to root damage and difficulty in water uptake. Only AMP applied separately did not lead to significant changes in dry weight levels in maize seedlings.

3.2. Interaction of DIC, IBU and AMP and Their Mixtures on the Content of Photosynthetic Pigments

All plants are counted among the so-called primary producers and form the basis of food chains. This is due to their possession of photosynthetic pigments, i.e., chlorophylls and carotenoids, which allow plants to carry out the process of photosynthesis, i.e., the conversion of light energy into energy that is accumulated in the form of chemical compounds. For the photosynthetic process to function properly, an optimal amount of chlorophyll a and chlorophyll b, properly constructed PSI and PSII photosystems, and carotenoids as protective compounds are needed. Otherwise, damage to the photosystems occurs, mainly due to the formation of excessive amounts of oxygen free radicals (ROS). Therefore, photosynthetic pigment analysis is a very common part of any environmental study [17,20,23,29,32,41,42,44].

In the discussed studies on the effects of DIC, IBU and AMP and their mixtures, we also determined the levels of all photosynthetic pigments in maize seedlings (Figure 3; Table 3).

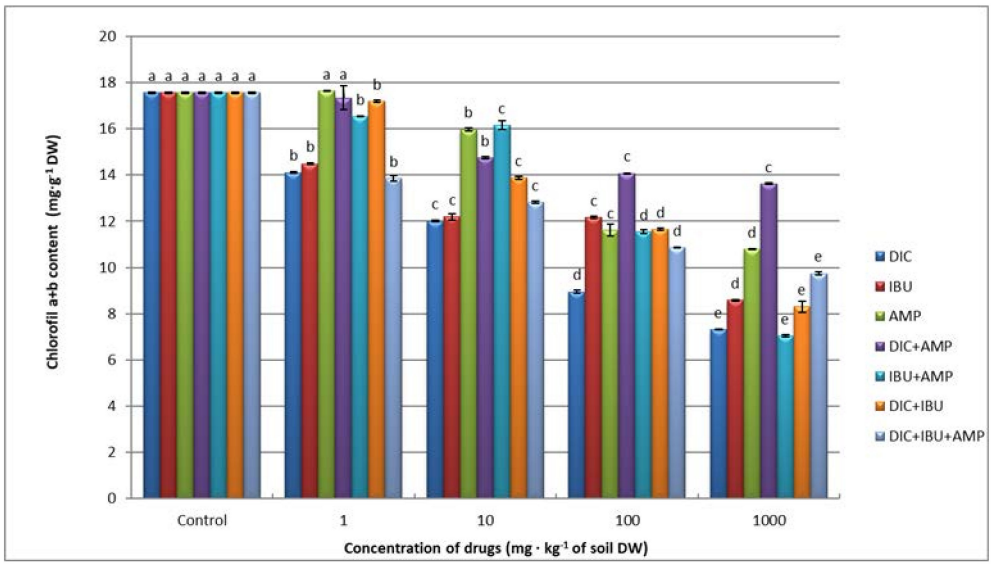

Figure 3. Changes in total chlorophyll content in maize seedlings grown on soil contaminated with DIC, IBU, AMP and mixtures of these drugs. Data are means ± SD from four independent experiments. Values denoted by the same letters do not differ statistically at $p < 0.05$.

Table 3. Interaction of DIC, IBU and AMP and their mixtures on the content of chlorophyll a, chlorophyll b, carotenoids and the values of Chla/Chlb and Chl(a+b)/Car ratios in maize seedlings. Data are means ± SD from four independent experiments. Values denoted by the same letters in the columns do not differ statistically at $p < 0.05$.

Concentration of Drugs [mg kg^{-1} of Soil DW]	DIC	IBU	AMP	DIC + AMP	IBU + AMP	DIC + IBU	DIC + IBU + AMP
			Chlorophyll a [mg g^{-1} DW]				
0	11.246 ± 0.026a	11.246 ± 0.026a	11.246 ± 0.026a	11.246 ± 0.026a	11.246 ± 0.026a	11.246 ± 0.026a	11.246 ± 0.026a
1	8.679 ± 0.029b	9.387 ± 0.023b	10.952 ± 0.035b	10.863 ± 0.029b	10.429 ± 0.018b	10.628 ± 0.016b	8.386 ± 0.072b
10	7.456 ± 0.034c	7.502 ± 0.091d	10.065 ± 0.035c	9.585 ± 0.039c	10.418 ± 0.142b	8.609 ± 0.044c	7.749 ± 0.025c
100	5.570 ± 0.051d	7.705 ± 0.037c	7.438 ± 0.158d	8.681 ± 0.017d	7.342 ± 0.043c	7.258 ± 0.021d	6.966 ± 0.021d
1000	4.932 ± 0.024e	5.767 ± 0.028d	6.847 ± 0.022e	8.469 ± 0.003e	4.784 ± 0.013d	5.522 ± 0.156e	6.151 ± 0.030e
			Chlorophyll b [mg g^{-1} DW]				
0	6.310 ± 0.017a	6.310 ± 0.017a	6.310 ± 0.017b	6.310 ± 0.017b	6.310 ± 0.017a	6.310 ± 0.017b	6.310 ± 0.017a
1	5.430 ± 0.021b	5.098 ± 0.014b	6.683 ± 0.018a	6.143 ± 0.066b	6.110 ± 0.024b	6.564 ± 0.034a	5.486 ± 0.049b
10	4.552 ± 0.028c	4.687 ± 0.034c	5.903 ± 0.031c	5.164 ± 0.020d	5.731 ± 0.022c	5.262 ± 0.026c	5.074 ± 0.026c
100	3.377 ± 0.017d	4.469 ± 0.021d	4.163 ± 0.100d	5.389 ± 0.012c	4.209 ± 0.055d	4.396 ± 0.038d	3.883 ± 0.013d
1000	2.376 ± 0.014e	2.807 ± 0.008e	3.944 ± 0.024e	5.149 ± 0.026d	2.244 ± 0.043e	2.780 ± 0.076e	3.584 ± 0.042e
			Chlorophyll a/chlorophyll b				
0	1.783 ± 0.006b	1.783 ± 0.006c	1.783 ± 0.006a	1.783 ± 0.006b	1.783 ± 0.006bc	1.783 ± 0.006b	1.783 ± 0.006a
1	1.598 ± 0.008d	1.841 ± 0.007b	1.639 ± 0.008d	1.768 ± 0.021b	1.705 ± 0.012d	1.621 ± 0.009d	1.529 ± 0.003c
10	1.638 ± 0.013c	1.601 ± 0.008e	1.705 ± 0.006c	1.856 ± 0.006a	1.818 ± 0.014b	1.636 ± 0.004cd	1.527 ± 0.006c
100	1.649 ± 0.007c	1.724 ± 0.007d	1.787 ± 0.006a	1.611 ± 0.004d	1.744 ± 0.017cd	1.651 ± 0.012c	1.794 ± 0.010a
1000	2.076 ± 0.019a	2.055 ± 0.0015a	1.736 ± 0.014b	1.645 ± 0.008c	2.132 ± 0.037a	1.986 ± 0.008a	1.716 ± 0.015b
			Carotenoids [mg g^{-1} DW]				
0	3.486 ± 0.012a	3.486 ± 0.012a	3.486 ± 0.012a	3.486 ± 0.012a	3.486 ± 0.012a	3.486 ± 0.012a	3.486 ± 0.012a
1	2.716 ± 0.005b	2.873 ± 0.010b	3.519 ± 0.004a	3.315 ± 0.009b	3.091 ± 0.018b	3.365 ± 0.015b	2.708 ± 0.016b
10	2.392 ± 0.012c	2.379 ± 0.024c	3.158 ± 0.005b	2.947 ± 0.017c	3.118 ± 0.074b	2.717 ± 0.009c	2.634 ± 0.173b
100	1.735 ± 0.008d	2.360 ± 0.007c	2.312 ± 0.041c	2.759 ± 0.013d	2.286 ± 0.014c	2.291 ± 0.012d	2.105 ± 0.014c
1000	1.356 ± 0.006e	1.588 ± 0.008d	2.119 ± 0.008d	2.622 ± 0.006e	1.383 ± 0.015d	1.624 ± 0.043e	1.952 ± 0.014c
			Chlorophyll (a + b)/carotenoids				
0	5.037 ± 0.013c	5.037 ± 0.013d	5.037 ± 0.013b	5.037 ± 0.013c	5.037 ± 0.013b	5.037 ± 0.013a	5.037 ± 0.013bc
1	5.194 ± 0.020b	5.042 ± 0.010d	5.012 ± 0.010b	5.130 ± 0.013b	5.194 ± 0.021a	5.110 ± 0.035a	5.123 ± 0.019ab
10	5.019 ± 0.026c	5.123 ± 0.006c	5.057 ± 0.022ab	5.005 ± 0.014c	5.147 ± 0.017ab	5.105 ± 0.021a	5.060 ± 0.028bc
100	5.156 ± 0.016b	5.159 ± 0.012b	5.017 ± 0.024b	5.102 ± 0.017b	5.052 ± 0.064b	5.089 ± 0.049a	5.020 ± 0.055c
1000	5.388 ± 0.020a	5.401 ± 0.018a	5.092 ± 0.020a	5.351 ± 0.031a	5.082 ± 0.090ab	5.112 ± 0.013a	5.154 ± 0.031a

The study found a decrease in the levels of all photosynthetic pigments in maize seedlings. The observed decreases in the content of chlorophylls and carotenoids were directly proportional to the magnitude of the concentration of drugs and their mixtures in the soil. The largest, more than 50% decrease in the content of all photosynthetic pigments, was observed not only for NSAIDs but also some mixtures—IBU + AMP and DIC + IBU—which also led to a drastic reduction in the level of chlorophylls and carotenoids. AMP and the binary mixture DIC + AMP had the least effect on the levels of these pigments. The proper functioning of photosystems requires an adequate ratio of chlorophyll a to chlorophyll b. In our study, we found that after applying the highest concentrations of drugs and their mixtures (1000 mg kg^{-1} of soil DW), there was a significant decrease in the value of the chla/chlb ratio, resulting from a decrease in the level of chlorophyll a. This may indicate a disturbance in the functioning of photosystems and, consequently, lead to a decrease in the efficiency of the photosynthetic process. A protective role for photosystems is played by carotenoids, which, as small-molecule oxidants, lead the process of ROS scavenging. Analyzing the values of the Chl (a+b)/Car ratio, it is clear that all drugs and their mixtures introduced into the soil at concentrations equal to 1000 mg kg^{-1} of soil DW lead to a significant increase in the value of this indicator. This is due to a decrease in the level of carotenoids, which may suggest a situation in which the protection system is inefficient and plants remain under oxidative stress.

As if to corroborate the described research results are the results of scientific papers proving the inhibitory effect of drugs on the level of photosynthetic pigments in terrestrial and aquatic plants. Many papers deal with the effects of NSAIDs, such as diclofenac, naproxen, ketoprofen, ibupron, indomethacin and acetylsalicylic acid, which led to a decrease in the levels of chlorophylls and carotenoids in spring barley and maize seedlings, cabbage, lettuce, peas, onions, chickpeas, lentils and beans [17,23,32,41,42,45]. Similar changes were also found by Opriş et al. [44], who analyzed the content of photosynthetic pigments in wheat seedlings exposed to the antibiotics amoxicillin, ampicillin, penicillin G, ceftazidime, ceftriaxone, tetracycline, doxycycline, ciprofloxacin and erythromycin. In contrast, analysis of the effects of drugs on photosynthetic pigment content in aquatic plants was the subject of a study by Wang et al. [20]. The authors found an inhibition of chloroplast growth and a decrease in carotenoid content in green algae *Scenedesmus obliquus* under the influence of ibuprofen, ketoprofen and acetylsalicylic acid. In contrast, a decrease in chlorophyll content in green algae *Scenedesmus rubescens* due to exposure of these algae to IBU was found in their study by Moro et al. [29]. A very graceful aquatic plant for all kinds of ecotoxicological studies is the *Lemna minor*. In the case of the *L. minor*, Kummerová et al. [46] found a more than 50% decrease in the levels of all photosynthetic pigments after a 10-day exposure of this plant to diclofenac and paracetamol. A similar decrease in the chlorophyll content of the tiny *L. minor* after treatment with diclofenac, paracetamol and chlorpromazine was also observed by Alkimin et al. [47]. At the same time, the author found that the effect of these drugs on the content of photosynthetic pigments depended not only on the type of drugs and the dose used but also on the species or variety of the plant.

3.3. Chlorophyll Fluorescence

Chlorophyll fluorescence is increasingly being used in modern environmental studies to determine the degree of impact of stress factors on plant metabolism. Its great importance is due to the fact that it is a non-invasive, rapid, but at the same time, accurate method. It allows for assessing changes in photosynthetic processes, in particular, PSII functionality and electron transfer rates in plants treated with various xenobiotics, including pharmaceuticals. Chlorophyll fluorescence parameters, especially initial (null) fluorescence (F_0) and maximum fluorescence after dark adaptation (F_m), are commonly used to assess PSII activity, as they are particularly sensitive to stress [20,48].

In the present study, we also used the measurement of chlorophyll fluorescence as an indicator of the degree of effect of DIC, IBU and AMP and mixtures of these drugs on

the efficiency of the photosynthetic process in maize seedlings. The results show that the application of AMP separately, in a binary mixture DIC + AMP and in a ternary mixture DIC + IBU + AMP did not lead to any changes in chlorophyll fluorescence parameters. In contrast, a slight decrease in the magnitude of such parameters as F_v/F_m and F_v/F_0 were observed for both applied NSAIDs, their mixture DIC + IBU and the mixture IBU + AMP. This was especially evident after applying the highest concentrations (1000 mg kg^{-1} of soil DW) of both drugs and their mixtures. The observed decrease in these two chlorophyll fluorescence indices with respect to the control may indicate a disruption in the photosynthetic process in maize seedlings (Table 4).

Table 4. Effect of DIC, IBU and AMP and their mixtures on changes in the magnitude of basic chlorophyll fluorescence parameters in maize seedlings. Data are means ± SD from four independent experiments. Values denoted by the same letters in the columns do not differ statistically at $p < 0.05$.

Concentration of Drugs [mg kg^{-1} of Soil DW]	DIC	IBU	AMP	DIC + AMP	IBU + AMP	DIC + IBU	DIC + IBU + AMP
				F_0			
0	198.80 ± 6.46ab	198.80 ± 6.46b	198.80 ± 6.46a	198.80 ± 6.46a	198.80 ± 6.46a	198.80 ± 6.46a	198.80 ± 6.46a
1	198.00 ± 2.58ab	191.50 ± 5.26b	180.75 ± 10.72a	195.25 ± 7.04a	176.50 ± 4.43c	190.50 ± 8.06b	194.00 ± 14.58a
10	195.50 ± 2.38b	192.25 ± 4.27b	190.00 ± 12.03a	197.25 ± 5.74a	180.00 ± 6.83bc	192.50 ± 3.11ab	192.25 ± 7.04a
100	192.25 ± 3.86b	199.00 ± 7.44b	193.00 ± 23,65a	191.00 ± 8.04a	193.25 ± 6.60ab	187.50 ± 8.43b	185.00 ± 11.22a
1000	207.00 ± 3.56a	213.00 ± 7.53a	207.00 ± 17.34a	188.00 ± 8.60a	191.75 ± 2.09abc	209.75 ± 14.61a	191.75 ± 4.19a
				F_m			
0	955.0 ± 25.9a	955.0 ± 25.9ab	955.0 ± 25.9a	955.0 ± 25.9a	955.0 ± 25.9a	955.0 ± 25.9a	955.0 ± 25.9a
1	949.5 ± 7.8ab	907.5 ± 44.7b	865.3 ± 56.2a	956.3 ± 39.2a	835.3 ± 42.1c	904.0 ± 29.8a	934.5 ± 37.6ab
10	895.0 ± 28.7b	925.0 ± 17.8ab	870.8 ± 92.1a	954.3 ± 6.1a	861.3 ± 34.5bc	911.0 ± 80.2a	952.5 ± 18.5ab
100	932.8 ± 32.8ab	969.0 ± 20.0a	903.0 ± 66.5a	890.0 ± 46.9a	930.3 ± 23.4ab	882.0 ± 40.5a	881.5 ± 51.2b
1000	910.8 ± 26.4ab	930.0 ± 27.2ab	958.8 ± 63.9a	888.5 ± 59.8a	871.5 ± 64.9bc	902.8 ± 75.0a	914.8 ± 28.6ab
				F_v			
0	765.2 ± 24.8a	765.2 ± 24.8a	765.2 ± 24.8a	765.2 ± 24.8a	765.2 ± 24.8a	765.2 ± 24.8a	765.2 ± 24.8a
1	751.5 ± 7.9ab	708.5 ± 53.6a	664.5 ± 82.2a	761.0 ± 32.4a	661.3 ± 33.8b	713.5 ± 24.2a	735.5 ± 50.4a
10	704.3 ± 24.2b	732.8 ± 15.2a	685.8 ± 73.7a	757.0 ± 2.45a	681.3 ± 28.6b	764.0 ± 30.0a	760.3 ± 11.8a
100	718.0 ± 30.8ab	770.0 ± 12.7a	710.0 ± 43.2a	699.0 ± 39.9a	737.0 ± 17.2ab	694.5 ± 32.4a	696.5 ± 40.0a
1000	703.8 ± 27.4b	717.0 ± 19.9a	751.8 ± 76.9a	700.5 ± 51.4a	674.8 ± 61.2a	695.5 ± 57.0a	723.0 ± 24.6a
				F_v/F_m			
0	0.791 ± 0.007a	0.791 ± 0.007a	0.791 ± 0.007a	0.791 ± 0.007a	0.791 ± 0.007a	0.791 ± 0.007a	0.791 ± 0.007a
1	0.791 ± 0.003a	0.791 ± 0.006a	0.790 ± 0.006a	0.796 ± 0.002a	0.791 ± 0.008a	0.789 ± 0.006a	0.761 ± 0.049a
10	0.787 ± 0.008ab	0.792 ± 0.003a	0.787 ± 0.009a	0.793 ± 0.005a	0.790 ± 0.004a	0.798 ± 0.007a	0.798 ± 0.004a
100	0.791 ± 0.005a	0.795 ± 0.004a	0.787 ± 0.011a	0.785 ± 0.005a	0.792 ± 0.002a	0.787 ± 0.002a	0.790 ± 0.001a
1000	0.772 ± 0.008b	0.771 ± 0.002b	0.782 ± 0.032a	0.786 ± 0.005a	0.778 ± 0.008b	0.770 ± 0.006b	0.786 ± 0.002a
				F_v/F_0			
0	3.806 ± 0.169a	3.806 ± 0.169a	3.806 ± 0.169a	3.806 ± 0.169a	3.806 ± 0.169a	3.806 ± 0.169a	3.806 ± 0.169a
1	3.796 ± 0.070a	3.802 ± 0.138a	3.774 ± 0.138a	3.897 ± 0.052a	3.800 ± 0.015a	3.747 ± 0.130a	3.750 ± 0.589a
10	3.696 ± 0.171ab	3.812 ± 0.080a	3.711 ± 0.193a	3.840 ± 0.113a	3.785 ± 0.086a	3.970 ± 0.178a	3.957 ± 0.092a
100	3.785 ± 0.117a	3.871 ± 0.085a	3.699 ± 0.235a	3.659 ± 0.112a	3.815 ± 0.059a	3.703 ± 0.047a	3.765 ± 0.017a
1000	3.401 ± 0.162b	3.367 ± 0.039b	3.667 ± 0.596a	3.723 ± 0.115a	3.516 ± 0.160b	3.359 ± 0.112b	3.769 ± 0.053a

An analysis of the available literature on changes in the basic parameters of chlorophyll fluorescence indicates that the decrease in their values found is a clear indicator of damage to PSII photosystems, which consequently leads to disruption of photosynthetic electron transport between PSI and PSII [19]. The authors found this by studying the effects of ibuprofen, ketoprofen and acetylsalicylic acid on the photosynthetic performance of green algae *Scenedesmus obliquus*. A more than 50% reduction in the values of chlorophyll fluorescence parameters in green algae *Chlamydomonas reinhardtii* compared to the control was also found by Majewska et al. [3], who exposed these algae to DIC. Under these conditions, the authors observed an almost complete cessation of photosynthesis. An even greater decrease in the values of the chlorophyll fluorescence parameters in the *L. minor* under DIC was found in their studies by Kummerová et al. [46] and Hájková et al. [49]. At the same time, Zezulka et al. [32] proved that by using DIC in the cultivation of onion, lettuce, pea, tomato and maize, only the C4 plant-maize reacts with a reduction in the values of chlorophyll fluorescence indices to the presence of these NSAIDs in the soil. Chlorophyll

fluorescence studies are also being conducted in the case of antibiotic contamination of soils. Zhang et al. [48] proved that the introduction ofloxacin into the growth environment of tomatoes causes very large phytotoxicity effects, reflected not only by a large decrease in the values of the basic parameters of chlorophyll fluorescence but also by the breakdown of chloroplasts and chlorophyll degradation. The number of research results now being published describing changes in chlorophyll fluorescence means that this analysis is rapidly becoming one of the most important indicators depicting the degree of environmental impact on plants.

4. Conclusions

The study shows that DIC, IBU, AMP and their binary mixtures and the ternary mixture, to a small extent, have toxic effects on the growth and development of the early stages of common maize, with greater phytotoxicity shown by both NSAIDs, especially when used separately. Ampicillin, on the other hand, was virtually harmless to maize seedlings. This was evidenced by changes in the levels of both fresh and dry weight, the height of aboveground parts and changes in root length. The absence of phytotoxicity is further evidenced by small changes in the content of all photosynthetic pigments and basic chlorophyll fluorescence indicators. The phytotoxicity of the drugs, as well as their mixtures, was practically revealed only after the application of their highest concentrations (1000 mg kg^{-1} of soil DW), which will certainly never appear in the environment.

However, it should be remembered that pharmaceuticals constitute what is known as permanent and persistent environmental pollution due to the fact that they are constantly being delivered to the environment since their diverse chemical structure does not allow them to be completely removed in current wastewater treatment plants. Some of the drugs tend to settle in sewage sludge, while others are dissolved substances in treated wastewater; hence, the pathways to aquatic and soil environments stand open for them. In turn, they can easily be taken up by crops, causing crop contamination. Therefore, it seems necessary to strive to understand the effects of drugs and their metabolites on biochemical and physiological processes, which can explain many of the changes in the growth and development of plants grown in contaminated environments. This could make it possible to safeguard agricultural crops from other global contaminants, such as drugs and their residues, which is of enormous importance for human health.

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