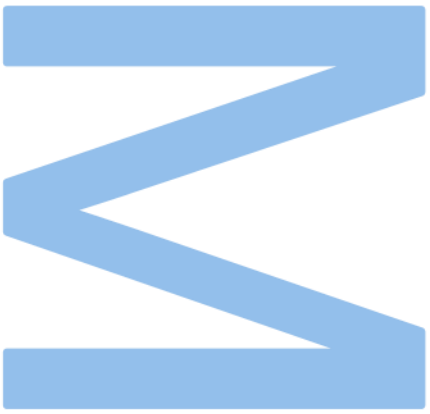
Study of the effectiveness of two natural amendments in mitigating the availability of Cu in agricultural viticulture soil

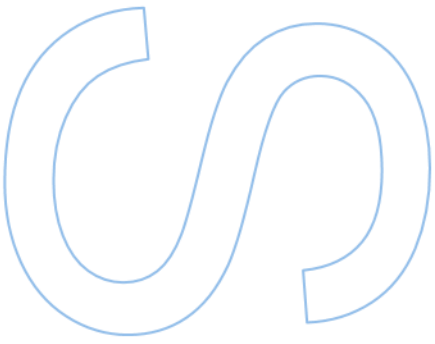
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Resumo

Durante muitos anos, a utilização de fungicidas à base de cobre na videira foi recorrente para o tratamento e controlo do míldio. Este facto resultou numa preocupação emergente com a contaminação do solo por este elemento e, consequentemente, com a contaminação dos ecossistemas adjacentes, o que levou à necessidade de estudar técnicas de remediação de solos contaminados. Devido à variabilidade dos componentes do solo, a remediação nem sempre é eficiente, o que cria uma questão sobre o que pode ser implementado para tornar o solo menos vulnerável à contaminação por cobre.

Este estudo investigou a eficácia de dois corretivos naturais, a casca de pinheiro e a concha de mexilhão, na mitigação da disponibilidade de cobre em solos de viticultura. Para o efeito, foi selecionado solo de uma exploração vitivinícola, a Quinta de Azevedo, em Barcelos, caracterizado pela equipa em estudos anteriores, e por ter sido considerado um solo com elevada vulnerabilidade à contaminação com Cu. O solo foi recolhido, seco e peneirado (<4 mm) e depois incubado com cobre através da mistura bordalesa durante 4 semanas. Após este período, foram adicionados os corretivos, casca de pinheiro e concha de mexilhão, ambos triturados. A quantidade utilizada de cada corretivo para cada ensaio foi de 3 g/kg de solo, com um período adicional de incubação de 4 semanas, com o objetivo de comparar a eficácia de cada material no sequestro de Cu, uma vez que estudos anteriores demonstraram que ambos têm potencial para reduzir a mobilidade deste elemento no solo.

Os parâmetros de pH, condutividade, matéria orgânica e teor de água só foram medidos apenas nas réplicas de controlo sem cobre para avaliar o efeito dos corretivos nas propriedades do solo. Em seguida, foram efetuados três testes ecotoxicológicos, o ensaio de reprodução com oligoquetas da espécie *Eisenia fetida*, o ensaio de crescimento de plantas utilizando a dicotiledónea *Medicago sativa* e o teste de crescimento com *Lemna minor*, uma planta aquática, usado para avaliar a função de retenção do solo. Com estes ensaios, a eficácia dos corretivos na imobilização do Cu no solo foi medida para perceber se os mesmos reduziam a biodisponibilidade e mobilização do Cu para a fase aquosa do solo.

Esta dissertação explora o impacto das conchas de mexilhão e da casca de pinheiro nas propriedades do solo. A concha de mexilhão aumenta efetivamente o pH^{KCl} do solo,

o que demonstra potencial para a correção de solos alcalinos, e a casca de pinheiro induz um pH ácido, com um impacto mínimo na matéria orgânica. Embora a casca de pinheiro diminua a condutividade elétrica, não influencia significativamente a capacidade de retenção de água. Em ensaios ecotoxicológicos, ambos mostram um impacto limitado na toxicidade induzida pelo cobre em organismos aquáticos. Relativamente aos organismos terrestres, a concha de mexilhão reduz o impacto do cobre nas oligoquetas, enquanto ambas as alterações atenuam a toxicidade do cobre na *Medicago sativa*. Os efeitos diferenciados observados sugerem a importância de considerar a composição, a concentração e a interação dos corretivos com as amostras ao longo do tempo. Este estudo sublinha os potenciais benefícios da combinação de vários aditivos para melhorar a remediação do solo, incentivando a uma maior exploração na otimização das estratégias de remediação.

Palavras-chave: contaminação com cobre, remedição de solo, casca de pinheiro, casca de mexilhão

Abstract

For many years, the use of copper-based fungicides was quite recurrent in grapevines for the treatment and control of downy mildew. This resulted in an emerging concern about soil contamination by metals throughout the application of the fungicide to the vines and, consequently, contamination of the adjacent ecosystems, raising the need to study remediation techniques for contaminated soils. Due to the variability of soil components, remediation is not always efficient, thus a more detailed evaluation is needed to assess materials and methods that can be implemented to make the soil less vulnerable to copper contamination.

This study investigates the effectiveness of two natural amendments, pine bark and mussel shell, in mitigating the availability of copper in agricultural viticulture soil.

For this purpose, it was selected a soil from a viticulture farm, Quinta de Azevedo in Barcelos. The soil was collected, dried and sieved (<4 mm) and then incubated with copper via Bordeaux mixture for 4 weeks. After incubation crushed pine bark and mussel shell were added. The amount used of each material was 3 g/kgsoil, followed by an incubation period of 4 weeks, with the objective of comparing the effectiveness of each amendment in the sequestration of Cu, as previous studies have shown their potential for reducing copper mobility.

The parameters of pH, conductivity, organic matter content and water content were only measured in the control without copper to check for the effects of the addition of the amendments on soil properties.

Then, three ecotoxicological tests were carried out, the reproduction test of oligochaetes with the species *Eisenia fetida*, the plant emergence and growth test using the dicotyledonous species *Medicago sativa* and the growth inhibition test with the species *Lemna minor*, an aquatic plant, to assess the soil retention function. These tests gather the efficacy of the amendments to reduce the bioavailability and mobility of copper to the aqueous phase of the soil.

This dissertation explores the impact of mussel Shells and pine Bark amendments on soil properties. The mussel shell effectively increases soil pH^{KCl}, showcasing potential for alkaline soil correction and the pine bark induces acidic pH, with a minimal impact on organic matter. While pine bark decreases electrical conductivity, it does not significantly influence water holding capacity. In ecotoxicological assays, both show limited impact

on copper-induced toxicity in aquatic organisms. For terrestrial organisms, mussel shell reduces copper impact on earthworms, while both amendments mitigate copper toxicity in *Medicago sativa*. The nuanced effects observed highlight the importance of considering amendments' composition, concentration, and their interaction over time. This study underscores the potential benefits of combining multiple amendments for enhanced soil remediation, paving the way for further exploration in optimizing remediation strategies.

Keywords: copper contamination , soil remediation, pine bark, mussel shell

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1. Introduction

The over-exploitation of the Earth's resources, directly related to the growth of the world population and the efforts to sustain it, has been a global and growing concern over the last few years. There has been a need to recognize our impact on the environment and its natural resources and find ways to reduce it, in order to achieve a balance between the persistence of earth resources and our needs. In this sense, in the eighties, the World Commission on Environment and Development from the United Nations, proposed a global agenda, the Brundtland Report - "Our Common Future," to propose a "call for action" by defining long term environmental strategies to achieve certain goals on sustainable development. The definition of sustainable development was defined as "the development capable of meeting the needs of the present generation without compromising the ability to meet the needs of future generations" (United Nations, 1987). From the report, the first universal Agenda for Sustainable Development was born, with 17 objectives structured around five major pillars of critical importance: people, planet, partnerships, peace and prosperity. The aim was to stand for all those that are influenced or are influential on sustainable development to work together towards a collective goal by the year 2030. (United Nations, 2015a). In line with the goals, several strategies have appeared, one of which is the European Green Deal, a comprehensive action plan presented by the European Commission to make the European Union (EU) climate-neutral by 2050 and promote sustainable growth. One of the key components of the European Green Deal is the commitment to protect and restore the environment, including soil, as it plays a crucial role in supporting ecosystems and agriculture. Actions toward soil conservation and improvement in the European Green Deal include (Commission on Environment, 1987):

- Zero Pollution Action: Striving to reduce soil contamination and minimize the use of hazardous chemicals;
- Biodiversity Strategy: Halting biodiversity loss and restoring ecosystems, including soil;
- Farm to Fork Strategy: Promoting sustainable agriculture, reducing pesticide and fertilizer use, and supporting organic farming;
- Circular Economy Action: Encouraging recycling and reducing waste to limit land degradation;

- European Soil Partnership: Collaborating with the Global Soil Partnership to combat soil degradation;
- Sustainable Agriculture: Reforming the Common Agricultural Policy (CAP) to focus on sustainable land and soil management;
- Land Use Planning: Integrating land use and spatial planning to reduce soil sealing and protect agricultural land;

These actions collectively aim to preserve and enhance soil quality for sustainable agriculture and ecosystems within the EU.

Soil, the "*top layer of the Earth's crust, formed by mineral particles, organic matter, water, air and living organisms"*, is "*the interface between earth, air and water, and hosts most of the biosphere*" (COMMITTEE AND THE COMMITTEE OF THE REGIONS, 2006) and the base for supporting livelihoods. It is considered to be a non-renewable resource due to the fact that is not recoverable within the human lifespan, or even during the human species existence. The soil is responsible for various ecological and socio-economic functions and services for humanity (FAO, 2002) such as:

- Provision of food and fiber: FAO reports write that 38% of the global land surface is occupied by agricultural settings, one-third of that being used as cropland while the remaining is meadows and pastures. As the population grows, the cropland area per capita is decreasing and being over explored resulting in its degradation.
- Support of forest: The forest is the main producer of several raw materials needed by industry, such as wood and pharmaceutical ingredients.
- Provision of water: Soil regulates the hydrological cycle, slowing surface runoff in periods of high rainfall and allowing percolation and storage and the recharge of aquifers and other watercourses.
- Climate regulation: The soil conducts various gas exchanges with the atmosphere, including oxygen, nitrogen, and carbon. It regulates the biogeochemical cycles of these elements and allows a healthy balance between the pedosphere and the atmosphere. In addition, the soil is one of the largest reservoirs of carbon on the planet.
- Support of infrastructures: The soil is the primary element when it comes to the construction of buildings, highways, routes and other infrastructures, necessary for human activities including recreational and cultural ones.

- Support of biodiversity: Soil serves as a habitat, food source, nesting and shelter for a great diversity of species from microorganisms to invertebrates, vertebrates and plants, both below ground and above ground.

In order to achieve the proper functioning of the ecosystem and allow the soil to support its natural and ideal properties like described before, it must have quality and present health. Soil health is a critical concept that reflects the overall condition of a specific soil, and it is closely linked to the soil's capacity to perform a range of vital functions that support life, ecosystems, agriculture, and human activities. By maintaining and enhancing soil health, we can ensure the sustainability of these functions, promote environmental well-being, and meet the growing demands of a global population like the definition explains as "*to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation*" (Doran & Zeiss, 2000).

Europe is now estimated to have 60-70% of its soil in an unhealthy state, incapable of supplying any type of service, resulting in economic losses of around 50 billion euros per year and ecosystem losses that put the well-being of millions of citizens at risk (Karlen et al., 1997a). The soil is exposed to various threats that jeopardize its functioning, being the main causes for the soil degradation process the following (Stolte et al., 2016):

- Soil sealing can be due to the permanent covering of land and its soil with impermeable artificial material, such as asphalt and concrete, results in a disruption of the hydrological cycle, gases exchanges and carbon sequestration. Urban sprawl is the main human driving force for sealing but has also a significant role on land use changes, taking soil from being used for other purposes.
- Soil erosion is the displacement of topsoil from land surface through water, wind, or tillage. Erosion is mainly caused by inappropriate land use or management practices, which leaves the soil increasingly exposed and vulnerable.
- Bad agricultural practices that may account for soils infertility due to the loss of organic matter, nutrients needed by crops, loss of the biodiversity of the soil and even contamination by the use of fungicides, insecticides, and fertilizers.
- Soil contamination, i.e., the occurrence of pollutants of natural or anthropic origin in the soil above a certain level that causes the deterioration of soil functions (ESDAC, 2023). Contamination can be point, linear or diffuse and the sources can be the most diverse, from the application of products in agriculture and livestock, to mining, industrial discharges, road traffic, etc. Contaminants are also diverse, metals, mineral oils, organic compounds, aromatic hydrocarbons, etc,

which makes the recovery and treatment of each soil even more difficult. Contaminated sites are hazardous for terrestrial and aquatic ecosystems and can have implications for human health through direct exposure, ingestion, and inhalation, depending on the contaminant (Panagos et al., 2013). In Europe, the most represented group of contaminants on soil and groundwater are the metals being 37.3% of all possible contaminants (European Environment Agency, 2009). The primary sources of metals can have various origins such as more natural ones like the natural weathering of rocks and minerals and volcanic activity but also, human induce ones, with industrial activities, landfills sites and agricultural practices.

Finding the ideal soil condition requires the necessary data and appropriate indicators for the current state of the soil and its future use. Thus, in order to assess the health status of a given soil, several indicators can be used, whether physical, chemical, or biological (Doran & Zeiss, 2000). Indicators are proprieties sensitive to disturbances and allow to determine the quality and health of each soil, and if they are performing their functions correctly, meaning that the ideal properties vary with the final purpose of the soil, like agriculture, pasture, construction sites, etc. As described by Doran & Parkin, the indicators must integrate the physical, chemical and biological proprieties of the soil, be accessible to multiple users and integrated in an existing database, easy to understand and sensitive to the soil management (Doran & Parkin, 2015).

The physical and chemical indicators include parameters such as color, texture, structure, density, pH, content of organic matter and conductivity. The biological parameters can be measured with direct studies, which evaluate the activity from various organisms, their behavior and distribution when subjected to different conditions (Karlen & Stott, 1994). They provide insights into the activity and diversity of soil organisms, which play a crucial role in nutrient cycling, organic matter decomposition and overall soil quality. Some important biological indicators are soil enzyme activity, microbial diversity and respiration, and earthworm abundance (Martinez & Ortega-Blu, 2010):

While these indicators play a crucial role in assessing soil health, they have certain limitations. They may not provide a comprehensive understanding of the full extent of soil degradation or contamination. Therefore, in-depth ecotoxicological testing becomes necessary. Ecotoxicological testing goes beyond the scope of these indicators by examining the toxic effects of contaminants on soil organisms and the potential risks they pose to ecosystems and human health. These tests evaluate the bioavailability of

contaminants, considering factors like exposure time, transfers between soil and organisms, bioaccumulation, and specific contaminant-organism interactions.

Ecotoxicology, as an interdisciplinary science, focuses on studying the intricate interactions between environmental chemicals and biota (Fent, 2004). This field examines critical factors such as the bioavailability of chemicals and their potential toxicity to various organisms and becomes significant in cases where phytopharmaceutical products are applied to combat specific issues, such as fungi and microorganisms causing plant diseases. While these products aim to address these concerns, they can inadvertently introduce toxic effects into soil and aquatic ecosystems by leaching contaminants into aquatic systems.

In response to the potential unintended consequences of such products, ecotoxicological tests are carried out. These tests encompass various categories, including those targeting aqueous soil elutriates and indirect tests, as well as terrestrial tests specifically designed to evaluate the entire soil matrix (Wilke et al., 2008). Both aqueous and terrestrial fractions are considered in these assays, as contaminants, due to their bioavailability and mobility, not only affect the soil solution but can also be transported to water resources, subsequently impacting aquatic organisms. This comprehensive approach ensures a thorough assessment of the potential risks posed by these substances on both soil and water ecosystems.

To ensure consistency and validity in the results of ecotoxicological tests, standardization is of paramount importance (Römbke, 2018). Several organizations provide protocols for conducting these tests, with ISO - International Organization for Standardization - and OECD - Organization for Economic Co-operation and Development - being two prominent entities in this regard. ISO, a non-governmental organization with 169 member countries, is dedicated to the development and dissemination of international standards, which offer practical guidance on agreed-upon methods and solutions for specific objectives. Similarly, the OECD focuses on the establishment of evidence-based international standards, fostering a common framework for conducting ecotoxicological assessments. This standardization helps researchers and environmental experts compare and validate the results, contributing to a more comprehensive understanding of the potential ecological risks posed by various substances in both soil and water ecosystems.

Terrestrial ecotoxicological assays require the involvement of various soil organisms. The soil comprises several microhabitats with microorganisms, plants, invertebrates, and

some vertebrates, making it essential to understand how these organisms will be affected by the tests being conducted (Daam et al., 2019). Distinct species exhibit varying levels of sensitivity to the same elements, with some being more tolerant than others. The trophic level also needs to be taken into account, as some species depend on others, which can create a cascading effect in the ecosystem (Posthuma et al., 2002). This diversity of organisms and trophic interactions is vital to comprehensively assess the potential ecological impacts of substances on soil and water ecosystems.

Various soil organisms can be utilized in ecotoxicological tests, as recommended by ISO. These organisms encompass:

- Soil Microorganisms: These tests focus on processes like nitrogen mineralization and nitrification. They also involve determining microbial biomass and assessing enzymatic activity, such as dehydrogenase activity.
- Soil Mesofauna: Tests involve evaluating the reproduction of soil-dwelling organisms like *Enchytraeus albidus* and *Folsomia candida*.
- Soil Macrofauna: These tests assess parameters like mortality, reproduction, and avoidance behavior in organisms like *Eisenia fetida*, which is commonly known as the red wiggler worm.
- Plants: Ecotoxicological assessments include the examination of plant emergence and growth, specifically focusing on both mono- and dicotyledonous plants.

Using this array of organisms allows researchers to comprehensively evaluate the potential ecological impacts of various substances on soil and water ecosystems.

One of the advantages of ecotoxicological tests is that they are able to respond to the bioavailability of contaminants. We can define bioavailability as the measure of the amount of a given contaminant present in soil that can be absorbed or metabolized by humans or other ecological receptors, or that is available to interact with biological systems (Doble & Kruthiventi, 2007). Thus, bioavailability is not only related with the concentration of a given contaminant, but it depends also on the organism under study and the specific contaminant. In addition, external factors such as exposure time, transfers between the soil and the organism, bioaccumulation, soil characteristics have to be considered (Avaliação da biodisponibilidade de Metais, 2012).

However, there are some limitations related to the testing with ecotoxicological assays:

- Real-world relevance: these tests are typically conducted in a controlled laboratory environment, which does not fully represent the complexity and variability of the natural world (Forbes et al., 2011);
- Limited Species Focus: Ecotoxicological tests often concentrate on a restricted number of species and may not account for potential impacts on entire ecosystems (Noyes et al., 2009);
- Short Duration or Acute Tests: Acute tests, which are short-term assessments, may not detect long-term effects (Ankley et al., 2010);
- Assessment of Individual Substances: These tests usually evaluate individual substances, whereas organisms in the real environment may be exposed to multiple contaminants. In such cases, chemical methods may be necessary to assess the available fraction of specific contaminants for particular receptors or their mobility in the soil (Xu et al., 2015).
- Sensitivity to Physical and Chemical Parameters: The sensitivity of test organisms to various physical and chemical factors can vary (Brock et al., 2000);
- Difficulty in Extrapolation: Extrapolating the results from laboratory tests to real-world field conditions can be challenging (Korkaric et al., 2019);
- Lack of Data for Certain Contaminants: Data may be insufficient for assessing certain contaminants (Boxall et al., 2012);

However, even with the limitations, these tests not only allow the assessment of the quality, contamination status and health of the soil but also build the basis of the action plan in terms of recovering the ecosystem.

1.1. Soil remediation

As already mentioned, soil is a non-renewable resource and is under various threats, and various soil remediation, restoration and rehabilitation techniques have started to be developed, with the most proper technique being applied to each scenario of soil degradation (United Nations, 2015b).

- Remediation

This technique refers to the removal or isolation of contaminants in the ecosystem. It aims to mitigate the risks associated with humans and the environment and includes several in-situ techniques and ex-situ techniques (Deuren et al., 2002):

  - Engineering-based - they stand out for being mostly faster, however they imply a higher cost. They involve soil transportation, soil washing, air injection and

extraction of vapors, chemical oxidation, adsorption by activated carbon, membrane processes, electrokinetic, soil burial and sealing, among others.

- Biological - are more flexible and adaptable methods to site conditions, some examples include natural attenuation, bio ventilation, land farming, phytoremediation, bioremediation, and composting. Biological methods could be complemented with soil amendment with different organic materials or substances aimed at reducing the bioavailability of contaminants and supporting the performance of the biological agents (Park et al., 2011). When immobilized, the metals are not available to the soil biota. Although this reduces the risk of the contaminants entering food chains by plant uptake, or of being mobilized by leaching or soil erosion, this also compromises the success of phytoextraction by plants. Organic amendments can be considered in order to influence the soil regarding the main goal. This can include contaminant degradation, boosting microbial activity, binding and immobilizing the contaminant or even increasing it so that it can be up taken by plants.

- Restoration

Restoration is the process of returning the ecosystem to an approximation of the original conditions before any physical or chemical disturbance. It is based on the ecological recovery of the site.

- Rehabilitation

Rehabilitation aims at recreating ecosystem processes and services but does not imply ecological and biodiversity restoration, i.e., it only makes the site functional for at least one type of soil use.

2. Viticulture in Portugal

Portugal is located in the extreme southwest of the Iberian Peninsula, bordering Spain, and the Atlantic Ocean, combines the ideal climatic, geographic and mesological characteristics so that it can be a country with a lot of economic use of agricultural resources. These factors support the production of ingredients that are part of an admirable diet, the Mediterranean diet, which was classified as Intangible Cultural Heritage of Humanity of 2013 (UNESCO, 2013).

The northern region of Portugal exhibits distinctive edaphoclimatic conditions that significantly influence agriculture and viticulture. This region features a unique blend of a maritime and Mediterranean climate. The coastal areas, such as Minho, experience a

maritime influence with milder temperatures and consistent rainfall throughout the year, creating fertile soils. Portugal has an associated viticultural heritage, with the maintenance and production of vines as a business capable of producing a large economic flow to the country. In the latest statistics published by the Institute of Vine and Wine, in the period of 2022/2023, the amount of wine produced in the whole country was around 6.8 million hectoliters (Instituto da Vinha e do Vinho, 2023).

2.1. Downy mildew in viticulture

One of the main causes of grapes losses is downy mildew, an oomycete introduced by maritime explorations in the 19th century, when the specie was imported to Europe, with rootstocks of American vines, that caused the invasive pathogen to spread across all Europe, reaching almost every wine producing country worldwide, making it the most damaging disease of grapevine (Fontaine et al., 2021). *Plasmopara viticola*, the grape downy mildew pathogen, has a lifecycle that starts with overwintering in infected grape leaves on the ground. In spring, oospores within these leaves germinate to form sporangia, which, when wet, release zoospores that can infect healthy grapevine leaves. The pathogen grows within the vine tissue, causing symptoms of downy mildew. Secondary infections may occur, and the disease can also affect grape clusters. Toward the end of the growing season, the pathogen produces oospores, completing the lifecycle (Burruano, 2000).

The symptoms are characterized by translucent spots on the upper side of the leaf at an early stage, after sporulation of the fungus on the lower side in the form of whitish spots. *P. viticola* takes away nutrients from the plant which results in the leaves drying out and falling off. In a more advanced stage, it can cause brown spots that compresses the berries and turn the grapes brown, thus making the harvest unproductive and unsuitable for sale (Fontaine et al., 2021).

For many centuries, the solution to problems associated with pests and diseases was the removal of infected plants, however, with technological advances, other solutions began to be developed. As viticulture has a high economic relevance in several countries, the use of fungicides has been the immediate solution used by farmers to prevent the infection of their crops. With the worsening and deregulation of climate conditions worldwide, plantations are more vulnerable to pathogenic agents that cause economic and environmental losses and therefore it is necessary to use more phytopharmaceutical products which may lead to the chemical and biological imbalance of the soil and its biota.

The most used plant protection product (PPP) to combat downy mildew is the Bordeaux mixture, which is classified as a fungicide, as it is capable of inhibiting the growth of fungi and oomycetes and it is used as both protective/preventive and as a curative agent. The use of this product dates back to the 19th century, where it was discovered in the Bordeaux region (France) by Pierre-Marie-Alexis Millardet. The professor of botany studied the effect of the mixture of copper sulphate and water because he found that it was able to control the growth of mildew, thus suggesting that it could have fungicidal properties. After 3 years of testing and experimenting, the botanic professor published his results in a journal of agricultural practices and thus the Bordeaux mixture became known as the first fungicide to have a global scale of use. (Editors of Encyclopedia, 2019). The market for products against mildew grew significantly and currently there are multiple fungicides with different active substances, however there is no alternative to copper in the context of organic viticulture.

2.2. Copper

Copper, symbol Cu with atomic number 29, is a transition metal. Cu is characterized by its high density, excellent electrical and thermal conductivity, and remarkable malleability and ductility. It melts at around 1,984 degrees Celsius and boils at approximately 2,562 degrees Celsius. Cu finds wide applications in electrical wiring, plumbing, alloys, and various industrial processes.

Cu occurs naturally in plants and animals as a micronutrient, which is essential for their proper functioning and overall well-being. This micronutrient is vital for the health of both plant and soil ecosystems. Within the realm of living organisms, Cu is incorporated into numerous organic molecules, such as proteins and enzymes, which are indispensable for various physiological processes. One of the central roles of Cu in living beings is its active involvement in essential metabolic functions. It participates in controlling the synthesis of DNA and RNA, the fundamental building blocks of life. Furthermore, Cu is a key player in critical biological processes, including photosynthesis, the process by which plants convert light energy into chemical energy, and plant respiration, which is essential for energy production (Kabata-Pendias, 2010).

When its present in excess, Cu alters physiological and chemical processes of plants, resulting in damage at the root level and inhibition of nutrient absorption. It also creates imbalance in soil biogeochemical cycle (US Department of Health and Human Services, 2022):

- Impact on Microbial Communities: Excess Cu can lead to a reduction in microbial diversity and activity. This disruption in the microbial community can have far-reaching consequences, as it impairs their ability to carry out essential biogeochemical processes, such as nutrient cycling and organic matter decomposition.
- Soil pH and Organic Matter Changes: High Cu levels in the soil can lead to reduced soil pH, making it more acidic, and reduce soil organic matter, which is essential for soil health and plant growth. This decrease in organic matter can disrupt soil structure, water retention, and nutrient availability.
- Reduce Plant Productivity: Excess Cu can act as a stressor, leading to stunted plant growth and reduced crop yields. In addition to hampering plant development, these unhealthy or undersized plants produce less plant litter—organic matter in the form of fallen leaves, stems, and other plant materials.

With the constant use of the copper-based fungicides, the metal accumulates in the soil, especially in the upper layer which results in its potential bioavailability to various organisms (Brun et al., 2001).

The bioavailability of Cu in soil is not a static parameter but varies significantly based on the soil's physical and chemical properties characteristics of the soil. These properties encompass a range of variables, such as soil pH, redox potential, texture, mineral composition (including the types and content of clays and oxides of elements like iron, aluminum, and manganese), cation exchange capacity (the soil's ability to retain and exchange ions). In addition, the presence of other metals, the temperature of the soil, the soil's water content, and various other environmental factors can influence microbial activity. The activity of soil microorganisms plays a significant role in determining the bioavailability of Cu in the soil. When these factors are considered collectively, they create a complex interplay that affects Cu bioavailability and its impact on the soil and ecosystems (Adriano, 1986). The toxic effect of Cu is mostly observed in soils with acidic pH, which usually exhibit low organic matter decomposition, due to the lack of conditions for the growth of decomposer communities and poor plant growth. The soil structure usually also shows little aggregation between soil particles, resulting in low permeability and high probability of leaching (Camargos, 2005).

2.3. Soil contamination with copper: possible solutions

By relating the toxic properties of Cu to the constant use of fungicides for the treatment of mildew, it is perceivable that we are facing a problem of soil contamination

with this metal in vineyard soils. There have been some political efforts to reduce the use of Cu, which is the list of candidate substances to be replaced at European level, related to its accumulative potential and lack of degradability in soil (Official Journal of the European Union, 2009).

However, when circumstances preclude the use of copper-based treatments, organic vineyard management faces a dilemma in finding suitable alternatives that are equally effective. The unavailability of copper-based treatments and the urgent need for alternative solutions for mildew control bring to the forefront the pressing issue of contaminated soils in vineyards. As a consequence of historical copper usage, some vineyard soils may have accumulated elevated copper levels, potentially leading to soil contamination. This contamination can have detrimental effects on soil health, microbial diversity, and overall, the well-being of the vineyard. Addressing contaminated soil requires thoughtful intervention strategies, including soil testing, remediation techniques, and sustainable soil management practices. Realistically, the implementation of these techniques can be complex, especially in regions with thousands of hectares of vineyards. Additionally, local farmers may not be fully aware of the problem or may be resistant to some proposed approaches. It is essential to promote awareness among farmers about the potential risks associated with soil contamination and to engage them in discussions about effective remediation strategies.

In this context, a study conducted in 2010 sheds light on the role of organic correctives in the bioremediation of soils polluted with metals, offering valuable insights that can contribute to the development of sustainable solutions for both mildew control and soil quality improvement in vineyards. Park et al., 2011 reached the conclusion that employing organic amendments serves to diminish the bioavailability of metal(loid)s. This reduction is achieved through adsorption and complexation reactions, which subsequently curtail the transfer of these substances via plant uptake and leaching.

2.4. Experimental framework

The research conducted in this dissertation explores the potential utilization of soil amendments to mitigate the presence of copper (Cu) in soils contaminated as a result of fungicide applications for mildew treatment in vineyards. In this context, two distinct amendments were selected based on findings from a prior investigation conducted by Fernández-Calviño et al., 2018. Their study focused on remediating copper-contaminated mining soil using a blend of half crushed pine bark and half crushed mussel

shell, assessing their impact on microbial growth and the potential reduction of bioavailable copper.

Building upon this foundation, the primary objective of this research was to assess the effectiveness of pine bark – later defined as PB - and mussel shell – later defined as MS - when used individually in immobilizing Cu. This assessment involved the monitoring of a range of parameters, including ecotoxicological, chemical, and physical aspects, in the soils amended with these materials. The aim was to evaluate the capacity of PB and MS to mitigate the mobility and potential ecological impact of copper in the treated soils. The tested hypothesis was supported by previous works that described the following:

- Pine Bark when added to the soil, pine bark can adsorb and complex with metals like copper, and increase the organic matter content in the long term (Cutillas-Barreiro et al., 2014);
- Mussel Shell also can retain metals and is able to change the pH of the soil to more basic, decreasing metal's availability (Garrido-Rodríguez et al., 2013a).

In a circular economy perspective, both amendments can be generated sustainably since they can be considered wastes resulting from other activities. PB is generated as a residue or byproduct of various activities and industries, such as timber harvesting and lumber processing, pulp and paper industry, biomass and bioenergy production and tree farming. MS is a residue or byproduct of various activities like seafood processing, aquaculture and seafood consumption.

Hence, the objective of this study was to subject natural soil samples contaminated with copper to individual amendments with PB and MS, and subsequently assess their performance using ecotoxicological tests. This approach aimed to gain insights into the effects of these amendments on metal bioavailability since there was six different concentrations of Cu present on soil samples. The ecotoxicological assays included three tests: two assessing the entire soil matrix with terrestrial species and one evaluating the soil's retention function with aquatic species. The latter was conducted using elutriates from the amended soil. The inclusion of an aquatic test was essential to determine the potential impact of copper leaching into water tables or mobilization through runoff, thus addressing concerns about the ecological consequences of copper contamination in aquatic environments.

By analyzing the physical-chemical properties of the soil and conducting ecotoxicological assays on aquatic and terrestrial organisms, our research seeks to elucidate the potential of these amendments in enhancing soil health and reducing Cu toxicity. Through this

investigation, we aim to contribute valuable insights into sustainable soil management practices for viticultural regions, addressing environmental concerns and promoting the circular economy utilization of waste materials.

3. Case study

3.1. Study area – Region of Vinhos Verdes

Situated in northwestern Portugal at an elevation below 700 meters, this region encompasses approximately 16,000 hectares of vineyards. It is geographically demarcated by natural boundaries, with the River Minho to the north, the River Douro to the south, the Peneda, Geres, and Marão mountain ranges to the east, and the vast expanse of the Atlantic Ocean to the west.

The region's climate is moderated by its orographic features and the configuration of the river network. It is marked by mild temperatures, with minimal extremes of heat or cold, and experiences a relatively high annual precipitation pattern, averaging around 1200 mm. Several factors, such as the proximity to the coast, the increasing altitude from the coast to the interior, the river network, soil heterogeneity, and unique microclimates associated with different geographical zones, delineate the geographical area of 'Vinho Verde' production into a distinct zone. Within this framework, nine sub-regions are situated. The specific sub-region under consideration in this study, Cávado, is characterized by its low thermal amplitudes, lower altitude, and greater exposure to maritime winds, given its location within hydrographic basins (IVV, 2023).

3.1.1. Quinta de Azevedo

The study area is found on the northern region of Portugal, in a city called Barcelos. “Quinta de Azevedo” has a history that goes back to the beginnings of Portugal until 1982, where it was acquired by Sogrape. It has a total area of forty-one hectares and stands out for production of “Vinho Verde.” (SOGRAPE, 2021)

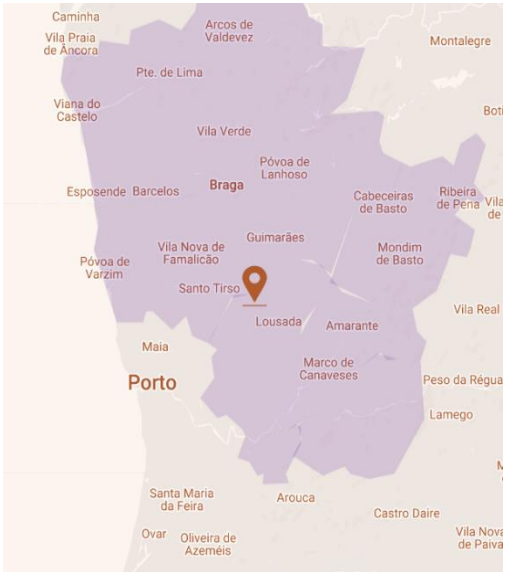




Figure 10. Location of Quinta do Azevedo (Google Maps) on the right and Photograph of Quinta do Azevedo on the left.

4. Experimental design

4.1. Sample collection and pre-treatment

The soil was collected from a plot of Quinta de Azevedo in the first 10 cm top layer, at an equidistant distance from the vines, and mixed to obtain a composite batch of soil. Soil was transported to the laboratory, dried at room temperature and sieved. The fraction lower than 4mm was stored for the experiments.

4.2. Soil contamination

The soil samples were contaminated with Cu, in the form of Bordeaux mixture, at six different concentrations: 0 mg/kg, 72 mg/kg, 111 mg/kg, 173 mg/kg, 268 mg/kg and 416 mg/kg. The table below shows the quantities of the mixture used to achieve the proper concentrations of Cu in the soil. The amount of water added to all the samples was 906.30 ml, based on calculations of the soil's water holding capacity up to 50%. Each batch of soil (for each concentration) was rewetted throughout the four weeks.

Table 1. Cu quantities used to achieve the desired concentration.

Contamination Concentration (mg/kg)	Bordeaux Mixture (g)
72	1,08
111	1,68
173	2,60
268	4,03
416	6,24

The samples were incubated for four weeks. At the end, for each Cu concentration, each batch of soil was divided into 3 different treatments, one contaminated only with Cu, another contaminated with Cu with the addition of MS and the last contaminated with Cu and amended with PB. The amendments were added individually, being a ratio of 3 g of each amendment per 1 kg of soil. This procedure was performed in duplicate. After being incubated for four additional weeks, the soil was tested for the ecotoxicological assays.

4.3. Physical and chemical characteristics of the soil

All the characterization of the soil samples was performed on the < 2 mm soil fraction, manually sieved.

Soil's pH was measured on both water and potassium chloride (KCl) 1 M suspensions (1:5 v/v), following the ISO 0390 standard. The samples were mechanically shaken for 1 hour and left to stand for the same time period. The pH was then measured in the supernatant of each sample using a previously calibrated pH meter, the Multiparameter Meter from Edge, HI2020-02.

The electrical conductivity (EC) was performed in the previous pH soil: water suspension (1:5 v/v) after resting for 24 hours. Then, the EC was measured in the supernatant using a conductivity meter, The Multiparameter Meter from Edge, HI2020-02.

The water holding capacity (WHC) of the soil was calculated according to (ISO, 1998) using plastic flasks with a filter paper replacing the bottom. It is determined by measuring the amount of water a soil sample can retain after saturation, as well as the mass of the soil when completely dry. The water holding capacity is then expressed as a percentage of the soil's dry mass:

$$WHC\ (dry\ mass\ \%) = \frac{(Mass\ of\ saturated\ soil - Mass\ of\ oven\ dried\ soil)}{(Mass\ of\ oven\ dried\ soil)}\ x\ 100$$

The soil organic matter content (OM) was also calculated by the loss of organic mass compared before and after the samples were ignited. The samples were first placed in an oven for 24 hours at 105ºC, and the dry weight recorded, afterwards samples were ignited in a furnace at a temperature of 550ºC for 2 hours (European Committee for Standardization, 2021).

After that, it is possible to calculate the total OM content (%):

$$Total\ OM\% = \frac{(Soil\ 105ºC - Soil\ 550ºC)}{(Soil\ 105ºC)}\ x\ 100$$

The physical and chemical parameters were analyzed in triplicate, for each treatment and for each soil batch.

5. Ecotoxicological assays

To evaluate the effect of the different soil amendments, tested individually, three different ecotoxicological assays were performed, one of them using aquatic organisms to assess the soil retention function and the other two with terrestrial organisms to assess the soil habitat function.

For the aquatic assays, soil elutriates were prepared for each soil treatment and replicate. The elutriates were obtained from a 1:4 (w/v) suspension of the soil samples, in 12 ml of calcium chloride solution. The tubes were mechanically shaken for 24 hours and then centrifuged at 3900 rpm, for 5 minutes. The supernatant was used for the ecotoxicological assays.

5.1. Aquatic Organisms Tests

5.1.1. *Lemna Minor* growth inhibition assay

The *Lemna minor* assay, followed the OECD guideline 221 (OECD, 2006b) and it is aimed at quantifying the growth of the plant, when exposed to different conditions. The plants were taken from a laboratory culture maintained under controlled conditions as referred to in the annex three of the OECD guideline. At the start of the assay, the plants and corresponding fronds were counted and selected. For each elutriate replicate plus the CTL of the ecotoxicological test with the culture medium, were prepared three of the wells with three plants with three visible fronts each, obtained from the same culture.

From the same culture, three plants, with a total of nine fronds, were randomly obtained in triplicate and dried at 60ºC, to estimate the initial dry biomass added to each well in the beginning of the test. For each soil sample from the trials 3 controls, represented in the following figure as (A) with Steinberg medium were prepared, for the validation of the assay. The 3 replicates represented in the following figure as (B) were the elutriates of the controls of the incubated soil elutriates.

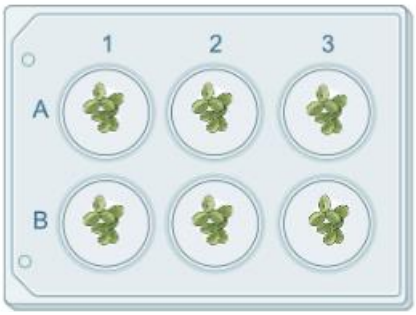


Figure 13. Representation of a 6-plate used in the *Lemna Minor* growth inhibition assay, where A has Steinberg Medium, and B has soil elutriates.

All the plates were placed on a climatic chamber for seven days at 20±2ºC with continuous lighting, and at the end of the assay the fronds were counted and dried at 60ºC until weight stabilization. These variables were necessary to calculate the percent inhibition of growth rate using the following equation:

$$\%\, I_r = \frac{(\mu c - \mu t)}{\mu c}\, x\, 100$$

Where:

- $\%\, Ir$: percent reduction in yield;
- μc : final biomass minus starting biomass for the control;
- μt : final biomass minus starting biomass for the treatment group.

And:

$$\%\, I_y = \frac{(bc - bt)}{bc}\, x\, 100$$

Where:

- $\%\, y$: percent reduction in yield;
- bc : final biomass minus starting biomass for the control;

- bt : final biomass minus starting biomass for the treatment group.

5.2. Terrestrial Organisms

5.2.1. Earthworm *Eisenia fetida* reproduction test

The earthworm assay followed the OECD guideline 222 (OECD, 2016), and had the goal of assessing the reproduction performance of earthworms when exposed to the different soil treatments, the Cu contamination and the Cu contamination with the individually added amendments.

For the terrestrial assays, an artificial soil was prepared according to the OECD protocol nº 222, being composed by a mixture (m/m) of 70% sand, 20% kaolin and 10% sphagnum peat, with a pH_{KCl} adjusted to 6.0 ± 0.5. This soil was used as a control for assays validation purpose.

In adherence to the OECD guideline, specifically annex four, adult earthworms were selected based on a weight criterion ranging from 0.30 to 0.60 grams, and the presence of a clitellum was also considered. These organisms were sourced from cultures that were meticulously maintained under laboratory conditions. Subsequently, the chosen earthworms underwent a one-day acclimatization period in OECD artificial soil before initiating the testing procedures. This rigorous selection and acclimatization process aligns with standardized protocols to ensure the reliability and reproducibility of the experimental results.

In each experimental replicate, batches of ten adult organisms were carefully weighed, subjected to a thorough wash using deionized water, and subsequently introduced into plastic containers. These containers were filled with a soil sample totaling 500 grams, which had been appropriately moistened with 5 to 6 milliliters of deionized water, administered through a spray bottle. The lids of each box were perforated in order to allow gases to exchange. During the assay and once per week earthworms were fed with about 5g of horse manure sieved at < 2 mm, and the moisture content was adjusted. The boxes were incubated in a climatic chamber at controlled temperature 20.5ºC, 73.6%rH (relative humidity) and 365 μE m^2/s of light intensity. There were 3 replicates for each soil sample and 5 controls with OECD soil. The mortality and growth of adults were measured after four weeks of exposure, which were taken out of the boxes and washed with deionized water.

The test replicates were incubated for another four weeks. In the end, the test boxes were placed in a hot water bath at 60ºC, to force the juveniles to move upwards in the soil, which were then counted.

The results obtained were:

- Adult mortality (%) in each replicate at the end of the first 4 weeks of incubation by the total number of adults;

5.2.2. Terrestrial Plant Test with *Medicago Sativa*: Seedling Emergence and Seedling Growth Test

The terrestrial plant assay, following the OECD guideline 208 (OECD, 2006a), aimed at assessing the emergence of seeds and the growth of both the aerial part and of the roots of seedlings growing in each soil replicate. The artificial OECD soil, described above, was used as the control for validation purposes.

For the assay, 20 seeds of *Medicago sativa* were added to each plastic pot, each having 200 g of soil sample, after being sterilized for 7 minutes with 70% (v/v) ethanol followed by 20% (v/v) commercial bleach and then washed with deionized water. The pots had a cotton rope that contacted another plastic pot in the bottom, which was used for watering the samples, which happened until the end of the assay. In the experimental setup, a total of five replicates were meticulously prepared for the control group, following the guidelines outlined by the OECD. Additionally, three replicates were established for each soil sample obtained from the incubation experiment. Upon reaching a germination rate of 50% for the control group seeds, a culling process was initiated to retain only 10 plants, thereby mitigating potential competition effects. Subsequently, all pots, both control and experimental, were carefully placed in a growth chamber. The growth chamber maintained controlled environmental conditions, including a temperature of 22 ºC, humidity levels set at 70%, and a photoperiod of a minimum of 16 hours of light. The light intensity was regulated at 350 µE m²/s, ensuring a standardized and controlled environment for the ongoing plant growth and incubation experiment.

The results obtained were:

- Fresh and dry biomass of seedlings both the shoot and root growing in each replicate;

5.3. Data Analysis

All the statistical analysis was done in the GraphPad 8 program.

All the results (both from physical and chemical data and ecotoxicological assays) were submitted to one-way ANOVA in order to compare the non-amended soil with the soil amended with PB or MS in order to identify statistically significant differences. Dunnett's multiple comparisons test was used when significant differences were recorded. Considering the *Lemna minor* trial, it was not possible to perform a statistical analysis as they performed only 2 soil batch replicates.

6. Results and Discussion

6.1. Physical-chemical parameters

In order to understand how amendments could be effective in soil remediation, and in particular in improving soil physical-chemical properties, some analyses were performed.

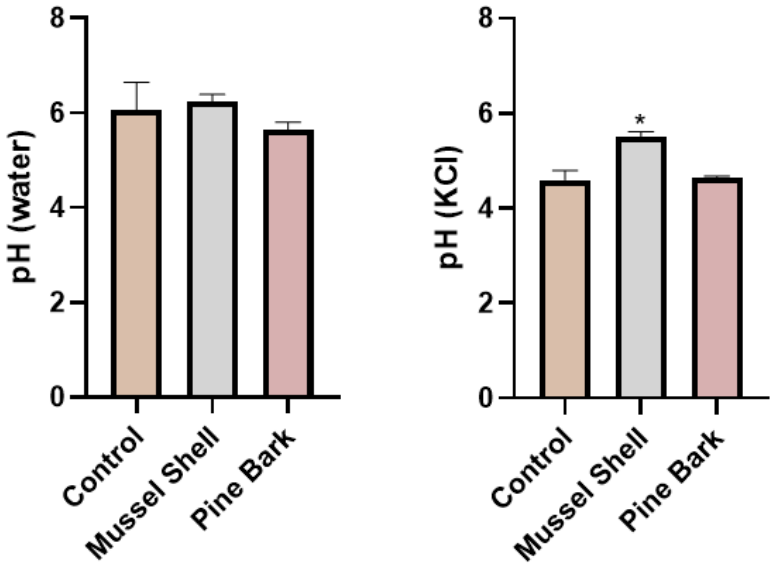


Figure 14. Average values of pH_{KCl} and pH_{H2O} for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the three replicates of each treatment. Statistically significant differences from the control ($p \leq 0.05$) are marked with a *

According to the Figure 3. Average values of pH_{KCl} and pH_{H2O} for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the three replicates of each treatment. Statistically significant differences from the control (p

≤ 0.05) are marked with a *, it is possible to conclude that for the pH_W, no significant differences were caused by the amendment of soil both with MS and PB. For pH_{KCl}, MS has caused a statistically significant increase of this parameter compared to the control.

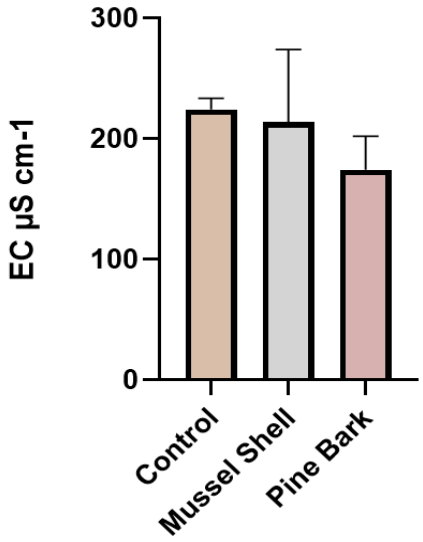


Figure 15. Mean values for the Electric Conductivity for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment.

The MS is a natural source of calcium carbonate ($CaCO_3$), a substance used for liming the soil, when an alkaline substance that neutralizes H+ is used, so it was predictable that the addition of this element would cause a pH increase. The selection of MS for this study stems from their abundant availability as a byproduct of economic activities, particularly the seafood industry, and their composition, notably rich in calcium carbonate. This calcium carbonate not only elevates soil pH but also exhibits an affinity for metal ions, forming the basis for an effective biosorption process in mitigating metal contamination in the soil. (Garrido-Rodríguez et al., 2013b). The addition of PB was expected to lower the pH, due to the organic acids such as humic and fulvic acids (Valentín et al., 2010), which can be explained by its known acidity when milled (Burner & Pote, 2010). Despite a similar effect was registered in our study, the decrease was not statistically significant.

In terms of electric conductivity, as shown in Figure 4. Mean values for the Electric Conductivity for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment., the PB appears to have caused a decrease in soil electrical conductivity. However, there were no statistically significant differences between treatments, likely due to some data variability which can be explained by the incubation period, which was likely short. Conductivity is

a parameter of interest in soil quality, as it assesses the presence of ions in the soil solution. When pine bark is added to the soil, it can interact with the soil solution and influence the mobility and availability of ions. If certain ions are immobilized or sequestered by the pine bark, it can lead to a decrease in EC. PB may influence the soil's ion exchange capacity and water movement, affecting the leaching process and may be use as an amendment (Weil & Brady, 2017).

Regarding the percentage of organic matter in the three treatments, as shown in Figure 5. Mean values for the percentage of Organic Matter for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment.

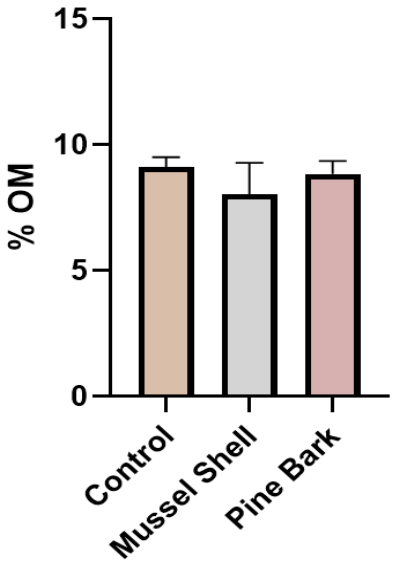


Figure 16. Mean values for the percentage of Organic Matter for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment.

Figure 6. Figure 5, no significant differences were recorded. The organic matter present in the soil is directly proportional to the quality of the soil as it improves its fertility, structure and well-being (Weil & Brady, 2017). It was expected that due to its composition, the addition of PB would have a positive influence on the OM content, but the difference between the sample and the control is not very noticeable. The absence of an increase in organic matter (OM) content could be linked to the quantity of pine bark added in this study. Previous research has demonstrated a considerable enhancement in organic matter content when higher amounts (specifically 96g) of pine bark were

employed. This suggests that the impact of pine bark on organic matter content might exhibit a dose-dependent relationship, and adjusting the quantity in future experiments could potentially yield more substantial increases in soil organic matter (Fernández-Calviño et al., 2018).

The apparent slight decrease in organic matter after adding MS may be due to the shift towards a more alkaline pH. This change could have potentially slowed down the activity of the soil microbial community, leading to a decrease in the breakdown of organic materials and, as a result, a decline in soil organic matter (Weil & Brady, 2017).

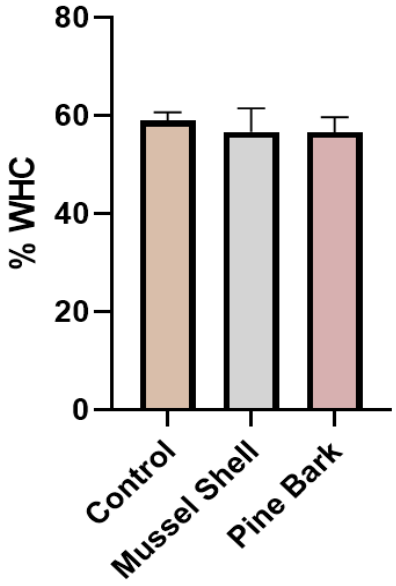


Figure 19. Mean values for the percentage of Water Holding Capacity for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment.

The soil water holding capacity is shown in Figure 6. Mean values for the percentage of Water Holding Capacity for soil without and with amendments (mussel shell or pine bark). Error bars represent standard deviation for the tree replicates of each treatment.

Figure 8. AFigure 6. No statistical differences were found between the treatments. WHC is an important soil property that varies according to the soil's characteristics like the porosity since the higher the water content, the more filled the pores will be, which affects the soil structure by increasing aggregates (Hillel, 2004). An increase in soil WHC was mainly expected for the amendment with the PB, however the richness of this materials in resins, may have the opposite effect, i.e., reduce water repellency instead of absorbing

water. A study highlights significant variations in hydraulic properties among PB sources, impacting moisture release and hydraulic conductivity (K). These differences result from variances in source material, processing, and cultural practices. For example, bark from source X rapidly reduces K, limiting water movement, while source Y exhibits a gradual reduction, indicating sustained water conductivity. These differences are attributed to different source material, processing and aging. Recognizing these distinctions is crucial for designing effective irrigation schedules and adapting water management strategies to the specific properties of each pine bark source (Altland et al., 2018).

6.2. Ecotoxicological assay – Aquatic organisms

Lemna minor serves as a model species for freshwater plants and, based on its growth evaluated through the analysis of different parameters such as number of fronds and weight, it can indicate the hazard of certain substances or environmental matrices. In this case, the growth inhibition rate was calculated based on the number of fronds and dry weight. The following figures represent the results obtained for *Lemna minor* growth inhibition assay. Figure 7. Average percentage of inhibition of growth rate based on the number of fronds for Lemna minor exposed to elutriates of soils contaminated with copper without and with amendments (mussel shell or pine bark). Error bars correspond to the standard error for two experimental duplicates (two incubations).

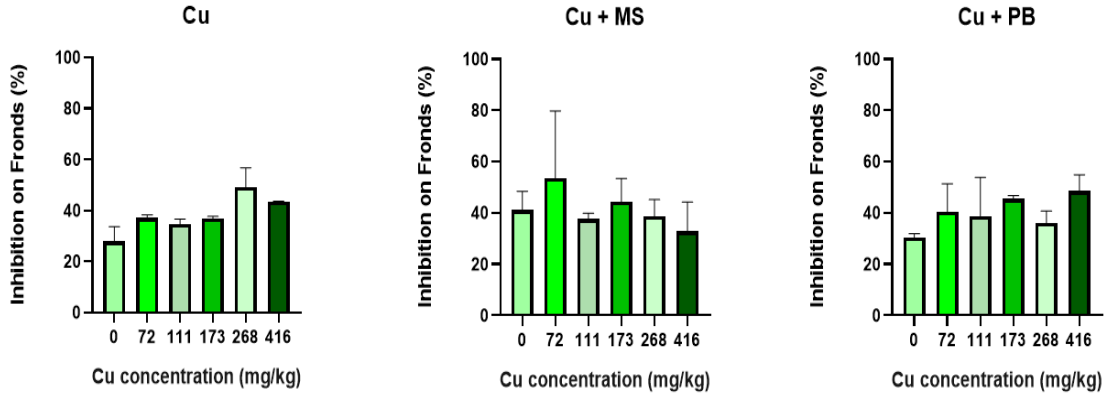


Figure 7. Average percentage of inhibition of growth rate based on the number of fronds for Lemna minor exposed to elutriates of soils contaminated with copper without and with amendments (mussel shell or pine bark). Error bars correspond to the standard error for two experimental duplicates (two incubations).

two experimental duplicates (two incubations).

two experimental duplicates (two incubations).

Figure 10. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Mussel Shell. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.Figure 7 describes growth inhibition based on the number of fronds and Figure 8. Average percentage of inhibition of growth rate based on dry biomass for Lemna minor exposed to elutriates of soil contaminated with copper without and with

amendments (mussel shell or pine bark). Error bars correspond to the standard error for two experimental duplicates (two incubations).

two experimental duplicates (two incubations).

Figure 7. Figure 8 the same endpoint but based on dry weight.

Looking at the first group, referring to inhibition in the fronds, the addition of copper did not have a significant inhibitory effect on the specie. No statistically significant differences in plants growth inhibition were recorded when the soil was amended both with PB and MS, however, the addition of MS seemed to be responsible for higher inhibition rates. The addition of PB did not change the inhibitory effect of Cu.

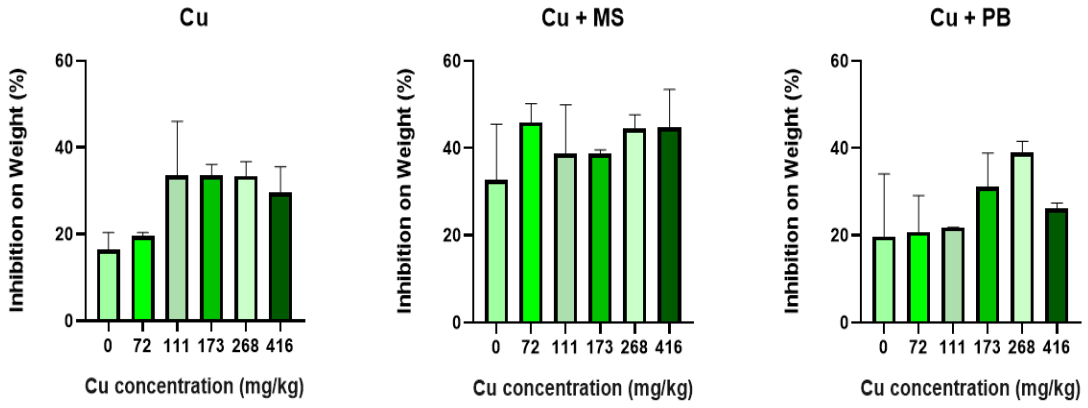


Figure 8. Average percentage of inhibition of growth rate based on dry biomass for Lemna minor exposed to elutriates of soil contaminated with copper without and with amendments (mussel shell or pine bark). Error bars correspond to the standard error for two experimental duplicates (two incubations).

In the samples relating to inhibition by weight, in the first case with Cu, the addition of copper like the previous samples, did not have a significant inhibitory effect on the plant growth. The diagram shows that the addition of MS seemed to be responsible for higher inhibition rates. The addition of PB did not change the inhibitory effect of Cu. The fact that the Cu + MS control seemed to cause a highest growth inhibition when compared with the exposure to Cu alone, suggests that the MS is likely causing changes in the availability of nutrients, including Cu to a level that may affect plants, and it might be related to the increase of pH. MS may also have a negative impact on Lemna growth because of the release of calcium, despite being an important nutrient for plants, when

in excess can create an imbalance or even alter the availability of other nutrients and therefore inhibit growth (LAZAR, 2003).

Overall, the amendments did not have a remarkable effect on reducing the toxicity of the elutriates from Cu contaminated soils. This may be related to the amendment's concentrations or the limited time of their contact with the soil (only 1 month).

6.3. Ecotoxicological assay – Terrestrial organisms

For the reproduction assay with *Eisenia fetida*, the results are shown on the following Figure 9. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.

Figure 11. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Pine Bark. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.Figure 9, Figure 10. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Mussel Shell. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.

Figure 9. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.Figure 10 and Figure 11. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Pine Bark. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.

Figure 12. Average growth inhibition percentages regarding biomass above soil of *Medicago Sativa* growing on soil amended with different Cu concentrations with and without the amendment with MS and PB. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.Figure 11. Reproduction inhibition was calculated in relation to the CTL with the artificial OECD soil. The reproduction of earthworms was slightly stimulated in the natural soil in comparison to the CTL with OECD, what demonstrates that the soil in itself did not compromise the performance of earthworms.

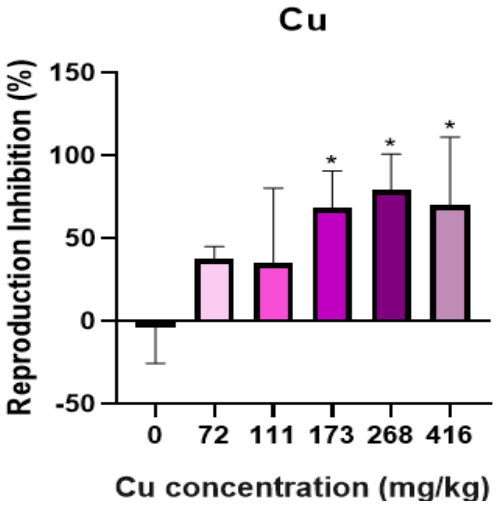


Figure 9. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.

For earthworms exposed to soil contaminated with Cu, the addition of copper did not have a significant inhibitory effect on the reproduction of earthworms up to a copper concentration of 111 mg/kg.

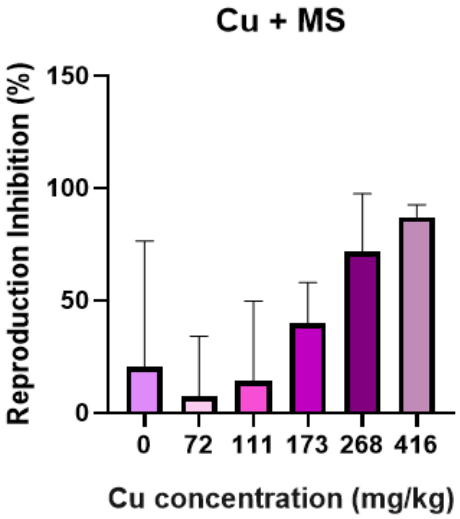


Figure 10. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Mussel Shell. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.

Considering the addition of MS, this did not cause any statistically significant change on reproduction inhibition. Great variability of the data is contributing for this result. The supply of calcium may have had an impact on the growth and reproduction of earthworms, since it is a truly relevant element on earthworm's metabolism and it may

have also influenced nutrient's availability in the soil (García-Montero et al., 2013). No statistically significant differences were recorded.

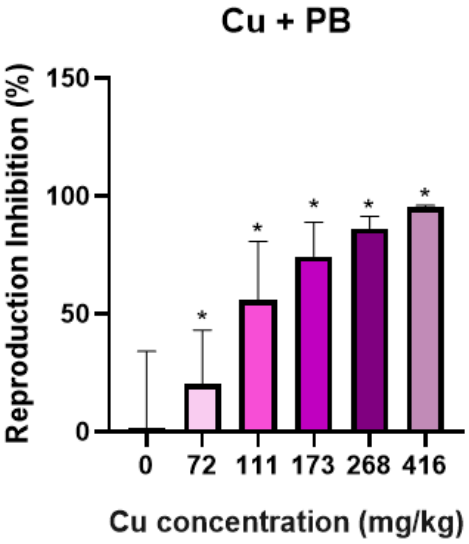


Figure 22. Average reproduction inhibition for *Eisenia fetida* exposed to different Cu concentrations. when added Pine Bark. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates. Statistically significant differences between samples and the control, considering $p \leq 0.05$, are marked with * above bars.

The graph above for the addition of PB to soil contaminated with Cu shows a gradual increase in the inhibition rate as a function of the increase in Cu concentration. In this case significant reproduction effects were registered even for the lowest concentration of Cu (72 mg/kg) suggesting that not only the Cu but also the PB has affected earthworms' reproduction inhibition. The increased toxicity of PB amended soils to earthworms may be related to their lower tolerance to more acidic pH. The PB could also have a toxic component for the species.

Taking into account the terrestrial plant *Medicago sativa*, two inhibition rates were calculated, one based on dry biomass above soil Figure 12. Average growth inhibition percentages regarding biomass above soil of *Medicago Sativa* growing on soil amended with different Cu concentrations with and without the amendment with MS and PB. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.

Figure 13 Average growth inhibition percentages regarding root growth of *Medicago Sativa* growing on soil amended with different Cu concentrations with and without the amendment with MS and PB. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.Figure 12, and the other on the root biomass,

Figure 13 In this way, the three study scenarios, with and without amendments, are grouped according to shoot and root data.

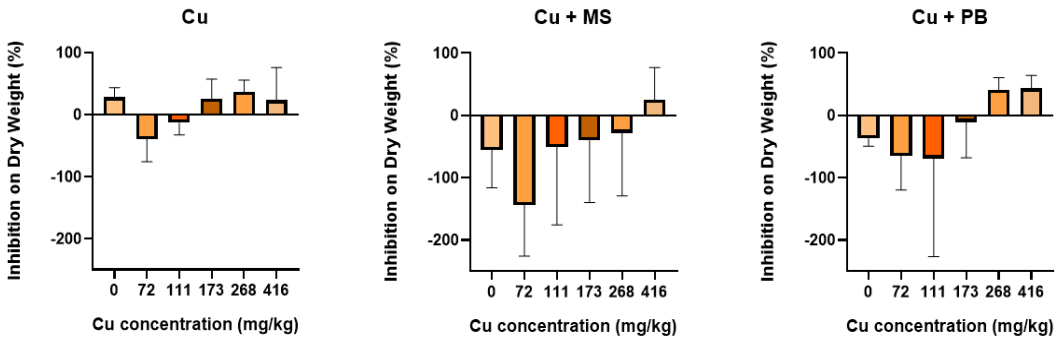


Figure 25. Average growth inhibition percentages regarding biomass above soil of *Medicago Sativa* growing on soil amended with different Cu concentrations with and without the amendment with MS and PB. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.

The first graph shows that the inhibition caused by the addition of copper was not significant. The inhibition caused by the addition of copper was not significant and was reversed with the addition of the amendments. Both MS and PB amendments stimulated shoot growth at the lowest copper concentrations.

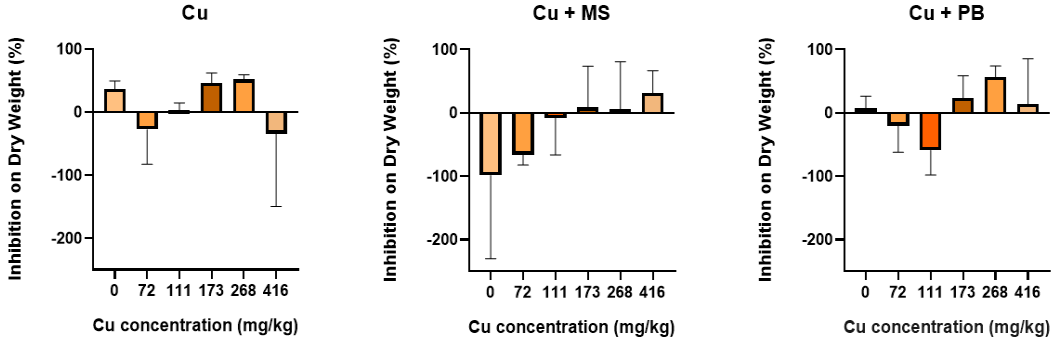


Figure 28 Average growth inhibition percentages regarding root growth of *Medicago Sativa* growing on soil amended with different Cu concentrations with and without the amendment with MS and PB. Error bars correspond to the standard error, for the assays repeated for the experimental triplicates.

In the first graph of figure 13, inhibition caused by the addition of copper was not significant. With the addition of MS, it can be seen that root growth was stimulated and the inhibition registered at the two concentrations mentioned above was reduced. With the addition of PB, there was stimulation at 72 mg/kg and 111 mg/kg, while the inhibition at 173 mg/kg of Cu was also mitigated. No statistically significant differences were recorded. The stimulatory effect of the amendments was not as evident as recorded for roots.

Taking this trial into account, it is possible to see that to a certain level of Cu the addition of both materials MS and PB reduced Cu toxicity in the soil and boosted growth both in the shoot and the roots. This may be related to the fact that the pH has risen with MS, as the plant thrives best in a neutral environment. The availability of calcium is also especially important, especially for the health of the roots and the plant, and since MS is a source of this, it is related to the boost in plant growth (Fredeen, 1989). This can also be related to WHC, since it has a dominant influence in the growth in the roots, and this, together with other factors, can further boost its growth. Taking PB into account, its addition may have been positive due to the increase in OM, which encourages greater retention of important nutrients for the plant. The fact that it also promotes soil structure could lead to a healthier environment for the roots.

The study was carried out to understand the efficiency and role of each of the amendments (MS and PB) in Cu immobilization in soil, and the results obtained did not prove their remediation potential, except in the terrestrial plant test. The addition of PB can contribute with some toxic substances to the soil, while the addition of MS can alter the availability of nutrients, creating an imbalance.

The existing body of research highlights the potential of both MS and PB as bio-absorbents, attributing their efficacy to various mechanisms such as binding sites, pH alterations, promotion of microbial activity, and increased organic matter. Notably, previous studies have employed larger quantities and longer durations for these amendments, potentially explaining their enhanced effectiveness over time. Individually, MS and PB exhibit favorable characteristics for copper retention in the soil. Combining the two may further amplify their efficiency, given their demonstrated unique modes of action.

MS, aligned with principles of the circular economy, has witnessed increasing recognition. The substantial growth in aquatic production, particularly from 2000 to 2019, has led to a significant waste stream, notably shellfish shells. Approximately 10 million tons of shells are discarded in landfills or oceans, impacting ecosystems. However, recognizing their untapped potential in various sectors, including agriculture, medicine, pharmacy, and the food industry, could transform this waste into valuable resources (Summa et al., 2022). The application of shell waste, owing to its ability to neutralize acidic soils effectively, presents a cost-efficient soil amendment with the potential to mitigate metal mobility, particularly copper.

PB, identified as a bio-absorbent, has demonstrated the capacity to retain various metals in the soil, albeit in smaller quantities. This suggests a potential threshold mass, defining the point at which PB serves as an effective soil improver (Cutillas-Barreiro et al., 2014). Integrating the strengths of MS and PB, considering their distinct attributes, provides a promising avenue for enhancing soil management practices with a focus on metal retention and overall environmental sustainability.

The most viable solution for immobilizing Cu could be to combine the two elements, as has already been done in another study, where larger quantities of between 6 and 96 grams were used and added to soil. The mixture contributed to boosting the growth of fungi and bacteria, which in turn increased the OM rate, increasing the pH and reducing the availability of Cu in the soil (Fernández-Calviño et al., 2018).

7. For future consideration

In conclusion, this study has provided insights into the potential of PB and MS amendments in reducing Cu toxicity in soil.

The study revealed certain limitations that warrant consideration. Firstly, the brief incubation period of the soil amendments may have constrained the discernible changes in soil parameters. Additionally, the reliance on a singular concentration for the soil amendments introduces a potential limitation, urging a nuanced interpretation of the findings. To advance the depth and applicability of future research endeavors, it is suggested to integrate assays that encompass a broader spectrum of species. Exploring the sustained efficacy of soil amendments over extended periods will provide a more comprehensive understanding. Consideration of varying quantities of amendments is vital for a nuanced and encompassing analysis. Employing chemical methods to quantify available copper in both elutriates and soil adds a layer of precision to the assessment. Furthermore, investigating the repercussions of alterations in different soil types is essential to broaden the spectrum of scenarios studied. Avoiding sample drying in subsequent studies is advised to accurately evaluate potential impacts on microbial activity and discern resulting alterations.

This work serves as a starting step for more comprehensive studies, showcasing not only the economic and social viability of the technique but also its potential as an environmentally sound practice. The promising aspects extend beyond the immediate scope, particularly in the application of amendments such as PB and MS in real-world scenarios. Exploring the scalability, economic feasibility, and broader ecological impacts

of these amendments becomes paramount. As we endeavor to develop effective and environmentally friendly soil remediation practices, it aligns seamlessly with the principles of the circular economy and sustainable agriculture. The noteworthy potential for recurrence within the country and across Europe underscores the significance of advancing research in this direction.

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Annex

Summary table with pH values

pH H_2O	Cu (mg/kg)	average	SD	Coefficient of variation
1ª incubation	0	5,69	0,070711	1,24271842
2ª incubation		5,765	0,021213	0,367965367
3ª incubation		6,73	0,311127	4,622986385

pH KCl	Cu (mg/kg)	Average	SD	Coefficient of variation
1ª incubation	0	4,66	0,056569	1,213917221
2ª incubation		4,375	0,077782	1,777868478
3ª incubation		4,755	0,275772	5,799613978

pH H_2O	Cu (mg/kg) + MS	Average	SD	Coefficient of variation
1ª incubation	0	6,39	0,084853	1,327900059
2ª incubation		6,08	0	0
3ª incubation		6,24	0,042426	0,679910367

pH KCl	Cu (mg/kg) + MS	Average	SD	Coefficient of variation
1ª incubation	0	5,605	0,219203	3,91084928
2ª incubation		5,525	0,176777	3,199578195
3ª incubation		5,415	0,021213	0,391748909

pH H_2O	Cu (mg/kg) + PB	Average	SD	Coefficient of variation
1ª incubation	0	5,82	0,325269	5,588816484
2ª incubation		5,61	0,19799	3,529231706
3ª incubation		5,495	0,049497	0,90077297

pH KCl	Cu (mg/kg) + PB	Average	SD	Coefficient of variation
1ª incubation	0	4,68	0,169706	3,626188621
2ª incubation		4,625	0,106066	2,29331929
3ª incubation		4,585	0,035355	0,771108813

Summary table with EC values

EC	Cu (mg/kg)	Average	SD	Coefficient of variation
1ª incubation	0	220,8	3,818376618	1,729337237
2ª incubation		217,5	6,646803743	3,056001721
3ª incubation		234,75	6,01040764	2,560344043

EC	Cu (mg/kg) + MS	Average	SD	Coefficient of variation
1ª incubation	0	146,5	5,656854249	3,861333959
2ª incubation		260,65	0,636396103	0,244157339
3ª incubation		234,7	7,778174593	3,314092285

EC	Cu (mg/kg) + PB	Average	SD	Coefficient of variation
1ª incubation	0	168,75	5,161879503	3,058891557
2ª incubation		204,65	7,707463915	3,766168539
3ª incubation		149,95	5,161879503	3,442400469

Summary table with OM values

OM	Cu (mg/kg)	Average	SD	Coefficient of variation
1ª incubation	0	57,26355354	0,502267272	0,877115095
2ª incubation		60,74381219	2,274293288	3,744074014
3ª incubation		58,9398299	16,41029013	27,8424457

OM	Cu (mg/kg) + MS	Average	SD	Coefficient of variation
1ª incubation	0	59,4122521	10,58960494	17,8239413
2ª incubation		51,17689214	10,0204983	19,58012275
3ª incubation		59,48479238	0,864344697	1,453051548

OM	Cu (mg/kg) + PB	Average	SD	Coefficient of variation
1ª incubation	0	55,78368259	2,365212442	4,239971855
2ª incubation		60,00950955	4,072178043	6,785887893
3ª incubation		54,04932337	1,732536913	3,205473825