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The Impact of Soil and Water Pollutants Released from Poultry Farming on the Growth and Development of Two Plant Species

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Abstract: Intensive poultry production may result in substantial emissions of pollutants into the environment, including pharmaceuticals and other chemicals used in poultry farming. The objective of this study was to verify the presence of ciprofloxacin, enrofloxacin, carbamazepine, metoclopramide, trimethoprim, diflufenican, flufenacet, and p,p′-DDE in soil and water in the immediate vicinity of a poultry manure heap. The influence of soil contaminants on the growth and selected physiological parameters of seed peas and common duckweed (as indicator plants) was tested. It has been proven that the cultivation of pea plants on soil coming from the close proximity of a heap of manure results in a deterioration of both morphological parameters (root length, shoot length) and physiological parameters (chlorophyll absorption, aminolevulinic acid dehydrogenase (ALAD) activity, aminolevulinic acid (ALA) content, lipid peroxidation, mitochondrial damage or production of HSP70 proteins). Similarly, water extracts from cultivated soils had a significant effect on duckweed, and it was found that contaminant leachates are indeed detectable in soil, groundwater, and deep water. Special attention should, therefore, be paid to the location, methods of storage, and use of poultry fertilizer.

Keywords: poultry manure; *Lemna minor* L.; *Pisum sativum* L.; lipid peroxidation; mitochondrial damage; chlorophyll

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1. Introduction

The dynamic growth of the world population means that in 2050, it will reach 9.7 billion citizens [1], and covering the global demand for food will be even more challenging [2]. One of the key products of animal origin consumed worldwide is poultry meat [3]. In developed countries, poultry is the preferred source of animal protein due to its ease of preparation. Moreover, due to the high protein content with relatively low-fat content, poultry meat is considered a healthy food choice. In developing countries, poultry meat is mainly consumed for economic reasons, as it is much cheaper than other types of meat [4]. In 2021, the world's production of poultry meat was 137.8 million tonnes [4]. The countries with the largest share of poultry production in 2021 were the USA (22 million tonnes), China (23 million tonnes), and Brazil (14 million tonnes) [5]. In the European Union, the production of poultry meat in 2021 amounted to 13.7 million tonnes. Poland, where over two million tonnes of poultry meat were produced in 2021 [5], is the largest producer of poultry in the EU. It is predicted that in 2050, the global demand for poultry meat will increase by 121% [2]. Despite the fact that poultry is a valuable source of dietary protein, its production affects the environment to a much greater extent than the production of other types of food [6]. Poultry farms emit pollutants to all components of the environment: air, soil, surface waters, and ground waters, thus affecting humans, animals, and plants.

Intensive poultry farming requires appropriate conditions in poultry houses [7], e.g., fresh air or optimal temperature. Vertical and horizontal mechanical fans are commonly used in buildings [8], resulting in increased emissions of pollutants into the atmosphere. The largest groups of air pollutants include dust (fragments of leather, bedding, and fodder) and odors (ammonia, volatile organic compounds, hydrogen sulfide) [9]. In addition, odors from poultry houses attract insects that cause diseases and are a nuisance for humans and animals [10].

On the other hand, poultry houses may provide fertilizer to stimulate crop production. However, animal manure can be a source of soil contamination by pharmaceuticals that are used for animal production. Usually, antimicrobials in soil and water are detected in trace concentrations; however, even small quantities of these pollutants are considered an environmental risk factor as they promote the development of antibiotic resistance, which is one of the greatest threats to human health [11]. Other groups of compounds used in chicken farming include antiparasitic, anti-inflammatory, sedative, diuretic, and anesthetic substances [12]. Their residues are also detected in animal waste (e.g., manure) and in all components of the environment. A detailed overview of pollutants resulting from intensive poultry farming and their impact on the environment and human health is presented in the work of Gržinić et al. [13]. Significant knowledge gaps in assessing the impact of contaminants on soils have been demonstrated [13]; research, especially on pharmaceutical residues on agricultural land, is extremely important in the context of implementing the objectives of a few European initiatives, including the new EU Soil Strategy [14].

Of course, pharmaceuticals are not the main chemical contaminants of crop fields. The use of pesticides is required in modern, intense crop production. Zhou et al. [15] indicate that most of the doses of the pesticide used end up in the environment as pollution. As a result, pesticide residues are determined in air, soil, and water samples, but also in plant and animal tissues, and thus, they can be found in fertilizers and animal feed. Mahugija et al. [16] showed the presence of pesticides in corn seeds intended for chicken feed, while Wang et al. [17] detected pesticide residues in chicken feces.

Runoff from such fields can become a source of surface and groundwater pollution. Furthermore, toxic substances have the capacity to be absorbed by aquatic and crop plants, leading to their circulation within the ecosystem [18]. The concentration of the main toxins in the environment varies seasonally and depends on the physicochemical properties of soils, the amount of manure applied, weather conditions (especially rainfall), and the degree of toxin degradation [19].

Safe storage and disposal of animal wastes is one of the biggest problems of intensive poultry farming [20]. In Europe, 1.4 billion tonnes of animal manure are generated annually [21]. Due to its physicochemical properties, manure is used as an organic fertilizer [22]. However, the amount of manure produced usually exceeds the amount needed to fertilize the surrounding fields, which means that manure needs to be stored [13]. In 2010, only 30% of poultry farms in Europe had manure storage facilities, while in 2016–2019, 90% of manure landed directly on farmland without prior treatment [22]. Fertilization of soil with untreated manure can lead to the accumulation of contaminants in the soil and their entry into plant tissues [23]. Accumulated xenobiotics in plant tissues, even at low concentrations, induce changes at both morphological, biochemical, and genetic levels [12]. The storage of manure piles in open spaces and the use of untreated manure in fields pose a risk of contamination not only of soils but also of surface waters and air [24]. River pollution is largely caused by runoff and leachate from surrounding fields fertilized with manure [25] and poses significant risks to aquatic organisms, including plants. Xenobiotics from the soil can also migrate to groundwater, a source of drinking water [26].

Intensive poultry farming helps to meet the ever-increasing demand for animal protein. On the other hand, it can be a source of environmental pollution, thus affecting the health of local residents, farm workers, and consumers. The objective of this paper was to analyze groundwater and soil pollution resulting from intensive poultry production. Additionally, the study examined the impact of soil contamination on plants grown under strictly controlled laboratory conditions. The pea plants were cultivated in samples of soils that were either fertilized with poultry manure or were collected in the immediate vicinity of a manure heap. Soil leachates were also prepared from these samples to assess their effects on the physiological and biochemical parameters of *Lemna minor* L. It was assumed that chlorophyll absorption, aminolevulinic acid dehydrogenase (ALAD) activity, aminolevulinic acid (ALA) content, lipid peroxidation, mitochondrial damage, or production of HSP70 proteins provide a comprehensive characteristic of the physiological status of plants.

2. Materials and Methods

2.1. Sampling Site and Soil Characteristics

Soil samples were collected from cultivated fields located at close distance to the broiler chicken farm (approx. 300 m), located in the Warmian-Masurian Voivodeship, near Nidzica. The two soils, S1 (sandy loam: sand—68%, silt—29%, clay—3%) and S2 (loamy sand: sand—76%, silt—22%, clay—2%), were collected from the surface (0–30 cm) layer of the agricultural land in an early spring 2021, soil S1 from the arable field regularly fertilized with the manure and soil S2 from the field in the close distance from the manure pile (See map 1). For each sampling site, six subsamples were collected from an area of 1 m^2, homogenized on the site after the removal of the upper layer of organic vegetative materials, and mixed to provide a bulked sample for the site. The soils were analyzed for the particle size distribution, pH, total organic carbon and nitrogen, available phosphorus, potassium, and mineral nitrogen. Particle size distribution was measured using laser diffraction method using the Mastersizer 2000 apparatus with Hydro UM attachment (Malvern Panalytical, Malvern, UK) [27]. The pH was measured potentiometrically in a suspension of 1 mol $\times$ L^{-1} KCl (1:2.5 (m $\times$ V^{-1})) (ISO 10390, 2005). Total organic carbon (TOC) content was determined after sulfochromic oxidation, followed by the titration of the excess of $K_2Cr_2O_7$ with $FeSO_4(NH_4)_2SO_4 \times 6H_2O$ (PN-ISO 14235, 2003). Total nitrogen content (TN) and total carbon content (TC) were determined in Vario Macro Cube CN apparatus (Elementar Analysensysteme GmbH, Langenselbold, Germany) after dry combustion according to ISO 13878 (1998) and ISO 10694 (1995) methods, respectively. Available phosphorus (P) was measured using the Egner–Riem colorimetric method after soil extraction with calcium lactate using a Lambda 45 UV-VIS spectrophotometer (PerkinElmer Inc., Waltham, MA, USA). Available potassium (K) was analyzed by atomic absorption spectrometry using AAnalyst 800. Mineral nitrogen (N-NO_3 and N-NH^4) was measured after soil extraction with 1 mol $\times$ L^{-1} K_2SO_4 by continuous segmented flow spectrometry using a QuAAtro39 analyzer (Seal-Analytical, Norderstedt, Germany) [28].

2.2. Sampling Water from the Piezometer

Piezometers were used for collecting underground water samples. In the first stage, a rotary geotechnical drilling rig type H16S was used. The second stage of drilling work consisted of the use of a drilling rig using the so-called drilling fluid to extract the sediments to the surface and secure the borehole against self-backfilling. During the drilling works, the presence of an aquifer in the sandy sediment was found at a depth of 26–45 m below the surface of the land, with a taut water table stabilizing at a depth of 21–17 m below the surface. In the piezometer, a mesh filter was installed in the aquifer, and PVC pipes with a diameter of 100 mm were brought to the surface.

A submersible pump of the Omnigena 3T23 type, supplied with 230 V electricity from a diesel power generator, was lowered into the test hole of the piezometer. The submersible pump pumped underground water to the surface with the flow rate of Q = 52 liters per minute. Before collecting water samples, purification pumping was carried out. A representative sample was collected after pumping out 1 m^3 of underground water. Water was collected in glass bottles cleaned with organic solvents (first with acetone and then with methanol) and transported within 5 h to the laboratory where the sample was analyzed.

2.3. Pea Growth Conditions

200 g of soil was added to pots of 9 cm × 9 cm: S1—from a field regularly fertilized with manure, S2—from a field near a heap of manure, control 3–350 mL of horticultural perlite. Four pea seeds (*Pisum sativum* L., cv. Cysterski) were placed in each pot and cultivated for 3 weeks at 23 °C/16 °C day/night, 16/8 h photoperiod in 8 klx light intensity. The plants in perlite were periodically supplemented with a solution of 50% MS minerals [29] (two doses 50 mL each). The experiment was repeated three times.

2.4. Preparation of Soil Extracts and Cultivation of Duckweed

In order to prepare soil extracts, 10 g of soil regularly fertilized with manure (sample S1) and taken from the vicinity of the manure heap (sample S2) were weighed into a plastic, sterile container. 100 mL of deionized water was then added to the containers. The containers were shaken at 100 RPM on a laboratory shaker (DLAB Scientific) for 72 h. The extracts were then filtered using a paper filter into 100 mL glass jars. 50% Murashige and Skoog medium [29] was used as a control. Then, 10 shoots of duckweed (*Lemna minor* L.) were placed in each jar using a laminar chamber. Duckweed was grown for 7 days at 25 °C/17 °C day/night, with a photoperiod of 16/8 h in 3.4 klx light intensity. The experiment was carried out in 3 replicates.

2.5. Plant Growth Analysis

Pea root and stem lengths were measured using the ImageJ software (1.54 g version). The number of pea lateral roots and the area of the second leaf were determined using a VHX-7000 digital microscope (Keyence, Osaka, Japan). The number of plants and the area of duckweed fronds were determined in accordance with the OECD protocol for *Lemna* sp. [30]. The area of fronds and the number of plants were measured using a VHX-7000 digital microscope with dedicated software (Keyence, Osaka, Japan) at 20× magnification.

2.6. Isolation and Measurement of Chlorophyll Absorption

Chlorophyll was isolated according to the method of Rydzyński et al. [31] with minor modifications. 500 mg of peas leaves or duckweed stems were homogenized with a pestle and mortar in 5 mL of methanol (Fisher Chemicals, Hampton, NH, USA). The homogenate was centrifuged at 1500× *g*. The resulting supernatant was diluted 6-fold with methanol, and the absorbance at λ = 664 nm was measured using a UV/Vis spectrophotometer (CE2021 2000 Series, Cecil Instruments, Ltd., Cambridge, UK). Chlorophyll concentration was calculated according to the Lambert-Beer law using the molar extinction coefficient of 66,600 $M^{-1} \times cm^{-1}$ for chlorophyll in methanol, according to Seely and Jensen [32].

2.7. Aminolevulinic Acid Dehydrogenase (ALAD) Activity

ALAD was extracted according to the method of Jiao et al. [33] with modifications. Duckweed or pea leaves samples (500 mg fresh weight) were ground in liquid nitrogen with a cold mortar and pestle in 5 mL extraction buffer containing 0.05 M Tris-HCl pH 8.2 and 0.1 mM DTT (Sigma-Aldrich, Poznań, Poland). The homogenate was filtered through gauze and then centrifuged at 10,000× *g* for 30 min at 4 °C. The supernatant was taken, and 1 mL of the supernatant was incubated with 0.27 mL ALA (concentration 1 mg × mL^{-1}), 1.35 mL 0.05M Tris-HCl pH 8.2 with 0.1 mM DDT, 0.08 mL 0.2 M $MgCl_2$ at 37 °C for 1 h. The reaction was stopped by adding 0.3 mL of 3M trichloroacetic acid (TCA). After cooling, the samples were centrifuged at 2000× *g* for 10 min. To 1 mL of the supernatant was added 1 mL of Ehrlich's reagent (4-(Dimethylamino)benzaldehyde, Sigma-Aldrich, St. Louis, MO, USA). After 10 min, the absorbance was measured (Cecil spectrophotometer, CE2021 2000 Series) at λ = 555 nm. ALAD activity was expressed as the amount of porphobilinogen (PBG) formed. The molar extinction coefficient of 6.2 × 104 $M^{-1} \times cm^{-1}$ was used for the calculations according to [34]. The result was expressed per 100 pieces of duckweed or one piece of pea.

2.8. Aminolevulinic Acid (ALA) Content

ALA content was determined according to the method of Jiao et al. [33]. 500 mg of pea or duckweed samples were ground in liquid nitrogen in a cold mortar and 3 mL of acetate buffer, pH 4.6. The homogenate was centrifuged at 10,000× *g* for 20 min at 4 °C. Then, four drops of ethyl acetate (Chempur, Piekary Śląskie, Poland) were added to 1 mL of the supernatant and boiled for 15 min. After cooling, 4 mL of Ehrlich's reagent (4-(Dimethylamino)benzaldehyde, Sigma-Aldrich, USA) was added to the samples. After 15 min, the absorbance was measured at λ = 533 nm (Cecil spectrophotometer, CE2021 2000 Series). ALA concentration was calculated according to Averina et al. [35], using the molar extinction coefficient for ALA 6.8×10^4 $M^{-1} \times cm^{-1}$. ALA concentration was expressed in nmol per 100 pieces of duckweed or one piece of pea.

2.9. Lipid Peroxidation—TBARS Test

The TBARS test was performed according to the method of Hodges et al. [36] with minor modifications. 500 mg of duckweed or pea material was homogenized in a mortar and pestle in 3 mL of 80% ethanol. The samples were centrifuged at 3000× *g* for 10 min. To 1 mL of the supernatant, 1 mL of a mixture of 20% trichloroacetic acid (TCA) and 5% thiobarbituric acid (TBA) was added. The samples were heated at 95 °C for 30 min. After cooling, the samples were centrifuged at 3000× *g* for 10 min. The absorbance was read at 400, 532, and 600 nm (Cecil spectrophotometer, CE2021 2000 Series). Malondialdehyde (MDA) concentration was calculated according to Hodges et al. [36] and expressed per 100 duckweed plants or one pea plant.

2.10. Assessment of Mitochondrial Damage—WST-1 Test

Mitochondria were isolated according to the method of Heckman et al. [37] with modifications. 500 mg of peas or duckweed were homogenized in liquid nitrogen in a mortar with the addition of 2 mL of a mixture with pH 7.6 and the following composition: 350 mM mannitol (Chempur, Poland), 30 mM Mops (3-(N-Morpholino) propanesulfonic acid sodium salt (Sigma-Aldrich, Poland), 1 mM EDTA (Ethylenedinitrilotetraacetic acid; Merck, Warszawa, Poland) with the addition of insoluble PVPP (Polyvinylpolypyrrolidone; Merck, Poland) (1.8 g × 100 mL^{-1} of the mixture) and L-cysteine (Sigma-Aldrich, Poland) (0.34 g × 100 mL^{-1} of the mixture). The homogenate was centrifuged at 4732× *g* for 2 min at 4 °C. The resulting supernatant was centrifuged at 18.207× *g* for 5 min at 4 °C. The supernatant was discarded, and 1 mL of the mixture was added to the pellet. The composition was 300 mM mannitol, 20 mM Mops, and 1 mM EDTA with pH 7.2. Centrifuged at 4732× *g* for 2 min at 4 °C. The supernatant was transferred to new tubes, and 0.6 M sucrose solution was added. Centrifuged at 9583× *g* for 20 min at 4 °C. The supernatant was discarded, and the pellet was dissolved in a mixture composed of 250 mM sucrose and 30 mM Mops of pH 7.2. Mitochondrial damage was assessed using commercial kit WST-1 (Cayman Chemicals, Ann Arbor, MI, USA). 100 µL of sample was incubated with 10 µL of WST-1 reagent for 2 h at 37 °C according to the manufacturer's protocol. The absorbance at λ = 450 nm was measured. In order to compensate for the readings for the natural color of the plant extract, the absorbance was measured at λ = 620 nm, according to Krupka et al. [38]. Mitochondrial damage was assessed as a reference (100% viable mitochondria) using the absorbance value of the control sample.

2.11. Protein Isolation and ELISA for HSP70 Proteins

Proteins were isolated using the method of Isaacson et al. [39] with minor modifications. 500 mg of peas or duckweed were homogenized in liquid nitrogen in a cold mortar with 3 mL of 10% TCA in acetone. The extracts were incubated at −20 °C for 24 h. Then, the extracts were centrifuged for 30 min at 5000× *g*. The pellet was washed with 2 mL of cold acetone and centrifuged at 5000× *g* for 10 min at 4 °C. Washing with acetone was repeated twice. The pellet was dried and dissolved in TBS buffer containing 250 mM Tris and 1.37 M NaCl. HSP70 protein content was determined using an ELISA kit (EIAab Science, China). The ELISA was performed according to the manufacturer's protocol. The absorbance at λ = 450 nm was measured using an Infinite 200 PRO spectrophotometer (Tecan, Männedorf, Switzerland). Sample diluent was used as blank. The concentration of HSP70 proteins was read from the prepared standard curve according to the manufacturer's protocol and expressed in ng × mL^{-1} per 100 pieces of duckweed or one piece of pea.

2.12. Pesticide and Pharmaceutical Analyses

Soil and water samples were frozen immediately after collection and stored at –60 °C. Using an analytical balance, 1 g of soil sample was weighed each time into a 5 mL vial in three replicates. Then, 4 mL of dichloromethane was added to each sample and shaken for 24 h (6× *g*). The obtained extracts were centrifuged (2381× *g* for 10 min), and the organic phases were collected and subsequently concentrated to a volume of 100 µL under a stream of nitrogen. Extracts were injected into a gas chromatograph equipped with a tandem mass spectrometer (GC-MS/MS Shimadzu Corp., Kyoto, Japan) for qualitative analysis. A mass spectrometer equipped with a triple quadrupole as a detector (MS-TQ8040, Shimadzu Corp., Japan) was chosen in order to increase the accuracy of screening, eliminate errors in substance identification, and reduce the impact of matrix effects.

The chromatographic separations were performed on a ZebronTM (ZB-5MSi) capillary column with a length of 30 m, an internal diameter of 0.25 mm, and a stationary phase thickness of 0.25 µm supplied by Phenomenex (Torrance, CA, USA). The temperature of the injector was set at 250 °C. For all analyses, an injection volume of 2 µL was selected. Helium (5.0 purity) supplied by Air Products (Poland) was used as the carrier gas, with constant flow at 1 mL × min^{-1}. The oven temperature program was from 40 °C to 290 °C (with a ramp rate of 10 °C/min.) and held at 290 °C for 2 min. The mass spectrometer was operated in full scan mode in the range of 45–450 *m/z* with a solvent delay of 3 min. The operating conditions of the mass spectrometer were as follows: ion source temperature 220 °C, interface temperature (transfer line to tandem MS) 300 °C, ionization voltage 70 eV, and emission current 150 µA. The system was controlled and operated using the GCMS Real Time Analysis software (GCMSsolution version 4.11, Shimadzu Corp., Kyoto, Japan). Chromatographic data were processed using GCMS Postrun Analysis software (GCMSsolution version 4.11, Shimadzu Corp., Japan). Identification of compounds was performed using a similarity search in the National Institute of Standards and Technology MS databases (NIST 11 and NIST11s). For the screening of 433 pesticide residues in the analyzed samples, the extensive Smart Pesticides Database software (version 1.03, Shimadzu Corp., Kyoto, Japan) with MRM Optimization Tool was used.

2.13. Statistics

The analyses of plant growth parameters were performed in 10 repetitions. Lipid peroxidation, mitochondrial damage, and HSP70 protein content were determined in triplicates. The results were analyzed using the Statistica program 11 and a one-way ANOVA test. Differences between groups were analyzed using Tukey's post hoc test with probability $p \leq 0.05$.

3. Results and Discussion

3.1. Analysis of Soils

Pollutants emitted by poultry farms end up in water, air, and soil. They can infiltrate soils from the air, but improper manure storage or application is more often the source of contamination. During animal production, tons of animal waste are generated in each breeding cycle [20]. Manure management strategies in the European Union assume its use in the surrounding agricultural fields as an organic fertilizer [22], but the amount of manure produced usually exceeds the amount needed to fertilize the surrounding fields, which means that it needs to be stored [13]. To minimize the risk of contaminants from manure getting into the soil or waters, it should be processed and stored in special buildings, closed or open lagoons, or places with impermeable pads [40]. The spreading of contaminated manure to the fields causes the penetration of micropollutants into the soil, thus affecting changes in the properties and deterioration of soil quality. Leachate and runoff from improperly stored animal manure also pose a threat to the surrounding fields. Soil contamination is one of the greatest threats to sustainable agriculture [41]; therefore, the soil quality in areas affected by intensive poultry farms should be constantly monitored. Two soils collected from farmlands located near chicken farms were analyzed and characterized (Table 1). Soil S1 is regularly fertilized with manure, while soil 2 (S2) is taken from the field closest to the manure heap (at a distance of approx. 3 m; see map 1 (Scheme 1)).

Table 1. Selected properties of tested soils.

Sample Name/Parameter	pH_{KCl}	TOC	TN	TC/TN	Available P	Available K	$N\text{-}NO_3$	$N\text{-}NH_4$
Soil S1	5.7	9.6	1.22	7.89	16.7	43.5	4.38	4.89
Soil S2	4.9	12.8	1.33	9.60	40.4	43.2	11.49	34.97

TOC—total organic carbon ($g \times kg^{-1}$); TN—total nitrogen content ($g \times kg^{-1}$); available P ($mg\ P_2O_5 \times 100\ g^{-1}$); available K ($mg\ K_2O \times 100\ g^{-1}$); mineral nitrogen ($mg \times kg^{-1}$).

Scheme 1. Map 1. The map of sampling locations. The colors are different because various plants are growing in neighboring fields. Dark green points next to the manure heap correspond to a grove.

Both soils tested were characterized by low content of total organic carbon (TOC = 9.6 $g \times kg^{-1}$ and 12.8 $g \times kg^{-1}$ for soil 1 and soil 2, respectively), which reflected the general characteristics of the majority of Polish agricultural soils [42] and was slightly below the optimal threshold level for light soils in Europe [43]. Furthermore, in both soils, the TC/TN ratio was below 10; such low TC/TN values were previously reported by Ukalska-Jaruga et al. [44] for arable soils and suggested the predominance of soil organic carbon decomposition processes in the soil. Another important threat to soil quality is soil acidification, which was identified as a serious land degradation problem [45].

The pHKCl of soil S1 was equal to 5.7 (Table 1), while soil S2 was more acidic (pHKCl = 4.9). Acidification of the soil (pH < 5.5) significantly reduces crop yields (e.g., peas) and the abundance and activity of bacteria involved in the transformation of organic compounds in soils; it inhibits many soil beneficial processes, e.g., nitrification or the fixation of free nitrogen. In acidic soils, rhizobium bacteria involved in atmospheric nitrogen fixation by the roots of legumes are reduced [46]. Under low pH conditions, the uptake of nutrients by plants is reduced (N, P, K, Ca); at the same time, the availability of many micronutrients (Zn, Mn, Fe) is increased, reaching even toxic levels [43] The high concentration of Fe^{2+}, Mn^{2+} as well as Al^{3+} and H^+ ions in very acidic and acidic soils also has toxic effects on the roots of the cultivated plants, resulting in a shortening of roots and a reduction in the surface area of the root system. These changes reduce the efficiency of water and nutrient uptake and consequently reduce plant growth [43,46]. Acidic soils are also characterized by increased mobilization and bioavailability of heavy metals [45]. Other inorganic soil pollutants include nitrates and phosphates. Although these compounds are plant nutrients and are not considered toxic, their high concentrations can cause environmental problems. Phosphorus extractability was rather high; available P in S1 was equal to the average content of this nutrient in Polish soils (16.7 mg $P_2O_5 \times 100$ g^{-1}, [47]), the amount of available P in S2 was almost 2.5-fold higher and reached a value of 40.4 mg $P_2O_5 \times 100$ g^{-1}). It is highly probable that this large difference resulted from the phosphate leakage from the manure heap. Soils significantly differed in mineral nitrogen content (sum of N-NO_3 and N-NH_4): 9.27 mg $\times$ kg^{-1} and 46.46 mg $\times$ kg^{-1} in soil S1 and S2, respectively. Soil 1 had similar levels of ammonium and nitrate N, while soil S2 was dominated by N-NH_4 (34.97 N-NH_4 mg $\times$ kg^{-1}). During rain, the nitrogen and phosphorus compounds present in manure are leached directly into the soil. Excessive amounts of phosphate in soils limit the uptake of iron and zinc by plants ([48], which results in symptoms of deficiency of these elements. Plants are the primary source of zinc for humans, and a deficiency of this element in plants can lead to a corresponding deficiency in humans. This problem has already been known and considered widespread and significant for 60 years [49]. Nowadays, in the post-pandemic era, it is particularly important as zinc increases human resistance to bacteria and viruses.

The largest group of soil pollutants, however, are organic pollutants. Due to their persistence, potential for bioaccumulation, and mobility in the environment, they are considered a global environmental problem [50]. The largest group of organic pollutants emitted by intensive poultry farming are pharmaceuticals. Although the use of antibiotics in poultry farms in Poland is subject to legal regulations, the scale of their use is not fully known. Consumption reports are based on the value of sales obtained from pharmaceutical companies [9]. Therefore, there is a need to monitor the presence of pharmaceuticals in the soil surrounding poultry houses. Soil S1 and soil S2 were screened for the presence of pharmaceuticals. No pharmaceutical residues were detected in the soil regularly fertilized with manure. In the soil taken from a field located near a heap of manure, the presence of various classes of pharmaceuticals was found (Table 2). Enrofloxacin, ciprofloxacin, and trimethoprim, found in soil S2, are broad-spectrum antibiotics. They are given to fight bacterial infections in chickens [51]. Although the concentrations of the compounds detected were low, their presence in the soil indicates the routine use of these antibiotics on farms. Moreover, the detection of pharmaceuticals in soil located near manure storage piles suggests that manure is the source of these compounds in the environment. Pharmaceuticals present in the soil can be taken up by plants and thus enter the food chain. Additionally, pharmaceuticals present in the environment, even at low concentrations, contribute to the spread of antibiotic resistance, thus posing a risk to human health [52]. In Poland, 26, 52, 36% of *Salmonella* spp. strains isolated from farm animals in 2020 showed resistance to ampicillin, ciprofloxacin, and tetracycline, respectively. Among *E. coli* strains, 78, 73 and 68% of strains, respectively, showed resistance to these antibiotics [53]. Antibiotics entering the environment as contaminants of manure, even at low concentrations, can affect the composition of the soil and plant microbiome [52]. Changes in the composition of the soil microbiota, resulting from significant environmental contamination with antibiotics, may cause disturbances in the species composition of the phyllosphere of plants growing on contaminated soils. Soto-Giron et al. [54] indicate that agricultural practices have the greatest impact on the formation of the plant phyllosphere. However, the plant phyllosphere is usually represented by those bacteria that develop best in a given environment. The presence of antibiotics in the environment causes selection pressure, which results in the growth and development of pathogenic and antibiotic-resistant bacteria while inhibiting the development of commensal bacteria [55]. It can, therefore, be assumed that the phyllosphere of plants growing on soils containing antibiotics will be rich in pathogenic and antibiotic-resistant strains, which poses a risk to consumers' health. In addition to antibiotics, residues of metoclopramide, an antiemetic drug that improves peristalsis in the digestive system, were also found in soil S2. This compound is given to chickens to stop the defecation process and keep the chicken houses more hygienic poultrydvm.com (accessed on 12 April 2023). In soil S2, residues of carbamazepine—a derivative of dibenzazepine, used as an anxiolytic and sedative—were also determined. In veterinary medicine, dibenzoazepines are administered to reduce stress in animals during transport and to eliminate behavioral problems [12]. However, the environmental consequences of the presence of these compounds are still unknown.

Table 2. Classes of pharmaceuticals detected in soil samples collected from the field near the manure pile.

Compound Name	Pharmaceutical Class
Ciprofloxacin	Fluoroquinolone antibiotic
Enrofloxacin	Fluoroquinolone antibiotic
Carbamazepine	Anticonvulsant and anxiolytic drug
Metoclopramide	Antiemetic drug
Trimethoprim	Antibiotic

Pesticide and herbicide residues were also detected in soil S2 (Table 3). Their presence in the soil was not the result of the emission of pollutants from chicken houses but was probably due to the wide use of these compounds in crops in the surrounding fields. Particularly alarming is the presence of the *p,p′*DDE compound in the soil—a decomposition product of the organochlorine pesticide DDT. WHO indicates DDT as a carcinogenic compound for humans [56]) and chronic exposure to this compound causes lung, liver, breast, and kidney cancer [56]. Despite the ban on its use since the 1980s, residues of DDT and its decomposition products are still detected in most agricultural soils in Poland. DDT level in Polish soils is, on average, 0.064 mg/kg^{-1}, sometimes reaching 0.120 $mg \times kg^{-1}$ [57].

Table 3. Pesticides detected in soil samples collected from the field located near the manure pile.

Compound Name	Action
Diflufenican	Inhibitor of carotenoid biosynthesis
Flufenacet	Herbicide, blocks enzymes that catalyze the biosynthesis of very long chain fatty acids
p,p′-DDE	Insecticide from the group of chlorinated hydrocarbons

Organochlorine pesticides, as a result of deposition from the atmosphere and as runoff from agricultural fields, also pollute surface waters [58], posing a threat to aquatic organisms. DDT is lipophilic and easily accumulates in the cuticle [59], making aquatic plants with a lipid cuticle particularly vulnerable.

3.2. Analysis of Groundwater Collected from the Vicinity of the Farm

Groundwater is the main source of fresh water for people around the world. One-third of the world's population uses groundwater as a source of drinking water [60]. Intensive agriculture is one of the factors deteriorating the quality of groundwater, thus contributing to the deepening of the water crisis [61], and pollutants from chicken houses emitted to soils (including medicines) may also pollute groundwater. Pharmaceuticals present in the soil undergo various transformations, like degradation, adsorption to soil particles, and transport to surface and groundwater [62] as a result of runoff and leaching from soils and manure. The degree of adsorption of pharmaceuticals in soil depends on the molecular structure of the compound, the polarity of the compound, the soil organic matter content, and the type of soil [62]. The soils in our study were characterized by a low organic carbon content (<1.2 $g \cdot kg^{-1}$), resulting in a low capacity of the soil to retain contaminants and their leaching into the deeper layers of the soil profile and/or groundwater. In addition, in soils with different pH (the case of this research), pharmaceuticals can occur in different ionic forms [63], characterized by different adsorption abilities. Weather conditions play an important role in the transfer of pharmaceuticals into the soil [64].

Rainwater that penetrates surface layers of soil can carry pharmaceuticals to deeper layers and contaminate groundwater. This contaminated water can be discharged into surface waters and seep into deep waters, which are often used as a source of drinking water [64]. The presence of pharmaceuticals in drinking water raises concerns about their safety [65], particularly in areas where pharmaceuticals are routinely used on farms. Therefore, it is important to monitor groundwater around these farms. Groundwater samples collected at a depth of 26 m have revealed the presence of various classes of pharmaceuticals, including fluoroquinolones such as ciprofloxacin and enrofloxacin. Ciprofloxacin is the most commonly detected antibiotic in European groundwater, often found in high concentrations near pig farms and manure-spreading sites [66]. In fact, farms have been identified as the main source of ciprofloxacin and enrofloxacin in water [67]. Ciprofloxacin is highly soluble in water and mobile due to its hydrophilic nature. However, its degradation rate slows down significantly under anaerobic conditions in groundwater, and it remains largely unchanged [26]. Given its widespread occurrence in groundwater, it is important to monitor its concentrations in drinking water as well [68].

Small concentrations of trimethoprim were also determined in the groundwater from the vicinity of the farms (Table 4). This compound is often detected in groundwaters in Europe [66], including those located in the vicinity of agricultural soils fertilized with manure. Burke et al. [69] showed the presence of trimethoprim in groundwater in a manure-spreading area. This compound was detected in 11 wells, and the determined concentrations ranged from 5 to 12 $ng \times L^{-1}$.

Table 4. Classes of pharmaceuticals detected in a groundwater sample collected from the area of intensive poultry farming.

Compound Name	Pharmaceutical Class
Ciprofloxacin	Fluoroquinolone antibiotic
Enrofloxacin	Fluoroquinolone antibiotic
Carbamazepine	Anticonvulsant and anxiolytic drug
Lincomycin	Antibiotic from the group of lincosamides
Metoclopramide	Antiemetic drug
Tetracycline	Tetracycline group antibiotic
Trimethoprim	Antibiotic

The presence of lincomycin was also detected in the groundwater (Table 4). This compound has not been previously detected in soil samples. However, its presence in groundwater in the vicinity of farms indicates the use of these compounds in chicken farming. Kuchta et al. [70] showed that the accumulation of lincomycin in soil is transient. Lincomycin residues have been detected in groundwater samples by other researchers [71], suggesting a high leaching potential of this compound.

Tetracycline residues were also detected in the groundwater (Table 4) but not in soils collected from the vicinity of chicken farms (Table 2). Tetracyclines are one of the most commonly used classes of veterinary antibiotics, and approximately 20–70% of the antibiotic dose enters the environment in unchanged form in urine and feces [72]. They are hydrophilic compounds characterized by very low desorption capacity. As soil pH increases, the ability of tetracyclines to adsorb to soil decreases [72]. In alkaline soils, the risk of tetracycline leaching into groundwater is, therefore, greater. Tetracyclines are regularly determined in groundwater samples. The highest concentration of tetracycline was determined in groundwater in the USA (500 ng $\times$ L^{-1}), collected from the vicinity of pig farms [73]. Additionally, Szekeres et al. [74] showed the presence of tetracycline in groundwater from the vicinity of farms and suggested improper management of animal waste as a source of this. Antibiotics taken up with drinking water enter the trophic chains. Chronic exposure, even to low concentrations of antibiotics, can cause health problems. Sub-therapeutic doses of antibiotics in food and water are especially dangerous for children [75]. Studies have shown that exposure to antibiotics in the early stages of life is associated with obesity in school-aged children [76]. Constant intake of antibiotics also contributes to disturbances in the composition of the intestinal microbiota. Disturbances in the composition of the digestive system microbiota are a risk factor for IBS, type II diabetes [77], and Crohn's disease [78]. Additionally, even low concentrations of antibiotics in groundwater pose a risk of antibiotic-resistant genes and strains being selected [26]. Andrade et al. [79] showed that 80% of strains isolated from groundwater show multidrug resistance, which is a threat to human health and life around the world.

In addition to antibiotics, residues of carbamazepine and metoclopramide were determined in groundwater as well as in soils collected from the vicinity of the farms. Carbamazepine is a persistent environmental pollutant that is resistant to degradation [80]. Therefore, its concentrations are very often determined in groundwater. Loos et al. [81] found that carbamazepine was detected in 42% of samples taken from 164 locations in 23 European countries, with a maximum concentration of 390 ng $\times$ L^{-1}. However, most studies on carbamazepine concentrations in groundwater have been carried out in urban areas. Carbamazepine concentrations in the vicinity of intensive poultry farms have not been reported so far. Additionally, metoclopramide has not been previously determined in the area affected by intensive farms. However, the presence of these compounds in groundwater indicates their wide use in animal husbandry.

3.3. Analysis of Morphological and Biochemical Parameters of Common Pea Pisum sativum L.

The stem length of peas grown in manure-treated soil did not change from the control (Figure 1A). A decrease in stem length was observed in peas grown on soil collected from the vicinity of the manure heap (a decrease of 15% compared to the control) (Figure 1A). Similar results were obtained for the length of the main root (27% decrease compared to control) (Figure 1B), the number of lateral roots (26.5% decrease compared to control) (Figure 1C), and second leaf area (24.5% decrease % compared to control) (Figure 1D). These results suggest that the pollutants present in the soil are taken up by plants and induce a stress reaction in them, thus impairing plant growth. Although the concentrations of micropollutants (pharmaceuticals and pesticides) in the soil collected from the vicinity of the manure heap were low (Tables 2 and 3), their synergistic effect is toxic to plants.

Pollutants emitted by intensive poultry farming can accumulate in plant tissues, negatively affecting the morphological and biochemical parameters of plants. Due to human intervention, the number of stress factors to which plants are exposed is significantly increasing [82]. Groups of pollutants toxic to plants include pesticides, heavy metals, and pharmaceuticals residues. Increasingly, it is indicated that environmental stress factors negatively affect the plant microbiome [83], which determines the health and productivity of plants [84]. Intensive agricultural production results in many plants being deprived of microorganisms responsible for the production of key metabolites and vitamins [85]. Sustainable management of environmental resources increasingly relies, therefore, on our understanding of the interactions between various pollutants present in the soil [86] and the synergistic effects of various anthropogenic stress factors. This issue is rapidly gaining attention in the field of plant physiology research [83]. In order to assess the potentially toxic effects of groups of pollutants generated during intensive poultry farming on plants, the morphological and biochemical parameters of pea *Pisum sativum* L. growing on soil regularly fertilized with chicken manure (S1) and located in close proximity to a heap of manure (S2) were assessed. The control was peas grown on perlite (S3). Environmental stress factors cause oxidative stress in plants, which is the result of the accumulation of reactive oxygen species (ROS) in plant tissues. The increase in the level of ROS in plant cells affects many different physiological and biochemical functions, which results in a decrease in plant growth and yield ([87]. Thus, disturbances in the morphological parameters of plants may be considered a visible biomarker of oxidative stress in plants.

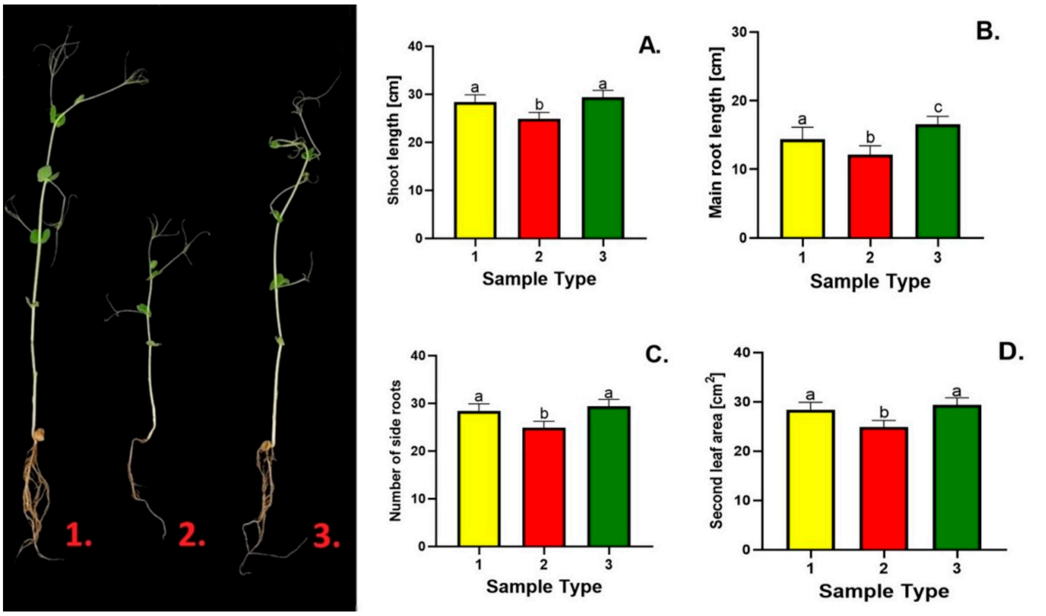

Figure 1. General appearance of peas: 1—pea grown on soil regularly fertilized with manure, 2—pea grown on soil taken from the vicinity of a manure heap, 3—pea grown on horticultural perlite-controland morphological parameters (**A**) shoot length [cm], (**B**) length of the main root [cm], (**C**) number of side roots, (**D**) area of the second leaf [cm^2]. 1 (yellow column)—pea grown on soil regularly fertilized with manure, 2 (red column)—pea grown on soil taken from the vicinity of a manure heap, 3 (green column)—pea grown on horticultural perlite—control. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

Chlorophyll is one of the key factors determining proper plant growth. Reduction in its concentration in a plant is a good stress biomarker [88]. The concentration of chlorophyll in plants grown on the soil collected from the vicinity of the manure heap decreased by 26% compared to the control (Table 5). A decrease in the content of chlorophyll in plants exposed to anthropogenic environmental factors has been repeatedly observed. The reduction in chlorophyll content in plants treated with antibiotics was demonstrated by Rydzyński et al. [31], Margas et al. [89] as well as Krupka et al. [38]. However, in most studies assessing the impact of anthropogenic stress factors on chlorophyll, high concentrations of toxic compounds are used. In the soil collected from the vicinity of manure heaps, the concentrations of drugs and pesticides were not determined; however, their presence was verified, and the reduction in chlorophyll content in plants was found.

Chlorophyll degradation may be the result of stress factors already at the stage of its biosynthesis [90,91]. The fundamental reaction of the chlorophyll biosynthesis pathway is the condensation of two molecules of δ-aminolevulinic acid (ALA) to porphobilinogen. This reaction is catalyzed by the enzyme ALAD (aminolevulinic acid dehydrogenase) [92]. Jiao et al. [33] indicated that the activity of the ALAD enzyme is a factor determining the concentration of chlorophyll in the plant [33]. In plants grown in soil regularly fertilized with manure, ALAD activity decreased by 24% compared to the control (Figure 2A). On the other hand, in plants growing on soil taken from the vicinity of the manure, the activity of the ALAD enzyme decreased by 48% (Figure 2A). The decrease in ALAD activity was correlated with the decrease in ALA content (Figure 2B). Therefore, it can be unequivocally stated that even small concentrations of pollutants present in the soil affect chlorophyll already at the stage of its synthesis, thus causing a decrease in its concentration in plants. Reducing the activity of the ALAD enzyme in plants has been demonstrated under the influence of bisphenol [33] and heavy metals [92]. On the other hand, a decrease in ALA content has been demonstrated under the influence of ciprofloxacin, erythromycin, and sulfamethoxazole [93]. So far, however, no studies have been conducted on ALAD activity and ALA content in plants exposed to several stress factors simultaneously.

Table 5. Concentration of chlorophyll [M] in pea (1—peas grown on soil regularly fertilized with manure, 2—peas grown on soil taken from the vicinity of manure heaps, 3—peas grown on horticultural perlite).

Sample Type	Chlorophyll Concentration [M]
Soil S1	$1.5 \times 10^{-5\,a}$
Soil S2	$1.18 \times 10^{-5\,b}$
Control S3	$1.59 \times 10^{-5\,a}$

Values marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

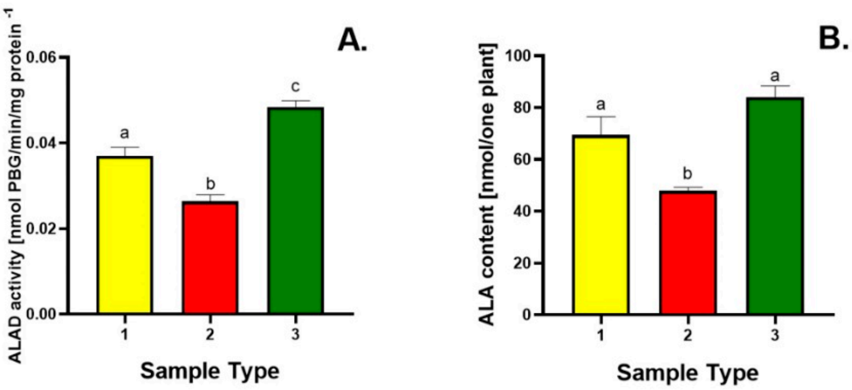

Figure 2. ALAD activity [nmol PGB/min/mg protein] (**A**) and ALA content [nmol × one plant^{-1}] (**B**) in peas grown on soil regularly fertilized with manure—1 (yellow column); collected from the vicinity of manure heaps—2 (red column) and the horticultural perlite—3 (green column). Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

Malonaldehyde (MDA) content is a commonly used parameter for measuring lipid peroxidation in cell membranes. Its concentration increases under conditions of oxidative stress [94]. Lipid peroxidation is a damaging process that affects membrane properties, causes protein degradation, and reduces ion transport capacity, ultimately leading to cell death [95]. Increased levels of MDA have previously been demonstrated in plants treated with antibiotics [38], p,p′-DDE [96], and heavy metals. In peas grown on soil taken from the vicinity of the manure heap, the concentration of MDA increased 4-fold (Figure 3A). The contaminants present in the soil caused, therefore, the destruction of cell membranes. Similar results were obtained with mitochondrial damage (Figure 3B). Mitochondria are organelles that are particularly vulnerable to organic pollutants, including pharmaceuticals [38]. Pesticides also damage the mitochondria. However, most of the studies on the effects of pesticide residues on mitochondria have been carried out in animals. The effect of these compounds on plant mitochondria has been known very poorly [97]; it is possible, however, that the decrease in mitochondrial viability may turn out to be an important indicator of biochemical changes in plants caused by the synergistic effect of pollutants emitted by intensive poultry farms.

HSP70 proteins are also essential components of the plant's response to stress. Their main role is to protect cells from oxidative stress by stabilizing membrane proteins and removing abnormal and damaged proteins [98]. Although they accumulate in the greatest amount during heat stress, HSP70 proteins are a biomarker of various stress reactions, including those induced by anthropogenic pollution [38]. An increase in the content of these proteins was observed both in plants growing on soil regularly fertilized with manure and growing on soil collected from the vicinity of manure heaps (Figure 3C). These results suggest that in plants growing on soil regularly fertilized with manure, a stress reaction was also triggered, although no residues of toxic factors were determined in this soil. These plants showed no changes at the morphological and biochemical level; therefore, the increase in the content of HSP70 proteins seems to be an effective mechanism for protecting plant cells from damage.

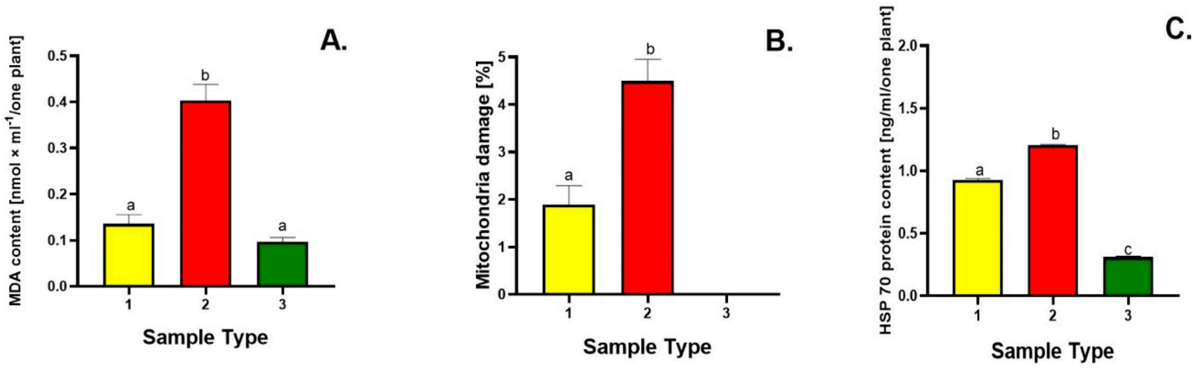

Figure 3. Biochemical parameters (**A**) concentration of MDA [nmol × mL^{-1} × one $plant^{-1}$], (**B**) damage to mitochondria [%], (**C**) concentration of HSP70 proteins [ng × mL × one $plant^{-1}$]) of pea growing on soil regularly fertilized with manure—1 (yellow column), collected from the vicinity of manure heaps—2 (red column) and on horticultural perlite—3 (green column; plants grown in perlite did not exhibit any symptoms of mitochondrial damage). Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

3.4. Analysis of Morphological and Biochemical Parameters of the Small Duckweed *Lemna minor* L.

In order to assess the impact of soil pollutants on the condition of surface waters, the soil was washed with redistilled water, and duckweed was grown in this effluent (see Section 2.4). Surface water—including rivers, ponds, and lakes—is still the source of drinking water for 159 million people in the world. Polluted water contributes to the deaths of 842,000 people a year [99]. Intensive agriculture is indicated as the main source of surface water pollution [1]. Groups of pollutants emitted to surface waters include, e.g., nitrates and phosphates, released from soils fertilized with excessive amounts of manure [1]. Consumption of water rich in nitrates contributes to the occurrence of methemoglobinemia, a fatal disease mainly affecting children under 6 months of age [100]. In addition to the direct consequences for human health, nitrates and phosphates present in water cause cyanobacterial blooms, which have negative effects on aquatic ecosystems [101]. In Europe, 50–80% of released nitrates and phosphates reach surface waters as a result of agricultural practices. Pharmaceuticals and their metabolites also pose a serious threat to the quality and safety of surface waters. Poor manure management practices and excessive use of manure in fields result in drugs entering the surrounding surface waters as a result of runoff and leachate. Park et al. [102] showed the presence of lincomycin and sulfamethoxazole in a stream near a manure heap. The presence of lincomycin, trimethoprim, and sulfamethoxazole has also been demonstrated in the nearby river [102]. Antibiotics have also been identified in manure leachate in an experiment simulating rainfall [102]. A study by Barrios et al. [103] also showed the presence of antibiotics in runoff from soil fertilized with manure. Tetracycline was also determined in the surface runoff from soil fertilized with manure, and its concentration was 2.79–35.97 μg × L^{-1} [104]. Meng et al. [105] showed the presence of 23 antibiotics in rivers from rural areas, which are a source of drinking water for local residents. In addition to antibiotic residues, antibiotic resistance genes were also detected in environmental samples [103]. Pesticides are another large group of surface water pollutants. Although their presence in surface waters may not be a direct result of their use in livestock farming, odors from chicken houses attract insects that can infest crops grown in the surrounding fields [106]. Therefore, in areas affected by intensive poultry farming, it becomes necessary to use pesticides in the fields. Pesticides are detected in waters in Europe, China, and the USA [107] as well as in Africa and South America [108]. Pollution of the aquatic environment with pesticides is, therefore, a global problem. Particularly alarming is the presence of organochlorine pesticides (including DDT) in water reservoirs, characterized by high biomagnification capabilities. DDT, as a lipophilic compound, is easily accumulated in lipid structures [59]. Aquatic plants covered with a lipid cuticle [59] and aquatic animals that accumulate DDT in adipose tissue [109] are particularly vulnerable to its effects. To monitor the quality of surface waters and study the impact of pollutants present in water on living organisms, toxicity tests for duckweed *Lemna minor* L. are used [110]. The analysis of morphological and biochemical parameters of duckweed is used to assess both the presence and toxicity of micropollutants in surface waters.

In order to demonstrate that pollutants emitted as a result of intensive poultry farming penetrate into surface waters and affect aquatic organisms, the morphological and biochemical parameters of duckweed *Lemna minor* L. growing on filtrates of soils regularly fertilized with manure (S1) and collected from the vicinity of manure heaps were assessed (S2). MS medium (S3) was used as a control. The OECD recommends the use of morphological parameters to assess the toxicity of micropollutants present in water [30]. The number of plants growing on filtrate 2 decreased by 50% (Figure 4A). Similar results were obtained by Krupka et al. [38]. Tetracycline at 2.5 mM resulted in a 48% reduction in plant numbers. Despite the fact that a high concentration of tetracycline was used in that study, the results described coincide with our current results. Various water contaminants can act synergistically, leading to stress symptoms in plants comparable to those caused by higher concentrations of compounds acting alone. Contaminant-caused disturbances were also demonstrated by comparing the area of duckweed fronds. Plants growing on filtrate number one had a 59% larger frond area compared to the control (Figure 4B). Park et al. [111] determined high concentrations of phosphorus in aqueous extracts of chicken manure. Li et al. [112] indicate that 69% of the phosphorus present in manure is soluble in water and thus easily gets into the environment. Phosphorus is an element that stimulates root growth, thus improving plant growth [13]. The root length of plants growing on filtrate 1 averaged 21 cm, while the plants grown on MS medium had an average root length of 7.8 mm (Figure 4C). Duckweed growing on filtrate 2 was characterized by both a smaller frond area (Figure 4B) and root length (Figure 4C) compared to the control, which indicates the influence of toxic factors present in the water on plant growth.

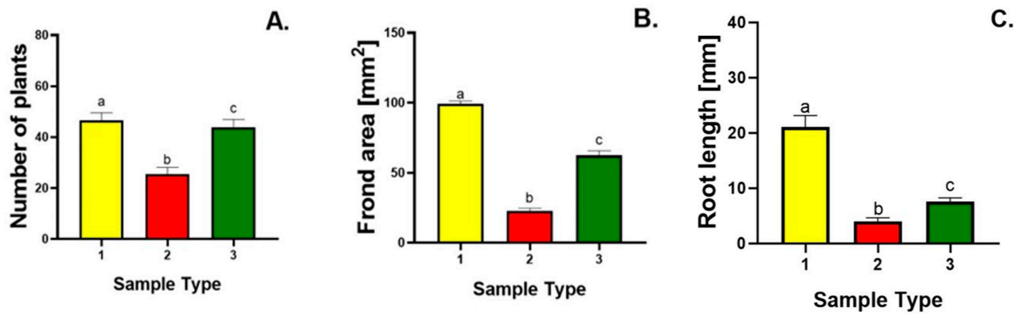

Figure 4. Morphological parameters (**A**) number of plants, (**B**) area of fronds [mm^2], (**C**) root length [mm]; 1 (yellow column)—duckweed cultivated on the filtrate of soil regularly fertilized with manure, 2 (red column)—duckweed grown on filtrate of soil taken from the vicinity of the manure heap, 3 (green column)—duckweed grown on MS medium. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

Another parameter of the *Lemna minor* L. toxicity test is the measurement of the chlorophyll content. Sackey et al. [113] showed low concentrations of chlorophyll in duckweed growing on a medium with the addition of leachate from landfills contaminated with various groups of micropollutants. Similar results were obtained for duckweed grown on soil filtrate taken from the vicinity of manure. In these plants, the concentration of chlorophyll decreased by 33% (Table 6); in addition, chlorosis of the fronds was observed (Figure 5).

Table 6. The concentration of chlorophyll [M] in duckweed (S1—duckweed cultivated on the filtrate of soil regularly fertilized with manure, S2—duckweed grown on filtrate collected from the vicinity of a manure heap, S—duckweed grown on MS medium).

Sample Type	Chlorophyll Concentration [M]
S1	$1.70 \times 10^{-5\,a}$
S2	$1.01 \times 10^{-5\,b}$
S3	$1.53 \times 10^{-5\,a}$

Values marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

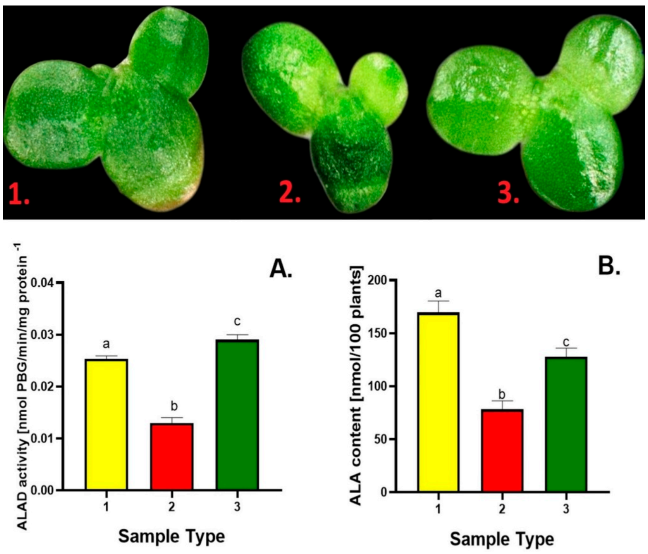

Figure 5. General appearance of duckweed: 1—duckweed grown on the filtrate from soil regularly fertilized with manure, 2—duckweed growing on the filtrate of soil taken from the vicinity of manure heap, 3—duckweed grown on MS medium) and ALAD activity [nmol PGB × min × mg protein $^{-1}$] (**A**) and ALA content [nmol × 100 plants^{-1}] (**B**) in duckweed growing on the filtrate of soil regularly fertilized with manure—1 (yellow column), collected from the vicinity of manure heap—2 (red column) and on the medium of MS—3 (green column). Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

The decrease in chlorophyll content was also correlated with decreased ALAD enzyme activity (2-fold decrease in activity) (Figure 5A) and lower ALA concentration (Figure 5B). Most studies on ALAD activity and ALA content concern the impact of single stress factors. Our results indicate that these parameters can also be used in the assessment of the toxicity of many water pollutants to *Lemna minor* L.

Krupka et al. [35] indicated that MDA content, HSP70 protein concentration, and mitochondrial damage are important parameters in assessing the toxicity of micropollutants present in water towards *Lemna minor* L. Plants growing on filtrate number 2 showed a 4-fold increase in MDA content (Figure 6A), suggesting damaged cell membranes in these plants. Similar results were obtained for peas grown on soil collected from manure heaps (Figure 3A). Mitochondria are organelles sensitive to micropollutants (especially antibiotics).

Duckweed cells growing on filtrate number 2 showed 7% mitochondrial damage (Figure 6B), and duckweed cells growing on filtrate number 1 showed 5% mitochondrial damage (Figure 6B), which is consistent with the results obtained for peas. Both plants growing on filtrate 1 and 2 showed induction of the HSP70 proteins (Figure 6C), indicating that these plants were initiating a stress response. *Lemna minor* L. toxicity tests are particularly useful in testing water samples in which the content of micropollutants has not been determined [100]. The physiological response of the duckweed is evidence of the presence of toxic components in these tests.

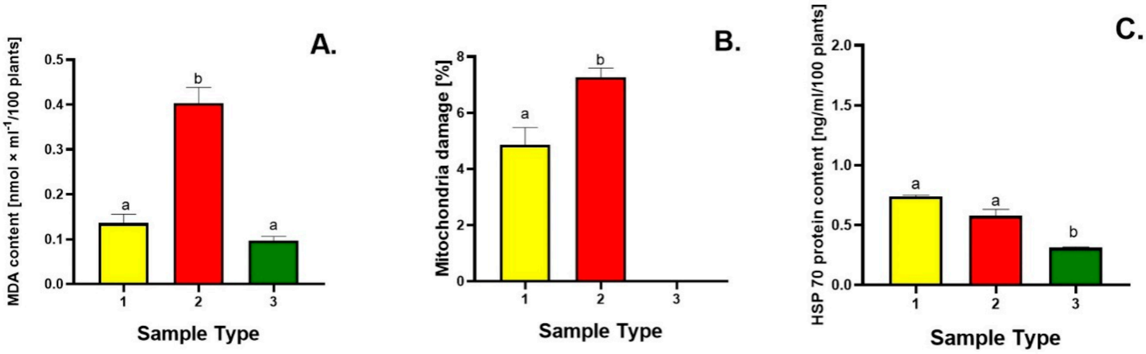

Figure 6. Biochemical parameters (**A**) concentration of MDA [nmol $\times$ mL^{-1} $\times$ 100 plants^{-1}], (**B**) damage to mitochondria [%], (**C**) concentration of HSP70 proteins [ng $\times$ mL^{-1} $\times$ 100 plants^{-1}]) of duckweed *Lemna minor* L. growing on the filtrate of soil regularly fertilized with manure—1 (yellow column), collected from the vicinity of manure heap—2 (red column) and on perlite rinsed with the minerals of 50% MS medium—3 (green column). Duckweed grown on perlite with minerals of 50% MS medium did not exhibit any symptoms of mitochondrial damage. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test).

4. Conclusions

The fertilizer from poultry farms can have a negative impact on plant growth and physiological status (demonstrated by decreases in ALAD activity and chlorophyll concentration, as well as increases in stress proteins HSP70, mitochondria damage, and the content of MDA, as indicators of membrane damage). The results show that special attention should be paid when making environmental decisions on the methods of storage of poultry manure and the use of such fertilizer in plant production. Particular attention should be paid to the impact on all environmental components, in particular soil, surface, and underground waters.

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