EDAPHOS

D2.2 – Report on initial ecotoxicity and ecosystem services assessment

WP2 – Task 2.2

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EDAPHOS

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Executive summary

The deliverable constitutes a synthetic report summarizing the preliminary results of the ecotoxicological and ecological analyses as well as ecosystem services (EES) studies conducted within the WP2-Task 2.2 of the EDPAHOS project. The data presented in this deliverable includes the outcomes of a battery of ecotoxicological tests and ecological indicators performed on soil samples collected from five out of the seven case studies (CS1, CS3, CS4, CS6 and CS7) constituting the core focus of the EDAPHOS project. Additionally, the deliverable outlines the assumptions and the methodology approach to assess ESS, which will be applied to all CS's as part of further work in WP2-Task 2.2, as well as in WP4 and WP5 of the project. The ESS Evaluation will be performed to assess the ability of restored soils to provide varied benefits to humans, such as environment.

The overarching goal of the deliverable is to provide information on the initial ecological and ecotoxicological potential of soils at the analysed sites (CS1, CS3, CS4, CS6 and CS7) before the commencement of nature-based solution (NBS) activities. Thus, the presented results provide an ecological and ecotoxicological characterization of the CS's soils, which, due to elevated concentrations of different types of contaminants (e.g. trace elements, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons) represent a potential for environmental risk, and as the preliminary results show, the current contamination levels not only pose a severe ecological hazard but also hinder the establishment and development of natural habitats for both flora and fauna.

The ecotoxicological and ecological analysis were performed in the initial phase of the project prior to the implementation of NBS and is intended to serve as a baseline for subsequent evaluations.

The obtained data will enable the assessment of the effectiveness of NBS applied within the project (WP3) and provide insights into the extent of environmental risk reduction achieved through these technologies. By establishing a comprehensive reference point, this study ensures a robust framework for the long-term monitoring of soil quality improvements and ecosystem recovery. A comprehensive soil monitoring including ecotoxicological and soil function analysis will then be conducted along the NBS implementation for the different CS.

The results obtained within WP2-Task 2.2 will then be used as key input data for the Environmental Risk Assessment (ERA), which will be performed in WP2-Task 2.3. The site-specific risk assessment method applied in the EDAPHOS project is based on the TRIAD concept, which integrates data concerning source of pollution, pathways, exposure, and their effects. This approach enables a more detailed interpretation of data by integrating multiple lines of evidence (i.e. chemical, ecotoxicological and ecological), ultimately leading to a more accurate and ecologically relevant risk characterization of the examined sites.

Keywords

Ecotoxicity, Soil quality, Ecological indicators, Environmental Risk Assessment, Ecosystem Services Evaluation

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Abbreviations and acronyms

Acronym	Description
ARS	Soil Arylsulfatase
BF	Bacterial Feeders
CS	Case study
EAP	Environment Action Programme
EI	Enrichment Index
ERA	Environmental Risk Assessment
ESS	Ecosystem Services
EESS	Ecosystem Services Evaluation
FF	Fungal Feeders
GIG-PIB/CMI-NRI	Główny Instytut Górnictwa – Państwowy Instytut Badawczy/Central Mining Institute – National Research Institute
INERIS	Institut national de l'environnement industriel et des risques/National Institute of Industrial Environment and Risks
NBS	Nature Based Solutions
NUTS	Nomenclature of Territorial Units for Statistics
OECD	Organisation for Economic Co-operation and Development
OPF	Obligate Plant Feeders
P	Predators
PAHs	Polycyclic Aromatic Hydrocarbons
PHs	Petroleum Hydrocarbons
QA	Quality Assurance
QAPA	Quality Assurance Project Plan
QC	Quality Control
SI	Structure Index
TE	Trace Elements
U.S. EPA	United States Environmental Protection Agency

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1 Introduction

1.1. Objective of the ecotoxicological analysis and ecosystem services evaluation.

The protection and restoration of soil functions constitute one of the key elements of the European Union's environmental policy. Strategic documents, such as the EU Soil Strategy for 2030 (COM/2021/699 final) and the Nature Restoration Law (2024/1991), emphasize the role of soils as a fundamental component of ecosystem functioning and an essential resource for food production, water retention, and carbon sequestration. These documents highlight the necessity of undertaking actions aimed at protecting and rehabilitating degraded soils, including the restoration of their natural structure and functions. Additionally, the Environmental Liability Directive (Directive 2004/35/EC) and the forthcoming Soil monitoring law (EU directive on the pathway to healthy soils by 2050 trilogue negotiations ongoing*)* stress the importance of prevention and effective management of contaminated sites to mitigate the negative impacts of anthropogenic activities.

The assessment of soil quality cannot rely solely on chemical analyses and the determination of individual contaminant concentrations. Despite their importance, the determined or threshold values of chemical substances do not reflect the scale of the actual risk as well as extent of contamination's impact on ecosystems. Therefore, other parameters and indicators such as biological ones and enzymatic tests could play a crucial role in evaluating the effects of contaminated soil on living organisms. Ecotoxicological assessments employ organisms representing different trophic levels, ranging from soil microorganisms and plants to soil-dwelling and aquatic animals, enabling a holistic evaluation of ecosystem-wide contamination effects. Living organisms, unlike chemical methods, allow for the detection of interactions between contaminants, including additive and synergistic effects, which can significantly enhance the toxicity of individual substances. This aspect is essential for determining the real environmental threat, as such interactions cannot be predicted based solely on chemical analysis. The most reliable assessment of soil degradation can only be obtained through an integrated approach—by combining chemical analysis with biological tests (ecotoxicological and ecological), ultimately facilitating the development of effective remediation and rehabilitation strategies.

Therefore, it is crucial that the assessment of the degree of the current impact of contaminated areas (CS) on the environment be conducted not only by evaluating the degree of contamination caused by the presence of various groups of pollutants but, most importantly, through a comprehensive ecotoxicological and ecological analysis. This analysis should be based on a dedicated battery of bioassays, providing an accurate representation of the real effects of contaminated soil on selected test species.

Therefore, within the EDAPHOS project, in WP2 (Task 2.2), efforts have been undertaken to assess the impact of contaminated soils on selected organisms included in the bioassay battery. The results obtained from analyses conducted during the initial phase of the EDAPHOS project, presented in this report (D2.2), as well as those that will be obtained based on the analysis of soil samples after the remediation process carried out as a result of further project work (updated D2.2), will provide insights into the actual adverse effects on organisms resulting from exposure to the analysed soil samples. Ultimately, the findings will be utilized for environmental risk assessment based on the TRIAD methodology (Task 2.3).

Additionally, as part of Task 2.2, efforts have been undertaken to assess ESS in the analysed areas (5 CS). This approach is most commonly applied to estimate the benefits that can be provided by natural or semi-natural ecosystems while emphasizing the necessity of biodiversity protection. Within Task 2.2, in areas where phytoremediation techniques based on Nature-Based Solutions (NBS) are implemented, an ESS evaluation procedure will be introduced to assess the effects of remedial actions applied to the analysed anthropogenically degraded sites. The primary objective of ESS assessment is to provide key information that, based on predefined scenarios for the potential redevelopment of restored areas, will facilitate the development of site-specific strategies and plans for the remediation and rehabilitation of contaminated sites.

This deliverable D2.2 presents the methodology for ESS valuation, developed in accordance with European guidelines outlined in CICES v5.1 (https://cices.eu). The first step in the evaluation of ESS involves defining the spatial scale of the study and mapping relevant ecosystem types. In the subsequent stage, various redevelopment scenarios will be formulated, and relevant ESS indicators will be applied to identify revitalization strategies that deliver the greatest environmental benefits. Task T4.2 builds upon the work conducted in T2.2 by advancing towards the economic valuation of ESS, whereas Task T4.3 encompasses a cost-effectiveness analysis of remediation methods based on Nature-Based Solutions (NBS).

Ultimately, the results obtained and presented in the final report will enable a comprehensive cost-benefit comparison of different revitalization strategies applied to the analysed sites.

1.2. Scope of the study

A comprehensive suite of bioassays was employed to conduct the ecotoxicological evaluation, incorporating a diverse range of test organisms representing various trophic levels, endpoints and exposure duration. These included bacteria, plant and invertebrate species, ensuring a multidimensional assessment of the soil ecotoxicity.

The ecological analyses encompassed extracellular enzymatic activities evaluations and the soil capability to degrade organic matter as well as nematofauna diversity, providing critical data on the functional status, soil global metabolisms and the biodiversity of the soils collected from the different CS. The application of a diverse set of bioassays enhances the reliability of the study by integrating responses from multiple biological indicators, thereby facilitating a thorough understanding of the ecological risks associated with the contaminated sites.

A complete characterization of the conducted bioassays and ecological indicators is presented in the following document sections.

Finally, a description of the developed methodology for ESS evaluation on the EDAPHOS CS is presented in the second part of this document.

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2 Soil sampling and preparation

2.1 Sampling site characterization

2.1.1 Location and description of sampling sites

The characteristics of the CS analysed as part of Task 2.2 are presented in the following chapter. Analysed areas represent different pedo-climatic ecological and social territories in the EU. The selected case studies (CS) cover the following climatic zones in Europe were:

a) **Atlantic:** cool summers relative to latitude, cool but not cold winters, a more temperate climate through the year with high precipitation levels.

CS1 - Carrières sous Poissy (FR) – Regeneration of an abandoned agricultural area contaminated by TE and PAH (lead: UBFC)

The site is located in the northwest region of Paris, the CS 1 site is part of a large agricultural area covering approximately 300 hectares, owned by the SYE (Seine & Yvelines Environnement). The location of the area corresponds to the coordinates 48°57'39.7"N 2°02'09.6"E. The total surface area for the experimental study covers approximately 8000 m².

The site was previously used for growing vegetables, and the soil organic matter was increased by the use of sewage from Paris. The use of sewage resulted in significant soil contamination with TE. In March 2000, a ban on the sale of fruit and vegetables was introduced, but the level of pollution in the area remained high. The area is infamously known as the "sea of waste", spanning 38,000m³ of rubble, asbestos and other pollutants that have accumulated over the years across more than 330 ha of land. One of the potential perpetrators in fuelling the waste is the Interdepartmental Syndicate for the Sanitation of the Parisian Agglomeration (SIAAP), a party involved in the complaint regarding the soil pollution made by the town hall in 2020. However, the syndicate itself transports and treats wastewater, rainwater and industrial water from a large part of the Parisian agglomeration. The spreading of raw or partially treated wastewater has led to diffuse pollution of the soil with Cd, Pb, and Zn, as well as PAHs and pesticides commonly used in agricultural practices. Currently some plots of the land function as residential areas, however the concentrations of the TE in the soil are observed. Especially Pb is suspected to be causing a significant health hazard in the area, based on the multiple diagnosis of lead poisoning, especially in children aged 0-6 (Ravagnan, Poli, Uras, 2019).

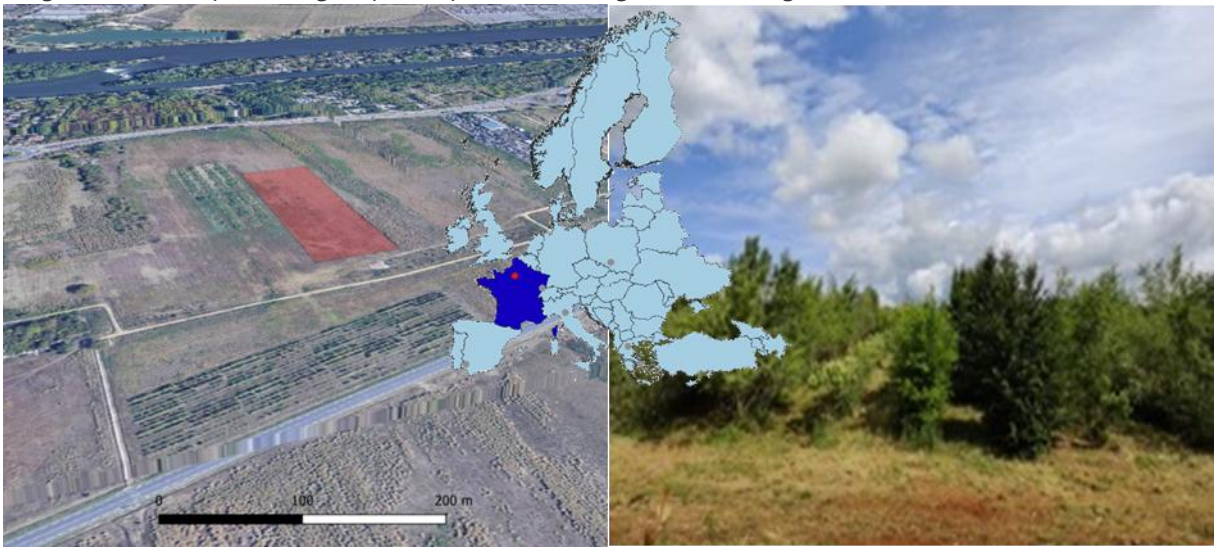

Figure 1. Localisation and images of the CS 1.

b) **Mediterranean North and South:** short precipitation periods, long, hot and dry summers, long growing season and air temperatures favourable for growing a wide range of crops

CS3 - Odiel basin Area (ESSP) – Restoration of highly contaminated mining areas under Mediterranean climate (lead: CSIC)

The site is located east of the city of Nerva, province of Huelva. The localization of the area corresponds to the coordinates 37°41'26" N 6°34'3" W. The total surface area for the experimental study covers approximately 5000 m^2. The Iberian Peninsula lies one of the world's largest sulphide mining areas, spanning over 12,000 hectares, with a history of exploitation dating back to the third millennium BC. Centuries of mining activity have left behind numerous abandoned mine sites, notorious for generating severe metallic pollution through acid mine drainage (Millán-Becerro et al., 2024). This has significantly degraded the ecological and chemical quality of the surrounding water bodies. The primary contaminants found in high concentrations include arsenic (As), lead (Pb), along with high contributions of copper (Cu) and mercury (Hg). The specific study area covers 2 hectares within the Atalaya Mine property, which holds active mining rights. The site is located in the area of Odiel River basin, which is also heavily polluted by acid mine drainage leachates and phosphate fertilizer industry effluents. Currently the Odiel River basin is subject to strong social and legislative pressures due to the future construction of the Alcolea reservoir intended for irrigation, although considering the severe acid mine drainage pollution as well as the need to comply with the European Water Framework Directive (WFD,2000/60/CE), the current state of the area heavily excludes the possibility of fulfilling the directives or ecological goals set for the development.

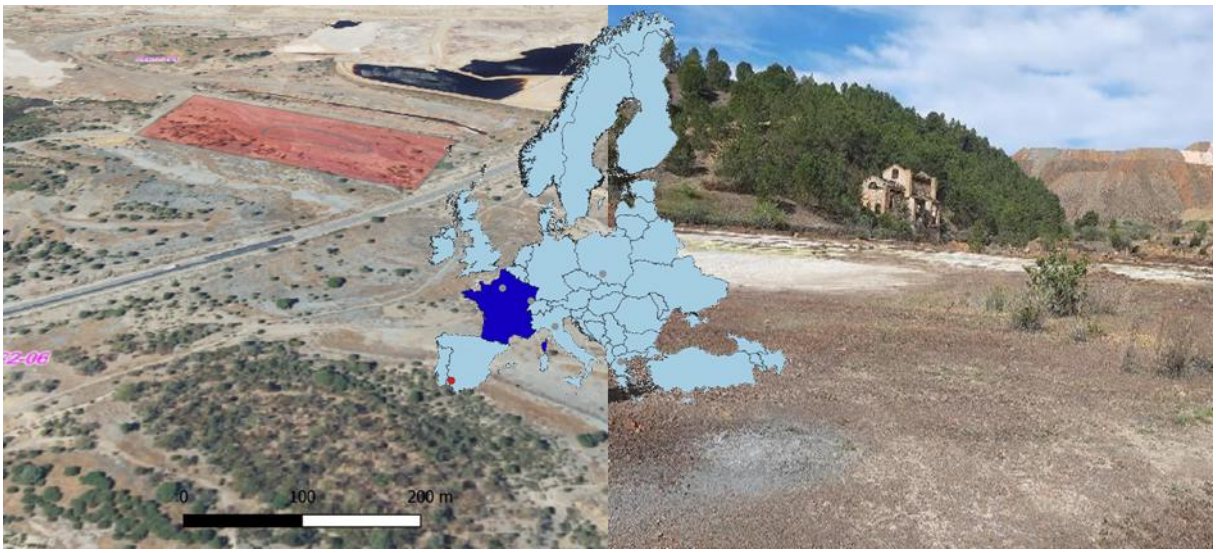

Figure 2. Localisation and images of the CS 3

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CS7 - Lavrio (GR) – Agroforestry for soil remediation at an old metallurgical (lead: CRES).

The site is located north of the Lavrio, a town in southeastern part of Attica, Greece. The localization of the area corresponds to the coordinates 37°44'1.04"N 24° 2'40.68"E. The total surface area for the experimental study covers approximately 2300 m^2.

An industrial area, near the Aegean Sea port, rich in metal ore such as Pb, Mn and Cd. The area has a long history of exploitation, due to the presence of silver ore in the ancient times, before the mines devolved into sites copper mining and other minerals and metals have been discovered in the area. Systematic exploitation of the mineral resources during the mining activities has led to a significant TE pollution in the soil (Demetriades, 2010). The area of the coast is also facing an increased pollution due to a heightened traffic of the water transport as the port is available for container shipping, ferry transport and yachting. Recent studies have discovered the soil, weeds and olives grown in the area contain high levels of potentially toxic elements including Zn, Pb, As, Cu, Sb, Cd and Ag (Antoniadis et al., 2022). The metallurgical processing wastes also present in the Lavrio urban area and their subsequent movement in aerial and fluvial processes as well as anthropogenic processes have increased the pollution of lead in residual soil. The bioaccessibility of elements such as Pb and As is reflected in the composition of blood, urine and teeth structure across both children and adults, which sparks a significant health hazard.

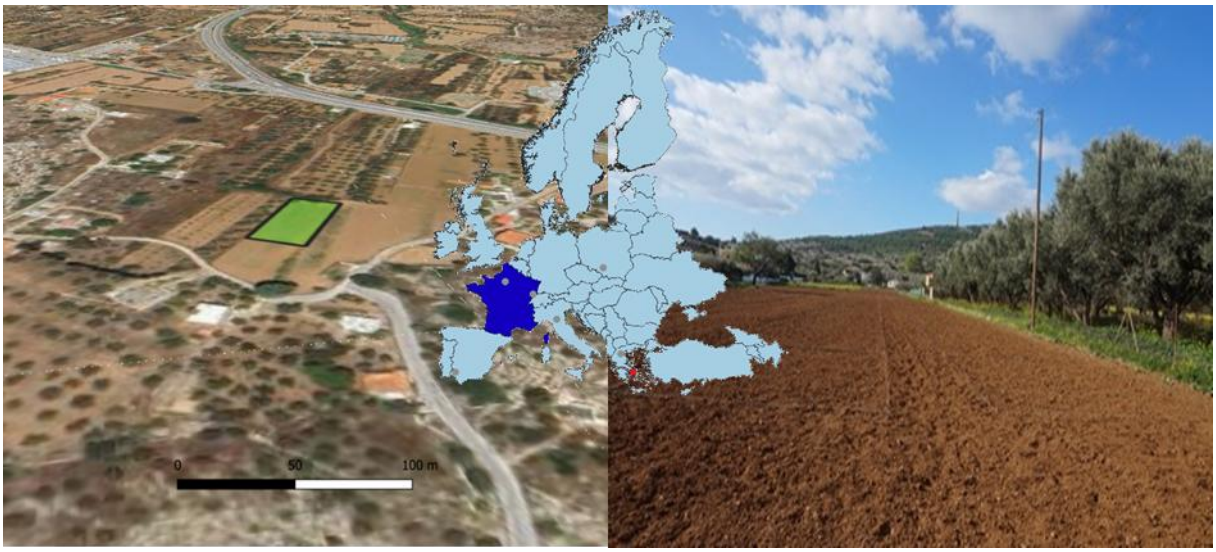

Figure 3. Localisation and images of CS 7

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c) **Continental:** high temperatures in summer and very low in winter, followed by relatively high precipitation.

CS4 - Upper Silesian Coal Basin, Silesian Voivodship (PL) – Phytoremediation techniques to restore the soil ecosystem of a post-mining area (lead: GIG-PIB)

The site is located east of the city Miasteczko Śląskie in the northern part of Upper Silesia region. The localization of the area corresponds to the coordinates 50°30'11.14"N 18°54'46.87"E The total surface area for the experimental study covers approximately 4000m².

The Upper Silesian Coal Basin (USCB) within the Silesian Voivodeship is located in southern Poland. The area of CS4 presents a significant challenge due to the high concentration of metals and metalloids contamination resulting from industrial activities conducted in the area. The CS4 is located within the Silesian Voivodeship, which is the most environmentally degraded region in Poland due to industrial activities, including heavy industry, mining and other related sectors. The importance of the problem is given by the fact that the total area of degraded and devastated land in Poland is approximately 3,463,374 ha (2017), which gives an area of 16.3 m² per Polish citizen (GUS 2017). In Silesia Voivodeship alone, the area of degraded and devastated terrains exceeded 11,300 ha, including more than 6000 ha in the central part of the region (Gasidło, 2019). However, the actual area occupied by brownfields in the region is much larger. The area has been heavily contaminated due to the proximity of industrial pollution sources, which has resulted in high concentration of As, Pb, Zn, and Cd, as well as high contributions of Cu and Mn in the soil. These pollutants have severely compromised the soil ecosystem, needing remediation efforts to restore ecological health and functionality. The total surface of the contaminated area is 36 300 m².

Figure 4. Localisation and images of the CS 4.

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CS6 - Vieux-Charmont (FR) – Soil regeneration and involving the larger public (lead: UBFC).

The site is located north of the Montbéliard, a town in the Doubs department in the Bourgogne-Franche-Comté (BFC) region in eastern France, about 13 km from the border with Switzerland. Localization of the area corresponds to the coordinates 47°31'15.8"N, 6°50'23.8"E. The total surface area for the experimental study covers approximately two hectares. Industrial wasteland where car industries left a significant soil TE contamination due to an old and potent accumulation of waste of a metallurgic site. The BFC Region exemplifies a broader issue with industrial wastelands across the area, totalling 150 hectares. This specific site is heavily polluted with a mix of contaminants, including As, Cd, Pb, Zn, and PAHs. The contamination levels present significant environmental challenges, affecting soil quality and potentially posing risks to local ecosystems and human health.

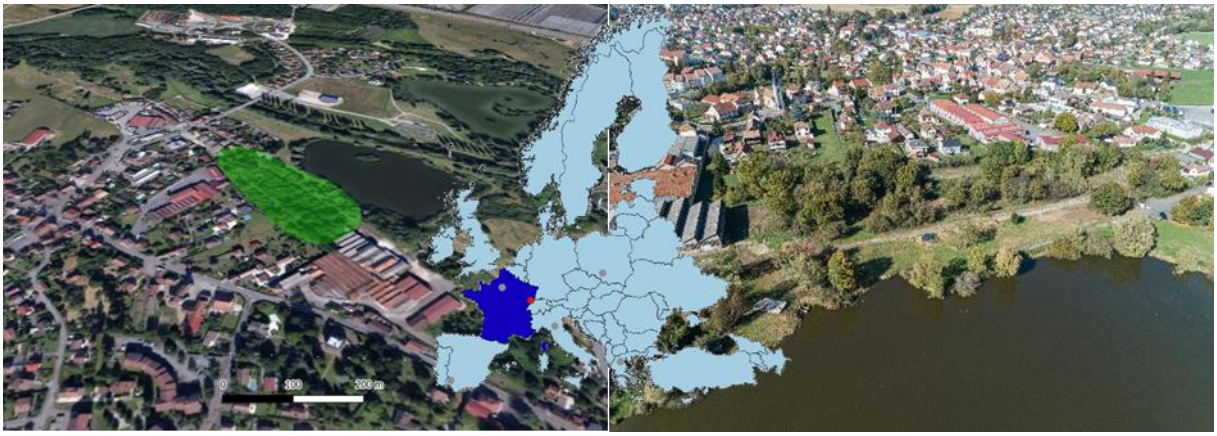

Figure 5. Localisation and images of the CS 6.

Each CS is oriented to target a specific pollution problem that needs a tailored approach, although the sites selected are diverse in nature and representative of common and relevant pollution problems found throughout EU.

2.2 Soil samples preparation

2.2.1 Soil sample collection and handling

2.2.1.1 Soil sampling -methodology approach

This section describes the protocol used to collect the soil from each CS at the beginning of the project. This protocol was designed to collect enough soil in the relevant conditions for further ecotoxicological, ecological and soil function analyses.

Because soils can be highly variable in terms of physico-chemical parameters and contamination, even over short distances, it is often insufficient to collect soil at just one location. It is instead preferable to prepare so-called "soil composite samples" which are a mixture of several sub-samples collected from multiple locations (plots) along the site surface area and then mixed to form a single "soil composite sample". Consequently, 3 soil composite samples were produced on each CS site. For each CS, the studied area corresponds to the location for further NBS implementation. On this area each composite sample was established by mixing up to 10 sub-samples following a sampling-grid and a "zig-zag" sampling pattern established according to each site specificity.

For each soil composition sample, the collected sub-samples were treated as follow:

- the soil surface (first 5 cm) was removed using a shovel or a pickaxe on a plot of approximatively 80cm to 80cm
- 8 kg of raw soil were collected using a shovel at a depth of approximatively 30 cm
- the collected raw soil was cleared of rocks and remaining plants roots and then sieved at 4 mm on tarpaulin to obtained 5 kg of prepared soil
- the overall quantity of prepared soil (50 – 60 kg) from the subsamples was then mixed using a shovel
- the soil composite sample was then weighted and distributed into different bags (big bag or Ziplock) for direct shipping in the different laboratory.

Once collected on site, the different bags were kept at room temperature or in icebox until being transferred to the respective laboratories. Once transferred to the respective laboratories the soil samples were stored as described in the table below.

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Table 1 Handling and storage condition for the ecotoxicity, ecological and soil function analyses.

Storage condition	Quantity needs (per soil composite)	Test performed
- room temperature - up to 1 month	~40 kg - 8 kg - 8 kg - 8 kg - 8 kg - 0,5 kg - 0,5 kg - 0,5 kg	 Worm acute toxicity test Worm reproduction test Plant root inhibition test Plant growth test Nematodes growth and reproduction test Daphnia magna toxicity test (soil leachates) Microalgae growth inhibition test (soil leachates)
- 4°C until analyses - up to 2 weeks	~2 kg - 1 kg - 0,5 kg - 0,5 kg	 Organic matter decomposition capability Nematode diversity index Microbial and fungal diversity (qPCR)
- Frozen (-20°C) - up to 2 months	~2 kg (subdivided in small bags of 200 g)	Enzyme activities (hydrolases)

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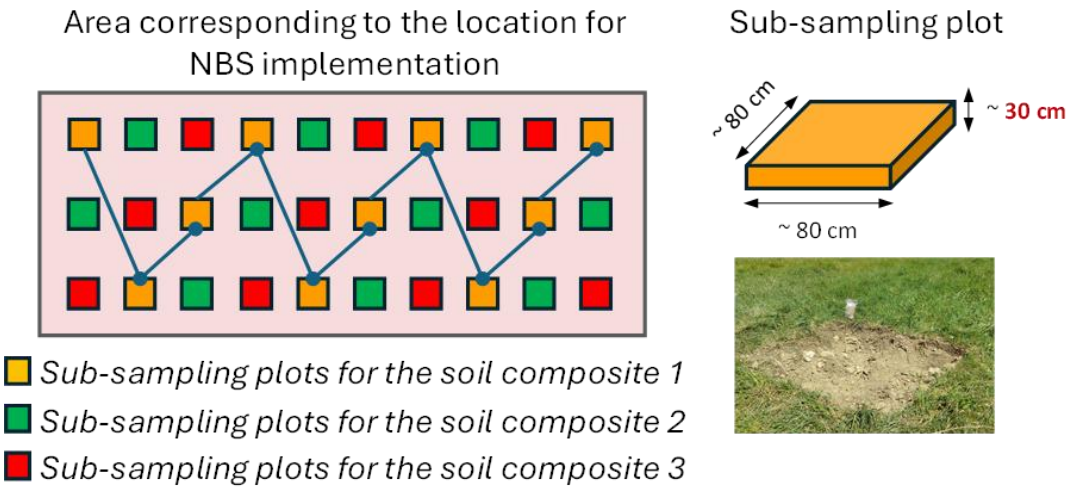

Figure 6 EDAPHOS "zig-zag" sampling pattern applied on the area corresponding to the location for NBS implementation and sub-sampling plot characteristics.

Figure 7. EDAPHOS soil sampling protocol applied on the different CS for ecotoxicological, ecological and soil function analyses. a. GPS position recording for each sub-sample plot, b. soil surface removing and raw soil collection, c. soil preparation, d. composite homogenization, e. soil weighting and f. handling bag preparation.

In addition, each sub-sample was collected apart for further physico-chemical and hyperspectral analyses (WP1).

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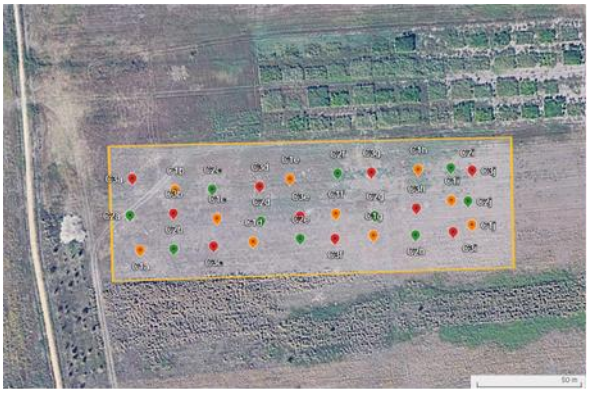
2.2.1.2 Soil sampling methodology for CS1 (Carrières-sous-Poissy)

The soil sampling campaign for ecotoxicological, ecological, and physico-chemical analysis for the CS1 area (Carrières-sous-Poissy) was conducted on July 2024. Within the analysed CS1 area, a research plot with a total area of 11 687 square meters were designated for sampling purposes. Within this plot, 30 sampling points were evenly distributed. At these points, on-site measurements were performed, and soil material was collected to prepare three individual and representative soil composites (CS1-C1; CS1- C2; CS1-C3).

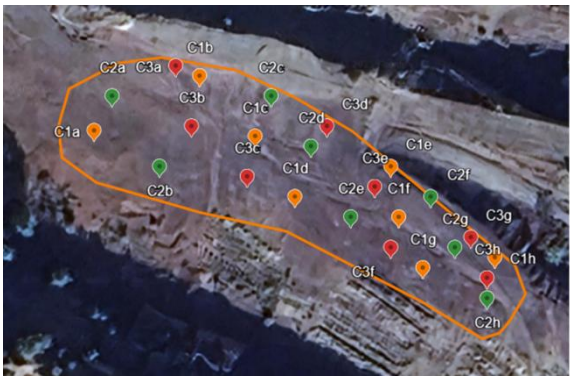


CS1_Carrière sous Poissy			Total surface of the studied area : 11 687,1 m²		
C1	GPS coord.	C2	GPS coord.	C3	GPS Coord.
C1_a	48°57'35"N 2°02'16"E	C2_a	48°57'35"N 2°02'16"E	C3_a	48°57'36"N 2°02'16"E
C1_b	48°57'36"N 2°02'17"E	C2_b	48°57'35"N 2°02'17"E	C3_b	48°57'35"N 2°02'17"E
C1_c	48°57'35"N 2°02'18"E	C2_c	48°57'36"N 2°02'18"E	C3_c	48°57'35"N 2°02'18"E
C1_d	48°57'35"N 2°02'19"E	C2_d	48°57'35"N 2°02'19"E	C3_d	48°57'36"N 2°02'19"E
C1_e	48°57'36"N 2°02'20"E	C2_e	48°57'35"N 2°02'20"E	C3_e	48°57'35"N 2°02'20"E
C1_f	48°57'35"N 2°02'21"E	C2_f	48°57'36"N 2°02'21"E	C3_f	48°57'35"N 2°02'21"E
C1_g	48°57'35"N 2°02'22"E	C2_g	48°57'35"N 2°02'22"E	C3_g	48°57'36"N 2°02'22"E
C1_h	48°57'36"N 2°02'23"E	C2_h	48°57'35"N 2°02'23"E	C3_h	48°57'35"N 2°02'23"E
C1_i	48°57'35"N 2°02'23"E	C2_i	48°57'36"N 2°02'23"E	C3_i	48°57'35"N 2°02'23"E
C1_j	48°57'35"N 2°02'24"E	C2_j	48°57'35"N 2°02'24"E	C3_j	48°57'36"N 2°02'24"E

Figure 8. Locational grid of soil sampling points for CS1.

2.2.1.3 Soil sampling methodology for CS3 (Odiel basin Area)

The soil sampling campaign for ecotoxicological, ecological, and physico-chemical analysis for the CS3 area (Odiel basin Area) was conducted on April 2024. Within the analysed CS3 area, a research plot with a total area of 1910 square meters was designated for sampling purposes. Within this plot, 24 sampling points were evenly distributed. At these points, on-site measurements were performed, and soil material was collected to prepare three individual and representative soil composites (CS3-C1; CS3- C2; CS3-C3).



CS3_Odiel basin Area			Total surface of the studied area : 1907,4 m²		
C1	GPS coord.	C2	GPS coord.	C3	GPS Coord.
C1_a	37.690709°N 6.567504°W	C2_a	37.690767°N 6.567467°W	C3_a	37.690817°N 6.567333°W
C1_b	37.690800°N 6.567283°W	C2_b	37.690650°N 6.567367°W	C3_b	37.690717°N 6.567300°W
C1_c	37.690700°N 6.567167°W	C2_c	37.690767°N 6.567133°W	C3_c	37.690633°N 6.567183°W
C1_d	37.690600°N 6.567083°W	C2_d	37.690683°N 6.567050°W	C3_d	37.690717°N 6.567017°W
C1_e	37.690650°N 6.566883°W	C2_e	37.690567°N 6.566967°W	C3_e	37.690617°N 6.566917°W
C1_f	37.690567°N 6.566867°W	C2_f	37.690600°N 6.566800°W	C3_f	37.690517°N 6.566883°W
C1_g	37.690483°N 6.566817°W	C2_g	37.690517°N 6.566750°W	C3_g	37.690533°N 6.566717°W
C1_h	37.690500°N 6.566667°W	C2_h	37.690433°N 6.566683°W	C3_h	37.690467°N 6.566683°W

Figure 9. Locational grid of soil sampling points for CS3.

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2.2.1.4 Soil sampling methodology for CS4 (upper Silesia Coal Basin)

The soil sampling campaign for ecotoxicological, enzymatic, and physico-chemical analysis for the CS4 area (Upper Silesia Coal Basin) was conducted on May 2024. Within the analyzed CS4 area, a research plot with a total area of 10100 square meters was designated for sampling purposes. Within this plot, 30 sampling points were evenly distributed. At these points, on-site measurements were performed, and soil material was collected to prepare three individual and representative soil composites (CS4-C1; CS4- C2; CS4-C3).

CS4_Silesia Coal Basin			Total surface of the studied area : 10 092,5 m²		
C1	GPS coord.	C2	GPS coord.	C3	GPS Coord.
C1_a	50°30'10"N 18°54'49"E	C2_a	50°30'11"N 18°54'49"E	C3_a	50°30'11"N 18°54'49"E
C1_b	50°30'11"N 18°54'48"E	C2_b	50°30'10"N 18°54'48"E	C3_b	50°30'11"N 18°54'48"E
C1_c	50°30'11"N 18°54'48"E	C2_c	50°30'11"N 18°54'48"E	C3_c	50°30'10"N 18°54'48"E
C1_d	50°30'10"N 18°54'47"E	C2_d	50°30'11"N 18°54'47"E	C3_d	50°30'12"N 18°54'47"E
C1_e	50°30'12"N 18°54'47"E	C2_e	50°30'10"N 18°54'47"E	C3_e	50°30'11"N 18°54'47"E
C1_f	50°30'11"N 18°54'46"E	C2_f	50°30'12"N 18°54'46"E	C3_f	50°30'10"N 18°54'46"E
C1_g	50°30'11"N 18°54'46"E	C2_g	50°30'11"N 18°54'46"E	C3_g	50°30'12"N 18°54'46"E
C1_h	50°30'12"N 18°54'45"E	C2_h	50°30'10"N 18°54'45"E	C3_h	50°30'11"N 18°54'45"E
C1_i	50°30'11"N 18°54'45"E	C2_i	50°30'12"N 18°54'45"E	C3_i	50°30'10"N 18°54'44"E
C1_j	50°30'10"N 18°54'44"E	C2_j	50°30'11"N 18°54'44"E	C3_j	50°30'12"N 18°54'44"E

Figure 10. Locational grid of soil sampling points for CS4.

2.2.1.5 Soil sampling methodology for CS6 (Vieux-Charmont)

The soil sampling campaign for ecotoxicological, enzymatic, and physico-chemical analysis for the CS6 area (Vieux-Charmont) was conducted on April 2024. Within the analyzed CS6 area, a research plot with a total area of 260 square meters was designated for sampling purposes. Within this plot, 15 sampling points were evenly distributed. At these points, on-site measurements were performed, and soil material was collected to prepare three individual and representative soil composites (CS6-C1; CS6- C2; CS6-C3).

CS6_Vieux Charmont			Total surface of the studied area : 260,8 m2		
C1	GPS coord.	C2	GPS coord.	C3	GPS Coord.
C1_a	47.521524°N 6.840902°E	C2_a	47.521540°N 6.840857°E	C3_a	47.521508°N 6.840930°E
C1_b	47.521572°N 6.840882°E	C2_b	47.521535°N 6.840973°E	C3_b	47.521553°N 6.840933°E
C1_c	47.521597°N 6.840998°E	C2_c	47.521618°N 6.840957°E	C3_c	47.521617°N 6.840927°E
C1_d	47.521660°N 6.841007°E	C2_d	47.521667°N 6.840962°E	C3_d	47.521647°N 6.841007°E
C1_e	47.521697°N 6.841018°E	C2_e	47.521693°N 6.841047°E	C3_e	47.521728°N 6.841035°E

Figure 11. Locational grid of soil sampling points for CS6.

2.2.1.6 Soil sampling methodology for CS7 (Lavrio)

The soil sampling campaign for ecotoxicological, enzymatic, and physico-chemical analysis for the CS7 area (Lavrio) was conducted on June 2024. Within the analyzedCS7 area, a research plot with a total area of 1513 square meters was designated for sampling purposes. Within this plot, 30 sampling points were evenly distributed. At these points, on-site measurements were performed, and soil

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material was collected to prepare three individual and representative soil composites (CS7-C1; CS7-C2; CS7-C3).

CS7_Lavrio				Total surface of the studied area : 1512,6 m²	
C1	GPS coord.	C2	GPS coord.	C3	GPS Coord.
C1_a	37.733917°N 24.044936°E	C2_a	37.733937°N 24.044872°E	C3_a	37.733967°N 24.044817°E
C1_b	37.733944°N 24.044784°E	C2_b	37.733887°N 24.044920°E	C3_b	37.733902°N 24.044853°E
C1_c	37.733877°N 24.044825°E	C2_c	37.733918°N 24.044751°E	C3_c	37.733847°N 24.044882°E
C1_d	37.733813°N 24.044848°E	C2_d	37.733840°N 24.044778°E	C3_d	37.733869°N 24.044727°E
C1_e	37.733841°N 24.044691°E	C2_e	37.733778°N 24.044822°E	C3_e	37.733798°N 24.044768°E
C1_f	37.733767°N 24.044733°E	C2_f	37.733805°N 24.044682°E	C3_f	37.733737°N 24.044806°E
C1_g	37.733717°N 24.044777°E	C2_g	37.733718°N 24.044708°E	C3_g	37.733757°N 24.044650°E
C1_h	37.733727°N 24.044602°E	C2_h	37.733660°N 24.044747°E	C3_h	37.733672°N 24.044672°E
C1_i	37.733642°N 24.044632°E	C2_i	37.733687°N 24.044557°E	C3_i	37.733610°N 24.044723°E
C1_j	37.733570°N 24.044687°E	C2_j	37.733595°N 24.044595°E	C3_j	37.733637°N 24.044517°E

Figure 12. Locational grid of soil sampling points for CS7.

2.2.2Physico-chemical characterization of soil samples

For each of the analysed areas, the tables below present the average values of selected soil indicators, and the concentrations of TE determined in the soil components.

Table 2 Physico-chemical characterization of soil components from the CS1 area.

№	pH	WHC	As	Cd	Cr	Cu	Fe	Ni	Pb	Sn	Zn
Unit	[-]	[%]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Value	7,3	38	<LQ (5,0)	3,9	52,9	70,6	7913,3	18,2	123,0	12,0	330,3

Table 3 Physico-chemical characterization of soil components from the CS3 area.

№	pH	WHC	As	Cd	Cr	Cu	Fe	Ni	Pb	Sn	Zn
Unit	[-]	[%]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Value	3,5	21	928,8	10,6	5,9	1593,3	436000	5,6	9603,3	112,0	420,8

Table 4 Physico-chemical characterization of soil components from the CS4 area.

№	pH	WHC	As	Cd	Cr	Cu	Fe	Ni	Pb	Sn	Zn
Unit	[-]	[%]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Value	7,2	68	70,7	38,5	64,9	185,6	29933,3	35,3	1356,7	25,7	3056,7

Table 5 Physico-chemical characterization of soil components from the CS6 area.

№	pH	WHC	As	Cd	Cr	Cu	Fe	Ni	Pb	Sn	Zn
Unit	[-]	[%]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Value	6,8	95	35,6	7,5	409,3	140,9	66266,7	177,0	6186,7	6340,0	15166,7

Table 6 Physico-chemical characterization of soil components from the CS7 area.

№	pH	WHC	As	Cd	Cr	Cu	Fe	Ni	Pb	Sn	Zn
Unit	[-]	[%]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]	[mg/kg]
Value	7,8	35	610,6	28,6	271,2	135,3	44500,0	195,8	6623,3	<LQ (2,5)	4840,0

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2.2.3Quality assurance and control measures

The study has been performed in compliance with the quality assurance (QA) and quality control (QC) measures developed by the U.S Environmental Protection Agency (US EPA), which ensure the precision and accuracy of data collection. Quality assurance focuses on the process of the analysis in all aspects with regards to preventing or/and minimizing the occurrence of errors in the measurement. The project plan based on the Quality Assurance Project Plan (QAPP) and included documentation of procedures, study design, monitoring effort data management and analysis as well as the specific QC measures undertaken during the study. The activities described in the QAPP define data quality objectives of the chemical analysis and the design of the QC system, to measure the quality of the generated data.

Quality control is a set of steps or activities that aim to ensure all the quality requirements are being met in other to determine the validity of the specific sampling and analytical approach. The constant monitoring of the laboratory operations and results was essential in order to ensure the results were reliable enough to be released. In order to fulfill the general principles of the QAQC for contaminant monitoring program, the data quality objectives were established prior to sample collection analysis, the samples were then collected, processed and analysed according to scientifically valid standardized procedures while maintaining and securing the samples at all times. A record schedule was kept in order to ensure traceability of the data from the collected samples, and the data quality was assessed and documented, concluding in a report with complete and accurate results.

In the field, the sample was procured with the use of appropriate permits, through an active collection process with the use of mechanical and human power. To ensure sample integrity after collection, it was preserved by limiting contamination loss and eliminating the risk of cross-contamination with other samples through the use of good practices such as using nitrile gloves when handling the samples, avoiding using tools contaminated with grease, oils, diesel fuels, gasoline or other pollutants, and not handling any foods, drinks or substances such as insecticides, repellents or cosmetics near the collected samples. Each sample was then secured for field reporting with a unique identification tag or code marking the sample containers.

Target contaminants were monitored using analytical methods based on the criteria such as technical merit (methods specified for targets of concerns, in this case TE including mostly TE), sensitivity (method detection and quantification limits should be efficiently low to allow reliable quantification of the target elements), data quality (with adequate accuracy and precision allowing the analytical data to be of an appropriate quality for further release) and cost-efficiency.

The data was verified by the laboratory personnel in order to ensure lack of errors and anomalies and that the results were returned for repeated analysis where the original results have failed to be obtained. The results are further reported in the form of electronic data, in this instance deliverables, in formats of spreadsheets or PDF files with supporting data. The data is then validated by project personnel outside of the laboratory or a third party skilled in data review procedures.

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3 Ecotoxicological and ecological Analysis

3.1 The importance of ecotoxicological and ecological analysis in assessing contaminated soil quality

In urban regions, land can be contaminated by industrial or mining activities which cause soil pollution including contamination of the areas with substances such as TE, petroleum hydrocarbons (PHs), PAHs and pesticides as a result of sewage production, waste treatment, agricultural waste disposal and fertilization or chemical leakage. Surface layers of the soils become the main sinks for pollutants, either physical, airborne or existing in surface and underground reservoirs. Among them TE prove frequent and persistent, with the ability to accumulate in the organisms living in the contaminated areas and cause negative side effects such as increased toxicity, physical changes (suffocation, reduced access to light), changes to parameters of the environment (pH, reduction of oxygen concentrations, crop degeneration) and deterioration of health in all living organisms.

One method of investigating the ecotoxicity of contaminants in soil is the implementation of ecotoxicological analysis involving species from different trophic levels and different type of endpoints (acute and chronic), mainly bioassays on bacteria, higher plants earthworms or nematodes. The assessment of phytotoxicity test is based on germination and seedling growth of the chosen terrestrial plant (in this case Oats species *Avena sativa* and white mustard species *Sinapis alba*) and the bioassay involves the survival and reproduction tests of the nematodes (in this case *Caenorhabditis elegans*) and earthworm species (in this case *Eisenia fetida*).

Invertebrates such as earthworms are considered suitable organisms to assess the adverse effects of soil pollution through ecotoxicological analysis because of a variety of factors such as continuous exposure to the chemicals through their outer tissues which are heavily dependent on defoliation process. In ecotoxicology, there is a number of tests including the earthworms, such as the avoidance, acute and chronic tests. In this case, acute and chronic tests were chosen due to the nature of the experiment and the properties of the soil. Acute tests measure the survival capacity of a species in a given environment while the chronic tests assess the ability of the organisms to reproduce in the adverse biological conditions and produce a healthy and living offspring which helps to monitor if life can thrive in conditions of abnormal pollution levels.

With regards to phytotoxicity, TE have been reported to affect germination process, the percent of germinated seeds, seedling development, root elongation, stalk growth, the morphology and physiology of the seed which later on can affect the plant. The plants grown on the soils polluted by contaminants such as TE are assessed through the measurement of their stalk, root, and green and dry biomass.

In addition to ecotoxicological endpoints, ecological indicators are necessary to provide information on the global status of a contaminated soil regarding its function in terms of metabolisms and support for biodiversity. The approach proposed in EDAPHOS is to combine complementary tests that provide an overview of the soil global metabolisms through extracellular enzyme activities characterisation, the soil capability to degrade organic matter and the diversity of soil microfauna (nematodes).

3.2 Objective and scope of the ecotoxicological analysis

One of the major challenges of the 21st century is the management of pollution levels in natural environments, including the issue of TE contaminated soils. In Europe, the soil contaminated by TE makes for up to 1,2 km^2, which is 28,3% of the entire soil of the European Union. The magnitude of the problem and extent of the issue have been brought to attention by the European Union mandated framework of the 8th Environment Action Programme (EAP), which includes ensuring the "soil protection and remediation strategies of contaminated sites." The EDAPHOS project, along the „Soil Deal For Europe" propagated by the European Union, aims to develop and demonstrate NBS for the sustainable remediation of contaminated soils in a way such methods could be introduced into real-life scenarios as an efficient and cost-effective method.

A potential solution to TE contamination would be remediation techniques using NBS. One of such methods is phytoremediation, which could serve as a form of an environmental engineering, using selected plant species to remediate and rehabilitate soils polluted by industrial, mining and other anthropogenic influences. One of the key strategies explored in the context of phytoremediation is testing selective, fast-growing plant species for their metal accumulating and nitrogen fixing abilities. This is a first step in developing a co-cropping system where different kinds of plant species could efficiently remediate contaminated soil, at the same time addressing the enhancement of ESS, including biodiversity, biomass production, C sequestration and bioeconomic applications.

The aim of the ecotoxicological analysis is to measure and evaluate the combined effects of different contaminants and their bioavailability in the soils recovered from polluted areas, by performing appropriate tests on representative species of the (micro)fauna and flora. The advantage of combining chemical and biological approaches is that it allows monitoring the level of toxicity of those contaminants as they are absorbed and converted by living organisms. As a result, the direct toxicity of a contaminant or a mixture of pollutants is revealed, moreover, the various specific effect across different species can be observed.

The laboratory tests consisted of chemical tests and biological experiments. The chemical analysis of the content of the plant biomass serves to detect the amounts of pollutants absorbed in the biomass of the tested plant species, including substances such as TE. The biological analysis involved a range of tests such as biochemical tests on soil bacteria dehydrogenase activity (*Arthrobacter globiformis),* plant root inhibition tests, plant emergence and growth tests, nematodes growth and reproduction tests and earthworm acute (survival) and chronic (reproduction) tests, all of which were divided between GIG-PIB and INERIS. The biological experiment focuses on testing selected species under real-world conditions to address soil contamination and aims to improve soil functionality. The species were selected according to OECD guidelines, on the account of the widespread natural occurrence, lifespan, and across plant-life, germination availability across all seasons.

The contaminated soil on which bioassays were applied, covers different pedo-climatic ecological and social areas in the EU, while being inclusive of the seven EU Nomenclature of Territorial Units for Statistics level 2 (NUTS2) regions. The selected case studies (CS) cover the following climatic zones in Europe: a) Atlantic (CS1 - Carrières sous Poissy (FR)), b) Mediterranean North and South (CS3 - Odiel basin Area (ESSP), CS7 - Lavrio (GR)), and c) Continental (CS4 - Upper Silesian Coal Basin,

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Silesian Voivodship (PL), CS6 - Vieux-Charmont (FR)). Up to date, the growth properties of both species have been tested across the soils CS1 (FR), CS3 (ES), CS4 (PL), CS6 (FR) and CS7 (GR).

The aim of this work was to measure the levels of soil ecotoxicity across the different species and soil sample origins to achieve a comparison with regards to accumulation of TE in biomass growth of different organisms, and to study exposure from CS to generate hazard and soil functionality data along the NBS remediation processes. Considering the duration of the EDAPHOS project, the current data generated and presented will be updated after three years of treatment by the NBS.

3.3 Ecotoxicological analyses

The following sections detail the protocols of the different bioassays performed to characterize the ecotoxicity of the soil samples from the different case studies of the EDAPHOS project.

Tested soils

The test samples comprised soils contaminated by human activities, collected from various study sites (CS) representing different climatic and ecological conditions. This report examined soils from the following study sites:

- CS1 - Carrières sous Poissy (FR)
- CS3 - Odiel Basin Area (ES)
- CS4 - Upper Silesian Coal Basin, Silesian Voivodeship (PL)
- CS6 - Vieux-Charmont (FR)
- CS7 - Lavrio (GR)

See section 2.1 for detailed CS characterization and 2.2 for the soil sampling procedure.

All ecotoxicological bioassays and ecological indicators were performed for the three individually prepared soil composite samples see section 2.2.1). In accordance with the research procedure established between the GIG-PIB and INERIS laboratories, for each soil composite sample, independent measurement series were performed within each ecotoxicological test. Depending on the applied procedure (ISO standards/OECD guidance document), each series included three to five replicates.

Battery of bioassays

The table below provides a summary of the conducted ecotoxicological bioassays. These tests follow standardized methodologies and focus on specific organisms and endpoints to evaluate the environmental impact.

Table 7 Battery of ecotoxicological bioassay

Bioassay	Analytical Standard	Endpoints
Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis*	ISO 18187:2024	Inhibition of dehydrogenase activity - % inhibition
Determination of the effects of pollutants on soil flora	ISO 11269-1:2012	Inhibition of root growth- % reduction in root elongation
Worms (*Eisenia fetida*) acute ecotoxicity test	ISO 11268-1:2012	Acute toxicity to *Eisenia fetida*/ - % mortality (measured on days 7 and 14) Behavioural changes- inability to dig into the soil, lying motionless, anomalous decomposition rate of deceased organisms, attempting to escape the soil by climbing on the lid (recorded on days 7 and 14) Morphological changes - open wounds (observed throughout the test period)
Determination of the toxic effect of sediment and soil samples on growth, fertility, and reproduction of *Caenorhabditis elegans*	ISO 10872:2020	Nematodes survival, growth inhibition, and inhibition of reproduction - % survival, % growth inhibition, % reproduction inhibition
Plant emergence and growth test	OECD 208	Seed germination efficiency - % germination success (assessment on days 1, 3, 5, and 7) Shoot Growth Inhibition Index -% inhibition of shoot growth, biomass evaluation of aerial plant parts(g) on day 15
Worms (*Eisenia fetida*) reproduction test	ISO 11268-2:2023	Number of offspring - total number of juveniles per adult (assessed on days 28 and 60)

The ecotoxicological assays presented in this table provide standardized methods for assessing the impact of contaminants on various biological systems. The results contribute to understanding the long-term consequences of soil pollution on soil organisms and support regulatory decision-making in environmental protection.

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3.3.1 Contact test for solid samples using the dehydrogenase activity of *Arthrobacter globiformis* (ISO 18187:2024)

3.3.1.1 Test protocol & standards

The protocol used for the soil sample toxicity assessment using *A. globiformis* was performed following the recommendation of the ISO 18187:2024 standard. This standard specifies a rapid contact-test protocol to measure the inhibition of the dehydrogenase activity (DHA) in *A. globiformis*, adapted for assessing the effect of water-soluble and solid matter bounded non-volatile contaminants in natural samples, such as soils.

The principle of this assay relies on the reduction of resazurin into resorufin that is measured by fluorescence and used as a proxy of the DHA. Whenever a solid sample inhibits the *A. globiformis* DHA, the level of resorufin production and, hence, the emitted fluorescence is reduced, indicating the toxicity of the soil sample.

3.3.1.2 Test methodology

Selection and description of test species

A. globiformis is a ubiquitous and non-pathogenic aerobic soil bacterium that synthesizes an extracellular enzyme during different metabolic processes (Thomson et al. 1986), which activity was suggested to be a potential indicator of the effect of contaminants on solid samples (Rönnpagel et al. 1998).

For this test, *A. globiformis* (Conn 1982) Conn and Dimmick 1947 (strain number ATCC 8010) was used. The strain was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ) GmbH and cultured under 30 ± 1 °C and 150 rpm into liquid media as described in ISO 18187:2024. Fresh bacterial cultures were then lyophilized into individual vials and maintained at -20°C before to be used for contact test.

Sample preparation (Soil, Controls, etc.)

Prior to the test, the different soil composite samples were sieved at 2 mm. A standard natural soil (Lufa 2.2), sieved at 2mm, was used as a control soil. The water content of each soil sample was adjusted to 20% using deionized water. This adjustment was calculated according to the original water content of samples.

One day before the start of the assay, 600 mg of pre-moistened soil (20% water content) and control soil were weighted into a 24-wells microplate at keep at ambient temperature. Four replicates per treatment were used. At the start of the test 0,6 ml of sterilized deionized water was added in all replicates. As natural dehydrogenase activity can occur in natural soil, a pre-treatment of the tested soils was done to suppressed as far as possible this activity (deactivation step). This pre-treatment is done, just before the start of the test, by heating the prepared soil samples and the control soil in a water bath at (85 ± 2) °C for 10 min. Afterward, the samples are cooled down to ambient temperature for 15 min in iced water bath. Blank control samples are used to indicate the activity level of dehydrogenase in natural samples and to ensure the efficiency of the deactivation step.

Detailed procedure for conducting the test

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Preparation of the *A. globiformis* inoculum

A vial of freeze-dried bacteria was reconstituted with 0.5 ml of sterilized iced cold water and placed at 6°C for 30 min. The suspension was then transferred into 30 ml of sterilized growth medium and warmed at 30°C.

Incubation and fluorescence measurement

A volume of 0.4 ml of reconstituted inoculum was added in each replicate. The plates were incubated for 2h in the dark at a temperature of 30°C and shake at a speed of 150 rpm using an incubator (THERMOstar, BMG LABTECH). Afterwards, a resazurin solution (0.8 ml) was added in each well of the microplate and the production of resorufin was directly measured using a microplate fluorimeter (emission at 590 nm, excitation at 535 ± 20 nm). This measurement was repeated every 15 min for a period of 1h.

Determining the percentage of inhibition of DHA

For all measurement periods, the average fluorescence value obtained for each blank was subtracted to the fluorescence value of the respective soil samples exposed to the test organism inoculum. This allows calculating the relative fluorescence of each treatment by rejecting the own fluorescence of the substrates tested.

Then the slope of the relative fluorescence for each replicate and treatment (controls and samples) is calculated using a linear regression according to the relative fluorescence values measured between 15 min and 45 min. Percentage of DHA is then calculated by comparison of the slope obtain after exposure to the tested soils samples, relative to the slope obtain in the control soil.

3.3.2 Determination of the effects of pollutants on soil flora / Part 1: Method for the measurement of inhibition of root growth (ISO 11269-1:2012)

3.3.2.1 Test protocol & standards

The determination of the inhibition of higher plants root growth was performed according to the recommendation of the ISO 11269-1. This standard describes a method for the determination of the effects of contaminated soils or contaminated samples on the root elongation of terrestrial plants. The test method described in this part of ISO 11269 can be used to compare soils, to monitor changes in their activity or to determine the effect of added chemicals or materials (compost, sludge, waste). Pregerminated seeds are exposed to the test material under controlled conditions. After the growth period, the lengths of the roots of the test plants exposed to the soil samples are compared with those of the plants in the control soil.

3.3.2.2 Test methodology

Selection and description of test species

Uncoated (insecticides or fungicides) *Avena sativa* (spring oat, KWS Uranie) seeds were used in this study. *A. sativa* is a monocotyledonous plant species which is recommended for this assay.

Sample preparation (Soil, Controls, etc.)

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Prior to the test, the different soil composite samples were sieved at 4 mm. Each sample was prepared by mixing it thoroughly and adding deionized water to an amount required to achieve 70 % of the maximum water holding capacity. Plastic plant plots (polypropylene, 400 ml) were filled with 300 g to 400 g (depending on the soil density) of the moistened soil samples or control soil (up to 10 mm below the upper edge of the plant plot). Four replicates were prepared per soil sample/control. A standard natural soil (Lufa 2.3) was used as a control soil for this study.

Detailed procedure for conducting the test

Avena Sativa seeds were pregerminated in a Petri dish, on a bed of paper moistened with deionized water, until the radical had just emerged (after 72 hr at 20°C, in absence of light).

The pregerminated seeds with a radical less than 2 mm length were then planted in each plant pot previously filled with moistened soil (six seeds per pot), approximately 10mm beneath the surface of the soil. To prevent dehydration and avoid watering during the test, the plots were placed individually in polyethylene bags.

The pots were then incubated in a phytotron culture chamber (Fitron type SGC 120, Weiss Technik France), under controlled light (16-hour light / 8-hour darkness; 2500 lux), temperature (20 ± 2 °C during the day, 16 ± 2 °C during the night) and humidity for 72 h (70 ± 5 % during light periods and dark periods). After the growth period, the soil was carefully removed from each pot manually and the plants were separated and washed with deionized water. The longest root length of each plant was then measured.

The mean root length (6 plants per pot, 4 pots per condition) of the plants exposed to the soils sample were compared with the mean root length obtained in the control soil. Results are expressed as a percentage of inhibition relative to the control plants.

3.3.3 Effects of pollutants on earthworms / Part 1: Determination of acute toxicity to *Eisenia fetida* (ISO 11268-1:2012)

3.3.3.1 Test protocol & standards

The protocol used for the soil sample toxicity assessment using *Eisenia fetida* was performed following the recommendation of the ISO 11268-1:2012 standard. This standard defines a test protocol to measure the toxicity of the substances present in the soil through the effect of said chemicals on the living organisms such as *E. fetida*. The principle of this assay relies on the mortality and mortality rate of the *E. fetida* organisms. The higher the mortality or/and mortality rate of earthworms, the more potent the toxicity of the trace elements in the soil. For the test to be considered valid, the mortality in the controls should not exceed 10 % at the end of either test.

3.3.3.2 Test methodology

Selection and description of test species

E. fetida is a species commonly inhabiting soil rich in organic matter and possesses susceptibility to chemicals that resembles that of true soil-inhabiting species. *E. fetida* has a short life-cycle, hatches from cocoons in 3-4 weeks and reaches maturity in 7-8 weeks at 20°C. The species is prolific with each worm producing 2-5 cocoons per week from each of which emerge several worms, it is available commercially and can be bred easily in a wide range of organic waste materials. Cocoons can be

purchased commercially or distributed from a central source to ensure the same strain is used. The selection criteria for the test stated all specimens used were adult, between 2 months to 1 year old with a fully developed clitellum, that the culture used was of a relatively homogenous age structure (individuals not differing in age by more than four weeks) and that there had been maintained a relatively uniform size between the smallest and largest specimen in a single vessel.

The worms selected for the test were acclimatized for at least one day with a special type of artificial soil substrate. During this period, the worms were fed on the same food that was used in the test. Groups of 10 worms were weighted individually, randomly assigning the groups to the containers at the start of the test. The worms were washed prior to weighing (with deionised water) and the excess water was removed by placing the worms briefly on filter paper. The wet mass of individual worms was between 300 and 600 mg for *E. fetida.*

Experimental conditions (e.g., temperature, humidity, light conditions)

The study was conducted under controlled 16-hours light - 8 hours dark cycles with illumination at 400-800 lux, at a room temperature of 20±2°C. The worms were kept in test containers made of polyethylene terephthalate of maximum capacity 500m and a cross-sectional area of approximately 100cm2 for moist substrate depth of 5-6cm with 300-400 g dry substrate mass added. The container cover design permitted gaseous exchange between the substrate and the atmosphere and access to light (transparent and perforated), while preventing the specimens from escaping.

Sample preparation (Soil, Controls, etc.)

The control substrate consisted of soil with defined parameters. The test substrates comprised soils contaminated by human activities, collected from various study sites (CS) representing different climatic and ecological conditions. All soils were sieved to a fraction ≤2 mm. An artificial soil was used in this test with the following composition (based on dry weights, dried to a constant weight at a 105°C should contain: 10% sphagnum peat (as close to pH 5,5 to 6,0 as possible, no visible plant remains finely ground, dried to measured moisture content); 20% kaolin clay (kaolinite content preferably above 30%); 0,3 to 1,0% calcium carbonate ($CaCO_3$, pulverised, analysis grade) to obtain an initial pH of 6,0 with a differential of 0,5; 70% air-dried quartz sand (depending on the amount of $CaCO_3$ required), predominantly as fine sand with more than 50% of the particles between 50 and 200 microns.

Detailed procedure for conducting the test

Test procedure

The soil was mixed with deionized water to obtain 40-60% of the maximum water holding capacity. Prepared soil was added to 500ml containers and 10 weighted worms were placed on the surface. Healthy worms burrowed into the substrate immediately and any worms remaining on the surface after 15 min. were defined as damaged and were replaced. If replaced, the substituted and new worms were weighted. The containers were closed and kept in the experimental conditions.

A loading of 10 earthworms was placed into a recommended amount of 500g of moist mass of each type of soil (CS1 - CS7), each soil including three samples (G1 - G3), each sample four series (S I - S IV) and each series four repeats (R 1 - R 4). The control for the test included four repeats of the artificial soil, moisturized as described above. Preparation of the worms involved washing them in water,

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wiping clean and placing them on absorbent paper for a short period to allow excess water to drain. To avoid systematic errors in distributing the worms to the test containers the homogeneity of the test population was determined by individually weighing 20 worms sampled randomly from the population from which the test worms were taken. Having ensured the homogeneity, batches of worms were then selected, weighted and assigned to the test containers using a randomization procedure.

Feeding

Any food of a quality shown to be suitable for at least maintaining worm weight during the test was considered acceptable. The test animals were fed mixtures of blended vegetables and fruits. Approximately 5g of food was mixed into the top layer of the soil in each container and moistened with deionized water (about 5-6ml per container). Food was provided twice a week, roughly every 3-4 days during the 4-week test period. If food remained uneaten, the ration was reduced to avoid fungal growth or molding. The adults were removed from the soil on day 28 of the test.

Test duration and measurements

On day 7 the living adult worms were observed and counted to record survival. The soil with worms was transferred from the container into a clean tray and after counting the specimens, they were returned into the container with the same soil and provided food and water. Any unusual behaviour (e.g. inability to dig into the soil, lying motionless, anomalous decomposition rate of deceased organisms, attempting to escape the soil by climbing on the lid) and in morphology (e.g. open wounds) were also recorded. After counting all worms were returned into their containers, fed and watered.

On day 14 the living adult worms were counted and weighted. The containers were emptied onto a clean tray to facilitate search and extraction without causing damage. Extracted, the specimens were washed in deionized water and briefly placed on a filter paper to empty their contents and remove excess water before weighing them. Any worms not found at this time were recorded as dead, since it was to be assumed that such worms have died and decomposed prior to the assessment. Adult worms were humanely euthanized, by rapid freezing at -80°C or cryopreservation.

3.3.3.3 Data Interpretation and analysis methodology

Characteristics of the measurable effects of the test

Several endpoints were defined and analyzed based on the percentages between the investigated soil and the artificial control substrate, such as: mortality; changes in behaviour (e.g. inability to dig into the soil, lying motionless, anomalous decomposition rate of deceased organisms, attempting to escape the soil by climbing on the lid); changes in morphology (*e.g.* open wounds).

3.3.4Determination of the toxic effect of sediment and soil samples on growth, fertility and reproduction of *Caenorhabditis elegans* (Nematoda) (ISO 10872:2020)

3.3.4.1 Test protocol & standards

The determination of the toxicity toward the juvenile organisms of the species *C. elegans*, exposed to the environmental sample over a period of 96 h. The test was performed according to the

recommendation of the ISO 11269-1 which specifies a method for determining the toxicity of environmental samples on the growth, fertility and reproduction of the nematode

3.3.4.2 Test methodology

Selection and description of test species

Nematodes are among the most abundant soil invertebrates (>10^6 individuals/m^2) (Yeates, 2003), and they play key roles in decomposition, energy flows, nutrient cycling, and microbial regulation (Sochova et al., 2006; Neher, 2001). Therefore, these organisms are well acknowledged as environmental indicators for assessing the toxicity of chemicals and the quality of soils.

The species used for the growth and reproduction toxicity test is *C. elegans* (variety Bristol, strain N2), a free-living transparent nematode about 1 mm in length, microbivorous, with a short life cycle (~3 days). C. elegans is diploid and hermaphroditic. In the laboratory, the population of *C. elegans* is maintained in a climatic chamber (20°C, obscurity) laying on nematode growth medium agar plates previously spread with *E. coli* (OP50) as food. Strain of *C. elegans* (variety Bristol, strain N2) and strain of *E. coli (OP50)* were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA).

Experimental conditions (e.g., temperature, humidity, light conditions)

The test was performed in a climatic chamber, under controlled environmental conditions, at a temperature of 20 ± 1 °C, in the dark.

Sample preparation (Soil, Controls, etc.)

Prior to the test, the different soil composite samples were sieved at 2 mm. A standard natural soil (Lufa 2.2), sieved at 2mm, was used as a control soil. A quantity of 500 mg of pre-moistened soil and control soil were then weighted into a 24-wells microplate. A mixed volume of M9-medium including the needed food quantity (freshly prepared E. Coli OP50 concentrated suspension at 60 000 FAU) is then added to adjust soil moisture to 80 % of the maximum water holding capacity and stir with a spatula to achieve homogenous material. The so-prepared microplates were then stored at 4°C to avoid loss of moisture until the introduction of juveniles C. elegans and the start of the test.

Detailed procedure for conducting the test

The test starts with worms in the first juvenile stage (approx. 250 µm to 350 µm length). In order to obtain worms synchronized to this life stage, stock of *C. elegans*, maintained on agar plate were rinsed with M9-medium. The suspension was then filter through a cascade of filter gauze of 5 µm and 10 µm mesh size to retain larger juveniles and adults. The first-stage juveniles (J1) were then collected in the filter suspension. The length of 30 J1 collected randomly were measured to obtain the mean initial length of the introduced exposed test organisms.

After allowing the microplates temperature to equilibrate to room temperature, ten first-stage juveniles (J1; test organisms) were collected from the filter suspension and transferred into each prepared soil sample using a micropipette. The microplates were then sealed and incubated for 96h in a climatic chamber at 20°C ± 1 °C, in the dark. After 96h, a volume of 0.5 ml of a solution of rose Bengal was added in each well to stain the nematodes and the microplate were heated in a drying oven for 10 min at 80 °C. Each sample were then rinsed and the content transferred into centrifugation tubes. The nematodes population (10 adults and the J1 produced during the test) was then separated from the soil using a Ludox suspension and successive centrifugation. Nematodes populations were

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then collected in the supernatant and transferred into a ward counting wheel to determine the number of offspring (reproduction endpoint), to measure the adult size and to check for internal eggs (growth and fertility endpoint) under binocular microscope equipped with a numerical camera and using a dedicated image analyses software for morphometrics analyses (Saisam software, Microvision instruments).

Nematodes survival, growth inhibition and inhibition of the reproduction of the organisms exposed to the soil composite samples were then calculated by comparison with the results obtained in the control soil.

3.3.5Plant emergence and growth test/Terrestrial Plant test:Seedling Emergence and Seedling Growth Test (OECD 208)

3.3.5.1 Test protocol & standards

The study is conducted in accordance with OECD 208 guidelines, which define standard procedures for assessing the impact of chemical substances on plant emergence and growth. The test evaluates the toxicity of substances in a soil environment, determining potential effects on both cultivated and wild plants.

3.3.5.2 Test methodology

Selection and description of test species

Test species were selected considering taxonomic diversity, widespread occurrence, and sensitivity to chemical exposure in the tested soils. Selection criteria included:

- Availability of uniform seeds with a reliable germination rate.
- Feasibility of conducting tests under laboratory conditions with reproducible results.
- Sensitivity to contaminants, including TE and other stress factors.
- Compliance with growth conditions specified in the test.

Among the species used for toxicological tests, White Mustard (*Sinapis alba*) was chosen as a representative of dicotyledons, and Oats (*Avena sativa*) as an example of monocotyledons.

Detailed procedure for conducting the test

For each soil sample (3 composites per CS), the test was conducted in four independent measurement series, with four replicates in each series (4 pots/replicate = 16 pots/series). The test was conducted using 500 ml pots. At the beginning of the test, all pots were filled with soil. Then, 10 seeds of the appropriate species (*Sinapis alba / Avena sativa*) were introduced into each pot, gently pressed to a depth of 0.5 mm and covered with soil. Throughout the test, soil moisture was maintained at 70%, and temperature and lighting conditions were controlled.

For both test species, the study was conducted under controlled environmental conditions, including:

- assessment of seed germination efficiency on days 1, 3, 5, and 7 of the test;
- measurement of plant shoot length on day 15.

Experimental conditions (e.g., temperature, humidity, light conditions)

The study was conducted under controlled environmental conditions, including:

- temperature: 25 ± 3 °C during the day, 20 ± 3 °C during the night
- humidity: 70 ± 5 % during light periods and 90 ± 5 % during dark periods
- photoperiod: 16-hour light / 8-hour darkness
- light intensity: 350 ± 50 µE/m²/s

Plants were grown in non-porous plastic pots, ensuring proper seed distribution to prevent overpopulation

3.3.5.3 Data Interpretation and analysis methodology

The test results were subjected to statistical analysis, where the mean, median, and standard deviation were determined. The final effect of the tested soils on plant emergence and growth was expressed as the % efficiency of seed germination on days 1, 3, 5, and 7, as well as the % stimulation (inhibition) of shoot growth in test plants after 14 days relative to the control group. The biomass of the aerial parts of the tested plants was also evaluated.

3.3.6 Worms reproduction test / Part 1: Determination of acute toxicity to *Eisenia fetida/Eisenia Andrei* (ISO 11268-2:2023)

3.3.6.1 Test protocol & standards

The protocol used for the soil sample toxicity assessment using *Eisenia fetida* was performed following the recommendation of the ISO 11268-2:2023 standard. This standard defines a test protocol to measure the toxicity of the substances present in the soil through the effect of said chemicals on the reproductive abilities of living organisms such as *E. fetida.* The principle of this assay relies on the number of produced offspring of the *E. fetida* organisms. The lower the number of offspring produced, the more potent the toxicity of the trace elements in the soil. For the test to be considered valid, each replicate in the controls must have produced ≥ 30% juveniles by the end of either test, the coefficient of variation of reproduction must have been ≤ 30% and adult mortality over the initial 4 weeks must have been ≤ 10%.

3.3.6.2 Test methodology

Selection and description of test species

E. fetida is a species commonly inhabiting soil rich in organic matter and possesses susceptibility to chemicals that resembles that of true soil-inhabiting species. *E. fetida* has a short life-cycle, hatches from cocoons in 3-4 weeks and reaches maturity in 7-8 weeks at 20°C. The species is prolific with each worm producing 2-5 cocoons per week from each of which emerge several worms, it is available commercially and can be bred easily in a wide range of organic waste materials. Cocoons can be purchased commercially or distributed from a central source to ensure the same strain is used. The selection criteria for the test stated all specimens used were adult, between 2 months to 1 year old with a fully developed clitellum, that the culture used was of a relatively homogenous age structure (individuals not differing in age by more than four weeks) and that there had been maintained a relatively uniform size between the smallest and largest specimen in a single vessel.

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The worms selected for the test were acclimatized for at least one day with a special type of artificial soil substrate. During this period, the worms were fed on the same food that was used in the test. Groups of 10 worms were weighted individually, randomly assigning the groups to the containers at the start of the test. The worms were washed prior to weighing (with deionised water) and the excess water was removed by placing the worms briefly on filter paper. The wet mass of individual worms was between 300 and 600 mg for *E. fetida* and between 250 and 600 mg for *E. andrei*.

Experimental conditions (e.g., temperature, humidity, light conditions)

The study was conducted under controlled 16-hours light - 8 hours dark cycles with illumination at 400-800 lux, at a room temperature of 20±2°C. The worms were kept in test containers made of polyethylene terephthalate of maximum capacity 500m and a cross-sectional area of approximately 100cm2 for moist substrate depth of 5-6cm with 300-400 g dry substrate mass added. The container cover design permitted gaseous exchange between the substrate and the atmosphere and access to light (transparent and perforated), while preventing the specimens from escaping.

Sample preparation (Soil, Controls, etc.)

The control substrate consisted of soil with defined parameters. The test substrates comprised soils contaminated by human activities, collected from various study sites (CS) representing different climatic and ecological conditions. All soils were sieved to a fraction ≤2 mm. An artificial soil was used in this test with the following composition (based on dry weights, dried to a constant weight at a 105°C should contain: 10% sphagnum peat (as close to pH 5,5 to 6,0 as possible, no visible plant remains finely ground, dried to measured moisture content); 20% kaolin clay (kaolinite content preferably above 30%); 0,3 to 1,0% calcium carbonate ($CaCO_3$, pulverised, analysis grade) to obtain an initial pH of 6,0 with a differential of 0,5; 70% air-dried quartz sand (depending on the amount of $CaCO_3$ required), predominantly as fine sand with more than 50% of the particles between 50 and 200 microns.

Detailed procedure for conducting the test

Test procedure

The soil was mixed with deionized water to obtain 40-60% of the maximum water holding capacity. Prepared soil was added to 500ml containers and 10 weighted worms were placed on the surface. Healthy worms burrowed into the substrate immediately and any worms remaining on the surface after 15 min. were defined as damaged and were replaced. If replaced, the substituted and new worms were weighted. The containers were closed and kept under the experimental conditions.

A loading of 10 earthworms was placed into a recommended amount of 500 g of moist mass of each type of soil (CS1 - CS7), each soil including three samples (G1 - G3), each sample four series (S I - S IV) and each series four repeats (R 1 - R 4). The control for the test included four repeats of the artificial soil, moisturized as described above. Preparation of the worms involved washing them in water, wiping clean and placing them on absorbent paper for a short period to allow excess water to drain. To avoid systematic errors in distributing the worms to the test containers the homogeneity of the test population was determined by individually weighing 20 worms sampled randomly from the population from which the test worms were taken. Having ensured the homogeneity, batches of

worms were then selected, weighted and assigned to the test containers using a randomization procedure.

Feeding

Any food of a quality shown to be suitable for at least maintaining worm weight during the test was considered acceptable. The test animals were fed mixtures of blended vegetables and fruits. Approximately 5 g of food was mixed into the top layer of the soil in each container and moistened with deionized water (about 5-6 ml per container). Food was provided twice a week, roughly every 3-4 days during the 4-week test period. If food remained uneaten, the ration was reduced to avoid fungal growth or moulding. The adults were removed from the soil on day 28 of the test.

Test duration and measurements

On day 28 the containers with the soil and worms were emptied onto a fresh tray. All adult worms were removed and all cocoons counted. Adult worms were humanely euthanized, by rapid freezing at -80°C or cryopreservation.

The soil was then returned to the containers (minus the adult worms but containing any cocoons that have been produced). The soil was then incubated for 4 additional weeks under the same test conditions except that feeding only took place once at the start of this phase of the test. At the end of the second 4-week period, the number of juveniles hatched from the cocoons in the test soil and cocoon numbers were determined through observation and extraction. All signs of harm or damage to the worm were recorded throughout the test period. At termination of the test, hatched juveniles were counted and humanely euthanized, by rapid freezing at -80°C or cryopreservation.

3.3.7 Fresh water algal growth inhibition test with unicellular green algae (ISO 8692:2012)

3.3.7.1 Test protocol & standards

Hydro-soluble contaminants and they potential toxicity toward aquatic unicellular green algae (primary producer) were determined on soil leachates according to the ISO 8692:2012 standard protocol. This document specifies a method for the determination of the growth inhibition of unicellular green algae by substances and mixtures contained in water, by wastewater or mixture leachates.

3.3.7.2 Test methodology

Selection and description of test species

Microalgae are an essential component of the aquatic nutrient chain and a major producer in the aquatic environment. They play a crucial role in preserving the balance of most aquatic environments and are necessary for the appropriate structure and operation of the overall ecosystem. Because of its high sensitivity to chemicals and it relevance in aquatic ecosystem, the freshwater chlorophyceae *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella subcapitata*) was used as the test organism in the algal growth inhibition test.

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Sample preparation (Soil, Controls, etc.)

Soil leachates were produced according to ISO 21268-2:2019 (leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil-like materials). Briefly, the soil composite samples were sieved at 2 mm and a quantity of 100 g (dry mass basis) was placed in glass bottle previously rinsed with deionized water. A volume of 200 ml of $CaCl_2$ solution (0.001 mol.L-1) was added. The bottles were agitated for 24h at room temperature using a roller table setup at 20 rotations/min. After 24h, the liquid and solid phase were separated by centrifugation (30 min, 20000 G). The supernatant was then filtrated using a nylon/polyamide filter and then filtrated again at 0.45 µm. The soil leachates were stored 24 h at 4°C until the start of the algae growth inhibition test.

Detailed procedure for conducting the test

The tests were conducted in 24-well microplate fulfil with 2.5 ml of soil composite sample leachates supplemented with micronutrients and the algae inoculum at a concentration of 10^4 cells/ml. Four replicates were prepared per condition. The algae growth medium (OCDE 201) inoculated with the same algal inoculum was used as a negative control. The microplates were then incubated in a climatic chamber at 22°C ± 1 °C under continuous orbital agitation (125 rotation per minute) for 72-hr. Algal biomass was measured daily by quantifying the chlorophyll in vivo in all control and test vessels at excitation and emission wavelength of 460/670 nm using a microplate reader (VICTOR™ X3, PerkinElmer). The algae growth rate was finally calculated, and the growth inhibition was determined by comparison with the results obtained in the negative control.

3.3.8 Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) — Acute toxicity test (ISO 6341:2012)

3.3.8.1 Test protocol & standards

Hydro-soluble contaminants and they potential toxicity toward aquatic micro-invertebrates were determined on soil leachates according to the ISO 6341:2012 standard protocol. This document specifies a procedure for the determination of the acute toxicity of chemicals, waters and waste waters to the water flea *D. magna* Straus.

3.3.8.2 Test methodology

Selection and description of test species

Crustaceans are of interest from the ecotoxicological point of view because they are primary consumers and a major component of the zooplankton in aquatic ecosystems. Among crustaceans, the genus Daphnia is widely distributed in aquatic environments worldwide. It is an important link between primary producers and consumers of higher trophic levels. *Daphnia magna* is internationally recognized as a standard experimental genus because of its rapid propagation, short life cycle. These organisms can be seen as a crucial model organism due to its sensitivity to various environmental stressors and its ecological relevance. The organisms used in this study were neonates (aged between 2 and 24 h) obtained by a cyclical parthenogenesis in a continuous laboratory breeding.

Sample preparation (Soil, Controls, etc.)

Soil leachates were produced according to ISO 21268-2:2019 (Leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil-like materials). Briefly, the soil composite samples were sieved at 2 mm and a quantity of 100g (dry mass basis) was placed in glass bottle previously rinsed with deionized water. A volume of 200 ml of $CaCl_2$ solution (0,001 mol.L-1) was

added. The bottles were agitated for 24h at room temperature using a roller table setup at 20 rotations/min. After 24h, the liquid and solid phase were separated by centrifugation (30 min, 20000 G). The supernatant was then filtrated using a nylon/polyamide filter. The soil leachates were stored 24 h at 4°C until the start of the *D. magna* immobilization test. A reconstituted water (ISO medium) was used as a negative control.

Detailed procedure for conducting the test

A volume of 10 ml of soil composite sample leachates or ISO medium were added to glass tubes and five neonates were transferred in each tube. Five replicates were used to have a group of 20 individuals per condition. The tubes were incubated in a climatic chamber at 20°C ± 1 °C, in the dark for 48 h. After a test period of 24 h and 48 h, the number of immobile D. magna was determined in each tube. The percentage of immobilization in soil composite sample leachates was calculated by comparison with the results obtained in the negative control replicates.

3.4 Ecology and soil functions analyses

3.4.1Measurement of enzyme activity patterns in soil samples (ISO 20130:2018)

3.4.1.1 Test protocol & standards

The measurement of the extracellular hydrolase activities in soil were performed according to the ISO 20130:2018 standard document. This document specifies a method for the measurement of several hydrolase activities (arylamidase, arylsulfatase, β-galactosidase, α-glucosidase, β-glucosidase, N-acetyl-glucosaminidase, acid- and alkaline- phosphatases, urease) in soil samples, using colorimetric substrates.

3.4.1.2 Test methodology

Selection and description of test species

Extracellular enzymes in soil are responsible for the degradation of organic macromolecules and their mineralization. These enzyme in soil play key roles in the biodegradation of organic macromolecules and are generally produced by microorganisms. These enzymes typically hydrolyse polymers into smaller subunits that can be taken into the cell for microorganisms such bacteria and/or fungi. At an ecological level, these microbial extracellular enzymes are responsible for much of the nutrient mineralization and organic matter decomposition that occurs in natural environments. The monitoring of such kind of enzyme activities important in the biodegradation of organic compounds and mineralization of carbon, nitrogen, phosphorus and sulphur in soil may reveal harmful effects caused by chemicals and other anthropogenic impacts.

The glucosidase (α or β) and galactosidase enzymes are important for cellulose degradation and catalyse the hydrolysis of cellulose to glucose, which provide an available carbon substrate for microorganisms uptake and assimilation (Eivazi et Tabatabai, 1988). Other enzymes, such as N-acetylglucosaminidase (NAG), are involved in the hydrolyse of chitin and peptidoglycan to produce glucosamine.

The phosphatases (acid or alkaline) release soluble inorganic phosphate groups from organophosphates, essentially mineralizing phosphate and making it available for use by most microorganisms.

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The soil arylsulfatase (ARS) is an important enzyme that controls the acquisition of organic sulphur and thus the soil sulphur cycling. ARS catalyses the hydrolysis of organic sulphate esters to sulphate-S (Tabatabai and Bremner, 1970) and thus soil ARS activity reflect the turnover and cycling of S in soil.

The enzyme arylamidase catalyses the hydrolysis of an N-terminal amino acid from peptides, amides or arylamides in soils organic matter. This enzyme plays a major role in the nitrogen (N) mineralization in soils. Finally, urease enzyme has an important role in the occurrence of accessibility of N for plant growth in N cycle.

Detailed procedure for conducting the test

Prior to the test, the different soil composite samples were sieved at 4 mm. The sieved soil was homogenized, and triplicate of exactly 4 g were weighed and deposited into flat bottom flask (30 to 60 ml). A volume of 25 ml of deionized water and cross shaped stirring bars were added. Containers were closed and homogenized for 10 min on orbital agitator (250 min^{-1}). Each soil suspension was then transferred in 96-well microplates (125 µl per well; 50 for measurement of urease), in four replicates. Three wells are analytical points, and one well was used as "blank" condition to reveal chemical interactions with soil compounds.

Soil suspension samples were allowed to react with specific artificial substrates which were then added to the previously prepared wells. The microplates were incubated during specific times at 25 °C ± 2 °C or 37 °C ± 2 °C, depending on the enzyme reaction followed. After the incubation, the different reactions were stopped, and the plates were centrifuged. The supernatants were transferred into new plates and the intensities of the coloration (corresponding to the quantity of end-product produced by the reaction) were measured using a 96-well microplate spectrophotometer (VICTOR™ X3, PerkinElmer) at specific wavelength. The enzyme activity is subsequently determined. Specific characteristics of each enzyme reaction are summarized in the table below.

Table 8 Set of enzymatic reaction performed within the analyse

Enzyme	substrates	Incubation duration (min)	Incubation temperature (°C)	End-product	Wavelength used for absorption measurement
α-Glucosidase	4-nitrophenyl α-D-glucopyranoside	60	37	Para-nitrophenol	450
β-Galactosidase	p-nitrophenyl β-D-galactopyranoside	120	37	Para-nitrophenol	450
β-Glucosidase	4-nitrophenyl β-D-glucopyranoside	60	37	Para-nitrophenol	450
N-acetyl-glucosamidase	para-nitrophenyl N-acethyl β-D glucopyranoside	60	37	Para-nitrophenol	450
Arylamidase	L-leucine β-naphthylamide hydrochloride	120	37	β-naphthylamine	540
Urease	Urea	180	25	Ammonium chloride	650
Acid phosphatase	4-nitro-phenylphosphate disodium salt hexahydrate	30	37	Para-nitrophenol	450
Alkaline phosphatase	4-nitro-phenylphosphate disodium salt hexahydrate	30	37	Para-nitrophenol	450

Enzyme	substrates	Incubation duration (min)	Incubation temperature (°C)	End-product	Wavelength used for absorption measurement
Arylsulfatase	Potassium 4-nitrophenyl sulfate	240	37	Para-nitrophenol	450

3.4.1.3 Data Interpretation and analysis methodology

Results are expressed as milliunit for one gram of dry soil corresponding to nmole of PNP, β-naphthylamine or ammonium chloride released per minute and per gram of soil dry mass of sample.

3.4.2Determination of organic matter decomposition in contaminated soil (ISO 23265:2022)

3.4.2. 1 Test protocol & standards

The ability of soil microorganisms to decompose lignin cellulosic material provides evidence that the microbial population in soil is active in organic matter decomposition and carbon cycling. The capability of the soil composite samples to degrade organic matter were tested according to the ISO 23265 standard protocol. This document specifies a method applicable to natural soils and soil materials of unknown quality to estimate the organic matter decomposition by determining the kinetic of the degradation of a standard cellulosic materials.

The protocol applied in the present study used sterilized filter paper disks are used as a source of organic matter. The ability of the soil composite sample to degrade the cellulose filter paper disk was recorded every 2 weeks up to 2 months.

3.4.2.2 Test methodology

Detailed procedure for conducting the test

Prior to the test, the different soil composite samples were sieved at 2 mm. Each soil sample was prepared by mixing it thoroughly and adding sterilized deionized water to an amount required to achieve 70% of the maximum water holding capacity.

A quantity of 10 g of soil (dry mass basis) was then added to a 50 ml sterile centrifuge tube. A pre-weighting filter paper was then placed on the top of the first soil layer and then recovered by a second layer of 10 g of soil (dry mass basis). The tubes were then closed using a cap allowing the gases exchange. Fives replicates were prepared per soil composite sample and four time point were prepared: after 15, 30, 45 and 60 days. The tubes were incubated in a climatic chamber at 20°± 2 °C, in the dark.

At each time point, 5 tubes were taken randomly. The soil and filter paper were then separated. The filters were then gently rinsed using a soft paintbrush and the soil-free filters were transferred to the pre-weighed aluminium weigh-boat and oven dry at 105 °C for 24 h. After drying, weigh-boats were transferred to a desiccator and allow to cool down for a minimum of 20 min before to be weighted on an analytical balance. The mass of the dried filter papers was finally recorded, and the percentage mass loss of each filter paper was calculated.

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3.4.3Nematofauna diversity analyses

3.43 1 Test protocol & standards

The nematofauna diversity analyses were performed by ELISOL environment laboratory. The method is based on the ISO NF 23611-4 standard protocol. This document specifies a method for sampling and handling free-living nematodes from terrestrial field soils as a prerequisite for using them as bio-indicators (e.g. to assess the quality of a soil as a habitat for organisms).

Nematode community structure integrates information of the soil micro-food web (microbial compartment, microfauna and mesofauna) which is responsible for the decomposition and mineralization of nutrients through organic matter transformation. The abundance and diversity of nematodes provides insights into the soil biological functioning as they occupy different levels of the soil food web (Ekschmitt et al., 2001). The calculation of various indices, based on the abundance and composition of nematode communities, is here used to assess nutrient flows, environmental stability or the diversity of organisms in the soil.

3.4.3 2 Test methodology

The analyses were performed on fresh collected soil composites samples; keep at cool temperature no more than 3 days before the organisms extraction and analyses. Nematodes were extracted from 150 - 200 g of soil composite fresh samples using an elutriation system. The nematodes were then counted using a binocular microscope. After fixing and transferring to mass slides, the composition of soil nematofauna was determined at family or genus level through microscopic observation at 400x magnification.

Nematode density was recorded as the total number of individuals per 100 g dry material. Each nematode was allocated to a colonizer-persister (cp) class according to Bongers (1990) and assigned to one of the five trophic groups defined by Yeates et al. (1993): bacterial feeders (BF), fungal feeders (FF), predators (P = carnivores (Ca) + omnivores (Om)), obligate plant feeders (OPF) and facultative plant feeders (FPF). The nematofauna diversity (Shannon, richness), the maturity indexes (for free-living taxa - MI, and for plant parasitic taxa - PPI), the Enrichment Index (EI) and Structure Index (SI) were calculated according to Bongers (1990) and Ferris et al. (2001). The Enrichment Index and the Structure Index, both based on the indicator importance of functional guilds of nematodes, are descriptors of food web condition. The results obtained were compared to a specific referential (ELIPTO® module EDITO V 1.1; 2019) and used to calculate an integrated index which indicates the global soil quality (good, limited, degraded).

4 Test results

4.1. Data interpretation and effect representation

To comprehensively present all results of the analyses conducted within Task 2.2, graphical summaries of the test outcomes were prepared. These visual representations allow for capturing and conveying a complete picture of the soil composite sample ecotoxic effects, global metabolisms and ecology. The results of the ecotoxicological analyses, soil function assessment, and ecological tests, were transformed and visually represented using color-coded scales as described below:

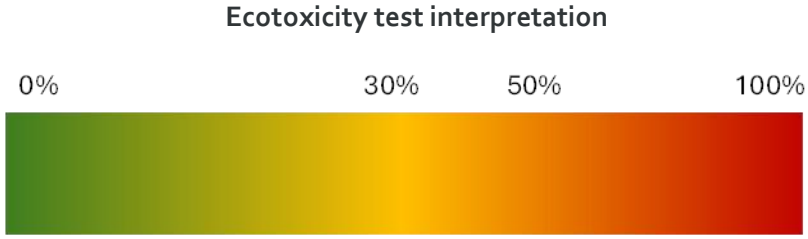

Figure 13. Color-coded scale for ecotoxicity test interpretation (% of effect observed, relative to the control soil).

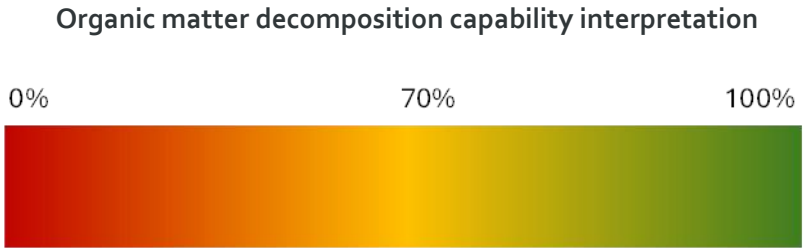

Figure 14. Color-coded scale for organic matter decomposition capability interpretation (% of degradation observed).

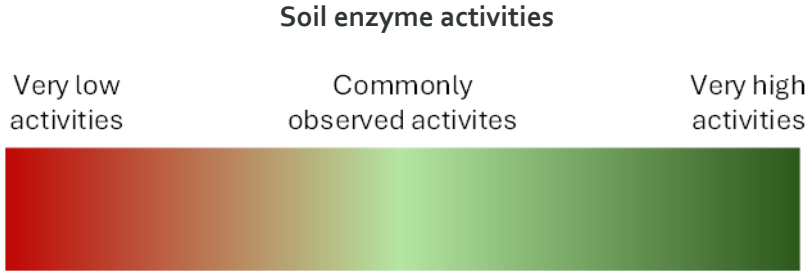

Figure 15. Color-coded scale for soil enzyme activities.

4.2 Cumulative assessment of soil sample toxicity

In the following subsections, for each analysed CS, the toxicity of the soil composites has been assessed individually.

4.2.1 Soil samples toxicity – CS1

A summary of the results of the ecotoxicological analysis, as well as for soil function and ecological indicators for CS1 is presented respectively in Figure 16 and Figure 17.

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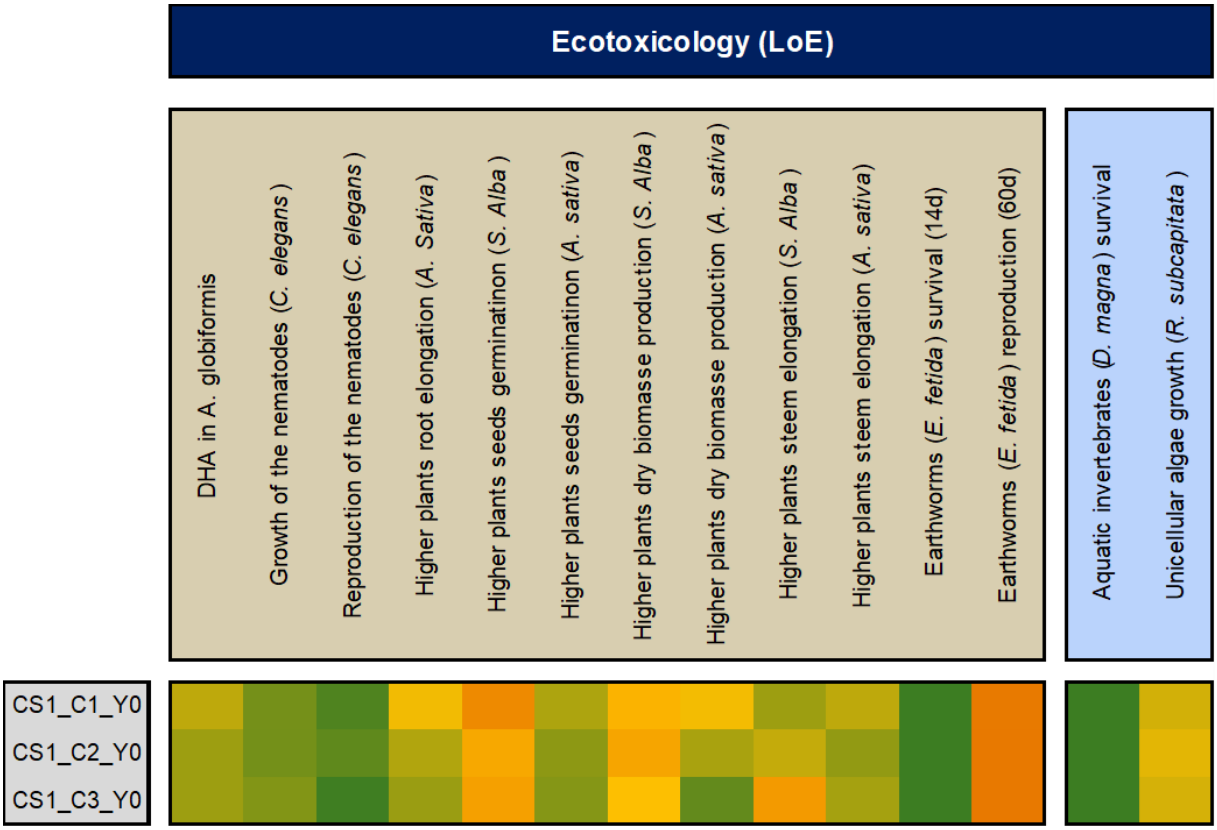

Figure 16 Summary results of ecotoxicological tests performed for CS1.

The data produced for CS1 indicates that the soil composite samples had no significative toxic effect on the bacteria (*A. globiformis*) dehydrogenase activity, as well as the growth and reproduction of nematodes (*C. elegans*). In the same way, according to the tests conducted on oligochaetes (*E. fetida*), no acute adverse effects were observed. However, chronic exposure to the soil composite sample have led to pronounced negative effects highlighted by a reduction in reproductive output, specifically a decrease in the number of cocoons (>50% of inhibition by comparison with the to the control group).

Beside these results, slight effects were observed on plant seed germination, plant root growth and general biomass production. The effects were more pronounced on the dicotyledonous specie (*S. Alba*) than on the monocotyledonous one (*A. sativ*a). The highest level of toxicity was observed during the phytotoxicity tests with the toxic effects ranging from 6-50%, however there is a clear distinction between *S. alba* and *A. sativa* species, where the latter had shown more resistance to the contamination in aspects of germination and growth. Regarding *S. alba*, the seeds germination was affected by more than 40% on average, while *A. sativa* by less than 20%. The dry biomass was affected respectively by 35% and 17% for *S.alba* and *A. sativa* and similarly, the stem elongation was affected by the soil toxicity by 27% and 5,5% for both species.

Considering earthworms (*E. fetida*), the reproduction was clearly affected (>50% of inhibition), although soil contamination had shown no adverse effects on the earthworm survival. The disparity between those results may come from the comparison with the control soil, but also the delicate nature of the cocoons observed during the chronic test, which were extremely prone to the level of moisture in the soil and during the control extraction while being counted.

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In addition to these observations, the toxicity of the soil leachate appeared to be limited on aquatic species, with no effect recorded on micro-invertebrates mobility (*D. magna*) and a slight growth inhibition observed on unicellular microalgae (*R. subcapitata*).

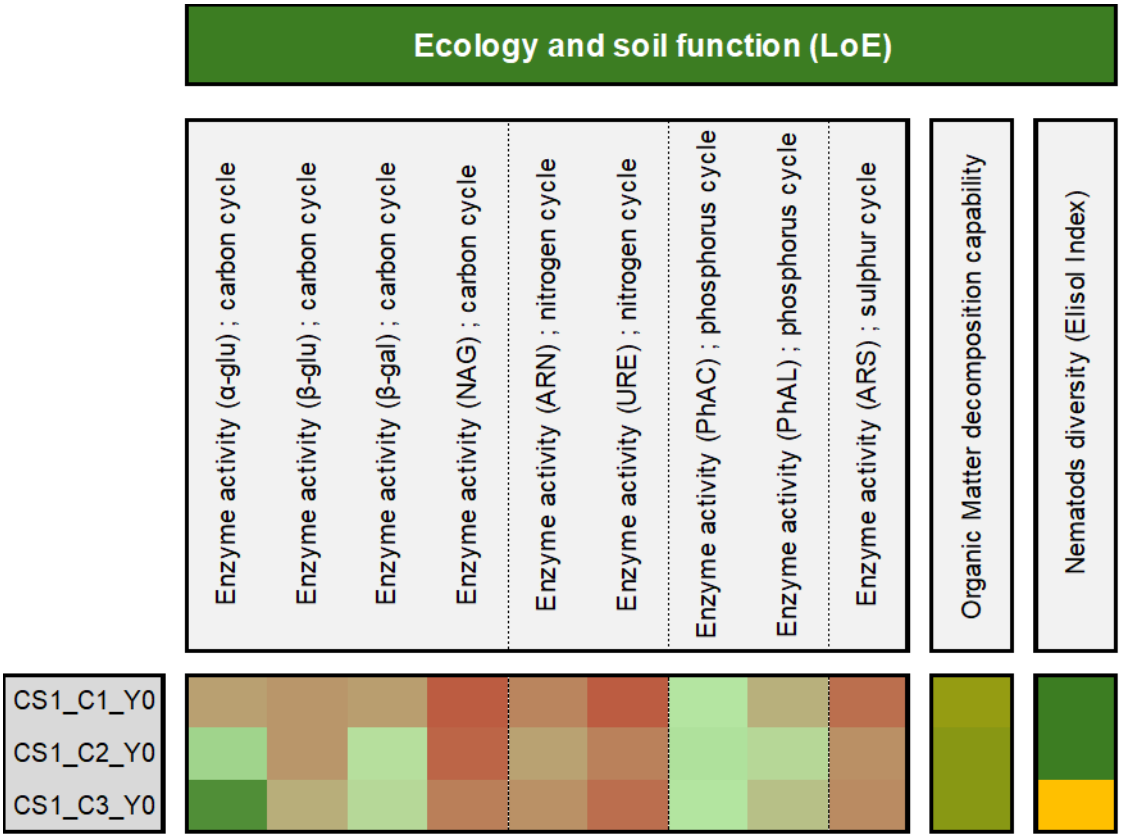

Figure 17 Summary results of ecology and soil function tests performed for CS1.

Concerning the soil global metabolisms, the enzyme activities measured in CS1 soil composite samples were generally below the usually measured enzyme activities in uncontaminated soils. Especially, the activities measured for both enzymes involved in the nitrogen cycle (urease and arylamidase) for some enzyme involved in the carbon cycle (N-acetyl-glucosamidase, β-galactosidase) and the enzyme involved in the sulphur cycle (arylsulfatase) were representative of low activities.

The tested soil has shown a good capability to degrade organic matter which is indicated by a percentage of cellulose degradation above 80% at the end of the organic matter decomposition test.

The nematofauna index calculated according to the analyses of the nematofauna density, diversity (Shannon index) and the structure of the trophic network indicate a limited to good soil environment for the soil collected on the CS1 site.

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4.2.2 Soil samples toxicity – CS3 (Odiel basin Area)

A summary of the results of the ecotoxicological analysis, as well as for soil function and ecological indicators for CS3 is presented respectively in Figure 18 and Figure 19.

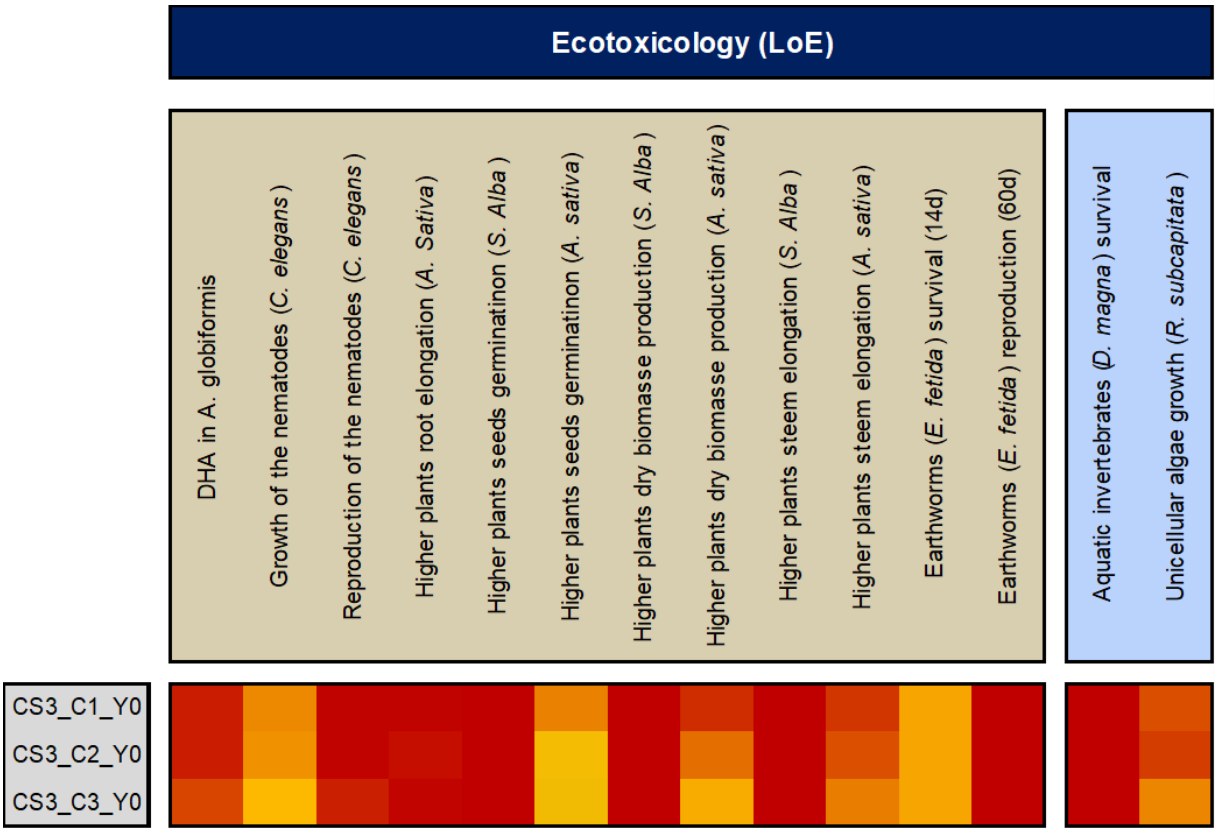

Figure 18 Summary results of ecotoxicological tests performed for CS3.

The results obtained with soil composite sample of CS3 revealed pronounced toxic effects whatever the species tested, or the endpoint analysed. For these soil sample, we have recorded an extreme inhibition of DHA in bacteria (>80%), of the root growth and the biomass production in higher plant, a pronounced inhibition of the growth of the invertebrates as well as an extreme inhibition of the reproduction of the invertebrates.

In particular, the growth of the nematodes (*C. elegans*) and the survival of the earthworm (*E. fetida*) showed more than 40% of inhibition. The reproductive of these organisms were also highly affected, with an average inhibition more than 95% for both species.

The phytotoxicity tests had shown uniform results within the *Sinapis alba* species where the soil toxicity has reached 100% regarding all aspects of the higher plants tests, including seeds germination, stem elongation and dry biomass production. While *A. sativa* fared had shown better resistance to the soil contamination, the root elongation was also completely affected by the soil toxicity, with the seed germination, stem elongation and dry biomass production being affected by around 37%, 69% and 60% respectively.

Finally, the aquatic invertebrate species *D. magna* survival and unicellular algae species *R. subcapitata* growth were highly inhibited, with the former succumbing to the soil eluate ecotoxicity of 100% and the latter being affected close to 60%.

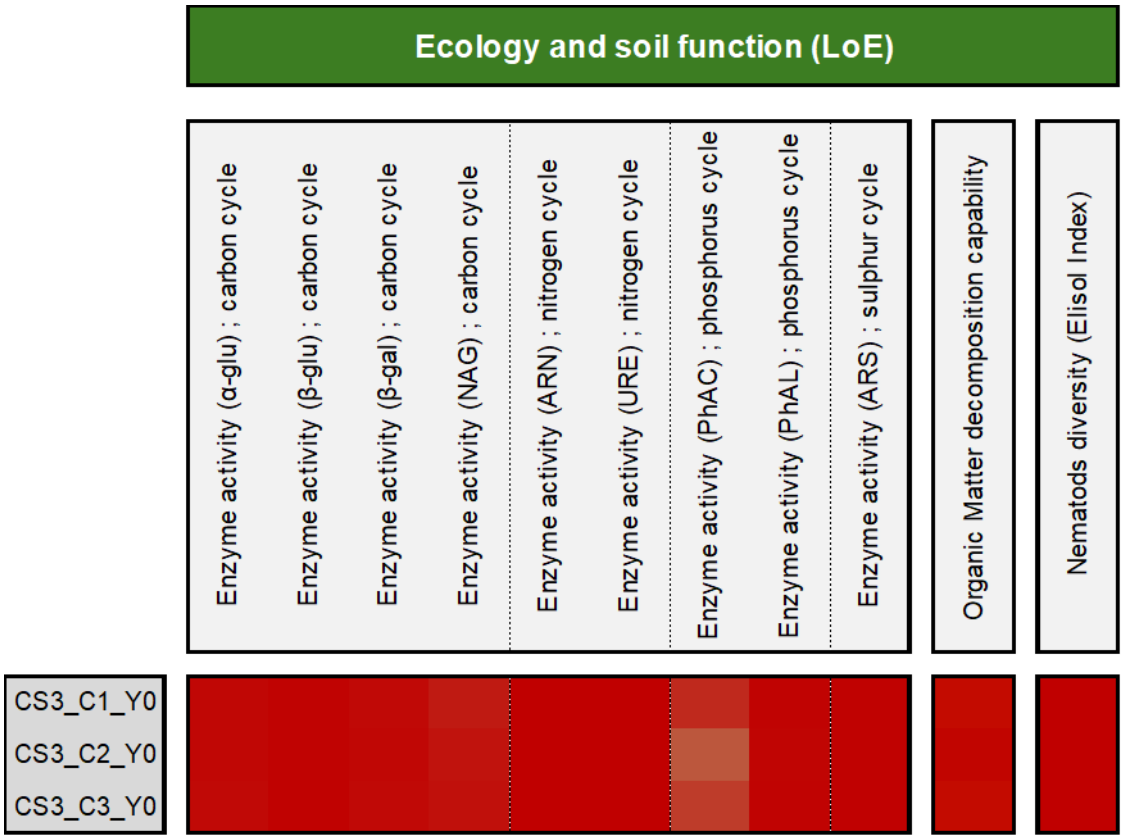

Figure 19 Summary results of ecology and soil function tests performed for CS3.

Concerning the soil metabolisms, both enzyme activities and the organic matter decomposition capability indicator pointed out the highly degraded profile of the soil composite samples collected in the CS3. The enzyme activities were close to zero, whatever the enzyme measured, and the results obtained for organic matter decomposition test pointed out the inability of this soil to degrade organic matter.

In accordance with the very high ecotoxicity measured and the highly degraded metabolisms, the nematofauna index calculated according to the analyses of the nematofauna density, diversity (Shannon index) and the structure of the trophic network is representative of an extremely degraded soil environment.

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4.2.3Soil samples toxicity – CS4 (Upper Silesia Coal Basin)

A summary of the results of the ecotoxicological analysis, as well as for soil function and ecological indicators for CS4 is presented respectively in Figure 20 and Figure 21.

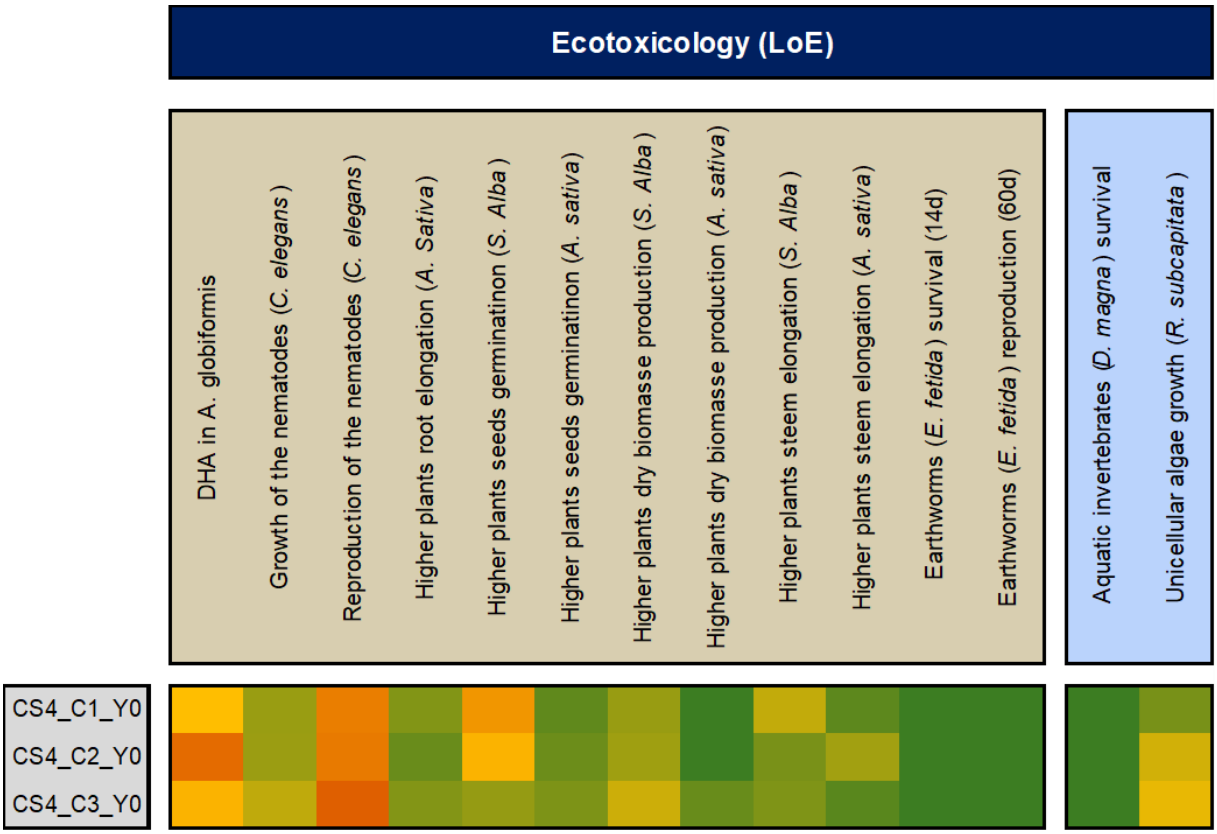

Figure 20 Summary results of ecotoxicological tests performed for CS4.

Concerning the global soil contamination, the data presented shows the soil toxicity measured in CS4 had not reached beyond 65% overall, although across the other samples, the effect of the soil toxicity on the survival, growth and reproduction of invertebrates is significantly lower than in the other samples.

The results showed that an average effect close to 40% of inhibition of the DHA of the bacterial species *A. globiformis.* Considering the nematodes (*C. elegans*), the soil toxicity had slight effect on their growth (<20%), while the reproduction functions were more affected by the soil contamination (close to 60% of growth inhibition). No significant effect was observed on the earthworms (*E. fetida*) whatever the endpoint observed (survival or reproduction).

The phytotoxicity tests ranged from 0-46% inhibition, although the most affected was the seeds germination of the *S. alba* species (around 30% of inhibition). Similarly to the previous case studies, *A. sativa* fared better than *S. alba*. Considering seed germination, the toxic effects reached only ~8% in *A. sativa.* Stem elongation for *S. alba* and *A. sativa* was 13% and 8%, while the dry biomass production was 17% and 7% respectively

The ecotoxicity of soil leachate were limited as no acute ecotoxic effect were observed on the micro-invertebrates (*D. magna*) and only a slight inhibition of the unicellular algae *R. subcapitata* were recorded.

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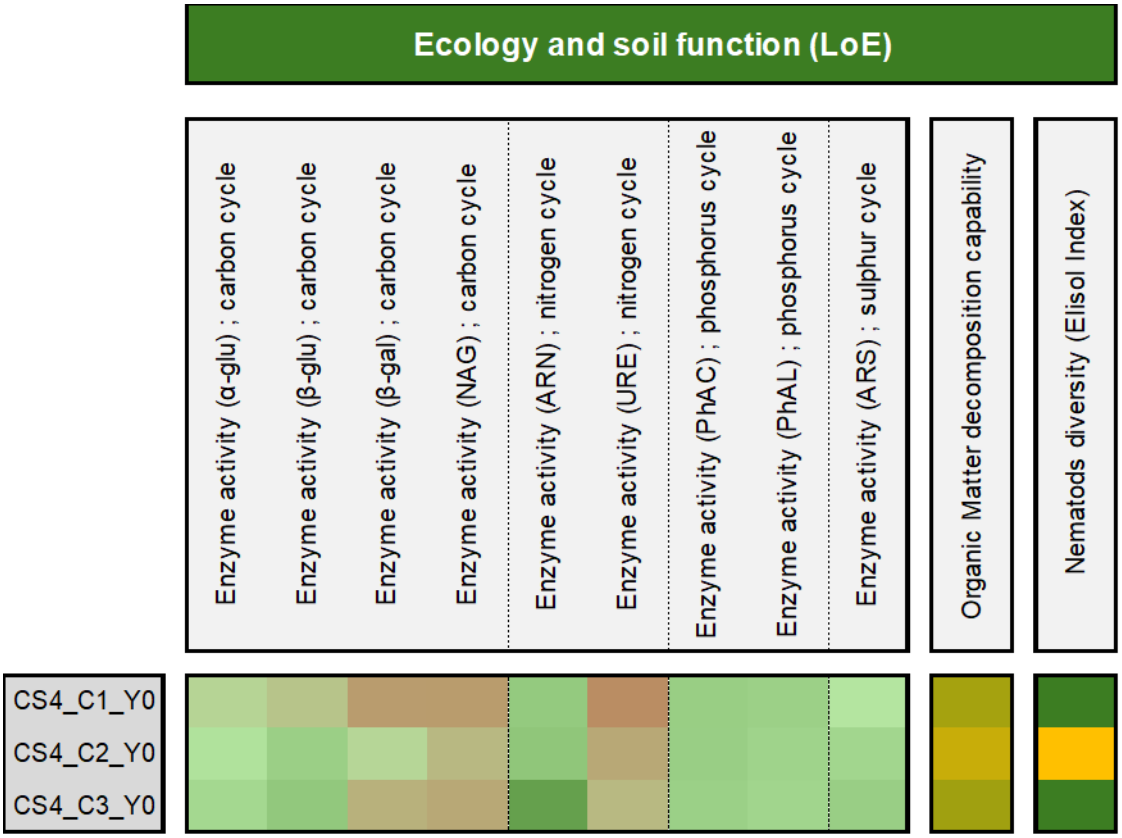

Figure 21 Summary results of ecology and soil function tests performed for CS4

Concerning the soil metabolisms, the enzyme activities measured in the soil composite samples collected on the CS4 site were representative to commonly used to slightly lower activities (N-acetyl-glucosaminidase and urease) by comparison with uncontaminated sites.

The tested soil has shown a good capability to degrade organic matter which is indicated by a percentage of cellulose degradation close or above 80% at the end of the organic matter decomposition test.

The nematofauna index calculated according to the analyses of the nematofauna density, diversity and the structure of the trophic network indicate a limited to good soil environment for the soil collected on the CS4 site.

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4.2.4 Soil samples toxicity – CS6 (Vieux-Charmont)

A summary of the results of the ecotoxicological analysis, as well as for soil function and ecological indicators for CS6 is presented respectively in Figure 22 and Figure 23.

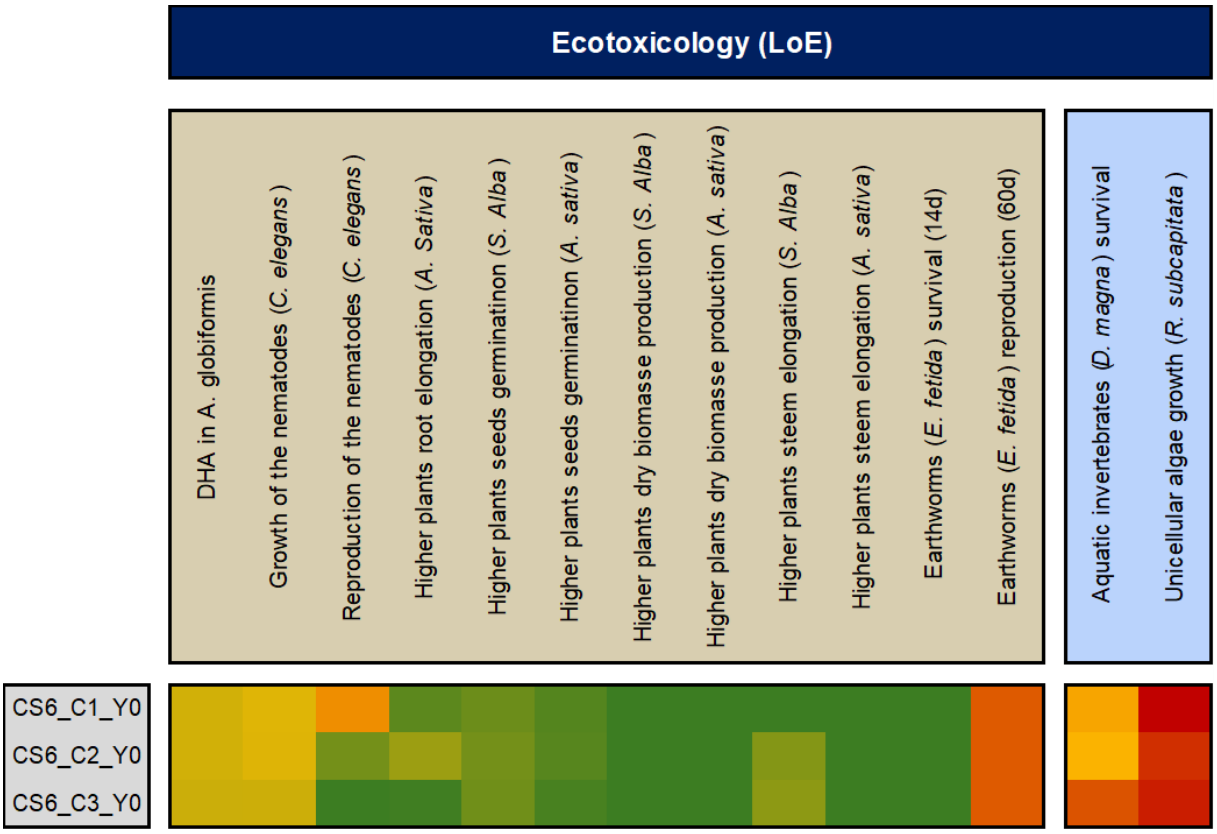

Figure 22 Summary results of ecotoxicological tests performed for CS6.

The data produced for CS6 indicates that the soil composite samples had no visible toxic effects towards higher plants, whatever the endpoint analysed: germination, stem growth, root growth, biomass accumulation, and dry biomass production). In the same way, according to the tests conducted on oligochaetes (*E. fetida*), no acute adverse effects were observed, as the test organisms generally showed an increase in the biomass.

However, looking to the chronic toxicity, potential negative effects were detected, indicated by a significative reduction in reproductive output, specifically a decrease in the number of cocoons produced after 60 days (> 50% by comparison with the control soil).

In addition, the aquatic ecotoxicity of the eluate appeared as unique, with a pronounced ecotoxicity measured on both microinvertebrates and unicellular microalgae while low to moderate toxicity were generally observed considering the terrestrial bioassays on higher plant.

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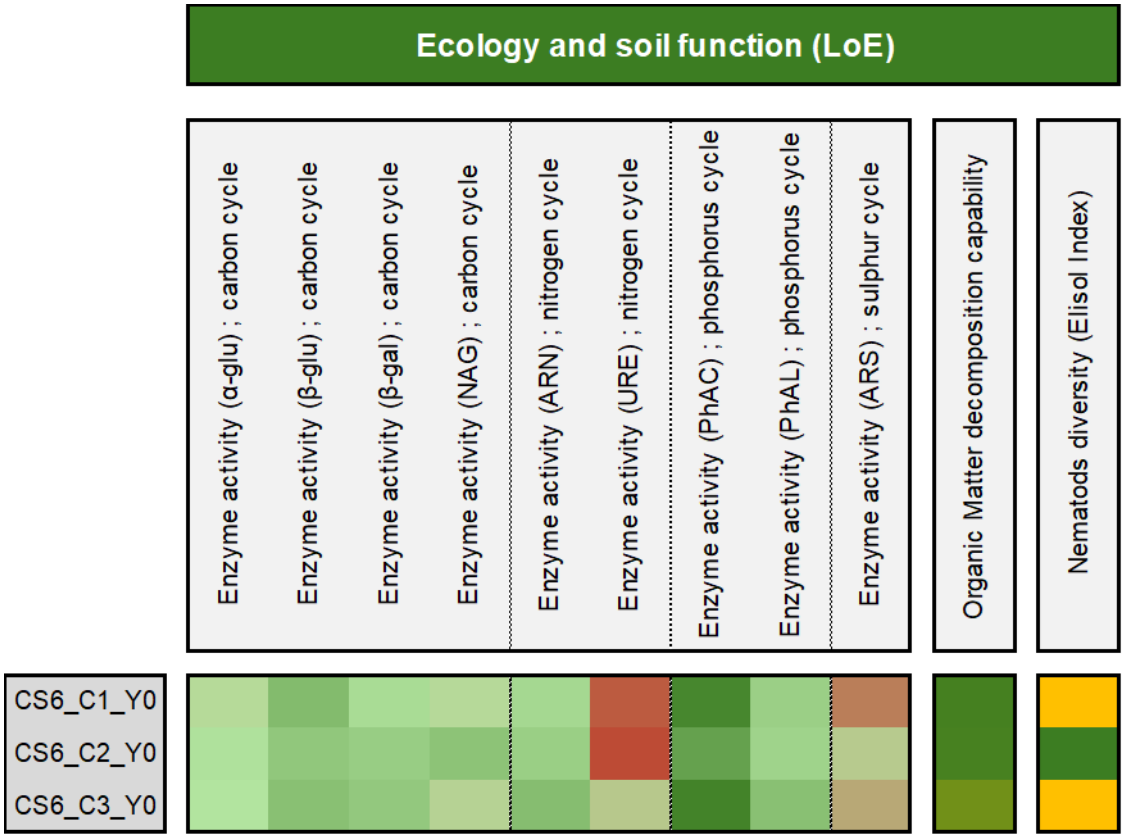

Figure 23 Summary results of ecology and soil function tests performed for CS6.

Concerning the soil metabolisms, the enzyme activities measured in the soil composite sample collected on the CS6 site were representative to commonly used to slightly lower activities by comparison with uncontaminated sites.

The tested soil has shown a good capability to degrade organic matter which is indicated by a percentage of cellulose degradation close or above 80% at the end of the organic matter decomposition test.

The nematofauna index calculated according to the analyses of the nematofauna density, diversity and trophic group structure indicate a limited to good soil environment for the soil collected on the CS6 site.

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4.2.5Soil samples toxicity – CS7 (Lavrio)

A summary of the results of the ecotoxicological analysis, as well as for soil function and ecological indicators for CS7 is presented respectively in Figure 24 and Figure 25.

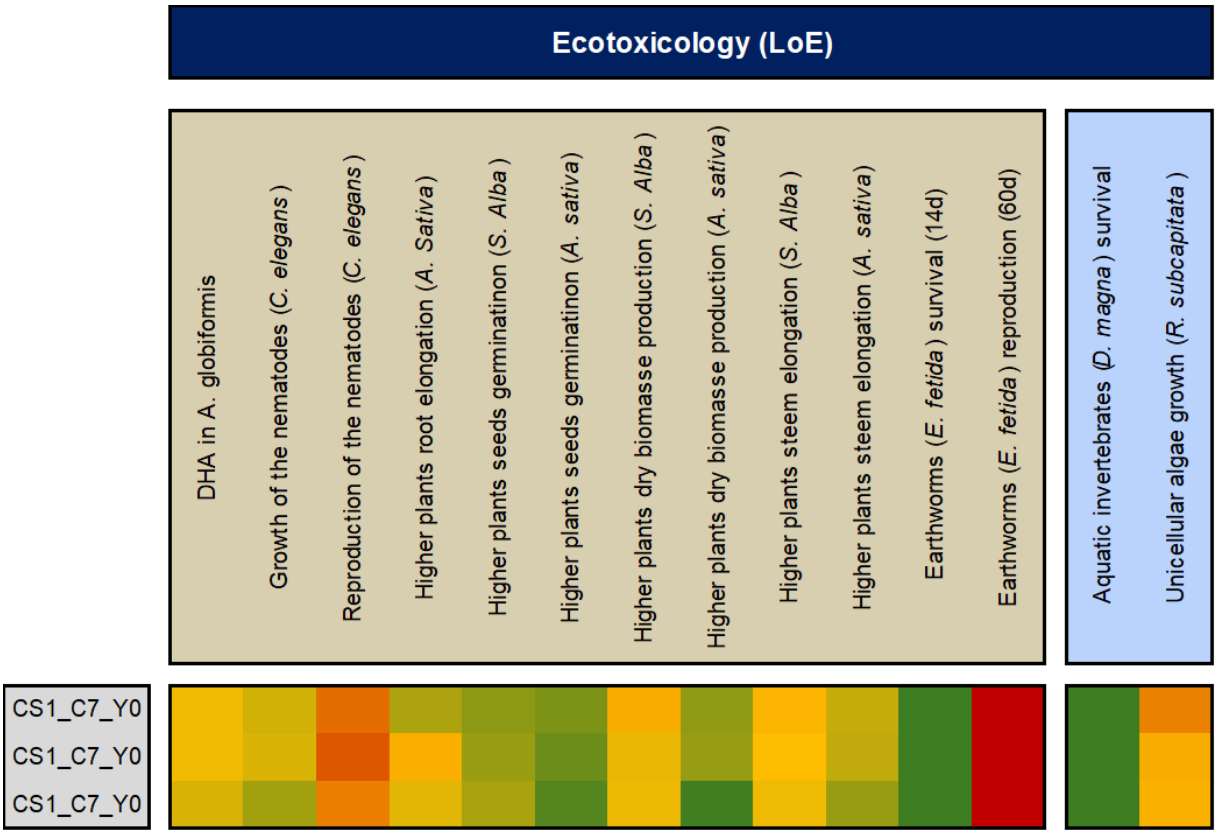

Figure 24 Summary results of ecotoxicological tests performed for CS7

The data produced for CS7 indicates that the soil composite samples had a potential for significant ecotoxicity towards bacteria, nematodes, higher plant and earthworms. More specifically, the soil composite samples have induced an inhibition of the DHA of the bacteria (*A. globiformi)* close to 30% by comparison with the control groups. While no acute toxicity was recorded on earthworms (*E. fetida*), a total inhibition of the reproduction was recorded. For this soil composites no survival of either offspring or the cocoons were observed. The chronic exposures to nematodes also indicate that this soil sample led to pronounced inhibition of the reproduction (>50% by comparison with the control groups). Considering the phytotoxicity tests, *S. alba* had yet again shown the signs of stronger inhibition after exposure to the soil composite sample, faring worse than *A. sativa* regarding the seeds germination, the stem elongation and the dry biomass production.

Finally, after exposure to the soil eluate the survival of the aquatic invertebrate species (*D. magna*) was not affected, while the growth of the unicellular algae species (*R. subcapitata*) was inhibited at a level of about 40%.

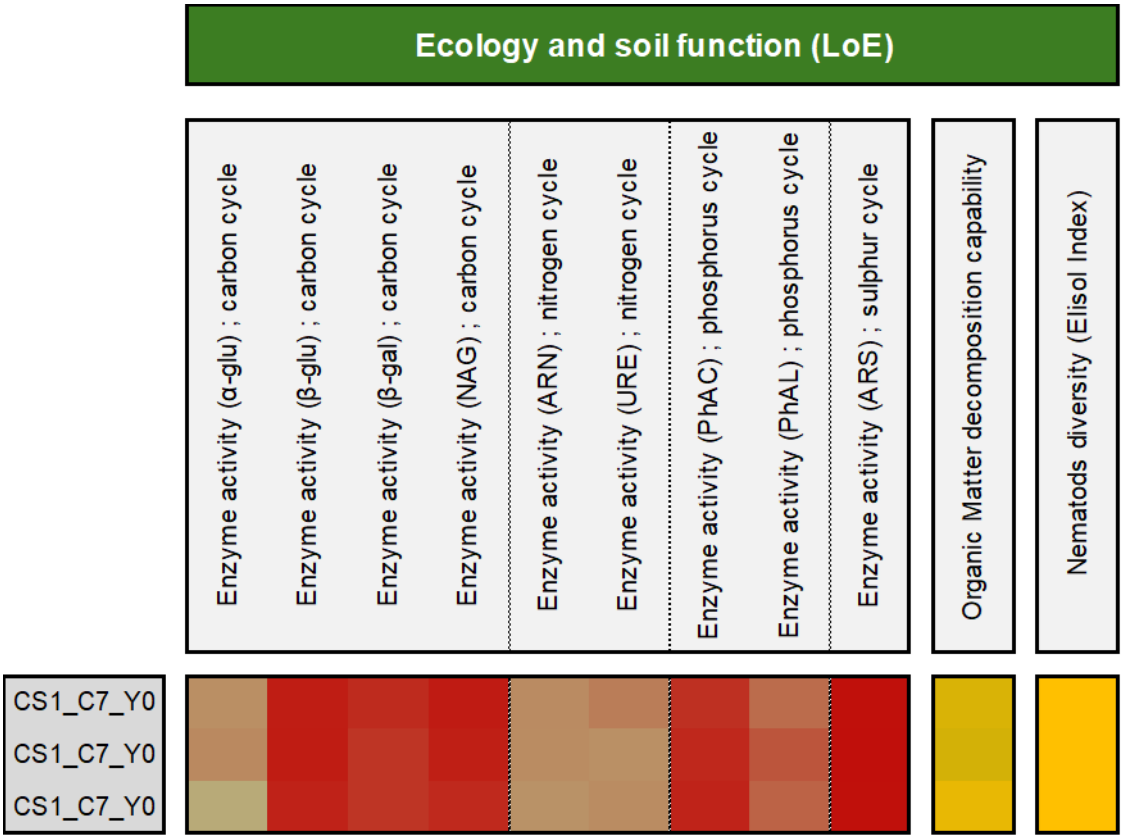

Figure 25 Summary results of ecology and soil function tests performed for CS7

Concerning the soil metabolisms, both enzyme activities and the organic matter decomposition capability indicator pointed out the highly degraded profile of the soil composite samples collected in the CS7. Whatever the enzymes considered, the activities measured were lower to the activities usually measured in uncontaminated soil. The results obtained for organic matter decomposition test pointed out the limited ability of this soil to degrade organic matter.

The nematofauna index calculated according to the analyses of the nematofauna density, diversity (Shannon index) and the structure of the trophic network is representative of a degraded soil environment.

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4.2 Ecotoxicity integrated index

To synthesize the results obtained with the battery of ecotoxicological tests, and to facilitate the interpretation of all these data, an integrated index was calculated based on the different approaches proposed in the literature (Hartwell 1997; Bombardier and Bermighan 1999; Vindimian et al. 1999; Ahlf and Heise 2005) and on the method published by Manzo et al. (2014). This method is a quantitative approach, that allow to consider several ecotoxicological descriptors, such as the type of endpoints, the severity if each endpoint, the relevance of the environmental matrices, and that integrates the overall toxicity results, coming from each tested matrix, into a synthetic toxicity index. This approach calculates the "ecotoxicity level" of an environmental matrix, weighting the kind of endpoint observed, the kind of environmental matrix analysed, and the accordance level among the test results. The toxicity test battery integrated index (TBI) is then calculated and compared to a specific scale proposed by Manzo et al. (2014). Figure 26 synthetizes the TBI index calculated for each soil composite samples from the different CS studied.

According to this approach, the first aspect worth highlighting is the consistence of the results obtained for the different soil composite samples. This consistency confirms the effectiveness and reliability of the methodology applied during the sampling and composite preparation stages. The closely aligned results obtained for the three composites from each CS demonstrate that the collected samples are reliable and can be individually considered representative of the entire analysed area, which can be crucial in case when it is necessary to limit the number of analysed samples, for example, due to high costs or time-consuming nature of the analyses.

Based on the cumulative toxicity value expressed as the TBI index, it can be concluded that any of the tested soil samples were representative of a soil with absence of ecotoxicological risk. The soil composite samples from CS1, CS4 and CS6 are categorised as soils with medium to high level of ecotoxicological risk while the soil from CS7 and CS3 are categorised as soil with high and very high level of ecotoxicological risk, respectively. Among the different CS, the soil composite samples from CS4 can be considered as the less ecotoxic ($14<TBI<16$) when the soil composite samples from CS3 were the most ecotoxic ($65>TBI>81$).

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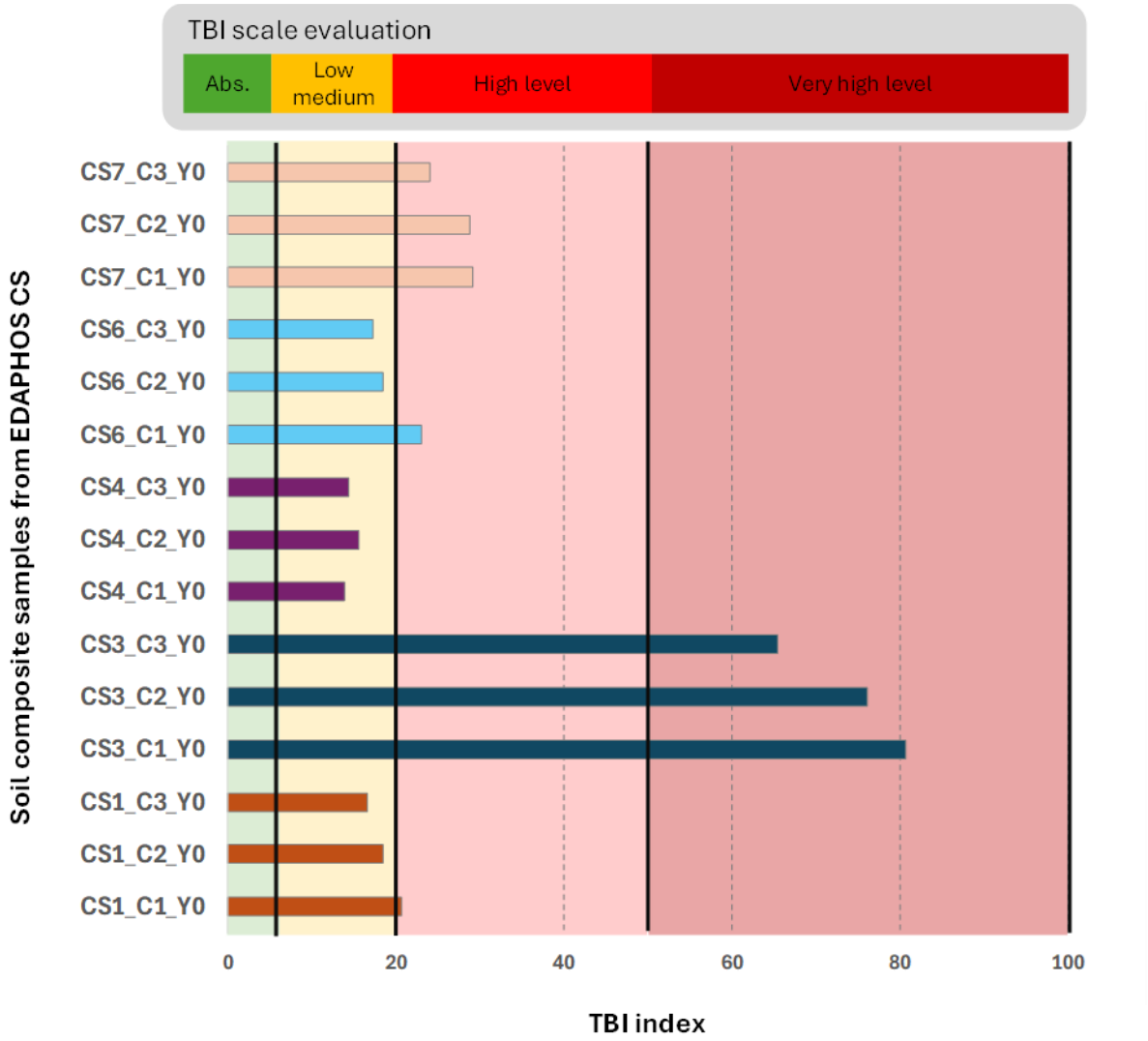

Figure 26 Toxicity Test Battery integrated Index (TBI) calculated based on the method proposed by Manzo et al. (2008, 2014). The scale indicates in green (TBI index < 5), the absence of ecotoxicological risk ; in yellow a low to medium ecotoxicological risk (5<TBI<20) ; in light red, a high level of ecotoxicological risk (20<TBI<50) and in dark red, a very high level of ecotoxicological risk (TBI>50).

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5 Ecosystem Services Evaluation

5.1 Methods and approaches for ecosystem service assessment

Ecosystem services and biodiversity are closely related to human well-being and planetary health, yet they are significantly affected by human activities, including changes in land use. Various methods have been developed to evaluate ESS. Assessing the market prices of ESS is the main way for assessing provisioning services that have a direct market value (such as timber, or food). This method relies on existing economic and market data and allows for the direct valuation of ESS in monetary value (Ghermandi, 2010). In the case of regulatory and cultural services, there is not always a direct reference to monetary value and other indirect evaluation methods have to be applied. The identification and assessment of the processes, which contribute to the ecosystem output is required. The most reliable source of data for regulating services is field survey measurements (e.g., measurement of biomass carbon content). Such methods require considerable time and usually cover small areas (local-scale evaluation). To use ESS evaluation in decision- and policy-making processes (e.g., spatial planning), needs to consider information regarding the relationship between ESS supply and demand (Syrbe, and Grunewald 2017. Klaus et 2024). This requires analysis on a larger spatial scale. Utilizes satellite or aerial imagery to assess land cover, vegetation health, and ecosystem conditions. This method can provide large-scale data on habitat extent, changes over time, and the distribution of services like carbon storage or temperature regulation (Schuster,et al., 2024, Sierka,, & Pierzchała, 2022). In the case of cultural services indirect valuation of benefits is used. For example, assessing how much people can spend money or time to visit the area with nature-based recreation value, reflecting the value of recreational opportunities provided by ecosystems (Vallecillo et al., 2019). In many cases using mathematical models and formulas to simulate or calculate ecosystem processes and predict the delivery of services under various ecosystem types is an effective way of ESS evaluating (Horea 2015).

Considering the diverse characteristics of different types of ESS and the need for evaluation on a scale beyond the boundaries of the analysed area (case study area) very often approaches integrate various data sources and methods to assess ESS has to be applied.

The purpose of this work is to develop a methodology for evaluating ESS for soil-contaminated sites in relation to the effectiveness of remediation activities and the selection of the most beneficial way of redevelopment.

5.2EES methodology adopted for EDAPHOS Project

The developed EES assessment approaches for the evaluation of NBS restoration actions, was developed based on the results from the RECOVER project. The potential to deliver ESS depends on land cover use, so the first element of the assessment is to conduct a baseline mapping of relevant ecosystem types. The spatial scope of the analysis covers area, where the planned restoration action has impact on ecosystem systems. The range of mapping should also identify demand for benefits deliver by ecosystems. For comparisons different strategies of contaminated sites remediation and rehabilitation action the scenario method was used. The suitable ESS indicators will be applied to proper quantification of ESS deliver by different redevelopment action. A detailed description of each step of developed ESS methodology is provided below.

1.Baseline mapping of relevant ecosystems

The first step of this task is determined the spatial scale and geographical coverage of areas where the planned activities may cause impact on economic environmental and social aspects. ESS mapping studies will be set on the administrative units that include all identified negative environmental impacts of contaminated sites under consideration. In the next step, CORINE land cover classes will be used to delineate different ecosystem types of land cover in the study areas.

2.Formulating alternative rehabilitation scenarios

Taking into account the recommendations for future planning as well as the need to decrease negative environmental impact and support improve socio-economic outcomes and to catalyse the development of new jobs, different types of land rehabilitation and ecosystem restoration actions will be proposed in order to generate different redevelopment scenarios.

3.Selection of suitable indicators

The aim of the step is to select the suitable indicators that will allow a proper quantification of every ESS involved on area impacted by in contaminated site. In addition, the indicators chosen should also allow the benefits of future land use to be identified.

The following criteria will be considered in order to select the most suitable indicators:

a. Stakeholders-relevant and meaningful: indicators should send a clear message and provide information at a level appropriate for management decision-making by assessing changes in the status of ESS.
b. Acceptance and intelligibility: the power of an indicator depends on its broad acceptance both by project partners and stakeholders
c. ESS-relevant: indicators should address key properties of ESS or related issues as pressures, state, impacts and responses.
d. Cause-effect relationship: information on cause-effect relationships should be achievable and quantifiable in order to link pressures, state and response indicators. These relationship models allow scenario analysis and represent the basis of the ecosystem approach.
e. Spatial coverage: indicators should ideally be relevant for area influenced by degraded site.
f. Country comparison: as far as possible, it should be possible to make valid comparisons between countries using the indicators selected.

4. Assessment of ESS provision for different rehabilitation scenarios

The use of the ESS approach allows to the detail exploring of consequences each of scenario taking into consideration benefits that could be generated by ecosystem.

The Common International Classification of Ecosystem Services (CICES, 2018) V5.1 will be used to make the assessment of the ESS of each case-study.

For each relevant land cover the three main section categories (provisioning services, regulating and maintenance services, and cultural services) will be considered and divided into main types of output or process.

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5.3 Preliminary results for selected CS

As part of preliminary analyses, baseline mapping of relevant ecosystems at the appropriate boundaries and formulating alternative rehabilitation scenarios for CS 4: Upper Silesian Coal Basin, Silesian Voivodship (PL) were carried out. Preliminary Catalogue of Indicators for ESS assessment was also set.

5.3.1 Baseline mapping of relevant ecosystems

The study area covers about 3,5 ha and is characterized by increased levels of contamination in the soil (In particular, As, Cd, Pb and Zn). Due to the shallow level of groundwater (1-2 m), this area may affect the quality of water within the first aquifer. It can also negatively affect the quality of the watercourse located in the immediate vicinity. Considering the area of the land and local conditions, it was assessed that the negative environmental impact of the area in question does not extend beyond the administrative boundaries of the Miasteczko Śląskie district.

For carrying out mapping of ecosystem within setting boundaries the European database of land use CORINE Land Cover (CLC). The CLC map was refined map based on current orthophotometric maps and UVA observations of terrain case study adjacent areas. Spatial analyses were performed in software 3.32.1-Lima. The result of the baseline mapping of relevant ecosystems is presented in the figure below.

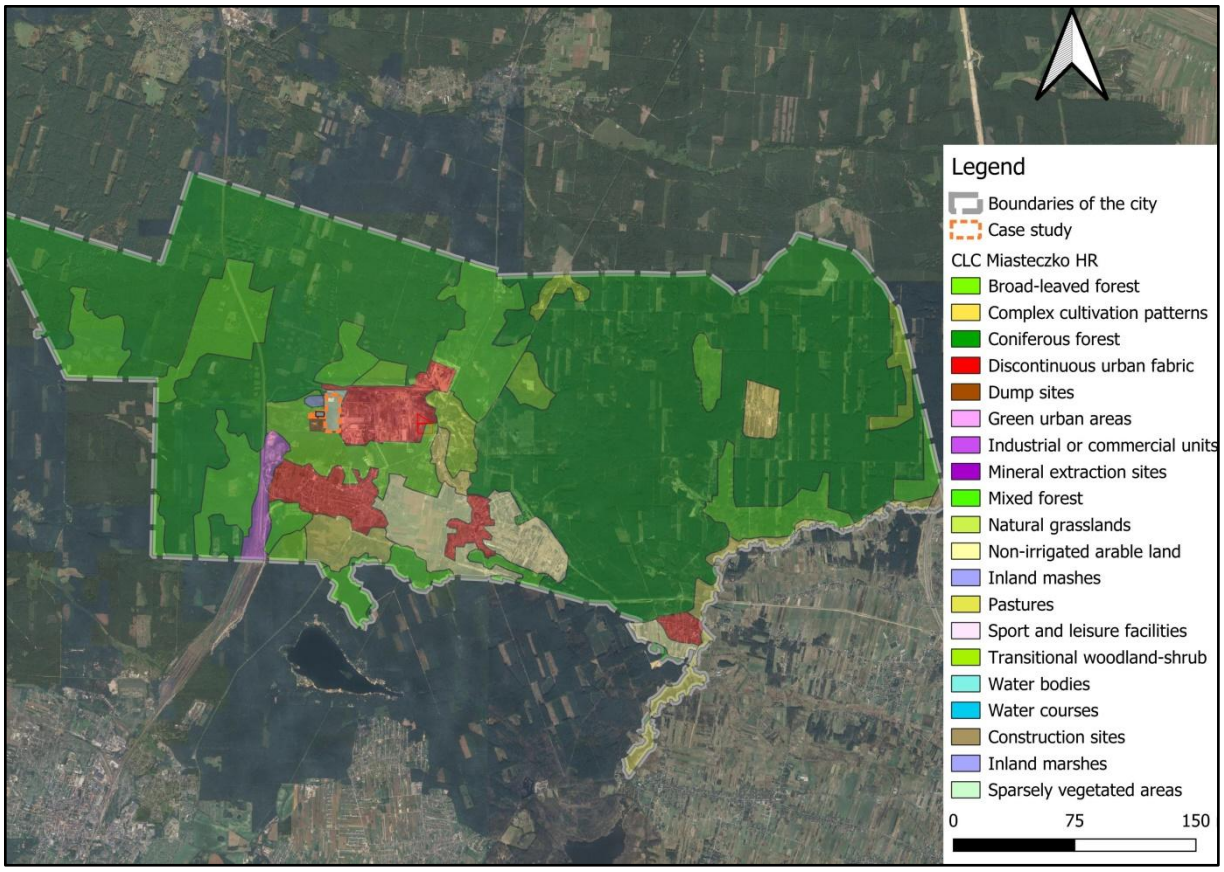

Figure 27 CLC classes in Miasteczko Śląskie district over the orthoimage of the area.

5.3.2 Formulating alternative rehabilitation scenarios

For the formulation of alternative development scenarios, the following information and aspects were taken into consideration:

- Current state: area covered by low vegetation, surrounded by forest, within 1km no residential area
- Spatial planning: area intended for renewable energy installations, production buildings and facilities
- Previous land cover: inland marshes, broad leaved forest
- Facilities harmful to the environment in close vicinity: Hazardous waste dump – containing Zn 2-5%, 5-20% Pb, Cd 5-15% and Tl. The landfill is isolated leachate water is treated in a treatment plant.
- Zinc and lead smelter: the significant emitter of CO_2 and pollutants to air significant impact on thermal emissivity
- Road accessibility: access by technical road
- Social aspects Unemployment in 2023 year was 4.4% % compared to 3.6% % in the region
- Surface water quality: Stream Woda Graniczna with exceeded limits for As, Cd, Pb, Zn and Tl.
- Soil contamination: exceedances of soil contamination limits in relation to land use are shown in the table below.

Table 9 Exceedances of contaminant limits in soil.

Contamination	Max value [mg/kg]	Threshold [mg/kg]	
		Industrial unit	Forest
As	176	<100	<50
Cd	92	<15	<10
Pb	1448	<600	<500
Zn	6528	<2000	<1000

Based on the above considerations, the following development scenarios were proposed:

I. Scenario I Photovoltaic panel farm.
Expected benefits: reduction in CO_2 emissions

II. Scenario II Industrial units.
Expected benefits: reduction in CO_2 emissions, increase economic growth, reducing the unemployment rate

III. Willow plantations
Expected benefits: improving soil, water and air quality, provision of biomass for energy purposes, temperature and flood regulating

The visuals for each scenario are presented in the figures below.

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Figure 28 Visualisation of scenario I - photovoltaic panel farm.

Figure 29 Visualisation of scenario II - Industral units.

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Figure 30 Visualisation of scenario III - Industral units.

Taking into account the identified negative impacts of the analyzed site and the expected benefits of the different development scenarios, the following and ESS indicators were selected:

- Air purification – ability of ecosystems to suspended dust reduction (particulate matter – PM10)
- Mitigation of climate change - CO_2 capture and storage.
- Local cooling –the ability of ecosystems to regulate temperature
- Flood regulation – decreasing of surface water runoff
- Cultural services – interactions with natural environment
- Mediation of solid/liquid wastes – waste storage capacity
- Solar energy production - potential for deliver electric power from solar power
- Biomass production for bioenergy or raw materials

5.4 Preliminary Catalogue of Indicators

The valuation of ESS that may be provided by (CS) areas following the remediation process constitutes a key focus of three work packages (WP2, WP4, and WP5) within the EDAPHOS project. To ensure methodological consistency and establish a unified framework for the assessment of ESS, a standardized set of indicators has been developed. This framework is based on a series of analyses conducted within Task 2.2 and Task T4.1. The selected indicators will be utilized in subsequent project activities to enhance the robustness and comparability of the ESS valuation process.

The table below presents a comprehensive set of indicators for the valuation of ESS, selected as part of Task 2.2 and Tasks T4.1 and T4.2, conducted respectively within WP 2 and WP4. These indicators have been carefully identified to ensure a robust and multidimensional assessment of ESS provision and their socio-environmental relevance. It should be noted that tasks related to ESS will be carried out in parallel within WP2, WP4, and WP5.

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Table 10 List of ESS indicators based on CICES V5.1

CICES V5.1 / 01.01.2018 -includes water							
Section	**Division**	**Group**	**Class**	**Code**	**Class type**	**V4.3 Equivalent**	**Code(4.3)**
Provisioning (Biotic)	Biomass	Cultivated terrestrial plants for nutrition, materials or energy	Fibres and other materials from cultivated plants, fungi, algae and bacteria for direct use or processing (excluding genetic materials)	1.1.1.2	*Material by amount, type, use, media (land, soil, freshwater, marine)*	*Fibres and other materials from plants, algae and animals for direct use or processing*	*1.2.1.1*
	Genetic material from all biota (including seed, spore or gamete production)	Genetic material from plants, algae or fungi	Seeds, spores and other plant materials collected for maintaining or establishing a population	1.2.1.1	*By species or varieties*	*Not recognised in V4.3*	*N/A*
Regulation & Maintenance (Biotic)	Transformation of biochemical or physical inputs to ecosystems	Mediation of wastes or toxic substances of anthropogenic origin by living processes	Bio-remediation by microorganisms, algae, plants, and animals	2.1.1.1	*By type of living system or by waste or subsistence type*	*Bio-remediation by micro-organisms, algae, plants, and animals*	*2.1.1.1*
	Transformation of biochemical or physical inputs to ecosystems	Mediation of wastes or toxic substances of anthropogenic origin by living processes	Filtration/sequestration/storage/accumulation by micro-organisms, algae, plants, and animals	2.1.1.2	*By type of living system, or by water or substance type*	*Filtration/sequestration/storage/accumulation by micro-organisms, algae, plants, and animals* *And* *Filtration/sequestration/storage/accumulation by ecosystems*	*2.1.1.2 & 2.1.2.1*
	Transformation of biochemical or physical inputs to ecosystems	Mediation of nuisances of anthropogenic origin	Visual screening	2.1.2.3	By type of living system	*Mediation of smell/noise/visual impacts*	*2.1.2.3*
	Regulation of physical, chemical, biological conditions	Regulation of soil quality	Decomposition and fixing processes and their effect on soil quality	2.2.4.2	*By amount/concentration and source*	*Decomposition and fixing processes*	*2.3.3.2*
	Regulation of physical, chemical, biological conditions	Atmospheric composition and conditions	Regulation of temperature and humidity, including ventilation and transpiration	2.2.6.2	*By contribution of type of living system to amount, concentration or climatic parameter*	*Micro and regional climate regulation &Ventilation and transpiration*	*2.3.5.2 & 2.2.3.2*

Cultural (Biotic)	Direct, in-situ and outdoor interactions with living systems that depend on presence in the environmental setting	Physical and experiential interactions with natural environment	Characteristics of living systems that enable activities promoting health, recuperation or enjoyment through passive or observational interactions	3.1.1.2	*By type of living system or environmental setting*	*Physical use of land-/seascapes in different environmental settings*	*3.1.1.2*
	Direct, in-situ and outdoor interactions with living systems that depend on presence in the environmental setting	Intellectual and representative interactions with natural environment	Characteristics of living systems that enable education and training	3.1.2.2	*By type of living system or environmental setting*	*Educational*	*3.1.2.2*

Table 11 List of ESS indicators based on CICES V5.1- abiotic extention.

CICES V5.1 01.01.2018 - Abiotic Extension (includes water)							
Section	**Division**	**Group**	**Class**	**Code**	**Class type**	**V4.3 Equivalent**	**Code (4.3)**
Provisioning (Abiotic)	Water	Ground water for used for nutrition, materials or energy	Ground water (and subsurface) used as a material (non-drinking purposes)	4.2.2.2	By amount & source	Ground water as source of energy	1.2.2.2
	Non-aqueous natural abiotic ecosystem outputs	Mineral substances used for nutrition, materials or energy	Mineral substances used for material purposes	4.3.1.2	Amount by type	Solid	N/A
	Non-aqueous natural abiotic ecosystem outputs	Non-mineral substances or ecosystem properties used for nutrition, materials or energy	Solar energy	4.3.2.4	Amount by type	Solar	N/A
Regulation & Maintenance (Abiotic)	Regulation of physical, chemical, biological conditions	Maintenance of physical, chemical, abiotic conditions	Maintenance and regulation by inorganic natural chemical and physical processes	5.2.2.1	Amount by type	Maintenance of physical, chemical, abiotic conditions	N/A

Further work on the valuation of ESS within Task 2.2 will focus on 5/6 indicators selected from the catalogue presented above, in particular, taking into account indicators with codes:

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- 1.1.1.2 – biomass production (provisioning)
- 1.2.1.2 – genetic material by species or varieties (provisioning)
- 2.1.1.1 – regulation of soil quality (regulation)
- 2.1.1.2 – mitigation of climate change/ carbon dioxide sequestration (regulation)
- 2.1.1.2 – air quality regulation (regulation)
- 2.1.2.3 – transformation of biochemical or physical inputs to ecosystem (regulation)
- 2.2.6.2 – temperature regulation (regulation)
- 3.1.1.2 – direct in-situ interactions (regulation)
- 3.1.2.2 – educational (culture)
- 4.3.2.4 – energy properties (provisioning)

These indicators have been chosen based on their ability to most accurately reflect the potential environmental benefits achievable under each of the three scenarios developed for each CS. This targeted selection ensures that the assessment effectively captures the key ecological and socio-economic outcomes associated with different remediation and land-use strategies (3 developed scenarios).

The remaining indicators, depending on data availability, will be utilized in subsequent research activities conducted within WP4 and WP5.

6. Conclusions and recommendations

6.1. Summary of key findings

The results presented in this deliverable (D2.2), derived from the initial phase of the study including ecotoxicological test as well as ecology and soil function analysis, provide key information for the baseline assessment of soil toxicity in the analysed areas prior to the commencement of remediation activities. These preliminary results provide an essential reference point, allowing for the evaluation of changes induced by remediation efforts and forming the foundation for subsequent stages of the project.

Based on the comprehensive analysis of all obtained results, the soil composites from CS3 (Odiel Basin Area) demonstrated the highest level of ecotoxicity. A comparably elevated toxicological response was observed in soil function and ecological assessment tests performed for the soil composites from CS7 (Lavrio). However, in contrast to CS3 (Odiel Basin Area), CS7 (Lavrio) exhibited a lower degree of toxicity towards the plant and animal organisms utilized in the biotest battery. A significant increase in the ecotoxic response was observed only in the case of CS7 in the reproduction test. The soil significantly affected the inhibition of the natural reproductive processes of the test organisms *(E. fetida*). Regarding the soil samples from the analysed areas CS4, CS6, and CS7, the highest ecotoxicological responses (inhibition levels) recorded across the full range of parameters measured in the phytotest (*A. sativa, S. alba*) did not exceed levels of 45 %, 12%, and 35%, respectively, for CS4, CS6, and CS7, while in the case of CS4 and CS6, the toxicity measured for most parameters did not exceed 10%. In contrast to the tests using higher plants, for those performed on *C. elegans* and *A. globiformis*, the obtained toxicity levels amounted to 61% (CS4), 25% (CS6), and 30% (CS7), which proves the high sensitivity of the applied species to the type of contaminants present in the analysed soil composites.

Based on the cumulative toxicity value expressed as the TBI index, the highest level of toxicity was observed in soil samples collected from CS3 (Odiel Basin Area) (Very high level). Additionally, a high level of toxicity was recorded for samples from CS7 (Lavrio). The findings from the conducted analyses allowed for the classification of soil samples from CS1 (Carrieres sous Poissy) CS4 (Upper Silesia Coal Basin) and CS6 (Vieux-Charmont) as a low to medium ecotoxic.

Regarding soil metabolism, both enzyme activity and the organic matter decomposition capability indicator performed worst for CS3 and CS7, indicating respectively a highly and significantly degraded profile of the soils. For these indicators, enzyme activities in the soil composites from the CS3 area were close to zero, regardless of the enzyme measured ; the results obtained from the OM decomposition test indicated the inability of this soil to degrade OM. Regarding soil metabolism, the enzyme activities measured in the composite soil samples collected from the CS4 and CS6 sites were representative of commonly occurring levels or slightly lower compared to uncontaminated areas. An alternative situation was observed for the soil composites from the CS1 area, where the measured enzyme activity levels were generally lower than the typically recorded enzymatic activities in uncontaminated soils. This was particularly evident for the activity of enzymes involved in the nitrogen cycle, some enzymes participating in the carbon cycle (NAG), and those related to the sulfur cycle.

The results obtained within Task 2.2 confirm that the anthropogenic contaminants present in the analysed areas (CSs) pose a significant risk to selected terrestrial species (tested terrestrial plant and animal organisms), as well as to aquatic environment representatives. This is particularly crucial in the

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context of the potential leaching of contaminants or their migration into groundwater and subsurface water. Given the European Union's strategic environmental protection priorities, including the *EU Soil Mission*, it is essential to implement effective measures to mitigate such risks. The preservation of natural habitats and the restoration of soil health are key objectives under the EU's environmental framework, emphasizing the need for sustainable land management practices and remediation strategies. Reducing contamination-related threats aligns with the broader European policies aimed at maintaining biodiversity, protecting ESS, and ensuring long-term soil resilience.

6.2. Recommendations for further project research

The results obtained within Task 2.2 will then be utilized for a comprehensive environmental risk assessment (ERA) based on the TRIAD methodology (Task 2.3). This integrative approach enables a robust and scientifically sound evaluation of actual environmental risks by synthesizing data from physico-chemical, ecotoxicological and ecological analyses. Additionally, it considers potential contaminant transport and migration pathways, as well as synergistic and antagonistic interactions between pollutants.

Given the complexity of contamination dynamics, this methodological framework ensures a more precise identification of ecological hazards and supports the formulation of effective remediation strategies. The significance of analyses conducted within Task 2.2 extends beyond site-specific assessments, as it contributes to the overarching objectives of the EDAPHOS project by providing a scientifically validated foundation for sustainable soil management and remediation planning.

Additionally, the methodology for ESS presented in this deliverable (D2.2.), initially tested for CS4, will be further adapted and implemented in the next phase of Task 2.2 to assess ESS for the remaining CS. This analysis will be based on a set of indicators, which has been included in this document as an open catalogue serving as a key outcome of Task 2.2, Task 4.1 and Task 4.2. In the subsequent stage of the project, scenario modelling will be conducted for the analysed CS, taking into account socio-economic, administrative, and environmental conditions. This process will explore potential land-use pathways for the studied sites, facilitating informed decision-making for their sustainable management and revitalization. For each CS, three potential land-use scenarios will be developed, within which ESS will be evaluated based on the indicators outlined in the presented catalogue. The final results of the ESS evaluation conducted for the analysed CS will be presented in the updated version of D2.2.

The evaluation of ESS plays a crucial role in soil restoration and the revitalization of degraded areas, as it provides a scientific basis for assessing the environmental and socio-economic benefits of various land management strategies. By integrating ESS evaluation into the decision-making framework, this approach enhances the effectiveness of remediation efforts (WP3) and supports the long-term sustainability of post-remediation land use.

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Attachment

Attachment 1 Photographic documentation of the germination and growth efficiency test of higher plants (*Avena sativa*) for CS1.

Sample	**CS1 - Carrières sous Poissy (FR)**		
Test species: *Avena sativa*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

Attachment 2 Photographic documentation of the germination and growth efficiency test of higher plants (*Avena sativa*) for CS3.

Sample	**CS3 - Odiel Basin Area (ESSP)**		
Test species: *Avena sativa*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

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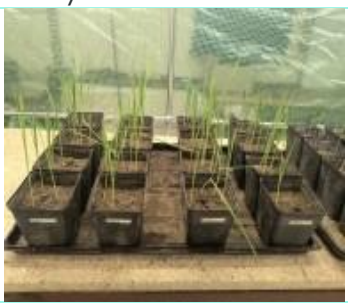
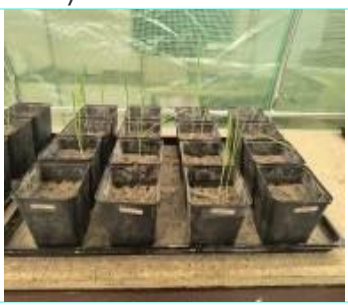
Attachment 3 Photographic documentation of the germination and growth efficiency test of higher plants (*Avena sativa*) for CS4.

Sample	**CS4 - Upper Silesian Coal Basin, Silesian Voivodeship (PL)**		
Test species: *Avena sativa*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

Attachment 4 Photographic documentation of the germination and growth efficiency test of higher plants (*Avena sativa*) for CS6.

Sample	**CS6 - Vieux-Charmont (FR)**		
Test species: *Avena sativa*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

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Attachment 5 Photographic documentation of the germination and growth efficiency test of higher plants (*Avena sativa*) for CS7.

Sample	**CS7 - Lavrio (GR)**		
Test species: *Avena sativa*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			
CONTROL	DAY1	DAY 7	DAY14
			

Attachment 6 Photographic documentation of the germination and growth efficiency test of higher plants (*Sinapis alba*) for CS1.

Sample	**CS1 - Carrières sous Poissy (FR)**		
Test species: *Sinapis alba*			
SERIE S I	DAY1	DAY 7	DAY14
			
SERIE S II	DAY1	DAY 7	DAY14
			
SERIE S III	DAY1	DAY 7	DAY14
			

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Attachment 7 Photographic documentation of the germination and growth efficiency test of higher plants (*Sinapis alba*) for CS3.

Sample	**CS3 - Odiel Basin Area (ESSP)**		
Test species: *Sinapis alba*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

Attachment 8 Documentation of the germination and growth efficiency test of higher plants (*Sinapis alba*) for CS4.

Sample	**CS4 - Upper Silesian Coal Basin, Silesian Voivodeship (PL)**		
Test species: *Sinapis alba*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			

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Attachment 9 Photographic documentation of the germination and growth efficiency test of higher plants (*Sinapis alba*) for CS6.

Sample	**CS6 - Vieux-Charmont (FR)**		
Test species: *Sinapis alba*			
SERIE S I	DAY1	DAY 7	DAY14
			
SERIE S II	DAY1	DAY 7	DAY14
			
SERIE S III	DAY1	DAY 7	DAY14
			

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Attachment 10 Photographic documentation of the germination and growth efficiency test of higher plants (*Sinapis alba*) for CS7.

Sample	**CS7 - Lavrio (GR)**		
Test species: *Sinapis alba*			
SERIES I	DAY1	DAY 7	DAY14
			
SERIES II	DAY1	DAY 7	DAY14
			
SERIES III	DAY1	DAY 7	DAY14
			
CONTROL	DAY1	DAY 7	DAY14
			

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Attachment 11 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for CS1.

Sample	CS1 -	
Test species: Earthworms (*Eisenia fetida*)		
SERIES I	DAY 28	DAY 60
	 	
SERIES II	DAY 28	DAY 60
	 	
SERIES III	DAY 28	DAY 60
	 	
SERIES IV	DAY 28	DAY 60
	 	

Attachment 12 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for CS3.

Sample	**CS3 - Odiel Basin Area (ESSP)**		
Test species: Earthworms (*Eisenia fetida*)			
SERIES I	DAY 28		
	None	*Lack of cocoons*	*None*
SERIES II	DAY 28		
	None	*Lack of cocoons*	*None*
SERIES III	DAY 28		
	None	*Lack of cocoons*	*None*
SERIES IV	DAY 28		
		*Lack of cocoons*	

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Attachment 13 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for CS4.

Sample	**CS4 - Upper Silesian Coal Basin, Silesian Voivodeship (PL)**	
Test species: Earthworms (*Eisenia fetida*)		
SERIES I	DAY 28	DAY 60
		 
SERIES II	DAY 28	DAY 60
		 
SERIES III	DAY 28	DAY 60
		 
SERIES IV	DAY 28	DAY 60
		 

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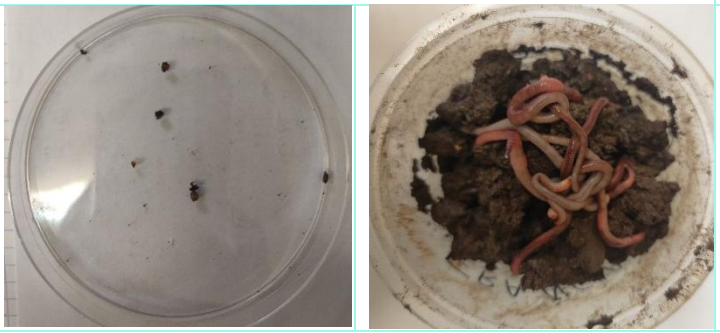
Attachment 14 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for CS6.

Sample	**CS6 - Vieux-Charmont (FR)**	
Test species: Earthworms (*Eisenia fetida*)		
SERIES I	DAY 28	DAY 60
		
SERIES II	DAY 28	DAY 60
		
SERIES III	DAY 28	DAY 60
		
SERIES IV	DAY 28	DAY 60
		

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Attachment 15 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for CS7.

Sample	CS7 - Lavrio (GR)		
Test species: Earthworms (*Eisenia fetida*)			
SERIES I	DAY 28		
		Lack of cocoons	
SERIES II	DAY 28		
		Lack f cocoons	
SERIES III	DAY 28		
		Lack of cocoons	
SERIES IV	DAY 28		
		Lack of cocoons	

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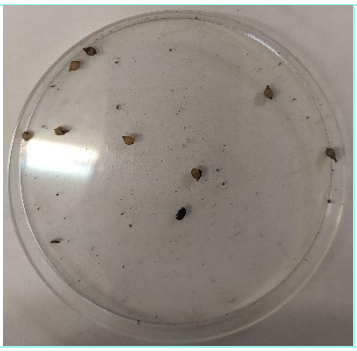
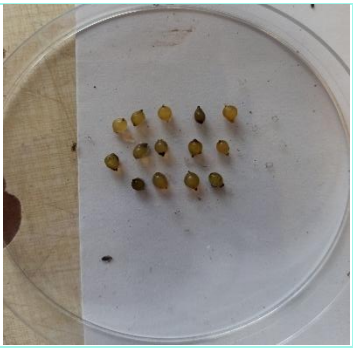
Attachment 16 Photographic documentation of the earthworm reproduction test (*Eisenia fetida*) for control.soil.

Sample	Control soil		
Test species: Earthworms (*Eisenia fetida*)			
SERIES I	DAY 28		DAY 60
			
SERIES II	DAY 28		
			
SERIES III	DAY 28		
			
SERIES IV	DAY 28