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Reakcja ekofizjologiczna roślin na zanieczyszczenie środowiska antybiotykami

Ecophysiological response of plants to environmental pollution by antibiotics

Praca wykonana
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KOPIE PUBLIKACJI TWORZĄCYCH PODSTAWĘ ROZPRAWY DOKTORSKIEJ WRAZ Z OŚWIADCZENIAMI WSPÓŁAUTORÓW 5

WYKAZ SKRÓTÓW

3 - PGADH – dehydrogenaza 3-fosfoglicerynianowa

ALA – kwas aminolewulinowy

ALAD – dehydrataza kwasu aminolewulinowego

APX – peroksyadaza askorbinianowa

ASV (ang. *aplicon sequence variant*) – wariant sekwencji aplikonu

ATP – adenozynotrójfosforan

CAT – katalaza

DTT – ditiotreitol

EDTA – kwas etylenodiaminotetraoctowy

HSP70 (ang. *heat – shock proteins*) – białka szoku cieplnego

MOPS – kwas 3 – (N – morfolino)propanosulfonowy

MDA – malondialdehyd

NADH – dinukleotyd nikotynoamidoadeninowy

NGS – (ang. *next – generation – sequencing)* – sekwencjonowanie nowej generacji

ODCZYNNIK EHRLICHA – dimetyloaminobenzaldehyd rozpuszczony w etanolu i stężonym kwasie solnym

PBS – sól fizjologiczna buforowana fosforanem

PDX – peroksydaza pirogalolowa

PGK – kinaza 3-fosfoglicerynianowa

PMSF – fluorek fenylometylosulfonylu

PVDF – polifluorek winylidenu

PVPP – poliwinylopolipirolidon

SDS – dodecylosiarczan sodu

RuBisCO – karboksylaza/oksygenaza rybulozo-1,5-bisfosforanu

RuBP – rybulozo-1,5-bisfosforan

TBA – kwas tiobarbiturowy

TCA – kwas trichlorooctowy

WYKAZ PUBLIKACJI TWORZĄCYCH PODSTAWĘ ROZPRAWY DOKTORSKIEJ

Publikacja 1.

Krupka M, Michalczyk DJ, Piotrowicz – Cieślak AI. 2022. Effects of antibiotics on the photosynthetic apparatus of plants. *Journal of Plant Interactions* 17: 96 – 104. DOI: 10.1080/17429145.2021.2014579.

IF[1]: 3,200; MEiN[2]: 70

Publikacja 2.

Krupka M, Michalczyk DJ, Žaltauskaite J, Sujetoviene G, Głowacka K, Grajek H, Wierzbicka M, Piotrowicz – Cieślak AI. 2021. Physiological and biochemical parameters of common duckweed *Lemna minor* after the exposure to tetracycline and the recovery from this stress. *Molecules* 26: 6765. DOI: 10.3390/molecules26226765.

IF[1]: 4,927; MEiN[2]: 140

Publikacja 3.

Krupka M, Michalczyk DJ, Piotrowicz – Cieślak AI. 2024. Critical stages in pea photosynthesis impaired by tetracycline as an environmental contaminant. *Journal of Plant Research,* 137: 1049 **–** 1059. DOI: 10.1007/s10265-024-01580-x.

IF[1]: 2,700; MEiN[2]: 100

Publikacja 4.

Krupka M, Olkowska E, Klimkowicz – Pawlas A, Łęczyński L, Tankiewicz M, Michalczyk DJ, Wolska L, Piotrowicz – Cieślak AI. 2024. The impact of soil and water pollutants released from poultry farming on the growth and development of two plant species. *Agriculture* 14: 87. DOI: 10.3390/agriculture14010087.

IF[1]: 3,300; MEiN[2]: 100

Publikacja 5.

Krupka M, Piotrowicz – Cieślak AI. 2024. Optimization of the method for isolating bacterial DNA from the aboveground part of lettuce. *International Journal of Molecular Sciences* 25: 8513. DOI: 10.3390/ijms25158513.

IF[1]: 4,900; MEiN[2]: 140

Materiał przygotowany do publikacji 6 (MPP 6).

The impact of tetracycline on the soil microbiome and the rhizosphere of lettuce (*Lactuca sativa* L.)

Sumaryczny IF – 19,027

Sumaryczna liczba punktów MEiN – 550

[1]IF – wskaźnik Impact Factor czasopisma w roku publikacji

[2]MEiN – liczba punktów na podstawie komunikatu Ministra Edukacji i Nauki w sprawie wykazu czasopism naukowych w roku publikacji

STRESZCZENIE

W obliczu rosnącego zaniepokojenia wpływem zanieczyszczeń środowiskowych, w tym antybiotyków, na ekosystemy, niniejsza rozprawa doktorska skupia się na badaniach dotyczących wpływu tetracykliny na rośliny oraz ich mikrobiom. Składa się z pięciu publikacji i materiału przeznaczonego do publikacji, które mają na celu zbadanie molekularnych biomarkerów uszkodzeń i regeneracji roślin poddanych działaniu tetracykliny, wpływu tego antybiotyku na aparat fotosyntetyczny, optymalizacji metody izolacji DNA z filosfery roślin, a także analizy składu mikroorganizmów w glebie oraz ryzosferze roślin uprawianych w środowisku zanieczyszczonym.

Zasadniczym celem pracy było poszukiwanie molekularnych biomarkerów uszkodzeń i regeneracji roślin traktowanych tetracykliną. Przegląd literatury ujawnił braki w dotychczasowych badaniach, szczególnie dotyczących wpływu tetracykliny na biosyntezę chlorofilu oraz aktywność enzymów kluczowych dla fotosyntezy. Aby uzupełnić tę lukę, przeprowadzono szereg eksperymentów, w których oceniano wpływ tetracykliny na zawartość chlorofilu w roślinach. Stwierdzono, że traktowanie tetracykliną prowadzi do istotnego spadku stężenia chlorofilu w roślinach, co jednoznacznie wskazuje na toksyczny wpływ tego antybiotyku na aparat fotosyntetyczny.

W ramach dalszych badań zastosowano rzęsę drobną, której wzrost monitorowano przez 7 dni w obecności różnych stężeń tetracykliny (1 mM, 2,5 mM, 10 mM). Wyniki pokazały również wyraźny spadek zawartości chlorofilu, sięgający 64% przy najwyższym stężeniu. Dodatkowo, badania nad grochem wykazały, że nawet niewielkie stężenia tetracykliny w glebie (5 mg/kg) powodują spadek zawartości chlorofilu o 17%, a przy stężeniu 500 mg/kg spadek wynosił 28%. Zidentyfikowane zostały także negatywne skutki działania tetracykliny na szlak biosyntezy chlorofilu, z obniżoną aktywnością kluczowego enzymu – dehydratazy kwasu aminolewulinowego (ALAD).
W badaniach na grochu zidentyfikowano również wpływ tetracykliny na karboksylazę/oksygenazę rybulozo 1,5 - bisfosforanu (RuBisCO), kluczowego w procesie fotosyntezy. Wykazano, że tetracyklina zmniejsza zawartość dużej podjednostki RuBisCO oraz jego aktywność, co jest poważnym zagrożeniem dla efektywności fotosyntezy. Zmiany te były istotne w kontekście zarówno stężeń tetracykliny, jak i długości czasu ekspozycji roślin na ten związek.

Kolejnym istotnym aspektem badanym w publikacjach było zjawisko uszkodzeń komórkowych. U rzęsy drobnej wykazano, że akumulacja wolnych rodników wzrasta w odpowiedzi na działanie tetracykliny, co prowadzi do uszkodzeń błon komórkowych i mitochondriów. Wyniki wskazują na wzrost stężenia malondialdehydu (MDA), co jest wskaźnikiem peroksydacji lipidów, oraz na uszkodzenia mitochondrialne, co potwierdzono testem WST-1.

Oprócz badań nad uszkodzeniami, analizowano również zdolności regeneracyjne roślin. Zastosowane wysokie stężenia tetracykliny (1 mM, 2,5 mM, 10 mM) ujawniły, że rośliny były w stanie odzyskać zdolności wzrostowe po usunięciu tetracykliny z pożywki. Analizowane markery, takie jak HSP70 oraz aktywność enzymów antyoksydacyjnych, wykazały, że mechanizmy regeneracyjne były aktywowane w odpowiedzi na stres.

Kolejny kluczowy cel pracy dotyczył optymalizacji metod izolacji DNA z filosfery roślin. Przeanalizowano 50 różnych protokołów, co pozwoliło na zidentyfikowanie najefektywniejszych parametrów izolacji DNA, takich jak ilość użytego materiału, sposób lizy komórkowej oraz metody zbierania komórek

bakteryjnych. Wyniki wykazały, że liza mechaniczno-chemiczna w połączeniu z sonikacją i filtracją membranową prowadzi do uzyskania DNA o wysokiej jakości, co jest istotne w badaniach mikrobiomu.

W kontekście analizy składu mikroorganizmów, badania wykazały, że tetracyklina znacząco wpływa na skład mikrobiomu glebowego oraz ryzosfery sałaty. Analiza taksonomiczna przed i po aplikacji tetracykliny ujawniła, że dominującym typem bakterii były Proteobacteria, z obecnością rodzin zdolnych do remediacji gleb. Wyniki wskazują na istotne zmiany w składzie mikroorganizmów w odpowiedzi na obecność tetracykliny, co ma potencjalne implikacje dla jakości gleb i upraw.

Przeprowadzone badania dostarczają istotnych informacji na temat wpływu tetracykliny na rośliny oraz ich mikrobiom. Zidentyfikowane biomarkery uszkodzeń i regeneracji mogą być stosowane do oceny ekotoksyczności środowiskowej oraz potencjału fitoremediacji roślin. Badania nad mikrobiomem i jego odpowiedzią na zanieczyszczenia oferują nowe podejścia do zrozumienia interakcji między roślinami a ich środowiskiem, co może mieć istotne znaczenie dla przyszłych strategii ochrony środowiska i zrównoważonego rozwoju rolnictwa.

Wyniki pracy mają również praktyczne zastosowanie w monitorowaniu zanieczyszczeń w ekosystemach rolniczych, przyczyniając się do rozwoju metod oceny jakości gleb i upraw oraz skutków stosowania antybiotyków w rolnictwie. Dalsze badania mogą skoncentrować się na długoterminowych skutkach działania tetracykliny oraz poszukiwaniu metod minimalizacji jej negatywnego wpływu na ekosystemy.

Słowa kluczowe: tetracyklina, fotosynteza, fitotoksyczność, regeneracja, mikrobiom roślin

ABSTRACT

In response to growing concerns about the impact of environmental pollutants, including antibiotics, on ecosystems, this doctoral dissertation focuses on investigating the effects of tetracycline on plants and their microbiomes. The work comprises five publications and additional unpublished material aimed at exploring molecular biomarkers of damage and regeneration in plants exposed to tetracycline, the antibiotic's impact on the photosynthetic apparatus, optimization of DNA isolation methods from the phyllosphere, and the analysis of soil and rhizosphere microbiome composition in contaminated environments.

The primary goal of this research was to identify molecular biomarkers of plant damage and recovery in response to tetracycline exposure. A literature review revealed significant gaps in current knowledge, particularly regarding tetracycline's effects on chlorophyll biosynthesis and the activity of key photosynthetic enzymes. To address these gaps, a series of experiments evaluated the impact of tetracycline on plant chlorophyll content. It was determined that tetracycline exposure leads to a significant reduction in chlorophyll concentration, demonstrating the antibiotic’s toxic effects on the photosynthetic system.

Further experiments involved monitoring the growth of *Lemna minor* over seven days in the presence of various tetracycline concentrations (1 mM, 2.5 mM, and 10 mM). Results showed a pronounced decline in chlorophyll content, with a reduction of up to 64% at the highest concentration. Additionally, studies with pea plants revealed that even low tetracycline concentrations in soil (5 mg/kg) reduced chlorophyll levels by 17%, while concentrations of 500 mg/kg caused a 28% decline. Negative effects on chlorophyll biosynthesis pathways were identified, including decreased activity of the key enzyme δ-aminolevulinic acid dehydratase (ALAD).

Research on pea plants also examined the impact of tetracycline on ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), a critical enzyme in photosynthesis. Findings showed that tetracycline reduces the abundance of RuBisCO's large subunit and its activity, posing a significant threat to photosynthetic efficiency. These changes correlated with both tetracycline concentration and exposure duration.

Another focus of the research was cellular damage mechanisms. In *Lemna minor*, tetracycline exposure increased free radical accumulation, leading to membrane and mitochondrial damage. Markers such as elevated malondialdehyde (MDA) levels indicated enhanced lipid peroxidation, while mitochondrial damage was confirmed using the WST-1 assay.

In addition to studying damage mechanisms, the research also investigated plant regeneration capacities. High tetracycline concentrations (1 mM, 2.5 mM, and 10 mM) revealed that plants could recover growth capacity after tetracycline removal from the medium. Biomarkers such as HSP70 and antioxidant enzyme activity indicated activation of regeneration mechanisms in response to stress.

A crucial objective of the dissertation involved optimizing DNA isolation methods from the plant phyllosphere. Fifty different protocols were analyzed, leading to the identification of optimal parameters for DNA isolation, including the amount of starting material, cell lysis methods, and bacterial cell collection techniques. Results showed that a combination of mechanical-chemical lysis with sonication and membrane filtration yielded high-quality DNA, critical for microbiome studies.

Microbiome analyses demonstrated that tetracycline significantly alters the composition of soil and rhizosphere microbial communities in lettuce. Taxonomic

analysis before and after tetracycline application revealed that Proteobacteria dominated, including families capable of soil remediation. Results highlighted substantial shifts in microbial composition in response to tetracycline, with potential implications for soil quality and crop health.

These studies provide essential insights into the effects of tetracycline on plants and their microbiomes. The identified damage and regeneration biomarkers can be used to assess environmental ecotoxicity and the phytoremediation potential of plants. Microbiome studies offer new perspectives on plant-environment interactions, which are crucial for future strategies in environmental protection and sustainable agriculture.

The findings also have practical applications for monitoring pollution in agricultural ecosystems, contributing to the development of methods for assessing soil and crop quality as well as the impacts of antibiotic use in agriculture. Future research could focus on the long-term effects of tetracycline exposure and methods for mitigating its adverse environmental impacts.

Keywords: tetracycline, photosynthesis, phytotoxicity, regeneration, plant microbiome

1. Wstęp

Koncepcja „One Health" (Jedno Zdrowie) jest holistycznym podejściem, które uwzględnia wzajemne powiązania między zdrowiem ludzi, zwierząt, roślin a ich środowiskiem (WHO, 2024). Rośliny odgrywają kluczową rolę w tej koncepcji na wielu poziomach, ponieważ ich prawidłowy stan fizjologiczny wpływa na zdrowie całego ekosystemu (Al–Eitan i in., 2023). Jednym z największych zagrożeń dla roślin są antropogeniczne zanieczyszczenia środowiska, w tym antybiotyki, które, choć stosowane w medycynie ludzkiej i weterynaryjnej, w największych ilościach wykorzystywane są w sektorze produkcji zwierzęcej (Scaria i in., 2021). W 2020 roku największe zużycie antybiotyków odnotowano w Chinach (43 tys. ton), Brazylii (7533 ton) i Stanach Zjednoczonych (6596 ton). W Europie najwięcej antybiotyków zużyto w Hiszpanii, Włoszech i Niemczech (Sriram i in., 2021). Polska również znajduje się w czołówce krajów o wysokim zużyciu antybiotyków, zwłaszcza w sektorze produkcji drobiu (Pyzik i in., 2024). Niepełny metabolizm antybiotyków w organizmach żywych (Kümmerer, 2009), nadmierne stosowanie oraz brak skutecznych metod usuwania prowadzą do ich obecności w środowisku, gdzie stają się zanieczyszczeniami (Otia i Dubey, 2024). Główną drogą rozprzestrzeniania się antybiotyków do środowiska jest stosowanie obornika zwierzęcego jako nawozu organicznego w rolnictwie (Tasho i in., 2016). Stosowanie nawozów organicznych może również prowadzić do rozprzestrzeniania się antybiotyków w środowisku wodnym (Gržinić i in., 2023), gdyż leki obecne w glebie mogą migrować do wód powierzchniowych i gruntowych (Zhi i in., 2019). Szczególny niepokój budzi obecność w środowisku tetracykliny, która z uwagi na swoje szerokie spektrum działania, łatwość podawania i niski koszt produkcji jest często stosowana w produkcji zwierzęcej (Gudda i in., 2023). Ze względu na długi okres półtrwania i rozpuszczalność w wodzie, tetracyklina charakteryzuje się dużą mobilnością i trwałością w środowisku (Gudda i in., 2023). Obecność tetracykliny w środowisku wpływa na organizmy inne niż docelowe, w tym na rośliny i związane z nimi mikroorganizmy (Rocha i in., 2021).

Skład mikrobiomu roślin ma kluczowe znaczenie dla ich zdrowia, wzrostu i odporności na stres (Compant i in., 2019; Trivedi i in., 2020). Coraz więcej badań wskazuje również na związek między mikrobiomem roślin a zdrowiem człowieka. Rośliny spożywane na surowo mogą stanowić źródło zarówno pożytecznych, jak i patogennych mikroorganizmów (Soto–Giron i in., 2021; Wicaksono i in., 2023), które

kolonizują organizm ludzki, i określane są mianem „jadalnego mikrobiomu roślin" (Soto–Giron i in., 2021; Wicaksono i in., 2023). Badania dotyczące mikrobiomu roślin uprawianych w środowisku zanieczyszczonym tetracykliną są wciąż nieliczne i obejmują jedynie kilka gatunków (Zhang i in., 2019; Li i in., 2022). Rola świeżych warzyw i owoców w kształtowaniu mikrobiomu człowieka wciąż pozostaje niedoceniana.

Wraz z rozwojem metod sekwencjonowania nowej generacji (NGS, ang. *next generation sequencing*), rośnie zainteresowanie badaniami molekularnymi dotyczącymi mikrobiomu roślin. Choć metody NGS są bardzo specyficzne, konieczne jest unikanie zanieczyszczeń oraz degradacji materiału genetycznego. Jest to szczególnie istotne w przypadku próbek o niskiej zawartości drobnoustrojów, takich jak filosfera (nadziemne części roślin) (Fricker i in., 2019). Dotychczas nie przeprowadzono badań nad optymalizacją metod izolacji DNA z filosfery roślin, a dostępne protokoły izolacji nie są jednolite (Zhang i in., 2021). Kluczowe jest opracowanie powtarzalnej i optymalnej metody izolacji bakteryjnego DNA z liści, co ma istotne znaczenie zarówno dla badań fizjologii roślin, jak i zdrowia konsumentów.

Antybiotyki obecne w środowisku stanowią także bezpośrednie zagrożenie dla roślin, ponieważ są one pobierane z gleby i wody (Azanu i in., 2016; Rocha i in., 2021). Wpływ tetracykliny na rośliny został do tej pory głównie zbadany na poziomie morfologicznym. Wykazano, że tetracyklina powoduje m.in. redukcję długości korzenia (Han i in., 2021; Li i in., 2023), zmniejszenie liczby komórek oraz powierzchni walca osiowego (Li i in., 2023), zahamowanie wzrostu łodyg (Margas i in., 2016; Yagoubi i in., 2023) i liści (Matamoros i in., 2022). Tetracyklina wykazuje również fitotoksyczność wobec roślin wodnych, prowadząc do zmniejszenia biomasy i powierzchni liści (Baciak i in., 2016). Antybiotyki pobierane przez rośliny mogą ulegać akumulacji, degradacji lub transformacji do metabolitów w tkankach roślinnych (Bártíková i in., 2015), co sugeruje ich potencjalne wykorzystanie w procesach fitoremediacji zanieczyszczonych środowisk (Zhou i in., 2024).

Ocena zdolności roślin do remediacji antybiotyków wymaga analizy parametrów morfologicznych oraz fizjologiczno-biochemicznych, co pozwoli na zidentyfikowanie mechanizmów toksyczności tych związków oraz zdolności roślin do regeneracji (Sikorski i in., 2024). Dotychczas badania nad regeneracją roślin prowadzono głównie po traktowaniu ich herbicydami (Burns i in., 2015; Zaltauskaite i Kacienie, 2020). Toksyczność tetracykliny wobec roślin na poziomie komórkowym jest słabo zbadana,

zaś markery uszkodzeń i regeneracji komórek roślinnych nie są dobrze poznane. Miejscem docelowym działania tetracykliny w roślinach jest prawdopodobnie chloroplast (Wang i in., 2015). Wpływ antybiotyków na aparat fotosyntetyczny został szczegółowo omówiony w pracy Krupka i in. (2022). Rydzyński i in. (2019) wykazali, że tetracyklina bezpośrednio degraduje chlorofil do feofityny, natomiast pełny mechanizm jej działania w aparacie fotosyntetycznym nie jest znany. Antybiotyki blokujące biosyntezę białek bakteryjnych mogą być szczególnie toksyczne dla chloroplastów, które posiadają bakteryjne mechanizmy transkrypcji i translacji (Liu i in., 2011; Kasai i in., 2004). Tetracyklina może również degradować białka odpowiedzialne za biosyntezę chlorofilu oraz inne białka chloroplastowe, w tym karboksylazę/oksygenazę rybulozo-1,5-bisfosforanu (RuBisCO), który jest kluczowy dla wydajności fotosyntezy (Muhie, 2022). Choć Margas i in. (2016) wykazali, że tetracyklina zmniejsza zawartość aktywazy RuBisCO, aktywność samego enzymu nie była dotąd przedmiotem badań.

Kompleksowa ocena wpływu tetracykliny na rośliny jest kluczowa z perspektywy jakości roślin, fitoremediacji zanieczyszczeń oraz bezpieczeństwa żywnościowego, co pośrednio wpływa na zdrowie ludzi i jakość ich życia. Analiza wpływu tetracykliny na morfologię i regenerację tkanek jest istotna dla określenia możliwości wykorzystania roślin do oczyszczania środowiska z antybiotyków oraz zapobiegania wprowadzaniu zanieczyszczeń do łańcucha pokarmowego ludzi.

Biorąc pod uwagę powyższe, ujęte w niniejszej pracy doktorskiej badania przeprowadzono **celem:**

1. Dostarczenia nowych danych dotyczących wpływu tetracykliny na aparat fotosyntetyczny, ze szczególnym uwzględnieniem biosyntezy chlorofilu i aktywności RuBisCO.
2. Poszukiwania molekularnych i morfologicznych biomarkerów uszkodzeń i regeneracji roślin traktowanych tetracykliną.
3. Optymalizacji metody izolacji DNA bakterii z filosfery roślin spożywanych na surowo.
4. Zbadania składu mikroorganizmów zasiedlających glebę, ryzosferę i filosferę roślin uprawianych w środowisku zanieczyszczonym.

2. Hipotezy i zadania badawcze

Tetracyklina jest jednym z najczęściej wykorzystywanych i wykrywanych w środowisku antybiotykiem. Stanowi zagrożenie dla organizmów, wobec których nie jest kierowana. Dostępne dane literaturowe wskazują na wciąż ograniczoną wiedzę dotyczącą interakcji pomiędzy roślinami a tetracykliną, szczególnie na poziomie molekularnym. W związku z powyższym, sformułowano następujące **hipotezy badawcze:**

1. Tetracyklina negatywnie wpływa na aparat fotosyntetyczny roślin, zmniejszając aktywność RuBisCO oraz zaburzając biosyntezę chlorofilu.
2. Parametry morfologiczne, zawartość białek szoku cieplnego (HSP70), aktywność enzymów antyoksydacyjnych, uszkodzenia błon komórkowych oraz mitochondriów są biomarkerami zarówno uszkodzenia jak i regeneracji roślin traktowanych tetracykliną.
3. Rośliny wykazują zdolność do regeneracji po traktowaniu tetracykliną.
4. Optymalizacja metody izolacji DNA bakterii z filosfery roślin zwiększa ilość i poprawia jakość uzyskiwanego materiału genetycznego.
5. Środowiskowe stężenia tetracykliny znacząco zmieniają mikrobiom gleby i ryzosfery sałaty uprawianej w zanieczyszczonej glebie.

W celu weryfikacji postawionych hipotez, zaplanowano następujące **zadania badawcze**:

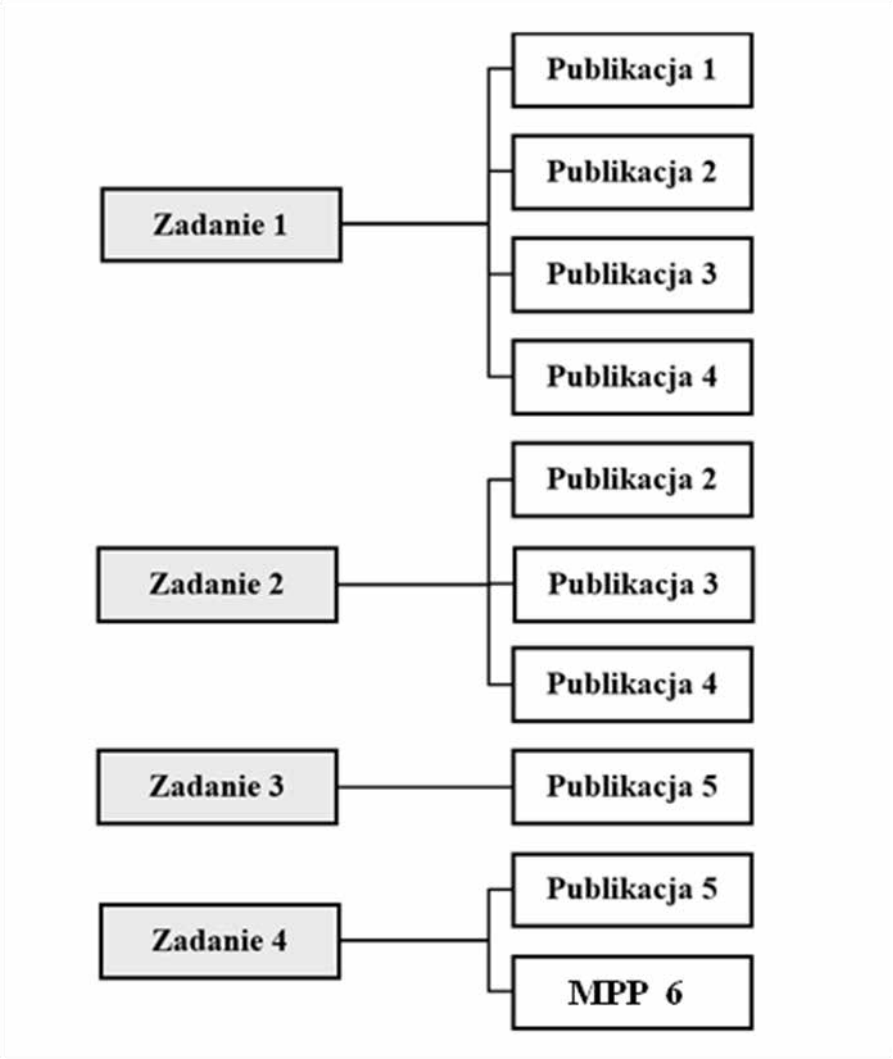
Zadanie 1 – Określenie wpływu tetracykliny na aparat fotosyntetyczny roślin.

Zadanie 2 – Poszukiwanie morfologicznych, fizjologicznych i biochemicznych markerów uszkodzeń i regeneracji roślin traktowanych tetracykliną.

Zadanie 3 – Optymalizacja metody izolacji DNA bakterii z filosfery roślin.

Zadanie 4 – Analiza składu mikroorganizmów ryzosfery i filosfery roślin uprawianych w zanieczyszczonym środowisku.

Schemat poszczególnych zadań badawczych oraz powiązanych z nimi publikacji przedstawiono na rysunku 1.

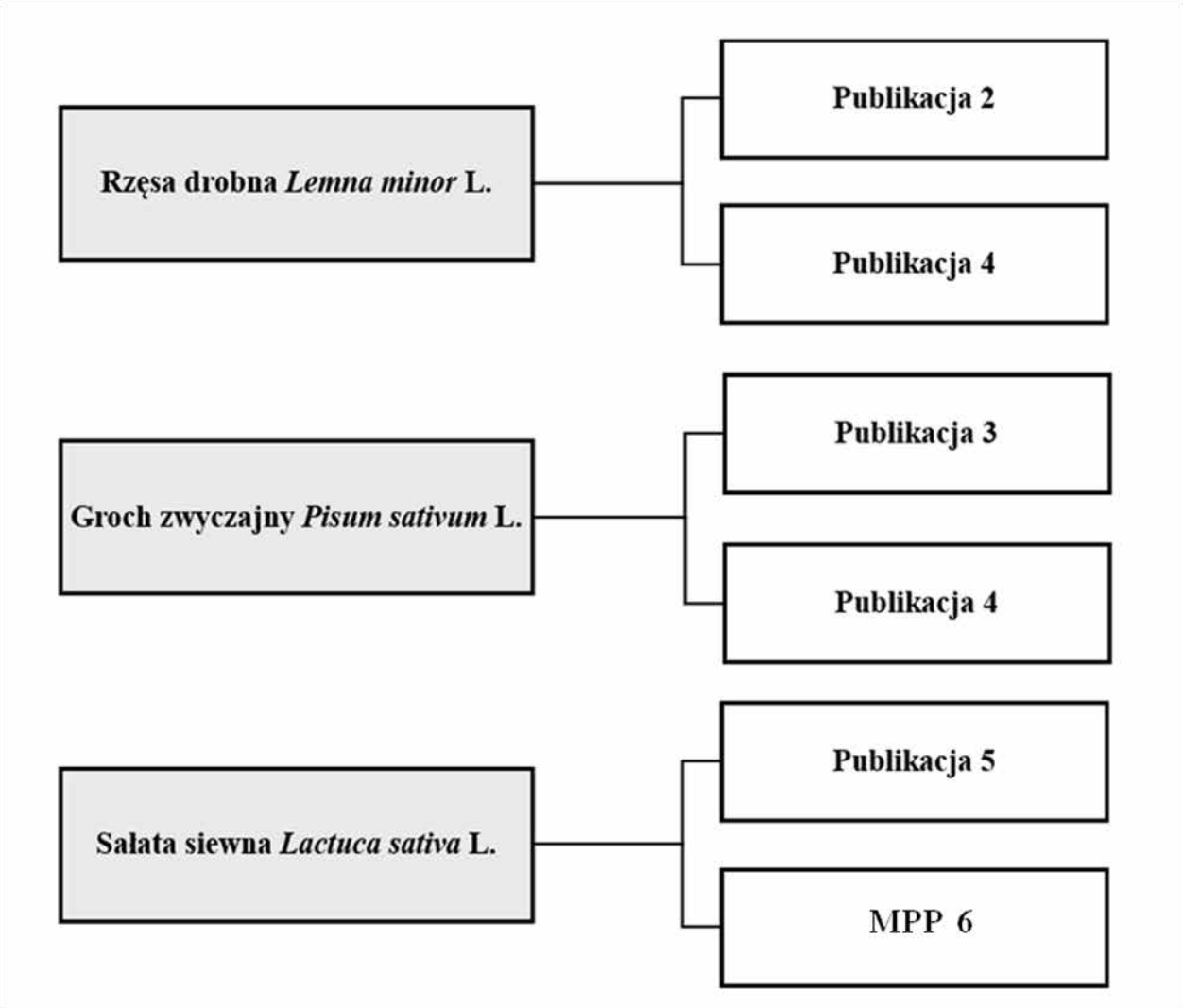


Rysunek 1. Schemat zadań badawczych zrealizowanych w ramach rozprawy doktorskiej oraz powiązanych z nimi publikacji.

3. Materiały i metody

3.1. Materiał badawczy

Materiał badawczy w niniejszej rozprawie doktorskiej stanowiły 3 gatunki roślin: rzęsa drobna (*Lemna minor* L.), groch zwyczajny (*Pisum sativum* L.) odmiana Cysterski oraz sałata siewna (*Lactuca sativa* L.) odmiana Takoda. Wykaz gatunków roślin wykorzystanych jako materiał badawczy oraz powiązanych z nimi publikacji przedstawiono na rysunku 2.



Rysunek 2. Wykaz gatunków roślin wykorzystanych jako materiał badawczy wraz z powiązanymi z nimi publikacjami.

3.1.1. Warunki hodowli rzęsy drobnej *Lemna minor* L.

W badaniach wykorzystano akseniczne kultury rzęsy drobnej prowadzone w Katedrze Fizjologii, Genetyki i Biotechnologii Roślin Uniwersytetu Warmińsko – Mazurskiego w Olsztynie. W **publikacji 2** rzęsę hodowano przez 7 dni w płynnej 50% pożywce Murashige i Skoog (MS), w kontrolowanych warunkach temperatury i oświetlenia. Próbę kontrolną stanowiła rzęsa hodowana przez 7 dni na pożywce MS, bez dodatku tetracykliny. Próby badawcze stanowiła rzęsa drobna hodowana na pożywce MS z dodatkiem tetracykliny o stężeniach: 1, 2,5 i 10 mM (faza ekspozycji). Po 7 dniach hodowli rośliny przeniesiono do świeżej pożywki MS bez dodatku tetracykliny (faza regeneracji). Parametry morfologiczne, fizjologicznie i biochemiczne roślin analizowano po 7 dniach hodowli, a następnie ponownie po 7 dniach wzrostu na świeżej pożywce, bez dodatku antybiotyku. W **publikacji 4** rzęsę drobną hodowano na ekstraktach z gleb pobranych z okolic intensywnych ferm drobiu. Ekstrakty przygotowano z: a) gleby pobranej z okolic pryzmy obornika, b) pobranej z pola uprawnego regularnie nawożonego obornikiem kurzym. Pola zlokalizowane były w województwie Warmińsko – Mazurskim. Szczegółową charakterystykę gleb oraz sposób pobierania i przygotowania prób przedstawiono w **publikacji 4**.

3.1.2. Warunki uprawy grochu zwyczajnego *Pisum sativum* L.

W **publikacji 3** do doniczek dodano glebę ogrodniczą oraz roztwór tetracykliny o stężeniach 0, 5, 50 i 500 mg/kg. Próbę kontrolną stanowiły rośliny podlewane wodą destylowaną. Nasiona grochu umieszczono w każdej doniczce bezpośrednio po dodaniu antybiotyku. Rośliny uprawiano w szklarni w kontrolowanych warunkach temperatury i oświetlenia. Po trzech tygodniach uprawy rośliny przeznaczono do dalszych analiz. W **publikacji 4** do doniczek dodano: a) glebę pobraną z pola regularnie nawożonego obornikiem, b) glebę pobraną z pola w pobliżu pryzmy obornika, oraz c) perlit ogrodniczy. Nasiona grochu umieszczono w każdej doniczce i uprawiano w kontrolowanych warunkach szklarniowych. Po 3 tygodniach uprawy rośliny przeznaczono do dalszych analiz.

3.1.3. Warunki uprawy sałaty siewnej *Lactuca sativa* L.

W **publikacji 5** nasiona sałaty wysiano do doniczek zawierających glebę pobraną z pola uprawnego nawożonego obornikiem kurzym. Rośliny uprawiano następnie w komorach klimatycznych w kontrolowanych warunkach temperatury i oświetlenia. Po 8 tygodniach liście oddzielono sterylnie nad powierzchnią gleby, wymieszano (aby

uzyskać jednorodną próbkę) i poddano analizie. W **MPP 6** do doniczek zawierających sadzonki sałaty dodano tetracyklinę o stężeniach 5 mg/kg i 25 mg/kg. Próbę kontrolną stanowiła sałata podlewana wodą destylowaną. Po 8 tygodniach uprawy w komorach klimatycznych rośliny wyjęto z doniczek, a liście i korzenie oddzielono sterylnie. Do analiz pobrano również glebę z doniczek oraz glebę ściśle przylegającą do korzenia (ryzosfera).

3.2. Metody

Wykaz metod stosowanych w niniejszej rozprawie doktorskiej przedstawiono w Tabeli 1.

Tabela 1. Wykaz metod stosowanych w badaniach oraz powiązanych z nimi publikacji

Oznaczenie	Metoda	Publikacja
Parametry morfologiczne roślin	Protokół OECD, metody mikroskopowe, metody biometryczne, program ImageJ	Publikacja 2, 3 i 4
Izolacja i pomiar chlorofilu	Metoda spektrofotometryczna	Publikacja 2, 3 i 4
Identyfikacja wolnych rodników	Mikroskopia konfokalna	Publikacja 2
Aktywność enzymów antyoksydacyjnych	Metody spektrofotometryczna	Publikacja 2
Zawartość malondialdehydu – peroksydacja lipidów w błonach komórkowych	Metoda spektrofotometryczna z kwasem trichlorooctowym i kwasem tiobarbiturowym	Publikacja 2 i 4
Izolacja błon komórkowych	Metoda ekstrakcji dwufazowej	Publikacja 2
Zawartość azotanów i azotynów w błonach komórkowych	Metoda spektrofotometryczna	Publikacja 2
Izolacja mitochondriów	Metoda wirowania	Publikacja 2 i 4

	różnicowego	
Aktywność dehydrogenazy mitochondrialnej	Metoda spektrofotometryczna z odczynnikiem WST−1	Publikacja 2 i 4
Zawartość białek HSP70	Test ELISA	Publikacja 2 i 4
Aktywność enzymu dehydratazy kwasu aminoleweulinowego	Metoda spektrofotometryczna	Publikacja 3 i 4
Zawartość kwasu aminolewelinowego	Metoda spektrofotometryczna	Publikacja 3 i 4
Zawartość podjednostki karboksylazy/oksygenazy rybulozo-1,5-bisfosforanu	Elektroforeza SDS−PAGE	Publikacja 3
Aktywność karboksylazy/oksygenazy rybulozo-1,5-bisfosforanu	Metoda spektrofotometryczna	Publikacja 3
Optymalizacja protokołu izolacji DNA bakterii filosfery	Przegląd literatury	Publikacja 5
Izolacja DNA bakterii z gleby, ryzosfery i filosfery	Metody izolacji z użyciem lizy mechanicznej oraz lizy enzymatycznej	Publikacja 5 i MPP 6
Określenie ilości i czystości DNA	Metoda spektrofotometryczna (NanoDrop)	Publikacja 5 i MPP 6
Sekwencjonowanie genu 16S rRNA	Sekwencjonowanie Nowej Generacji (NGS)	Publikacja 5 i MPP 6
Analiza taksonomiczna mikrobiomu gleby, ryzosfery i filosfery	Oprogramowanie QIIME 2	Publikacja 5 i MPP 6
Wizualizacja wyników	Program GraphPad Prism 8.0.1, Oprogramowanie	Publikacja 2, 3, 4, 5 i MPP 6

	ImageLab (Bio–Rad)	
Analizy statystyczne	Wybrane testy statystyczne programu Statistica 11 i GraphPad Prism 8.0.1	Publikacja 2, 3, 4, 5 i MPP 6

3.2.1. Parametry morfologiczne roślin

Parametry morfologiczne roślin oceniano przy wykorzystaniu metod biometrycznych, mikroskopu Keyence VHX–7000 z dedykowanym oprogramowaniem oraz programu ImageJ (wersja 1,54 g). Parametry morfologiczne rzęsy drobnej badano zgodnie z protokołem OECD dla *Lemna* sp. (OECD, 2006). Szczegółową metodykę przedstawiono w **publikacjach 2, 3** i **4**.

3.2.2. Izolacja i pomiar chlorofilu

Chlorofil z roślin izolowano zgodnie z metodyką Rydzyński i in. (2017). Rośliny homogenizowano w alkoholu etylowym lub metylowym (patrz publikacja 2, 3 i 4), a po odwirowaniu supernatant rozcieńczono i wyznaczono maksimum absorbcji za pomocą spektrofotometru UV/Vis. Stężenie chlorofilu obliczono na podstawie prawa Lamberta–Beera, stosując molowe współczynniki ekstynkcji zgodnie z Seely i Jensen (1965). Szczegółową metodykę przedstawiono w **publikacjach 2, 3** i **4**.

3.2.3. Identyfikacja wolnych rodników

Korzenie rzęsy analizowano pod kątem obecności reaktywnych form tlenu (ROS) przy użyciu dioctanu 2,7-dichlorodihydrofluoresceiny (H_2DCFDA). Rośliny inkubowano w roztworze H_2DCFDA w buforze PBS w ciemności przez 30 minut, po czym bufor zastąpiono świeżym PBS. Po barwieniu próbki analizowano za pomocą konfokalnego mikroskopu skaningowego (Leica TCS SP5). Szczegółową metodykę przedstawiono w **publikacji 2**.

3.2.4. Aktywność enzymów antyoksydacyjnych

Rośliny homogenizowano na lodzie w buforze fosforanowym z dodatkiem poliwinylopolipirolidonu (PVPP), kwasu etylenodiaminotetraoctowego (EDTA) i

fluorku fenylometylosulfonylu (PMSF). Próbki odwirowano, a następnie zebrano supernatant. Aktywność enzymów oznaczano spektrofotometrycznie. Aktywność katalazy (CAT) oznaczono w mieszaninie reakcyjnej buforu fosforanowego z nadtlenkiem wodoru (H_2O_2) zgodnie z Aebi (1984). Mierzono absorbancję przez 10 minut przy 240 nm. Aktywność peroksydazy askorbinianowej (APX) oznaczono w mieszaninie reakcyjnej z buforem fosforanowym, askorbinianem i H_2O_2, mierząc absorbancję przy 290 nm przez 10 minut zgodnie z Murshed i in. (2008). Aktywność peroksydazy pirogalolowej (PDX) oznaczano w mieszaninie reakcyjnej z buforem fosforanowym, pyrogallolem i H_2O_2, mierząc absorbancję przy 420 nm zgodnie z Chance i Maehly (1955) oraz Radić i in. (2006). Szczegółową metodykę przedstawiono w **publikacji 2**.

3.2.5. Oznaczanie zawartości malondialdehydu (MDA) w błonach komórkowych

Zawartość malondialdehydu (MDA) badano zgodnie z Hodges i in. (1999) z modyfikacjami. Rośliny homogenizowano na lodzie w 0,1% kwasie trichlorooctowym (TCA) i odwirowano. Do supernatantu dodano 50% etanol, inkubowano przez 30 min i odwirowano. Procedurę powtórzono dwukrotnie. Do supernatantu dodano następnie mieszaninę 20% TCA oraz 0,5% kwasu tiobarbiturowego (TBA). Ekstrakty inkubowano w 95 °C przez 30 min a następnie szybko schłodzono i odwirowano. Supernatant pobrano i zmierzono absorbancję przy 525 nm oraz 600 nm zgodnie z Hodges i in. (1999). Szczegółową metodykę przedstawiono w **publikacjach 2** i **4**.

3.2.6. Izolacja błon komórkowych i oznaczanie zawartość azotanów i azotynów

Błony komórkowe izolowano z komórek roślinnych wykorzystując metodę ekstrakcji dwufazowej. Materiał roślinny homogenizowano w buforze Tris-HCl z dodatkiem EDTA. Homogenat zawieszono w buforze Tris-HCl z dodatkiem $MgCl_2$. Przygotowano dwufazowy roztwór, mieszając dekstran z buforem Tris-HCl i glikol polietylenowy przygotowany w tym samym buforze. Próbki zawieszono w roztworze dwufazowym i inkubowano przez 24 godziny w 4 °C. Błony zebrano z granicy faz. Oznaczanie zawartości azotanów i azotynów przeprowadzono przy wykorzystaniu kitu Cayman Chemicals, wykorzystując odczynnik Griessa. Absorbancję próbek mierzono przy 540 nm, a do określenia stężenia azotanów i azotynów użyto wyznaczonej krzywej wzorcowej. Szczegółową metodykę przedstawiono w **publikacji 2**.

3.2.7. Izolacja mitochondriów i oznaczanie aktywność dehydrogenazy mitochondrialnej

Mitochondria z komórek roślinnych izolowano zgodnie z metodą Heckmana i in. (2002). Tkankę roślinną homogenizowano roztworem zawierającym mannitol, MOPS, EDTA, PVPP i L-cysteinę o pH 7,6. Homogenat przefiltrowano, a następnie odwirowano przy prędkości 4732 × g przez 5 minut w 4 °C. Supernatant ponownie pobrano i odwirowano przy prędkości 18,207 × g przez 5 minut w 4°C. Supernatant odrzucono, a osad przemywano roztworem o pH 7,2 z dodatkiem mannitolu, MOPS i EDTA. Do osadu dodano następnie roztwór 0,6 M sacharozy i odwirowano. Osad rozpuszczono w roztworze sacharozy z dodatkiem MOPS. Aktywność dehydrogenazy mitochondrialnej badano przy użyciu odczynnika WST−1 (Cayman Chemicals). Próbki inkubowano z odczynnikiem przez 2 godziny w temperaturze 37 °C, po czym mierzono absorbancję zgodnie z protokołem producenta. Szczegółową metodykę przedstawiono w **publikacjach 2** i **4**.

3.2.8. Izolacja białek i zawartość białek HSP70

Białka izolowano metodą Isaacsona i in. (2006). Tkanki homogenizowano w ciekłym azocie w mieszaninie 10% TCA rozpuszczonego w acetonie, a następnie inkubowano przez 24 godziny w – 20 °C. Po wirowaniu otrzymany osad przemywano kilkukrotnie acetonem i ponownie wirowano. Osad osuszono i rozpuszczono w buforze TBS zawierającym Tris oraz NaCl. Zawartość białek HSP70 oznaczono przy użyciu zestawu ELISA (EIAab Science, Wuhan) zgodnie z protokołem producenta, mierząc absorbancję przy 450 nm. Stężenie HSP 70 obliczono na podstawie krzywej wzorcowej. Szczegółową metodykę przedstawiono w **publikacjach 2** i **4.**

3.2.9. Aktywność dehydratazy kwasu aminolewulinowego (ALAD)

Dehydratazę kwasu aminolewulinowego (ALAD) izolowano metodą Jiao i in. (2015). Rośliny homogenizowano w ciekłym azocie w buforze Tris-HCl z dodatkiem ditiotreitolu (DTT). Homogenat odwirowano i pobrano supernatant. Do supernatantu dodano kwas aminolewulinowy (ALA), Tris-HCl, DTT i $MgCl_2$ następnie inkubowano w 37 °C przez 1 godzinę. Reakcję zatrzymano przez dodanie TCA, po czym próbki schładzano i ponownie wirowano. Do supernatantu dodano odczynnik Ehrlicha i zmierzono absorbancję przy λ = 555 nm. Szczegółową metodykę przedstawiono w **publikacjach 3** i **4**.

3.2.10. Zawartość kwasu aminolewulinowego (ALA)

Zawartość kwasu aminolewulinowego **(**ALA) oznaczano metodą Jiao i in. (2015). Rośliny homogenizowano w ciekłym azocie z buforem octanowym o pH 4,6. Homogenat odwirowano i pobrano supernatant. Do supernatantu dodano octan etylu i gotowano przez 15 minut. Po schłodzeniu dodano odczynnik Ehrlicha. Po 15 minutach inkubacji mierzono absorbancję przy λ = 533 nm. Szczegółową metodykę przedstawiono w **publikacjach 3** i **4**.

3.2.11. Izolacja białek z liści i elektroforeza SDS – PAGE

Białka izolowano zgodnie z metodą Li i in. (2009). Liście homogenizowano w ciekłym azocie, a następnie dodano mieszaninę izolacyjną zawierającą β-merkaptoetanol, Tris-HCl i glicerol. Próbki odwirowano i pobrano supernatant. Supernatant wymieszano z roztworem rozpuszczającym, który zawierał dodecylosiarczan sodu (SDS), β-merkaptoetanol i glicerol, aby uzyskać końcowe stężenie białka wynoszące 2 mg/mL. Próbki inkubowano w 100 °C przez 5 minut, a następnie schłodzono. Elektoforezę SDS–PAGE przeprowadzono w systemie Mini PROTEAN TetraSystem (Bio–Rad) w dwóch etapach: 80 V przez 10 minut i 140 V przez 1 godzinę. Po elektoforezie żele barwiono w 0,25% roztworze Coomassie Brilliant Blue przez 12 godzin, a następnie odbarwiano. Zdjęcia żeli zdigitalizowano za pomocą aparatu Gel Doc EZ Imager (Bio–Rad). Analizę elektrofotogramów przeprowadzono w oprogramowaniu ImageLab (Bio–Rad), gdzie obliczono stężenie dużej podjednostki RuBisCO na podstawie intensywności pików odpowiadających zidentyfikowanym białkom. Identyfikacja dużej podjednostki RuBisCO została przeprowadzona przy użyciu komercyjnego RuBisCO (Sigma–Aldrich, USA), który przygotowano identycznie jak próby właściwe. Szczegółową metodykę przedstawiono w **publikacji 3**.

3.2.12. Aktywność enzymu karboksylazy/oksygenazy rybulozo-1,5-bisfosforanu (RuBisCO)

Karboksylazę/oksygenazę rybulozo-1,5-bisfosforanu **(**RuBisCO) izolowano z liści zgodnie z metodą Tavarini i in. (2016). Liście homogenizowano w ciekłym azocie z dodatkiem mieszaniny o składzie Tricine–KOH, EDTA, β-merkaptoetanol, PMSF oraz PVPP. Próbki wirowano i pobrano supernatant. Aktywność RuBisCO badano w

mieszaninie reakcyjnej zawierającej bicynę, $MgCl_2$, DTT, $NaHCO_3$, adenozynotrójfosforan (ATP), dinukleotyd nikotynoamidoadeninowy (NADH), rybulozo-1,5-bisfosforan (RuBP), kinazę 3-fosfoglicerynianowa (PGK) oraz dehydrogenazę 3-fosfoglicerynianową (3-PGADH). Do mieszaniny dodano wyizolowany enzym i rejestrowano widmo absorbcji w zakresie długości fal od 310 nm do 370 nm przez 40 minut. Temperaturę reakcji wynosiła 25 °C, a pomiar absorbancji wykonywano co 30 sekund, uzyskując łącznie 80 pomiarów. Aktywność RuBisCO obliczono zgodnie z metodą Sales i in. (2020). Szczegółową metodykę przedstawiono w **publikacji 3**.

3.2.13. Optymalizacja protokołu separacji komórek bakterii i izolacji DNA bakterii z liści

W celu wyboru optymalnej metody separacji komórek z liści przetestowano następujące parametry wstępnego przygotowania próbek: ilość materiału wyjściowego, etap sonikacji, metodę zbierania komórek bakteryjnych (wirowanie lub filtracja membranowa) oraz metodę przygotowania membrany (membrana pocięta na drobne fragmenty lub wykorzystana w całości). Warianty eksperymentalne dobrano na podstawie przeglądu literatury, wykorzystując słowa kluczowe „filosfera", „bakterie", „izolacja DNA" oraz „16S rRNA". Liście sałaty przygotowano zgodnie z metodą Zhou i in. (2019) wprowadzając odpowiednie modyfikacje. Przygotowanie próbek polegało na umieszczeniu 5 g (A) lub 50 g (B) liści w pojemniku i dodaniu 45 mL sterylnego roztworu buforu fosforanowego o pH 7,4. Próbki poddano sonikacji przez 7 minut (C) lub pozostawiono bez sonikacji (D). Następnie próby mieszano przez 1 godzinę przy 180 RPM w temperaturze 30 °C. Aby oddzielić fragmenty liści próby przefiltrowano przez gazę. Uzyskany roztwór poddano wirowaniu przy 7500 RPM przez 30 minut (E) lub filtracji membranowej przez sterylne membrany z polifluorku winylidenu (PVDF) o porowatości 0,22 μm (F). Eksperymenty przeprowadzono łącznie w 24 wariantach. Szczegółowy schemat eksperymentów przedstawiono w **publikacji 5**. Osady uzyskane podczas wirowania oraz membrany przechowywano w temperaturze –80 °C do dalszej analizy.

3.2.14. Izolacja DNA

W **publikacji 5** izolację DNA bakterii filosfery przeprowadzono z uzyskanego osadu lub membran PVDF, korzystając z komercyjnie dostępnych zestawów: DNeasy

Power Pro Soil Kit (Qiagen Inc., USA) który stosuje lizę mechaniczno–chemiczną oraz Genomic Mini AX Soil Spin (A&A Biotechnology, Gdańsk), który stosuje lizę enzymatyczną, zgodnie z protokołami producentów. W **MPP 6** DNA izolowano z gleby i ryzosfery przy użyciu zestawu DNeasy Power Pro Soil Kit (Qiagen) zgodnie z protokołem producenta. Ilość wyizolowanego DNA mierzono za pomocą spektrofotometru NanoDrop 1000 (NanoDrop Technologies, USA). Czystość wyizolowanego DNA sprawdzono na podstawie parametrów OD 260/280 i OD 260/230. Uzyskany materiał genetyczny zawieszono w buforze Tris-HCl o pH 7 i przechowywano w temperaturze –20 °C do dalszej analizy. Szczegółową metodykę przedstawiono w **publikacjach 5** i **MPP 6**.

3.2.15. Sekwencjonowanie genu 16S rRNA

Analiza metagenomowa prób gleby, ryzosfery i filosfery została przeprowadzona przez firmę zewnętrzną (Genomed S.A., Warszawa). Analiza populacji bakterii została przeprowadzona na bazie hiperzmiennego regionu V3–V4 genu 16S rRNA. Do amplifikacji wybranego regionu i przygotowania biblioteki zostały użyte primery 341F oraz 785R. Reakcję PCR przeprowadzono z uzyciem Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, USA), zgodnie z zaleceniami producenta. Sekwencjonowanie przeprowadzono na aparacie MiSeq, w technologii paired-end (PE), 2x300nt, z użyciem zestawu v3 Illumina. Analiza taksonomiczna została przeprowadzona pakietem oprogramowania QIIME 2 w oparciu o bazę sekwencji referencyjnych Silva 138. Wykorzystano również pakiet DADA2, który pozwolił na wyszczególnienie sekwencji pochodzenia biologicznego od nowo powstałych w procesie sekwencjonowania. Pakiet ten wykorzystano również do wyodrębnienia unikalnych sekwencji biologicznego pochodzenia tzw. sekwencji ASV (ang. *amplicon sequence variant*).

4. Wyniki

Zadanie 1 – Określenie wpływu tetracykliny na aparat fotosyntetyczny roślin

Szczegółowe wyniki badań oraz ich omówienie zostało przedstawione w publikacjach:

Publikacja 1. *Krupka i in. Effects of antibiotics on the photosynthetic apparatus of plants. J. Plant Interact. 2022 doi: 10.1080/17429145.2021.2014579.*

Publikacja 2. *Krupka i in. Physiological and biochemical parameters of common duckweed Lemna minor after the exposure to tetracycline and the recovery from this stress. Molecules 2021 doi: 10.3390/molecules26226765.*

Publikacja 3. *Krupka i in. Critical stages in pea photosynthesis impaired by tetracycline as an environmental contaminant. J Plant Res. 2024 doi: 10.1007/s10265-024-01580-x.*

Publikacja 4. *Krupka i in. The impact of soil and water pollutants released from poultry farming on the growth and development of two plant species. Agriculture 2024 doi: 10.3390/agriculture14010087.*

W ramach przeglądu literatury szczegółowo przeanalizowano dostępne badania dotyczące wpływu tetracykliny na aparat fotosyntetyczny roślin (**Publikacja 1**). Stwierdzono, że pomimo rosnącego zainteresowania tym tematem, brakuje kompleksowych badań, które obejmowałyby wszystkie kluczowe aspekty, takie jak wpływ tetracykliny na biosyntezę chlorofilu oraz na aktywność enzymów niezbędnych do procesu fotosyntezy. W szczególności, dotychczasowe badania nie uwzględniały szeroko interakcji tetracykliny z mechanizmami biosyntezy chlorofilu i z enzymami takimi jak karboksylaza/oksygenaza rybulozo-1,5-bisfosforanu (RuBisCO), co pozostawiało luki w wiedzy na temat pełnych konsekwencji działania tego antybiotyku na rośliny.

Odpowiadając na te braki, w pierwszym etapie badano zawartość chlorofilu w roślinach traktowanych tetracykliną. W celu lepszego zrozumienia jej wpływu, przeprowadzono eksperymenty z rzęsą drobną, rosnącą przez 7 dni na pożywce z dodatkiem tetracykliny o stężeniach: 1 mM, 2,5 mM oraz 10 mM (**Publikacja 2**). Wyniki tych badań były jednoznaczne – stwierdzono wyraźny spadek zawartości chlorofilu, wynoszący odpowiednio 41%, 52% i 64%.

Dalsze badania przeprowadzono na grochu uprawianym w glebie zanieczyszczonej tetracykliną (**Publikacja 3**). Wykazano, że nawet niewielkie stężenia tego antybiotyku, takie jak 5 mg/kg, obecne w środowisku, powodują spadek zawartości

chlorofilu o 17%. Zwiększenie stężenia tetracykliny w glebie do 50 mg/kg i 500 mg/kg wywołało dalsze obniżenie zawartości chlorofilu o odpowiednio 20% i 28%. To jasno wskazuje, że nawet niskie stężenia tetracykliny mają istotny wpływ na zdolność roślin do fotosyntezy.

Jednym z kluczowych odkryć opisanych w **publikacji 3** był wpływ tetracykliny na szlak biosyntezy chlorofilu. U grochu traktowanego tetracykliną zaobserwowano znaczne obniżenie poziomu kwasu aminolewulinowego (ALA), będącego jednym z prekursorów chlorofilu. Co istotne, efekt ten był widoczny już przy niskich stężeniach antybiotyku (5 mg/kg). Jednocześnie wykazano zmniejszenie aktywności dehydratazy kwasu aminolewulinowego (ALAD), kluczowego enzymu zaangażowanego w biosyntezę chlorofilu, po zastosowaniu wyższych stężeń tetracykliny (50 mg/kg i 500 mg/kg). Odkrycia te dowodzą, że tetracyklina może zaburzać kluczowe etapy szlaku biosyntezy chlorofilu, co negatywnie wpływa na zdolności fotosyntetyczne roślin.

Aby sprawdzić, jak te zjawiska zachodzą w naturalnych warunkach środowiskowych, przeprowadzono dodatkowe badania na roślinach uprawianych w glebie pobranej z obszarów intensywnej hodowli drobiu, a także na ekstraktach wodnych z tych gleb (**Publikacja 4**). W wykazanych badaniach stwierdzono obecność tetracykliny w wodach gruntowych w okolicach ferm, gdzie głównym źródłem zanieczyszczeń były odcieki z gleb nawożonych obornikiem. Obserwowano zmniejszoną aktywność ALAD zarówno u roślin uprawianych w glebie regularnie nawożonej obornikiem kurzym, jak i u roślin rosnących w glebie pobranej z okolic pryzm obornika. Zmniejszoną zawartość ALA stwierdzono także w grochu uprawianym w tej glebie, a podobne wyniki uzyskano dla rzęsy drobnej hodowanej na ekstrakcie wodnym. Wyniki te jednoznacznie potwierdzają, że zarówno zawartość ALA, jak i aktywność ALAD są czułymi wskaźnikami toksycznego wpływu tetracykliny na aparat fotosyntetyczny roślin.

Przegląd literatury (**Publikacja 1**) wykazał brak wcześniejszych badań nad wpływem tetracykliny na enzym RuBisCO, który odgrywa kluczową rolę w procesie fotosyntezy. Ten temat podjęto po raz pierwszy w **publikacji 3**, gdzie przeprowadzono badania elektroforetyczne. Wykazano, że tetracyklina powoduje zmniejszenie zawartości dużej podjednostki RuBisCO w liściach grochu o 6,5%, 11% oraz 35%, w zależności od stężenia tetracykliny w glebie (odpowiednio 5 mg/kg, 50 mg/kg i 500 mg/kg). Jednak bardziej wrażliwym parametrem okazała się aktywność RuBisCO, która spadła o 19%, 37% i 47% w roślinach traktowanych tetracykliną.

Aby potwierdzić te wyniki oraz zweryfikować dokładność metod spektroskopowych, zbadano spadek absorbancji NADH w czasie 40 minut, w zakresie długości fal 310 – 370 nm, z maksimum absorpcji przy 340 nm. Badania wykazały, że u roślin traktowanych antybiotykiem spadek absorbancji był znacznie wolniejszy, co jednoznacznie wskazuje na inhibicję aktywności RuBisCO. Na podstawie tych wyników stwierdzono również, że standardowy czas pomiaru reakcji (10 minut) stosowany w większości prac może być zbyt krótki i prowadzi do błędnych wyników. Zaproponowano wydłużenie tego czasu do 20 minut. Dodatkowo, przeprowadzono walidację metody, określając optymalne warunki dla reakcji: temperaturę 25 °C oraz stężenie RuBisCO wynoszące 25 µg/ml roztworu.

Rozbudowane badania nad wpływem tetracykliny na procesy fotosyntezy, w tym na szlak biosyntezy chlorofilu i aktywność RuBisCO, pozwoliły na lepsze zrozumienie toksycznych efektów tego antybiotyku na rośliny oraz weryfikację przydatności stosowanych metod badawczych.

Zadanie 2 – Poszukiwanie morfologicznych, fizjologicznych i biochemicznych markerów uszkodzeń i regeneracji roślin traktowanych tetracykliną

Szczegółowe wyniki badań oraz ich omówienie zostało przedstawione w publikacjach:

Publikacja 2. *Krupka i in. Physiological and biochemical parameters of common duckweed Lemna minor after the exposure to tetracycline and the recovery from this stress. Molecules 2021 doi: 10.3390/molecules26226765.*

Publikacja 3. *Krupka i in. Critical stages in pea photosynthesis impaired by tetracycline as an environmental contaminant. J Plant Res. 2024 doi: 10.1007/s10265-024-01580-x.*

Publikacja 4. *Krupka i in. The impact of soil and water pollutants released from poultry farming on the growth and development of two plant species. Agriculture 2024 doi: 10.3390/agriculture14010087.*

Oprócz analizy uszkodzeń aparatu fotosyntetycznego, poszukiwano innych markerów stresu w roślinach poddanych działaniu tetracykliny, badając parametry morfologiczne, fizjologiczne i biochemiczne w roślinach wodnych i uprawnych. Wykazano, że na poziomie morfologicznym toksyczność tetracykliny u rzęsy drobnej objawia się zmniejszeniem liczby roślin, powierzchni zredukowanych pędów (*ang. fronds*) oraz świeżej masy (**Publikacja 2**). W przypadku grochu traktowanego tetracykliną w stężeniach 50 mg/kg i 500 mg/kg zaobserwowano skrócenie korzenia, łodygi oraz zmniejszenie powierzchni liści (**Publikacja 3**). Oznacza to, że zmiany

morfologiczne są pierwszymi widocznymi oznakami toksycznego wpływu tetracykliny i wskazują na potencjalne zmiany fizjologiczno–biochemiczne.

W roślinach rzęsy drobnej wykazano, że akumulacja wolnych rodników (ROS) w korzeniach wzrasta wraz ze stężeniem tetracykliny (**Publikacja 2**). Badania nad systemem antyoksydacyjnym wykazały również znaczący wzrost aktywności peroksydazy askorbinianowej (APX) oraz niewielki wzrost aktywności katalazy (CAT) w komórkach rzęsy traktowanej tetracykliną w stężeniach 2,5 mM i 10 mM. Wzrastała również zawartość białek HSP70 (o 131% i 123% dla stężeń odpowiednio 2,5 mM oraz 10 mM), co dowodzi, że aktywacja mechanizmów antyoksydacyjnych stanowi komórkowy marker stresu wywołanego antybiotykiem. Nadmiar wolnych rodników, w połączeniu z niewydolnością mechanizmów ich neutralizacji, prowadzi do uszkodzeń komórkowych.

Poszukując komórkowych markerów uszkodzeń wywołanych tetracykliną, zbadano uszkodzenia błon komórkowych i mitochondriów w komórkach rzęsy drobnej (**Publikacja 2**). Obserwowano wzrost zawartości malondialdehydu (MDA) po ekspozycji na tetracyklinę (2,5 mM i 10 mM), co wskazuje na peroksydację lipidów i uszkodzenie błon komórkowych. Po raz pierwszy zbadano zawartość azotanów i azotynów jako markerów uszkodzeń błon komórkowych, wykazując, że zawartość tych związków w błonach komórek rzęsy wzrasta o 80% przy stężeniu tetracykliny 10 mM w porównaniu do prób kontrolnych, co potwierdza skuteczność tej metody oceny uszkodzeń błon komórkowych. Zbadano również wpływ tetracykliny na mitochondria komórek roślinnych, stosując test WST–1, który mierzy aktywność dehydrogenazy mitochondrialnej. Wykazano, że tetracyklina w stężeniach 1 mM, 2,5 mM i 10 mM powoduje uszkodzenia mitochondriów w komórkach rzęsy drobnej, odpowiednio na poziomie 37%, 38% i 80%. Wyniki te wskazują, że mitochondria są wyjątkowo wrażliwe na działanie tetracykliny.

Wybrane markery zastosowano następnie do oceny parametrów fizjologiczno–biochemicznych roślin uprawianych na glebach z rejonów intensywnej hodowli drobiu oraz ich ekstraktach (**Publikacja 4**). U grochu uprawianego w glebie pobranej z okolic pryzmy obornika oraz u rzęsy hodowanej na ekstrakcie tej gleby odnotowano czterokrotny wzrost zawartości MDA w komórkach, wzrost stężenia białek HSP70 oraz niewielkie uszkodzenia mitochondriów, co było skorelowane z

zahamowaniem wzrostu roślin. Wyniki te potwierdzają skuteczność wybranych markerów w badaniach ekotoksykologicznych próbek środowiskowych.

Kolejna część badań obejmowała poszukiwanie markerów regeneracji roślin traktowanych tetracykliną. Po raz pierwszy zbadano zdolności regeneracyjne rzęsy drobnej po ekspozycji na antybiotyki, uwzględniając parametry morfologiczne, fizjologiczne i biochemiczne (**Publikacja 2**). Wysokie stężenia antybiotyków (1 mM, 2,5 mM oraz 10 mM) pozwoliły na określenie maksymalnych poziomów, przy których rośliny są w stanie się regenerować. Wykazano, że po 7 dniach hodowli na pożywce bez dodatku tetracykliny rzęsa odzyskuje wzrost, co objawia się zwiększeniem liczby roślin, powierzchni zredukowanych pędów oraz świeżej masy, nawet po ekspozycji na najwyższe stężenia tetracykliny. Wzrost aktywności enzymów antyoksydacyjnych (CAT, APX, PDX) oraz poziomu białek HSP70 w komórkach rzęsy wskazuje na aktywację systemu antyoksydacyjnego jako mechanizmu regeneracji. Zbadano również akumulację ROS w korzeniach rzęsy w fazie regeneracji, stwierdzając jej obniżenie, choć wartości te pozostają wyższe niż w próbie kontrolnej.

Analiza markerów uszkodzeń błon komórkowych i mitochondriów wykazała zmniejszenie zawartości MDA o 45%, 31% oraz 19% (w stosunku do fazy ekspozycji), co sugeruje regenerację struktur błon komórkowych. Mitochondria potwierdziły swoją wrażliwość na tetracyklinę – mimo wyeliminowania antybiotyku z pożywki, stwierdzono kontynuację uszkodzeń tych organelli.

Przeprowadzone badania pozwoliły wskazać markery zarówno uszkodzeń, jak i regeneracji roślin poddanych działaniu tetracykliny, co potwierdza ich przydatność w ocenie ekotoksyczności oraz regeneracyjnych zdolności roślin. Wyniki sugerują również potencjał zastosowania tych markerów do oceny zdolności roślin do fitoremediacji antybiotyków, przyczyniając się do rozwoju metod monitorowania ekotoksyczności środowiskowej.

Zadanie 3 – Optymalizacja metody izolacji DNA bakterii z filosfery roślin

Szczegółowe wyniki oraz ich omówienie przedstawiono w publikacji:

Publikacja 5. *Krupka M., Piotrowicz – Cieślak A.I. Optimization of the method for isolating bacterial DNA from the aboveground part of lettuce. IJMS 2024 doi: 10.3390/ijms25158513.*

W niniejszej pracy doktorskiej przeprowadzono badania nad wpływem tetracykliny na mikrobiom roślin, w tym innowacyjne badania nad optymalizacją metody izolacji DNA z filosfery, co jest nowatorskim podejściem w tej dziedzinie. W oparciu o analizę 50 protokołów izolacji DNA bakterii z liści, wytypowano i zoptymalizowano kluczowe parametry przygotowania prób: ilość materiału (liści sałaty), etap sonikacji, sposób zebrania komórek bakteryjnych (wirowanie vs. filtracja membranowa) oraz sposób przygotowania membrany. Oceniono również wpływ metod lizy komórkowej – enzymatycznej oraz mechaniczno–chemicznej – na jakość i ilość pozyskanego DNA. Do oceny DNA wykorzystano spektrofotometrię (parametry A260/280, A260/230 oraz pomiar przy $\lambda = 260$ nm).

Eksperyment przeprowadzono w 24 wariantach, szczegółowo opisanych w **publikacji 5**. Wyniki pokazały, że liza enzymatyczna nie sprawdziła się w izolacji DNA z liści sałaty (warianty 1–12), a uzyskane DNA było zanieczyszczone (A260/280 od 1,2 do 1,54; A260/230 od 0,57 do 1,11). W eksperymentach 8 i 9 uzyskano największe stężenie DNA (10,3 i 11,5 ng/µl), jednak nie spełniało ono wymogów jakościowych (A260/280 poniżej 1,8). Stwierdzono, że metody lizy enzymatycznej nie są optymalne do izolacji DNA mikrobiomu filosfery. Natomiast liza mechaniczno–chemiczna pozwoliła na uzyskanie DNA bakterii o odpowiednich parametrach jakościowych (A260/280 pomiędzy 1,6 a 1,9; A260/230 pomiędzy 0,91 a 1,87), przy stężeniach od 5,4 ng/µl do 38,8 ng/µl.

Badania wykazały, że sonikacja istotnie zwiększa stężenie wyizolowanego DNA, jednak nie jest jedynym kluczowym czynnikiem. Kolejny etap, polegający na zebraniu i koncentracji komórek bakteryjnych, potwierdził, że filtracja membranowa pozwala na uzyskanie większej ilości DNA w porównaniu do wirowania. Kluczowym elementem okazało się przygotowanie membrany – zastosowanie membrany pociętej na małe fragmenty znacząco zwiększyło ilość wyizolowanego DNA (38,08 ng/µl) w porównaniu do pełnej membrany (24,29 ng/µl). Najlepsze wyniki uzyskano stosując lizę mechaniczno–chemiczną, sonikację i filtrację membranową (eksperymenty 20 i 21) oraz zwiększając ilość materiału roślinnego do 50 g.

Aby potwierdzić jakość wyizolowanego DNA, przeprowadzono sekwencjonowanie genu 16S rRNA na próbkach DNA o najlepszych parametrach (eksperyment 21). Wyniki sekwencjonowania potwierdziły wysoką specyficzność i skuteczność izolacji: 99,8% sekwencji przypisano do królestwa bakterii, a tylko 0,2%

stanowiło chloroplastowe DNA *Lactuca sativa* L., co świadczy o niskiej współekstrakcji chloroplastowego DNA. Wyniki te jasno wskazują, że opracowany protokół izolacji DNA z liści sałaty pozwala uzyskać materiał genetyczny o wysokiej jakości i odpowiednim stężeniu, przydatny do badań molekularnych.
Niniejsze badania stanowią istotny pierwszy etap w pogłębianiu wiedzy na temat mikrobiomu roślin jadalnych i przyczyniają się do rozwoju tej dziedziny nauki.

Zadanie 4 – Analiza składu mikroorganizmów roślin uprawianych w zanieczyszczonym środowisku

Szczegółowe wyniki oraz ich omówienie przedstawiono w publikacjach:

Publikacja 5. *Krupka M., Piotrowicz – Cieślak A.I. Optimization of the method for isolating bacterial DNA from the aboveground part of lettuce. IJMS 2024 doi: 10.3390/ijms25158513.*
MPP 6. *The impact of tetracycline on the soil microbiome and the rhizosphere of lettuce (Lactuca sativa L.).*

Analizy metagenomowe objęły próbki gleby zarówno bez tetracykliny, jak i z dodatkiem antybiotyku w stężeniach 5 i 25 mg/kg. Przebadano także ryzosferę sałaty uprawianej na tych glebach, ponieważ sałata jest jednym z najczęściej spożywanych na surowo warzyw. Analiza taksonomiczna gleby przed aplikacją tetracykliny wykazała, że dominującym typem bakterii były Proteobacteria (43,17%). Zidentyfikowano wśród nich dwie klasy: Alphaproteobacteria i Gammaproteobacteria, stanowiące odpowiednio 30,32% i 12,85% wszystkich zidentyfikowanych klas. W grupie Alphaproteobacteria wykryto rodziny Micropepsaceae (7,46%), Rhizobiaceae (4,56%) – odpowiedzialne za asymilację i obieg azotu – oraz Devosiaceae (3,97%), zdolne do remediacji gleb zanieczyszczonych. Wśród Gammaproteobacteria zidentyfikowano rząd Xanthomonadales (3,39%), który obejmuje zarówno patogeny roślinne i ludzkie, jak i bakterie środowiskowe. Drugą pod względem liczebności grupą były Bacteroidota. W ich obrębie dominowała klasa Bacteroidia (17,91%) z rodzinami Flavobacteriaceae (6,68%), Chitinophagaceae (4,59%) i Sphingobacteriaceae (3,13%). Trzecim najliczniejszym typem bakterii były Actinobacteriota (9,35%), z obecnością klasy Actinobacteria (7,86%). Firmicutes stanowiły 3,06% wszystkich zidentyfikowanych typów, a inne typy – Verrucomicrobiota, Planctomycetota, Myxococcota i

Patescibacteria – stanowiły odpowiednio 7,34%, 4,03%, 2,98% i 2,72% wszystkich bakterii. Szczegółowy skład gleby przed i po aplikacji tetracykliny opisano w **MPP 6**.

Dodatek tetracykliny wpłynął istotnie na skład mikrobiomu gleby zarówno przy stężeniu 5 mg/kg, jak i 25 mg/kg. Dominującą grupą bakterii stały się Actinobacteriota (22%), których liczebność w glebie traktowanej tetracykliną znacznie wzrosła. W klasie Actinobacteria zidentyfikowano rzędy Micrococcales (6,66%) i Propionibacteriales (3,88%) oraz rodziny Micrococcaceae (2,57%) i Nocardoidiaceae (3,86%), zdolne do degradacji związków aromatycznych, co może tłumaczyć wzrost ich liczebności w obecności tetracykliny. Może być to również związane z występowaniem antybiotykooporności, co będzie przedmiotem dalszych badań. W glebie traktowanej tetracykliną zaobserwowano zmniejszenie liczebności bakterii Proteobacteria (o 21%) oraz wzrost liczebności Firmicutes (wzrost o 11,6% przy 5 mg/kg i o 14,7% przy 25 mg/kg). W glebie tej pojawiły się także bakterie Chloroflexi i Gemmatimonadota, nieobecne w próbach bez dodatku tetracykliny, oraz zmniejszyła się liczebność Bacteroidota (o 12%). Wyniki wskazują, że nawet niewielkie stężenia tetracykliny (5 mg/kg) powodują znaczące zmiany w mikrobiomie gleby, co może wpływać na jej funkcjonowanie. W glebie traktowanej tetracykliną zidentyfikowano również groźny patogen ludzki *Mycobacterium tuberculosis*, wywołujący gruźlicę, którego nie wykryto w próbach kontrolnych. Jego obecność może sugerować, że tetracyklina sprzyja przeżywalności tego patogenu w glebie. Odkrycie to wymaga dalszych badań, aby zrozumieć mechanizmy przetrwania *M. tuberculosis* w glebie oraz potencjalne zagrożenia dla zdrowia publicznego.

Efekt działania tetracykliny na mikrobiom ryzosfery sałaty był mniej wyraźny niż w glebie. Na podstawie danych ASV wykazano 38,7% wspólnych ASV między mikrobiomem gleby i ryzosfery sałaty uprawianej bez dodatku tetracykliny, podczas gdy w ryzosferze sałaty traktowanej tetracykliną o stężeniach 5 mg/kg i 25 mg/kg wspólnych ASV było odpowiednio 12,4% i 21%. Pokazuje to, że mikrobiom ryzosfery kształtowany jest przez inne czynniki, a nie tylko przez mikrobiom gleby. W ryzosferze sałaty wykazano obecność bakterii należących do 11 typów, z dominacją Proteobacteria, Bacteroidetes i Actinobacteria, także po aplikacji tetracykliny. W ramach typu Proteobacteria zidentyfikowano klasy Alphaproteobacteria i Gammaproteobacteria oraz rzędy Rhizobiales, Micropepsales, Caulobacterales, Burkholderiales i Xanthomonadales. Obfitość rzędu Burkholderiales rosła wraz ze wzrostem stężenia tetracykliny. Typ Bacteroidota w ryzosferze bez antybiotyku

reprezentowany był przez klasę Bacteroidia oraz rodzinę Flavobacteriaceae, które przeżyły w ryzosferze pomimo zmniejszenia ich liczebności w glebie zanieczyszczonej. Największe zmiany dotyczyły bakterii typu Firmicutes, których liczebność w ryzosferze rosła wraz ze wzrostem stężenia tetracykliny. Wzrost liczebności Firmicutes po ekspozycji na tetracyklinę w glebie i ryzosferze wskazuje na potrzebę monitorowania tego taksonu w roślinach rosnących w zanieczyszczonym środowisku. Uzyskane wyniki nie wskazują jednak na obecność patogenów w ryzosferze sałaty, a szczegółowy skład mikrobiomu ryzosfery przed i po aplikacji antybiotyku przedstawiono w **MPP 6**. Dane wykazują, że tetracyklina (5 mg/kg i 25 mg/kg) znacząco zmienia mikrobiom gleby na różnych poziomach taksonomicznych, a efekty te są widoczne już przy niższych stężeniach. Dodatek tetracykliny powoduje również niewielkie zmiany w mikrobiomie ryzosfery sałaty, jednak obecność patogenów w glebie traktowanej tetracykliną budzi obawy i wymaga dalszych badań.

W **publikacji 5** przeanalizowano mikrobiom liści sałaty uprawianej w glebie pobranej z okolic intensywnych hodowli drobiu. Stwierdzono, że dominują na nich bakterie z typów: Proteobacteria, Bacteroidetes, Actinobacteria i Verrucomicrobiota, a mniej licznie reprezentowane są bakterie Acidobacteriota, Firmicutes i Myxococcota. Skład mikrobiomu na innych poziomach taksonomicznych oraz obecność genów oporności na antybiotyki w filosferze będą przedmiotem dalszych badań, ze względu na istotność dla fizjologii roślin oraz zdrowia konsumentów.

5. Podsumowanie i wnioski wynikające z uzyskanych wyników badań

1. Tetracyklina wywiera toksyczny wpływ na aparat fotosyntetyczny roślin, szczególnie zaburzając szlak biosyntezy chlorofilu oraz aktywność kluczowych enzymów, takich jak karboksylaza/oksygenaza rybulozo-1,5-bisfosforanu (RuBisCO) i dehydrataza kwasu aminolewulinowego (ALAD). Obniżenie zawartości chlorofilu i jego prekursora (kwas aminolewulinowy; ALA) a także zahamowanie aktywności ALAD i RuBisCO, prowadzi do zmniejszenia wydajności procesu fotosyntezy, nawet przy niskich stężeniach tetracykliny. Wyniki te wskazują na możliwość trwałego osłabienia zdolności fotosyntetycznych roślin narażonych na zanieczyszczenie tetracykliną w środowisku.

2. Aktywność ALAD i RuBisCO, oraz zawartość chlorofilu i kwasu aminolewulinowego, mogą stanowić czułe markery ekotoksykologiczne umożliwiające ocenę wpływu tetracykliny na rośliny.

3. Dostosowanie warunków badawczych, takich jak wydłużenie czasu pomiaru aktywności RuBisCO, zwiększa precyzję pomiarów i poprawia wiarygodność wyników. Wyniki te przyczyniają się do rozwoju bardziej efektywnych metod monitorowania wpływu antybiotyków w środowisku na rośliny, co ma istotne znaczenie w kontekście oceny zanieczyszczeń i ich wpływu na ekosystemy.

5. Tetracyklina wywołuje istotne zmiany na poziomie morfologicznym, fizjologicznym i biochemicznym u roślin, obejmujące akumulację wolnych rodników, peroksydację lipidów błon komórkowych oraz uszkodzenia mitochondriów. Wybrane parametry biochemiczne, takie jak wzrost zawartości malondialdehydu i białek stresu HSP70, oraz aktywność enzymów antyoksydacyjnych (np. peroksydazy askorbinianowej) okazały się czułymi markerami toksycznego wpływu tetracykliny. Wyniki te podkreślają przydatność tych wskaźników w badaniach ekotoksykologicznych i ocenie negatywnego wpływu antybiotyków na środowisko.

4. Rzęsa drobna wykazuje znaczne zdolności regeneracyjne po usunięciu tetracykliny z pożywki, wyrażające się odbudową parametrów morfologicznych i zmniejszeniem poziomu wolnych rodników oraz malondialdehydu. Aktywacja systemu antyoksydacyjnego stanowi kluczowy mechanizm regeneracji, choć mitochondria

pozostają strukturą szczególnie wrażliwą na trwałe uszkodzenia. Wskazanie markerów zarówno uszkodzeń, jak i regeneracji roślin w odpowiedzi na tetracyklinę dostarcza wartościowych narzędzi do oceny zdolności roślin do fitoremediacji antybiotyków i ich potencjalnego wykorzystania w procesach oczyszczania środowiska.

6. Opracowanie i optymalizacja protokołu izolacji DNA z filosfery roślin przy użyciu lizy mechaniczno-chemicznej, sonikacji i filtracji membranowej stanowi skuteczną metodę uzyskiwania materiału genetycznego o wysokiej jakości i ilości. Badania dowodzą, że zastosowanie tych procedur pozwala uzyskać DNA o właściwych parametrach (A260/280 i A260/230), co umożliwia wiarygodne analizy molekularne mikrobiomu roślin, minimalizując jednocześnie współekstrakcję DNA chloroplastowego.

7. Sekwencjonowanie genu 16S rRNA potwierdziło wysoką specyficzność opracowanego protokołu izolacji DNA, który zapewnia wysoką wydajność ekstrakcji bakterii oraz niską obecność zanieczyszczeń pochodzących z roślinnego DNA. Wyniki te stanowią fundament do bardziej zaawansowanych badań nad mikrobiomem roślin jadalnych, otwierając nowe perspektywy w rozwoju nauki o interakcjach między roślinami a ich mikrobiotą, co może mieć zastosowanie w rolnictwie i ekologii.

8. Dodatek tetracykliny do gleby w stężeniu 5 i 25 mg/kg znacząco zmienia skład mikrobiomu glebowego, zwłaszcza poprzez wzrost obfitości Actinobacteriota i Firmicutes oraz zmniejszenie udziału Proteobacteria i Bacteroidota. Zmiany te świadczą o zdolności wybranych taksonów do przetrwania w obecności antybiotyku, co może być związane z mechanizmami antybiotykooporności. Wyniki te wskazują na potencjalne zagrożenie dla funkcji ekosystemów glebowych, gdzie antybiotyk może sprzyjać wzrostowi bakterii o właściwościach degradujących, jak również patogenów, takich jak *Mycobacterium tuberculosis*, które nie występowały w glebie wolnej od tetracykliny.

9. Wpływ tetracykliny na mikrobiom ryzosfery sałaty jest mniejszy w porównaniu do mikrobiomu gleby, co sugeruje dodatkowe czynniki kształtujące skład bakteryjny w ryzosferze. Zidentyfikowano jednak wzrost liczebności Firmicutes w ryzosferze w miarę wzrostu stężenia tetracykliny w glebie, co może wskazywać na ich znaczenie w adaptacji mikrobiomu roślinnego do środowisk zanieczyszczonych. Obserwacja ta sugeruje, że monitoring ryzosfery roślin uprawianych w glebach skażonych tetracykliną

może być istotny dla zapewnienia bezpieczeństwa żywnościowego i zdrowia publicznego.

10. Zintegrowanie wyników badań dotyczących wpływu tetracykliny na ekosystemy roślinne, mikrobiomy glebowe oraz ryzosferę roślin stanowi ważne odniesienie dla koncepcji *One Health*. Tetracyklina, przenikając do środowiska, nie tylko znacząco zakłóca procesy fotosyntetyczne roślin, ograniczając ich wydajność, ale także zmienia skład mikrobiomu gleby i ryzosfery poprzez selektywne wspieranie mikroorganizmów odpornych na antybiotyki oraz patogenów, takich jak *Mycobacterium tuberculosis*. Te zmiany w ekosystemach mają kluczowe implikacje nie tylko dla zdrowia roślin, ale i dla zdrowia ludzi i zwierząt, z uwagi na ryzyko przenoszenia patogenów z gleb zanieczyszczonych do łańcucha pokarmowego. Badania te podkreślają konieczność monitorowania środowisk skażonych antybiotykami, rozwijania metod detekcji markerów ekotoksykologicznych i działania na rzecz zapobiegania skażeniom gleby, co przyczynia się do ochrony zdrowia w skali globalnej.

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SPIS TABEL I RYSUNKÓW

Rysunek 1. Schemat zadań badawczych zrealizowanych w ramach rozprawy doktorskiej oraz powiązanych z nimi publikacji.

Rysunek 2. Wykaz gatunków roślin wykorzystanych jako materiał badawczy wraz z powiązanymi z nimi publikacjami.

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Effects of antibiotics on the photosynthetic apparatus of plants

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ABSTRACT

Antibiotics as environmental pollutants can affect both the morphology and physiology of higher plants. The first visible symptoms of damage caused by antibiotics are chlorotic changes resulting from the degradation of chlorophyll and the impairment of the chlorophyll biosynthesis pathway, followed by damage to PS II and disturbances in the electron flow between the photosystems. Research on the effects of antibiotics, on plants has recently intensified, however, our understanding of this problem is still far from complete. This paper provides a review of current data on the impacts antibiotics on plant photosynthetic apparatus.

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1. Introduction

Modern animal production methods include extensive use of antibiotics. Moreover, the Covid-19 pandemic, although caused by viruses, has further increased the antibiotic use in medicine. Antibiotics are not completely metabolized in the human and animal body, in fact most of the dose taken up is excreted unchanged, thus polluting the environment (Kümmerer 2009). The antibiotics most frequently used in animal production and veterinary medicine include tetracyclines, penicillins, and sulphonamides, with their consumption amounting to 33%, 25%, and 11% of all antibiotics used, respectively (EMA 2016). In 2020, the countries with the highest antibiotic consumption rates were China (43024 tonnes), Brazil (7533 tonnes), and the United States (6596 tonnes) (Sriram et al. 2021).

Although the presence of antibiotic residues in soils and waters is an undisputed fact, there is still no legal requirement to monitor the concentration of antibiotics in the environment. The concentration and persistence of pharmaceuticals in water and soil depends on the physicochemical properties of the drug itself and the properties of the matrix (matrix type, pH) and temperature (Polianciuc et al. 2020). For example, the concentration range of tetracycline detected in soil fertilized with natural fertilizers was 10–15 $\mu g \times kg^{-1}$ soil (Minden et al. 2017), while Hamscher et al. (2002) indicate a concentration range of 86–172 $mg \times kg^{-1}$ soil, depending on the depth of sampling. References to antibiotic contents in plants and the aqueous environments (surface water, ground water, drinking water, waste water) are given in the Appendices (Tables 1 and 2). Most studies into the effects of antibiotics on living organisms are focused on animals. The effects of antibiotics on physiological and biochemical processes in plants have been documented but are still poorly understood. Most reports suggest that photosynthesis is one of those areas of plant metabolism that are most rapidly and most severely affected by antibiotic residues. Therefore, this study aims to review the literature concerning the effect of antibiotics on the photosynthetic apparatus of higher plants.

2. The effect of antibiotics on morphology and chlorophyll content in higher plants

Antibiotics in the environment can have both a positive and a negative effect on plants. A positive effect (hormesis) occurs at very low concentrations in the environment, whereas high concentrations of antibiotics result in toxic effects. The first visible symptom of antibiotic toxicity to plants is a change in the color of the photosynthetic organs. Changes in the leaves' color into light green or yellow under the influence of antibiotics were observed in pea (Margas et al. 2016), *Lemna gibba* (Brain et al. 2004), and *Lemna minor* (Sikorski et al. 2014; Baciak et al. 2016). The overall chlorophyll content is a significant parameter in the assessment of antibiotics impacts on the photosynthetic apparatus. It was demonstrated that plant exposure to tetracycline at a concentration of 12.84 µM (EC 50) results in 50% inhibition of chlorophyll a synthesis in *Lemna minor* L., while EC50 for chlorophyll b was observed at a concentration of 0.26 µM (Baciak et al. 2016). The inhibition of chlorophyll synthesis under the influence of tetracycline was also examined by Rydzyński et al. (2017). It was demonstrated that after 10 days of growth with the addition of tetracycline at a concentration of 90 $mg \times kg^{-1}$ of soil, chlorophyll a content in yellow lupin (*Lupinus luteus*) seedlings decreased by 80% in young, developing leaves. In older leaves, chlorophyll degradation was estimated at 68% in relation to the control. Similar results were obtained for spinach *Spinacia oleracea* (Rydzyński et al. 2019) and *Iberis sempervirens* (Di Marco et al. 2014). However, low concentrations of antibiotics may even support the chlorophyll biosynthesis process by affecting nucleic acids and chloroplast proteins, thus reducing the activity of chlorophyllase, and preventing the degradation of chlorophyll (Liu et al. 2013). The phenomenon of

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positive effects of a stress factor on organisms is called hormesis (Agathokleous et al. 2018). Hormesis in the plant-antibiotic system was first described by Migliore et al. (2000) in *Lythrum salicaria* L. treated with fluoroquinols. Hormetic effects of antibiotics were also demonstrated in *Raphanus sativus* L., *Cucumis sativus* L., *Lactuca sativa* L. (Migliore et al. 2003). Liu et al. (2011) showed an increase in chlorophyll content in *Phragmites australis* induced by low concentrations of antibiotics ($0.1–1\ \mu g \times L^{-1}$). Although the mechanism of antibiotic-induced hormesis is not yet understood at the molecular level, production of Reactive Oxygen Species (ROS) seems to be the essential element of this biphasic dose–response pathway. Small amounts of ROS accumulated under stress activate cellular signaling pathways leading to adaptive/beneficial responses (Duarte-Sierra et al. 2020).

Additionally, in antibiotic-treated plants, disturbances in the chlorophyll a to chlorophyll b ratio were observed, as well as the chlorotic and necrotic spots manifesting chlorophyll degradation. Under physiological conditions, the ratio of chlorophyll a to b contents in cryptogamic plants is approximately 3:1, with chlorophyll b accounting for 25–35% of the overall chlorophyll content. An decrease in chlorophyll b content in plants treated with antibiotics results in disturbances in electron flow between both photosystems (Liu et al. 2013). Changes in the chlorophyll a to chlorophyll b ratio were demonstrated in the brittle willow (*Salix fragilis*) treated with sulfadimethoxine (Michelini et al. 2012). An increase in the chlorophyll a/b ratio was also demonstrated in *Spirodela polyrhiza* under the influence of amoxicillin (Singh et al. 2018). Disturbances in the chlorophyll a/b ratio are a good biomarker that enables an assessment of the condition of the photosynthetic apparatus in plants exposed to stress factors (Li et al. 2011b).

The changes in chlorophylls content are accompanied by disturbances in chloroplast structure. The effect of bactericidal compounds on plant photosynthetic apparatus is very probable in view of the fact that chloroplasts are considered descendants of cyanobacteria (Cavalier-Smith 2000). In lower plants, systems responsible for chloroplast division have been identified that originate from the bacterial peptidoglycan synthesis pathway. This pathway is the target site for β-lactam antibiotics, which act by interfering with bacterial peptidoglycan polymerization. In *Physcomitrella patens*, after ampicillin treatment, disturbances in chloroplast division and consequent formation of so-called macrochloroplasts were observed. In *Euglena gracilis* cells treated with chloramphenicol, a disruption of chloroplast ultrastructure was observed, probably resulting from the inhibition of expression of proteins responsible for lamella structure formation.

Although genes of the peptidoglycan synthesis pathway have not been found in plastids of higher plants, murE and murG genes, involved in the peptidoglycan synthesis pathway in bacteria, have been identified in the *Arabidopsis thaliana* nuclear genome (Katayama et al. 2003).

3. The effect of antibiotics on chlorophyll synthesis

In order for the antibiotic to act on chloroplasts, it has to enter them. Aminoglycoside antibiotics get into the chloroplast via an iron transporter (MAR1, multiple antibiotic resistance 1), which is probably involved in cellular iron homeostasis and allows opportunistic entry of many antibiotics into the chloroplast (Conte et al. 2009). After penetrating the chloroplast, the antibiotics may disturb its functioning as early as at the chlorophyll biosynthesis stage. Chlorophyll biosynthesis consists of 16 reactions catalyzed by 16 different enzymes that are encoded by more than 20 genes (Beale 2005). The process of chlorophyll synthesis starts with the formation of glutamyl-tRNA and subsequent modifications of the glutamyl-derived moiety into six main precursors: δ-aminolevulinic acid, porphobilinogen, uroporphyrinogen III, protoporphyrinogen IX, Mg-protoporphyrin IX and protochlorophyllide. The end products of this process are both chlorophyll a and chlorophyll b (Brusslan and Peterson 2002). To date, the effects of antibiotics on the specific enzymes of chlorophyll biosynthesis have not been determined. A general schematic of chlorophyll biosynthesis is shown in Figure 1. The sites where antibiotic effects have been studied so far are shown. However, determining the effect of antibiotics on specific reactions is very difficult. It is known that the enzymes involved in chlorophyll biosynthesis are encoded by nuclear genes (Beale 2005). One could therefore expect that antibiotics should have no effect on chlorophyll biosynthesis. However, many protein complexes of the chloroplasts are co-coded by both chloroplast and nuclear genes (Larkin et al. 2003; von Gromoff et al. 2006; Koussevitzky et al. 2007). Koussevitzky et al. (2007) showed that the expression of a nuclear gene associated with chlorophyll synthesis can be inhibited by some feedback signaling pathways when a chloroplast gene expression is inhibited. It is likely that chlorophyll biosynthesis is first inhibited at the level of the chloroplast gene expression and then there is also an indirect effect on the nuclear genes expression.

δ-Aminolevulinic acid (ALA) is the first compound of the chlorophyll biosynthesis pathway and thus a chlorophyll precursor (Reinbothe et al. 2010). A decrease in ALA content was observed in algae *Selenastrum capricornutum* treated with erythromycin, ciprofloxacin, and sulfamethoxazole, however, the ciprofloxacin treatment resulted chiefly in

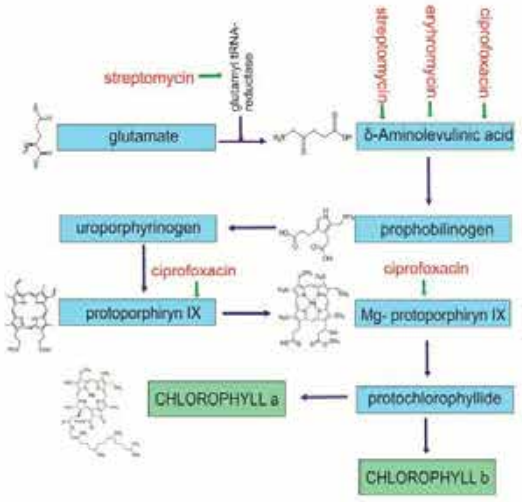


Figure 1. The chlorophyll biosynthesis stages and sites of antibiotics action: 1 – streptomycin, 2 – erythromycin and 3 – ciprofloxacin.

reduced contents of protoporphyrin IX and protochlorophyllide (Liu et al. 2011). Erythromycin inhibits the production of proteins by reducing the chloroplast gene expression and binding to the small subunit of the ribosome (Liu et al. 2011). A decreased ALA content was also demonstrated in soybean (*Glycine max*) treated with bisphenol at a concentration of 1.5 mg × L^{-1}. On the other hand, increasing the bisphenol concentration to 17.2 and 50 mg × L^{-1} stimulated the accumulation of δ-aminolevulinic acid (Jiao et al. 2015). A similar effect was demonstrated under the influence of thermal stress in the seeds of cucumber and common wheat (Tewari and Tripathy 1998).

In contrast, streptomycin (an aminoglycoside targeting the 30S subunit of the ribosome) caused an increase in ALA content as well as a change of chloroplast shape and the disappearance of their ribosomes (Yaronskaya et al. 2007). The accumulation of ALA in plant cells results in the generation of free radicals (Noriega et al. 2007).

Oxidative stress, and thus the accumulation of free radicals, is a common pathway of plant responses to environmental stress. The accumulation of free radicals under the influence of antibiotics was demonstrated in pea (Margas et al. 2019), yellow lupin (Orzoł and Piotrowicz-Cieślak 2017), and thale cress (*Arabidopsis thaliana*) (Gudiño et al. 2018; Xu et al. 2020), ginger (Lv et al. 2020) and many other plants.

4. The effect of antibiotics on the photosynthetic apparatus

The main stages of photosynthesis are light reactions in photosystems I and II (including in the later case the Hill reaction) and CO_2 assimilation in the Calvin cycle. Photosystem I (PSI) is a large membrane protein complex and consists of two essential parts: the core complex, also called the reaction center complex (RC), where most of the light capture and charge separation reactions take place, and the light-harvesting complex I (LHCI), which serves as an additional antenna system (Chitnis 2001). The plant supercomplex PSI-LHCI consists of 19 known protein subunits and approximately 200 non-covalently bound cofactors (Jensen et al. 2007). Photosystem II (PSII) is also a large supramolecular pigment–protein complex embedded in the thylakoid membrane (Barber 2003), but it mainly occurs in a dimeric form, and each monomer consists of at least 27–28 subunits organized into a core complex and antenna system (Dekker and Boekema 2005). The core contains, among others, proteins D1, D2, CP47, and CP43 that coordinate the molecule of chlorophyll a and form the internal antenna (Shi and Schröder 2004). PSII in addition to its bioenergetic and catalytic role in the breakdown of water and oxygen release (Hill reaction) supports the electron transport and is an essential control element in photosynthesis (Geiken et al. 1998). Ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), on the other hand, is a key control in the Calvin cycle (Udenigwe et al. 2017). Photosynthesis is one of the main sources of reactive oxygen species (Asada 2006), and the presence of antibiotics and their derivatives taken up by plants further enhances this effect (Di Marco et al. 2014). PSII and PSI control the mode of photosynthetic electron flow (Iwai et al. 2010). The effect of antibiotics on photosynthesis is not fully understood. Studies on the direct effect of antibiotics are shown in Figure 2. Most of the claims about the effect of antibiotics on photosynthesis are conjectural. What we do know, however, is that antibiotic action leads to H_2O_2 accumulation in chloroplasts, and this results in damage to the D1 protein in PSII, which is essential for coordinating electron flow between subsequent electron carriers. Hydrogen peroxide in plant cells may result in the inhibition of the D1 protein repair processes, thus leading to irreversible damage to PS II and the inhibition of Hill reaction by 3% and 73% depending on antibiotic concentration (Kummerová et al. 2006; Hájková et al. 2019). Inhibition of Hill reaction may be due to the inhibition of chloroplast gene expression and, consequently, the synthesis of proteins responsible for electron flow. Wall et al. (2004) demonstrated in *Arabidopsis thaliana* the presence of DNA gyrase, an enzyme that catalyzes the DNA helix twisting, characteristic of Procaryota. Antibiotics, by affecting the gyrase activity, may cause damage in chloroplasts inhibiting the DNA replication process (Evans-Roberts et al. 2016). Treating tobacco (*Nicotana tabacum*) with antibiotics targeting DNA gyrase (quinolone nalidixic acid and aminocoumarin novobiocin) resulted in the virtually immediate inhibition of plant growth (Heinhorst et al. 1985). Inhibition of the inclusion of thymidine in pea chloroplasts was observed (Mills et al. 1989). The consequences of nalidixic acid and novobiocin application included also some disturbances that were not directly related to the biosynthesis of plastid DNA. Lam and Chua (1987) demonstrated the inhibition of the transcription process in pea chloroplasts under the influence of novobiocin. Following the treatment of thylakoids with various antibiotics, a disturbance was demonstrated in the chloroplast translation process, caused by a decreased number of Lhcb transcripts (that encode the subunits of the light-harvesting LHC II complex). Moreover, phosphorylation of the proteins contained in the LHC II complex (CP43, D1/D2) was demonstrated in these plants following the application of streptomycin and cyclophosphamide (Mulo et al. 2003). The inhibition of the protein translation process in pea chloroplasts under the influence of tetracycline was demonstrated by Kasai et al. (2004). These results show that certain antibiotics may disrupt the transcription and translation processes in plants, thus affecting the proteins contained in the photosynthetic apparatus and leading to multiple damaging effects. Conte et al. (2009) suggest that aminoglycoside antibiotics inhibit chloroplast translation by attacking rRNA of the ribosomal small subunit. Similarly, in prokaryotic organisms aminoglycoside antibiotics also bind to rRNA (16S rRNA) in the small subunit of the ribosome (Recht et al. 1999). Kasai et al. (2004) indicates that because chloroplasts possess bacterial transcription and translation systems the inhibitory effect of tetracycline on plant (pea) chloroplast activity in protein synthesis can probably be attributed to a specific interaction of the antibiotic with the 70S ribosome.

The effect of antibiotics on chloroplast proteins was demonstrated by Margas et al. (2019). In pea seedlings, following the application of tetracycline, an increase was noted in the content of protective proteins involved in the photosynthesis process, inter alia the HCF136 factor involved in the process of stabilizing the PS II. A reduction in the transcription rate of photosynthesis-related nuclear genes, such as Lhcb (chlorophyll-binding protein) and RbcS (ribulose diphosphatocarboxylase small chains) was also demonstrated (Mulo et al. 2003). The chlorophyll-binding protein Lhcb builds the main antenna complex and is present as trimer built from homologous binding proteins

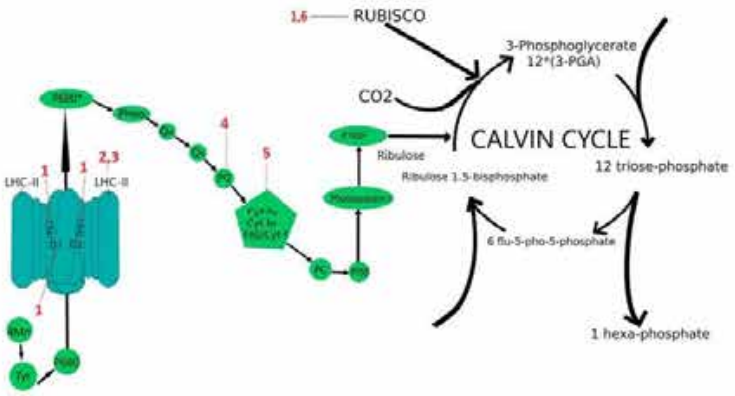


Figure 2. Sites of action of antibiotics the deteriorative effect of which in photosynthesis was shown. 1 – azithromycin; 2 – streptomycin; 3 – cyclophosphamide; 4 – fluoroquinolone; 5 – quinolones; 6 – tetracycline.

Lhcb1, Lhcb2 and Lhcb3. LHCII trimers occur in several homo- and heterotrimeric configurations, depending on their location and function. In addition to the trimeric LHCII with PSII (Wei et al. 2016), there are also smaller monomeric antenna complexes: Lhcb4 (CP29), Lhcb5 (CP26) and Lhcb6 (CP24) (Caffarri et al. 2009; Sacharz et al. 2017). However, a detailed elucidation of the antibiotic-induced changes within the antenna complex is still lacking. The emergence of thylakoid protective proteins was also noted. Yoon et al. (2020) demonstrated the degradation of most of the chloroplast structural proteins in *Brassica campestris* treated with erythromycin (5 mg × L^{-1}). Neither PS II proteins CP47 or CP43 nor PS II protein D1 were identified.

Most studies focus on the examination of damage to photosystem II under the influence of antibiotics. A disturbance in the FV/FM (variable fluorescence to maximum fluorescence) ratio is a good measure of the PS II damage. This coefficient reflects quickly and accurately the degree of damage to PS II. A decrease in this coefficient was noted in the common duckweed under the influence of oxytetracycline. A decrease in this parameter value below 0.85 indicates the PS II photoinhibition caused by the action of antibiotics. A decrease in the FV/FM coefficient value was also demonstrated following the treatment of ginseng with tetracycline (Liu et al. 2018). A significant reduction in the FV/FM value was also demonstrated in the leaves of lettuce (*Lactuca sativa* L. var Ramosa Hort) (Yang et al. 2020a) and *Lemna minor* L. (Kummerová et al. 2006). Damage to PS II under the influence of ibuprofen was demonstrated by González-Naranjo et al. (2015). The authors examined the PS II quantum efficiency (ΦPSII) and photochemical quenching in sorghum (*Sorghum bicolor*). A reduction in these parameters under the influence of ibuprofen was demonstrated. A reduction in the quantum efficiency of photosynthesis was also demonstrated in *Microcystis aeruginosa* exposed to erythromycin. The disturbance in this parameter value is related to the increase in the proportion of oxidized P700 deprived of donors from the PS II (Deng et al. 2014).

Apart from structural damage to PSII and PSI photosystems, antibiotics also cause disturbances in electron flow between the photosystems. Based on measurements of the ETR (electron transport rate) coefficient, a disturbance in electron flow between photosystems was demonstrated in cyanobacterium *Microcystis aeruginosa* treated with erythromycin. At the same time, it was indicated that PS II was more sensitive to damage. On the other hand, however, increased activity of the cyclic electron transport was noted (Deng et al. 2014). A disturbance in the ETR coefficient was also demonstrated in the leaves of *Salix fragilis* L. treated with sulphonamides (Michelini et al. 2012). At the same time, no disturbances in the FV/FM coefficient were noted, which suggests the normal activity of the photosynthetic apparatus and the probable activation of alternative photosynthetic mechanisms. Similar results were obtained for isolated spinach chloroplasts following the application of quinolone antibiotics (although DNA gyrase is generally considered the main target of this group of inhibitors). The authors demonstrated a disturbance in electron flow via PS II to the cytochrome b6/f complex (Reil et al. 2001). A disturbance in the electron flow was also demonstrated in *Synechocystis* sp. following the application of amoxicillin – a member of the β-lactam group of antibiotics, normally targetting the bacterial cell wall biosynthesis system (Pan et al. 2008). Measurements of fluorescence demonstrated the inhibition of electron transport on the donor site of PS II, which resulted in the accumulation of P680+, an oxidized form of P680. What is more, the application of amoxycillin at a concentration of 150 mg × L^{-1} resulted in complete degradation of PS II. Moreover, the effect of amoxycillin on the acceptor site of PS II was demonstrated as well.

Aristilde et al. (2010) demonstrated the effect of fluoroquinolone antibiotics on the thylakoids isolated from spinach chloroplasts by indicating the exact locations where nalidixic acid and ciprofloxacin bond in PS II. It was demonstrated that fluoroquinolone antibiotics had a chemical structure typical of PS II reaction center inhibitors on the plastoquinone side. Nalidixic acid forms two hydrogen bonds on the side of subunit L of plastoquinone QB molecule, thus resulting in disturbances in electron flow. It was also demonstrated that ciprofloxacin indirectly interfered with the photosynthetic apparatus structures by reducing the rate of energy flow from the chlorophyll-harvesting antennas to reaction centers. The increasing ciprofloxacin concentration led to an increase in the Fo parameter which reflects the amount of excited chlorophyll. Under these

conditions, energy is not transferred to the PS II reaction center. The authors indicate that the indirect interference of ciprofloxacin with the photosynthetic apparatus is not due to the damages in the chloroplast structures but to the disturbances in the course of photosynthesis. Tests on spinach chloroplasts were also conducted using an antibacterial substance (trachylobane-19-oic acid). This substance interferes with the spinach chloroplast PS II at the level of plastoquinone QA and QB molecules as well as at the LHC II level, thus resulting in the inhibition of photosystem II (Hernández-Terrones et al. 2003).

Although responses of the light phase of photosynthesis to contamination with antibiotics are relatively well documented, there are only a few studies concerning the impacts of antibiotics on the Calvin phase of photosynthesis. Significant parameters in the assessment of the efficiency of photosynthesis dark phase include the photosynthesis rate (Pn), transpiration rate (Tr), stomatal conductance (Gs), and the intercellular CO_2 concentration. These parameters were examined by Li et al. (2011a) in cereal (*Triticum aestivum* L.) seedlings treated with oxytetracycline. Significant disturbances of these parameters at each oxytetracycline concentration were demonstrated compared to the control. These disturbances may be due to the decreased degree of carbon assimilation in the plant or disturbances in electron flow. Opriş et al. (2013) also demonstrated a disturbance in the Gs parameter value under the influence of tetracycline in *Triticum aestivum* L. Photosynthetic parameters were also examined in *Pontederia cordata* during heavy metal stress. The authors note that the disturbances in photosynthetic parameters (particularly Tr) may result from the accumulation of heavy metals in the leaves (Xin et al. 2020). On the other hand, however, Hájková et al. (2019) demonstrated that the disturbances to photosynthetic parameters in *Lemna minor* L. treated with diclofenac do not result from the direct damage to PS I but from disturbances in electron flow in PS II.

The efficiency of the photosynthetic apparatus, measured by chlorophyll a fluorescence transient, is a very sensitive indicator for the assessment of the degree of plant damage due to abiotic stress. It was shown that the electron transfer in the intersystem chain to the terminal electron acceptors on the PSI acceptor side decreases (Strasser et al. 2004). At the same time, it is concluded that chlorophyll a fluorescence measurement is a sensitive and reliable method to detect and quantify changes in PSII and PSI (Chen et al. 2017). Siedlewicz et al. (2020) demonstrated that concentrations of 1, 4 and 8 µg/ml oxytetracycline cause a large increase in the absorption of the light harvesting complex in the active center of the reaction and efficient dissipation of energy in the form of heat. Although the authors did not observe an effective utilization of the energy absorbed in the reaction center and transported for use in photosynthesis, there was always an increase in the effective antenna size, which is directly correlated with the chlorophyll concentration.

The enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) which catalyzes the binding of CO_2 to ribulose-1,5-diphosphate is a key component of the Calvin cycle, the crucial reaction of photosynthesis dark phase. A decrease in the RuBisCo protein content in pea seedlings under the influence of tetracycline was demonstrated by Margas et al. (2016). Decline of RuBisCo enzyme activity under the influence of antibiotics results from the inhibition of the expression of rbcL genes which encode the large RuBisCo subunit (Liu et al. 2014). Complete degradation of the large RuBisCo subunit was demonstrated in *Brassica campestris* under the influence of erythromycin. Interestingly, at the same concentration, a small RuBisCo subunit was not completely degraded, yet its content decreased.

5. The direct effect of antibiotics on chlorophyll

The direct effect of antibiotics on chlorophyll is also very poorly understood. In contrast, the effect of heavy metals (Kondzior and Butarewicz 2018; Houri et al. 2020; Yang et al. 2020b) or acids (Koca et al. 2007; Du et al. 2017; Hu et al. 2021) on chlorophyll degradation has been thoroughly investigated. The chlorophyll degradation pathway called the PAO consists of four main steps. The first step in chlorophyll degradation is the detachment of phytol from the chlorophyll molecule by chlorophyllase (Hörtensteiner 2006); the product of the reaction is chlorophyllide. The next step is the removal of the magnesium ion by chelating molecules, leading to the formation of pheophorbide (Hörtensteiner and Kräutler 2011). The third step of chlorophyll degradation is the opening of the porphyrin ring of pheophorbide. This reaction results in the formation of fluorescent chlorophyll catabolites (FCCs). The enzyme involved in the third step is pheophorbide oxygenase a (PAO). The final step of the pathway is the conversion of fluorescent products to non-fluorescent chlorophyll catabolites (NCCs) (Hörtensteiner and Kräutler 2011). Chlorophyll degradation pathway and possible tetracycline site of action is presented in Figure 3. Pheophytin, a chlorophyll molecule lacking magnesium but containing phytol, can also be the product of chlorophyll degradation (Schelbert et al. 2009).

This type of chlorophyll degradation proceeds particularly easily in an acidic environment. In vitro studies by Rydzyński et al. (2019) demonstrated that the product of chlorophyll degradation by tetracycline is pheophytin (Figure 3) and this is regardless of the ambient pH. The reaction, however, takes place much faster in an acidic environment than in a neutral one. It was confirmed by the analyses of absorption and fluorescence spectra of the 'pure' chlorophyll degradation product (pheophytin) – the emergence of new bands at wavelengths of 506 and 536 mm, the hypsochromically shifted fluorescence spectrum, the overlap of the absorption and fluorescence spectra of the chlorophyll degradation product with the spectra of commercially purchased pheophytin, and the disappearance of fluorescence. Moreover, the obtained fluorescence lifetime for the product is identical to that for the commercially purchased pheophytin.

There are two mechanisms of chlorophyll degradation to pheophytin by tetracycline hydrochloride: (1) the loss of Mg^{2+} ions from the chlorophyll molecule due to the presence of H^+ ions in the solution (a result of acidifying the environment by tetracycline hydrochloride), (2) the removal of Mg^{2+} ions directly from chlorophyll by tetracycline which bonds Mg^{2+} from chlorophyll. Tetracycline without HCl ions directly removes magnesium from the chlorophyll molecule, yet the reaction proceeds over a longer period. The removed magnesium bonds to the tetracycline molecule in two locations: one Mg^{2+} ion binds to the tetracycline BCD ring (at low Mg^{2+} concentrations in the solution) and a second Mg^{2+} ion bonds to the A ring (at higher Mg^{2+}

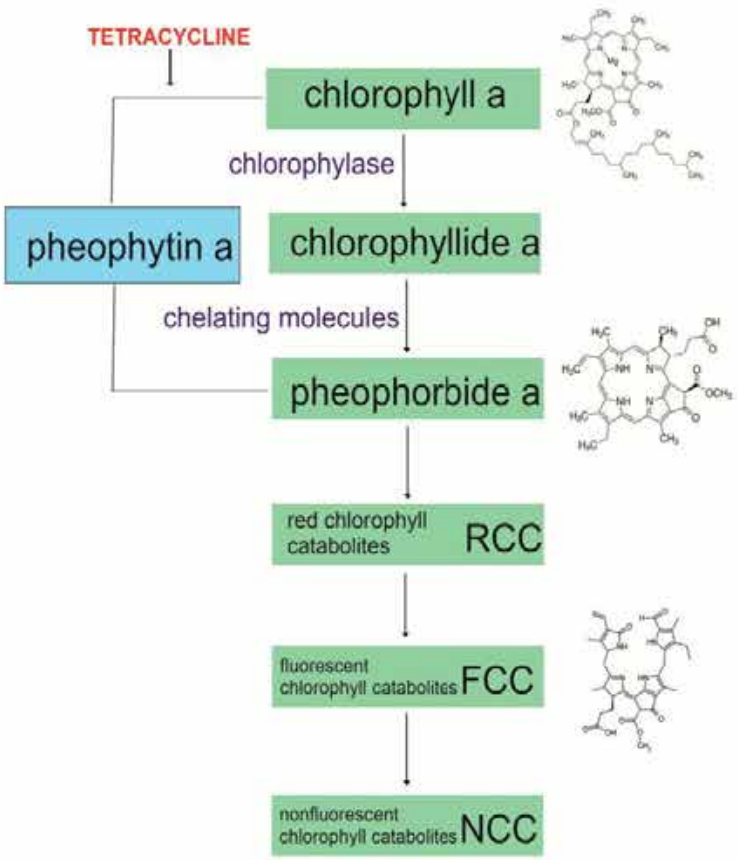


Figure 3. Chlorophyll degradation pathway and possible tetracycline site of action.

concentrations in the solution). Chlorophyll degradation leads to changes in the energy of absorbed and emitted light quanta by the formed Cd-Chl, a decrease in the amount of absorbed light quanta, and a reduction in the chlorophyll fluorescence intensity. The considerable reduction in chlorophyll concentration in plants due to its degradation by tetracycline and the changes in its spectroscopic properties (e.g. a change in energy levels) impair the energy transfer in chlorophyll antennas, and thus contribute to a reduction in photosynthesis efficiency.

6. Conclusion

The presence of antibiotics in the environment is undoubtedly one of the factors causing concerns. The worldwide consumption of antibiotics is steadily increasing, and the COVID-19 pandemic is even enhancing this problem. So far, the effects of antibiotics on plants have rather been documented than thoroughly understood. Antibiotic-caused disturbances arise in plants at various levels – damages to chlorophylls a and b, shifts in the quantitative ratio of these pigments, impairment of chlorophyll biosynthesis pathway, damages to other components of photosystems (PSII in particular), generation of ROS, impairment of electron transfer between both photosystems, inhibition and breakdown of RuBisCo, changes in numbers, sizes and structure of chloroplasts (inhibition of chloroplast divisions, formation of macrochloroplasts, reduced internal membrane system, disappearance of chloroplast ribosomes), in some cases inhibition of chloroplast DNA replication, transcription and translation, down regulation of chloroplast-related nuclear genes. Some of these effects cannot be easily predicted based on the data on antibiotic effects on microorganisms (e.g. gyrase inhibitors in some studies affected not only the plastid DNA replication but also the electron flow to the cytochrome b6/f complex). Plant responses to antibiotic pollution have undoubtedly become a vital area of plant and environmental research.

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Oświadczam, że w pracy autorstwa **Krupka M, Michalczyk DJ, Piotrowicz – Cieślak AI** pod tytułem **Effects of antibiotics on the photosynthetic apparatus of plants** opublikowanej w ***Journal of Plant Interactions* 2021, 17: 96 - 104** mój wkład merytoryczny (85%) w jej przygotowanie polegał na sformułowaniu założenia pracy przeglądowej, określiłam zakres tematyczny oraz kluczowe obszary analizy. Przeprowadziłam selekcję materiałów źródłowych – szczegółową kwerendę literaturową, analizując i wybierając najbardziej wartościowe i aktualne publikacje. Poddałam krytycznej analizie i syntezie zebrane materiały – wykonałam szczegółową analizę zgromadzonych źródeł, identyfikując kluczowe teorie, koncepcje oraz odkrycia naukowe. Przygotowałam strukturę pracy oraz jej treści merytoryczne – zaplanowałam i opracowałam logiczny układ rozdziałów pracy, zapewniając spójność tematyczną oraz przejrzystość poszczególnych sekcji. Opracowałam rysunki oraz inne elementy graficzne, które stanowią integralną część pracy. Wykonałam redakcję techniczną oraz formalną pracy dbając o spójność terminologiczną, stylistyczną oraz o poprawność językową tekstu.

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Publikacja 2

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Physiological and Biochemical Parameters of Common Duckweed *Lemna minor* after the Exposure to Tetracycline and the Recovery from This Stress

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Abstract: In this study, the ability of *Lemna minor* L. to recover to normal growth, after being degraded in a tetracycline-containing medium, was extensively investigated. The plants were exposed to tetracycline (TC) at concentrations of 1, 2.5, and 10 mM. Subsequently, their physiological status was analysed against the following criteria: rate of plant growth; free radical accumulation; antioxidant enzyme activity; chlorophyll content; HSP70 protein content; cell membrane permeability, and mitochondrial activity. The study showed that duckweed can considerably recover from the damage caused by antibiotics, within a week of cessation of stress. Of the plant properties analysed, mitochondrial activity was the most sensitive to antibiotic-induced disturbances. After transferring the plants to a tetracycline-free medium, all plant parameters improved significantly, except for the mitochondrial activity in the plants grown on the medium containing the highest dose of tetracycline. In the plants treated with this antibiotic at the concentration of 10 mM, the proportion of dead mitochondria increased and was as high as 93% after one week from the beginning of the recovery phase, even after the transfer to the tetracycline-free medium.

Keywords: enzyme activity; chlorophyll; membrane damage; mitochondrial activity; HSP70 protein content; free radicals

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1. Introduction

Water pollution is a serious global problem that makes it necessary to constantly monitor the quality of water resources. In 2015, 663 million people used unsafe drinking water, 2.1 billion people worldwide lacked access to safe water at home, and 4.4 billion lacked safe sanitation [1]. Contaminated water is a major cause of many diseases and deaths [2]; the World Health Organization indicates that more than 3.4 million people die every year due to diseases related to contaminated water [3]. In India, 80% of health problems are due to water quality [4]. Moreover, 80% of Pakistan's population is forced to use unsafe drinking water, due to a lack of safe and healthy water sources. Anthropogenic activities cause water-borne diseases that account for about 80% of all diseases in Pakistan, and are responsible for 33% of deaths [5]. The problem of water quality is particularly relevant in developing countries, but it affects developed countries as well. The Water Quality Report for the US published in 2017 indicated that 46% of rivers, 21% of lakes, and 18% of coastal waters surveyed were considered polluted [6]. This is very disturbing data, given that most people in the US still drink water from surface systems.

Water pollution affects entire populations of both plants and animals in aquatic biotopes. The majority of this pollution is due to human activity and includes industrial, agricultural, and municipal wastes. Antibiotics are a newly identified but particularly dangerous variety of water pollutant. Their threat stems from the potential adverse effects on non-target organisms and an increased bacterial resistance [7]. In 2020, the countries with the highest antibiotic consumption rates were China (43,024 tonnes), Brazil (7533 tonnes) and the United States (6596 tonnes) [8]. China had the highest rate of antibiotic consumption, increasing by almost 300% between 2000 and 2015. European countries, due to their smaller populations, had lower levels of antibiotic consumption, but in 2020, Spain (1804 tonnes), Italy (1057 tonnes) and Germany (773 tonnes) consumed the most antibiotics.

Antibiotics are not completely metabolised in the human and animal body; in fact, most of the ingested dose is released unchanged, polluting the environment [9]. Tetracyclines are among the antibiotics most used in animal and veterinary production [10]. The widespread use of tetracycline results from its low production costs and a wide spectrum of activity [11]. However, similarly to most antibiotics, tetracycline is poorly absorbed in the digestive tract of animals, with up to 60% of the dose being excreted in faeces, and up to 30% in urine [12]. The antibiotics excreted by the animals eventually find their way to the fields, and thus they enter the groundwater. The tetracycline content in manure is about 23 mg $\times$ kg^{-1} [13], whereas in pig slurry it is about 100 mg $\times$ kg^{-1} [14]. Tetracycline is found in surface water, groundwater and drinking water. The average concentrations of tetracycline in rivers ranges from 0.021 µg $\times$ L^{-1} (Soeste River, Germany) to 13.6 µg $\times$ L^{-1} (Wangyang River, China) [15,16]. The highest concentration of tetracycline was found in Brazilian rivers (110.1 µg $\times$ L^{-1}) [17]. The mean concentration of tetracycline in lakes ranged from 0.004 to 1.05 µg $\times$ L^{-1} [18,19]. Moreover, tetracycline was also detected in groundwater (0.184 µg $\times$ L^{-1}) [20] and in drinking water (0.027 µg $\times$ L^{-1}) [21]. Another class of antibiotics have also been detected worldwide in seawater, lake and river water, groundwater, and even drinking water. The presence of antibiotics in drinking water, even in trace amounts, is particularly dangerous. Detailed data on the presence of antibiotics in water are presented in the Supplementary Materials (Table S1). It is likely that this presence in drinking water is due to the lack of antibiotic removal capacity in municipal drinking water infrastructure and wastewater treatment plants [22]. Antibiotics and their metabolites become a significant threat to the ecosystems, as they are fed from municipal and industrial wastewater into waterways on a regular basis. The presence of antibiotics in the environment causes toxic effects in the organisms against which they are not targeted, e.g., plants. It has also been shown that tetracycline degrades chlorophyll in plants [23], disrupts the flow of electrons in photosystems [24], and causes the overproduction of free radicals and peroxidation of membrane lipids [25]. These disturbances result in the inhibition of plant growth.

Lemna is an excellent indicator plant for the assessment of water quality, among others, due to its ability to absorb harmful substances present in water. It accumulates heavy metals [26], nitrogen [27], phosphorus [28], and even antibiotics [29]. In ecotoxicological studies, the duckweed is recommended [30] as a test organism due to its simple structure, small size, vegetative reproduction, and short multiplication period; the doubling time for plant numbers is as low as 48 h. The high rate of *Lemna* multiplication has resulted in a wide range of its potential biotechnological applications, including biofuel [31,32], animal feed [33], and even human food production [34].

The scientific literature on *Lemna*, however abundant, does not contain data on the recovery of these plants from exposure to antibiotic-contaminated environments. Therefore, the aim of this work was to evaluate the ability of lesser duckweed to recover after growth in a liquid medium contaminated with tetracycline. High doses of tetracycline were used in this study in order to determine the upper limit of antibiotic concentration, below which plant recovery is possible. The experiments described here provide the basis for predictions of severity, as well as the durability of the damages, caused in duckweed by antibiotics. We assessed the response of duckweed (*Lemna minor* L.) to antibiotic exposure and antibiotic

removal at both physiological and biochemical levels, considering the following criteria: rate of plant growth; free radical accumulation; antioxidant enzyme activity; chlorophyll content; HSP70 protein content; cell membrane permeability, and mitochondrial activity.

2. Results and Discussion

The effect of tetracycline present in the medium (at concentrations of 1, 2.5, and 10 mM) on the recovery of duckweed after TC removal was investigated. The plants were grown for seven days on a tetracycline-containing medium (exposure phase) and then for seven days without tetracycline (recovery phase). The recovery capacity was evaluated by assessing the physiological and biochemical parameters, including: the rate of plant growth; frond surface area; dry and fresh weight; free radical accumulation; antioxidant enzyme activity; chlorophyll content; HSP70 protein content; cell membrane permeability and mitochondrial activity.

2.1. Growth Parameters

The OECD [30] recommends the use of growth parameters in the assessment of chemicals toxicity to *Lemna minor*. Tetracycline with a concentration range of 1 to 10 mM showed the toxicity by a reduction in the number of plants in each treatment (Figure 1A); however, the removal of tetracycline resulted in a distinct resumption of growth. Plant exposure to 2.5 mM TC caused a 48% reduction in the number of plants compared with the control, whereas the removal of TC resulted in recovery, so the ultimate reduction in plant number, at the end of the experiment, was only 8%.

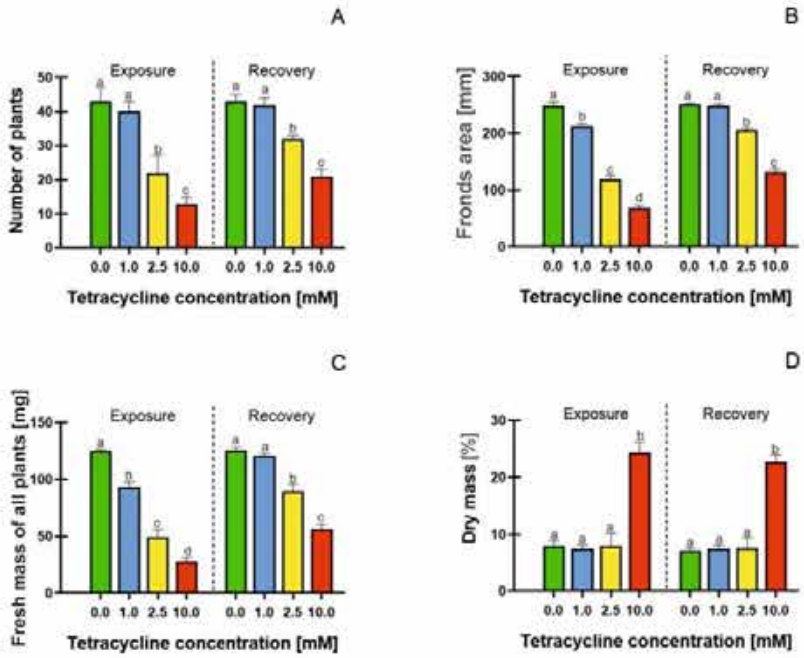


Figure 1. Growth parameters: number of plants (**A**), frond area (mm) (**B**), fresh mass of all plants (mg) (**C**), dry mass (%) (**D**) in *Lemna minor* subjected to tetracycline and in the recovery (resumed growth after 7 days of culture in antibiotic-free medium). Means denoted with different letters are significantly different ($p \leq 0.05$) across the described groups: average ± standard deviation.

Tetracycline at 10 mM concentration caused a 70% inhibition of growth at the end of the exposure phase; however, plants subjected to this treatment were also able to resume growth in an antibiotic-free medium, as culture on the medium without any TC

decreased the tetracycline-induced growth inhibition by 42%. In order to discuss the recovery capacity of the lesser duckweed in more detail, the EC50 index (the concentration at which 50% inhibition of growth or other analysed parameters occurs) was determined. For the increase in *Lemna* number, this was determined as 1.944 mM tetracycline (Table 1). The EC50 parameter is usually assessed for animals [35]. The use of the EC50 parameters in this study allowed us to compare the changes in recovery capacity and recovery rate of duckweed, after the exposure to different concentrations of TC. The EC50 factor estimated for the combined TC exposure and recovery treatment was higher by 0.571 mM, compared with the value obtained before the recovery stage started. Our results clearly indicated that the EC50 parameter increased when the plant regeneration stage was added, i.e., when the tetracycline-treated plants were transferred to an antibiotic-free medium. The largest increase in EC50 (by 75%) was noted when the dry weight was analysed. The dry weight seemed, therefore, to be the plant-quality parameter which was least sensitive to tetracycline. On the other hand, the frond number was found to be the most sensitive indicator of damage caused by the temporary plant exposure to tetracycline. Adding the regeneration stage resulted in an EC50 increase of 29%, in the case of this parameter. In other words, in the case of frond numbers, the effect of the tetracycline removal was least pronounced, compared with the fronds area, fresh mass and dry mass (Table 1).

Table 1. EC50 values for morphological in *Lemna minor* L. exposure (subjected to tetracycline) and recovery (resumed growth after 7 days of culture in antibiotic-free medium).

	EC50 Value (Exposure) [mM]	EC50 Value (Recovery) [mM]
Number of fronds	1.944	2.515
Fronds area	1.724	2.850
Fresh mass	1.431	2.447
Dry mass	3.825	6.715

In the literature, the EC50 values for *Lemna minor* plant growth have very rarely been analysed, and the results obtained are inconsistent. The reported EC50 values for the growth of duckweed in tetracycline-containing media range from 2.38 µM [36] to 4.42 µM [29]. The difficulty in determining EC50 is probably due to the difficulty in determining the concentration at which plant death will occur. Our results indicated that tetracycline was toxic to plants even at moderate concentrations, but *Lemna* showed a good capacity to recover after the removal of the toxic agent. In plants grown in a tetracycline-containing medium, the frond surface area was reduced (Figure 1B), with particular severity in the medium containing 10 mM TC (3-fold drop compared with the control). The calculated EC50 value for frond surface area was 1.724 mM tetracycline (Table 1), which increased to 2.850 after 7 days of recovery in the tetracycline-free medium; thus, the regeneration capacity was more than 60%. Baciak et al. [29] also observed a systematic decrease in the frond area of *Lemna minor* treated with increasing concentrations of TC. Moreover, Brain et al. [37] showed that biomass is a sensitive index for the assessment of antibiotic toxicity to *Lemna* sp. The reduction in the fresh weight of the plants correlated with the accumulation of antibiotics in their tissues. Hussain et al. [38] indicated that antibiotics are accumulated in roots and leaves, and thus disrupt plant growth. Tetracycline accumulation in pea leaves was shown by Margas et al. [39]. Di Marco et al. [40] showed tetracycline accumulation in *Iberis sempervirens* L., indicating an apoplastic distribution of the antibiotic in the plant. Rydzyński et al. [41] showed tetracycline accumulation (123 µg × fresh weight $^{-1}$) in yellow lupin seedlings, resulting in a reduction in plant fresh weight. These results were confirmed by Rydzyński et al. [23]. The authors showed tetracycline accumulation in spinach seedlings, which correlated with a decrease in fresh weight content. Our results clearly indicate that plant fresh weight decreased significantly in samples treated with 2.5 and 10 mM tetracycline, with 60 and 76% decreases, respectively, compared with the control (Figure 1C). However, the removal of the antibiotic promoted

an increase of 120% in plant fresh weight, within seven days of culture. The EC50 for fresh weight was 1.431 mM tetracycline, which increased to 2.447 mM at the end of the recovery stage of the experiment. The fresh weight was, therefore, among the investigated morphological parameters most sensitive to damage.

A significant increase in dry matter content of 300% was found in the plants treated with the highest concentration of tetracycline (Figure 1D). This change reflected the loss of water by plants, as demonstrated by the increased dry mass to frond area ratio (Figure 1B–D). The values of this ratio ranged from 0.032 in control, to 0.359 in 10 mM tetracycline treatment at the end of the exposure stage; this decreased at the recovery stage, so that it ranged from 0.028 in control, to 0.172 in 10 mM tetracycline treatment. Similar results were obtained by Rydzyński et al. [41], showing a 400% increase in dry matter of tetracycline-treated plants. The increase in dry matter content of antibiotic-treated plants was probably due to the impaired water uptake of the plant, resulting in tissue dehydration [42,43]. It may also have resulted from an increase in cell wall rigidity. Schopfer [44], in a study of maize coleoptiles, found that hydrogen peroxide inhibited the elongation of these organs and decreased the extensibility of their cell walls. He also demonstrated that the increase in cell wall rigidity resulted from the peroxidase-catalysed cross-linking of the cell wall phenolics, although the precise identification of the phenolic components was not carried out.

An analysis of the growth parameters showed that the duckweed had a high regeneration potential, when transferred to the medium without TC. Similar results were obtained by Zaltauskaite et al. [45], who treated duckweed with a sulfonylurea herbicide. The authors demonstrated that *Lemna minor* was able to regenerate after the stress factor was removed, and also indicated that the 7 days growth time in the toxicant-free medium may have been too short to achieve full-plant recovery; this is also consistent with our results. The results presented in this paper show that all growth parameters, number of plants, frond area, and fresh and dry weight, increased by about 40% after the transfer of the plants to the tetracycline-free medium, with the most visible improvements concerning the dry weight.

2.2. *Effect of Tetracycline on Chlorophyll Content*

Chlorophyll content is one of the key factors in determining plant growth. The analysis of the chlorophyll content was carried out by analysing the absorption spectra at $\lambda = 664$. According to Lamber–Beer's law, the absorbance is described by the relation: $A(\lambda) = \varepsilon(\lambda)lc$, where $\varepsilon(\lambda)$ is the molar extinction coefficient at wavelength λ, l is the thickness of the absorbing layer, and c is the molar concentration. The molar extinction coefficient for chlorophyll is 69 400 M^{-1} cm^{-1} in ethanol at $\lambda = 664.7$ nm, according to Seely and Jensen [46].

The chlorophyll content in the duckweed that was not treated with tetracycline was 1.574×10^{-5} M (Table 2). In the plants subjected to the lowest concentration of tetracycline (c = 1 mM) during the exposure phase, the absorbance at $\lambda = 664$ nm decreased from A = 1.09 to A = 0.64 (Figure 2A). Thus, the chlorophyll content at the end of this phase decreased to 0.918×10^{-5} M (Table 2). For 2.5 mM of TC, the absorbance decreased to 0.56 (chlorophyll content was 0.749×10^{-5}), for 10 mM of TC to A = 0.37, and the chlorophyll content dropped to 0.571×10^{-5} M.

The reduction of the chlorophyll content in the plants subjected to tetracycline treatment has been observed repeatedly [23,41,47]. Margas et al. [47] have shown that in peas exposed to 250 mg $\times$ L^{-1} tetracycline, a 37% decrease in chlorophyll content occurred. Moreover, a decrease in the absorbance of chlorophyll was also observed by Rydzyński et al. [41], who analysed the leaves of yellow lupin growing on soil containing tetracycline. These authors showed a decrease in chlorophyll concentration in every sample tested. The reduced chlorophyll concentration was particularly evident in the young leaves. Our current results indicate that the tetracycline treatment also reduced the concentration of chlorophyll in *Lemna*, and the intensity of this effect was related to the antibiotic concen-

tration (Table 1). However, transferring the plants to a medium without tetracycline clearly resulted in the recovery of chlorophyll absorbance (Figure 2B). The increase in chlorophyll absorbance resulted from the increase in its concentration; for plants treated with 1 mM TC, there was an increase in absorbance to A = 0.937 (up 43%), and for 2.5 mM TC to A = 0.711 (31%), whereas for 10 mM TC it only slightly increased to A = 0.552 (29%).

Table 2. Chlorophyll a content in *Lemna minor* subjected to tetracycline treatment (exposure) and in the recovery (resumed growth after 7 days of culture in antibiotic free medium).

Tetracycline Concentration [mM] during 7 Days	Chlorophyll Concentration [M]	
	Exposure	Recovery
0	1.574×10^{-5}	1.574×10^{-5}
1	0.918×10^{-5}	1.315×10^{-5}
2.5	0.749×10^{-5}	0.988×10^{-5}
10	0.571×10^{-5}	0.742×10^{-5}

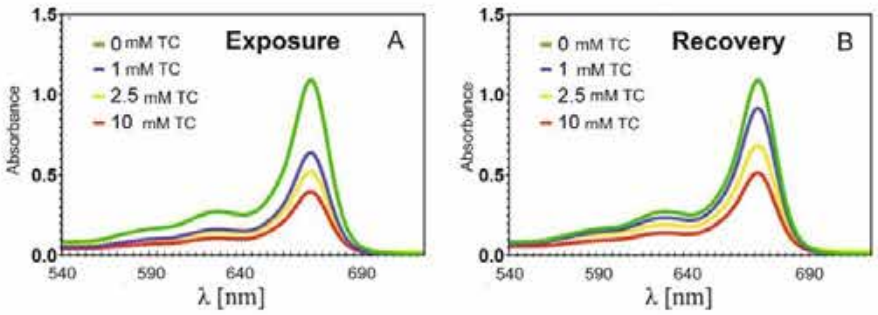


Figure 2. (**A**) Changes in absorption spectra of chlorophyll in *Lemna minor* subjected to tetracycline treatment and (**B**) in the recovery phase (1 week of growth in a tetracycline-free medium).

This demonstrates that the chlorophyll concentration in the plants growing after the tetracycline removal did not reach the same values as in the control. Therefore, it can be stated that the degradation of chlorophyll in the plants treated with pharmaceuticals was at least partially reversible, but seven days was too short a time for full recovery to occur.

As indicated by Rydzyński et al. [23], the decrease in chlorophyll content in plants treated with antibiotics may result from the direct degradation of chlorophyll, and from the formation of its degradation products. The authors conducted a study on the direct effect of tetracycline on chlorophyll, and showed shifts in the chlorophyll absorption bands as well as the appearance of new absorption bands, due to the formation of chlorophyll degradation products, including pheophytin. The model proposed by the authors for the degradation of chlorophyll by tetracycline involved the removal of a magnesium ion from the porphyrin ring. The energy transport in chlorophyll antennae was then disrupted and thus photosynthetic efficiency was reduced [23].

2.3. Free Radical Accumulation and Antioxidant Enzyme Activities

The H_2DCFDA was used as a marker for the total ROS production. H_2DCFDA is taken up by the cells, then deacetylated by intracellular esterases to DCFH. DCFH can be oxidized to fluorescent DCF by hydrogen peroxide, lipid peroxides and reactive nitrogen species (RNS), and also by peroxidases even in the absence of hydrogen peroxide [48,49]. Therefore, this assay is best applied as a qualitative marker of cellular oxidant stress [50], but should be carried out in parallel with peroxidase activity [51]. Oxidative stress, leading to the formation of reactive oxygen species (ROS), lies at the origin of nearly all plant stresses. Although ROS are also formed under physiologically normal conditions, mainly

as byproducts of photosynthesis and respiration, environmental stresses can cause ROS overproduction [52]. The prevalence of free radical accumulation over free radical scavenging mechanisms is the core of oxidative stress [53]. In tetracycline-treated plants, ROS accumulation was observed (Figure 3). The fluorescence intensity, associated with the presence of ROS, increased with increasing tetracycline concentration; thus, the highest ROS accumulation was shown in plants treated with the highest tetracycline concentration.

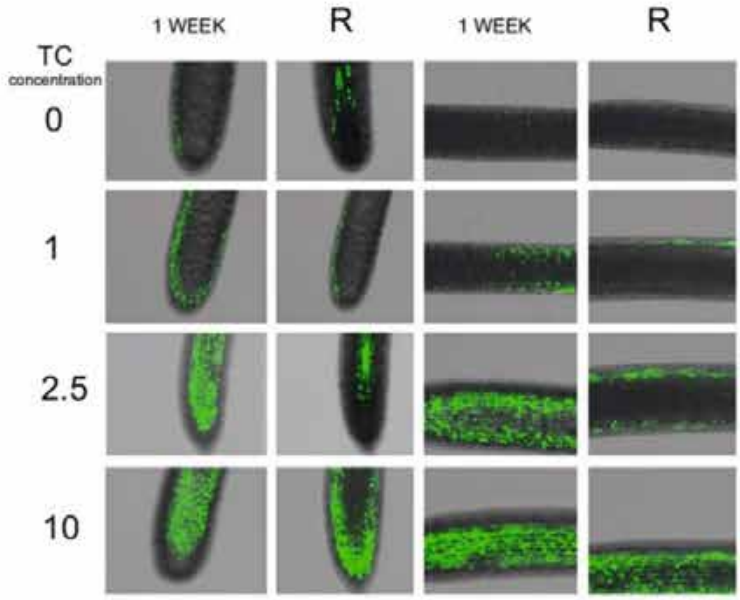


Figure 3. Free radicals (green colour) in roots (500×) of *Lemna minor* subjected to tetracycline (1 week) and in the recovery phase (R) (resumed growth after 7 days of culture in antibiotic-free medium).

Similar results were obtained by Margas et al. [47], showing free radical accumulation in the roots of peas (*Pisum sativum* L.) treated with tetracycline, as well as Orzoł et al. [54], in the root of yellow lupin treated with levofloxacin.

To date, no studies have been conducted on the free radical accumulation in plants recovering from tetracycline treatment. After transferring the tetracycline-treated duckweed to a medium without tetracycline, a reduced accumulation of ROS was observed (Figure 3) in each test sample. However, the concentration of ROS was still higher than in the control sample. In addition, it was observed that ROS accumulated mainly in the apical part of the root, whereas during the tetracycline-exposure phase, ROS accumulated in both the apical and the middle part of the root. It is known that ROS can modify the structure of cell walls, and even determine the size of the meristematic zone of roots by regulating the cell cycle [55,56]. Differences in the spatial distribution of ROS in root tips during the recovery from the TC treatment (10 mM) may suggest the mechanism of root growth inhibition by tetracycline and the recovery from this stress [56]. Our results indicated that free radical scavenging occurred in the plants transferred to the medium without TC, suggesting the activation of free radical scavenging mechanisms. One of the mechanisms for the removal of free radicals from cells was the activation of antioxidant enzymes. Catalase (CAT) is an enzyme that directly converts hydrogen peroxide to water and oxygen [57].

During the exposure phase, increased catalase activity was only observed after treatment with 1 mM tetracycline in the medium: a 30% increase in comparison with the control (Figure 4A). However, a significant increase in catalase activity was observed during the recovery phase (Figure 4A); the enzyme activity increased by 120, 140, and 200%, for tetracycline 1, 2.5 and 10 mM treatments, respectively, compared with plants analysed

directly after the exposure phase. The increase in catalase activity was also expressed by the EC50 parameter that elevated from 2.711 mM (at the end of the exposure phase) to 6.506 mM in the recovery phase (Table 3). Drobniewska et al. [58] carried out a similar study, investigating the recovery of *Lemna minor* L. treated with sulfadimethoxine; however, the authors did not show an increase in CAT activity during the recovery phase.

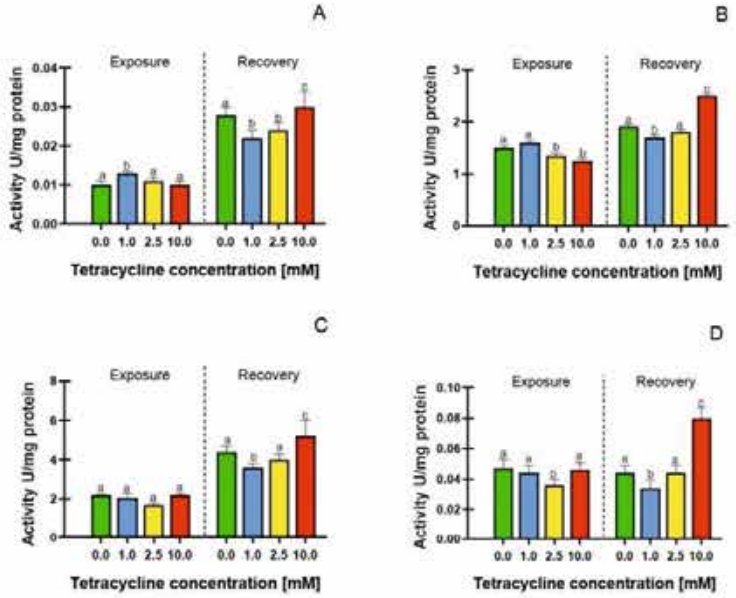


Figure 4. Catalase activity (**A**), ascorbate peroxidase (**B**), pyrogallol peroxidase (**C**), glutathione reductase (**D**) in *Lemna minor* subjected to tetracycline (exposure) and in the recovery phase (R) (resumed growth after 7 days of culture in an antibiotic-free medium). Means denoted with different letters are significantly different ($p \leq 0.05$) across the described groups: average ± standard deviation.

Table 3. EC50 values for catalase activity (CAT), ascorbate peroxidase (APX), pyrogallol peroxidase (PDX), glutathione reductase (GR) in *Lemna minor* subjected to tetracycline (exposure) and in the recovery (R) (resumed growth after 7 days of culture in antibiotic free-medium).

Antioxidant Enzymes	EC50 Value (Exposure) [mM]	EC50 Value (Recovery) [mM]
Catalase activity	2.711	6.506
Ascorbate peroxidase	2.173	5.231
Pyrogallol peroxidase	9.253	5.098
Glutathione reductase	1.612	3.002

Ascorbate peroxidase (APX) is an enzyme that catalyses the decomposition of hydrogen peroxide, using ascorbate as an electron donor. This enzyme is particularly active in chloroplasts [41]. Ascorbate peroxidase (APX) activity was expressed as 1 U/mg protein. During the exposure phase, the enzyme activity decreased in the plants treated with tetracycline at concentrations of 2.5 mM (15% decrease in activity) and 10 mM (22% decrease in activity). APX was also the most sensitive antioxidant enzyme (EC50 value = 2.173 mM). Similar results were obtained by Gomes et al. [24]. These authors showed that in *Lemna minor* L. treated with ciprofloxacin, there was a decrease in APX activity with a concomitant accumulation of ROS, suggesting a clear dominance of ROS accumulation over ROS removal mechanisms.

During the recovery phase, we observed a general increase in enzyme activity (and an increase in the EC50 parameter to 5.231 mM), especially in the plants treated with the 10 mM antibiotic. In these samples, the APX activity increased by 66%, compared with the control plants (Figure 4B). Similar results were also obtained for peroxidase (PDX) (Figure 4C); however, no significant differences in peroxidase activity were observed during the exposure phase (the EC50 value for this parameter was 9.253 mM). In the recovery phase, however, a large increase in the enzyme activity was already visible in the samples treated with the lowest concentration of tetracycline (an increase in PDX activity of 66% compared with the control). In plants subjected to tetracycline at a concentration of 2.5 mM, peroxidase activity increased by 81%, whereas in plants treated with the highest tetracycline concentration, it increased by 136% compared with the control. The increase in PDX activity was correlated with a decrease in EC50 values to 5.098 mM. POX activity was higher than that of APX, though the tetracycline-induced changes in the activities of these enzymes were more notable in the case of APX. However, it could be clearly seen that the pattern of the changes was the same, and some similarities could be seen with the CAT response as well. So, our data show that TC affects hydrogen peroxide metabolism in the plant. However, no clear dependence on the TC concentration could be detected, i.e., there was no significant statistical relationship between concentration and response. Moreover, the moderate decrease in particular enzyme activity in the treatments of 2.5–10 mM TC concentrations did not necessarily indicate adverse ROS effects, because several enzymes were involved in hydrogen peroxide scavenging.

Glutathione reductase (GR) is the factor responsible for maintaining adequate levels of reduced glutathione (GSH) in the cell. Reduced GSH is responsible for the cellular control of reactive oxygen species [59]. We recorded an increase in GR activity only in the plants undergoing recovery from the 10 mM tetracycline treatment. The highest concentration of tetracycline resulted in an almost two-fold increase in GR activity in the recovery phase (Figure 4D). GR activity was mainly detected in the photosynthesising tissues. In our results, we showed a positive correlation between the GR activity in the recovery phase and the increased chlorophyll concentration. The exposure to moderate TC concentrations (1–2.5 mM) led to a gradual reduction in GR activity, though the treatment of 10 mM GR was similar to that of the control. After the recovery period, GR activity remained lower than the control only in the treatment of 1 mM TC. The GR level in the *L. minor* pre-exposed to 2.5–10 mM showed a complete recovery in this ASC/GSH cycle enzyme, indicating an efficient antioxidant defense system. A moderate recovery was also recorded in the other ASC/GSH cycle enzymes.

The increased activity of antioxidant enzymes is a good biomarker of a plant's stress response. On the other hand, the prevalence of free radical accumulation over free radical scavenging mechanisms may lead to permanent structural damage to the plant cell. Liu et al. [25] reported that in ginger cells treated with 500 mg $\times$ L^{-1} tetracycline, an increase in malondialdehyde (MDA) concentration occured, despite the concurrent increase in CAT and PDX activity. The authors indicated that the increased enzyme activity was apparently not sufficient for free radical scavenging. Consequently, membrane lipid peroxidation occurred, leading to the accumulation of MDA as the peroxidation product. However, knowing that DCFH can be oxidized to fluorescent DCF by peroxidases even in the absence of hydrogen peroxide, it is possible that the increased amount of ROS in the roots in the recovery phase was caused by the peroxidases.

2.4. Malondialdehyde (MDA) Content, Cell Membrane Damage, Mitochondrial Activity and HSP70 Protein Content

The MDA content measured at the end of the exposure phase increased significantly as the tetracycline concentration increased to 2.5 and 10 mM, by 196 and 346%, respectively, compared with the control (Figure 5A). Liu et al. [25] also showed lipid peroxidation in ginseng cells treated with 500 mg $\times$ L^{-1} tetracycline, reporting a 2.5-fold increase in MDA content, compared with the control. On the other hand, Nunes et al. [60] observed no significant differences in MDA content in *Lemna minor* L. and *Lemna gibba* L. treated

with fluoroquinolone antibiotics. The authors suggested that both plants were able to cope with oxidative stress by eliminating ROS, thus protecting cell membranes from lipid peroxidation, probably through catalase activity. Such an effect was also demonstrated in this paper, in plants undergoing the recovery phase. We detected a decrease in MDA content of 45%, 31%, and 19%, compared with the exposure phase (Figure 5A), with an increase in antioxidant enzyme activity and a decrease in free radical accumulation.

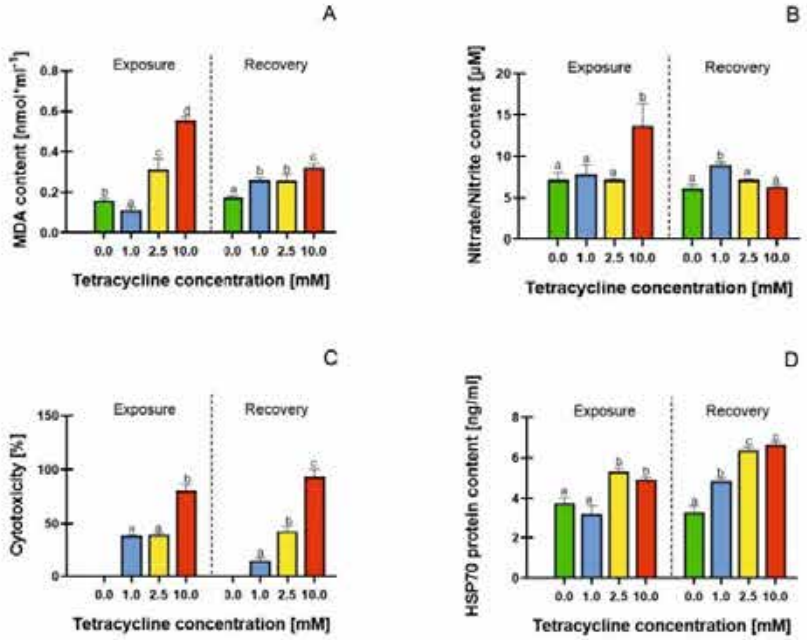


Figure 5. Malondialdehyde (MDA) content (**A**), cell membrane damage (**B**), mitochondrial cytotoxicity (**C**), and HSP70 protein content (**D**) in *Lemna minor* subjected to tetracycline (exposure) and in the recovery phase (resumed growth after 7 days of culture in an antibiotic free medium). Means denoted with different letters are significantly different ($p \leq 0.05$) across the described groups: average ± standard deviation.

In addition to the peroxidative damage to the cell membranes, we investigated the effect of antibiotics on cell membrane integrity by determining nitrate and nitrite. In plants, nitrate and nitrite are involved in nitric oxide synthesis. Nitric oxide (NO) is a signalling molecule responsible, among other things, for vegetative development or ion homeostasis. However, under stress conditions, NO accumulation induces toxic effects in plants [61]. During the exposure phase, a significant increase in nitrate and nitrite content occurred in plants treated with TC at the highest concentration, showing a 89% increase compared with the control (Figure 5B). Valderrama et al. [62] showed the increased production of NO and the accumulation of its derivatives in olive leaves under salt stress. The accumulation of reactive nitrogen species was demonstrated by Kummerova et al. [63] in *Lemna minor* L. treated with diclofenac. The overproduction and accumulation of NO outside cell membranes is the first symptom for the loss of membrane integrity in cells undergoing programmed cell death [64]. Therefore, it can be assumed that in plants treated with tetracycline at a concentration of 10 mM, there was a loss of cell membrane integrity and consequently the onset of cell apoptosis; however, transferring plants to a tetracycline-free medium reduced this effect by 46%.

The assessment of mitochondrial viability can be an important parameter in the evaluation of biochemical changes induced by antibiotics in plants. To assess the sta-

tus of mitochondria, we used the WST-1 assay based on the cleavage of tetrazolium salt to formazan, through the activity of dehydrogenases present in mitochondrial membranes. In the control samples, we did not observe mitochondrial damage. Increasing the concentrations of tetracycline caused 37, 38 and 80% mitochondrial toxicity (Figure 5C). Kummerova et al. [63] showed a reduced mitochondrial activity in *Lemna minor* L. treated with diclofenac. Our study similarly indicated that mitochondria may be the site of antibiotic direct action in plants. The effect of antibiotics on mitochondria was also described by Geisler et al. [65], who treated potatoes with antimycin A, and showed a reduction in NADH dehydrogenase activity in leaves. Gomes et al. [66] showed that in *Lemna minor* L. treated with ciprofloxacin there was a disruption of the mitochondrial electron transport chain. These results suggested that the antibiotic-induced impairment of mitochondria may result from both structural damages in mitochondrial membranes and the disruption of electron transport. Moreover, the oxidative damage to the plant's mitochondria may result from the accumulation of free radicals correlates with the insufficient activity of antioxidant enzymes [67]. When the duckweeds were transferred to a tetracycline-free medium, a 23% reduction in the frequency of dead mitochondria was observed. The highest concentration of tetracycline caused a 93% mitochondrial cytotoxicity, measured during the recovery phase (Figure 5C). This was the only parameter that continued to increase after the removal of TC from the medium.

HSP 70 proteins are also essential components of the plant stress response. Their main role is to protect cells from oxidative stress by stabilizing membrane proteins and removing abnormal and damaged proteins [68]. Although they accumulate in the highest amount during heat stress, they are a biomarker of the stress responses induced by other factors as well, including anthropogenic factors. HSP proteins have been identified in various cellular structures, e.g., in chloroplasts, cell nuclei, the cytoplasm, cell membranes and mitochondrial membranes [68]. HSP proteins are involved in repair processes and are a good biochemical marker of the plant's stress response. A significant increase in HSP 70 protein content was observed when tetracycline was added to the medium at concentrations of 2.5 and 10 mM. The HSP 70 protein content in these samples was 51.86 and 48.71 ng $\times$ mL^{-1}, respectively (131 and 123% increase, compared with the control). Gorovits et al. [69] also showed an increase in HSP 70 content in tomato leaves treated with pharmaceuticals, in the concentration range of 10–100 µg $\times$ L^{-1}. Even the lowest concentration of pharmaceuticals (10 µg $\times$ L^{-1}) induced HSP 70 proteins in leaves. In contrast, no increase in HSP 70 proteins was observed in the roots. Margas et al. [39] and Ziółkowska and Piotrowicz-Cieślak [70], on the other hand, observed a reduction in HSP 70 proteins in pea roots under the influence of pharmaceuticals. Das et al. [71] indicated that in soybeans under heat stress, increased levels of HSP 70 proteins activated a cascade of proteins responsible for protecting the photosynthetic apparatus and chloroplasts.

We observed an increase in HSP 70 protein content by 26, 25 and 34%, in the plants recovering from TC 1, 2.5 and 10 mM treatments, respectively (Figure 5D). The increase in HSP 70 content in the recovery phase correlated with a decrease in free radical accumulation, and with an increase in the chlorophyll concentration in plants. Considering the above aspects, it can be assumed that HSP 70 proteins play an important role in the repair processes in antibiotic-treated plants.

3. Materials and Methods

3.1. Plant Material and Culture Conditions

Axenic cultures of duckweed (*Lemna minor* L.) maintained in the Department of Plant Physiology, Genetics and Biotechnology of the University of Warmia and Mazury in Olsztyn, were used. Ten specimens of plants were cultured for 7 days in glass jars with the capacity of 200 mL, in 100 mL of liquid 50% Murashige and Skoog (MS) medium at 25 °C/17 °C, day/night temperatures, 16/8 h photoperiod, with daylight intensity 3.4 klx (fluorescent lamp Osram L36W/77 Fluora). Additionally, the medium contained tetracycline added at concentrations of 0, 1, 2.5 and 10 mM. After 7 days of culture in

the tetracycline treatment, the plants were transferred to a fresh medium with the same content as above, only without the tetracycline (recovery phase). The plant responses were analysed after 7 days of tetracycline treatment and then again after 7 days of the recovery phase.

3.2. Analyses of Growth, Frond Area, Dry and Fresh Weight

The number of plants, total frond area and dry and fresh weight were determined according to the OECD protocol for *Lemna* sp. [30].

3.3. Isolation and Absorption Measurements of Chlorophyll

The plants (0.3 g) were homogenized in a mortar with 5 mL of 96% ethanol. After centrifugation at 1700× *g* for 15 min, the pellets were washed with the solvent five times, and the ethanol solutions from all extraction stages were combined. The supernatants were diluted five times; then, they were subjected to analyses of absorption spectra using a Carry 300 UV-Visible Spectrophotometer (Varian, Inc., Victoria, Australia) according to Rydzyński et al. [72].

3.4. Presence of Free Radicals (ROS)

Control seedlings and seedlings grown on tetracycline containing media were analysed to detect the presence of reactive oxygen species (ROS). To detect ROS, 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich, Poland) was used. Plants were placed in H_2DCFDA solution in 0.1 M phosphate-buffered saline (PBS, Merck, Poland) in the dark. After 30 min, the buffer was replaced with fresh PBS. After staining, the samples were analysed by confocal laser-scanning microscopy (Leica TCS SP5) and the Leica Application Suite 2.0.2 build 2038. The following excitation and emission wavelengths were used in the experiment: 488 nm excitation and 515–565 nm emission.

3.5. Enzyme Activity

The plant extracts were prepared on ice. The plants were then ground in liquid nitrogen, using a porcelain mortar and pestle. For antioxidative enzymes, plants were homogenized in 0.05 M K-phosphate buffer (pH 7.0), containing 2% (*w*/*v*) PVPP, 0.4 mM EDTA, 0.2 mM PMSF by Retsch Mixer Mill MM400 (Germany). The samples were centrifuged for 20 min at 12,000× *g* at 4 °C. The supernatant was then carefully collected, and the pellet discarded.

The catalase activity was determined spectrophotometrically (SPECTROstar Nano), in a reaction mixture containing 50 mM phosphate buffer, pH 7 and 15 mM H_2O_2. The absorbance was measured for 10 min at room temperature, at 240 nm according to Aebi [73]. One unit corresponded to a reduction of 1 µmol H_2O_2 in 1 min.

The ascorbate peroxidase activity was determined spectrophotometrically in a 1 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.35 µM ascorbate and 10 µM H_2O_2. APX activity was determined by following the decrease in absorbance at 290 nm for 10 min at room temperature, according to Murshed et al. [74]. One unit corresponded to a reduction of 1 µmol H_2O_2 in 1 min.

Pyrogallol peroxidase activity was determined spectrophotometrically (λ = 420 nm) in a reaction mixture containing 100 µL of 1% pyrogallol (2,3-Dihydroxyphenol, Merck, Poland), 2 mL of 0.1 M 50 mM phosphate buffer, pH 6, 50 µL of supernatant and 20 µL of 0.06% H_2O_2. The rate of increase in absorbance was measured at room temperature at 420 nm. One unit corresponded to 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at room temperature according to Chance and Maehly [75] and Radić et al. [76].

Glutathione reductase activity was determined with a spectrophotometer (CECIL, CE2021 2000 SERIES, Cambridge, United Kingdom) in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH (β-Nicotinamide adenine dinucleotide phosphate, Sigma-Aldrich, Poland) and 0.5 mM GSSG (L-Glutathione oxidized, Merck, Poland). The rate of decrease in absorbance was measured at room

temperature at 340 nm according to Murshed et al. [77]. One unit corresponds to the oxidation of 1µM NADPH in 1 min.

3.6. Lipid Peroxidation—TBARS Assay

In order to assess lipid damage, the method of Hodges et al. [78] with modifications was used. An amount of 0.4 g of tissue was homogenized in a cold porcelain mortar and pestle (on ice) in 4 mL 0.1% trichloroacetic acid (TCA, Sigma-Aldrich, Poland). The extracts were centrifuged at 5000× *g* for 10 min. Then 1 mL of 50% ethanol solution was added, the extracts were incubated for half an hour, and centrifuged at 5000× *g* for 10 min. The procedure was repeated twice. An amount of 1 mL of supernatant was taken and a mixture of 20% trichloroacetic acid and 0.5% thiobarbituric acid (TBA, Sigma-Aldrich, Poznań, Poland) was added. The extracts were heated in a water bath at 95 °C for 30 min. The extracts were ice-cooled and centrifuged at 5000× *g* for 10 min. Absorbance was measured at 525 nm and 600 nm as a reference using a TCA/TBA mixture. The assay was performed on plants collected after the antibiotic exposure and the recovery phase. The results were presented as the content of malondialdehyde (MDA, [nmol × mL^{-1}]). The MDA content was calculated according to the method of Heath and Packer [79], using the molar extinction coefficient MDA 155 $mM^{-1} \times cm^{-1}$.

3.7. Assessment of Damages to Cell Membranes

Cell membranes were isolated from *Lemna minor* fronds according to Jett et al. [80]. An amount of 0.4 g plant material was homogenized in a 10 mM Tris/HCl, pH 7.5 buffer, containing 2 mM EDTA. The homogenate was centrifuged at 5000× *g* for 10 min. Tris/HCl buffer, pH 7.4, containing 1 mM $MgCl_2$ was added, and after 24 h the extracts were centrifuged at 100× *g* for 5 min. An amount of 5 mL aliquots from the upper and bottom phases were collected and centrifuged at 10,000× *g* for 10 min. A two-phase solution was then prepared by mixing 9.68 g of dextran (Sigma-Aldrich, Poznań, Poland) suspended in 100 mL Tris/HCl buffer, containing EDTA, pH 7.5, and 7 g of polyethylene glycol (Sigma-Aldrich, Poznań, Poland), suspended in 100 mL of the same buffer. The samples were suspended in the two-phase solution and allowed to sit at 4 °C for 24 h. After 24 h, 40 µL samples were aliquots to a 96-well multiplate. Next 20 µL assay buffer was added to each well. Ten µL of a feshly prepared 1 mM NADPH solution was added, and the plate was kept at room temperature for 60 min. After the incubation 10 µL of the lactate dehydrogenase (LDH) was added and 10 µL of a cofactor solution. Reagent 1, and the next 50 µL Griess Reagent 2 were added. The absorbances of the samples were measured at 540 nm. The standard curve was used to determine the nitrate concentration [µM].

3.8. Assessment of Mitochondrial Damages—WST-1 Test

Mitochondria were isolated essentially according to Heckman et al. [81]. Homogenization was carried out in a pH 7.6 buffer with the following composition: 350 mM mannitol, 30 mM Mops (3- (N-Morpholino) propanesulfonic acid sodium salt (Sigma-Aldrich, Poznań, Poland), 1 mM EDTA (Ethylenedinitrilotetraacetic acid; Merck, Warsaw, Poland) with the addition of 1.8 g of insoluble PVPP (Polyvinylpolypyrrolidone; Merck, Warsaw, Poland) and 0.34 g of L-cysteine in 100 mL. An amount of 0.4 g of tissue was ground in 5 mL of ice-cold buffer in a mortar. The homogenate was filtered and then centrifuged at 4732× *g* for 2 min at 4 °C. The supernatant was removed and centrifuged at 18.207× *g* for 5 min at the same temperature. The supernatant was discarded, and the pellet was washed with 1 mL of a pH 7.2 buffer containing 300 mM mannitol, 20 mM Mops and 1 mM EDTA. After a 2 min centrifugation at 4732× *g* the supernatant was transferred to new tubes, and a 0.6 M sucrose solution was added. The samples were centrifuged at 9583× *g* for 20 min. The supernatant was discarded, and the pellet was dissolved in pH 7.2 buffer, containing 250 mM sucrose and 30 mM Mops.

An assessment of mitochondrial damages was performed using the cellular cytotoxicity assay with the WST-1 reagent (Cayman Chemical). An amount of 100 µL of sample and

10 µL of WST-1 reagent solution were placed in a 96-well plate. The plate was incubated at 37 °C (the optimum temperature for conversion of tetrazolium salt to formazan) for 2 h. The absorbance of the samples was measured at 440 and 620 nm. The 620 nm absorbance was used to correct the readings for the natural hew of the extracts. The absorbance of the unheated sample was used as a blank. Mitochondrial cytotoxicity was calculated according to the formula:

$$cytotoxicity = \frac{Abscontrol - Abssample}{Abscontrol} \times 100\% \quad (1)$$

where: Abs control = the reference wavelength at 620 nm recommended by manufacturers of some cytotoxicity assay kits (Merck; Product No. CELLPRO-RO, BioChain Institut, WST-1 Cell Proliferation Assay Kit). Abs sample = absorbance of the test sample at 440 nm

3.9. Protein Isolation and HSP70 Protein Content

Proteins were isolated using the method of Isaacson et al. [82], with minor modifications. The tissue (400 mg) was ground in a cold mortar in 4 mL of 10% TCA in acetone. The extracts were transferred to Eppendorf tubes and stored at −20 °C for 24 h. The extracts were then centrifuged for 30 min at 5000× *g*. The extracts were purified by adding 4 mL of cold acetone. The pellet washing was repeated twice, followed by centrifugation for 10 min at 4 °C, at a speed of 5000× *g*. The pellet was dried at room temperature and then suspended in a TBS buffer containing 250 mM Tris, 1.37 M NaCl. The HSP70 protein content was determined using ELISA kit (EIAab Science, Wuhan, China). Then, 100 µL of protein samples were applied to a 96-well plate and then incubated at 37 °C. Further steps were carried out following the manufacturer's protocol, and the plate was incubated again at 37 °C for an hour. Next, the wells were washed again, the substrate was applied, and the reaction was carried out at 37 °C for 20 min. The absorbance at the 450 nm wavelength was measured. The sample Diluent solution was used as a blank.

3.10. Statistics

All the tests were carried out in triplicates. The results were analysed in the Statistica program using the ANOVA (univariate) test. The differences between the trials were analysed using Tukey's post-hoc test at the significance level $p \leq 0.005$.

4. Conclusions

Our studies firmly suggested that the tetracycline contamination of water leads to manifold disturbances in the metabolism of *Lemna minor* L., including: water balance; photosynthetic apparatus (chlorophyll); respiration (mitochondrial dehydrogenase activity); membrane lipid peroxidation; accumulation of free radicals and the activation of free radical scavenging mechanisms. On the other hand, duckweed shows a considerable capacity to recover from intoxication with modetate doses (up to 2.5 mM approx. 1 g × L^{-1}) of tetracycline. A significant improvement in the physiological status of the plants was observed within one week of the transfer to a tetracycline-free medium. However, the damages to the mitochondria caused by high doses of tetracycline tended to accumulate, even after the plants were transferred to an antibiotic-free medium. A wide range of plant-stress responses were probed in the experiments described, so it was not possible to go deeply into the mechanism of each of them. However, the data obtained should be helpful for predicting the outcomes of transient, accidental contamination of water reservoirs with tetracycline, one of the most widespread antibiotic pollutants of water. The data should also provide a useful framework for similar analyses in other aquatic plants and for future, more in-depth analyses.

Supplementary Materials: The following are available online. Table S1: Antibiotic contents [µg × L^{-1}] in river water, drinking water, groundwater, sea and lake water [83–125].

Author Contributions: Conceptualization, A.I.P.-C.; methodology, M.K., K.G., J.Ž., G.S. and D.J.M.; software, M.K.; validation, M.K., K.G. and H.G.; formal analysis, M.K., H.G., J.Ž., G.S. and M.W.;

investigation, M.K., H.G., K.G., J.Ž. and G.S.; writing—original draft preparation, A.I.P.-C., and M.K.; writing—review and editing, A.I.P.-C., M.K., D.J.M. and H.G.; visualization, M.K., D.J.M. and J.Ž.; supervision, A.I.P.-C.; project administration, A.I.P.-C.; funding acquisition, A.I.P.-C. All authors have read and agreed to the published version of the manuscript.

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I hereby declare that in the paper authored by **M. Krupka, D.J. Michalczyk, J. Žaltauskaite, G. Sujetoviene, K. Głowacka, H. Grajek, M. Wierzbicka, and A.I. Piotrowicz-Cieślak,** entitled *"Physiological and biochemical parameters of common duckweed Lemna minor after exposure to tetracycline and recovery from this stress"* published in ***Molecules* 2021, 26: 6765,** my substantive contribution to its preparation consisted of the analysis of antioxidant enzyme activity.

I also give my consent for the above-mentioned work to be submitted by M.Sc. Eng. Magdalena Krupka as part of her doctoral dissertation, presented as a thematically coherent collection of scientific articles published in peer-reviewed journals.

I declare that the independent and identifiable contribution of the candidate (65%)—M.Sc. Eng. Magdalena Krupka—comprises the following elements: meticulous planning of the entire research process, considering the specificity of the scientific problem and the research objectives; selection of optimal research methods and tools, which enabled the execution of the research objectives with a high level of scientific reliability and accuracy; ensuring that the chosen research techniques were appropriately aligned with the defined scope and scale of the study, facilitating precise data collection and analysis; independent performance of certain laboratory experiments according to the pre-established methodology; detailed documentation of each stage of the research, including the preparation of comprehensive notes, reports, and protocols from conducted experiments; rigorous analysis of the collected data using appropriate analytical and statistical tools, which yielded reliable and significant results; formulation of key conclusions that formed the basis of the primary theses of the work; scientific interpretation of results, situating the findings within a broader research context and the relevant literature; independent writing of the manuscript, including clear presentation of the conclusions, detailed justification of results, and their interpretation; creation of a cohesive and logical description of the research methodology, results, and their discussion in the context of previous studies in the literature; preparation of illustrations and tables that depict the research results and highlight key information, enhancing the clarity and visual impact of the findings.

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Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr inż. Magdalenę Krupkę** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych. Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata (65%) **mgr inż. Magdaleny Krupki** który polegał na precyzyjnym zaplanowaniu całego procesu badawczego, z uwzględnieniem specyfiki problemu naukowego oraz celów badania. Doborze optymalnych metod i narzędzi badawczych, które umożliwiły realizację założeń badawczych na wysokim poziomie rzetelności naukowej i dokładności. Zapewnieniu, by wybrane techniki badawcze były dostosowane do założonego zakresu i skali badań, co pozwoliło na precyzyjne zbieranie oraz analizę wyników. Samodzielnym wykonaniu niektórych doświadczeń laboratoryjnych, zgodnie z wcześniej przygotowaną metodologią. Dokumentowaniu każdego etapu badania, w tym sporządzeniu szczegółowych notatek, raportów oraz protokołów z przeprowadzonych eksperymentów. Dokładnym opracowaniu zebranych danych, z zastosowaniem odpowiednich narzędzi analitycznych i statystycznych, co pozwoliło na uzyskanie rzetelnych i znaczących wyników. Wyciąganiu kluczowych wniosków, które stały się fundamentem głównych tez pracy. Przeprowadzeniu naukowej interpretacji wyników, co umożliwiło osadzenie uzyskanych wyników w szerszym kontekście badawczym oraz literaturze przedmiotu. Samodzielnym napisaniu manuskryptu pracy, który obejmował przejrzyste przedstawienie uzyskanych wniosków, szczegółowe uzasadnienie wyników oraz ich interpretację. Stworzeniu spójnego i logicznego opisu metodologii badawczej, wyników oraz ich omówienia w kontekście dotychczasowych badań z zakresu literatury przedmiotu. Stworzeniu rysunków i tabel ilustrujących wyniki badań oraz przedstawiających kluczowe informacje, co ułatwiło przejrzystość prezentacji wyników i zwiększyło wartość wizualną pracy.

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A. Piotrowicz-Cieślak

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podpis

Publikacja 3

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REGULAR PAPER – ECOLOGY/ECOPHYSIOLOGY/ENVIRONMENTAL BIOLOGY

Critical stages in pea photosynthesis impaired by tetracycline as an environmental contaminant

Magdalena Krupka[1] · Dariusz J. Michalczyk[1] · Agnieszka I. Piotrowicz-Cieślak[1]

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Abstract

The widespread use of antibiotics in intensive animal husbandry, and the agricultural utilization of manure from such farms, imposes a significant burden on the environment. Consequently, the effects of antibiotics should be studied not only in animals and humans but also in all components of biocenoses and agrocenoses. In our study, we analyze the impact of four different concentrations of tetracycline present in soil (0, 5, 50, and 500 mg/kg of soil) on the growth and key photosynthesis parameters of pea seedlings: chlorophyll concentration, aminolevulinic acid concentration, aminolevulinic acid dehydrogenase activity, and ribulose bisphosphate carboxylase-oxygenase (RuBisCO) activity. At the lowest tetracycline concentration, chlorophyll content decreased by 13% compared to the control (0 tetracycline), while at the highest antibiotic concentration, it decreased by as much as 27%. Similarly, the decrease in aminolevulinic acid (a chlorophyll precursor) concentration was significant, amounting to 34%. However, the activity of the dehydrogenase enzyme, which consumes this precursor, decreased even more drastically by 51%, indicating significant disturbances in the light phase of photosynthesis. However, the activity of RuBisCO in pea plants subjected to tetracycline was even more severely affected, dropping by 58%, 69%, and 70% in soils with increasing concentrations of tetracycline. The reduction in enzyme activity could only partially be explained by a less pronounced decrease in the quantity of RuBisCO (large subunit) protein, which amounted to 6.5%, 11%, and 35% for tetracycline concentrations of 5, 50, and 500 mg/kg of soil, respectively.

Keywords Pea · Ribulose bisphosphate carboxylase-oxygenase activity · Tetracycline

Introduction

Soil pollution is a serious environmental issue. Recently, antibiotics and their metabolites have been detected in soils. The widespread use of pharmaceuticals in animal production is one of the main causes of the entry of these compounds into the environment (Gržinić et al. 2023). In intensive and mass animal production, antibiotics are used for therapeutic, preventive, and growth promotion purposes (Muaz et al. 2018). Although the use of antibiotics as growth promoters has been banned in EU countries, they are still administered therapeutically to the entire herds instead of individual animals (FAO 2021). In Asian countries, there are legal regulations regarding the use of antibiotics in animal production, but these substances are still widely available and used, among others as growth promoters (Shao et al. 2021). According to the CDEEP report, the annual consumption of antibiotics by livestock will exceed 200,000 tons by 2030. The incomplete metabolism of these drugs in animal bodies results in the excretion of approximately 40–90% of the administered dose in an active form in urine and feces (Polianciuc et al. 2020). Tetracycline (TC) is one of the most commonly used antibiotics in animal production, and its residues are found in agricultural soils worldwide (Conde-Cid et al. 2020). The application of untreated manure to fields containing tetracycline residues is one of the main sources of this antibiotic in soil. The lack of effective methods for removing antibiotics in wastewater treatment plants means that irrigation of fields with processed

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waste water may also lead to the antibiotics entering soil. Tetracycline present in the soil is taken up and accumulated in plant tissues (Tasho and Cho 2016), causing morphological and biochemical changes (Rocha et al. 2021).

Numerous studies indicate that photosynthesis is the most sensitive metabolic process to the action of antibiotics (Krupka et al. 2022). Antibiotics can enter chloroplasts, possibly with the help of MAR1 transporters (Conte and Lloyd 2010; Margas et al. 2019), thereby directly affecting the photosynthetic apparatus. The first visible symptoms of damage to the photosynthetic apparatus include leaf yellowing (Margas et al. 2019; Rydzyński et al. 2017). At the biochemical level, the effects of antibiotics on the photosynthetic apparatus are not yet fully understood. Several studies suggest that one of the causes of damage to the photosynthetic apparatus may be the accumulation of reactive oxygen species (ROS) in chloroplasts. Hájková et al. 2019 suggested that a 10-minute exposure to pharmaceuticals results in an increase in ROS content in chloroplasts. The accumulation of ROS then leads to lipid peroxidation in thylakoid membranes (Hájková et al. 2019). Proteins associated with photosystem II are particularly sensitive to ROS accumulation (Gomes et al. 2020). A detailed characterization of photosystem damage under the influence of antibiotics is presented in Krupka et al. (2021).

Tetracyclines inhibit bacterial protein synthesis by binding to the bacterial 30 S ribosome subunit (Scaria et al. 2021). Some studies suggest that antibiotics that block bacterial protein biosynthesis are particularly toxic to the photosynthetic apparatus (Liu et al. 2011) due to the fact that chloroplasts have bacterial transcription and translation mechanisms (Kasai et al. 2004). In addition to directly affecting the proteins involved in photosystems, antibiotics likely also affect the chlorophyll biosynthesis pathway (Krupka et al. 2022). The chlorophyll biosynthesis pathway involves 16 reactions catalyzed by 16 enzymes encoded by more than 20 different genes (Beale 2005). Chloroplast protein complexes are encoded by both nuclear and chloroplast genes (Yoon et al. 2020). Koussevitzky et al. (2007) demonstrated that the expression of a nuclear gene involved in chlorophyll synthesis can become inhibited when the chloroplast gene expression is not occurring. Therefore, antibiotics can affect chlorophyll biosynthesis at the very beginning of the biosynthetic pathway once they enter the chloroplast. However, most studies neglect this aspect. Another aspect of the impact of antibiotics on photosynthesis is their effect on the dark phase of photosynthesis. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is a key enzyme of the Calvin cycle, catalyzing the binding of CO_2 to ribulose-1,5-bisphosphate (RuBP). The activity of this enzyme is a key factor determining the efficiency of photosynthesis. Margas et al. (2019) demonstrated a decrease in RuBisCO content in plants exposed to tetracycline, although its activity in plants treated with tetracycline has not yet been investigated. Most studies on the impact of antibiotics on photosynthesis are speculative in nature, and interactions between antibiotics and the photosynthetic apparatus are still not fully understood. Most of the available studies on antibiotic interactions in the photosynthetic apparatus refer to chlorophyll degradation and consequent visible changes at the morphological level. Leaf yellowing is a visible end-effects of tetracycline-induced damage, but does not provide information on the molecular and biochemical mechanisms leading to these changes. The mechanism of tetracycline-induced chlorophyll degradation has already been studied (Rydzyński et al. 2019). However, to fully understand the effects of tetracycline on the photosynthetic apparatus, it is necessary to identify the exact sites of tetracycline action in plants, such as chlorophyll biosynthesis and the Calvin cycle. Therefore, the aim of this study is to assess how tetracycline affects key biochemical and molecular elements of the photosynthetic process of peas (*Pisum sativum* L. cv. Cysterski) growing in soil contaminated with tetracycline at concentrations of 5, 50, and 500 mg/kg. Additionally, the utility of spectrophotometric methodology for assessing the activity of RuBisCO in pea seedlings was evaluated. To the best of our knowledge, such studies are rare in the literature. Photosynthetic parameters such as chlorophyll precursor content, chlorophyll biosynthetic pathway enzyme activity and RuBisCO enzyme activity will allow a multi-stage evaluation of the effect of tetracycline on the photosynthetic apparatus of plants. This will allow identification of the key steps at which tetracycline interferes with photosynthesis and an understanding how this interference leads to the negative effects observed in plants. Tetracyclines are antibiotics routinely detected in agricultural soils. Their concentrations in soils usually range from 5 to 25 mg/kg (Zheng et al. 2020), whereas in manure, concentrations exceed 700 mg/kg (Santás-Miguel et al. 2022). Tetracycline is a poorly degraded antibiotic that is constantly released into the environment (Amangelsin et al. 2023), so higher concentrations were also used in the study.

Materials and methods

Pea growth conditions

200 g of soil was added to 9 cm × 9 cm pots. Tetracycline at concentrations of 5, 50 and 500 mg/kg was added to pots. Tetracycline hydrochloride was added as an aqueous solution in 15 ml distilled water according to Margas et al. (2019). The control group were pots without tetracycline watered with 15 ml distilled water. Four pea seeds were

placed in each pot immediately after the addition of the antibiotics. Plants were grown in a greenhouse at 23 °C/16°C day/night, 16/8 h photoperiod in 8 klx light intensity. After 3 weeks the plants were harvested for further analysis.

Pea growth analysis

Pea growth analysis included germination (%), root length (cm), stem length (cm) and leaf area (cm^2). For the germination test, the seeds were surface sterilized with a 1% sodium hypochlorite solution for 10 min and then thoroughly rinsed with distilled water. 10 seeds were placed in pots and transferred to the greenhouse with 23 °C/16°C day/night temperature in 8 klx light intensity. Seeds were considered germinated if the root reached a length of at least 2 mm. For root and stem length, after 3 weeks of growth, plants were gently pulled from the soil and rinsed with water. The roots were straightened without damaging them and their length was measured with a ruler to 1 mm accuracy. Stems were measured from the base (the point where the root turns into a stem) to the apex, using a ruler to 1 mm accuracy. The leaf area was determined using a VHX-7000 digital microscope equipped with image analysis software (Keyence, Osaka, Japan).

Isolation and measurement of chlorophyll absorption

Chlorophyll was isolated according to the method of Rydzyński et al. (2017) with minor modifications. 500 mg of pea leaves were homogenized with a pestle and mortar in 5 ml of methanol (Fisher Chemicals). The homogenate was centrifuged at 1500 × g. The resulting supernatant was diluted 6 – fold with methanol. The absorption spectra of solutions were measured using TECAN Infinite 200 PRO. Chlorophyll a, b and total chlorophyll concentration was calculated according to Porra et al. (1989). Additionally, calculations were performed based on the absorption at $\lambda = 664$ nm according to the Lambert-Beer law, using the molar extinction coefficient of 66,600 M/cm for chlorophyll in methanol according to Seely and Jensen (1965).

Aminolevulinic acid dehydrogenase (ALAD) activity

ALAD was extracted according to the method of Jiao et al. (2015) with minor modifications. 500 mg of pea leaves was ground in liquid nitrogen in a cold mortar and 5 ml of a mixture containing 0.05 M Tris-HCl pH 8.2 and 0.1 mM DTT (Sigma – Aldrich, USA). The homogenate was filtered through gauze, then centrifuged at 10,000 × *g* for 30 min at 4°C and the supernatant was taken. 0.27 ml ALA (concentration 1 mg/ml), 1.35 ml 0.05 M Tris-HCl pH 8.2 with 0.1 mM DDT, 0.08 ml 0.2 M $MgCl_2$ were added to 1 ml of the supernatant,. The mixture was incubated at 37 °C for 1 h. The reaction was stopped by adding 0.3 ml of 3 M trichloroacetic acid (TCA). After cooling, the samples were centrifuged at 2000 × *g* for 10 min. 1 ml of Ehrlich's reagent (4-(dimethylamino) benzaldehyde, Sigma – Aldrich, USA) was added to 1 ml of the supernatant After 10 min, the absorbance was measured (Cecil spectrophotometer, CE2021 2000 Series) at $\lambda = 555$ nm. ALAD activity was expressed as the amount of porphobilinogen (PBG) formed per 1 min per mg of protein per cm^2 of leaves. The molar extinction coefficient of 6.2×10^4 M/cm was used for the calculations according to Kumar and Charan (1998).

Aminolevulinic acid (ALA) content

ALA content was determined according to the method of Jiao et al. (2015). 500 mg of pea leaves 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, 4 drops of ethyl acetate (Chempur) were added to 1 ml of the supernatant and boiled for 15 min in a water bath. 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 $\lambda = 533$ nm (Cecil spectrophotometer, CE2021 2000 Series). ALA concentration was calculated according to Averina et al. (2010), using the molar extinction coefficient for ALA 6.8×10^4 M/cm. ALA concentration was expressed in nmol per cm^2 of leaves.

Leaf protein isolation and SDS-PAGE electrophoresis

Leaf protein isolation was carried out according to Li et al. (2009) with minor modifications. 500 mg of pea leaves were homogenized in liquid nitrogen with mortar and pestle. Then, 3 ml of isolation buffer containing 5 mM β – mercaptoethanol (Sigma – Aldrich, USA), 50 mM Tris – HCl (pH 8) and 12.5% (v/v) glycerol (Chempur) was added. The samples where then centrifuged at 1500 × *g* for 15 min at 4 °C. Protein concentration in the resulting supernatant was carried out using Bradford assay (1976) using bovine serum albumin (Sigma – Aldrich, USA) as a standard.

Resulting supernatants were then mixed with dissolving solution containing 2% SDS (w/v), 4% β – mercaptoethanol (v/v) and 10% glycerol (v/v), so as to obtain the final protein concentration of 2 mg/ml. The samples were incubated in a dry bath for 5 min at 100 °C. The samples were cooled down and 40 µg of proteins were loaded to a 10% separating and 5% concentrated polyacrylamide gel (7.0 × 10.0 cm). SDS-PAGE electrophoresis (Mini PROTEAN TetraSystem; Bio-Rad) was run at 80 V for 10 min and 140 V for 1 h.

After electrophoresis the gels were stained in 0.25% Coomassie Brilliant Blue G-250 (Sigma – Aldrich, USA) solution for 12 h. The gels were destained until the background was colourless. Gel images were digitized with Gel Doc EZ Imager (Bio-Rad). Electrophorograms were analyzed and RuBisCO large subunit concentration was calculated with ImageLab software (Bio – Rad) using peaks intensity, corresponding to indentified proteins. The identification of RuBisCO large subunit was carried out using commercial RuBisCO (Sigma – Aldrich, USA). The commercial RuBisCO was prepared in the same way as proteins samples using dissolving solution and loaded to a polyacrylamide gel.

Isolation and measurement of RuBisCO activity

RuBisCO was isolated from pea leaves according to Tavarini et al. (2016) with minor modifications. 500 mg of pea leaves were ground with liquid nitrogen in mortar and pestle. Then 3 ml of isolation mixture containing 100 mM Tricine – KOH (pH 8.0), 1 mM (EDTA), 1% β – mercaptoethanol (v/v), 1 mM phenylmethylsulfonyl (PMSF), and 5% polyvinylprrolidone (PVP) (w/w of sample fresh weight) was added. Samples where then centrifuged at 12,000 × *g* for 20 min at 4 °C. Protein content in the resulting supernatant was measured by Bradford assay (1976) using bovine serum albumin (Sigma – Aldrich, USA) as a standard.

RuBisCO activity was assayed in a reaction mixture containing 100 mM bicine (pH 8.0), 10 mM $MgCl_2$, 0.2 mM EDTA, 5 mM dithiothreitol (DTT), 40 mM $NaHCO_3$, 4 mM ATP, 0.2 mM NADH, 0.2 mM RuBP, one enzyme unit of 3-phosphoglycerate kinase (PGK) and one enzyme unit of glyceraldehyde 3-phosphate dehydrogenase (3-PGADH). To measure RubisCO activity, a method based on the NADH-linked assay was used. This test is based on RuBP carboxylation and the formation of 3-phosphoglyceric acid (3-PGA). 3-PGA is then converted to 1,3 PGA by 3-phosphoglycerate kinase (PGK) using ATP. 1,3 PGA is then reduced to glyceraldehyde-3-phosphate (G3P) with oxidation of NADH to NAD^+ by glyceraldehyde 3-phosphate dehydrogenase (3-PGADH). A schematic of the reaction is shown in Fig. 1. 50 µl of enzyme was added to the mixture (final concentration of RuBisCO in reaction mixture was 25 µg/ml) and absorbance spectra were recorded within the wavelength range 310–370 nm for 40 min using the Carry 300 UV Visible Spectrophotometer (Varian, Inc). The reaction temperature was monitored using a laboratory thermometer and held at 25 °C. The reaction was initiated by the addition of RuBP. Single measurement was recorded every 30 s (80 measurements were conducted). RuBisCO activity was calculated according to Sales et al. (2020), using the molar extinction coefficient for NADH, ε = 6220 M/cm. RuBisCO activity was expressed as µmol NADH/ min/mg protein.

Validation of RuBisCO activity measurement method

In order to validate the method, enzyme activity was measured by maintaining different reaction temperatures: 15 °C, 18 °C, 20 °C, 25 °C and 40 °C using previously described method. Final concentration of RuBisCO in reaction mixture was 25 µg/ml. In addition, different concentrations of RuBisCO were used to measure activity: 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml. Temperature was maintained at 25 °C. Absorbance spectra were recorded within the wavelength range 310–370 nm for 40 min using the Carry 300 UV Visible Spectrophotometer (Varian, Inc) for 40 min. RuBisCO activity was calculated according to Sales et al. (2020), using the molar extinction coefficient for NADH, ε = 6220 M/cm. RuBisCO activity was expressed as µmol NADH/ min/mg protein.

Statistics

The analyses of plant growth parameters were performed in 10 repetitions. Aminolevulinic acid (ALA) content, aminolevulinic acid dehydrogenase (ALAD) activity, chlorophyll content, RuBisCO content and activity were determined in triplicates. The results were analysed using the Statistica program 11 and a one-way ANOVA test. Differences between groups were analysed using Tukey's post-hoc test with probability $p \leq 0.05$.

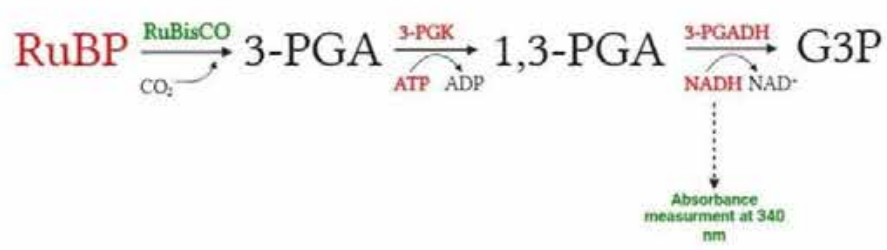


Fig. 1 A schematic of the reaction for determining RuBisCO activity

Table 1 Growth parameters of pea grown on soil with 0, 5, 50 and 500 mg/kg tetracycline addition. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test)

Tetracycline concentration in soil [mg/kg]	Germination [%]	Root length [cm]	Shoot length [cm]	Total leaf area [cm^2]
0	98 ± 0.3 **a**	16.5 ± 2.3 **a**	14.5 ± 1.4 **a**	136.0 ± 7.8 **a**
5	98 ± 0.3 **a**	15.9 ± 2.5 **a**	14.1 ± 1.1 **a**	134.1 ± 5.6 **a**
50	88 ± 0.4 **b**	10.4 ± 1.5 **b**	13.1 ± 1.0 **a**	125.7 ± 5.2 **b**
500	77 ± 0.4 **c**	9.5 ± 0.9 **b**	12.8 ± 0.9 **b**	122.3 ± 4.2 **b**

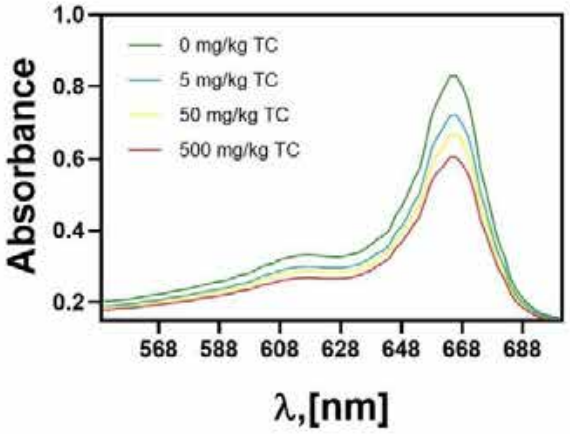


Fig. 2 Absorption spectra of chlorophyll isolated from pea grown on soil

Results and discussion

The study examined the effect of tetracycline at concentrations of 5, 50 and 500 mg/kg on pea, with particular attention paid to photosynthetic parameters. The utility of the spectrophotometric method for assessing the activity of RuBisCO was also evaluated in this study. Furthermore, the obtained results were verified through the evaluation of selected photosynthetic parameters, including chlorophyll concentration, aminolevulinic acid (ALA) concentration, aminolevulinic acid dehydrogenase (ALAD) activity, and the content of isolated RuBisCO enzyme.

The first visible symptoms of the action of antibiotics in plants include changes at the morphological level. A general appearance of pea plants is shown in Fig. S1. Fig. S1 presents properly developed seedlings. It is important to note that significant changes in seed germination were observed after treatments with 50 and 500 mg/kg tetracycline (Table 1). The germination rate decreased by 12% and 23%, respectively. Cheong et al. (2020) also demonstrated reduced germination in plants treated with chlorotetracycline. Tetracycline at concentrations of 50 and 500 mg/kg caused a reduction in root length by 6.1 and 9 cm, respectively (Table 1). A decrease in shoot length was observed in plants treated with the highest tetracycline concentration. Xie et al. (2011) also observed a reduction in growth, expressed as a decrease in root and stem length, in wheat treated with higher concentrations of tetracycline (up to 300 mg/l). The authors state that the root is more sensitive to tetracycline action than the shoot, which was confirmed by our results. A change in the color of photosynthetic organs is the first symptom of tetracycline action on the photosynthetic apparatus (Krupka et al. 2022). At the morphological level, the effect of tetracycline in plants may also be expressed as a reduction in leaf area. The total leaf surface area of pea plants growing in soil contaminated with tetracycline at concentrations of 50 and 500 mg/kg decreased by 7.6% and 10%, respectively, compared to the control (Table 1).

Changes in the morphology of photosynthetic organs in plants cultivated in antibiotic-contaminated soils result from alterations in the photosynthetic apparatus. One of the key factors determining proper plant and organ growth is chlorophyll. The direct mechanism of chlorophyll degradation by tetracycline was demonstrated by Rydzyński et al. (2019). The authors showed that the degradation product of chlorophyll resulting from the action of tetracycline is pheophytin, and the mechanism of this process involves the direct removal of the Mg^{2+} ion from chlorophyll by tetracycline. The removed magnesium binds to the tetracycline molecule at two sites - the BCD ring and the A ring, depending on the magnesium concentration (Rydzyński et al. 2019).

Our results indicate that the lowest tetracycline concentration (5 mg/kg) led to a decrease in chlorophyll a absorbance at $\lambda = 665$ nm from $A = 0.83$ to $A = 0.72$ (Fig. 2) (a 17% decrease in chlorophyll concentration in the plant) (Table 2). These findings clearly demonstrate that even low concentrations of tetracycline, as sometimes detected in the environment, cause chlorophyll degradation. Increasing the tetracycline concentration 500 mg/kg resulted in a decrease in absorbance to $A_{665} = 0.6$, respectively (Fig. 2), and thus a reduction in chlorophyll a concentration by 20% and 28% (Table 2).

Some studies indicate that tetracycline can block the synthesis of both chlorophyll a and b by inhibiting enzyme biosynthesis. A decrease in chlorophyll b content in tetracycline-treated *Lemna minor* was demonstrated by Baciak et al. (2016). However, our results indicate a slight decrease in chlorophyll b concentration in peas (Table 2). It can therefore be concluded that chlorophyll a is much more sensitive to tetracycline-induced degradation, than chlorophyll b.

Table 2 Chlorophyll a, b, total chlorophyll content (mg/cm^2) and chlorophyll a/b ration in pea grown on soil with 0, 5, 50 and 500 mg/kg tetracycline addition. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test)

Tetracycline concentration in soil [mg/kg]	Chlorophyll a content [μg/cm^2]	Chlorophyll b content [μg/cm^2]	Total chlorophyll [μg/cm^2]	Chlorphyll a/b ratio
0	0.47 ± 0.01 **a**	0.26 ± 0.006 **a**	0.73 ± 0.012 **a**	1.79 ± 0.056
5	0.39 ± 0.006 **b**	0.24 ± 0.006 **b**	0.63 ± 0.01 **b**	1.67 ± 0.035
50	0.39 ± 0.006 **b**	0.24 ± 0.012 **b**	0.63 ± 0.015 **b**	1.62 ± 0.104
500	0.34 ± 0.015 **c**	0.25 ± 0.006 **b**	0.60 ± 0.01 **c**	1.37 ± 0.089

The chlorophyll a/b ratio is a good parameter for assessing the state of the photosynthetic apparatus in plants. Any disturbances of this parameter may indicate damage of the photosynthetic apparatus (Krupka et al. 2022). Our results indicate that tetracycline in a concentration range 5–500 mg/kg caused a disturbance in the chlorophyll a/b ratio (Table 2).

To demonstrate at which stage tetracycline may interfere with the photosynthesis, the activity of an enzyme involved in chlorophyll biosynthesis (ALAD) was examined. However, our results indicate that despite the inhibition of ALAD activity (Fig. 3a) in plants treated with higher tetracycline, there was also a reduction in the concentration of ALA (Fig. 3b). Similar results were obtained by Liu et al. (2014) when treating *Selenastrum capricornutum* with antibiotics. The authors pointed out that erythromycin, as an antibiotic inhibiting bacterial protein synthesis, exhibits high toxicity towards chlorophyll precursors, even at low concentrations. As a mechanism of erythromycin's toxicity towards chlorophyll precursors, the authors identified the inhibition of chloroplast gene expression, and subsequently, indirectly, nuclear genes responsible for chlorophyll biosynthesis (Liu et al. 2014). Tetracycline, as an antibiotic inhibiting protein synthesis, may also exhibit a similar mechanism of toxicity. However, further research is needed to address this issue.

RuBisCO is the most widely occurring plant protein, consisting of 8 large and 8 small subunits (Abdulbaki et al. 2022). The small subunits are encoded by the nuclear gene family rbcS, while the large subunits are chloroplast-encoded, by the rbcL gene (Wostrikoff and Stern 2007). Although the impact of pharmaceuticals on the structure and activity of the RuBisCO enzyme is only partially understood, Liu et al. (2014) demonstrated reduced expression of the rbcL gene in the chloroplasts of *Microcystis aeruginosa* algae treated with antibiotics. Similar results were obtained by Yoon et al. (2020) when treating *Brassica campestris* with erythromycin at a concentration of 5 mg/kg. As an antibiotic inhibiting protein synthesis, erythromycin reduced the expression level of proteins involved in photosynthesis, including the large subunit of RuBisCO, leading to a decrease in its content. The down regulation of the rbcL gene was identified as the cause of the reduced protein content (Yoon et al. 2020).

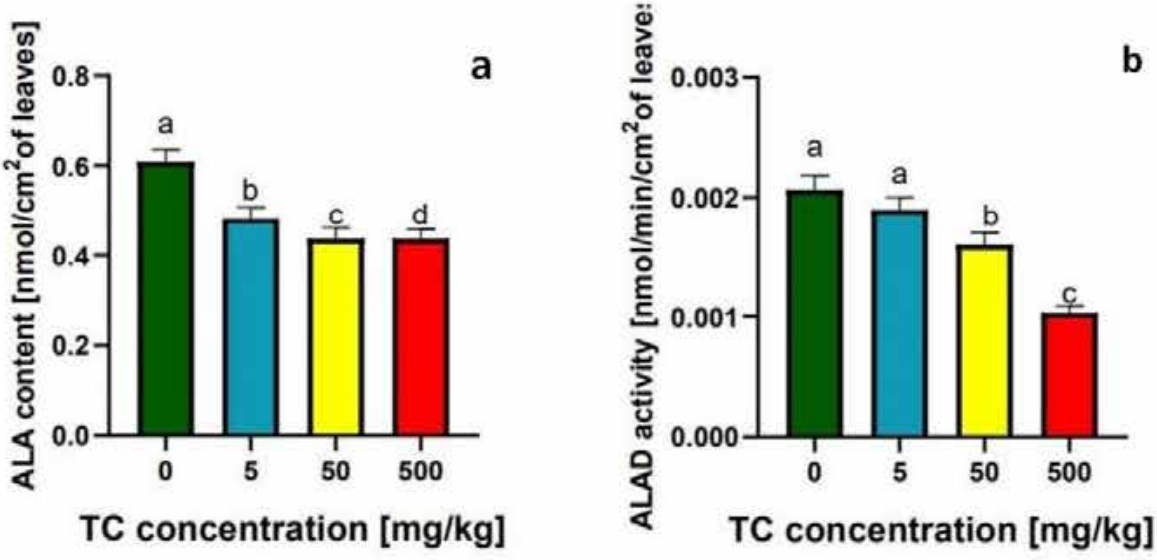


Fig. 3 δ-Aminolevulinic acid (ALA) content [nmol/cm^2 of leaves] (**a**) and acid dehydrogenase (ALAD) activity [nmol PGB/min/mg protein/cm^2 of leaves] (**b**) in peas grown on soil without tetracycline and 5, 50 500 mg/kg tetracycline addition. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test)

Tetracycline, as an antibiotic inhibiting protein synthesis, likely exhibits a similar mechanism of action.

The content of the large subunit of RuBisCO (LSU) in plants treated with tetracycline was assessed in this work using SDS-PAGE electrophoresis (Fig. 4). To properly assess the quality of the isolated enzyme from peas, we conducted an SDS-PAGE analysis (Fig. 4b), whose quality we evaluated by calculating the area of the obtained electrophoretic bands after conversion to peaks (Fig. 4a). The most abundant leaf protein fraction was in the 50–55 kDa mass range (Fig. 5), with a content of 17 µg/ml (Table 2). This identification of this protein fraction as the large subunit of RuBisCO was confirmed by comparison to a commercial enzyme. In the commercially purchased RuBisCO, the area of the LSU was 1802, and for the LSU isolated after applying tetracycline at concentrations of 0, 5, 50, and 500 mg/kg, the areas were 1387, 1294, 1228, and 896, respectively. However, the total area of all proteins (calculated from Fig. 4a) isolated was approximately 4,000,000 area units for all five variants, including the commercial RuBisCO. The analyzed protein was used at the same concentration for all variants, which fully confirms the good quality of the isolated enzyme. The application of tetracycline at concentrations of 5, 50, and 500 mg/kg resulted in a decrease in LSU content by 6.5%, 11%, and 35%, respectively, compared to the control (Fig. 4c). Cohen et al. (2005) indicate that the removal of one subunit can influence the expression of other subunits in the complex, thereby affecting the enzyme's activity.

The activity of RuBisCO is a crucial factor determining the efficiency of the photosynthesis process. Under abiotic stress conditions, one of the factors leading to reduced enzyme activity is the degradation of RuBisCO activase. RuBisCO activase acts as a chaperone responsible for changing the enzyme's conformation, thereby regulating its function (Waheeda et al. 2023). Margas et al. (2019) demonstrated a decreased concentration of RuBisCO activase in pea seedlings treated with tetracycline; however, the impact of tetracycline on the activity of this enzyme is not well-documented. Therefore, both the decrease in LSU content and the degradation of activase may affect RuBisCO activity. Our current results indicate, however, that tetracycline caused a decrease in RuBisCO activity more pronounced than the drop in RuBisCo quantity in pea leaves. The application of an antibiotic in the soil at a concentration of 5 mg/kg resulted in a 19% decrease in enzyme activity compared to the control (Table 3). Increasing the concentration of tetracycline to 50 and 500 mg/kg resulted in a decrease in RuBisCO activity by 37% and 47% (Table 3). Figure 5a-d shows the decrease in absorbance over time (40 min). The decrease in absorbance at 340 nm over time indicates the progression of the reaction catalysed by RuBisCO. A decrease in absorbance at $\lambda = 340$ nm from $A = 0.672$ to $A = 0.471$ was observed in control pea, indicating high enzymatic

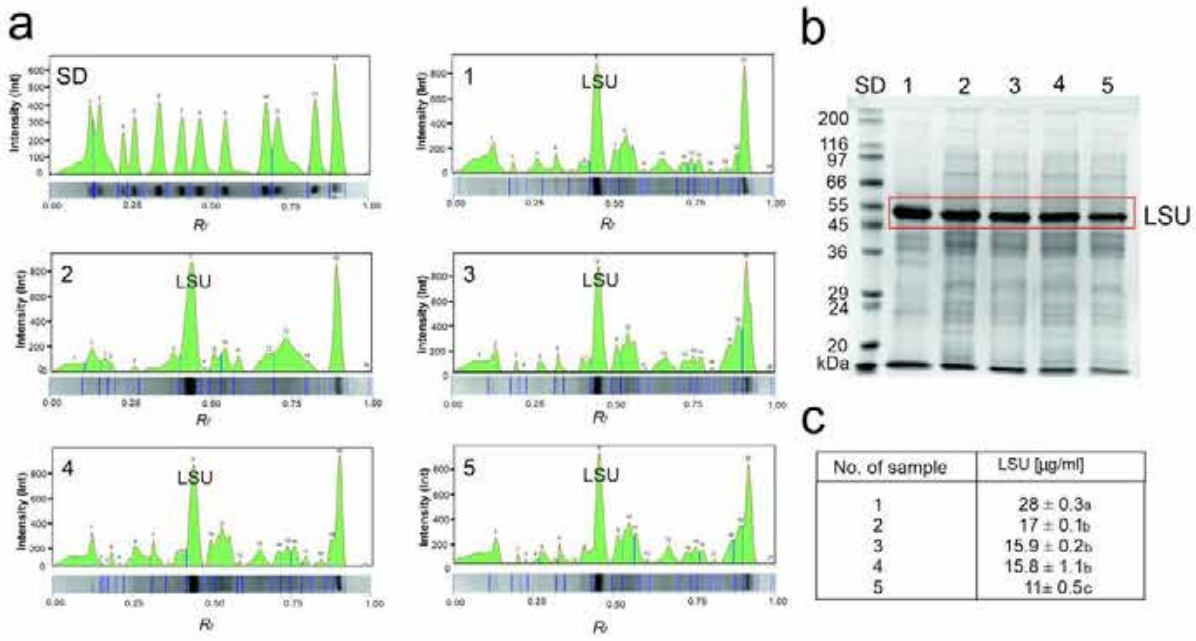


Fig. 4 **a** - Intensity of individual proteins; **b** - SDS – PAGE protein pattern and large subunit concentration (LSU) of RuBisCO; **c** – Content of large subunit concentration (LSU); SD – standard of protein, 1 – commercial RuBisCO, 2 – RuBisCO isolated from pea grown on soil without tetracycline, 3 – RuBisCO isolated from pea grown on soil with 5 mg/kg tetracycline addition, 4 – RuBisCO isolated from pea grown on soil with 50 mg/kg tetracycline addition, 5 – RuBisCO isolated from pea grown on soil with 500 mg/kg tetracycline addition

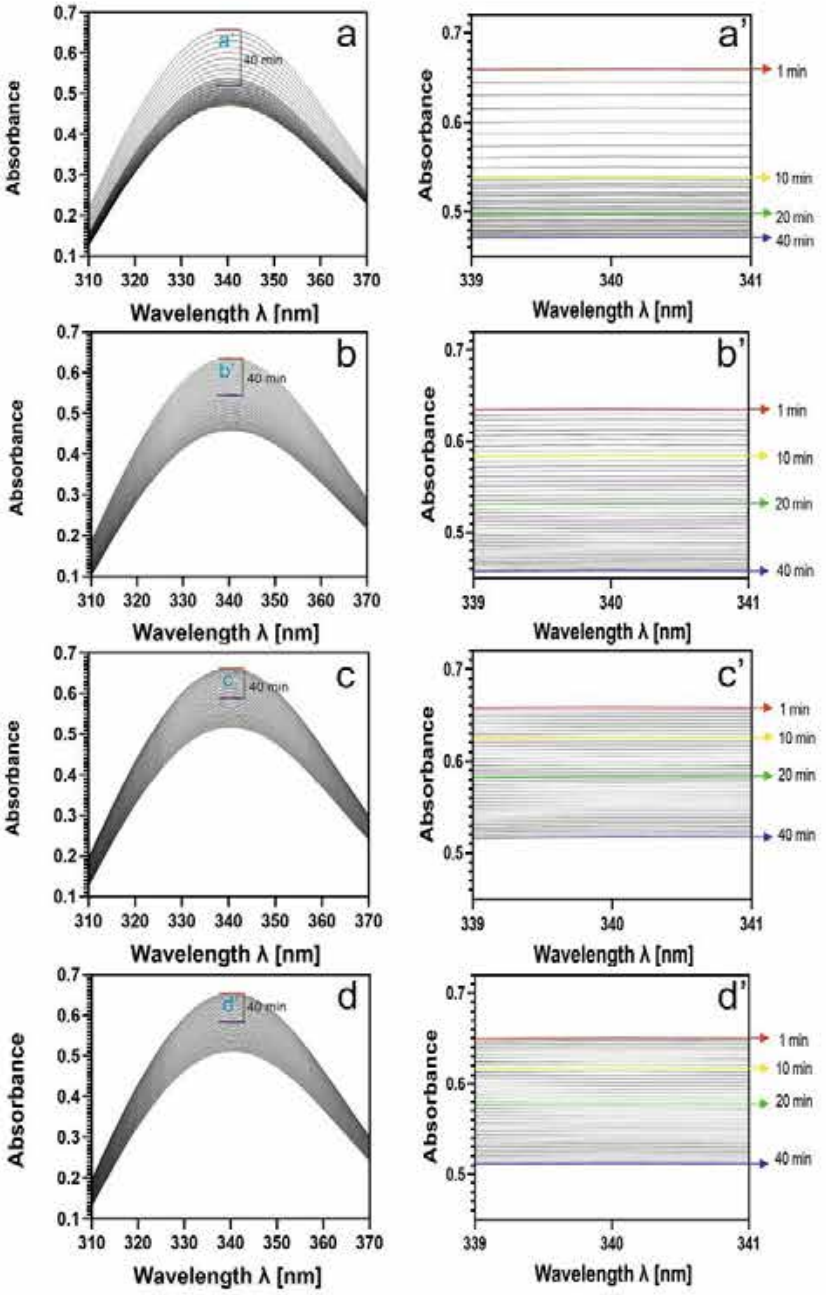


Fig. 5 Absorption spectra of NADH (80 measurements) in the wavelength range of 310–370 nm for determining RuBisCO activity from peas grown on soil contaminated with tetracycline at doses of 0 (**a**), 5 (**b**), 50 (**c**), and 500 (**d**) mg/kg. The rectangles marked on these figures refer to NADH absorption in the narrow range of 339 to 341 nm, whose data are presented separately in panels **a'**, **b'**, **c'**, and **d'**. Absorption measurements were taken over 40 min

activity. After treatment with tetracycline, the decrease in absorbance was slower, suggesting an inhibitory effect of tetracycline on RuBisCO activity. For pea treated with 5 mg/kg tetracycline, a decrease in absorbance at $\lambda = 340$ nm from $A = 0.636$ to $A = 0.459$ was observed (Fig. 5b). Increasing the concentration of tetracycline to 50 mg/kg resulted in a decrease in absorbance from $A = 0.662$ to $A = 0.518$. The highest tetracycline concentration caused a decrease in absorbance at $\lambda = 340$ nm from $A = 0.653$ to $A = 0.511$ (Fig. 5d). Therefore, even low concentrations of tetracycline, as found in the environment, lead to reduced RuBisCO activity, thereby influencing a decrease in photosynthetic efficiency. The linear decrease in absorbance at 340 nm is shown in Fig. 5a'-d'. The graph can be used to validate the assay method by demonstrating a consistent linear relationship under controlled conditions. Smaller

Table 3 Activity [µmol NADH/min/mg protein] of RuBisCO isolated from pea grown on soil with 0, 5, 50 and 500 mg tetracycline/kg of soil after 10 and 20 min. RuBisCO activity were measured using NADH-linked microtiter plate-based assay: GAPDH–GlyPDH. Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test)

Tetracycline mg/kg	Activity of RuBisCO [µmol NADH/min/mg protein]		
	10 min	20 min	Linear decline in absorbance
0	0.234 **a**	0.181 **a**	0.234 **a**
5	0.098 **b**	0.099 **b**	0.099 **b**
50	0.064 **c**	0.072 **c**	0.072 **c**
500	0.062 **c**	0.069 **c**	0.070 **c**

distances between lines may suggest that tetracycline inhibits the enzyme activity (Fig. 5b'–d'). The NADH absorption between two measurements for RuBisCO isolated from plants grown on soil uncontaminated with tetracycline was 0.02, whereas for plants from soil contaminated with the highest dose of tetracycline, the decrease in absorption between two measurements was only 0.00085, which we observe clearly as a densification of the lines. Comparing the control samples (Fig. 5a') with those treated with tetracycline, we observe a significant reduction in the rate of absorbance decrease in the treated samples, indicating an inhibitory effect of tetracycline on RuBisCO activity.

In this study, RuBisCO activity was investigated using a spectrophotometric method, employing absorbance spectrum measurements. Spectrophotometric method can be applied to this enzyme as an alternative to the more prevalent radioactive methods utilizing ^{14}C to monitor the rate of product formation. Although the radioactive method is considered accurate and specific, its application requires appropriate training and adherence to strict safety regulations (Koussevitzky et al. 2007). The method used in this study provides a rapid and effective alternative for assessing RuBisCO enzyme activity. Most studies utilizing the spectrophotometric method for RuBisCO activity recommend measuring the reaction for 10 min. To determine the reaction time, measurements were taken every 30 s, resulting in 80 measurements. Based on the absorbance spectra measurements (Fig. 5a-d), it was demonstrated that a 10-minute measurement time is too short to assess RuBisCO activity accurately. The enzymatic reaction concluded after 20 min of measurement (Fig. 5a-d). Therefore, using a 10-minute activity measurement may lead to obtaining inaccurate results (Table 3).

Validation of the method for the determination of RuBisCO activity was performed in this study. According to Sales et al. (2020), the concentration of unpurified RuBisCO in the reaction should vary between 10 and 40 µg/ml. Concentrations exceeding this range may limit the sensitivity of

Table 4 RuBisCO activity depending on temperature (15 °C, 18 °C, 20 °C, 25 °C, 40 °C) and RuBisCO concentration (100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml). Means marked with different letters differ significantly ($p \leq 0.05$) between groups (ANOVA, Tukey HSD test)

Temperature		
	Activity of RuBisCO [µmol NADH/min/mg protein]	Linear decline in absorbance
15 °C	0.100	0.104
18 °C	0.145	0.150
20 °C	0.164	0.170
25 °C	0.236	0.200
40 °C	0.096	0.100
RuBisCO concentration		
	Activity of RuBisCO [µmol NADH/min/mg protein]	Linear decline in absorbance
100 µg/ml	0.027	0.111
50 µg/ml	0.099	0.207
25 µg/ml	0.234	0.200
12.5 µg/ml	No activity	0.004
6.25 µg/ml	No activity	0

NADH-linked assays (Sales et al. 2020). Our results indicate that the application of higher enzyme concentrations in the reaction (50–100 µg/ml) results in lower enzyme activity (Table 4). The use of lower concentrations – 6.25 µg/ml – 12.5 µg/ml – does not allow for the determination of enzymatic activity (Table 4). Another factor significantly affecting the efficiency of the reaction is temperature. Our results indicate that the highest activity of RuBisCO is achieved at a temperature of 25 °C. Lower and higher values result in a significant decrease in the efficiency of the reaction (Table 4).

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Data availability The raw data will be available at the University of Warmia and Mazury web page.https://bazawiedzy.uwm.edu.pl/info/researchdata/UWMd130cd42364e484d90bc3af029dba69c?r=researchdata&ps=20&tab=&title=Szczeg%25C3%25B3%25C5%2582y%2Brekordu%2B%25E2%2580%2593%2BDane%2Bbadawcze%2B%25E2%2580%2593%2BUniwersytet%2BWarmi%25C5%2584sko-Mazurski&lang=pl

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Nauki Biologiczne

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OŚWIADCZENIE

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Oświadczam, że w pracy autorstwa **Krupka M, Michalczyk DJ, Piotrowicz – Cieślak AI** pod tytułem **Critical stages in pea photosynthesis impaired by tetracycline as an environmental contaminant** opublikowanej w ***Journal of Plant Reaserch* 2024, 137: 1049-1059** mój wkład merytoryczny w jej przygotowanie obejmował samodzielne wykonanie szczegółowych analiz zgodnych z przyjętą metodologią badawczą. Polegał na uwzględnieniu czynników mogących wpływać na wyniki, co pozwoliło na uzyskanie rzetelnych danych i ich późniejszą interpretację. Opracowaniu wysokiej jakości rysunków, wykresów i tabel ilustrujących kluczowe wyniki badań, co pozwoliło na ich czytelne i przystępne przedstawienie. Zastosowaniu przejrzystych schematów graficznych, które umożliwiły efektywne przedstawienie danych oraz wniosków wynikających z przeprowadzonych analiz. Dbałości o estetykę i precyzję wykonania grafik, co podkreśliło naukową wartość wyników oraz umożliwiło lepsze ich zrozumienie przez odbiorców. Przeprowadzeniu krytycznej analizy uzyskanych wyników, co pozwoliło na omówienie ich znaczenia oraz określenie ich miejsca w kontekście dotychczasowych badań i literatury przedmiotu. Sformułowaniu spostrzeżeń dotyczących wyników, które wskazują na ich potencjalne praktyczne i teoretyczne zastosowania, co zwiększa wartość poznawczą pracy. Dokonaniu korekt i dostosowań metody badawczej w celu jej optymalizacji, co wpłynęło na poprawę jakości i precyzji uzyskanych wyników. Przeanalizowaniu i potwierdzeniu, że metoda spełnia wymagania badawcze, co stanowiło podstawę do dalszych działań analitycznych oraz wyciągnięcia trafnych wniosków. Wyciągnięciu kluczowych zależności i wzorców wynikających z przeprowadzonych analiz, co stanowiło istotny fundament dla dalszej interpretacji danych. Zwięzłym przedstawieniu, jakie znaczenie mają uzyskane wyniki oraz jak mogą być zastosowane w dalszych badaniach.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny wkład na poziomie 80%.

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Przewodniczący Rady Naukowej Dyscypliny

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OŚWIADCZENIE współautora

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Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr inż. Magdalenę Krupkę** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata na poziomie 80%. Udział **mgr inż. Magdaleny Krupki** obejmował samodzielne wykonanie szczegółowych analiz zgodnych z przyjętą metodologią badawczą. Obejmował uwzględnienie czynników mogących wpływać na wyniki, co pozwoliło na uzyskanie rzetelnych danych i ich późniejszą interpretację. Opracowaniu wysokiej jakości rysunków, wykresów i tabel ilustrujących kluczowe wyniki badań, co pozwoliło na ich czytelne i przystępne przedstawienie. Zastosowaniu przejrzystych schematów graficznych, które umożliwiły efektywne przedstawienie danych oraz wniosków wynikających z przeprowadzonych analiz. Dbałości o estetykę i precyzję wykonania grafik, co podkreśliło naukową wartość wyników oraz umożliwiło lepsze ich zrozumienie przez odbiorców. Przeprowadzeniu krytycznej analizy uzyskanych wyników, co pozwoliło na omówienie ich znaczenia oraz określenie ich miejsca w kontekście dotychczasowych badań i literatury przedmiotu. Sformułowaniu spostrzeżeń dotyczących wyników, które wskazują na ich potencjalne praktyczne i teoretyczne zastosowania, co zwiększa wartość poznawczą pracy. Dokonaniu korekt i dostosowań metody badawczej w celu jej optymalizacji, co wpłynęło na poprawę jakości i precyzji uzyskanych wyników. Przeanalizowaniu i potwierdzeniu, że metoda spełnia wymagania badawcze, co stanowiło podstawę do dalszych działań analitycznych oraz wyciągnięcia trafnych wniosków. Wyciągnięciu kluczowych zależności i wzorców wynikających z przeprowadzonych analiz, co stanowiło istotny fundament dla dalszej interpretacji danych. Zwięzłym przedstawieniu, jakie znaczenie mają uzyskane wyniki oraz jak mogą być zastosowane w dalszych badaniach.

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A. Piotrowicz-Cieślak

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podpis

Publikacja 4

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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} × 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} × 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 com-

pounds 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 $N\text{-}NO_3$ and $N\text{-}NH_4$): 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 $N\text{-}NH_4$ (34.97 $N\text{-}NH_4$ 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 × 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 × 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.

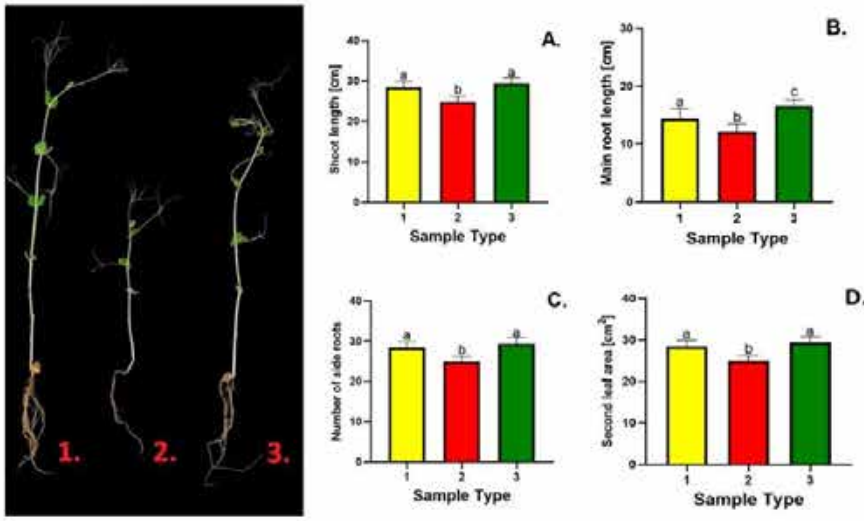


Figure 1. General appearance of peas: 1—pea grown on soil regularly fertilized with manure, 2—pea grown on soil taken from the vicinity of a manure heap, 3—pea grown on horticultural perlite-controland morphological parameters (**A**) shoot length [cm], (**B**) length of the main root [cm], (**C**) number 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	1.5×10^{-5} a
Soil S2	1.18×10^{-5} b
Control S3	1.59×10^{-5} a

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

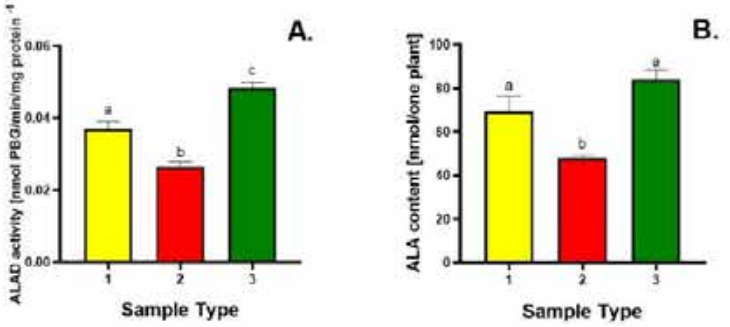


Figure 2. ALAD activity [nmol PGB/min/mg protein] (**A**) and ALA content [nmol × one plant^{-1}] (**B**) in peas grown on soil regularly fertilized with manure—1 (yellow column); collected from the vicinity of manure heaps—2 (red column) and the horticultural perlite—3 (green column). Means marked with 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.

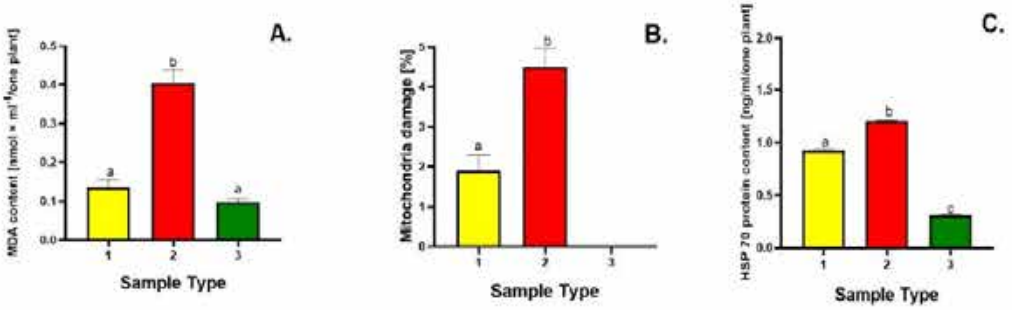


Figure 3. Biochemical parameters (**A**) concentration of MDA [nmol × mL^{-1} × one $plant^{-1}$], (**B**) damage to mitochondria [%], (**C**) concentration of HSP70 proteins [ng × mL × one $plant^{-1}$]) of pea growing on soil regularly fertilized with manure—1 (yellow column), collected from the vicinity 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.

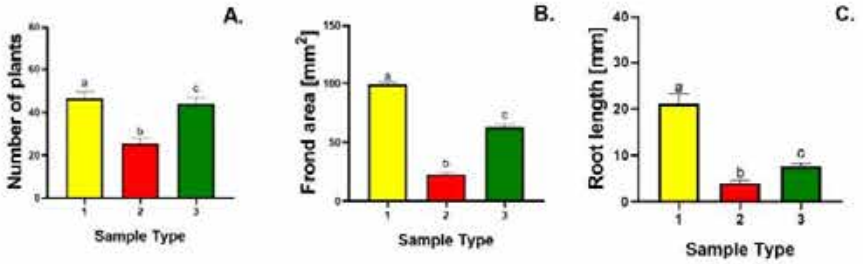


Figure 4. Morphological parameters (**A**) number of plants, (**B**) area of fronds [mm^2], (**C**) root length [mm]; 1 (yellow column)—duckweed cultivated on the filtrate of soil regularly fertilized with manure, 2 (red column)—duckweed grown on filtrate of soil taken from the vicinity of the manure 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).

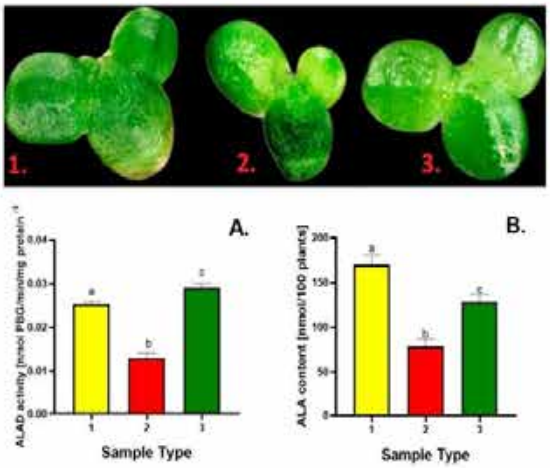


Figure 5. General appearance of duckweed: 1—duckweed grown on the filtrate from soil regularly fertilized with manure, 2—duckweed growing on the filtrate of soil taken from the vicinity of manure heap, 3—duckweed grown on MS medium) and ALAD activity [nmol PGB × min × mg protein $^{-1}$] (**A**) and 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.

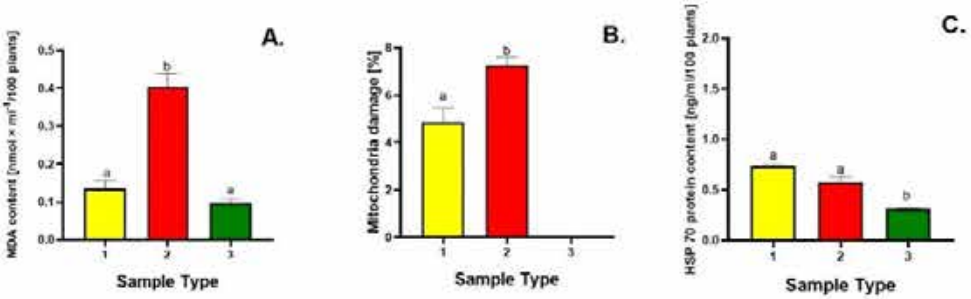


Figure 6. Biochemical parameters (**A**) concentration of MDA [nmol × mL^{-1} × 100 $plants^{-1}$], (**B**) damage to mitochondria [%], (**C**) concentration of HSP70 proteins [ng × mL^{-1} × 100 $plants^{-1}$]) of duckweed *Lemna minor* L. growing on the filtrate of soil regularly fertilized with manure—1 (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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Przewodniczący Rady Naukowej Dyscypliny

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Publikacja 5

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Brief Report

Optimization of the Method for Isolating Bacterial DNA from the Aboveground Part of Lettuce

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Abstract: Developing an effective method for isolating bacterial genetic material from plants is a relatively challenging task and often does not yield adequately prepared material for further analyses. Previous studies often overlook connections, primarily focusing on laboratory investigations. With advancements in high-throughput sequencing techniques, we can now revisit and delve deeper into these interactions. Our study focuses on the initial phase of these investigations: genetic material isolation. Extracting bacterial DNA from aboveground plant parts, known as the phyllosphere, poses a significant challenge due to plant-derived contaminants. Existing isolation protocols frequently yield inconsistent results, necessitating continuous refinement and optimization. In our study, we developed an effective isolation protocol employing mechanical–chemical lysis, sonication, and membrane filtration. This approach yielded high-quality DNA at a concentration of 38.08 ng/µL, suitable for advanced sequencing applications. Our results underscore the effectiveness and necessity of these methods for conducting comprehensive microbiological analyses. Furthermore, our research not only lays the groundwork for further studies on lettuce microbiota, but also highlights the potential for utilizing our developed protocol in investigating other plants and their microbiomes.

Keywords: phyllosphere; plant; mechanical–chemical lysis; bacteria

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1. Introduction

The "One Health" concept describes the interactions between humans, animals, plants, and their environment, emphasizing the interdependence of these components for public health maintenance [1]. Microorganisms are a key element of this concept, and act as a bridge between these components, impacting the health of all three domains of life [2]. Soil serves as the primary environmental habitat for microorganisms, forming the basis of the microbiological ecosystem [3]. However, the greatest threat to soil bacteria comes from antibiotics and their metabolites, which are routinely detected in agricultural soils. Even low concentrations of antibiotics in soils can lead to declines in certain bacterial groups and induce antibiotic resistance, posing a serious public health threat. Projections indicate that, by 2050, antibiotic resistance will become the leading cause of death in the human population [4].

To date, most research has focused on the spread of antibiotic resistance in hospital environments [5]. However, the "One Health" concept has highlighted the presence of antibiotic-resistant strains in other environmental components, including food products. Most studies on microorganisms and antibiotic resistance genes (ARGs) transmitted through the food chain pertain to animal-derived products [6–11]. Changes in dietary habits and the popularity of vegan and vegetarian diets have made raw plant products an important element of the human diet [12]. Unfortunately, the role of fresh vegetables and fruits in the spread of ARGs is still marginalized [5].

The concept of edible plant microbiota is relatively new and assumes that fresh vegetables and fruits can be sources of microorganisms, both pathogenic and beneficial,

that colonize the human body [13]. The Centers for Disease Control and Prevention (CDC) in the USA indicates that 46% of foodborne infections are associated with the consumption of fresh vegetables and fruits [14]. On the other hand, some beneficial bacteria have representatives in both the gut and plant microbiomes, suggesting that bacteria colonizing plants may perform beneficial functions in the human body [15].

Understanding the composition and function of the microbiota of fresh vegetables and fruits is therefore important from both consumer and public health perspectives. The aboveground parts (phyllosphere) of lettuce, one of the most commonly consumed raw vegetables, are colonized by numerous microorganisms [16]. Studies on the phyllosphere of plants, particularly those growing in polluted environments, are still rare in the literature.

Only a small fraction of the microorganisms colonizing plants can be cultured on traditional media [17]. The development of high-throughput sequencing methods allows for both the identification and study of complex microbial populations [18]. Although sequencing methods are highly specific, the material being studied must not be contaminated or degraded. Eliminating contaminants is especially important for samples with low microbial content, such as those from the phyllosphere [19]. Therefore, developing a repeatable and optimal method for isolating bacterial DNA from leaves is crucial.

Although many protocols for the separation and isolation of DNA from the phyllosphere are available, they are not consistent [20]. A typical isolation protocol consists of several stages: the preparation of plant material, the separation of bacterial cells, the lysis of bacterial cells, and DNA purification [21]. Sample preparation involves shaking plant fragments with a PBS solution [22] or NaCl [23]. Bacterial cells are then collected by centrifugation [24] or membrane filtration [22,25]. Some protocols recommend cutting the membrane into small fragments, while others use the entire membrane for isolation. Most available protocols for isolating DNA from phyllosphere bacteria recommend using commercial kits for isolating genomic DNA from soil. These kits mainly use enzymatic or mechanical lysis [21] and differ in their effectiveness in lysing specific microorganisms [19].

Differences between sample preparation and isolation protocols can introduce errors in the taxonomic analysis of the phyllosphere microbiome, and even cause the loss of certain bacterial groups in a sample. Moreover, a large portion of the available protocols pertain to ready-to-eat plant products, which undergo preparation processes before sale that may lead to the transfer of ARGs to the final product [5], and thus do not reflect real environmental conditions.

The aim of this study was to compare protocols for the separation of bacteria and the isolation of bacterial DNA from the phyllosphere of lettuce grown in soil collected from a field. Developing a repeatable and effective method for isolating DNA from plant samples is the first and most important step in molecular studies of microorganisms colonizing lettuce. Understanding the lettuce microbiota can provide significant insights into plant–microbe interactions and potential human health impacts, in line with the principles of the "One Health" concept.

2. Results and Discussion

Sequencing methods require genetic material of appropriate quantity and quality [26]. Although advancements in next-generation sequencing (NGS) methods allow for accurate results from small amounts of genetic material, a DNA concentration above 10 ng/µL is recommended to ensure optimal performance [27]. One of the most influential factors affecting the quantity and quality of the obtained material is the isolation method [28]. Isolating bacterial DNA from environmental samples, including the phyllosphere, presents particular challenges due to the presence of contaminants that can interfere with PCR methods [21,29].

The choice of an appropriate protocol is crucial for isolating genetic material with the correct quantitative and qualitative parameters, enabling further analysis. Most available protocols for isolating DNA from phyllosphere bacteria utilize mechanical lysis. However, Yang et al. [21] indicate that mechanical lysis can cause fragmentation and a subsequent

loss of genetic material. On the other hand, using milder enzymatic lysis carries the risk of introducing contaminants from plant material [21], significantly affecting the quality parameters of the isolated DNA.

Our results indicate that enzymatic lysis does not yield DNA of adequate quality, as demonstrated in experiments 1–12, where the OD 260/280 ratios ranged from 1.2 to 1.54 (Table 1). Similar results were obtained by Yang et al. [21] when isolating DNA from the phyllosphere of alfalfa and by Baturo-Cieśniewska et al. [30] when isolating genomic DNA from soybean rhizosphere. These findings underscore the limitations of enzymatic lysis in producing high-quality DNA from phyllosphere samples.

Table 1. Effects of lysis method (enzymatic, mechanical–chemical) and extraction conditions on yield [ng/µL], total yield [ng], and purity [$A_{260/280}$, $A_{260/230}$] of bacterial DNA isolated from lettuce phyllosphere. Small letters represent groups of significant difference.

Number of Experiment	DNA [ng/µL]	DNA [ng]	A260/280	A260/230
Enzymatic lysis				
1	3.94 ± 0.70 a	194 ± 39.4 a	1.40 c	0.77 b
2	6.41 ± 0.95 b	327 ± 45.0 b	1.50c	1.10d
3	7.45 ± 0.95 b	378 ± 47.0 b	1.48 c	0.97 c
4	3.14 ± 0.69 a	160 ± 35.8 a	1.20 a	0.75 b
5	4.86 ± 1.02 b	241± 53.9 b	1.34 b	1.00 c
6	4.19 ± 1.03 a	206 ± 53.7 a	1.50 b	1.11 d
7	5.56 ± 1.19 b	271 ± 59.0 b	1.30 b	0.70 b
8	10.30 ± 1.38 c	521 ± 70.5 c	1.60 d	1.40 e
9	11.50 ± 1.00 c	575 ± 54.7 c	1.53 c	1.13 d
10	5.57 ± 0.70 b	281 ± 36.4 b	1.30 b	0.57 a
11	6.90 ± 0.65 b	348 ± 32.9 b	1.40 b	0.73 b
12	6.99 ± 1.16 b	346 ± 60.5 b	1.54 c	0.77 b
Mechanical–chemical lysis				
13	6.07 ± 0.81 b	306 ± 42.0 b	1.60 d	1.12 d
14	9.50 ± 1.35 c	470 ± 69.8 c	1.75 f	1.79 g
15	9.98 ± 1.20 c	490 ± 57.4 c	1.90 g	1.50 f
16	5.48 ± 0.69 b	276± 35.8 b	1.68 f	1.45 f
17	6.81 ± 0.84 b	345 ± 42.5 b	1.85 g	1.78 g
18	6.84 ± 0.75 b	342 ±39.6 b	1.90 g	1.62 g
19	5.67 ± 0.84 b	280 ± 43.2 b	1.78 f	1.56 f
20	24.29 ± 4.32 d	1205 ± 226.6 d	1.83 g	1.56 f
21	38.08 ± 4.28 f	1929 ± 210.4 f	1.85 g	1.81 g
22	5.84 ± 0.93 b	301 ± 37.7 b	1.76 f	1.74 g
23	8.41 ± 1.39 c	420 ± 73.9 c	1.84 g	0.91 c
24	9.20 ± 0.93 c	461 ± 49.6 c	1.90 h	1.87 h

Another parameter for assessing the quality of isolated genetic material is the A260/230 ratio, which should range between 1.8 and 2.2 [31]. In our study, this parameter was very low (0.57–1.11 in experiments 1–12 with enzymatic lysis), suggesting contamination of the isolated genetic material. The highest DNA concentrations (10.3 and 11.5 ng/µL) were obtained in experiments 8 and 9, where samples were prepared through sonication and membrane filtration with an initial material amount of 50 g. However, these samples

did not meet quality standards (OD 260/280 below 1.8) (Table 1). Thus, despite multiple sample-preparation steps, enzymatic lysis methods are not suitable for isolating DNA from the phyllosphere microbiome.

Yang et al. [21] demonstrated that mechanical lysis allows the obtainment of genetic material with good quality parameters, although the DNA yield is lower compared to enzymatic lysis. Our results show that using mechanical–chemical lysis produced genetic material with better quality parameters compared to enzymatic lysis (Table 1). The purity parameters ranged from 1.6 to 1.9 (A260/280) and 0.91 to 1.87 (A260/230). DNA was obtained in a range of concentrations from 5.4 ng/ µL to 38.08 ng/µL (Table 1). These findings indicate that the cell lysis method is not the only factor determining the amount of genetic material obtained. The phyllosphere, compared to the rhizosphere, is an environment with a lower abundance of microorganisms [27]. Therefore, obtaining large amounts of DNA requires additional sample-preparation methods.

One approach that significantly increases the concentration of isolated DNA is sonication. Sonication is widely used for separating bacterial cells from soil [32]. Most phyllosphere microbiome isolation protocols also recommend sonication for separating bacterial cells from leaves [27,33]. Our results indicate that sonication yields higher DNA concentrations, although it is not the sole factor for obtaining the appropriate amount of genetic material. The next step involves collecting and concentrating bacterial cells. Our results show that membrane filtration yields more DNA than cell collection by centrifugation (Table 1).

The preparation method of the membrane for isolation is also crucial. Both cut [34] and whole membranes [22] are used in phyllosphere DNA isolation protocols. Using membranes cut into small fragments results in significantly higher DNA yields (38.08 ng/µL) compared to whole membranes (24.29 ng/µL) (Table 1). This suggests that the increased surface area of cut membranes enhances the efficiency of DNA recovery. The best quantitative and qualitative results were obtained using mechanical–chemical lysis, sonication, and membrane filtration (experiments 20 and 21), along with increasing the amount of initial material to 50 g. This combination allowed for the isolation of DNA with the desired quality and concentration, demonstrating that integrating multiple preparation steps is essential for optimizing DNA extraction from phyllosphere samples.

To further confirm the quality of the isolated DNA and its utility in molecular studies, 16S rRNA gene sequencing was performed. This procedure involved the PCR amplification of a selected region of the 16S rRNA gene, followed by sequencing of the resulting products. The DNA with the best quantitative and qualitative parameters (experiment 21, Table 1) was used for 16sRNA sequencing. The sample was prepared using sonication and membrane filtration (cut membrane), and the amount of tissue was 50 g (Table 2). In total, we obtained 84,068 raw-read pairs from the sequencing run. Of these sequences, 99.85% were successfully assigned to the bacterial kingdom, demonstrating the high specificity and effectiveness of our DNA extraction and sequencing protocols in targeting bacterial DNA. This high assignment rate underscores the robustness of our method in isolating bacterial genetic material while minimizing contamination from non-bacterial sources. The relative abundance of bacterial communities is presented in Figure 1.

Table 2. Overview of experiments for bacterial DNA isolation from lettuce phyllosphere.

Number of Experiment	Experimental Steps
1	5 g of lettuce leaf, sonication, centrifugation, enzymatic lysis
2	5 g of lettuce leaf, sonication, membrane filtration, entire membrane, enzymatic lysis
3	5 g of lettuce leaf, sonication, membrane filtration, cut membrane, enzymatic lysis
4	5 g of lettuce leaf, without sonication, centrifugation, enzymatic lysis

Table 2. *Cont.*

Number of Experiment	Experimental Steps
5	5 g of lettuce leaf, without sonication, membrane filtration, entire membrane, enzymatic lysis
6	5 g of lettuce leaf, without sonication, membrane filtration, cut membrane, enzymatic lysis
7	50 g of lettuce leaf, sonication, centrifugation, enzymatic lysis
8	50 g of lettuce leaf, sonication, membrane filtration, entire membrane, enzymatic lysis
9	50 g of lettuce leaf, sonication, membrane filtration, cut membrane, enzymatic lysis
10	50 g of lettuce leaf, without sonication, centrifugation, enzymatic lysis
11	50 g of lettuce leaf, without sonication, membrane filtration, entire membrane, enzymatic lysis
12	50 g of lettuce leaf, without sonication, membrane filtration, cut membrane, enzymatic lysis
13	5 g of lettuce leaf, sonication, centrifugation, mixed lysis
14	5 g of lettuce leaf, sonication, membrane filtration, entire membrane, mixed lysis
15	5 g of lettuce leaf, sonication, membrane filtration, cut membrane, mixed lysis
16	5 g of lettuce leaf, without sonication, centrifugation, mixed lysis
17	5 g of lettuce leaf, without sonication, membrane filtration, entire membrane, mixed lysis
18	5 g of lettuce leaf, without sonication, membrane filtration, cut membrane, mixed lysis
19	50 g of lettuce leaf, sonication, centrifugation, mixed lysis
20	50 g of lettuce leaf, sonication, membrane filtration, entire membrane, mixed lysis
21	50 g of lettuce leaf, sonication, membrane filtration, cut membrane, mixed lysis
22	50 g of lettuce leaf, without sonication, centrifugation, mixed lysis
23	50 g of lettuce leaf, without sonication, membrane filtration, entire membrane, mixed lysis
24	50 g of lettuce leaf, without sonication, membrane filtration, cut membrane, mixed lysis

In our study, the most abundant groups were Proteobacteria, Bacteroidetes, Actinobacteria, and Verrucomicrobiota (Figure 2). Meanwhile, Trivedi et al. [35] indicated that plants are most commonly colonized by bacteria belonging to Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria. Moreover, we identified 84 reads (0.2006%) attributed to *Lactuca sativa* L. chloroplast DNA. This low percentage indicates that our method effectively limited the co-extraction of chloroplast DNA. Chloroplast contamination is a common issue in plant-associated microbiome studies, as plant tissues often contain high amounts of chloroplast DNA, which can interfere with the accurate profiling of bacterial communities [36]. The minimal presence of chloroplast DNA in our results highlights the precision of our extraction process, confirming that the majority of the isolated DNA was of bacterial origin. The reduction in chloroplast DNA contamination is particularly significant because it allows for a more accurate representation of the bacterial community within the phyllosphere. Chloroplast DNA can overshadow bacterial sequences, leading to skewed

or incomplete data. By minimizing chloroplast DNA extraction, our method enhances the reliability of subsequent metagenomic analyses, providing a clearer and more detailed picture of the bacterial populations present on lettuce leaves.

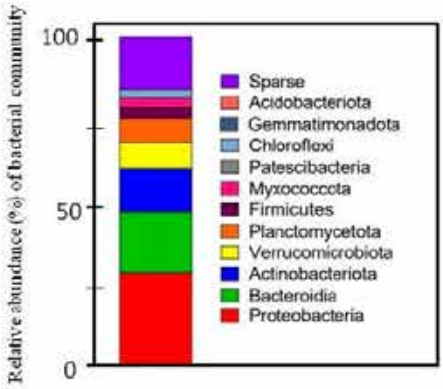


Figure 1. Relative abundance (%) of bacterial community. Taxons that made up less than 5% of total classified reads were grouped and labeled as "Sparse".

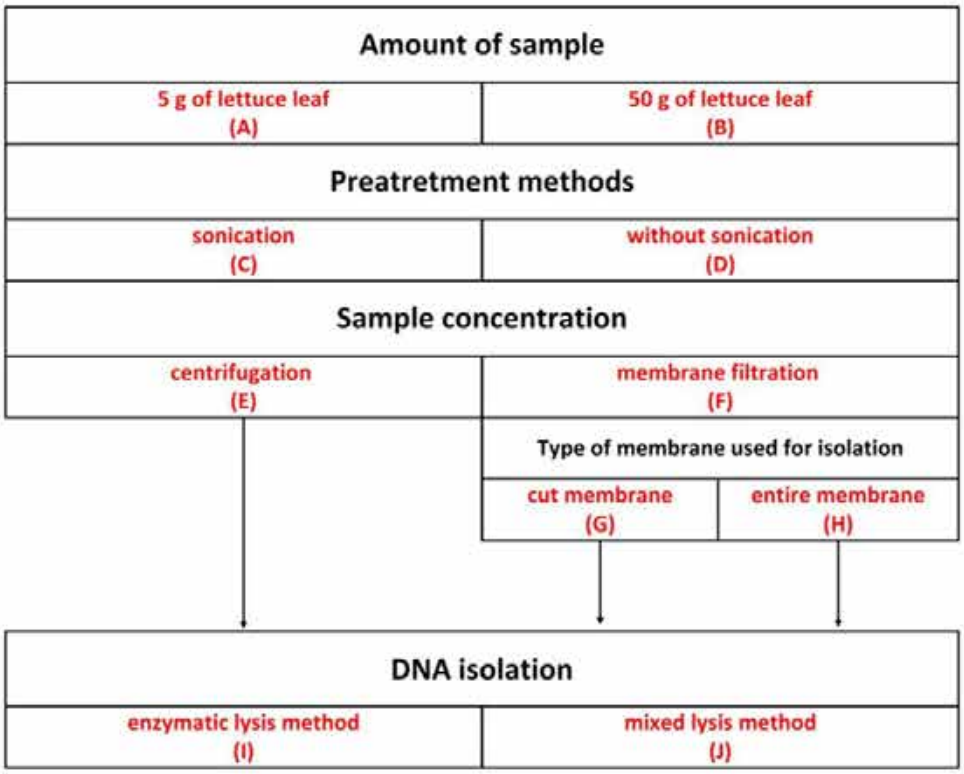


Figure 2. Overview of experimental steps of bacterial DNA isolation from lettuce phyllosphere.

3. Materials and Methods

3.1. Lettuce Cultivation Conditions

Soil was collected from an agricultural field regularly fertilized with manure, located in the Warmian–Masurian Voivodeship. After collection, the samples were transported to

the laboratory and stored at 4 °C. Detailed soil characteristics are presented in [37]. Seeds of lettuce (*Lactuca sativa* L. var. Takoda) were sown in seedling pots measuring 24 × 24 mm. The plants were then grown for 4 weeks in climate chambers (POL-EKO, Wodzisław Śląski, Poland) at 18 °C/14 °C day/night, with a 16/8 h photoperiod and a light intensity of 8000 lx. Subsequently, 2 kg of soil was added to 17 cm diameter pots, and the lettuce seedlings were transferred (one seedling per pot). The plants were cultivated for another 4 weeks under the same conditions. After 4 weeks, the leaves were cut with sterile scissors 3 cm above the soil surface, mixed (to obtain a homogeneous sample), and analyzed.

3.2. Bacterial Cell Separation from Lettuce Leaves

To select the optimal method for separating cells from leaves, the following parameters of preliminary sample preparation were tested: the amount of starting material, the sonication step, the method of collecting bacterial cells (centrifugation or membrane filtration), and the preparation method of the membrane. Experimental variants were chosen based on a literature review using the keywords "phyllosphere", "bacteria", "DNA isolation", and "16S RNA". The scheme of the conducted experiments is shown in Figure 2. Lettuce leaves were prepared according to [38] with modifications.

Sample Preparation

An amount of 5 g (A) or 50 g of leaf (B) was placed in a glass bottle, and sterile 0.01 M PBS (phosphate-buffered saline) at pH 7.4 was added (45 mL per 5 g of leaf). Samples were sonicated (Sonic-3, 310 W, 40 KHz, POLSONIC Pałczyński, Warsaw, Poland) (C) for 7 min according to [38] or left without sonication (D). The bottles were then shaken for 1 h at 180 RPM on a laboratory shaker (DLAB Scientific, Senai, Malaysia) at 30 °C. To separate the leaf fragments, the bottle contents were filtered through gauze. Subsequently, 50 mL of the resulting liquid was centrifuged at 7500 RPM for 30 min (E) according to [18], or subjected to membrane filtration through sterile PVDF membranes with a 0.22 µm pore size and 47 mm diameter (Durapore, Merck Millipore, Burlington, MA, USA) (F), according to [38]. The pellets obtained during centrifugation and the membranes were stored at −80 °C until further analysis.

3.3. DNA Isolation

Each pellet was suspended in Tris-HCl buffer at pH 7, while PVDF membranes were cut into small fragments using sterile scissors (G) or left whole (H). DNA isolation was performed on the resulting pellet or PVDF membranes using the following commercially available kits: DNeasy Power Pro Soil Kit (Qiagen Inc.Germantown, MD, USA), which utilizes both mechanical and chemical lysis, and Genomic Mini AX Soil Spin (A&A Biotechnology, Gdansk, Poland), which uses enzymatic lysis (J), according to the manufacturers' protocols. The experimental scheme is presented in Figure 2.

3.4. Experimental Variants, Quantity, and Quality of Isolated DNA

The planned experiments were conducted with 24 variants (Table 2). The quantity of isolated DNA was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, NC, USA). The purity of the isolated DNA was assessed using the OD 260/280 and OD 260/230 parameters (NanoDrop Technologies, Wilmington, NC, USA).

3.5. Next-Generation Sequencing

To confirm that the isolated genetic material originated from bacteria, next-generation sequencing (NGS) was performed. The metagenomic analysis of the phyllosphere samples was conducted by an external company (Genomed S.A., Warsaw, Poland). The bacterial population analysis was based on the hypervariable V3-V4 region of the 16S rRNA gene. Specific primer sequences 341F and 785R were used for the amplification of the selected region and library preparation. The PCR reaction was carried out using the Q5 Hot Start

High-Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, MA, USA), following the manufacturer's recommendations. Sequencing was performed on a MiSeq instrument using paired-end (PE) 2 × 300 nt technology with the Illumina v3 kit. Bioinformatic analysis, ensuring taxonomic classification, was conducted using the QIIME 2 software package (version 2017.6.0) based on the Silva 138 reference sequence database. The DADA2 package was also employed to distinguish biological origin sequences from those newly formed during the sequencing process. This package was additionally used to extract sequences of unique biological origin, known as amplicon sequence variants (ASVs).

3.6. Statistical Analyses

All experiments were conducted in 10 replicates. One-way ANOVA with Tukey's post hoc test was used to assess the differences in DNA concentration between the extraction methods. Statistical analyses were performed using GraphPad Prism 8.0.1.

4. Conclusions

In this work, we achieved the optimization of our DNA extraction protocol, including specific steps such as mechanical–chemical lysis, sonication, and membrane filtration. These steps were designed not only to maximize bacterial DNA yield, but also to selectively target bacterial cells and reduce the carryover of plant DNA. The success of these techniques in reducing chloroplast DNA contamination while retaining high bacterial DNA integrity and quantity is crucial for downstream applications, such as metagenomic sequencing and microbial diversity studies. Overall, the effective limitation of chloroplast DNA co-extraction and the high percentage of bacterial DNA obtained reflect the methodological rigor and the potential applicability of our approach to other plant microbiome studies. This method can serve as a reliable protocol for researchers aiming to study the complex interactions within the phyllosphere microbiome, ensuring that the focus remains on microbial communities rather than being confounded by plant DNA.

Author Contributions: Conceptualization, M.K. and A.I.P.-C.; methodology, M.K.; software, M.K.; validation, M.K.; formal analysis, M.K.; writing—original draft preparation, M.K. and A.I.P.-C.; writing—review and editing M.K. and A.I.P.-C.; visualization, M.K.; supervision A.I.P.-C.; project administration, A.I.P.-C. All authors have read and agreed to the published version of the manuscript.

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OŚWIADCZENIE

autora rozprawy doktorskiej

Oświadczam, że w pracy autorstwa **Krupka M, Piotrowicz – Cieślak AI** pod tytułem **Optimization of the method for isolating bacterial DNA from the aboveground part of lettuce** opublikowanej w ***International Journal of Molecular Sciences* 2024, 25: 8513** mój wkład merytoryczny w jej przygotowanie obejmował opracowanie szczegółowego harmonogramu badań. Wkład merytoryczny polegał na określeniu kluczowych parametrów badawczych, co pozwoliło na kontrolę istotnych zmiennych oraz uzyskanie rzetelnych wyników. Zoptymalizowaniu stosowanej metody pod kątem specyficznych wymagań analizy filosfery, co obejmowało dobór parametrów eksperymentalnych w sposób, który maksymalizował precyzję i powtarzalność wyników. Przeprowadzeniu serii testów i kalibracji narzędzi badawczych, co umożliwiło dopasowanie metody do badanej materii i uzyskanie wiarygodnych danych. Weryfikacji skuteczności zoptymalizowanej metody w porównaniu z metodami stosowanymi dotychczas, co potwierdziło jej wyższość w zakresie dokładności oraz wydajności. Selekcji i dogłębnej analizie najnowszych publikacji, które stanowiły podstawę teoretyczną dla opracowywanej metody i dostarczyły cennych danych porównawczych. Szczegółowej analizie oraz porównaniu alternatywnych metod analizy filosfery stosowanych w literaturze przedmiotu, co pozwoliło na wyciągnięcie wniosków dotyczących ich mocnych i słabych stron. Wypracowaniu unikalnego podejścia do analizy filosfery, które łączyło elementy innych metod, tworząc rozwiązanie bardziej dostosowane do specyficznych wymagań tego badania. Przeprowadzeniu testów potwierdzających, że opracowana metoda spełnia wymogi dokładności, powtarzalności i czułości, istotne w analizie filosfery. Sporządzeniu procedur badawczych i zaleceń praktycznych dotyczących wdrożenia tej metody w przyszłych badaniach.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje mój indywidualny na poziomie 90%.

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Oświadczam, że w pracy autorstwa **Krupka M, Piotrowicz – Cieślak AI** pod tytułem **Optimization of the method for isolating bacterial DNA from the aboveground part of lettuce** opublikowanej w ***International Journal of Molecular Sciences* 2024, 25: 8513** mój wkład merytoryczny w jej przygotowanie polegał na opracowaniu koncepcji pracy, krytycznej ocenie pracy i przygotowaniu ostatecznej wersji manuskryptu.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez **mgr inż. Magdalenę Krupkę** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów naukowych opublikowanych w czasopismach naukowych. Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład kandydata na poziomie 90%. Wkład **mgr inż. Magdaleny Krupki** obejmował opracowanie szczegółowego harmonogramu badań. Zidentyfikowaniu kluczowych parametrów badawczych, co pozwoliło na kontrolę istotnych zmiennych oraz uzyskanie rzetelnych wyników. Zoptymalizowaniu stosowanej metody pod kątem specyficznych wymagań analizy filosfery, co obejmowało dobór parametrów eksperymentalnych w sposób, który maksymalizował precyzję i powtarzalność wyników. Przeprowadzeniu serii testów i kalibracji narzędzi badawczych, co umożliwiło dopasowanie metody do badanej materii i uzyskanie wiarygodnych danych. Weryfikacji skuteczności zoptymalizowanej metody w porównaniu z metodami stosowanymi dotychczas, co potwierdziło jej wyższość w zakresie dokładności oraz wydajności. Selekcji i dogłębnej analizie najnowszych publikacji, które stanowiły podstawę teoretyczną dla opracowywanej metody i dostarczyły cennych danych porównawczych. Szczegółowej analizie oraz porównaniu alternatywnych metod analizy filosfery stosowanych w literaturze przedmiotu, co pozwoliło na wyciągnięcie wniosków dotyczących ich mocnych i słabych stron. Wypracowaniu unikalnego podejścia do analizy filosfery, które łączyło elementy innych metod, tworząc rozwiązanie bardziej dostosowane do specyficznych wymagań tego badania. Przeprowadzeniu testów potwierdzających, że opracowana metoda spełnia wymogi dokładności, powtarzalności i czułości, istotne w analizie filosfery. Sporządzeniu procedur badawczych i zaleceń praktycznych dotyczących wdrożenia tej metody w przyszłych badaniach.

A. Piotrowicz-Cieślak

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podpis

Materiał przygotowany do publikacji 6 (MPP 6)

The impact of tetracycline on the soil microbiome and the rhizosphere of lettuce (*Lactuca sativa* L.)

The impact of tetracycline on the soil microbiome and the rhizosphere of lettuce (*Lactuca sativa* L.)

Abstract: The impact of tetracycline on the soil and rhizosphere microbiome of lettuce was analyzed. Soil was collected from an agricultural field regularly fertilized with manure, and tetracycline was added at two concentrations (5 mg/kg and 25 mg/kg). In untreated soil, dominant bacteria included *Proteobacteria* (43.17%), *Bacteroidota* (17.91%), and *Firmicutes* (3.06%). Tetracycline addition caused significant shifts in microbiome composition, notably increasing *Actinobacteriota* (22%) and favoring *Mycobacterium tuberculosis* (low concentration) and *Mycobacterium holsaticum* (high concentration). *Proteobacteria* decreased by 21%, possibly indicating antibiotic resistance development. An increase in *Firmicutes*, particularly *Bacillales*, suggested a selection for resistant strains. In the lettuce rhizosphere, tetracycline-induced changes were less pronounced than in soil. *Proteobacteria* remained dominant, but taxa like *Burkholderiales* and *Chitinophagales* increased in response to tetracycline. The rise in chitin-degrading bacteria might result from fungal overgrowth linked to the bacteriostatic effects of tetracycline. Pathogens such as *M. tuberculosis*, observed in the soil, were not detected in the lettuce rhizosphere.

Keywords: bacteria; plants; soil and rhizosphere microbiome

1. Introduction

Plants are a habitat for 10^4-10^{10} bacteria per gram of plant material [1]. The largest groups of bacteria inhabiting plants belong to the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* [2]. These bacteria colonize the roots (rhizosphere), leaves (phyllosphere), fruits (carposphere), and internal plant tissues (endosphere). While many bacterial species inhabiting plants are considered pathogens, the plant microbiome also plays a crucial role in plant health, growth, and stress resistance [3]. However, understanding the role and composition of microorganisms inhabiting plants is still far from complete [4], and the factors shaping the plant microbiome are not fully defined. Increasing research suggests that microorganisms inhabiting plant tissues can colonize the human body [5]. One of the significant factors affecting the human microbiome is diet [6]. Until now, most attention has been focused on microorganisms from fermented foods [1] and the presence of pathogens in animal-derived products [7]. The "One Health" concept recently highlighted plants as a link between microorganisms and the human body. Wicaksono et al. [5] conducted the first study on the colonization of the gut microbiome by bacteria associated with vegetables and fruits, showing that strains inhabiting fresh vegetables and fruits are detected in human intestines and form a functional part of the human microbiome [5]. The largest groups of bacteria inhabiting plants, such as genera *Bacteroidetes* and *Firmicutes*, are dominant in the human intestine and play an important role in the process of digestion and energy absorption [8]. As the major dominants in core microbiota, they are the key regulators of body homeostasis, involving both intestinal and extra-intestinal effects by influencing many physiological functions such as metabolism, maintenance of barrier homeostasis, inflammation, and hematopoiesis [9]. Maintaining the right proportion of *Bacteroides* and *Firmicutes* strains is important, especially when it comes to combating obesity and related metabolic disorders. The literature suggests that a high *Firmicutes*/*Bacteroidetes* (F/B) ratio may be associated with a higher risk of metabolic syndrome, diabetes, and obesity [10]. Therefore, microbiological control of vegetables and fruits and understanding their microbiome composition during cultivation are crucial for ensuring food safety and protecting consumer health. Soil is the largest reservoir of environmental microorganisms and serves as the source of the microbiome for the plants grown in it [11]. The greatest threat to environmental microorganisms is antibiotics, which are still widely used in commercial livestock production. These compounds are used both prophylactically and therapeutically. By 2030, the estimated use of these pharmaceuticals in

animal production will exceed 100,000 tons [12]. Only a small portion of antibiotics is effectively metabolized in animal bodies. Most of the administered dose is excreted directly by the animal through urine and feces [13]. Unprocessed waste containing antibiotic residues is then applied as organic fertilizer to agricultural fields [13], introducing antibiotics into the soil. Most research on plant microbiomes focuses on manure as a source of antibiotics, microorganisms, and antibiotic resistance genes (ARGs). In addition to manure, a significant source of antibiotics in agricultural soils is irrigation water, agricultural waste, sewage sludge, and spread from nearby livestock farms [14]. However, the direct impact of veterinary antibiotics present in the soil on plant microbiota composition is often overlooked and only fragmentarily understood. Plants actively recruit their microbiome from the surrounding environment, with microorganisms typically transported horizontally from the soil and rhizosphere through vascular tissues to the aerial parts of the plant [15]. Changes in the composition of microorganisms inhabiting the soil and rhizosphere can thus induce changes in the endosphere and phyllosphere of plants [16], negatively impacting plant health and raising concerns about the transfer of pathogens and ARGs to edible plant parts. The first step in studying plant microbiomes should, therefore, be the assessment of the composition and diversity of microorganisms inhabiting the soil and rhizosphere, particularly in plants growing in environments contaminated with antibiotics. Tetracycline is one of the most commonly used veterinary antibiotics due to its broad spectrum of activity, low cost, and ease of administration [17]. Tetracycline antibiotic residues are detected in most agricultural soils [18]. Thus, the aim of this study was to assess the structure of the microbiome in tetracycline-contaminated soil and the rhizosphere of lettuce (*Lactuca sativa* L.) grown in soil with the addition of tetracycline. This study also aimed to understand the ecological consequences of antibiotics in the soil environment, such as changes in microorganism biodiversity and potential effects on plants and agricultural ecosystems. The hypothesis was formulated that tetracycline, even in small, environmentally detectable concentrations, significantly alters the microbiome structure of the soil and the rhizosphere of lettuce grown in it. Therefore, it will be possible to gain a better understanding of how tetracycline affects key microorganisms in the soil and rhizosphere, which is important both from the perspective of plant physiology and public health.

2. Results and Discussion

Organic fertilizers are commonly used in agricultural production due to their low cost and high nutrient content, which improve soil quality and crop yield. Both manure and activated sludge can contain antibiotic residues that affect the soil microbiome composition and induce antibiotic resistance in the soil [19]. To simulate real environmental conditions, soil collected from a field regularly fertilized with chicken manure was used as the control sample in this study. Although the soil was confirmed to be free of antibiotics, previous work by our team detected tetracycline in nearby groundwater [20]. Pharmaceuticals undergo various transformations in soil, and the degree of drug adsorption to soil particles depends on the chemical structure, polarity of the drug, and the soil's physicochemical properties. The soil used in this study had a low organic carbon content (<1.2 g/kg), resulting in a reduced ability to retain contaminants and an increased risk of leaching into deeper soil layers and/or groundwater [20]. The presence of tetracycline in the groundwater indicates its use in poultry farming, suggesting that the microbiome of soil fertilized with manure from these farms would be altered. A total of 92,009, 68,855, and 40,611 paired reads (pairs joined) were obtained for soil samples S1, S2, and S3, respectively. A phylogenetic summary of the results is presented in Table 1.

Table 1. Percentage of reads assigned to appropriate taxonomic levels for analyzed samples. S1 – soil without tetracycline addition; S2 – soil with 5 mg/kg tetracycline addition; S3 – soil with 25 mg/kg tetracycline addition.

Sample	Kingdom	Phylum	Class	Order	Family	Genus	Species
S1	100	99.99	99.96	99.47	96.15	76.45	9.607
S2	100	99.93	99.86	98.78	94.24	80.43	6.89
S3	100	99.98	99.86	98.88	95.08	82.86	6.78

Alphaproteobacteria and *Gammaproteobacteria*, constituting 30.32% and 12.85% of all identified classes, respectively. Within *Alphaproteobacteria*, three families were identified: *Micropepsaceae* (7.46%) and *Rhizobiaceae* (4.56%), responsible for nitrogen fixation and cycling in nature, and *Devosiaceae* (3.97%), which have the ability to remediate contaminated soils [21]. Within *Gammaproteobacteria*, the order *Xanthomonadales* (3.39%) was identified. The *Xanthomonadales* group includes both plant and human pathogens, as well as non-pathogenic environmental bacteria [22]. Species within the genus *Stenotrophomonas* from *Xanthomonadales* group are multidrug-resistant opportunistic pathogens that are responsible for nosocomial infections in immunodeficient patients. *Stenotrophomonas maltophilia* is among the top ten pathogens causing nosocomial infections. The main cases of this bacterium being isolated are: bacteremia, wound infection, meningitis, urinary tract infections, and pneumonia [23,24].

The second most abundant bacterial group at the phylum level was *Bacteroidota* (Figure 1A). Within this phylum, the class *Bacteroidia* (17.91%) and three families were identified: *Flavobacteriaceae* (6.68%), *Chitinophagaceae* (4.59%), and *Sphingobacteriaceae* (3.13%). The presence of these bacteria has been previously reported in agricultural soils in Poland [25]. Special attention is given to the occurrence of the *Flavobacteriaceae* family and the genus *Flavobacterium*. These bacteria participate in the degradation of complex organic compounds, such as pesticides and insecticides [26], and are considered indicators of good soil quality [25]. These bacteria are also important components of the rhizosphere and phyllosphere microbiomes [27]. On the other hand, a positive correlation between *Flavobacteriaceae* and the transmission of antibiotic resistance genes in soils, including those for tetracycline resistance, has been demonstrated [28]. *Flavobacterium* bacteria can also cause infections in humans and animals [26]. *Flavobacterium* species can cause disease in fish; human infections are extremely rare. Only a few cases of opportunistic infections have been described in humans, including pneumonia, septicemia, and spontaneous bacterial peritonitis caused by species: *Flavobacterium ceti* and *Flavobacterium lindanitolerans* [29-32].

The third most dominant bacterial group at the phylum level was *Actinobacteriota* (9.35%). Among them, the class *Actinobacteria* (7.86%) was identified. However, the abundance of this bacterial group in the soil was lower than the values reported in agricultural soils [26]. Results obtained by Minkina et al. [33] indicate that fertilization of soils with chicken manure increases the abundance of bacteria from the *Proteobacteria* and *Bacteroidetes* groups while decreasing the abundance of *Actinobacteria*, which aligns with our findings. *Proteobacteria* and *Bacteroidetes*, along with *Firmicutes*, are components of the chicken microbiome [34]. *Firmicutes* are among the most commonly found bacterial groups in animal manure. However, our results show that in soil fertilized with manure, *Firmicutes* accounted for only 3.06% of all identified phyla. The lower abundance of *Firmicutes* in soils may be due to their reduced ability to utilize carbohydrates from manure compared to *Proteobacteria* [35]. Laconi et al. [36] suggest that the increase in *Firmicutes* abundance in manure-fertilized soils

may only be a temporary effect. Additionally, the abundance of *Firmicutes* in soils depends on the soil's physicochemical properties and agricultural practices [37].

Bacteria from the phyla *Verrucomicrobiota*, *Planctomycetota*, *Myxococcota*, and P*atescibacteria* accounted for 7.34%, 4.03%, 2.98%, and 2.72% of all identified phyla, respectively. The taxonomic profile of the soil at the phylum and class levels is presented in Figure 1. The taxonomic profile of the soil at the order and family levels is presented in Table 2.

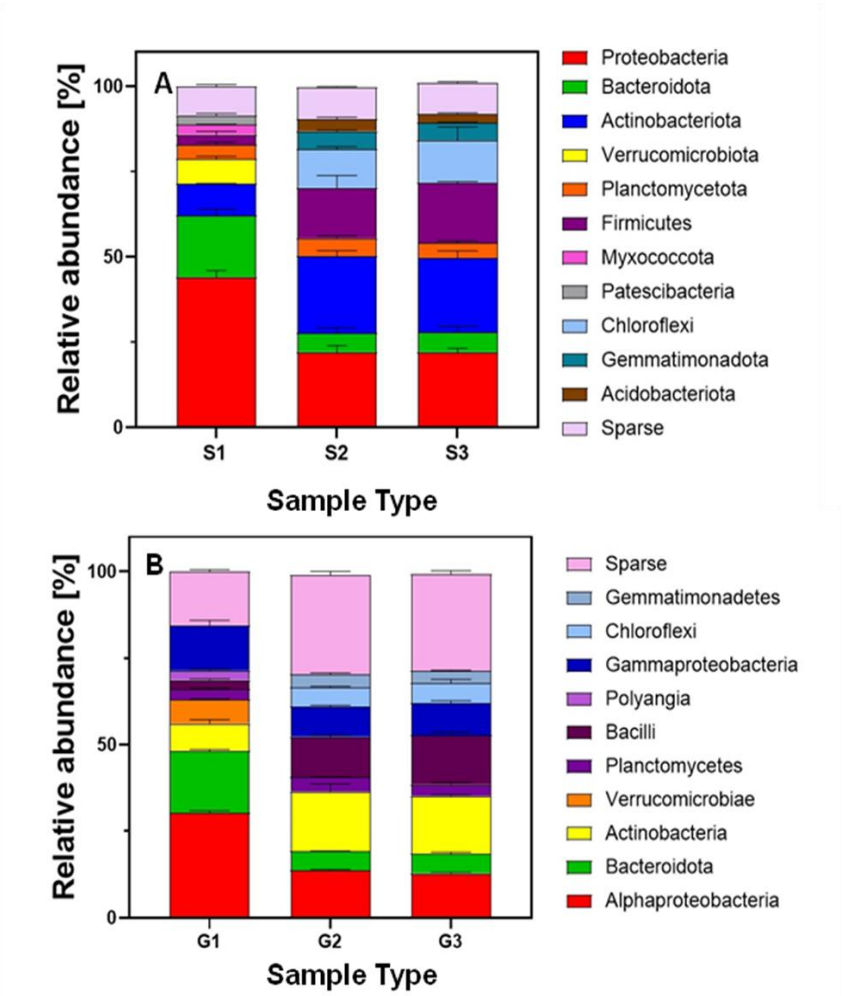


Figure 1. Relative abundance (%) of the soil bacterial community at the phylum (A) and class (B) levels. S1 – soil without tetracycline addition; S2 – soil with 5 mg/kg tetracycline addition; S3 – soil with 25 mg/kg tetracycline addition. Taxa that comprised less than 5% of the total classified reads were grouped and labeled as "Sparse".

The addition of tetracycline caused significant changes in the soil microbiome composition, and this effect was not dependent on the concentration of the antibiotic (Figure 1). Both lower (5 mg/kg) and higher concentrations of the antibiotic (25 mg/kg) resulted in similar soil microbiome compositions, which significantly differed from the microbiome of soil without the addition of antibiotics. The most dominant bacterial group at the phylum level was *Actinobacteriota* (22%), whose abundance in tetracycline-treated soils increased significantly compared to soil without the antibiotic (Figure 1A). The presence of *Actinobacteriota* in the soil is ecologically important as these bacteria are involved in the metabolism of organic matter and exhibit remediation capabilities [38]. On the other hand, many members of this phylum are significant human pathogens [39. Within the *Actinobacteria* class, two orders were identified – *Micrococcales* (6.66%) and *Propionibacteriales* (3.88%) – as well as two families – *Micrococcaceae* (2.57%) and Nocardoidiaceae (3.86%). *Micrococcaceae* bacteria have the ability to degrade aromatic compounds in soils [40], which explains their increased abundance

in tetracycline-treated soil. Fan et al., [41] also indicate that *Micrococcaceae* are carriers of tetracycline resistance genes in the soil–rhizosphere–plant internal tissues system, and this family is particularly rich in mobile genetic elements. *Nocardoidiaceae* bacteria also have remediation capabilities and are detected in contaminated environments [42. Studies show that these bacteria can degrade various xenobiotics – including ibuprofen [43], sulfamethoxazole [44], and carbamazepine [45]. An increase in the abundance of antibiotic-degrading bacteria in soils may be a factor in the spread of antibiotic resistance [46]. *Nocardia* spp. bacteria can also cause infections in humans, such as nocardiosis, including skin and pulmonary infections, especially in immunocompromised individuals [47]. In soil treated with 5 mg/kg tetracycline, the presence of *Mycobacterium tuberculosis*, a member of the Actinobacteria class, was also detected. *Mycobacterium tuberculosis* is a dangerous human pathogen causing tuberculosis. In 2022, 10.6 million people worldwide contracted tuberculosis, and 1.3 million patients died [48]. *Tuberculosis* bacteria are primarily transmitted through aerosol, in direct contact with an infected person. However, Velayati et al. [49] indicate that tuberculosis bacteria can also be transmitted without contact with an infected person. Small droplets of sputum (1-3 μm) coughed out by the patient after losing water turn into droplet nuclei and then into bacterial dust, which stays in the air for a long time in the form of an aerosol. Due to the typical structure of mycobacteria rich in lipids in cell wall, the strong tolerance of tuberculosis bacteria benefits the survival for 10 months in dry sputum and 5 months in water [50]. *M. tuberculosis* has previously been isolated from sewage sludge, wastewater, and soils [51], with patient excrement being indicated as a source of pathogens. To date, few studies have assessed the transmission of *M. tuberculosis* from the environment to humans, and the sources of these pathogens in soil are not fully understood. The detection of this pathogen in tetracycline-treated soil raises particular concerns. *M. tuberculosis* is known to exhibit antibiotic resistance [52], and multidrug-resistant tuberculosis is one of the most serious public health threats [48]. Although this pathogen was not detected in soil treated with the highest concentration of tetracycline, *Mycobacterium holsaticum* was identified. The genus *Mycobacterium* includes over 140 species, and many new species have been described from the environment in recent years [53]. *Mycobacterium holsaticum* is rarely reported as a cause of human infections. This strain was first isolated from clinical cases in Germany [54]. There are also case reports identifying this microorganism as a causative agent of infections. Verghese et al. [55] described a case of intestinal infection caused by *M. holsaticum*. Lima et al. [56] isolated *M. holsaticum* from the respiratory tract of patients with pulmonary tuberculosis, highlighting the need for identification of potential infections by bacteria not belonging to the *Mycobacterium tuberculosis* complex (non-tuberculosis bacteria)[56]. In soil treated with the lowest concentration of tetracycline, *Actinomadura geliboluensis* was also detected. Although its pathogenicity in humans has not been reported to date, it was isolated from a patient with pneumonia in 2023 [57].

In tetracycline-treated soil, a 21% decrease in the abundance of *Proteobacteria* was observed (Figure 1A). Xiong et al. [58] also reported a reduction in the abundance of *Proteobacteria* in soils treated with tetracycline. The authors suggest that the decrease in *Proteobacteria* abundance in response to tetracycline treatment indicates the occurrence of antibiotic resistance [58]. In our case, however, this aspect requires further investigation. Among *Proteobacteria*, a decrease in the abundance of *Rhizobiales* and B*urkholderiales* orders was observed. The reduction in these orders may have significant consequences for soil health and plant growth, as these bacteria play key roles in soil ecosystems. Tetracycline-treated soils showed a significant increase in the abundance of *Firmicute*s bacteria (with an increase of 11.6% and 14.7% in soils with 5 and 25 mg/kg tetracycline, respectively). Blau et al. [59] demonstrated an increase in this taxon in soils fertilized with manure supplemented with doxycycline, indicating that *Firmicutes* are responsible for the spread of ARGs in the environment. Within the *Firmicutes* phylum, one class – *Bacilli* – and one order – *Bacillales* – were identified (Figure 1B, Table 2). *Bacillales* bacteria perform many important functions in soils, including participation in nutrient cycling and exhibiting remediation properties [60]. On the other hand, an increase in the abundance of these taxa following tetracycline application may indicate the selection of antibiotic-resistant strains. Within the *Bacillales* order, two families – *Planococcaceae* and B*acillaceae* – were identified (Table 2). The presence of

Chloroflexi bacteria was also observed in tetracycline-treated soils (Figure 1), which were not identified in soil without the antibiotic addition. Although the exact function of these bacteria in soil is unknown, they survive in low-fertility soils [61]. In soils treated with antibiotics, a decrease in the abundance of *Bacteroidota* (by 12% compared to soil without tetracycline) was also observed. These bacteria are an important group of soil microorganisms, and a reduction in their abundance may have negative consequences for both the soil and the plants grown in it. In tetracycline-treated soils, *Gemmatimonadota* bacteria were also identified (Figure 1A). These bacteria are widely distributed in the environment. Their cosmopolitan distribution in various soils suggests that they utilize a broad range of nutrients [62]. Their presence in tetracycline-treated soils was also demonstrated by Zheng et al. [63]. The increased abundance of these bacteria in tetracycline-treated soils may be related to their use of tetracycline as a carbon source. Li et al. [64] demonstrated that *Gemmatimonadota* bacteria were carriers of antibiotic resistance genes in soil treated with sulfamethoxazole.

Table 2. Relative abundance (%) of soil bacterial community on order and family level. S1 – soil without tetracycline addition, S2 – soil with 5 mg/kg tetracycline addition, S3 – soil with 25 mg/kg tetracycline addition.

Order	S1	S2	S3
Rhizobiales	13.16±0.97	7.51±0.57	6.70±0.40
Micropepsales	7.46±0.56	<1±0.09	6.54±0.39
Burkholderiales	7.29±0.89	3.68±0.41	3.94±0.54
Flavobacteriales	6.8±0.85	<1±0.08	<1±0.08
Chitinophagales	5.34±0.4	<1±0.09	<1±0.10
Opitutales	3.87±0.37	<1±0.09	<1±0.08
Sphingobacteriales	3.42±1.18	<1±0.03	<1±0.06
Xanthomodales	3.39±0.78	<1±0.03	<1±0.04
Bacillales	<1±0.06	10.25±0.33	12.58±0.37
Micrococcales	<1±0.06	6.66±0.43	6.54±0.36
Thermomicrobiales	<1±0.11	5.06±0.99	5.36±0.37
Gemmatimonadales	<1±0.07	3.78±0.19	3.5±0.06
Gitt-GS	<1±0.05	2.87±1.1	<1±0.06
Propionibacteriales	<1±0.05	<1±0.44	3.92±0.25
Family	**S1**	**S2**	**S3**
Rhizobiaceae	4.56±0.48	2.79±0.39	<1±0.10
Micropepsaceae	7.46±0.56	<1±0.10	<1±0.05
Flavobacteriaceae	6.68±1.59	<1±0.04	<1±0.04
Chitiniphagaceae	4.59±0.09	<1±0.07	<1±0.08
Devosiaceae	3.97±0.82	<1±0.06	<1±0,04
Comamonadaceae	3.84±0.31	<1±0.06	<1±0.04

Opitutaceae	3.81±0.18	<1±0.06	<1±0.05
Sphinhobacteriaceae	3.13±0.12	<1±0.05	<1±0.03
Planococcaceae	<1±0.1	5.93±0.89	7.31±0.88
Bacillaceae	<1±0.07	4.3±0.26	5.26±0.27
Nocardioidaceae	<1±0.1	3.86±0.48	3.91±0.06
Gemmatimonadaceae	<1±0.06	3.77±0.71	3.51±0.2
Gitt-GS	<1±0.06	2.87±0.60	2.99±0.35
Micrococcaceae	<1±0.07	2.57±0.06	2.58±0.24

Changes in the composition of soil microorganism populations can also lead to changes in the rhizosphere microbiome. The rhizosphere is an environment particularly rich in microorganisms. Rhizosphere bacteria play a crucial role in maintaining plant health [65] by improving the availability of nutrients, including nitrogen [66] and phosphorus [67]. Additionally, rhizosphere bacteria can produce plant hormones (auxins, cytokinins, and gibberellins) that stimulate plant growth and development [21]. Furthermore, they protect plants from pathogens by producing antibiotic substances and inducing systemic resistance. Increasingly, research highlights the role of fresh vegetables and fruits in shaping the human microbiome [68] is one of the most commonly consumed raw vegetables [69], with a global cultivation area of 1.24 million hectares [70]. Therefore, it is important to study the microbiome of plants grown in environments contaminated with antibiotics. A total of 56,862, 69,185, and 44,995 paired reads (pairs joined) were obtained for the rhizosphere microbiome of lettuce grown in soil without antibiotic addition (R1), with 5 mg/kg tetracycline addition (R2), and with 25 mg/kg tetracycline addition (R3), respectively. A phylogenetic summary of the results is presented in Table 3.

Table 3. Percentage of reads assigned to appropriate taxonomic levels for analyzed samples. R1 – rhizosphere of lettuce grown on soil without tetracycline addition, R2 – rizosphere of lettuce grown soil with 5 mg/kg tetracycline addition, R3 – rizosphere of lettuce grown on soil with 25 mg/kg tetracycline addition.

Sample	Kingdom	Phylum	Class	Order	Family	Genus	Species
R1	100	100	99.97	99.34	95.87	76.67	13.6
R2	100	99.97	99.94	99.26	95.00	76.72	5.35
R3	100	99.89	99.84	99.13	95.96	79.05	13.43

Although tetracycline significantly alters the composition of microorganisms inhabiting contaminated soils, this effect is not as pronounced in the rhizosphere. The impact of antibiotics on rhizosphere microorganisms is influenced by factors such as the interaction between antibiotic molecules and root exudates [71] as well as the physical and chemical properties of the soil [72]. Besides soil microorganism composition, other factors shaping the plant rhizosphere include soil type, pH [73], and plant developmental stage [4]. Žiarovská et al. [74] demonstrated that the composition of microorganisms varies depending on the lettuce species. Additionally, the seed microbiome may influence the final composition of the rhizosphere microorganism population [75]. Based on ASV data, it was found that only 12.4% and 21% of

ASVs in the rhizosphere microbiome of lettuce treated with tetracycline at concentrations of 5 mg/kg and 25 mg/kg, respectively, originated from the soil (Figure 2). In control samples (S1, R1), 38.7% of ASVs were common between the soil and rhizosphere (Figure 2). Under stress conditions, plants alter the chemical composition of root exudates, attracting microorganisms that support plant health [76]. Huang et al. [77] found that rhizosphere bacteria under antibiotic-induced stress enhance their community structure by dispersing antibiotics in the soil and favoring the growth of key species that support plant growth [77]. Therefore, the final composition of the rhizosphere microbiome depends on specific interactions between antibiotics, the soil, and the plants.

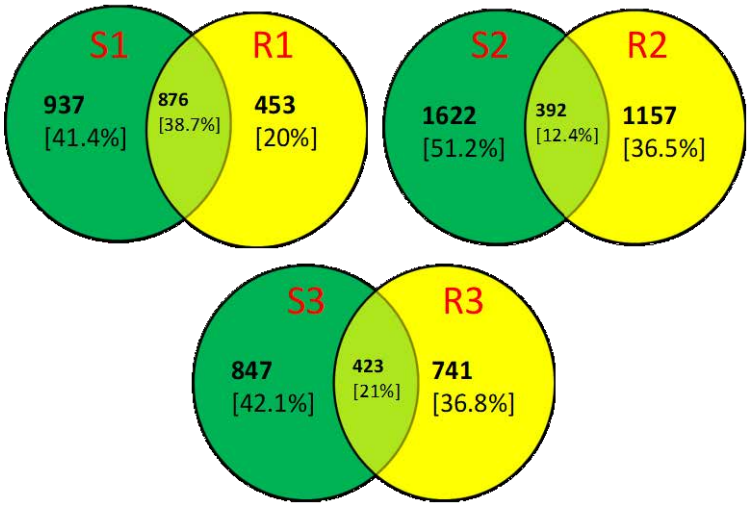


Figure 2. Shared ASVs between soil and lettuce rhizosphere. S1 – soil without tetracycline addition, S2 – soil with 5 mg/kg tetracycline addition, S3 – soil with 25 mg/kg tetracycline addition, R1 – rizopshere of lettuce grown on soil without tetracycline addition, R2 – rizosphere of lettuce grown soil with 5 mg/kg tetracycline addition, R3 – rizosphere of lettuce grown on soil with 25 mg/kg tetracycline addition.

In the lettuce rhizosphere, bacteria from 11 phyla were identified: *Proteobacteria*, *Bacteroidota*, *Actinobacteriota*, *Verrucomicrobiota*, *Planctomycetota*, *Firmicutes*, *Myxococcota*, *Patescibacteria*, *Chloroflexi*, *Gemmatimonadota*, and *Acidobacteriota*, in varying proportions (Figure 3). Pandiyan et al. [78] indicate that at the phylum level, plant microbiomes show significant similaritis. However, lower taxonomic levels are shaped by environmental conditions and differ from each other. In the lettuce rhizosphere, the relative abundance of *Proteobacteria* was higher compared to the soil (Figure 1, Figure 2). Similar results were also obtained by Schreiter et al. [73]. The plant rhizosphere is preferentially colonized by *Proteobacteria*, *Bacteroidetes*, and *Actinobacteriota* because these bacteria actively utilize root exudates [79]. These taxa also dominate within roots [3]. Our results indicate *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* as dominant bacterial phyla in the lettuce rhizosphere (Figure 2), including those treated with tetracycline. At the phylum level, the most dominant bacterial group in each sample was *Proteobacteria* (Figure 3), and the addition of tetracycline did not significantly alter the structure of this bacterial group. Within *Proteobacteria*, two classes were identified: A*lphaproteobacteria* and *Gammaproteobacteria* (Figure 3), five orders: R*hizobiales*, *Micropepsales*, *Caulobacterales, Burkholderiales*, *Xanthomonadales*, and five families: *Rhizobiaceae*, *Devosiaceae*, *Xanthobacteraceae*, *Comamonadaceae* and R*hodanobacteraceae* (Table 4). These bacteria perform diverse and complex functions in the soil ecosystem, positively influencing plant health and growth. Plant Growth-Promoting Bacteria (PGPB) play a key role in maintaining plant health, especially in contaminated environments. However, there is growing concern about the presence of antibiotic resistance genes among these bacteria, particularly within the *Rhizobiales* and B*urkholderiales* taxa [80]. These taxa constituted 13.43% and 5.30% of the identified orders in the rhizosphere of R1 and R2, respectively. Among *Burkholderiales*, an increase in abundance was observed in the rhizosphere with rising tetracycline concentrations in the soil. According to Wicaksono et al. [5], *Burkholderiales*

bacteria associated with vegetables and fruits are a functional component of the human microbiome. This taxon includes both health-promoting species and pathogens, as well as antibiotic-resistant strains. Among them, *Burkholderia cepacia* is a plant phytogen and is known as a hardy and versatile organism. Over the past two decades it has emerged as a pathogen in the cystic fibrosis (CF) community, with devastating effects. Pulmonary colonisation can lead to an accelerated decline in lung function. *B. cepacia* is inherently resistant to multiple antibiotics and highly transmissible and virulent strains have been identified [81]. Therefore, the presence of these bacteria in plants growing in contaminated environments should be closely monitored. The *Bacteroidota* phylum in the rhizosphere of lettuce grown without antibiotic addition was represented by the class *Bacteroidia* (Figure 3), order *Flavobacteriales*, family *Flavobacteriaceae* (Table 4), and genus *Flavobacterium*. Žiarovská et al. [74], also identified the genus Flavobacterium as one of the most abundant in the lettuce rhizosphere. These bacteria likely play a role in maintaining plant health and are considered growth-promoting taxa [27]. They are also widely distributed in the plant phyllosphere. Our results suggest that these bacteria survive in the rhizosphere of plants growing in tetracycline-contaminated environments, despite a decrease in their abundance in contaminated soils. Although these taxa have a positive impact on plants, their ability to survive in antibiotic-contaminated environments raises concerns about the spread of antibiotic resistance. These bacteria are considered carriers of tet(A) resistance genes among bacterial communities [82]. In the rhizosphere of plants R2 and R3, an increase in the abundance of the *Chitinophagales* taxon was also observed. The increase in chitin-degrading taxa in the rhizosphere of lettuce treated with antibiotics may be related to the bacteriostatic effect of tetracycline and the associated fungal overgrowth [59]. The *Actinobacteriota* phylum was represented by bacteria from the orders *Micrococcales* and *Propionibacteriales* and the family *Nocardioidaceae* (Table 4). The presence of these bacteria has been previously reported in soil treated with tetracycline. However, in the rhizosphere of plants treated with tetracycline, the presence of *Mycobacterium tuberculosis*, which was present in the soil, was not observed. The most significant changes in the rhizosphere due to tetracycline were observed in *Firmicutes* bacteria (Figure 3). The abundance of this phylum increased in the rhizosphere of lettuce grown in tetracycline-contaminated soil, and this increase was proportional to the rising concentration of the antibiotic. *Firmicutes* are widely distributed in the lettuce microbiome [83], and the phyllosphere (aboveground plant parts) is typically represented by microorganisms that thrive in the given environment [78]. Zhang et al. [84] demonstrated a positive correlation between Firmicutes and the presence of the tetX gene in the rhizosphere. The increase in the abundance of this taxon in the plant rhizosphere may be related to the occurrence of antibiotic resistance. However, this aspect requires further investigation. The *Gemmatimonadota* phylum has also been associated with the presence of tetracycline resistance genes (tetT) [85]. An increase in the abundance of this taxon was observed in the rhizosphere of lettuce treated with the highest concentration of tetracycline (Figure 3). Increasing evidence suggests that minimally processed, raw lettuce can be a pathway for the introduction of pathogens and ARGs into the human body [86]. However, our results do not indicate the presence of pathogens in the lettuce rhizosphere. It should be noted that only a few percent of strains in the lettuce rhizosphere were identified in this study. However, the detection of *M. tuberculosis* in tetracycline-treated soil is concerning. The possibility of this pathogen entering plant tissues should be investigated. The detailed composition of microorganisms inhabiting the rhizosphere of lettuce grown in tetracycline-contaminated environments at the phylum and class levels is shown in Figure 3. The taxonomic profile of the rhizosphere of lettuce treated with tetracycline at the order and family levels is presented in Table 4.

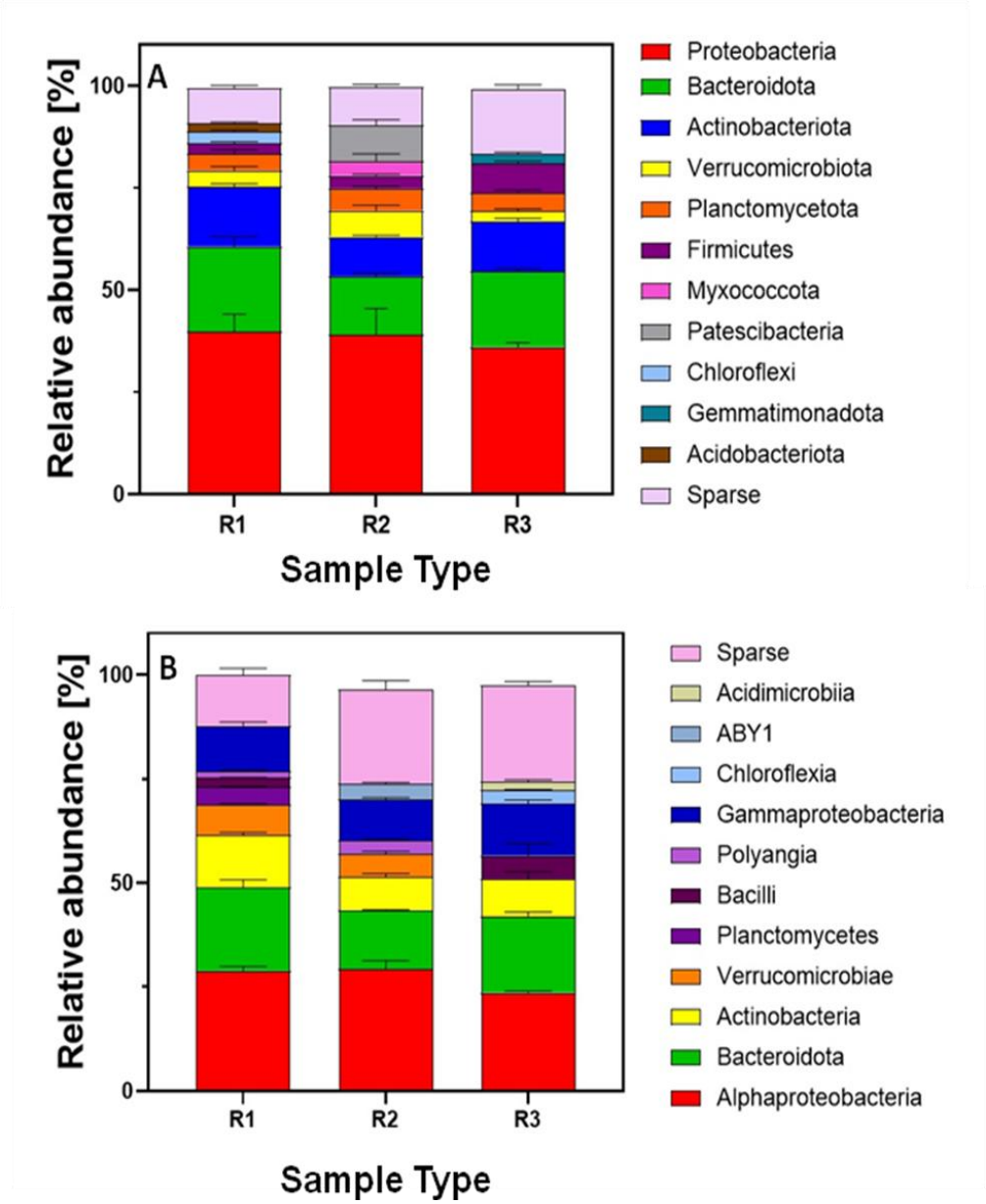


Figure 3. Relative abundance (%) of the lettuce rhizosphere bacterial community at the phylum (A) and class (B) levels. R1 – lettuce grown in soil without tetracycline addition, R2 – lettuce grown in soil with 5 mg/kg tetracycline addition, R3 – lettuce grown in soil with 25 mg/kg tetracycline addition. Taxa that made up less than 5% of the total classified reads were grouped and labeled as "Sparse".

Table 4. Relative abundance (%) of the rhizosphere bacterial community at the order and family levels. R1 – rhizosphere of lettuce grown in soil without tetracycline addition, R2 – rhizosphere of lettuce grown in soil with 5 mg/kg tetracycline addition, R3 – rhizosphere of lettuce grown in soil with 25 mg/kg tetracycline addition.

Order	R1	R2	R3
Rhizobiales	14.94±0.65	13.43±0.74	11.69±0.44
Micropepsales	4.14±0.35	4.63±1.09	<1±0.09
Burkholderiales	4.94±0.81	5.29±0.13	6.52±0.62

Flavobacteriales	10.4±0.48	13.24±0.45	10.46±1.72
Chitinophagales	4.45±1.00	4.95±1.15	4.35±0.85
Opitutales	<1±0.07	<1±0.09	<1±0.10
Sphingobacteriales	<1±0.07	4.38±0.17	<1±0.06
Xanthomodales	<1±0.06	<1±0.07	<1±0.12
Bacillales	<1±0.06	<1±0.08	5.05±0.39
Micrococcales	3.79±0.24	<1±0.08	3.37±0.75
Gemmatimonadales	<1±0.08	<1±0.07	<1±0.10
Candidatus Magasanikbacteria	<1±0.06	3.72±1.1	<1±0.08
Propionibacteriales	<1±0.10	<1±0.2	3.02±0.41
Caulobacterales	3.45±0.63	4.96±1.97	3.09±0.56
Family	**R1**	**R2**	**R3**
Rhizobiaceae	5.13±0.34	3.31±0.67	3.79±0.37
Micropepsaceae	4.14±0.27	4.63±0.96	<1±0.14
Flavobacteriaceae	10.16±0.94	10.13±0.13	10.18±1.96
Chitiniphagaceae	4.12±1.03	3.79±0.21	3.71±0.39
Devosiaceae	4.52±0.71	4.55±0.75	3.36±0.43
Comamonadaceae	2.97±0.12	<1±0.13	3.47±0.92
Sphinhobacteriaceae	<1±0.05	4.07±0.52	<1±0.05
Planococcaceae	<1±0.17	<1±0.19	2.78±0.36
Nocardioidaceae	3.00±0.12	3.01±0.05	3.18±0.66
Gemmatimonadaceae	<1±0.12	3.77±1.21	3.51±0.5
Xanthobacteraceae	3.25±0.39	<1±0.14	2.97±0.25
Comamonadaceae	2.97±0.37	<1±0.10	<1±0.08
Caulobacteriaceae	<1±0.23	4.73±1.95	2.73±0.64

4. Materials and Methods

4.1. Lettuce Growing Conditions

The soil was collected in early spring 2021 from an agricultural field before the application of a fresh batch of manure. The field had been regularly fertilized with chicken manure in previous growing seasons. The agricultural field is located in the Warmian-Masurian Voivodeship. After collection, the samples were transported to the laboratory and stored at 4 °C before analysis. The soil was confirmed to be free of antibiotics. A detailed characterization of the soil is presented in the work by Krupka et al. [20]. Seeds of lettuce (*Lactuca sativa* L. var. Takoda) were sown in seed trays measuring 24 × 24 mm. The plants were then grown for 4 weeks in climate chambers (POL-EKO, Wodzisław Śląski, Poland) at a temperature of 20 °C/15 °C day/night, with a 16/8 h photoperiod, and a light intensity of 8000 lx. Subsequently, 2 kg of

soil was added to pots with a diameter of 17 cm, and lettuce seedlings (one seedling per pot) were transplanted. The plants were then watered with 15 ml of an aqueous solution of tetracycline-HCl (Sigma-Aldrich, USA) at concentrations of 5 mg/kg (S2) and 25 mg/kg (S3). According to Zhang et al. [63] 2020, tetracycline is detected in concentrations ranging from 5 to 25 mg/kg in areas with increased agricultural activity. The control group consisted of lettuce grown in soil without tetracycline (S1), watered with distilled water. A total of 5 replicates were obtained for each treatment. The plants were grown for an additional 4 weeks under the same conditions before further analyses were conducted.

4.2.DNA Isolation from Soil and Rhizosphere

Plants were removed from the pots, and the leaves and roots were separated using sterile scissors. To minimize local variability in bacterial populations, 10 lettuce plants were cultivated for each treatment. Soil tightly adhering to the roots was considered the rhizosphere Rhizosphere samples were collected from each plant and pooled to create a single, homogeneous composite sample representative of the rhizosphere. A similar approach was applied to soil samples to obtain a representative soil sample. DNA was isolated from 250 mg of soil and rhizosphere using the DNeasy Power Pro Soil Kit (Qiagen INC, USA), following the manufacturer's protocol. The quantity of isolated DNA was measured using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). The purity of the isolated DNA was checked using the OD 260/280 and OD 260/230 parameters (NanoDrop Technologies, Wilmington, USA). The resulting genetic material was suspended in Tris-HCl buffer at pH 7 and stored at -20°C until further analysis. DNA isolation was performed in 5 replicates.

4.3. Metagenomic Analysis of Soil and Rhizosphere Samples

The metagenomic analysis of the samples was conducted by an external company (Genomed S.A., Warsaw). Bacterial population analysis was based on the hypervariable V3-V4 region of the 16S rRNA gene. Specific primer sequences 341F and 785R were used for the amplification of the selected region and library preparation. The PCR reaction was performed using Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs Inc., Ipswich, USA), according to the manufacturer's recommendations. Sequencing was performed on a MiSeq system using paired-end (PE) technology, 2x300nt, with an Illumina v3 kit. Bioinformatic analysis, which ensured the classification of reads to the species level, was conducted using the QIIME 2 software package based on the Silva 138 reference sequence database. The DADA2 package was also used to differentiate between biologically derived sequences and those newly generated during sequencing. This package was also utilized to extract unique biologically derived sequences, known as ASV (amplicon sequence variants). For taxonomic analysis, three independent replicates were prepared. A one-way analysis of variance (ANOVA) was conducted to evaluate significant differences between bacterial communities in the rhizosphere and bulk soil. Post hoc tests were also applied.

4. Conclusions

1. Tetracycline at concentrations of 5 and 25 mg/kg significantly alters the soil microbiome at various taxonomic levels, and this effect is noticeable even after applying the lowest concentration of the antibiotic.
2. The presence of *M. tuberculosis* in soil treated with 5 mg/kg tetracycline may suggest that tetracycline affects the microbiome in a way that favors the survival of this pathogen in the environment. This finding requires further investigation to understand the mechanisms allowing *M. tuberculosis* to persist in soil environments, potential transfer to plant tissues, and public health risks.
3. The addition of tetracycline did not induce significant changes in the taxonomic profile of the rhizosphere microorganisms. This suggests that plants may influence the stabilization of the rhizosphere microbiome.
4. The increased abundance of Firmicutes bacteria after tetracycline treatment in soil and rhizosphere suggests that this taxon should be particularly monitored in plants growing in antibiotic-contaminated environments.

The use of tetracycline and other antibiotics in agriculture can lead to lasting changes in the soil microbiome, which may have long-term consequences for soil ecosystem health and crop plants. Given the observed changes, further research is necessary to better understand the mechanisms underlying microorganism adaptation to tetracycline and their long-term effects on soil ecosystems and plant health. This study serves as a preliminary investigation for more advanced research, including the detection of antibiotic resistance genes in plant tissues, which will significantly contribute to protecting consumers who eat fresh, unprocessed lettuce leaves.

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