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Ecological approach to evaluate the effects of antibiotic and heavy metal contamination on ecosystems

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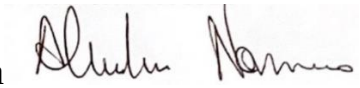
Dott. Alessandra Narciso

Firma 

Coordinatore del corso
Prof. Massimiliano Fenice

Firma

Tutor
Dott. Anna Barra Caracciolo

Firma

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ABSTRACT

Soil contamination (e.g. heavy metals) has been recognized by the EU Soil Strategy as one of the main threats to soil quality. Moreover, since 2004 the *Wildlife Conservation Society* has defined the "One Health concept" recognizing that the ecosystem, human and animal health are inextricably linked. This approach has been adopted by the World Health Organization for facing the antimicrobial resistance issue (AMR): *resistant organisms can spread quickly through health-care facilities, animals, food and the environment (soil and water), making the treatment of certain infections in people and animals more challenging, and increasing the risk of disease spread, severe illness and death*.

Assessing the effects of contaminants in agroecosystems, which are highly impacted by anthropogenic pollution, is an important starting point. In fact, antibiotics and heavy metals are ubiquitous soil contaminants due to agricultural practices, such as the use of organic amendments (which may contain antibiotic residues) and antifungal products (which contain copper). Both copper (Cu) and antibiotics (ABs) are known to show toxic effects on microorganisms, however it depends on their environmental concentration. Moreover, an additional concern to be considered is if co-presence of Cu and ABs can increase their deleterious effects, including AMR spread. This complex environmental issue needs to be studied with an ecological and One-Health approach, which includes evaluations of pollutant fate and of their effects at different biological hierarchical levels (e.g. population, community and ecosystem).

In this context, the overall purpose of this thesis was to evaluate effects of antibiotic and heavy metal contamination on a test organism (e.g. *A. fischeri*), a natural soil microbial community and non-target organism, such as lettuce (*Lactuca sativa*), using an ecological approach.

The first work (DOI:10.3390/antibiotics12091471) is a review paper on antibiotic direct and indirect effects on non-target terrestrial organisms, considering potential ecotoxicological effects and possible impacts on associated microbiomes.

The second work (DOI:10.1093/femsec/fiad125) aimed to evaluate direct effects of various mixtures consisting of three antibiotics (sulfamethoxazole, ciprofloxacin and chlortetracycline) and copper, using the *Aliivibrio fischeri* bacterium as model organism, in order to identify possible additive, synergic and antagonistic effects.

The third work (DOI:10.1007/s00253-024-13324-x) evaluated the effects of sulfamethoxazole and copper on soil natural microbial communities in a soil amended with organic fertilizers (i.e. cattle manure and digestate). The effects were evaluated in terms of antibiotic resistance gene spread and effects on the natural soil microbial community and lettuce plant grown on it in presence/absence of the two contaminants.

INTRODUCTION

Soil and water are limited natural resources and for this reason, their quality and availability are essential for overall biota and agricultural productivity (Ashraf and Mohd Hanafiah 2019). The continuous increase in human population has led to a strong pressure on these no-renewable resources (Bayabil et al. 2022). The growing need of cultivation productivity in the last decades has caused nutrients and various chemicals accumulation in agricultural soils and from them to water bodies (Bayabil et al. 2022). Moreover, the effects of anthropogenic environmental contamination can be exacerbated by climate change (e.g. prompt temperature variations and sudden atmospheric phenomenon), (Saidon et al. 2024).

The Food and Agriculture Organization of the United Nations (FAO) has stated that global production of industrial chemicals has been increasing since the last two decades, reaching 2.3 billion of tons per year and growing by another 85% by the 2030 is expected (Zhao et al. 2024), with very concerning contamination scenarios which pose serious risks for ecosystems (Liu et al. 2023). An important aspect to be considered is the type of environmental matrix contaminated, which plays an important role in the bioavailability of nutrients and pollutants. Water bodies are known as one of the most sensitive and vulnerable environmental compartments considering that they can be sinks of high load of available pollutants which can act directly on aquatic organisms or indirectly influencing the equilibrium in ecosystems (Vasilachi et al. 2021). On the contrary, soil can adsorb or bind contaminants immobilizing contaminant due to its constituents such as size of soil particles and quantity of organic matter. Soil capacity to retain contaminants is due to physico-chemical properties such as pH, particle charge, moisture and oxidation-reduction potential which make it possible to display strong or weak chemical interactions (electrostatic attraction, complexation, ion exchange) with them. For this reason, some contaminants can remain in the soil solid phase for a long time and others be removed by soil water by run-off and leaching contaminating surface water or groundwater (Sarkar et al. 2021). Finally, contaminants immobilized in stable conditions can become available by extreme or sudden environmental phenomena (Biswas et al. 2018).

The 2030 Agenda for Sustainable Development has provided strategies for avoiding risks and impacts of soil degradation and water exploitation (Savarese and Falconi 2024). The European Commission has also recognized the importance of soil and water qualities making them central themes of the European Green Deal. In particular, the Farm to Fork Strategy aims to promote a transition to sustainable food systems and plans to achieve a significant increase in organic production in Europe, limiting of the usage of chemicals in agriculture and mitigating the impacts

of the climate change on soil and water (Molitorisová and Burke 2023). In accordance with soil health, the EU soil strategy for 2030 (European Commission 2021), a key part of the EU biodiversity strategy for 2030, has posed attention on degradation of soils and has called for change of energy sources from carbon like to renewable ones, a better waste management and strategies to improve biodiversity. Soil degradation issue is highly connected to its contamination. In fact, the need of new strategies and substantial progresses in the remediation of contaminated environmental matrices were addressed by the EU Action Plan: 'Towards Zero Pollution of Air, Water and Soil' (Fig. 1), (Savarese and Falconi 2024).

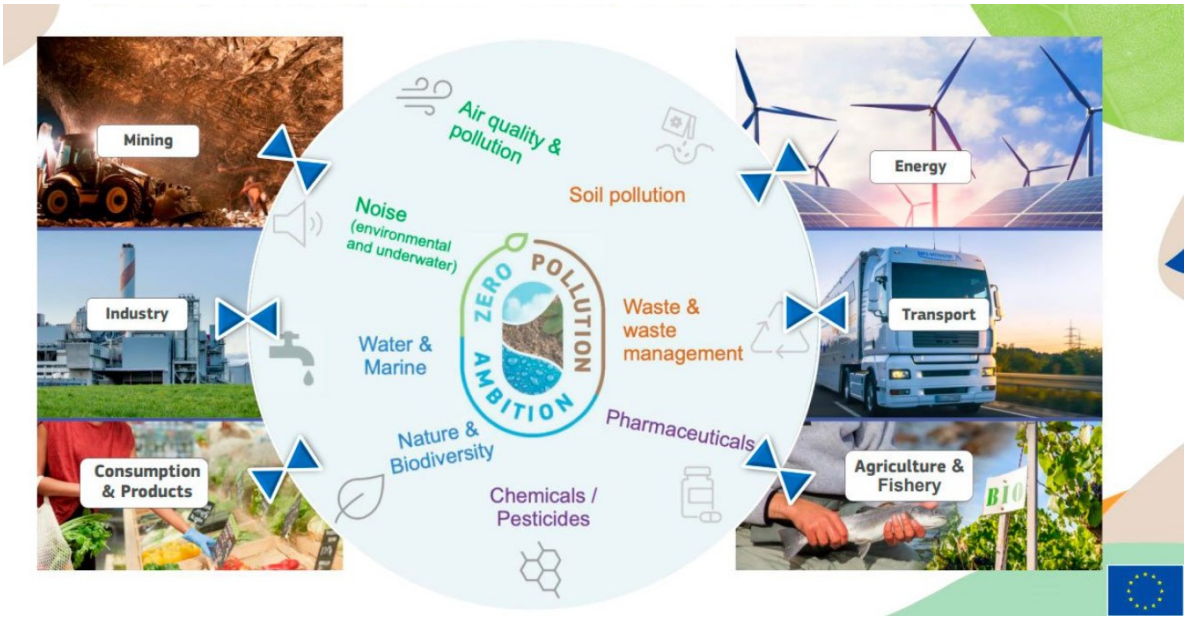

Figure 1. Graphical representation of *EU Action Plan: 'Towards Zero Pollution for Air, Water and Soil'*, adopted by the European Parliament's Committee on the Environment, Public Health and Food Safety (ENVI).

In line with the European strategies, the One Health approach is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals and ecosystems (Prata et al. 2022) and promote an integrative, interdisciplinary and transdisciplinary approach to face the issues on human health, food, energy and environment (Fig. 2), (Queenan et al. 2017; Prata et al. 2021).

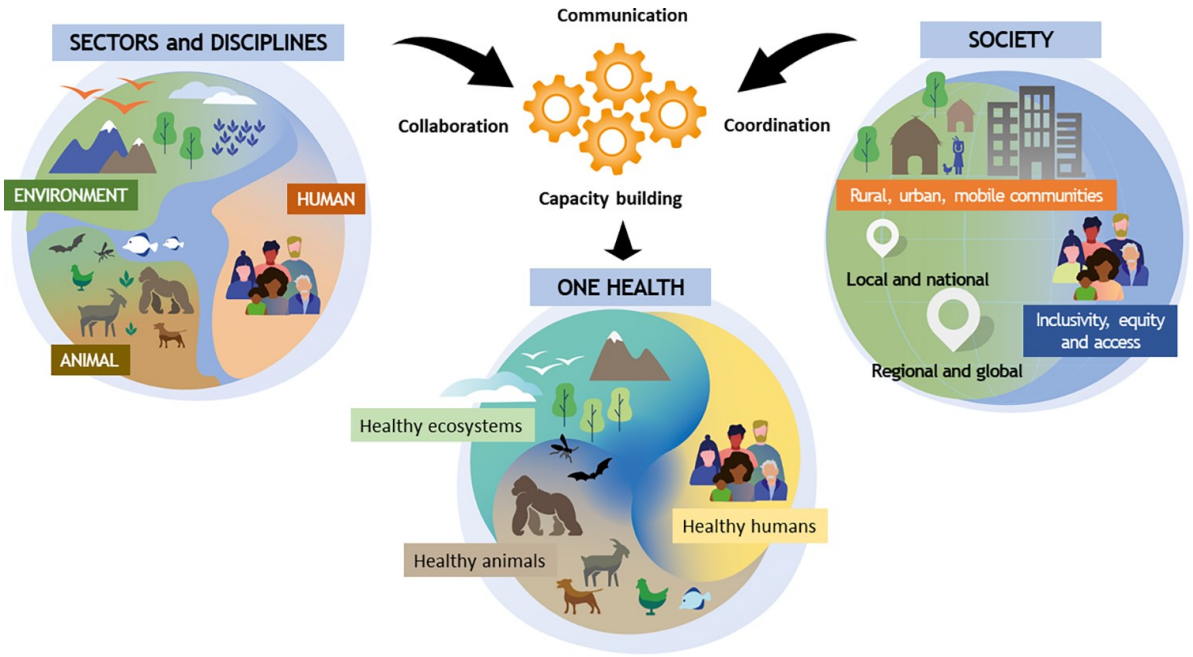

Figure 2. Graphic representation of One Health concept developed by the One Health High-Level Expert Panel (OHHLEP, Adisasmito et al. 2022).

Soil contamination is a complex phenomenon and for this reason it needs to be deeply investigated with an ecological approach, considering pollutant fate and effects at different biological hierarchical levels (Clements 2000; Fischer et al. 2013).

Agroecosystems and main sources of contamination

Agroecosystems are ecological systems modified by human beings for producing food, fibre or other agricultural products (Conway 1987). An agroecosystem differs from natural ecosystems (e.g. freshwater and terrestrial ecosystems), which are supported by solar energy, for several aspects: use of subsidiary energy for plant cultivation such as application of chemical fertilisers and pesticides, artificial irrigation, use of agricultural machineries with consumption of fuels, a reduction in biodiversity for favouring selected monoculture crops or farm animals. Consequently, conventional agriculture has multiple impacts on the environment and climate. Farming practices over-exploit natural resources, with significant soil and water consumption (Hossain et al. 2020). Soil quality depends on its intrinsic characteristics (e.g. pH, texture, mineralogy and organic matter content), however agricultural practices can alter significantly them, causing its degradation (JRC, 2020). The EU Soil Strategy (European Commission 2021) identified the main threats to soil quality in Europe, such as compaction (e.g. due to use of heavy machinery), erosion (e.g. for loss of organic matter), acidification (acid rain), salinization (e.g. through saline intrusion phenomena), organic matter and biodiversity loss and contamination by inorganic and organic compounds (by intensive use of chemical fertilizers and pesticides), (Fig. 3), (Bünemann et al. 2018; European Commission et al. 2020).

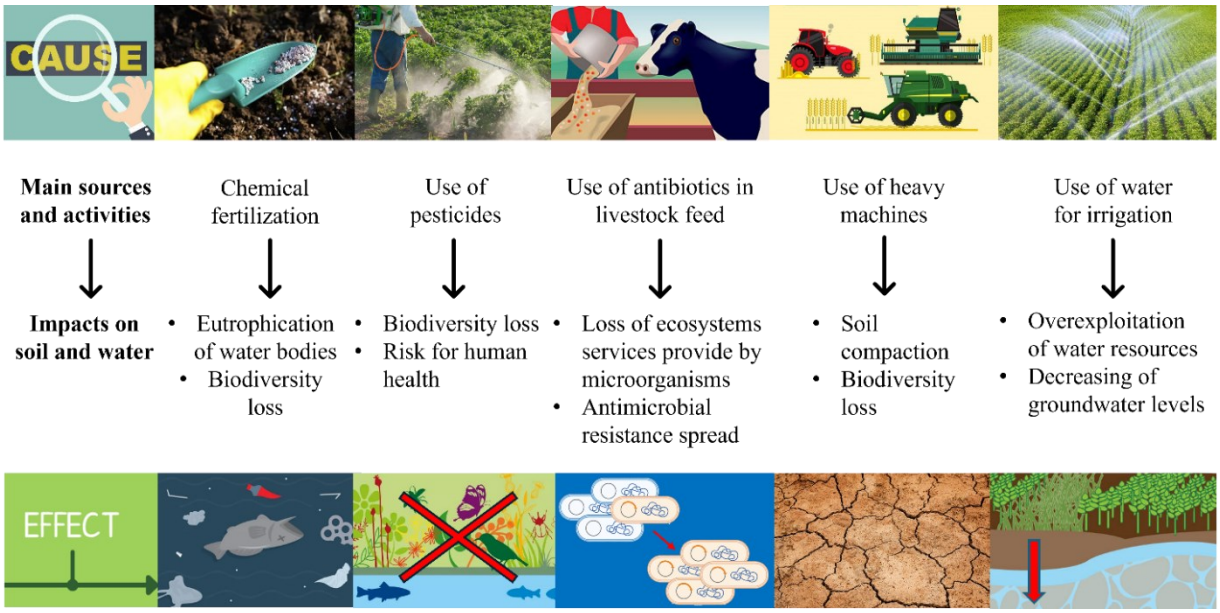

Figure 3. Illustration of the main agriculture impacts on environment. Modified from EEA report - The European environment - state and outlook 2020.

In the last years, organic agriculture has been developing as an alternative practice to conventional ones for minimizing the use of chemicals and promote organic amendment re-use, in line with the circular economy approach and with organic farming regulation (Regulation (EU) 2018/848). For example, livestock manure can be used as a soil organic fertilizer, however its application rate needs to consider its nitrogen content in order to avoid possible unwanted nitrogen release. Manure

application can improve soil fertility thanks to the addition of organic matter, microorganisms and nutrients to soils (Barra Caracciolo et al. 2020; De Carolis et al. 2024). On the other hand, in the recent years the possibility that veterinary pharmaceuticals used to treat animal diseases can be found in manure and in turn in amended soils has been evidenced (Barra Caracciolo et al. 2020; Muhammad et al. 2020; Barra Caracciolo et al. 2022; Caneschi et al. 2023). Among them antibiotics such as sulphonamides, fluoroquinolones and tetracyclines (Ghimpețeanu et al. 2022) are frequently found in agricultural soils. Indeed, after their administration, antibiotics (ABs) are not completely metabolized and are excreted by urine or faeces (Barra Caracciolo et al. 2020; Visca et al. 2021). A further concern raises from the possibility that ABs from organic amendment fertilized soils transfer through roots to plant edible parts (e.g. leaves and fruits), (Pan and Chu 2017; Geng et al. 2022). Currently, ABs and eventual antibiotic resistance bacteria (ARB), which reach soil from organic amendments, are considered ubiquitous contaminants. AB residues can affect negatively natural microbial communities, which play a key role in fundamental ecological processes, such as the maintenance of ecosystem quality. At the same time, ABs can stimulate development and spread of antibiotic resistance. Indeed, ABs and other resistance cofactors (e.g. heavy metals) can promote the development of ARGs in chromosomes or their transfer through mobile genetic elements between pathogens and natural bacteria and vice versa favoured. In turn, ARGs can be transferred from environmental bacteria to animal and human associated microorganisms (microbiomes), (Grenni and Barra Caracciolo 2024).

In recent decades, several livestock farms have developed innovative practices of management and valorisation of their agro-zootechnical waste, such as the use of manure for producing biogas, a renewable energy, through anaerobic digestion plants. A by-product of this process is the digestate, which can be used as an organic fertilizer, in line with circular economy approach. In particular, cattle manure digestate has been shown to be a good fertilizer (Visca et al. 2021; Visca et al. 2022; Barra Caracciolo et al. 2022). Comparing a manure used as feed for an aerobic digestor and the digestate obtained from it, in the latter higher amounts of plant micro and macro-nutrient and lower pathogens were found. Moreover, antibiotic residues and ARGS present were also removed or significantly decreased (Mazzurco Miritana et al. 2022; Barra Caracciolo et al. 2022; De Carolis et al. 2024).

Several heavy metals (HMs) are essential micronutrients for microorganisms and plants but, at the same time, they are among the most common soil contaminants, present in high amounts in agroecosystems (Fagnano et al., 2020). They can accumulate in agricultural soils forming strong chemical interactions with soil particles (Udeigwe et al. 2011; Ye et al. 2017; Ukalska-Jaruga et al. 2023) or can be bioavailable and be up-taken by edible plants, with the potential to be

bioaccumulated in biota feeding on them, with detrimental consequences (Antoniadis et al. 2017; Barra Caracciolo and Terenzi 2021; Qvarforth et al. 2022). Among HMs, copper is an essential micro-element, even if its excessive amounts can be deleterious for biota (Ochoa-Herrera et al. 2011; Barra Caracciolo and Terenzi 2021; Ramos-Zúñiga et al. 2023). In fact, copper sulphate, is widely applied to vineyards as a pesticide to prevent and treat plant antifungal disease and it can also be used as antibacterial protector for livestock (Fagnano et al. 2020). Microorganisms, due to their high capacity of adaptation and plasticity, display several mechanisms to face and resist to copper and other heavy metals (Barra Caracciolo and Terenzi 2021), including generic resistance mechanisms (e.g. efflux pumps) which can also be useful to face antibiotics and favour antibiotic resistance spread in environment (cross-resistance phenomenon), (Virieux-Petit et al. 2022; Murray et al. 2024).

Role of Soil Natural Microbial Communities in contaminant removal

Soil microorganisms, thanks their high plasticity and capacity to respond and to recover from disturbances provide numerous regulation ecosystem services such as cycling of nutrients, and degradation of organic matter and removal of contaminants (Fig. 4), (Barra Caracciolo et al. 2015b; Philippot et al. 2021). Moreover, some soil microorganisms (e.g. cyanobacteria) can sequester carbon and mitigate the climate change and others can detoxify soil from deleterious chemicals, preventing water compartment contamination (Sarkar et al. 2021; Hou 2023). A high diversity of soil microbial communities is crucial in deterring pathogen establishment and growth. In fact, microbial communities with greater diversity may be more likely to include antagonists or competitors that are particularly effective at suppressing pathogen spread (Fig. 4), (Garbeva et al. 2004; Tilman et al. 2014; Samaddar et al. 2021).

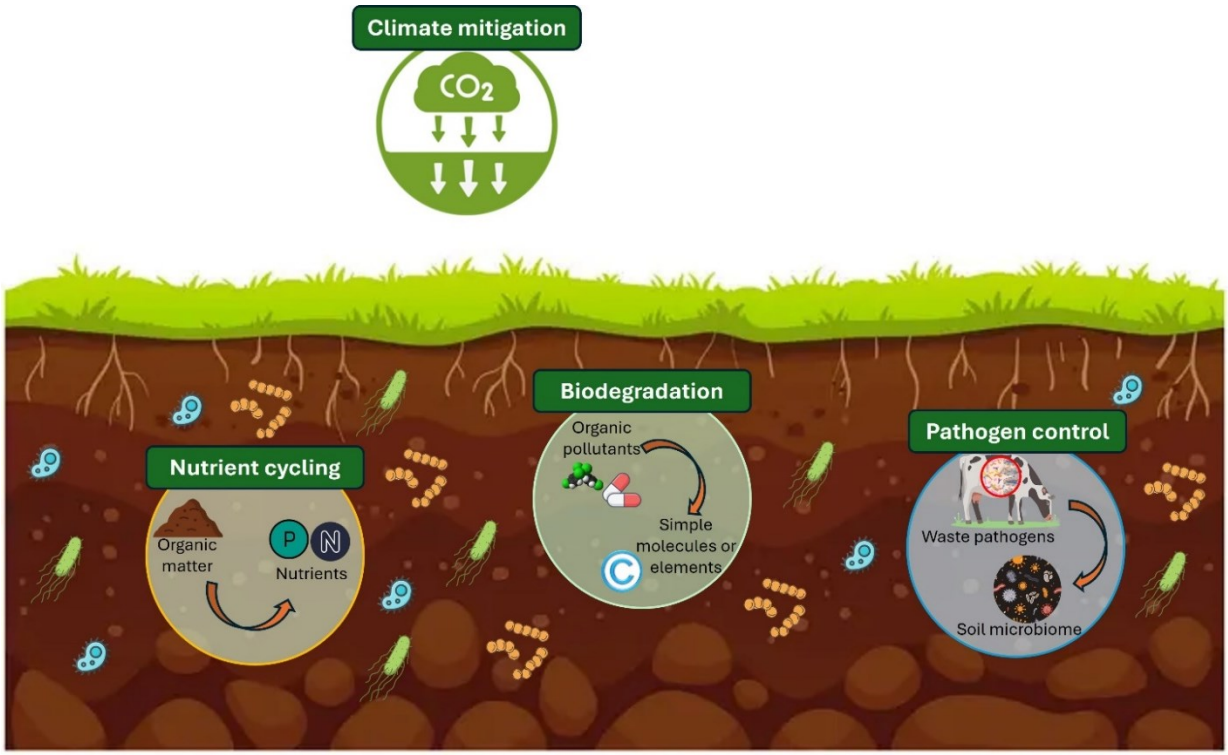

Figure 4. Examples of regulation ecosystems functions provided by soil natural microorganisms.

Microorganisms can degrade and remove a wide range of chemicals due to their versatility and multiple type of metabolisms, which is still in a large part unknown (Barra Caracciolo et al. 2015b; Barra Caracciolo et al. 2015a; Waschina et al. 2016; Zhang and Zhang 2022; Alidoosti et al. 2024a). Several microorganisms can bio-transform organic contaminants and convert toxic molecules into other compounds, which can further be degraded by other microorganisms and be eliminated from the environment with mineralization processes (Bala et al. 2022; Alidoosti et al. 2024b). Microorganisms can adapt to different conditions, including extreme environments such as acidophiles which can survive in very low pH soil or water bodies, psychrophiles that have the capacity to live in extreme cold climate and the halophiles that are favoured by saline environments (Hurtado-Bautista et al. 2021; Ayilara and Babalola 2023).

Soil microbial communities and their response to antibiotics and heavy metals

Soil microbial communities display a huge variety of mechanisms for removing inorganic or organic contaminants, including antibiotics (Barra Caracciolo et al. 2015b; Fouad et al. 2022; Zhang and Zhang 2022). Antibiotics can have bacteriostatic actions, inhibiting the microbial growth, or bactericidal one, killing microorganisms (Zhang and Cheng 2022). In order to assess the effects of an antibiotic on natural microorganisms, is important to assess which is its minimum inhibitory concentration (MIC), i.e. the lowest concentration which can inhibit the growth of a microorganism or the minimum bactericidal concentration (MBC), i.e. the lowest concentration which prevents the growth of a microorganism (Andrews 2001; Kowalska-Krochmal and Dudek-

Wicher 2021; Rauseo et al. 2021). In particular, if environmental antibiotic residues correspond to its MBC, it can affect microbial biodiversity, eliminating one or more microbial populations and their corresponding functions. On the contrary, if antibiotic concentrations are lower than MIC, ABs can favour the development of resistance mechanisms. In the latter case the antibiotic concentration is defined as minimum selective concentration (MSC), environmental threshold concentration which selects for resistant bacteria (Le Page et al. 2017; Chow et al. 2021; Rauseo et al. 2021). Occurrence of microbial populations capable to resist to an antibiotic means that they can spread antibiotic resistance genes (ARGs) among different species through horizontal gene transfer (HGT) mechanisms. Several ARGs located on mobile genetic elements (MGEs) such as plasmids or conjugative transposons are facilitated to be transferred horizontally to other microorganisms. The transfer between different species can occur in conditions with high cell densities of plasmid donors and recipients, nutrient availability, and various selective pressures (Hudson et al. 2017; Blau et al. 2018). Plasmids offer new adaptive tools to bacteria for host populations and provide fitness advantages under several selective conditions, including pollutants and/or antibiotic exposure (Smalla et al. 2015; Shintani et al. 2020). However, plasmid carriage also implies a metabolic burden that, in the absence of selective pressure, represents a fitness cost for the microorganisms (Rajer and Sandegren 2022). Therefore, depending on the ecology of a microorganism, the carriage is convenient only if the fitness cost is balanced by the benefit of resistance in specific environmental conditions. Otherwise microorganisms can re-adapt to the new conditions (e.g. environmental pressures relieved or no longer metabolically advantageous) and lost the un-needed plasmid during bacterial cell division (Carroll and Wong 2018).

Antimicrobial resistance mechanisms can be categorized in three groups characterized by levels of growing specific resistance to a particular molecule. The first level is the formation of biofilms to protect from and overcome the action of antimicrobial agents. The second level consists in modification of cell walls, cell membranes and the expression of efflux pumps. If antimicrobial agents overcome these two first resistance levels and enter cytoplasm, bacteria develop a third line of defence, involving gene regulation and antibiotic resistance genes spread in a bacterial community (Zhou et al. 2015). The antimicrobial resistance genes generally found in an environment depend on current or previous exposure of a specific matrix (e.g. water, soil and air) to their corresponding antibiotics. However, ARB and ARG occurrence and persistence in natural environments is a complex phenomenon, only partially known and that needs to consider that ABs are generally present with other contaminants (e.g. heavy metals) and/or stress factors (Grenni and Corno 2019; Maurya et al. 2020).

Heavy metals are ubiquitous soil contaminants and their presence in concentrations higher than is natural poses a risk because of their toxicity, bioaccumulation and biomagnification (Memon and Schröder 2009; Alloway BJ 2013; Barra Caracciolo and Terenzi 2021). They are normally present in ecosystems as "trace elements" (Tchounwou et al. 2012; Ancona et al. 2020), since many minerals and rocks can contain and release them through erosion and water dissolution. Some heavy metals are essential micronutrients for plants, microorganisms (key co-factors of several enzyme reactions) and organisms at higher trophic levels (e.g. copper, zinc, iron, nickel). Other HMs (e.g. mercury, cadmium, lead and arsenic) are non-essential elements (Tchounwou et al. 2012; Ali et al. 2019). Unlike organic substances, they are non-biodegradable and can become toxic for biota if present in higher concentrations than natural ones, or in case of chronic exposures (Igiri et al. 2018; Ali et al. 2019; Vardhan et al. 2019). Several types of generic or specific resistance can be expressed by microorganisms (Barra Caracciolo and Terenzi 2021). The most common bacterial responses to excess of heavy metals are modifications in cell membranes and development of efflux pumps, for one or more heavy metals (Chandrangsu et al. 2017). Bacteria can also limit metal ion excess activating the expression of specific sets of genes in regulons, which are typically controlled by a metal-sensing regulatory transcription factor, such as metallo-regulatory proteins. HM homeostasis primarily relies on modulating their transport into and out of a cell. However, adaptation to metal is a complex phenomenon and can pose an intoxication risk for the bacterial cell (Becker and Skaar 2014; Chandrangsu et al. 2017). In the case of copper, several bacterial genera (e.g. *Acinetobacter* or *Pseudomonas*) can bio-adsorb copper on cell membranes and immobilized it (Barra Caracciolo and Terenzi 2021). Moreover, Cu can induce activation of the cop operon which codifies for metallo-regulatory proteins involved in its homeostasis (Odermatt and Solioz 1995; Reyes-Caballero et al. 2011; Chandrangsu et al. 2017).

Recent works report that HMs can contribute to promotion of antibiotic resistance through co-selection mechanisms (Fig. 5), (Mazhar et al. 2021; Gillieatt and Coleman 2024). These phenomena occur when genes codifying for resistance to both antibiotics and metals are located on the same mobile genetic elements (co-resistance), or a single resistance mechanism (e.g. an efflux pump) confers resistance to both antibiotics and biocides/metals (cross-resistance), (Ozawa et al. 2023; Wang et al. 2024b). Several studies have demonstrated co-occurrence of metal resistance genes (MRGs) and multiple antibiotic resistance genes (ARGs) in the same plasmids (Li et al. 2015; Li et al. 2017; Congilosi et al. 2022; Wang et al. 2024b). In addition, another mechanism, the co-regulation one, has been described as a co-selection phenomenon. In this case a single bacteria regulator (a protein involved in communication with cell external environment and control of bacterial homeostasis) acts controlling the transcriptions of a panel of genes

conferring resistance to various substrates such as Cu and ABs (Mc Carlie et al. 2020; Virieux-Petit et al. 2022; Huo et al. 2024). For example, high concentrations of periplasmic Cu, can activate the *Psuedomonas aeruginosa* two-component system CopR/S, responsible for an indirect down-regulation of OprD synthesis, resulting in resistance to the imipenem carbapenem antibiotic (Ducret et al. 2016; Virieux-Petit et al. 2022).

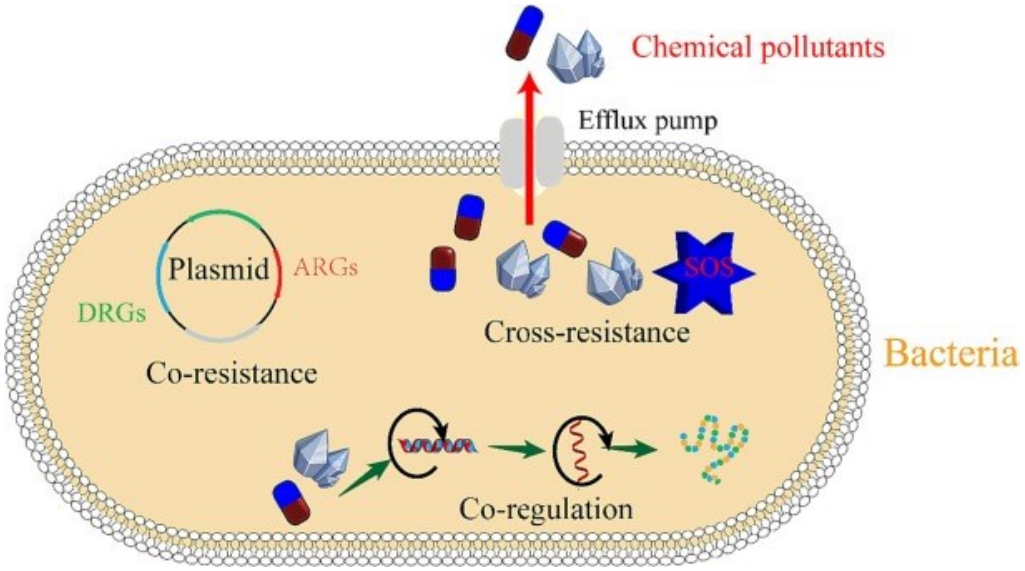

Figure 5. Illustration of the main co-selection mechanisms (co-resistance, cross-resistance and co-regulation) in a bacterial cell. ARGs: Antibiotic resistance genes; DRGs: other drug resistance genes (Huo et al. 2024).

Antibiotic and heavy metal effective concentrations

HMs are regulated as pollutants in water by the EU Water Frame Directive and in food by the EU 2023/915 Regulation (EU) (maximum admissible levels for lead, cadmium, mercury, inorganic tin and arsenic). However, in the case of soil, the EU legislation provides heavy metal concentration threshold limits only for agricultural soils and regarding "the Ecolabel criteria for growing media and soil improvers" (EU 2022/1244 Commission Decision). In the case of Cu, the concentration limit is 200 mg/kg for both soil improvers and growing media. Moreover, the European Commission has adopted the EU 2018/1981 Regulation to restrict copper use in agricultural soils. In particular, the use of plant protection products containing copper compounds has been restricted to a maximum application rate of 28 kg/ha of copper over a period of 7 years (i.e. on average 4 kg/ha/year).

The Italian legislation (Dlgs 152/2006) provides threshold concentration limits for HMs in contaminated soils, with different values depending on their use (e.g. for Cu: 600 mg/kg for industrial and 120 mg/kg for green areas) and other limits (e.g. for Cu: 200 mg/Kg) for agricultural areas (DM 56/2019).

On the other hand, currently, there are not legal limits in soils for emerging contaminants including antibiotics (Gomes et al. 2017; Sardiña et al. 2019; Chaturvedi et al. 2021).

To establish threshold limits for a compound, it is necessary to know its intrinsic toxicity, its action mechanisms and target sites of action inside a cell (McCarty et al. 2020; Schwenk and Burr 2021). Moreover, depending on its intrinsic chemical structure, and properties a chemical can differently interact with a specific soil matrix (Hong et al. 2023).

First of all, the direct effects of a contaminant on biota can be evaluated using ecotoxicological tests which rely on model organisms at different trophic levels (Zaghloul et al. 2020). Ecotoxicological bioassays make it possible to determine the Effective Concentration (EC), generally expressed as mg/L or mg/kg) of a specific chemical compound which causes a particular effect on exposed organisms, (Forfait-Dubuc et al. 2012; Clasen and de Moura Lisbôa 2019). An EC refers to a specific endpoint (i.e. a biological parameter used as a quantitative indicator of the effect of a chemical on an organism) and to an exposure time(Ratte et al. 2003; Palma et al. 2016). Indeed, an effect can be acute (short-term exposure), or sub-chronic or chronic (long-term exposure), (Ratte et al. 2003; Clasen and de Moura Lisbôa 2019; Yuan et al. 2024b). Chronic tests evaluate effects related to a toxic exposure more than 50% of an organism life. Sub-chronic tests evaluate effects related to a toxic exposure less than 10% of an organism life, (APAT 2006). Sub-lethal effects can include both endpoints such as growth and behaviour inhibition and molecular biomarkers (early warning) such as a change in expression of a biological molecule, (APAT 2006). EC generally refers to EC_{50}, i.e. a chemical concentration which causes an effect in half of the exposed population. Ecotoxicological tests generally rely on standard procedures, and this makes it possible to compare different chemicals with same test and to obtain ranks of intrinsic toxicity. Chemical toxicity can be evaluated in single solutions or in mixture. In the case of an environmental matrix, it is also possible to assess the overall toxicity, capturing effects of all possible contaminants and metabolites available (including possible transformation products or not known chemicals), (Goutam Mukherjee et al. 2022; Mariani et al. 2022; Albert and Bloem 2023).

ECs are evaluated in the case of the terrestrial ecosystems, considering both soil and aquatic test organisms, because a contaminant can act its deleterious action directly on the solid matrix or owing to run-off or leaching phenomena on water bodies. Many ecotoxicological tests on soil can be performed in accordance with the standard "UNI EN 2004 leaching test", simulating the “worst-case scenario of leaching of a substance from soil to water (Schultz et al. 2002; Patrolecco et al. 2020).

It is known that heavy metals are toxic at high concentrations for plants (generally from 150 mg/kg and depending on their bioavailability), (Chiou and Hsu 2019; Diaconu et al. 2020). In particular, copper excess can induce a plant stress response with an increase in production of reactive oxygen species (early-warning indicators), (Xu et al. 2024).

Cu is toxic at high concentrations also for soil microorganisms. Shaw et al. (2020) have reported an inhibition of 50% in bacterial respiration and proteolysis activity at concentrations of 483 mg/kg and 663 mg/kg, respectively, although it caused changes in microbial composition at 200 mg/kg. HMs, including copper, have also accumulation capacity in biota (Ali et al. 2019; Ali et al. 2023).

On the other hand, knowledge of antibiotic effects on non-target organisms and in terrestrial environments are quite scarce so far. Cycoń et al. (2019) have reported direct effects on plant early growth and earthworm abundance at relatively high concentrations (from 10 mg/kg to 500 mg/kg). However, the same authors found sub-lethal stress responses (e.g. reactive oxygen species super production and immune cell system variations) at low AB environmental concentrations (2 mg/kg and 0.47 mg/kg, respectively), (Parente et al. 2021; Jin et al. 2022). Another concern regards possible effects on microbiomes associated to plants (Barra Caracciolo et al. 2022) and other terrestrial organisms because they contribute to plant and organism health. In fact, complex organisms, including plants and invertebrates, can be considered as holobionts, i.e. meta-organisms including both the macroscopic host and its symbiotic associated microbiome (Bosch 2012; Youle et al. 2013; Cassidy and Tholey 2014; Hassani et al. 2018).

In the context of revising overall data available on direct and indirect effects of antibiotics on soil organisms, the first work presented in this PhD thesis is a review focused on possible effects of antibiotics on natural organisms belonging to terrestrial ecosystems. This review (published in ***Antibiotics***) focuses on AB impacts on plant, invertebrates and their associated microbiomes. Different endpoints are reported such as lethal effects, mortality and growth inhibition and effects at molecular level (early warning) such as detrimental effects on enzyme activities or activation of oxidative stress responses. ABs can also cause shifts in microbiome composition compromising important symbiotic functions of a holobiont.

Overview of Direct and Indirect Effects of Antibiotics on Terrestrial Organisms

Alessandra Narciso, Anna Barra Caracciolo, Chiara De Carolis

Abstract:

Antibiotics have made it possible to treat bacterial infections, which were in the past untreatable and consequently fatal. Regrettably, their use and abuse in human and veterinary medicine increased occurrence of antibiotic residues in environment with consequent development of antibiotic resistance. The spread of antibiotic resistance bacteria and genes is not limited to

nosocomial environments, but it also involves water and soil ecosystems. The environmental presence of antibiotics and antibiotic resistance genes is a hot topic, but their direct and indirect effects on natural biota are still not clarified. A particular concern involves presence of antibiotics in agroecosystems due to the application of agro-zootechnical waste (e.g. manure and biosolids), which can introduce antibiotic residues and resistance genes in soils. This review provides an insight of recent findings of AB effects on terrestrial organisms, focusing on plant and invertebrates. Among the most observed impacts, the direct effects in terms of mortality and organism growth have been found mostly at amounts higher than environmental ones. Soil invertebrates are more sensitive to antibiotics compared to plants, but several plant physiology mechanisms (sub-lethal effects) can be also influenced at antibiotic concentrations close to environmental ones (µg/kg). For example, plants show oxidative stress responses such as production of reactive oxygen species (ROS). ROS can be used as biomarkers of AB presence and early-warning of possible effects. Finally, effects on plant or invertebrate can be due to changes in their associated microbiome structure and this can have negative influence in several physiological functions, such as plant or animal capacity to respond to stress from pests or chemicals.

antibiotics

MDPI

Review

Overview of Direct and Indirect Effects of Antibiotics on Terrestrial Organisms

Alessandra Narciso [1,2], Anna Barra Caracciolo [1,*] and Chiara De Carolis [1,3]

[1] Water Research Institute, National Research Council (IRSA-CNR), SP 35d, km 0.7 Montelibretti, 00010 Rome, Italy; alessandra.narciso@irsa.cnr.it (A.N.); chiara.decarolis@irsa.cnr.it (C.D.C.)
[2] Department of Ecological and Biological Sciences, Tuscia University, Largo dell'Università s.n.c., 01100 Viterbo, Italy
[3] Department of Environmental Biology, La Sapienza' University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
* Correspondence: barracaracciolo@irsa.cnr.it

Abstract: Antibiotics (ABs) have made it possible to treat bacterial infections, which were in the past untreatable and consequently fatal. Regrettably, their use and abuse among humans and livestock led to antibiotic resistance, which has made them ineffective in many cases. The spread of antibiotic resistance genes (ARGs) and bacteria is not limited to nosocomial environments, but also involves water and soil ecosystems. The environmental presence of ABs and ARGs is a hot topic, and their direct and indirect effects, are still not well known or clarified. A particular concern is the presence of antibiotics in agroecosystems due to the application of agro-zootechnical waste (e.g., manure and biosolids), which can introduce antibiotic residues and ARGs to soils. This review provides an insight of recent findings of AB direct and indirect effects on terrestrial organisms, focusing on plant and invertebrates. Possible changing in viability and organism growth, AB bioaccumulation, and shifts in associated microbiome composition are reported. Oxidative stress responses of plants (such as reactive oxygen species production) to antibiotics are also described.

Keywords: antibiotic environmental exposure; microbiomes; antibiotic resistance genes; plants; soil fauna; stress response

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1. Introduction

Antibiotics (ABs) comprise a wide variety of substance classes designed to kill or inhibit microorganisms and their occurrence in ecosystems can significantly alter natural microbial communities [1,2]. Moreover, AB effects on non-target organisms, especially terrestrial ones, are not well known. ABs are used in large quantities to treat or prevent human and animal diseases and in several countries for animal growth promotion [1]. ABs may be natural products, typically bacterial secondary metabolites, semi-synthetic derivatives of natural products, or synthetic substances. Currently 250 different antibiotics are registered for use in human and veterinary medicine [2–4]. Once administered, only a part of an AB is metabolized in a treated organism and between 10–100% of this kind of chemical can be excreted in unchanged form or as a metabolite [5]. Consequently, ABs can reach wastewater treatment plants (human origin) and natural environments (livestock origin). Common agricultural practices, such as organic fertilization through manure or biosolid applications, and reclaimed water irrigation [6–8], also introduce AB residues to terrestrial ecosystems [9]. Moreover, from soil, antibiotics can reach surface water via runoff and/or be leached to groundwater [10,11]. AB residues in organic waste, such as manure, sewage sludge or biosolids, can range from a few ng/kg to mg/kg soil [6,12–14] and agricultural soils are recognized to be an AB reservoir [14,15]. Currently, ABs are considered widespread emerging environmental micro-contaminants because they are present in both water and soil [16–18].

AB occurrence in ecosystems can directly alter natural microbial community biodiversity and functioning, with consequences for nutrient cycling and biodegradation of contaminants [16,19–23]. Several studies have underlined the decrease in microbial diversity and inhibition of microbial growth and activities, with consequences for the ecological functions involved in key nutrient transformation [24–27]. On the other hand, AB residues can modify natural microbial communities, by exerting a selective pressure and promoting the spread of antibiotic resistance gene in ecosystems [28–30], with consequences for human and animal health because of antibiotics becoming ineffective [31,32].

Microorganisms are ubiquitous and live in close association with plants and animals, including humans. Animals and plants are no longer viewed as autonomous entities, but rather as "holobionts" [33–35], composed of a host plus all of its symbiotic and non-symbiotic microorganisms (microbiomes).

Microbiomes are fundamental to nearly every aspect of host form, function and fitness, including in traits that once seemed untouched by microbiology: behaviour [36–38], sociality [39,40], and the origin of species [31]. The conviction that microbiology has a central role in the life sciences has been growing, and microbial symbiosis is becoming a central branch of knowledge in the life sciences [41–43]. For this reason, it is crucially important to increase our knowledge of the direct and indirect effects (e.g., on microbiomes) of ABs.

2. Environmental Concentrations of Antibiotics in Soils

AB concentrations in the environment vary; their prescribed dosages, treatment times and drug metabolism in a treated organism influence environmental loads. In turn, AB residues in ecosystems depend on their possible degradation. The biodegradation of an antibiotic is linked to the presence of microbial populations resistant to its detrimental effects [44] and at the same time with an acquired ability to degrade it [45–47]. Abiotic parameters (such as temperature, water and oxygen availability, and organic matter content) can affect biodegradation [47]. ABs introduced into soils can persist for long periods due to their recalcitrance to degradation or their continuous introduction into the environment (pseudo-persistence), [48]. Indeed, antibiotic residues have different environmental fates in soils depending on their intrinsic characteristics (e.g., solubility and photostability) and they can migrate to water bodies by run-off or leaching or be adsorbed to soil [8,15]. ABs in soils have spatial and temporal variations due to land use, human disturbance, climatic conditions and biological activities [14,49–51]. The co-presence of various antibiotics and/or other biocides in soil can influence AB degradation and contribute to the overall toxic effects [3,11,52].

Tetracyclines (TCs), fluoroquinolones (FQs), sulfonamides (SFs), are among the most frequently antibiotics detected in terrestrial ecosystems, because they are widely prescribed in both human and veterinary medicine [53–55].

Tetracyclines (TCs), a class of broad-spectrum antibiotics that have as target the bacterial ribosomes [56], have been found at high concentrations in animal manure [12,13,57] and particularly in pig manure [51,58]. Hu et al. [6] detected oxytetracycline (OTC), tetracycline (TET) and chlortetracycline (CTC) in pig manure at concentrations up to 183.5 mg/kg, 43.5 mg/kg and 26.8 mg/kg, respectively. Moreover, a soil fertilized with this manure was analysed and OTC (2.68 mg/kg), TET (0.11 mg/kg) and CTC (1.08 mg/kg) residues were also found, showing how pig manure is an AB source [6,51].

Fluoroquinolones are used to treat various human and animal diseases as they inhibit bacterial DNA synthesis/replication by targeting DNA gyrase and topoisomerase [59]. FQs are known to be persistent compounds [59–61]. Ciprofloxacin (CIP), enrofloxacin (ENR) and norfloxacin (NOR) are commonly found in animal excreta. In poultry litter these antibiotics have been detected at concentrations up to 45.6 mg/kg, 1420.8 mg/kg and 225.4 mg/kg for CIP, ENR and NOR, respectively [62]. Other authors found FQs at higher amounts in pig (CIP: 34 mg/kg, ENR: 33.3 mg/kg and NOR: 5.5 mg/kg) than cattle manure

(CIP: 29.6 mg/kg; ENR: 46.7 mg/kg and NOR: 2.76 mg/kg [60]. Soil contamination from CIP, ENR and NOR can reach values up to 5.6 mg/kg [14,51,63].

Finally, Sulfonamides act inhibiting nucleic acid synthesis and interfering with folic acid metabolism [51]. Sulfadiazine (SD), and Sulfamethoxazole (SMX), have been also detected in manure, but at lower concentrations (ranging from μg/kg to mg/kg) than FQs [14,51,64]. Consequently, SMX residues in agricultural soil have been found only up to 90 μg/kg [14,51,65,66]. This is because sulfonamides are intrinsically less persistent than FQs. For example, Rauseo et al. [67], in a soil amended with cattle manure, evaluated SMX halving in only 7 days. In this study, the antibiotic sulfamethoxazole, added at an initial high concentration (20 mg/kg) in a soil amended with anaerobically digested cattle manure, displayed an initial detrimental effect on the microbial abundance. At the same time, it promoted a prompt increase in the prevalence of the *intI1* gene (known proxy for environmental antibiotic resistance). A decrease in Sulfamethoxazole over time in a digestate-amended soil was associated with an increase in microbial abundance, suggesting a selection of resistant microbial populations able to biodegrade it.

AB residues in the environment can affect ecosystems in different ways. However the effects on non-target organisms and particularly terrestrial organisms have been poorly investigated so far [68–72]. There is particular interest in the possible effects on the microbiomes of plants and soil fauna. It has been recently recognized that microbiomes associated with plants and other terrestrial organisms contribute to their health status and a biocide can alter these positive interactions [73–75].

The present study gives an overview of the direct and indirect effects of ABs, by considering different trophic (e.g., microorganisms, plants and soil fauna) and biological hierarchical levels (from molecular to ecosystem levels), and single species (e.g., plant, worm) and synergic interactions between species (e.g., plant-microbiome system), and ecosystems [76–78].

3. Antibiotic Effects on Terrestrial Plants

The possibility that antibiotics can cause alterations in plant germination, growth and physiology, and act as an abiotic stress has been recently investigated [79–81].

The evaluation of the direct effects of antibiotics on plant germination and growth can be performed applying ecotoxicological studies relying on standard methods (e.g., ISO 11269-2:2012 [82]), using both model target plant species (e.g., *L. sativum*) and common vegetables (e.g., lettuce, rice), and/or soil microcosms [30]. Moreover, the impact of antibiotics can also be evaluated by considering the molecular targets of vegetal cells, in terms of plant genotoxicity [83,84].

For example, Liu et al. [85] evaluated the effects of six antibiotics (chlortetracycline, tetracycline, tylosin, sulfamethoxazole, sulfamethazine) on early growth (germination and root elongation) of different plant species (sweet oat, rice, and cucumber). They evaluated effective concentrations (ECs), in terms of EC_{50}, (EC on 50% of the seedlings tested). Chlortetracycline had the highest negative impact on *Oryza sativa* (EC_{50}: 16 mg/L) and sulfamethoxazole on *Cucumis sativus* (EC_{50}: 8 mg/L). The authors concluded that acute phytotoxic effects varied with the antibiotics and plant species tested.

In another study, Pan et al. [86] assessed whether there was inhibition of root elongation of several edible plants (i.e., lettuce, tomato, cucumber, and carrot) by different antibiotics (tetracycline, sulfamethazine, norfloxacin, erythromycin and chloramphenicol). *Cucumis sativus* was found to be less sensitive to tetracycline (EC_{50}: 34.8 mg/L) than chlortetracycline [86]. This result can be ascribed to the presence of the chloride in CTC, which is a known toxic element [87,88]. Among all plants, *Daucus carota* was found to be the most sensitive and tetracyclines to be the most toxic antibiotics for all species tested (Table 1).

More recently, Mukhtar et al. [89] compared five antibiotics, such as levofloxacin, ofloxacin, ciprofloxacin, ampicillin, and amoxicillin separately (at 10 mg/kg each) or combined with different types of organic amendment (i.e., rice husk, poultry litter), to evaluate their acute and chronic effects on *Oryza sativa*. Ciprofloxacin and ofloxacin displayed an

acute effect, evaluated in terms of the germination index (IG%). Levofloxacin had only a chronic effect (% Growth inhibition at 4 months), with a decrease in shoot length and biomass. The same authors demonstrated that these effects were alleviated if the organic amendments were present [89].

The overall ecotoxicological results here reported suggest that concentrations effective on plant early growth range from 10 mg/L to values higher than 500 mg/L (Table 1), and these values are much higher than soil residual concentrations, excluding a risk for plant development in a real environmental contamination scenario.

However, the impact of antibiotics has also been evaluated by considering plant genotoxicity, such as DNA damage (e.g., single or double strand breakage). Evans-Roberts et al. [90] hypothesized that the biocide activity of fluoroquinolones, which have bacterial DNA gyrases (an enzyme involved in replication and/or repair in prokaryotes) as targets, can also inhibit chloroplast and mitochondrial enzymes involved in DNA replication. This is because they are similar and have an archetypal prokaryotic enzymatic structure [90,91]. Indeed, Mukhtar et al. [89], performed an ecotoxicological test like the comet assay (OECD 489:2014), and demonstrated that, at 7 days of exposure, fluoroquinolone antibiotics (10 mg/L) caused significant DNA damage to *Oryza sativa* root tips, in comparison with other antibiotics (ampicillin and amoxicillin). On the other hand, the use of organic amendments (e.g., rice husk, poultry litter) reduced antibiotic negative impacts, as mentioned above for the germination endpoint.

Cheong et al. [92] performed a seedling growth test with lentil beans, rice, and *Napa cabbage* and found that sulfamethoxazole, sulfathiazole, sulfadiazine and sulfamethazine had significant effects (at 5 mg/L) on root elongation of the species tested. Because sulfonamides inhibit bacterial dihydropteroate synthase (DHPS), by blocking microbial folate biosynthesis, this result could be ascribed to the effect of these ABs on plant folate metabolism. In fact, these plants have a DHPS similar to the bacterial one.

Considering the detrimental effects of antibiotic residues in soil ecosystems, particular concern also arises about the possibility that plants can uptake, through their roots, antibiotic residues from contaminated soils and bioaccumulate them in their tissue. Consequently, antibiotics can be accidentally ingested by herbivores, entering food chains. This possibility can also involve humans, who consume fresh vegetables [14,93].

Albero et al. [81] simulated exposure conditions in lettuce plant (*Lactuca sativa*) pots by adding organic amendment and spiking a mixture of enrofloxacin, ciprofloxacin, sulfamethazine, sulfamethoxazole and doxycycline, chlortetracycline hydrochloride and lincomycin, (at a concentration of 2.5 mg/kg each). Fluoroquinolones, sulfonamides and lincomycin were detected in plant tissues one month after exposure. Sulfamethoxazole, sulfamethazine and lincomycin were found at the highest concentrations in lettuce shoots with uptake amounts of 0.044, 0.021 and 0.051 mg/kg respectively. These results suggested the various ABs have different bioaccumulation potential in plant tissue, presumably due to their different intrinsic chemical characteristics.

In another study, Cheng et al. [94] evaluated sulfamethoxazole bioaccumulation in lettuce plants, using three different concentrations (100; 200; 300 mg/kg). The antibiotics found in lettuce plants at 120 days of exposure were positively correlated with the initial amount spiked in the soil.

3.1. Antibiotics and Oxidative Stress in Plants

The possibility that antibiotics can cause alterations in plant growth and physiology, and act as an abiotic stress has been described in several studies.

The presence of antibiotics can interfere with the photochemical phases of photosynthesis and with electron flows, (both linked to ATP production). A decrease in photosynthesis rates has a consequent impact on plant growth [80]. Negative effects on photosynthesis and an impairment of antioxidant metabolism lead to an increase in the production of reactive oxygen species (ROS) [95]. In response to ROS stress, plants produce enzymes such as catalase (CAT), superoxide dismutase (SOD) and peroxidase (POD) to counteract

their presence [80]. In many cases, although there is an increase in production of enzymes, which degrade ROS, this response may be not sufficient to maintain a balance between ROS production and their degradation, and oxidative damage and a consequent growth inhibition occur [80].

Li et al. [79] tested oxytetracycline and enrofloxacin at various concentrations (5, 10, 20, 40 and 80 mg/L) on *Triticum aestivum* L. (wheat), evaluating the plant response in terms of seedling growth, root elongation and antioxidants. No toxic effects were found on seed germination, however, a reduction in root length, fresh weight and surface area was observed, depending on the AB tested (Table 2). From the lowest concentrations of 5 mg/L of ENR and 10 mg/L of OTC, a reduction in plant growth was found. Moreover, the authors also observed that ENR and OTC with concentrations higher than 20 mg/L caused a significant ($p < 0.05$) increase in CAT, SOD, POD activity and malondialdehyde (MDA) content in shoots and roots. The decrease in root and seedling growth was presumably ascribed to a high ROS production. Moreover, at all ENR concentrations tested, an increase in shoots and roots of abscisic acid (ABA), a plant hormone which plays a key role in multiple plant functions, including dealing with environmental stress, was observed. In the case of OTC an increase of ABA content was found only in roots and from 10 mg/L. ABA can be considered an early-warning biomarker.

Khan et al. [96] analysed the effects on *Brassica rapa* ssp. Chinensis grown in a soil with tetracycline or oxytetracycline or norfloxacin, (100 mg/kg each antibiotic). They found effects on plant growth, chlorophyll, and antioxidant activities. In the TET, OTC, and NOR contaminated soils they found a decrease in plant height of 20%, 12%, and 20%, respectively; fresh weight of 11%, 12%, and 7.0%, respectively and Fv/Fm (Chlorophyll fluorescence parameter) of 9%, 6%, and 2%, respectively, (compared to control plants). Moreover, antibiotic stress increased antioxidant enzyme activities and MDA in shoots of treated plants.

In another study, Jin et al. [97] tested different concentrations (2, 10, 20, 50 mg/L) of ENR, NOR and levofloxacin (LEV) to assess the toxic effects of these quinolones on *Arabidopsis thaliana*. At 7 days, the growth of *A. thaliana* was significantly inhibited, and the leaves turned yellow at 10 mg/L of the antibiotics. The treatment with 50 mg/L ENR resulted in a 33% reduction in fresh leaf weight compared to a control. They observed higher ion leakages of 2.54 (LEV), 2.68 (NOR) and 3.17 (ENR) times than the control at 5.0 mg/L of the antibiotics. Chlorophyll fluorescence parameter (Fv/Fm) values decreased with the increase in AB concentrations. At 2, 10, 20, 50 mg/L of antibiotics, a respective decrease in 5.33%, 56.69%, 57.92%, 73.22% for ENR; 3.42%, 6.42%, 50.96%, 61.48% for NOR; 3.01%, 6.15%, 36.07% for LEV in aged leaves, was observed. In this study, higher relative ROS levels led to an increase in MDA content, with enrofloxacin showing the highest effect. The MDA content in the 50 mg/L ENR treatment was 1.17 times higher than that of levofloxacin.

Table 1. Antibiotic effects on plant early growth (germination, biomass, length) and physiology.

Antibiotic	Plant Species	Effect	Reference
Sulfamethoxazole (SMX) Sulfathiazole (STZ) Sulfadiazine (SDZ) Sulfamethazine (SMZ)	*Brassica campestris* *Lens culinaris* *Oryza sativa*	primary root length (EC_{50}: 5 mg/L)	[92]

Table 1. *Cont.*

Antibiotic	Plant Species	Effect	Reference
ciprofloxacin (CIP) levofloxacin (LEV) ofloxacin (OFL) amoxicillin (AMX) ampicillin (AMP)	*Oryza sativa*	LEV (10 mg/kg): significant decrease in root/shoot length and biomass reduction CIP (10 mg/kg): significant reduction in seedling vigour index CIP and AMX (10 mg/kg each): significant decrease in root/shoot length and maturity stage. OFL and LEV (10 mg/kg each): reduction in P assimilation All ABs (10 mg/kg each) showed genotoxicity at root tips (DNA damage, evaluated by the comet assay).	[89]
tetracycline (TET) sulfamethazine (SMZ) norfloxacin (NOR) erythromycin (ERY) chloramphenicol (CLP)	1. *Lactuca sativa* 2. *Daucus carota* 3. *Cucumis sativus* 4. *Lycopersicon esculentum*	Root elongation—TET 1. EC_{10}: 0.11 mg/L; EC_{50}: 14.4 mg/L 2. EC_{10}: 0.26 mg/L; EC_{50}: 10.3 mg/L 3. EC_{10}: 0.43 mg/L; EC_{50}: 34.8 mg/L 4. EC_{10}: 0.1 mg/L; EC_{50}: 11.6 mg/L Root elongation—SMZ 1. EC_{10}: 1.94 mg/L; EC_{50}: 157 mg/L 2. EC_{10}: 25 mg/L; EC_{50} > 300 mg/L 3. EC_{10} > 300 mg/L; EC_{50} > 300 mg/L 4. EC_{10}: 5.83 mg/L; EC_{50} > 300 mg/L Root elongation—NOR 1. EC_{10}: 0.61 mg/L; EC_{50}: 49 mg/L 2. EC_{10}: 13 mg/L; EC_{50}: 109 mg/L 3. EC_{10}: 0.93 mg/L; EC_{50}: 75 mg/L 4. EC_{10}: 1.1 mg/L; EC_{50}: 32 mg/L Root elongation—ERY 1. EC_{10}: 0.85 mg/L; EC_{50}: 69 mg/L 2. EC_{10}: 8.6 mg/L; EC_{50} > 300 mg/L 3. EC_{10}: 22.5 mg/L; EC_{50} > 300 mg/L 4. EC_{10}: 25.9 mg/L; EC_{50} > 300 mg/L Root elongation—CLP 1. EC_{10}: 2.52 mg/L; EC_{50}: 204 mg/L 2. EC_{10}: 102 mg/L; EC_{50} > 300 mg/L 3. EC_{10}: 10.2 mg/L; EC_{50} > 300 mg/L 4. EC_{10}: 29 mg/L; EC_{50} > 300 mg/L	[86]
chlortetracycline (CTC) tetracycline (TET) tylosin (TYL) sulfamethoxazole (SMX) sulfamethazine (SMZ)	1. *Oryza sativa* 2. *Cucumis sativus* 3. *Cichaorium endivia*	Root elongation—CTC 1. EC_{10}: 0.2 mg/L; EC_{50}: 16 mg/L 2. EC_{10}: 8 mg/L; EC_{50}: 39 mg/L 3. EC_{10}: 0.7 mg/L; EC_{50}: 48 mg/L Root elongation—TET 1. EC_{10}: 14 mg/L; EC_{50}: 57 mg/L 2. EC_{10}: 16 mg/L; EC_{50}: 69 mg/L 3. EC_{10}: 8 mg/L; EC_{50}: 203 mg/L Root elongation—TYL 1. EC_{10}: 19 mg/L; EC_{50}: 141 mg/L 2. EC_{10} > 500 mg/L; EC_{50} > 500 mg/L 3. EC_{10}: 217 mg/L; EC_{50} > 500 mg/L Root elongation—SMX 1. EC_{10}: 16 mg/L; EC_{50}: 69 mg/L 2. EC_{10}: 0.1 mg/L; EC_{50}: 8 mg/L 3. EC_{10} > 300 mg/L; EC_{50} > 300 mg/L Root elongation—SMZ 1. EC_{10}: 2 mg/L; EC_{50}: 37 mg/L 2. EC_{10}: 6 mg/L; EC_{50}: 45 mg/L 3. EC_{10}: 6 mg/L; EC_{50} > 300 mg/L	[85]

Table 1. *Cont.*

Antibiotic	Plant Species	Effect	Reference
oxytetracycline (OTC) enrofloxacin (ENR)	*Triticum aestivum* L.	OTC (10 mg/L) Reduction in root length (18.6%), biomass (19.8%), surface area (24.8%) and an increase in abscisic acid (ABA) content ENR (5 mg/L) Reduction in root length (29.6%), biomass (32.5%), surface area (35%) and an increase in abscisic acid (ABA) content	[79]
tetracycline (TET) oxytetracycline (OTC) norfloxacin (NOR)	*Brassica rapa* ssp. *chinensis*	TET (100 mg/Kg) 20% plant height reduction; 11% biomass reduction; 9% Fv/Fm reduction OTC (100 mg/Kg) Reduction in plant height (12%); fresh weight (12%) and Fv/Fm (6%) NOR (100 mg/Kg) Reduction in plant height (20%), fresh weight (7%) and chlorophyll fluorescence parameter (Fv/Fm) (2%)	[96]
enrofloxacin (ENR) norfloxacin (NOR) levofloxacin (LEV)	*Arabidopsis thaliana*	ENR Reduction in fresh leaf weight (33%, with 50 mg/L), in chlorophyll fluorescence parameter (Fv/Fm) (5.33%, 56.69%, 57.92%, 73.22% with a concentration of 2, 10, 20, 50 mg/L) and increase in ion leakage (3.17%, with 5.0 mg/L) NOR Increase in ion leakage (2.68%, with 5.0 mg/L) and a reduction in chlorophyll fluorescence parameter (Fv/Fm) (3.42%, 6.42%, 50.96%, 61.48% at a concentration of 2, 10, 20, 50 mg/L) LEV Increase in ion leakage (2.54% with 5.0 mg/L) and a reduction in chlorophyll fluorescence parameter (Fv/Fm) (3.01%, 6.15%, 36.07% at a concentration of 2, 10, 20, 50 mg/L) The MDA content at 50 mg/L ENR was 1.17 times higher than LEV treatment group.	[97]

3.2. Antibiotic Effects on Plant-Microbiome System

Soil antibiotic contamination can not only affect plant growth and physiology, but also influence its associated microbiome. Microorganisms are present both on and inside plant tissues, and especially at root level (rhizosphere). The plant microbiome comprises the rhizosphere, phyllosphere (mainly leaves) and endosphere (bacteria which live inside plant tissue). Healthy plants host symbiotic and non-symbiotic rhizo-epiphytic and/or endophytic microorganisms, which do not cause diseases, but support the host nutritionally, by stimulating germination and growth, or helping plants to overcome biotic or abiotic stress [98,99]. Consequently, it is often hard to distinguish any antibiotic effects on a plant from those on its associated microbiome. Moreover, ABs can select antibiotic resistant bacteria and promote the spread of antibiotic resistance genes (ARGs) among soil microorganisms and plant-microbiomes [3,29].

Zhang et al. [100] demonstrated a shift and an increase in ARGs in a lettuce-associated microbiome grown on manure-amended soils. The poultry or cattle manures used in this pot experiment were characterized by a high beta-lactams and tetracycline presence (antibiotics commonly administered in livestock farming). The authors found a change in plant microbiome composition: *Gammaproteobacteria* became dominant in the phyllosphere and *Alphaproteobacteria* in the rhizosphere. Bacteria multi-resistant to these antibiotics were

found, particularly at root level (Table 2). Moreover, the same authors found a correlation between some phyla (*Firmicutes*, *Chloroflexi*, *Gemmatimonadetes*, *Acidobacteria*) and the ARGs for aminoglycoside, tetracycline, sulfonamide and beta-lactam resistance. *Proteobacteria* were found to be correlated with a vancomycin resistance gene.

In another study, Wen et al. [101] found a significant correlation between a lettuce microbial community (phyllosphere and endosphere) and tetracycline ARGs. As in the previously cited study, the dominant bacteria class was *Proteobacteria*, and it was correlated with the *tetA* gene. Moreover, the *Chelativorans* genus (*Proteobacteria*) found in the endosphere was correlated to the *intI1* gene (Table 2).

Recently, Yin et al. [102] performed a microcosm experiment with cherry radish simulating an antibiotic treatment for controlling plant pathogens. Streptomycin (STR) or oxytetracycline (OTC), at 1.45 mg/kg each, were added at 21 days of plant growth. The application of the antibiotic was repeated each 6 days for four times. At 44 days of plant growth most of the pots were sampled and eight were maintained with added antibiotic concentrations of 14.5 mg/kg each, in line with agricultural practices for large biomass plants [102]. The ABs did not affect radish growth, and OTC stimulated plant development. However, they found a significant AB accumulation in the radish tissues, mainly in the leaves and fruits at 74 days. Moreover, a shift in the plant microbiome was observed: *Proteobacteria* and *Actinobacteria* increased in leaves and roots. *Cyanobacteria* increased in the rhizosphere.

In a recent study, Barra Caracciolo et al. [30] demonstrated that enrofloxacin and ciprofloxacin bioaccumulated in lettuce leaves grown on manure; moreover, antibiotics resistance genes (*ARGs*) were also found. In particular, the *aac-(6′)-lb-cr* gene related to fluoroquinolone resistance was detected as the most abundant one, followed by *sul1* (for sulfonamides resistance). The same study compared two organic amendments, that is cattle manure and its corresponding digestate. Lettuce plants grown on digestate-amended soil showed a significantly lower AB bioaccumulation and ARG presence than those grown in cattle-manure soil. This study showed how organic amendments can potentially transfer ABs and ARGs to edible plants and then to animals and humans feeding on them. Consuming fresh vegetables which contain AB residues and ARGs can be a risk for organisms and their microbiomes [55].

Finally, an AB presence in a plant-microbiome system can also make plants more sensitive/susceptible to various environmental disturbances [71,103].

Table 2 summarizes data from studies on antibiotic and ARG presence in soil and related plants (soil-plant systems) and/or on the effects in terms of microbial composition shifts.

Table 2. Antibiotics and ARGs found in soil-plant systems (1–4 columns) and effects in terms of soil and plant microbiome shifts (5–6 columns).

Antibiotics		ARGs		Microbial Composition		References
Soil	Plant	Soil	Plant Microbiome	Soil	Plant Microbiome	
ABs: 2.5 mg/kg each enrofloxacin (ENR) ciprofloxacin (CIP) sulfamethazine (SMZ) sulfamethoxazole (SMX) doxycycline (DOX) chlortetracycline hydrochloride (CTCC) lincomycin (LIN)	Lettuce: SMX: 0.044 mg/kg fresh weight LIN: 0.051 mg/kg fresh weight SMZ: 0.021 mg/kg fresh weight	-	-	-	-	[81]

Table 2. *Cont.*

Antibiotics		ARGs		Microbial Composition		References
Soil	**Plant**	**Soil**	**Plant Microbiome**	**Soil**	**Plant Microbiome**	
Sulfamethoxazole (SMX) 100 mg/kg 200 mg/kg 300 mg/kg	Lettuce: 0.084 mg/kg 0.181 mg/kg 0.503 mg/kg	*sul1* *sul2* *tetM* *tetA/P* *tet34* *tetG1* *tetG2* *qnrS1* *qnrS2* *cmlA1* *floR*	-	Control soil microbial community dominated by: *Gaiella*, *Streptomyces*, *Sphingomonas* Soil + SMX dominated by: *Lysobacter*, *Bacillus*	-	[86]
-	-	beta-lactam, aminoglycoside, MLSB, tetracycline-sulphonamide, FCA, vancomycin, MGEs	beta-lactam, aminoglycoside, MLSB, tetracycline, sulphonamid, FCA, vancomycin, MGEs	Soil: *Actinobacteria* and *Deltaproteobacteria* increase, *Cyanobacteria* decrease in AB presence	Root endophytes: *Alphaproteobacteria* increase and *Gammaproteobacteria* decrease; Phyllosphere: *Actinobacteria* decrease	[100]
Swine manure added to the soil and doxycycline (DOX): 84.02 µg/kg sulfamethoxazole (SMX): 86.41 µg/kg tilmicosin (TIL): 69.37 µg/kg	Lettuce	*tetA*; *tetG*; *tetM*; *tetX*, *intI1*	Phyllosphere: *sul2*; *tetA*; *tetG*; *tetM*; *tetQ*; *ermC* *intI1*; *intI2* Endosphere: *tetQ*; *tetL*; *tetA*; *tetO*; *tetX*; *intI1*; *intI2*	*Fluviicola*, *Cohnella*, *Alcanivorax* bacteria correlated with ARGs	Phyllosphere: *Pseudomonas*, *Clostridium_IV* correlated with ARGs Endosphere: *Chelativorans*, *Halomonas*	[101]
ABs: 7.5 mg/kg each Sulfamethoxazole (SMX) Ciprofloxacin (CIP) Enrofloxacin (ENR)	Lettuce: CIP bioaccumulation, with significantly higher values in manure amended than digestate condition	*sul2* *aac-(6′)-Ib-cr* *qepA*	Rhizosphere: *sul2* *aac-(6′)-Ib-cr* *qepA* Phyllosphere: *sul1* *sul2* *aac-(6′)-Ib-cr* *qepA* *sul1* *tnpA*	Significant increase in *Bacilli* and *Bacteroida* in antibiotic and manure amended conditions Significant decrease in *Actinobacteria* and *Alphaproteobacteria*	Rhizosphere: significant decrease in *Actinobacteria*, *Alphaproteobacteria* and *Bacilli* in antibiotic and manure amended conditions Phyllosphere: significant increase in *Bacilli* and *Gammaproteobacteria* in antibiotic and manure amended conditions	[30]

Table 2. *Cont.*

Antibiotics		ARGs		Microbial Composition		References
Soil	**Plant**	**Soil**	**Plant Microbiome**	**Soil**	**Plant Microbiome**	
Oxytetracycline (OTC) Streptomycin (STR) Calculated soil concentration of 59.45 mg/kg at 74 days	Cherry radish: Significantly higher presence of STR in plant tissue in comparison with OTC Phyllosphere: STR 0.3 mg/kg OTC 0.003 mg/kg Fruits: STR 0.2 mg/kg OTC 0.005 mg/kg Soil STR 0 mg/kg OTC 0.007 mg/kg	-	-	At 74 days no more *Firmicutes* detected and general decrease in *Actinobacteria.* Increase in *Poteobacteria* and *Chloroflexi* in OTC condition; decrease in *Actinobcteria.* Increase in *Gemmatimonadetes* in STR condition	At 74 days Phyllosphere: decrease in *Bacterioidetes.* Increase in *Cyanobacteria* and decrease in *Firmicutes* in antibiotic conditions. Fruits: increase in *Actinobacteria* in OTC condition. Root Endophytes: increase in *Chloroflexi.* Increase in *Proteobacteria* and *Actinobacteria* in OTC and STR conditions, respectively	[102]

4. Effect on Terrestrial Invertebrates

As mentioned above, environmental exposure to antibiotics can influence natural organism growth in different ways. It is still unclear if antibiotics are directly harmful for terrestrial invertebrates, or if they act indirectly by affecting their associated microbiomes [104–106]. Antibiotics can display different effects depending on their action mechanisms and on the type of terrestrial organisms exposed [107]. Clarifying which antibiotics are the most detrimental for the most susceptible organisms in an ecosystem has great significance in evaluating the potential ecological hazards of these compounds. Soil fauna (e.g., earthworms), which play a central role in soil nutrient bioavailability by contributing to soil fertility, can be impacted by antibiotic residues from human and organic waste. For example, earthworms can bioaccumulate antibiotics through the ingestion of contaminated fine particles or absorb them through their skin [108].

Zhao et al. [109] exposed earthworms to a multi-antibiotic-contaminated agricultural soil derived from a long-term manure exposure. Twelve antibiotics belonging to the quinolones, tetracyclines and sulfonamide categories were identified, and their concentrations ranged from 0.015 to 0.33 µg/kg for each substance. The results showed a decline in earthworm abundance with increased antibiotic concentrations. Tetracyclines, ofloxacin and sulfamethazine were negatively correlated with earthworm abundance, but only sulfamethazine and oxytetracycline caused a significant decrease in earthworm biomass. A depletion in abundance of earthworms, which can increase soil fertility through their activity and metabolism [110–112] can lead to a decrease in nutrient availability for plants, influencing vegetal growth negatively [112].

Parente et al. [113] exposed *Eisenia andrei* earthworms to a soil amended with different quantities of poultry litter contaminated by fluoroquinolones. Ciprofloxacin (CIP) and enrofloxacin (ENR) maximum concentrations in the poultry litter were 6.74 and 23.6 mg/kg, respectively. The authors observed that all individuals tested escaped the contaminated soil, showing so called "avoidance behaviour" [114,115] at 48 h of exposure and for all the concentrations tested. Moreover, they estimated a lethal concentration (LC_{50}) at 7 and 14 days and found a significant decrease in biomass. Moreover, dead worms, at the end

of the test, showed morphological changes, such as swelling, partition and bottlenecks. The authors also performed chronic tests [116,117], which highlighted a significant effect on worm reproduction (Table 3). Moreover, they found sub-lethal effects on the immune system, in terms of amoebocyte and eleocyte cell variation (e.g., cell density, feasibility and typing). The overall effective concentrations found are reported in Table 3.

Table 3. Antibiotic ecotoxicity on terrestrial invertebrates.

Antibiotic	Species	Effect	References
chlortetracycline (CTC) oxytetracycline (OTC) doxycycline (DOX) tetracycline (TET) norfloxacin (NOR) ofloxacin (OFL) lomefloxacin (LOM) ciprofloxacin (CIP) enrofloxacin (ENR) sulfamethoxazole (SMX) sulfamerazine (SMR) sulfamethazine (SMZ)	*Eisenia fetida*	All ABs decreased earthworm abundance ($p < 0.05$) SMZ and OTC also decreased earthworm biomass	[109]
ciprofloxacin (CIP) enrofloxacin (ENR)	*Eisenia Andrei*	CIP LC_{50} (7 days): 0.25 mg/kg LC_{50} (14 days): 0.19 mg/kg biomass decrease (14 days) at 0.27 mg/kg Effect on reproduction at 0.14 mg/kg Immune system cell variation between 0.04 to 14 mg/kg ENR LC_{50} (7 days): 0.89 mg/kg LC_{50} (14 days): 0.67 mg/kg biomass decrease (14 days) at 0.94 mg/kg Effect on reproduction at 0.47 mg/kg Immune system cell variation between 0.12 to 0.47 mg/kg	[113]

Ma et al. [118] studied the bioaccumulation of oxytetracycline, as a model for a commonly used antibiotic for livestock, in the *E. crypticus* earthworm body. The environmental concentration used was 10 mg/kg and animals were exposed to the antibiotic for 21 days. The antibiotic concentration significantly increased in body tissues (45.65 mg/kg) and was significantly higher than in the soil (0.45 mg/kg), showing its bioconcentration. Moreover, the same authors demonstrated a deep change in the *E. crypticus* gut microbiome after antibiotic exposure. *Proteobacteria* relative abundance was affected by oxytetracycline and, in particular, the *Moraxellaceae* family significantly decreased from 15.6% to 2.64%. On the contrary, *Planctomycetes* relative abundance increased, in particular the *Isosphaeraceae* family (from 16.9% to 28.5%). This study showed how the earthworm microbiome can be altered by ABs and can be considered a very sensitive indicator of pollution by them.

Ding et al. [119] chronically exposed the *Drawida gisti* earthworm to a soil amended with either sewage sludge, chicken manure or inorganic fertilizers. The authors analysed the microbiome diversity and ARGs in the earthworm gut. In particular, they evaluated multiple drug resistance, (i.e., the insensitivity or resistance of a microorganism to administered antimicrobials which were structurally unrelated and had different molecular targets) [120]. They found the highest gut microbiome multiple drug resistance, beta-lactam resistance and MLSB (Macrolide-Lincosamide-Streptogramin B) resistance in the chicken manure condition. In the condition with the sewage sludge, the authors reported that beta-lactam resistance increased, while in the chicken manure condition tetracycline resistance increased, in line with the different amendment origin [121]. It is known that TCs because

are the most common antibiotics administrated to poultry are found in high amount in poultry manure [122,123].

The latter mentioned studies show that not only antibiotic residues, but also ARGs in soil and organic fertilizers may alter the antibiotic resistome in the gut microbiome of soil fauna, making it an additional reservoir of ARGs in terrestrial ecosystems (Table 4) [119].

Table 4. Antibiotic effects on terrestrial invertebrates and their commensal/associated microbiome.

Antibiotics		Organism Microbiome ARGs	Organism Microbiome Microbial Composition	References
Soil Concentration	Organism Concentration			
oxytetracycline (OTC) 10 mg/kg	*Enchytraeus crypticus*: At 21 days 45.65 mg/kg	-	OTC: decrease in *Proteobacteria* relative abundance, *Moraxellaceae* family from 15.6% to 2.64%. *Planctomycetes* relative abundance increase, Isosphaeraceae family from 16.9% to 28.5%.	[118]
tetracycline (TET) oxytetracycline (OTC) chlortetracycline (CTC) doxycycline (DOX) sulfamethoxazole (SMX) sulfadiazine (SDZ) sulfaquinoxaline (SQX) sulfamonomethoxine (SMT) sulfaclozine sodium (SLC) sulfadimethoxine (SMN) sulfameter (SMT) sulfamerazine (SMR) norfloxacin (NOR) ciprofloxacin (CIP) ofloxacin (OFL) enrofloxacin (ENR) roxithromycin (RXM)	*Drawida gisti*	Chicken manure condition: multiple drug resistance, beta-lactam resistance, MLSB (Macrolide-Lincosamide-Streptogramin B) resistance and tetracycline resistance Sewage sludge condition: beta-lactam resistance	-	[119]

Zhu et al. [124] performed a more complex experiment using soil fauna, agricultural soils and manure. The authors analysed the changes in the gut microbiome composition of *Collembola*, *Nematodes* and *Enchytraeids*. They found a significant change in all invertebrate microbiomes in response to manure exposure. Notably, ARGs abundance was found to be significantly higher in manure conditions compared to control ones, in all taxa tested.

5. Conclusions

Several experimental data show how ABs can have deleterious effects on terrestrial organisms such as plants and soil fauna.

Antibiotics can potentially inhibit seed germination and plant growth; however, the effective concentrations are generally higher than the residual ones found in environment. On the other hand, most common categories of ABs can induce sub-lethal effects at molecular level, such as expression of enzymes linked to stress or DNA damage at concentrations close to environmental ones. The effects found at plant molecular level can be considered biomarkers and early-warning of antibiotic presence in environments. Terrestrial invertebrates have been found more sensitive to ABs than plants. It is still unclear if effects of

ABs on soil organisms can be attributed only to the similarity of some target enzymes to bacterial enzymes, or also to the changes of their associated microbiomes. Particular concern relies on the possible presence of AB in crop species because they can have a reduction in productivity and transfer both antibiotics and ARGs to humans and livestock which feed on them. Further studies are needed to clarify these aspects and can be very useful to increase knowledge on ABs and support national and international plans to combat antibiotic resistance. For example, EU National Plans to Combat Antibiotic Resistance have been created with the aim of providing strategic lines and operational indications to face the emergency of antibiotic resistance in the coming years, following a multidisciplinary approach and a One Health vision, promoting constant international data comparison.

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Abbreviations

amoxicillin (AMX); ampicillin (AMP); antibiotic resistance genes (ARGs); antibiotics (ABs); chloramphenicol (CLP); chlortetracycline (CTC); chlortetracycline hydrochloride (CTCC); ciprofloxacin (CIP); doxycycline (DOX); enrofloxacin (ENR); erythromycin (ERY); fluoroquinolones (FQs); levofloxacin (LEV); lincomycin (LIN); norfloxacin (NOR); ofloxacin (OFL); oxytetracycline (OTC); streptomycin (STR); sulfadiazine (SD); sulfamethazine (SMZ); sulfamethoxazole (SMX); sulfonamides (SFs); tetracycline (TET); tetracyclines (TCs); tylosin (TYL).

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Overall objective of the experimental research

The overall objective of the PhD thesis was to improve knowledge on the impact of pollutants on terrestrial ecosystems. In particular, two classes (antibiotics and heavy metals) of ubiquitous contaminants, introduced in soil through agricultural practices, have been studied. The ecological approach applied made it possible to assess their toxicity at different hierarchical levels of biological complexity (i.e. population, community and ecosystem).

Firstly, ecotoxicological bioassays using as test organism a population of the *Aliivibrio fischeri* bacterium were performed. This experimental part made it possible to investigate the contaminants in single solution, binary, ternary and quaternary mixture in order to identify their intrinsic toxicity, effective concentrations (EC_{20}, EC_{50}) and their possible synergic, antagonistic and additive effects (in terms of Toxic Units). Moreover, the overall toxicity of a real contaminated soil was also evaluated.

Subsequently, microcosm experiments were set-up, using a contaminated agricultural soil, to evaluate the effects of adding organic amendments (manure and digestate) on the structure and activity of the microbial community. Laboratory microcosms represent ecosystem models in which a portion of the natural environment (i.e. contaminated soil) was circumscribed and studied. They contained natural microbial communities maintained under controlled environmental conditions (e.g. temperature, light, humidity), but corresponding to natural ones. The capacity of the soil microbial community, both to resist contaminants (in terms of antibiotic resistance gene spread) and to remove the antibiotic sulfamethoxazole, was evaluated.

Moreover, the contaminant and amendment effects were studied on the lettuce plant-microbiome system, with particular regard to rhizosphere microbiome.

This type of approach made it possible to more firmly establish the causal relationship between antibiotic and copper alone and in mixture and their effects on communities, in particular using microbial communities and plants, as they represent important key to understand the impacts of environmental and anthropogenic factors on ecosystems.

Experimental part

Effects of antibiotic and heavy metal mixture on biota

The agricultural contamination scenario is quite complex because mixtures of contaminant present and for the fact that some of them are regulated, however for others there are not yet legal threshold limits (emerging ones). Moreover, contaminants can interact each other, forming new compounds which can act worsening or buffering the effects of one of them (Altenburger et al. 2018; Goutam Mukherjee et al. 2022). Indeed, the World Health Organization highlighted the need for considering mixture in environmental risk assessment and regulation (Kortenkamp et al. 2019). Regarding soil matrices, it is necessary to consider not only the possible interactions between two or more substances, but also with soil particles and their constituents, as well as organic matter. In fact, these interactions influence the bioavailability of contaminants (Ramakrishnan et al. 2011; Ortega-Calvo et al. 2015).

Knowledge on effects of mixtures of antibiotics and copper has very scarce, however some recent works have evaluated these effects on aquatic organisms (Jijie et al. 2023; Zhang et al. 2024). The authors highlighted the importance in evaluating different mixture combinations considering various concentrations of each compound in a mixture. In fact, even if antibiotics in environment can be found at low concentrations (ng-µg/L), they can interact with copper forming new complexes (Zhang et al. 2012) and displaying synergic effects on organisms (Wang et al. 2022). In particular, Jijie et al. (2023) evaluated single and combined effects of ciprofloxacin (CIP, 5 mg/L) and lead (Pb, 50 µg/L) on zebrafish behaviour, oxidative stress, and essential element content. In all investigated endpoints, the mixture treatment produced more damage compared with single treatments. Moreover, Zhang et al. (2024a) investigated the ecotoxicity of ciprofloxacin-copper (CIP-Cu) mixtures at different water environmental realistic concentrations (0.14-4.41 µg/L of CIP and 0.5-6.5 µg/L of Cu) for the *Microcystis aeruginosa* cyanobacterium. The authors recorded a higher effect of a mixture compared with the single contaminants if the copper concentration exceeded 2.6 mg/L (corresponding to EC_{10}).

In general, the fate and behaviour of chemical mixtures and, in particular, of Cu and ABs in soil, are still poorly investigated (Cipullo et al. 2018). A protective regulation comparable to the EU Water Frame Directive (WFD) still not exists and consequently soil does not receive the same level of legal protection in the EU as air and water. Only on 5 July 2023, the Commission tabled a proposal for a directive on “soil monitoring and resilience” ('Soil Monitoring Law'), (European Commission 2023). The long-term objective of the proposed directive is to have all soils across the EU in healthy condition by 2050, as outlined in the EU soil strategy.

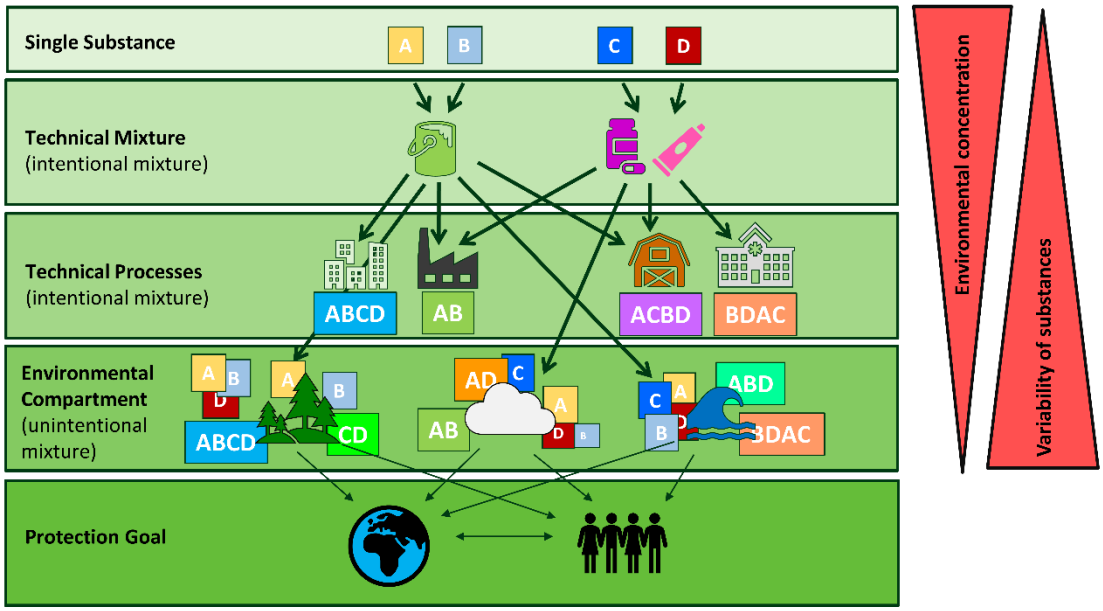

Figure 6. Illustration of different levels and complexity of environmental contamination by chemical mixtures. (Modified from Hassold et al. (2021).

In general, for studying and categorizing combined effects of chemicals, two approaches can be used: the "whole mixture" and "component-based" one (Backhaus and Faust 2012; Altenburger et al. 2018; Kortenkamp et al. 2019; Tralau et al. 2021).

The "whole mixture" directly assesses the toxicity of a chemical mixture, with the advantage of considering all chemicals in a mixture, but the limitation of being only applicable to the mixture investigated, and with no way to extrapolate the results to apply it to different exposure scenarios.

The "component-based" approach establishes the toxicity of a mixture on the basis of the effects of its components, following the two concepts of potential interaction among chemicals: Concentration Addition (CA) and Independent Action (IA). The first assumes a similar activity and a common site of action for all the substances in the mixture and proposes a differentiation in additive effects (if the observed effect is equal to the sum of the effects of the single compounds), synergic effects (if the observed effect is higher in comparison with the sum of the effects of the two compounds) and antagonistic ones (if the observed effect is lower compared with the sum of the effects two molecules alone). The IA implies that the substances act on different molecular targets that led to a common toxicological effect. The CA model can be considered a worst-case scenario, and it is usually employed to assess the toxicity of mixtures thanks to the ready availability of the data required to implement the model and the good toxicity predictions it usually provides (Backhaus and Faust 2012; Riva et al. 2019).

In the second work presented in this PhD thesis, published in **FEMS Microbiology Ecology**, the effects of three antibiotics (Sulfamethoxazole - SMX, Ciprofloxacin - CIP and Chlortetracycline -

CTC) and copper (Cu, Copper sulphate) alone or in different combinations (binary, ternary and quaternary mixtures) were investigated on the *Aliivibrio fischeri* bacterium.

Application of the Aliivibrio fischeri Bacterium Bioassay for Assessing Single and Mixture Effects of Antibiotics and Copper

Alessandra Narciso, Anna Barra Caracciolo, Paola Grenni, Jasmin Rauseo, Luisa Patrolecco, Francesca Spataro, Livia Mariani

Abstract:

The present work aimed to increase knowledge on effects of SMX, CIP and CTC and Cu chemical mixture, using the *Aliivibrio fischeri* test organism. For this purpose, the UNI EN ISO 11348-3:2019 standard ecotoxicological test was performed and various ECs (EC_{20} and EC_{50}) of these chemicals were evaluated in single, binary, ternary and quaternary solutions. Finally, the toxicity of water extracts from a soil contaminated by both the three antibiotics and copper was also evaluated. This approach made it possible to assess potential AB and Cu interactions and additive/synergic/antagonistic effects of the various mixtures.

The ecotoxicological test was performed using the Microtox® analyser (Model 500, Modern Water, UK), a luminometer which records bacterial bioluminescence. Using the connected specific software (Microtox Omni® V 4.2, Milan, Italy), it is possible to compare measured data in a control condition and in presence of a chemical. Effects of single solutions and various mixtures were reported as EC_{50}, (concentration which causes 50% bioluminescence inhibition). The various EC_{50s} were also expressed as Toxic Unit ($TU=1/EC_{50}$).

The mixture TU values measured, were then compared with the predicted TU values, in accordance with the Concentration Addition model (CA).

If the TU measured were equal to those calculated (TU=CA), an additive effect was confirmed and the CA model was met; if the TU values were higher than CA ones (TU>CA), the mixtures displayed synergic effects; finally, the mixtures displayed antagonistic effects if the TUs were lower than CA values (TU<CA).

Copper (EC_{50}=0.78 mg/L) resulted the most toxic compound for *Aliivibrio fischeri* followed by chlortetracycline (EC_{50}=3.64 mg/L). Mixtures generally displayed additive effects. Interestingly, the binary mixture of copper and ciprofloxacin showed a synergic effect and both the binary and ternary mixtures containing chlortetracycline showed antagonistic effects. The water extracts of a contaminated soil did not show any toxicity, evidencing its buffering capacity versus both copper and antibiotics.

The *A. fischeri* bacterium was very sensitive in recording single and combined toxic effects of the chemical compounds tested, demonstrating as the bioluminescence inhibition can be a reliable biosensor.

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Application of the *Aliivibrio fischeri* bacterium bioassay for assessing single and mixture effects of antibiotics and copper

Alessandra Narciso [1,2], Anna Barra Caracciolo [1,*], Paola Grenni [1], Jasmin Rauseo [3], Luisa Patrolecco [3], Francesca Spataro [3], Livia Mariani [1]

[1]Water Research Institute, National Research Council (IRSA-CNR), SP 35d, km 0.7 Montelibretti 00010 (Rome), Italy
[2]Department of Ecological and Biological Sciences, Tuscia University, Largo dell'Università s.n.c., 01100 Viterbo, Italy
[3]Institute of Polar Sciences - National Research Council (ISP-CNR), SP 35d, km 0.7 Montelibretti 00010 (Rome), Italy
*Corresponding author. Water Research Institute, National Research Council (IRSA-CNR), SP 35d, km 0.7 Montelibretti 00010 (Rome), Italy.
E-mail: barracaracciolo@irsa.cnr.it
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Abstract

The *Aliivibrio fischeri* bioassay was successfully applied in order to evaluate the acute effect of sulfamethoxazole (SMX), ciprofloxacin (CIP), chlortetracycline (CTC) and copper (Cu), alone or in binary, ternary, and overall mixture. The toxicity results are reported in terms of both effective concentrations, which inhibited 50% of the bacterium bioluminescence (EC_{50}%), and in Toxic Units (TUs). The TUs were compared with predicted values obtained using the Concentration Addition model (CA). Finally, the toxicity of water extracts from a soil contaminated by the three antibiotics (7 mg Kg^{-1} each) in the presence/absence of copper (30 mg Kg^{-1}) was also evaluated. Copper was the most toxic chemical (EC_{50}: 0.78 mg L^{-1}), followed by CTC (EC_{50}: 3.64 mg L^{-1}), CIP (96 mg L^{-1}) and SMX (196 mg L^{-1}). Comparing the TU and CA values of the mixtures, additive effects were generally found. However, a synergic action was recorded in the case of the CIP+Cu co-presence and antagonistic effects in the case of CTC+Cu and the ternary mixture (containing each antibiotic at 0.7 mg L^{-1}), were identified. Soil water extracts did not show any toxicity, demonstrating the buffering ability of the soil to immobilize these chemicals.

Keywords: acute toxicity; additive; antagonistic effects; chemical mixture; contaminated soil; synergic

Introduction

Owing to the growing impact of human activities, ecosystems are being continuously exposed to a mixture of contamination by both legacy (e.g. polycyclic aromatic hydrocarbons: PAHs and heavy metals: HMs) and emerging contaminants (e.g. pharmaceuticals, including antibiotics), (Cipullo et al. 2018, Yang et al. 2021, Finizio et al. 2022). Microorganisms (*Bacteria*, *Archaea* and microfungi) have a primary role in organic matter decomposition, nutrient cycling, primary production and removal of contaminants, contributing to soil and water purification processes (Saccà et al. 2017). The maintenance of several regulating ecosystem services is linked to microbial diversity and functioning (Barra Caracciolo and Grenni 2021; Saccà et al. 2017). Recovery from contamination is only possible if toxic chemicals do not hamper microbial activity. In particular, prokaryotic cells display a huge variety and versatility and include both taxa able to resist and degrade contaminants, but at the same time bacterial species very sensitive to different environmental stressors. Traditionally, pathogenic microorganisms have been used as indicators of faecal contamination (Cabral 2010), but microorganisms offer an unexplored variety of microbial species with different functions which can be used to assess ecosystem quality with different approaches. The identification of natural microbiological indicators and their use as test organisms in bioassays capable of responding to different stressors can be very useful for providing a global view of contamination effects (Karimi et al. 2017). In this context, the *Aliivibrio fischeri* (*γ-proteobacteria* class and formerly classified as *Vibrio fischeri*) is a test organism very sensitive to various environmental contaminants (Parvez et al. 2006, Abbas et al. 2018). *Aliivibrio fischeri* is a bioluminescent marine bacterium, which lives naturally in symbiosis with various abyssal marine animals (Chavez-Dozal et al. 2012, Abbas et al. 2018) and in particular with the bobtail squid (e.g. *Euprymna scopoles*). The bioluminescence is regulated via cell density dependent induction of luciferase-gene expression, in a quorum sensing regulation (Chavez-Dozal et al. 2012, Brodl et al. 2018). Because bacterial bioluminescence can be promptly inhibited by various toxicants, this bacterium showed itself to be a suitable organism for evaluating the toxicity of chemicals. In fact, *A. fischeri* has been successfully used in ecotoxicological evaluations and as a method complementary to chemical monitoring (Wieczerzak et al. 2016, Abbas et al. 2018, Mariani et al. 2020), which showed it to be a very sensitive organism at low contaminant concentrations. For example, polychlorobiphenyls, polycyclic aromatic hydrocarbons, heavy metals and anionic surfactants cause toxicity (50% bioluminescence inhibition) at concentrations between 0.1 to 1.4, 0.2 to 13.6, 0.2 to 4.5 and 1.72 to 3.52 mg L^{-1} respectively, (Salizzato et al. 1998, Mariani et al. 2006, McCready et al. 2006, Tsiridis et al. 2006, Khan et al. 2012, Lee et al. 2013, Mansour et al. 2015). Interestingly, in the case of an anionic surfactant, sodium lauryl ether sulfate, a direct relationship between bioluminescence

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inhibition and its chemical concentration in real matrices has also been demonstrated (Mariani et al. 2020).

The *A. fischeri* bioassay has also been applied to various contaminated and impacted matrices, such as effluents (Farré et al. 2008), sewage sludge (Farré and Barceló 2003), landfill leachate (Thomas et al. 2009) and soil and sediment water extracts (Cillari et al. 2011, Piva et al. 2011, Mariani et al 2020, Domínguez et al. 2023). However, knowledge of the effects of multi-contaminated environments on this sensitive bacterium is still scarce and few ecotoxicological data are available for antibiotics (ABs) and HM co-occurrence (Tong et al. 2015, Liu et al. 2022).

Antibiotics and heavy metals are among the most common contaminants found in natural environments in chemical mixtures. Agricultural practices contribute significantly to AB and HM multi-contamination. Human and veterinary antibiotics are organic compounds which are not fully metabolised by the organisms treated and are excreted via faeces and urines. Consequently, they reach water and soil ecosystems through wastewater treatment plants, organic fertilizer applications (e.g. biosolid, manure), and reclaimed water used for irrigation purposes (Ortiz de García et al. 2014, Barra Caracciolo et al. 2020, Polianciuc et al. 2020). Owing to their biocide properties, they can directly affect natural microbial communities by killing or inhibiting natural bacterial populations involved in key ecosystem functioning or by promoting antibiotic-resistant bacteria (Grenni et al. 2018, Visca et al. 2022). Sulfamethoxazole (SMX), ciprofloxacin (CIP) and chlortetracycline (CTC) are among the most widely used ABs, in both human and veterinary medicine, and their residues are commonly found in agricultural soils (Park and Choi 2008, Visca et al. 2022, Zhang et al. 2022). SMX is a widely used sulfonamide antibiotic, which can be degraded in relatively few days (Wang and Wang 2018, Rauseo et al. 2019, Rauseo et al. 2021). SMX residual concentrations have been found in wastewater, surface water and soils, with the highest concentrations (up 0.1 mg Kg^{-1}) in agricultural soils (Chen et al. 2014, Kuppusamy et al. 2018, Kairigo et al. 2020). The fluoroquinolone CIP has an intrinsic environmental persistence (Girardi et al. 2011, Barra Caracciolo et al. 2018, Visca et al. 2021) and is also the main metabolite of enrofloxacin, a widely used antibiotic for livestock diseases (Trouchon and Lefebvre 2016). Owing to its recalcitrance to biodegradation, CIP tends to accumulate in solid matrices, reaching concentrations of up to 5.6 mg Kg^{-1} in agricultural soils (Martinez-Carballo et al. 2007, Pan and Chu 2017, Cycoń et al. 2019, Kairigo et al. 2020). CTC is a tetracycline frequently used in livestock treatment and found in soils at concentrations ranging from μg Kg^{-1} to mg Kg^{-1}. For example, Zhang et al. (2022) reported a maximum concentration of 43.75 μg Kg^{-1} in different manure-amended soils and Cycoń et al. (2019) 12.9 mg Kg^{-1} in agricultural soil.

Copper (Cu) is commonly found in agricultural soils because it can be used as a foliar pesticide and applied (up to 4 Kg ha^{-1} y^{-1}) to vineyards and horticultural crops to prevent fungal attacks and other plant diseases (Dhanasekaran et al. 2012, Fagnano et al. 2020, Kumar et al. 2021). The recent literature data reported copper concentrations from 30 to 300 mg Kg^{-1} in Italian agricultural soils and up to 800 mg Kg^{-1} in other European ones (Fagnano et al. 2020, Tamm et al. 2022, Zamulina et al. 2022).

Although copper is a microelement necessary for all living organisms, including microorganisms, it can, if present at concentrations higher than those necessary, have adverse effects on biota (Mincarelli et al. 2019, Lu et al. 2022, Pande et al. 2022). For example, Cu can interfere with the functioning of several natural soil microbial enzymes (Ochoa-Herrera et al. 2011, Nunes et al. 2016, Barra Caracciolo and Terenzi 2021). Many works have also reported that HMs (including Cu) can boost resistant bacteria (Icgen and Yilmaz 2014, Ladomersky and Petris 2015, Di Cesare et al. 2016, Glibota et al. 2019). Indeed, several resistance mechanisms for a number of antibiotics and copper are located in the same mobile genetic elements (Martínez-Bussenius et al. 2017, Coclet et al. 2020). In light of the above, the scientific community has been paying attention to the evaluation of the overall effects of chemical mixtures on biota and looking for valid tools to assess the overall toxicity (Escher et al. 2020). The environmental risk assessment of multi-contaminated matrices, involving the prediction and assessment of additive/synergistic/antagonistic effects of chemical mixtures on ecosystems and on human health (in the light of the One-Health concept), is a future challenge for science.

In this work, the toxic effects of SMX, CIP, CTC, and Cu as single compounds or in binary, ternary and overall mixtures were evaluated using the *A. fischeri* bacterium. Moreover, water extracts from a soil contaminated by antibiotics, copper or a mixture of ABs and Cu were analysed and tested to evaluating the overall effects in real environmental matrices.

Materials and methods

Single solutions and mixtures to evaluate toxicity of chemicals

The main SMX, CIP, CTC and copper physicochemical properties are reported in Table 1.

Single antibiotic solutions were prepared by dissolving standard powders (sulfamethoxazole, ciprofloxacin, and chlortetracycline hydrochloride Vetranal Analytical Standards >98.0% HPLC purchased from Sigma-Aldrich, Germany) in a mixture of methanol (MeOH) and ultrapure water (1 : 99 v/v) for SMX, in NaCl aqueous solution (2%) acidified with formic acid (0.1%) for CIP and in ultrapure water for CTC, to reach a final concentration of 420, 500 and 100 mg L^{-1}, for SMX, CIP and CTC, respectively.

A single copper solution (31.25 mg L^{-1}) was prepared by dissolving $CuSO_4 \cdot 5H_2O$ (purchased from Sigma-Aldrich, Germany) in ultrapure water.

Moreover, three binary mixtures (SMX+Cu, CIP+Cu, CTC+Cu) consisting of one antibiotic (0.7 mg L^{-1}) combined with copper (3 mg L^{-1}) were performed.

Two ternary mixtures with each antibiotic at 0.7 mg L^{-1} (SMX+CIP+CTC_0.7) or 7 mg L^{-1} (SMX+CIP+CTC_7) were prepared.

Finally, two overall mixtures were also prepared using the same low (0.7 mg L^{-1}) or high (7 mg L^{-1}) AB concentrations used for the ternary mixture with the addition of copper at 3 mg L^{-1} (SMX+CIP+CTC_0.7+Cu_3) or 30 mg L^{-1} (SMX+CIP+CTC_7+Cu_30), respectively.

In the cases of the ternary and overall mixtures, the amounts used (3 mg L^{-1} for Cu and 0.7 mg L^{-1} for each AB) were based on the effective copper concentrations (experimentally determined in this work) and environmental water antibiotic concentrations found in literature (Chen et al. 2014, Kuppusamy et al. 2018).

The most concentrated mixtures (7 mg L^{-1} for each AB and 30 mg L^{-1} for Cu) were also used for soil contamination in order to simulate a "worst case scenario" in agroecosystems (Cycoń et al. 2019, Fagnano et al. 2020, Bombaywala et al. 2021).

The experimental solutions tested are summarized in Table S1. All the solutions were diluted (dilution factor two) to obtain seven different concentrations (Table S2) for determining the effective concentrations (ECs) with *A. fischeri* bioassay for each chemical (EC_{50}) and mixtures ($EC_{50\ mixture}$).

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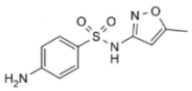
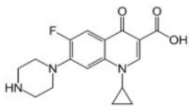
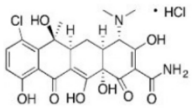
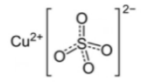
Table 1. Main physicochemical properties of the sulfamethoxazole (SMX), ciprofloxacin (CIP), chlortetracycline hydrochloride (CTC) antibiotics and copper (Cu).

Antibiotic	Molecular Structure	Molecular Weight (g mol^{-1})	Water Solubility (mg L^{-1})	Log K_{ow}	K_{OC} (L kg^{-1})	Ref.
Sulfamethoxazole: SMX		253.28	610 (37 °C)	0.89	258	Figueroa-Diva et al. 2010, Wang and Wang 2018, Ilyas et al. 2021
Ciprofloxacin: CIP		331.34	86 (25 °C)	0.28	35.51	Breda et al. 2009, Van Doorslaer et al. 2014
Chlortetracycline hydrochloride: CTC		515.34	8600 (25 °C)	2.07	32.3	Schwartzman et al. 1979, Ma et al. 2015, Kim et al. 2023
Heavy metal						
Copper sulphate: Cu		159.61	230 500	-2.2	6.124	National Center for Biotechnology Information 2023

For EC_{50} values obtained for mixture solutions, the influence of each chemical component to the mixture toxicity can be calculated considering the amount tested of each AB (0.7 or 7 mg L^{-1}) or Cu (3 or 30 mg L^{-1}) multiplied by the corresponding $EC_{50\ mixture}$ (%). In this way, the effective concentration of each single chemical in a mixture (expressed as mg L^{-1}) can be obtained, in accordance with how reported in SCHER, SCCS, SCENIHR, (2012).

Soil contaminated by antibiotics and copper

An agricultural sandy-clay-loam soil (60% sandy, 18% clay, 22% loam, USDA 1987) with 1.09% organic carbon, 0.116% total nitrogen and a residual copper concentration of 2.8 mg Kg^{-1}, was collected from an organic farm located close to Rome (Italy). The soil did not contain antibiotic residues. The soil was contaminated, adding ABs and Cu at concentrations close to those found in agricultural soils (Cycoń et al. 2019, Fagnano et al. 2020).

Four experimental conditions (500 g each) were set up:

- Soil spiked with a copper solution to reach a final copper concentration of 30 mg Kg^{-1}: **SCu**
- Soil spiked with a ternary mixture containing the three antibiotics (SMX+CIP+CTC) to reach a final soil concentration for each AB of 7 mg Kg $^{-1}$: **SABs**
- Soil spiked with an overall mixture containing the three antibiotics (SMX+CIP+CTC) and copper to reach a concentration of 7 mg Kg $^{-1}$ for each AB and 30 mg Kg^{-1} for Cu: **SCuABs**
- Uncontaminated soil, as a control: **S**

Each condition was performed in triplicate. The final soil humidity was about 30%, corresponding to 45% of the soil water holding capacity (WHC_{max} 66%). Soil pH was 6.5 and adding chemical solutions did not change its value.

Soil sub-samples (40 g each) were collected from each replicate condition (SCu, SABs, SCuABs, S) 6 h after the spiking and soil water extracts (elutriates with a liquid/solid ratio of 1 : 10) were performed in accordance with the leaching test protocol (UNI EN 12457–2 : 2004, Hubálek et al. 2007, Marguí et al. 2016). Elutriates in fact simulated a possible leaching of chemicals from soil to water. The soil water extracts were used for analytical determinations and tested with *A. fischeri*. In the latter case, elutriates were filtered through a 0.45 μm cellulose acetate filter Whatman, (as required by UNI EN 14735 : 2005).

Aliivibrio fischeri bioassays

The toxicity effects of solutions and water extracts from soil contaminated with ABs and copper, were assessed with the luminescent bacterium *A. fischeri*, in accordance with the ecotoxicological standardised procedure (UNI EN ISO 11348–3 : 2019). The bioassays were performed on each single chemical (intrinsic toxicity), and on different combinations of them to record potential synergic, additive or antagonistic effects.

The freeze-dried bacteria (strain NRRL B-11177, batch number BL10970422) used in the tests, were rehydrated with a reconstitution solution (purchased from Ecotox LDS s.r.l., Milan, Italy) to achieve a ready-to-use bacterial suspension. The blank (saline solution, 20 g L^{-1} NaCl) and reference toxicants ($ZnSO_4 \cdot 7H_2O$ for inorganic and 3,5-dichlorophenol for organic chemicals) were used in all tests as the negative and positive controls, respectively. Before each test, the pH of samples was measured and corrected, if necessary (range 6.0–8.0), using an HCl (0.1 M) or NaOH (0.1 M) solution. The reagents (methanol and formic acid in different percentages from 1% to 5% in distilled water) used for AB solubilization, were also tested to exclude any ecotoxicity for *A. fischeri*.

The toxicity effect (endpoint) was the percentage (%) of bioluminescence inhibition resulting from the bacterium exposure to each sample after 15 and 30 min.

A sample is considered toxic if cause a bioluminescence inhibition >20% (qualitative threshold) in comparison with the

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bioluminescence produced by *A. fischeri* in optimal life condition (blank), (Persoone et al. 2003). In order to quantify the amount of each sample which cause a toxic effect, the effective concentration (EC) has to be determined. The EC can be expressed also as Toxic Unit (TUs).

Bioluminescence was measured using the Microtox® analyser (Model 500, Modern Water, UK) and Effective Concentrations (ECs) and toxic units (TUs) values were calculated with a specific software (Microtox Omni® V 4.2, Milan, Italy).

The EC_{20} or EC_{50}, causing 20% or 50% of the bioluminescence inhibition (Microtox Analyzer-basic test), were evaluated for each chemical alone (SMX, CIP, CTC, and Cu), or in binary (SMX+Cu, CIP+Cu, CTC+Cu), ternary (SMX+CIP+CTC_0.7 and SMX+CIP+CTC_7) and overall mixtures (SMX+CIP+CTC_0.7+Cu_3 and SMX+CIP+CTC_7+Cu_30). Each solution was diluted (1 : 2) to obtain seven decreasing concentrations to be tested (see Table S1).

The EC values were expressed as mg L^{-1} for each single chemical and as a percentage (%) of the maximum concentration tested for the mixtures. The 95% confidence limits were also calculated.

Finally, the potential toxicity of soil water extracts from the three different experimental conditions (SCu, SABs and SCuABs) and the uncontaminated soil (S) was also assessed with this bioassay.

All bioassays were repeated at least three times and the validity criterion (CV%: standard deviation/mean × 100 <20%) was also verified for each test (Persoone et al. 2003, Environment Canada 2007).

Toxic units

The toxicity results at 30 min were also reported in terms of Toxic Units (TUs), which are dimensionless and make possible a comparison between the various toxicity results and different mixture compositions. The results for single solutions (TU) and mixtures ($TU_{mixture}$), were calculated using the following formula (Persoone et al. 2003):

$$TU = [1/EC_{50}] \times 100$$

Moreover, the Concentration Addition ($CA_{mixture}$) model, considered the conservative worst case (Finizio et al. 2022), was used to obtain the predicted toxicity of each binary, ternary and overall mixtures.

The predicted $CA_{mixture}$ was calculated as follows:

$$\sum c_a/EC_{50a} + c_b/EC_{50b} + \ldots c_n/EC_{50n}$$

c = concentration (mg L^{-1}) of an antibiotic or copper in the mixture considered; EC_{50} = single effective concentration (mg L^{-1}) of an antibiotic or copper.

For the same mixture, if a $TU_{mixture}$ was equal to the predicted $CA_{mixture}$, the additive toxicity effect was confirmed; if a $TU_{mixture}$ was lower than the $CA_{mixture}$, antagonistic effects were present and if a $TU_{mixture}$ was higher than $CA_{mixture}$, synergic effects were found (Fong et al. 2018, Drzymała and Kalka 2020).

Total microbial abundance of *A. Fischeri*

The average total microbial number (N. total cell mL^{-1}) of *Aliivibrio fischeri* occurring in the bacterial suspension used for the tests was evaluated with the epifluorescence direct count method. Aliquots (100 μL each x 3 replicates) of *A. fischeri* were fixed in a buffered formaldehyde solution and stained using DAPI (4′,6-diamidino-2-phenylindole), as the DNA fluorescent intercalant dye. The microbial cells were counted with a Leica DM4000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). Further details on this method are reported in other works (Barra Caracciolo et al. 2005, Barra Caracciolo et al. 2010, Barra Caracciolo et al. 2013). Pictures were elaborated by Leica Application Suite X Software (version 3.3).

Antibiotic and copper analytical determination in soil water extracts

Soil water extracts were analysed to determine the analytical concentration of SMX, CIP and CTC. The extraction/purification procedure was carried out using SPE (Solid Phase Extraction) equipped with Oasis Hydrophilic-Lipophilic Balance (HLB, Waters-Milford, USA) cartridges (6 ml, 200 mg), in accordance with Spataro et al. (2019). Briefly, the cartridges were activated with 5 ml of MeOH and 5 ml of ultrapure water (18 MΩ cm^{-1} quality, Millipore- Bedford, USA) passed at a flow rate of 5 ml min^{-1}. Soil water extracts were then passed into the cartridges and, after drying them for 60 min, elution was performed with a mixture of MeOH, acetonitrile (ACN, HPLC grade, VWR-Radnor, USA), and acetic acid (Sigma Aldrich, Germany) (40 : 40 : 20 v/v). The eluates were finally evaporated with a gentle nitrogen stream and reconstituted with 0.5 ml of a mixture of ACN and ultrapure water (50 : 50 v/v) acidified with formic acid at pH 3.

The analytical determination of the target antibiotics was performed with high-performance liquid chromatography (HPLC, column oven mod. LC-100, micropump series 200, Perkin Elmer, USA) interfaced with a triple quadrupole mass spectrometer (MS/MS, mod. API 3000, AB Sciex, Germany). The injection loop was 20 μL and the chromatographic Gemini column (150 × 4.6 mm, 5 μm RP C 18, Phenomenex, France) connected with an equally packed guard column were both maintained at 25 °C (Spataro et al. 2019).

SMX, CIP and CTC elution was carried out in gradient mode at a flow rate of 0.3 ml min^{-1}. The mobile phase was composed of MeOH (Phase A) and ultrapure water (phase B), both acidified with 0.1% of formic acid. The gradient profile started with phase A at 10%. It then increased from 10% to 90% in 10 min. and finally returned to 10% in 15 min. The MS/MS parameters set for the determination of SMX, CIP and CTC are reported in Table 2.

The ESI (Electrospray Ionization) operated with a curtain gas 12 units, nebulizer gas 14 units and source temperature 400 °C. Nitrogen was used as both the collision and drying gas.

The quantification of antibiotics was based on the m/z ion ratios and a comparison of the retention times (RTs) of product ions/fragments in the samples with those of each standard, considering criteria differences of 0.2 min. Two calibration curves (five points) at lower (0.5, 1.0, 2.0, 2.5, and 5.0 ng L^{-1}) and higher (5, 20, 50, 100, and 500 ng L^{-1}) concentration ranges were considered, indicating the good linearity of the method (R^2 > 0.98). The recoveries of SMX, CIP and CTC were also evaluated using ultrapure water artificially spiked at different concentrations (50, 200, and 500 ng L^{-1}) and ranging between 82% and 99%. The detection limits (LODs) of SMX, CIP and CTC were calculated in accordance with the IUPAC method (Thompson et al. 2002) and were 2.1, 5, and 4 ng L^{-1}, respectively. Quantification limits were set at three times the LODs.

Moreover, copper concentrations in soil water extracts were measured using an inductively coupled plasma optical emission spectrometry (ICP-OES, 5800 Agilent technologies, USA). The quantification was performed by interpolation using a calibration curve obtained by diluting a 1 g L^{-1} stock solution into six

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Table 2. MS/MS parameters set for the analytical determination of SMX, CIP and CTC. RT: retention time; ESI mode means positive (+) or negative (−) ionization mode; DP: declustering potential; CE: collision energy.

Compound	RT (min)	ESI mode	Ion spray Voltage (KV)	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EC (eV)
SMX	6.4	+	5	254.1	92	42	39.0
					108.1		39.4
					156.0		22.8
CIP	14.5	+	5	332.2	245.2	50	33.6
					288.2		26.5
CTC	7.8	+	5	478.9	442.9	43.2	20.4
					462.4		29.1

Table 3. Average effective concentrations (EC_{20} and EC_{50}, mg L^{-1} ± s.e.) of sulfamethoxazole (SMX), ciprofloxacin (CIP), chlortetracycline (CTC) and copper (Cu) in single solutions assessed with *A. fischeri* bioassays.

Chemical	EC_{20} (mg L^{-1}± s.e.) 15 min	30 min	EC_{50} (mg L^{-1}± s.e.) 15 min	30 min
SMX	30.50 ± 5.77	34.53 ± 11.57	179.21 ± 55.24	194.06 ± 25.44
CIP	35.54 ± 5.45	17.06 ± 3.24	139.15 ± 9.05	96.93 ± 16.26
CTC	1.71 ± 0.02	1.41 ± 0.14	5.04 ± 0.07	3.64 ± 0.21
Cu	0.55 ± 0.02	0.52 ± 0.05	1.41 ± 0.09	0.78 ± 0.05

standard solutions. The wavelengths (nm) used for Cu were 327.395 (Barreto et al. 2021, Iannelli et al. 2022).

Statistical analysis

The ecotoxicological and chemical results are reported as average ± standard errors of triplicate analysis of triplicate samples. One-way ANOVA (using the *aov* function) together with the *pairwise.wilcox.test* function, as the post-hoc test (Benavoli et al. 2016), was performed to find any significant differences between the single, binary, ternary or overall mixture and contaminated water soil extract samples. The statistical significance level was at least 0.05 ($P \leq 0.05$, confidence interval 95%).

Results and discussions

Effects of antibiotics and copper alone or in mixtures

The effects of the single chemicals (intrinsic toxicity) on the bacteria *A. fischeri*, expressed as effective concentrations (EC_{20} and EC_{50}), are reported in Table 3. The EC_{50} values showed sulfamethoxazole (SMX) to be less toxic than ciprofloxacin (CIP) and the latter less toxic than CTC ($P < 0.05$). The highest CTC toxicity can be ascribed to its chemical structure, as it is a very complex chemical composed of four aromatic rings and two chlorides (see Table 1). Recently some authors have discussed the literature data on the ecotoxicological effects of several antibiotics on different organisms, including bacteria (Kovalakova et al. 2020). Jiang et al. (2014) reported cyanobacteria to be very sensitive to fluoroquinolones and sulfonamides, with EC_{50} values of 0.1 mg L^{-1} for CIP and 1 mg L^{-1} for SMX, confirming the higher toxicity of CIP than SMX. The EC_{50} values higher (lower toxicity) for *A. fischeri* than *Microcystis aeruginosa* can be ascribed to the different experimental exposure times (30 min vs 7 days), as the contact time is a factor influencing the action of CIP and SMX (Robinson et al. 2005). On the other hand, the tetracycline toxicity (EC_{50}: 1.5 mg L^{-1}) reported for *M. aeruginosa* (Kovalakova et al. 2020) was similar to that of CTC found in our work for *A. fischeri* and this supports the hypothesis that CTC's effect is immediate. The highest toxicity of CTC is due to its complex structure and toxic chlorine presence. Other authors have reported EC_{50} values (bioluminescence inhibition endpoint) for CTC (Wei et al. 2018), CIP (Załęska-Radziwiłł et al. 2014) and SMX (Majewsky et al. 2014, Drzymała and Kalka 2020) similar to ours, confirming both that CTC is the most toxic antibiotic and the reliability of the *A. fischeri* bioassay if it is performed following the standard procedure.

Regarding copper (Cu), the toxicity responses of *A. fischeri* pointed to the highest intrinsic toxicity among the chemicals tested, with EC_{50} values corresponding to 1.41 mg L^{-1} and 0.78 mg L^{-1} at 15 and 30 min of exposure time, respectively (Table 3). These results are in accordance with other authors (Mansour et al. 2015), who reported a similar value (EC_{50} at 15 min of 2.84 mg L^{-1}). Moreover, copper has been found to be toxic for several microbial functional groups at concentrations in the order of a few mg L^{-1}. In fact, Ochoa-Herrera et al. (2011) found copper inhibiting 50% (IC_{50}) of wastewater microorganism metabolic activity. They found IC_{50} values of 0.95 mg L^{-1}, 3.5 mg L^{-1}, 4.6 mg L^{-1} and 26.5 mg L^{-1} for denitrifying, fermentative, aerobic glucose-degrading heterotrophs and nitrifying bacteria, respectively.

The toxicity of the binary mixtures (0.7 mg L^{-1} for each antibiotic; 3.0 mg L^{-1} for copper) showed similar $EC_{50\ mixture}$ (%) values (Table S2) in the SMX+Cu and CIP+Cu conditions. Copper maintained its toxicity with SMX presence, while it increased slightly with CIP. The binary combination of chlortetracycline and copper (CTC+Cu) instead decreased ($p < 0.05$) in overall toxicity, (Fig. 1). The latter result might be ascribed to a complexation between Cu and CTC, which presumably decreased their bioavailability (Zhao et al. 2013), in accordance with the fact that tetracycline contains electron-donor groups able to form strong complexes with metal ions (Gu et al. 2007). This phenomenon and the consequent decrease in toxicity have also been found in the case of the cyanobacterium *Synechocystis sp.* (You et al. 2022) and in wastewater microorganisms (Pulicharla et al. 2015, 2017).

Excluding AB synergic effects, the ternary mixture of the three antibiotics at 0.7 mg L^{-1} each (SMX+CIP+CTC_0.7) did not show toxicity (data not shown); this result was presumably due to the fact that all the concentrations tested (0.7 mg L^{-1})

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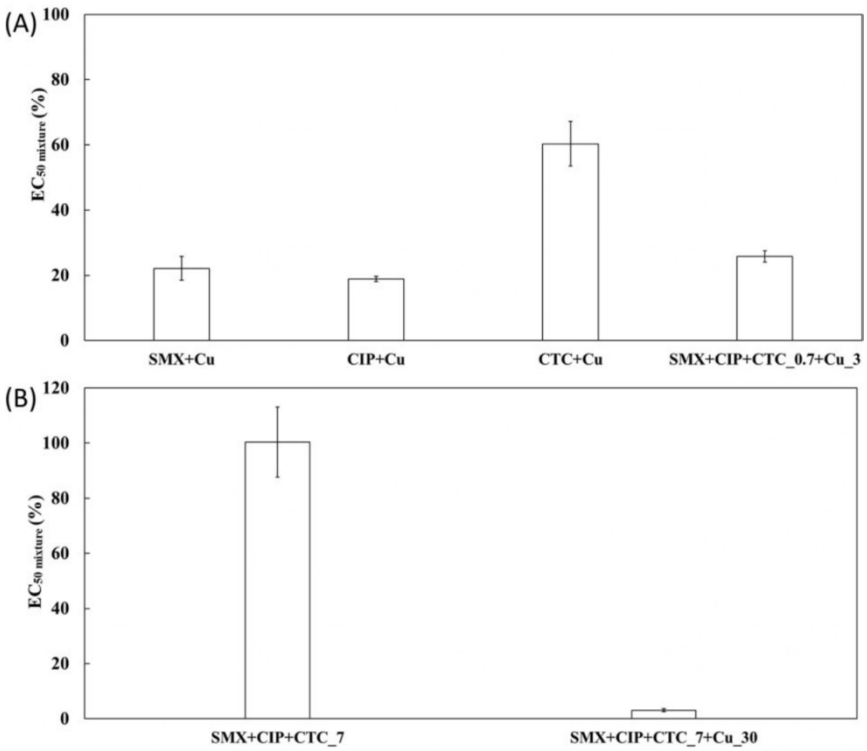

Figure 1. Effective concentrations ($EC_{50\ mixture}$, %) at 30 min assessed with *A. fischeri* of: (A) Binary and overall mixtures at low concentrations (AB: 0.7 mg L^{-1} each; Cu: 3 mg L^{-1}); (B) Ternary and overall mixtures at high concentrations (AB: 7 mg L^{-1} each; Cu: 30 mg L^{-1}).

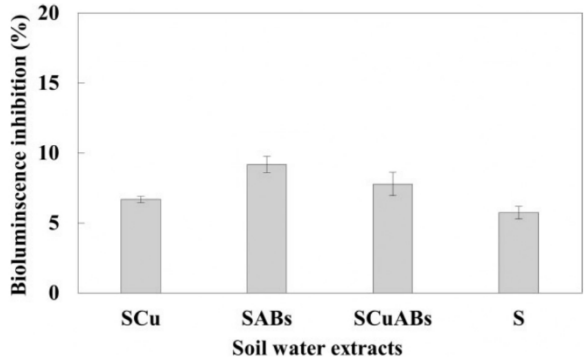

Figure 2. Effect (bioluminescence inhibition % of *A. fischeri* at 30 min) of soil water extracts from the different experimental conditions. SCu: soil+Cu; SABs: soil+ABs, SCuABs: soil+Cu+ABs and S: uncontaminated soil. Cu: copper; ABs: SMX+CIP+CTC.

were below the effective intrinsic ones for all the ABs (Table 3). Unlike the SMX+CIP+CTC_0.7 ternary mixture, copper in the SMX+CIP+CTC_0.7+Cu_3 overall mixture caused toxicity with a $EC_{50\ mixture}$ value of 25.8% (Fig. 1A). This result means that the copper influenced the mixture toxicity (25.8% of 3 mg L^{-1} copper = 0.77 mg L^{-1}), in accordance with its intrinsic toxicity value (Cu single solution EC_{50}= 0.78 mg L^{-1}).

In addition, the ternary mixture of the three antibiotics at 7 mg L^{-1} each (SMX+CIP+CTC_7) showed toxicity with an $EC_{50\ mixture}$ value (at 30 min) corresponding to the maximum tested concentrations (Fig. 1B). The latter result can be ascribed to the fact that CTC was present in the mixture at a concentration close to its effective one (CTC single solution EC_{50}=3.64 mg L^{-1}). Consequently, chlortetracycline drove the toxicity of this mixture.

Finally, the overall mixture (SMX+CIP+CTC_7+Cu_30) showed a higher toxicity ($EC_{50\ mixture}$ value of 3%) than the corresponding ternary mixture (SMX+CIP+CTC_7) without copper (Fig. 1B). This was in line with the additive effect of copper (3.0% of 30 mg L^{-1}= 0.92 mg L^{-1}).

Table 4 reports the above-mentioned results as Toxic Units (TUs) at 30 min of the single chemicals and mixtures. The TUs measured in the binary, ternary and overall mixtures are compared with the predicted toxicity values calculated with the Concentration addition (CA) model. The comparison of measured vs predicted values shows how the CA model was generally a good prediction of the combined effects, as reported in other works (Drzymała and Kalka 2020, Finizio et al. 2022). However, in a few cases, synergic and antagonistic effects were observed. Interestingly, a synergic effect was found in the CIP+Cu mixture (Table 4). The fact that ciprofloxacin alone at residual concentrations (0.7 mg L^{-1}) was not toxic, but with copper (3 mg L^{-1}) the binary mixture toxicity increased due to synergic effects, is particularly concerning because this result does not exclude that this phenomenon can occur in natural ecosystems impacted with similar amounts of contaminants (Mutiyar and Mittal 2014, Perlatti et al. 2021). Moreover, the latter result is in accordance with a recent work, which found deleterious synergic effects from the fluoroquinolone enrofloxacin in the co-presence of copper on soil microbial biomass and enzymatic activities (Yang et al. 2021).

An unexpected antagonistic effect was observed in the binary mixture comprising the most toxic chemicals (CTC+Cu, Table S3 and Table 4); the latter result suggests a possible complexation between the copper and the antibiotic, which reduced their bioavailability (Zhao et al. 2013). On the other hand, the fact that SMX+CIP+CTC_0.7+Cu_3 toxicity was different from the corresponding binary solution (CTC+Cu), containing the same

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Table 4. Mean toxicity values (± s.e.) obtained as Toxic Unit (TU: single solution or $TU_{mixture}$: mixtures) and Concentration Addition ($CA_{mixture}$) for the different solutions.

Solutions	TU or $TU_{mixture}$ ± s.e.	$CA_{mixture}$ ± s.e.	Effect
SMX	2.99 ± 0.34	-	-
CIP	5.26 ± 0.52	-	-
CTC	27.52 ± 0.92	-	-
Cu	80.77 ± 5.29	-	-
SMX+Cu	4.61 ± 0.45	3.87 ± 0.78	additive
CIP+Cu	5.30 ± 0.13	3.88 ± 0.78	synergic*
CTC+Cu	1.67 ± 0.11	4.06 ± 0.76	antagonistic*
SMX+CIP+CTC_0.7	0.13 ± 0.00	0.20 ± 0.02	antagonistic*
SMX+CIP+CTC_7	1.06 ± 0.20	1.72 ± 0.99	additive
SMX+CIP+CTC_0.7+Cu_3	3.89 ± 0.15	4.07 ± 0.76	additive
SMX+CIP+CTC_7+Cu_30	33.04 ± 3.21	40.72 ± 7.63	additive

The comparison between the $TU_{mixture}$ and $CA_{mixture}$ values makes it possible to categorize toxic effects (Effect) of mixtures, such as additive, synergic, antagonistic ones. The star symbol (*) highlights significant differences ($p < 0.05$) between $TU_{mixture}$ and $CA_{mixture}$ values.
For acronyms: see Table 2.

Table 5. Average concentrations and standard errors ($\mu g\ L^{-1}$ ± s.e.) of sulfamethoxazole (SMX), ciprofloxacin (CIP), chlortetracycline (CTC) and copper (Cu) in soil water extracts.

Conditions	Concentrations ($\mu g\ L^{-1}$ ± s.e.) SMX	CIP	CTC	Cu
SABs	0.106 ± 0.000 001	0.00	0.050 ± 0.000 001	< 20.0
SCu	0.00	0.00	0.00	< 20.0
SCuABs	0.153 ± 0.000 001	0.00	0.00	< 20.0
S	n.d.	n.d.	n.d.	< 20.0

SABs: Soil+SMX+CIP+CTC; SCu: Soil+copper; SCuABs: Soil+copper+SMX+CIP+CTC; S: uncontaminated soil. For antibiotics and copper concentrations in soil: see Materials and methods, "Contaminated soil with antibiotics and copper" paragraph.

amounts of CTC and Cu, can be due to a binding competition between CIP and CTC and Cu. This phenomenon has been described in other works (Lu et al. 2014, Ma et al. 2020, Wang et al. 2021).

Comparing CA and TU values for the ternary mixture of the three antibiotics at 0.7 mg L^{-1} each (SMX+CIP+CTC_0.7), an antagonistic effect (in accordance with the absence of toxicity discussed above) was observed (Table 4). On the contrary, in the case of the ternary mixture of the three antibiotics at 7 mg L^{-1} each (SMX+CIP+CTC_7), an additive effect was detected.

These results suggest that different chemical bindings among the chemicals may occur, which can depend on each chemical's intrinsic characteristics, but also on their concentration, showing the complexity of the interactions in mixtures.

Effect of soil water extracts

The water extracts from the soil contaminated with copper at concentrations of 30 mg Kg^{-1} (SCu) and of antibiotics at 7 mg Kg^{-1} (SABs), and with a Cu and AB co-presence (SCuABs) did not show any toxicity (bioluminescence inhibition < 20%) for the *A. fischeri* bacteria (Fig. 2). This result could be due to the soil buffering capacity, which can adsorb organic contaminants and immobilise heavy metals (Villen-Guzman et al. 2015), avoiding their leaching and preventing their reaching water. Indeed, soil lithology can significantly influence the release of contaminants in the aqueous phase and consequently affect the toxic response of *A. fischeri* (Mariani et al. 2020) and other aquatic organisms (Barra Caracciolo et al. 2017, Grenni et al. 2018, Mariani et al. 2022), which are the most sensitive to pollutants (Amoatey and Baawain 2019, Kovalakova et al. 2020). For example, depending on soil lithology and mineralogical composition, the anionic surfactant sodium lauryl ether sulphate (SLES) was found in different amounts in the eluviates, with significantly lower concentrations and no ecotoxicological effects, in fine texture as compared to sandy soils (Barra Caracciolo et al. 2019, Finizio et al. 2020).

Antibiotic and copper analytical determination in soil water extracts

In accordance with the ecotoxicological results, antibiotics and copper in elutriates (Table 5) were negligible and far below their effective concentrations (EC_{50}, see Table 3). These results confirm the soil capacity to adsorb antibiotics (Vasudevan et al. 2009, Antilen et al. 2016) and to form a complex with copper. It is generally recognized that, through its components (e.g. organic matter and fine fraction), soil is able to decrease HM mobility and potential toxic effects on microorganisms (Bengtsson et al. 2006, Terekhova et al. 2021, Ortega et al. 2022).

Interestingly, the absence of chlortetracycline residues in elutriates in the co-presence of copper (SCuABs, Table 5), would confirm a possible complexation of the latter with CTC. Indeed, binding of tetracycline to divalent metal cations has been suggested to explain their adsorption to organic matter, minerals, and soils (Figueroa et al. 2004).

The fact that the elutriates from the soil contaminated by copper at 30 mg Kg^{-1} did not show any toxicity for *A. fischeri*, does not exclude the risk that higher amounts, which can accumulate in soils, can exert detrimental actions. Literature data report copper in some agricultural soils at concentrations of up to 800 mg Kg^{-1} and higher than those tested in this work (Fagnano et al. 2020, Tamm et al. 2022). Other works showed that copper present in such high amounts had deleterious effects on microorganisms, plants and plant-microbiota (Narendrula-Kotha et al. 2019; Barra Caracciolo and Terenzi 2021, Pande et al. 2022). Particular concern

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arises for coarse textured soils, with low organic carbon where copper cannot be complexed (Maisto et al 2011, Liu et al. 2018, Angelaki et al 2022) and can exert its toxic action on microorganisms (Terekhova et al. 2021) and run-off to water in concentrations higher than the effective ones. For example, copper residues have been detected in water at concentrations higher than the EC_{50} (0.78 mg L^{-1}) found in this work (Mutia et al. 2012, Liu et al. 2020, Parihar et al. 2020), showing how this HM can be a hazard for microorganisms and aquatic sensitive species, such as microalgae (Cavalletti et al. 2022). Moreover, it is important to highlight that the co-presence of other contaminants (e.g. copper and ciprofloxacin, as shown in our work) can increase the toxicity of this heavy metal. Our results are in line with those of other authors (Tsiridis et al. 2006).

Total microbial number

The total bacterial number counted with an epifluorescence microscope was 8×10^7 cells mL^{-1}. This means that the toxic response refers to a very high bacterial number, with statistically significant samples, which makes this bioassay particularly reliable. Fig. S1 shows a photo of the *A. fischeri* cell suspension used for the bioassays.

Conclusions

In this work, the ecotoxicological effects of antibiotics and copper in single or mixture solutions were evaluated at increasing complexity in order to simulate environmental scenarios of mixture contamination, and the possible leaching from a multi-contaminated soil to water.

Among the evaluated compounds, copper and chlortetracycline hydrochloride have been demonstrated to be the most toxic chemicals. Interestingly, the copper toxicity (EC_{50}: 0.78 mg L^{-1}, close to the EU Water Directive threshold limit of 0.1 mg L^{-1}) in most of mixtures followed the concentration addition model. However, a synergistic effect in CIP+Cu and an antagonistic one in the case of the CTC+Cu binary mixtures were also found.

The application of the *A. fischeri* bioassay showed very sensitive in detecting the single and combined toxic effects of the chemicals tested, demonstrating as the bioluminescence inhibition can be a reliable biosensor. This study contributed to increase knowledge of the additive, synergic and antagonistic effects related to mixtures, which is currently considered mandatory for a more realistic environmental risk assessment.

Author contributions

Alessandra Narciso (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing), Anna Barra Caracciolo (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing), Paola Grenni (Conceptualization, Data curation, Formal analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – review & editing), Jasmin Rauseo (Data curation, Formal analysis, Methodology, Visualization, Writing – original draft), Luisa Patrolecco (Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing), Francesca Spataro (Data curation, Formal analysis, Methodology, Visualization, Writing – original draft), and Livia Mariani (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data is available at FEMSEC *Journal* online.

Conflicts of interest: The authors have no conflicts of interest to declare.

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Impacts of antibiotics and heavy metals on natural microbial communities of soil and associated with edible plants

Antibiotic residues and heavy metals in soil can go through sorption/desorption and transformation processes (both biotic and abiotic) which have the potential to affect soil and soil-microbiome plant system, (Kim et al. 2015; Ali et al. 2019; Cycoń et al. 2019). Antibiotics and heavy metals if present in soil pore water (bioavailable fraction) may be taken up by plant root system, bioaccumulate in plant tissues and, subsequently, enter food webs (Salam 2020; Shu et al. 2024). The latter phenomenon can have public health implications, especially regarding antimicrobial resistance spread (Christou et al. 2017). It has been recognized that ARB and ARG spread in soil can be promoted by agricultural practices, such as the application of biosolid or manure, reclaimed water irrigation or aerosols. From agricultural soils ARGs can be transferred to microbiome-plant system resistomes and in turn to through edible plants to animals and humans (Koutsoumanis et al. 2021; Musiyiwa et al. 2024). Hence, exploring the transmission mechanism of ARGs in plant-associated microorganisms is crucial to improve knowledge on ARG pollution and transmission to humans and animals in the framework of the One Health concept (Fig. 7), (Chen et al. 2023).

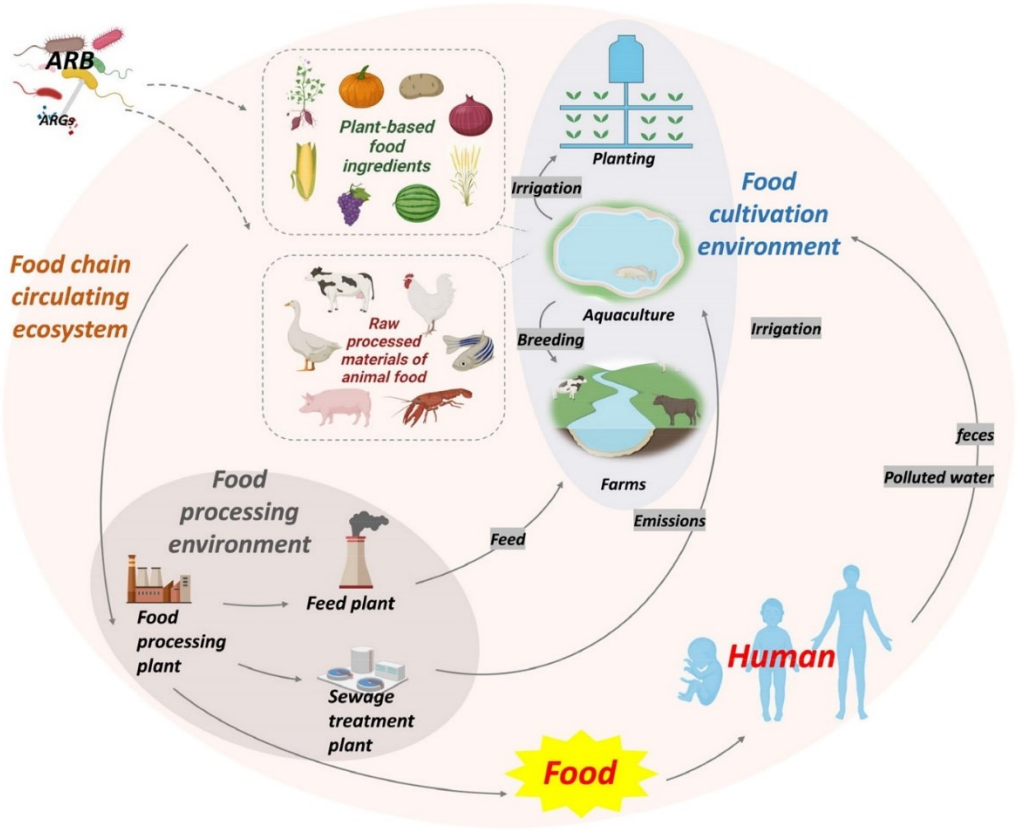

Figure 7. Graphical representation of the transfer and propagation cycle of ARGs (Antibiotic resistance genes) and ARB (Antibiotic resistant bacteria) in the environment through food chains. (Modified from Yuan et al. 2024).

Plant microbiomes connected with antibiotic resistance bacteria from water, soil and air can acquire ARGs (Chen et al. 2023). Antibiotic-resistant bacteria (ARB) in rhizosphere can also

communicate with endophytic ones, and because the latter are not eliminated during the washing of edible vegetables, they might be vectors of transmission of antibiotic resistance to consumers (Scaccia et al. 2021). ARGs in plants can also be acquired from air in phyllosphere bacteria. For example, leaves of field-grown lettuce are exposed to a high number of bacteria originating from dust and soil particles transported by wind and from irrigation water or rainwater, which could already contain resistant bacteria or transfer soil particles to leaves by splash erosion. Furthermore, ARB may be transported to crops on-field by different shuttles like insects, livestock, birds, wild animals, and agricultural equipment (Alegbeleye et al. 2018; Blau et al. 2019).

Rhizosphere is considered a hot spot of both microbial activity and bacterial HGT, which, thanks to root growth and exudate production, make it possible to harbour different resistome from bulk soils, potentially buffering contaminant effects (Blau et al. 2019; Mayans et al. 2024; Lin et al. 2024). If ARG spread is promoted more in the rhizosphere than in the bulk soil is a controversial issue. Some studies (Xie et al. 2018; Huang et al. 2022) have reported that rhizosphere is a habitat which can facilitate proliferation and spread of ARGs due to a high degree of bacterial interactions. Other ones (Blau et al. 2019; Wu et al. 2019) have found an inhibition of ARG transmission because root exudates provide nutrients for microorganisms and can favour both contaminant degradation and face adverse conditions (Blau et al. 2019; Banerjee and van der Heijden 2023; Lü et al. 2024). For example, Blau et al. (2019) have demonstrated a higher increase in AGRs in bulk soils amended with manure than in rhizosphere; moreover an additional effect for some ARGs codifying for efflux pumps against other antibiotics or transposons (*tetA* and *tnpA*) was found if the bulk soil was spiked with doxycycline. The same phenomenon was observed in another study which found a significantly lower abundance of *sul1* and *sul2* resistance genes in a maize rhizosphere compared with bulk soil, after a soil treatment with manure from pigs treated with sulfadiazine (Jechalke et al. 2013).

Interestingly, some recent works showed that organic fertilizer application altered the type and amount of root secretions, as signalling molecules which can markedly affect microbial activity and abundance of ARGs carried by microbes (Chen et al. 2023; Wang et al. 2024a; Guan et al. 2024). Among rhizosphere microorganisms, *Clostridium* (Blau et al. 2019), *Bacillus*, *Pseudomonas* and *Streptomyces* genera (Lin et al. 2024), were identified as ARG biomarker and carriers of multi-drug resistance. However, it remains poorly understood how and which factors drive differences in ARGs abundance in rhizosphere and bulk soils.

In any case, the co-presence of HMs and ABs in bulk soil pose a continuous selective pressure on microorganisms accelerating ARB proliferation and their prevalence (Poole 2017; Fu et al. 2023; Zhang et al. 2023). In particular, a recent work (Fu et al. 2023) studied the effects of cadmium and

sulfadiazine on a soil microbial community structure. In co-presence of the two contaminants, the authors found an increase in *Cloroflexi* and *Bacteroidetes,* which comprise pathogenic bacteria and possible carriers and transmitters of ARGs, in comparison with the control and the condition with only cadmium or only the antibiotic. Moreover, the cadmium and sulfadiazine combination has stimulated the expression of ARGs directly and indirectly by promoting the integron (*intI1*) expression.

The combined effects of oxytetracycline and cadmium on a rhizosphere microbial community composition has also been evaluated (Guo et al. 2021). The authors evidenced a shift in the community in the two-contaminant co-presence if compared to the control one, with an increase in *Proteobacteria* and *Bacteroidetes*. The *Clostridium* genera*,* which comprises pathogenic species and correlated with ARG transfer (Michaelis and Grohmann 2023; Shen et al. 2024) also increased.

Current studies regarding soil microbial community response to multi-contamination by antibiotics and heavy metals and comparisons between bulk soil and rhizosphere microorganisms are not sufficient to make general considerations. It is fundamental to consider multiple factors which can maintain or promote ARG spread among natural microbial populations, in order to find solutions for halting this phenomenon.

A starting point is to assess effects of antibiotics and copper through soil microcosms using samples collected from agricultural areas in order to simulate realistic environmental scenarios. Indeed, laboratory microcosms are ecosystem models in which a portion of the natural environment (soil or water) is circumscribed and then studied. They contain natural microbial communities maintained under controlled environmental conditions (e.g. temperature, light, humidity), but corresponding to natural ones. This type of approach makes it possible to more firmly establish the causal relationship between a toxicant and its effects on communities, in particular microbial ones, as they represent an important key to understanding the impacts of environmental and anthropogenic factors on ecosystems (Barra Caracciolo et al. 2013).

In this context, the third work (published on ***Applied Microbiology and Biotechnology***), evaluated the effects of SMX and copper on a natural microbial community from an agricultural soil amended with cattle manure or digestate. The effects were assessed in terms of microbial abundance, viability, activity and biodiversity. Moreover, the relative abundance of the *sul1*, *sul2* and *intI1* genes was also evaluated.

Effects of Sulfamethoxazole and Copper on the Natural Microbial Community from a Fertilized Soil

Alessandra Narciso, Paola Grenni, Francesca Spataro, Chiara De Carolis, Jasmin Rauseo, Luisa Patrolecco, Gian Luigi Garbini, Ludovica Rolando, Maria Adelaide Iannelli, Maria Angeles Bustamante, Cristina Alvarez-Alonso, Anna Barra Caracciolo

Abstract:
This work evaluated the effects of sulfamethoxazole and copper on a natural microbial community in an agricultural soil amended with cattle manure or its digestate. Various microcosms were set-up with soil amended with manure or digestate and spiked with Cu (30 mg/kg) alone or with SMX (7 mg/kg). *Lactuca sativa* was planted in half of the experimental set in order to compare the bulk soil and rhizosphere microbial community response to contaminant co-presence.

The mixture effects on the microbial community were evaluated in terms of abundance (epifluorescence direct counts), viability (epifluorescence live/dead method) and dehydrogenase activity. The microbial diversity was assessed by NGS. Moreover, the qPCR was used for quantifying the *sul1*, *sul2*, *intI1*and rRNA16s genes.

Cattle manure or digestate addition caused a prompt rise in microbial activity, on the other hand introduced some ARGs. The SMX antibiotic was almost completely biodegraded in 36 days in the amended conditions and ARGs generally decreased over time. Interestingly, the *sul2* gene increased at 36 days in Cu and SMX co-presence, showing a copper strong co-selection for this ARG. Moreover, copper affected negatively plant growth and microbial activity, except in soil amended with digestate. In fact, digestate favoured microbial activity, biodiversity and lettuce growth both in presence and in absence of copper. Overall, lettuce favoured rhizosphere microbial biodiversity and classes (e.g., *Alphaproteobacteria*) and genera (e.g., *Bacillus*) denoting a good-quality soil increased.

The application of organic amendments such as manure and digestate can introduce both ARB and ARGs. However, digestate not only contain a lower amount of ARGs, but owing to its composition is a very good fertilizer which not only promoted lettuce growth but also mitigated the overall copper negative effects.

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ENVIRONMENTAL BIOTECHNOLOGY

Effects of sulfamethoxazole and copper on the natural microbial community from a fertilized soil

Alessandra Narciso[1,2] · Paola Grenni[1,3] · Francesca Spataro[3,4] · Chiara De Carolis[1,5,6] · Jasmin Rauseo[3,4] · Luisa Patrolecco[3,4] · Gian Luigi Garbini[1] · Ludovica Rolando[1] · Maria Adelaide Iannelli[3] · Maria Angeles Bustamante[7] · Cristina Alvarez-Alonso[7] · Anna Barra Caracciolo[1]

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Abstract

Cattle manure or its digestate, which often contains antibiotic residues, can be used as an organic fertilizer and copper (Cu) as a fungicide in agriculture. Consequently, both antibiotics and Cu are considered soil contaminants. In this work, microcosms were performed with soil amended with either manure or digestate with Cu and an antibiotic (sulfamethoxazole, SMX) co-presence and the planting of *Lactuca sativa*. After the addition of the organic amendments, a prompt increase in the microbial activity and at the same time of the *sul1* and *intI1* genes was observed, although ARGs generally decreased over time. In the amended and spiked microcosms, the microbial community was able to remove more than 99% of SMX in 36 days and the antibiotic did not bioaccumulate in the lettuce. Interestingly, where Cu and SMX were co-present, ARGs (particularly *sul2*) increased, showing how copper had a strong effect on resistance persistence in the soil. Copper also had a detrimental effect on the plant-microbiome system, affecting plant biomass and microbial activity in all conditions except in a digestate presence. When adding digestate microbial activity, biodiversity and lettuce biomass increased, with or without copper present. Not only did the microbial community favour plant growth, but lettuce also positively influenced its composition by increasing bacterial diversity and classes (e.g., *Alphaproteobacteria*) and genera (e.g., *Bacillus*), thus indicating a good-quality soil.

Key points

- *Cattle digestate promoted the highest microbial activity, diversity, and plant growth*
- *Cattle digestate counteracted detrimental contaminant effects*
- *Cu presence promoted antibiotic cross-resistance in soil*

Keywords Lettuce · Plant-microbiome system · Antibiotics · Cattle manure · Cattle manure digestate · ARGs

Introduction

Antibiotics (ABs) and heavy metals (HMs) are widely used in agriculture for different purposes, such as treating animal disease and crop pests, respectively. It has been generally recognized that ABs, owing to their incomplete metabolism in treated organisms, are excreted through feces and urine and can be found as active residues in wastewater, surface water, sediment, and soils (Zhang et al. 2018a; Barra Caracciolo et al. 2020b). Consequently, the application of manure

✉ Francesca Spataro
francesca.spataro@cnr.it

[1] Water Research Institute, National Research Council (IRSA-CNR), Montelibretti, 00010 Rome, Italy

[2] Department of Ecological and Biological Sciences, Tuscia University, 01100 Viterbo, Italy

[3] National Biodiversity Future Center (NBFC), Palermo, Italy

[4] Institute of Polar Sciences-National Research Council (ISP-CNR), Montelibretti, 00010 Rome, Italy

[5] Department of Environmental Biology, Sapienza University of Rome, 00185 Rome, Italy

[6] Institute of Agricultural Biology and Biotechnology, National Research Council (IBBA-CNR), Montelibretti, 00010 Rome, Italy

[7] Centro de Investigación e Innovación Agroalimentaria y Agroambiental (CIAGRO-UMH), Miguel Hernandez University, Orihuela, 03312 Alicante, Spain

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or biosolids as organic fertilizers to agricultural soils can unintentionally introduce antibiotics, often in mixtures with other contaminants such as heavy metals (Kuppusamy et al. 2018; Bünemann et al. 2024).

AB occurrence in environments represents a significant concern for ecosystems and human health, as highlighted by the "One Health Concept" (White and Hughes 2019; Prata et al. 2022) which states that animal and human health is linked to that of their environment. In fact, AB residues in ecosystems can promote the spread of antibiotic resistance between resistant bacteria and natural microorganisms (Ben et al. 2019; Samreen et al. 2021; Narciso et al. 2023a) and from them to animal and human microbiomes through contaminated water or edible vegetal species (Gudda et al. 2020; Soto-Giron et al. 2021; Barra Caracciolo et al. 2022).

ABs are emerging and ubiquitous micropollutants and, owing to their biocide properties, they can kill or inhibit natural microbial communities involved in key ecosystem functioning (Grenni et al. 2018; Cycoń et al. 2019; Sodhi et al. 2021; Narciso et al. 2023a). In addition, particularly at below minimum inhibitory concentrations, they act as selective agents which can increase antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in natural ecosystems (Berendsen et al. 2018; Barra Caracciolo et al. 2020b, 2022; Singh et al. 2021).

Sulfamethoxazole (SMX) is a sulfonamide antibiotic among the most widely used in both human and veterinary medicine, and its residues are frequently detected in organic fertilizers and agricultural soils (Conde-Cid et al. 2020; Vieublé Gonod et al. 2022; Visca et al. 2022; Patyra et al. 2024). SMX is not an intrinsically persistent compound (e.g., its halving in soil is ca. 7 days, Rauseo et al. 2019; Garbini et al. 2022) but its continuous use and release determine its pseudo-persistence in water (Patrolecco et al. 2018) and soil (Wu et al. 2024). Indeed, sulfonamide resistance genes are very common in the environment and for this reason are considered resistance determinants in water, livestock waste, and manure-amended agricultural soils (Ben et al. 2019; He et al. 2020; Radu et al. 2021).

Most antibiotic resistance genes (ARGs), including *sul* genes, are located on mobile genetic elements, such as plasmids, which favour their horizontal transfer between different species (Pinilla-Redondo et al. 2018; Dimitriu 2022). Plasmids can contain gene cassettes, which confer resistance not only to ABs but also to other contaminants (e.g., HMs) or environmental stresses (Chaturvedi et al. 2021; Huang et al. 2021). For this reason, not only ABs but also other factors can favour ARG persistence among natural bacterial populations (Feng et al. 2021). Indeed, antibiotic resistance has been frequently found in metal-tolerant bacteria, and this can contribute to a reservoir of transferable antibiotic resistance genes in the environment (Nath et al. 2019; Glibota et al. 2019).

Several HMs are essential micronutrients for biota, but their occurrence at concentrations higher than the natural ones (due to anthropogenic pollution) can exert toxic effects (Masmoudi et al. 2013; Ali et al. 2019; Kumar et al. 2021; Barra Caracciolo and Terenzi 2021).

As a trace element, Cu is a co-factor in several animal, plant, and bacterial enzymes (Hänsch and Mendel 2009; Kaur et al. 2023). Cu is also a biocide in the high amounts widely applied on vineyards and horticultural crops (up to 4 kg/ha/y) for preventing fungal attacks and other plant disease (Fagnano et al. 2020). Cu concentrations in agroecosystems can range from 20 up to 800 mg/kg, as, for example, in South European agricultural soils impacted by long-term fungicide applications (Panagos et al. 2018; Fagnano et al. 2020; Zamulina et al. 2022; Tamm et al. 2022). The EU Reg. 1981/2018 limits the use of copper compounds in agriculture to an average of 4 kg/ha per year, and an Italian decree (DM 46/2019) indicates thresholds of 200 mg/kg in soils (Tóth et al. 2016).

Recent studies suggest that Cu can increase the toxic effects of ABs and/or favour ARB, but the conditions and amounts causing this phenomenon need to be investigated (Perković et al. 2022). For example, a recent work showed that a mixture of copper (3 mg/L) and SMX (0.7 mg/L) displayed an additive ecotoxicological effect on the *A. fischeri* bacterium, compared with that obtained from single solutions (with only copper or sulfamethoxazole) (Narciso et al. 2023b). In another work, Kang et al. (2018) demonstrated that ARGs (comprising those involved in sulfonamide resistance) and mobile genetic elements (MGEs, as *intI1*) increased significantly in a soil microbial community treated with different copper concentrations (from 50 to 1000 mg/kg).

Microbial community structure (biodiversity) and activity, including the capability to respond to contamination with different strategies (e.g., developing resistance and/or capability to degrade and remove toxic compounds), depend on a plethora of site-specific environmental conditions. Among them, temperature is a critical variable modulating microbial development and activity (Burman and Bengtsson-Palme 2021) and affecting contaminant fate and dissipation. For example, sulfonamide degradation in the composting of chicken manure was found to increase when passing from mesophilic (30–40 °C) to thermophilic (50–60 °C) conditions (Lin et al. 2017). Indeed increasing temperatures not only influenced microbial community activity and composition but also favoured AB dissipation by promoting abiotic hydrolysis processes (Mitchell et al. 2014; Yang et al. 2021).

Light is another abiotic factor, which can influence AB transformation, especially for photosensitive molecules such as SMX. Although the primary role of microbial degradation in SMX removal has been demonstrated, sulfamethoxazole dissipation increases significantly in the presence of light

(Lai et al. 2011; Poirier-Larabie et al. 2016; Patrolecco et al. 2018).

For evaluating AB and ARG environmental fate and persistence, it is therefore necessary to take into consideration different abiotic and biotic factors, including possible sudden temperature changes, due to ever more frequent extreme weather events (Li et al. 2023; Jampani et al. 2024). For example, recent studies have reported that, if high temperatures unexpectedly persist, they can both promote the survival of gut bacterial populations and increase the possibility of ARG spreading from pathogens to natural bacterial populations (MacFadden et al. 2018). On the other hand, temperatures higher than the gut bacteria optimal ones (ca. 37 °C) can select the mesophilic and thermophilic bacteria involved in composting and anaerobic digestion processes and possibly in AB degradation (Mitchell et al. 2015; Lin et al. 2017; Visca et al. 2022).

Another complex aspect to be considered is the relationships between AB concentrations and their ARGs. For example, Selvam et al. (2012b) reported that sulfadiazine and chlortetracycline degraded completely, in 3 and 14 days, respectively, of composting and this process decreased *tet* genes during the mesophilic stage. On the contrary, sulfamethoxazole gene (*sul1*, *sul2*, *dfrA1*, *dfrA7*) abundances were not influenced by a sulfadiazine presence (initially spiked at 2–20 mg/kg and then completely removed), and these genes were still present after its degradation (up to 21–28 days). The authors hypothesized that the high *sul* persistence might be due to the possible presence of antibiotic metabolites or high mobility of these genes among bacteria. The authors concluded that temperature is essential for reducing ARGs during the thermophilic phase because it selects for different microbial populations and resistance mechanisms (Selvam et al. 2012a). In a biogas plant, operating in mesophilic conditions (33–35 °C) and fed with zootechnical waste, it was found that both antibiotics (sulfamethoxazole, ciprofloxacin, and enrofloxacin) and their ARGs decreased during the 45-day anaerobic digestion process. In particular, SMX was removed by up to 100%, ENR up to 84%, and CIP up to 92%. At the same time, ARGs (including *sul1*, *sul2*, and *intI1*) declined significantly (up to 80%) in the digestate samples (Visca et al. 2021).

Overall, sulfamethoxazole ARGs (such as *sul1* and *sul2*) can be influenced by temperature variations. Some authors simulated a biological treatment of dairy lagoon water by incubating some samples at 4° and 20 °C in both aerobic and anaerobic conditions. In aerobic conditions, s*ul1* had the highest abundance at 20 °C and at day 10 and then decreased significantly; on the other hand, *sul1* at 4 °C (although with lower values) persisted for 100 days. In anaerobic conditions, a similar temperature effect was found, with a general lower ARG presence than in aerobic conditions. Interestingly, *sul2* increased over time at 20 °C, with the highest level at 60 days, in both aerobic and anaerobic conditions. This phenomenon was not found at 4 °C in anaerobic conditions (Pei et al. 2007; Lin et al. 2017).

Another key aspect to be considered in environmental studies is contaminant bioavailability. In fact, not only ABs but also heavy metals (including copper) can have different effects on microorganisms depending on their bioavailability in a contaminated matrix. Heavy metals can form stable complexes with soil organic matter (Kim et al. 2015; Barra Caracciolo and Terenzi 2021), and soluble or non-soluble fractions of organic carbon (OC) can influence antibiotic-soil and copper-soil interactions. For example, copper can bound strongly to clay minerals and form both insoluble and soluble organic complexes with OC (Shao et al. 2020). Moreover, in the presence of an external carbon source, increases in adsorption and complexation of Cu can occur (Bolan et al. 2003). Interestingly, some authors found that fulvic acids resulted particularly in decreasing plant uptake and toxicity of heavy metals at high concentrations (25 mg/kg), because of their strong binding affinity (Shahid et al. 2012; Canellas et al. 2015).

AB and ARG persistence in the environment is a very complex issue, which depends on the combination of various biotic and abiotic factors, including multicontamination, which can act as opposite selective forces on natural microbial communities. In this context, this work aimed at evaluating the possible effects of SMX alone or in a copper co-presence on a natural soil microbial community in the presence/absence of organic amendments (manure or digestate) and lettuce plants. The soil microbial community was assessed in terms of abundance (DAPI counts), activity (dehydrogenase), structure (NGS), and ARGs (qPCR). Moreover, SMX dissipation in the soil and lettuce biomass was also evaluated at the end of the experiment (36 days).

Materials and methods

Sampling site and organic amendments

An agricultural sandy-clay-loam (60% sandy, 18% clay, 22% loam, USDA 1987) soil was collected from an organic farm close to Rome. The samples were collected from the surface layer (0–20 cm) using a shovel, and an initial characterization for organic carbon, total nitrogen, heavy metals, and microbial abundance was performed.

The manure and digestate used as organic amendments came from a cattle manure biogas plant located in a livestock farm in central Italy, and their characteristics are described in detail in previous works (Visca et al. 2021, 2022; Mazzurco Miritana et al. 2022). All the parameters analyzed showed values which met the European Regulation limits for fertilizing products (EU 1009/2019).

Microcosm set-up

Soil microcosms (plant pots containing 1-kg soil each) were set up with different experimental conditions. The pots contained soil spiked with the antibiotic sulfamethoxazole (7 mg/kg) and copper sulfate (30 mg/kg) and were amended with manure (1% w/w) or digestate (1% w/w). Half of the experimental conditions were performed in the presence of lettuce plants (*Lactuca sativa*). Moreover, 36 microcosms were performed without the SMX antibiotic (in the presence/absence of the organic amendments) in order to assess the possible influence of copper alone on the soil microbial community.

The 18 experimental conditions performed are summarised as follows in Table 1.

Each experimental condition was performed in three replicates. The experiment was maintained for 36 days in a greenhouse (from 1st May 2022), and each microcosm was watered daily to keep the same soil moisture. The initial temperature was 23.7 ± 2.8 °C, in line with average spring values at this latitude. From day 14, the temperature rapidly increased to 30.4 ± 15.5 °C in the following days, reaching an average temperature of 32.8 °C with variations between a minimum temperature of 22.2 °C and a maximum one of 43.3 °C, at day 18. The average temperature remained at 30 ± 2 °C until the end of the experiment (day 36).

Two samplings were performed, at the start (3 h) and at the end (36 days) of the experiment.

Table 1 Summary of the 18 experimental conditions. *Cu* copper, *SMX* sulfamethoxazole, *P Lactuca sativa*, *M* manure, *D* digestate

Experimental condition	Acronym
Soil + manure + Cu + SMX	SMCA
Soil + digestate + Cu + SMX	SDCA
Soil + Cu + SMX	SCA
Soil + manure + Cu	SCM
Soil + digestate + Cu	SCD
Soil + Cu	SC
Soil + manure	SM
Soil + digestate	SD
Control soil	S
Soil + manure + Cu + SMX + *L. sativa*	SMCA_P
Soil + digestate + Cu + SMX + *L. sativa*	SDCA_P
Soil + Cu + SMX + *L. sativa*	SCA_P
Soil + manure + Cu + *L. sativa*	SCM_P
Soil + digestate + Cu + *L. sativa*	SCD_P
Soil + Cu + *L. sativa*	SC_P
Soil + manure + *L. sativa*	SM_P
Soil + digestate + *L. sativa*	SD_P
Control soil + *L. sativa*	S_P

At the end of the experiment, plant roots developed throughout the soil inside each pot, and, consequently, the soil results refer to "rhizosphere soil."

For each condition, destructive samplings were performed in three replicates. Different aliquots of soil were collected and used for microbiological analyses or chemical determinations.

Soil physicochemical characterization and amendment organic carbon analyses

The soil pH and electrical conductivity (EC) were measured in a water solution (1:2.5 and 1:5 soil to distilled water (w/v) ratio, respectively) (Allison and Moodie 1965). The soil water content was determined with a gravimetric analysis, following official methods (DM 13/09/1999 and s.m.i.). Aliquots of air-dried samples (15–20 mg each) were used for the total organic carbon (OC) and nitrogen (N) analyses, using an elemental carbon analyser (Carlo Erba NA 1500 series 2 C/H/N/O/S). Further details are described in previous works (Barra Caracciolo et al. 2020a).

The water-soluble organic carbon was determined in water extracts of 1:10 (w/v) and 1:50 (w/v) of soil samples and amendments respectively, after filtration through a synthetic filter with a pore diameter of 0.45 µm, by using an automatic analyser for liquid samples (TOC-V CSN Analyzer, Shimadzu) (Bustamante et al. 2012). The extractable organic carbon (C_{EX}) was determined in organic amendments (manure and digestate) by dissolving sample aliquots in 0.1 M NaOH. The fulvic acid–like carbon (C_{FA}) amount after the precipitation of the humic acid–like carbon (C_{HA}) one was found to be pH 2.0 (Sánchez-Monedero et al. 1996). The C_{HA} was calculated by subtracting the C_{FA} from the C_{EX}.

Chemicals and reagents

Pure solvents (HPLC grade), such as methanol (MeOH), acetone (ACT), acetonitrile (ACN), and hydrochloric acid (37%, HCl), were purchased from VWR (Radnor, PA, USA). Formic acid (98–100%) for LC–MS LiChropur™, used to acidify solvents and composing the mobile phase for the analytic determinations, was purchased from Sigma-Aldrich (Steinheim, Germany). The pH of the mobile phase was adjusted with a portable pH meter (HANNA Instruments, Woonsocket, RI USA). A Milli-Q Millipore system (Bedford, MA, USA) produced the ultrapure water (18 MΩ/cm quality).

SMX vetranal analytical standards were from Merck KGaA (Darmstadt, Germany). Deuterated SMX (SMX-d4, Clearsynth) was used as the internal standard.

$CuSO_4 \cdot 5H_2O$ was purchased from Sigma-Aldrich, Germany, and used to prepare a copper solution (30 mg/L) in ultrapure water.

An individual stock solution (100 mg/L) was prepared by dissolving the AB standard powder in methanol and storing it in the dark at − 20 °C. A working standard solution was prepared at each sampling time by diluting the stock solution with water and then stored at 4 °C.

The mixture of SMX and $CuSO_4$ was prepared by dissolving 2.5 mg of SMX in MeOH (50 ml) to obtain a final concentration of 50 mg/L and stored at − 20 °C. Daily working standard solutions of the antibiotic were obtained by dilution of the stock solution with a mixture of ultrapure water: MeOH (1:1 v/v) and stored at 4 °C. Waters Oasis Hydrophilic–Lipophilic Balance (HLB) cartridges (6 ml, 1 g) were from Waters (Milford, MA, USA). The inert material used to fill the extraction cells was diatomaceous earth (Dionex™ ASE™ Prep DE) purchased from Thermo Scientific (Waltham, MA, USA).

Chemical determinations

The antibiotic extraction procedure was performed using a SPE (solid-phase extraction) equipped with Oasis Hydrophilic-Lipophilic Balance (HLB, Waters, Milford, USA) cartridges (6 ml, 200 mg), in line with previous work (Spataro et al. 2019). Briefly, the cartridges were activated with 5 ml of MeOH and 5 ml of ultrapure water (18 MΩ/cm quality, Millipore, Bedford, USA) passed at a flow rate of 5 ml/min. Soil water extracts were passed into the cartridges, and their elution was performed with a mixture of MeOH, acetonitrile (ACN, HPLC grade, VWR-Radnor, USA), and acetic acid (Sigma Aldrich, Germany) (40:40:20 v/v/v). The samples were then evaporated with a gentle nitrogen stream and reconstituted with 0.5 ml of a mixture of ACN and ultrapure water (50:50 v/v) acidified with formic acid at pH 3. SMX analytical determination was performed with high-performance liquid chromatography (HPLC, column oven mod. LC-100, micropump series 200, Perkin Elmer, USA) interfaced with a triple quadrupole mass spectrometer (MS/MS, mod. API 3000, AB Sciex, Germany). The operative conditions were as follows: injection loop of 20 µl and the chromatographic column was Gemini column (150×4.6 mm, 5 µm RP C 18, Phenomenex, France) (Spataro et al. 2019). SMX elution was carried out in gradient mode at a flow rate of 0.3 ml/min. The mobile phase was composed of MeOH (phase A) and ultrapure water (phase B), both acidified with 0.1% of formic acid. The gradient profile started with phase A at 10%. It then increased from 10 to 90% in 10 min and finally returned to 10% in 15 min. The ESI (electrospray ionization) operated with 12 units of curtain gas, 14 units of nebulizer gas, and source temperature of 400 °C. Nitrogen was both collision and drying gas. The AB quantification was based on the *m*/*z* ion ratios and a comparison of the retention times (RTs) of product ions/fragments in the samples with those of each standard, considering criteria differences of 0.2 min. Two calibration curves (five points) at lower (0.5, 1.0, 2.0, 2.5, and 5.0 ng/L) and higher (5, 20, 50, 100, and 500 ng/L) concentration ranges were considered, indicating the good linearity of the method ($R^2 > 0.98$). The SMX recoveries were also evaluated using ultrapure water artificially spiked at different concentrations (50, 200, and 500 ng/L) and ranging between 82 and 99%. The detection limits (LODs) for SMX were calculated following the IUPAC method (Thompson et al. 2002) and were 2.1, 5, and 4 ng/L, respectively. Quantification limits were set at three times the LODs.

Copper concentrations were measured using an inductively coupled plasma optical emission spectrometer (ICP-OES, 5800 Agilent technologies, USA). The quantification was performed by interpolation using a calibration curve obtained by diluting a 1 g/L stock solution into six standard solutions. The wavelengths (nm) used for Cu were 327 and 395 (Barreto et al. 2021; Iannelli et al. 2022).

Total microbial number, cell viability, and dehydrogenase activity

The total microbial number (N. cells/g soil) of each sample was analyzed in formaldehyde-fixed soil aliquots (1 g each), using the epifluorescence direct count method, which relies on DAPI (4′,6-diamidino-2-phenylindole), as the DNA fluorescent intercalant. Samples were processed as detailed in previous works (Barra Caracciolo et al. 2005, 2013, Barra Caracciolo et al. 2015b).

Cell viability (% live cells/live + dead) was assessed in non-fixed soil samples using two fluorescent dyes, SYBR Green II and propidium iodide (Sigma-Aldrich Germany), to discriminate between viable (green) and dead (red) cells, as previously detailed (Grenni et al. 2014).

Both the total microbial cell number and the percentage of viable cells were counted under a Leica DM 4000B fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

Finally, the N. of live cell abundance (N. live cells/g soil) was obtained by multiplying each total microbial abundance datum (N. total cell/g) by the corresponding cell viability one (% live cells/live + dead), as reported in other works (Amalfitano et al. 2008; Garbini et al. 2022).

Dehydrogenase activity (DHA) was evaluated in 6-g soil samples, using a colorimetric method based on the quantification of 2,3,5-triphenyl formazan (TPF) compound produced from the reduction of 2,3,5-triphenylte-trazolium-chloride (Grenni et al. 2009, 2012). The microbial activity (expressed as TPF/g soil) was measured using a Multiskan Sky Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

All the microbiological analyses were performed in at least three replicates for each experimental condition and sampling.

Microbial DNA extraction and quantification of ARGs

Total DNA was extracted from each soil sample using a DNeasy PowerSoil kit (Qiagen, USA) following the procedure described by Barra Caracciolo et al. (2022). Further details are provided in the Supplementary Information. The DNA extracted was used for both the qPCR and NGS analyses.

Quantitative PCR was used to quantify two SMX-resistance genes (*sul1* and *sul2*) and the class 1 integron-integrase gene (*intl1*). The *16S rRNA* gene copy numbers were determined for calculating the relative abundance of the resistance genes targeted in the samples. The qPCR procedure used is described in detail by Visca et al. (2022). Further description of the method is reported in the Supplementary Information. The quantitative PCR data were expressed as the ratio of ARG or *intI1* gene copy number per 16S copy number to evaluate the relative abundance of each target gene in the bacterial community.

Phylogenetic characterization of the microbial community

Aliquots of the DNA extracted (15 ng/µl) from each replicate were used for NGS. The V3–V4 region of *16S rRNA* genes was amplified with the 341F and 805R primers (Table S1, Supplementary Information) for the identification of the prokaryotic community (*Bacteria* and *Archaea*). The method used followed the procedure previously described (Callahan et al. 2016; Bolyen et al. 2019; Mazzurco Miritana et al. 2022; Garbini et al. 2023). Further details are provided in Supplementary Information. Alpha-diversity indices (Shannon, Evenness, and Chao1) were also estimated.

The Illumina Miseq sequencing raw data are deposited in the NCBI (National Center for Biotechnology Information) database (accession number: PRJNA1153537).

Lettuce biomass

L. sativa plants were sampled at the end of the experiment, washed with sterile MilliQ water to remove any soil particles, weighed (aerial parts + roots), and put in an air-forced oven at 60 °C for 72 h to evaluate the dry biomass.

Statistical analyses

All the statistical analyses were performed using R (4.3.1version https://www.r-project.org).

The microbiological and chemical results are reported as average values ± standard errors of at least three replicates and were compared with each other with ANOVA using the *aov* function. More specifically, if the ANOVA test indicated the significative difference among results obtained from different conditions, the Tukey honestly significant difference test (*TukeyHSD* function) was applied to evaluate the paired comparison of experimental condition values. A significance threshold of 0.05 was considered ($p < 0.05$). To detect any significant differences between the MGE and ARGs in the experimental conditions, a non-parametric one-way ANOVA (Kruskal–Wallis) was performed using the *kruskal.test* function combined with the *pairwise.wilcox.test* function as a post hoc test (Benavoli et al. 2016). The microbial community alpha diversity was analyzed using the Evenness and Shannon diversity indices, while the Chao1 index (Chao et al. 2005) was used as an estimator of potential richness.

The effect of the amendments, SMX and Cu, and *Lactuca sativa* on the prokaryotic communities in the different conditions was evaluated using the principal coordinate analysis (PCoA) based on the Bray–Curtis distance estimated using the function *vegdist* (*Vegan* package of R, https://doi.org/10.32614/CRAN.package.vegan).

A multivariate ANOVA with permutations (PERMANOVA) was applied to assess significance. Pairwise PERMANOVA was performed using the function *pairwise.perm.manova* from the *RVAideMemoire* package to evaluate the significance of ASV changes in the prokaryotic composition in the different experimental conditions.

Most abundant classes and genera from each experimental condition were selected, and their relative abundance was estimated using the *Phyloseq* package (https://code.bioconductor.org/browse/phyloseq/). ASV abundances of the selected genera were normalized by *z*-score and displayed in a heatmap generated by the *ComplexHeatmap* package. In the heatmap, genera and experimental conditions were grouped in accordance with hierarchical clustering dendrograms.

Network analyses were performed to visualize correlations between ARGs, MGE, SMX, and microbial genera relative abundances. Spearman's rank correlations ($\rho > 0.75$ and $p < 0.01$) were used to build a correlation matrix on which to base the network analysis (*Hmisc* r package). The network visualization was carried out on

the interactive "Gephi" platform (Gephi Version 0.10.1, https://gephi.org/).

Results

Manure, digestate, and soil physicochemical characterization

Table 2 reports the main characteristics of the manure and digestate (e.g., pH, electrical conductivity, total nitrogen (N), total organic carbon (OC), water-soluble C, extractable C (fulvic and humic acids)) used in this experiment. Both organic amendments contained residual concentrations of SMX (ca. 0.045 mg/kg).

The agricultural soil used for the experiment had an organic carbon content of 1.05% ± 0.05 and nitrogen of 0.33% ± 0.00 and a pH of 6.8 (Table S2). The highest increase in soil organic carbon and pH was observed in the case of digestate-amended soils (SD, SCD, and SDCA) (Table S2).

Chemical determinations

The SMX antibiotic almost disappeared over the 36 experimental days in all conditions, from 99.4% in SCA to 99.5% in SDCA. Adding plant did not promote any significant further antibiotic removal (Table 3).

Considering the bioaccumulation factors ($BAF = SMX_{leaves}/SMX_{soil3h}$), negligible residual concentrations of sulfamethoxazole were found in the lettuce leaves. The BAF values were always lower than 1, excluding SMX bioaccumulation in the edible part of the lettuce (Table 3).

The initial amount of copper in the soil was 21.97 ± 1.7 mg/kg; with addition of the copper solution, its concentration increased to 30.66 ± 1.8 mg/kg. Interestingly, in the planted microcosms, higher amounts (average values 43.31 ± 0.7 mg/kg) of copper than in the corresponding un-planted conditions (SCA, SMCA, SDCA) were observed at 36 days, showing how lettuce, through its roots, concentrated it inside its rhizosphere.

Live cell abundance and dehydrogenase activity

Figure 1a shows how amendments promoted live cell abundance (N. of live cells/g soil). However, copper and SMX had an initial detrimental effect on cell numbers ($p < 0.05$, Table S3) in all conditions where they were co-present (SCA, SMCA, and SDCA). At day 36, microbial abundances were significantly higher than in the control (S) in all conditions ($p < 0.05$), with the highest values in the amended rhizosphere. The amendments also promoted overall dehydrogenase activity, and this effect was amplified by the plant presence (36 days) (Fig. 1b). However, only in the digestate planted microcosms (SD_P, SCD_P, and SDCA_P) did DHA show values that were always significantly higher ($p < 0.05$, Table S4) than in the corresponding un-planted conditions.

Quantification of ARGs

Manure and digestate introduced both *intI*1 and *sul1* genes to the amended soils. Overall, when adding manure, a significantly higher (Kruskal Wallis $p < 0.05$, Table S5) ARG abundance was found than in digestate-amended conditions. The *intI1* gene was initially the most abundant, but at 36 days, it had almost disappeared in all conditions (Fig. 2). At the same time, a general decrease in *sul1* was also observed (except in the conditions where copper was present), and a positive correlation ($p < 0.05$) between *sul1* and *intI1* was found.

Table 2 Physicochemical and chemical characterization of the cattle manure and digestate

	Manure	Digestate
pH	6.8 ± 0.3	7.6 ± 0.0
Electrical conductivity (μS/cm)	4.5 ± 0.0	1.4 ± 0.0
Total N (%)	1.8 ± 0.1	1.5 ± 0.1
Total organic C (%)	41.3 ± 0.7	32.8 ± 0.3
Water-soluble C (%)	6.7 ± 0.0	1.2 ± 0.4
Humic acid–like C (%)	12.7 ± 0.1	6.7 ± 0.0
Fulvic acid–like C (%)	1.1 ± 0.0	3.5 ± 0.1

Table 3 SMX residues (%) in soil (3 h and 36 days) and in leaves (36 days) and the corresponding bioaccumulation factor (BAF)

	3 h (mg/kg)	Soil 36 days (mg/kg)	Leaves 36 days (mg/kg)	BAF
SMCA	7.01 ± 0.01	0.0430 ± 0.001	-	-
SDCA	7.01 ± 0.03	0.0329 ± 0.00	-	-
SCA	7.01 ± 0.01	0.0450 ± 0.001	-	-
SMCA_P	7.01 ± 0.01	0.0419 ± 0.0004	0.263 ± 0.000	0.038
SDCA_P	7.01 ± 0.03	0.0377 ± 0.0004	0.096 ± 0.000	0.014
SCA_P	7.01 ± 0.01	0.0425 ± 0.001	0.399 ± 0.000	0.057

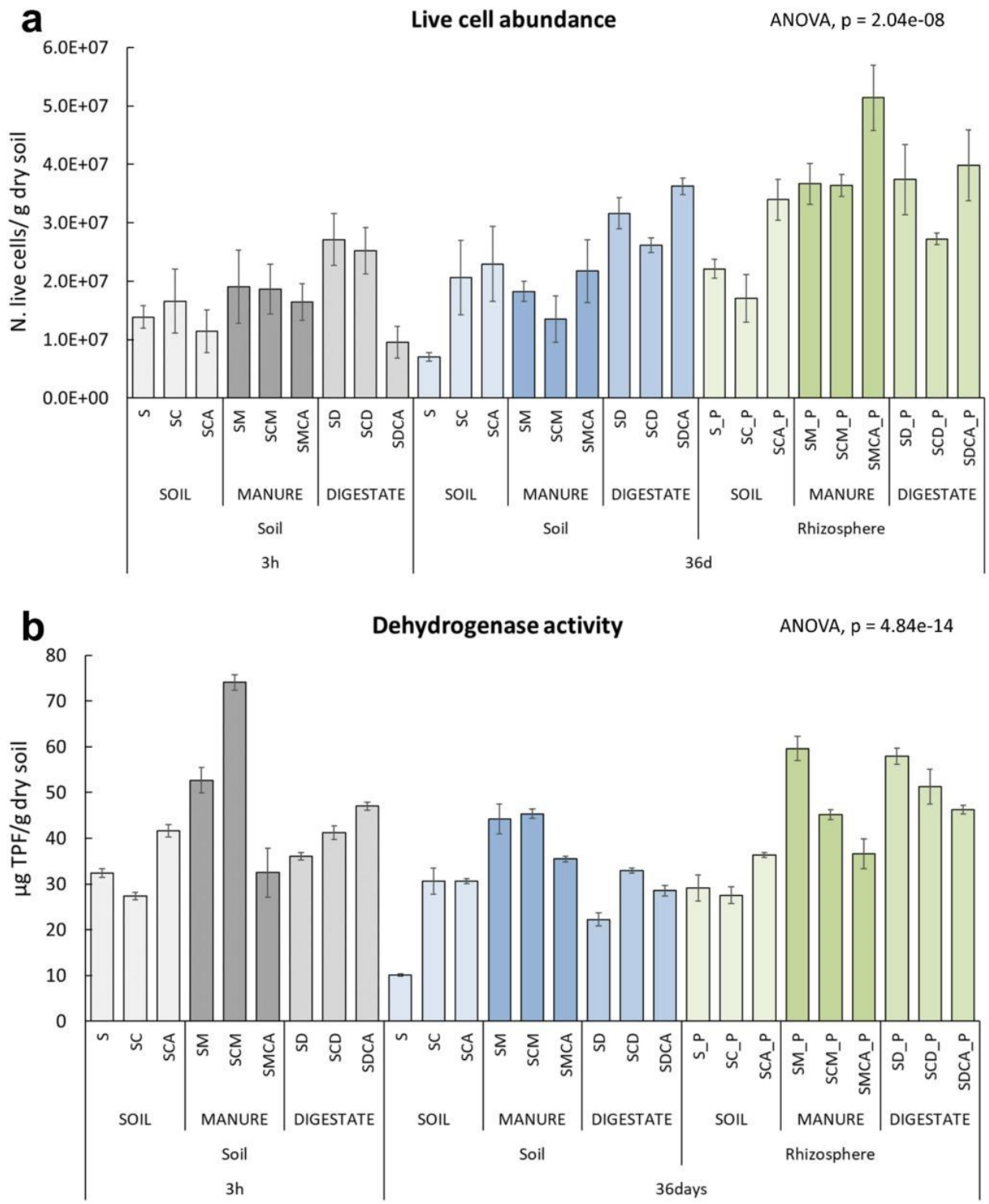

Fig. 1 **a** Live cell abundance (N. live cells/g dry soil). **b** Dehydrogenase activity (µg TPF/ g dry soil). Data are means of three independent replicates. The vertical bars represent the standard errors. The post hoc tests are reported in detail in Supplementary Material (Table S3 and Table S4)

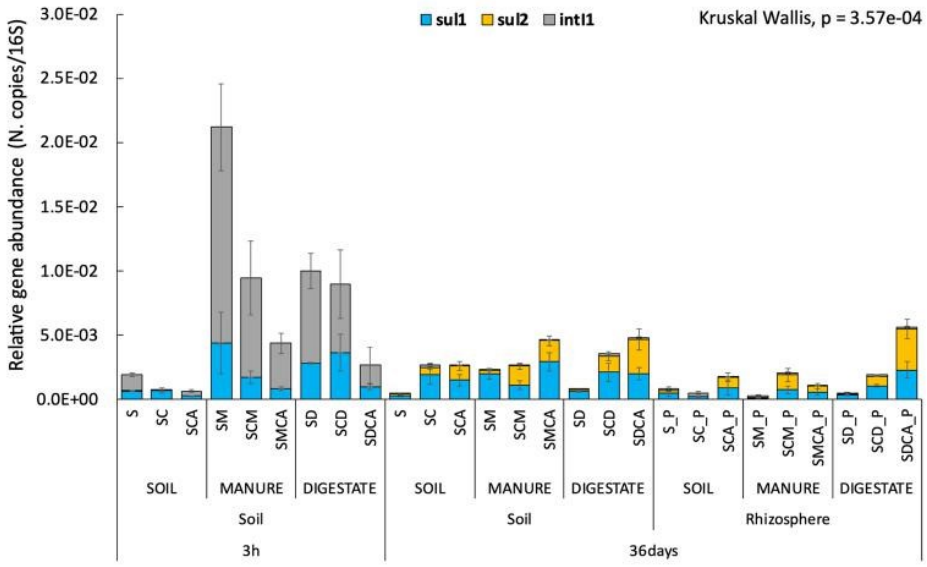

Fig. 2 Relative gene abundances (ARGs or MGE/16S) in the various experimental conditions. The post hoc test is reported in detail in Supplementary Material (Table S5)

Indeed, the relative highest values ($p < 0.05$) of ARGs (*sul1* + *sul2*) were with a copper and sulfamethoxazole co-presence in both the soil (SMCA and SDCA) and rhizosphere (SMCA_P, SDCA_P at 36 days). Moreover, comparing to the control soil (S), a higher relative abundance of *sul1* + *sul2* was found where copper was present (SC and SCA at 36 days).

Biodiversity and phylogenetic characterization of the microbial community

The prokaryotic community was characterized by sequencing the 16S gene in the various experimental conditions. Table 4 reports the diversity (Shannon, Evenness, and Chao1) indices, based on ASV abundance.

The Shannon and Evenness values were significantly different ($p < 0.05$) between the SMX and Cu co-presence conditions (SCA, SMCA, and SDCA) and the other conditions (S, SC, SM, SMC, SD, SDC) and between the initial and the final sampling ($p < 0.05$). The Evenness values were also significantly different ($p < 0.05$), between the amended (manure or digestate) conditions in co-presence of SMX and Cu (SMCA and SDCA) and the non-amended ones (SCA).

The Chao1 index values showed a significant difference between the initial and final sampling ($p < 0.05$) for all conditions. Interestingly, Chao1 was found to be lower ($p < 0.05$) in the SCM condition at 3 h in comparison with S and SM at 3 h. At 36 days, the SC value was significantly lower than those found in S, SCA, SMCA, SCD, and SDCA.

Finally, the diversity values were generally higher in a plant presence (rhizosphere) in all conditions, confirming the positive effect of the rhizosphere on microbial community structure and activity.

The principal coordinate analysis (PCoA) of the ASV distribution did not show significant differences between the presence and absence of SMX and/or Cu. At 36 days, there were significant differences (PERMANOVA, p-value < 0.001, $R = 0.89$, Table S6) between amended and un-amended conditions and between plant and un-planted ones (PERMANOVA, p-value < 0.001, $R = 0.94$). In the absence of plants, the soil microbial communities were significantly different (PERMANOVA, p-value < 0.001, $R = 0.84$, Table S7) between manure and digestate at 36 days. In a plant presence, the effect of the amendments was not observed (Fig. 3).

As regards the microbial community at class level, *Actinobacteria* (32.4%), *Gammaproteobacteria* (14.01%), and *Alphaproteobacteria* (11.5%) were predominant in the control soil (S at 3 h). *Bacteroidia* were found to be relatively more abundant in SC (12.2%) and SCA (11.3) than in the S microcosms, whereas *Bacilli* only in SC (11.6%). In manure-amended conditions, *Bacteroidia* were also in a relatively high number and in the co-presence of SMX and Cu (SMCA) were the dominant group (19%). An overall description of the classes (%) in the various conditions can be found in Fig. 4 and Table S8.

At 36 days, a general increase in *Actinobacteria* was observed in all experimental conditions (Fig. 4b, c), with relative percentages ranging from 27.3% (SM_P) and 28.5% (SCD_P) in a plant presence to 40.04% (SM) and 42.7% (SCM). Moreover, a sharp decrease in *Bacteroidia*, with the lowest values in SM and SCA (1.05% and 1.54%, respectively), was observed.

At the genus level (Fig. 5), S was initially (3 h) characterized by a high abundance of the *Streptomyces* (10.53%) and *Sphingomonas* (8.05%) genera; at 36 days, *Streptomyces*

Table 4 Diversity indices (Shannon, Evenness, and Chao1)

Experimental condition			Average values			Standard errors		
			Shannon	Evenness	Chao1	Shannon	Evenness	Chao1
3 h	Soil	S	9.57	0.927	1410.35	0.23	0.00	296.66
		SC	9.06	0.895	1166.26	0.41	0.03	98.28
		SCA	9.61	0.929	1365.05	0.09	0.00	105.70
		SM	9.69	0.929	1409.55	0.03	0.00	45.59
		SCM	9.01	0.921	902.75	0.12	0.00	82.25
		SMCA	9.51	0.924	1314.21	0.13	0.00	160.84
		SD	9.43	0.922	1236.46	0.15	0.00	132.34
		SCD	9.61	0.928	1354.93	0.10	0.00	114.90
		SDCA	9.55	0.927	1315.32	0.06	0.00	54.66
36 days	Soil	S	8.54	0.915	675.77	0.19	0.00	93.22
		SC	8.07	0.921	443.99	0.07	0.00	24.37
		SCA	8.50	0.920	618.61	0.08	0.00	48.30
		SM	8.10	0.879	625.98	0.20	0.00	103.74
		SCM	8.53	0.909	742.21	0.38	0.00	178.20
		SMCA	8.89	0.916	855.74	0.06	0.00	47.48
		SD	8.18	0.921	494.19	0.24	0.00	78.30
		SCD	8.66	0.906	785.57	0.19	0.00	106.86
		SDCA	8.88	0.923	813.74	0.13	0.00	73.76
	Rhizosphere	S_P	9.42	0.908	1396.23	0.09	0.00	130.58
		SC_P	9.29	0.917	1212.35	0.25	0.00	277.56
		SCA_P	9.30	0.912	1302.72	0.32	0.00	302.46
		SM_P	9.37	0.918	1219.18	0.06	0.00	48.78
		SCM_P	9.14	0.897	1240.20	0.19	0.00	196.34
		SMCA_P	9.29	0.918	1151.64	0.05	0.00	44.66
		SD_P	9.47	0.917	1322.11	0.11	0.00	103.92
		SCD_P	9.28	0.911	1203.83	0.12	0.00	154.68
		SDCA_P	9.55	0.922	1378.43	0.14	0.00	181.18

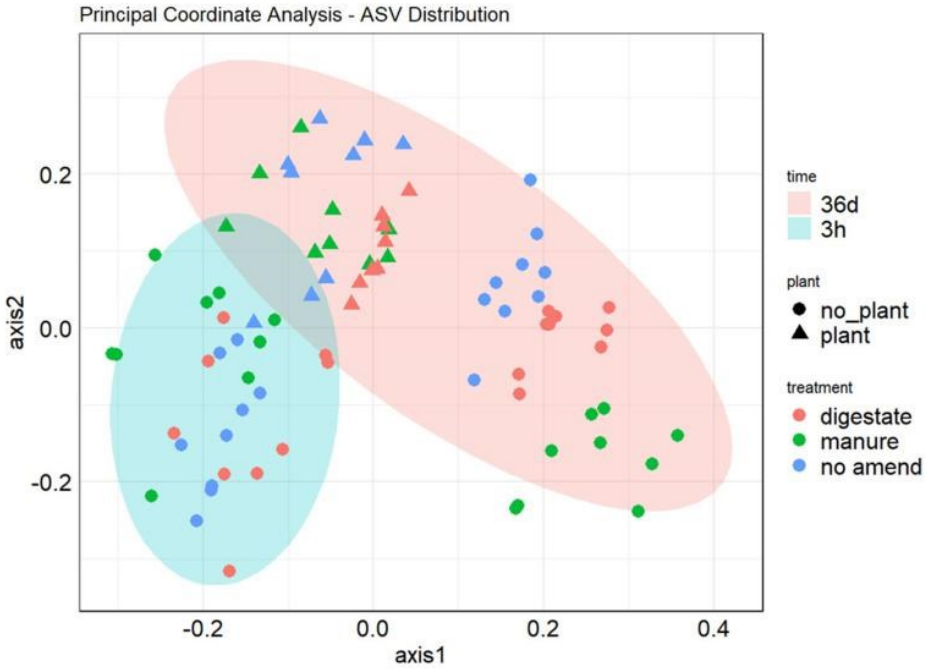

Fig. 3 Principal coordinate analysis (PCoA) based on the Bray–Curtis distance matrix and calculated on ASV distribution. Red points indicate the digestate-amended conditions, green points the manure-amended conditions, and blue ones the no amended conditions. The circle shape indicates no plant presence. The pink ellipse indicates the initial sampling time (3 h) and the light blue one the finale sampling time (36 days)

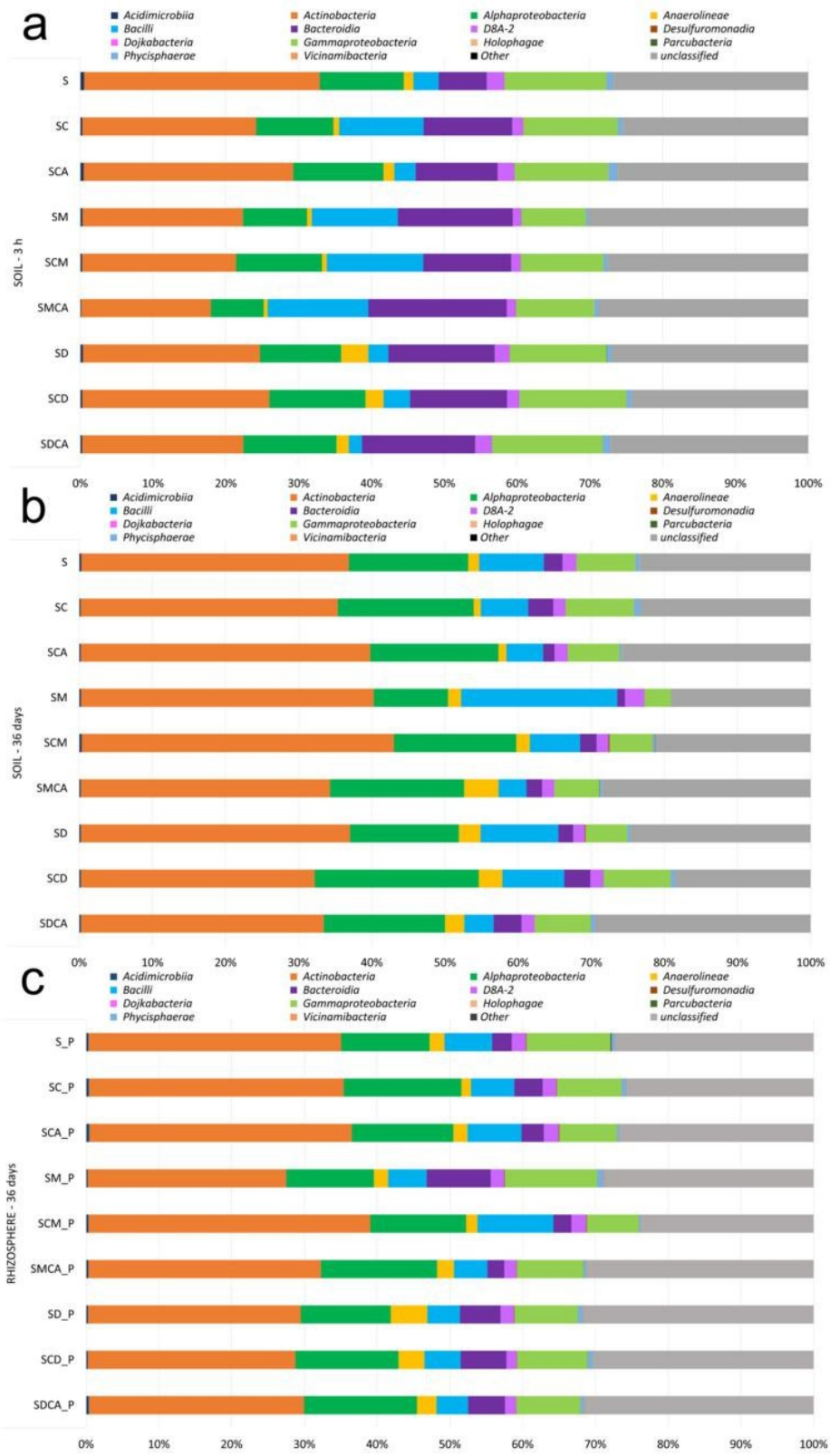

Fig. 4 Class relative abundances (% ASV) with an average presence > 1% in each experimental condition at 3 h (**a**), soil at 36 days (**b**), and rhizosphere at 36 days (**c**). Data are means of three independent replicates

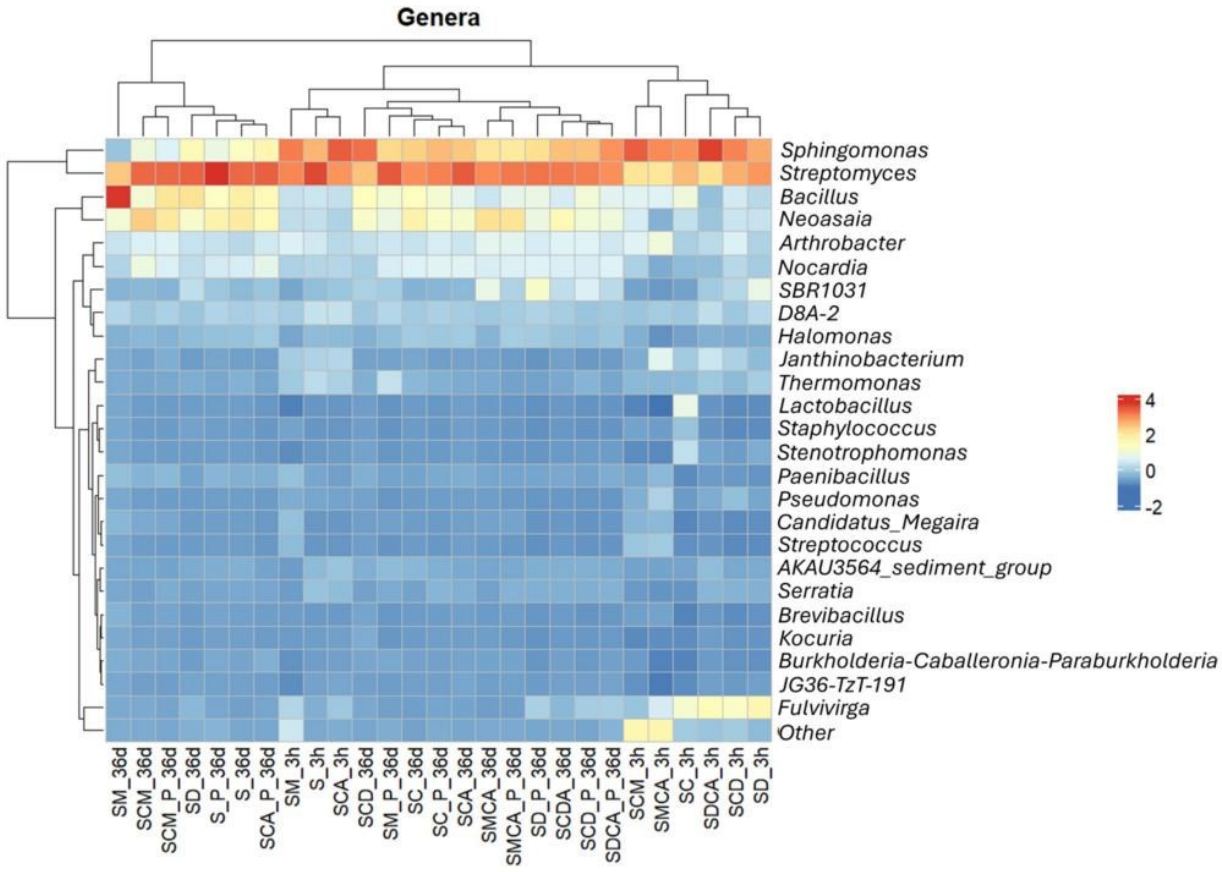

Fig. 5 Heatmap for prokaryotic relative abundances at genus level in the different conditions and at the sampling times (3 h and 36 days). Genera and conditions were grouped in accordance with a hierarchical clustering dendrogram, at the top and the left side of the heatmap. Data are means of three independent replicates

increased and *Sphingomonas* decreased in both the soil and rhizosphere (S and S_P). Copper spiking (SC) caused a prompt increase in *Bacillus* (*Bacilli*), *Fulvivirga* (*Bacteroidia*), and *Lactobacillus* (*Bacilli*) and a decrease in *Streptomyces* and *Sphingomonas*. In a similar way, an SMX and Cu co-presence (SCA) negatively influenced *Streptomyces*, but favoured *Sphingomonas*.

Adding digestate (SD, SCD, and SDCA at 3 h) induced an increase in *Fulvivirga* (*Bacteroidia*), but at 36 days, the same genus decreased in the same conditions in both the rhizosphere and soil (SD, SCD, SDCA, SD_P, SCD_P, and SDCA_P). Moreover, the *SBR1031* genus, belonging to the *Anaerolineae* class, increased in the presence of digestate at 3 h and became more abundant at 36 days compared with the control soil (S). The same genus also increased in the SMCA un-planted condition at 36 days.

On the contrary, adding manure caused a halving of *Streptomyces* and *Sphingomonas* compared to S at 3 h. At 36 days, a strong increase in *Bacillus* and *Neoasaia* was detected in the SM condition, as well as a decrease in *Sphingomonas*. On the other hand, in the manured condition (SM_P), the relative abundance values of other genera (*Sphingomonas*, *Streptomyces*, *Bacillus*, and *Neoasaia*) turned out to be similar to the initial ones. Moreover, a manure presence at 36 days favoured the *Nocardia* genus (*Alphaproteobacteria* class) in copper conditions (SCM, SMCA), especially in the absence of lettuce.

The principal component analysis (PCA), using overall data as variables, explained 47% of the total variance (Supplemental Fig. S1). Dimension 1 was significantly correlated ($p < 0.05$, Table S9) at 3 h with some bacterial genera such as *Staphylococcus*, *Streptococcus*, and *Pseudomonas* and ARGs (e.g., *intI1*) and at 36 days with *Nocardia*, *Streptomyces*, and *Neoasaia*, which in turn were positively correlated with other parameters such as *sul2* and copper. On the other hand, other genera (e.g., *Arthrobacter* and *Bacillus*) are on the opposite side of SMX.

The network analysis (including SMX, Cu, and 26 microbial genera) was performed to evaluate the relationships between the contaminants and the main bacterial genera in the soil and rhizosphere amended with manure or digestate at 36 days. Supplemental Fig. S2 shows how manure or digestate differently affected the microbial community. For example, in manure conditions, copper was in the same cluster as *Streptococcus* and *Staphylococcus* and *JG36-TzT* in the same cluster as *Stenotrophomonas*. In digestate conditions, SMX was linked to *Spingomonas* and *Halomonas* and ARGs were in the same cluster as copper and *Pseudomonas*.

Lettuce biomass

When adding copper and an antibiotic, a detrimental effect on lettuce biomass was observed, except in the digestate-amended conditions (Fig. 6). Indeed, the significantly highest ($p < 0.05$, Table S11) biomass values were found in the digestate (SD_P, SCD_P, and SDCA_P) conditions, whether or not copper or an antibiotic was present.

Discussion

The fact that SMX was dissipated in the soil studied shows how microbial community degradative populations were already present in the soil. SMX removal from soil has been reported in other works. For example, a 90% SMX decrease at 60 days (initial concentration 20 mg/kg) in soil amended with a cattle manure digestate and maintained at 20 °C was found also by Rauseo et al. (2019). A previous work reports the disappearance of 85% of SMX in 46 days (at ca. 26°C) in soil amended with manure or digestate (initial concentration 7.5 mg/kg soil) with no differences between bulk soil and lettuce rhizosphere (Barra Caracciolo et al. 2022). In our case, the higher degradation rate (> 99% in 36 days) compared to other works can be ascribed to the higher experimental temperature and daylight hours occurring in the greenhouse in the second part of the experimental period. Even if the initial temperature was ca. 22 °C, over time, it rose, with a sudden temperature increase (up to 40 °C on 24 May) and a seasonal increase in daylight hours. The positive influence of high temperatures and light on SMX dissipation has been reported in other works (Patrolecco et al. 2018; Archundia et al. 2021).

The SMX residues found in the lettuce leaves showed how it was not bioaccumulated in this edible plant; in fact, its BAF values were always lower than one. Comparable results for SMX were obtained by Barra Caracciolo et al. (2022). The negligible tendency of SMX to be assimilated by plants has also been demonstrated by other authors (Mullen et al. 2019; Stando et al. 2022). Cheng et al. (2020), in a 49-day experiment, found about 0.08 mg/kg of SMX in cabbage biomass, from an initial soil concentration of 5 mg/kg.

When adding digestate, the lowest residual concentrations of SMX in both soil and leaves were found, and the rhizosphere microbial activity and plant biomass were the highest (Figs. 2 and 6), in line with what is reported in other works (Rauseo et al. 2019; Rolando et al. 2023; De Carolis et al. 2024), because organic carbon, nutrients, and microbial populations were introduced with it (Innangi et al. 2017; Garbini et al. 2022, 2023). Moreover, digestate, increasing soil pH, presumably decreased copper availability and toxicity and promoted close and positive relationships between the plant and its microbiome (Barra Caracciolo et al. 2015a, 2022; Ancona et al. 2017; Di Lenola et al. 2018; Adedayo et al. 2022). Indeed, with digestate present, the positive and synergic relationships established in the lettuce-microbiome "metaorganism" (Berg et al. 2016; Hassani et al. 2018; Compant et al. 2019; Schmidt and Saha 2021) were effective in improving overall

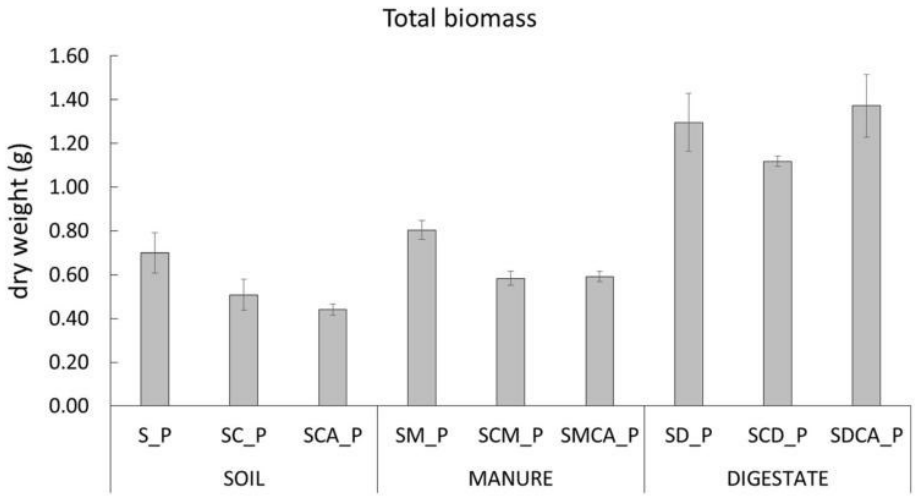

Fig. 6 Total (aerial + root) biomass (dry weight, g) of the lettuce plants under experimental different conditions at the end of the experiment (36 days)

soil quality and plant productivity. The digestate contained a higher amount of fulvic acids than manure, and this can have also favoured possible complexation processes between copper and antibiotics and this organic component, as described in other works (Qiu et al. 2007; Xu et al. 2017).

Although copper is an essential plant micronutrient, vegetal species can be affected by concentrations higher than is essential (Barra Caracciolo and Terenzi 2021) and microorganisms are more sensitive to it, even at concentrations of a few milligrams (Ochoa-Herrera et al. 2011; Narciso et al. 2023b). Copper, therefore, affected the rhizosphere microbiome (except in the case of digestate), as also demonstrated by the lower biodiversity values found in both un-amended (S_P, SC_P, and SCA_P) and manure-amended soils (SM_P, SCM_P, and SMCA_P).

In line with our results, a recent work reported both a negative impact from copper (at concentrations similar to ours) and a positive effect from digestate on lettuce growth. In particular, the chlorophyll content, leaf numbers, and shoot height were higher in all digestate conditions (including with copper present) than manure and unamended ones (De Carolis et al. 2024).

The effectiveness of the digestate in increasing rhizosphere microbial biodiversity and activity even with antibiotic presence has been found in other works (Singh et al. 2007; Buée et al. 2009; Barra Caracciolo et al. 2015a, 2022; Garbini et al. 2022).

It was evident that not only the microbial community favoured plant growth but also lettuce influenced its overall composition (Figs. 3 and 4c). For example, *Alphaproteobacteria* became the second dominant group in all the planted conditions and this result was in accordance with the presence in this class of several bacteria associated with the rhizosphere (e.g., nitrogen-fixing and nitrifying bacteria), in line with other works (Mendes et al. 2013; Barra Caracciolo et al. 2015a; Qiao et al. 2017; Di Lenola et al. 2018; Ling et al. 2022). Moreover, at 36 days, *Actinobacteria* were always in lower percentages than in the corresponding un-planted conditions and this was in line with a general increase in *Proteobacteria* in the rhizosphere, in particular in the amended conditions without copper (SM_P and SD_P). A *Proteobacteria* presence reflects an overall improvement in soil characteristics; in fact, they are dominant in good-quality state soils (Mocali et al. 2013; Godoi et al. 2014; Barra Caracciolo et al. 2015a, 2020a; Ancona et al. 2017).

Moreover, whether or not lettuce was present, digestate conditions generally favoured an increase in the percentage of the chemoorganotrophic *Anaerolineae*, which are known to favour nutrient recycling and soil quality (Xia et al. 2016; Freches and Fradinho 2024). On the other hand, the low organic carbon content (%) and dominance of *Actinobacteria* in the agricultural soil used in this work show that it was degraded, presumably because of its excessive use for agricultural purposes.

The analysis of the microbial community structure also confirmed that copper and sulfamethoxazole acted as a stressor for some microbial groups such as *Actinobacteria* and *Bacilli*, selecting, at 36 days, inside these classes, antibiotic-resistant bacteria and copper-tolerant ones. Indeed, *Actinobacteria*, which comprise not only sensitive bacteria but also several genera are able to resist unfavourable habitats and antibiotic and copper tolerant species (Shaw et al. 2020; Barra Caracciolo and Terenzi 2021), increased at 36 days in SC and SC_P. In particular, *Streptomyces*, the most abundant genus found at 3 h in the control soil (S), promptly decreased with the additions of copper, antibiotics, and amendments (SC, SCA SM, SD). Its subsequent increase at 36 days was possible thanks to its marked plasticity and capacity to deal with antibiotics and heavy metals (Hoff et al. 2018; Brangsch et al. 2022; Sedeek et al. 2023), with a selection of resistant strains (Benimeli et al. 2011; Fróes et al. 2012; Rammali et al. 2022).

The increase in the *Bacillus* genus is also in line with the presence of several multidrug-resistant species, able to counteract various metals, including copper (San et al. 2015; Tiwari et al. 2016; Barra Caracciolo and Terenzi 2021; Damle et al. 2021), and AB toxic effects (Haque et al. 2024).

On the other hand, other microbial groups, such as *Bacteroidia*, were not affected by adding contaminants and this was in line with their capacity to resist their toxicity (Niestępski et al. 2019b). Indeed, *Bacteroidia* comprise several antibiotic resistant species, including animal and human pathogens (Niestępski et al. 2019a).

Interestingly, *Alphaproteobacteria* not only increased with a plant presence, but some genera, e.g., *Sphingomonas* and *Neoasaia*, were found in higher percentages in a copper and antibiotic presence than in the other conditions, in accordance with their capacity to be copper resistant (Altimira et al. 2012; Shen et al. 2021). Although the *Neoasaia* genus (*Alphaproteobacteria*) has not been widely studied so far, its capacity to grow with an acidic pH and in degraded soils, developing a variety of resistance mechanisms, has been reported (Wang et al. 2015).

Moreover, *Arthrobacter* which remained quite stable over the experimental time have been reported to resist several heavy metals and to degrade a high variety of xenobiotic compounds (Pathak et al. 2020; Zhang et al. 2021; Wang et al. 2023).

Overall, when adding organic amendments, *Bacilli*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Bacteroidia*, which include bacteria able to resist and degrade sulfonamides, increased in accordance with other works (Reis et al. 2020; Readyhough et al. 2021; Wang et al. 2021; Barra Caracciolo et al. 2022) and with the fact that both the manure and digestate contained SMX residues. In particular,

Bacillus and *Sphingomonas* are able to denitrify organic compounds and have also been reported to be SMX-resistant genera and capable of degrading this antibiotic (Cheng et al. 2020; Barra Caracciolo et al. 2022). The amendments also introduced *Fulvivirga* (common in gut microbiomes), which has been reported to be able to transform complex molecules, including pharmaceuticals (Wardman et al. 2022).

The fact that *Nocardia* increased at 36 days in SCM and SMCA showed how copper and manure favoured a significantly higher number of potential resistant pathogenic bacteria. Indeed, the *Nocardia* genus is capable of resisting metals and antibiotics (Nouioui et al. 2020) and many species are pathogens (e.g., the etiological agent of nocardiosis in humans and a variety of animals) (Brown-Elliott et al. 2006; Yasuike et al. 2017).

The organic amendments introduced not only microorganisms but also antibiotic-resistant bacteria, with a significantly higher relative abundance of ARGs in manure than in digestate, as demonstrated in other recent works (Checcucci et al. 2020; Visca et al. 2022; Barra Caracciolo et al. 2022), and this confirms that manure, if used as an organic fertilizer, is a potential ARG source (He et al. 2020; Ibekwe et al. 2023).

The overall reduction in *intI1* and *sul1* in the amended conditions at 36 days is in accordance with other works and was generally related to an AB decrease (Rauseo et al. 2019; Yang et al. 2021; Garbini et al. 2022). The *intI1* and *sul1* genes are generally positively related, and this fact is ascribable to their location on the same plasmid (Domínguez et al. 2019; de los Santos et al. 2021). On the contrary, *sul2* increased at 36 days and particularly in a copper co-presence, showing how this gene was not strictly linked to SMX but also to other stressors (Enne 2004; Bean et al. 2009), such as heavy metals (Mazhar et al. 2021; Gao et al. 2022). The *sul2* gene is generally located on a small plasmid which has a broad host range (Enne 2004; Visca et al. 2022; Barra Caracciolo et al. 2022) and different genetic mobility mechanisms, which confer bacteria several fitness advantages (Bean et al. 2009; Zhou et al. 2021). Indeed, Zhang et al. (2018b) demonstrated that sub-inhibitory copper concentrations (ranges of 0.005–0.05 mg/kg) can cause an increase in ARG horizontal transfers by raising cell membrane permeability and conjugation-related genes.

When adding copper to the soil, the detrimental effects of antibiotics on the microbial community were boosted, making bacterial populations potentially more sensitive to SMX and this result is in line with other authors' findings (Liu et al. 2016; Orta-Rivera et al. 2023). The fact that the highest values of *sul2* were detected at the end of the experiment in an SMX and copper co-presence confirms that Cu can be a co-factor contributing to sulfamethoxazole resistance gene persistence (cross-resistance), as recognized in other works (Glibota et al. 2019; Mazhar et al. 2021; Wu et al. 2022; Congilosi et al. 2022).

The network analysis supports the fact that the amendments had a different impact on the overall microbial community. In manure conditions, copper was linked to *Streptococcus* and *Staphylococcus* because they are both antibiotic- and metal-resistant (Price and Boyd 2020; Akbari et al. 2022) genera. Moreover, *JG36-TzT* and *Stenotrophomonas* were found in the same cluster thanks to their capacity to live in an acidic soil, with a heavy metal, and to degrade xenobiotics (Glibota et al. 2019; Köhler et al. 2021). In digestate conditions, SMX was linked to *Sphingomonas* and *Halomonas*, presumably because they were able to degrade it (Cheng et al. 2020; Barra Caracciolo et al. 2022; He et al. 2024). Finally, it is very interesting to highlight that copper is in the same cluster as the ARGs and *Pseudomonas*, and this can explain the relative increase in *sul2* at 36 days with an antibiotic co-presence.

On the other hand, if we consider the conditions with exclusively manure (SM) or digestate (SD), which reproduce real agricultural scenarios, ARGs decreased strongly at 36 days, with the lowest values with digestate present and comparable to the control soil (S). At the same time, because the copper concentrations used in this work were in line with those commonly found in agricultural soils, its residues can have a serious impact on the spreading of antibiotic resistance genes, confirming both the antibiotic cross-resistance phenomenon (Chapman 2003; Zhang et al. 2019) and the possible negative effect of copper on plants (De Carolis et al. 2024).

Our work shows how antibiotic resistance is a dynamic and "silent phenomenon," which can suddenly increase among soil microbial populations not only when adding organic amendments (which contain ARB and AB residues) but also in response to agricultural practices such as the use of copper as a fungicide (cross-resistance). Overall, manure and digestate had a different microbiological and chemical composition, improving soil quality and structure in different ways, adding different microbial populations and pathogenic genera, and organic matter components, useful nutrients for plants, and making copper bioavailable in different ways as well as, in the case of the digestate-amended soil, buffering toxic contaminant synergic effects. On the other hand, the plants in the amended conditions also contributed to increasing soil quality, thanks to synergic interactions with their rhizosphere, including a decrease in overall ARGs. Finally, cattle digestate was confirmed to be not only a suitable organic fertilizer but also a nature-based solution for limiting contaminant effects and ARG spreading.

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Data availability All data supporting the findings of this study are available within the paper and in its Supplementary information.

Declarations The authors declare no competing interests. The authors declare that they agree with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute/organization where the work has been carried out before the work is submitted and the research study was performed without involving human participants and/or animals.

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The Effects of Different Combinations of Cattle Organic Soil Amendments and Copper on Lettuce (cv. *Rufus*) Plant Growth

Chiara De Carolis [1,2,3], Valentina Iori [1], Alessandra Narciso [2], Davide Gentile [1,4], Barbara Casentini [2], Fabrizio Pietrini [5], Paola Grenni [2], Anna Barra Caracciolo [2] and M. Adelaide Iannelli [1,*]

[1] Institute of Agricultural Biology and Biotechnology—National Research Council (CNR-IBBA), SP 35d, 9, Montelibretti, 00010 Rome, Italy; chiara.decarolis@uniroma1.it (C.D.C.); valentina.iori@cnr.it (V.I.); davide.gentile@uniroma1.it (D.G.)
[2] Water Research Institute, National Research Council (CNR-IRSA), SP 35d, 9, Montelibretti, 00010 Rome, Italy; alessandra.narciso@irsa.cnr.it (A.N.); barbara.casentini@irsa.cnr.it (B.C.); paola.grenni@irsa.cnr.it (P.G.); anna.barracaracciolo@irsa.cnr.it (A.B.C.)
[3] Department of Environmental Biology, 'Sapienza' University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[4] Department of Biology and Biotechnology 'Charles Darwin', Sapienza University of Rome, P.le Aldo Moro 5, 00185 Rome, Italy
[5] Research Institute on Terrestrial Ecosystems—National Research Council (CNR-IRET), SP 35d, 9, Montelibretti, 00010 Rome, Italy; fabrizio.pietrini@cnr.it
* Correspondence: mariaadelaide.iannelli@cnr.it

Abstract: In modern agricultural production, cattle manure waste recovery is considered as a sustainable approach to agricultural waste management, reducing environmental pollution and chemical fertilizer use. This study aimed to investigate the effects of manure and digestate derived from a pilot-scale livestock waste-recycling system, in combination with a low copper concentration as a fungicide, on the physiological response of lettuce cv *Rufus* (*Lactuca sativa* L.) plants and the associated soil microbiome. A five-week microcosm experiment was conducted in a greenhouse under environmental conditions. Lettuce plant performance was assessed in terms of biomass, leaf area index, photosynthetic activity, chlorophyll measurements, lipid peroxidation, total phenolic content, and nutrient uptake. The results suggested that incorporating digestate into the potting soil mix significantly enhanced crop yields compared to the control and manure treatments. The soil microbial activity increased in the presence of fertilizers, improving the soil chemical and biological properties. The addition of copper negatively affected the growth and physiological performance of the lettuce plants under both the control and manure-treated conditions, except for those grown in the presence of digestate, where copper accumulation was reduced. These findings highlight the potential of growing horticultural crops using organic fertilization through livestock waste anaerobic digestate, establishing a waste-to-food recycling system.

Keywords: manure; digestate; copper; lettuce; plant growth; nutrient uptake

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1. Introduction

As the human population increases, so does the demand for food, leading to a significant increase in food waste and its environmental impact. Over the last 50 years, worldwide, the use of inorganic fertilizers has significantly increased agricultural production. However, the use of organic fertilizers could be a sustainable way to reuse both organic wastes and manure from livestock farms in order to increase food production. Animal feces and urine contain high percentages of nitrogen (N) (55–95%), phosphorous (P), and potassium (K), which are essential macronutrients for animals, plants, and bacteria [1].

Although livestock waste is a valuable resource and can be used as a biofertilizer, it must be managed correctly to avoid environmental negative impacts, such as water and soil contamination (e.g., excess of organic nutrients and presence of trace micropollutants

as antibiotics), air pollution (greenhouse gas emissions), and human health problems (spread of pathogens) [1]. A growing number of intensive livestock farms are turning to anaerobic digestion (AD) technology to solve the problem concerning the disposal of the huge volumes of manure and slurry produced every day. The produced biogas and digestate provide significant energy and fertilizer benefits [2]. Anaerobic digestion is an efficient process in which organic matter breaks down naturally in the absence of oxygen and takes place in a reactor, where the resulting greenhouse gases can be reused rather than released into the atmosphere [1]. The by-product of the AD process is digestate, which can be employed as a biofertilizer returning nutrients to the soil through natural cycles and replacing inorganic fertilizer inputs. Thus, AD can be considered as a process that reduces the use of fossil fuels and inorganic fertilizers, lowers greenhouse gas emissions, and provides a highly efficient method for recycling resources, in line with green and circular economy projects [1,3].

Previous studies have demonstrated that digestate can be a source of macro and micronutrients useful for improving soil quality, promoting biological activity, and improving physiological responses in crop growth [4–6]. Nevertheless, digestate, due to its chemical compounds, can also cause slow growth and early senescence in crop plants [7].

Copper (Cu) is an essential micronutrient for plant growth and metabolism, related to many physiological and biochemical processes in plants [8,9]. As a cofactor for many enzymes, Cu plays a key role under stress conditions. Copper is also associated with oxidative phosphorylation, protein trafficking, signal regulation, and lipid and iron metabolism [10]. Indeed, Cu deficiencies can alter several functions of plant metabolism and its excessive concentration can generate phytotoxicity, resulting in the overproduction of reactive oxygen species (ROS) and causing damage to carbohydrates, lipids, proteins, and DNA in respiration, photosynthesis, and enzyme activity that can result in growth and productivity inhibition and imbalances in nutrient uptake [11,12]. Actually, owing to different agricultural practices, such as the use of manure as fertilizer (especially of pig origin) and copper application as a pesticide in vineyards, this metal can accumulate in high concentrations in soils and can become a pollutant [8,9].

In particular, Cu has been applied to vineyards, orchards, and horticultural crops since the late 19th century at doses of several kilograms per hectare to prevent downy mildew [9]. Copper's antifungal properties were demonstrated in the laboratory in the 1950s, and nowadays, its use is also authorized in organic farming. Copper-based fungicides, such as the Bordeaux mixture, consist of copper (II) sulphate ($CuSO_4$) and quicklime (CaO). Indeed, the average Cu concentration in European agricultural soils has been increasing and it has been estimated to be between 30 and 50 mg kg^{-1} [8]. The use of copper in agricultural soils has been restricted by the European Commission (EU Reg. 1981/2018) to minimize its potential accumulation in the soil and exposure to non-target organisms, such as plants and animals, so a maximum of 28 kg ha^{-1} may be applied for plant protection over a 7-year period, with an annual limit of 4 kg ha^{-1} [9].

Possible copper mobility from soil to plant tissue raises several concerns, including possible adverse effects on herbivorous organisms and humans, which ingest edible plants [9,13].

Lettuce (*Lactuca sativa* L.), an annual crop belonging to the Asteraceae family, is among the world's most widely cultivated and consumed vegetables and it is rich in nutrients as well as vitamins, calcium, and fiber. It also has a short growing season, characteristics for which it is considered a model vegetable species [14,15]. Moreover, lettuce is a biological species recommended for soil toxicity testing by the OECD (1984) to evaluate the potential impact of using natural products to limit contaminants and plant growth [16].

The present study aimed to investigate the effects of the application of different combinations of cattle organic soil amendments, manure (M), or digestate (D), and low concentrations of copper on the growth of lettuce plants in terms of biometric (i.e., shoot biomass, shoot height, number of leaves, and surface leaf area), photosynthetic (i.e., chlorophyll content and fluorescence), and biochemical (i.e., lipid peroxidation and total phenol

content) parameters, and the uptake of macro- and micro-nutrients highlighting the copper element. Furthermore, the response of the associated soil microbial community (i.e, soil total microbial abundance and soil microbial activity) was analyzed.

2. Materials and Methods

2.1. Plant, Soil Materials, and Experimental Design

Lettuce (*Lactuca sativa* L.), as one of the most economically important leafy vegetables, was chosen as the model plant species. Twenty-four seedlings of lettuce (cv. "Rufus"), with a height of about 4 cm, were purchased from a local hatchery. The seedlings were transplanted into plastic pots (1 seedling/pot) (1.4 L capacity), previously filled with 900 g of soil. Before transplanting, cattle manure (1% *w*/*w*) or digestate (1% *w*/*w*) was homogenously added to the soil alone or in combination with 30 mg kg^{-1} of copper sulphate ($CuSO_4$ $5H_2O$). All residual vegetable matter and stones were manually removed from the soil, and it was air-dried before setting up the microcosm experiment. Distilled water was used to obtain a 29.8% soil humidity, corresponding to 45% of the soil water-holding capacity. Soil was originally collected from an organic local farm, while manure and digestate were obtained from a biogas plant located on a cattle farm in an agricultural area in central Italy. The main physico-chemical characteristics of the soil, manure, and digestate, such as the pH, total organic carbon (Corg) and nitrogen (N) (Table S1), and elements content (Table S2), were measured according to the methods described in [17]. Afterwards, the seedlings were placed in a greenhouse located within the National Research Council (CNR) (42°10′ N, 12°63′ E) in Montelibretti (Rome, Italy), and grown for five weeks (from May to June 2022), where there was a daily average temperature of 26–31 °C during the day and 12–17 °C at night (Figure 1).

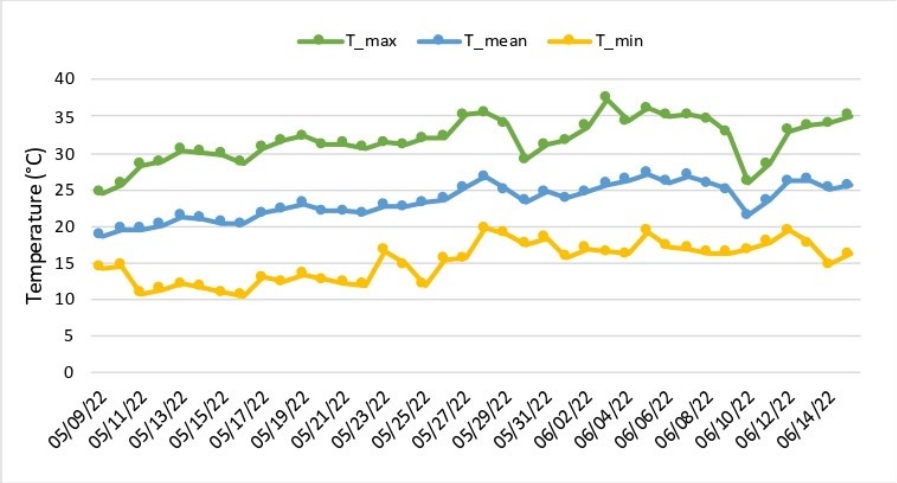


Figure 1. Temperatures observed during the experimental period in the greenhouse: Maximum (T_max), mean (T_mean) and minimum (T_min).

The soil moisture was maintained at a constant level by daily replenishing lost water with distilled water. A systematic experimental design was established to evaluate the effect of the two amendments used and of the added copper on the plant growth and microbial community response, several experimental conditions were established (Table 1).

Table 1. Experimental conditions and corresponding acronyms.

Conditions	Experimental Acronym
Soil	S
Soil + Cu	S+Cu
Soil + Manure	M
Soil + Manure + Cu	M+Cu
Soil + Digestate	D
Soil + Digestate + Cu	D+Cu

A schematic representation of the experimental design is described in Figure 2.

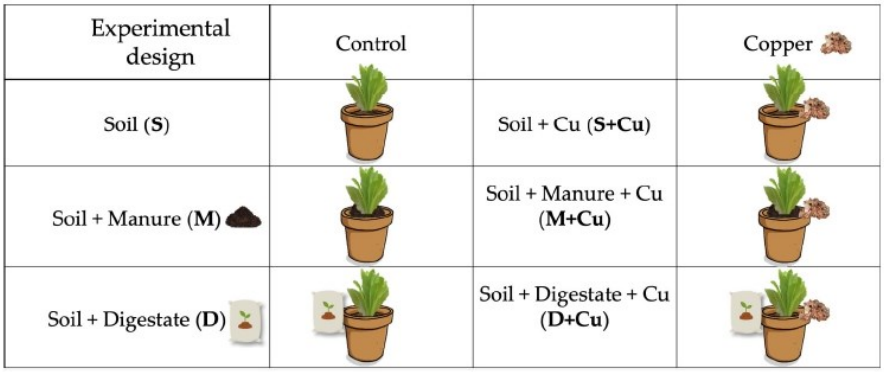
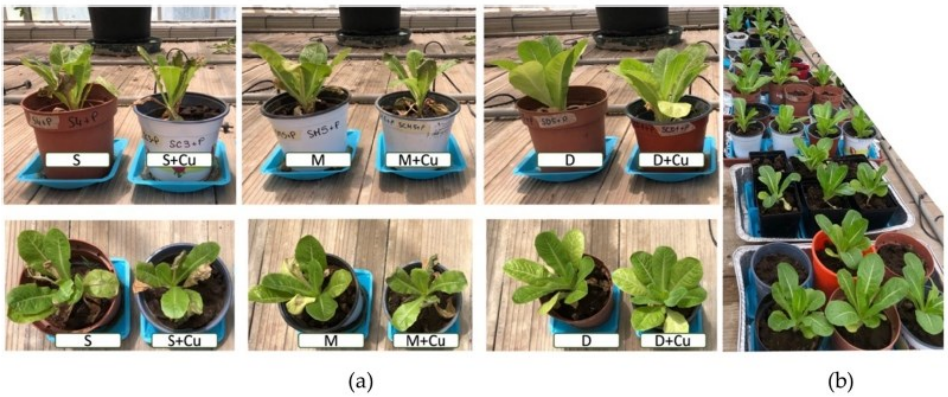


Figure 2. Representation of the experimental design. Lettuce production during five weeks under different soils: soil alone, cattle manure (1% *w*/*w*) and digestate (1% *w*/*w*), both homogenously added to the soil alone, and the in combination with 30 mg kg^{-1} copper sulphate ($CuSO_4$ $5H_2O$). All treatments were tested in quadruplicates, i.e., twenty-four experimental units were used.

Figure 3 shows images of lettuce plants at harvest for each treatment (a) and the layout of the experiment in the greenhouse investigating lettuce growth in different soil conditions (b).



(a) (b)

Figure 3. (**a**) One representative lettuce plant (side and top view) from each treatment at harvest: S, soil alone; S+Cu, soil and copper; M, manure; M+Cu, manure and copper; D, digestate; D+Cu, digestate and copper and (**b**) The layout of the experiment in the greenhouse for growing lettuce in different soil conditions.

After five weeks, the lettuce plants were harvested and stored for further analyses. Moreover, aliquots of the rhizosphere soil samples were collected for measurements of the microbiological parameters.

2.2. Plant Growth Analyses

At the end of the experiment, the height (cm) of the lettuce plants and the number of leaves (N° leaves) were measured to assess the plant growth. To quantify the plant shoot biomass, the lettuces were cut from the base with scissors and then weighed. The dry weight (DW) of the lettuce samples was obtained by placing leaves in an oven at 60 °C until a constant mass was reached. The leaf dry mass was then related to its leaf area, calculated using image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA), to obtain the specific leaf area (SLA, $cm^2\ g^{-1}$ DW). The growth parameters were measured on four biological replicates from each condition (Table 1).

2.3. Determination of Chlorophyll

Photosynthetic pigments of the lettuce plants (0.1 g) were extracted in 96% (*v*/*v*) ethanol for 72 h at room temperature in the dark. After centrifugation at 3000× *g* for 10 min at 4 °C, the absorbance of the supernatant was read spectrophotometrically at 663 and 645 nm (Lambda 35 UV/VIS, Perkin Elmer, Norwalk, CT, USA) [18]. The total chlorophyll content was assessed according to the equations described by Lichtenthaler et al. [19] and the results are expressed in mg of the total chlorophyll per gram of fresh weight plant tissue (mg g^{-1} FW).

2.4. Chlorophyll Fluorescence Analysis

The chlorophyll *a* fluorescence transient (OJIP transients) was measured on the fully expanded leaves of the *Lactuca sativa* plants using a portable fluorometer (FluorPen FP 110-LM/D, Photon System Instruments, Drasov, Czech Republic). The measurements were performed on 60 min dark-adapted leaves with leaf clips, and the fluorescence intensity was measured after the application of a saturating light pulse of 3000 µmol $m^{-2}\ s^{-1}$. These measurements were taken on 10 plants (one leaf per plant) per treatment (S, S+Cu, M, M+Cu, D, and D+Cu), around midday (between 10.00 a.m. and 12.00 p.m.). The obtained data were used in the JIP test [20] to determine various bioenergetic parameters of PSII photochemistry (Table 2).

Table 2. Abbreviations, formulas, and definitions of the JIP-test parameters.

Basic Parameters Calculated from the Extracted Data	
F_0	Fluorescence intensity at 50 µs (O step)
F_{300}	Fluorescence intensity at 300 µs
F_J	Fluorescence intensity at 2 ms (J step)
F_I	Fluorescence intensity at 30 ms (I step)
F_m	Maximal fluorescence intensity (P step)
$F_v = F_m - F_0$	Maximal variable fluorescence
$V_J = (F_J - F_0)/(F_m - F_0)$	Variable fluorescence at J step
$M_0 = 4\ (F_{300} - F_0)/(F_m - F_0)$	Approximated initial slope of the fluorescence transient, expressing the rate of RCs' closure
Specific energy fluxes (per RC: QA-reducing PSII reaction center), in ms^{-1}	
$ABS/RC = M_0 \times (1/V_J) \times [1/(F_v/F_m)]$	Absorption per active reaction center
$TR_0/RC = M_0 \times (1/V_J)$	Trapping per active reaction center
$ET_0/RC = M_0 \times (1/V_J) \times (1 - V_J)$	Electron transport per active reaction center
$DI_0/RC = (ABS/RC) - (TR_0/RC)$	Dissipation per active reaction center
Quantum yields and efficiencies	
$TR_0/ABS = F_v/F_m = \varphi P_0 = (F_m - F_0)/F_m$	Maximum quantum yield of PSII photochemistry
$ET_0/TR_0 = \psi_0 = (F_m - F_J)/(F_m - F_0)$	Probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A
$ET_0/ABS = \varphi E_0 = \varphi P_0 \times \psi E_0$	Quantum yield of electron transport
$F_v/F_0 = TR_0/DI_0 = (F_m - F_0)/F_0 = \varphi P_0/(1 - \varphi P_0)$	Maximum ratio of quantum yields of photochemical and concurrent non-photochemical processes in PSII
$F_0/F_m = DI_0/ABS = \varphi D_0$	Maximum quantum yield for energy dissipation at the antenna level
Performance index	
$PI_{ABS} = [\varphi P_0\ (V_J/M_0)] \times [\varphi P_0/(1 - \varphi P_0)] \times [\psi E_0/(1 - \psi E_0)]$	Performance index (potential) for energy conservation from photons absorbed by PSII to the reduction of intersystem electron acceptors

2.5. Measurement of Lipid Peroxidation Level

Lipid peroxidation was determined by a measurement of the malondialdehyde (MDA) content according to the modified method of Hodges et al. [21]. Frozen samples (0.1 g) were homogenized in a pre-cooled mixer mill (TissueLyser LT, Quiagen, Hielden, German) with 1 mL of 0.1% trichloroacetic acid (TCA) and centrifuged for 10 min at 13,000× *g* at 4 °C. An assay mixture containing 0.4 mL of supernatant, 1 mL of 0.5% (*w*/*v*) thiobarbituric acid (TBA) in 20% (*w*/*v*) TCA, and 1 mM ethylenediamine tetraacetic acid (EDTA) was incubated at 80 °C for 30 min and then quickly cooled in an ice bath. Then samples were centrifuged at 13,500× *g* for 5 min at 4 °C, and the absorbance of the supernatant was measured at 532 nm and 600 nm with a Thermo Multiskan FC Microplate Photometer (Thermo Fisher Scientific; Waltham, MA, USA). Values corresponding to non-specific absorption at 600 nm were subtracted. The MDA concentration was calculated using the extinction coefficient (ε = 155 mM^{-1} cm^{-1}). The lipid peroxidation levels are expressed as nanomoles of MDA per gram of fresh weight plant tissue (mg g^{-1} FW).

2.6. Estimation of Total Phenolic Content

Frozen leaves (0.5 g), homogenized in a pre-cooled mortar and pestle, were extracted in 5 mL of 80% (*v*/*v*) ethanol, placed on a shaker for 24 h in the dark at 25–30 °C, then centrifuged at 4 °C for 25 min at 4000× *g*. The total phenolic content was quantified using Folin–Ciocalteu (FC) reagent, with gallic acid as the standard according to the modified protocol of Bernabé-Antonio et al. [22]. Briefly, 20 µL of extract was mixed with 20 µL of 10% FC reagent and 160 µL of 1M Na_2CO_3. After 20 min of incubation in the dark, the absorbance was measured at 765 nm using a Thermo Multiskan FC Microplate Photometer (Thermo Fisher Scientific; Waltham, MA, USA). The total phenolic content is expressed as mg of gallic acid equivalents (GAE)/g of fresh weight plant tissue (mg GAE g^{-1} FW) using the gallic acid calibration curve (0.1–0.75 mg mL^{-1}, R^2 = 0.97).

2.7. Chemical Analysis

To quantify the chemical element contents in the plants and soils, both oven-dried lettuce leaves and soil samples were weighed and mineralized with a nitric-acid-based microwave digestion method using an EXCEL Microwave digestion system (Preekem Scientific Instruments Co., Ltd., Shanghai, China). Mineralization was performed by treating 250 mg of dried lettuce leaves with 4 mL HNO_3 65% (*w*/*w*), 2 mL H_2O_2 30% (*v*/*v*), and 3 mL of distilled water. Regarding the metal concentration in the soil, 0.2 g of dried soil samples was digested with 5 mL of HNO_3 65% (*w*/*w*) and 1 mL of HCl 37% (*v*/*v*). Samples were then filtered and analyzed. The determination of elements was performed using inductively coupled plasma optical emission spectrometry at 327.395 nm (ICP-OES, Agilent 5800, USA-LOD = 0.02 mg L^{-1}).

2.8. Bioaccumulation Factor of Copper in Lettuce Plants

The bioaccumulation factor (BAF) was calculated to evaluate the ability of the lettuce plants to extract and accumulate copper from the soil to shoot [23]:

$$\text{BAF} = \text{Cu content in plant shoot (mg kg}^{-1}\text{ DW)}/\text{Cu content in soil (mg kg}^{-1}\text{ DW)} \quad (1)$$

2.9. Tolerance Index of Lettuce Plants toward Copper

The tolerance index (Ti) was measured to assess the growth of the lettuce plants in Cu-contaminated soil (Cu) compared to the control soil (S) [24]:

$$\text{Ti \%} = (FW_{Cu}/FW_S) \times 100 \quad (2)$$

where FW_{Cu} is the fresh weight (g) of shoot lettuce grown in Cu-contaminated soil and FW_S is the fresh weight (g) of shoot lettuce grown in non-contaminated soil.

2.10. Microbiological Analyses of Soil Community

Microbiological analyses of the soil community were performed at the end of the experiment, after 36 days. The total microbial abundance was measured in the soil samples (1 g), previously fixed in 2% formaldehyde (*v*/*v*), by the direct epifluorescence count method with a Leica DM LB 30 epifluorescence microscope and using DAPI (4′,6-Diamidino-2-phenylindole dihydrochloride) dye as a fluorescent DNA intercalant [25,26]. The total microbial abundance is expressed as N. cells g^{-1} dry soil.

Microbial activity was detected by measuring the dehydrogenase activity (DHA) in the soil samples (6 g) using tetrazolium salt, 2,3,5-triphenyl-tetrazolium chloride (TTC) as an artificial terminal hydrogen acceptor in the electron transport chain. TTC was reduced to red-colored triphenylformazan (TPF). TPF was extracted using an organic solvent (ethanol), and the color intensity of the extract was quantified after 24 h at 37 °C in the dark [27] with a Thermo Multiskan FC Microplate Photometer (Thermo Fisher Scientific; Waltham, MA, USA). The intensity of the color was directly proportional to the concentration of the produced TPF. Soil dehydrogenase activity is expressed as μg TPF g^{-1} dry soil.

2.11. Statistical Analysis

All collected data were statistically analyzed by one-way analysis of variance (ANOVA) using R software (version 4.1.2 https://www.r-project.org; accessed on 4 December 2023). Differences among the different treatment means were evaluated by post hoc Tukey's Honest Significant Difference test (Tukey HSD) with a significance level of $p < 0.05$. In the figures, the results are presented as box plots with the median value displayed as a line in the middle of the box. Maximum and minimum observations are shown as the endpoint of the upper and lower whiskers, respectively, and outliers are plotted as dots when present. The box plots were made using R ggplot2 package (https://cran.r-project.org/web/packages/ggplot2/citation.html; accessed on 4 December 2023), In the tables, the results are expressed as mean values ± standard deviations ($n = 4$). The relationships among the plant growth, biochemical parameters, and microbiological data in the soil were analyzed using Principal Component Analysis (PCA).

3. Results and Discussion

3.1. Plant Growth Analyses

The effects of different combinations of cattle organic soil amendments and copper on the lettuce plants growth were analyzed by evaluating the shoot biomass production (Figure 4). In the presence of manure (M) and digestate (D), the plant biomass increased significantly (by 30% and 140%, respectively) compared to the control plants (S). It is worth noting that the lettuces grown on D soil showed the highest shoot biomass. A comparison across separate studies may be quite difficult due to the influence of several variables such as different compositions of biochar, manure or digestate, tested plants and soil, and pot experiment conditions. Nevertheless, our findings were in accordance with Kwoczynski et al. [6], who reported a two-fold increase in the yield of lettuce plants (*Lactuca sativa* L.) grown in the presence of 5% (*w*/*w*) digestate compared to controls [6]. Similarly, the addition of digestate to soil caused an enhancement in biomass yield also in eggplant (*Solanum melongena* L.) [28], ryegrass (*Lolium perenna* L.) [29], tomato (*Solanum lycopersicum* L.) [30], and forage grass (*Lolium rigidum* L.) [31], resulting in an important source of nutrients. The increase in lettuce shoot biomass might be associated with a slow release of nutrients from the digestate that occurred during the development of the crop, as suggested by Mortola et al. [5]. The addition of a low Cu concentration to the soil (S+Cu) and manure (M+Cu) resulted in a significant reduction in shoot biomass compared to the soil (S) and manure (M) (32.1% and 30.9%, respectively), highlighting a harmful impact of copper sulphate (30 mg kg^{-1}) on biomass production. Instead, no significant difference was shown in the shoot biomass of the lettuce plants grown in the presence of digestate with or without copper. These results are in line with those reported by Gharbi et al. [32] and Shams et al. [33], who observed a decline in the biomass production of lettuce

plants exposed to increasing Cu concentrations (100–1000 mg kg^{-1}) for 15 days and 50 and 100 mg kg^{-1} Cu for 30 days, respectively.

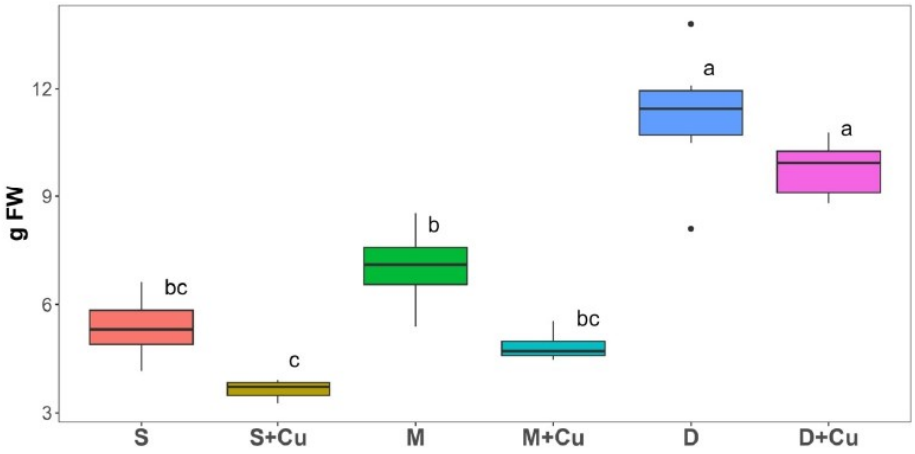


Figure 4. Shoot biomass (g FW) of lettuce plants grown for five weeks in soil (S), manure (M), and digestate (D) and with the addition of copper (Cu). Box plots labelled with different letters indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05). In the box-plots, the upper and lower whisker endpoints represent the maximum and minimum observations, respectively, outliers are shown as dots. $n = 4$.

No significant difference in plant height was observed between the soil (S), manure (M), and digestate (D) treatments. A significant downward trend in plant height was detected in S+Cu and M+Cu, at 19.12% and 11.36%, respectively, compared to the S and M conditions. Instead, the addition of copper to digestate significantly increased the height of the lettuce plants (3.8%) (Table 2). The number of leaves significantly increased in the presence of M and D, at 40.9% and 46.5%, respectively, with respect to the lettuce plants grown in the S. Copper addition to S, M, and D did not cause changes in the numbers of leaves compared to the respective uncontaminated soil improvers (Table 3). It is to be noted that the numbers of lettuce leaves in S+Cu were lower than those detected for the plants grown in M+Cu and D+Cu. Considering the specific leaf area (SLA) parameter, no significant difference was observed between the S, M, and D conditions. Accordingly, the addition of copper did not induce any effect on the SLA values (Table 3). The analyzed growth parameters (height, number of leaves, and SLA) were consistent with the previously measured biomass results. Previous studies have indicated that the use of soil organic fertilizer contributes to the better development of crop plants such as lettuce [14], eggplant, Shanghai cabbage [34], and tomato [35]. This might be ascribed to the high nutrient and organic matter contents of amendments, which optimize both the deficit and excess nutrients in the soil [4]. The addition of a low copper amount adversely affected lettuce growth both in the soil and in manure, interfering with plant development and associated metabolic processes [10].

Table 3. Shoot height (cm), leaf density (N° leaves), and specific leaf area (SLA, cm^2 g^{-1} DW) for lettuces plants collected from different soil conditions: S, M, and D; and in presence of copper (Cu): S+Cu, M+Cu, and D+Cu.

	S	S+Cu	M	M+Cu	D	D+Cu
Height	14.6 ± 1.2^{ab}	11.8 ± 0.8^{c}	14.3 ± 1.0^{ab}	12.9 ± 1.9^{bc}	14.5 ± 1.2^{ab}	15.1 ± 1.0^{a}
N° leaves	11.9 ± 1.1^{b}	11.1 ± 0.4^{b}	16.5 ± 0.5^{a}	15.9 ± 1.2^{a}	17.4 ± 0.5^{a}	16.3 ± 1.1^{a}
SLA	2.9 ± 0.2^{a}	3.0 ± 0.5^{a}	2.6 ± 0.9^{a}	2.9 ± 0.4^{a}	3.0 ± 0.4^{a}	2.9 ± 2.0^{a}

Means ± SD with different letters in the vertical columns indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05) ($n = 4$).

3.2. Chlorophyll Content

A downward trend in the chlorophyll content was observed under the M (9%) treatment compared with S (Figure 5). The addition of copper significantly reduced the chlorophyll content both in S+Cu and M+Cu (18,48% and 12.48%, respectively) compared to their respective uncontaminated soil improvers. A reduction in the photosynthetic pigment content suggested a significant inhibition of photosynthesis, thus affecting the plant growth, as reported by Slamet et al. [36] Indeed, the pattern observed in the plant biomass production (Figure 5) was consistent with that concerning the chlorophyll content (Figure 5). The addition of copper to the soil and manure induced chlorophyll and lettuce biomass reductions, even if the Cu concentration was lower than that reported in the literature (from 200 mg kg^{-1}), which causes toxicity [33]. In agreement with the study by Li et al. [37], low copper concentrations (25–50 mg kg^{-1}) might induce a hormesis effect and a consequent reduction in biomass and pigment contents. The chlorophyll content was significantly increased in lettuce plants grown with D (32%) with respect to S (Figure 5). No significant difference was highlighted in the chlorophyll content measured in the lettuce plants grown with digestate in the absence and presence of copper. The increase in chlorophyll content in the lettuce plants grown with digestate and copper was 30% with respect to S (Figure 5). Chlorophyll is an essential pigment for plant growth and a higher chlorophyll content is usually linked to an increase in plant biomass accumulation. Digestate contains available nitrogen and phosphorus, therefore, its use can stimulate photosynthesis, thus enhancing the chlorophyll content and biomass in a range of vegetable crops, including lettuce (*Lactuca sativa* L.) [14], tomato (*Solanum lycopersicum* L.) [38], Spring Wheat (*Triticum aestivum* L.) [39], and aubergine (*Solanum melongena* L.) [28].

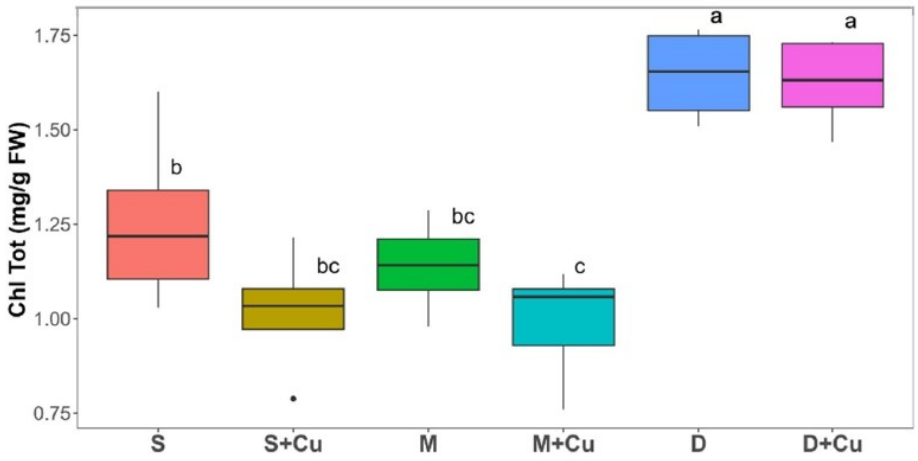


Figure 5. Total chlorophyll content (mg g^{-1} FW) of lettuce plants grown for five weeks in soil (S), manure (M), and digestate (D) and with the addition of copper (Cu). Box plots labelled with different letters indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05). In the box-plots, the upper and lower whisker endpoints represent the maximum and minimum observations, respectively, outliers are shown as dots. $n = 4$.

3.3. Chlorophyll Fluorescence Analysis

In order to study the effect of the soil amendments on the photosynthetic performance of the lettuce plants exposed to a low Cu concentration, the bioenergetic parameters obtained from the JIP test were measured. In particular, some structural and functional parameters are presented in the form of a radar plot (Figure 6), where all values have been normalized to those obtained from the control plants.

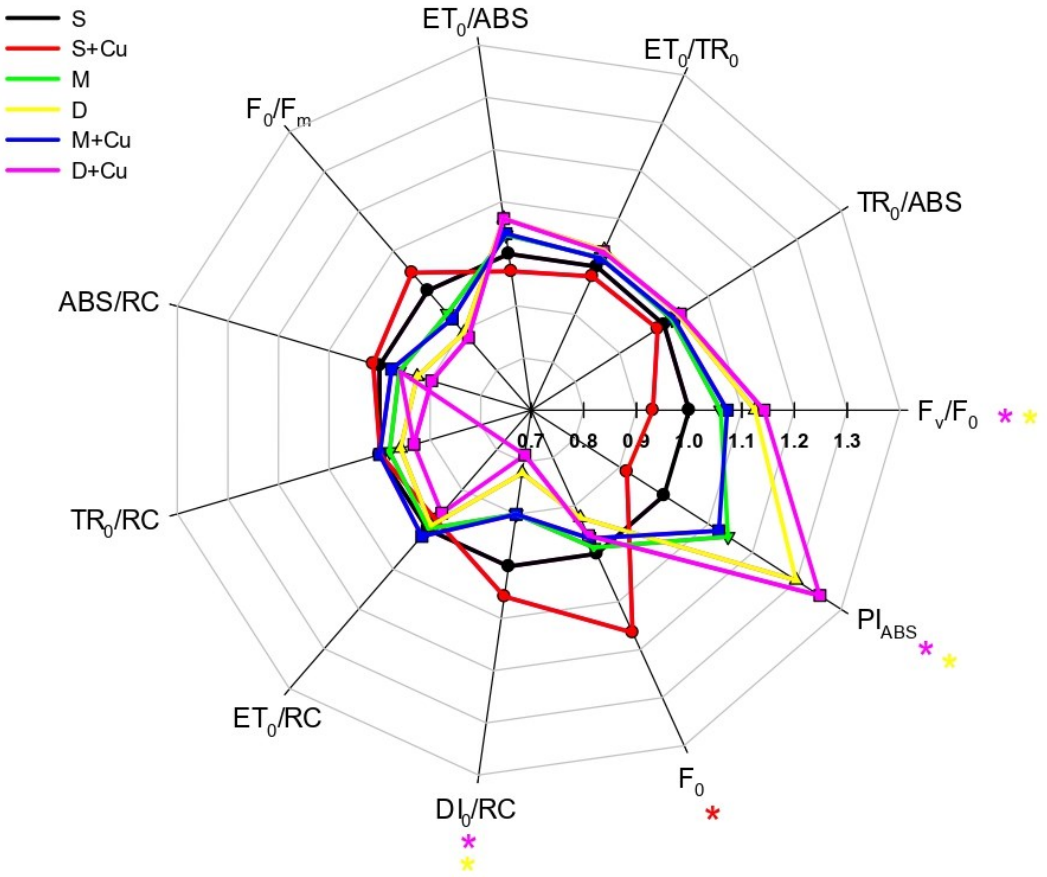


Figure 6. Changes in the shape of the radar plot JIP-test parameters induced by different amendment treatments with or without the addition of a low copper concentrations applied to *Lactuca sativa* plants and measured at the end of the treatment. The data are the average of ten replicates and report the values with respect to plants grown in the control condition (S = control = 1). Red, pink, and yellow asterisks indicate significant differences (LSD test, $p < 0.05$) between control plants (S) and those exposed to S+Cu, D, or D+Cu, respectively.

A chlorophyll a fluorescence-transient analysis is a useful method for examining the physiological aspects of structure and activity, particularly in the PSII [20]. It has been extensively employed to assess damage to the plant photosynthetic system caused by various types of stress [40]. The exposure of lettuce plants to a low copper concentration, as in S+Cu, caused a slight change in most of the parameters analyzed, except for F_0, which exhibited a significant increase compared to the control (S) plants (Figure 6). As reported in the literature, the absence of an F_0 variation indicates a good ability of the treated plants to maintain the efficiency of energy transfer between the pigments of the antenna and the PSII reaction center without structural damage at the photosystem level [41]. However, an increase in F_0 can be interpreted as indicating irreversible damage to PSII caused by uncontrolled dissipation of heat that produces an excess of excitation energy [42,43], while a decrease in F_0 is, on the contrary, an indication of a high-energy dissipation in the minor antenna [44]. The data relating to the specific energy fluxes, such as the absorption flux per reaction center (ABS/RC), trapped energy flux per reaction center (TR_0/RC), electron transport flux per reaction center (ET_0/RC), and dissipated energy flux per reaction center (DI_0/RC), measured to determine the photosynthetic performance of the active PSII reaction centers of the lettuce plants subjected to Cu treatment (S+Cu), did not show significant variations with respect to the control plants (Figure 6). Nevertheless, although copper

treatment was found to not significantly impact the other analyzed parameters in the S+Cu plants, such as the maximum quantum yield of primary photochemical reactions (TR_0/ABS), the maximum quantum yield for electron transport (ET_0/ABS), the probability that an electron moves further than Q_A (ET_0/TR_0), and the light energy used for thermal energy dissipation (F_0/F_m), both the (F_v/F_0) and the performance index PI_{ABS} showed a slight decrease compared to the control, indicating a reduction in the efficiency of water splitting by the PSII and the overall vital state of the plant [45].

The exposure of lettuce plants to manure amendments in the soil, without or with the addition of a low copper concentration (M or M+Cu, respectively), resulted in a partial improvement in the plant physiological status. Compared to the plants grown in the S+Cu treatment, some of the analyzed parameters in the plants grown under the M and M+Cu treatments showed a positive impact on the plant viability but did not significantly differ from the control plants. These results are consistent with the biomass and chlorophyll content data, which indicate similar grown patterns across the treatments (S, S+Cu, M, and M+Cu).

On the contrary, the exposure of the lettuce plants to digestate amendments in soil, without or with the addition of a low copper concentration (D or D+Cu, respectively), significantly enhanced the plant physiological performance.

Several chlorophyll fluorescence parameters measured in the plants grown under the D or D+Cu treatments were found to be significantly different from those of the control plants (S), indicating a beneficial effect on the plant viability due to the digestate amendment.

Indeed, in our study, the functionality of both photosystems I and II was noticeably higher in the D- and D+Cu-treated lettuce plants, as indicated by the increase in the performance index PI_{ABS} and in the F_v/F_0 values compared to the control [14]. Additionally, the decrease in the dissipated energy flux per reaction center (DI_0/RC) indicated a greater ability to use the energy by the active RCs [46]. The data on the biomass and chlorophyll content confirm these findings, showing an increase in biomass production and pigment content in the plants treated with D or D+Cu compared to the controls.

The results obtained in this experiment are consistent with previous studies that demonstrate the positive impact of digestate addition on crop growth [47–49]. Notably, significant increases in biomass have been reported for wheat (*Triticum aestivum* L.) [50], giant Napier (*Pennisetum purpureum* Schumach) [51], tomato (*Solanum lycopersicum* L.), and cucumber (*Cucumis sativus* L.) [52].

3.4. Measurement of Lipid Peroxidation Levels

The malondialdehyde (MDA) content increased significantly in the manure (M) and digestate (D) conditions (by 32% and 86%, respectively) compared to the control condition (S), (Table 3). Malondialdehyde is a natural by-product of cell membrane lipid peroxidation, usually analyzed to assess the damage caused by oxidative stress in plant tissues [14]. The addition of low concentrations of copper significantly reduced the MDA content in the lettuces grown in S+Cu (22%) compared to soil (S). In the M+Cu condition, the measured MDA values were significantly reduced (66%) when compared with M, and no significant difference was detected compared to the control (S), highlighting a partial improvement of the plant physiological performance, strictly consistent with previous results concerning the chlorophyll fluorescence analysis. The MDA content in the lettuces planted in D+Cu did not differ significantly from the digestate (D) conditions, maintaining high MDA levels. If MDA levels persist to be high, irreversible modifications to proteins and nucleic acids occur. However, if the scavenging system composed of enzymatic and non-enzymatic systems synergistically works to maintain intra- and extracellular redox homoeostasis, MDA increases, and its maintenance may represent an acclimation process rather than damage, as suggested by Morales et al. [53] and Liang et al. [54]. Consequently, MDA might act as a protection factor exerting a positive role by activating the regulatory genes involved in plant defense and development according to Tagnon et al. [55]. Additional parameters, such as growth measurements and chlorophyll fluorescence analyses, in plants grown

under the D and D+Cu treatments showed a beneficial effect on plant vitality due to the presence of digestate when compared to the S and M conditions with and without copper.

3.5. Estimation of Total Phenolic Content

Lettuces of different varieties have different total phenol contents due to genetic characteristics and different agronomic practices, such as fertilizer application, which could influence the nutritional status of the crop, modulating the antioxidant and phenol contents [56]. Phenols are secondary metabolites used by plants, along with antioxidants, to defend against oxidative or abiotic stresses [28]. Lettuces grown with digestate (D) and digestate plus copper (D+Cu) showed the highest content of total phenolic content, (20% and 23% respectively) compared to the control condition (S). The phenolic content in the lettuce plants did not change significantly between the different soil conditions, with or without copper addition (Table 4). These findings agreed with the results obtained for cucumber [57] and lettuce plants [58], where organic fertilizers enhanced the total phenol content, since they are rich in carbon and phenols, and stimulated plant antioxidant activity. Phenols can be found in organic fertilizer from the biological degradation of several xenobiotic substances, like pesticides, or naturally occurring aromatic amino acids and polymers in plant materials such as lignin, tannins, and humic acids [28].

Table 4. Measurement of lipid peroxidation levels (MDA, nmol MDA g^{-1} FW) and estimation of total phenolic content (TPC, mg GAE g^{-1} FW) for lettuces plants collected from different soil conditions: S, M, and D; and in presence of copper (Cu): S+Cu, M+Cu, and D+Cu.

	S	S+Cu	M	M+Cu	D	D+Cu
MDA	5.0 ± 0.6^{c}	3.9 ± 0.4^{d}	6.6 ± 0.7^{b}	4.3 ± 0.5^{cd}	9.3 ± 0.5^{a}	9.5 ± 1.3^{a}
TPC	0.30 ± 0.05^{ab}	0.32 ± 0.07^{a}	0.31 ± 0.01^{a}	0.35 ± 0.03^{a}	0.36 ± 0.07^{a}	0.37 ± 0.05^{a}

Means ± SD with different letters in the vertical columns indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05). $n = 4$.

3.6. Copper Determination, Bioaccumulation Factor, and Tolerance Index in Lettuce

Copper is an essential element for plant nutrition, needed as a cofactor for many enzymatic activities catalyzing redox reactions and for photosynthetic functions. Excess copper induces high levels of reactive oxygen species (ROS) and negatively affects the photosystem during photosynthesis [10]. About Cu concentration in soil any differences were detected in S, M and D (Table S2). Although copper addition increased its concentration in different soils, no significant difference was observed between them (Table S2). The lettuce plants grown under the control condition (S) showed the lowest Cu accumulation (1.1 ± 0.2 mg kg^{-1}) (Table 5). Compared to the S condition, in lettuce plants grown with M and D, a marked increase in Cu concentration was observed, following the order of D > M > S. The addition of a low Cu concentration to different soils (S+Cu, M+Cu, and D+Cu) induced a significant enhancement in copper accumulation in the lettuce plants, following the order of M+Cu > D+Cu > S+Cu > S.

Table 5. Copper content in leaves (Cu, mg kg^{-1} DW), bioaccumulation factor (BAF) of Cu (BAF_{Cu}), and tolerance index (Ti) for lettuces plants collected from different soil conditions: S, M, and D; and in presence of copper (Cu): S+Cu, M+Cu, and D+Cu.

	S	S+Cu	M	M+Cu	D	D+Cu
Cu	1.1 ± 0.1^{c}	3.3 ± 0.3^{b}	2.9 ± 0.4^{b}	5.7 ± 0.4^{a}	4.2 ± 0.2^{b}	3.9 ± 0.2^{b}
BAF_{Cu}	0.04 ± 0.01^{c}	0.14 ± 0.06^{ab}	0.23 ± 0.09^{a}	0.19 ± 0.07^{ab}	0.15 ± 0.03^{ab}	0.10 ± 0.03^{ab}
Ti%	100 ± 0.00^{b}	53 ± 7^{d}	105 ± 6^{b}	77 ± 13^{c}	134 ± 10^{a}	121 ± 2^{a}

Means ± SD with different letters in the vertical columns indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05). $n = 4$.

Based on the Cu contents in the plant shoot and soil, the bioaccumulation factor (BAF) and tolerance index (Ti) were calculated to evaluate the metal uptake and plant tolerance to Cu stress. The highest BAF was detected in the lettuce plants grown with M soil (0.23 ± 0.09 mg kg^{-1}) compared to the different uncontaminated soils (Table 5). The addition of a low Cu concentration caused a significant enhancement in the BAF value in the lettuces grown with S+Cu compared to the S condition, not statistically different from those occurring in the M+Cu and D+Cu soils. It is well known that the BAF value depends on the tested metal, plant species, soil pollution level, and soil acidity, as reported by Sipter et al. [58,59]. Nevertheless, in this study, the BAF value in the plant leaves was <1, revealing that the lettuces were poor accumulators [60,61] (Table 5). The plant capability to limit Cu's damaging effects was very different among soil conditions, as evidenced by the tolerance index (Ti) (Table 5). The lettuce plants grown in D+Cu soil showed the highest Ti values with respect to the S+Cu and M+Cu conditions, even though the respective BAF values were similar. These results are consistent with data concerning plant growth and physiological parameters, suggesting that the presence of digestate in the soil reduced Cu's phytotoxic effect, improving the plant physiological status. It is worth noting that the lowest Ti was observed in the lettuces planted in S+Cu, which corresponded to a 3.5-fold increase in the BAF value compared to the S conditions and a decrease in the growth parameters and chlorophyll content. Although the Cu concentration at which toxic effects become evident is plant-species- and soil-type-dependent [62], our findings agree with previous studies where plants with Ti values of <100 experienced stress due to metal contamination along with a net reduction in plant biomass. Conversely, the plant species with a T_i of >100 developed tolerance along with a net biomass increase and improved physiological performance [63,64].

3.7. Macro- and Micro-Nutrient Content in Lettuce Plants

It is well established that plant growth and nutritional quality are related to mineral accumulation. Macronutrients such as Ca, K, Mg, P, and S carry out important functions in several metabolic pathways, including photosynthesis, growth, and stress tolerance, and are required in large quantities by plants [65]. Compared to macronutrients, micronutrients (Cu, Fe, Mn, Na, Zn, Al, Cd, Cr, Ni, and Si) are required in relatively lesser amounts and, likewise, they play key roles in plant growth and metabolism [66]. The levels of chemical elements recorded in the lettuce plants used in this study were all below the WHO safe limit of the selected heavy metals in vegetables for human consumption [67]. Macro- and micronutrient uptake in the lettuce leaves was reported as a radar plot (Figure 7), where all values were normalized to those obtained from the control plants (S) (Table S4). In general, the uptake of macro- and micronutrients varied significantly in the lettuce plants treated with different soil amendments in the absence or presence of a low Cu concentration, resulting to be higher in plants grown in S+Cu. Compared to the control plants (S), the presence of the amendments mainly affected the uptake of micronutrients, such as Cr, Ni, Cu, and Cd. In particular, under the M treatment, the leaves of lettuce plants showed reductions to 36.7% and 41.7% in Ni and Cd concentrations, respectively, whereas an enhancement was observed in Cr and Cu uptake (up to 45.7% and 91%, respectively) with respect to the control (S). Instead, in the lettuce plants exposed to D, marked reductions were detected in Ni and Cd uptake (up to 50% and 66%, respectively) whereas Cr and Cu concentrations increased to 25% and more than twice, respectively, compared to the control (S). It is worth noting that, under the D and D+Cu treatments, the plants showed significant decreases also in Al, Zn, Na, Mn, and Fe uptake (13–16%, 14–20%, 10–12%, 14–17%, and 17–19%, respectively). As described in the literature, the uptake of inorganic nutrients and metals is dependent on both the metal and soil, and strongly influenced by the amount of water transpired by the plant. As a consequence of the rise in temperature and the subsequent increase in transpiration, there is the potential for an increase in the transfer of copper from the soil to the plant [16]. The reduction in micronutrients in the lettuce leaves could likely be ascribed to their lower solubility due to the higher digestate pH compared

to the soil one (Table S1), as reported by Olszyk et al. [68], who observed a decrease in Fe, Mn, and Zn concentrations, mainly, in lettuce plants grown in the presence of several biochars as amendments. Similarly, a reduction in Fe uptake was detected in baby leaf lettuce under different digestate treatments [69,70]. The addition of a low Cu concentration (M+Cu and D+Cu) caused in the plant leaves a two-fold increase in Cu uptake with respect to the control (S). Moreover, as reported previously, our findings highlighted that the Cu concentration was higher in the plants grown in M+Cu compared to those exposed to S+Cu and D+Cu.

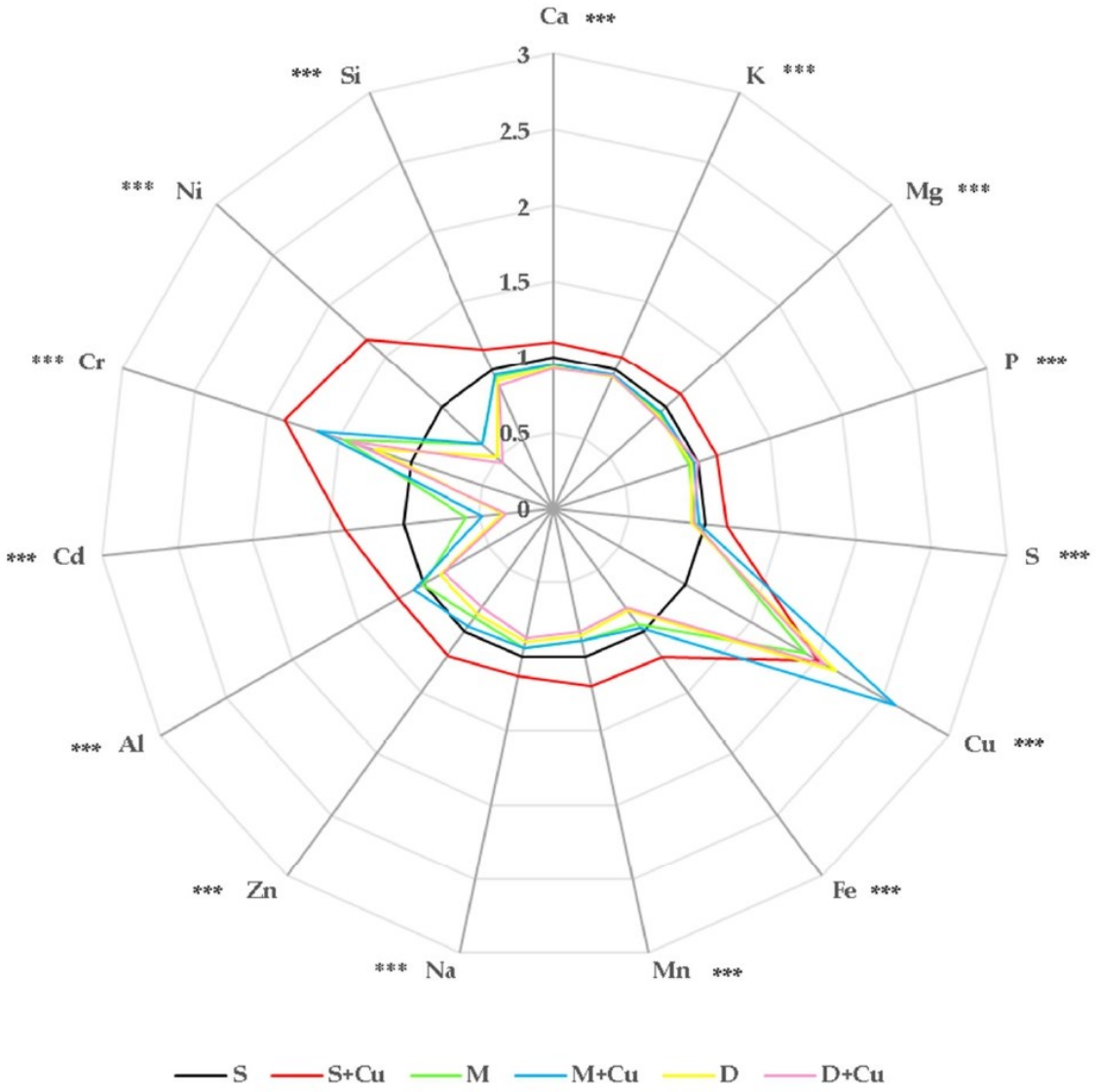


Figure 7. Changes in the shape of the radar plot relative to plant uptake of macro- and micronutrients induced by different amendment treatments with or without the addition of a low copper concentrations applied to lettuce plants and measured at the end of the treatment. The data are the average of four replicates and report the values with respect to plants grown in the control condition (S = control = 1). Asterisks indicate significant differences (Tukey HSD, $p < 0.05$) between control plants (S) and those exposed to M, M+Cu, D, and D+Cu.

3.8. Microbiological Analyses of Soil Community

At the end of the experiment, after five weeks, the total microbial abundance was not significantly different between the tested soils (S, M, and D) (Figure 8a). The addition of a low Cu concentration significantly reduced the microbial abundance in the S+Cu soil (36%) whereas a significant enhancement was observed in the M+Cu soil (35%) compared to S and M, respectively. The higher microbial abundance in M+Cu with respect to S+Cu could be ascribed to the presence of copper-resistant bacteria in the manure [71]. Moreover, organic soil amendments could immobilize metals, reducing their bioavailability, due to the presence of humic acids able to chelate a wide variety of metals, including copper [72]. No significant difference in microbial abundance was highlighted between the D and D+Cu soils.

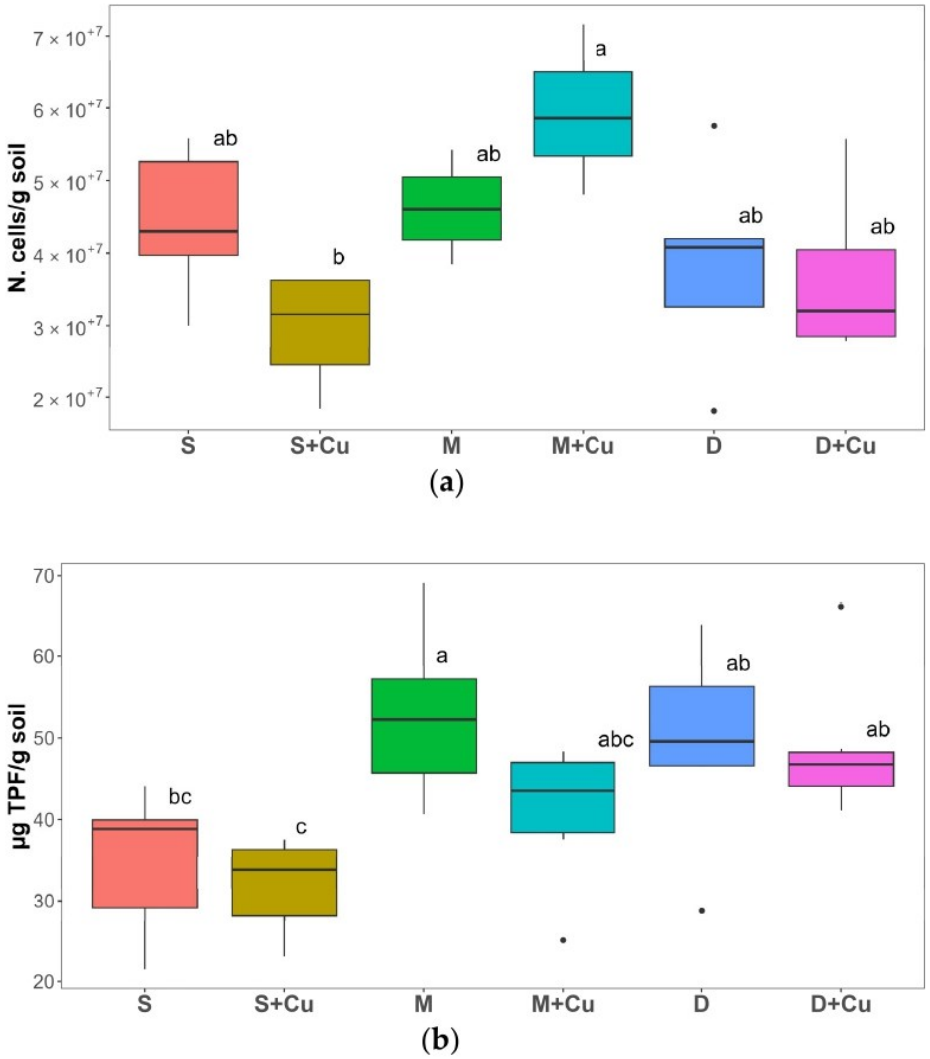


Figure 8. (**a**) Soil total microbial abundance (N. cells g^{-1} dry soil) and (**b**) soil microbial activity (ug TPF g^{-1} dry soil) measured in soil (S), manure (M), and digestate (D) and with the addition of copper (Cu) after five weeks of experimentation. Box plots labelled with different letters indicate significant differences between different treatments (ANOVA followed by Tukey HSD, p value ≤ 0.05). In the box-plots, the upper and lower whisker endpoints represent the maximum and minimum observations, respectively, outliers are shown as dots. $n = 4$.

Both cattle organic soil amendments, manure (M) and digestate (D), showed higher soil microbial activity, 51.24% and 41.31%, respectively, when compared to the non-amended soil (S). Following Cu addition (S+Cu, M+Cu, and D+Cu), a downward trend in dehydrogenase activity was observed compared to the respective soils without amendments (S, M, and D, respectively) (Figure 8b). Dehydrogenase activity (DHA) is a biomarker related to soil fertility and measures the metabolic state of soil microbial communities. In addition, this enzyme reflects the total range of oxidative activity of soil microorganisms [73]. Soil microflora contributes to the improvement of plant physiological performance status. Any application of a toxicant that might affect the growth of soil microorganisms can induce alterations in the general activity of enzymes, such as dehydrogenase [74]. As reported in previous works, DHA activity is increased by the addition of organic amendments in the soil [73], such as the use of manure to fertilize winter rye, potato, oats, barley, and red clover in crop rotations. Heavy metals, including copper, might negatively affect soil bacterial communities by reducing their populations, changing their structure and diversity, decreasing soil enzyme activity, and interfering in plant–soil–metal associations [70].

3.9. Principal Component Analysis

The first two components of the PCA analysis explained 68.3% of the overall variance in the lettuce dataset (Dim 1: 43.1%; Dim 2: 25.2%) (Figure 9). The contribution (contrib) values of the plant performance parameters showed that shoot biomass, MDA, total chlorophyll, number of leaves, DHA, and DAPI were well represented in Dim1 and Dim2, in contrast with SLA, TPC, and the copper content in leaves (Figure 9a). A correlation analysis of the plant performance parameters (Figure S1) demonstrated that shoot biomass, MDA, total chlorophyll, number of leaves, and DHA were significantly positively correlated with Dim1, while SLA, TPC, DAPI, and leaves' copper content were significantly negatively correlated with Dim1. A correlation analysis of Dim2 revealed that TPC, DAPI, DHA, and leaves' number correlated positively, whereas SLA, copper content in leaves, shoot biomass, MDA, and Chl Tot were negatively correlated. The outcomes of the PCA clearly discriminated the samples according to soil composition, showing six clusters, which reflected the different soil conditions plus the control (S) (Figure 9b). The profile of the lettuce plants grown in D and D+Cu clustered in a Dim1-positive direction, while those grown in S, S+Cu, and M+Cu clustered in a Dim1-negative direction. Plants grown in D and D+Cu were separated from the control (S) and manure (M) plants grown with or without copper towards number of leaves, shoot biomass, Chl tot, MDA, and DHA. Similarly, separation of the control (S) and manure (M) plants grown with or without copper was observed towards TPC, copper content in leaves, SLA, and DAPI. Treatment with D and D+Cu resulted in a better physiological performance in the lettuce plants with respect to the manure treatments and control conditions. The second axis (Dim2) was most closely related to DAPI and TPC, and the samples segregation on Dim2 showed that manure had the highest microbial abundance.

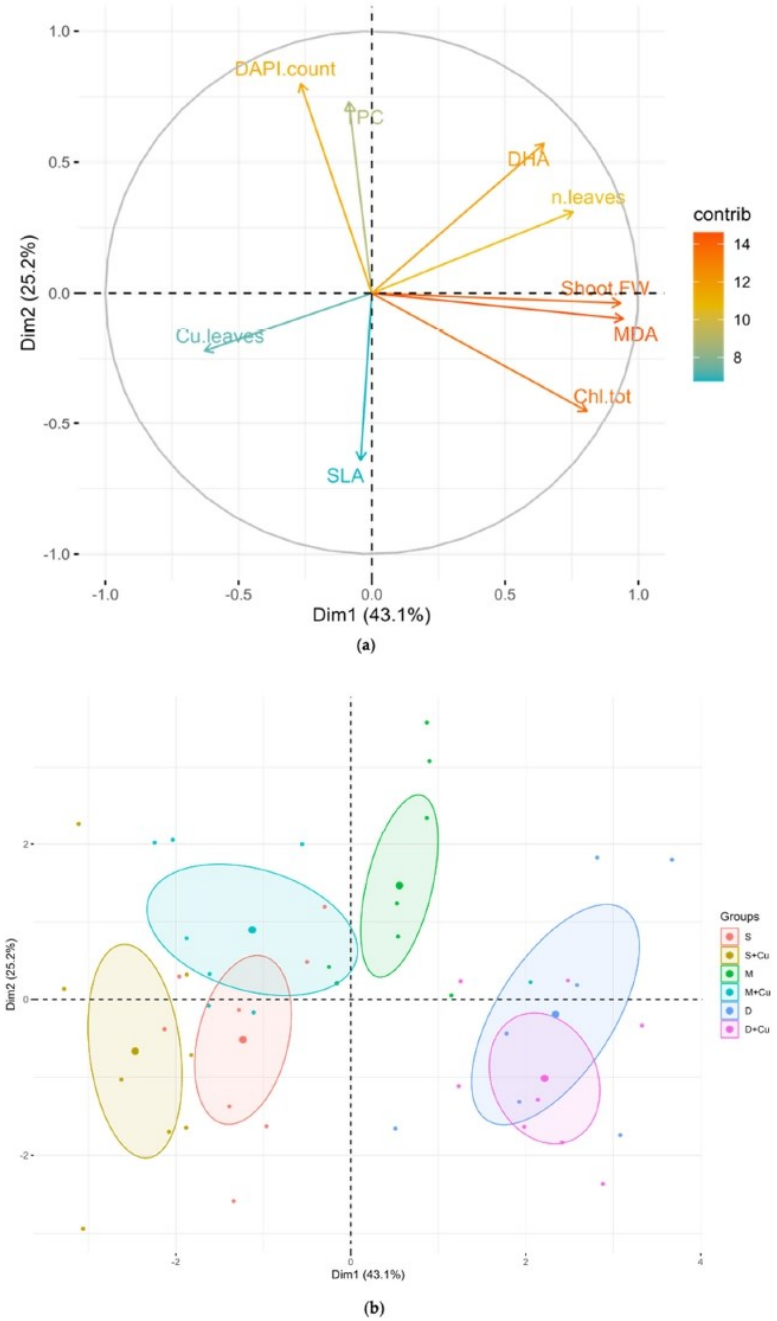


Figure 9. (**a**) Variable correlation plot, constructed using the following variables: shoot fresh biomass (Shoot FW), total chlorophyll content (Chl Tot), specific leaf area (SLA), malondialdehyde content (MDA), copper content in leaves (Cu leaves), leaves number (n.leaves), total phenolic content (TPC), soil total microbial abundance (DAPI count), and soil microbial dehydrogenase activity (DHA). The length of each vector represents the contribution of the corresponding variable on the principal component (Dim1 and Dim2), while the direction of each vector indicates the correlation between the variable and the principal components. Vector colors represent the contribution of the corresponding variable to the overall variability. (**b**) Principal component analysis (PCA) showing samples' segregation by soil composition (S, S+Cu, M, M+Cu, D, and D+Cu). Data points are grouped into colored ellipses representing a 95% confidence interval. Enlarged points within ellipses represent the centroids of the sample distributions grouped by soil composition. The legend provides a color code for each group.

4. Conclusions

This study investigated the effects of growing lettuce plants with different combinations of cattle organic soil amendments, cattle manure (M) or digestate (D), and low concentrations of copper, like those found in agricultural soils used for the cultivation of horticultural species treated with, i.e., copper-based fungicides. The results showed that both manure and digestate affected the lettuce plants very differently. While manure showed negative effects overall, the application of digestate resulted in an increased yield and improved the photosynthetic performance. Digestate application might have buffered the decrease in soil pH caused by fertilization, potentially mitigating soil acidification compared to manure amendment. In the M and M+Cu conditions, a decreased lettuce growth, a higher Cu bioaccumulation, and a reduced tolerance index were associated with increasing the Cu concentration in the soil, suggesting an increased level of Cu phytotoxicity in the plants and a detrimental impact compared to the plants grown in digestate and in the digestate-copper amended soil.

Our greenhouse pot study aligns with existing research on digestate's potential, but field trials are necessary for real-world applicability.

In low- and middle-income countries, digestate offers a more advantageous alternative to manure for replacing chemical fertilizers. As a renewable and inexpensive source of nutrients and energy derived from organic materials, it promotes a circular economy. Large-scale validation is needed to confirm the benefits of anaerobic digestion for a circular economy, as shown by research on energy and fertilizer production from various organic materials.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments11070134/s1; Table S1: Main physico-chemical characteristics of soil, manure and digestate; Table S2: Elements content (mg kg^{-1}) in different soils; Table S3: Elements content in leaves of lettuce plants grown in different soil conditions; Figure S1: Correlation between the variable and the principal components in PCA analyses.

Author Contributions: Conceptualization, P.G., A.B.C. and M.A.I.; methodology, P.G., A.B.C. and M.A.I.; validation, C.D.C. and M.A.I.; formal analysis, C.D.C., A.N., B.C., F.P., D.G. and V.I.; investigation C.D.C., A.N. and F.P.; resources, P.G., A.B.C., F.P. and M.A.I.; writing—original draft preparation, C.D.C., F.P., V.I. and M.A.I.; writing—review and editing, C.D.C., A.N., B.C., F.P., V.I., P.G., A.B.C. and M.A.I.; visualization, C.D.C., F.P., V.I. and M.A.I.; supervision, M.A.I.; funding acquisition, A.B.C. and M.A.I. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All the data supporting the reported results can be found in this article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Use of the *Heterocypris incongruens* bioassay for assessing ecotoxicity of soils containing the anionic surfactant sodium lauryl ether sulphate (SLES)

Livia Mariani [a], Paola Grenni [a,*], Anna Barra Caracciolo [a,*], Tanita Pescatore [a], Francesca Spataro [b], Jasmin Rauseo [b], Alessandra Narciso [a,c], Ludovica Rolando [a], Luisa Patrolecco [b]

[a] Water Research Institute, National Research Council (IRSA-CNR), Rome, Italy
[b] Institute of Polar Sciences, National Research Council (ISP-CNR), Rome, Italy
[c] Ecology and Biology Dept., Tuscia University, Viterbo, Italy

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ABSTRACT

The production and consumption of surfactants are constantly increasing, and huge amounts are found in the environment as contaminants. Surfactants are widely used in domestic and industrial applications, including in tunnel-mechanized excavation of large-scale infrastructures (e.g. highways and railways). In the latter case, the commercial products are foaming agents containing the anionic surfactant sodium lauryl ether sulphate (SLES). Foaming agents are necessary for enhancing Tunnel Boring Machine-Earth Pressure Balance (TBM-EPB) performance. Consequently, there are SLES concentrations in excavated soils of which large quantities can have harmful effects on biota. SLES toxicity in the aquatic environment is well known; on the contrary, knowledge of its effects on soil organisms is quite limited. In order to better understand SLES ecotoxicity in soil, the standardized bioassay (ISO 14371:2012) with the crustacean *Heterocypris incongruens*, living in the soil–water interface, was used. The lethal concentrations of standard SLES (LC_{15}: 120; LC_{50}: 140 mg/L, respectively) and of three common commercial products used as foaming agents (LC_{50} varying from 275 to 3810 mg/L) were evaluated. Subsequently, the crustacean acute and sub-chronic effects (mortality and growth inhibition) were assessed in seven different excavated soils conditioned with various commercial products. In addition, SLES concentrations in each soil and in their soil water extract were also determined. Mortality was not recorded in soils conditioned with foaming agents; however, a growth inhibition (c.a. 61.6 %) was found for soils with a high fine fraction and conditioned with high amounts ($\geq$2 L/m^3 soil) of foaming agents. *H. incongruens* proved to be an appropriate bioassay for assessing the possible effects of high SLES concentrations in soil, especially when this surfactant is bound to soil.

1. Introduction

Surfactants are surface-active agents able to reduce the surface tension of compounds. Their chemical structure contains both a hydrophilic (head) and a lipophilic (tail) group. Thanks to this structure, they are amphiphilic molecules with a high affinity for both polar (e.g. water) and non-polar (e.g. hydrocarbons, ethers, and esters) solvents (Aslam et al., 2021; Cornwell, 2018; Massarweh and Abushaikha, 2020; Ying, 2006; Zhu et al., 2017). Owing to their properties, surfactants are widely used as anticorrosive materials in personal care products and cosmetics, household products, paint, textiles, dyes, polymers, foodstuffs, agrochemicals, and petroleum products (Aslam et al., 2021; Barra Caracciolo et al., 2017; Zhu et al., 2017), and also in remediation research for improving oil contaminant removal from soil (Massarweh and Abushaikha, 2020; Ying, 2006). Due to their widespread use, production has been growing in recent years, with an estimated output of 16.60 million tons in 2022 (Smithers, 2017). Surfactants in which the hydrocarbon tail originates from vegetable oils or fats, such as palm oil or tallow, belong to the so-called oleo-chemicals, while, when the source of the hydrocarbon is petroleum or gas, the surfactants belong to the so-called petrochemicals (Lara-Martín et al., 2005). In the latter case, they can have impurities, which in some cases are not mentioned in their chemical datasheets.

Anionic surfactants, which represent 60 % of global production of

* Corresponding authors.
E-mail addresses: paola.grenni@irsa.cnr.it (P. Grenni), barracaracciolo@irsa.cnr.it (A. Barra Caracciolo).

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surfactants (Karray et al., 2016), have a negative charge on their hydrophilic end. Their ecotoxicity in the aquatic environment is well known (Barra Caracciolo et al., 2017; Grenni et al., 2018; Mariani et al., 2020, 2006; Mousavi and Khodadoost, 2019). Among anionic surfactants, sodium lauryl ether sulphate (SLES) is the main component of a wide variety of commercial products used as foaming agents for mechanized tunnelling in Europe and worldwide. SLES can be obtained from petroleum or from plants (Farias et al., 2021), with the latter making it a more environmentally friendly product.

SLES is an ethoxylated surfactant, and is in particular a sodium salt produced through the ethoxylation of the alkyl sulphate sodium lauryl sulphate (SLS) (Cserháti et al., 2002). This generates an ethoxylate, which is transformed into a half ester of sulphuric acid and neutralized to achieve SLES. Depending on both the length of the predominantly linear aliphatic hydrocarbon chain and the length or number of components of the ethoxy chain, SLES can have a different CAS number (Barra Caracciolo et al., 2017; Fernandes et al., 2020).

SLES in some foaming products has a hydrocarbon chain length from C10 to C16 (alcohols, C10-16, ethoxylated, sulphates, sodium salts), with a CAS number 68585-34-2; in those where it is reported as C12-C14 (alcohols, C12-14, ethoxylated, sulphates, sodium salts), its CAS number is 68891-38-3. In other foaming products, SLES is called 2-dodecoxyethyl hydrogen sulphate and the related CAS number is 9004-82-4. The molar mass of SLES is around 420 g/mol, considering three ethoxylate groups (274.35 g/mol for its basic structure plus 44.05 g/mol for each ethoxylate group) (Cowan-Ellsberry et al., 2014).

In the commercial products used as foaming agents SLES is able to lower soil adhesion to the metallic excavating surfaces of tunnelling machines (Carigi et al., 2020). The tunnelling sector has been growing in view of new and expanding transport infrastructure needs, e.g. for high-speed railways, supporting sustainable development, in line with the EU 8th Environment Action Programme (2021–2030) goals. Tunnels are a large-scale infrastructure that has to be built with an eye to environmental protection at all their construction phases. This is particularly important as huge amounts of tunnel debris have to be recycled (e.g., about 1.5 millions of excavated soil was produced for a tunnel of about 7.5 km in Italy; Tunnel, 2020), avoiding production of waste. In Italy, the excavated soil containing surfactant residues (spoil material) can be considered a by-product if it meets legislative criteria (e.g., Italian Decree 120/2017), i.e. specific inorganic and organic chemicals below fixed threshold values in soil (Padulosi et al., 2019). Regarding commercial foaming agents, the same Decree only requires that they do not contain any substance classified as hazardous in EU Regulation 1272/2008 (Classification, Labelling and Packaging). Indeed, most commercial products do not contain hazard chemicals listed in Annex VI of the CLP. However, this classification does not exclude that high amounts of chemicals not considered hazardous (e.g., SLES) can have a toxic effect. Considering that SLES could bound to soil (Finizio et al., 2020), toxic effect could occur not only for the aquatic environment, but also for the terrestrial one.

Several aquatic (e.g., microalgae, bacteria, crustaceans and fish) and to a lesser extent terrestrial organisms (e.g., worms, plants) have been used for assessing the anionic surfactant SLES ecotoxicity. Aquatic organisms are more sensitive to SLES residues than terrestrial ones (Barra Caracciolo et al., 2017; Finizio et al., 2020; Galli et al., 2019; Grenni et al., 2018; Sibila et al., 2008). For this reason, the Italian Ministry of Environment has required site-specific environmental assessments to be performed before the construction of several railway or highway tunnels. These assessments evaluated the SLES concentrations and the toxicity of the commercial products, conditioned soils and their soil water extracts (Grenni et al., 2018; Mariani et al., 2020; Patrolecco et al., 2020). This evaluation was crucial for rendering the final destination for the spoil material in a green area close to a water body safe (Mariani et al., 2020). In this context, the identification of a test organism for assessing overall spoil material toxicity can be very useful.

The crustacean ostracod *Heterocypris incongruens*, which lives in soil/sediment–water interfaces, can be appropriate for this goal. *H. incongruens* also has the characteristic of having a cosmopolitan environmental distribution and it is an important member of meiofauna (Fryer, 1993; Kim et al., 2015; Martens and Behen, 1994; Mesquita-Joanes et al., 2012; Pieri et al., 2020). In particular, *H. incongruens* occurs in river sediment, groundwater, and stagnant and temporary waters in contact with soil, and is considered a keystone species for evaluating biotic changes produced by anthropogenic activities (Bellin et al., 2021; Ruiz et al., 2013). The direct contact of this benthic test species with the non-dissolved toxicants adsorbed on sediment/soil particles and/or the ingestion of the latter makes it very suitable for the evaluation of contaminated matrices (Burton, 1991; Burton and Ingersoll, 1994). *H. incongruens* is also considered a bioindicator of the overall toxicity of solid matrices, including sediment and soil (Chial and Persoone, 2002; Santorufo et al., 2012; Sevilla et al., 2014; Watanabe et al., 2008).

In accordance with the standard procedure (ISO 14371:2012), this test organism has been used for evaluating the toxicity of sediments and soils (Mierzwa-Hersztek et al., 2016; Pandey et al., 2019; Santorufo et al., 2012; Wang et al., 2009) and more recently for spoil material (Patrolecco et al., 2020). Data on SLES intrinsic toxicity (e.g. LC_{15} and LC_{50}) for *H. incongruens* have not been published so far. In this regard, this work reports the use of *H. incongruens* to evaluate the ecotoxicity of the anionic surfactant SLES and of three foaming agents (containing SLES) currently used in tunnelling for the construction of railways. Moreover, the same organism was tested in soils excavated at real sites and conditioned with the three commercial foaming products. Finally, SLES concentrations in soil and soil water extracts were analysed for evaluating SLES amount leached from soils to water compartment and, therefore, assessing SLES bound to soil. This key information is useful in ecotoxicological test that use an organism in direct contact with non-dissolved toxicants adsorbed on sediment/soil, such as the *H. incongruens* test.

2. Materials and methods

2.1. Chemicals and commercial products containing SLES

The anionic surfactant sodium lauryl ether sulphate (standard SLES, CAS: 68585-34-2, purity 71.92 %), was purchased from BOC Sciences (US, Canada) and used as the reference compound for the methylene blue active substances assay (MBAS).

Three commercial products (P1, P2 and P3) were selected using the geotechnical slump cone tests (Carigi et al., 2020), which demonstrated their suitability for conditioning seven soils coming from three construction sites (Supporting Information, Table S1). The products consisted of water solutions of the anionic surfactant SLES at various concentrations (P1: 10–20 %; P2: 5–10 %; P3: 25–50 %; data from their chemical data sheets). The main characteristics of standard SLES and of the commercial products are reported in Table 1.

2.2. Soils conditioned with commercial foaming products

Seven different soils (S1-S7) with various lithologies (Supplementary Materials, Table S1), were sampled from three real tunnel construction sites (denominated A, B and C) for high-speed railway lines located in Central and Southern Italy. Soil samples (at least three replicates of 5 kg) were collected from the conveyor belt of the Tunnel Boring Machine-Earth Pressure Balance (TBM-EPB) which transports spoil material out of the tunnel. Each soil was conditioned by the TBM-EPB with one or two commercial foaming products (P1, P2 or P3) in different amounts, expressed as Treatment Ratio, (TR L/m^3). The TR values, previously selected with geotechnical slump cone tests (Carigi et al., 2020; Martinelli et al., 2019; Peila, 2014; Peila et al., 2007), ranged from 0.34 L/m^3 to 2.5 L/m^3. The TR values (Table 2) depended on the foaming product characteristics, excavated soil lithology (Supplementary Materials, Table S1), geological conditions and mechanical and functional

Table 1
Main characteristics of standard SLES and foaming agents. Percentages (%) of the anionic surfactant sodium lauryl ether sulphate (SLES) and CAS number derived from their chemical data sheets. Synonyms (based on the hydrocarbon chain lengths) and number of the ethoxylate groups in standard SLES and in the commercial foaming products derived from ECHA website (https://echa.europa.eu).

Standard and Foaming Products	SLES (%)	SLES CAS no.	Synonyms	No. ethoxylate groups
SLES standard	71.92	68585-34-2	Alcohols, C10-16, ethoxylated, sulphates, sodium salts	1–3
P1	10–20	68891-38-3	Alcohols, C12-14, ethoxylated, sulphates, sodium salts	1–2.5
P2	5–10	9004-82-4	2-dodecoxyethyl hydrogen sulphate	1–12
P3	25–50	68891-38-3	Alcohols, C12-14, ethoxylated, sulphates, sodium salts	1–2.5

Table 2
Excavated soils from three sites (A, B and C) conditioned with various commercial foaming products (P1, P2 or P2 + P3), TR: Soil Treatment Ratio (L/m^3) and corresponding acronym.

Construction sites	Soil	Foaming Product	TR (L/m^3)	Conditioned Acronyms
A	S1	P1	0.36	**S1 + P1_0.36**
			2.00	**S1 + P1_2.00**
			2.50	**S1 + P1_2.50**
		P2	0.62	**S1 + P2_0.62**
	S2	P1	0.95	**S2 + P1_0.95**
		P2 + P3	0.98 + 0.27	**S2 + P2 + P3_1.25***
	S3	P1	1.48	**S3 + P1_1.48**
		P2 + P3	1.60 + 0.38	**S3 + P2 + P3_1.98***
B	S4	P1	0.34	**S4 + P1_0.34**
		P2	0.69	**S4 + P2_0.69**
	S5	P1	0.34	**S5 + P1_0.34**
		P2	0.69	**S5 + P2_0.69**
C	S6	P1	2.00	**S6 + P1_2.00**
			2.50	**S6 + P1_2.50**
	S7	P1	1.50	**S7 + P1_1.50**

*1.25 and 1.98 values correspond to the sum of TRs used for P2 and P3, as described below:
S2 + P2 + P3_1.25: P2 0.98 L/m^3 + P3 0.27 L/m^3; the P2:P3 rate is 3.65:1.00.
S3 + P2 + P3_1.98: P2 1.60 L/m^3 + P3 0.38 L/m^3; the P2:P3 rate is 4.21:1.00

properties of the tunnel boring machine (Carigi et al., 2020; Peila, 2014). The overall fifteen conditioned soils and corresponding acronym are listed in Table 2. Aliquots of unconditioned soils (S1-S7) were also analysed as controls.

Each excavated conditioned sample and control was analysed for SLES concentration in soil and in its corresponding soil water extract. The same samples were used for the ecotoxicological tests with *Heterocypris incongruens*.

2.3. Preparation of soil water extracts from soil samples

Soil water extracts (elutriates) were prepared in a 1:10 ratio between soil and distilled water in accordance with the UNI EN 12457-2:2004 standard procedure. It was used to simulate the leaching processes of SLES from soil to water.

For each soil conditioning, at least three replicate samples (100 g each) were prepared.

2.4. Determination of SLES in commercial products, soils and elutriates

The SLES concentration was determined in the three commercial products (P1, P2, P3), conditioned and unconditioned soils and their corresponding soil water extracts (elutriates). The method used was previously published in Patrolecco et al. (2020). Briefly, SLES was extracted from solid phase matrices (about 2 g of conditioned or unconditioned soil) with a Pressurized Liquid Extractor (PLE, E-916, BUCHI Italia s.r.L, Cornaredo, Italia) using methanol as the extraction solvent, and operating at the following conditions: 100 °C; 3 extraction cycles; 100 % flush volume and 60 s purge time (Patrolecco et al., 2020). The extraction was done in three replicates for all experimental conditions.

The aqueous phase samples such as the PLE extracts (in methanol), elutriates and commercial product solutions were processed for SLES concentration by using the optimized Methylene Blue Active Substances (MBAS) method (Patrolecco et al., 2020). The reaction between SLES and methylene blue forms a blue complex salt, which is extracted by chloroform. The SLES concentrations were then quantified by spectrophotometric measurements (Perkin–Elmer Lambda 25 UV–VIS spectrophotometer) at 650 nm using the equation ($y = 0.5425x - 0.0342$; $R^2 = 0.998$) resulting from the standard calibration curve (0.05–4 mg/L SLES).

The detection limit (LOD), and the recovery for the PLE extraction were 0.013 mg/L (IUPAC, 1999) and 96.3 ± 1.4 %, respectively.

2.5. Ecotoxicological tests with Heterocypris incongruens

The direct-contact test with the Ostracodtoxkit F™ (MicroBioTests Inc., Mariakerke, Belgium) was performed for evaluating the intrinsic toxicity (LC_{15} and LC_{50}) of SLES and of the P1, P2 and P3 commercial products and the potential toxic effect of the excavated soils. The tests were executed in accordance with the Ostracodtoxkit procedure and the standard (ISO 14371:2012) protocol, using *H. incongruens* batches n. HI200519 and HI120416. The reference toxic compound used was $CuSO_4{\cdot}5H_2O$.

Briefly, 10 ostracod neonates from cysts hatched in 52 h were added to each test chamber (6 replicates for each condition) with 2 mL of algal food (*Scenedesmus* spp., batch n. SS190821F and SS190820F), 2 mL of standard freshwater (MicroBioTests Inc., Mariakerke, Belgium), and 1 g of standard sediment or soil sample. All tests were performed with a standard sediment (blank) as the negative control. Unconditioned soils were also tested.

The acute and sub-chronic effect (mortality and growth inhibition) were recorded after exposition (six days at 25 °C in the dark) to samples. Both results (mean value of six replicates) were expressed as a percentage (%).

Samples were considered toxic if the ostracod mortality was > 20 %, or/and the growth inhibition was > 30 % (ISO 14371:2012; Persoone et al., 2003). The mortality was reported as Abbot mortality (with the Abbott's correction %, Abbott, 1925). As required by the standard procedure, growth inhibition (GIn%) was calculated only in samples that displayed a mortality rate of the exposed organisms < 30 %. The latter is the limit condition for recording the inhibition of growth on a representative number of surviving organisms (ISO 14371:2012).

The Leica S9i stereomicroscope and the LAS V4.12 software (Leica, Microsystems Co., Ltd., Weztlar, Germany) were used for measuring the ostracod length at start and end of test.

At the end of test, validity criteria were verified in accordance with the ISO 14371:2012 standard procedure. In the blank (standard sediment), ostracod mortality should not be higher than 20 % and the organism length should have increased by a factor of 1.5 (Persoone et al.,

2003). Moreover, before the tests, the sensitivity of ostracod batches was verified assessing the lethal concentration (6d LC_{50}) to $CuSO_4{\cdot}5H_2O$ (reference compound).

Data were considered valid and reproducible if the coefficient of variation (CV %) was in the 20–30 % range (Persoone et al., 2003). All data were analysed with the US EPA Benchmark Dose Software (BMDS), Version 2.1.

2.5.1. Lethal concentrations of SLES standard and commercial products

The toxicity of SLES (standard SLES), of each single product (P1, P2 and P3) and a combination of them (P2 + P3) were assessed using the *H. incongruens* test. The ecotoxicity was expressed in terms of lethal concentration for 15 % (LC_{15}) and 50 % (LC_{50}) organisms exposed to SLES standard and foaming products, after six consecutive days.

For LC_{15} and LC_{50} tests, stock solutions using standard freshwater (SW) for SLES standard (71.92 %, technical grade) and commercial products (P1, P2, P3 and P2 + P3) were prepared at double concentration, as foreseen by ISO and the kit procedure. Moreover, in order to compare the toxicity of the commercial products and standard SLES, all stock solutions were prepared to obtain the same final SLES concentration (1 g/L SLES in standard fresh water, expressed as g_{SLES}/L_{SW}). Serial dilutions (four or five from stock solutions, see Table 3) of products in SW and one blank (only SW) were prepared in triplicate and each test was performed three times.

The LC_{15} and LC_{50} values (at 6 days) and their 95 % confidence limits were calculated with the Trimmed-Spearman Karber and Probit statistical methods (Hamilton et al., 1977; Sparling, 2016).

2.5.2. Ecotoxicological tests on excavated soils

Bioassays were also performed on real environmental matrices (excavated soils) consisting of seven soils conditioned with one or two commercial products (foaming agents; Table 2). In particular, the mortality and growth inhibition endpoints were recorded and compared with both standard sediment (blank) and unconditioned soil (used as the control). The evaluation of unconditioned soil is a crucial point for excluding any possible toxic effect of the soil matrix characteristics on test organisms. In fact, extreme pH values (Ruiz et al., 2013) or fine textured soil (Casado-Martinez et al., 2016) can be harmful for some crustaceans, such as ostracods. The effects of the conditioned soils were therefore calculated net of the effects found in the unconditioned soils.

3. Results and discussions

3.1. SLES concentrations in commercial foaming products, soils and elutriates

The SLES concentrations were determined for each commercial foaming product and the results are in Table 4. For each foaming agent, SLES percentages were in the range reported in the corresponding Chemical data sheets (Table 1).

Table 3
Stock solution and test concentrations of standard SLES or foaming agent product (P1, P2, P3 and P2 + P3) tested with *H. incongruens* to evaluate the Lethal Concentrations (LC_{15} and LC_{50}). Stock solutions are expressed as grams of substance (SLES) or product (P1-P3) in standard fresh water (g product/L_{SW}). Test concentrations refer to SLES/product concentration to which the ostracods were exposed, considering the dilution due to adding algal food, as required by the ISO procedure.

SLES Standard/ Foaming Product	Stock solution (g product/L_{SW})	Test concentrations (mg product/L_{SW})
SLES	1	75; 100; 125; 150; 175
P1	5.65	495; 565; 705; 990; 1130
P2	15.56	2725; 3500; 3890; 4280; 4670
P3	1.69	255; 265; 275; 295; 380
P2 + P3_1.25*	6.18	775; 1080; 1390; 1855
P2 + P3_1.98*	5.52	690; 965; 1240; 1655

* For P2 + P3 conditions see Table 2.

Table 4
SLES concentration (%) measured in each foaming product.

Foaming Product	SLES measured (%)
P1	17.7 ± 0.71
P2	6.94 ± 0.77
P3	60.10 ± 0.79
P2 + P3_1.25*	18.48 ± 0.76
P2 + P3_1.98*	17.13 ± 0.72

* For P2 + P3 conditions see Table 2.

The SLES concentrations were also determined in the excavated soils conditioned with the P1, P2, P3 and P2 + P3 foaming products. Fig. 1 reports the SLES concentration in the excavated soil and in its corresponding soil water extract. For each soil, the product (P) used and the applied treatment ratio (L/m^3) is reported.

The SLES concentrations (mg/kg, Fig. 1) measured in the excavated soils were in line with the predicted environmental concentrations (PEC) calculated considering each TR value, the percentage of SLES inside a specific product and soil density (Supplementary materials, Table S2). The highest SLES values (c.a. 200 mg/kg) were found for S1 and S6 conditioned with the highest TR values of P1 (2.5 L/m^3). Although the TR value influenced the SLES amount in soil (a positive correlation was found between TR and SLES in soil with $r = 0.9545$ and $p < 0.01$), this was not the case of elutriates. In fact, SLES concentrations in soil water extracts were quite variable (ranging from 0.2 to 11.3 mg/L) and the highest values (7.5–11.3 mg/L in S6 and S7, respectively) did not necessarily correspond to the highest use of foaming agents in terms of TRs. Indeed, soil water extracts were performed to check for a possible leaching of SLES from soil to water. It is known that the leaching of a chemical from a soil depends on its intrinsic solubility and the soil lithology and mineralogy (Finizio et al., 2020). SLES leaching therefore varied depending on the characteristics of each soil and product (see Table 1 and Supplementary Materials, Table S1). In particular, in the sandy-loam or silty-loam soils (S4, S5, S6 and S7), in which the fine fraction (clay) was less present, the highest amounts of SLES were found in the elutriates, in accordance with previous studies (Barra Caracciolo et al., 2019; Finizio et al., 2020; Mariani et al., 2020; Patrolecco et al., 2020).

3.2. Ecotoxicological tests with Heterocypris incongruens

All the test validity criteria foreseen by the ISO method were met. In fact, in the standard sediment samples (blank), the mean length of the ostracods was > 1.5 times compared to neonate lengths at the start of tests (Chial and Persoone, 2003) and mortality was < 20 % for all tests. Moreover, the test reproducibility was always verified (CV < 20 %). The average LC_{50} value for the ostracod batches tested with the reference toxic compound ($CuSO_4{\cdot}5H_2O$) was 6.03 ± 1.4 mg/L, in the range of 2.21–9.37 mg/L determined by the International Interlaboratory Comparison Protocol Toxkit (Terekhova et al., 2018).

3.2.1. Lethal concentrations of SLES and commercial products

The Lethal Concentrations of SLES and commercial products expressed as LC_{15} and LC_{50} are reported in Table 5.

The LC_{50} value of *H. incongruens* for SLES standard was 141.7 mg/L. This is the first work to report lethal effects of SLES on this ostracod so far. The value was higher than acute exposures recorded for other freshwater crustaceans [e.g. *Gammarus pulex*, 48 h-IC_{50}: 39.24 mg/L, Singh et al. (2002); *Daphnia magna*, 24 h-LC_{50}: 7.2–7.4 mg/L (chemical datasheet of SLES Standard and ECHA database for CAS 68891-38-3, ECHA, 2022; Barra Caracciolo et al., 2017)].

The LC_{50} shows this ostracod to be less sensitive than other aquatic organisms (Barra Caracciolo et al., 2017; Grenni et al., 2018), but more

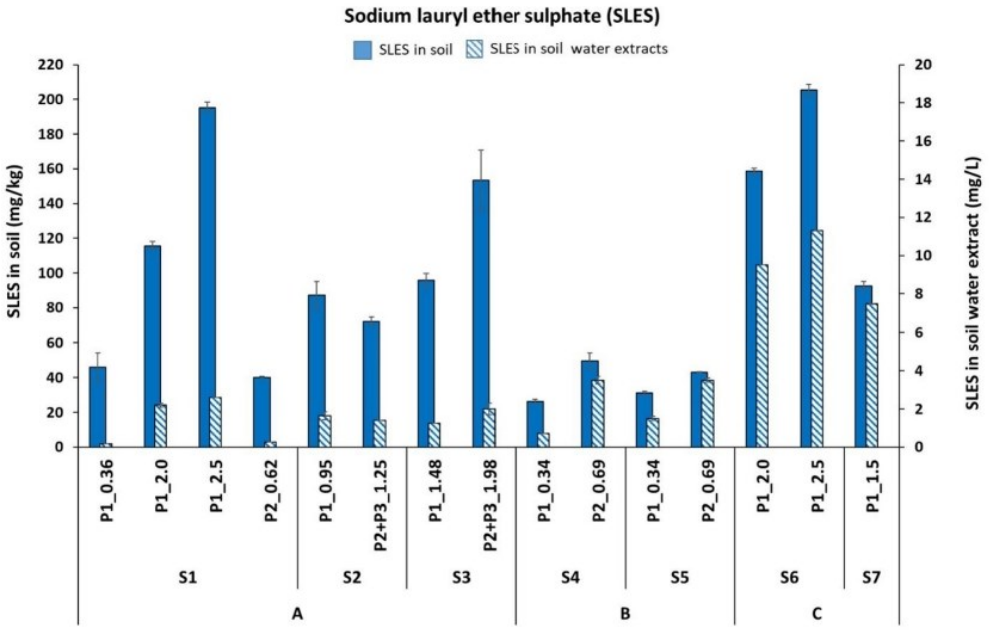

Fig. 1. SLES concentration in the conditioned soils (mg/kg) and in their soil water extracts (mg/L). A, B and C: construction sites. S1-S7: soils analysed in this work and conditioned with P1, P2, P3 or P2 + P3 foaming agents. The number below each bar refers to the treatment ratio (TRs, L/m3) applied. For further details on the conditioned soils, see Table 2.

Table 5
Lethal concentrations (LC_{15} and LC_{50}, mean values ± standard error) obtained for standard SLES and for each commercial foaming product. For each product, the SLES concentration (mg $_{SLES}$/L) affecting 50 % of organisms was calculated, considering the SLES % measured in each product.

SLES Standard/ Foaming Product	LC_{15} mg $_{product}$/L ± s.e.	LC_{50} mg $_{product}$/L ± s.e.	SLES concentration in each product corresponding to LC_{50} mg$_{SLES\ inside\ product}$/L
SLES standard	118.3 ± 6.7	141.7 ± 8.8	–
P1	588.3 ± 17.4	743.3 ± 20.3	125
P2	3,383.3 ± 51.8	3,810.0 ± 50.7	250
P3	263.3 ± 1.7	275.0 ± 2.9	165
P2 + P3_1.25*	761.7 ± 14.5	895.0 ± 15.0	150
P2 + P3_1.98*	726.7 ± 22.1	876.7 ± 10.9	150

* For P2 + P3 conditions see Table 2.

sensitive to SLES than other terrestrial ones. For example, a recent work reports the *Eisenia foetida* earthworm and *Lepidium sativum* plant display LC_{50s} of 402 mg/kg and 433 mg/kg, respectively (Finizio et al., 2020). Salvatori et al. (2021) reported phytotoxicity effects for *Lepidium sativum* L. from higher concentrations of SLES (1000–1200 mg/kg), which are not realistic for excavated soils (Barra Caracciolo et al., 2021, 2019; Galli et al., 2019; Mariani et al., 2020; Patrolecco et al., 2020). Other tests performed on *Zea mays* L. confirmed the low sensitivity to SLES of plants (Salvatori et al., 2021). The intermediate sensitivity of *H. incongruens*, between aquatic and terrestrial organisms, makes it a suitable biondicator for the soil–water interface.

Interestingly, the various commercial products affected *H. incongruens* differently and this was in accordance with their SLES percentage (see Table 1). However, P2 showed the lowest toxicity (3,383 and 3,810 mg/L for LC_{15} and LC_{50}, respectively), with a SLES content of 250 mg/L, much higher than the LC_{50} of the standard SLES (142 mg/L). The lowest toxicity of P2 is ascribable to the higher number of ethoxylated groups if compared to other products (Table 1), as reported by other authors (Cornwell, 2018; Fernandes et al., 2020). However, a key point is that SLES in P2 was obtained from vegetal material. Indeed, the anionic surfactant SLES can be derived from different material sources, either animal or plant origin, or from petroleum (Pironti et al. 2020; Farias et al. 2021) and this can affect its intrinsic toxicity. This is an important aspect to be considered in environmental studies, and shows how, even if with the same chemical formula and labelling, some synthetic chemicals can affect biota differently because of their different origin. This result highlights the usefulness of ecotoxicological tests for evaluating the environmental impact of a chemical.

3.2.2. Ecotoxicity of the excavated soils

The acute and sub-chronic toxic responses of *Heterocypris incongruens* to unconditioned soils and soils conditioned with foaming agents are summarized in Fig. 2 and Fig. 3 and in Supplementary Materials (Table S3).

The red line represents the threshold toxicity values (20 %) for acute effect (mortality), as reported by ISO 14371:2012.

No acute effects (mortality) were recorded for *H. incongruens* exposed to S1, S2 and S3 (site A) and to S6 and S7 (site C) unconditioned and conditioned soils (Fig. 2 and Table S3). In fact, the mortality values were < 20 % for ostracods exposed both to unconditioned and conditioned soils from these construction sites. Conversely, unconditioned soils from site B (S4 and S5) showed acute toxic effects with 63–67 % mortality (Fig. 2A and Table S3). The latter result was presumably due to soil acidity (pH about 5, Table S1). In fact, the soils from site B had pH values below the tolerance range indicated for this ostracod species (pH 5.5–10; Ruiz et al., 2013).

The unconditioned and conditioned soils from sites A and C that revealed a mortality < 30 % (mortality limit condition for recording the growth inhibition, ISO 14371:2012) were subject to the sub-chronic test (growth inhibition). Some unconditioned soils displayed a sub-chronic effect (Fig. 3A).

The red line represents the threshold toxicity values (30 %) for the sub-chronic effect.

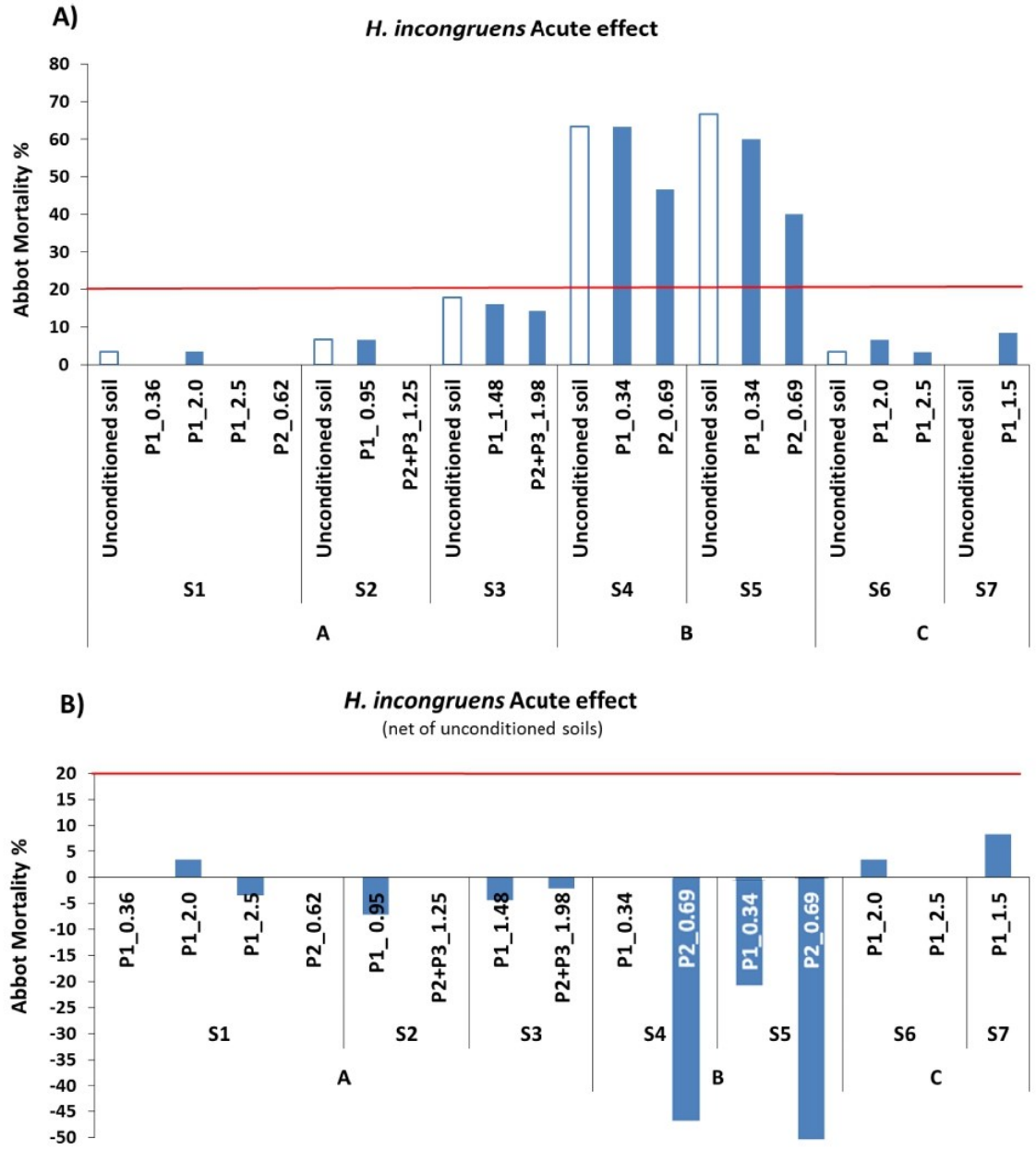

Fig. 2. Acute effects (Abbott's mortality) on *H. incongruens*. A) Acute effects for all soil samples (conditioned and unconditioned one). B) Acute effects of conditioned soil samples calculated net of the effects found in the unconditioned soils. The red line represents the threshold toxicity values (20%) for acute effect (mortality), as required by ISO 14371:2012. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In particular, S1, S2 and S6 soils caused from 34 % to 83 % growth inhibition (compared to the ostracod growth in standard sediment, Fig. 3A). These effects were ascribable to the excavated soil characteristics (Supplementary Materials, Table S1) and in particular to the soil fine texture component, which presumably affects the crustacean growth, as also found by other authors (Casado-Martinez et al., 2016; Patrolecco et al., 2020). Interestingly, the sub-chronic effect was not observed in the conditioned soil (Fig. 3B), except for S1 conditioned with the P1 foaming product (S1 + P1_2.0; S1 + P1_2.5) at high treatment ratios (TRs 2.0 and 2.5 L/m^3), with a growth inhibition > 61 %.

Although S1 and S6 conditioned soils, treated with the same foaming agent (P1) and at the same TR (L/m^3 soil) and therefore containing similar SLES concentrations in soil (Fig. 1), they showed a different sub-chronic effect (Fig. 3B). SLES leached in a higher amount in S6 compared to S1, verified by the different SLES concentration in soil water extracts (Fig. 1), therefore SLES was more bound to S1 excavated soil than to S6 one. This fact was due to the different adsorption of the anionic surfactant to soil, depending on soil texture characteristics of S1 and S6 (Supplementary Material, Table S1), as also reported in our previous studies (Patrolecco et al., 2020; Grenni et al., 2018). The sub-chronic effect recorded for S1 was mainly due to the direct contact of organism with a high SLES amount adsorbed (non-leached) on soil.

Specifically, soil water extracts (elutriates) obtained from S6 conditioned with P1 had higher amounts of SLES (9.50 or 11.30 mg/L SLES for TR 2.00 and 2.50, respectively) than from S1 (P1_2.0: 0.17 mg/L; P1_2.5: 2.20 mg/L), showing the different adsorption capacity of these two soils.

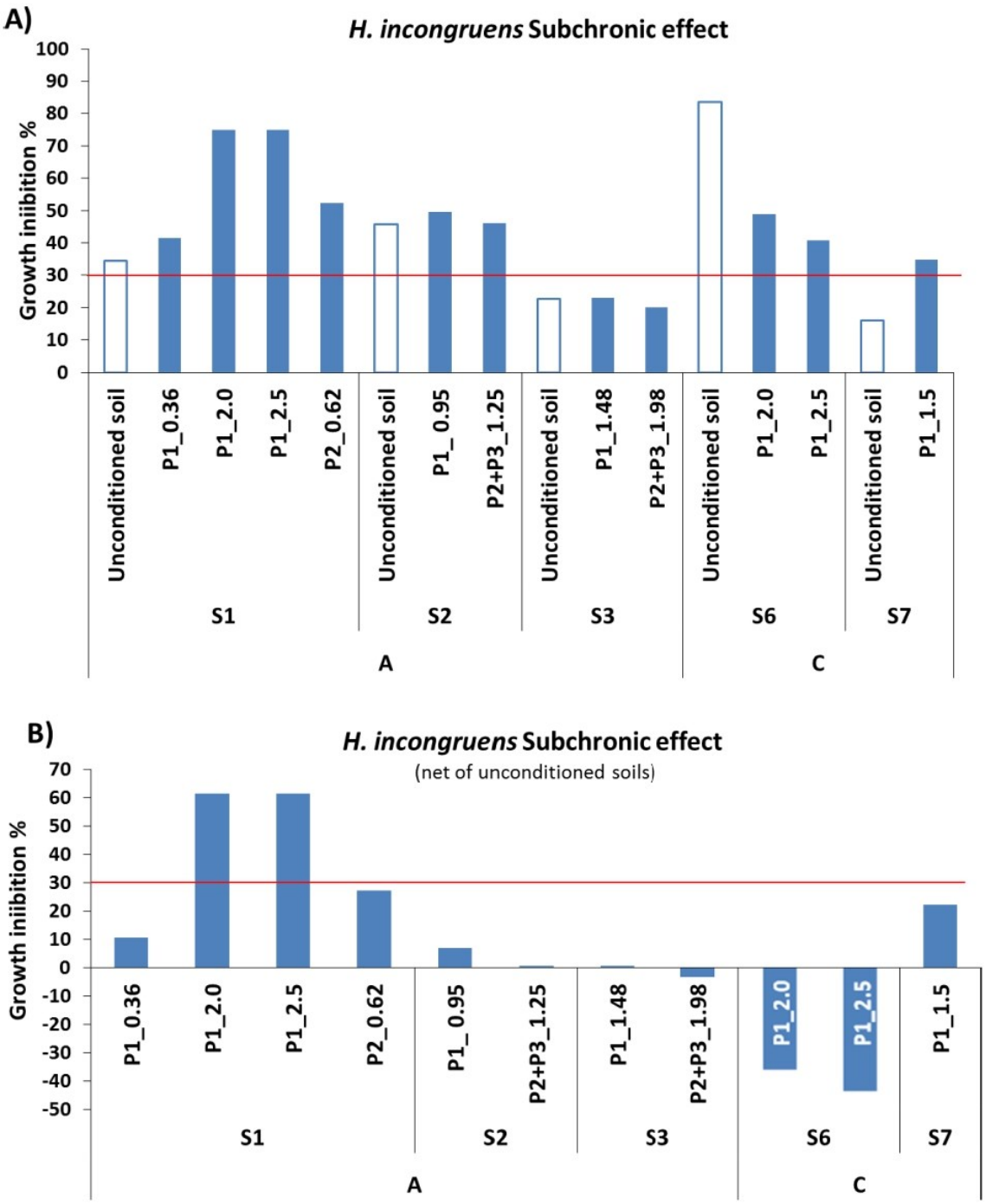

Fig. 3. Sub-chronic effects (growth inhibition) on *H. incongruens*. A) Effects for all soil samples (conditioned and unconditioned one). B) Effects for conditioned soil samples calculated net of unconditioned soil effect (see also Supplementary Materials, Table S3). Since S4 and S5 mortality values were > 30 % (Fig. 2), these samples were subject to the sub-chronic test. The red line represents the threshold toxicity values (30 %) for the sub-chronic effect. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This fact confirm the value of *H. incongruens* test as a valid tool for chemicals with different behaviour (e.g., leaching) depending on soil texture. A higher toxic response of *H. incongruens* to pollutants bound to solid phase particles of a matrix has been found by other authors (Chial and Persoone, 2003; Ruiz et al., 2013). To confirm this result, other previous studies did not find sub-chronic ecotoxicity for *H. incongruens* from high concentrations of SLES in excavated soil, if a high SLES amount was found in the elutriates (Patrolecco et al., 2020).

The *H. incongruens* test can be used to evaluate both water and soil ecotoxicity (Chial and Persoone, 2003; Wieczorek and Baran, 2022), including in a test battery (Patrolecco et al., 2020; Radziemska et al., 2019; Urbaniak et al., 2020). *H. incongruens* has the advantage of a direct contact with the matrix (liquid–solid phase) where the overall toxicity is recorded. Moreover, *H. incongruens* is a benthic organism and,

consequently, more exposed to the compounds adsorbed to soil and to the toxicity released by a substrate if compared to planktonic organisms and other crustaceans commonly used in ecotoxicology. *H. incongruens* test is less labour intensive, less space demanding and cost-effective if compared to others (e.g., *Hyalella azteca* assay), and does not depend on the continuous culturing and maintenance of the test biota (Chial and Persoone, 2002; Chial and Persoone, 2003; Belgis et al., 2003; ; De Cooman et al., 2015). Moreover, it has the advantage of obtaining a sub-chronic endpoint (growth inhibition) in only 6 days, with reproducible and reliable results (thanks to crustacean short-life cycles and a large number of organisms used).

The overall results of this work show this ostracod to be suitable for evaluating soil toxicity, as it is sensitive to different soil texture and adsorption characteristics, which in turn influence the amount of a chemical bound to soil; the *H. incongruens* test can be a useful complement to other bioassays on leachates. Although large amounts of excavated soil could be potentially contaminated by foaming agents, SLES chemical determinations and ecotoxicological tests for the re-use of spoil material are not currently foreseen by the legislation, but they would be desirable, especially in the case of high values of TR applied.

4. Conclusions

This work reports new values and contribute to full-fill data gaps of the intrinsic ecotoxicity for standard SLES and commercial products containing it. The *Heterocypris incongruens* bioassay has been shown a valid tool for assessing the ecotoxicity of real matrices such as excavated soils conditioned with foaming agents containing SLES, considering organism tolerance range for pH and soil texture. *H. incongruens* is an appropriate choice, compared to other crustaceans which live exclusively in freshwater (e.g. *Daphnia magna*) and other terrestrial test organisms, for estimating overall soil toxicity (solid matrix and its water extract).

The sensitivity of the ostracod *Heterocypris incongruens* to different environmental conditions and anthropogenic impacts, plus the fact that it is an organism naturally found in both soil and water, makes this micro-crustacean not only a good test organism, but also a bioindicator.

CRediT authorship contribution statement

Livia Mariani: Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Paola Grenni:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Anna Barra Caracciolo:** Supervision. **Tanita Pescatore:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Francesca Spataro:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Jasmin Rauseo:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Alessandra Narciso:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Ludovica Rolando:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing. **Luisa Patrolecco:** Conceptualization, Methodology, Data curation, Writing – original draft, Investigation, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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CONCLUSIONS

Overall antibiotics and copper interacted in various mixture showing additive, synergic and antagonistic effects, depending on each antibiotic characteristic, as shown using the *Aliivibrio fischeri* as the bacterium model organism. Interestingly, contaminant effects in the real soil were partially buffered due to interactions between soil matrix and chemicals.

The soil microcosm experiments showed how antibiotics affected microorganisms in different ways, selecting for resistant bacteria able to degrade it and promoting antibiotic resistance gene spread. The application of organic amendments introduced differently ARB and ARGs. Manure carried a higher amount of ARGs than cattle manure digestate. Some antibiotic resistance genes (e.g. *intI1*) tended to decrease with antibiotic dissipation. Others persisted (e.g. *sul2*) over time, particularly in copper co-presence and other stressors (e.g. high temperature), confirming co-selection phenomena. However, ARGs were generally in lower amounts the lettuce rhizosphere, showing the strong positive effect of the plant-microbiome system.

Copper showed toxicity for the lettuce plant-microbiome system decreasing overall plant biomass, however adding cattle manure digestate its deleterious action was buffered, and an antagonist effect was found in SMX presence. In general, cattle manure digestate can be considered a better organic fertilizer than manure for different aspects.

In conclusion, although direct and indirect effects of contaminant mixtures are hard to determine, the ecological approach, using ecotoxicological tests (for assessing direct effects of contaminants) and microcosms for studying site-specific soil matrices and their interactions with biota, made it possible to improve knowledge on these complex ecosystems. Further studies on the effects of ABs and HMs are desirable to improve knowledge on the responses of soil microorganisms, plants and soil fauna to these contaminant threats and to provide realistic environmental risk assessments for soil.

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