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*Rozprawa doktorska*

**Wpływ stresorów środowiskowych na wybrane lądowe bezkręgowce Lumbricidae  
(Oligochaeta, Clitellata) i Helicidae (Pulmonata, Gastropoda)**

***The impact of environmental stressors on selected terrestrial invertebrates Lumbricidae  
(Oligochaeta, Clitellata) and Helicidae (Pulmonata, Gastropoda)***

Rozprawa doktorska wykonana w Katedrze Zoologii i Ekologii Zwierząt

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## Spis treści

|     |                                                                                                                         |     |
|-----|-------------------------------------------------------------------------------------------------------------------------|-----|
| 1.  | Streszczenie.....                                                                                                       | 5   |
| 2.  | Summary .....                                                                                                           | 7   |
| 3.  | Wprowadzenie teoretyczne .....                                                                                          | 9   |
| 4.  | Cele badań i hipotezy badawcze .....                                                                                    | 13  |
| 5.  | Materiał i metody .....                                                                                                 | 15  |
| 6.  | Omówienie wyników badań i dyskusja.....                                                                                 | 23  |
| 7.  | Podsumowanie i wnioski .....                                                                                            | 40  |
| 8.  | Bibliografia .....                                                                                                      | 44  |
| 9.  | Kopie opublikowanych prac wchodzących w skład cyklu publikacji .....                                                    | 56  |
| 10. | Oświadczenia doktoranta oraz współautorów dotyczących ich wkładu<br>w przygotowanie opublikowanych prac naukowych ..... | 137 |
| 11. | Aneks .....                                                                                                             | 151 |

## 1. Streszczenie

Bezkręgowce lądowe takie jak dżdżownice Lumbricidae i ślimaki Helicidae są organizmami biowskaźnikowymi. Skałę płonną potraktowano jako potencjalne źródło metali ciężkich w środowisku. Ponieważ dżdżownice żyją w glebie, a ślimaki podobnie jak dżdżownice mają kontakt z glebą przez powłoki ciała i układ pokarmowy, przeprowadzono analizę właściwości fizykochemicznych i biologicznych gleby z depozytem i bez depozytu skały płonnej, która wykorzystywana jest jako materiał w rekultywacji. Obecność skały płonnej w glebie wpływała na jej parametry fizykochemiczne i aktywność biologiczną, przy czym efekt ten zależał głównie od sposobu użytkowania rolniczego i rodzaju pokrywy roślinnej terenu. Obserwowano transfer siedmiu metali (Cr, Ni, Cd, Ba, Pb, Zn i Cu) w różnych konfiguracjach lokacyjnych, glebowych, roślinnych i zwierzęcych, którego kluczowym elementem był depozyt skały płonnej. Wybrane metale wpływały na gospodarkę lipidową dżdżownic, prowadząc do zmian w składzie i oksydacji kwasów tłuszczowych. Z zaobserwowanymi zmianami w składzie i oksydacji kwasów tłuszczowych zsynchronizowane były: aktywność enzymatycznych (SOD i CAT) i poziom nieenzymatycznych (TBARS) biomarkerów stresu oksydacyjnego. Wpływ metali na badane parametry fizjologiczne u dżdżownic zależał od czasu trwania depozytu i sposobu użytkowania terenów z depozytem skały płonnej. U dżdżownic pochodzących z nieużytku z długoletnim depozytem (powyżej 10 lat) skały płonnej, zaobserwowano najmniej korzystną odpowiedź fizjologiczną na poziomie peroksydacji kwasów tłuszczowych. Stwierdzono, że użytkowanie terenów w formie upraw zbożowych lub łąk utrzymywanych w dobrej kulturze rolnej, obniżało w percepcyjnym fizjologicznie zakresie negatywny wpływ metali na badane dżdżownice. Wybrane metale wpływały na odpowiedź fizjologiczną u ślimaków, która była związana ze zmianami w składzie biomarkerowych kwasów tłuszczowych, wskaźnikiem peroksydacji i wskaźnikiem nienasycenia kwasów tłuszczowych, w tym na potencjalną peroksydację biomarkerowych kwasów tłuszczowych. U ślimaków pozyskanych ze stanowiska z depozytem skały płonnej zaobserwowano zauważalny spadek zawartości wielonienasyconych kwasów tłuszczowych w porównaniu z próbami terenowymi bez jej depozytu, a także z próbami laboratoryjnymi, co może sugerować potencjalną peroksydację kwasów tłuszczowych. W przypadku ślimaków, zastosowanie podejścia łączącego pozyskanie materiału ze środowiska z eksperymentalnym suplementowaniem metalami w warunkach laboratoryjnych (kontrolowanych) umożliwiło zwiększenie uniwersalności proponowanych biomarkerów fizjologicznych. Podejście to

daje także możliwość oceny wpływu pośrednich czynników środowiskowych na biomarkerowe kwasy tłuszczowe u tych mięczaków. Wykonane analizy potwierdziły zasadność wykorzystania wybranych bezkręgowców lądowych jako źródła biomarkerów odpowiedzi biologicznej na wybrane metale, co wskazuje na ich potencjał i możliwe zastosowanie w badaniach ekotoksykologicznych.

**Słowa kluczowe:** dżdżownice, Lumbricidae, ślimaki, Helicidae, skała płonna, metale ciężkie, biomarkerowe kwasy tłuszczowe

## 2. Summary

Terrestrial invertebrates such as Lumbricidae earthworms and Helicidae snails are bioindicator organisms. Waste rock was considered a potential source of heavy metals in the environment. Because earthworms live in the soil, and snails, like earthworms, have contact with the soil through their integuments and digestive tracts, an analysis of the physicochemical and biological properties of soil with and without waste rock deposits was conducted, which is used as a material in reclamation. The presence of waste rock in the soil affected its physicochemical parameters and biological activity, with this effect depending mainly on the type of agricultural use and the type of vegetation cover in the area. The transfer of seven metals (Cr, Ni, Cd, Ba, Pb, Zn, and Cu) was observed across various location, soil, plant, and animal configurations, with the waste rock deposit serving as a key element. Selected metals influenced the lipid metabolism of earthworms, leading to changes in the composition and oxidation of fatty acids. The observed changes in the composition and oxidation of fatty acids were synchronized with the activity of enzymatic (SOD and CAT) and levels of non-enzymatic (TBARS) biomarkers of oxidative stress. The effect of metals on the physiological parameters studied in earthworms depended on the duration of the deposit and the land use of areas with a waste rock deposit. In earthworms originating from wasteland with a long-term deposit (over 10 years) of waste rock, the least favorable physiological response was observed at the level of fatty acid peroxidation. It was found that land use in the form of cereal crops or meadows maintained in good agricultural condition reduced, within a physiologically perceptible range, the negative impact of metals on the studied earthworms. Selected metals influenced the physiological response in snails, which was associated with changes in the composition of biomarker fatty acids, the peroxidation index, and the unsaturation index of fatty acids, including potential peroxidation of biomarker fatty acids. Snails collected from a site with a waste rock deposit showed a noticeable decrease in polyunsaturated fatty acid content compared to field samples without a deposit, as well as to laboratory samples, which may suggest potential fatty acid peroxidation. In the case of snails, the use of an approach combining the collection of material from the environment with experimental metal supplementation under laboratory (controlled) conditions made it possible to increase the versatility of the proposed physiological biomarkers. This approach also allows for the assessment of the impact of indirect environmental factors on biomarker fatty acids in these mollusks. The analyses conducted confirmed the validity of using selected terrestrial invertebrates as a source of

biomarkers of biological response to selected metals, indicating their potential and possible application in ecotoxicological studies.

**Keywords:** earthworms, Lumbricidae, snails, Helicidae, waste rock, heavy metals, biomarker fatty acids

### 3. Wprowadzenie teoretyczne

W badaniach ekologicznych określających relacje organizm-środowisko, ale też w monitoringu środowiska często wykorzystywane są bezkręgowce lądowe, w tym dżdżownice (Oligochaeta, Lumbricidae) (Traczewska, 2011; Munhoz i Beriot, 2025) ślimaki (Gastropoda, Helicidae) (de Vaufleury i in., 2006; Traczewska, 2011; Parmar i in., 2016; Hamed i in., 2025). Charakteryzują się one szybkim rozwojem i są wrażliwe na fizykochemiczne stresogenne zmiany w środowisku (Vranković i in., 2020). Zwierzęta bezkręgowce ze względu na bezpośredni kontakt z glebą i roślinnością, mają istotne znaczenie w procesie transferu zanieczyszczeń, w tym metali ciężkich (Gimbert i in., 2006; Madoz-Escande i Simon, 2006; El-Gendy i in., 2021). Zawartość metali ciężkich w środowisku stanowi kluczowy czynnik determinujący ich akumulację w bezkręgowcach lądowych (Hobbelen i in., 2006; Shen i in., 2026). W zależności od potrzeb organizmu, metale te, mogą być magazynowane lub z niego usuwane (Ardestani i in., 2014). Metalami niezbędnymi, a jednocześnie mikroelementami są między innymi cynk (Zn), miedź (Cu), chrom (Cr) i nikiel (Ni). Ich podwyższone stężenie może być toksyczne, ze względu na przekroczenie fizjologicznego zapotrzebowania organizmu (Sizmur i Hodson, 2009; Lev i in., 2010; Yadav i in., 2023). Subprogowe dawki tych pierwiastków są niezbędne do życia, co wskazuje, że nawet niewielkie ich stężenia mogą indukować zauważalne zmiany fizjologiczne (Richardson i in., 2020). Natomiast wśród metali nieistotnych, a jednocześnie toksycznych nawet w niskich stężeniach, znajdują się między innymi kadm (Cd), ołów (Pb) i bar (Ba). Wszystkie wymienione pierwiastki klasyfikowane są jako metale ciężkie, które w zależności od stężenia i czasu ekspozycji mogą być potencjalnie toksyczne dla organizmów żywych (Sizmur i Hodson, 2009; Lev i in., 2010; Yadav i in., 2023).

Spośród wielu zwierząt bezkręgowych, w ekosystemach lądowych kluczową rolę odgrywają dżdżownice. Ich obecność wpływa na właściwości gleby, zarówno fizyczne, chemiczne, jak i biologiczne, dlatego często określa się je mianem „inżynierów ekosystemów” (Shi i in., 2017; Kooch i in., 2025; Shen i in., 2026). Dzięki zdolności do detoksykacji, regulowania i eliminowania nadmiaru metali ciężkich, przyczyniają się do oczyszczania gleby (Sivakumar, 2015; Yadav i in., 2023). Uczestniczą w procesie rozkładu materii organicznej, co czyni je istotnym elementem funkcjonowania ekosystemów. Pełnią ważną funkcję w łańcuchu troficznym, łącząc system podziemny z nadziemnym, a jednocześnie stanowiąc pożywienie dla licznych kręgowców, w tym np. kretów czy niektórych ptaków (Sivakumar, 2015; Shi i in., 2017). Dżdżownice są stale narażone na

obecność zanieczyszczeń w środowisku glebowym (Velki i Ečimović, 2016). Do ich organizmu metale przedostają się zarówno poprzez bezpośredni kontakt z glebą, jak i przez ich pobieranie wraz z pokarmem (Sivakumar, 2015). Pełnią rolę bioindykatorów, dzięki ich zdolności do akumulowania zanieczyszczeń glebowych w tym metali, co umożliwia tym samym monitorowanie zanieczyszczenia gleby oraz ocenę wpływu tych polutantów (Zhang i in., 2009; Georgescu i in., 2011; Velki i Ečimović, 2016).

Ważną część lądowej fauny bezkręgowej stanowią także ślimaki lądowe, zwłaszcza gatunki z rodziny Helicidae. Pełnią one kluczową rolę w łańcuchu troficznym jako konsumenci I rzędu (Kowalczyk-Pecka i in., 2015). Jednocześnie są cennymi organizmami wskaźnikowymi potencjalnego transferu zanieczyszczeń do wyższych poziomów troficznych (Vranković i in., 2020). Ze względu na szerokie występowanie ślimaków lądowych w środowisku, a także ich zdolność do akumulacji zanieczyszczeń, mięczaki te stanowią ważny element w biomonitoringu jako organizmy biowskaźnikowe (Côte i in., 2015; Dummee i in., 2015; Hamed i in., 2025). Szczególnie cennym gatunkiem ślimaka jest wstężyk gajowy *Cepaea nemoralis* (Linnaeus, 1758), charakteryzujący się szerokim zasięgiem występowania, niewielkimi rozmiarami i szybkim metabolizmem (Kowalczyk-Pecka i in., 2015). Ślimaki lądowe wykazują zdolność wykrywania obecności metali w pożywieniu, co skutkuje ograniczeniem jego spożycia przy wysokim stężeniu zanieczyszczeń i przechodzeniem w stan estywacji (Nowakowska i in., 2016; Kowalczyk-Pecka i in., 2017a; El-Gendy i in., 2021). Jest to forma uśpienia będąca strategią przetrwania w warunkach braku pokarmu i innych stresorów środowiskowych np. wysokiej temperatury (Wang i in., 2015). W tym stanie ich metabolizm ulega spowolnieniu (Storey i Storey, 2010; Wang i in., 2015). Mimo to, przy niskich stężeniach metali ich obecność może pozostać niewykryta, co pozwala tym mięczakom nadal pobierać pokarm i prowadzi do akumulacji metali w ich organizmach. Tolerancja tych zwierząt na obecność metali jest stosunkowo wysoka, co prawdopodobnie wynika z działania metalotionein oraz mechanizmów wewnątrzkomórkowego magazynowania i wydalania (El-Gendy i in., 2021).

Istotnym elementem badań środowiskowych jest gleba, która stanowi złożone miejsce życia wielu organizmów lądowych - od mikroorganizmów (np. bakterii, grzybów), przez drobne bezkręgowce (np. dżdżownice, ślimaki), aż po kręgowce (np. krety) (Giovannetti i in., 2023; Eisenhauer i in., 2026). Gleba jest miejscem bezpośredniego kontaktu organizmów z obecnymi w niej zanieczyszczeniami, w tym metalami. Pomiędzy tymi organizmami zachodzą zróżnicowane interakcje biotyczne, w tym symbioza, komensalizm i drapieżnictwo (Giovannetti i in., 2023; Eisenhauer i in., 2026). Na glebę oraz organizmy

żywe, w tym rośliny i zwierzęta oddziałują zanieczyszczenia występujące w środowisku (Hueso-González i in., 2018; El-Gendy i in., 2021). Do najczęściej wymienianych należą zanieczyszczenia nieorganiczne (np. metale ciężkie) i organiczne (np. pestycydy, węglowodory) (Rašović, 2025). Mogą one przenikać do organizmów zwierzęcych przez kontakt z powłokami ciała (np. skórą, nabłonkiem) oraz drogą pokarmową, w tym z pobieraną wodą i pokarmem (Sivakumar, 2015; Radwan i in., 2020). Jednym z potencjalnych źródeł metali w środowisku może być skała płonna, stanowiąca odpad górniczy powstający w procesie wydobywania węgla kamiennego (Tong i in., 2022). Skała płonna składowana jest głównie na hałdach (Tong i in., 2022; Mazurek i in., 2025), a także wykorzystywana jako materiał do rekultywacji wyrobisk poeksploatacyjnych zlokalizowanych na polach, łąkach i nieużytkach (Gorczyca i Dutka, 2015; Joniec i in., 2025). W wyniku wietrzenia chemicznego skały może dojść do uwolnienia jonów metali, co warunkuje ich dostępność dla organizmów żywych (Tong i in., 2022).

Wiele badań laboratoryjnych skupia się na oddziaływaniu pojedynczych metali ciężkich (Kowalczyk-Pecka i in., 2017a, 2018), lecz w rzeczywistości w środowisku bezkręgowce lądowe narażone są na oddziaływanie wielu czynników (El-Gendy i in., 2021). Obecność jednego polutanta może wpływać na sposób wiązania i biodostępność innego, co z kolei zmienia jego dostępność dla organizmów (Velki i Ečimović, 2016). Analogicznie, w organizmach współwystępowanie zanieczyszczeń może oddziaływać na procesy detoksykacji, a tym samym modyfikować toksyczność poszczególnych substancji (Velki i Ečimović, 2016). Ze względu na wrażliwość organizmów na stres indukowany różnymi czynnikami antropogenicznymi, analiza ich bezpośredniego oddziaływania jest kluczowa dla zrozumienia mechanizmów działania tych stresorów (Velki i Ečimović, 2016). W tym celu stosuje się biomarkery, będące mierzalnymi odpowiedziami biologicznymi organizmu na ekspozycję na zanieczyszczenia i wynikające z niej skutki (Velki i Ečimović, 2016). Zmiany powodowane przez stresory środowiskowe są głównie efektem stymulacji reakcji oksydacyjnych (Chandran i in., 2005). Procesy oksydacji są inicjowane przez różnorodne stresory środowiskowe, w tym biologiczne (np. patogeny) (Ghosh, 2018), fizyczne (np. temperatura, wilgotność, napowietrzenie) (Edwards i Arancon, 2022) i chemiczne (np. metale ciężkie, pestycydy) (Uwizeyimana i in., 2017). Oksydacja dotyczy przede wszystkim lipidów i białek (Musakhanian i in., 2022). W przypadku lipidów szczególne znaczenie ma peroksydacja lipidów, w tym fosfolipidów wchodzących w skład błon intra- i extracelularnych (Singh i in., 2023; Chakraborty i in., 2024). Proces ten dotyczy utleniania kwasów tłuszczowych, głównie wielonienasyconych kwasów tłuszczowych (PUFA), które

są podatne na działanie wolnych rodników (Singh i in., 2023; Chakraborty i in., 2024). Peroksydacja kwasów tłuszczowych stanowi ważny biomarker reakcji organizmu na stresory środowiskowe. Umożliwia ocenę stopnia uszkodzeń oksydacyjnych spowodowanych przez czynniki zewnętrzne, w tym metale obecne w środowisku (Singh i in., 2023; Chakraborty i in., 2024). Skład kwasów tłuszczowych u dżdżownic wykazuje ich znaczną różnorodność (Sampedro i in., 2006). Wyjątkowy skład steroli i kwasów tłuszczowych obserwuje się także u mięczaków (Özogul i in., 2005), a zmiany w profilu kwasów tłuszczowych stanowią jeden z biomarkerów stanu fizjologicznego tych zwierząt (Kowalczyk-Pecka i in., 2017b). W odpowiedzi na działanie stresorów organizmy żywe aktywują mechanizmy obronne, w tym enzymatyczne, przede wszystkim dysmutazę ponadtlenkową (SOD) i katalazę (CAT) (Roubalová i in., 2015; Chaitanya i in., 2016; Wang i in., 2021; Chakraborty i in., 2024; Fallahzadeh i in., 2026). Efektywność tych mechanizmów ulega ograniczeniu pod wpływem wysokiego poziomu polutantów lub ich długotrwałego oddziaływania. Nawet przy niższych stężeniach może dochodzić do progresji oksydacji, w tym inicjacji procesów autooksydacyjnych (Ahmad, 1995; Fallahzadeh i in., 2026).

Do badań wybrano dżdżownice z rodziny Lumbricidae i ślimaki - wstężyk gajowy - ponieważ posiadają ważne cechy takie jak: szybki rozwój, powszechne występowanie, wysoka wrażliwość na czynniki stresogenne oraz bezpośredni kontakt z glebą i roślinnością jako konsumenci I rzędu. To czyni je efektywnymi bioindykatorami transferu zanieczyszczeń, w tym metali ciężkich, w ekosystemach lądowych. Natomiast ślimaki lądowe z glebą mają głównie pośredni kontakt, poprzez powłoki ciała, jak i układ pokarmowy. Istotnym elementem pracy jest podkreślenie jej nowatorskiego charakteru. Dotychczas nie prowadzono badań łączących skałę płonną jako potencjalne i środowiskowo dostępne źródło metali ciężkich, z próbą standaryzacji wykorzystania bezkręgowców lądowych jako organizmów biowskaźnikowych oraz jednocześnie jako źródła biomarkerów fizjologicznych. W niniejszej pracy podejście to zostało zintegrowane, co stanowi jej istotne *novum* badawcze. Ponadto przeprowadzono ocenę użyteczności wybranych organizmów takich jak ślimaki, jako źródła biomarkerów ekspozycji na wybrane metale, zarówno w warunkach naturalnych, jak i laboratoryjnych (kontrolowanych).

## 4. Cele badań i hipotezy badawcze

### Cele badań

Sformułowano następujące cele badań:

1. Określenie właściwości fizykochemicznych i biologicznych gleb w których zdeponowano skałę płonną (**Publikacja P1**).
2. Oznaczenie zawartości wybranych metali ciężkich w skałe płonnej, glebie, cieple dżdżownic Lumbricidae (**Publikacja P2**), częściach zielonych roślin (**Publikacja P3**) i stopie ślimaków lądowych Helicidae na przykładzie wstężyka gajowego *Cepaea nemoralis* (**Publikacja P3**).
3. Ocena wpływu wybranych metali zdeponowanych w środowisku na kwasy tłuszczowe, aktywność dysmutazy ponadtlenkowej (SOD) i katalazy (CAT) oraz na poziom substancji reagujących z kwasem tiobarbiturowym (TBARS) u przedstawicieli Lumbricidae, a także wytypowanie grupy kwasów tłuszczowych, które mogą być wykorzystane jako biomarkery skutków fizjologicznych oddziaływania polutantów środowiskowych (**Publikacja P2**).
4. Określenie wpływu wybranych metali jako zmiennych środowiskowych na panel biomarkerowych kwasów tłuszczowych u wstężyka gajowego pozyskanego ze środowiska i suplementowanego w warunkach laboratoryjnych (**Publikacja P3**).
5. Opracowanie panelu biomarkerów fizjologicznych u dżdżownic i ślimaków (**Publikacja P2, P3**).

### Hipotezy badawcze

W niniejszej rozprawie doktorskiej weryfikowano następujące hipotezy badawcze:

1. Obecność skały płonnej w glebie wpływa negatywnie na właściwości fizykochemiczne i biologiczne gleby, w tym na stan populacji mikroorganizmów.
2. Istnieje transfer wybranych metali ciężkich w środowisku - z gleby do zwierząt bezkręgowych - których źródłem jest skała płonna zdeponowana w wyrobiskach piasku.
3. Wybrane metale jako czynniki stresogenne, wpływają na gospodarkę lipidową dżdżownic Lumbricidae, będących organizmami biowskaźnikowymi, prowadząc do zmian w składzie i oksydacji kwasów tłuszczowych oraz na aktywność SOD, CAT i poziom TBARS w ich tkankach.

4. Ekspozycja ślimaków Helicidae np. wstężyk gajowy *Cepaea nemoralis* jako organizmów biowskaźnikowych na wybrane metale prowadzi do zwiększonej potencjalnej peroksydacji lipidów.
5. Kwasy tłuszczowe (długość łańcucha: C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3 i C22:2 n-6), są biomarkerami dżdżownic poddanych wpływowi metali pochodzących ze skały płonnej.
6. Kwasy tłuszczowe (długość łańcucha: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6 i C22:5 n-3), są biomarkerami ekspozycji ślimaków lądowych na metale zarówno w warunkach oddziaływania czynników w środowisku, jak i laboratoryjnych.

## 5. Materiał badawczy i metody

### Materiał badawczy

Materiał badawczy wykorzystany do realizacji celów badawczych i weryfikacji hipotez, szczegółowo opisano w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej - poniżej przedstawiono uproszczony jego opis:

**Publikacja nr 1 (P1):** Garbacz A., Nowak A., Marzec-Grządział A., Przybyś M., Gałązka A., Jaroszuk-Ścisł J., Grzywaczewski G. (2025). Impact of coal waste rock on biological and physicochemical properties of soils with different agricultural uses. *Sustainability* 17(6), 2603. <https://doi.org/10.3390/su17062603>

Materiałem badawczym były gleby ryzosferowe typu bielcowego, powstałe z piasków gliniasto-lekkich, które pobierano z rekultywowanych wyrobisk wypełnionych skałą płonną i miejsc kontrolnych bez jej depozytu. Stanowiska badawcze zlokalizowane były we wschodniej Polsce, na Polesiu Zachodnim. Próbkę gleby pobrano z 20 punktów po 50 g każda, o łącznej masie 1 kg, z sześciu miejsc o porównywalnych uprawach. Gleba została przesiana przez sito z oczkami o rozmiarze 5 mm. Następnie materiał badawczy przechowywano w warunkach sterylnych w celu dalszych badań.

Charakterystyka stanowisk badawczych:

- stanowiska z depozytem skały płonnej (SP) i czasem oddziaływania: pole użytkowane rolniczo - uprawa gryki *Fagopyrum esculentum*, depozyt SP powyżej 10 lat; pole użytkowane rolniczo - uprawa owsa *Avena sativa*, depozyt SP przez 2 lata; nieużytek, depozyt SP powyżej 10 lat.
- stanowiska bez depozytu skały płonnej (stanowiska kontrolne): pole użytkowane rolniczo - uprawa gryki; pole użytkowane rolniczo - uprawa owsa; pole nieużytkowane rolniczo (nieużytek).

**Publikacja nr 2 (P2):** Garbacz A., Kowalczyk-Pecka D., Kurska W. (2025). Fatty acids in Lumbricidae as biomarkers of *in situ* metals exposure. *Sustainability*, 17(17), 8076. <https://doi.org/10.3390/su17178076>

Materiał badawczy stanowiły skała płonna, gleba i dżdżownice z rodziny Lumbricidae, pozyskane z miejsc poddanych rekultywacji wyrobisk piasku z wykorzystaniem skały płonnej. Stanowiska badawcze różniły się sposobem użytkowania

rolniczego: pole, łąka, nieużytek i zlokalizowane były we wschodniej Polsce, na Polesiu Zachodnim. Z każdego stanowiska zebrano 30 dżdżownic, podzielonych na pięć grup, po sześć dżdżownic w każdej. Dżdżownice charakteryzowały się zbliżonymi rozmiarami ciała (6-8 cm), z wyraźnie zaznaczonym siodełkiem, co sugerowało na ich podobny wiek rozwojowy. Po uzyskaniu materiału badawczego, dżdżownice pozostawiono bez pożywienia na 24 h, w celu oczyszczenia układu pokarmowego. Następnie zamrożono w temperaturze -80°C, celem ich dalszej analizy laboratoryjnej.

Opis stanowisk badawczych:

- stanowiska z depozytem skały płonnej (SP) i czasem oddziaływania: pole użytkowane rolniczo - uprawa gryki *Fagopyrum esculentum*, depozyt SP powyżej 10 lat; pole użytkowano rolniczo - uprawa owsa *Avena sativa*, depozyt SP przez 2 lata; łąka I - depozyt SP powyżej 1 roku; łąka II - depozyt SP poniżej 1 roku; pole nieużytkowane rolniczo (nieużytek) I - depozyt SP powyżej 10 lat; nieużytek II - depozyt SP poniżej 1 roku.
- stanowiska bez depozytu skały płonnej (stanowiska kontrolne): pole - uprawa gryki; pole - uprawa owsa; łąka I; łąka II; nieużytek I; nieużytek II.

**Publikacja nr 3 (P3):** Garbacz A., Kursa W., Kowalczyk-Pecka D. (2026). Effect of deposition of Cr, Ba, Ni, Zn, and Cu on biomarker fatty acids as indirect indicators of peroxidation in *Cepaea nemoralis* (Helicidae) *in situ* and *in vivo* (in laboratory conditions). *Ecotoxicology and Environmental Safety*, 312, 119932. <https://doi.org/10.1016/j.ecoenv.2026.119932>

W badaniach wykorzystano ślimaki lądowe z rodziny Helicidae - wstężyk gajowy *Cepaea nemoralis*.

Doświadczenie terenowe: Wstężyki gajowe pozyskano z dwóch stanowisk badawczych zlokalizowanych we wschodniej Polsce, na Polesiu Zachodnim. Stanowiska badawcze:

- stanowisko z depozytem skały płonnej powyżej 1 roku, ze zbiorowiskami ziołoroślowymi *Filipendulion ulmariae*.
- stanowisko bez depozytu skały płonnej (stanowisko kontrolne), z roślinnością muraw napiaskowych *Koelerio glaucae-Corynepheretea canescentis*.

Ślimaki zbierano metodą „na upatrzonego” o zbliżonych rozmiarach (szerokość muszli 2,3-2,4 cm) z wywiniętą wargą, co wskazywało na podobny wiek i ich dojrzałość.

Z każdego stanowiska zebrano 30 ślimaków, podzielonych na pięć grup, po sześć ślimaków. Z tych samych lokalizacji pozyskano także rośliny stanowiące naturalny pokarm wstężyków gajowych. Po zebraniu materiału badawczego ślimaki pozostawiono przez 24 h bez dostępu do pożywienia w celu oczyszczenia układu pokarmowego, po czym zamrożono w temperaturze  $-80^{\circ}\text{C}$  do dalszej analizy. Doświadczenie laboratoryjne polegało na wykorzystaniu wstężyków gajowych, pozyskanych z czystej/kontrolnej hodowli prowadzonej w vivarium. W każdej grupie eksperymentalnej wykorzystano 30 ślimaków, podzielonych na pięć grup, po sześć ślimaków w każdej. Utworzono dwie grupy kontrolne - początkową (I-CG) i końcową (II-CG). Do badań zastosowano metodykę zgodną z publikacją Kowalczyk-Pecka i in. (2017c). Ślimaki z grup badawczych utrzymywano przez sześć tygodni w komorze hodowlanej BIOGENET, w perforowanych pojemnikach HDPE (ang. *High-Density Polyethylene*), zapewniających dobrostan zwierząt. Zwierzęta karmiono dwa razy w tygodniu pożywką w formie galaretki (wg metodyki Laskowski i Hopkin (1996) zmodyfikowanej przez Kowalczyk-Pecka i in. (2017c)) na bazie agaru (Difco), z liofilizowanym, sproszkowanym korzeniem marchwi, bez fungicydu. Dla każdej grupy badawczej przygotowywano 10 ml każdorazowo przed podaniem. Oszacowano na podstawie wcześniejszych doświadczeń, że ta porcja pożywki zostanie całkowicie zjedzona, bez strat. Ślimaki otrzymywały *per os* (za pomocą pipety) 10  $\mu\text{l}$  (na ślimaka) roztworu zawierającego określone jony metali. Wykorzystano sole następujących metali, wszystkie o czystości do analizy (cz.d.a.): Cr ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ , Avantor Performance Materials), Ni ( $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ , Merck), Zn ( $\text{ZnCl}_2$ , Avantor Performance Materials) i Cu ( $\text{CuSO}_4$ , Avantor Performance Materials). Przygotowano niezależne roztwory każdej soli w stężeniach: 0,0005, 0,00075 i 0,001 mg Met/ml wody redestylowanej (grupy oznaczone jako 0.5, 0.75 i 1.0, w zależności od stężenia). Ze względu na dużą toksyczność rozpuszczalnych związków baru, jako źródło Ba użyto  $\text{BaSO}_4$  (o czystości cz.d.a., Merck) w postaci stałej i w proporcji odpowiadającej pozostałym metalom, dodawano do agaru stanowiącego bazę pożywki. Utworzono trzy grupy badawcze, którym suplementowano mieszaniny wszystkich wymienionych jonów metali w równych proporcjach w identycznych stężeniach (Mix 0.5; Mix 0.75 i Mix 1.0). Po sześciu tygodniach ślimaki ze wszystkich grup pozostawiono na 24 h bez pożywienia w celu oczyszczenia przewodu pokarmowego, a następnie zamrożono w temperaturze  $-80^{\circ}\text{C}$  do dalszej analizy.

## Metody badań

Metodykę wykorzystaną do realizacji zadań badawczych wraz z urządzeniami i odczynnikami szczegółowo opisano w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej - poniżej przedstawiono uproszczony zakres przeprowadzonych badań:

- 1) Określenie właściwości fizykochemicznych prób gleby (P1)
  - Sucha masa. Naważkę 10 g świeżej masy gleby suszono w 105°C (3 cykle po 8 h).
  - pH<sub>H2O</sub>, pH<sub>KCl</sub>. Zawiesiny 1 g świeżej masy gleby w dH<sub>2</sub>O i 1M KCl (1:2,5) mierzono potencjometrycznie (pH-metr CP-505, Elmetron).
  - Zawartość kwasów huminowych wg Alef i Nannipier (1995). Kwasy huminowe ekstrahowano ze 100 g gleby 0,5M NaOH, wytrącono 6M HCl, osad suszono w 80°C (3 cykle po 8h).
  - Zawartość całkowitego węgla organicznego metodą Tiurina wg Alef i Nannipier (1995). Naważkę 0,5 g suchej masy gleby gotowano 5 min z 0,2 g Ag<sub>2</sub>SO<sub>4</sub> i 10 ml 0,4N K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>, po czym miareczkowano 0,2N solą Mohra wobec difenyloaminy do zmiany barwy.
- 2) Określenie ogólnej liczebności mikroorganizmów hodowalnych (P1)
  - Przygotowanie posiewów. Naważkę 5 g świeżej masy gleby wytrząsano w 45 ml dH<sub>2</sub>O (15 min, 120 obr./min, 20°C), rozcieńczenia dziesiętne wysiewano na podłoża; hodowle (pkt. 2-3) inkubowano 7 dni w 20°C.
  - Liczebność grzybów mikroskopowych wg Martin (1950) na podłożu Martina z 1% streptomycyną i 1% różem bengalskim.
  - Liczebność bakterii kopiotroficznych i oligotroficznych wg Alef i Nannipier (1995) na podłożu PYS z wyciągiem glebowym (100 ml/l), odpowiednio nierozcieńczonym i rozcieńczonym 100-krotnie.
- 3) Określenie liczebności grup fizjologicznych mikroorganizmów hodowalnych (P1)
  - Celulolityczne wg Mahdi i in. (2011) na podłożu CMC (z karboksymetylocelulozą); strefy hydrolizy wybarwiono 0,1% czerwieni Kongo i odbarwiono 1M NaCl (po 15 min).
  - Amylolityczne wg Khokhar i in. (2011) na podłożu ze skrobią; strefy amylolizy wizualizowano płynem Lugola.
  - Proteolityczne wg Chandran i in. (2014) na podłożu z odtłuszczonym mlekiem (strefy przejaśnień).

- Rozpuszczające fosforany wg Kurek i in. (2004) na podłożu PS z osobno sterylizowanymi  $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$  (10 g/100 ml) i  $\text{CaCl}_2$  (22 g/100 ml) (strefy przejaśnień).
  - Oporne na metale ciężkie wg Schlegel i in. (1991), Truyens i in. (2016) na podłożu Schlegel 284 z mieszaniną osobno sterylizowanych roztworów: 0,4 mM  $\text{CdSO}_4 \cdot 3\text{H}_2\text{O}$ , 0,8 mM  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0,6 mM  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  i 0,4 mM  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ .
  - Wytwarzające siderofory wg Schwyn i Neilands (1987) na podłożu CAS (chromazurol S,  $\text{FeCl}_3$ , HDTMA) z hydrolizatem kazeiny i  $\text{CaCl}_2$ .
- 4) Określenie aktywności biologicznej gleby (P1)
- Aktywność dehydrogenazy wg Casida i in. (1964). 3 g gleby inkubowano z  $\text{CaCO}_3$  i 3% TTC; TPF ekstrahowano 96% etanolem i mierzono przy 485 nm (wszystkie pomiary absorbancji mierzono spektrofotometrem mikro płytkowym Infinite 200 PRO, Tecan).
  - Aktywność fosfatazy kwaśnej i zasadowej wg Tabatabai i Bremner (1969). 1 g gleby inkubowano z toluenem (15 min), buforem MUB (pH 5,5 lub 11,0) i 100 mM *p*-NPP (Merck); uwolniony *p*-NP mierzono przy 410 nm przy użyciu spektrofotometru mikro płytkowego Infinite 200 PRO (Tecan).
- 5) Wyznaczenie fizjologicznego profilu gleby (P1)
- CLPP (ang. *Community Level Physiological Profiling*) wg Wolinska i in. (2017), Németh i in. (2021). Zawiesinę gleby (100 µl/dołek) inkubowano na płytkach Biolog EcoPlate™ w 20°C, mierząc OD<sub>590</sub>, co 24 h przez 8 dni używając spektrofotometru mikro płytkowego Infinite 200 PRO (Tecan). Obliczono wskaźniki ekofizjologiczne: AWCD (ang. *Average Well Colour Development*; średnia skorygowana OD<sub>590</sub> 31 substratów), Substrate Richness (R; OD<sub>590</sub> > 0,25 po 96 h) i Shannon index (H;  $H = -\sum P_i \ln P_i$ ).
- 6) Populacja bakterii w próbach gleby (P1). DNA izolowano z 0,5 g gleby zestawem FastDNA™ SPIN Kit for Soil (MP BIOMEDICAL). Pliki fastq przetwarzano pakietem DADA2 (1.14) (Callahan i in., 2016) w R (3.6.0) (R Core Team, 2016) (przecięcie 20 końcowych zasad F i R, maxN = 0, maxEE = 2, truncQ = 2; *learnErrors*, *dada*, *removeBimeraDenovo*). Taksonomię przypisano algorytmem IDTAXA (Murali i in., 2018) względem bazy RDP v18 (ang. *Ribosomal Database Project*), dane opracowano w pakiecie *phyloseq* (1.22.3) (McMurdie i Holmes, 2013) po usunięciu DNA chloroplastowego lub mitochondrialnego (Liu i in., 2021). Dane zdeponowano

w NCBI SRA (PRJNA1087279).

- 7) Mineralizacja prób (P2, P3). Próby ze skałą płonną, dżdżownicami (P2), częściami zielonymi roślin i stopą wstężyków gajowych (P3), rozdrobniono i zhomogenizowano, a próby gleby przesiano (P2). Do analiz wykorzystano 0,5 g próbki. Mineralizację w  $\text{HNO}_3$  (Suprapur-Merck) przeprowadzono w piecu mikrofalowym (CEM Mars Xpress;  $210^\circ\text{C}/7\text{ atm}$ ), a uzyskane mineralizaty rozcieńczono wodą demineralizowaną ( $0,055\text{ }\mu\text{S/cm}$ ) (P2, P3).
- 8) Oznaczenie zawartości wybranych metali ciężkich (P2, P3)
  - Analiza ICP-MS (ang. *Inductively Coupled Plasma-Mass Spectrometry*). W próbach skały płonnej, gleby i dżdżownic oznaczono zawartość Cr, Ni, Cd, Ba, Pb (P2), natomiast w częściach zielonych roślin i stopie wstężyków gajowych - Cr, Ni, Ba (P3). Analizy wykonywano zgodnie z procedurą CLB/ESA/5/2019 (wersja 3), z wykorzystaniem wzorców Ultra Scientific. Gazem stosowanym do wytworzenia plazmy był argon (Messer). Otrzymane roztwory analizowano na spektrometrze masowym ze wzbudzeniem w plazmie indukcyjnie sprzężonej (ICP Mass Spectrometer Varian MS-820). W analizie nie stosowano komory reakcyjnej (CRI). Zastosowano sterowanie jakością badań poprzez pomiar próbki ślepej, próbki podwójnej oraz certyfikowanego materiału odniesienia „NIST-1577c Bovine Liver” (National Institute of Standards & Technology) (P2, P3).
  - Analiza FAAS (ang. *Flame Atomic Absorption Spectrometry*). W próbach skały płonnej, gleby, dżdżownic (P2) oraz części zielonych roślin i stopy wstężyków gajowych (P3) oznaczono zawartość Zn i Cu. Analizy wykonano zgodnie z procedurą CLB/ASA/2/2019 (wersja 4) z wykorzystaniem certyfikowanych wzorców i 2%  $\text{HNO}_3$  jako matrycy. Roztworami podstawowymi był Zn ( $10\text{ mg/dm}^3$ ) i Cu ( $20\text{ mg/dm}^3$ ) firmy CPA Chem. Otrzymane roztwory analizowano na spektrometrze absorpcji atomowej z atomizacją w płomieniu (FAAS Spectrometer Varian, SpektrAA 280FS z autosamplerem SPS-3 i diluterem SIPS) (P2, P3).
- 9) Ekstrakcja lipidów i otrzymywanie kwasów tłuszczowych (P2, P3). Próby z dżdżownicami (P2) i stopą wstężyków gajowych (P3), rozdrobniono i zhomogenizowano. Do analiz wykorzystano 100 mg tłuszczu. Zmydlenie przeprowadzono dodając metanolowego roztworu KOH (Avantor Performance Materials), a estryfikację wykonano z udziałem metanolowego roztworu  $\text{BF}_3$  (Thermo Scientific). Estry kwasów tłuszczowych otrzymano zgodnie z procedurą CLB/GC/3/2019 (wersja 4). Próbkę estrów kwasów tłuszczowych analizowano przy

użyciu chromatografu gazowego (Varian 450-GC z autosamplerem CP-8400, detektorem FID). Gazem nośnym był hel. Spośród przeanalizowanych 35 kwasów tłuszczowych wybrano panel biomarkerowych kwasów tłuszczowych. Obliczono sumy nasyconych kwasów tłuszczowych (SFA), wielonienasyconych kwasów tłuszczowych (PUFA) i jednonienasyconych kwasów tłuszczowych (MUFA) (P2, P3).

- 10) Wskaźnik peroksydacji (PI) i wskaźnik nienasycenia (UI) (P2, P3). Obliczono wskaźniki wykorzystując wzór opracowany przez Hulbert i in. (2007):

$$PI = 0,025 \times (\% \text{ kwasów tłuszczowych monoenowych}) + 1 \times (\% \text{ kwasów tłuszczowych dienowych}) + 2 \times (\% \text{ kwasów tłuszczowych trienowych}) + 4 \times (\% \text{ kwasów tłuszczowych tetraenowych}) + 6 \times (\% \text{ kwasów tłuszczowych pentaenowych}) + 8 \times (\% \text{ kwasów tłuszczowych hekseenowych}) \quad (1)$$

$$UI = 1 \times (\% \text{ kwasów tłuszczowych monoenowych}) + 2 \times (\% \text{ kwasów tłuszczowych dienowych}) + 3 \times (\% \text{ kwasów tłuszczowych trienowych}) + 4 \times (\% \text{ kwasów tłuszczowych tetraenowych}) + 5 \times (\% \text{ kwasów tłuszczowych pentaenowych}) + 6 \times (\% \text{ kwasów tłuszczowych hekseenowych}) \quad (2)$$

- 11) Analiza enzymatycznych biomarkerów stresu oksydacyjnego w dżdżownicach – aktywność dysmutazy ponadtlenkowej (SOD) i katalazy (CAT) (P2) wg Atli i Grosell (2016). Zmierzono absorbancję SOD (550 nm, 1 min) i CAT (240 nm, 1 min) przy użyciu spektrofotometru mikropłytkowego (Varioskan™ LUX Thermo Scientific™).
- 12) Analiza nieenzymatycznych biomarkerów stresu oksydacyjnego w dżdżownicach – substancje reagujące z kwasem tiobarbiturowym (TBARS) (P2) wg Radwan i in. (2010a, 2010b). Absorbancję zmierzono (532 nm, 1 min) przy użyciu spektrofotometru mikropłytkowego (Varioskan™ LUX Thermo Scientific™).
- 13) Analizy statystyczne (P1, P2, P3). W publikacji P1 wykonano analizy statystyczne z wykorzystaniem oprogramowania RStudio dla systemu Windows w wersji 2024.09.0+375 (Posit, PBC, GNU Affero General Public License v3). Wszystkie eksperymenty przeprowadzono w trzech powtórzeniach biologicznych. Wyniki przedstawiono jako wartości średnie z odchyleniem standardowym (SD). Dane poddano jednoczynnikowej analizie wariancji (ANOVA), a następnie zastosowano test post hoc Tukeya dla wielokrotnych porównań przy  $p < 0,05$  (P1). W publikacjach P2 i P3 dane poddano analizie statystycznej przy użyciu oprogramowania Statistica w wersji 13.3 (1984–2017 TIBCO Software INC, Palo Alto, Kalifornia, USA). Każda grupa została potraktowana jako pojedyncze powtórzenie biologiczne (P2, P3). Normalność rozkładu danych oceniono testem Kołmogorowa–Smirnowa (P2), testem

Shapiro-Wilka (P3), a do zbadania równości wariancji zastosowano test jednorodności wariancji Levene'a. Zastosowano ANOVA (jednoczynnikową i dwuczynnikową) i test post hoc Tukeya. Zależności liniowe określono za pomocą współczynnika korelacji Pearsona (r) przy poziomie istotności  $p \leq 0,01$ , interpretując je zgodnie ze skalą Matyja (2014). Przeprowadzono analizę skupień (aglomeracja, grupowanie obiektów i cech) przy użyciu danych standaryzowanych względem zawartości biomarkerowych kwasów tłuszczowych, wartości wskaźnika PI i wskaźnika UI (P2, P3) oraz zawartości CAT, SOD i TBARS. Dla zmiennych związanych z Pb, wykonano pełną macierz korelacji i zastosowano procedurę Benjaminiego-Hochberga ( $FDR = 0,01$ ) (Benjamini i in., 2001) (P2).

## 6. Omówienie wyników badań i dyskusja

### 6.1. Określenie właściwości fizykochemicznych i biologicznych gleb w których zdeponowano skałę płonną (Publikacja P1)

#### 6.1.1. pH gleby, zawartość węgla organicznego i kwasów huminowych w glebie

W celu oceny właściwości fizykochemicznych analizowanych prób gleb oznaczono odczyn ( $\text{pH}_{\text{H}_2\text{O}}$ ,  $\text{pH}_{\text{KCl}}$ ), zawartość węgla organicznego i zawartość kwasów huminowych. Dodatek skały płonnej do gleby z pola z gryką spowodował alkalizację gleby - wartości  $\text{pH}_{\text{H}_2\text{O}}$  były w zakresie 7,5-8,0. Wśród wszystkich analizowanych próbek, gleby te wykazywały najwyższe wartości  $\text{pH}_{\text{H}_2\text{O}}$  i  $\text{pH}_{\text{KCl}}$ . W uprawie owsa zaobserwowano podobną tendencję w przypadku  $\text{pH}_{\text{H}_2\text{O}}$  (zalkalizowanie gleby), jednak odnotowano spadek pH w KCl. Odmienny efekt wystąpił w glebach nieużytkowanych rolniczo z obecnością skały płonnej, gdzie zaobserwowano wyraźne zakwaszenie podłoża. W przeprowadzonych badaniach wynika, że długoletni depozyt (powyżej 10 lat) skały płonnej skutkował czterokrotnym wzrostem zawartości węgla organicznego w glebach nieużytkowanych i około 30% wzrostem w glebie z uprawą gryki. Przeciwnie, w glebie z dwuletnią ekspozycją na skałę płonną przy uprawie owsa odnotowano czterokrotne zmniejszenie zawartości węgla organicznego. W glebach, na których występował depozyt skały płonnej, stwierdzono spadek zawartości kwasów huminowych w porównaniu z glebami pozbawionymi tego depozytu (próby kontrolne). Zjawisko to było szczególnie widoczne w glebach użytkowanych rolniczo, zwłaszcza w przypadku uprawy owa, podczas gdy w glebach nieużytkowanych spadek ten był niewielki (publikacja P1 - ryc. 2). Z kolei badania przeprowadzone przez Kompała-Bąba i in. (2021) wykazały, że podłoża z hałd pokopalanianych porośniętych roślinami jednoliściennymi (wiechlina spłaszczona *Poa compressa* –  $\text{pH}_{\text{H}_2\text{O}}$   $6,88 \pm 0,15$  i  $\text{pH}_{1\text{MKCl}}$   $6,26 \pm 0,16$ ; trzcinnik piaskowy *Calamagrostis epigejos*  $\text{pH}_{\text{H}_2\text{O}}$   $6,13 \pm 0,25$  i  $\text{pH}_{1\text{MKCl}}$   $5,46 \pm 0,28$ ) i roślinami dwuliściennymi (marchew uprawna *Daucus carota*  $\text{pH}_{\text{H}_2\text{O}}$   $6,44 \pm 0,14$  i  $\text{pH}_{1\text{MKCl}}$   $5,95 \pm 0,19$ ; podbiał pospolity *Tussilago farfara*  $\text{pH}_{\text{H}_2\text{O}}$   $5,62 \pm 0,35$  i  $\text{pH}_{1\text{MKCl}}$   $5,15 \pm 0,38$ ) miały wyższe wartości  $\text{pH}_{\text{H}_2\text{O}}$  i  $\text{pH}_{1\text{MKCl}}$  w porównaniu do podłoża z hałd bez roślinności ( $\text{pH}_{\text{H}_2\text{O}}$   $4,35 \pm 0,24$  i  $\text{pH}_{1\text{MKCl}}$   $3,90 \pm 0,27$ ).

### **6.1.2. Liczebność mikroorganizmów glebowych**

Dodatek skały płonnej (SP) do gleby z pola gryką (depozyt SP powyżej 10 lat) nie spowodował znaczących zmian w liczebności bakterii kopiotroficznych i oligotroficznych, grzybów i mikroorganizmów zdolnych do rozpuszczania fosforanów, jednak zaobserwowano niewielki wzrost liczebności mikroorganizmów celulolitycznych, amylolitycznych i proteolitycznych. W glebie z uprawą owsa z dwuletnim depozytem skały płonnej, odnotowano spadek liczebności wszystkich grup mikroorganizmów. Podobne zależności stwierdzono w glebie nieużytkowanej z ponad dziesięcioletnim okresem ekspozycji w porównaniu z glebą bez depozytu skały płonnej, lecz z wyjątkiem grzybów mikroskopowych, których liczebność nie uległa znacząco zmianie. W efekcie zmiany w składzie mikrobiomu mogą być uznawane za jeden z kluczowych wskaźników wpływu badanego odpadu. W przypadku mikroorganizmów opornych na metale ciężkie można wyraźnie dostrzec odmienne zależności w glebach użytkowanych i nieużytkowanych rolniczo. W glebach uprawnych (gryka, owies) z depozytem skały płonnej ich liczebność malała, natomiast w glebach nieużytkowanych pozostawała zbliżona do gleby kontrolnej lub nieznacznie wzrastała. Na szczególną uwagę zasługuje zaobserwowany spadek liczebności mikroorganizmów zdolnych do kompleksowania metali z wykorzystaniem silnych chelatorów, takich jak siderofory. Zjawisko to występowało zarówno w glebach uprawnych (gryka, owies), jak i w glebach nieużytkowanych z depozytem skały płonnej, w porównaniu z próbami kontrolnymi. Największy spadek liczebności tych mikroorganizmów (o 1,5-2,0  $\log_{10}/\text{gDW}$ ) zauważono w glebach pozyskanych z nieużytku z obecnością odpadu (publikacja P1 - ryc. 3-4). Jednym z mechanizmów interakcji mikroorganizmów na metale jest zdolność do chelatowania metali. Zachodzi ten proces przy udziale związków kompleksujących - niespecyficznych, takich jak kwasy, polimery cukrowe i aminokwasowe oraz specyficznych, takie jak siderofory (Kachel i in., 2021). Występowanie metali ciężkich w glebie wpływa na liczebność mikroorganizmów (Boshoff i in., 2014; Truyens i in., 2016), co znajduje odzwierciedlenie w badaniach własnych.

### **6.1.3. Aktywność enzymów glebowych**

W przypadku uprawy gryki aktywność dehydrogenazy pozostawała na zbliżonym poziomie w obu badanych glebach. Odmienny efekt zaobserwowano w glebie z depozytem skały płonnej z uprawy owsa i nieużytku, gdzie stwierdzono znaczący spadek aktywności enzymu. W glebie z uprawy owsa aktywność dehydrogenazy była obniżona prawie stukrotnie - z około 2  $\mu\text{g TPF/h/gDW}$  do 0,02  $\mu\text{g TPF/h/gDW}$ , natomiast w glebie

nieużytkowanej odnotowano około czterokrotny spadek (publikacja P1 - ryc. 5). Dehydrogenaza jest jednym z najczęściej stosowanych wskaźników aktywności biologicznej - enzym uczestniczący w procesach oddychania komórkowego wszystkich organizmów. Wykorzystywana jako czuły parametr odzwierciedlający wpływ czynników stresowych na mikroorganizmy glebowe (Kumar i Tarafdar, 2003). Trudno jednoznacznie odnieść wyniki badań własnych do piśmiennictwa z podobnego zakresu, choć badania Kompała-Bąba i in. (2021) wykazały, że aktywność dehydrogenazy była wyższa w podłożach pozyskanych z hałd pokrytych roślinami jednoliściennymi i dwuliściennymi w porównaniu do podłoży z hałd pozbawionych roślinności.

Z uwagi na kluczową rolę fosforu, oceniono aktywność fosfatazy kwaśnej i fosfatazy zasadowej. We wszystkich próbkach gleby z dodatkiem skały płonnej odnotowano obniżenie aktywności zarówno fosfatazy kwaśnej, jak i fosfatazy zasadowej, przy czym, największy spadek (ponad czterokrotny) wystąpił w glebie z uprawą owsa (publikacja P1 - ryc. 5). Fosfatazy są enzymami, które pełnią istotną funkcję w glebie, ponieważ stymulują one przemiany organicznych związków fosforu w nieorganiczne fosforany, bezpośrednio dostępne dla roślin i organizmów glebowych. W związku z tym, mogą być dobrym wskaźnikiem potencjału mineralizacji fosforu organicznego (Gałązka i Kocoń, 2015). Wyniki te są zgodne z obserwacjami Joniec i in. (2025), którzy stwierdzili, że aktywność fosfatazy kwaśnej była najniższa na stanowisku z depozytem skały płonnej w porównaniu z innymi typami siedlisk bez depozytu tego odpadu (łąka, pole uprawne, torfowiska, las liściasty). Porównanie wyników badań własnych z dostępnymi publikacjami jest trudne, ze względu na zakres badań. Jednak badania przeprowadzone Kompała-Bąba i in. (2021) wykazały, że aktywność fosfatazy zasadowej była wyższa w podłożach pozyskanych z hałd pokrytych roślinami jednoliściennymi i dwuliściennymi, niż w podłożach z hałd bez roślinności. Natomiast aktywność fosfatazy kwaśnej była wyższa w podłożach z hałd pokrytych roślinami jednoliściennymi, a niższa z hałd porośniętych roślinami dwuliściennymi w porównaniu do podłoży z hałd bez roślinności.

#### **6.1.4. CLPP – Biolog EcoPlate™**

W przypadku wskaźnika AWCD (ang. *Average Well Colour Development*) dla prób gleby (z depozytem i bez depozytu skały płonnej) pobranych z pola z gryką i owsa wykorzystanie substratu rozpoczęło się po 48 h inkubacji, natomiast w próbach z nieużytku z depozytem skały płonnej spowodowało znaczne opóźnienie w tempie wykorzystania substratu do 96 h inkubacji. W obu badanych glebach z uprawy gryki, wartości AWCD

pozostawały zbliżone przez cały okres analizy, podczas gdy w glebie z uprawą owsa średnia wartość tego wskaźnika była niższa w próbach gleby z depozytem skały płonnej. Zaobserwowane opóźnienie w rozpoczęciu wykorzystania substratu w glebie nieużytkowanej z obecnością odpadu utrzymywało się przez cały okres inkubacji - nawet po 192 h wciąż pozostawało poniżej wartości notowanych w glebie bez depozytu. Analizy wykazały, że dodatek skał płonnych do gleb nie spowodował znaczących zmian w wartości Shannon index (H) w glebach uprawnych (gryka i owies). Wyraźny spadek tego wskaźnika (z  $H=3,1$  do  $H=2,6$ ) stwierdzono w glebie z nieużytku z ponad dziesięcioletnim depozytem odpadu w porównaniu do kontroli. Uzyskane wyniki potwierdzają, że rodzaj użytkowania gleby ma duże znaczenie. Analiza wskaźnika R (ang. *Substrate Richness*) wykazywała tendencję zbliżoną do obserwowanej dla AWCD. W próbach gleb z uprawą gryki i owsa odnotowano zwiększone wykorzystanie poszczególnych substratów po 48 h inkubacji. Natomiast w glebie nieużytkowanej z depozytem skały płonnej proces ten był opóźniony. Po 96 h inkubacji różnica we wskaźniku R wynosiła 10 dla gleby pozyskanej z nieużytku z depozytem odpadu i 24 dla gleby kontrolnej, tendencja ta utrzymywała się w dalszych okresach inkubacji (publikacja P1 - ryc. 7). Wskaźnik AWCD odzwierciedla aktywność metaboliczną, wskaźnik R liczbę zużytych substratów, a wskaźnik H wskazuje na stan zróżnicowania populacji mikroorganizmów (Majewska i Jaroszuk-Ściśeł, 2017). Aktywność metaboliczna mikroorganizmów zależy od właściwości gleby, lecz także od odmian roślin, jak i rodzaju uprawy, co potwierdzono w badaniach Wolińska i in. (2020).

#### **6.1.5. Zróżnicowanie mikrobiomu gleby**

Analiza danych sekwencjonowania bakterii wykazała obecność 241 ASV (ang. *Amplicon Sequence Variant*), z których 151 zidentyfikowano na poziomie rodzajów, należących do 74 rodzin, 32 rzędów, 16 klas i 11 typów. Analiza metastatu wykazała, że największą liczbę unikalnych ASV odnotowano w glebie z uprawą owsa, niepodlegającej wpływowi skały płonnej. Najmniejszą liczbę unikalnych ASV stwierdzono w glebie z nieużytku pozbawionego depozytu skały płonnej oraz w glebie z pola z gryką z ponad dziesięcioletnim jej depozytem. W porównaniu z glebą bez depozytu odpadu, w glebie z jego depozytem z pola z gryką stwierdzono ponad trzykrotne, a w glebie z pola z owsem około dwukrotne zmniejszenie liczby unikalnych ASV. Odmienne zależności stwierdzono w przypadku gleby z nieużytku na której znajdowała się skała płonna, gdzie zaobserwowano prawie dwukrotne zwiększenie liczby ASV (publikacja P1 - ryc. 8). Wskaźniki Shannon Alpha i Simpson Alpha, obliczone na podstawie danych z sekwencjonowania 16S rRNA,

potwierdziły wpływ sposobu użytkowania rolniczego na różnorodność mikrobiologiczną gleby. Ich wartości, niezależnie od gatunku uprawianych roślin (gryka, owies), były wyraźnie wyższe niż w glebie z nieużytków (publikacja P1 - ryc. 9). Dominującymi typami były Proteobacteria, a następnie Acidobacteria i Actinobacteria. Przeprowadzone badania pokazały, że we wszystkich glebach z depozytem skały płonnej zwiększyła się liczebność Proteobacteria. W glebie nieużytkowanej pod wpływem odpadu zaobserwowano spadek liczebności Actinobacteria (publikacja P1 - ryc. 8). Obserwacje Zhang i in. (2024) wykazały, że bakterie Actinobacteria przeważały w skałach towarzyszących pokładowi węgla. Z kolei Lin i in. (2024) wykazali, że gleba na terenie kopalni węgla w Chinach również charakteryzowała się dominacją Proteobacteria, a następnie Actinobacteria i Acidobacteria, co potwierdza obecność odnotowanych bakterii należących do wyróżnionych typów w badaniach własnych.

W przeprowadzonych badaniach w glebie z uprawy gryki i owsa stwierdzono znaczną obecność rodzaju *Sphingomonas*, bakterii o szerokim potencjale biostymulującym, ochronnym i biokontrolnym, wspierających wzrost roślin w warunkach stresu biotycznego i abiotycznego (Kim i in., 1998; Asaf i in., 2020). W glebie pochodzącej z uprawy gryki i owsa, w której znajdował się depozyt skały płonnej, stwierdzono zmniejszenie liczebności bakterii z rodzajów *Mycobacterium* i *Streptomyces* w porównaniu z próbkami gleby bez obecności tego depozytu. W próbach gleby z nieużytku zawierającego depozyt skały płonnej stwierdzono bardzo niską liczebność bakterii z rodzajów *Clostridium*, *Rhizobium* i *Blastococcus*, przy czym była ona najniższa spośród wszystkich analizowanych gleb użytkowanych rolniczo. W glebie pozyskanej z uprawy gryki z depozytem tego odpadu, odnotowano dodatkowo zmniejszenie liczebności bakterii z rodzajów *Rhizobium*, *Methylobacterium* i *Streptosporangium* w porównaniu z glebą z uprawy gryki bez jego dodatku (publikacja P1 - ryc. 8). Zjawisko to jest niepożądane, ponieważ diazotroficzne bakterie z klasy Alphaproteobacteria z rodzaju *Rhizobium* i *Methylobacterium* mogą zasiedlać glebę, jak i wewnątrz tkanek roślinnych. Mikroorganizmy te pełnią istotną rolę m.in. w stymulowaniu wzrostu roślin poprzez wiązanie azotu, zwiększanie biodostępności żelaza i fosforu, redukcję stężenia etylenu oraz indukcję odporności roślin (Zhang i in., 2021; Palberg i in., 2022). Natomiast bakterie z rodzaju *Streptosporangium*, zaliczane do typu Actinobacteria, cechują się m.in. wysoką aktywnością metaboliczną i zdolnością do syntezy enzymów uczestniczących w rozkładzie materii organicznej, a także źródłem naturalnych antybiotyków (Vaddavalli i in., 2015).

## **6.2. Oznaczenie zawartości wybranych metali ciężkich w skale płonnej, glebie, ciele dżdżownic Lumbricidae (Publikacja P2), częściach zielonych roślin (Publikacja P3) i stopie ślimaków lądowych Helicidae na przykładzie wstężyka gajowego *Cepaea nemoralis* (Publikacja P3)**

Przeprowadzone analizy pozwoliły na zestawienie i porównanie stanowisk potencjalnie obciążonych sedymencem antropogenicznym (skałą płonną) ze stanowiskami kontrolnymi bez depozytu skały płonnej. Badania wykazały obecność wybranych metali (Cr, Ni, Ba, Pb, Zn i Cu) w skale płonnej (Publikacja P2). Uzyskane wyniki znajdują potwierdzenie w opublikowanych publikacjach (Baran i in., 1993; Kujawska, 2018). Po przedostaniu się do środowiska, metale te ulegały transferowi do gleby, a następnie do dżdżownic.

Jak wynika z wykonanych badań najwyższe zawartości Ba i Zn, a najniższe Pb i Ni, odnotowano w próbkach skały płonnej ze wszystkich badanych stanowisk (publikacja P2 - tab. 2). Pomimo braku Cd w skale płonnej, jego obecność w glebie i dżdżownicach najprawdopodobniej wynikała z depozycji atmosferycznej (opady atmosferyczne) (publikacja P2 - tab. 2-5). Najwyższe zawartości Pb i Zn odnotowano w skale płonnej z pola z gryką (depozyt SP powyżej 10 lat), Cr z łąki I (depozyt SP powyżej 1 roku), natomiast Ba, Ni i Cu z nieużytku II (depozyt SP poniżej 1 roku). Z kolei najniższe zawartości Cr i Ni stwierdzono w odpadzie pozyskanym z nieużytku I (depozyt SP powyżej 10 lat), Cr i Ba z pola z owsem (depozyt SP przez 2 lata), Pb i Zn z łąki II (depozyt SP poniżej 1 roku), a Cu z łąki I (depozyt SP powyżej 1 roku) (publikacja P2 - tab. 2). W analizowanych próbkach gleby i dżdżownic pochodzących ze wszystkich badanych stanowisk stwierdzono najwyższe zawartości Zn, a najniższe Cd (publikacja P2 - tab. 3-5). Najwyższe zawartości Pb, Ni, Cu oraz Zn stwierdzono w glebie pochodzącej z nieużytku I obciążonego wieloletnim depozytem skały płonnej (powyżej 10 lat). Najwyższe stężenie Cd odnotowano w glebie z pola z gryką (depozyt SP powyżej 10 lat). Z kolei najwyższe stężenia Cr i Ba wystąpiły w glebie z łąki I, na której depozyt skały płonnej utrzymywał się ponad rok (publikacja P2 - tab. 3). Najwyższe zawartości metali, takich jak Pb, Cd, Zn i Cu, odnotowano w dżdżownicach z nieużytku I, gdzie skała płonna była deponowana przez okres ponad 10 lat (długoletni depozyt). Z kolei najwyższe stężenia Cr, Ba i Ni zaobserwowano w dżdżownicach pobranych z łąki I, na której odpad ten znajdował się przez okres przekraczający rok (publikacja P2 - tab. 5). Na zawartość analizowanych metali wpływały

( $p \leq 0,01$ ) wszystkie pojedyncze czynniki (skała płonna, miejsce poboru próbek), a także interakcja między nimi. Stężenia metali w glebie były nietoksyczne, dostępne dawki nie były dla nich letalne, co potwierdza przeżywalność tych bezkręgowców lądowych. Dżdżownice są ważnym elementem łańcucha troficznego, jako konsumenci I rzędu. Powszechnie uznawane są za bioindykatory, ponieważ akumulują zanieczyszczenia, w tym metale (Zhang i in., 2009; Georgescu i in., 2011; Sivakumar, 2015; Shi i in., 2017).

Przeprowadzone badania wykazały, że wybrane metale, takie jak Cr, Ni, Ba, Zn i Cu występowały w częściach zielonych roślin i stopie wstężyków gajowych. Porównanie wyników ze stanowiska zawierającego ponad roczny depozyt skały płonnej ze stanowiskiem kontrolnym (bez depozytu skały płonnej), wykazało, że wyższe zawartości Cr, Ba, Ni i Zn występują w częściach zielonych roślin i stopie ślimaków ze stanowiska z depozytem odpadu. W przypadku Cu odnotowano wyższe stężenia tego pierwiastka w częściach zielonych roślin, a niższe w stopie ślimaków ze stanowiska z depozytem skały płonnej (powyżej 1 roku) niż w próbach kontrolnych (publikacja P3 - tab. 1). Analiza statystyczna wykazała istotny wpływ ( $p \leq 0,01$ ) poszczególnych czynników (obecność skały płonnej, rodzaj badanego materiału), jak i ich interakcji na zawartość badanych metali. W publikacji P2 wykazano obecność metali (np. Cr, Ni, Ba, Zn i Cu) m.in. w skale płonnej, glebie i dżdżownicach. Analizowane stanowiska w publikacji P2 obejmowały również dwa stanowiska opisane w publikacji P3. To z kolei sugeruje możliwość potencjalnego transferu metali ze skały płonnej, przez glebę, do części zielonych roślin, a następnie do organizmów konsumentów takich jak wstężyki gajowe. W eksperymencie laboratoryjnym w grupach suplementowanych pojedynczymi metalami stwierdzono wyraźną tendencję wzrostu ich zawartości w stopie ślimaków wraz ze wzrostem stężenia dawek. Analogiczny efekt zaobserwowano w przypadku mieszaniny metali, gdzie wzrost stężenia powodował wzrost zawartości wszystkich analizowanych metali w stopie ślimaków (publikacja P3 - ryc. 1). Warto zwrócić uwagę na tego typu organizmy biowskaźnikowe, ponieważ ślimaki są pokarmem dla kręgowców znajdujących się na wyższym poziomie troficznym (Vranković i in., 2020).

### **6.3. Ocena wpływu wybranych metali zdeponowanych w środowisku na kwasy tłuszczowe, aktywność dysmutazy nadadtlenkowej (SOD) i katalazy (CAT) oraz na poziom substancji reagujących z kwasem tiobarbiturowym (TBARS) u przedstawicieli Lumbricidae, a także wytypowanie grupy kwasów tłuszczowych, które mogą być wykorzystane jako biomarkery skutków fizjologicznych oddziaływania polutantów środowiskowych (Publikacja P2). Opracowanie panelu biomarkerów fizjologicznych u dżdżownic (Publikacja P2)**

Dżdżownice z rodziny Lumbricidae stanowią cenne źródło biomarkerów, w związku z tym zaproponowano dotychczas nieopisywany w piśmiennictwie następujący panel biomarkerowych kwasów tłuszczowych: C12:0 (kwas laurynowy); C14:0 (kwas mirystynowy); C16:0 (kwas palmitynowy); C18:0 (kwas stearynowy); C20:0 (kwas arachidowy, kwas eikozanowy); C21:0 (kwas heneikozanowy); C24:0 (kwas lignocerynowy, kwas tetrakozanowy); C16:1 n-7 (kwas palmitooleinowy); C18:1 n-9c (kwas oleinowy) + C18:1 n-9t (kwas elaidynowy); C20:1 n-9 (kwas cis-11-eikozenowy); C18:2 n-6c (kwas linolowy) + C18:2 n-6t (kwas linolelaidonowy); C18:3 n-3 (kwas  $\alpha$ -linolenowy); C20:2 n-6 (kwas cis-11-14-eikozadienowy); C20:3 n-3 (kwas eikozatrienowy); C20:4 n-6 (kwas arachidonowy); C20:5 n-3 (kwas cis-5,8,11,14,17-eikozapentaenowy, EPA) i C22:2 n-6 (kwas cis-13-16-dokozadienowy). Współcześnie poszukuje się powszechnie występujących organizmów biowskaźnikowych, które mogą dostarczać użytecznych biomarkerów umożliwiających ocenę wpływu potencjalnych polutantów na organizmy żywe. Porównując zaproponowany panel biomarkerowych kwasów tłuszczowych u dżdżownic z panelem opracowanym dla innych bezkręgowców lądowych, np. ślimaków (Kowalczyk-Pecka i in., 2017a, 2017c), stwierdzono różnice. Panel biomarkerowych kwasów tłuszczowych u ślimaków lądowych obejmuje następujące kwasy: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2; C20:4 n-6; C20:5 n-3; C22:4 n-6; i C22:5 n-3 (Kowalczyk-Pecka i in., 2017a, 2017c). Tkanki dżdżownic charakteryzują się większym udziałem nasyconych kwasów tłuszczowych (SFA), co powoduje, że panel biomarkerowych kwasów tłuszczowych zawiera ich więcej niż w przypadku ślimaków.

Badania wykazały, że u dżdżownic zasiedlających stanowiska z depozytem skały płonnej stwierdzono wyższą zawartość SFA i jednonienasyconych kwasów tłuszczowych

(MUFA), a mniejszą zawartość wielonienasyconych kwasów tłuszczowych (PUFA), w porównaniu z pierścienicami pozyskanymi ze stanowisk bez depozytu skały płonnej. Najwyższe zawartości SFA i MUFA odnotowano u dżdżownic z nieużytku I z długoletnim oddziaływaniem skały płonnej (powyżej 10 lat) i pola z owsem z dwuletnim depozytem tego odpadu. Podczas, gdy PUFA dominowały w pierścienicach pozyskanych ze stanowisk bez depozytu skały płonnej z łąki I i łąki II oraz nieużytku II. Najniższe zawartości SFA i MUFA wykazano u bezkręgowców glebowych pobranych ze stanowisk bez depozytu skały płonnej - z łąki I i łąki II oraz w przypadku SFA pozyskanych jeszcze z nieużytku II. Natomiast najniższą zawartość PUFA stwierdzono w dżdżownicach z nieużytku I z długoletnim oddziaływaniem skały płonnej (powyżej 10 lat) i pola z owsem z dwuletnim depozytem skały płonnej (publikacja P2 - tab. 6, ryc. 2). Porównując zawartość metali i biomarkerowych kwasów tłuszczowych w dżdżownicach pozyskanych ze stanowisk z depozytem i bez depozytu skały płonnej, stwierdzono, że w bezkręgowcach pobranych ze stanowisk z depozytem tego odpadu była większa zawartość metali (Pb, Cd, Cr, Ba, Ni, Zn, Cu) oraz większa zawartość biomarkerowych kwasów tłuszczowych, takich jak C12:0, C14:0, C16:0, C18:0, C20:0, C21:0, C24:0, C16:1 n-7, C18:1 n-9c + C18:1 n-9t i C20:1 n-9. Jednocześnie zaobserwowano obniżoną zawartość biomarkerowych kwasów tłuszczowych, takich jak C18:2 n-6c + C18:2 n-6t, C18:3 n-3, C20:2 n-6, C20:3 n-3, C20:4 n-6, C20:5 n-3 i C22:2 n-6 (publikacja P2 - tab. 5-6, ryc. 2). Wyniki badań sugerują, że niektóre procesy peroksydacyjne mogły ulegać zatrzymaniu na MUFA. Możliwe, że w środowisku występowały czynniki ograniczające rozpad PUFA, czyli nastąpiła inwersja do MUFA a nie koniecznie do SFA. PUFA ogrywają kluczową rolę w utrzymaniu prawidłowej przepuszczalności błon komórkowych, zwłaszcza w zakresie równowagi pomiędzy kwasami n-6 i n-3, niezbędnej dla prawidłowego funkcjonowania błon komórkowych i błon lizosomów (Puca i in., 2008). Oprócz funkcji ochronnej PUFA w zachowaniu integralności błony komórkowej, niektóre z tych kwasów wykazują właściwości przeciwbakteryjne (Kowalczyk-Pecka i in., 2017a).

W dżdżownicach pochodzących z lokalizacji z obecnością depozytu skały płonnej wartości wskaźnika peroksydacji (PI) i wskaźnika nienasycenia (UI) były niższe w porównaniu z lokalizacji kontrolnych, czyli ze stanowisk pozbawionych tego depozytu. Najwyższą wartość PI zaobserwowano u dżdżownic pochodzących z łąki II (bez depozytu skały płonnej) i UI z łąki I (bez depozytu skały płonnej). Najniższe wartości obu wskaźników odnotowano u dżdżownic z nieużytku I z długoletnim depozytem skały płonnej (powyżej 10 lat) (publikacja P2 - tab. 5-6, ryc. 2). Wskaźnik PI określa całkowitą zawartość kwasów

tłuszczowych w błonach komórkowych, natomiast wskaźnik UI odzwierciedla liczbę wiązań podwójnych w błonach (Hulbert i in., 2007, 2014). Ekspozycja na metale wpływa na skład kwasów tłuszczowych, prowadzi do peroksydacji lipidów i uszkadza błony komórkowe (Wang i in., 2007b; Oves i in., 2016; Yadav i in., 2023).

W celu weryfikacji przydatności zaproponowanego panelu biomarkerowych kwasów tłuszczowych u dżdżownic, oznaczono enzymatyczne (SOD - dysmutaza ponadtlenkowa; CAT - katalaza) i nieenzymatyczne (TBARS - substancje reagujące z kwasem tiobarbiturowym) biomarkery stresu oksydacyjnego. We wszystkich analizowanych próbach dżdżownic pozyskanych ze stanowisk z depozytem skały płonnej stwierdzono obniżoną aktywność SOD i CAT oraz podwyższony poziom TBARS, w porównaniu z pierścienicami zasiedlającymi stanowiska kontrolne, czyli bez depozytu skały płonnej. Najwyższą aktywność SOD zaobserwowano w dżdżownicach pobranych ze stanowiska bez depozytu odpadu - z łąki II, natomiast najniższą ze stanowisk z długoletnim oddziaływaniem skały płonnej (powyżej 10 lat) - z pola z gryką i nieużytku I. Najwyższą aktywność CAT wykazano w próbach z pola z gryką bez depozytu skały płonnej, a najniższą z nieużytku I z ponad dziesięcioletnim oddziaływaniem skały płonnej. Najwyższy poziom TBARS stwierdzono w dżdżownicach z nieużytku I (depozyt skały płonnej powyżej 10 lat), a najniższy u bezkręgowców glebowych pozyskanych ze stanowisk bez depozytu skały płonnej z łąki I i łąki II (publikacja P2 - tab. 5-6, ryc. 2). SOD ma właściwości antyoksydacyjne, katalizuje reakcję dysmutacji anionów ponadtlenkowych ( $O_2^-$ ) do tlenu cząsteczkowego ( $O_2$ ) oraz nadtlenu wodoru ( $H_2O_2$ ). Enzym ten chroni komórki przed uszkodzeniami wywołanymi przez reaktywne formy tlenu. Z kolei CAT jest enzymem rozkładającym nadtlenek wodoru do wody i tlenu ( $O_2$ ), zapobiega gromadzeniu się tego szkodliwego związku chemicznego (Kamińska i in., 2024). TBARS to produkty uboczne utlenienia lipidów, a ich oznaczanie pozwala na ocenę poziomu peroksydacji lipidów w badanych tkankach (Osowska i Rogulska, 2023). Ekspozycja na metale aktywuje w organizmach enzymatyczne i nieenzymatyczne mechanizmy obronne (Roubalová i in., 2015; Chaitanya i in., 2016; Wang i in., 2021; Chakraborty i in., 2024). Nawet niewielkie, subprogowe dawki metali w środowisku, mogą indukować w organizmie zmiany fizjologiczne, np. zaburzenia aktywności enzymów antyoksydacyjnych, świadczące o występowaniu stresu oksydacyjnego (Nowicka, 2022).

#### **6.4. Określenie wpływu wybranych metali jako zmiennych środowiskowych na panel biomarkerowych kwasów tłuszczowych u wstężyka gajowego pozyskanego ze środowiska i suplementowanego w warunkach laboratoryjnych (Publikacja P3). Opracowanie panelu biomarkerów fizjologicznych u ślimaków (Publikacja P3).**

W celu oceny wpływu polutantów na wstężyki gajowe, stanowiące bardzo użyteczne źródło biomarkerów, szczególną uwagę zwrócono na panel biomarkerowych kwasów tłuszczowych: C16:0 (kwas palmitynowy); C18:0 (kwas stearynowy); C23:0 (kwas trikozanowy); C18:1 n-9 (kwas oleinowy); C20:1 n-9 (kwas cis-11-eikozenowy); C18:2 n-6 (kwas linolowy); C18:3 n-3 (kwas  $\alpha$ -linolenowy); C20:2 n-6 (kwas cis-11-14-eikozadienowy); C20:4 n-6 (kwas arachidonowy); C20:5 n-3 (kwas cis-5,8,11,14,17-eikozapentaenowy, EPA); C22:4 n-6 (kwas dokozaetraenowy) i C22:5 n-3 (kwas dokozaetraenowy) (Kowalczyk-Pecka i in, 2017a, 2017c). Przeprowadzone badania wykazały, że w stopie wstężyków gajowych pozyskanych ze stanowiska z depozytem skały płonnej odnotowano wyższą zawartość nasyconych kwasów tłuszczowych (SFA: C16:0, C18:0, C23:0) i jednonienasyconych kwasów tłuszczowych (MUFA: C18:1 n-9 i C20:1n-9), w porównaniu ze ślimakami ze stanowiska pozbawionego obecności tego depozytu. Jednocześnie zaobserwowano niższe zawartości wielonienasyconych kwasów tłuszczowych (PUFA: C18:2 n-6, C18:3 n-3, C20:2 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6 i C22:5 n-3) w stopie ślimaków pobranych ze stanowiska z depozytem skały płonnej niż bez jej depozytu (publikacja P3 – ryc. 2-3). Wyraźny spadek PUFA zaobserwowano w próbach badawczych laboratoryjnych, w których organizmy suplementowano dawkami Cr (0.75;1.0), Ni (0.5; 0.75; 1.0) i Ba (0.5; 0.75; 1.0) (publikacja P3 - tab. 2, ryc. 2). Porównanie uzyskanych wyników z doświadczenia laboratoryjnego i terenowego pozwoliło stwierdzić, że w przypadku SFA i MUFA wyższe zawartości odnotowano jedynie dla Cr (1.0), Ba (1.0) i Ni (1.0) w porównaniu ze ślimakami ze stanowiska kontrolnego (bez depozytu skały płonnej). Jednakże zawartości te pozostawały niższe niż w próbach pobranych ze stanowiska z depozytem skały płonnej. Dla PUFA wyraźne różnice wystąpiły jedynie przy Ba (1.0) i Ni (1.0), gdzie zawartość była niższa niż w stopie wstężyków gajowych ze stanowiska bez depozytu skały płonnej, lecz wyższa niż u ślimaków pobranych ze stanowiska z obecnością tego odpadu. W próbach kontrolnych laboratoryjnych stwierdzono niższe zawartości SFA i MUFA oraz wyższe PUFA w porównaniu zarówno do prób kontrolnych i badawczych

pozyskanych ze środowiska (publikacja P3 - tab. 2, ryc. 2-3). To może sugerować potencjalną peroksydację kwasów tłuszczowych. Zauważono także wzrost PUFA i spadek SFA i MUFA dla wszystkich zastosowanych dawek Zn (0.5; 0.75; 1.0) i Cu (0.5; 0.75; 1.0), a także dla najniższej dawki Cr (0.5) (publikacja P3 - tab. 2, ryc. 2). Subprogowe dawki pierwiastków, takich jak Zn, Cu i Cr, są niezbędne do życia. Co oznacza, że nawet niewielkie ich stężenia, nie powodując ostrej toksyczności czy śmierci, mogą jednak indukować zauważalne zmiany fizjologiczne. Na profil kwasów tłuszczowych ślimaków występujących w środowisku naturalnym, może oddziaływać nie tylko transfer samych metali, lecz również potencjalnie w pewnym stopniu obecność kwasów tłuszczowych pochodzących potencjalnie z mikrobiomu jelitowego (Huyan i in., 2022; Jiao i in., 2025). Preferencje pokarmowe ślimaków mogą stanowić jeden z mechanizmów ograniczających narażenie na zanieczyszczenia, gdyż zwierzęta te potrafią ograniczyć spożycie zanieczyszczonego pokarmu. Dodatkowo, w środowisku naturalnym, organizmy mogą spożywać pokarm zawierający substancje bioaktywne, które wpływają na peroksydację kwasów tłuszczowych. Przykładem może być witamina C, która pełni istotną rolę w nieenzymatycznej obronie antyoksydacyjnej (Doseděl i in., 2021). Przypuszczać można, że poza metalami, wpływ na gospodarkę lipidową, szczególnie na przetrwanie i strukturę długołańcuchowych kwasów tłuszczowych PUFA mają także inne czynniki obecne w środowisku naturalnym. Należą do nich: czynniki fizyczne (np. temperatura) (Edwards i Arancon, 2022), chemiczne (np. pestycydy) (Uwizeyimana i in., 2017) i biologiczne (np. patogeny) (Ghosh, 2018).

Zastosowanie analizy materiału biologicznego pochodzącego z terenu i laboratorium stanowi istotną wartość metodologiczną, ponieważ umożliwia porównanie reakcji ślimaków w środowisku z reakcjami uzyskanymi w warunkach kontrolowanych. Większość badań prowadzonych w warunkach laboratoryjnych skupia się na oddziaływaniu pojedynczych metali, jednak w środowisku naturalnym, ślimaki są narażone na działanie złożonych mieszanin zanieczyszczeń (Amiard-Triquet i Amiard, 1998). Wiele gleb zanieczyszczonych zawiera kilka pierwiastków jednocześnie, co może prowadzić do występowania efektów synergistycznych lub antagonistycznych między poszczególnymi metalami (Nahmani i in., 2007). Badania oparte na próbach środowiskowych pozwalają uwzględnić złożone interakcje między organizmami a otoczeniem, ujawniając reakcje na zanieczyszczenia, które mogą być niedostrzegalne w badaniach eksperymentalnych prowadzonych w warunkach kontrolowanych. Takie podejście umożliwia uzyskanie bardziej obiektywnego obrazu wpływu polutantów na środowisko. Porównanie wyników statusu biomarkerowych kwasów tłuszczowych uzyskanych z doświadczenia laboratoryjnego i terenowego stanowi istotny

element weryfikacji wyników otrzymanych z eksperymentu laboratoryjnego w odniesieniu do rzeczywistych warunków życia organizmów. Doświadczenia prowadzone w warunkach laboratoryjnych nie zawsze odzwierciedlają pełną złożoność interakcji środowiskowych, co może skutkować zbyt uproszczoną interpretacją wyników (Lesel, 1976; Crane i in., 2007).

W stopie wstężyków gajowych pochodzących ze stanowiska z obecnością depozytu skały płonnej wartości zarówno wskaźnika peroksydacji, jak i wskaźnika nienasycenia były niższe niż w porównaniu ze stanowiska bez depozytu (publikacja P3 – ryc. 2-3). Porównanie uzyskanych wyników z doświadczenia laboratoryjnego i terenowego wykazało, że tylko dla Ni (1.0) i Ba (1.0) wartości PI były niższe niż w wstężykach gajowych pozyskanych ze stanowiska bez depozytu skały płonnej, lecz wyższe w próbach badawczych pobranych ze stanowiska z jej depozytem. Analogiczną tendencję w przypadku wskaźnika UI odnotowano jedynie dla Cr (1.0). Dodatkowo, dla Ba (0.5) i Ni (1.0) wartości wskaźnika UI były niższe zarówno względem prób pobranych ze stanowiska z i bez depozytu skały płonnej (publikacja P3 - tab. 2, ryc. 2-3). Na podstawie analizy składu kwasów tłuszczowych możliwe jest obliczenie wskaźnika PI, który odzwierciedla teoretyczną podatność składu lipidów błonowych na proces peroksydacji (Hulbert i in., 2014; Kowalczyk-Pecka i in., 2017b).

## **6.5. Podsumowanie i perspektywa wykorzystania wyników badań w praktyce**

### **6.5.1. Publikacja P1**

Wpływ depozytu skały płonnej na właściwości fizykochemiczne i biologiczne gleb zależał przede wszystkim od rodzaju użytkowania terenu (nieużytki, pola uprawne) i rodzaju roślinności (rośliny: jednoliścienne, dwuliścienne). W mniejszym stopniu zależał od okresu trwania depozytu tego odpadu. Porównując wyniki uzyskane z gleb z depozytem i bez depozytu skały płonnej pochodzących z nieużytków i pól uprawnych oraz gleb z roślinnością jednoliścienną w porównaniu z roślinnością dwuliścienną, zaobserwowano ewidentne zmiany właściwości fizykochemicznych i biologicznych w glebie z nieużytków oraz z roślinnością jednoliścienną. W glebach rolniczych prawdopodobnie zachodziły intensywne procesy bioremediacyjne, w których mechanizmy fitoremediacji były wspomagane przez aktywność mikroorganizmów ryzosferowych.

Analizowane parametry umożliwiają skuteczne monitorowanie stanu gleby, co ma kluczowe znaczenie dla zrozumienia zarówno krótkoterminowego, jak i długoterminowego wpływu skały płonnej na środowisko glebowe.

### 6.5.2. Publikacja P2

Uzyskane wyniki wskazują, że dżdżownice zasiedlające stanowiska poddane długoletniemu oddziaływaniu skały płonnej (powyżej 10 lat) wykazywały najbardziej niekorzystną odpowiedź fizjologiczną pod względem peroksydacji kwasów tłuszczowych. Krótsza ekspozycja depozytu skutkowała poprawą analizowanych parametrów u dżdżownic. Oprócz okresu trwania depozytu, również rodzaj użytkowania terenów miał wpływ na status fizjologiczny dżdżownic. Użytkowanie terenów w formie upraw rolnych np. zbóż lub łąk utrzymywanych w dobrej kulturze rolnej obniżało w percepcyjnym fizjologicznie zakresie negatywne oddziaływanie metali na badane bezkręgowce glebowe. To mogło być spowodowane mniejszą kumulacją oraz ograniczonym transferem metali w systemie gleba-roślina (korzenie)-dżdżownice na terenach użytkowanych rolniczo, w porównaniu z nieużytkami, gdzie wieloletnia roślinność sprzyjała ich akumulacji i silniejszemu oddziaływaniu metali na dżdżownice.

Zastosowanie zintegrowanego modelu potencjalnego transferu wybranych metali w środowisku lądowym łączącego ogniwa troficzne w całościowy schemat potencjalnego transferu metali (skała płonna - gleba - dżdżownice), jest szerszym podejściem w stosunku do tradycyjnych analiz skupiających się jedynie na glebie.

Wytypowanie grupy kwasów tłuszczowych, które mogą być wykorzystywane jako użyteczne biomarkery skutków fizjologicznych oddziaływania polutantów środowiskowych otwiera nowe możliwości wykorzystania dżdżownic jako bioindykatorów. Dodatkowo poszukiwanie biomarkerów odpowiedzi biologicznej u bezkręgowców ma istotne znaczenie w ekotoksykologii, także przy subprogowych dawkach polutantów środowiskowych.

### 6.5.3. Publikacja P3

Zaobserwowano możliwość transferu wybranych metali obecnych na stanowisku z depozytem skały płonnej. U wstężyków gajowych pozyskanych ze stanowiska z depozytem skały płonnej stwierdzono najmniej korzystną odpowiedź fizjologiczną, charakteryzującą się zauważalnym spadkiem zawartości PUFA (najniższa zawartość PUFA) i wzrostem zawartości SFA (najwyższa zawartość SFA) w porównaniu z próbami ze stanowiska bez depozytu skały płonnej oraz próbami kontrolnymi i badawczymi laboratoryjnymi. Taki układ zmian może sugerować potencjalną peroksydację kwasów tłuszczowych. Proces peroksydacji kwasów tłuszczowych u ślimaków bezpośrednio może wpływać na zaburzenia struktury błon prowadząc subkomórkowo do zaburzenia procesów oddechowych, a międzykomórkowo np. do zakłócenia przepływu jonów między

komórkami. Ponadto możliwe jest wyodrębnienie grupy kwasów tłuszczowych, które mogą być użytecznymi biomarkerami narażenia ślimaków lądowych na te zmienne w warunkach środowiskowych, jak i laboratoryjnych.

Doświadczenia przeprowadzone w warunkach laboratoryjnych dają możliwość precyzyjnego kontrolowania poszczególnych zmiennych. Natomiast badania zrealizowane z wykorzystaniem materiału biologicznego pochodzącego z terenu odzwierciedlają rzeczywiste zależności między organizmem a środowiskiem. Takie komplementarne podejście zwiększa wiarygodność uzyskanych wyników oraz może być z powodzeniem stosowane w praktycznej ekotoksykologii.

## **6.6. Weryfikacja hipotez naukowych**

### **6.6.1. Weryfikacja hipotezy 1. Obecność skały płonnej w glebie wpływa negatywnie na właściwości fizykochemiczne i biologiczne gleby, w tym na stan populacji mikroorganizmów.**

Obecność skały płonnej w glebie powodowała zmiany odczynu gleby, prowadząc do zakwaszenia nieużytków oraz istotnie statystycznego spadku zawartości substancji humusowych. Dodatek skały płonnej istotnie statystycznie obniżał liczebność grzybów i bakterii proteolitycznych w glebie z pola z owsem. Ponadto obserwowano również spadek liczebności bakterii kopiotroficznych, oligotroficznych, celulolitycznych, amylolitycznych, rozpuszczania fosforanów, aktywności dehydrogenazy w glebie pola z owsem i nieużytku. Dodatek skały płonnej istotnie statystycznie obniżał liczebność bakterii produkujących siderofory i aktywność dehydrogenazy w glebie z pola z gryką, pola z owsem i nieużytku. Obniżał również liczebność bakterii opornych na metale w glebie z pola z gryką. Istotność statystyczną oceniono na poziomie  $p < 0,05$ , test ANOVA z post hoc Tukey.

### **6.6.2. Weryfikacja hipotezy 2. Istnieje transfer wybranych metali ciężkich w środowisku - z gleby do zwierząt bezkręgowych - których źródłem jest skała płonna zdeponowana w wyrobiskach piasku.**

Uzyskane wyniki wskazują, że zwiększona zawartość metali w glebach z depozytem skały płonnej znajduje istotne statystycznie potwierdzenie w ich podwyższonej zawartości w organizmach dżdżownic. Do oceny tej zależności zastosowano dwuczynnikową analizę wariancji (ANOVA) uzupełnioną jednoczynnikową analizą wariancji (ANOVA) oraz testy wielokrotnych porównań Tukeya, a istotność statystyczną oceniano na poziomie  $p \leq 0,01$ .

**6.6.3. Weryfikacja hipotezy 3. Wybrane metale jako czynniki stresogenne, wpływają na gospodarkę lipidową dżdżownic Lumbricidae, będących organizmami biowskaźnikowymi, prowadząc do zmian w składzie i oksydacji kwasów tłuszczowych oraz na aktywność SOD, CAT poziom TBARS w ich tkankach.**

Analiza dwuczynnikowa ANOVA wykazała istotny statystycznie wpływ stanowiska na profil wybranych kwasów tłuszczowych, wskaźnik PUFA, PI i UI oraz aktywność SOD, CAT i poziom TBARS. Analiza korelacji Pearsona wykazała ponadto liczne istotne statystycznie zależności pomiędzy zawartością metali a badanymi parametrami, przy czym kierunek i siła zależności były zróżnicowane w zależności od metalu i parametru. Istotność statystyczną oceniano na poziomie  $p \leq 0,01$ .

**6.6.4. Weryfikacja hipotezy 4. Ekspozycja ślimaków Helicidae np. wstężyk gajowy *Cepaea nemoralis* jako organizmów biowskaźnikowych na wybrane metale prowadzi do zwiększonej potencjalnej peroksydacji lipidów.**

Uzyskane wyniki wskazały istotne statystycznie zwiększenie PI i SFA u ślimaków związanych z terenami ze zdeponowaną skałą płonną, przy czym w warunkach laboratoryjnych szczególnie wyraźny wzrost PI obserwowano po ekspozycji na Zn i Cu. Hipotezę zweryfikowano na podstawie analizy różnic wartości indeksu PI i SFA pomiędzy grupami ślimaków, analizy wariancji z testem Tukeya oraz oceny zależności liniowych pomiędzy zawartością metali a PI, SFA i pozostałymi parametrami profilu kwasów tłuszczowych. Istotność statystyczną oceniano na poziomie  $p \leq 0,01$ .

**6.6.5. Weryfikacja hipotezy 5. Kwasy tłuszczowe (długość łańcucha: C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3 i C22:2 n-6), są biomarkerami dżdżownic poddanych wpływowi metali pochodzących ze skały płonnej.**

Na podstawie zastosowania dwuczynnikowej analizy wariancji (ANOVA) uzupełnionej jednoczynnikową analizą wariancji (ANOVA) oraz testy wielokrotnych porównań Tukeya, przy istotności statystycznej na poziomie  $p \leq 0,01$  wykazano istotne zróżnicowanie profilu kwasów tłuszczowych dżdżownic w zależności od obecności skały płonnej i stanowiska. Obecność skały płonnej oraz lokalizacja stanowiska istotnie

różnicowały profil kwasów tłuszczowych dżdżownic, przy czym występował również istotny efekt interakcji tych czynników.

**6.6.6. Weryfikacja hipotezy 6. Kwasy tłuszczowe (długość łańcucha: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6 i C22:5 n-3), są biomarkerami ekspozycji ślimaków lądowych na metale zarówno w warunkach oddziaływania czynników w środowisku, jak i laboratoryjnych.**

Na podstawie analizy wariancji (one-way ANOVA) z testem post-hoc Tukeya HSD, korelacji liniowej Pearsona pomiędzy zawartością kwasów tłuszczowych (FA), indeksami PI i UI a zawartością metali (Cr, Ba, Ni, Zn i Cu) potwierdzona istotne zróżnicowanie profilu kwasów tłuszczowych u wstężyków gajowych w odpowiedzi na ekspozycję na Cr, Ba, Ni, Zn i Cu, zarówno w warunkach środowiskowych, jak i laboratoryjnych. Zmiany składu FA oraz indeksów PI i UI wskazują na ich potencjalną przydatność jako biomarkerów ekspozycji ślimaków na metale. Istotność statystyczną oceniano na poziomie  $p \leq 0,01$ .

## 7. Podsumowanie i wnioski

### Podsumowanie i wnioski ogólne

W praktycznej ekotoksykologii bardzo pożądane są możliwe do szybkiego przeprowadzenia wielopłaszczyznowe analizy, łączące fizyko-chemiczno-biologiczne uwarunkowania *in situ* ze zwierzętami wykazującymi w krótkim czasie, rejestrowalną odpowiedź fizjologiczną. Ze względu na to, że gleba stanowi środowisko życia dżdżownic, a ślimaki również mają z nią kontakt, dokonano analizy jej właściwości fizykochemicznych i biologicznych w wariantach z udziałem i bez udziału depozytu skały płonnej. Uzyskane wyniki wskazują, że obecność skały płonnej wpływała na te właściwości gleby, przy czym zakres zmian zależał przede wszystkim od sposobu użytkowania rolniczego i rodzaju pokrywy roślinnej (P1).

Przeprowadzone badania obejmują wieloaspektowe spektrum łączące skałę płonną jako potencjalne źródło metali, z próbą standaryzacji wykorzystywania wybranych bezkręgowców lądowych (dżdżownice, ślimaki) zarówno jako organizmów biowskaźnikowych, jak i źródła biomarkerów fizjologicznych. Połączenie tych aspektów stanowi nowatorski wkład w rozwój badań z zakresu ekotoksykologii (P2, P3).

Wykonane analizy potwierdziły zasadność wykorzystania wybranych bezkręgowców lądowych jako źródła biomarkerów odpowiedzi biologicznej na wybrane metale (P2, P3). W przypadku wstężyków gajowych zastosowanie podejścia obejmującego pozyskanie materiału ze środowiska oraz suplementowanie metalami w warunkach laboratoryjnych pozwoliło na zwiększenie wiarygodności i uniwersalności proponowanych biomarkerów fizjologicznych (P3).

### Podsumowanie i wnioski szczegółowe

- 1) W glebie z uprawą gryki z depozytem skały płonnej stwierdzono najwyższe wartości  $\text{pH}_{\text{H}_2\text{O}}$  i  $\text{pH}_{1\text{MKCl}}$  w porównaniu z glebą z uprawy owsa i nieużytków. Zaobserwowano wzrost zawartości węgla organicznego w glebie z uprawą gryki i nieużytku z depozytem skały płonnej. Natomiast w glebie z uprawą owsa z depozytem odnotowano czterokrotne zmniejszenie zawartości węgla organicznego. Zawartość kwasów huminowych była niższa we wszystkich glebach z depozytem skały płonnej (gryka, owies, nieużytek) w porównaniu do gleby bez jej depozytu (P1).
- 2) Aktywność dehydrogenazy w glebie z uprawą gryki, zarówno z dodatkiem skały płonnej, jak i bez niej, utrzymywała się na zbliżonym poziomie, zaś w glebie z uprawy

owsa i nieużytku z jej depozytem była niższa niż w glebie bez depozytu. We wszystkich analizowanych glebach z depozytem skały płonnej w stosunku do gleb bez jej depozytu zaobserwowano spadek aktywności zarówno fosfatazy kwaśnej, jak i fosfatazy zasadowej (P1).

- 3) Dodatek skały płonnej do gleby z pola z gryką nie spowodował znaczących zmian w liczebności bakterii kopiotroficznych i oligotroficznych, grzybów mikroskopowych i mikroorganizmów zdolnych do rozpuszczania fosforanów, jednak zaobserwowano niewielki wzrost liczebności mikroorganizmów celulolitycznych, amylolitycznych i proteolitycznych. W glebie z uprawą owsa z depozytem skały płonnej odnotowano spadek liczebności wszystkich grup mikroorganizmów. Podobne zależności stwierdzono w glebie nieużytkowanej z depozytem tego odpadu w porównaniu z glebą bez jego obecności, lecz z wyjątkiem grzybów, których liczebność nie uległa znacząco zmianie (P1).
- 4) W glebie z depozytem skały płonnej z pola z gryką stwierdzono ponad trzykrotne, a w glebie z pola z owsem około dwukrotne zmniejszenie liczby unikalnych ASV w porównaniu z glebą bez depozytu skały płonnej. Odwrotną sytuację stwierdzono w przypadku gleby z nieużytku na której znajdowała się skała płonna, gdzie zaobserwowano prawie dwukrotne zwiększenie liczby ASV. Wskaźniki Shannon Alpha i Simpson Alpha potwierdziły wpływ sposobu użytkowania rolniczego na różnorodność mikrobiologiczną gleby. Ich wartości, niezależnie od gatunku uprawianych roślin (gryka, owies), były wyraźnie wyższe niż w glebie z nieużytków (P1).
- 5) Dla dżdżownic zaproponowano nieopisywany dotychczas w piśmiennictwie panel biomarkerowy obejmujący kwasy: C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3 i C22:2 n-6 (P2). Natomiast w przypadku ślimaków lądowych zaproponowano panel biomarkerowych kwasów tłuszczowych, takich jak: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6 i C22:5 n-3. Panel ten może służyć jako użyteczny zestaw biomarkerów skutków biologicznych u ślimaków na wybrane zmienne środowiskowe zarówno w warunkach środowiskowych, jak i laboratoryjnych (P3).
- 6) Skład panelu biomarkerowych kwasów tłuszczowych u dżdżownic wykazuje częściowe różnice w porównaniu z panelem opracowanym dla ślimaków lądowych.

Tkanki dżdżownic charakteryzują się większą zawartością nasyconych kwasów tłuszczowych w porównaniu ze ślimakami u których dominują w składzie wielonienasycone kwasy tłuszczowe. Przekłada się to na większy udział nasyconych kwasów tłuszczowych w panelu biomarkerowym dżdżownic (P2, P3).

- 7) U dżdżownic pochodzących z nieużytku z długoletnim (powyżej 10 lat) depozytem skały płonnej, zaobserwowano najmniej korzystną odpowiedź fizjologiczną na poziomie peroksydacji kwasów tłuszczowych. Wraz z wydłużeniem okresu trwania depozytu odnotowano wzrost nasilenia peroksydacji kwasów tłuszczowych u tych bezkręgowców (P2).
- 8) Na status fizjologiczny dżdżownic wpływał nie tylko okres trwania depozytu skały płonnej, ale również rodzaj użytkowania terenów z depozytem skały płonnej. Użytkowanie terenów w formie upraw zbożowych lub łąk utrzymywanych w dobrej kulturze rolnej obniżało w percepcyjnym fizjologicznie zakresie negatywny wpływ metali na badane pierścienice (P2).
- 9) Dla oceny przydatności panelu biomarkerowych kwasów tłuszczowych u dżdżownic zbadano enzymatyczne (SOD, CAT) i nieenzymatyczne (TBARS) biomarkery stresu oksydacyjnego, których aktywność, czy poziom były zsynchronizowane z zaobserwowanymi zmianami w składzie i oksydacji kwasów tłuszczowych (P2).
- 10) U wstężyków gajowych pozyskanych ze stanowiska z depozytem skały płonnej zaobserwowano najmniej korzystną reakcję fizjologiczną. Przejawiała się ona zauważalnym spadkiem zawartości wielonienasyconych kwasów tłuszczowych w porównaniu z próbami terenowymi bez depozytu skały płonnej, a także próbami kontrolnymi i badawczymi laboratoryjnymi, co może sugerować potencjalną peroksydację kwasów tłuszczowych. Ponadto w próbach kontrolnych bez depozytu skały płonnej stwierdzono zauważalne zmniejszenie zawartości wielonienasyconych kwasów tłuszczowych w porównaniu z próbami kontrolnymi laboratoryjnymi (P3).
- 11) Zaobserwowano transfer wybranych metali zdeponowanych w środowisku, którego kluczowym elementem był depozyt skały płonnej (P2, P3).
- 12) Po oznaczeniu stężenia zanieczyszczeń w naturalnym siedlisku ślimaków lądowych, warto przeprowadzić badania porównawcze w laboratorium, suplementując badane substancje w warunkach kontrolowanych. Takie podejście pozwala ocenić wpływ pobocznych czynników środowiskowych, np. klimatycznych lub różnych substancji, które mogą działać antagonistycznie lub synergistycznie na badane parametry u tych zwierząt (P3).

- 13) Skała płonna może być traktowana jako potencjalne źródło zanieczyszczeń, takich jak metale. Czynniki te powodowały fluktuacje wewnątrzustrojowe u dżdżownic z rodziny Lumbricidae (P2) i ślimaków *Cepaea nemoralis* (P3), co było związane z obserwowanymi zmianami w składzie biomarkerowych kwasów tłuszczowych, a także ze zmianami wartości wskaźnika peroksydacji i wskaźnika nienasylenia (P2, P3).
- 14) Na podstawie przeprowadzonych badań nie można jednoznacznie stwierdzić, czy wpływ skały płonnej jest korzystny czy niekorzystny dla badanych bezkręgowców lądowych. Niektóre metale w niskich dawkach (Zn, Cu) chronią wielonienasycone kwasy tłuszczowe u ślimaków przed procesem utleniania (P3). Badania te stanowią jedynie podstawę do dalszych, bardziej szczegółowych analiz obejmujących różne poziomy organizacji i struktury organizmów żywych.

## Podsumowanie

Na podstawie przeprowadzonych badań stwierdzono, że zarówno dżdżownice, jak i ślimaki lądowe stanowią cenne organizmy biowskaźnikowe oraz źródło użytecznych biomarkerów fizjologicznych wykorzystywanych w praktycznej ekotoksykologii. Badane bezkręgowce lądowe charakteryzują się szybkim rozwojem, powszechnym występowaniem oraz wysoką wrażliwością na czynniki stresogenne, a także pozostają w bezpośrednim kontakcie z glebą i roślinnością. Zastosowanie obu grup organizmów umożliwia uzyskanie pełniejszego obrazu odpowiedzi biologicznej na oddziaływanie wybranych polutantów. Jednak na wyniki prób pozyskanych *in situ* mogą wpływać także uwarunkowania środowiskowe, w szczególności czynniki klimatyczne oraz różnice w dostępności i składzie pokarmu. W związku z tym zasadne wydaje się prowadzenie równoległych badań obejmujących zarówno pozyskanie materiału badawczego ze środowiska, jak i eksperymenty laboratoryjne w warunkach kontrolowanych, z celową suplementacją badanych substancji. To podejście pozwala na bardziej wiarygodną interpretację wyników. Uzyskane rezultaty mogą stanowić podstawę do dalszych, perspektywicznych badań w zakresie praktycznej ekotoksykologii. W przyszłości warto rozszerzyć ten kierunek o analizę biomarkerów molekularnych, w tym ekspresji genów kodujących enzymy cytochromu P450, biomarkerów stresu oksydacyjnego, np. aktywność peroksydazy glutationowej i S-transferazy glutationowej, a także biomarkerów neurotoksyczności, takich jak aktywność acetylocholinesterazy.

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## **9. Kopie opublikowanych prac wchodzących w skład cyklu publikacji**

## Article

# Impact of Coal Waste Rock on Biological and Physicochemical Properties of Soils with Different Agricultural Uses

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**Abstract:** During the mining process in mines, a problem arises with the formation of coal post-mining waste, which is waste rock. It is often stored by mines on various types of land to manage the resulting spoil. However, this is not without its impact on the soil. In this study, we determined the biological and physicochemical properties of rhizosphere soils of the podzolic type, subjected to waste rock reclamation and without the influence of waste rock (control), differing in the type of agricultural use and type of plant cover: field-monocotyledonous (oat cultivation), field-dicotyledonous (buckwheat cultivation), and wasteland covered with very species-poor vegetation. Research has shown that long-term cultivation (buckwheat) contributed to the elimination (leveling out) of the microbial and biochemical differences. The addition of waste rock significantly reduced the number of microorganisms synthesizing siderophore, especially on wasteland (decreased by 1.5 log<sub>10</sub>/gDW). The abundant presence of the genera *Acidocella* and *Acidiphilum*, absent in wasteland without waste rock, in the unused soil under the influence of waste rock was strongly associated with the effect of lowering the pH by waste rock in soil not used for agriculture. Increased levels of 77 types of bacteria were observed in samples from buckwheat cultivation compared to wasteland. The number of microorganisms resistant to heavy metals as well as microorganisms capable of producing specific Fe-binding ligands—siderophores—decreased under the influence of waste rock. Moreover, the dehydrogenase activity in long-term cultivation both under the influence of waste rock and without its influence was at a similar level. In contrast, an almost 100-fold decrease in dehydrogenase activity was observed in soils with oat cultivation and a more than 4-fold decrease in acid phosphatase (ACP) and alkaline phosphatase (ALP) activity. These parameters provide an effective system for monitoring soil health, from inexpensive and fast methods to advanced and precise techniques. The results can be applied to solve the problems associated with coal mining wastes by developing methods for their use in soils with long-term agricultural use.

**Keywords:** waste rock; agricultural land reclamation; mining environmental hazards; soil enzymatic activity; physiological fingerprint; bacterial community



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## 1. Introduction

Over the last two hundred years, human activity has significantly influenced changes in the Earth's surface. For many millennia, humans have been changing and shaping terrestrial ecosystems through agriculture, urbanization, industrial activities, and technical barriers (roads, railroads, fences, etc.). Fragmentation and homogenization involve more than 50% of habitats [1–3]. This in turn affects the biodiversity of many groups of living organisms [4,5]. However, there are areas such as Polesie in Eastern Europe where the diversity of species and natural habitats is still well preserved. Polesie is a special area with primeval wetlands that have been continuously accumulating, e.g., CO<sub>2</sub>, for over 11,000 years [6,7] and hosts a very well-preserved biodiversity, called the Amazon of Europe [8,9]. Many areas of Polesie are protected, but are simultaneously degraded and threatened by decades of land improvement, peat and hard coal exploitation, and other plans to change natural ecosystems [10–12].

In Poland, in the area of Western Polesie, hard coal mining has been operating since 1982 [13]. Waste rock is stored next to the Bogdanka Mine, but part of the waste rock is used to harden local roads and fill excavations after sand mining sites as a reclamation method [14–16]. There are cases of waste rock being stored in the immediate vicinity of protected areas, such as Polesie National Park, Natura 2000 Area (e.g., Jeziora Uściwierskie PLH060009), Polesie Landscape Park, and the UNESCO International Biosphere Reserve “Western Polesie” [15]. Thus far, the significance of the impact of waste rock on protected areas has not been determined, which is why it is important to monitor its storage sites in and near protected areas. Mining exploitation also causes destruction of the area through changes in topography, leads to changes in the existing flora and fauna, affects the deterioration of air quality, and causes changes in hydrological systems and the quality of surface water [17]. Long-term contact with air and water can lead to the oxidation process, which in turn can lead to intensive process desulfurization, especially from inorganic sulfur (occurring in the form of pyrite) involving the formation of Fe<sub>2</sub>O<sub>3</sub> on pyrite surfaces and strong chemical and physical changes with significant consequences for the environment, such as an increase in the content of oxygen-containing functional groups as well as the hydrophilicity and adsorption of water molecules [18]. Therefore, cyclical wetting, for example, by rainfall, may to some extent affect the hydrophobic properties of the material and the soil as a result of interaction with water [19].

The economic development of countries is significantly related to the exploitation of mineral resources [17,20]. The role of the mining industry, including the development of mines, is a very important and strategic part of the economic development of many countries [21]. On the one hand, the mining industry provides work for thousands of people, contributing to social well-being, but on the other hand, the exploitation of resources has a negative impact on the natural environment [12]. The development of the mining industry causes improper use of soil and changes in the landscape [22]. Other empirical studies indicate that mineral extraction is also associated with socio-economic benefits, while contributing to regional economic growth and employment. On the other hand, mine closures negatively affect the lives of local communities and local economic entities related to services [23,24]. In addition, the mining industry is crucial to the global energy transformation in the case of developing countries. Resource extraction contributes significantly to the development and economic stability of more than 80 countries [25]. On the one hand, the functioning of the mining industry causes socio-environmental conflicts. However, the limited availability of some fossil fuels poses a threat to energy security, and on the other hand, it is necessary to achieve a sustainable energy future [23,26]. However, technological progress in the use of energy from renewable sources is a component of sustainable development [26–28]. Changing the forms of energy acquisition and the new

technologies developing at the same time are referred to as the energy revolution [29]. One of the perspectives is local and regional development strategies based on this type of energy source. This developing energy industry can improve the living conditions of the population and at the same time is a sustainable response to conflicts and environmental problems [26,27,30]. While economic development is essential to improving living standards, it must be balanced with environmental sustainability to ensure long-term prosperity. Of the 42 European countries, Poland is roughly in the middle of the European ecological footprint in terms of the human development index, despite the fact that a large share of energy comes from fossil fuels. Policymakers must implement and monitor policies that promote sustainable development [31]. Ensuring a balance among society, the environment, and the economy is defined by the idea of sustainable development [17,32]. In particular, the idea of sustainable development improves the quality of life for a large part of the world's population, in all its forms: social, economic, environmental, and therefore it is still a key requirement during the 21st century [26,30]. The protection and maintenance of terrestrial and aquatic ecosystems and the preservation of biodiversity affect human well-being, hence the validity of the idea of sustainable development [33].

An important part of solving problems related to mining activities is identifying adverse effects on environmental conditions in the impact zone [17]. Additionally, mining activities, along with the development of related infrastructure, negatively affect the landscape and local ecosystems. Threats resulting from mining activities include reduced availability, contamination and depletion of groundwater, large-scale disruptions to hydrological and geological systems, noise pollution, mine spills, excess waste, deforestation, habitat changes, aridification and desertification, loss of landscapes and aesthetic degradation, reduction in and loss of biodiversity, threats to food security, soil pollution, floods, soil erosion, reduction in and limitation of hydrological connectivity, and air pollution [34]. An important threat is waste rock used for the recultivation of degraded areas [35]. Waste rock presence in the soil leads to changes in the population of microorganisms, which, depending on the concentration of metals and the period of exposure to contamination, results in a strong reduction in the total abundance of microorganisms and the creation of a population of microorganisms adapted to contamination [36,37].

At the same time, degradation conditions vary depending on the area and should be considered in detail due to strong physical (water, soil) and biological (microorganisms, fauna, flora) pollution and other components of ecosystems [17,35,38]. When using waste rock for reclamation, the reclamation procedures should be adapted to specific field conditions [39]. Moreover, when used, they must be monitored for at least five years, and the responsibility rests with the same company (or government) that must carry out the reclamation [17,35,38]. Considering the scope of the potential effects of the use of waste rock, it is highly justified to monitor the biological condition of soils, especially those used for agriculture, because of the potential possibility of contamination.

It is necessary to monitor the biological condition of soils exposed to various types of contamination from coal mining waste, particularly in soils used for agriculture, because the plants grown in these areas are a direct threat to human health. At the same time, such comprehensive research will allow the identification of the best indicators of environmental hazards from waste rock contamination. Studies concerning the impact of various factors on the restoration of vegetation cover on coal mine heaps have shown that the biotic characteristics of the soil substrate and the plant–soil microbiota interaction are more important for vegetation development than abiotic parameters, and that the activity of microorganisms related to nutrient cycling is particularly important in such difficult ecosystems [40]. Moreover, the introduction of waste into the soil to cause soil remediation must also have an impact on fertility and ecosystem productivity through its impact on

biological activity, which consists of biochemical processes carried out by living organisms, mainly soil-inhabiting microorganisms [41]. Groups of microorganisms differing in their requirements in relation to the amount of carbon and nitrogen compounds that develop mainly at low (oligotrophs) and high (copiotrophs) concentrations of these elements can be distinguished. Most often, there is a clear dominance of bacterial oligotrophs [42]. There are also microorganisms capable of degrading organic compounds such as protein, starch, and cellulose, as well as making elements available: phosphorus via phosphate dissolution metals such as Fe, but also heavy metals via non-specific (e.g., acids) and specific (siderophores) chelating compounds [43]. In coal seams and associated rocks, the composition of the microbial community depends on the composition of nutrient elements (i.e., C, S, P, and N) and elements such as Fe and As [44]. The main indicator of soil biological activity is the enzyme participating in the respiration process of all organisms, dehydrogenase. This indicator is commonly used to assess the factors that exert adverse effects on soil microorganisms [43]. EcoPlate Biolog<sup>®</sup> tests to determine ecophysiological indicators are useful tools for monitoring biodiversity changes. The usefulness of measuring the activity of this enzyme as an ecological test has been confirmed by numerous studies on the changes in biological activity in various cultivation and fertilization systems in the case of soil contamination with heavy metals, pesticides, or petroleum compounds [45]. The problem of contamination of soils by coal mining waste occurs worldwide, but so far only a few research teams have analyzed such soils in terms of changes in soil properties (pH and especially biochemical and biological parameters) (Table 1).

**Table 1.** Characteristics of agricultural land affected by coal mine waste.

| Location                                              | Type of Development/Cultivation                                                            | Predominant Microbial Groups/<br>Enzymatic Activity                                                                                                                  | Literature |
|-------------------------------------------------------|--------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Muli coal mine site in Qinghai, China                 | Grasslands                                                                                 | Proteobacteria (~32%), Actinobacteria (~28%), Acidobacteria (~15%)                                                                                                   | [46]       |
| Coal mine drainage from Onyeama, Nigeria              | Derelict coal fields                                                                       | Proteobacteria (50.8%) and Bacteroidetes (18.9%) dominate the bacterial community, Ascomycota fungi (60.8%) and Ciliophora (12.6%) dominate the eukaryotic community | [47]       |
| Coal fly ash soil amendment in Hebei province, China  | Corn                                                                                       | Acidobacteria (77.05%), Sphingomonas (25.60%), Nitrospira (20.78%), Streptomyces (11.32%), Gaiella (10.20%)                                                          | [48]       |
| Coal mine spoil heaps in the Silesian Uplands, Poland | Grasses ( <i>Poa compressa</i> , <i>Calamagrostis epigejos</i> )                           | pH, dehydrogenase, and alkaline phosphatase higher than in bulk soil; acid phosphatase lower than in bulk soil                                                       | [49]       |
| Coal mine spoil heaps in the Silesian Uplands, Poland | Forbs ( <i>Daucus carota</i> , <i>Tussilago farfara</i> )                                  | pH, dehydrogenase, alkaline phosphatase, and acid phosphatase higher than in bulk soil                                                                               | [49]       |
| Bulianta coal mine in Yijin Horo Banner, China        | <i>Pinus sylvestris</i> , <i>Prunus sibirica</i> , and <i>Hippophae rhamnoides</i> forests | Soil organic carbon content higher after 15 years of vegetation restoration than after 5 and 10 years                                                                | [50]       |

We hypothesized that fields subjected to waste rock reclamation are a threat to protected areas (e.g., national park) and agricultural crops, and their presence in the soil affects the state of the microorganism population and the biological and physicochemical properties of the soil. In monitoring the condition of soils, it is necessary to give simultaneous attention to both the physicochemical and biological properties, including the enzymatic activity of the soil and, in particular, the abundance of the main systematic and physiological groups of microorganisms, the physiological profile and diversity of soil microorganisms, and the balance between these parameters. We assumed that the parameters analyzed are helpful in monitoring the condition of the soils and in selecting the appropriate agricultural use of polluted soils for specific crops.

The aim of this study was to determine the biological and physicochemical properties of soils subjected to reclamation using waste rock, which was used for agricultural purposes in protected areas. This study provides a comparison of complex changes in soil properties (their microbiological, biochemical, and physical parameters) between agriculturally managed soils and wasteland influenced by the impact of waste rock, as well as between soils subjected to the short-term and long-term impact of coal mine waste rock. There is also a complete lack in the literature on comprehensive analyses of post-coal waste in affected soils on the diversity of the microbiota by cultivating and genetic methods, nor comparisons of ecophysiological indicators reflecting the metabolic activity of the soil microbiota.

## 2. Materials and Methods

### 2.1. Soil Collection Site

The research material consisted of rhizosphere soils of the podzolic type formed from light loamy sand subjected to waste rock reclamation and without the influence of waste rock (control), differing in the type of agricultural use and type of plant cover: field-monocotyledonous (oat cultivation), field-dicotyledonous (buckwheat cultivation), and wasteland covered with very species-poor ruderal vegetation (grasses, fescue *Festuca* sp., *Helichrysum arenarium*, *Oenothera* sp.). The research sites were located in eastern Poland, in the Lublin Voivodeship, in the communes of Urszulin (Włodawa County), and Siedliszcze (Chełm County) (Figure S1). Materials for research were collected from places where waste rock from a hard coal mine was deposited as part of the reclamation in 2010 Andrzejów (geographical coordinates: 51.39091659373544, 23.243677450864734) and Wola Wereszczyńska (51.422832965028725, 23.14591751618067) and in 2020 Chojeniec (51.212092390547845, 23.072456542552718). After reclamation, two sites (Andrzejów and Chojeniec) were returned for agricultural use and the third site (Wola Wereszczyńska) was an agricultural wasteland. One of the sites where waste rock was deposited was located only about 0.6 km from the border of Polesie National Park (Figure S1).

Two different points were sampled at each location (Figure 1). The samples were collected from fields with identical cropping histories:

A\_bu\_in—field with buckwheat, influenced (>10 years) by waste rock, Andrzejów

A\_bu\_win—field with buckwheat, without influence of waste rock, Andrzejów

Ch\_oat\_in—field with oats, influenced (2 years) by waste rock, Chojeniec

Ch\_oat\_win—field with oats, without influence of waste rock, Chojeniec

WW\_wa\_in—wasteland, influenced (>10 years) by waste rock, Wola Wereszczyńska

WW\_wa\_win—wasteland, without influence of waste rock, Wola Wereszczyńska

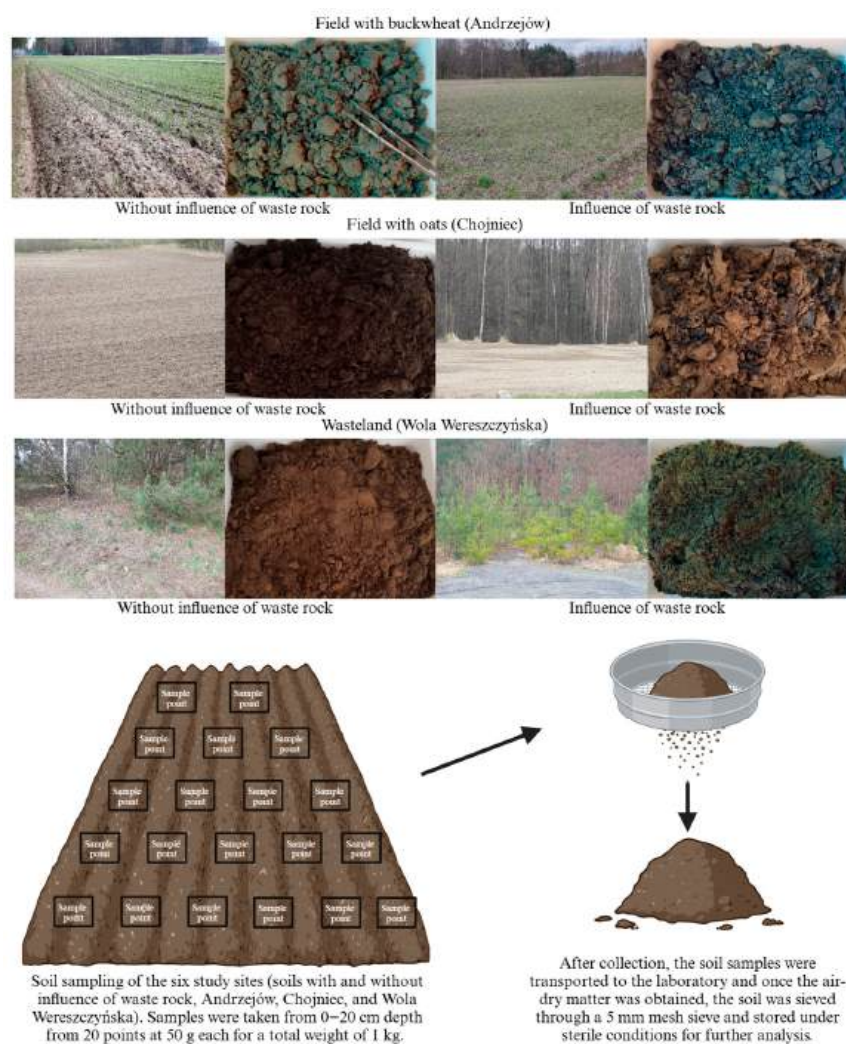


**Figure 1.** Flow chart of soil sampling and soil sampling locations. Created in BioRender.com, accessed on 24 November 2024.

Using the following list of methods (Table 2), the soil was subjected to microbiological, biochemical, ecopsychological and genetic analyses.

**Table 2.** Summary of methodology used in the study.

| Methodology          | Soil Sample | Main Information                                                                                                                                                                                       | Literature |
|----------------------|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Soil dry matter      | 10 g FW     | Dry in a laboratory dryer to constant dry mass: 3 cycles, 105 °C after 8 h                                                                                                                             | -          |
| pH                   | 1 g FW      | Preparation of soil suspensions at a ratio of 1:2.5 dH <sub>2</sub> O or 1M KCl * and pH determination/measured potentiometrically by glass electrode (with CP-505, Elmetron, Poland pH meter)         | -          |
| Humic acid content   | 100 g FW    | Humic acids extracted from the soil with 0.5M NaOH are precipitated from the solution with 6M HCl                                                                                                      | [51]       |
| Total organic carbon | 0.5 g DW    | 0.2 g of AgSO <sub>4</sub> and 10 mL of 0.4N potassium dichromate were added to the soil sample and boiled for 5 min. After cooling, the mixture was titrated with 0.2N Mohr's salt until bottle green | [51]       |

Table 2. Cont.

| Methodology                                                    | Soil Sample | Main Information                                                                                                                                                                                                                                                       | Literature |
|----------------------------------------------------------------|-------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| Fungal abundance                                               | 1 g FW      | The soil suspension was cultivated on Martin's substrate, with the incorporation of 1% Rose Bengal dye and 1% streptomycin                                                                                                                                             | [52]       |
| Copiotroph and oligotroph abundance                            | 1 g FW      | Determined on a PYS medium containing soil extract. For oligotrophic abundance, the PYS substrate was diluted 100 times                                                                                                                                                | [51]       |
| Abundance of cellulolytic microorganisms                       | 1 g FW      | Determined on CMC supplemented medium. Abundance was determined after inducing zones with 0.1% Congo Red and 1M NaCl.                                                                                                                                                  | [53]       |
| Abundance of amylolytic microorganisms                         | 1 g FW      | Determined on starch-supplemented medium. Abundance was determined after inducing zones with Lugol's liquid                                                                                                                                                            | [54]       |
| Abundance of proteolytic microorganisms                        | 1 g FW      | Determined on skimmed milk-supplemented medium. After incubation, the zones were visible as translucencies                                                                                                                                                             | [55]       |
| Abundance of microorganisms capable of solubilizing phosphates | 1 g FW      | Abundance was determined on Na <sub>3</sub> PO <sub>4</sub> + CaCl <sub>2</sub> supplemented medium. After incubation, the zones were visible as translucencies                                                                                                        | [56]       |
| Microorganisms resistant to heavy metals                       | 1 g FW      | Determined on Schlegel 284 medium supplemented with heavy metal solutions: CdSO <sub>4</sub> , NiCl <sub>2</sub> , CuSO <sub>4</sub> , ZnSO <sub>4</sub>                                                                                                               | [36,57]    |
| Microorganisms capable of synthesizing siderophores            | 1 g FW      | Determined on "blue" agar medium supplemented with CAS-Fe(III)-HDTMA complex                                                                                                                                                                                           | [58]       |
| Dehydrogenase activity                                         | 3 g FW      | Determined spectrophotometrically (485 nm) based on the conversion of TTC to formazan after 48 h incubation at 37 °C                                                                                                                                                   | [59]       |
| Phosphatase activity                                           | 1 g FW      | Determined spectrophotometrically (410 nm) based on the concentration of <i>p</i> -nitrophenol released from <i>p</i> -nitrophenyl phosphate at pH 5.5 for ACP and 11.0 for ALP                                                                                        | [60]       |
| Community level physiological profiling                        | 1 g FW      | The soil suspension was applied to an EcoPlate Biolog plate and incubated at 20 °C. Spectrophotometric measurements at 590 nm were performed every 24 h for 8 days; coefficients were calculated from the results: AWCD, substrate richness (R), and Shannon index (H) | [61,62]    |
| Bacterial community                                            | 0.5 g FW    | DNA was isolated with FastDNA™ SPIN Kit for Soil; results obtained were then subjected to bioinformatic and statistical analysis, obtaining a picture of the microbial population in the soil                                                                          | [63–65]    |

FW—fresh weight; DW—dry weight; CMC—carboxymethylcellulose; CAS—chromazurol S; ACP—acid phosphatase; ALP—alkaline phosphatase; AWCD—average well color development; HDTMA—hexadecyltrimethylammonium bromide. \* pH values measured in the water suspension correspond to the concentration of hydrogen ions present in the soil solution and the pH values measured in the soil suspension obtained in 1MKCl reflect the sum of the concentration of hydrogen ions present in the soil solution and hydrogen ions weakly bound to the solid fraction of the soil.

## 2.2. Determination of Dry Weight and pH Value

Soil dry matter was obtained by drying the soil at 105 °C for 8 h in three cycles in a laboratory dryer (MOV-112S, Sakata, Oizumi-Machi, Ora-Gun, Gunma Panasonic, Japan) until constant dry matter mass was obtained. The soil pH was assessed in water and 1M KCl at a ratio of 1:2.5 (CP-505, Elmetron, Poland).

## 2.3. Total Organic Carbon Content and Humus Content

The humic acid content was determined in 100 g of the sieved soil. To the weights, 100 mL of 0.5M NaOH was added and shaken for 2 h at RT. The suspension was centrifuged at 10,000 rpm for 15 min (MPW 350-R, Warsaw, Poland) and filtered through a cellulose filter to obtain clear supernatant. The supernatant was acidified with 50 mL of 6M HCl and allowed to precipitate humic acids for 2 h. The suspension was deacidified at 10,000 rpm for 15 min, and the resulting precipitate was dried at 80 °C for 8 h in three cycles until a stable dry mass was obtained. Total organic carbon content was determined using the Tiuren method. Air-dried soil was ground in a mortar to silty dust and then weighed into Erlenmeyer flasks (0.5 g); 0.2 g of AgSO<sub>4</sub> was added to each flask, and 10 mL of 0.4N acidic potassium dichromate solution was also added. The mixture was placed on a hotplate (Ceran 11 A, Harry Gestigkeit GmbH, Düsseldorf, Germany) and subjected to constant uniform boiling for 5 min. Subsequently, the flasks were cooled and the condensate from the septum was rinsed with 5 mL of distilled water. The resulting solution was then titrated with 0.2N Mohr's salt solution (0.2M ferric ammonium sulfate) against diphenylamine as an indicator (10 drops per flask). The solution was then titrated until a bottle-green color was obtained [51].

## 2.4. Determination of Soil Microbial Abundance

The soil samples were sieved through a sieve with a mesh diameter of 5 mm. Then, 5 g of soil was transferred to 45 mL of water and shaken for 15 min at 120 rpm at 20 °C (Biosan PSU-10i, Józefów, Poland). Subsequently, a series of decimal dilutions were prepared, which were sown onto microbial media to assess the abundance of culturable microorganisms. All cultures were incubated at 20 °C for 7 days (Innova 4900, Edison, NJ, USA).

### 2.4.1. Fungal Abundance

Fungal abundance was determined on Martin's medium with the following composition: glucose 10 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), peptone 5 g·L<sup>-1</sup> (BTL, Łódź, Poland), KH<sub>2</sub>PO<sub>4</sub> 1 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), MgSO<sub>4</sub>·7H<sub>2</sub>O 1 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), and agar 15 g·L<sup>-1</sup> (Biomaxima, Lublin, Poland) supplemented with 1% streptomycin and 1% Rose Bengal after sterilization (3 mL/L) [52].

### 2.4.2. Copiotrophs and Oligotrophs Abundance

Copiotrophic abundance was determined on PYS medium with the following composition: glucose 1 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), peptone 0.2 g·L<sup>-1</sup>, yeast extract 0.1 g·L<sup>-1</sup> (BTL, Łódź, Poland), K<sub>2</sub>HPO<sub>4</sub> 0.4 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), MgSO<sub>4</sub>·7H<sub>2</sub>O 0.05 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), horticulture soil extract 100 mL/L, and agar 15 g·L<sup>-1</sup> (Biomaxima, Lublin, Poland). The abundance of oligotrophs was determined using a medium diluted 100 times [51].

### 2.4.3. Abundance of Cellulolytic Microorganisms

The abundance of cellulolytic microorganisms was determined on CMC Agar with the following composition: carboxymethylcellulose (CMC) 10 g·L<sup>-1</sup> (Sigma-Aldrich, St. Louis, MO, USA), K<sub>2</sub>HPO<sub>4</sub> 0.5 g·L<sup>-1</sup> (POCH, Gliwice, Poland), KCl 0.5 g·L<sup>-1</sup> (POCH, Gliwice, Poland), MgSO<sub>4</sub>·7H<sub>2</sub>O 0.2 g·L<sup>-1</sup> (Chempur, Piekary Śląskie, Poland), CaCl<sub>2</sub> 0.1 g·L<sup>-1</sup>

(POCH, Gliwice, Poland),  $(\text{NH}_4)_2\text{SO}_4$   $0.1 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland), and agar  $15 \text{ g}\cdot\text{L}^{-1}$  (Biomaxima, Lublin, Poland). After incubation, the cellulose decomposition zone was eluted using 0.1% Congo Red (Park Scientific, Northampton, UK) for 15 min. The excess dye was washed with 1M NaCl (POCH, Gliwice, Poland) for 15 min [53].

#### 2.4.4. Abundance of Amylolytic Microorganisms

The abundance of amylolytic microorganisms was determined on starch agar with the following composition: soluble starch  $10 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{KH}_2\text{PO}_4$   $0.5 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland),  $\text{K}_2\text{HPO}_4$   $0.5 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland),  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$   $0.2 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland),  $(\text{NH}_4)_2\text{SO}_4$   $0.2 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland), and agar  $15 \text{ g}\cdot\text{L}^{-1}$  (Biomaxima, Lublin, Poland). After incubation, amylolysis zones were induced using Lugol's liquid (POCH, Gliwice, Poland) [54].

#### 2.4.5. Abundance of Proteolytic Microorganisms

The abundance of proteolytic microorganisms was determined on milk agar with the following composition: meat extract  $15 \text{ g}\cdot\text{L}^{-1}$  (BTL, Łódź, Poland) and skimmed milk  $200 \text{ mL/L}$  (Łaciate, Mlekpól, Poland) [55].

#### 2.4.6. Abundance of Microorganisms Capable of Solubilizing Phosphates

The abundance of microorganisms capable of solubilizing phosphates was determined on PS medium with the following composition: glucose  $10 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland), asparagine  $1 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland), casein hydrolysate  $0.2 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$   $0.4 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland),  $\text{K}_2\text{SO}_4$   $0.2 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland), and agar  $15 \text{ g}\cdot\text{L}^{-1}$  (Biomaxima, Lublin, Poland) in 800 mL of  $\text{H}_2\text{O}$ . Solutions of  $\text{Na}_3\text{PO}_4\cdot 12\text{H}_2\text{O}$   $10 \text{ g}/100 \text{ mL}$  (POCH, Gliwice, Poland) and  $\text{CaCl}_2$   $22 \text{ g}/100 \text{ mL}$  (POCH, Gliwice, Poland) were sterilized separately and added after autoclaving [56].

#### 2.4.7. Microorganisms Resistant to Heavy Metals

The abundance of microorganisms resistant to heavy metals was determined on Schlegel 284 medium with the following composition: Tris-HCl  $6.06 \text{ g}\cdot\text{L}^{-1}$  (Sigma-Aldrich, Hamburg, Germany), NaCl  $4.68 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland), KCl  $1.49 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{NH}_4\text{Cl}$   $1.07 \text{ g}\cdot\text{L}^{-1}$  (Sigma-Aldrich, Hamburg, Germany),  $\text{Na}_2\text{SO}_4$   $0.43 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$   $0.2 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$   $0.03 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$   $0.04 \text{ g}\cdot\text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{Fe(III)NH}_4\text{Citrate}$   $10 \text{ mL/L}$  (solution  $48 \text{ mg}/100 \text{ mL}$ ) (Sigma-Aldrich, Hamburg, Germany), and agar  $20 \text{ g}\cdot\text{L}^{-1}$  (Biomaxima, Lublin, Poland). Prepared separately was C-mix containing sodium lactate (solution 50%)  $0.7 \text{ mL/L}$ , glucose  $0.52 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland), D-gluconic acid sodium salt  $0.66 \text{ g}\cdot\text{L}^{-1}$  (Sigma-Aldrich, Hamburg, Germany), fructose  $0.54 \text{ g}\cdot\text{L}^{-1}$  (Sigma-Aldrich, Hamburg, Germany) and sodium succinate  $\cdot 6\text{H}_2\text{O}$   $0.81 \text{ g}\cdot\text{L}^{-1}$  (Sigma-Aldrich, Hamburg, Germany). Heavy metal solutions were prepared and sterilized separately:  $0.4 \text{ mM}$   $\text{CdSO}_4\cdot 3\text{H}_2\text{O}$  (Sigma-Aldrich, Hamburg, Germany),  $0.8 \text{ mM}$   $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$  (Sigma-Aldrich, Hamburg, Germany),  $0.6 \text{ mM}$   $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$  (Sigma-Aldrich, Hamburg, Germany), and  $0.4 \text{ mM}$   $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$  (Sigma-Aldrich, Hamburg, Germany). After sterilization, all solutions were mixed together [36,57].

#### 2.4.8. Microorganisms Capable of Synthesizing Siderophores

The abundance of microorganisms capable of synthesizing siderophores was determined on CAS agar with the following composition: glucose  $4.0 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland),  $\text{KH}_2\text{PO}_4$   $3 \text{ g}\cdot\text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland), NaCl  $0.5 \text{ g}\cdot\text{L}^{-1}$

(POCH, Gliwice, Poland),  $\text{NH}_4\text{Cl}$   $1 \text{ g} \cdot \text{L}^{-1}$  (POCH, Gliwice, Poland),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   $0.2 \text{ g} \cdot \text{L}^{-1}$  (Chempur, Piekary Śląskie, Poland), and agar  $15.0 \text{ g} \cdot \text{L}^{-1}$  (Biomaxima, Lublin, Poland) in 860 mL of 0.1M PIPES buffer (Merck, Darmstadt, Germany). Solutions of 10% acidic casein hydrolysate (POCH, Gliwice, Poland) (30 mL), 0.01 M  $\text{CaCl}_2$  (POCH, Gliwice, Poland) (10 mL), and a dark blue solution of CAS-complex (100 mL) prepared by mixing 60.5 mg chromazurol S (CAS) (Fulka, Göteborg, Sweden) (50 mL), 1 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  (POCH, Gliwice, Poland) in 10 mM HCl (POCH, Gliwice, Poland) (10 mL), and 72.9 mg of detergent (hexadecyltrimethylammonium bromide (HDTMA) (Sigma-Aldrich, Hamburg, Germany) (40 mL)) were sterilized separately and added after autoclaving [58].

### 2.5. Dehydrogenase Activity

Three grams of soil was weighed into Falcon tubes, and 2 mL of  $\text{CaCO}_3$  (POCH, Gliwice, Poland) suspension ( $0.6 \text{ g} \cdot \text{mL}^{-1}$ ), 1 mL of 3% TTC (Alfa Aesar, Ward Hill, MA, USA), and distilled water (1.5 mL) were added. The mixture was thoroughly mixed and incubated for 48 h at  $37^\circ\text{C}$  (Panasonic MOV-112S, Sakata, Japan). The formazan (TPF) produced was extracted with 5 mL 96% ethanol. The resulting mixture was centrifuged at 10,000 rpm for 15 min (MPW 350-R, Warsaw, Poland), and absorbance was measured at 485 nm using an Infinite 200 PRO TECAN microplate spectrophotometer (Tecan, Grödig, Austria). Dehydrogenase activity was expressed as  $\mu\text{g TPF/h/gDW}$  [59].

### 2.6. Phosphatase Activity

Acid phosphatase (ACP) and alkaline phosphatase (ALP) activities were determined in the tested soils. One gram of soil was weighed into Falcon tubes, and 200  $\mu\text{L}$  of toluene was added and allowed to stand for 15 min. After this time, 4 mL of modified universal buffer (MUB) with pH 5.5 (for ACP) and 11.0 (for ALP) and 1 mL of 100 mM *p*-nitrophenylphosphate (*p*-NPP) (Merck, Darmstadt, Germany) were added to the tubes. The mixture was incubated for 3 h at  $37^\circ\text{C}$  (Panasonic MOV-112S, Sakata, Japan). After this time, 1 mL of 0.5M  $\text{CaCl}_2$  (POCH, Gliwice, Poland) and 4 mL of 0.5M NaOH (POCH, Gliwice, Poland) were added to the tubes. The mixture was thoroughly mixed and centrifuged at 10,000 rpm for 15 min (MPW 350-R, Warsaw, Poland). The concentration of released *p*-nitrophenol (*p*-NP) was determined at 410 nm using an Infinite 200 PRO TECAN microplate spectrophotometer (Tecan, Grödig, Austria). Activity was expressed as  $\mu\text{mol p-NP/h/g DW}$  [60].

### 2.7. Community Level Physiological Profiling—EcoPlate Biolog®

The Biolog EcoPlate™ system (Biolog Inc., Hayward, CA, USA) was used to determine variability in the biodiversity of soil microbial populations. One gram of soil was weighed into 99 mL of distilled water and shaken for 15 min at 120 rpm at  $20^\circ\text{C}$ . One hundred microliters of the resulting suspension was transferred to each well of a 96-well Biolog EcoPlate™. The plates were incubated for 8 days at  $20^\circ\text{C}$  (Innova 4900, Edison, NJ, USA) and spectrophotometric measurement at 590 nm ( $\text{OD}_{590}$ ) was performed every 24 h using an Infinite 200 PRO TECAN microplate spectrophotometer (Tecan, Grödig, Austria) [61,62]. The average well color development (AWCD), substrate richness (R), and Shannon index (H) were calculated using the following formulae:

$$\text{AWCD} = \frac{\sum_{i=1}^N \text{OD}_i}{N} \quad (1)$$

where AWCD—average well color development,  $\sum_{i=1}^N \text{OD}_i$ —Sum of  $\text{OD}_{590}$  from all wells corrected for the control well, and N—the number of substrates ( $N = 31$ ).

Substrate richness (**R**) was calculated from the number of substrates used, where  $ABS_{590} > 0.25$  after 96 h of incubation:

$$H = - \sum_{i=1}^N P_i \ln P_i \quad (2)$$

where  $H$ —Shannon index,  $\ln P_i$ —the natural logarithm of the ratio of the  $OD_{590}$  of the well to the sum of the  $OD_{590}$  for all the wells, and  $N$ —the number of substrates ( $N = 31$ ).

## 2.8. Bacterial Community

DNA was isolated using a commercially available kit (FastDNA™ SPIN Kit for Soil, MP BIOMEDICAL, Burlingame, CA, USA). For processing of demultiplexed fastq files, the DADA2 (1.14) package [66] in R software (3.6.0) [67] was used. Based on the quality plots, the last 20 forward and 20 reverse bases were trimmed off (filter parameters:  $\max N = 0$ ,  $\max EE$  for both reads = 2,  $\text{truncQ} = 2$ ). The next steps of analysis were *learnErrors*, *dada*, and *removeBimeraDenovo*. For taxonomy assignment, the latest version of the modified RDP (Ribosomal Database Project) v18 database [63] was used by applying *IDTAXA* [68]. The results were input into the *phyloseq* (1.22.3) package [64] (chloroplast or mitochondrial DNA were deleted). Statistical and graphical analysis were prepared using the *microeco* package (RStudio for Windows version 2024.09.0+375 (Posit, PBC, GNU Affero General Public Licence v3) [65]. Data were deposited in the NCBI SRA database in bioproject no. PRJNA1087279.

## 2.9. Statistical Analysis

All experiments were conducted in 3 biological replicates. Data are presented as mean values with standard deviation (SD). The results were subjected to analysis of variance (one-way ANOVA) followed by Tukey's post hoc test for multiple comparisons at  $p < 0.05$ . Statistical analysis and the Shannon and Simpson tests were performed using the open-source software RStudio for Windows version 2024.09.0+375 (Posit, PBC, GNU Affero General Public License v3).

# 3. Results

## 3.1. Soil pH Variability and Organic Carbon and Humus Content in Soil

The addition of waste rock to the soils affected both pH values (Figure 2). The addition of waste rock to buckwheat culture fields alkalinized the soils to a pH level of  $\sim 7.5$ – $8.0$  (Figure 2A,D). These soils also showed the highest pH levels compared with the other samples tested. Soil acidification was most affected by the waste rock in the wasteland, where an up to 2-fold decrease in soil pH values was observed (Figure 2C,F). In the field with oat farming, the effect of waste rock on soil pH levels varied more strongly. Soil alkalization was observed in water (Figure 2B) and a decrease in pH in KCl (Figure 2E).

The addition of waste rock to the soils was observed to cause an increase in organic carbon content in the case of buckwheat cultivation (1.5%) and wasteland (4-fold increase) (Figure 2G,I). Organic C decreased to  $1 \mu\text{g/g}$  only in fields with oat cultivation (Figure 2H). However, the change in organic C content did not translate into the level of humic substances in the soil, where a decrease in the content of these compounds was observed in all fields compared to the control (Figure 2J,K,L). The wasteland was additionally characterized by the lowest humic substances content in the fields, without the influence of waste rock (Figure 2L).

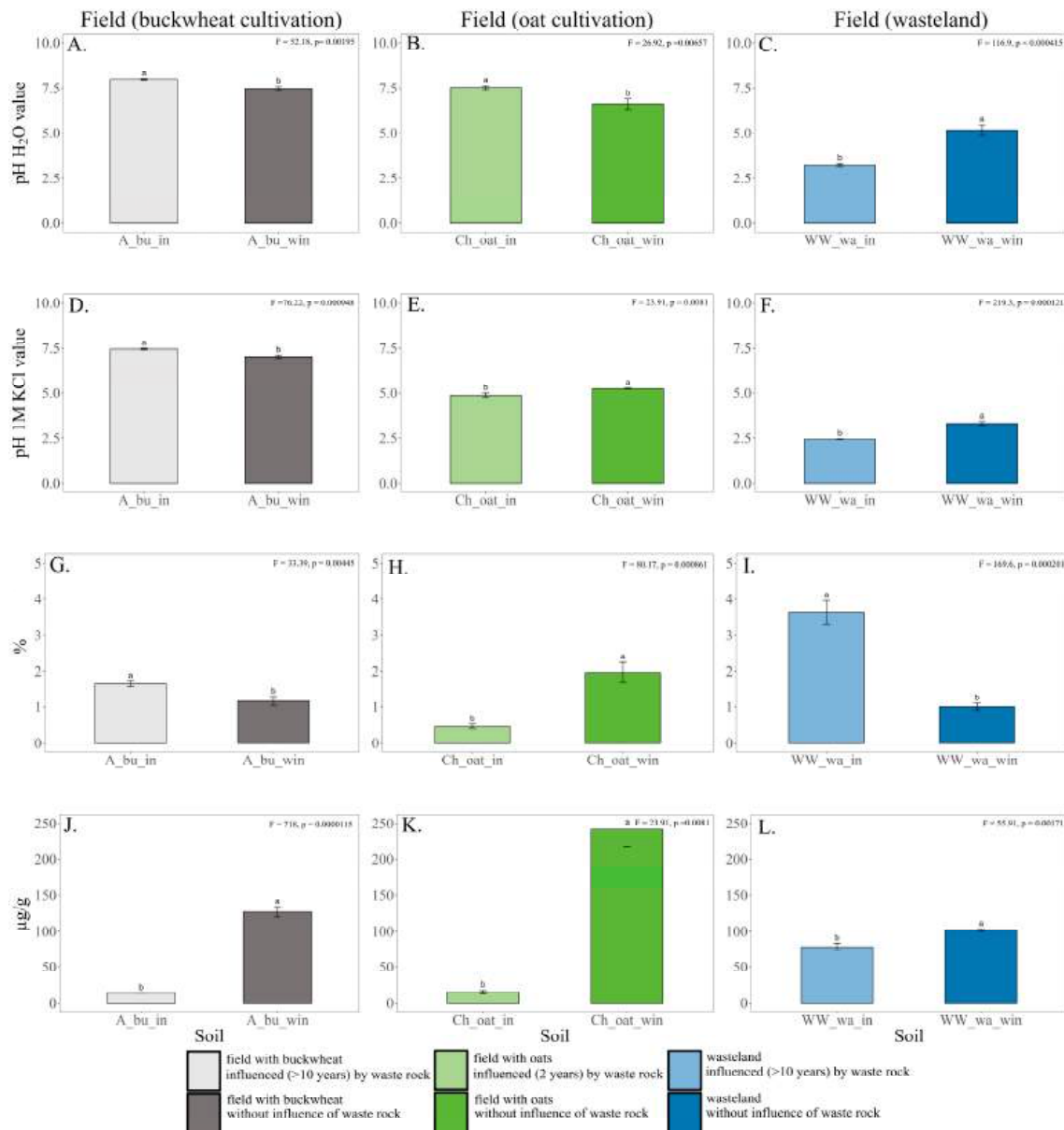


**Figure 2.** The pH value of soils, organic C content, and humic substances in the samples: field (buckwheat cultivation), field (oat cultivation), field (wasteland). pH value determined in water: (A–C) and in 1M KCl: (D–F); org. C (%): (G–I). and humus (μg/g): (J–L). ANOVA statistical analysis with Tukey’s HSD significance test. The different letters above the charts indicate statistical differences ( $p < 0.05$ ). Note/abbreviation: with influence (in), without influence (win) of waste rock.

### 3.2. Abundance of Soil Microorganisms

The addition of waste rock did not significantly affect the abundance of all microbial groups in the buckwheat cultivation field, where the average abundance was  $\sim 5.5 \log_{10}/\text{gDW}$  for fungi and  $7.5\text{--}7.7 \log_{10}/\text{gDW}$  for copiotrophs and oligotrophs, respectively (Figure 3A,D,G). On the other hand, in the soil from oat cultivation, a reduction in the abundance of all microbial groups by about  $1\text{--}1.5 \log_{10}/\text{gDW}$  was observed (Figure 3B,E,H). A similar situation was observed for the abundance of copiotrophs and oligotrophs in wasteland, where the influence of waste rock caused a decrease in the abundance of these groups of microorganisms to the level of  $6.2 \log_{10}/\text{gDW}$  and only the abundance of fungi remained at the same level (Figure 3C,F,I).

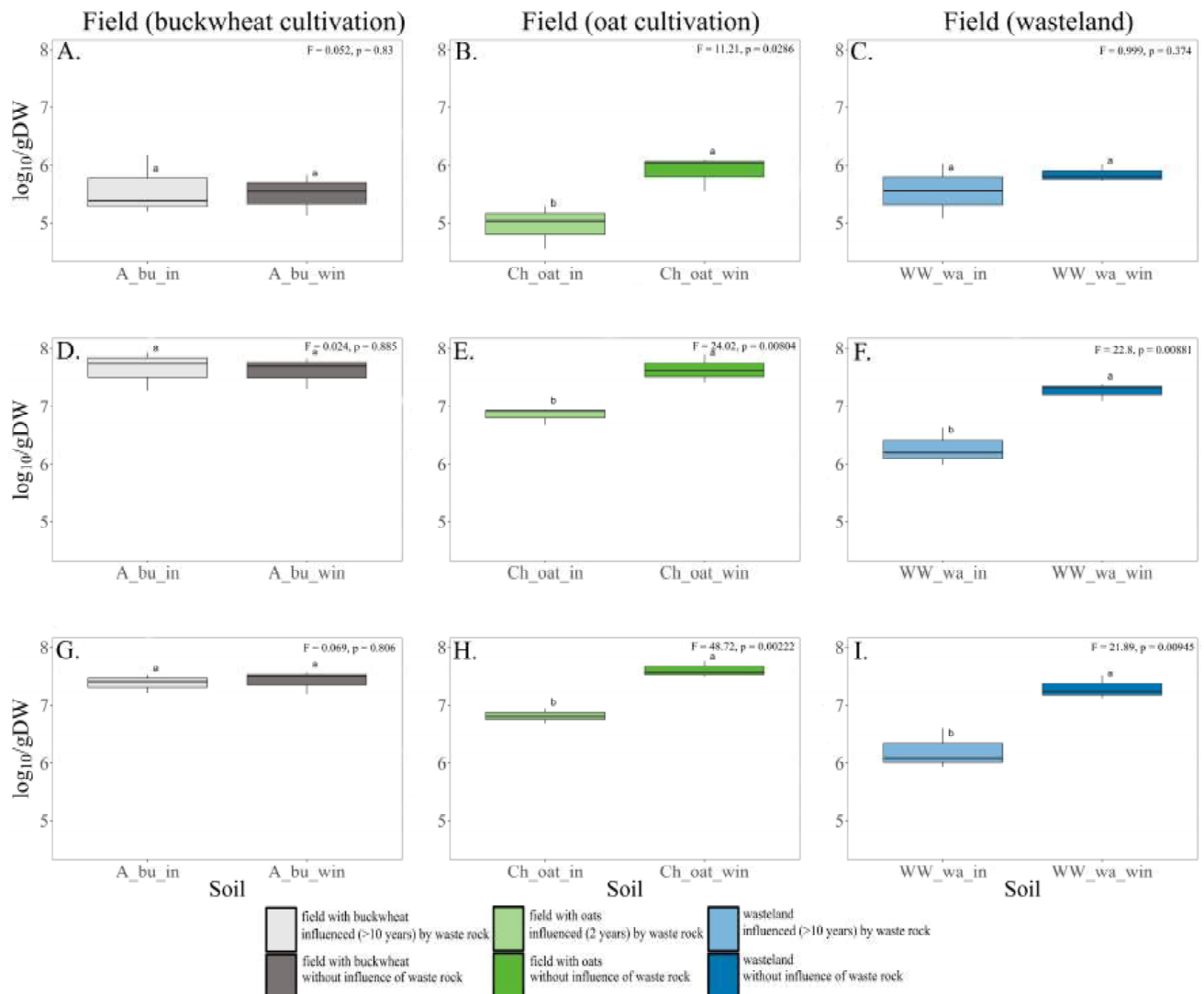


**Figure 3.** Abundance of main groups of microorganisms in soils: field (buckwheat cultivation), field (oat cultivation), field (wasteland). Fungal abundance: (A–C); copiotroph abundance: (D–F); oligotroph abundance: (G–I). ANOVA statistical analysis with Tukey's HSD significance test. The different letters above the charts indicate statistical differences ( $p < 0.05$ ). Note/abbreviation: with influence (in), without influence (win) of waste rock.

High variability in the abundance of individual physiological groups of microorganisms was observed depending on the type of cultivation and addition of waste rock to the soil (Figure 4). The addition of waste rock to the soil had no effect on the abundance of microorganisms in the four main physiological groups during the long-term buckwheat cultivation (Figure 4A,D,G,I). In contrast, the opposite situation was observed for oats cultivation and wasteland, where the effect of waste rock was much shorter. A significant decrease in the abundance of microorganisms from the four main physiological groups was observed in both soils. The greatest impact of the addition of waste rock was on cellulolytic and phosphate-solubilizing microorganisms, where the decrease in abundance was at the level of 1.5–2  $\log_{10}/\text{gDW}$  (Figure 4B,C,K,L).

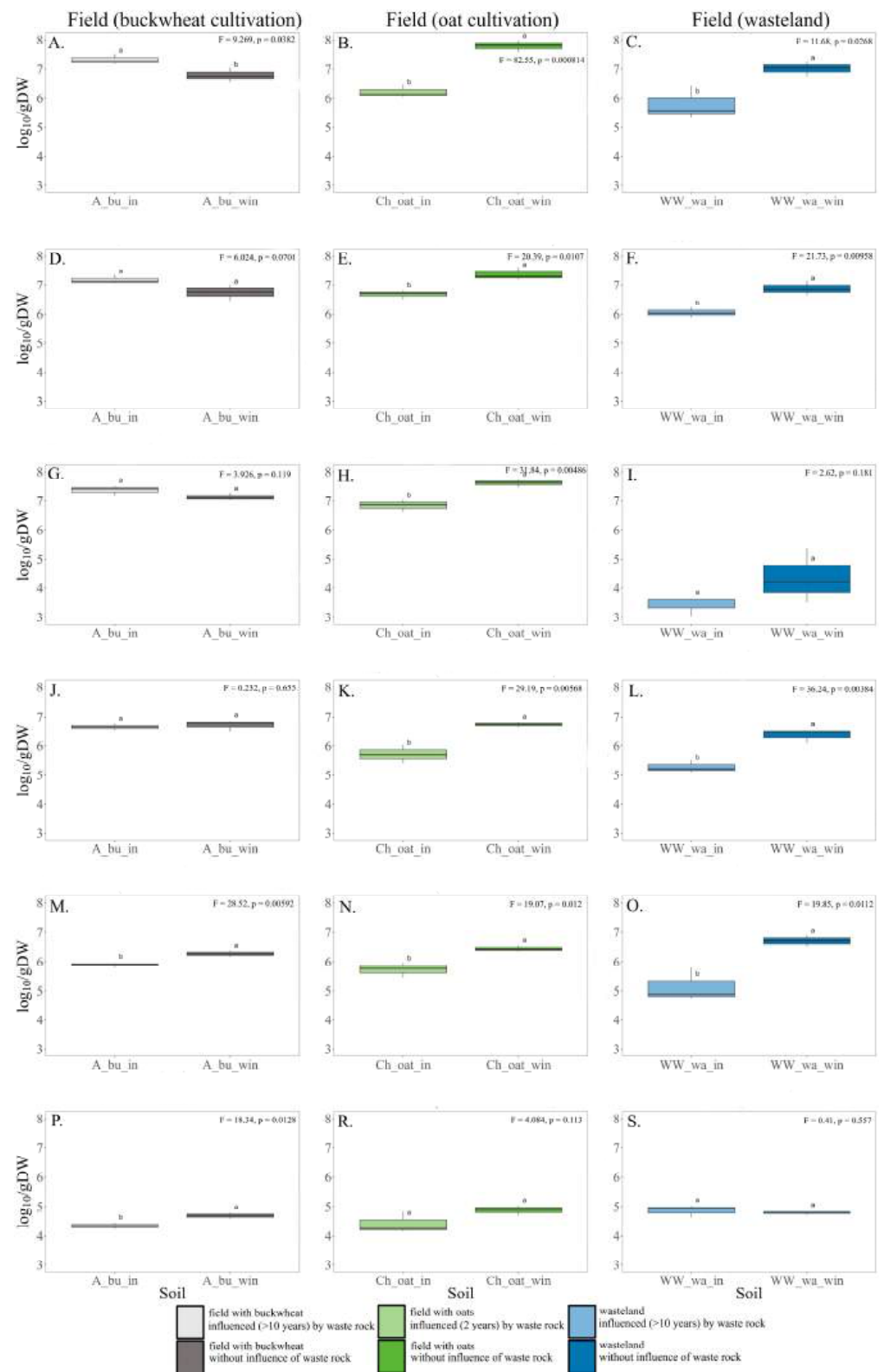


**Figure 4.** Abundance of microorganisms from different groups of soil nutrient cycles and abundance of microorganisms resistant to heavy metals and capable of synthesizing siderophores: field (buckwheat cultivation), field (oat cultivation), field (wasteland). Cellulolytic microorganisms: (A–C); amylolytic microorganisms: (D–F); proteolytic microorganisms: (G–I); phosphate-solubilising microorganisms: (J–L); microorganisms capable of synthesizing siderophores: (M–O); microorganisms resistant to heavy metals: (P–S). ANOVA statistical analysis with Tukey’s HSD significance test. The different letters above the charts indicate statistical differences ( $p < 0.05$ ). Note/abbreviation: with influence (in), without influence (win) of waste rock.

The addition of waste rock to soils also decreased the abundance of microorganisms capable of synthesizing siderophores. The greatest decrease was observed in wasteland ( $1.5 \log_{10}/\text{gDW}$ ) (Figure 4M,N,O). A similar situation was observed for heavy metal-resistant microorganisms in fields with buckwheat and oat cultivation, where a statistically significant decrease in the abundance of this group of microorganisms was observed (Figure 4P,R). In the case of the wasteland, the abundance of this group of microorganisms remained at the same level as in both soils (Figure 4S).

### 3.3. Soil Enzyme Activity

For buckwheat cultivation, dehydrogenase activity was at the same level ( $\sim 0.5 \mu\text{g TPF/h/gDW}$ ) in both soils (Figure 5A). In contrast, a significant decrease in the activity of these enzymes was observed in the soil under the influence of waste rock from oat cultivation and wasteland (Figure 5B,C). An almost 100-fold decrease in the activity of this enzyme was observed in soils under oat cultivation, from  $\sim 2 \mu\text{g TPF/h/gDW}$  to  $0.02 \mu\text{g TPF/h/gDW}$  (Figure 5B).

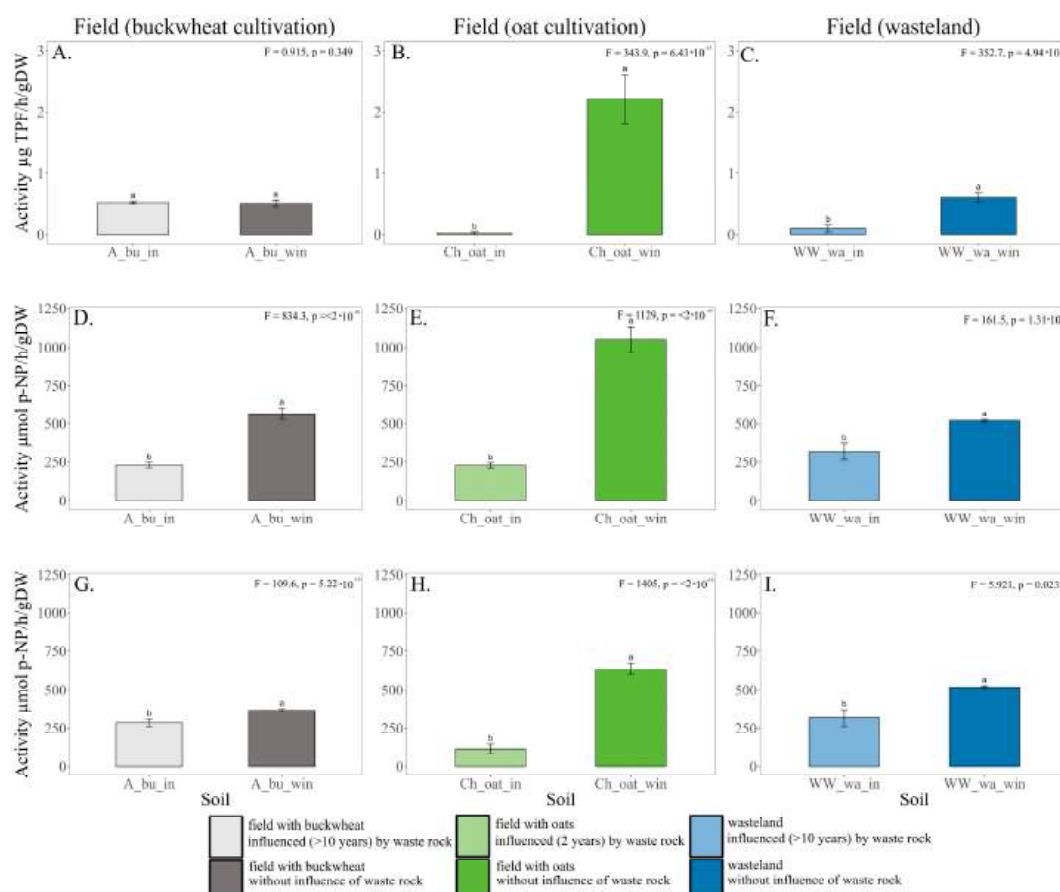


**Figure 5.** Dehydrogenase activity and phosphatase activity: field (buckwheat cultivation), field (oat cultivation), field (wasteland). Dehydrogenase activity: (A–C); acid phosphatase (ACP): (D–F); alkaline phosphatase (ALP): (G–I). ANOVA statistical analysis with Tukey’s HSD significance test. The different letters above the charts indicate statistical differences ( $p < 0.05$ ). Note/abbreviation: with influence (in), without influence (win) of waste rock.

A decrease in the activity of both acid phosphatase (ACP) and alkaline phosphatase (ALP) in soils under the influence of waste rock addition was observed in all the samples tested (Figure 5D–I). The greatest decrease in the activity of both enzyme groups was observed in oat cultivation, where a more than 4-fold decrease in the activity of these enzymes was observed (Figure 5E,H). In the context of ACP, the activity reduction observed

in both buckwheat and wasteland soils due to the impact of waste rock was twice as significant (Figure 5D,F).

The relationships between individual soil physicochemical properties, abundance of individual microbial groups, and soil enzyme activities were tested using principal component analysis (PCA) (Figure 6). For buckwheat soils, principal components Dim1 and Dim2 explained the largest percentage of the variance, with a total of 80.4%, with Dim1 and Dim2 accounting for 58.9% and 21.5%, respectively. For these soils, the studied parameters were not closely correlated with each other. Phosphatase activity, humic substances content, and the abundance of metal-resistant microorganisms and those capable of synthesizing siderophores were most strongly correlated with soils without the influence of waste rock; however, these parameters also showed strong correlations. However, the pH, TOC, and abundance of proteolytic, amylolytic, and cellulolytic microorganisms collared more strongly with soils under the influence of the waste rock. The other tested parameters were separated and showed less correlation with both the described parameters and the type of soil tested (Figure 6A). In the case of soils under oat cultivation, principal components Dim1 and Dim2 explained the largest percentage of the variance, a total of 94.6%, with Dim1 and Dim2 accounting for 88.6% and 6%, respectively. For these soils, differentiation was also observed between the samples with and without the influence of waste rock. However, in the case of these soils, the tested parameters collared more with each other and were shifted toward the soils without the influence of waste rock. Only the pH value in H<sub>2</sub>O differed from the other parameters studied and shifted toward soils under the influence of waste rock (Figure 6B). For soils from wasteland, principal components Dim1 and Dim2 explained the largest percentage of the variance, a total of 86.4%, with Dim1 and Dim2 accounting for 75.8% and 10.6%, respectively. A similar relationship was observed between the soils with and without the influence of waste rock in the PCA distribution, as in the case of oat cultivation. Similarly, most of the parameters studied showed a correlation with each other and with soils without the influence of waste rock. In contrast, the TOC content and abundance of metal-resistant microorganisms shifted toward soils under the influence of waste rock (Figure 6C).

### 3.4. Community Level Physiological Profiling (CLPP)—EcoPlate Biolog<sup>®</sup>

For buckwheat and oat cultivation, substrate utilization with EcoPlate Biolog started after 48 h of incubation (Figure 7A,B). In the case of wasteland, the addition of waste rock caused a significant delay in the rate of substrate utilization until 96 h of incubation (Figure 7C). In buckwheat cultivation, very similar levels of AWCD were observed in both soils tested (AWCD<sub>560</sub> = 0.387 and AWCD<sub>560</sub> = 0.432 after 48 h), which persisted throughout the analysis period (Figure 7A). In contrast, for oats, the mean AWCD was lower in soils under the influence of waste rock (AWCD<sub>560</sub> = 0.329 after 48 h) than in soils without the influence (AWCD<sub>560</sub> = 0.454 after 48 h) (Figure 7B). The aforementioned delay in the onset of substrate utilization in the waste rock-influenced wasteland persisted throughout the incubation period, where even after 192 h was not at the control soil level (Figure 7C).

The addition of waste rock to the soils did not affect the Shannon diversity index in either buckwheat or oat cultivation soils, which remained at a similar level of  $H = 3.0$  (Figure 7D,E). Only in the case of soils from the wasteland was there a significantly statistical decrease in the H-index in soils influenced by waste rock, from  $H = 3.1$  to  $H = 2.6$  (Figure 7F).

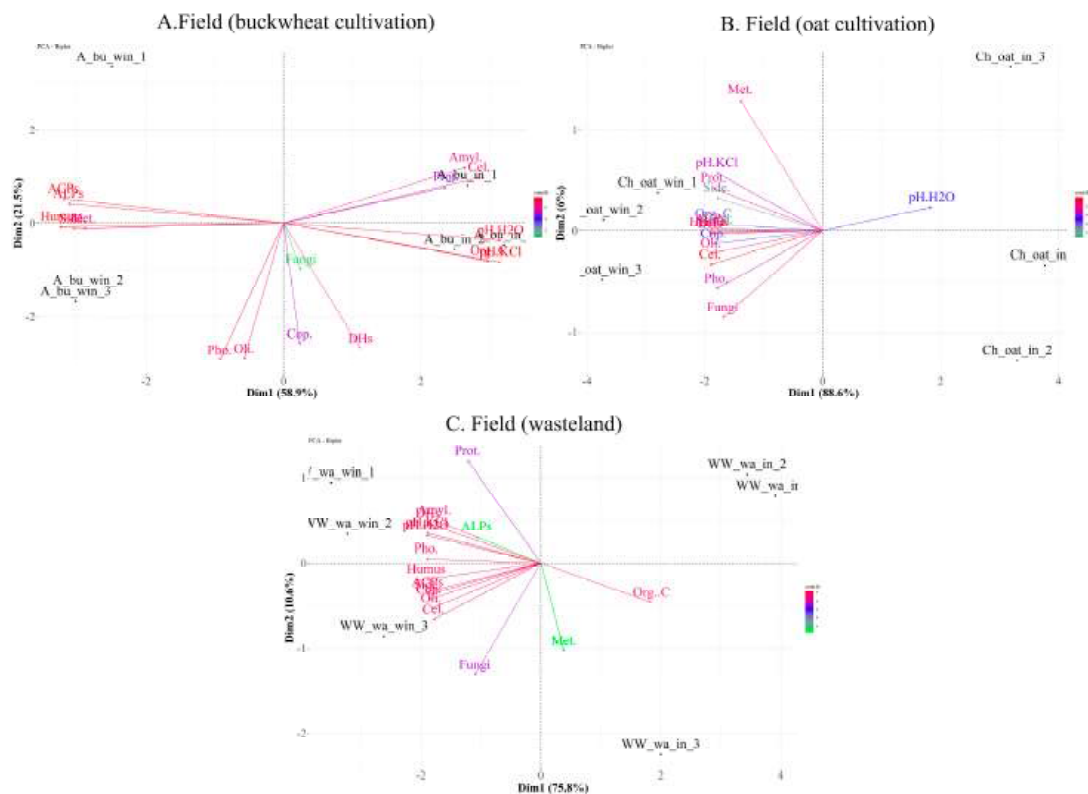


**Figure 6.** Biplot of the principal component analysis (PCA) describing correlations between pH (H<sub>2</sub>O and 1 M KCl), fungi (Fungi), copiotrophs (Cop.), oligotrophs (Oli.), microorganisms capable of synthesizing siderophores (Side.), cellulolytic microorganisms (Cel.), amylolytic microorganisms (Amyl.), proteolytic microorganisms (Prot.), phosphate-solubilizing microorganisms (Pho.), microorganisms resistant to heavy metals (Met.), TOC (Org. C), humic substances (Humus), dehydrogenase (DHs), and phosphatases (ACPs, ALPs) in soil samples from (A) field (buckwheat cultivation), (B) field (oat cultivation), and (C) field (wasteland). ANOVA statistical analysis with Tukey's HSD significance test,  $p < 0.05$ . Note/abbreviation: with influence (in), without influence (win) of waste rock.

The R-factor showed a similar trend to the AWCD, where soils under buckwheat and oat also showed an increase in the utilization of individual substrates after 48 h of incubation (Figure 7G,H,J,K). Again, soils from wasteland under the influence of waste rock showed a delay in the utilization of individual substrates compared to soil without the influence of waste rock (Figure 7M,N). After 96 h of incubation, the variation in the degree of substrate utilization between the tested soils was assessed (Figure 7F,L,O). In the case of buckwheat cultivation, no statistically significant change in substrate utilization was observed between the test soils, and this trend persisted up to 192 h of incubation (Figure 7G,H,I). In contrast, the R-factor for soils from oat crops was statistically different after 96 h of incubation, and was always 2–3 points lower in samples influenced by waste rock on subsequent incubation days (Figure 7L). The greatest variability in substrate utilization was observed in the wasteland, where the difference between R after 96 h of incubation was  $R = 10$  for soils influenced by waste rock and  $R = 24$  for soils without influence (Figure 7O). This relationship persisted over the subsequent incubation periods (Figure 7M,N).

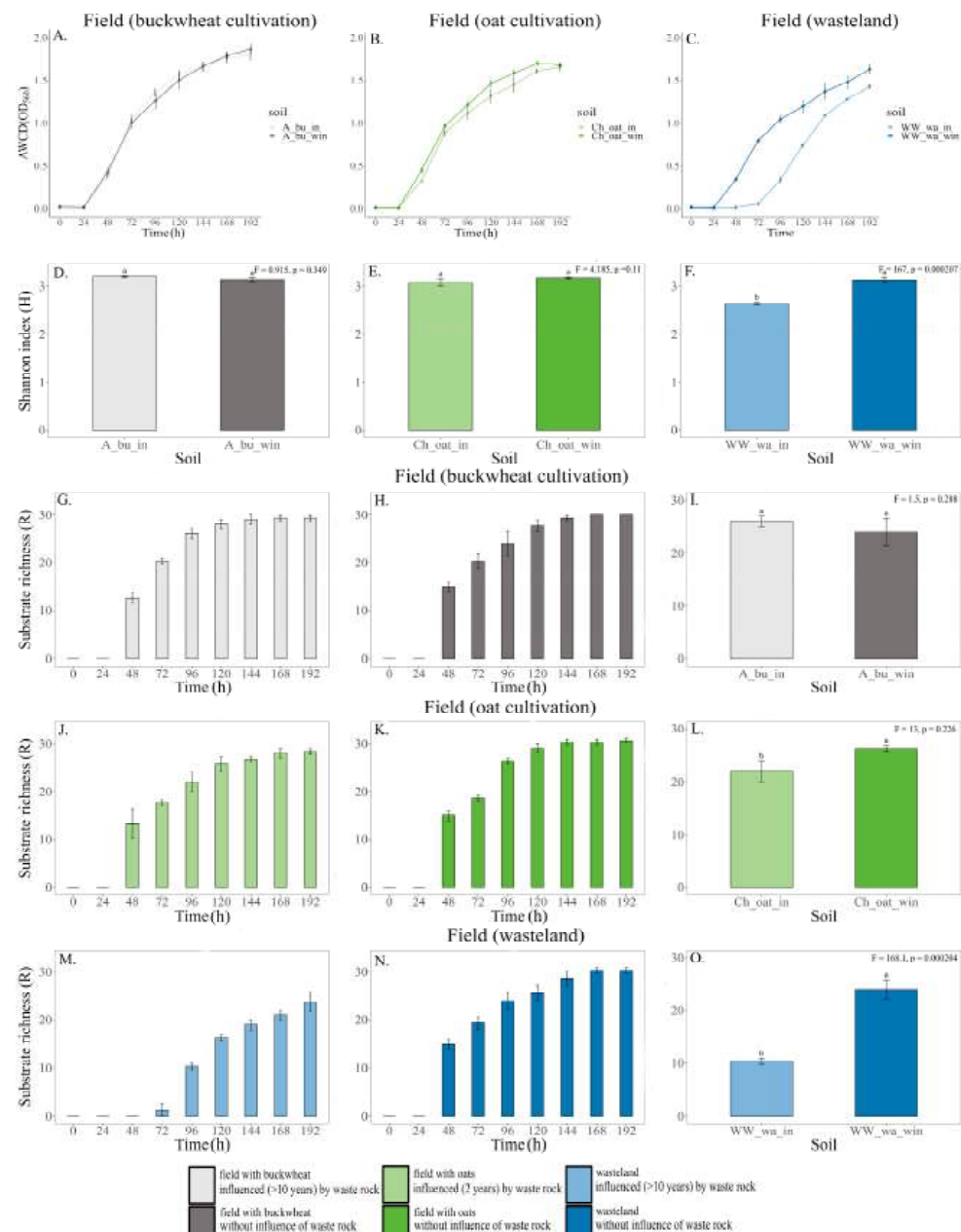
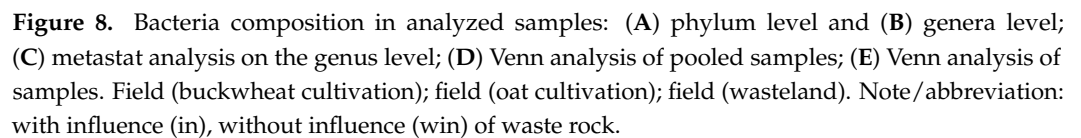


**Figure 7.** Change in average well color development (AWCD) and Shannon index (H), substrate richness (R): field (buckwheat cultivation), field (oat cultivation), field (wasteland). AWCD: (A–C); Shannon index: (D–F); substrate richness: (G–O). Substrate richness (R) after 96 h incubation between samples with and without influence of waste rock: (I) field (buckwheat cultivation), (L) field (oat cultivation), (O) field (wasteland). ANOVA statistical analysis with Tukey’s HSD significance test. The different letters above the charts indicate statistical differences ( $p < 0.05$ ). Note/abbreviation: with influence (in), without influence (win) of waste rock.

### 3.5. Bacterial Community

Analysis of bacteria sequencing data revealed the presence of 241 amplicon sequence variants (ASVs) of which 151 were identified on genera level, belonging to 74 identified family taxa, 32 identified order taxa, 16 identified class taxa, and 11 phyla taxa. The most abundant identified genus was *Sphingomonas*, followed by *Mycobacterium* (Figure 8B). *Micrococcaceae*, *Bradyrhizobiaceae*, and *Acetobacteraceae* were the most abundant on family taxa. The most abundant phyla were *Proteobacteria* followed by *Acidobacteria*, and *Actinobacteria* (Figure 8A).



A pooled analysis revealed an abundance of 94 unique bacterial ASVs, most of which were present in samples from oat cultivation, and the core microbiome was represented by 70 ASVs (Figure 8D). The highest number of unique bacterial ASVs was observed in the field with oats cultivation, without influence of waste rock; the lowest in wasteland, without influence of waste rock, and in the field with buckwheat, under the influence of waste rock (Figure 8E).

Alpha diversity indexes calculated based on data from 16S rRNA sequencing showed differences between samples from buckwheat and oat cultivation samples compared to wasteland (Figure 9A,B).

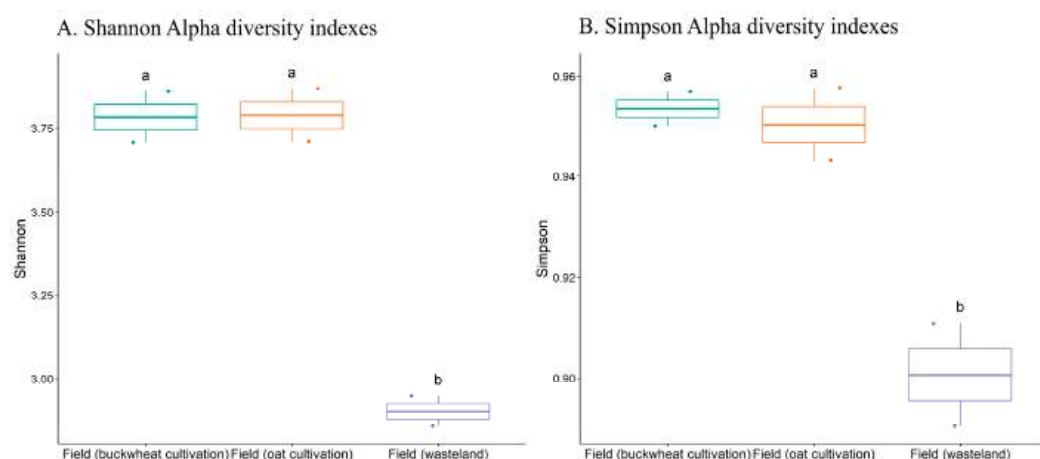


**Figure 9.** (A) Shannon Alpha diversity indexes and (B) Simpson Alpha diversity indexes in analyzed samples: field (buckwheat cultivation); field (oat cultivation); field (wasteland).

## 4. Discussion

### 4.1. Coal Mining Waste Rock and the Problem of Its Management

A separate group of hazards that can lead to negative effects on the environment is waste (coal waste, coal refuse, tailings, rock bank) from the coal industry. Coal waste stored in heaps/piles can be the cause of serious pollution of surface and groundwater and acid drainage of soils, as metallic elements such as iron, manganese, and aluminum are released from this waste in a soluble available form [69], as well as air pollution as a result of the release of toxic compounds during the spontaneous combustion of this waste.

In eastern Poland, one of the largest generators of mining waste is the Bogdanka Mine, which produces approximately 2 million tons of waste per year [70]. Among mining wastes, mining and tailings are distinguished [71]. After separation of the mining spoil, waste rock is produced, among other materials, which is used for land reclamation [14–16]. The presence of this waste in environmentally protected areas is particularly problematic for areas described as the “European Amazon”.

### 4.2. Acid Mine Drainage (AMD) and pH-Decreasing Effect of Waste Rock

The sulfur content of coal mined in Bogdanka [72] is significant and acid mine drainage (AMD) may be formed in the coal mining waste at this mine, contributing to the release of metals, including dangerous heavy metals, from the waste rock into the soil [73,74]. A very pronounced pH-decreasing effect of waste rock was observed in soils not used for agriculture. It has been shown that the cultivation of both monocots and dicotyledonous plants reduced the effect of waste rock and even caused a slight increase in this parameter.

The introduction of waste rock to buckwheat cultivation resulted in soil alkalization, reaching  $\text{pH}_{\text{H}_2\text{O}}$  levels in the range of 7.5–8.0. These soils also had the highest  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{1\text{MKCl}}$  values compared to other samples tested (Figure 2A,D). Russell et al. (2024) [75] studies showed that adding mining waste to the soil and the presence of willows (*Salix matsudana* Koidz.  $\times$  *S. alba* L. “Austree”) increased the pH of the soil solution. A higher percentage of mining waste (together with trees) led to a higher pH of the soil solution. Kompała-Baba et al. (2021) [49] showed that soil from mine dumps covered with grasses (*Poa compressa*, *Calamagrostis epigejos*) and forbs (*Daucus carota*, *Tussilago farfara*) had higher  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{1\text{MKCl}}$  compared to soil from dumps without vegetation.

An interesting result of the perennial exposure waste rock impact was a 4-fold increase in the organic carbon content in soils unused for agriculture and about 30% in the cultivation of buckwheat, and at the same time the opposite effect (a 4-fold reduction in the organic C content) was observed in soil with a two-year exposure to waste rock and with the

cultivation of oats. The AMD, as shown by Oyetibo et al. (2021) [47] for the Onyeama coal mine in Nigeria, contained sulfate ( $313.0 \pm 15.9 \text{ mg}\cdot\text{L}^{-1}$ ), carbonate ( $253.0 \pm 22.4 \text{ mg}\cdot\text{L}^{-1}$ ), and nitrate ( $86.6 \pm 41.0 \text{ mg}\cdot\text{L}^{-1}$ ). This AMD may cause the pollution load index for toxic metals and metalloids to rise to very high values ( $3110 \pm 942 \text{ mg}\cdot\text{L}^{-1}$ ) with a particular risk for release of lead and cadmium and subsequently arsenic, nickel, cobalt, iron, and chromium [47]. However, the microbial communities of these contaminated sites affected by coal waste rock and AMD can actively address these threats, and changes in the composition of the microbiome are therefore one of the best indicators of the impact of these wastes.

#### 4.3. Need to Monitor Biotic and Abiotic Parameters of Soils Treated with Waste Rock

A good solution to avoid the disposal of mining waste seems to be the introduction of this waste in relatively small quantities into wasteland and agricultural land, which could improve the structure of these soils or at least would not have negative environmental effects or inhibit plant growth [76]. However, the use of such a method of disposal of waste rock requires great caution and monitoring the impact of the rock on the condition of the soil, its abiotic and biotic components, and the yield and physiological state of plants. A very good indicator of soil health is the composition, diversity, and abundance of these soils [77]. The results of research on the impact of waste rock, which is a waste rock used for soil reclamation, clearly indicate the need to simultaneously monitor the physical, chemical, and biological parameters of soils along with the state of the soil microbiota in soils used for agriculture and wasteland, which react more strongly to changes in parameters than rhizosphere soils to the addition of waste rock.

#### 4.4. Soil and Plant Microbiota as an Indicator of the Impact of Waste Rock

The biological activity of the environment consists of enzymatic activity (dehydrogenase and phosphatase activity are the main indicators of this activity) and the abundance, physiological activity, and diversity of the main representatives of the microbiota, including oligotrophic and copiotrophic bacteria (adapted to low and high nutrient concentrations, respectively) and microscopic fungi, along with their abundance ratio [45,78,79]. Soil is an environment of diverse organisms interacting with each other, among which the most abundant are microorganisms belonging to the domains of bacteria (Eubacteria) and archaea (Archaea), and to microscopic fungi representing Eukaryota [80]. Many members of the soil microbiota have the ability to colonize the tissues of soil plants and animals, which is their endophytic development for part or the entire life cycle. Among the microbial endophytes of higher organisms, we distinguish both symbionts and parasites, which often develop in the soil as saprotrophs or survive in this environment in spore form [81]. All soil organisms should be considered as a holobiont, that is, a set of organisms that jointly respond to environmental factors [77]. A strong and statistically significant decrease in the abundance of microorganisms was observed with a 2-year exposure of the cereal rhizosphere—oats in the soil. A significant decrease in the abundance of the soil microbiota groups tested—fungi, copiotrophic and oligotrophic bacteria, microorganisms with cellulolytic, amylolytic, and proteolytic activity, and phosphate-dissolving microorganisms—was observed in soils treated with waste rock when oats were grown on them or when they were wastelands covered with mixed meadow plants (i.e., with long-term waste rock exposure). In the soil from oat cultivation, a decrease in the abundance of microorganisms by  $1\text{--}1.5 \log_{10}/\text{gDW}$  was observed. On the other hand, on wasteland, the influence of waste rock reduced the abundance of copiotrophs and oligotrophs to  $6.2 \log_{10}/\text{gDW}$ , while the abundance of fungi did not change. Long-term cultivation (buckwheat) contributed to the elimination (leveling out) of the microbial and biochemical differences between the soil under the influence of waste rock and without its influence. The addition of waste rock did not significantly

affect the abundance of microorganisms in buckwheat cultivation, reaching values of about  $5.5 \log_{10}/\text{gDW}$  for fungi and  $7.5\text{--}7.7 \log_{10}/\text{gDW}$  for copiotrophs and oligotrophs.

The presence of heavy metals in the soil, especially in an accessible form, leads to changes in the population of microorganisms, manifested, depending on the concentration of metals and the period of exposure to contamination, by a strong reduction in the overall number of microorganisms and the formation of populations of microorganisms adapted to contamination [36,37]. One of the mechanisms of microorganism interaction with metals contained in artificial and natural substrates (soil) is the ability to chelate metals through non-specific (acids, sugar, and amino acid polymers) and specific (siderophores) complexing compounds [45]. Different relationships could be clearly observed in the case of microorganisms resistant to heavy metals in soils used and not used for agriculture. A significant decrease in the number of microorganisms resistant to heavy metals was recorded in cultivated fields, both in the rhizosphere of monocotyledonous and dicotyledonous plants, regardless of the period of waste rock exposure. On the other hand, in soil not used for agriculture, there was no decrease at all, and even a slight, statistically insignificant, increase in the number of this group of microbiota was observed. It is very interesting in our research that microorganisms capable of complexing metals with the use of strong chelators—siderophores—in cultivated soils treated with waste rock were slightly lower than in those cultivated soils that were not treated with waste rock. However, the greatest decrease in the abundance of microorganisms capable of synthesizing siderophores was recorded on wasteland under the influence of waste rock ( $1.5 \log_{10}/\text{gDW}$ ).

#### 4.5. Enzyme Activity as a Reflection of the Influence of Bedrock on Overall Soil Activity

The activity of dehydrogenase in the soil of the rhizosphere of monocotyledonous plant—oats was about three times higher than in the soil of the rhizosphere of the dicotyledonous plant—buckwheat, and soil not used for agriculture. The rhizosphere soil from oats exposed to waste had more than 100-times lower dehydrogenase activity ( $\sim 0.02 \mu\text{g TPF}/\text{h}/\text{gDW}$ ) than the oat rhizosphere soil without waste rock ( $\sim 2 \mu\text{g TPF}/\text{h}/\text{gDW}$ ), and about 4-times less dehydrogenase activity was observed in wasteland soil, whereas in buckwheat cultivation rhizosphere soil, the addition of waste rock did not affect the level of dehydrogenase activity. Very interestingly, were the activity of dehydrogenase (DHA), which in long-term cultivation both under the influence of waste rock and without the influence of waste rock was at a similar level, while much lower activity in soil from short-term cultivation (oats) with waste rock than in soil not under the influence of this rock resulted additionally from a high activity in the soil without the influence of waste rock. As in the case of dehydrogenase activity, the activity of acid and alkaline phosphatase was highest in the rhizosphere of oats not treated with waste rock and was approximately four times higher than that in the rhizosphere of oats under the 2-year term influence of waste rock. In the case of phosphatase activity in the rhizosphere of buckwheat and wastelands (with the long-term impact of waste rock), a decrease in activity was recorded in soils under the influence of waste rock, but the decrease in alkaline phosphatase activity was very small. This is well illustrated in Figure 5. Part of the bacterial microbiota in the waste rock is capable of degrading coal hydrocarbon pollutants and generating hydrogen using formate dehydrogenase enzyme [82]. Kompala-Bąba et al. (2021) [49] showed that the activity of soil enzymes (dehydrogenase, alkaline phosphatase) was higher in soils from hard coal mine spoil heaps covered with vegetation than in those without plants. In turn, the activity of acid phosphatase was lower in soil from hard coal mine spoil heaps covered with grasses (*Poa compressa*, *Calamagrostis epigejos*), and higher in soil from hard coal mine spoil heaps overgrown with forbs (*Daucus carota*, *Tussilago farfara*) compared to soil from hard coal mine spoil heaps without vegetation.

#### 4.6. Changes in Ecophysiological Parameters as Marker of the Influence of Waste Rock

The physiological activity of microorganisms reflects their ability to participate in various stages of SOM degradation and in the circulation of biogenic elements [83]. The substrate utilization capacity of environmental microorganisms determined by the standard Biolog MicroPlate™ system on EcoPlates enables the determination of the physiological profile and ecophysiological indicators such as AWCD (reflecting overall activity) and the Shannon index (reflecting diversity), and is a very sensitive tool for tracking changes in metabolic activity and diversity occurring in the soil environment under the influence of abiotic and biotic factors [84]. The high metabolic activity in the use of various substrates is conducive to the adaptation of both bacteria and fungi to changing soil conditions and to the colonization of the roots and interior of plants, often by endophytes with properties of plant growth-promotion (PGP), and these properties seem to be largely universal and not limited to specific plant species [85,86]. In all three rhizospheres tested, the AWCD index in the first days of incubation was lower when the soil was under the influence of waste rock, which was particularly evident in soil not used for agriculture, where the rate was close to zero by the 72nd hour. The Shannon index (H), which was significantly lower in the case of wasteland under the influence of waste rock than in the case without the addition of waste rock, is a very clear confirmation that the type of soil use is essential in reducing the impact of waste rock on the physiological activity of the soil microbiome. The values of the R index after 96 h of incubation in non-agricultural soil with waste rock were more than twice lower than in wasteland without the influence of this rock, but also in the cultivation of oats it was slightly, but statistically significantly lower. Similar relationships were indicated by the CLPP analysis, which showed a significant reduction in the metabolic activity of microorganisms in unused soil (wasteland) under the influence of rock, which is indicated by a very low AWCD index—substrate utilization and other indicators calculated on the basis of substrate utilization capacity: Shannon index and, in particular, richness. Similarly, as Wolińska et al. (2020) [87] showed, CLPP proved to be a very sensitive indicator of inhibition of activity reflected by substrate consumption indicators: the AWCD, Shannon index, and richness index, especially, decreased in non-agricultural soil under the influence of waste rock, but the richness index decreased significantly in soil under oat cultivation.

#### 4.7. Soil Organic Matter and Humic Acids as a Marker of Waste Rock Impact on Soil

Abiotic factors cause changes in the soil environment, affecting plants, animals, and their microbiota directly, but mainly indirectly. Plants, in turn, interact with the soil microbiota through rhizodeposits (e.g., root secretions and root border cells) [88]. Higher organisms, both plants and animals, are not only the source of soil organic matter (SOM), but also participate in its degradation, transformation, and nutrient cycling. However, the microbiota inhabiting the soil and the higher organisms that thrive in the soil play a fundamental role in the transformation of SOM. The equilibrium of the soil environment is reflected in the maintenance of its basic physicochemical parameters (pH, organic C and N content and the ratio of these values, concentration of humic acids being the final product of SOM transformations) and biological activity [89]. It was also noted that in soils treated with waste rock used for agricultural purposes for the cultivation of both monocotyledonous and dicotyledonous plants, the decrease in the content of humic acids was very strong, while in the case of wastelands, in which the content of humic acids was the lowest, it was small. In the cultivated soil, the humic acid (KH) content decreased under the influence of the waste rock, but the highest difference from the soil without waste rock observed in short-term oat cultivation was due to the high KH content in the rhizosphere soil of oats, as was the case with dehydrogenase activity.

#### 4.8. Microbiome Diversity as a Marker of Waste Rock Impact on Soil

Our own analysis of bacteria sequencing data showed that differences in the composition of bacteria composition both on genera and phylum level. It was shown that the composition, which was almost identical in cultivated soils, both buckwheat and oats, underwent a marked change under the influence of post-mine rock. Certainly, changes in the abundance and diversity of the microbiota in coal mine waste are influenced by strongly reduced pH, which contributes to the appearance of tolerant microorganisms belonging to both bacteria and fungi adapted to the acidic environment [90,91].

Zhang et al. (2024) [92] noted that different parts of the mine are dominated by different groups of bacteria; Actinobacteria as functional bacteria dominate the rocks accompanying the coal beds and Proteobacteria tolerate poor environments and dominate parts such as mine roadways. Regardless of the type of cultivation, relative abundance of *Proteobacteria* increased in soil with the addition of waste rock. In soil not used for agriculture, waste rock caused a decrease in the relative abundance of *Actinobacteria* and a marked increase in the relative abundance of *Acidobacteria*, which in waste rock soils used for agriculture accounted for approximately 30% of RA of this group of bacteria in wastelands. In the non-cultivated soil, the proportion of *Proteobacteria* increases under the influence of the waste rock to a level similar to that in the cultivated soil of both plants under the influence of rock, and the share of *Actinobacteria* was much lower, and in addition to waste rock in unused soil, the level of *Acidobacteria*. In the wasteland influenced by rock, the abundance of *Actinobacteria* was reduced 3-fold and without rock influence 2-fold compared to cultivated soils. This is well illustrated in Figure 8A. A study by Lin et al. (2024) [46] showed that the soil from grasslands at the Muli coal mine site in Qinghai was also characterized by the dominance of *Proteobacteria* (~32%), followed by *Actinobacteria* (~28%) and *Acidobacteria* (~15%). The microbiome of drains from the Onyeama coal mine in Nigeria was also distinguished by the dominance of *Proteobacteria* (50.8%) among the bacteria, and the second dominant group, but with only an 18.9% share of the microbiome, was *Bacteroidetes* [47]. Similarly to the research on waste from Bogdanka Mine, there was a significant predominance of *Ascomycota* fungi, which in Nigeria constituted as much as 60.8% of the eukaryotic community. It has been shown that the bacterial community of hyperaccumulator plants (*Noccaea caerulea*), capable of accumulating heavy metals such as Pb and Zn, growing on two heavy metal-polluted sites in Belgium, comprised mainly *Proteobacteria* (seeds) and *Actinobacteria* in the bulk soil [93]. Plants growing in non-agriculturally unused areas, on poor soils and under stress conditions, shows that *N. caerulea* provides an excellent model system to study the mechanisms of heavy metal hyperaccumulation and abundant bacterial and fungal endophytic microbiota [94]. Plants, and especially hyperaccumulator plants, through phytoremediation mechanisms, can significantly contribute to reducing the effects of contamination with both heavy metals and petroleum derivatives, and hyperaccumulator plants, which usually appear naturally in such areas and therefore can be abundant in agriculturally unused wasteland areas, can also support the restoration of these areas to agricultural use [95]. Wolińska et al. (2020) [87] demonstrated that microbial metabolic activity depended on soil properties, varieties of plants, and type of cultivation. In this study, the biodiversity of rhizosphere soils of plants (various varieties of wheat), grown on podzolic soil in a no-tillage system, was determined by two techniques of next generation sequencing (NGS, meta-barcoding of 16S rRNA community) and with the use of Biolog tests to determine the community level physiological profiling (CLPP). This relationship was also confirmed in the study of monoculture of maize by Gałazka and Grządziel (2018) [96]. In this study, the authors showed that fungal diversity was changed under the influence of different cultivation techniques; techniques of maize cultivation and season were important factors that could

influence the biochemical activity of soil. Other authors have also confirmed microbial diversity and physical properties depending on the type of soil [97]. The highest biological activity and larger microbial diversity were found in Gleyic Phaeozem, Rendzic Leptosol, and Fluvic Cambisol. This study indicates that a specific edaphone of soil microorganisms may be of great importance when assessing the quality of soil and improving soil health. This effect is particularly important for agricultural soils that are threatened by ongoing land degradation. Changes in the bacterial community are observed in the context of soil pollution [98]. The soils contaminated with oil were characterized by the highest biodiversity indexes. *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* were strongly correlated with biological activity in these soils. Families of *Alphaproteobacteria* were also dominant, including *Bradyrhizobiaceae*, *Rhizobiaceae*, *Rhodobacteraceae*, *Acetobacteraceae*, *Hyphomicrobiaceae*, and *Sphingomonadaceae*. Gałazka et al. (2020) [98] showed that the long-term contamination of soil changes bacterial communities and their metabolic activity.

The composition of the bacterial community in non-agricultural soil under the influence of bedrock differed significantly from other soils, and in particular from wasteland soils, without the influence of rock. The abundant presence of the genera *Acidocella* and *Acidphilum*, absent in wasteland without rock, in the unused soil under the influence of waste rock was strongly associated with the effect of lowering the pH by waste rock in soil not used for agriculture. Significantly lower abundances of *Clostridium*, *Rhizobium*, and *Blas-tococcus* were recorded in the wasteland soil influenced by waste rock. A very strong impact of waste rock in unused soil on the composition of microorganisms is evidenced by the lack of detection of common genera in long-term cultivated soil and untreated soil treated with waste rock. The metatest analysis made it possible to show the level of individual genera in specific types of use, with particular the influence of buckwheat and oat cultivation on the dominance of *Devosia* genera, and only buckwheat on the genera *Micro-lunatus* and *Blasococcus*. On the other hand, in soils unused to cultivation, the genera *Jatrophihabitans* and *Dictyobacter* clearly dominated, and the genera of diazotrophic bacteria, *Mesorhizobium* and *Bradyrhizobium*, were eliminated. Very high numbers of *Sphingomonas* were found in the rhizospheres of buckwheat and oat crops. The abundance of *Mycobacterium* and *Streptomyces* decreased significantly under the influence of the rock.

Coal mining waste in the soil with buckwheat cultivation contributed to the dominance of *Sphingomonas*, but also such genera as *Stenotrophobacter*, *Microvirga*, and *Skermanella* in the bacterial community, which are absent in the rhizosphere of oats subjected to the action of waste rock. In addition to these three genera, in this oat rhizosphere influenced by the rock, bacteria of the genera were also present in very low numbers: *Hyphomicrobium*, *Dictyobacter*, *Rhodococcus*, *Blastococcus*, and *Streptosporangium*. Bacteria from genus *Sphingomonas*, commonly found in the soil and as endophytes in plant tissues, have great remedial, bio-stimulatory (through, among other things, the production of auxins and gibberellins), and biocontrol potential to improve plant growth under biotic and abiotic stress conditions such as salinity, drought, and heavy metal pollution [99,100]. In the rhizosphere of buckwheat with waste rock, a significant decrease in the abundance of *Rhizobium*, *Methylobacterium*, and *Streptosporangium* was observed in relation to the rhizosphere of buckwheat without waste rock. This is very unfavorable, as bacteria belonging to diazotrophic *Alphaproteobacteria* genera *Rhizobium* and *Methylobacterium* can colonize not only rhizosphere soil, but also plant tissues and play a significant role in promoting plant growth through nitrogen-fixing processes, making Fe and P available, reducing ethylene levels, producing phytohormones, and inducing plant resistance [101,102]. *Streptosporangium*, belonging to *Actinobacteria*, are characterized by high metabolic activity and the ability to produce enzymes that degrade organic matter and numerous antibiotics [103].

Simpson and Shannon alpha diversity indexes also clearly indicate that diversity is high and very similar for soils on which cultivation was carried out, regardless of whether it was buckwheat or oats, while many times lower diversity was found in soils without agricultural cultivation. Venn analysis indicates that the core microbiome was represented by 70 unique bacterial ASVs and at a level similar for common ASVs in soils with buckwheat and oat cultivation, and no common ASVs were found between unused soil and soil with buckwheat cultivation. Shannon and Simpson alpha diversity indexes calculated based on data from 16S rRNA sequencing indicated a huge impact on the type of agricultural use on microbial diversity, with their value, regardless of the plant species cultivated, significant and significantly higher than in soil without cultivation. Almost twice as many unique bacterial ASVs (19) were found in non-agricultural soil than in soils with buckwheat (30) and especially oats (35). The largest, more than a 3-fold decrease in unique bacterial ASVs in soil with the influence of waste rock in relation to soil without effect, was found in the rhizosphere of buckwheat, and a 2-fold decrease in the rhizosphere of oats was observed. In contrast, the complete opposite situation of an increase (almost 2-fold) in ASV numbers in soil influenced by waste rock was observed in the case of a wasteland.

The obtained results indicate that the adverse impact of the coal mining industry is related not only to the mineral itself [39], but to a large extent to the waste rock from this industry. Therefore, there is uncertainty about the attempt to manage this waste from an agricultural point of view. The present research indicates that this waste has a strong effect on the composition of the soil microbiome. At the same time, in soils used for agricultural purposes, the impact of waste rock on the rhizosphere microbiome is much smaller than on the microbiome of wastelands, where the vegetation cover is poor and the impact of root secretions is weak. Comprehensive analysis of the biotic and abiotic parameters of the soils affected by mining waste made it possible to ascertain that very dynamic changes were taking place in the composition of the microbiome and its metabolic activity, as reflected in the values of the ecological indicators. The structure of the soil microbiome, the biochemical processes carried out by the microbiota, and its biodiversity under the influence of the waste rock, leads to a state of equilibrium characteristic of soils not subjected to the impact of waste rock when the soils are used agriculturally and when the process is prolonged. Continuous assessment of microbiome changes is essential to understand the short- and long-term impacts of waste on soil ecosystems, ensuring the implementation of effective reclamation strategies [104].

## 5. Conclusions

In this article, the biological and physicochemical properties of soils subjected to reclamation with waste rock were examined, in comparison to soils that were not exposed to this type of mining waste. The soils differed in the type of agricultural use and vegetation cover, including field crops—monocotyledons (oats) and dicotyledons (buckwheat)—and wastelands with poor vegetation. Additionally, the soils differed in the duration of the waste rock impact. The following conclusions can be drawn from this study:

- (1) When monitoring the impact of waste rock on the formation, changes, and disturbances of the composition of the microbiome, it is worth combining the indicators of catabolic, ecophysiological (Biolog EcoPlate), and genetic fingerprinting with the determination of physicochemical parameters and indicators of biological activity of soil determined on the basis of determining the activity of soil enzymes and the abundance of various groups of microorganisms cultivated in soils to obtain data on multidirectional changes occurring in the environment, soil structure, and quality, as well as the physiological state, yield, and elemental composition of plants.

- (2) The influence of waste rock on physical parameters, biological and biochemical activity, and the composition of the soil microbiota depended mainly on the method of agricultural use (wastelands, cultivated fields), the type of vegetation cover (monocotyledonous plants, dicotyledonous plants), and, to a much lesser extent, on the duration of waste rock impact. In agriculturally used soil, there is most likely a strong influence of bioremediation processes combining phytoremediation mechanisms supported by the activity of rhizosphere microorganisms, which are characterized by high metabolic activity.
- (3) The introduction of waste rock to soils had a significant effect on pH. The use of these rocks in buckwheat cultivation caused soil alkalization, reaching  $\text{pH}_{\text{H}_2\text{O}}$  levels in the range of ~7.5–8.0. These soils were also characterized by the highest  $\text{pH}_{\text{H}_2\text{O}}$  and  $\text{pH}_{1\text{MKCl}}$  values compared to the other soil samples tested.
- (4) Research has shown that long-term cultivation (buckwheat) contributed to the elimination (leveling out) of microbiological and biochemical differences. The addition of waste rock did not significantly affect the abundance of microorganisms in buckwheat cultivation (~5.5  $\log_{10}/\text{gDW}$  for fungi and 7.5–7.7  $\log_{10}/\text{gDW}$  for copiotrophs and oligotrophs). In the soil from oat cultivation, a decrease in the abundance of microorganisms by 1–1.5  $\log_{10}/\text{gDW}$  was observed. On wasteland, the influence of waste rock reduced the abundance of copiotrophs and oligotrophs to 6.2  $\log_{10}/\text{gDW}$ , while the abundance of fungi remained unchanged.
- (5) The abundance of microorganisms resistant to heavy metals, as well as microorganisms capable of producing specific Fe-binding ligands—siderophores—decreased under the influence of waste rock. The greatest decrease in the number of microorganisms capable of synthesizing siderophores was observed on wastelands (1.5  $\log_{10}/\text{gDW}$ ). The combined analysis revealed an abundance of 94 unique bacterial ASVs, most of which were present in oat cultivation samples, and the core microbiome was represented by 70 ASVs.
- (6) Dehydrogenase activity in long-term cultivation both with and without the influence of waste rock was at a similar level, whereas in the soil from short-term cultivation (oats), it was significantly lower with waste rock (~0.02  $\mu\text{g TPF/h/gDW}$ ) than in the soil without its influence (~2  $\mu\text{g TPF/h/gDW}$ ). However, in all soil samples tested, a decrease in the activity of both acid phosphatase and alkaline phosphatase was observed due to the addition of waste rock. The most significant decrease in the activity of both enzyme groups was noted in oat cultivation, where the activity of these enzymes decreased more than 4-fold.
- (7) These results allow for the formulation of a recommendation for the introduction of agricultural cultivation in areas under the influence of waste rock. It is recommended to use pre-crop plants, which are intended to supply the soils mainly with elements such as nitrogen and phosphorus, such as phacelia, clover, or other plants entering symbiosis with diazotrophs, that is, plants from the *Fabaceae* family, but also crops such as mustard or buckwheat.

Waste rock is often used for the reclamation of fields and other agricultural areas. The methods used in this study are planned for application in analyzing the impact of waste rock on rapeseed crops, cereals (rye, wheat), and grass production in meadows.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su17062603/s1>, Figure S1: Location of the study area (▲—material collection points; —border of Polesie National Park; —border of UNESCO “West Polesie” Transboundary Biosphere Reserve).

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## **Supplementary materials**

# Impact of Coal Waste Rock on Biological and Physicochemical Properties of Soils with Different Agricultural Uses

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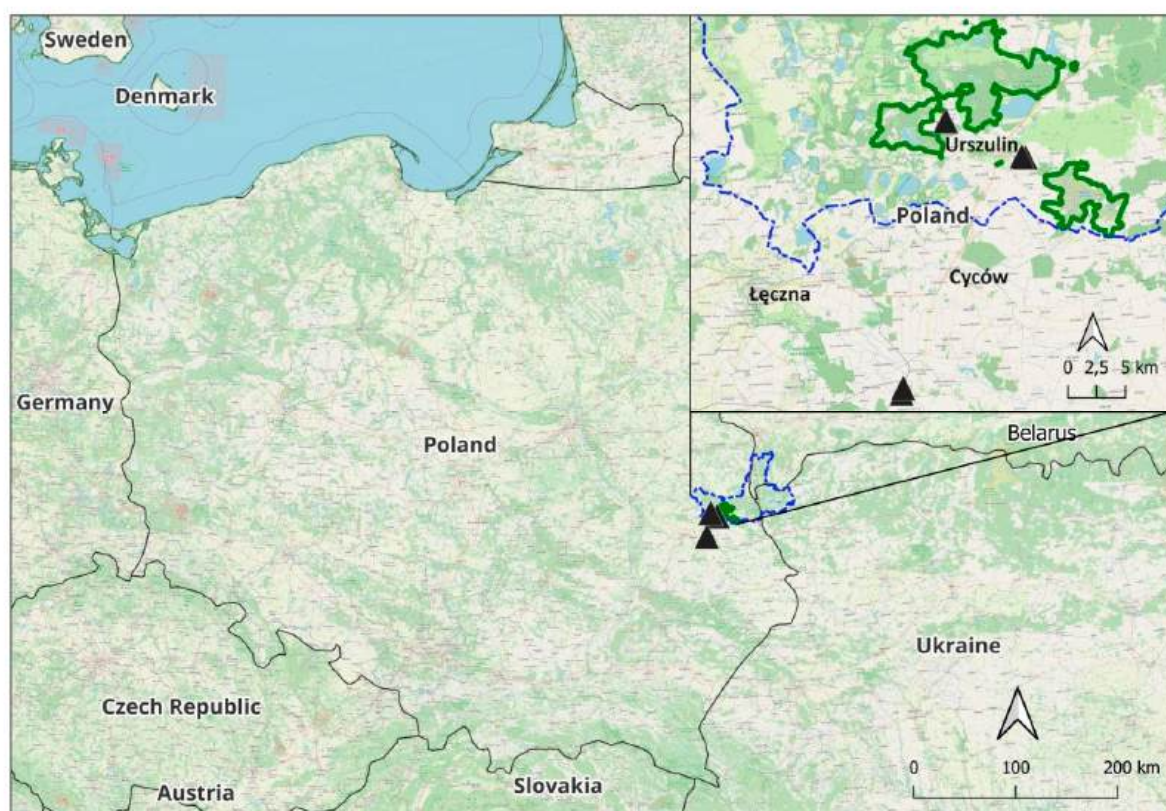


**Figure S1.** Located of study area (▲ – material collection points; — - border of Polesie National Park; — - border of UNESCO “West Polesie” Transboundary Biosphere Reserve).

Article

# Fatty Acids in Lumbricidae as Biomarkers of *In Situ* Metals Exposure

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## Abstract

Hard coal mining activity generates post-mining waste (waste rock). Waste rock is deposited in the environment in large quantities for reclamation of agricultural land. In this study, waste rock was treated as a potential source of metal pollutants. The research material (waste rock, soil, plant roots, and Lumbricidae earthworms) was obtained from sites that had been reclaimed using waste rock as well as sites without waste rock. From each site, 30 individuals ( $n = 30$ ) were collected, divided into five groups, 6 individuals each. Within the group, individuals were analyzed collectively. The study tested whether selected metals (Cr, Ni, Cd, Ba, Pb, Zn, and Cu) are present in waste rock and whether they can be transferred to the soil, plant root systems, and representatives of Lumbricidae, which are important bioindicators and a source of biomarkers. Particular attention was focused on the assessment of the effects of metals deposited *in situ* on fatty acids in representatives of Lumbricidae and on selecting a set of fatty acids that can be used as biomarkers of physiological effects, including oxidative stress. A panel of biomarker fatty acids was used, which included a panel of 17 biomarker fatty acids from 35 fatty acids analyzed. To confirm or disprove the usefulness of the biomarker fatty acid panel in earthworms, superoxide dismutase (SOD), catalase (CAT), and thiobarbituric acid reactive substances (TBARS) were determined. The study enabled an effective comparison of reference locations with locations potentially burdened with anthropogenic sediment. The results indicate that selected metals present in the waste rock are transferred to the soil, plant root systems, and soil organisms such as Lumbricidae. Selected metals affected the lipid metabolism of Lumbricidae as stressors, leading to changes in the composition and oxidation of fatty acids. The effect on the physiological state of Lumbricidae depended on the duration of the deposit and the type of use (field, meadow, wasteland) of the land with the waste rock deposit. In earthworms obtained from sites with waste rock deposits, higher contents of biomarker saturated fatty acids and biomarker monounsaturated fatty acids and lower contents of biomarker polyunsaturated fatty acids were found compared to earthworms obtained from sites without waste rock deposits. Only Pb (lead) showed a statistically significant correlation with all analyzed parameters in earthworms obtained from sites with waste rock deposits. The results have significant practical implications for environmental protection management. The proposed set of biomarker fatty acids in Lumbricidae can be used to assess the impact of pollutants and environmental monitoring.



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**Keywords:** waste rock; post-mining waste; agricultural land reclamation; invertebrates; earthworms

## 1. Introduction

Earthworms play a key role in terrestrial ecosystems. Owing to their positive effects on soil properties (i.e., physical, chemical, and biological) they are referred to as ecological engineers [1–4]. They are able to rid the soil of heavy metal contamination due to their ability to detoxify, regulate and eliminate excessive metals [5–7]. They take part in the decomposition of organic matter, thus performing an important role in the ecosystem. They are an important link in the food chain, as they connect the underground system to the above-ground system, serving as an important food source for numerous vertebrates, including birds [1,5,8,9]. Underground invertebrates are key components of the diet of nestlings [10]. Earthworms (Oligochaeta, Lumbricidae) in particular are an essential element of the food web and the basis of the diet of many species of birds feeding on invertebrates [11], and they are one of the most common groups of annelids in the diet of birds [12]. Invertebrates are a basic food source for many bird species of the agricultural landscape, and this food significantly influences their breeding success, as nestlings are fed on invertebrates. This food source supplies developing birds with protein essential to their growth and energy [13,14].

Earthworms function as bioindicators due to their ability to accumulate pollutants, such as metals. These invertebrates can be used to monitor soil contamination and assess the ecological effects of these pollutants [15–17]. Earthworms are used in standard toxicity tests, such as OECD 207 [18] and OECD 222 [19], and as model organisms in ecotoxicological studies [20]. Bioaccumulation of metals by earthworms can serve as an ecological indicator of the availability of metals [21]. A key factor influencing the accumulation of metals is their content in the environment [22]. Metals enter the body of the earthworm via direct contact with the soil, where they are present in the soil solution, or through ingestion, as they are present in food [5]. Depending on need, metals can either be retained or removed from the body [23]. Essential metals which are microelements include zinc (Zn), copper (Cu), chromium (Cr), and nickel (Ni), which can also be toxic in high concentrations, exceeding the physiological requirements of the organism, i.e., the essential levels for the normal course of metabolic processes. Essential metals are controlled and excreted, whereas non-essential metals can be detoxified through binding to organic ligands or sequestration in inorganic matrices. Metals that are non-essential and toxic even at low concentrations include cadmium (Cd), lead (Pb), and barium (Ba). All of these elements are heavy metals, and depending on the concentration and exposure time, they can be potentially toxic to living organisms [6,24–26]. Earthworms can reduce the availability of metals in the soil by accumulating and sequestering them in their tissues [27]. The availability of metals can be increased by the decomposition of earthworm tissues which have accumulated large amounts of these elements, and their metabolism has previously increased the bioavailability of metals for other organisms [25]. These invertebrates are able to survive in metal-contaminated soil, but field studies are needed for a better understanding of their physiological activity [28]. Earthworms are able to identify and pass over metal-contaminated soil, which may be an important indicator in the assessment of ecological risk [5].

Coal extraction is an important source of income in many countries. Earthworms are highly sensitive bioindicators of changes in the soil caused by factors originating in mining activity [29]. In some species of earthworms, despite low mortality, a significant reduction in the number of cocoons and juveniles has been observed [30]. Mining waste stored in the environment, including waste rock, poses a threat and affects the physico-chemical properties of soil [30–32]. The use of earthworms in programmes for monitoring the biological quality of soil has been suggested as a tool for assessing the biodiversity of this environment [30]. Given the sensitivity of organisms to stress associated with various

anthropogenic factors, in order to understand the impact of stressors, it is necessary to study their direct effects on organisms. This can be achieved using biomarkers, which are an organism's measurable biological responses to exposure to pollutants and their consequences [20]. Oxidation processes have been the subject of ecotoxicological studies for many years. They are initiated by various types of environmental stressors: biological, e.g., pathogens [33], physical, e.g., temperature, moisture, or aeration [34], and chemical, e.g., heavy metals or pesticides [35]. Oxidation primarily affects lipids and proteins [36]. Peroxidation of lipids, including phospholipids contained in intra- and extracellular membranes, affects fatty acids (FA), mainly polyunsaturated fatty acids (PUFA), which are attacked by free radicals [37,38]. Earthworms have a diverse range of FAs, of which the predominant acids are C20:4 n-6 and C18:2 n-6c among PUFAs; C16:0 and C18:0 among SFAs (saturated fatty acids); and C18:1 n-9c among MUFAs (monounsaturated fatty acids) [39]. Stressor induction activates defence mechanisms in living organisms: enzymatic, e.g., superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST), and non-enzymatic, e.g., glutathione (GSH) [38,40–42]. These mechanisms can become ineffective, e.g., due to excessive levels of pollutants or long-term exposure even at low concentrations, which can lead to the progression of oxidation, including the initiation of autoxidation processes [43]. Monitoring of the phenomenon of FA peroxidation in Lumbricidae *in situ* in response to the potential impact of environmental pollutants in the form of metals from waste rock has not previously been considered.

The research hypothesis posits the possibility of transfer of metals deposited in an anthropogenically polluted environment *in situ* (from waste rock, soil, and plant roots to invertebrate organisms of the family Lumbricidae) and that these metals, as stressors, affect lipid metabolism, leading to changes in the composition and oxidation of fatty acids and in the levels of SOD, CAT and TBARS in the tissues of these organisms. Therefore, these parameters can be treated as biomarkers of physiological status, including oxidative stress. The aim of the study was to assess the impact of selected metals deposited *in situ* on fatty acids, SOD, CAT and TBARS in representatives of Lumbricidae and to identify a set of fatty acids that can be used as biomarkers of the biological (physiological) impact of environmental pollutants *in situ*.

The research has significant practical implications for environmental monitoring and management. A novelty is the proposal of a panel of biomarker fatty acids in Lumbricidae as biomarkers of the biological (physiological) effects of environmental pollutants, which has not been widely used in environmental monitoring and management to date. The proposed panel opens up new possibilities for the use of Lumbricidae as bioindicators and broadens the perspective of biomonitoring, as it can be helpful in assessing the impact of pollution and complement existing methods of environmental monitoring. Another important element is the presentation of metal transfer from waste rock (post-mining waste deposited in the environment as part of land reclamation) to soil, root systems, and Lumbricidae as primary consumers, as well as the assessment of the impact of metals on biomarker fatty acids and enzymatic and non-enzymatic biomarkers of oxidative stress in Lumbricidae. This approach could be helpful in monitoring the state of the environment.

## 2. Materials and Methods

### 2.1. Experimental Design

The research material consisted of waste rock, soil, plant roots, and earthworms (Lumbricidae) obtained from sites that had been reclaimed using waste rock as well as sites without waste rock (Control), from a depth of up to 0.25 m below ground level. From each site, 30 individuals (n = 30) were collected, divided into 5 groups, 6 individuals each. Within the group, individuals were analyzed collectively. The specimens were of similar

size, i.e., 6–8 cm, suggesting similar age, with a distinct clitellum. The sites differed in the type of agricultural use: field, meadow, and wasteland. The sites were located in eastern Poland, in the Polesie region of the Lublin voivodeship (Table 1, Figure 1). The material was collected at sites where waste rock (WR) from a hard coal mine had been deposited for the purposes of reclamation, as well as in sites with no waste rock deposit. The research material was collected in 2022 (September).

**Table 1.** Sampling collection sites and group designations with and without waste rock deposits.

| Number of Maps | Sites                                                                                                                                                                                                    | Geographic Coordinates              | With or Without WR Deposit/<br>Time of WR Exposure | Research Material (Group)                                                                   |
|----------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|----------------------------------------------------|---------------------------------------------------------------------------------------------|
| 1.             | Buckwheat field<br>( <i>Fagopyrum esculentum</i> )                                                                                                                                                       | N: 51°23′27.16″;<br>E: 23°14′35.84″ | WR deposit > 10 years                              | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI10HFB<br>SI10HFB<br>RI10HFB<br>EI10HFB |
| 2.             | Oat field<br>( <i>Avena sativa</i> )                                                                                                                                                                     | N: 51°14′18.23″;<br>E: 23°05′15.98″ | WR deposit 2 years                                 | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI2FO<br>SI2FO<br>RI2FO<br>EI2FO         |
| 3.             | Meadow I<br>with <i>Filipendulion ulmariae</i> herb communities                                                                                                                                          | N: 51°14′06.32″;<br>E: 23°05′07.15″ | WR deposit > 1 year                                | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI1HMI<br>SI1HMI<br>RI1HMI<br>EI1HMI     |
| 4.             | Meadow II<br>with vegetation belonging to the <i>Molinio-Arrhenatheretea</i> class                                                                                                                       | N: 51°14′12.74″;<br>E: 23°05′10.93″ | WR deposit < 1 year                                | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI1LMII<br>SI1LMII<br>RI1LMII<br>EI1LMII |
| 5.             | Wasteland I<br>overgrown with pine and birch forest with dominant sand reed grass <i>Calamagrostis epigejos</i> and the presence of herb communities and clear-cut grasses <i>Epilobion angustifolii</i> | N: 51°25′22.12″;<br>E: 23°08′44.81″ | WR deposit > 10 years                              | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI10HWI<br>SI10HWI<br>RI10HWI<br>EI10HWI |
| 6.             | Wasteland II<br>with communities of ruderal thermophilic plants<br><i>Onopordion acanthii</i>                                                                                                            | N: 51°08′52.15″;<br>E: 23°01′32.98″ | WR deposit < 1 year                                | Waste rock<br>Soil<br>Plant roots<br>Earthworms<br>WI1LWII<br>SI1LWII<br>RI1LWII<br>EI1LWII |
| 7.             | Buckwheat field ( <i>Fagopyrum esculentum</i> )                                                                                                                                                          | N: 51°23′29.93″;<br>E: 23°14′31.36″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWFB<br>RWFB<br>EWFB                                   |
| 8.             | Oat field<br>( <i>Avena sativa</i> )                                                                                                                                                                     | N: 51°14′34.98″;<br>E: 23°05′23.72″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWFO<br>RWFO<br>EWFO                                   |
| 9.             | Meadow I<br>with vegetation from the <i>Calthion</i> association                                                                                                                                         | N: 51°19′36.42″;<br>E: 23°15′01.63″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWMI<br>RWMI<br>EWMI                                   |
| 10.            | Meadow II<br>with vegetation from the <i>Molinion</i> association                                                                                                                                        | N: 51°21′16.57″;<br>E: 23°14′49.31″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWMII<br>RWMII<br>EWMII                                |
| 11.            | Wasteland I<br>overgrown with pine and birch forest with vegetation belonging to the <i>Koelerio glaucae-Coryneporetea canescentis</i> class                                                             | N: 51°25′21.63″;<br>E: 23°08′47.66″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWWI<br>RWWI<br>EWWI                                   |
| 12.            | Wasteland II<br>with vegetation belonging to the <i>Koelerio glaucae-Coryneporetea canescentis</i> class                                                                                                 | N: 51°20′12.81″;<br>E: 23°13′57.74″ | Without WR deposit<br>(control)                    | Soil<br>Plant roots<br>Earthworms<br>SWWII<br>RWWII<br>EWWII                                |

Once the material was obtained, the earthworms were left without food for 24 h to clean out the digestive tract and remove food residues, minimizing the risk of sample contamination and ensuring reliable and comparable analysis results. The earthworms were then frozen at −80 °C for subsequent analysis.

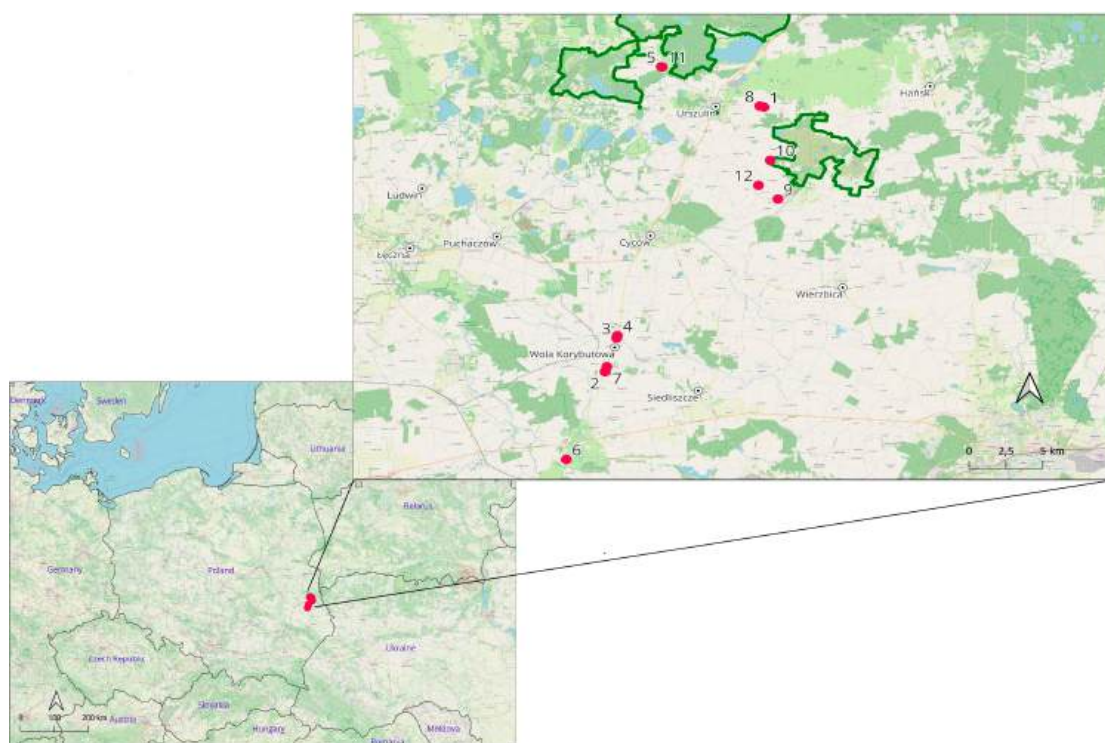


**Figure 1.** Distribution of sampling sites (●—material collection points; —border of Polesie National Park).

## 2.2. Determination of Selected Metals in Waste Rock, Soil, Plant Roots and Lumbricidae

Samples with waste rock, roots and earthworms were crushed and homogenized; soil samples were sifted and crushed. Samples weighing 0.5 g were used for the analyses. Samples were digested in the presence of  $\text{HNO}_3$  (Suprapur-Merck, Darmstadt, Germany) in a CEM Mars Xpress microwave (USA) ( $210\text{ }^\circ\text{C}/7\text{ atm}$ ). The digestates were diluted with demineralized water (conductivity  $0.055\text{ }\mu\text{S}/\text{cm}$ ).

### 2.2.1. Analysis by Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

The solutions were analyzed using the 820-MS ICP-MS mass spectrometer (USA) according to the CLB/ESA/5/2019 procedure (version 3). The following metals were analyzed: Cr, Ni, Cd, Ba, and Pb. The gas used to generate the plasma was argon (Messer, Germany) with 99.999% purity. Ultra Scientific (USA) standards with 99.999% purity were used. No reaction cell (CRI) was used. The settings were as follows: Plasma Flow— $16\text{ dm}^3/\text{min}$ , Nebulizer Flow— $0.98\text{ dm}^3/\text{min}$ , RF Power— $1.38\text{ kW}$ , Sampling Depth— $6.5\text{ mm}$ . The following isotopes were used:  $^{52}\text{Cr}$ ,  $^{60}\text{Ni}$ ,  $^{114}\text{Cd}$ ,  $^{137}\text{Ba}$ ,  $^{206}\text{Pb}$ ,  $^{207}\text{Pb}$  and  $^{208}\text{Pb}$ . Validation parameters: Cr (LOD-limit of detection =  $0.015\text{ mg}\cdot\text{kg}^{-1}$ , LOQ-limit of quantification =  $0.030\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 97%), Ni (LOD =  $0.022\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.044\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 98%), Cd (LOD =  $0.004\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.007\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 99%), Ba (LOD =  $0.038\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.076\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 97%), Pb (LOD =  $0.005\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.010\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 99%). Determinations were made by the standard curve method. The results were expressed in  $\text{mg}/\text{kg}$  fresh weight. Quality was controlled by measuring a blank sample, a double sample and certified reference material: NIST-1577c Bovine Liver (National Institute of Standards & Technology, Gaithersburg, MD, USA). Results are expressed as  $\text{mg}\cdot\text{kg}^{-1}$ .

### 2.2.2. Analysis by Flame Atomic Absorption Spectrometry (FAAS)

The solutions were analyzed using the Varian SpektrAA 280FS FAAS Spectrometer with the SPS-3 autosampler and SIPS diluter (Varian, Mulgrave, Australia). FAAS analysis was carried out according to the CLB/ASA/2/2019 procedure (version 4). The metals analyzed by FAAS were Zn and Cu. The stock solutions were Zn (10 mg/dm<sup>3</sup>) and Cu (20 mg/dm<sup>3</sup>) from CPA Chem (Stara Zagora, Bulgaria). The standards were certified according to ISO 17034 [44]. The operating parameters of the spectrometer were as follows: Zn (flame: acetylene/air, wavelength: 213.9 nm, slit width: 1.0 nm, lamp current: 5.0 mA); Cu (flame: acetylene/air, wavelength: 324.8 nm, slit width: 0.5 nm, lamp current: 4.0 mA). Validation parameters: Zn (LOD = 0.4 mg·kg<sup>-1</sup>, LOQ = 0.09 mg·kg<sup>-1</sup>, recovery = 106%), Cu (LOD = 1.3 mg·kg<sup>-1</sup>, LOQ = 2.6 mg·kg<sup>-1</sup>, recovery = 99%). The matrix was 2% HNO<sub>3</sub>. Results are expressed as mg·kg<sup>-1</sup>.

### 2.3. Lipid Extraction and Recovery of Fatty Acids from Lumbricidae

Samples of material were crushed and homogenized. Fat samples weighing 100 mg were used for the analyses. Saponification was carried out by adding a methanolic KOH solution (Avantor Performance Materials, Gliwice, Poland), and esterification with a methanolic BF<sub>3</sub> solution (Thermo Scientific, China). Esters of fatty acids were obtained according to the CLB/GC/3/2019 procedure (version 4). Esters were isolated with C<sub>6</sub>H<sub>14</sub> (Avantor Performance Materials, Gliwice, Poland) and salted out with a saturated NaCl solution (Avantor Performance Materials, Gliwice, Poland). The fatty acid ester sample was dried by adding anhydrous Na<sub>2</sub>SO<sub>4</sub> (Avantor Performance Materials, Gliwice, Poland), and chromatographic analysis was performed. Samples of fatty acid esters were analyzed using the Varian 450-GC gas chromatograph (Varian, Palo Alto, CA, USA) with the CP-8400 autosampler, an FID detector (temperature 270 °C), and the Select™ Biodiesel capillary column (Agilent Technologies, Santa Clara, CA, USA) for FAME (30 m 0.32 mm 0.25 µm). A 1177 Split/Splitless injector was used (temperature 250 °C). The initial temperature of the furnace column was 100 °C, and the final temperature was 240 °C. The stationary phase of the fused silica Select Biodiesel column for FAME was used, with helium as the carrier gas. The flow rate was 1.5 mL/min. The Galaxie™ Chromatography Data System was used to control the chromatograph and to collect, integrate and calculate the results. Among the 35 fatty acids analyzed, following initial assessment of the differences in the amounts of FAs between locations, a panel of biomarker FAs was selected: C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3; and C22:2 n-6. Total SFAs, PUFAs and MUFAs were calculated.

### 2.4. Peroxidation and Unsaturation Indices

Based on the fatty acid analysis, peroxidation (PI) and unsaturation (UI) indices were calculated. A formula developed by Hulbert et al. (2007) [45] was employed:

$$\text{Peroxidation index (PI)} = 0.025 \times (\% \text{ monoenoics}) + 1 \times (\% \text{ dienoics}) + 2 \times (\% \text{ trienoics}) + 4 \times (\% \text{ tetraenoics}) + 6 \times (\% \text{ pentaenoics}) + 8 \times (\% \text{ hexaenoics})$$

$$\text{Unsaturation index (UI)} = 1 \times (\% \text{ monoenoics}) + 2 \times (\% \text{ dienoics}) + 3 \times (\% \text{ trienoics}) + 4 \times (\% \text{ tetraenoics}) + 5 \times (\% \text{ pentaenoics}) + 6 \times (\% \text{ hexaenoics})$$

### 2.5. Analysis of Enzymatic Biomarkers of Oxidative Stress

Superoxide dismutase (SOD) activity was measured by the indirect method described by Atli and Grosell (2016) [46]. Absorbance was measured at 550 nm for 1 min using a

microplate spectrophotometer (Varioskan™ LUX Thermo Scientific™, Waltham, MA, USA). Results are expressed as Unit/mg protein.

Catalase activity (CAT) was measured using the method described by Atli and Grosell (2016) [46]. The absorbance decline was examined at 240 nm for 1 min using a microplate spectrophotometer (Varioskan™ LUX Thermo Scientific™, Waltham, MA, USA). Results are expressed as  $\mu\text{mol H}_2\text{O}_2/\text{mg protein}/\text{min}$ .

## 2.6. Analysis of Non-Enzymatic Biomarkers of Oxidative Stress—TBARS (Thiobarbituric Acid Reactive Substances)

The malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation. It was determined according to the method described by Radwan et al. (2010a, 2010b) [47,48]. Absorbance was measured at 532 nm for 1 min using a microplate spectrophotometer (Varioskan™ LUX Thermo Scientific™, Waltham, MA, USA). Results are expressed as nM of MDA/mg of wet tissue.

## 2.7. Statistical Analyses

The data were subjected to statistical analysis using Statistica version 13.3 software (1984–2017 TIBCO Software INC, Palo Alto, CA, USA). The normality was assessed using the Kolmogorov–Smirnov test, and the Levene’s homogeneity of variance test was applied to examine the equality of variances. Two-way ANOVA was used to assess the effect of the waste rock deposit (WR deposit, without WR deposit) and the type of land (buckwheat, oats, wasteland I and II, meadow I and II) as well as their interaction on the content of each FAs, PI and UI indices, SOD, CAT and TBARS. One-way ANOVA and Tukey’s multiple range tests were performed to compare all experimental groups and the Control one (sites without WR deposit). Lower and upper 95% confidence intervals (CI) were expressed in the original scale of measurement.

As part of the assessment of the linear relation between FAs and PI and UI indices, SOD, CAT and TBARS content and content of metals (Pb, Cd, Cr, Ba, Ni, Zn, Cu)

Pearson’s linear correlation coefficient ( $r$ ) were calculated at a significance level of  $p \leq 0.01$  and the interpretation was based on the following Matyja (2014) [49] scale: negligible correlation:  $0 < r < |0.1|$ ; weak correlation:  $|0.1| \leq r < |0.3|$ ; moderate correlation:  $|0.3| \leq r < |0.5|$ ; strong correlation:  $|0.5| \leq r < |0.7|$ ; very strong correlation:  $|0.7| \leq r < |0.9|$ ; nearly perfect correlation:  $|0.9| \leq r < |1|$ .

Cluster analyses (area and feature clustering) were conducted using data standardized relative to the FAs, PI and UI indices, SOD, CAT and TBARS content.

Additionally, the full correlation matrix was calculated for variables related to Pb to control the risk of the error in multiple comparisons. The Benjamini–Hochberg procedure was applied with a false discovery rate (FDR) set at 0.01. For each test, the Benjamini–Hochberg (BH) threshold was calculated as:

$$FDR (BH) \text{ thresholds} = \frac{i}{m} \cdot \alpha \quad (1)$$

where  $i$ —the rank of the test (1–31),  $m$ —the total number of tests (31), and  $\alpha = 0.01$ . Tests with  $p\text{-value} \leq FDR (BH)$  thresholds were considered statistically significant [50].

## 3. Results

### 3.1. Metals: Waste Rock—Soil—Plant Roots—Earthworms

Upon entering the environment, heavy metals contained in waste rock were transferred to the soil, plant roots, and organisms of the family Lumbricidae (Tables 2–5). In the waste rock (WR) samples collected from all sites, accumulation of barium (Ba) and zinc (Zn)

was highest, and that of lead (Pb) and nickel (Ni) was lowest, while cadmium (Cd) was not detected. The highest concentrations of individual elements in the waste rock were noted at the following sites: Pb and Zn—buckwheat field (WR > 10 years), Cr—meadow I (WR > 1 year), and Ba, Ni and Cu—wasteland II (WR < 1 year). The lowest content in WR was noted at the following sites: Cr and Ni—wasteland I (WR > 10 years), Cr and Ba—oat field (WR = 2 years), Pb and Zn—meadow II (WR < 1 year), and Cu—meadow I (WR > 1 year) (Table 2).

**Table 2.** Metals analyzed in waste rock obtained from sites with and without waste rock deposits.

| Metals + CI<br>[mg·kg <sup>−1</sup> ] | Groups                          |                                |                               |                                 |                                 |                                 | SEM  |
|---------------------------------------|---------------------------------|--------------------------------|-------------------------------|---------------------------------|---------------------------------|---------------------------------|------|
|                                       | WI10HFB                         | WI10HWI                        | WI2FO                         | WI1HMI                          | WI1LWII                         | WI1LMII                         |      |
| <b>Pb</b><br>[CI]                     | 30 <sup>b</sup><br>19.8–40.7    | 30 <sup>b</sup><br>25.2–34.0   | 24 <sup>ab</sup><br>20.0–28.2 | 20 <sup>ab</sup><br>16.0–23.0   | 16 <sup>a</sup><br>12.5–20.1    | 15 <sup>a</sup><br>9.8–19.7     | 1.36 |
| <b>Cd</b><br>[CI]                     | 0<br>-                          | 0<br>-                         | 0<br>-                        | 0<br>-                          | 0<br>-                          | 0<br>-                          | -    |
| <b>Cr</b><br>[CI]                     | 77 <sup>b</sup><br>59.8–94.3    | 26 <sup>a</sup><br>16.1–35.2   | 26 <sup>a</sup><br>18.5–32.9  | 82 <sup>b</sup><br>72.3–90.7    | 67 <sup>b</sup><br>612.0–71.5   | 66 <sup>b</sup><br>51.9–80.4    | 4.51 |
| <b>Ba</b><br>[CI]                     | 109 <sup>ab</sup><br>97.1–120.7 | 101 <sup>a</sup><br>88.3–112.5 | 91 <sup>a</sup><br>82.1–99.5  | 124 <sup>b</sup><br>112.5–136.3 | 158 <sup>c</sup><br>145.8–169.3 | 226 <sup>d</sup><br>209.5–241.6 | 8.68 |
| <b>Ni</b><br>[CI]                     | 29 <sup>b</sup><br>25.3–33.3    | 19 <sup>a</sup><br>16.0–21.9   | 26 <sup>ab</sup><br>23.8–29.0 | 46 <sup>c</sup><br>39.5–52.9    | 51 <sup>c</sup><br>47.3–54.6    | 26 <sup>ab</sup><br>22.2–30.4   | 2.21 |
| <b>Zn</b><br>[CI]                     | 101 <sup>c</sup><br>81.2–120.3  | 43 <sup>ab</sup><br>40.3–46.2  | 56 <sup>b</sup><br>49.6–62.0  | 54 <sup>b</sup><br>46.4–60.8    | 45 <sup>b</sup><br>40.4–49.0    | 26 <sup>a</sup><br>23.44–27.6   | 4.46 |
| <b>Cu</b><br>[CI]                     | 35 <sup>a</sup><br>29.6–40.5    | 46 <sup>b</sup><br>42.5–48.9   | 32 <sup>a</sup><br>23.3–34.8  | 30 <sup>a</sup><br>24.5–34.8    | 48 <sup>b</sup><br>43.1–52.8    | 45 <sup>b</sup><br>40.0–50.7    | 1.50 |

Groups: WI10HFB—WR (waste rock) deposit > 10 years, buckwheat field; WI10HWI—WR deposit > 10 years, wasteland I; WI2FO—WR deposit = 2 years, oat field; WI1HMI—WR deposit > 1 year, meadow I; WI1LWII—WR deposit < 1 year, wasteland II; WI1LMII—WR deposit < 1 year, meadow II. n = 30. CI—95% confidence interval (lower–upper); SEM—standard error of mean; <sup>a–d</sup>—values in rows with different letters differ significantly at the level  $p \leq 0.01$ ; significant at  $p \leq 0.01$ .

In the samples of soil, plant roots, and earthworms from all study sites, the highest accumulation was noted for Zn and the lowest for Cd (Tables 3–5). Specifically, the highest concentrations of Pb, Ni, Cu and Zn were noted in the soil from wasteland I with long-term waste rock deposit (WR > 10 years); the highest Cd accumulation was detected in soil from the buckwheat field (WR > 10 years); and Cr and Ba concentrations were highest in soil from the meadow I with a WR deposit of more than one year (WR > 1 year). The lowest soil concentrations of individual elements were observed in samples from the following sites without WR: Pb and Zn—meadow II, Cd and Ba—wasteland I, Cr—oat field, and Ni and Cu—buckwheat field. The content of the analyzed metals was influenced ( $p \leq 0.01$ ) by all single factors (WR, S), as well as the interaction between them (WR×S) (Table 3).

In the case of plant roots, accumulation of Pb, Cr, Ni and Cu was greatest in the samples collected from wasteland I (WR > 10 years), Cd and Ba in the samples from meadow II (WR < 1 year), and Zn in the samples from meadow II (−WR), where −WR means no WR deposit. The lowest concentrations of individual elements in the roots were observed at the following site without WR: Pb—wasteland II, Cd—buckwheat field, Cr and Ni—meadow I, Ba—wasteland I, and Zn and Cu—oat field. The content of the analyzed metals was influenced ( $p \leq 0.01$ ) by all single factors (WR, S), as well as the interaction between them (WR×S) (Table 4).

**Table 3.** Metals analyzed in soil obtained from sites with and without waste rock deposits.

| Metals + CI<br>[mg·kg <sup>−1</sup> ] |                                | Groups                        |                                |                               |                                |                               |                                 |                                |                                  |                                |                                  |                               | SEM   |
|---------------------------------------|--------------------------------|-------------------------------|--------------------------------|-------------------------------|--------------------------------|-------------------------------|---------------------------------|--------------------------------|----------------------------------|--------------------------------|----------------------------------|-------------------------------|-------|
|                                       |                                | SI10HFB                       | SWFB                           | SI10HWI                       | SWWI                           | SI2FO                         | SWFO                            | SI1HMI                         | SWMI                             | SI1LWII                        | SWWII                            | SI1LMII                       | SWMII |
| <b>Pb</b><br>[CI]                     | 9.8 <sup>c</sup><br>2.7–17.0   | 4.2 <sup>abc</sup><br>1.9–6.5 | 16.2 <sup>d</sup><br>13.3–19.1 | 3.0 <sup>ab</sup><br>1.5–4.4  | 8.3 <sup>bc</sup><br>4.7–11.7  | 3.7 <sup>abc</sup><br>0.7–6.8 | 5.2 <sup>abc</sup><br>1.3–9.0   | 0.9 <sup>a</sup><br>0.3–1.6    | 2.1 <sup>a</sup><br>0.4–3.7      | 1.1 <sup>a</sup><br>0.3–1.8    | 1.1 <sup>a</sup><br>0.3–2.0      | 0.9 <sup>a</sup><br>0.3–1.4   | 0.20  |
| <b>Cd</b><br>[CI]                     | 4.5 <sup>f</sup><br>3.7–5.3    | 0.2 <sup>a</sup><br>0.1–0.2   | 0.5 <sup>ab</sup><br>0.1–0.9   | 0.1 <sup>a</sup><br>0.1–0.1   | 0.4 <sup>ab</sup><br>0.3–0.5   | 0.1 <sup>a</sup><br>0.0–0.2   | 3.3 <sup>def</sup><br>2.8–3.7   | 1.2 <sup>abc</sup><br>0.8–1.6  | 3.5 <sup>ef</sup><br>2.1–4.7     | 2.3 <sup>cde</sup><br>0.8–3.8  | 2.9 <sup>de</sup><br>1.8–4.1     | 1.7 <sup>bcd</sup><br>1.3–2.2 | 0.16  |
| <b>Cr</b><br>[CI]                     | 10.9 <sup>d</sup><br>9.7–12.1  | 4.6 <sup>ab</sup><br>4.0–5.1  | 8.4 <sup>e</sup><br>7.2–9.6    | 3.8 <sup>a</sup><br>3.4–4.3   | 4.5 <sup>ab</sup><br>3.7–5.2   | 3.8 <sup>a</sup><br>3.1–4.6   | 15.0 <sup>f</sup><br>12.5–17.6  | 6.9 <sup>cd</sup><br>6.3–7.6   | 5.7 <sup>abc</sup><br>4.9–6.4    | 6.5 <sup>bcd</sup><br>5.5–7.5  | 6.0 <sup>abc</sup><br>5.6–6.3    | 5.4 <sup>abc</sup><br>4.6–6.1 | 0.27  |
| <b>Ba</b><br>[CI]                     | 19.5 <sup>b</sup><br>16.9–22.2 | 0.2 <sup>a</sup><br>0.1–0.2   | 1.2 <sup>a</sup><br>1.0–1.4    | 0.2 <sup>a</sup><br>0.1–0.2   | 1.6 <sup>a</sup><br>1.3–1.9    | 0.3 <sup>a</sup><br>0.3–0.4   | 27.5 <sup>c</sup><br>23.9–31.1  | 2.4 <sup>a</sup><br>2.0–2.7    | 19.5 <sup>b</sup><br>17.4–21.6   | 18.8 <sup>b</sup><br>17.3–20.3 | 25.6 <sup>c</sup><br>23.7–27.5   | 1.5 <sup>a</sup><br>1.3–1.7   | 0.08  |
| <b>Ni</b><br>[CI]                     | 6.0 <sup>cd</sup><br>5.2–6.8   | 1.9 <sup>a</sup><br>1.6–2.1   | 9.2 <sup>e</sup><br>7.9–10.4   | 1.9 <sup>a</sup><br>1.5–2.3   | 3.1 <sup>ab</sup><br>2.0–4.1   | 2.4 <sup>a</sup><br>2.0–2.9   | 6.5 <sup>d</sup><br>5.9–7.0     | 5.2 <sup>cd</sup><br>4.7–5.7   | 4.8 <sup>cd</sup><br>3.1–6.6     | 5.5 <sup>cd</sup><br>4.76–6.2  | 4.5 <sup>bc</sup><br>4.0–5.0     | 3.2 <sup>ab</sup><br>2.8–3.5  | 0.13  |
| <b>Zn</b><br>[CI]                     | 16.8 <sup>c</sup><br>14.6–19.0 | 1.6 <sup>ab</sup><br>8.3–14.9 | 25.3 <sup>e</sup><br>20.8–29.8 | 9.4 <sup>ab</sup><br>7.3–11.5 | 10.8 <sup>ab</sup><br>9.0–12.6 | 8.5 <sup>a</sup><br>7.4–9.7   | 13.9 <sup>bc</sup><br>13.0–14.9 | 10.3 <sup>ab</sup><br>8.3–12.3 | 12.7 <sup>abc</sup><br>10.8–14.6 | 11.5 <sup>ab</sup><br>7.1–15.8 | 11.7 <sup>abc</sup><br>10.4–13.1 | 8.1 <sup>a</sup><br>7.5–8.8   | 0.24  |
| <b>Cu</b><br>[CI]                     | 4.4 <sup>abc</sup><br>3.7–5.2  | 2.2 <sup>a</sup><br>1.7–2.6   | 25.6 <sup>e</sup><br>22.2–29.1 | 5.9 <sup>c</sup><br>4.2–7.6   | 3.6 <sup>abc</sup><br>2.7–4.4  | 2.6 <sup>ab</sup><br>2.1–3.2  | 5.3 <sup>bc</sup><br>4.6–6.0    | 4.1 <sup>abc</sup><br>3.0–5.2  | 4.5 <sup>abc</sup><br>3.2–5.8    | 4.0 <sup>abc</sup><br>3.3–4.7  | 4.4 <sup>abc</sup><br>4.0–5.0    | 3.8 <sup>abc</sup><br>3.4–4.2 | 0.15  |
| <b>Interactions</b>                   | <b>WR</b>                      | *                             | *                              | *                             | *                              | *                             | *                               | *                              | *                                | *                              | *                                | *                             | *     |
|                                       | <b>S</b>                       | *                             | *                              | *                             | *                              | *                             | *                               | *                              | *                                | *                              | *                                | *                             | *     |
|                                       | <b>WRxS</b>                    | *                             | *                              | *                             | *                              | *                             | *                               | *                              | *                                | *                              | *                                | *                             | *     |

Groups: SI10HFB—WR deposit > 10 years, buckwheat field; SWFB—without WR deposit, buckwheat field; SI10HWI—WR deposit > 10 years, wasteland I; SWWI—without WR deposit, wasteland I; SI2FO—WR deposit = 2 years, oat field; SWFO—without WR deposit, oat field; SI1HMI—WR deposit > 1 year, meadow I; SWMI—without WR deposit, meadow I; SI1LWII—WR deposit < 1 year, wasteland II; SWWII—without WR deposit, wasteland II; SI1LMII—WR deposit < 1 year, meadow II; SWMII—without WR deposit, meadow II. n = 60. CI—95% confidence interval (lower–upper); SEM—standard error of mean; WR—waste rock; S—sampling sites; WRxS—interaction of waste rock with the sampling site; <sup>a–f</sup>—values in rows with different letters differ significantly at the level  $p \leq 0.01$ ; \* significant at  $p \leq 0.01$ .

**Table 4.** Metals analyzed in plant roots obtained from sites with and without waste rock deposits.

| Metals + CI<br>[mg·kg <sup>−1</sup> ] |      | Groups                           |                                  |                                 |                                 |                                |                                |                                 |                                 |                                |                                   |                                 |                                | SEM  |
|---------------------------------------|------|----------------------------------|----------------------------------|---------------------------------|---------------------------------|--------------------------------|--------------------------------|---------------------------------|---------------------------------|--------------------------------|-----------------------------------|---------------------------------|--------------------------------|------|
|                                       |      | RI10HFB                          | RWFB                             | RI10HWI                         | RWWI                            | RI2FO                          | RWFO                           | RI1HMI                          | RWMI                            | RI1LWII                        | RWWII                             | RI1LMII                         | RWMII                          |      |
| <b>Pb</b><br>[CI]                     |      | 3.8 <sup>abc</sup><br>1.7–5.8    | 1.9 <sup>ab</sup><br>1.1–2.7     | 14.2 <sup>d</sup><br>10.3–18.0  | 6.5 <sup>c</sup><br>3.5–9.5     | 4.0 <sup>abc</sup><br>2.9–5.0  | 1.7 <sup>a</sup><br>1.2–2.3    | 3.9 <sup>abc</sup><br>1.1–6.7   | 2.2 <sup>ab</sup><br>1.0–3.4    | 5.8 <sup>bc</sup><br>4.7–7.0   | 1.7 <sup>a</sup><br>0.9–2.4       | 2.8 <sup>abc</sup><br>1.0–4.7   | 2.0 <sup>ab</sup><br>1.4–2.5   | 0.48 |
| <b>Cd</b><br>[CI]                     |      | 0.9 <sup>ab</sup><br>0.7–1.1     | 0.3 <sup>a</sup><br>0.1–0.5      | 1.4 <sup>ab</sup><br>0.8–2.0    | 0.5 <sup>ab</sup><br>0.3–0.8    | 0.7 <sup>ab</sup><br>0.5–1.0   | 0.5 <sup>ab</sup><br>0.3–0.6   | 2.9 <sup>b</sup><br>1.5–4.4     | 1.3 <sup>ab</sup><br>0.9–3.6    | 0.9 <sup>ab</sup><br>0.6–1.2   | 0.8 <sup>ab</sup><br>0.1–2.0      | 3.1 <sup>b</sup><br>0.6–5.5     | 2.3 <sup>ab</sup><br>0.3–4.2   | 0.17 |
| <b>Cr</b><br>[CI]                     |      | 4.5 <sup>bc</sup><br>3.4–5.6     | 3.7 <sup>abc</sup><br>2.4–5.1    | 37.0 <sup>f</sup><br>34.1–40.0  | 20.9 <sup>e</sup><br>17.8–24.0  | 6.5 <sup>c</sup><br>5.7–7.3    | 4.8 <sup>bc</sup><br>4.1–5.6   | 4.1 <sup>abc</sup><br>3.6–4.6   | 1.5 <sup>a</sup><br>0.9–2.2     | 3.3 <sup>ab</sup><br>2.9–3.8   | 2.9 <sup>ab</sup><br>2.2–3.7      | 10.5 <sup>d</sup><br>9.7–11.3   | 1.5 <sup>a</sup><br>1.5–1.6    | 1.31 |
| <b>Ba</b><br>[CI]                     |      | 8.7 <sup>b</sup><br>7.9–9.5      | 0.3 <sup>a</sup><br>0.3–0.4      | 0.6 <sup>a</sup><br>0.5–0.8     | 0.2 <sup>a</sup><br>0.1–0.2     | 0.8 <sup>a</sup><br>0.6–0.9    | 0.6 <sup>a</sup><br>0.5–0.7    | 9.7 <sup>b</sup><br>8.5–11.0    | 7.9 <sup>b</sup><br>6.5–9.3     | 24.0 <sup>c</sup><br>20.3–27.7 | 9.7 <sup>b</sup><br>7.6–11.7      | 48.4 <sup>d</sup><br>42.7–54.0  | 7.8 <sup>b</sup><br>6.3–9.2    | 1.75 |
| <b>Ni</b><br>[CI]                     |      | 13.3 <sup>c</sup><br>12.4–14.3   | 8.6 <sup>ab</sup><br>7.7–9.5     | 21.1 <sup>f</sup><br>18.9–23.4  | 18.6 <sup>de</sup><br>16.8–20.5 | 9.4 <sup>ab</sup><br>7.1–11.8  | 7.3 <sup>a</sup><br>6.2–8.3    | 11.4 <sup>bc</sup><br>10.4–12.4 | 7.1 <sup>a</sup><br>6.2–7.9     | 12.8 <sup>c</sup><br>11.0–14.5 | 9.0 <sup>ab</sup><br>7.8–10.3     | 17.2 <sup>d</sup><br>15.6–18.8  | 7.4 <sup>a</sup><br>6.3–8.5    | 0.61 |
| <b>Zn</b><br>[CI]                     |      | 27.0 <sup>bcd</sup><br>23.7–30.3 | 22.9 <sup>abc</sup><br>19.3–26.4 | 28.3 <sup>cd</sup><br>24.5–32.0 | 27.4 <sup>cd</sup><br>23.6–31.1 | 18.3 <sup>a</sup><br>16.6–20.1 | 18.3 <sup>a</sup><br>17.0–19.6 | 20.3 <sup>ab</sup><br>18.0–22.7 | 27.3 <sup>cd</sup><br>20.9–33.7 | 52.6 <sup>e</sup><br>47.9–57.2 | 24.5 <sup>abcd</sup><br>22.0–27.0 | 28.9 <sup>cd</sup><br>27.0–20.8 | 30.6 <sup>d</sup><br>28.6–32.5 | 1.17 |
| <b>Cu</b><br>[CI]                     |      | 10.4 <sup>ef</sup><br>8.2–12.6   | 7.0 <sup>bcd</sup><br>5.4–9.7    | 17.3 <sup>g</sup><br>15.4–19.2  | 5.4 <sup>abc</sup><br>4.4–6.5   | 4.9 <sup>ab</sup><br>3.8–6.0   | 3.8 <sup>a</sup><br>3.1–4.5    | 8.6 <sup>de</sup><br>7.4–9.9    | 8.5 <sup>de</sup><br>7.6–9.5    | 7.7 <sup>cd</sup><br>7.0–8.5   | 4.5 <sup>ab</sup><br>3.8–5.4      | 11.5 <sup>f</sup><br>10.2–12.7  | 8.6 <sup>de</sup><br>7.8–9.4   | 0.48 |
| Interactions                          | WR   | *                                | *                                | *                               | *                               | *                              | *                              | *                               | *                               | *                              | *                                 | *                               | *                              |      |
|                                       | S    | *                                | *                                | *                               | *                               | *                              | *                              | *                               | *                               | *                              | *                                 | *                               | *                              |      |
|                                       | WRxS | *                                | *                                | *                               | *                               | *                              | *                              | *                               | *                               | *                              | *                                 | *                               | *                              |      |

Groups: RI10HFB—WR deposit > 10 years, buckwheat field; RWFB—without WR deposit, buckwheat field; RI10HWI—WR deposit > 10 years, wasteland I; RWWI—without WR deposit, wasteland I; RI2FO—WR deposit = 2 years, oat field; RWFO—without WR deposit, oat field; RI1HMI—WR deposit > 1 year, meadow I; RWMI—without WR deposit, meadow I; RI1LWII—WR deposit < 1 year, wasteland II; RWWII—without WR deposit, wasteland II; RI1LMII—WR deposit < 1 year, meadow II; RWMII—without WR deposit, meadow II. n = 60. CI—95% confidence interval (lower–upper); SEM—standard error of mean; WR—waste rock; S—sampling sites; WRxS—interaction of waste rock with the sampling site; <sup>a–g</sup>—values in rows with different letters differ significantly at the level  $p \leq 0.01$ ; \* significant at  $p \leq 0.01$ .

**Table 5.** Metals analyzed in earthworms obtained from sites with and without waste rock deposits.

| Metals + CI<br>[mg·kg <sup>−1</sup> ] |                                | Groups                          |                                   |                                 |                                 |                                |                                 |                                  |                                 |                                |                                  |                                 | SEM   |
|---------------------------------------|--------------------------------|---------------------------------|-----------------------------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|----------------------------------|---------------------------------|--------------------------------|----------------------------------|---------------------------------|-------|
|                                       |                                | EI10HFB                         | EWFB                              | EI10HWI                         | EWWI                            | EI2FO                          | EWFO                            | EI1HMI                           | EWMI                            | EI1LWII                        | EWII                             | EI1LMII                         | EWMII |
| <b>Pb</b><br>[CI]                     | 3.5 <sup>cd</sup><br>1.6–5.3   | 1.0 <sup>ab</sup><br>0.6–1.4    | 5.3 <sup>e</sup><br>4.1–6.5       | 2.3 <sup>abc</sup><br>1.5–3.1   | 2.7 <sup>bc</sup><br>2.0–3.4    | 1.0 <sup>ab</sup><br>0.7–1.3   | 1.2 <sup>ab</sup><br>0.5–1.8    | 0.6 <sup>a</sup><br>0.4–0.8      | 3.5 <sup>cd</sup><br>1.7–5.3    | 2.1 <sup>abc</sup><br>1.8–2.4  | 1.0 <sup>ab</sup><br>0.5–1.5     | 0.6 <sup>a</sup><br>0.3–0.9     | 0.20  |
| <b>Cd</b><br>[CI]                     | 0.9 <sup>ab</sup><br>0.4–1.3   | 0.6 <sup>a</sup><br>0.0–1.1     | 2.5 <sup>c</sup><br>1.8–3.1       | 1.3 <sup>b</sup><br>0.6–2.0     | 0.8 <sup>ab</sup><br>0.7–0.9    | 0.5 <sup>a</sup><br>0.4–0.7    | 0.5 <sup>a</sup><br>0.4–0.6     | 0.3 <sup>a</sup><br>0.3–0.4      | 0.4 <sup>a</sup><br>0.2–0.6     | 0.4 <sup>a</sup><br>0.1–0.6    | 0.3 <sup>a</sup><br>0.3–0.4      | 0.2 <sup>a</sup><br>0.1–0.3     | 0.09  |
| <b>Cr</b><br>[CI]                     | 6.2 <sup>e</sup><br>5.3–7.1    | 1.2 <sup>ab</sup><br>0.8–1.5    | 6.0 <sup>de</sup><br>5.0–7.0      | 2.5 <sup>abc</sup><br>1.7–3.4   | 2.0 <sup>ab</sup><br>1.4–2.6    | 0.6 <sup>a</sup><br>0.5–0.6    | 12.1 <sup>f</sup><br>10.5–13.7  | 5.2 <sup>de</sup><br>3.4–7.1     | 2.8 <sup>bc</sup><br>1.5–4.0    | 1.5 <sup>ab</sup><br>0.9–2.1   | 4.2 <sup>cd</sup><br>3.7–4.7     | 3.1 <sup>bc</sup><br>2.7–3.6    | 0.41  |
| <b>Ba</b><br>[CI]                     | 2.6 <sup>c</sup><br>1.5–3.7    | 0.0 <sup>a</sup><br>0.0–0.01    | 0.0 <sup>a</sup><br>0.0–0.1       | 0.0 <sup>a</sup><br>0.0–0.0     | 0.0 <sup>a</sup><br>0.0–0.0     | 0.0 <sup>a</sup><br>0.0–0.0    | 4.0 <sup>d</sup><br>3.3–4.8     | 1.3 <sup>b</sup><br>0.4–2.3      | 2.7 <sup>c</sup><br>2.0–3.4     | 2.2 <sup>bc</sup><br>2.0–2.4   | 1.8 <sup>bc</sup><br>1.4–2.3     | 1.4 <sup>b</sup><br>1.2–1.7     | 0.18  |
| <b>Ni</b><br>[CI]                     | 1.9 <sup>c</sup><br>1.3–2.5    | 0.8 <sup>ab</sup><br>0.7–0.8    | 0.9 <sup>ab</sup><br>0.8–0.9      | 0.2 <sup>a</sup><br>0.1–0.2     | 0.1 <sup>a</sup><br>0.1–0.1     | 0.1 <sup>a</sup><br>0.1–0.1    | 3.1 <sup>d</sup><br>2.3–4.0     | 2.1 <sup>c</sup><br>1.6–2.5      | 1.6 <sup>bc</sup><br>0.6–2.5    | 0.2 <sup>a</sup><br>0.1–0.4    | 2.3 <sup>cd</sup><br>1.8–2.8     | 1.8 <sup>c</sup><br>1.6–2.0     | 0.14  |
| Metals + CI<br>[mg·kg <sup>−1</sup> ] |                                | Groups                          |                                   |                                 |                                 |                                |                                 |                                  |                                 |                                |                                  |                                 | SEM   |
|                                       |                                | EI10HFB                         | EWFB                              | EI10HWI                         | EWWI                            | EI2FO                          | EWFO                            | EI1HMI                           | EWMI                            | EI1LWII                        | EWII                             | EI1LMII                         | EWMII |
| <b>Zn</b><br>[CI]                     | 66.6 <sup>d</sup><br>59.0–74.1 | 50.4 <sup>bc</sup><br>46.5–54.4 | 115.9 <sup>f</sup><br>107.4–124.3 | 95.5 <sup>e</sup><br>88.1–102.9 | 57.9 <sup>cd</sup><br>52.9–63.0 | 34.6 <sup>a</sup><br>28.0–41.2 | 88.3 <sup>e</sup><br>72.2–104.5 | 45.3 <sup>abc</sup><br>37.0–53.7 | 36.7 <sup>ab</sup><br>32.5–41.0 | 33.6 <sup>a</sup><br>31.7–35.5 | 48.7 <sup>abc</sup><br>44.2–53.2 | 41.1 <sup>ab</sup><br>37.1–45.2 | 3.41  |
| <b>Cu</b><br>[CI]                     | 3.8 <sup>de</sup><br>2.9–4.6   | 1.7 <sup>ab</sup><br>0.9–2.5    | 4.3 <sup>e</sup><br>4.0–4.6       | 2.3 <sup>abc</sup><br>1.6–3.1   | 1.7 <sup>ab</sup><br>1.3–2.1    | 1.2 <sup>a</sup><br>0.8–1.6    | 3.8 <sup>de</sup><br>3.2–4.4    | 3.1 <sup>cd</sup><br>2.9–3.2     | 2.6 <sup>bc</sup><br>1.8–3.4    | 1.8 <sup>ab</sup><br>1.5–2.0   | 2.8 <sup>bcd</sup><br>2.5–3.1    | 2.6 <sup>bcd</sup><br>2.3–3.0   | 0.13  |
| <b>Interactions</b>                   | <b>WR</b>                      | *                               | *                                 | *                               | *                               | *                              | *                               | *                                | *                               | *                              | *                                | *                               |       |
|                                       | <b>S</b>                       | *                               | *                                 | *                               | *                               | *                              | *                               | *                                | *                               | *                              | *                                | *                               |       |
|                                       | <b>WRxS</b>                    | *                               | *                                 | *                               | *                               | *                              | *                               | *                                | *                               | *                              | *                                | *                               |       |

Groups: EI10HFB—WR deposit > 10 years, buckwheat field; EWFB—without WR deposit, buckwheat field; EI10HWI—WR deposit > 10 years, wasteland I; EWWI—without WR deposit, wasteland I; EI2FO—WR deposit = 2 years, oat field; EWFO—without WR deposit, oat field; EI1HMI—WR deposit > 1 year, meadow I; EWMI—without WR deposit, meadow I; EI1LWII—WR deposit < 1 year, wasteland II; EWWII—without WR deposit, wasteland II; EI1LMII—WR deposit < 1 year, meadow II; EWMII—without WR deposit, meadow II. n = 60. CI—95% confidence interval (lower–upper); SEM—standard error of mean; WR—waste rock; S—sampling sites; WRxS—interaction of waste rock with the sampling site; <sup>a–f</sup>—values in rows with different letters differ significantly at the level  $p \leq 0.01$ ; \* significant at  $p \leq 0.01$ .

The accumulation of metals Pb, Cd, Zn and Cu was greatest in earthworms from the wasteland I with a long-term deposit of waste rock (WR > 10 years). The highest concentrations of Cr, Ba and Ni were noted in earthworms from the meadow I with a waste rock deposit exceeding one year (WR > 1 year). The lowest concentrations of individual metals were detected in earthworms from the following locations without WR: Pb—meadow I, Cd—meadow II, Zn—wasteland II, Cu—oat field, and Cr, Ba and Ni—oat field. The content of the analyzed metals was influenced ( $p \leq 0.01$ ) by all single factors (WR, S), as well as the interaction between them (WRxS) (Table 5).

On the crop fields (buckwheat and oat) with a short-term deposit of waste rock, there was not enough time for the metals to be transferred from the plant roots to the earthworms, whereas on the long-term waste rock deposit on wasteland I with species-poor vegetation and in the meadows, plants were able to store certain metals, enabling their transfer to earthworms.

### 3.2. Biomarker FAs, Peroxidation (PI) and Unsaturation (UI) Indices, Enzymatic (SOD, CAT) and Non-Enzymatic (TBARS) Biomarkers of Oxidative Stress

A set of biomarker fatty acids (FA) in earthworms was proposed—C12:0 (lauric acid); C14:0 (myristic acid); C16:0 (palmitic acid); C18:0 (stearic acid); C20:0 (arachidic acid, eicosanoic acid); C21:0 (heneicosanoic acid); C24:0 (lignoceric acid, tetracosanoic acid); C16:1 n-7 (palmitoleic acid); C18:1 n-9c (oleic acid) + C18:1 n-9t (elaidic acid); C20:1 n-9 (cis-11-eicosenic acid); C18:2 n-6c (linoleic acid) + C18:2 n-6t (linolelaidic acid); C18:3 n-3 ( $\alpha$ -linolenic acid); C20:2 n-6 (cis-11-14-eicosadienic acid); C20:3 n-3 (eicosatrienic acid); C20:4 n-6 (arachidonic acid); C20:5 n-3 (cis-5,8,11,14,17-eicosapentaenic acid, EPA); and C22:2 n-6 (cis-13-16-docosadienic acid) (Table 6, Figure 2A).

The analyses showed that in earthworms obtained from the sites with waste rock, the content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) was higher than at the sites without waste rock, while that of polyunsaturated fatty acids (PUFAs) was lower. Content of SFAs and MUFAs was highest in earthworms from wasteland I (WR > 10 years) and oat field (WR = 2 years), while PUFAs dominated in the samples from meadow I (−WR), meadow II (−WR) and wasteland II (−WR). The SFA content was lowest in earthworms from meadow I (−WR), meadow II (−WR) and wasteland II (−WR); MUFA content in earthworms from meadow I (−WR) and meadow II (−WR); and PUFAs were lowest in specimens from wasteland I (WR > 10 years) and oat field (WR = 2 years) (Table 6, Figure 2B). The predominant fatty acids were C16:0 and C18:0 among SFAs; C18:1 n-9c + C18:1 n-9t among MUFAs; and C20:4n-6, C18:2 n-6c + C18:2 n-6t among PUFAs. Small amounts of FAs were noted for C20:0 and C24:0 among SFAs; C16:1 n-7 and C20:1 n-9 among MUFAs; and C20:5 n-3 among PUFAs (Table 6). The content of C14:0, C16:0, C18:0, C21:0 and C24:0 was highest in earthworms from wasteland I with a long-term deposit of waste rock (WR > 10 years). Content of C20:0 was highest in earthworms from oat field (WR = 2 years) and wasteland I (WR > 10 years), while C12:0 content was highest in samples from wasteland II (WR < 1 year). The content of C16:1 n-7 was highest in earthworms from wasteland I (WR > 10 year) and wasteland II (WR < 1 year) and lowest in those from the buckwheat field (−WR). The content of C18:1 n-9c + C18:1 n-9t was highest in earthworms from the oat field (WR = 2 years) and wasteland I (WR > 10 years) and lowest in earthworms from meadow I (−WR). The concentration of C20:1 n-9 was highest in earthworms from the buckwheat field (WR > 10 years) and lowest in those from wasteland I (−WR). In the case of PUFAs, the highest content of acids C18:2 n-6c + C18:2 n-6t, C18:3 n-3, and C20:3 n-3 was detected in earthworms from sites without waste rock: meadow I, meadow II and wasteland II. The content of C20:2 n-6 was highest in samples from the buckwheat field, wasteland II and meadow II, while that of C20:4 n-6 was highest in samples from meadow II and from wasteland I and II. The content of C20:5

n-3 was highest in earthworms from meadows I and II, while that of C22:2 n-6 was highest in samples from the buckwheat field and meadow II. The lowest concentrations of PUFAs C18:2 n-6c + C18:2 n-6t, C18:3 n-3, C20:3 n-3, C20:4 n-6, C20:5 n-3 and C22:2 n-6 were observed in earthworms from sites with long-term exposure to waste rock, i.e., the buckwheat field (WR > 10 years) and wasteland I (WR > 10 years). In addition, the content of C18:2 n-6c + C18:2 n-6t was low in earthworms from the oat field (WR = 2 years) (Table 6, Figure 2A). In earthworms from locations with waste rock, the peroxidation index (PI) and unsaturation index (UI) were lower than in the control, i.e., earthworms from locations without waste rock. The highest PI was noted in earthworms from meadow II (−WR), and the highest UI in those from meadow I (−WR). The values of both indices were lowest in earthworms from the wasteland I with a long-term deposit of waste rock (WR > 10 years) (Table 6, Figure 2B). These findings confirm that organisms from the habitats subjected to long-term exposure to waste rock (WR > 10 years) had the most unfavourable physiological response in terms of peroxidation of FAs, whereas deposits of shorter duration resulted in improvement of these parameters in annelids.

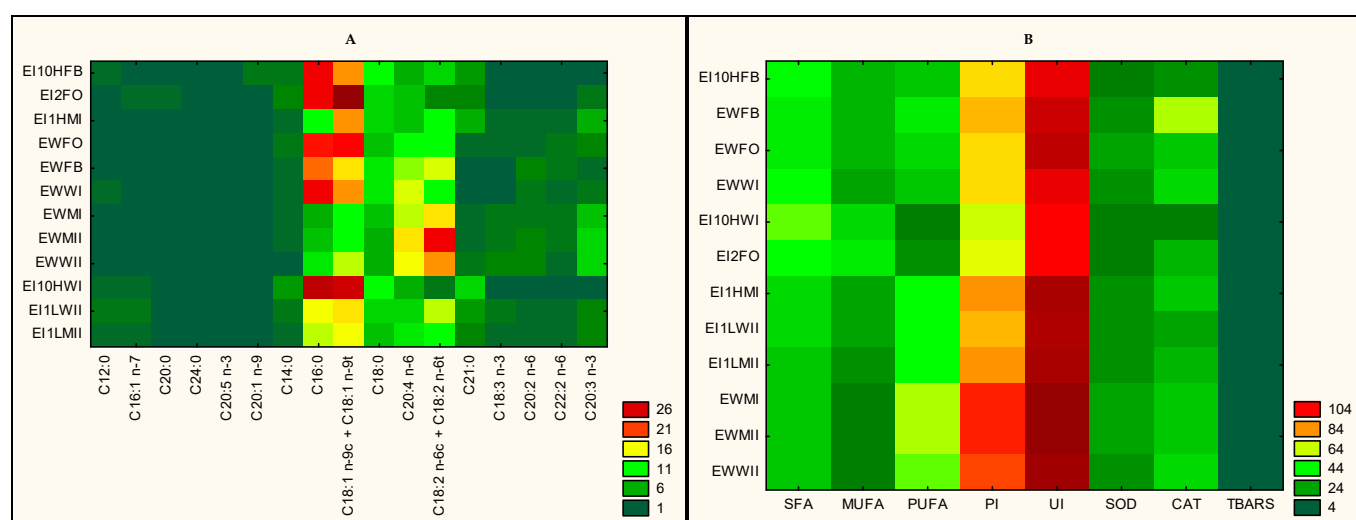


**Figure 2.** Heat map based on the analysis of the content of: (A) biomarker FAs; (B) SFA, MUFA, PUFA, PI, UI, SOD, CAT and TBARS in earthworms obtained from sites with and without waste rock deposits. Groups: EI10HFB—WR deposit > 10 years, buckwheat field; EWFB—without WR deposit, buckwheat field; EI10HWI—WR deposit > 10 years, wasteland I; EWWI—without WR deposit, wasteland I; EI2FO—WR deposit = 2 years, oat field; EWFO—without WR deposit, oat field; EI1HMI—WR deposit > 1 year, meadow I; EWMII—without WR deposit, meadow I; EI1LWII—WR deposit < 1 year, wasteland II; EWWII—without WR deposit, wasteland II; EI1LMII—WR deposit < 1 year, meadow II; EWMII—without WR deposit, meadow II. n = 60. Parameters: biomarker FAs [%]: C12:0—lauric acid; C14:0—myristic acid; C16:0—palmitic acid; C18:0—stearic acid; C20:0—arachidic acid, eicosanoic acid; C21:0—heneicosanoic acid; C24:0—lignoceric acid, tetracosanoic acid; C16:1 n-7—palmitoleic acid; C18:1 n-9c + C18:1 n-9t—oleic acid + elaidic acid; C20:1 n-9—cis-11-eicosenic acid; C18:2 n-6c + C18:2 n-6t—linoleic acid + linolelaidic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6—cis-11-14-eicosadienic acid; C20:3 n-3—eicosatrienic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenic acid, EPA; C22:2 n-6—cis-13-16-docosadienic acid. SFA [%]—saturated fatty acids, MUFA [%]—monounsaturated fatty acids; PUFA [%]—polyunsaturated fatty acids. PI—peroxidation index; UI—unsaturation index. SOD [Unit/mg protein]—superoxide dismutase; CAT [ $\mu\text{mol H}_2\text{O}_2/\text{mg protein}/\text{min}$ ]—catalase; TBARS [nM of MDA/mg of tissue]—thiobarbituric acid reactive substances.

**Table 6.** Biomarker fatty acids, peroxidation and unsaturation indices, enzymatic and non-enzymatic biomarkers of oxidative stress analyzed in earthworms obtained from sites with and without waste rock deposits.

| Parameters + CI              | Groups                           |                                  |                                 |                                  |                                  |                                  |                                  |                                 |                                   |                                  |                                  |                                 | SEM  |
|------------------------------|----------------------------------|----------------------------------|---------------------------------|----------------------------------|----------------------------------|----------------------------------|----------------------------------|---------------------------------|-----------------------------------|----------------------------------|----------------------------------|---------------------------------|------|
|                              | EI10HFB                          | EWFB                             | EI10HWI                         | EWWI                             | EI2FO                            | EWFO                             | EI1HMI                           | EWMI                            | EI1LWII                           | EWII                             | EI1LMII                          | EWMI                            |      |
| C12:0 [CI]                   | 1.0 <sup>bcd</sup><br>0.9–1.1    | 0.6 <sup>a</sup><br>0.4–0.8      | 1.2 <sup>d</sup><br>0.8–1.7     | 1.1 <sup>cd</sup><br>0.9–1.2     | 0.8 <sup>abc</sup><br>0.7–0.9    | 0.6 <sup>ab</sup><br>0.6–0.7     | 0.8 <sup>abc</sup><br>0.7–0.9    | 0.5 <sup>a</sup><br>0.3–0.7     | 2.7 <sup>f</sup><br>2.6–2.9       | 0.7 <sup>abc</sup><br>0.5–1.0    | 2.0 <sup>e</sup><br>1.8–2.1      | 0.5 <sup>a</sup><br>0.4–0.6     | 0.09 |
| C14:0 [CI]                   | 2.1 <sup>abc</sup><br>1.9–2.3    | 1.8 <sup>ab</sup><br>1.8–1.9     | 4.5 <sup>d</sup><br>2.5–6.3     | 1.1 <sup>ab</sup><br>0.3–3.3     | 3.9 <sup>cd</sup><br>3.2–4.4     | 2.8 <sup>bcd</sup><br>2.1–3.5    | 2.0 <sup>ab</sup><br>1.7–2.3     | 1.4 <sup>ab</sup><br>1.1–1.6    | 2.1 <sup>abc</sup><br>1.9–2.2     | 0.9 <sup>a</sup><br>0.7–1.1      | 1.5 <sup>ab</sup><br>1.4–1.7     | 1.1 <sup>ab</sup><br>0.9–1.4    | 0.16 |
| C16:0 [CI]                   | 24.8 <sup>g</sup><br>21.4–28.1   | 19.8 <sup>e</sup><br>18.7–20.3   | 27.2 <sup>g</sup><br>25.2–29.2  | 24.4 <sup>fg</sup><br>23.6–25.3  | 24.8 <sup>g</sup><br>23.2–26.4   | 21.1 <sup>ef</sup><br>19.2–23.1  | 10.7 <sup>bc</sup><br>8.4–13.0   | 5.7 <sup>a</sup><br>4.8–6.7     | 16.0 <sup>d</sup><br>14.5–17.4    | 8.2 <sup>ab</sup><br>6.6–9.7     | 14.0 <sup>cd</sup><br>12.9–15.0  | 6.0 <sup>a</sup><br>5.1–6.9     | 0.99 |
| C18:0 [CI]                   | 9.0 <sup>fg</sup><br>8.0–10.0    | 8.1 <sup>defg</sup><br>7.9–8.2   | 9.3 <sup>g</sup><br>8.4–10.1    | 8.3 <sup>efg</sup><br>7.9–8.7    | 7.7 <sup>bcd</sup><br>6.7–8.8    | 6.8 <sup>abcd</sup><br>6.3–7.3   | 7.8 <sup>cdef</sup><br>6.8–8.7   | 6.5 <sup>ab</sup><br>6.0–7.0    | 7.1 <sup>abcde</sup><br>6.9–7.2   | 6.0 <sup>a</sup><br>5.7–6.2      | 6.8 <sup>abc</sup><br>6.2–7.3    | 6.0 <sup>a</sup><br>5.7–6.2     | 0.15 |
| C20:0 [CI]                   | 0.6 <sup>bcd</sup><br>0.4–0.7    | 0.4 <sup>abc</sup><br>0.4–0.4    | 1.0 <sup>e</sup><br>0.8–1.1     | 0.7 <sup>cde</sup><br>0.6–0.8    | 1.0 <sup>e</sup><br>0.6–1.4      | 0.8 <sup>de</sup><br>0.7–1.0     | 0.7 <sup>cde</sup><br>0.5–1.0    | 0.2 <sup>a</sup><br>0.1–0.2     | 0.4 <sup>abc</sup><br>0.4–0.5     | 0.3 <sup>ab</sup><br>0.2–0.3     | 0.2 <sup>ab</sup><br>0.2–0.4     | 0.1 <sup>a</sup><br>0.1–0.2     | 0.04 |
| C21:0 [CI]                   | 4.8 <sup>e</sup><br>3.9–5.7      | 0.8 <sup>ab</sup><br>0.7–0.9     | 7.6 <sup>f</sup><br>6.3–9.0     | 0.7 <sup>a</sup><br>0.6–0.9      | 3.3 <sup>d</sup><br>2.7–3.9      | 1.4 <sup>abc</sup><br>0.9–1.9    | 5.5 <sup>e</sup><br>4.7–6.3      | 2.0 <sup>bc</sup><br>1.7–2.3    | 4.8 <sup>e</sup><br>4.5–5.2       | 2.3 <sup>cd</sup><br>2.1–2.6     | 3.4 <sup>d</sup><br>2.9–3.9      | 1.8 <sup>abc</sup><br>1.5–2.0   | 0.27 |
| C24:0 [CI]                   | 0.6 <sup>bc</sup><br>0.3–1.0     | 0.3 <sup>a</sup><br>0.2–0.3      | 0.8 <sup>c</sup><br>0.7–0.9     | 0.4 <sup>ab</sup><br>0.3–0.6     | 0.6 <sup>bc</sup><br>0.4–0.8     | 0.3 <sup>a</sup><br>0.2–0.4      | 0.4 <sup>ab</sup><br>0.3–0.5     | 0.2 <sup>a</sup><br>0.1–0.3     | 0.3 <sup>a</sup><br>0.3–0.4       | 0.2 <sup>a</sup><br>0.2–0.3      | 0.3 <sup>a</sup><br>0.2–0.4      | 0.2 <sup>a</sup><br>0.2–0.2     | 0.03 |
| SFA [CI]                     | 42.2 <sup>bcd</sup><br>34.9–49.5 | 37.0 <sup>abc</sup><br>33.7–40.3 | 52.1 <sup>e</sup><br>46.5–57.7  | 46.8 <sup>de</sup><br>41.2–51.8  | 45.1 <sup>cde</sup><br>41.3–48.9 | 36.8 <sup>abc</sup><br>31.5–42.2 | 33.3 <sup>a</sup><br>28.5–38.0   | 29.8 <sup>a</sup><br>27.1–32.6  | 35.5 <sup>ab</sup><br>32.8–38.1   | 28.5 <sup>a</sup><br>24.9–32.1   | 31.1 <sup>a</sup><br>28.4–33.9   | 28.4 <sup>a</sup><br>26.1–30.1  | 1.05 |
| C16:1 n–7 [CI]               | 0.8 <sup>bc</sup><br>0.6–1.0     | 0.3 <sup>a</sup><br>1.2–1.4      | 1.6 <sup>d</sup><br>1.4–1.8     | 1.0 <sup>bc</sup><br>0.8–1.1     | 1.6 <sup>d</sup><br>1.4–1.8      | 1.0 <sup>bc</sup><br>0.7–1.2     | 1.0 <sup>bc</sup><br>0.7–1.2     | 0.7 <sup>ab</sup><br>0.5–0.8    | 2.2 <sup>e</sup><br>1.9–2.5       | 0.8 <sup>bc</sup><br>0.6–1.1     | 1.1 <sup>c</sup><br>0.9–1.2      | 0.7 <sup>bc</sup><br>0.6–0.9    | 0.07 |
| C18:1 n–9c + C18:1 n–9t [CI] | 18.6 <sup>c</sup><br>17.2–19.9   | 16.2 <sup>bc</sup><br>15.4–17.0  | 26.8 <sup>d</sup><br>23.0–30.5  | 18.4 <sup>c</sup><br>16.1–20.9   | 31.5 <sup>e</sup><br>28.3–34.8   | 23.7 <sup>d</sup><br>20.6–26.8   | 18.5 <sup>c</sup><br>15.5–21.6   | 11.1 <sup>a</sup><br>9.0–13.1   | 16.8 <sup>bc</sup><br>15.5–18.2   | 13.7 <sup>ab</sup><br>13.7–14.7  | 15.4 <sup>abc</sup><br>14.2–16.6 | 11.3 <sup>a</sup><br>9.8–12.8   | 0.79 |
| C20:1 n–9 [CI]               | 2.4 <sup>d</sup><br>1.7–3.1      | 0.4 <sup>ab</sup><br>0.2–0.5     | 0.8 <sup>bc</sup><br>0.4–1.0    | 0.2 <sup>a</sup><br>0.2–0.3      | 0.7 <sup>abc</sup><br>0.5–0.9    | 0.3 <sup>ab</sup><br>0.2–0.5     | 0.8 <sup>abc</sup><br>0.5–1.0    | 0.4 <sup>ab</sup><br>0.2–0.5    | 0.9 <sup>c</sup><br>0.7–1.1       | 0.3 <sup>ab</sup><br>0.3–0.4     | 0.6 <sup>abc</sup><br>0.5–0.8    | 0.4 <sup>ab</sup><br>0.3–0.5    | 0.08 |
| MUFA [CI]                    | 27.8 <sup>ef</sup><br>26.8–28.8  | 26.7 <sup>de</sup><br>19.7–33.6  | 33.8 <sup>fg</sup><br>29.3–38.3 | 21.7 <sup>cde</sup><br>17.8–25.5 | 36.8 <sup>g</sup><br>32.9–40.7   | 27.8 <sup>ef</sup><br>24.1–31.4  | 20.6 <sup>bcd</sup><br>17.7–23.4 | 13.4 <sup>a</sup><br>11.2–15.7  | 20.2 <sup>abcd</sup><br>18.4–21.9 | 15.9 <sup>abc</sup><br>14.7–17.2 | 17.3 <sup>abc</sup><br>15.7–18.8 | 13.8 <sup>ab</sup><br>12.5–15.1 | 1.00 |
| C18:2 n–6c + C18:2 n–6t [CI] | 7.4 <sup>bc</sup><br>5.9–8.9     | 14.9 <sup>efg</sup><br>13.4–16.3 | 2.4 <sup>a</sup><br>2.1–2.8     | 9.3 <sup>cd</sup><br>7.9–10.6    | 3.2 <sup>ab</sup><br>2.3–4.1     | 11.5 <sup>cde</sup><br>9.3–13.8  | 11.8 <sup>ed</sup><br>7.7–15.8   | 16.7 <sup>fg</sup><br>14.8–18.5 | 14.0 <sup>ef</sup><br>12.9–15.1   | 18.6 <sup>g</sup><br>15.8–21.3   | 11.8 <sup>ed</sup><br>9.5–14.0   | 24.4 <sup>h</sup><br>21.7–27.1  | 0.80 |
| C18:3 n–3 [CI]               | 0.4 <sup>a</sup><br>0.3–0.4      | 0.7 <sup>ab</sup><br>0.6–0.7     | 0.4 <sup>a</sup><br>0.3–0.6     | 0.8 <sup>ab</sup><br>0.6–1.1     | 0.5 <sup>ab</sup><br>0.3–0.8     | 1.9 <sup>c</sup><br>1.5–2.2      | 1.1 <sup>b</sup><br>0.8–1.4      | 2.7 <sup>d</sup><br>2.1–3.2     | 2.3 <sup>cd</sup><br>1.9–2.6      | 3.5 <sup>e</sup><br>3.3–3.8      | 1.9 <sup>c</sup><br>1.5–2.2      | 2.7 <sup>d</sup><br>2.5–3.0     | 0.14 |
| C20:2 n–6 [CI]               | 0.4 <sup>a</sup><br>0.3–0.5      | 3.2 <sup>d</sup><br>2.4–4.1      | 0.3 <sup>a</sup><br>0.2–0.5     | 2.6 <sup>d</sup><br>2.6–3.0      | 0.7 <sup>ab</sup><br>0.5–1.0     | 1.5 <sup>bc</sup><br>1.3–1.7     | 1.1 <sup>abc</sup><br>0.8–1.4    | 2.8 <sup>d</sup><br>2.4–3.3     | 1.3 <sup>bc</sup><br>1.1–1.5      | 3.2 <sup>d</sup><br>2.9–3.5      | 1.8 <sup>c</sup><br>1.4–2.2      | 3.0 <sup>d</sup><br>2.7–3.4     | 0.14 |

Table 6. Cont.

| Parameters + CI   |                                   | Groups                            |                                |                                   |                                 |                                   |                                   |                                   |                                    |                                    |                                    |                                   | SEM  |
|-------------------|-----------------------------------|-----------------------------------|--------------------------------|-----------------------------------|---------------------------------|-----------------------------------|-----------------------------------|-----------------------------------|------------------------------------|------------------------------------|------------------------------------|-----------------------------------|------|
|                   |                                   | EI10HFB                           | EWFB                           | EI10HWI                           | EWWI                            | EI2FO                             | EWFO                              | EI1HMI                            | EWMI                               | EI1LWII                            | EWVII                              | EI1LMII                           |      |
| C20:3 n—3<br>[CI] | 0.9 <sup>a</sup><br>0.6–1.1       | 1.8 <sup>b</sup><br>1.6–2.1       | 0.9 <sup>a</sup><br>0.7–1.1    | 2.2 <sup>b</sup><br>1.9–2.6       | 2.1 <sup>b</sup><br>1.5–2.7     | 3.1 <sup>c</sup><br>2.7–3.4       | 6.0 <sup>d</sup><br>5.7–6.2       | 6.2 <sup>d</sup><br>6.1–6.4       | 3.2 <sup>c</sup><br>3.0–3.4        | 7.1 <sup>e</sup><br>6.8–7.5        | 3.4 <sup>c</sup><br>3.2–3.7        | 7.2 <sup>e</sup><br>6.9–7.5       | 0.29 |
| C20:4 n—6<br>[CI] | 5.9 <sup>a</sup><br>4.7–7.1       | 12.8 <sup>cd</sup><br>10.8–14.8   | 5.0 <sup>a</sup><br>4.1–5.9    | 14.1 <sup>cde</sup><br>11.6–16.6  | 6.1 <sup>a</sup><br>5.7–6.5     | 11.0 <sup>bc</sup><br>9.2–12.8    | 6.9 <sup>a</sup><br>5.7–8.1       | 13.8 <sup>cde</sup><br>11.8–15.9  | 7.8 <sup>ab</sup><br>5.5–10.2      | 15.2 <sup>de</sup><br>13.2–17.2    | 8.0 <sup>ab</sup><br>6.1–10.0      | 16.9 <sup>e</sup><br>13.8–20.0    | 0.55 |
| C20:5 n—3<br>[CI] | 0.3 <sup>a</sup><br>0.2–0.4       | 0.5 <sup>abc</sup><br>0.4–0.6     | 0.3 <sup>a</sup><br>0.1–0.4    | 0.7 <sup>bc</sup><br>0.5–0.8      | 0.4 <sup>abc</sup><br>0.3–0.6   | 0.7 <sup>bc</sup><br>0.6–0.7      | 0.4 <sup>ab</sup><br>0.2—0.6      | 0.7 <sup>c</sup><br>0.6–0.9       | 0.4 <sup>abc</sup><br>0.2–0.6      | 0.6 <sup>bc</sup><br>0.5–0.8       | 0.5 <sup>abc</sup><br>0.4–0.7      | 0.7 <sup>c</sup><br>0.6–0.9       | 0.02 |
| C22:2 n—6<br>[CI] | 0.7 <sup>a</sup><br>0.4–0.9       | 2.4 <sup>e</sup><br>2.0–2.8       | 0.5 <sup>a</sup><br>0.4–0.7    | 1.9 <sup>cde</sup><br>1.6–2.2     | 0.8 <sup>ab</sup><br>0.5–1.2    | 2.0 <sup>cde</sup><br>1.6–2.4     | 1.0 <sup>ab</sup><br>0.8–1.2      | 2.1 <sup>de</sup><br>1.7–2.6      | 1.4 <sup>bc</sup><br>1.1–1.7       | 1.9 <sup>cde</sup><br>1.6–2.2      | 1.5 <sup>bcd</sup><br>1.2–1.8      | 2.2 <sup>e</sup><br>1.8–2.7       | 0.09 |
| PUFA<br>[CI]      | 30.0 <sup>abc</sup><br>24.4–35.7  | 36.3 <sup>abc</sup><br>14.4–58.3  | 14.1 <sup>a</sup><br>60.1–65.7 | 31.5 <sup>abc</sup><br>12.1–51.0  | 18.1 <sup>ab</sup><br>9.2–27.1  | 35.4 <sup>abc</sup><br>18.7–52.1  | 46.2 <sup>abc</sup><br>31.2–61.2  | 56.7 <sup>c</sup><br>32.4–80.1    | 44.4 <sup>abc</sup><br>25.2–53.5   | 55.4 <sup>c</sup><br>37.1–73.6     | 51.6 <sup>bc</sup><br>36.6–66.7    | 57.8 <sup>c</sup><br>33.4–82.2    | 2.45 |
| PI<br>[CI]        | 72.8 <sup>b</sup><br>71.3–74.2    | 76.5 <sup>bc</sup><br>75.1–78.0   | 62.9 <sup>a</sup><br>93.1–99.7 | 73.8 <sup>b</sup><br>71.8–75.7    | 64.9 <sup>a</sup><br>62.5–67.3  | 75.9 <sup>bc</sup><br>73.8–77.1   | 80.7 <sup>cd</sup><br>77.9–83.5   | 92.2 <sup>e</sup><br>90.0–94.6    | 79.0 <sup>cd</sup><br>76.1–81.8    | 89.8 <sup>e</sup><br>86.8–92.7     | 83.0 <sup>d</sup><br>81.0–85.0     | 94.3 <sup>e</sup><br>91.3–97.3    | 1.26 |
| UI<br>[CI]        | 108.8 <sup>b</sup><br>105.9–111.7 | 119.9 <sup>c</sup><br>116.8–123.0 | 96.4 <sup>a</sup><br>93.1–99.7 | 111.7 <sup>b</sup><br>108.4–115.0 | 99.7 <sup>a</sup><br>94.9–104.4 | 120.7 <sup>c</sup><br>116.6–124.7 | 128.4 <sup>d</sup><br>126.0–130.9 | 139.7 <sup>f</sup><br>126.9–142.6 | 125.8 <sup>cd</sup><br>123.5–128.1 | 135.2 <sup>ef</sup><br>132.7–137.7 | 130.7 <sup>de</sup><br>128.6–132.8 | 139.1 <sup>f</sup><br>137.5–140.7 | 1.85 |
| SOD<br>[CI]       | 13.3 <sup>a</sup><br>12.4–14.1    | 19.6 <sup>c</sup><br>17.9–21.2    | 13.8 <sup>a</sup><br>13.014.7  | 18.8 <sup>c</sup><br>17.2–20.3    | 15.5 <sup>ab</sup><br>14.3–16.3 | 22.9 <sup>d</sup><br>21.3–24.6    | 18.9 <sup>c</sup><br>18.7–19.1    | 22.6 <sup>d</sup><br>21.5–23.8    | 17.9 <sup>bc</sup><br>17.1–18.8    | 18.4 <sup>c</sup><br>16.6–20.2     | 19.8 <sup>c</sup><br>18.5–21.2     | 23.4 <sup>d</sup><br>22.2–24.6    | 0.43 |
| CAT<br>[CI]       | 16.9 <sup>a</sup><br>14.2–19.5    | 56.3 <sup>f</sup><br>52.4–60.2    | 13.1 <sup>a</sup><br>11.7–14.5 | 34.2 <sup>e</sup><br>31.0–37.4    | 24.7 <sup>bc</sup><br>23.2–26.1 | 31.7 <sup>de</sup><br>30.1–33.4   | 29.3 <sup>cde</sup><br>24.6–34.1  | 31.2 <sup>de</sup><br>28.0–34.4   | 23.4 <sup>b</sup><br>22.3–24.6     | 34.9 <sup>e</sup><br>32.1–38.8     | 26.6 <sup>bcd</sup><br>24.0–29.3   | 31.8 <sup>de</sup><br>29.4–34.2   | 1.37 |
| TBARS<br>[CI]     | 1.6 <sup>e</sup><br>1.6–1.7       | 0.8 <sup>bc</sup><br>0.7–0.8      | 1.7 <sup>e</sup><br>1.6–1.9    | 0.8 <sup>bc</sup><br>0.8–0.9      | 1.0 <sup>cd</sup><br>0.8–1.1    | 0.6 <sup>ab</sup><br>0.5–0.7      | 1.1 <sup>d</sup><br>0.8–1.5       | 0.5 <sup>a</sup><br>0.4–0.5       | 1.0 <sup>cd</sup><br>0.8–1.7       | 0.8 <sup>bc</sup><br>0.7–0.8       | 0.8 <sup>bc</sup><br>0.8–0.8       | 0.5 <sup>a</sup><br>0.4–0.5       | 0.05 |
| Interactions      | WR                                | *                                 | *                              | *                                 | *                               | *                                 | *                                 | *                                 | *                                  | *                                  | *                                  | *                                 | *    |
|                   | S                                 | *                                 | *                              | *                                 | *                               | *                                 | *                                 | *                                 | *                                  | *                                  | *                                  | *                                 | *    |
|                   | WRxS                              | *                                 | *                              | *                                 | *                               | *                                 | *                                 | *                                 | *                                  | *                                  | *                                  | *                                 | *    |

Groups: EI10HFB—WR deposit > 10 years, buckwheat field; EWFB—without WR deposit, buckwheat field; EI10HWI—WR deposit > 10 years, wasteland I; EWWI—without WR deposit, wasteland I; EI2FO—WR deposit = 2 years, oat field; EWFO—without WR deposit, oat field; EI1HMI—WR deposit > 1 year, meadow I; EWMI—without WR deposit, meadow I; EI1LWII—WR deposit < 1 year, wasteland II; EWWII—without WR deposit, wasteland II; EI1LMII—WR deposit < 1 year, meadow II; EWMII—without WR deposit, meadow II. n = 60. Parameters: biomarker FAs [%]: C12:0—lauric acid; C14:0—myristic acid; C16:0—palmitic acid; C18:0—stearic acid; C20:0—arachidic acid, eicosanoic acid; C21:0—heneicosanoic acid; C24:0—lignoceric acid, tetracosanoic acid; C16:1 n–7—palmitoleic acid; C18:1 n–9c + C18:1 n–9t—oleic acid + elaidic acid; C20:1 n–9—cis-11-eicosenoic acid; C18:2 n–6c + C18:2 n–6t—linoleic acid + linolelaidic acid; C18:3 n–3— $\alpha$ -linolenic acid; C20:2 n–6—cis-11-14-eicosadienic acid; C20:3 n–3—eicosatrienic acid; C20:4 n–6—arachidonic acid; C20:5 n–3—cis-5,8,11,14,17-eicosapentaenic acid, EPA; C22:2 n–6—cis-13-16-docosadienic acid. SFA [%]—saturated fatty acids, MUFA [%]—monounsaturated fatty acids; PUFA [%]—polyunsaturated fatty acids. PI—peroxidation index; UI—unsaturation index. SOD [Unit/mg protein]—superoxide dismutase; CAT [ $\mu$ mol H<sub>2</sub>O<sub>2</sub>/mg protein/min]—catalase; TBARS [nM of MDA/mg of tissue]—thiobarbituric acid reactive substances. CI—95% confidence interval (lower–upper); SEM—standard error of mean; WR—waste rock; S—sampling sites; WRxS—interaction of waste rock with the sampling site; <sup>a–h</sup>—values in rows with different letters differ significantly at the level  $p \leq 0.01$ ; \* significant at  $p \leq 0.01$ .

To assess the usefulness of the biomarker FAs in earthworms, the activity of antioxidant enzymes—superoxide dismutase (SOD) and catalase (CAT)—was determined, as well as the level of lipid peroxidation end products (TBARS). SOD and CAT activity was reduced in all samples of earthworms from the sites with waste rock, accompanied by an increased TBARS level, in comparison with organisms from sites without waste rock. SOD activity was highest in earthworms from meadow II (−WR) and lowest in those from the buckwheat field (WR > 10 years) and wasteland I (WR > 10 years). CAT activity was highest in samples from the buckwheat field (−WR) and lowest in those from wasteland I with a long-term waste rock deposit (WR > 10 years). The TBARS value was highest in earthworms from wasteland I (WR > 10 years) and lowest in those from meadow I and meadow II (−WR) (Table 6, Figure 2B). The content of the analyzed biomarkers FAs, SFA, MUFA, PUFA, PI and UI indices, SOD, CAT and TBARS was influenced ( $p \leq 0.01$ ) by all single factors (WR,S), as well as the interaction between them (WRxS)

### 3.3. Pearson's Correlation

Pearson's correlation analysis was conducted between metals (Pb, Cd, Cr, Ba, Ni, Zn, Cu) and analyzed biomarker FAs, peroxidation (PI) and unsaturation (UI) indices, enzymatic (SOD, CAT) and non-enzymatic (TBARS) biomarkers of oxidative stress in earthworms obtained from sites with (Table 7) and without (Table 8) waste rock deposits.

**Table 7.** Pearson's correlation coefficient (r) between metals (Pb, Cd, Cr, Ba, Ni, Zn, Cu) and analyzed biomarker FAs, peroxidation (PI) and unsaturation (UI) indices, enzymatic (SOD, CAT) and non-enzymatic (TBARS) biomarkers of oxidative stress in earthworms obtained from sites with waste rock deposits.

|                                    | Pb                      | Cd                      | Cr                    | Ba                    | Ni                    | Zn                      | Cu                    |
|------------------------------------|-------------------------|-------------------------|-----------------------|-----------------------|-----------------------|-------------------------|-----------------------|
| <b>C12:0</b>                       | 0.3890 *<br>moderate    | 0.0663<br>negligible    | −0.0007<br>negligible | 0.2654<br>weak        | 0.1775<br>weak        | −0.0193<br>negligible   | 0.1773<br>weak        |
| <b>C14:0</b>                       | 0.5423 *<br>strong      | 0.6091 *<br>strong      | 0.0577<br>negligible  | −0.3199<br>moderate   | −0.2122<br>weak       | 0.3808 *<br>moderate    | 0.1788<br>weak        |
| <b>C16:0</b>                       | 0.6118 *<br>strong      | 0.6554 *<br>strong      | −0.1539<br>weak       | −0.4808 *<br>moderate | −0.4707 *<br>moderate | 0.5083 *<br>strong      | 0.0297<br>negligible  |
| <b>C18:0</b>                       | 0.6005 *<br>strong      | 0.6693 *<br>strong      | 0.2994<br>weak        | −0.1565<br>weak       | −0.0531<br>negligible | 0.7102 *<br>very strong | 0.4106 *<br>moderate  |
| <b>C20:0</b>                       | 0.4632 *<br>moderate    | 0.6007 *<br>strong      | 0.0943<br>negligible  | −0.3404 *<br>moderate | −0.3544 *<br>moderate | 0.5647 *<br>strong      | 0.0478<br>negligible  |
| <b>C21:0</b>                       | 0.6298 *<br>strong      | 0.4764 *<br>moderate    | 0.6133 *<br>strong    | 0.3661 *<br>moderate  | 0.3758 *<br>moderate  | 0.5082 *<br>strong      | 0.6990 *<br>strong    |
| <b>C24:0</b>                       | 0.7112 *<br>very strong | 0.6797 *<br>strong      | 0.2858<br>weak        | −0.1244<br>weak       | −0.1379<br>weak       | 0.6503 *<br>strong      | 0.4719 *<br>moderate  |
| <b>SFA</b>                         | 0.6941 *<br>strong      | 0.8242 *<br>very strong | 0.0105<br>negligible  | −0.4497 *<br>moderate | −0.3921 *<br>moderate | 0.6662 *<br>strong      | 0.1997<br>weak        |
| <b>C16:1 n−7</b>                   | 0.5759 *<br>strong      | 0.2840<br>weak          | 0.0355<br>negligible  | 0.0467<br>negligible  | −0.1015<br>weak       | 0.1659<br>weak          | 0.1615<br>weak        |
| <b>C18:1 n−9c +<br/>C18:1 n−9t</b> | 0.4989 *<br>moderate    | 0.5261 *<br>strong      | −0.0349<br>negligible | −0.4112 *<br>moderate | −0.4117 *<br>moderate | 0.4000 *<br>moderate    | −0.0488<br>negligible |
| <b>C20:1 n−9</b>                   | 0.4171 *<br>moderate    | 0.1512<br>weak          | 0.3584 *<br>moderate  | 0.3497 *<br>moderate  | 0.3384 *<br>moderate  | 0.1553<br>weak          | 0.4456 *<br>moderate  |
| <b>MUFA</b>                        | 0.5381 *<br>strong      | 0.5761 *<br>strong      | −0.0760<br>negligible | −0.4405 *<br>moderate | −0.4311 *<br>moderate | 0.3948 *<br>moderate    | −0.0090<br>negligible |

Table 7. Cont.

|                                | Pb                       | Cd                       | Cr                    | Ba                    | Ni                   | Zn                    | Cu                    |
|--------------------------------|--------------------------|--------------------------|-----------------------|-----------------------|----------------------|-----------------------|-----------------------|
| <b>C18:2 n–6c + C18:2 n–6t</b> | –0.6062 *<br>strong      | –0.6551 *<br>strong      | –0.1763<br>weak       | 0.2652<br>weak        | 0.2318<br>weak       | –0.6035 *<br>strong   | –0.2468<br>weak       |
| <b>C18:3 n–3</b>               | –0.3961 *<br>moderate    | –0.5514 *<br>strong      | –0.2366<br>weak       | 0.2718<br>weak        | 0.0695<br>negligible | –0.6683 *<br>strong   | –0.2536<br>weak       |
| <b>C20:2 n–6</b>               | –0.5832 *<br>strong      | –0.4221 *<br>moderate    | –0.4061 *<br>moderate | –0.1546<br>weak       | –0.1394<br>weak      | –0.4235 *<br>moderate | –0.4340 *<br>moderate |
| <b>C20:3 n–3</b>               | –0.5459 *<br>strong      | –0.5682 *<br>strong      | 0.1359<br>weak        | 0.4329 *<br>moderate  | 0.3069<br>moderate   | –0.4344 *<br>moderate | –0.0928<br>negligible |
| <b>C20:4 n–6</b>               | –0.5138 *<br>strong      | –0.3749 *<br>moderate    | –0.4203 *<br>moderate | –0.1919<br>weak       | –0.2052<br>weak      | –0.3982 *<br>moderate | –0.4175 *<br>moderate |
| <b>C20:5 n–3</b>               | –0.5936 *<br>strong      | –0.4037 *<br>moderate    | –0.3711 *<br>moderate | –0.1440<br>weak       | –0.1846<br>weak      | –0.4512 *<br>moderate | –0.4641 *<br>moderate |
| <b>C22:2 n–6</b>               | –0.6540 *<br>strong      | –0.4929 *<br>moderate    | –0.4704 *<br>moderate | –0.2271<br>weak       | –0.1448<br>weak      | –0.5314 *<br>strong   | –0.5315 *<br>strong   |
| <b>PUFA</b>                    | –0.3862 *<br>moderate    | –0.6185 *<br>strong      | 0.0257<br>negligible  | 0.3671 *<br>moderate  | 0.3318 *<br>moderate | –0.4514 *<br>moderate | –0.0698<br>negligible |
| <b>PI</b>                      | –0.6229 *<br>strong      | –0.6781 *<br>strong      | –0.0013<br>negligible | 0.3990 *<br>moderate  | 0.3757 *<br>moderate | –0.5845 *<br>strong   | –0.1122<br>weak       |
| <b>UI</b>                      | –0.6701 *<br>strong      | –0.7190 *<br>very strong | 0.0249<br>negligible  | 0.4405 *<br>moderate  | 0.4342 *<br>moderate | –0.6093 *<br>strong   | –0.1342<br>weak       |
| <b>SOD</b>                     | –0.7633 *<br>very strong | –0.5480 *<br>strong      | –0.2233<br>weak       | –0.0612<br>negligible | 0.1156<br>weak       | –0.5072 *<br>strong   | –0.3934 *<br>moderate |
| <b>CAT</b>                     | –0.5958 *<br>strong      | –0.4376 *<br>moderate    | –0.3692 *<br>moderate | –0.1992<br>weak       | –0.2099<br>weak      | –0.3471 *<br>moderate | –0.5437 *<br>strong   |
| <b>TBARS</b>                   | 0.7206 *<br>very strong  | 0.6463 *<br>strong       | 0.4280 *<br>moderate  | 0.1529<br>weak        | 0.1107<br>weak       | 0.6502 *<br>strong    | 0.5912 *<br>strong    |

C12:0—lauric acid; C14:0—myristic acid; C16:0—palmitic acid; C18:0—stearic acid; C20:0—arachidic acid, eicosanoic acid; C21:0—heneicosanoic acid; C24:0—lignoceric acid, tetracosanoic acid; C16:1 n–7—palmitoleic acid; C18:1 n–9c + C18:1 n–9t—oleic acid + elaidic acid; C20:1 n–9—cis-11-eicosenic acid; C18:2 n–6c + C18:2 n–6t—linoleic acid + linolelaidic acid; C18:3 n–3— $\alpha$ -linolenic acid; C20:2 n–6—cis-11-14-eicosadienic acid; C20:3 n–3—eicosatrienic acid; C20:4 n–6—arachidonic acid; C20:5 n–3—cis-5,8,11,14,17-eicosapentaenic acid, EPA; C22:2 n–6—cis-13-16-docosadienic acid; SFA—saturated fatty acids, MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index; SOD—superoxide dismutase; CAT—catalase; TBARS—thiobarbituric acid reactive substances; n = 30; \* significant at  $p \leq 0.01$ .

**Table 8.** Pearson’s correlation coefficient (r) between metals (Pb, Cd, Cr, Ba, Ni, Zn, Cu) and analyzed biomarker FAs, peroxidation (PI) and unsaturation (UI) indices, enzymatic (SOD, CAT) and non-enzymatic (TBARS) biomarkers of oxidative stress in earthworms obtained from sites without waste rock deposits.

|              | Pb                      | Cd                 | Cr                  | Ba                       | Ni                       | Zn                      | Cu                    |
|--------------|-------------------------|--------------------|---------------------|--------------------------|--------------------------|-------------------------|-----------------------|
| <b>C12:0</b> | 0.7165 *<br>very strong | 0.6165 *<br>strong | –0.1448<br>weak     | –0.2304<br>weak          | –0.5754 *<br>strong      | 0.6423 *<br>strong      | –0.0737<br>negligible |
| <b>C14:0</b> | –0.1872<br>weak         | 0.1917<br>weak     | –0.3305<br>moderate | –0.4120<br>moderate      | –0.1974<br>weak          | –0.1822<br>weak         | –0.3154<br>moderate   |
| <b>C16:0</b> | 0.3720<br>moderate      | 0.6245 *<br>strong | –0.5544 *<br>strong | –0.8450 *<br>very strong | –0.7225 *<br>very strong | 0.5674 *<br>strong      | –0.5100 *<br>strong   |
| <b>C18:0</b> | 0.3083<br>moderate      | 0.6316 *<br>strong | –0.2053<br>weak     | –0.7266 *<br>very strong | –0.3816<br>moderate      | 0.7325 *<br>very strong | –0.1656<br>weak       |
| <b>C20:0</b> | 0.3583<br>moderate      | 0.5446 *<br>strong | –0.5497 *<br>strong | –0.7228 *<br>very strong | –0.7421 *<br>very strong | 0.3933<br>moderate      | –0.4963 *<br>moderate |

Table 8. Cont.

|                                    | Pb                       | Cd                      | Cr                    | Ba                       | Ni                       | Zn                      | Cu                    |
|------------------------------------|--------------------------|-------------------------|-----------------------|--------------------------|--------------------------|-------------------------|-----------------------|
| <b>C21:0</b>                       | −0.1464<br>weak          | −0.5509 *<br>strong     | 0.2691<br>weak        | 0.7946 *<br>very strong  | 0.2911<br>weak           | −0.6538 *<br>strong     | 0.2014<br>weak        |
| <b>C24:0</b>                       | 0.4410<br>moderate       | 0.5652 *<br>strong      | −0.0123<br>negligible | −0.4781 *<br>moderate    | −0.4734 *<br>moderate    | 0.7543 *<br>very strong | −0.0628<br>negligible |
| <b>SFA</b>                         | 0.4376<br>moderate       | 0.8136 *<br>very strong | −0.2776<br>weak       | −0.7424 *<br>very strong | −0.5176 *<br>strong      | 0.7634 *<br>very strong | −0.2297<br>weak       |
| <b>C16:1 n−7</b>                   | 0.3165<br>moderate       | 0.1975<br>weak          | 0.0262<br>negligible  | 0.0405<br>negligible     | −0.3385<br>moderate      | 0.2054<br>weak          | 0.0266<br>negligible  |
| <b>C18:1 n−9c +<br/>C18:1 n−9t</b> | 0.2199<br>weak           | 0.3881<br>moderate      | −0.6156 *<br>strong   | −0.6950 *<br>strong      | −0.7230 *<br>very strong | 0.1711<br>weak          | −0.6619 *<br>strong   |
| <b>C20:1 n−9</b>                   | −0.3539<br>moderate      | −0.3852<br>moderate     | 0.1333<br>weak        | 0.0803<br>negligible     | 0.2376<br>weak           | −0.3860<br>moderate     | 0.0748<br>negligible  |
| <b>MUFA</b>                        | 0.0993<br>negligible     | 0.4147<br>moderate      | −0.6381 *<br>strong   | −0.7510 *<br>very strong | −0.6142 *<br>strong      | 0.1113<br>weak          | −0.6124 *<br>strong   |
| <b>C18:2 n−6c +<br/>C18:2 n−6t</b> | −0.4229<br>moderate      | −0.6337 *<br>strong     | 0.2862<br>weak        | 0.6775 *<br>strong       | 0.6158 *<br>strong       | −0.5369 *<br>strong     | 0.3185<br>moderate    |
| <b>C18:3 n−3</b>                   | −0.1199<br>weak          | −0.5308 *<br>strong     | 0.2854<br>weak        | 0.8425 *<br>very strong  | 0.2710<br>weak           | −0.6458 *<br>strong     | 0.2193<br>weak        |
| <b>C20:2 n−6</b>                   | 0.0262<br>negligible     | −0.0783<br>negligible   | 0.2798<br>weak        | 0.3509<br>moderate       | 0.3531<br>moderate       | 0.0524<br>negligible    | 0.3344<br>moderate    |
| <b>C20:3 n−3</b>                   | −0.1780<br>weak          | −0.5483 *<br>strong     | 0.4454<br>moderate    | 0.8820 *<br>very strong  | 0.5247 *<br>strong       | −0.5251 *<br>strong     | 0.4183<br>moderate    |
| <b>C20:4 n−6</b>                   | 0.1242<br>weak           | −0.1593<br>weak         | 0.2820<br>weak        | 0.4563<br>moderate       | 0.3495<br>moderate       | 0.0341<br>negligible    | 0.3388<br>moderate    |
| <b>C20:5 n−3</b>                   | −0.2069<br>weak          | −0.0139<br>negligible   | 0.2735<br>weak        | 0.3147<br>moderate       | 0.3039<br>moderate       | −0.0351<br>negligible   | 0.1591<br>weak        |
| <b>C22:2 n−6</b>                   | −0.4478<br>moderate      | −0.1844<br>weak         | 0.0657<br>negligible  | −0.1406<br>weak          | 0.2942<br>weak           | −0.0890<br>negligible   | 0.0248<br>negligible  |
| <b>PUFA</b>                        | −0.1535<br>weak          | −0.6050 *<br>strong     | 0.3274<br>moderate    | 0.4808 *<br>moderate     | 0.3697<br>moderate       | −0.3284<br>moderate     | 0.2210<br>weak        |
| <b>PI</b>                          | −0.3414<br>moderate      | −0.5888 *<br>strong     | 0.5556 *<br>strong    | 0.7966 *<br>very strong  | 0.6996 *<br>strong       | −0.5128 *<br>strong     | 0.5202 *<br>strong    |
| <b>UI</b>                          | −0.4632 *<br>moderate    | −0.6830 *<br>strong     | 0.5153 *<br>strong    | 0.7873 *<br>very strong  | 0.7123 *<br>very strong  | −0.6167 *<br>strong     | 0.4642 *<br>moderate  |
| <b>SOD</b>                         | −0.7414 *<br>very strong | −0.3476<br>moderate     | 0.2964<br>weak        | 0.0058<br>negligible     | 0.5492 *<br>strong       | −0.3516<br>moderate     | 0.2401<br>weak        |
| <b>CAT</b>                         | −0.0609<br>negligible    | −0.0119<br>negligible   | −0.3603<br>moderate   | −0.3239<br>moderate      | −0.1371<br>weak          | 0.0660<br>negligible    | −0.2586<br>weak       |
| <b>TBARS</b>                       | 0.7657 *<br>very strong  | 0.5712 *<br>strong      | −0.5169 *<br>strong   | −0.2889<br>weak          | −0.7934 *<br>very strong | 0.4795 *<br>moderate    | −0.4378<br>moderate   |

C12:0—lauric acid; C14:0—myristic acid; C16:0—palmitic acid; C18:0—stearic acid; C20:0—arachidic acid, eicosanoic acid; C21:0—heneicosanoic acid; C24:0—lignoceric acid, tetracosanoic acid; C16:1 n−7—palmitoleic acid; C18:1 n−9c + C18:1 n−9t—oleic acid + elaidic acid; C20:1 n−9—cis-11-eicosenic acid; C18:2 n−6c + C18:2 n−6t—linoleic acid + linolelaidic acid; C18:3 n−3— $\alpha$ -linolenic acid; C20:2 n−6—cis-11-14-eicosadienic acid; C20:3 n−3—eicosatrienic acid; C20:4 n−6—arachidonic acid; C20:5 n−3—cis-5,8,11,14,17-eicosapentaenic acid, EPA; C22:2 n−6—cis-13-16-docosadienic acid; SFA—saturated fatty acids, MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index; SOD—superoxide dismutase; CAT—catalase; TBARS—thiobarbituric acid reactive substances; n = 30; \* significant at  $p \leq 0.01$ .

Only Pb showed a statistically significant correlation with all analyzed parameters. Among the analyzed biomarker fatty acids, only C21:0 showed a positive, statistically significant correlation with all seven metals. For PUFA, PI, and UI, positive, statistically significant correlations were observed with Ba and Ni, while negative, statistically significant correlations were observed with lead (Pb, Cd, and Zn). An inverse relationship was observed for MUFA and PUFA, which showed an opposite trend. Antioxidant enzyme activity also showed significant correlations with metals. SOD was negatively, statistically significantly, correlated with Pb, Cd, Zn, and Cu, while CAT showed negative, statistically significant correlations with Pb, Cd, Cr, Zn, and Cu. TBARS was positively and statistically significantly correlated with Pb, Cd, Cr, Zn and Cu (Table 7).

Among the analyzed FA biomarkers, the highest number of statistically significant correlations with metals were shown by C16:0 and C20:0. PUFAs showed a positive, statistically significant correlation with Ba and a negative, statistically significant correlation with Cd. MUFAs were negatively, statistically significant correlated with Cr, Ba, Ni, and Cu. SFAs showed positive, statistically significant correlations with Cd and Zn, and negative, statistically significant correlations with Ba and Ni. PI and UI were positively, statistically significant correlated with Cr, Ba, Ni, and Cu. PI showed negative, significant correlations with Cd and Zn, while UI with Pb, Cd, and Zn. Among antioxidant enzymes, SOD was positively and statistically significantly correlated with Ni and negatively and statistically significantly correlated with Pb. TBARS showed positively and statistically significantly correlated with Pb, Cd, and Zn, and negatively and statistically significantly correlated with Cr and Ni. For CAT, no statistically significant correlations were found with any of the analyzed metals (Table 8).

The reliability of the results was confirmed using the Benjamini–Hochberg FDR correction, as the associations between Pb and fatty acid profiles as well as oxidative stress parameters remained statistically significant (Table 9). This indicates that they reflect genuine and biologically meaningful relationships.

**Table 9.** The full correlation matrix between Pb concentration and the other analyzed variables.

| Variable 1 | Variable 2                 | r       | p        | FDR (BH) Thresholds | Significant |
|------------|----------------------------|---------|----------|---------------------|-------------|
| Pb         | C12:0                      | −0.7633 | 0.0000 * | 0.0003              | **          |
| Pb         | C14:0                      | 0.7206  | 0.0000 * | 0.0006              | **          |
| Pb         | C16:0                      | 0.7112  | 0.0000 * | 0.0010              | **          |
| Pb         | C18:0                      | 0.6941  | 0.0000 * | 0.0013              | **          |
| Pb         | C20:0                      | 0.6727  | 0.0000 * | 0.0016              | **          |
| Pb         | C21:0                      | −0.6701 | 0.0000 * | 0.0019              | **          |
| Pb         | C24:0                      | −0.6540 | 0.0000 * | 0.0023              | **          |
| Pb         | SFA                        | 0.6298  | 0.0000 * | 0.0026              | **          |
| Pb         | C16:1 n−7                  | −0.6229 | 0.0000 * | 0.0029              | **          |
| Pb         | C18:1 n−9c +<br>C18:1 n−9t | 0.6118  | 0.0000 * | 0.0032              | **          |
| Pb         | C20:1 n−9                  | −0.6062 | 0.0000 * | 0.0035              | **          |
| Pb         | MUFA                       | 0.6005  | 0.0000 * | 0.0039              | **          |
| Pb         | C18:2 n−6c +<br>C18:2 n−6t | −0.5958 | 0.0000 * | 0.0042              | **          |
| Pb         | C18:3 n−3                  | −0.5936 | 0.0000 * | 0.0045              | **          |

Table 9. Cont.

| Variable 1 | Variable 2 | r       | p        | FDR (BH) Thresholds | Significant |
|------------|------------|---------|----------|---------------------|-------------|
| Pb         | C20:2 n–6  | −0.5832 | 0.0000 * | 0.0048              | **          |
| Pb         | C20:3 n–3  | 0.5759  | 0.0000 * | 0.0052              | **          |
| Pb         | C20:4 n–6  | −0.5459 | 0.0000 * | 0.0055              | **          |
| Pb         | C20:5 n–3  | 0.5423  | 0.0000 * | 0.0058              | **          |
| Pb         | C22:2 n–6  | 0.5381  | 0.0000 * | 0.0061              | **          |
| Pb         | PUFA       | −0.5138 | 0.0000 * | 0.0065              | **          |
| Pb         | PI         | 0.4989  | 0.0000 * | 0.0068              | **          |
| Pb         | UI         | 0.4768  | 0.0001 * | 0.0071              | **          |
| Pb         | SOD        | 0.4632  | 0.0002 * | 0.0074              | **          |
| Pb         | CAT        | 0.4171  | 0.0009 * | 0.0077              | **          |
| Pb         | TBARS      | 0.4052  | 0.0013 * | 0.0081              | **          |
| Pb         | CD         | −0.3961 | 0.0017 * | 0.0084              | **          |
| Pb         | Cr         | 0.3890  | 0.0021 * | 0.0087              | **          |
| Pb         | Ba         | −0.3862 | 0.0023 * | 0.0090              | **          |
| Pb         | Ni         | −0.1945 | 0.1363   | 0.0094              | -           |
| Pb         | Zn         | −0.0772 | 0.5579   | 0.0097              | -           |
| Pb         | Cu         | 0.0771  | 0.5582   | 0.0100              | -           |

\* Significance confirmed by Pearson's correlation analysis; \*\* Significance confirmed after Benjamini–Hochberg FDR correction; n = 60; significant at  $p \leq 0.01$ .

#### 4. Discussion

Ecotoxicological analyses of soil and animals most often make use of invertebrate organisms, especially Aschelminthes and gastropods [51]. Earthworms are constantly exposed to pollutants in the soil, via both ingestion and passive absorption through the cuticle, which predisposes them to assessment of the ecotoxicological role or impact of soil pollutants. Assessment of the environmental risk associated with soil pollutants is crucial to the protection of soil ecosystems. Earthworms are commonly used as model organisms in studies associated with ecotoxicology [20]. To determine the extent of the impact of potential pollutants on living organisms, widespread bioindicator organisms are currently sought as sources of economically, instrumentally, and constructively optimal biomarkers. Earthworms are a useful source of this type of biomarker, with a proposed novel panel of biomarker fatty acids: C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n–7; C18:1 n–9c + C18:1 n–9t; C20:1 n–9; C18:2 n–6c + C18:2 n–6t; C18:3 n–3; C20:2 n–6; C20:3 n–3; C20:4 n–6; C20:5 n–3; C22:2 n–6 and the peroxidation index (PI) and unsaturation index (UI).

Earthworms are an important element in the food chain because they connect underground and above-ground ecosystems [1,5,8]. Their presence in the food chain depends on various soil factors, such as fertilization, climate, plant species, and the interactions between these factors [52]. Metals are absorbed by soil due to its sorption properties [53]. Earthworms are sensitive to sub-threshold quantities of metals in the environment, which means that even minimal concentrations which do not cause acute toxicity or death can induce perceptible (by testing or visually *in vivo*) physiological changes, such as disturbances in the activity of antioxidant enzymes, indicative of the occurrence of oxidative stress. Upon entering the environment, metals (Cr, Ni, Ba, Pb, Zn, and Cu) present in waste rock are transferred to the soil, to root systems of plants, and to Lumbricidae repre-

sentatives. Despite the absence of Cd in waste rock, its presence in soil, plant roots, and earthworms suggests that it may originate from atmospheric deposition [54]. In the crop fields (buckwheat and oats) with deposited waste rock, the ability of earthworms to take up metals from the roots of annual plants was limited due to the short growing period; for this reason, the soil was the main source of these elements for invertebrates. In contrast, in the wasteland with a long-term deposit of waste rock, with poor vegetation, and in the meadows with this waste, plants were able to accumulate metals in their root tissue, which created a potential route of transfer of these elements to earthworms. The concentrations of metals present in the soil were nontoxic, as the organisms survived and the available amounts were not lethal to them.

Although bioaccumulation of metals in the food chain may not be harmful to earthworms, it induces detectable physiological responses, e.g., in the form of fatty acid peroxidation in these invertebrates and in organisms at higher trophic levels which feed on earthworms [5]. Absorption of metals by these organisms depends on numerous factors, such as the earthworm's species, habitat and choice of food; moreover, the speciation, bioavailability and mobility of metals are important factors in this process [5,55]. Metals can be processed in various ways depending on the defence strategies of organisms against their potential harmful effects. Depending on need, metals can either be retained or removed from an earthworm's body. Each earthworm species has its own unique method of protection against the negative effects of metals [23]. Earthworms are able to accumulate significant quantities of heavy metals, transferring them from areas with high concentrations to areas with a lower concentration [25,56]. On the other hand, they can restrict the mobility of metals by secreting mucus which binds soil and metal particles [56,57].

The main site of metal accumulation in earthworms is chloragogen tissue. Two routes of intracellular binding of metals in this tissue can be distinguished: pathway A and pathway B. Pathway A binds lead (Pb) and zinc (Zn) in insoluble form, replacing calcium (Ca), which leads to detoxification by accumulating and decreasing the availability of these metals at harmful concentrations. Pathway B has various functions depending on the metal concentration in the soil [25]. A key factor in determining the toxic effects of metals on organisms is metal-binding proteins, such as metallothioneins (MT), which play an important role in preventing the toxicity of metals. Two isoforms of metallothionein (MT1 and MT2) have been identified in earthworms; MT1 transports essential metals, such as Zn and Cu at nontoxic concentrations, while MT2 inactivates and detoxifies non-significant metals such as Cd and essential metals such as Zn when their concentrations exceed the toxic threshold [25,58,59]. However, long-term exposure to metals can lead to local adaptation, which can influence the responses of biomarkers in earthworms collected in the field in comparison to those bred in the laboratory [60]. Earthworms are capable of specific regulation of metal uptake [61]. Local adaptation and acclimatization can influence accumulation patterns, i.e., the means, rate, and level of accumulation of metals in their tissues, potentially leading to intraspecific differences [60,61]. Zn plays a dual role in the metabolism of earthworms of the family Lumbricidae, as a microelement and as a potential toxin at elevated concentrations. Whereas a low level of zinc is essential for various metabolic processes, supporting the growth and reproduction of earthworms [26], an elevated Zn level leads to significant weight loss, a reduced growth rate, and a reduction in body length in these invertebrates [62]. Earthworms are particularly sensitive to copper, which plays a dual role in the metabolism of earthworms, exerting both beneficial and harmful effects. Cu is an essential microelement in various metabolic processes and can exert a stimulatory effect at low concentrations [63]. It is crucial to enzymatic functions, including cytochrome P450s [64]. Excessive exposure to copper can lead to toxicity, because it is not degraded and accumulates in soils, potentially harming soil organisms [63]. Studies have shown

that high amounts of Cu have a negative impact on various levels of biological organization [35,63,65]. The lowest levels, such as lysosomes, are the most sensitive [63]. However, there are doubts regarding the impact of excessive amounts of Cu on the reproduction and development of earthworms [66]. The toxicity of copper increases the production of reactive oxygen species through mitochondrial dysfunction, which damages DNA and leads to apoptosis [67]. Soils in nature often contain a mixture of pollutants. Analysis of the effect of copper in polluted soils is complicated, because other pollutants can increase the sensitivity of organisms to copper or protect it against toxicity. Moreover, due to the 'ageing' process of metals, assessment of the impact of copper becomes more important, as its bioavailability and toxicity depend on the duration of its presence in the soil [63]. Cd, as a heavy metal, exerts toxic effects such as induction of oxidative stress, DNA damage, and carcinogenesis; it also affects the immune system by reducing the number of coelomocytes in earthworms [68,69]. It is one of the most harmful metals. Many studies have shown that the presence of Cd in soil negatively affects the growth and development of earthworms, leading to overproduction of reactive oxygen species and aldehydes. This induces oxidative stress, abnormal gene expression, and inhibition of growth and reproduction. Earthworms exposed to Cd reduce food intake in order to avoid toxic effects. Cadmium also affects metabolism, resulting in weight loss and damage to mitochondrial enzymes and peroxisomes, which accelerates their ageing. Most studies focus on elevated concentrations of Cd (10–1000 mg/kg) and their impact on the behaviour, growth and survival of these organisms [70–74]. The decrease in the body weight of earthworms induced by Cd is the result of their stress response, which leads them to reduce food intake in order to avoid toxins. Earthworms use this strategy to protect themselves from poisoning, including with heavy metals [74,75]. In a polluted environment, earthworms can adapt their metabolic processes to reduce the harmful impact of metals, which often results in weight loss. Cd can bind to specific protein thiols in the mitochondrial membrane, which alters mitochondrial permeability and leads to lipid peroxidation. This metal can also disrupt the functions of mitochondrial enzymes, e.g., citrate synthase, and can accelerate ageing of peroxisomes by increasing the activity of native proteases and enzymes of the glyoxylate cycle [74]. The reactions of earthworms to Pb pollution constitute a basis for the assessment of ecological risk [76,77]. This element is extremely persistent, with a retention time of 150–5000 years in soil. It serves no biological function, which means that there are no safe levels of its presence, and its effects can lead to irreversible damage [78]. Metabolomic studies, i.e., analyses of small molecules (metabolites) in the body, reveal that exposure to low levels of Pb in soil disturbs amino acid metabolism, energy metabolism, and osmotic balance, leading to oxidative stress and neurotoxic effects. Histopathological damage is observed as well [79]. Exposure to Pb inhibits the growth rate of earthworms, and higher concentrations are correlated with reduced growth [80]. Cr plays a complicated role in the metabolism of earthworms; it is present in six oxidation states, among which Cr (III) and Cr (VI) are the most stable forms. Cr (VI) is highly toxic and acts as a strong oxidant, leading to the production of reactive oxygen species (ROS) and cellular damage. It is able to penetrate the cell membrane, enter the cytoplasm, and react with intracellular structures [81]. Although it can be essential in small quantities for certain metabolic processes, higher concentrations are harmful, and its effects are complex. Exposure to chromium increases malondialdehyde and metallothionein levels, which indicates oxidative damage and stress in earthworms [82]. In earthworms exposed to soils with Cr at concentrations of 0.24–893 mg/kg, tissue changes can take place, such as cell fusion and a decrease in the thickness of the epidermis, even at the lowest concentrations [83,84]. Soil contaminated with chrome can also affect reproduction in earthworms [84,85]. Compared to other metals, data on nickel accumulation in earthworms is limited [86]. At low concentrations, Ni can stimulate the activity of

antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT), which play a key role in protecting cells against oxidative stress [87]. Podolak et al. (2011) [88] showed that earthworms from polluted areas have high accumulation potential, associated with an ineffective Ni regulation mechanism. Moreover, high Ni concentrations only minimally affected the mortality of adult earthworms, which means that accumulation remained high [86,89]. Hirano and Tamae (2010) [90], on the other hand, did not observe Ni uptake by earthworms and determined that it was non-bioaccumulative. Ni pollution can have toxic consequences, such as growth inhibition, problems with reproduction, changes in enzyme activity, and damage to DNA and tissues [91]. There is also little information on the effects of barium on earthworms, but it is known not to be an essential nutrient for these organisms. Barium can be highly mobile in soil, because it mainly associates with soil colloids through ion exchange [92–94]. In soluble form it is toxic for soil invertebrates. In nature, barium is primarily present in the form of barite ( $\text{BaSO}_4$ ), which is its most stable and insoluble form. Apart from sulfate, barium can also occur as calcium carbonate and in the form of soluble salts, which are rarely encountered in nature but can appear in soils polluted by industrial activity [92,95]. A study by Lamb (2013) [92] demonstrated that in barite-contaminated soils, the body weight of earthworms decreases, which is positively correlated with the barium concentration in the soil. In earthworms collected from locations with waste rock, two indicators, i.e., PI (peroxidation index), indicating the total content of fatty acids in cell membranes, and UI (unsaturation index), reflecting the number of double bonds in membranes [45,96], were lower than in the control group, i.e., earthworms from locations without waste rock. According to the results of the study, the most unfavourable physiological response in terms of peroxidation of fatty acids was observed in earthworms from habitats that had been exposed to waste rock for more than 10 years. As the duration of the waste rock deposit decreased, the physiological parameters in Annelida improved, which indicates that their status depends on the duration of contamination. Apart from the duration of the deposit, the type of land use was important as well; in wasteland, where accumulation and in some cases magnification of metals in the roots of perennial plants is possible, the potential transfer of pollutants to the soil and directly to organisms (by ingestion or through the skin) increases. This leads to disturbances in lipid metabolism and thus to changes in the composition and oxidation of fatty acids. In contrast, the use of land for crops (e.g., cereals) or meadows kept in good agricultural condition limited the negative impact of metals in terms of lipid metabolism in these invertebrates.

The study enabled an effective comparison of reference locations with locations potentially burdened by anthropogenic sediment. In the natural environment, many contaminated soils contain more than one metal, which can lead to synergistic effects between various pollutants. To enhance the reliability of the results for the bioavailability of metals, analyses have been carried out using contaminated soils, both in laboratory conditions and by collecting earthworms directly from contaminated areas [97]. The impact of metal mixtures and the responses of organisms are the result of biological activity, bioavailability, and the characteristics of biochemical processes taking place in specific taxa and specific organisms belonging to these taxa, as well as potential interactions between components of the mixture. These interactions can take place at various levels and may result in higher or lower toxicity than in individual substances. The presence of one pollutant in soil can influence the way another pollutant is bound, which in turn changes its availability for organisms. In organisms, a pollutant can affect the detoxification processes of another pollutant, which in turn can alter its toxicity for a given individual. In effect, the results of analyses of the impact of individual compounds may not reflect the actual impact of mixtures of pollutants present in the environment [20]. Due to the sensitivity of organisms to stress associated with pollutants, their direct impact on the organism can be determined

using biomarkers, which are valued as potential tools for assessment of ecological risk [1]. The use of biomarkers in field studies should take into account the possibility of local adaptation of earthworms which have been exposed to long-term pollution, because this can affect their biological reactions in comparison to laboratory samples. Individuals from contaminated areas can exhibit increased tolerance due to the potential occurrence of adaptive genes or altered defence mechanisms [60]. It is worth conducting *in situ* studies in combination with *in vitro* studies, with the closest possible levels of exposure to the pollutants analyzed, in order to limit the impact of additional environmental factors and facilitate precise analysis of selected physiological responses—in this case peroxidation of fatty acids, which can be particularly important when saturated fatty acids (SFAs) predominate.

The proposed set of biomarker FAs in earthworms (C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3; and C22:2 n-6) differs in some ways from the panel of biomarker FAs determined for other terrestrial invertebrates, e.g., in gastropod tissues (C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3) according to Kowalczyk-Pecka et al. (2017a, 2017b) [98,99]. Earthworm tissues naturally have a higher proportion and diversity of saturated fatty acids (SFAs), which means that the panel of biomarker FAs contains more SFAs than in the case of snails. Sampedro et al. (2006) [39] showed that earthworm tissues have higher content of PUFAs than of SFAs. In the present study, SFA and MUFA content was higher in earthworms from sites with waste rock than in the groups without waste rock, while PUFA content was lower. Moreover, in earthworms inhabiting sites with waste rock deposits, compared to sites without this deposit, a higher content of metals such as Pb, Cd, Cr, Ba, Ni, Zn, Cu and a higher content of biomarker fatty acids such as C12:0, C14:0, C16:0, C18:0, C20:0, C21:0, C24:0, C16:1 n-7, C18:1 n-9c + C18:1 n-9t and C20:1 n-9 was observed, with a simultaneous reduction in the content of biomarker fatty acids such as C18:2 n-6c + C18:2 n-6t, C18:3 n-3, C20:2 n-6, C20:3 n-3, C20:4 n-6, C20:5 n-3 and C22:2 n-6. The predominant FAs were C20:4n-6 and C18:2 n-6c + C18:2 n-6t among PUFAs; C16:0 and C18:0 among SFAs; and C18:1 n-9c + C18:1 n-9t among MUFAs. Small amounts were detected for FA C20:5 n-3 among PUFAs; C20:0 and C24:0 among SFAs; and C16:1 n-7 and C20:1 n-9 among MUFAs. PUFAs play a role in cell membrane permeability; in particular, the balance between n-6 and n-3 acids in cell membranes and lysosome membranes is essential to their normal functioning [100]. Apart from the protective function of PUFAs in preserving cell membrane integrity, some of these acids have antibacterial effects [99]. Some peroxidation processes have been shown to stop at monounsaturated fatty acids. There were probably factors that arrested the degradation of polyunsaturated acids, so that inversion to monounsaturated fatty acids took place, but not necessarily to saturated fatty acids. To verify the suitability of the panel of biomarker FAs in earthworms, enzymatic (superoxide dismutase and catalase) and non-enzymatic (thiobarbituric acid reactive substances) biomarkers of oxidative stress were determined. Superoxide dismutase (SOD) is an enzyme with antioxidant properties which catalyzes dismutation of superoxide anions ( $O_2^-$ ) to molecular oxygen ( $O_2$ ) and hydrogen peroxide ( $H_2O_2$ ), protecting cells against damage induced by these reactive oxygen species. Catalase (CAT) functions as an enzyme decomposing hydrogen peroxide into water and oxygen, preventing the accumulation of this harmful compound [101]. Thiobarbituric acid reactive substances (TBARS) are by-products of lipid peroxidation whose determination enables assessment of the level of lipid peroxidation in tissues [102]. The values of these biochemical parameters were consistent with the changes observed in the oxidation of fatty acids. From a practical perspective, this research could have significant implications for land reclamation and soil engineering. The analysis of biomarker fatty acids, as well as

enzymatic and non-enzymatic biomarkers of oxidative stress in earthworms, could provide a valuable tool for environmental monitoring, including processes related to reclamation activities. Integrating a biomarker-based approach into practical environmental monitoring framework could complement traditional methods of assessing environmental status, while also providing clearer guidelines for practical application in environmental management. Incorporating biomarkers of physiological status into soil engineering practices could be an important element of this approach, supporting the development of sustainable environmental monitoring and management strategies.

Earthworms, through their soil activity, influence the microbiological properties of soil, which is crucial for the stability of soil ecosystems. Their intestine creates a unique ecologic niche with stable conditions, in contrast to the surrounding environment. Bacteria in the digestive tract of earthworms represent those present in the soil only to a limited degree, i.e., mainly those that are resistant to the conditions prevailing there. This means that the microbiota of the digestive tract of these invertebrates is a selective portion of soil microorganisms [68]. Exposure to metals influences the composition of fatty acids, leads to lipid peroxidation, and damages cell membranes [6,103,104]. Heavy metals such as Pb and Cd deactivate key sulfhydryl groups, which induces oxidative stress [6]. *In situ*, the fatty acid profile can be affected not only by the transfer of metals themselves (waste rock, soil, and plant roots to invertebrates of the family Lumbricidae), but also to some extent by fatty acids from the intestinal microbiome.

Some of the observed patterns, such as increased SFA and decreased PUFA in earthworms from sites with waste rock deposits, may not solely reflect the influence of waste rock. Alternative explanations include differences in diet, variation in gut microbiome composition, and seasonality, which may also contribute to the observed variability. Despite detailed analysis of biomarker FA, PI and UI indices, and enzymatic and non-enzymatic biomarkers of oxidative stress, these potential confounding factors must be considered. Future studies could eliminate or control these confounding factors by using a controlled diet, monitoring the gut microbiome, and repeating experiments at different times of the year.

## 5. Conclusions

Earthworms are a source of useful biomarker fatty acids (FAs), and the proposed panel comprises C12:0; C14:0; C16:0; C18:0; C20:0; C21:0; C24:0; C16:1 n-7; C18:1 n-9c + C18:1 n-9t; C20:1 n-9; C18:2 n-6c + C18:2 n-6t; C18:3 n-3; C20:2 n-6; C20:3 n-3; C20:4 n-6; C20:5 n-3; and C22:2 n-6. The composition of this panel differs from the panel in gastropod tissues, as earthworm tissues naturally contain a greater number of diverse saturated fatty acids (SFAs) than gastropods. Therefore, the panel of biomarker FAs in earthworms contains more SFAs. In turn, selected metals contained in the waste rock, once released into the environment, were transferred to the soil, plant roots, and Lumbricidae. The potential primary source of metals for earthworms in fields was soil, while in wasteland and meadows, plant roots were sources as well. In crop fields (buckwheat and oat) with waste rock, there was not enough time for metals to be transferred from the roots of annual plants to earthworms. In contrast, on wasteland with a long-term deposit of waste rock, with very species-poor vegetation, and on meadows with waste rock, plants were able to store metals in their roots for a long time, even for many years, which enabled the potential transfer of these metals from plant roots to the body of earthworms. The type of use of land with a waste rock deposit and the duration of the deposit influenced the physiological status of the organisms. In wasteland, it was possible for metals to accumulate and sometimes magnify in the roots of perennial plants. This in turn could affect the transfer of pollutants to the soil and to the body of earthworms *per os* or *per cutis*, with pronounced negative physiological consequences. The use of the study sites for cereal cultivation

or meadows kept in good agricultural condition reduced the physiologically perceptible negative impact of metals on these invertebrates. The least favourable physiological response for Oligochaeta at the level of FA peroxidation was observed in organisms from the habitats that had been exposed to waste rock for more than 10 years. According to the duration of the deposit, i.e., the shorter the duration, the more favourable the physiological parameters in Annelida. Waste rock as an environmental stressor and a potential source of pollutants that cause disturbances in the functioning of Lumbricidae, influenced lipid metabolism and thus changes in the composition and oxidation of fatty acids treated as biomarkers of physiological status, including oxidative stress. To confirm or disprove the usefulness of the panel of biomarker FAs in earthworms, superoxide dismutase (SOD), catalase (CAT) and thiobarbituric acid reactive substances (TBARS) were determined; the levels of these parameters were consistent with the changes noted in the composition and oxidation of fatty acids. The results confirm that earthworms, like other invertebrates, are sensitive to small changes in the content of metals in the environment due to their position as primary consumers in the food chain. Invertebrates belonging to Lumbricidae can be a useful biomonitoring tool as effective bioindicators of the state of the soil environment. At the same time, waste rock can be a potential source and a theoretical and experimental basis for further, more extensive research. In future studies, it is planned to use microbiological and metagenomic analyses to assess the impact of metals deposited in the environment, a key element of which is the deposition of waste rock on terrestrial invertebrates.

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# Effect of deposition of Cr, Ba, Ni, Zn, and Cu on biomarker fatty acids as indirect indicators of peroxidation in *Cepaea nemoralis* (Helicidae) *in situ* and *in vivo* (in laboratory conditions)

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## ABSTRACT

The study determined the effect of selected environmental variables (Cr, Ni, Ba, Zn and Cu) on the potential peroxidation of biomarker fatty acids (FAs) in snails *Cepaea nemoralis*, both in *in situ* and *in vivo* (in laboratory conditions). The following panel of biomarker FAs was used: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3. Field studies included samples obtained from a location potentially affected by anthropogenic sediment, containing a deposit of waste rock, and from a location without this influence. Waste rock, which is mining waste produced during extraction of bituminous coal, was treated as a potential source of metals. The study tested whether metals deposited in an anthropogenically contaminated environment whose main element is a waste rock deposit can be transferred to the body of land snails. In laboratory conditions, Cr<sup>3+</sup>, Ni<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup> ions were administered to snails separately, per os, using a pipette, in a volume of 10 µl per snail, in the form of independent solutions of prepared salts (CrCl<sub>3</sub>·6H<sub>2</sub>O; NiSO<sub>4</sub>·6H<sub>2</sub>O; ZnCl<sub>2</sub>; CuSO<sub>4</sub>). Separate experimental groups were created for each metal, in which three metal concentrations were used: 0.0005 mg Met/ml redistilled water; 0.00075 mg Met/ml redistilled water; and 0.001 mg Met/ml redistilled water. Due to the high toxicity of soluble barium compounds, poorly soluble BaSO<sub>4</sub> was used as the source of this element, which in solid form, in an amount proportional to the other metals, was added to the agar constituting the base of the medium. Regardless of exposure to individual metals, three additional experimental groups were also created in which snails were supplemented with a mixture of the tested metal ions, administered together in equal proportions, corresponding to the three concentrations used. The results indicate that metals affect the physiological response in snails associated with changes in the composition of biomarker FAs, peroxidation (PI) and unsaturation (UI) indices, including potential lipid peroxidation. It is possible to identify a group of FAs which can be useful biomarkers of exposure of snails (Gastropoda, Pulmonata) to these variables, in both natural and laboratory conditions. The search for this type of biomarkers in invertebrates is important in ecotoxicology even in the case of subthreshold levels of environmental pollutants. When using snails as a source of biomarkers *in situ*, after determining the levels of the pollutants in their natural habitat, in order to determine the synergy or antagonism of these pollutants with elements of the environmental background, for example climate and soil factors, it is worth carrying out comparative testing in a laboratory using supplementation with the same substances in controlled conditions.

## 1. Introduction

The widespread occurrence of land snails in the natural environment and their ability to accumulate pollutants makes them an important element in biomonitoring and ecotoxicology (Beeby and Richmond, 2011; Côte et al., 2015; Das and Khangarot, 2011; Dummee et al., 2015).

Land snails are one of many invertebrate taxa used as bioindicator organisms and a source of biomarkers (de Vaufléury et al., 2006; Parmar et al., 2016). For organisms to be recognized as potential bioindicators of pollution, they must meet certain criteria, including the ability to accumulate xenobiotics in their tissues, widespread presence in a variety of environments, an important role in terrestrial food webs, ease of

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sample collection, and tolerance for metals and organic pollutants (Baroudi et al., 2020; Cofone et al., 2020; El-Gendy et al., 2021; Radwan et al., 2020, 2010a; Regoli et al., 2006). These animals are exposed to a variety of environmental pollutants, and understanding the routes of their exposure is key to assessing the toxicity of substances such as metals (de Vaufleury et al., 2006; El-Gendy et al., 2021). One possible source of metal pollutants is waste rock. This is mining waste arising during extraction of bituminous coal, accumulated on landfills (Tong et al., 2022), but it can also be deposited in smaller quantities on the surface of the earth, for example in fields, meadows, or wasteland (Joniec et al., 2025). Waste rock is a source of metals such as chromium (Cr), barium (Ba), nickel (Ni), zinc (Zn) and copper (Cu) (Kujawska, 2018). Exposure of invertebrates to metals and other environmental pollutants often leads to oxidative-reductive imbalance and increased production of reactive oxygen species (ROS). In invertebrate organisms, fatty acid oxidation is a sensitive marker of oxidative stress caused by environmental factors. The resulting ROS primarily attack polyunsaturated fatty acids (PUFAs) found in phospholipids, among other things, initiating the process of lipid peroxidation (Ayala et al., 2014; Tortajada-Pérez et al., 2025). This process occurs in stages and includes initiation, propagation, and termination phases, leading to the formation of end products such as malondialdehyde (MDA) (Ayala et al., 2014). In terrestrial ecosystems, invertebrates play a key role in the transfer of pollutants, and living in direct contact with soil and plants, they are particularly exposed to the effects of xenobiotics present in the polluted environment (El-Gendy et al., 2021; Gimbert et al., 2006; Madoz-Escande and Simon, 2006). Among these invertebrates, land snails, especially of the family Helicidae, are an important group, performing an important function in the food chain as primary consumers (Nica et al., 2012). They are valuable indicators of the potential transfer of pollutants to higher trophic levels (Nahmani and Lavelle, 2002; Vranković et al., 2020). Due to their ability to absorb environmental pollutants through contact, food intake, drinking, or breathing (Radwan et al., 2020), land snails are considered effective bioindicators of the state of the environment (Salih et al., 2021). Animal organisms are able to accumulate metals in two ways, actively or passively, and excess amounts can be either stored or excreted (Ardestani et al., 2014). Some metals, such as Zn, Cu, Cr, and Ni, are microelements essential for the proper functioning of the body. These elements participate in many metabolic processes, and their presence in appropriate amounts is a prerequisite for the proper functioning of biological processes. At the same time, excessive accumulation of these metals in tissues, exceeding the physiological needs of the body, can lead to toxic effects. On the other hand, metals that are not essential, include Ba, do not perform any significant metabolic function in organisms, but are toxic even at low concentrations. All of the above-mentioned elements are heavy metals, which, depending on their concentration and exposure time, can have a potentially harmful effect on living organisms (Boshoff et al., 2013; Kapranov et al., 2021). Animals employ various strategies of defence against the harmful effects of metals (Ardestani et al., 2014). Snails can accumulate substantial amounts of pollutants in their bodies, often without serious consequences (Holan et al., 2017; Itziou and Dimitriadis, 2012; Jordaens et al., 2006b, 2006a).

The grove snail *Cepaea nemoralis* (Linnaeus, 1758), a land snail with a wide range of occurrence, is a valuable material for ecotoxicological studies owing to its small size and rapid metabolism (Kowalczyk-Pecka et al., 2015). Land snails are able to detect metals in food, which they manifest by restricting food intake when pollutant concentrations are high and entering a state of aestivation (Chandran et al., 2005; El-Gendy et al., 2021; Kowalczyk-Pecka et al., 2017b; Nowakowska et al., 2016). This is a form of dormancy used as a survival strategy in conditions of food shortage or other environmental stressors, such as high temperature (Wang et al., 2015). During this process, the animals become inactive and stop feeding, and their metabolism slows down, allowing them to save energy more effectively (Storey and Storey, 2010; Wang et al., 2015). At low concentrations of metals, however, land snails may

be unable to recognize them and may continue feeding, allowing the metals to accumulate in their bodies. They exhibit relatively high tolerance for the presence of metals in food, which may be due to the action of metallothioneins – proteins that bind metal ions – and mechanisms of intracellular storage and excretion (El-Gendy et al., 2021). Despite their adaptive abilities, conclusive information on the toxicity of metals for these organisms is still lacking (Chandran et al., 2005; Kowalczyk-Pecka et al., 2017b; Nowakowska et al., 2016).

Snails are an example of organisms exhibiting a rapid physiological response to changing conditions in natural ecosystems or in laboratories. Changes in cellular processes, both structural and functional, which can be demonstrated in a specific way can be used as biomarkers (Kowalczyk-Pecka et al., 2017b). Biomarkers can be indicators or signals of measurable changes in biological systems at the molecular, biochemical, cellular, physiological, or behavioural level in response to pollutants (El-Gendy et al., 2021; Sogorb et al., 2014). Of particular importance are reactions taking place at the lowest, most sensitive levels of biological organization, as they are crucial to the organism's normal functioning (Gomot and Pihan, 1997; Notten et al., 2008; Swaileh and Ezzughayyar, 2000). Fatty acids can be used as biomarkers of the body's response to environmental stressors, especially anthropogenic factors, as their profile reflects both the metabolic state of the cell and adaptive changes occurring in response to environmental disturbances. Changes in lipid metabolism and fatty acid composition are used to assess the impact of pollutants, including metals, on living organisms and as an integrated biochemical response to exposure and accumulation of pollutants. Fatty acids are essential components of cells, performing key functions as energy sources, structural elements of cell membranes, or as metabolic and signaling mediators. The composition of membrane lipids is particularly sensitive to stressors and environmental changes due to their role in maintaining membrane integrity and functionality (Silva et al., 2017). Environmental stress can lead to selective changes in the proportion of saturated, monounsaturated, and polyunsaturated fatty acids, thereby affecting the physicochemical properties of membranes, such as their fluidity and permeability (El-Shenawy et al., 2012). However, the observed changes in the structure and proportion of individual fatty acids do not necessarily indicate definitive lipid peroxidation; they may also reflect disturbances in lipid homeostasis, including modifications in the composition of cell membranes or adaptive changes in their fluidity (Fadhlaoui et al., 2024).

Most studies associated with biomarkers in land snails have been carried out in laboratories. Biomarkers have rarely been analysed in natural conditions enabling assessment of the physiological state of a snail population (El-Gendy et al., 2021). They have primarily concerned determination of the levels of antioxidant enzymes in the bodies of snails (El-Shenawy et al., 2012; Vranković et al., 2020). In the natural environment, organisms are exposed to a variety of pollutants, which affects their physiology (El-Gendy et al., 2021). Although many laboratory studies focus on the effects of individual metals (Kowalczyk-Pecka et al., 2018, 2017a), snails in their natural environment are actually in contact with mixtures of pollutants. The effects of these mixtures of metals on the growth of snails can be described as additive, synergistic, or antagonistic (El-Gendy et al., 2021). *In situ* studies take into account the complex interactions between organisms and their surroundings, revealing effects of pollutants that may not be perceptible in laboratories. This type of study supplies realistic information about the impact of substances on the environment (Crane et al., 2007; Lesel, 1976). For this reason, an effective biomarker should be tested both in laboratories and in the field (Radwan et al., 2020). Comparison of FA status *in situ* and in laboratory conditions enables verification of laboratory results in relation to the actual living conditions of bioindicator organisms. The results of studies conducted under controlled laboratory conditions may not reflect the full complexity of the natural environment of organisms, leading to oversimplified conclusions (Crane et al., 2007; Lesel, 1976).

Environmental and physiological stressors have previously been assumed to induce lipid peroxidation processes, and the effects of these

processes are measured using standard biomarkers of physiological status. Changes in the fatty acid profile are one of the biomarkers of the physiological condition of snails (Kowalczyk-Pecka et al., 2017c). Molluscs have an unusual composition of sterols and fatty acids (Özogul et al., 2005). When we know the fatty acid composition, we can calculate the peroxidation index (PI), which can be used to determine the theoretical susceptibility of the lipid membrane composition to peroxidation (Hulbert et al., 2014; Kowalczyk-Pecka et al., 2017c).

The research hypothesis assumed that exposure of snails to selected metals leads to increased potential lipid peroxidation. It is possible to find a group of fatty acids that can serve as useful biomarkers of exposure of snails (Gastropoda, Pulmonata) to these variables in both natural (*in situ*) and laboratory (*in vivo*) conditions. In addition, in *in situ* conditions, metals deposited in an anthropogenically contaminated environment whose main element is a waste rock deposit can be transferred. Therefore, the aim of the study was to determine the effect of selected metals as environmental variables on the panel of biomarker fatty acids in snails *Cepaea nemoralis* (Gastropoda, Pulmonata), *in situ* and *in vivo* (in laboratory conditions).

## 2. Materials and methods

### 2.1. *In situ* experiment

Grove snails, which are land snails of the species *Cepaea nemoralis* (Helicidae, Pulmonata, Gastropoda), were used as the subject of the experiment. Snails for *in situ* analyses were obtained from two study sites located in eastern Poland, in the Polesie region in the Lublin Voivodeship, designated as follows: I – site with more than one year of waste rock (WR) deposition, with herb communities of the *Filipendulion ulmariae* alliance (geographic coordinates: N:51°14'06.32"; E:23°05'07.15") and II – site without WR deposit (Control), potentially 'anthropogenically pure', with vegetation of the class *Koelerio glaucae-Corynephoretea canescens* (geographic coordinates: N:51°20'12.81"; E:23°13'57.74"). The sites were meant to differ in their degree of human impact, including the size of the deposit of pollutants (selected metals) in the plants constituting the natural food of snails *C. nemoralis*. Snails of similar size were selected, 23–24 mm in width, which meant that they were very likely to be mature and of similar age. From each site, 30 snails *C. nemoralis* were collected by hand and divided into five groups of six snails each, so each group of six pooled snails was treated as a single biological replicate, producing one analytical data point. Consequently, all statistical analyses were conducted using  $n = 5$  independent replicates per treatment/site. Within each group, snails were analysed collectively. Snails collected from the field locations were designated I-SF and II-SF. Plants (designated I-P and II-P) were sampled from the same sites. The research material was collected in 2022 (September). After collection, the snails were left without food for 24 h to clean out their digestive tract, and then frozen at  $-80^{\circ}\text{C}$  for subsequent analysis.

### 2.2. *In vivo* experiment (in laboratory conditions)

For the *in vivo* analyses, groups the same size as the groups of field snails were used, that is, 30 snails *C. nemoralis*, divided into five groups with six individuals in each ( $n = 5$ ), obtained from an uncontaminated culture conducted in a vivarium. For the experiment in laboratory conditions, as in previous experiments (Kowalczyk-Pecka et al., 2017a), two control groups were established: I-CG – the initial control group and II-CG – the final control group.

Snails from the laboratory groups were kept in a BIOGENET growth chamber for six weeks in perforated high-density polyethylene (HDPE) containers ensuring the animals' welfare. The snails were fed twice a week on feed in the form of a jelly according to Laskowski and Hopkin (1996), cited by Kowalczyk-Pecka et al., (2017a) made from agar (Difco) with no fungicide, with freeze-dried, powdered carrot root. For each group, 10 ml of feed was prepared before each feeding. Based on

previous experiments, it was estimated that this portion would be eaten in its entirety, with no losses. The snails received *per os* (by pipette) 10  $\mu\text{l}$  (per snail) of a solution containing specific metal ions. Salts of the following metals were used, all of analytical grade purity (p.a.): Cr ( $\text{CrCl}_3 \cdot 6\text{H}_2\text{O} = \text{Cr(III)}$ , Avantor Performance Materials, Poland), Ni ( $\text{NiSO}_4 \cdot 6\text{H}_2\text{O} = \text{Ni(II)}$ , Merck, Germany), Zn ( $\text{ZnCl}_2 = \text{Zn(II)}$ , Avantor Performance Materials, Poland) and Cu ( $\text{CuSO}_4 = \text{Cu(II)}$ , Avantor Performance Materials, Poland). Independent solutions of each salt were prepared at concentrations of 0.0005, 0.00075 and 0.001 mg Met/ml redistilled water (groups designated 0.5, 0.75 and 1.0, respectively). Due to the high toxicity of soluble barium compounds for snails, the source of Ba used for the laboratory analyses was  $\text{BaSO}_4$  (Ba (II), Merck, Germany) of analytical grade purity (p.a.), which was added in solid form to the agar forming the basis of the feed in an amount proportional to that of the other metals. There were also three experimental groups whose diet was supplemented with a mixture of the above-mentioned metal ions in equal proportions at the three above-mentioned concentrations. Thus 20 experimental laboratory groups were established, including snails receiving chromium (groups designated Cr 0.5, Cr 0.75 and Cr 1.0), groups receiving barium (Ba 0.5, Ba 0.75 and Ba 1.0), groups receiving nickel (Ni 0.5, Ni 0.75 and Ni 1.0), groups receiving zinc (Zn 0.5, Zn 0.75 and Zn 1.0), and groups receiving copper (Cu 0.5, Cu 0.75 and Cu 1.0). The groups whose diet was supplemented with a mixture of metals were designated Mix 0.5, Mix 0.75 and Mix 1.0. The laboratory group of control snails (II-CG) received only the feed twice a week, without added metals. After six weeks, the snails in all groups were left without food for 24 h to clean out their digestive tract, and then frozen at  $-80^{\circ}\text{C}$  for subsequent biochemical analysis.

### 2.3. Determination of selected metals in plants, and the foot of snails

Samples with the green parts of plants and the soft tissues (foot) of snails were crushed and then homogenized. Analyses were performed on 0.5 g of sample. Samples were digested in  $\text{HNO}_3$  (Suprapur-Merck, Germany) in a CEM Mars Xpress microwave oven (USA) ( $210^{\circ}\text{C}/7\text{ atm}$ ). The digestates were diluted in demineralized water ( $0.055\text{ }\mu\text{S}/\text{cm}$  conductivity).

#### 2.3.1. ICP-MS analysis (inductively coupled plasma mass spectrometry)

The resulting solutions were analysed in an inductively coupled plasma mass spectrometer (Varian ICP Mass Spectrometer MS-820, USA). ICP-MS analysis was carried out according to the CLB/ESA/5/2019 procedure (version 3). ICP-MS was used to analyse Cr, Ni, and Ba. The gas used to create the plasma was argon (Messer, Germany) with 99.999 % purity. Ultra Scientific standards (Ultra Scientific, USA) with 99.999 % were used. No reaction cell (CRI) was employed. The settings were as follows: Plasma Flow –  $16\text{ dm}^3/\text{min}$ , Nebulizer Flow –  $0.98\text{ dm}^3/\text{min}$ , RF Power – 1.38 kW, Sampling Depth – 6.5 mm. The following isotopes of the elements were used:  $^{52}\text{Cr}$ ,  $^{60}\text{Ni}$  and  $^{137}\text{Ba}$ . Interferences for the isotopes  $^{52}\text{Cr}$  and  $^{60}\text{Ni}$  are taken into account automatically, based on the interference tables prepared for each isotope and entered into the device. Validation parameters: Cr (LOD – limit of detection =  $0.015\text{ mg}\cdot\text{kg}^{-1}$ , LOQ – limit of quantification =  $0.030\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 97 %), Ni (LOD =  $0.022\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.044\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 98 %), Ba (LOD =  $0.038\text{ mg}\cdot\text{kg}^{-1}$ , LOQ =  $0.076\text{ mg}\cdot\text{kg}^{-1}$ , recovery = 97 %). Determinations were made by the standard curve method. During the analysis, quality was controlled by measuring a blank sample, a double sample, and certified reference material: NIST-1577c Bovine Liver (National Institute of Standards & Technology, USA). Elemental contents in the CRM used: Cr -  $53\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ , Ni -  $44.5\text{ }\mu\text{g}\cdot\text{kg}^{-1}$ . Barium was not included in the reference material. Results are expressed as  $\text{mg}\cdot\text{kg}^{-1}$ .

#### 2.3.2. FAAS analysis (flame atomic absorption spectrometry)

The solutions were analysed in a flame atomic absorption spectrometer (Varian FAAS Spectrometer, SpektrAA 280FS with SPS-3 autosampler and SIPS diluter, Australia). FAAS analysis was carried

out according to the CLB/ASA/2/2019 procedure (version 4). The metals analysed by FAAS were Zn and Cu. The stock solutions were Zn (10 mg/dm<sup>3</sup>) and Cu (20 mg/dm<sup>3</sup>) sourced from CPA Chem (Bulgaria). The standards are certified in accordance with ISO 17034 (2016). The operating parameters of the spectrometer were as follows: Zn (flame: acetylene/air, wavelength: 213.9 nm, slit width: 1.0 nm, lamp current: 5.0 mA); Cu (flame: acetylene/air, wavelength: 324.8 nm, slit width: 0.5 nm, lamp current: 4.0 mA). The matrix was 2 % HNO<sub>3</sub>. Validation parameters: Zn (LOD = 0.4 mg·kg<sup>-1</sup>, LOQ = 0.09 mg·kg<sup>-1</sup>, recovery = 106 %), Cu (LOD = 1.3 mg·kg<sup>-1</sup>, LOQ = 2.6 mg·kg<sup>-1</sup>, recovery = 99 %). Results are expressed as mg·kg<sup>-1</sup>.

#### 2.4. Lipid extraction and recovery of fatty acids from snail feet

Samples of material were crushed and homogenized. The analyses were performed on 100 mg of fat. Saponification was carried out by adding a methanolic KOH solution (Avantor Performance Materials, Poland), and a methanolic BF<sub>3</sub> solution (Thermo Scientific, China) was used for esterification. The ampoule was placed in a water bath at a temperature of 85–95°C, brought to a boil, and kept at this temperature for 10 min. It was then cooled. Esters of fatty acids were obtained according to the CLB/GC/3/2019 procedure (version 4). Esters were isolated with C<sub>6</sub>H<sub>14</sub> (Avantor Performance Materials, Poland) and salted out with a saturated NaCl solution (Avantor Performance Materials, Poland). The fatty acid ester sample was dried by adding anhydrous Na<sub>2</sub>SO<sub>4</sub> (Avantor Performance Materials, Poland), and chromatographic analysis was performed. Recovery: 80–110 %. Samples of fatty acid esters were analysed using the Varian 450-GC gas chromatograph (USA) with the CP-8400 autosampler, an FID detector (temperature 270°C), and the Select™ Biodiesel capillary column for FAME (30 m 0.32 mm 0.25 µm). The 1177 Split/Splitless injector was used (temperature 250°C). The initial temperature of the furnace column was 100°C, and the final temperature was 240°C. The stationary phase of the fused silica Select Biodiesel column for FAME was used, with helium as the carrier gas. The flow rate was 1.5 ml/min. The standard used was Supelco 37 Component FAME Mix (Merck, Germany). The Galaxie™ Chromatography Data System was used to control the chromatograph and to collect, integrate and calculate the results. Among the 35 fatty acids analysed, a panel of biomarker FAs proposed by Kowalczyk-Pecka et al. (2017b) was selected: C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3. Total SFAs (saturated fatty acids), PUFAs (polyunsaturated fatty acids) and MUFAs (monounsaturated fatty acids) were calculated.

#### 2.5. Peroxidation and unsaturation indices

Based on the fatty acid analysis, peroxidation (PI) and unsaturation (UI) indices were calculated using a formula developed by Hulbert et al. (2007):

$$\text{Peroxidation index (PI)} = 0.025 \times (\% \text{ monoenoics}) + 1 \times (\% \text{ dienoics}) + 2 \times (\% \text{ trienoics}) + 4 \times (\% \text{ tetraenoics}) + 6 \times (\% \text{ pentaenoics}) + 8 \times (\% \text{ hexaenoics})$$

$$\text{Unsaturation index (UI)} = 1 \times (\% \text{ monoenoics}) + 2 \times (\% \text{ dienoics}) + 3 \times (\% \text{ trienoics}) + 4 \times (\% \text{ tetraenoics}) + 5 \times (\% \text{ pentaenoics}) + 6 \times (\% \text{ hexaenoics})$$

#### 2.6. Statistical analyses

The data were subjected to statistical analysis using Statistica software version 13.3 (1984–2017 TIBCO Software INC, Palo Alto, CA, USA). Normality of data distribution was assessed using the Shapiro–Wilk test, which is recommended for small sample sizes, and the Levene's homogeneity of variance test was applied to examine the

equality of variances. No missing values were present in the dataset. All observations were retained for statistical analyses; no data points were excluded as outliers. Due to the correlated nature of the analysed variables, formal multiple-testing corrections were not applied, and statistical significance was assessed at  $p \leq 0.01$ . Each group of six pooled snails was treated as a single biological replicate, producing one analytical data point. Consequently, all statistical analyses were conducted using  $n = 5$  independent replicates per treatment/site. Two-way ANOVA was used to assess the effect of the WR deposit (WR deposit > 1, no deposit), the type of research material (RM – snail foot, green plant parts), and their interaction on the content of metals (Cr, Ba, Ni, Zn, and Cu). One-way ANOVA and Tukey's multiple range tests were performed to compare all experimental groups and the Control group.

As part of the assessment of the linear relationship between FA content, PI and UI indices, and the content of metals (Cr, Ba, Ni, Zn, and Cu), Pearson's linear correlation coefficient ( $r$ ) was calculated at a significance level of  $p \leq 0.01$  and interpreted according to the following scale (Matyja, 2014):  $0 < r < |0.1|$  – negligible correlation;  $|0.1| \leq r < |0.3|$  – weak correlation;  $|0.3| \leq r < |0.5|$  – moderate correlation;  $|0.5| \leq r < |0.7|$  – strong correlation;  $|0.7| \leq r < |0.9|$  – very strong correlation;  $|0.9| \leq r < |1|$  – nearly perfect correlation. The results were presented in the form of a heat map using cluster analysis (area and feature clustering). Positive correlations are shown in red and negative correlations in blue; colour intensity reflects the strength of the relationship.

Cluster analyses (agglomeration and area and feature clustering) were conducted using data standardized (Z-scores) relative to FA content, PI and UI. Hierarchical agglomerative clustering was performed using single linkage and the Euclidean distance metric. This allowed us to assess the similarity between groups based on the minimum distance between cluster elements. The results were visualised in a dendrogram, where „branch” length reflects the degree of dissimilarity between samples or groups. Shorter branches indicate greater similarity, while longer branches indicate greater dissimilarity (Hinkle et al., 2003). The clustering analysis included samples from both *in situ* and *in vivo* experiments, encompassing all the experimental and control groups (I-SF, II-SF, I-CG and II-CG, as well as groups supplemented with various metal concentrations). The analysis aimed to visually identify groups with similar fatty acid profiles and PI/UI indices. Positive correlations are shown in red and negative correlations in blue; colour intensity reflects the strength of the relationship.

### 3. Results

#### 3.1. Metals (Cr, Ba, Ni, Zn, and Cu)

The analyses showed that the metals tested can accumulate in the green parts of plants, and tissues (foot) of *Cepaea nemoralis* snails. In *in situ* conditions, the highest chromium (Cr) concentration was noted in the green plant parts from the site with the waste rock deposit (WR) (I-P). Accumulation of barium (Ba), nickel (Ni) and zinc (Zn) was greatest in the foot of snails from the same location, while the highest copper (Cu) concentration was noted in the foot of snails from the site with no WR deposit (II-SF). The lowest concentrations of the elements were observed in the samples collected from the site with no WR deposit (II): Cr in the snail foot, Ba, Ni, Zn and Cu in the green plant parts. Comparison of the results revealed that in the green parts of plants and foot of snails from the site with the waste rock deposit, content of Cr, Ba, Ni and Zn was higher than at the site without it. In the case of Cu, higher concentrations were observed in the green plant parts from the location with the WR deposit than without it, while in the snail foot, Cu content was higher at the site with no waste rock deposit. The content of the metals was influenced ( $p \leq 0.01$ ) by both factors (WR and RM) individually, as well as the interaction between them (WRxRM) (Table 1).

In the groups whose diet was supplemented with individual metals, their content in the foot of snails *Cepaea nemoralis* showed a clear increasing tendency as the level of supplementation increased. An

**Table 1**Metals (Cr, Ba, Ni, Zn, and Cu) analysed in the foot of *Cepaea nemoralis* snails, green plant parts from sites with and without waste rock deposits (*in situ*).

| Groups |    |    | Metals [mg·kg <sup>-1</sup> ] ± SD |                         |                        |                          |                           | Interactions |    |       |
|--------|----|----|------------------------------------|-------------------------|------------------------|--------------------------|---------------------------|--------------|----|-------|
|        |    |    | Cr                                 | Ba                      | Ni                     | Zn                       | Cu                        | WR           | RM | WRxRM |
| I-SF   | I  | SF | 3.55 <sup>b</sup> ±0.52            | 73.83 <sup>c</sup> ±1.2 | 9.77 <sup>c</sup> ±0.9 | 103.53 <sup>b</sup> ±9.1 | 155.58 <sup>b</sup> ±7.3  | *            | *  | *     |
| I-P    |    | P  | 4.84 <sup>c</sup> ±0.7             | 0.76 <sup>a</sup> ±0.2  | 5.52 <sup>b</sup> ±0.5 | 92.59 <sup>b</sup> ±8.5  | 50.46 <sup>a</sup> ±5.1   | *            | *  | *     |
| II-SF  | II | SF | 2.05 <sup>a</sup> ±0.3             | 33.37 <sup>b</sup> ±3.5 | 8.46 <sup>c</sup> ±0.7 | 66.45 <sup>a</sup> ±5.0  | 201.13 <sup>c</sup> ±12.2 | *            | *  | *     |
| II-P   |    | P  | 4.17 <sup>bc</sup> ±0.4            | 0.12 <sup>a</sup> ±0.0  | 1.22 <sup>a</sup> ±0.3 | 51.58 <sup>a</sup> ±4.7  | 42.66 <sup>a</sup> ±3.6   | *            | *  | *     |
| SEM    |    |    | 0.258                              | 6.936                   | 0.745                  | 4.942                    | 15.656                    |              |    |       |

I-SF—snails- foot, WR deposit (waste rock); II-SF—snails- foot, without WR deposit; I-P—green plant parts, WR deposit; II-P—green plant parts, without WR deposit; SEM—standard error of mean; WR—waste rock; RM—research material; WRxRM—interaction of waste rock and research material; <sup>a-c</sup>— values in rows with different letters indicate significant differences among metals according to one-way ANOVA followed by Tukey's HSD test at  $p \leq 0.01$ ;  $n = 5$  per treatment/site; data expressed as mg·kg<sup>-1</sup> dry weight; \*significant at  $p \leq 0.01$ .

analogous effect was observed for the mixture of metals, as an increase in its concentration was associated with an increase in the content of all metals analysed in the snail foot. In the control groups, some differences were noted in the content of metals between the initial group (I-CG) and final group (II-CG). Accumulation of Cr, Zn and Cu was somewhat higher than in II-CG, while that of Ba and Ni was lower. In all experimental groups, irrespective of the level of supplementation with metals, the content of the elements was higher than in both control groups. At all analysed concentrations, supplementation with individual metals and with the mixture of metals significantly increased their content in the foot of *Cepaea nemoralis* relative to the control groups, with the greatest effect observed for the mixture of all metals, Cr, Ba, Ni, Zn, and Cu, applied at the highest concentration (Mix 1.0) (Fig. 1).

### 3.2. Biomarker FAs; peroxidation (PI) and unsaturation (UI) indices

The set of biomarker fatty acids (FAs) in snails *Cepaea nemoralis* comprised C16:0 (palmitic acid); C18:0 (stearic acid); C23:0 (tricosanoic acid); C18:1 n-9 (oleic acid); C20:1 n-9 (cis-11-eicosenoic acid); C18:2 n-6 (linoleic acid); C18:3 n-3 (α-linolenic acid); C20:2 n-6 (cis-11 14-eicosadienoic acid); C20:4 n-6 (arachidonic acid); C20:5 n-3 (cis-

5,8,11,14,17-eicosapentaenoic acid, EPA); C22:4 n-6 (docosatetraenoic acid); and C22:5 n-3 (docosapentaenoic acid) (Fig. 2a, Fig. 3, Table 2).

In both *in situ* and *in vivo* conditions, C18:0 was predominant among saturated fatty acids (SFAs), C20:4 n-6 and C18:2 n-6 among polyunsaturated fatty acids (PUFAs), and C18:1 n-9 among monounsaturated fatty acids (MUFAs) (Fig. 2a, Fig. 3, Table 2). In *in situ* conditions, higher content of C16:0, C18:0, C23:0, C18:1 n-9 and C20:1 n-9 and lower content of C18:2 n-6, C18:3 n-3, C20:2 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6 and C22:5 n-3 were noted in the foot of snails from the site with the waste rock deposit (I-SF) in comparison with snails collected from the control site without waste rock (II-SF) (Fig. 2a, Fig. 3). In *in vivo* conditions, analysis of metal concentrations showed the highest content of biomarker FAs (C16:0, C18:0, C23:0 and C18:1 n-9) for Cr 1.0, Ba 1.0, Ni 1.0, Zn 0.5, Cu 0.5 and Mix 0.75, and the lowest for Cr 0.5, Ba 0.5, Ni 0.5, Zn 0.75, Cu 1.0 and Mix 1.0. Content of C20:1 n-9 was highest for Cr 1.0, Ba 1.0, Ni 1.0, Zn 0.5, Cu 0.5 and Mix 1.0, and lowest in Cr 0.5, Ba 0.5, Ni 0.5, Zn 0.75, Cu 1.0 and Mix 0.5. Content of C18:2 n-6, C18:3 n-3 and C20:2 n-6 was highest for Cr 0.5, Ba 0.5, Ni 0.5, Zn 0.75, Cu 1.0 and Mix 0.5. Content of C18:2 n-6 and C18:3 n-3 was lowest for Cr 1.0, Ba 1.0, Ni 1.0, Zn 0.5, Cu 0.5 and Mix 0.75, but in the case of C20:2 n-6, the lowest content was noted for Cr 1.0, Ba 1.0, Ni 1.0, Zn 0.5, Cu 0.5 and Mix 1.0.

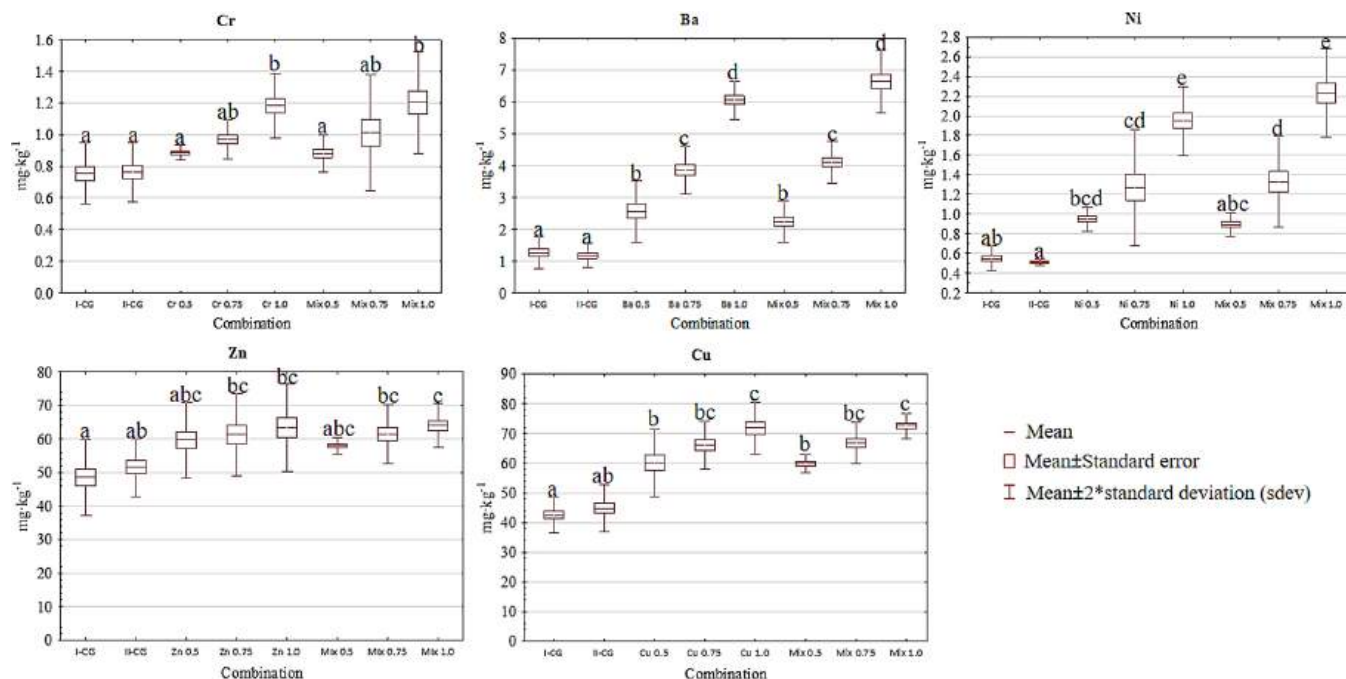


**Fig. 1.** Metals (Cr, Ba, Ni, Zn, and Cu), expressed as mg·kg<sup>-1</sup> dry weight, analysed in the foot of *Cepaea nemoralis* snails *in vivo* (in laboratory conditions). I-CG—initial control group; II-CG—final control group; data expressed as mg·kg<sup>-1</sup> dry weight; □ —mean ± standard error (SE); ▤ —mean ± 2\*standard deviation (SD); <sup>a-e</sup>— values in rows with different letters indicate significant differences among metals according to one-way ANOVA followed by Tukey's HSD test at  $p \leq 0.01$ ;  $n = 5$  per treatment; \*significant at  $p \leq 0.01$ .

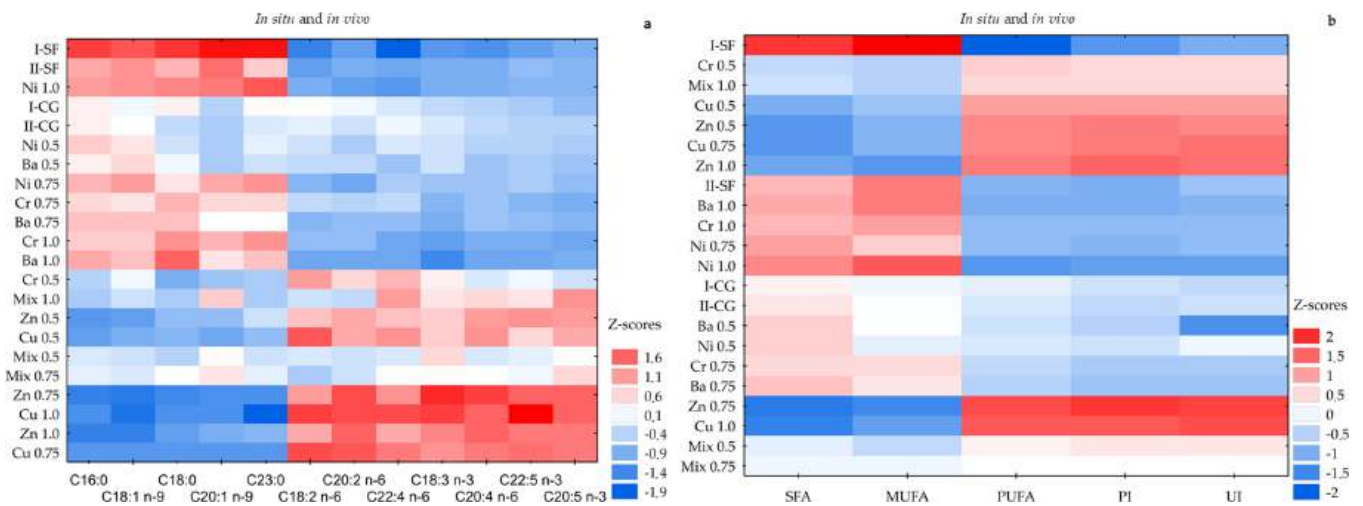


**Fig. 2.** Heat map based on analysis of the content of (a) biomarker FAs; (b) SFAs, PUFAs, MUFAs, PI, and UI in the foot of *Cepaea nemoralis* snails *in situ* and *in vivo* (in laboratory conditions). I-SF—snails- foot, WR deposit (waste rock) (*in situ*); II-SF—snails- foot, without WR deposit (*in situ*); I-CG—initial control group (*in vivo*); II-CG—final control group (*in vivo*). Biomarker FAs: C16:0—palmitic acid; C18:0—stearic acid; C23:0—tricosanoic acid; C18:1 n-9—oleic acid; C20:1 n-9—cis-11-eicosenoic acid; C18:2 n-6—linoleic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6—cis-11 14-eicosadienoic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenoic acid, EPA; C22:4 n-6—docosatetraenoic acid; C22:5 n-3—docosapentaenoic acid. SFA—saturated fatty acids; PUFA—polyunsaturated fatty acids; MUFA—monounsaturated fatty acids; PI—peroxidation index; UI—unsaturation index.

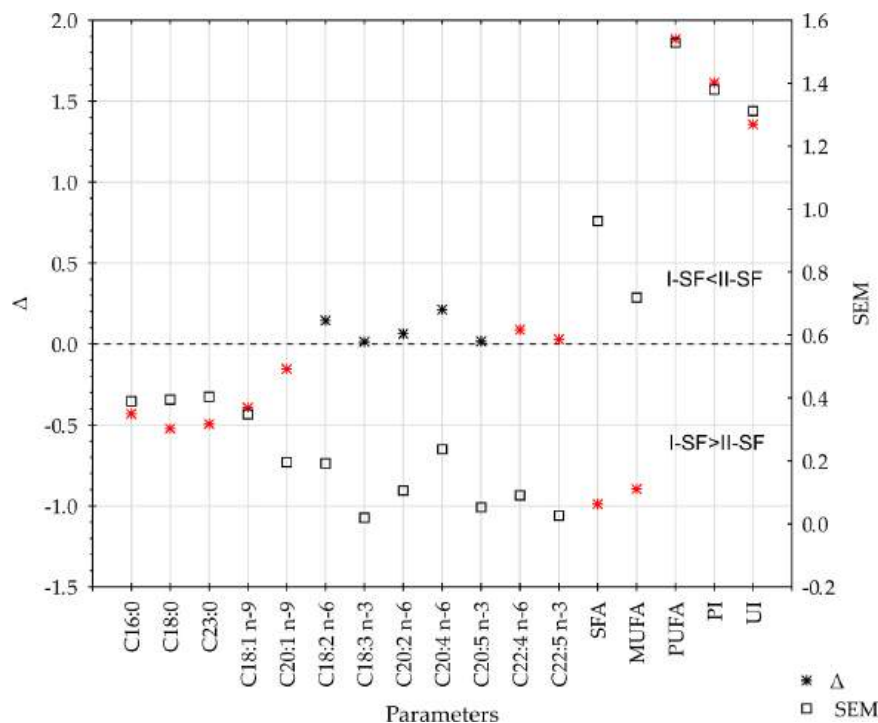


**Fig. 3.** The difference between the values of the biomarker fatty acids, peroxidation and unsaturation indices analysed in the foot of *Cepaea nemoralis* snails obtained from sites with and without waste rock deposits (*in situ*). I-SF—snails- foot, WR deposit (waste rock); II-SF—snails- foot, without WR deposit. Biomarker FAs: C16:0—palmitic acid; C18:0—stearic acid; C23:0—tricosanoic acid; C18:1 n-9—oleic acid; C20:1 n-9—cis-11-eicosenoic acid; C18:2 n-6—linoleic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6—cis-11 14-eicosadienoic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenoic acid, EPA; C22:4 n-6—docosatetraenoic acid; C22:5 n-3—docosapentaenoic acid. SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index;  $\Delta$ —the difference between the values of the analysed parameters for the I-SF and II-SF combinations ( $\Delta = \text{II-SF} - \text{I-SF}$ ); SEM—standard error of mean; \*—values in rows with red \* differ significantly at  $p \leq 0.01$ .

In the case of C20:4 n-6, C20:5 n-3, C22:4 n-6 and C22:5 n-3, the highest content was observed for Cr 0.5, Ba 0.5, Ni 0.5, Zn 0.75, Cu 1.0 and Mix 1.0, and the lowest for Cr 1.0, Ba 1.0, Ni 1.0, Zn 0.5, Cu 0.5 and Mix 0.5 (Fig. 2a, Table 2). To sum up, in the *in vivo* control samples (I-CG and II-CG), the content of C16:0, C18:0, C23:0, C18:1 n-9 and C20:1 n-9 was lower and that of C18:2 n-6, C18:3 n-3, C20:2 n-6, C20:4 n-6, C20:5 n-3,

C22:4 n-6 and C22:5 n-3 was higher than in both the experimental and control *in situ* samples (Fig. 2a, Fig. 3, Table 2).

The analyses showed that in *in situ* conditions, the content of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs) in the foot of snails *C. nemoralis* from the site with the waste rock deposit (I-SF) was higher than in the snails from the site without waste rock (II-SF),

Table 2

Biomarker fatty acids, peroxidation and unsaturation indices analysed in the foot of *Cepaea nemoralis* snails *in vivo* (in laboratory conditions).

| Parameters | Groups                  |                         |                         |                         |                         |                       |                         |                       |                       |                        |                      |                       |                      |                       |                        |                      |                      |                         |                         |                        | SEM   |
|------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-----------------------|-------------------------|-----------------------|-----------------------|------------------------|----------------------|-----------------------|----------------------|-----------------------|------------------------|----------------------|----------------------|-------------------------|-------------------------|------------------------|-------|
|            | I-CG                    | II-CG                   | Cr 0.5                  | Cr 0.75                 | Cr 1.0                  | Ba 0.5                | Ba 0.75                 | Ba 1.0                | Ni 0.5                | Ni 0.75                | Ni 1.0               | Zn 0.5                | Zn 0.75              | Zn 1.0                | Cu 0.5                 | Cu 0.75              | Cu 1.0               | Mix 0.5                 | Mix 0.75                | Mix 1.0                |       |
| C16:0      | 7.94 <sup>ef</sup>      | 8.02 <sup>f</sup>       | 6.28 <sup>c</sup>       | 8.37 <sup>fg</sup>      | 8.56 <sup>fg</sup>      | 7.98 <sup>ef</sup>    | 8.74 <sup>fgh</sup>     | 9.17 <sup>gh</sup>    | 8.56 <sup>fg</sup>    | 9.06 <sup>gh</sup>     | 9.47 <sup>h</sup>    | 4.57 <sup>ab</sup>    | 4.02 <sup>a</sup>    | 4.09 <sup>a</sup>     | 4.94 <sup>b</sup>      | 4.62 <sup>ab</sup>   | 4.34 <sup>ab</sup>   | 6.84 <sup>cd</sup>      | 7.16 <sup>de</sup>      | 6.07 <sup>c</sup>      | 0.189 |
| C18:0      | 18.33 <sup>fgh</sup>    | 17.20 <sup>def</sup>    | 16.22 <sup>abcd</sup>   | 19.21 <sup>hij</sup>    | 19.76 <sup>ij</sup>     | 17.87 <sup>efg</sup>  | 19.09 <sup>ghij</sup>   | 20.31 <sup>j</sup>    | 17.34 <sup>def</sup>  | 18.46 <sup>ghi</sup>   | 19.88 <sup>j</sup>   | 16.51 <sup>abcd</sup> | 15.32 <sup>a</sup>   | 15.76 <sup>abc</sup>  | 16.36 <sup>abcd</sup>  | 15.73 <sup>abc</sup> | 15.53 <sup>ab</sup>  | 17.03 <sup>cde</sup>    | 17.98 <sup>efgh</sup>   | 16.77 <sup>bcde</sup>  | 0.158 |
| C23:0      | 5.81 <sup>defg</sup>    | 5.35 <sup>cdef</sup>    | 4.74 <sup>bcd</sup>     | 6.26 <sup>efgh</sup>    | 7.16 <sup>hi</sup>      | 5.16 <sup>cde</sup>   | 5.69 <sup>cdef</sup>    | 6.55 <sup>fghi</sup>  | 5.43 <sup>cdef</sup>  | 7.04 <sup>ghi</sup>    | 7.78 <sup>i</sup>    | 5.16 <sup>cde</sup>   | 3.63 <sup>ab</sup>   | 4.42 <sup>bc</sup>    | 4.49 <sup>bcd</sup>    | 3.79 <sup>ab</sup>   | 2.80 <sup>a</sup>    | 5.14 <sup>cde</sup>     | 5.42 <sup>cdef</sup>    | 4.82 <sup>bcd</sup>    | 0.129 |
| SFA        | 36.69 <sup>def</sup>    | 37.23 <sup>efg</sup>    | 33.46 <sup>c</sup>      | 37.74 <sup>fgh</sup>    | 38.98 <sup>ghij</sup>   | 38.21 <sup>fghi</sup> | 38.74 <sup>fghij</sup>  | 39.35 <sup>hij</sup>  | 38.17 <sup>fghi</sup> | 40.05 <sup>ij</sup>    | 40.79 <sup>j</sup>   | 29.57 <sup>ab</sup>   | 27.93 <sup>a</sup>   | 30.62 <sup>b</sup>    | 30.75 <sup>b</sup>     | 29.68 <sup>ab</sup>  | 28.13 <sup>a</sup>   | 35.04 <sup>cd</sup>     | 35.24 <sup>cde</sup>    | 34.26 <sup>c</sup>     | 0.420 |
| C18:1 n-9  | 12.84 <sup>cd</sup>     | 13.14 <sup>cde</sup>    | 12.87 <sup>cd</sup>     | 13.85 <sup>def</sup>    | 14.62 <sup>efg</sup>    | 14.27 <sup>defg</sup> | 14.84 <sup>efg</sup>    | 14.94 <sup>fg</sup>   | 14.06 <sup>def</sup>  | 15.82 <sup>g</sup>     | 15.93 <sup>g</sup>   | 8.99 <sup>ab</sup>    | 7.66 <sup>a</sup>    | 8.01 <sup>ab</sup>    | 9.62 <sup>b</sup>      | 8.72 <sup>ab</sup>   | 7.37 <sup>a</sup>    | 11.76 <sup>c</sup>      | 12.00 <sup>c</sup>      | 11.72 <sup>c</sup>     | 0.282 |
| C20:1 n-9  | 3.25 <sup>abcde</sup>   | 3.16 <sup>abcd</sup>    | 3.03 <sup>abc</sup>     | 4.36 <sup>defg</sup>    | 4.73 <sup>fg</sup>      | 3.19 <sup>abcd</sup>  | 3.91 <sup>bcdef</sup>   | 4.28 <sup>defg</sup>  | 3.23 <sup>abcde</sup> | 4.83 <sup>fg</sup>     | 5.21 <sup>g</sup>    | 3.01 <sup>abc</sup>   | 2.35 <sup>a</sup>    | 2.75 <sup>ab</sup>    | 2.62 <sup>a</sup>      | 2.46 <sup>a</sup>    | 2.32 <sup>a</sup>    | 4.04 <sup>cdefg</sup>   | 4.25 <sup>defg</sup>    | 4.43 <sup>efg</sup>    | 0.097 |
| MUFA       | 19.77 <sup>fghi</sup>   | 20.33 <sup>ghij</sup>   | 18.11 <sup>def</sup>    | 21.45 <sup>ijk</sup>    | 23.29 <sup>kl</sup>     | 20.14 <sup>ghi</sup>  | 21.07 <sup>hij</sup>    | 24.28 <sup>lm</sup>   | 19.35 <sup>fgh</sup>  | 22.10 <sup>jk</sup>    | 25.28 <sup>m</sup>   | 16.64 <sup>bcd</sup>  | 14.75 <sup>a</sup>   | 15.26 <sup>ab</sup>   | 17.44 <sup>cde</sup>   | 16.64 <sup>bcd</sup> | 15.65 <sup>abc</sup> | 18.56 <sup>efg</sup>    | 19.85 <sup>fghi</sup>   | 18.03 <sup>def</sup>   | 0.296 |
| C18:2 n-6  | 11.76 <sup>cdef</sup>   | 11.53 <sup>bcde</sup>   | 13.43 <sup>fgh</sup>    | 10.87 <sup>abc</sup>    | 10.16 <sup>abc</sup>    | 10.87 <sup>abc</sup>  | 10.08 <sup>abc</sup>    | 9.56 <sup>a</sup>     | 11.07 <sup>abcd</sup> | 9.92 <sup>ab</sup>     | 9.83 <sup>ab</sup>   | 12.86 <sup>defg</sup> | 13.52 <sup>fgh</sup> | 13.22 <sup>efgh</sup> | 14.50 <sup>gh</sup>    | 14.69 <sup>h</sup>   | 14.92 <sup>h</sup>   | 11.26 <sup>abcd</sup>   | 10.63 <sup>abc</sup>    | 11.06 <sup>abcd</sup>  | 0.180 |
| C18:3 n-3  | 0.69 <sup>abcd</sup>    | 0.72 <sup>abcd</sup>    | 0.85 <sup>cde</sup>     | 0.55 <sup>ab</sup>      | 0.49 <sup>a</sup>       | 0.70 <sup>abcd</sup>  | 0.53 <sup>a</sup>       | 0.43 <sup>a</sup>     | 0.71 <sup>abcd</sup>  | 0.61 <sup>abc</sup>    | 0.54 <sup>ab</sup>   | 0.91 <sup>de</sup>    | 1.25 <sup>f</sup>    | 1.05 <sup>ef</sup>    | 0.93 <sup>de</sup>     | 1.04 <sup>ef</sup>   | 1.21 <sup>f</sup>    | 0.89 <sup>cde</sup>     | 0.81 <sup>bcd</sup>     | 0.86 <sup>cde</sup>    | 0.025 |
| C20:2 n-6  | 8.64 <sup>bcd</sup>     | 8.24 <sup>abc</sup>     | 9.33 <sup>cd</sup>      | 7.84 <sup>abc</sup>     | 7.43 <sup>ab</sup>      | 8.05 <sup>abc</sup>   | 7.47 <sup>ab</sup>      | 7.08 <sup>a</sup>     | 7.74 <sup>ab</sup>    | 7.07 <sup>a</sup>      | 6.92 <sup>a</sup>    | 9.93 <sup>de</sup>    | 11.153 <sup>e</sup>  | 10.85 <sup>e</sup>    | 9.95 <sup>de</sup>     | 10.92 <sup>e</sup>   | 11.16 <sup>e</sup>   | 8.25 <sup>abc</sup>     | 8.14 <sup>abc</sup>     | 8.06 <sup>abc</sup>    | 0.151 |
| C20:4 n-6  | 9.94 <sup>bcd</sup>     | 10.17 <sup>cd</sup>     | 10.83 <sup>de</sup>     | 8.97 <sup>abc</sup>     | 7.85 <sup>a</sup>       | 9.15 <sup>abcd</sup>  | 9.05 <sup>abcd</sup>    | 7.65 <sup>a</sup>     | 9.83 <sup>bcd</sup>   | 9.00 <sup>abc</sup>    | 8.16 <sup>ab</sup>   | 15.15 <sup>gh</sup>   | 18.01 <sup>j</sup>   | 17.05 <sup>ij</sup>   | 15.63 <sup>hi</sup>    | 16.06 <sup>hi</sup>  | 17.05 <sup>ij</sup>  | 10.74 <sup>cde</sup>    | 12.07 <sup>ef</sup>     | 13.43 <sup>fg</sup>    | 0.346 |
| C20:5 n-3  | 1.23 <sup>ab</sup>      | 1.53 <sup>ab</sup>      | 1.76 <sup>bc</sup>      | 1.04 <sup>ab</sup>      | 0.96 <sup>a</sup>       | 1.32 <sup>ab</sup>    | 1.13 <sup>ab</sup>      | 1.03 <sup>ab</sup>    | 1.42 <sup>ab</sup>    | 1.20 <sup>ab</sup>     | 1.14 <sup>ab</sup>   | 3.05 <sup>ef</sup>    | 3.54 <sup>f</sup>    | 3.30 <sup>f</sup>     | 2.96 <sup>ef</sup>     | 3.35 <sup>f</sup>    | 3.55 <sup>f</sup>    | 2.24 <sup>cd</sup>      | 2.56 <sup>de</sup>      | 3.163 <sup>ef</sup>    | 0.101 |
| C22:4 n-6  | 1.84 <sup>abcde</sup>   | 1.96 <sup>abcde</sup>   | 2.34 <sup>cdef</sup>    | 1.78 <sup>abcd</sup>    | 1.43 <sup>a</sup>       | 1.61 <sup>abc</sup>   | 1.55 <sup>ab</sup>      | 1.44 <sup>a</sup>     | 1.85 <sup>abcde</sup> | 1.66 <sup>abc</sup>    | 1.33 <sup>a</sup>    | 2.26 <sup>bcd</sup>   | 2.46 <sup>def</sup>  | 2.37 <sup>cdef</sup>  | 2.45 <sup>def</sup>    | 2.55 <sup>def</sup>  | 2.75 <sup>f</sup>    | 1.85 <sup>abcde</sup>   | 2.05 <sup>abcde</sup>   | 2.45 <sup>def</sup>    | 0.049 |
| C22:5 n-3  | 0.66 <sup>abc</sup>     | 0.68 <sup>abc</sup>     | 0.84 <sup>cd</sup>      | 0.57 <sup>a</sup>       | 0.56 <sup>a</sup>       | 0.65 <sup>abc</sup>   | 0.60 <sup>ab</sup>      | 0.53 <sup>a</sup>     | 0.71 <sup>abc</sup>   | 0.65 <sup>abc</sup>    | 0.58 <sup>a</sup>    | 1.16 <sup>e</sup>     | 1.30 <sup>e</sup>    | 1.22 <sup>e</sup>     | 1.00 <sup>d</sup>      | 1.27 <sup>e</sup>    | 1.54 <sup>f</sup>    | 0.81 <sup>bcd</sup>     | 0.85 <sup>cd</sup>      | 0.95 <sup>d</sup>      | 0.030 |
| PUFA       | 43.54 <sup>efg</sup>    | 42.44 <sup>def</sup>    | 48.44 <sup>i</sup>      | 40.81 <sup>de</sup>     | 37.73 <sup>bc</sup>     | 41.65 <sup>de</sup>   | 40.19 <sup>cd</sup>     | 36.37 <sup>ab</sup>   | 42.48 <sup>def</sup>  | 37.85 <sup>bc</sup>    | 33.93 <sup>a</sup>   | 53.79 <sup>jk</sup>   | 57.32 <sup>l</sup>   | 54.12 <sup>jk</sup>   | 51.81 <sup>j</sup>     | 53.68 <sup>jk</sup>  | 56.22 <sup>kl</sup>  | 46.39 <sup>ghi</sup>    | 44.92 <sup>fgh</sup>    | 47.71 <sup>hi</sup>    | 0.697 |
| PI         | 96.43 <sup>d</sup>      | 94.33 <sup>d</sup>      | 117.57 <sup>e</sup>     | 88.86 <sup>bcd</sup>    | 83.46 <sup>abc</sup>    | 92.37 <sup>cd</sup>   | 87.47 <sup>bcd</sup>    | 79.42 <sup>ab</sup>   | 96.43 <sup>d</sup>    | 82.37 <sup>abc</sup>   | 75.34 <sup>a</sup>   | 136.02 <sup>fg</sup>  | 151.26 <sup>h</sup>  | 141.42 <sup>g</sup>   | 128.96 <sup>f</sup>    | 136.58 <sup>fg</sup> | 144.91 <sup>gh</sup> | 115.45 <sup>e</sup>     | 109.93 <sup>e</sup>     | 118.36 <sup>e</sup>    | 2.428 |
| UI         | 134.30 <sup>abcde</sup> | 136.12 <sup>abcde</sup> | 155.19 <sup>defgh</sup> | 129.36 <sup>abcde</sup> | 123.54 <sup>abcde</sup> | 108.76 <sup>a</sup>   | 125.89 <sup>abcde</sup> | 120.31 <sup>abc</sup> | 142.30 <sup>bcd</sup> | 122.81 <sup>abcd</sup> | 113.43 <sup>ab</sup> | 174.20 <sup>ghi</sup> | 188.24 <sup>i</sup>  | 178.80 <sup>hi</sup>  | 168.05 <sup>fghi</sup> | 178.14 <sup>hi</sup> | 186.11 <sup>i</sup>  | 153.47 <sup>defgh</sup> | 147.14 <sup>cdefg</sup> | 156.05 <sup>efgh</sup> | 2.753 |

I-CG—initial control group; II-CG—final control group. Biomarker FAs [%]: C16:0—palmitic acid; C18:0—stearic acid; C23:0—tricosanoic acid; C18:1 n-9—oleic acid; C20:1 n-9—cis-11-eicosenoic acid; C18:2 n-6—linoleic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6—cis-11 14-eicosadienoic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenoic acid, EPA; C22:4 n-6—docosatetraenoic acid; C22:5 n-3—docosapentaenoic acid. SFA [%]—saturated fatty acids; MUFA [%]—monounsaturated fatty acids; PUFA [%]—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index; SEM—standard error of mean;

<sup>a-m</sup>— values in rows with different letters differ significantly at  $p \leq 0.01$ .

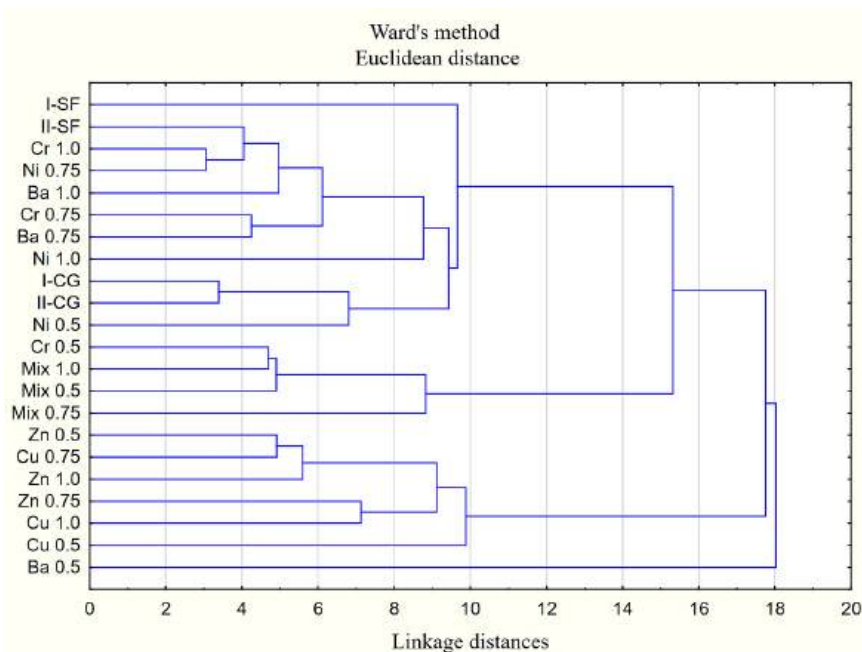


**Fig. 4.** Hierarchical dendrogram of samples utilizing Ward's clustering method with linkage distances *in situ* and *in vivo* (in laboratory conditions). I-SF—snails- foot, WR deposit (waste rock) (*in situ*); II-SF—snails- foot, without WR deposit (*in situ*); I-CG—initial control group (*in vivo*); II-CG—final control group (*in vivo*).

while the content of polyunsaturated fatty acids (PUFAs) was lower (Fig. 2b, Fig. 3). In *in vivo* conditions, the content of SFAs was highest for Ni 1.0, Ni 0.75 and Ba 1.0, and lowest for Zn 0.75 and Cu 1.0. MUFA content was highest for Cr 1.0, Ba 1.0 and Ni 1.0 and lowest for Zn (0.5, 0.75) and Cu (0.75, 1.0). PUFA content was highest for Zn (0.5, 0.75, 1.0) and Cu (0.75, 1.0) and lowest for Ni 1.0, Ba 1.0 and Cr 1.0 (Fig. 2b, Table 2).

Comparison of the results obtained *in vivo* with the *in situ* results showed that only for Cr 1.0, Ba 1.0 and Ni 1.0 was SFA and MUFA content higher than in the samples from the control site, such as without waste rock (II-SF). At the same time, these contents remained lower than in the samples collected from the site with WR (I-SF). A similar pattern was observed in the case of Ni 0.75 for SFA. In contrast, only for Ba 1.0 and Ni 1.0 was PUFA content lower than in the control (II-SF) but higher than in the experimental samples (I-SF). In the *in vivo* control samples (I-CG, II-CG), the content of SFAs and MUFAs was lower and PUFA content was higher than in the *in situ* experimental (I-SF) and control (II-SF) samples (Fig. 2b, Fig. 3, Table 2).

In *in situ* conditions, in the foot of snails *C. nemoralis* from the location with waste rock, the values of the peroxidation index (PI) and unsaturation index (UI) were lower than in snails from the location without waste rock (Fig. 2b, Fig. 3). In *in vivo* conditions, PI and UI were highest for Zn (0.5, 0.75, 1.0) and Cu (0.75, 1.0), while PI was lowest for Ni 1.0 and Ba 1.0, and UI was lowest for Ni 1.0 and Ba 0.5 (Fig. 2b, Table 2). Comparison of the *in vivo* values with the *in situ* results showed that only for Ni 1.0 and Ba 1.0 was the PI value lower than in the *in situ* control group (II-SF) and higher than in the *in situ* experimental samples from the site with waste rock (I-SF). Similarly, only for Cr 1.0 Ba 1.0, and Ni 0.75 were the UI values lower than in the *in situ* control group (II-SF) and higher than in the *in situ* experimental samples from the site with waste rock (I-SF). In addition, at Ba 0.5 and Ni 1.0, the UI values were lower than in the samples from the sites with waste rock (I-SF) and without it (II-SF). In the *in vivo* controls (I-CG and II-CG), the PI and UI values were higher than in the *in situ* experimental (I-SF) and control (II-SF) samples (Fig. 2b, Fig. 3, Table 2).

Cluster analysis carried out by Ward's method using Euclidean distance made it possible to determine the degree of similarity between *in situ* and *in vivo* samples (Fig. 4). The smallest linkage distances were

observed between samples Cr 1.0 and Ni 0.75, indicating high similarity.

### 3.3. Pearson's correlation

In *in situ* conditions, among the biomarker FAs analysed, C23:0 showed the greatest number of statistically significant correlations with metals. The most statistically significant correlations with the parameters tested were noted for Ba and Cr, while Ni showed no statistically significant correlations. In the case of PUFAs and PI, statistically significant negative correlations were found with Cr, Ba and Zn, and statistically significant positive correlations with Cu. MUFAs and SFAs were positively, significantly statistically correlated with Cr, Ba and Zn; in addition, for MUFAs there was a negative, statistically significant correlation with Cu. UI showed negative, statistically significant correlations with Ba and Zn, and positive statistically significant correlations with Cu (Fig. S1).

In *in vivo* conditions, among the biomarker FAs analysed, C20:1 n-9 showed the greatest number of statistically significant correlations with metals. Among metals, the most statistically significant correlations with the parameters analysed were noted for Cu and Zn. In the case of PUFAs and the PI and UI indices, there were positive statistically significant correlations with Zn, Cu and their applied concentrations, while SFAs and MUFAs showed negative, statistically significant correlations with the same metals and their applied concentrations (Fig. S2).

## 4. Discussion

Land snails of the family Helicidae, which are widespread in natural ecosystems, are a useful source of biomarkers and perform an important function in the food chain as primary consumers (Kowalczyk-Pecka et al., 2015). They also participate in the circulation of matter (Tomaz et al., 2018). Invertebrates themselves develop rapidly and have a short lifespan. They serve as bioindicators due to their ability to accumulate pollutants, including metals, and their sensitivity to even minimal changes in the environment, which is why they are used in ecotoxicological studies. Owing to their varied metal metabolism, involving elimination, biomagnification, and the capacity for detoxification, they play an important role in the further transfer of pollutants to higher

trophic levels (Gomot de Vaufléury and Pihan, 2000; Nahmani and Lavelle, 2002; Vranković et al., 2020). Biomagnification is the process of gradual increase in the concentration of pollutants in organisms occupying successive, higher trophic levels in the food chain (Danovaro et al., 2023). Detoxification, on the other hand, is the process by which the body neutralizes, metabolizes, and removes toxic substances (Dallinger, 2024; Dvorak et al., 2019; Yap and Al-Mutairi, 2025). In the present study, the metals such as Cr, Ni, Ba, Zn, and Cu were shown to be present in the research material (green plant parts, and foot of snails) collected from the sites with and without waste rock. A study by Garbacz et al. (2025a) showed the presence of metals, including Cr, Ni, Ba, Zn, and Cu, in waste rock, soil, plant roots, and earthworms. The metal content in waste rock and soil from the analysed sites was presented by Garbacz et al. (2025a). The authors showed that at the site with waste rock deposits, the metal content in waste rock was as follows: Cr – 82 mg·kg<sup>-1</sup>; Ni – 46 mg·kg<sup>-1</sup>; Ba – 124 mg·kg<sup>-1</sup>; Zn – 54 mg·kg<sup>-1</sup>; Cu – 30 mg·kg<sup>-1</sup>. In the soil from the same site, the following values were recorded: Cr – 15 mg·kg<sup>-1</sup>; Ni – 6.5 mg·kg<sup>-1</sup>; Ba – 27.5 mg·kg<sup>-1</sup>; Zn – 13.9 mg·kg<sup>-1</sup>; Cu – 5.3 mg·kg<sup>-1</sup>. However, at the site without waste rock deposits, the metal content in the soil was even lower than in the soil with deposits and amounted to: Cr – 6.5 mg·kg<sup>-1</sup>; Ni – 5.5 mg·kg<sup>-1</sup>; Ba – 18.8 mg·kg<sup>-1</sup>; Zn – 11.5 mg·kg<sup>-1</sup>; Cu – 4.0 mg·kg<sup>-1</sup> (Garbacz et al., 2025a). This, in turn, suggests the possibility of metal transfer to the green parts of plants and foot of snails. To determine the degree of impact of pollutants on land snails *C. nemoralis*, which are a very useful source of biomarkers, it is particularly worth focusing on the panel of biomarker FAs C16:0; C18:0; C23:0; C18:1 n-9; C20:1 n-9; C18:2 n-6; C18:3 n-3; C20:2 n-6; C20:4 n-6; C20:5 n-3; C22:4 n-6; and C22:5 n-3, as well as the peroxidation index (PI) and unsaturation index (UI). The study showed that in *in situ* and *in vivo* conditions, the predominant FAs were C20:2n-6, C20:4 n-6, and C18:2 n-6 among PUFAs (polyunsaturated fatty acids); C18:0 among SFAs (saturated fatty acids); and C18:1 n-9 among MUFAs (monounsaturated fatty acids).

Environmental stressors induce oxidation processes in organisms, leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and to the induction of autooxidation processes. PUFAs present in cell membranes are particularly susceptible to the effects of free radicals, as they undergo peroxidation (oxidation). Fatty acid peroxidation is an important biomarker of the response to environmental stressors, enabling assessment of the degree of oxidative damage induced by external factors, including environmental pollutants such as metals (Chakraborty et al., 2024; Singh et al., 2023). Differences in the accumulation of metals, both in the soft tissues of snails, including the foot or hepatopancreas, and in their shells, are due to the unique ability of individual snail species to regulate or accumulate metals and to their habitat conditions (Abdullah et al., 2007; Christopher et al., 2010; Vranković et al., 2020). They are able to sequester metals in the hepatopancreas, accumulating them in granules or binding them to metallothioneins. The foot can take part in the immobilization and detoxification of metals by binding them in the outer mucus layers and in tissues in direct contact with the ground. Secreted mucus can limit the absorption of metals into the body, and at the same time enable their removal from the surface of the body (Boshoff et al., 2013). Snails' ability to accumulate metals is influenced by the level of pollution, the digestive physiology of a given species, and the rate of absorption of metals, as well as the metabolism, body weight and age of the organism (Gawad, 2018; Vranković et al., 2020). Each organism has its own defence strategy against the harmful effects of metals (Ardestani et al., 2014; Kowalczyk-Pecka et al., 2017b). Snail defences rely on the detoxifying role of the hepatopancreas, restriction of consumption of contaminated food, estivation, and the ability to encapsulate and mechanically remove metals from the body (Kowalczyk-Pecka et al., 2017a). Apart from the special detoxifying role of the hepatopancreas, it is also able to produce enzymes and is regarded as a crucial site of numerous redox reactions involving high levels of activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT)

(Kowalczyk-Pecka et al., 2017b). At low concentrations in the natural environment, defense mechanisms such as detoxification and the activation of antioxidant systems appear to be less effective than in laboratory conditions. Land snails are able to detect elevated levels of metals in food (El-Gendy et al., 2021). The decreased metabolic rate during estivation allows them to save energy, extending their survival time (Wang et al., 2015).

The results of the study show that Zn, Cu, Cr, Ni, and Ba in *in situ* conditions (experimental group I-SF, influenced by waste rock, and control group II-SF, without waste rock) and Cr (0.75; 1.0), Ni (0.5; 0.75; 1.0) and Ba (0.5; 0.75; 1.0) in *in vivo* conditions (experimental group) were associated with changes in the composition of biomarker FAs and indices in snails *Cepaea nemoralis*. In the foot of snails from the site with the waste rock deposit, the content of SFAs and MUFAs was higher and that of PUFAs was lower than in the foot of snails from the site without waste rock. Alternatively, the observed variability may result from other mechanisms, such as dietary selection (Silva et al., 2017), decreased assimilation (Kowalczyk-Pecka et al., 2017b; Silva et al., 2017), or metabolic remodeling (El-Sikaily and Shabaka, 2024), rather than solely from peroxidation losses. Furthermore, the observed increases in SFAs and MUFAs and decreases in PUFAs may not solely reflect the influence of waste rock. Other explanations also include potential confounding factors such as differences in diet and seasonality (Garbacz et al., 2025a). A noticeable decrease in PUFA was observed in the *in vivo* experimental samples from snails compared to the *in vivo* control samples whose diet was supplemented with Cr (0.75; 1.0), Ni (0.5; 0.75; 1.0) and Ba (0.5; 0.75; 1.0). Analysis of the *in vivo* and *in situ* results revealed that only for Cr 1.0, Ba 1.0 and Ni 1.0 was the content of SFAs and MUFAs higher than in the samples from the site with no waste rock deposit. At the same time, these contents were lower than in the samples from the site with the waste rock deposit. A similar pattern was noted for Ni 0.75 in the case of SFAs. For PUFAs, marked differences were observed only at Ba 1.0 and Ni 1.0, for which the content was lower than in the samples from the site without waste rock but higher than in the samples from the location where it was present. Furthermore, the PI (peroxidation index), expressing the total content of fatty acids in the cell membrane, and UI (unsaturation index), reflecting the number of double bonds in the membranes (Hulbert et al., 2014, 2007), were lower in the snails from the site with the waste rock deposit than in the control group – snails from the location without waste rock. Comparison of the *in vivo* analyses with the *in situ* results shows that only for Ni 1.0 and Ba 1.0 were the PI values lower than in the *in situ* control group (II-SF) and higher than in the *in situ* experimental samples from the site with waste rock (I-SF). In the case of UI, a similar pattern was noted for Cr 1.0, Ba 1.0, and Ni 0.75. In addition, the UI values for Ba 0.5 and Ni 1.0 were lower than in the samples from the sites with and without waste rock. Interestingly, in laboratory conditions, an increase in PUFAs and a decrease in SFAs and MUFAs were observed for all applied concentrations of Zn and Cu and Cr 0.5. Subthreshold levels of the elements Zn, Cu and Cr are essential to life. This means that even small concentrations that do not cause acute toxicity or death can induce perceptible physiological changes (Garbacz et al., 2025a). Their demand for metals varies depending on the species, and both excessive intake and deficiency can be harmful for organisms (Vranković et al., 2020). Zinc plays many roles in metabolism, which can be divided into three main categories – catalytic, structural and regulatory functions – and is also a crucial component of the catalytic centres of metalloenzymes (Beigi and Mavarakis, 2015). The toxic effects of zinc affect various cellular processes, particularly the functioning of lipid membranes (Nowakowska et al., 2016; Valko et al., 2005). Zinc inhibits Na<sup>+</sup>K<sup>+</sup>ATPase, which is involved in transport through cell membranes, and in this way disrupts osmoregulatory processes in invertebrates (Kowalczyk-Pecka et al., 2017b). Copper is an element essential to the normal physiological functioning of snails, which they require as a component of haemocyanin (Boshoff et al., 2015; Kowalczyk-Pecka et al., 2017b). It also acts as a catalyst in the Fenton and Haber–Weiss reactions, facilitating the

transformation of superoxide anion and hydrogen peroxide into hydroxyl radical, which has strong oxidizing properties. Oxidative stress, which can result from the presence of copper, leads to DNA damage, lipid peroxidation, and protein modifications (Atli and Grosell, 2016; Kowalczyk-Pecka et al., 2017b; Radwan et al., 2010b, 2010a). Chromium plays an important role in regulating carbohydrate metabolism and enzymes in animals (Havel, 2004). In biological terms, chromium is a crucial element for animals because its reduced form Cr(III) is involved in glucose metabolism, performing the function of an active component of a factor influencing glucose tolerance. Some forms of chromium, however, can be harmful; for example, the oxidized form of Cr(VI) is 100–1000 times as toxic as Cr(III) (Cœurassier et al., 2000). In the case of nickel, exceeding allowable levels can be dangerous (de Roma et al., 2017). The toxicity of Cr is strongly dependent on its oxidation state. In this study, no direct speciation analyses of chromium in waste rock or exposures were performed, which is one of the limitations of the work. Nevertheless, based on the geological characteristics of the studied material, it can be assumed that Cr(III) was the dominant form of chromium. Waste rock is sedimentary rock with a mineral composition dominated by clay minerals (about 60 %) and mudstone, mainly quartz (about 30 %). Sandstone accounts for only about 10 % of the mineral composition of waste rock (Kujawska, 2018). For this reason, Cr in the form of Cr(III) was used in the *in vivo* experiment.

Very high nickel concentrations can be lethal for molluscs, with the consequences depending on their age (Reddy et al., 2011; Uvaeva et al., 2023), the hydrochemical conditions in their environment, the amount of food consumed, and their overall health condition and genetic traits (Oros and Gomoiu, 2010; Uvaeva et al., 2023; Zuykov et al., 2013). On the other hand, it is an essential element for the body in small quantities, playing a key role in haematopoiesis and reproduction (Uvaeva et al., 2023). In the present study, Ni applied at 0.75 and 1.0 had the most harmful effect in laboratory conditions, as SFAs and MUFAs increased, while PUFAs decreased. Among all concentrations of metals analyzed *in vivo*, the highest SFA content and lowest PUFA content, as well as the lowest PI and UI indices, were found for Ni (1.0).

The results of the *in vivo* analyses suggest that the observed responses to the metal mixtures used (Mix 0.5; Mix 0.75; Mix 1.0) may possibly reflect the balance between Zn/Cu and Ni/Ba. However, this interpretation is only a hypothesis due to the lack of a factorial design involving independent manipulation of each metal in the mixture. Additional uncertainty is introduced by the limited and difficult to estimate bioavailability of Ba in the form of BaSO<sub>4</sub>. For this reason, the observed relationships cannot be considered as evidence of the antagonistic roles of individual elements. These conclusions should be verified in future studies using factorial experiments or mixture-response modelling. In the field samples, on the other hand, even in the control sample, a noticeable decrease in PUFA content was observed compared to laboratory control samples, which may suggest potential peroxidation of fatty acids. The fatty acid profile in snails *in situ* can be affected not only by the transfer of the metals themselves, but also potentially to some degree by fatty acids from the intestinal microbiome, which has been observed in other invertebrates, such as earthworms (Garbacz et al., 2025a). It can be concluded from the data that even minimal differences in metal deposition in the field affect the physiological response in snails associated with changes in the composition of biomarker FAs, PI and UI indices, which are indirect indicators of lipid peroxidation, although metal concentrations do not differ significantly between samples taken from land reclaimed with rock waste and land without reclamation. Snails can restrict their consumption of polluted food, which is a natural phenomenon in these animals. Moreover, in the natural environment they can consume food containing other active substances, such as vitamin C, which is responsible for non-enzymatic antioxidant defence.

The foot of snails from the study sites contained high concentrations of Ba in comparison to the other elements. In contrast, the concentration of Ba in the green parts of plants was the lowest, as its compounds, for example barium salts, are very poorly soluble in water (Kujawska,

2018), and it is difficult for plants to take up. The Ba in the experimental sample most likely came from the waste rock, and the snails simply ingest it and incorporate it in their bodies. The high Ba content may have been due to absorption from the soil (Tardugno et al., 2023). Due to the high toxicity of soluble Ba compounds for snails, poorly soluble BaSO<sub>4</sub> was used as a source of this element in *in vivo* studies. The concentration of dissolved Ba in the medium was not determined due to the limited solubility of this salt. However, the low solubility of BaSO<sub>4</sub> may affect the internal bioavailability of Ba. In the land snail *Helix aspersa*, Ba has been shown to accumulate in significant amounts in the soft tissues and shell (Madoz-Escande and Simon, 2006). Snails regulate the Ba level by various physiological and biochemical mechanisms, especially in the context of interaction with calcium channels. Barium ions (Ba<sup>2+</sup>) block calcium channels of both the surface membrane and intracellular stores, that is, cell structures such as the smooth endoplasmic reticulum and mitochondria. Ba<sup>2+</sup> ions inhibit the release of Ca<sup>2+</sup> from these intracellular stores (Usachev and Mironov, 1989).

## 5. Future directions

It seems likely that in addition to metals, lipid metabolism – the survival and structure of long-chain PUFAs – is also influenced by other environmental factors in the natural environment – physical such as temperature (Edwards and Arancon, 2022), chemical such as pesticides (Uwizeyimana et al., 2017), or biological such as pathogens (Ghosh, 2018). PUFAs play an important role in maintaining cell membrane permeability, and the balance between n-6 and n-3 acids in cell and lysosome membranes is crucial to their function (Puca et al., 2008). Apart from the protective function of PUFAs involving preservation of cell membrane integrity, some of these acids exhibit antibacterial properties (Kowalczyk-Pecka et al., 2017b). Although most laboratory studies focus on the effect of single metals, snails in the natural environment often come into contact with mixtures of pollutants (Amiard-Triquet and Amiard, 1998). Many polluted soils in the natural environment contain more than one metal, which can lead to synergistic or antagonistic interactions between different pollutants (Nahmani et al., 2007). Abiotic factors, such as temperature, salinity, oxygen availability, and pH, influence physiological processes such as enzymatic activity in snails (Bula et al., 2017; Donham et al., 2022; Pathak et al., 2024; Saallah et al., 2020). According to de Almeida et al. (2007), (2004), a variety of environmental factors, such as seasonal changes, temperature, oxygen concentration, and the type of stressor, can lead to significant changes in the antioxidant defence system of invertebrates. In addition, increasing vehicular traffic and emissions of air pollutants pose a serious threat to environmental health (Vranković et al., 2020). The issues presented are prospective in nature and may indicate potential directions for further research.

## 6. Conclusions

Snails are a source of useful biomarkers of biological (physiological) effects. The proposed panel of biomarker fatty acids, including C16:0, C18:0, C23:0, C18:1 n-9, C20:1 n-9, C18:2 n-6, C18:3 n-3, C20:2 n-6, C20:4 n-6, C20:5 n-3, C22:4 n-6, and C22:5 n-3, can be used to assess the exposure of snails (Gastropoda Pulmonata) to selected environmental variables in the form of metals in both natural and laboratory conditions.

The possibility of transferring metals deposited in an anthropogenically polluted environment whose main element was a waste rock deposit, was observed. Waste rock can be treated a potential environmental stressor and a possible source of pollutants such as metals. This is associated with observed changes in the composition of biomarker fatty acids, peroxidation and unsaturation indices in snails *Cepaea nemoralis*, which are indirect indicators of lipid peroxidation.

Under *in situ* conditions with and without waste rock deposits, metals such as Zn, Cu, Cr, Ni and Ba, and under *in vivo* in laboratory conditions

in the experimental group, metals such as Cr (0.75; 1.0), Ni (0.5; 0.75; 1.0) and Ba (0.5; 0.75; 1.0) were associated with changes in the composition of biomarker fatty acids and indices in snails *Cepaea nemoralis*. *In situ*, snails *Cepaea nemoralis* from the site with the waste rock deposit, the least favourable physiological reaction was observed, which was manifested by a noticeable decrease in PUFA content (the lowest PUFA content) and at the same time an increase in SFA content (the highest SFA content) compared to the field and laboratory control samples and the experimental laboratory samples, which may suggest potential peroxidation of fatty acids. At the same time, in the field samples, even the control, a noticeable reduction in PUFA content was observed compared to control and experimental laboratory samples.

It cannot be definitively concluded from this study alone, whether the effect of waste rock is beneficial or harmful, because some metals at low concentrations, such as Zn and Cu, protect PUFAs against oxidation in snails *Cepaea nemoralis*.

The study was conducted in both field and laboratory conditions, which is an important advantage as it enables comparison of the reactions of snails in controlled conditions with those of snails present in the natural environment. Most studies of biomarkers in land snails have been carried out in laboratory conditions, which provides valuable information about the mechanisms of their reactions to metals. However, FAs have rarely been studied as biomarkers in field conditions, where the effects of multiple factors can be taken into account at the same time. In these conditions, the animals can be exposed to various pollutants simultaneously, which can lead to changes in their fatty acid profile and intensify FA peroxidation (El-Gendy et al., 2021). *In situ* studies take into account the complicated interactions between organisms and their environment, which makes it possible to detect the multi-faceted consequences of pollution that may not be visible in laboratory conditions. This approach offers a more realistic picture of the environmental impact of substances (Crane et al., 2007; Lesel, 1976). The results of the study indicate that an effective biomarker should be tested in both laboratory and field conditions, which is supported in the literature (Radwan et al., 2020). In addition, particular attention should be paid to the control sample in field studies because, as in ecotoxicological studies, it is an essential reference point, and environmental factors must be comparable with the experimental sample in the field. Combining the *in situ* and *in vivo* (in laboratory conditions) results is essential to a comprehensive understanding of the induction, course and consequences of oxidative stress. While laboratory experiments allow for controlled isolation of specific variables, *in situ* studies provide insight into the complex interactions between organisms and their natural habitat. This dual nature of the research increases the reliability of results concerning oxidative damage and fatty acid metabolism in molluscs and can be successfully employed in practical ecotoxicology.

#### CRedit authorship contribution statement

**Danuta Kowalczyk-Pecka:** Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Weronika Kurska:** Visualization. **Aleksandra Garbacz:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119932.

#### Data availability

Data will be made available on request.

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## **Supplementary materials**

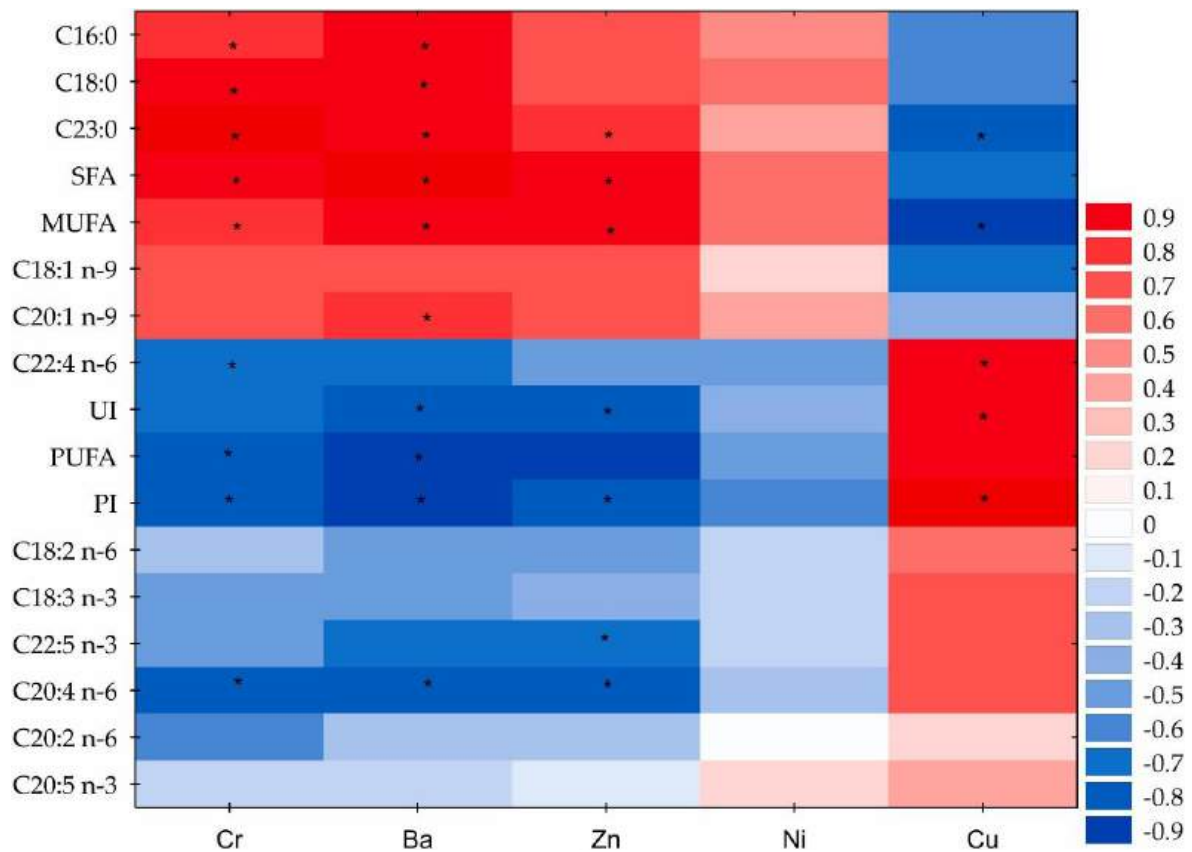


**Fig. S1.** The heatmap of Pearson's correlation coefficient ( $r$ ) between metals (Cr, Ba, Ni, Zn, and Cu) and biomarker FAs and peroxidation (PI) and unsaturation (UI) indices in the foot of *Cepaea nemoralis* snails (*in situ*). C16:0—palmitic acid; C18:0—stearic acid; C23:0—tricosanoic acid; C18:1 n-9—oleic acid; C20:1 n-9—cis-11-eicosenoic acid; C18:2 n-6—linoleic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6—cis-11 14-eicosadienoic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenoic acid, EPA; C22:4 n-6—docosatetraenoic acid; C22:5 n-3—docosapentaenoic acid; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index; Positive correlations are shown in red and negative correlations in blue; color intensity reflects the strength of the relationship; \*significant at  $p \leq 0.01$

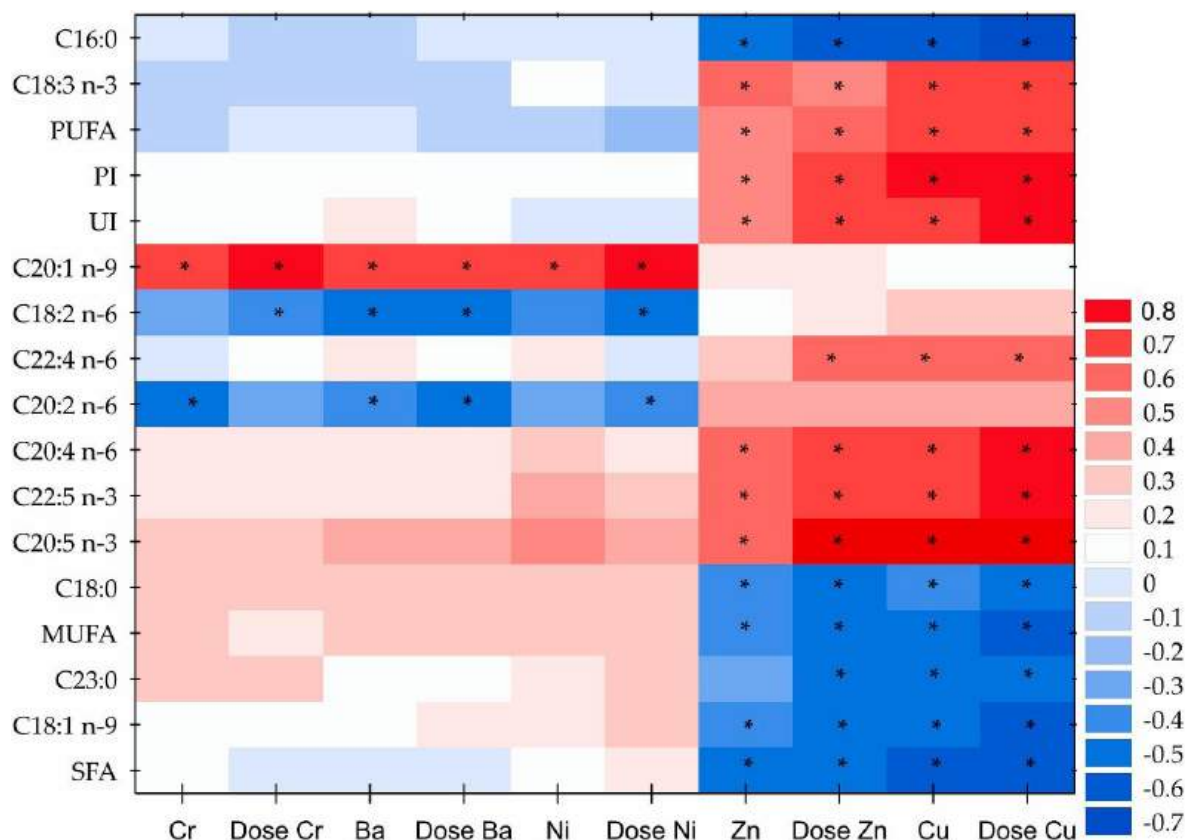


**Fig. S2.** The heatmap of Pearson's correlation coefficient (r) between metals (Cr, Ba, Ni, Zn, and Cu) and their applied concentrations and biomarker FAs and peroxidation (PI) and unsaturation (UI) indices in the foot of *Cepaea nemoralis* snails *in vivo* (in laboratory conditions). C16:0—palmitic acid; C18:0—stearic acid; C23:0—tricosanoic acid; C18:1 n-9—oleic acid; C20:1 n-9—cis-11-eicosenoic acid; C18:2 n-6—linoleic acid; C18:3 n-3— $\alpha$ -linolenic acid; C20:2 n-6— cis-11 14-eicosadienoic acid; C20:4 n-6—arachidonic acid; C20:5 n-3—cis-5,8,11,14,17-eicosapentaenoic acid, EPA; C22:4 n-6—docosatetraenoic acid; C22:5 n-3—docosapentaenoic acid; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; PI—peroxidation index; UI—unsaturation index; Positive correlations are shown in red and negative correlations in blue; color intensity reflects the strength of the relationship; \*significant at  $p \leq 0.01$

**10. Oświadczenia doktoranta oraz współautorów dotyczących ich wkładu w przygotowanie opublikowanych prac naukowych**

Lublin, 06 marca 2026 r.

mgr inż. Aleksandra Garbacz  
Katedra Zoologii i Ekologii Zwierząt  
Wydział Biologii Środowiskowej  
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**Rada Dyscypliny**  
**Nauki Biologiczne**  
**Uniwersytetu Przyrodniczego w Lublinie**

### **Oświadczenie o współautorstwie**

Niniejszym oświadczam, że mój udział w publikacji:

Garbacz A., Nowak A., Marzec-Grządziel A., Przybyś M., Gałązka A., Jaroszuk-Ścisel J., Grzywaczewski G. (2025). Impact of coal waste rock on biological and physicochemical properties of soils with different agricultural uses. Sustainability 17(6), 2603. <https://doi.org/10.3390/su17062603>

polegał na opracowaniu koncepcji badań, zebraniu materiału badawczego, wykonaniu badań (określenie właściwości fizyko-chemicznych prób gleby i aktywności biologicznej gleby, wyznaczenie wskaźników fizjologicznego profilowania gleby, określenie ogólnej liczebności mikroorganizmów hodowlanych i liczebności grup fizjologicznych mikroorganizmów hodowlanych, izolacja DNA), analizie wyników, sformułowaniu wniosków, współudziale w napisaniu tekstu manuskryptu i odpowiedzi na recenzje, redagowaniu publikacji, korespondencji z redakcją czasopisma, korekcie pracy po recenzjach, działaniach koordynacyjnych i organizacyjnych oraz pozyskaniu funduszy na badania.

Aleksandra Garbacz.....  
Podpis

Lublin, 06 marca 2026 r.

dr Artur Nowak  
Katedra Mikrobiologii Przemysłowej i Środowiskowej  
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polegał na opracowaniu metodyki, przeprowadzeniu analizy statystycznej, wizualizacji, analizowaniu i interpretacji wyników, współudziale w napisaniu tekstu manuskryptu i odpowiedzi na recenzje.



Podpis

Lublin, 06 marca 2026 r.

dr Anna Marzec-Grządziel  
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polegał na obróbce bioinformatycznej i analizowaniu danych, współudziale w napisaniu tekstu manuskryptu i przeprowadzeniu analizy statystycznej.

  
.....  
Podpis

Lublin, 06 marca 2026 r.

dr Marcin Przybyś  
Zakład Biotechnologii i Hodowli Roślin  
Instytut Uprawy Nawożenia i Gleboznawstwa  
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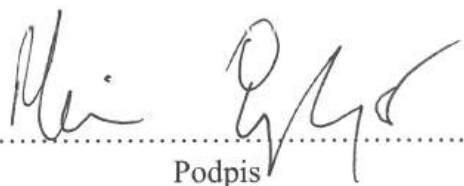
**Rada Dyscypliny  
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polegał na wykonaniu badań dotyczących analizy DNA mikroorganizmów występujących w badanych próbach gleb.

  
.....  
Podpis

Lublin, 06 marca 2026 r.

prof. dr hab. Anna Gałązka  
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Instytut Uprawy Nawożenia i Gleboznawstwa  
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polegał na współudziale w napisaniu tekstu manuskryptu.

..... Anna Gałązka .....  
Podpis

Lublin, 06 marca 2026 r.

dr hab. Jolanta Jaroszuk-Ścisiel, prof. uczelni  
Katedra Mikrobiologii Przemysłowej i Środowiskowej  
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Properties of Soils with Different Agricultural Uses. Sustainability 17(6), 2603.  
<https://doi.org/10.3390/su17062603>

polegał na opracowaniu koncepcji badań, korespondencji z redakcją czasopisma,  
współudziale w napisaniu tekstu manuskryptu i odpowiedzi na recenzje.



.....  
Podpis

Lublin, 06 marca 2026 r.

dr hab. Grzegorz Grzywaczewski, prof. uczelni  
Katedra Zoologii i Ekologii Zwierząt  
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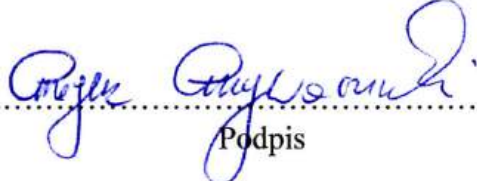
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polegał na opracowaniu koncepcji badań, udziale w zbieraniu materiału badawczego, analizowaniu danych i współudziale w napisaniu tekstu manuskryptu.

  
.....  
Podpis

Lublin, 06 marca 2026 r.

mgr inż. Aleksandra Garbacz  
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polegał na współudziale w opracowaniu koncepcji badań i metodyki, zebraniu materiału badawczego, wykonaniu badań, współudziale w analizie wyników, sformułowaniu wniosków, współudziale w napisaniu tekstu manuskryptu, udziale w napisaniu odpowiedzi na recenzje, redagowaniu publikacji, korespondencji z redakcją czasopisma, korekcie pracy po recenzjach, działaniach koordynacyjnych i organizacyjnych oraz pozyskaniu funduszy na badania.

*Aleksandra Garbacz*.....  
Podpis

Lublin, 06 marca 2026 r.

dr hab. Danuta Kowalczyk-Pecka, prof. uczelni  
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.....

Podpis

Lublin, 06 marca 2026 r.

dr inż. Weronika Kursa  
Katedra Ochrony Roślin  
Wydział Ogrodnictwa i Architektury Krajobrazu  
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**Rada Dyscypliny  
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polegał na przeprowadzeniu analizy statystycznej i wizualizacji wyników.



.....  
Podpis

Lublin, 06 marca 2026 r.

mgr inż. Aleksandra Garbacz  
Katedra Zoologii i Ekologii Zwierząt  
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aleksandra.garbacz@up.edu.pl

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Aleksandra Garbacz.....  
Podpis

Lublin, 06 marca 2026 r.

dr inż. Weronika Kursa  
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Wydział Ogrodnictwa i Architektury Krajobrazu  
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.....  
Weronika Kursa  
Podpis

Lublin, 06 marca 2026 r.

dr hab. Danuta Kowalczyk-Pecka, prof. uczelni  
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polegał na współudziale w opracowaniu koncepcji badań i metodyki, współudziale w analizie wyników oraz współudziale w napisaniu tekstu manuskryptu.

  
Podpis

## 11. Aneks

### Wykształcenie

- 2021-2026 r.**      **Szkoła Doktorska Uniwersytetu Przyrodniczego w Lublinie**  
Kształcenie w dyscyplinie: nauki biologiczne
- 2020-2021 r.**      **Uniwersytet Przyrodniczy w Lublinie**  
**Kierunek: Biotechnologia (studia II stopnia)**  
**Uzyskany tytuł zawodowy: magister inżynier**  
Tytuł pracy magisterskiej: „Ocena bezpieczeństwa bakteriologicznego hemolimfy ślimaków jadalnych z gatunku *Cornu aspersum* (Helicidae)”.
- 2018-2021 r.**      **Uniwersytet Przyrodniczy w Lublinie**  
**Kierunek: Biologia (studia I stopnia)**  
**Uzyskany tytuł zawodowy: licencjat**  
Tytuł pracy licencjackiej: „Lekowrażliwość i czynniki wirulencji bakterii *Escherichia coli* izolowanych od ptaków”.
- 2016-2021 r.**      **Uniwersytet Przyrodniczy w Lublinie**  
**Kierunek: Biotechnologia (studia I stopnia)**  
**Uzyskany tytuł zawodowy: inżynier**  
Tytuł pracy inżynierskiej: „Izolacja potencjalnych patogenów ze śluzu ślimaków *Cornu aspersum* Müller”.

### Stáže naukowe i mobilności zagraniczne

- sierpień–wrzesień 2024 r.**      **zagraniczny staż naukowy w ramach programu Erasmus+**  
Prešovská Univerzita v Prešove, Katedra Ekologii, Prešov, Słowacja
- lipiec 2024 r.**      **mobilność zagraniczna w ramach programu Erasmus+**  
Samsun Ondokuz Mayıs University: „Blended Intensive Program for Sustainable Agriculture”, Samsun, Turcja
- sierpień–wrzesień 2023 r.**      **zagraniczny staż naukowy w ramach programu Erasmus+**  
Universitätsmedizin Greifswald, Instytut Immunologii, Greifswald, Niemcy
- marzec–maj 2023 r.**      **krajowy staż naukowy w ramach II edycji programu „Staż za Miedzą” Związku Uczelni Lubelskich**

Katedra Mikrobiologii Przemysłowej i Środowiskowej,  
Uniwersytet Marii Curie Skłodowskiej w Lublinie, Lublin,  
Polska

## Zatrudnienie

**01.12.2023 r. do chwili obecnej** asystent, Katedra Zoologii i Ekologii Zwierząt, Wydział  
Biologii Środowiskowej, Uniwersytet Przyrodniczy  
w Lublinie

## Dorobek publikacyjny

- 1) Joniec J., Kwiatkowska E., Walkiewicz A., Grzywaczewski G., **Garbacz A.** (2025). Assessment of N-related microbial processes in the soil of the Polesie National Park and adjacent areas, including reclaimed land. *Journal of Environmental Management*, 373, 124002. <https://doi.org/10.1016/j.jenvman.2024.124002> (MNiSW = 200 pkt, IF = 9,2)
- 2) Kamińska A., **Garbacz A.**, Sadurski S. Katalaza i dysmutaza ponadtlenkowa jako wybrane enzymatyczne biomarkery stresu oksydacyjnego (2024). W: Wybrane zagadnienia z zakresu ochrony i zagrożeń środowiska. Tom 4, M. Babicz, B. Nowakowicz-Dębek, A. Gawryluk (red.). Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie, 85-91. <https://doi.org/10.24326/mon.2024.13> (MNiSW = 20 pkt, IF = 0,0)
- 3) **Garbacz A.** (2023). Zagrożenia siedlisk przyrodniczych Polesia przyczyną powstania zagrożenia produkcji żywności o podwyższonych walorach prozdrowotnych. W: Lokalne rasy zwierząt w ochronie bioróżnorodności i zachowaniu tradycji regionów, W. Chabuz, W. Sawicka-Zugaj (red.). Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie, 11-21. <https://doi.org/10.24326/mon.2023.8> (MNiSW = 20 pkt, IF = 0,0)
- 4) Kawalec M., **Garbacz A.**, Dec M. (2022). Mikrobiologiczne biosurfaktanty – rodzaje i zastosowanie w bioremediacji środowiska. W: Wybrane zagadnienia z zakresu ochrony i zagrożeń środowiska. Tom 2, M. Babicz, B. Nowakowicz-Dębek, K. Kropiwek-Domańska (red.). Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie, 87-94. <https://doi.org/10.24326/mon.2022.10> (MNiSW = 20 pkt, IF = 0,0)
- 5) **Garbacz A.** (2021). Zjawisko quorum sensing u bakterii. W: Wybrane zagadnienia z zakresu ochrony i zagrożeń środowiska, M. Babicz, B. Nowakowicz-Dębek (red.).

Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie, 20-26.  
<https://doi.org/10.24326/mon.2021.6> (MNiSW = 20 pkt, IF = 0,0)

- 6) Kawalec M., **Garbacz A.**, Dec M. (2021). Nietolerancja laktozy – przyczyny i diagnostyka. W: Wybrane zagadnienia z zakresu bromatologii, M. Babicz, K. Kropiwiec-Domańska (red.). Wydawnictwo Uniwersytetu Przyrodniczego w Lublinie, 58-65. <https://doi.org/10.24326/mon.2021.8> (MNiSW = 20 pkt, IF = 0,0)

**Liczba punktów MNiSW = 300; IF = 9,2**

## **Udział w konferencjach naukowych**

### **• Referaty wygłoszone na konferencjach naukowych**

- 1) **Garbacz A.** Wyzwania związane z zachowaniem różnorodności biologicznej na Polesiu. IV Międzynarodowa Multidyscyplinarna Konferencja Doktorantów Uniwersytetu Szczecińskiego „MKDUS 2.0”, 12 (19-21.06.2024 r.)
- 2) **Garbacz A.**, Grzywaczewski G. Analysis of the phenotypic diversity of the *Enterobacterales* microflora of the digestive system as a physiological biomarker of land snails of the genus *Cepaea* from Polesie. 3rd International PhD Students' Conference: Environment-Plant-Animal Product together with the International Workshops at the University of Life Sciences in Lublin (ICDSUPL), E008. <https://doi.org/10.24326/ICDSUPL3.E008> (24-26.04.2024 r.)
- 3) **Garbacz A.**, Grzywaczewski G. Analysis of the phenotypic diversity of *Enterobacterales* microflora digestive system as a physiological biomarker of *Lumbricus* sp. earthworms from the wetland ecosystems of Polesie. 2nd International PhD Student's Conference at the University of Life Sciences in Lublin, Poland: Environment-Plant-Animal-Product (ICDSUPL), E014. <https://doi.org/10.24326/ICDSUPL2.E014> (19-20.04.2023 r.)
- 4) **Garbacz A.** Kadm jako silnie toksyczny pierwiastek oddziałujący na zwierzęta. VII Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen. Sesja: Ekologia i Ochrona Środowiska, 30. ISBN 978-83-67376-00-6 (7-8.04.2022 r.)
- 5) **Garbacz A.** Ochrona gatunkowa ślimaków w Polsce. Ogólnopolska Konferencja Naukowa „Ochrona środowiska” (23.02.2022 r.)
- 6) **Garbacz A.** Pesticides as a serious threat to many animal species occurring in Polesie. Ogólnopolska Konferencja Naukowa „Globalne problemy” (16.02.2022 r.)
- 7) **Garbacz A.** Antropogeniczna degradacja ekosystemów torfowiskowych Polesia. Ogólnopolska Konferencja Naukowa „Ekosystemy” (10.02.2022 r.)

- 8) **Garbacz A.** Zmiany klimatyczne zagrożeniem dla zwierząt. Ogólnopolska Konferencja Naukowa „Zmiany klimatyczne” (23.11.2021 r.)
- 9) **Garbacz A.** Zatoczek łamliwy jako gatunek chroniony. Ogólnopolska Konferencja Naukowa „Ochrona roślin i zwierząt” (19.07.2021 r.)
- 10) **Garbacz A.,** Rachwał K. Toksyna botulinowa – mechanizm działania i zastosowanie. II Międzynarodowe Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”, 130 (15.04.2021 r.)
- 11) **Garbacz A.,** Dzięgiel B. Nutritional value of edible *Cornu* snail meat – a literature review. National Scientific Conference „Understand The Science” IV Edition, 44 (26.09.2020 r.)
- 12) **Garbacz A.** Jakość mikrobiologiczna mięsa ślimaków jadalnych. IX Kopernikańskie Sympozjum Studentów Nauk Przyrodniczych, 27 (19.09.2020 r.)
- **Postery zaprezentowane na konferencjach naukowych**
  - 1) **Garbacz A.,** Czepiel-Mil K., Kamińska A. Diversity of thrips fauna (Insecta, Thysanoptera) of two types of meadow communities in Pliszczyn. 5th International PhD Students’ Conference: Environment-Plant-Animal-Product together with the International Workshops at the University of Life Sciences in Lublin, Poland:, E008. <https://doi.org/10.24326/ICDSUPL5.E008> (22-24.04.2026 r.)
  - 2) **Garbacz A.,** Grzywaczewski G. The influence of selected anthropogenic factors on enzymatic biomarkers of oxidative stress in earthworms (Lumbricidae) and snails *Cepaea nemoralis* (Helicidae) Polesie. 4th International PhD Students’ Conference: Environment-Plant-Animal-Product (I-SDUPL) together with the Information campaign on the Lublin Digital Union (LUC) – digital solutions and artificial intelligence in medical, natural and technical sciences, E004. <https://doi.org/10.24326/ICDSUPL4.E004> (09.04.2025 r.)
  - 3) **Garbacz A.,** Grzywaczewski G. Analysis of non-enzymatic biomarkers of oxidative stress in earthworms (Lumbricidae) and snails *Cepaea nemoralis* (Helicidae) of Polesie as a result of the impact of selected anthropogenic factors. 4th International PhD Students’ Conference: Environment-Plant-Animal-Product (I-SDUPL) together with the Information campaign on the Lublin Digital Union (LUC) – digital solutions and artificial intelligence in medical, natural and technical sciences, E005. <https://doi.org/10.24326/ICDSUPL4.E005> (09.04.2025 r.)

- 4) **Garbacz A.**, Grzywaczewski G. Potencjał dżdżownic (Lumbricidae) w monitorowaniu zanieczyszczeń środowiska glebowego. V Ogólnopolska Konferencja Doktorantów Nauk Ścisłych i Przyrodniczych „Bio-Idea 5.0”, 58 (01.02.2025 r.)
- 5) **Garbacz A.**, Jaroszuk-Ściśeł J., Nowak A., Marzec-Grządziel A., Przybyś M., Słomka A., Gałązka A., Grzywaczewski G. Zróżnicowanie mikrobiomu bakteryjnego w glebach uprawnych i nieużytku uzupełnionych skałą płonną – odpadem po wydobyciu węgla kamiennego. VIII Ogólnopolskie Sympozjum Mikrobiologiczne „Metagenomy różnych środowisk”, 69 (17-18.06.2024 r.)
- 6) Kamińska A., **Garbacz A.**, Sadurski J. Proteomika – nowoczesna analiza białek. V Międzynarodowe Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”, 169 (18.04.2024 r.)
- 7) **Garbacz A.**, Grzywaczewski G. Czy idea zrównoważonego rozwoju zagraża środowisku? IV Ogólnopolska Konferencja Doktorantów Nauk Ścisłych i Przyrodniczych „Bio-Idea 4.0”, 63 (03.02.2024 r.)
- 8) **Garbacz A.**, Nowak A., Jaroszuk-Ściśeł J., Słomka A., Grzywaczewski G. Oddziaływanie skały płonnej z odpadów pokopalnianych na aktywność biologiczną gleb. 55 Jubileuszowa Konferencja Mikrobiologiczna „Mikrobiologia w badaniach środowiskowych – rys historyczny i perspektywy na przyszłość”, 55 (14-15.09.2023 r.)
- 9) **Garbacz A.** Zagrożenia bioróżnorodności Polesia jako element zagrożenia produkcji żywności o podwyższonych walorach prozdrowotnych. Konferencja Międzynarodowa „Rodzime rasy zwierząt jako ważny element ochrony bioróżnorodności, zachowania tradycji regionów oraz produkcji żywności o podwyższonych walorach prozdrowotnych” (14-15.06.2022 r.)
- 10) **Garbacz A.**, Jerzy Ziętek J. Isolation and identification of bacteria from the hemolymph of *Cornu aspersum* snails. 1st International PhD Student's Conference at the University of Life Sciences in Lublin, Poland: Environment–Plant–Animal–Product, A010. <https://doi.org/10.24326/ICDSUPL1.A010> (26.04.2022 r.)
- 11) Kawalec M., **Garbacz A.**, Dec M. Mięso z próbki – zalety i wady nowoczesnej technologii pozyskiwania mięsa. III Międzynarodowe Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”, 180. <https://doi.org/10.24326/mon.2022.2> (21.04.2022 r.)
- 12) Kawalec M., **Garbacz A.**, Dec M. Mykoproteina zamiennikiem mięsa. II Międzynarodowe Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”, 155 (15.04.2021 r.)

## **Aktywność popularyzatorska**

- 1) Współopiekun warsztatów „Gekon – jak rozpocząć hodowlę?” i „Tajemnice sześciu nóg – identyfikacja owadów” podczas Dnia Otwartego Uniwersytetu Przyrodniczego w Lublinie (19.03.2026 r.)
- 2) Współprowadzenie warsztatów „Twój pierwszy gekon” w ramach Nocy Biologów, Wydział Biologii Środowiskowej, Uniwersytet Przyrodniczy w Lublinie (16.01.2026 r.)
- 3) Współorganizator warsztatów „Akwarystyka dla początkujących – jak stworzyć własny, podwodny świat?” i „Klucz do świata owadów – jak je identyfikować?” podczas Lubelskiego Festiwalu Nauki (16.09.2025 r.)
- 4) Współopiekun warsztatów „Hodujemy owady egzotyczne” i „Hodujemy ślimaki egzotyczne” podczas Dnia Otwartego Uniwersytetu Przyrodniczego w Lublinie (03.04.2025 r.)
- 5) Współorganizator stoiska SKN Zoologów podczas Dnia Otwartego Uniwersytetu Przyrodniczego w Lublinie (03.04.2025 r., 07.03.2024 r.)
- 6) Współprowadzenie wykładu „Jak ocieplenie klimatu wpływa na krajowe owady?” w ramach Nocy Biologów, Wydział Biologii Środowiskowej, Uniwersytet Przyrodniczy w Lublinie (10.01.2025 r.)
- 7) Współprowadzenie warsztatów „Kolory w świecie owadów” i „Stawonogi wokół mnie – nie boję się!”. Stacjonarne warsztaty zoologiczne w ramach Nocy Biologów, Wydział Biologii Środowiskowej, Uniwersytet Przyrodniczy w Lublinie (12.01.2024 r.)
- 8) Współprowadzenie warsztatów „Co w wodzie piszczy? Wodny świat bezkręgowców. Stacjonarne warsztaty zoologiczne” w ramach Nocy Biologów, Wydział Biologii Środowiskowej, Uniwersytet Przyrodniczy w Lublinie (13.01.2023 r.)
- 9) Utworzenie na Facebooku strony Katedry Zoologii i Ekologii Zwierząt UP w Lublinie i sprawowanie funkcji administratora - promocja Katedry i popularyzacja wiedzy naukowej w mediach społecznościowych (od 13.12.2021 r. do chwili obecnej)
- 10) Czynny udział w organizacji Dnia Otwartego Uniwersytetu Przyrodniczego w Lublinie – SKN Biotechnologów „Biom” (07.03.2019 r., 08.03.2018 r.)

## **Aktywność organizacyjna**

- 1) Członek komitetu organizacyjnego V edycji Międzynarodowej Konferencji Doktorantów „5th International PhD Students' Conference: Environment-Plant-

Animal-Product together with the International Workshops at the University of Life Sciences in Lublin, Poland” (22-24.04.2026 r.)

- 2) Moderator sekcji “Health and Basic Sciences” podczas V edycji Międzynarodowej Konferencji Doktorantów „5th International PhD Students’ Conference: Environment-Plant-Animal-Product together with the International Workshops at the University of Life Sciences in Lublin, Poland” (22-24.04.2026 r.)
- 3) Członek komitetu organizacyjnego „Próbna Matura z Biologii na Uniwersytecie Przyrodniczym w Lublinie” (18.04.2026 r.)
- 4) Członek komitetu organizacyjnego „Próbna Matura z Biologii na Uniwersytecie Przyrodniczym w Lublinie” (12.04.2025 r.)
- 5) Członek Akademickiego Zespołu ds. Promocji i Wspierania Rekrutacji Uniwersytetu Przyrodniczego w Lublinie (od 19.03.2025 r. do chwili obecnej)
- 6) Członek komitetu organizacyjnego IV edycji Międzynarodowej Konferencji Doktorantów „4th International PhD Students’ Conference: Environment-Plant-Animal-Product (I- SDUPL) together with the Information campaign on the Lublin Digital Union (LUC) – digital solutions and artificial intelligence in medical, natural and technical sciences” (09.04.2025 r.)
- 7) Członek komitetu organizacyjnego III edycji Międzynarodowej Konferencji Doktorantów „3rd International PhD Students’ Conference: Environment-Plant-Animal-Product together with the International Workshops at the University of Life Sciences in Lublin, Poland (ICDSUPL)” (24-26.04.2024 r.)
- 8) Sekretarz Rady Doktorantów - działalność w Samorządzie Doktorantów Uniwersytetu Przyrodniczego w Lublinie (01.11.2022-31.01.2024 r.)
- 9) Członek komitetu organizacyjnego II edycji Międzynarodowej Konferencji Doktorantów „2nd International PhD Student’s Conference at the University of Life Sciences in Lublin, Poland: Environment – Plant – Animal – Product” (19-20.04.2023 r.)

### **Nagrody i wyróżnienia**

- 1) Laureat II edycji programu „Staż za Miedzą” Związku Uczelni Lubelskich. Przyznający nagrodę/wyróżnienie: Związek Uczelni Lubelskich (2023 r.)
- 2) III miejsce w konkursie na najlepszą pracę prezentowaną w sesji posterowej na III Międzynarodowym Sympozjum Studenckich Kół Naukowych „Środowisko-

Roślina-Zwierzę-Produkt”. Kawalec M., **Garbacz A.**, Dec M. „Mięso z próbki – zalety i wady nowoczesnej technologii pozyskiwania mięsa”. Przyznający nagrodę/wyróżnienie: Polskie Towarzystwo Technologów Żywności Oddział Lubelski (2022 r.)

- 3) II miejsce w konkursie za najlepszą pracę prezentowaną w sesji referatowej na II Międzynarodowym Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”. **Garbacz A.**, Kamila Rachwał R. „Toksyna botulinowa – mechanizm działania i zastosowanie”. Przyznający nagrodę/wyróżnienie: Polskie Towarzystwo Technologów Żywności Oddział Lubelski (2021 r.)
- 4) II miejsce w konkursie za najlepszą pracę prezentowaną w sesji posterowej na II Międzynarodowym Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”. Kawalec M., **Garbacz A.**, Dec M. „Mykroproteina zamiennikiem mięsa”. Przyznający nagrodę/wyróżnienie: Uniwersytet Przyrodniczy w Lublinie (2021 r.)
- 5) III miejsce w konkursie za najlepszą pracę prezentowaną w sesji posterowej na II Międzynarodowym Sympozjum Studenckich Kół Naukowych „Środowisko-Roślina-Zwierzę-Produkt”. Kawalec M., **Garbacz A.**, Dec M. „Mykroproteina zamiennikiem mięsa”. Przyznający nagrodę/wyróżnienie: Polskie Towarzystwo Technologów Żywności Oddział Lubelski (2021 r.)

### **Członkostwo w towarzystwie naukowym**

- 1) Członek Polskiego Towarzystwa Zoologów (od 22.11.2023 r. do chwili obecnej)

### **Członkostwo w Studenckim Kole Naukowym**

- 1) Opiekun sekcji bezkręgowców w Studenckim Kole Naukowym Zoologów (od 01.10.2024 r. do chwili obecnej)
- 2) Członek Studenckiego Koła Naukowego Biotechnologów „Biom” (2017-2021 r.)