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ENVIRONMENTAL REMEDIATION AND ECOTOXICOLOGICAL ASSESSMENTS OF XENOBIOTICS

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Abstract

Soil pollution is a serious, current and expensive threat to the planet's health; many xenobiotics, mostly of anthropogenic origin, affect soil's ability to provide ecosystem services essential to life.

Pollution studies require case-by-case approaches and may relate to contamination prevention, monitoring, or remediation techniques. This thesis moves within the macro-area of soil pollution addressed at different levels, aiming to prevent, understand or solve specific soil contamination events. In particular, the study focused on two main classes of xenobiotics: potentially toxic elements and pesticides.

Seven research projects were discussed to investigate three major issues: i) The application of several bioremediation techniques against Ni contamination in a disused quarry (Chapters 1 and 2), ii) Pesticide kinetics and ecotoxicity in different substrates (Chapters 3, 4 and 5), iii) Copper monitoring and ecotoxicity in vineyards soils (Chapters 6 and 7).

In the first case (i), the novelty lies in applying eco-sustainable techniques in solving a real contamination case on a carbonation lime; the main results show that sequestering minerals such as bentonite and zeolite and phytoremediation can immobilise the metal, thus containing the contamination.

Regarding the pesticide studies (ii), after an exhaustive review describes research performed in the last years to assess the impact of pesticide sub-lethal doses on soil microorganisms and non-target organisms in agricultural

soil ecosystems, two studies were conducted to evaluate adsorption and degradation parameters for three pesticides and the ecotoxicological effects of two insecticides, one natural and one synthetic, on earthworms and soil microbiota. Finding suggests different dynamics depending on pesticide and substrate tested and could contribute to sustainable management of chemicals in the environment: the soil amendment with cheap and available organic wastes could reduce the pesticide impact in the ecosystem. Earthworm activity helps the degradation of bioinsecticide, and the microbial community can adapt and change according to time and the presence of *Eisenia fetida*. Effects of the synthetic insecticide chlorpyrifos on earthworms occur early at the genotoxic level and on their growth, and in the longer term also on the reproductive activity.

Finally, the copper topic (iii) was tackled in the first step, monitoring two organically run vineyards. Then ecotoxicological tests were conducted at different sensitivity levels on earthworms. Ecotoxicological effects were assessed on natural soil with sub-lethal doses of copper, and early DNA damage on earthworms was evaluated. The original experimental design was proposed to detect bacterial community changes in soil and the earthworm's gut. Furthermore, the sensitivity of earthworms to copper was evaluated using reproduction and avoidance tests. The results showed a high ability of earthworms to avoid copper-contaminated soil even at the lowest concentrations and dose-dependent adverse effects on the reproductive outputs. Despite this, these annelids seem to recover genotoxic damage at intermediate copper concentrations in the soil, while irreversible damage and death have been highlighted beyond a certain threshold.

The metal accumulation in earthworms increased with the gain of soil copper concentrations until a limit was reached beyond which bioaccumulation remained almost constant, probably due to internal regulation systems. In the twenty-eighth-day experiment, total and bioavailable copper did not appear influenced by the presence of earthworms. To date, analyses of changes to the earthworm and soil microbiome are ongoing.

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Introduction

Environmental pollution

The concept of Environmental pollution is continually changing, and thanks to its ubiquity, giving it a determinate meaning is difficult (Ajibade *et al.* 2021). There are several definitions, some more specific than others; "The introduction by man, into the environment, of substances or energy liable to cause interference with legitimate uses of environment" (Holdgate 1979), "Disequilibrium condition from equilibrium condition in any system" (Singh 1991), "The contamination of the physical and biological components of the earth/atmosphere system to such an extent that normal environmental processes are adversely affected" (Krishna *et al.* 2017).

Since the 1940s, when rapid industrialisation and urbanisation occurred, environmental pollution has become a global problem that affects the three natural matrices such as air, water, soil, and that is linked directly to humanity and other life forms on earth (Merian 1984; Bradford 2018; Lv *et al.* 2019; Sulaymon *et al.* 2020). Many substances can affect environmental health, which could be named xenobiotics. Initially, the term xenobiotic comes from the Greek word *Xenos*, foreign or strange, and *bios*, which means life. Today xenobiotics are defined as any substance whose presence is considered unusual or in concentrations too high for the body or ecosystem under consideration; therefore, even a natural substance can be defined as such if it damages the organisms (Embrandiri *et al.* 2016; Vallero 2016).

Until now, the discussions about pollution have mainly been directed towards water resources, but xenobiotics must cross the soil to reach the aquifers in most cases (Rampanelli *et al.* 2021). Therefore, soil conservation is fundamental, considering that this environmental compart could buffer,

degrade, immobilise, detoxify, and trap xenobiotics, such as oil, pesticides, herbicides, and heavy metals, and keep them from entering groundwater (Spellman 2017).

Since World War II, pesticides have been intentionally released into the environment at a larger scale (Montanarella and Alva 2015).

Among xenobiotics, pesticides and heavy metals are the worst affecting soil health due to their interaction with non-target organisms and persistence (Castelo-Grande *et al.* 2010; Komárek *et al.* 2010; Puglisi 2012; Montanarella and Alva 2015; Ockleford *et al.* 2017; Yu *et al.* 2018).

Only a small percentage of pesticides reach the target organisms; their over-used, their long persistence and the lack of specificity put at risk the equilibrium of the ecosystems (Bünemann, Schwenke and Van Zwieten 2006; Carriger *et al.* 2006; Castelo-Grande *et al.* 2010; Komárek *et al.* 2010; Puglisi 2012; Popp, Pető and Nagy 2013; Ockleford *et al.* 2017).

As mentioned by Aktar et al. (2009), among the several pesticides categories, insecticides are generally the most acutely toxic class. Nowadays, biologically based pesticides are becoming more popular as they are safer than traditional pesticides.

About heavy metals, it is well known that some considered essential elements (cobalt, copper, iron, manganese, vanadium, and zinc) are indispensables in small quantities for various biochemical processes. The danger regarding heavy metals in the soil is that biodegradation cannot occur, and thus metals accumulate in the ecosystem (Inyinbor *et al.* 2018; Ali, Khan and Ilahi 2019). Moreover, metal concentrations above threshold levels affect the microbiological balance of soils and may reduce their fertility (Barbieri 2016).

As stated by Panagos et al. (2013), the management of contaminated sites is estimated to cost around 6 billion euros annually; despite this, scientists and world organisations recognise the need to act against pollution as a primary issue for the regions with mature industrial sectors and a well-developed regulatory framework (FAO and ITPS 2015; Lv *et al.* 2019). Moreover, correct management of natural resources and monitoring the ecosystem health by ecotoxicological studies are crucial tools to prevent pollution events (Jha *et al.* 2000).

Against pollution, it should begin with an understanding of the dynamics between the xenobiotic and the environment involved, study the characterisation of the polluted sites and, only after that it is possible to act directly with remediation technologies to reduce, contain or remove the contamination (Pierzynski, Vance and Sims 2005; Rodríguez-Eugenio, McLaughlin and Pennock 2018).

Remediation

In Italy, only some regions had specific legislation about managing contaminated sites. The regulation at the national level comes with the Legislative Decree n°2 of 5 February 1997, followed by the subsequent Ministerial Decree n° 471 of 25 October 1999 about specific administrative and technical procedures for identifying and managing contaminated sites. Then this regulation was revised and included under the environmental code (2006), currently in force with five technical annexes on risk assessment, characterisation, remediation techniques, and soil and groundwater screening values.

According to current legislation, the administrative procedure for contaminated sites identification and management is under the responsibility of municipalities and regions with the help of provinces.

The national remediation program started in 1998 with the creation of Contaminated sites of national interest (SIN) that are now under the direct responsibility of the Ministry of Environment. The management approach is the same for historical and new contamination, and the site-specific risk-assessment procedure is used to identify polluted sites.

There are many remediation strategies; among them, adsorption techniques are considered the most functional for removing heavy metals in terms of cost, simplicity of design, insensitivity to toxic pollutants and efficiency (Mohan *et al.* 2002; Amer, Khalili and Awwad 2010). Clay minerals, zeolites and metal oxides effectively remedied heavy metal contamination in various environmental matrices thanks to their high adsorbing capacity (Ugwu and Igbokwe 2019).

Attention has recently been focused on 'bioremediation' because of its cheapness and eco-friendly aspect (Eapen, Singh and D'Souza 2007). In this context, phytoremediation is a "green" and well-accepted technology that uses living plants, mostly hyperaccumulators and accumulators species, to remove different pollutants, especially heavy metals (Ali, Khan and Anwar Sajad 2013).

Phytoremediation modalities depend on the xenobiotic involved (chemical nature and properties) and the plant selected. It is possible to classify six different strategies (Favas *et al.* 2014). Phytodegradation (Phytotransformation), where the absorbed organic contaminant is broken down, metabolised or mineralised up to carbon dioxide and water through a

series of enzymatic reactions and metabolic processes (Schnoor *et al.* 1995). Phytostabilization (Phytoimmobilization) aims to avoid the mobilisation of organic and inorganic contaminants locked into humus or the lignin of the cell wall of the roots (Berti and Cunningham 2000). Phytovolatilisation occurs when plants can volatilise certain metals/metalloids and some organic compounds after the absorption by the roots system and conversion into non-toxic forms (Padmavathiamma and Li 2007). Phytoextraction (Phytoaccumulation, Phytoabsorption or Phytosequestration) is a technique applied against organic and inorganic pollutants (especially metals) that mainly uses hyperaccumulator plants. Phytofiltration, with plants in aquatic environments that absorbs, concentrate or precipitate the contaminants through their submerged organs, if the roots make the filtering activity, the term Rhizofiltration is used (Dhote and Dixit 2009). Finally, Rhizodegradation (Phytostimulation) occurs when the exudates from the root system enhance and promote the activity of the microorganisms in the rhizosphere, which can transform the organic pollutant. In many cases, the exudates contain enzymes for the contaminants' degradation.

Ecotoxicology

The mere use of chemical measures in assessing the potential for adverse toxic effects at polluted sites can underestimate or overestimate the required level of site cleanliness; thus, the employment of ecotoxicity tests can better assess the environmental impact (Wilson, Hatcher and Goudey 2002).

Moreover, current approaches to environmental pollution monitoring combine chemical-physical (SOM, aggregate stability, etc.) and biological

tools; thus, living organisms are used to assess soil health (Beiras 2018). It is in this field that ecotoxicology operates.

Ecotoxicology was born in the first half of the twentieth century following numerous disastrous pollution events that damaged human and environmental health worldwide (Vasseur 2020).

In the 1960s, Jean-Michel Jouany conceptualised ecotoxicology: a multidisciplinary science that combines ecology and toxicology aimed to protect the ecosystems by studying pollutants' harmful effects on living organisms at the individual, population and communities levels and the interrelations with the surrounding environment. This goal is generally achieved by assessing effects on single species of selected test organisms and extrapolating the obtained (no) effect concentrations to safe levels for populations and communities. In the ecotoxicological risk assessment of chemicals, such safe levels are compared with predicted or measured exposure levels to evaluate the possible risk for exposed ecosystems (van Gestel 2012).

The parameters commonly used to quantify the impacts of xenobiotics on organisms are defined as ecotoxicological endpoints and are represented by measurable effects through ecotoxicological assays. Interest in soil ecotoxicology is constantly increasing with the awareness of the impacts of contaminants on soil communities, species diversity and associated ecosystem services (Wong *et al.* 2018).

The toxicity tests for soil invertebrates were standardised by Organisation for Economic Co-operation and Development and the International Standardization Organization, starting with survival as the only endpoint (Table 1). Later, the reproduction performance and biomass change detection

were proposed as other endpoints, and more recently, avoidance response was introduced as a more effortless and sensitive endpoint for some xenobiotics.

Table 1. Toxicity test with soil invertebrates by van Gestel (2012).

Test organism	Species	Duration (days)	Endpoint	Guideline	Reference
Earthworms	*Eisenia fetida/ Eisenia andrei*	14	Survival	OECD 207 ISO 11268-1	OECD (1984) ISO (1993)
		28 (+28)	Reproduction	ISO 11268-2 OECD 222	ISO (1998) OECD (2004b)
		2	Avoidance	ISO 17512-1	ISO (2008a)
	Field test, different species	Up to 1 year	Species diversity; abundance	ISO 11268-3	ISO (1999b)
Enchytraeids	*Enchytraeus albidus,* other *Enchytraeus* species	21 (+21)	Survival, Reproduction	ISO 16387 OECD 220	ISO (2004) OECD (2004a)
		2	Avoidance	No standard guidelines	Amorim et al. (2008a,b)
Mollusca	*Helix aspersa*	28	Survival, Growth	ISO 15952	ISO (2006)
Mites	*Hypoaspis aculeifer*	14	Survival, Reproduction	OECD 226	OECD (2008)
	Platynothrus peltifer	14 70	Survival Reproduction	No standard guideline	Van Gestel and Doornekamp (1998)
	Oppia nitens	28	Reproduction	No standard guideline	Princz et al. (2010)
		2	Avoidance	No standard guideline	Owojori et al. (2011)
Isopods	*Porcellio scaber*	28	Survival, growth	No standard guideline	Hornung et al. (1998a,b)
	Porcellionides pruinosis	14	Survival, reproduction	No standard guidelines	Jänsch et al. (2005)
		2	Avoidance	No standard guidelines	Loureiro et al. (2005)
Collembola	*Folsomia candida Folsomia fimetaria*	28	Survival, Reproduction	ISO 11267 OECD 232	ISO (1999a); OECD (2009)
		2	Avoidance	ISO 17512-2	ISO (2011)
Insects	*Oxythyrea funesta*	14	Survival	ISO 20963	ISO (2005)
Carabid beetles	*Pterostichus oblongopunctatus; Poecilus cupreus*	Different durations	Adult or larval survival; adult behaviour, respiration	No standard guidelines	Schrader et al. (1998); Bednarska et al. (2010)

Spurgeon et al.(2003) describe the importance of earthworms among soil organisms in ecotoxicology from the analysis of many published papers. In soils, earthworms act synergistically with microbial communities (Bart *et al.* 2019), and both of them are commonly used in the ecotoxicological study because they represent a significant fraction of soil living biomass and play

an essential role in soil functioning (Pelosi, Barot and Capowiez 2014; Delgado-Baquerizo *et al.* 2016).

Eisenia fetida is the model earthworm species for routine toxicity testing because it is easy to maintain and breed in laboratory conditions (Edwards 2004).

Microorganisms living in soil are the primary agents responsible for key ecosystem processes and can be affected by pesticide contamination and interact with their degradation pathways and soil functions (Vasileiadis *et al.* 2013). Regarding soil microbiota, regulators and policymakers must consider both the negative and adaptation effect of xenobiotics (Wittebolle *et al.* 2009). As concluded in the report "Response of microbial organisms to pesticides", changes in microbial community structure should be analysed as early warnings of the real status of the environment. In fact, most methods considered in the literature for the effects of pesticides on soil microbes can indeed indicate if the community is different, not if its biodiversity is affected (Puglisi 2017).

It is possible to study the effects of a xenobiotic at different levels of biological organisation (molecular, cellular, individual, population, etc.) (Connon, Geist and Werner 2012). At the cellular level, genotoxicity is used as an early biomarker usually detected by comet assay (single gel electrophoresis assay), which is a sensitive and rapid method for DNA strand break detection in the individual cells of the studied organism (Fairbairn, Olive and O'Neill 1995; Albertini *et al.* 2000; Ramírez and Cuenca 2002). Whereas genomic impairment could be an early biomarker of effects at the individual level (Burcham 1999), it can be hypothesised that a higher level of DNA damage is associated with a greater impact at the population level. This

assumption has been verified in several studies that have observed decreased reproductive capacity parallel to genotoxicity (Hertel-aas *et al.* 2007; Bonnard *et al.* 2009; Hertel-Aas *et al.* 2011).

Objectives

Environmental pollution was the macro research area of this doctorate and has been addressed at different levels. All the studies focused on soil resources during the three years because this environmental compart could buffer, degrade, immobilise, detoxify, and trap pollutants, such as oil, pesticides, herbicides, and heavy metals, and keep them from entering groundwater supplies (Spellman 2017).

As reported in the introduction chapter, pollution studies require specific approaches for each case study and may relate to contamination prevention, pollution monitoring or the application of remediation techniques.

Therefore the objectives of this thesis, depending on the specific case studies, ranged from:

- studying several bioremediation techniques to lower the levels of nickel contamination of a disused quarry.
- understanding the kinetics of three pesticides in natural soil and organic biomix to assess if amendment with cheap and available organic wastes could reduce the impact of pesticides;
- evaluating the effects of sublethal doses of two insecticides on earthworm *Eisenia fetida* and microbial community as non-target soil organisms to provide more information for the prevention of toxicity phenomena;
- monitoring copper contamination levels throughout the years in vineyard soils and studying the ecotoxicological effects on earthworm *Eisenia fetida* and bacterial community to evaluate if this element could accumulate in soil and affect the earthworms and microbiota health.

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CHAPTER 1: Zeolite and bentonite as nickel sequestrants in carbonation lime coming from the sugar industry

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Abstract

A laboratory trial was performed to test the sequestration capacity of two minerals (bentonite and zeolite) at three initial concentrations (2.5, 5 and 10%) in order to counter water-soluble nickel (Ni) exceeding the Italian legal limit (10 μg L^{-1}) in carbonation lime disposed of in a field and sampled for an 85-day lab study. The results show a noticeable reduction in water soluble and bioavailable Ni in lime after the addition of sequestrants, especially at the dose of 5% bentonite or zeolite, thereby indicating a "ceiling effect" of the sequestrant, i.e., an increasing dose could reduce the adsorption capacity and be less effective.

The alkaline pH and the presence of organic matter could be the main factors affecting the good performance of sequestrant addition, causing an increase in the negative charge of the organic and mineral colloids and the formation of available Ni precipitates. The 85-day experiment seems to be sufficient to reach an adsorption equilibrium for water-soluble nickel, while for the bioavailable form, a longer period appears to be necessary.

Keywords: Nickel, Carbonation lime, Zeolite, Bentonite.

1.1 Introduction

A recent reference reported by the Joint Research Centre of the European Commission (Payá Pérez and Rodríguez Eugenio, 2018) indicates that there are nearly 2.8 million potentially contaminated sites in Europe and that the most frequent contaminants are heavy metals and mineral oils. Regarding heavy metals, much attention must be paid to their bioavailable form, which can be transferred between

the environmental compartments up to man through the food chain. The bioavailability of heavy metals in soils and solid waste materials can be noteworthily lowered by the formation of organic and inorganic complexes and by a change in pH and organic matter (OM) content. Therefore, long-term metal availability depends on the strength of the bonds with the soil components and by the release in the soil solution (Nachtegaal and Sparks, 2003). As for most metals, the toxicity of Nickel (Ni), which is the fifth most common element on the Earth, is dependent on the route of exposure and the solubility of a Ni compound (Coogan et al., 1989).

Several nickel species can be present in soils, such as water-soluble, exchangeable and complexed/adsorbed species; in soil solutions, nickel species are classified as free Ni^{2+} cations and organic and inorganic complexes. The complexation with dissolved OM is an important parameter to describe the solubility and mobility of nickel in soil and soil solution (Schaumlöffel, 2005).

In general, liming reduces the mobility of heavy metals in soils (McNear et al., 2007; Malinowska, 2017), inducing an increase in pH and favouring binding with mineral fractions. Concerning the remediation of heavy metal polluted matrices, several experiments have been made worldwide using zeolite (Vaca Mier et al., 2001; Querol et al., 2006; Diale et al., 2011; Shah et al., 2013) and/or bentonite (Ling et al., 2007; Akpomie and Dawodu, 2015; Tahervand and Jalali, 2017; Chaves et al., 2017) as sequestering materials.

The use of zeolites for pollution control depends on their ion-exchange capabilities (Edwards et al., 1999). Indeed this property of zeolites (especially clinoptilolites) has been exploited in the decontamination of heavy metals in soils (Shi et al., 2009) and sediment (Chiang et al., 2012). In 1996, Shanableh and Kharabsheh demonstrated that the addition of a natural zeolite to soil contaminated by Pb^{+2}, Cd^{+2} and Ni^{+2} reduced the leaching of the three metals by more than 97%. Chen et al. (2000) found that the addition of zeolite and/or calcium carbonate and manganese oxide to two soil types reduces the extractability of Pb and Cd in both soils. Castaldi et al. (2005), in an acidic soil cultivated with white lupin and contaminated at high concentrations

of Pb, Cd and Zn, found that the addition of a natural zeolite noticeably reduced the water-extractable form of the metals. Clinoptilolite is the most abundant naturally occurring zeolite, which is formed by crystalline aluminio-silicates with tetrahedra linked to each other at the corners by sharing their oxygen (Shi et al., 2009); the substitution of Si by Al in the tetrahedra generates a negative charge of the structure, which may give rise to high adsorption of cations (Mohamed, 2001). Moreover, the presence of channels and cages and the shape of their internal pore structure allow it to work as "molecular sieving" (Braschi et al., 2010).

As previously mentioned, bentonites are interesting adsorbent materials in reducing the mobility of heavy metals. Bentonite is a clay mainly composed of montmorillonite which is a 2:1 type aluminosilicate, with a negative charge on the surface generated by isomorphous substitutions of Al with Mg in the octahedral layer, which allows to adsorb cations (Kumpiene, 2010; Tahervand and Jalali, 2017). The adsorption could also occur on the adsorption sites available within interlayer space (Tabak et al., 2007). This property is mainly responsible for the great adsorptive power of bentonite, especially toward ions in solution (Rodríguez-Sarmiento and Pinzón-Bello, 2001). The bentonites that are used industrially are predominantly comprised of either sodium montmorillonite, calcium montmorillonite or, to a much lesser extent, hectorite (Murray, 2006).

The present paper is a part of a collaboration activity with Sadam Spa, Bologna, Italy, a sugar industry involved in an environmental recovery project of a disused quarry. According to the Italian Legislative Decree n.186 (2006), the recovery project was aimed to remodel the morphology of the quarry using lime from sugar refining as filling material. The following assessment of chemical pollutants in lime, conducted by Marche Regional Environmental Protection Agency (ARPAM), highlighted a contaminated area exceeding the legal limit of soluble Ni concentration (10 $\mu g\ L^{-1}$).

Evaluating the remediation potential of any material in a controlled experimental setting before field application in the contaminated site is an essential requirement to avoid largescale failure.

The objective of this study was to assess the sequestering capacity of two different minerals on carbonation lime in order to decrease the concentration of the soluble Ni below the legal limit set by the Italian Legislative Decree n.186 (2006).

1.2 Materials and methods

1.2.1 Lime

Carbonation lime disposed in the disused quarry located in the municipality of Monte Roberto (Italy) was sampled in the contaminated area mapped by ARPAM. Four square plots (2 m × 2 m) were delineated by placing their centroids where ARPAM measured a concentration of water-soluble Ni ranging from 26 to 32 μg L^{-1} at a depth of 3.00–3.20 m. Five samples were collected in each of the four plots at this depth. Carbonation limes were the residue of the industrial process of sugar juice purification, and they were made up of 90% calcium carbonate to which non-sugar components had been added. These lime had a clearly alkaline reaction (pH 8.9) and an OM content of 7%.

1.2.2 Sequestrants

In the present study, a natural zeolite and an activated bentonite with a mesh size of 100–500 μm, were used and compared. Zeolite was supplied by Chemia S.p.a., Terre del Reno, Ferrara, Italy, and the composition of the commercial product ZEOLIT was as follows: clinoptilolite 85–95%, calcite-mica clay minerals 4–12%, quartz and feldspar traces. The main physicochemical characteristics of the used zeolite were surface area of 54.3 $m^2 g^{-1}$; CEC of 80 meq/100 g and pH of 8.5 (sol. 1%).

Activated bentonite was supplied by Dal Cin Gildo S.p.a., Concorezzo, Monza-Brianza, Italy, and the composition of the commercial product SUPERBENTON DC was as follows: montmorillonite 85–90% and inert silica 1–4%. The main

physicochemical characteristics of the used bentonite were as follows: surface area of 88.0 m^2 g^{-1}, CEC of 150 meq/100 g and pH of 9.5 (sol. 5%).

1.2.3 Laboratory experiment

Samples were air dried and then thoroughly mixed to obtain a single homogeneous sample representative of the whole contaminated area. The substrate was treated with the two sequestrants using three different concentrations (2.5, 5 and 10% w/w). This resulted in seven treatments: three with bentonite (B), three with zeolite (Z) and a negative control without the addition of any material (C). Six replicates (1 Kg dry weight) per treatment were maintained at 25 °C with a moisture content of 30% (w/w). Water-soluble and bioavailable Ni in each treatment were measured at different times (0, 1, 6, 8, 22, 35 and 85 days). The doses of bentonite and zeolite and the sampling times fall within ranges employed in studies using these sequestrants for the remediation of soils contaminated by heavy metals, since the application of these sequestrants on contaminated carbonation lime has not been evaluated previously (Garau et al. 2007; Radziemska and Mazur 2016; Tahervand and Jalali, 2017).

1.2.4 Ni extraction and analysis

Water-soluble Ni was extracted with distilled water, (1 g/10 mL) according to Italian Legislative Decree n. 186 (2006), and bioavailable Ni was extracted with a solution of diethylenetriaminepentaacetic acid (DTPA), $CaCl_2$ and triethanolamine at pH 7.3, (1 g/2 mL) following the indications in the Italian Official Gazette n. 248 (1999). Analyses were performed by atomic absorption using an AA-6800 Series Spectrophotometer Shimadzu set at a wavelength of 232 nm.

1.2.5 Statistical analyses

The presence of significant differences (p value < 0.05) among treatments at each sampling time was assessed using a oneway analysis of variance (ANOVA). To further elucidate which differences were significant among pairs of treatments, the post hoc multiple pairwise-comparison Tukey HSD test (Tukey Honest Significant Differences significance 95%) was performed. Analyses were carried out with

RStudio software (R version 3.5.2). Assumptions of ANOVA were tested beforehand through Shapiro-Wilk test for normality and Levene Test for homogeneity of variance.

1.3 Results and discussion

Table 1.1 reports the concentration of the soluble Ni fraction, measured in the lime at each sampling time; data are the mean of six replicates with the respective standard deviation.

Significant differences between treatments and the control at all sampling times were found, indicating a noticeable effect of the addition of the two sequestrants on the reduction of the soluble Ni fraction, except at day 1 from treatment, when the two treatments at 10% concentration did not differ from the control. However, starting from day 6, significant differences were found even for these two treatments.

The effect of the addition of the two sequestrants was strong at all the concentrations tested. This is clearly shown in Table 1.3 where the percentage reduction in the soluble and bioavailable Ni during the experiment is reported. The reduction is calculated at each sampling time with respect to the initial concentration of Ni in the control sample. The data show a reduction in the soluble Ni varying from 13.4% for B10 at day 6 to 69.0% for B5 at day 85, with the highest values obtained at the end of the experiment for all the treatments, indicating that adsorption kinetics occurred over 85 days, and consisted in a progressive decrease in the soluble Ni fraction in the lime, reaching values near or below (B5) the legal limit fixed by Italian law (10 μg L^{-1}). At the end of the experiment, B5 was found to be the most active in the retention of water-soluble Ni with a reduction of 69.0%, and B10 the least active (reduction of 49.4%).

The trend in the decrease of the soluble Ni fraction is described in Fig. 1.1 A kinetic behaviour is evident for all treatments with a progressive decrease in the soluble fraction until day 85, when the treatments at 5% of sequestrant, for both B5 and Z5,

seemed to be the most efficient, with a final concentration below or near the legal limit, being 9.0 μg L^{-1} for B5 and 10.6 μg L^{-1} for Z5.

The kinetic trend in Fig. 1.1 shows that, even at day 1, the samples at 5% of sequestrant reached values near to the legal limit, and this condition remained stable during the entire period of the experiment, indicating a permanent adsorption of Ni on the internal sites of the two minerals which do not allow the metal to return to the soluble form.

In a review, Shi et al. (2009) investigated the potential of natural zeolite in the soil bioremediation of heavy metals and stated that increasing the pH value is a crucial factor, determining an increase in cation exchange capacity (CEC) on the zeolite surface due to the deprotonation of the surface unsaturated bond and a subsequent increase in the negative charge. The high pH value of the material in the present experiment (8.9) could have caused an immediately strong increase in the CEC of the added zeolite which in turn could have influenced a quick adsorption of soluble Ni on the mineral surfaces.

On the other hand, some authors have demonstrated the ability of zeolite and calcium hydroxide to reduce the soluble form of some heavy metals, like Cd, Pb and Zn, transforming them into unavailable forms (Chen et al., 2000; Castaldi et al., 2005).

In a recent research (Malinowska, 2017), the effect of liming and sewage sludge on the speciation in soil of Pb, Cr and Ni was observed in a laboratory experiment with addition of different concentration of sewage sludge and lime to a soil with a pH of 4.3. The content of Ni in different extracted fraction varied and was correlated with sludge doses. The highest amount of the metal was in the residual fraction while the amount in the mobile fractions was even small and further reduced by liming.

The carbonation lime used in the present experiment, rich in $CaCO_3$ and with high content of OM and high pH value, with the addition of the sequestrants, may have become a very active substrate for transforming the soluble form into unavailable forms of Ni.

A further observation regarding the data in Table 1.1 and the trends in Fig. 1.1 is that the two sequestrants seemed to behave in a similar way, showing values of soluble Ni at day 85 which are almost the same for the same concentration of the two minerals (B2.5-Z2.5, B5.0-Z5.0, B10.0-Z10.0). This may mean that, independently from the trend followed during the experiment, the final results for both zeolite and bentonite at the same concentration can be considered equivalent, and therefore the efficiency of the reduction in soluble Ni in the carbonation lime depends only on the concentration, and 5% seems to be the most efficient dose.

In a recent work (Akpomie and Dawodu, 2015), the influence of the adsorbent dose on the adsorption of Ni and Mn in a binary system consisting in a solution treated with different concentrations of a low-cost bentonite was evaluated; an increase in bentonite concentration determined greater adsorption, but the adsorption capacity (mg g^{-1}) of the adsorbent decreased when the dose increased due to the reduction in total surface area available, showing a "ceiling effect". This could explain why, in the present experiment, a dose of 5% for both zeolite and bentonite was the most efficient in removing the highest percentage of soluble and bioavailable Ni.

The main aim of this experiment was to try to reduce the concentration of the soluble Ni fraction to below the Italian legal limit after treatment with the sequestrants at an appropriate concentration. Considering the results obtained, it is possible to affirm that this goal could be reached with the addition of the bentonite used in the present experiment at a dose of 5%; the same dose of zeolite provided a similar result, with a concentration of soluble Ni very near to the legal limit (10.6 μg L^{-1}).

Table 1.2 reports the concentration of the bioavailable Ni fraction, measured in the lime at each sampling time; data are the mean of six replicates with the respective standard deviation.

The bioavailable fraction includes both soluble Ni and the Ni slightly adsorbed on the mineral sequestrants and on the OM of the carbonation lime. This fraction followed a particular trend, observable in Fig. 1.2, with an initial increase with respect to the control at day 1, followed by a strong decrease starting from day 6

which continued until the end of the experiment; on the contrary, the control sample remained at the same concentration for the entire period of the experiment.

The percentage of reduction in bioavailable Ni (Table 1.3) shows a negative number at day 1 for all treatments, indicating an initial increase in the bioavailable Ni in lime. This is likely due to an exchange of adsorption sites soon after the addition of sequestrants: the adsorption sites of sequestrants caused the initial desorption of Ni from OM-Ni complexes at day 1. The following adsorption of bioavailable Ni by zeolite and bentonite decreased its concentration throughout the experiment, achieving a considerable reduction of bioavailable Ni for all treatments at the end, varying from 60.4% for Z2.5 to 73.1% for B5 (Table 1.3).

The effect of several variables on the adsorption of heavy metals on absorbing minerals and clays in water, soil and solid substrates has been underlined in many reports, indicating pH, initial concentration and nature of the adsorbing substrate, initial concentration of heavy metals and temperature as the determining factors.

The great reduction in bioavailable Ni at day 6, which persisted until day 85, could be due to the above-mentioned factors; Nachtegaal and Sparks (2003) demonstrated that Ni can produce precipitates at the kaolinite-water interface and the addition of humic acids at higher concentrations increased adsorption; Ni uptake continued without reaching an apparent equilibrium. The carbonation lime used in the present experiment contains 7% of organic matter, which can positively influence the formation of precipitates with Ni on the surfaces of the two sequestrants, causing a long kinetic trend without reaching an apparent equilibrium after 85 days.

The effect of pH, temperature and adsorbent nature and concentration on the adsorption of Ni and Mn were studied in a binary system consisting in a solution treated with different concentrations of a low-cost bentonite (Akpomie and Dawodu, 2015). The effect of pH was clear and evident, showing an adsorption of the two metals from 20 to 60–70%, at a pH ranging from 2 to 8; a large number of active sites of bentonite are positively charged at low pH, becoming negative at higher pH

values, and it is likely that in the present experiment, the high value of pH in the lime favoured the significant reduction in the available and soluble Ni.

Querol et al. (2006) evaluated the effect of the addition of two different concentrations of an alkaline zeolite, synthesized using fly ashes, to a 25-cm topsoil (pH 3.5–3.9; illite 20%, montmorillonite 5%) polluted by some heavy metals including Ni. The addition of zeolite at 15 ton ha^{-1} and 25 ton ha^{-1} strongly reduced (80–90%) the mobility of the heavy metals, including Ni, in soil column experiments; the increase in soil pH (up to 7.5–8.0) caused by zeolite addition seemed to be the factor responsible for metal immobilization, favouring metal adsorption on illite surfaces and the precipitation of metal hydroxides. The sequestrant doses of the present experiment were in the same order as the above, and the effect of both the alkaline pH and the organic matter adsorption surfaces could have determined the drastic reduction in the soluble and bioavailable Ni, by adsorption on the negative double electric layer of the humic and fulvic acids and the formation of Ni hydroxides.

Diale et al. (2011) conducted a laboratory batch equilibrium experiment to study the adsorption behaviour of natural zeolite coming from the Western Cape (South Africa) with respect to some heavy metals, including Ni. The sorption capacity was high for all metals and fitted with linearly to the pseudo-second order kinetics model indicating that the process occurred in at least two steps. The kinetics behaviour of bioavailable Ni in Fig. 1.2 confirms the trend with a high increase of sosrption during the first 6 days, followed by a slight increase during the further 79 days.

1.4 Conclusions

The zeolitic and bentonitic materials used in the present experiment appeared to be an effective amendment to attenuate lime contamination by soluble and bioavailable Ni. The alkaline pH and the presence of organic matter could be the main factors affecting the good performance of sequestrant addition. The doses of 5% bentonite or zeolite proved to be the most effective, indicating a “ceiling effect” of the sequestrant, i.e., an increasing dose could reduce the adsorption capacity and be less

effective. The 85-day experiment seems to be sufficient to reach an adsorption equilibrium for the water-soluble form of Ni, while for the bioavailable form a longer period appears to be necessary. The carbonation lime used in the present experiment contains 7% of organic matter, which can positively influence the formation of precipitates with Ni on the surfaces of the two sequestrants, causing a long kinetic trend of the available Ni fraction without reaching an apparent equilibrium after 85 days.

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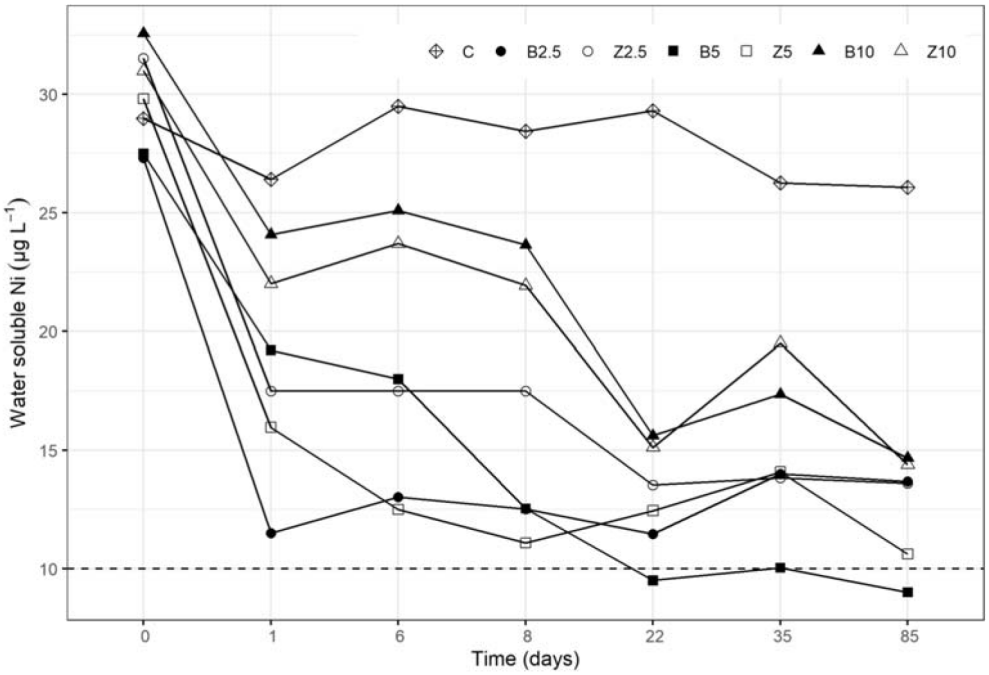

Figure 1.1 Trend of the soluble Ni fraction during the 85-day experiment for the different treatments (dashed line indicate the Italian legal limit for soluble Ni) (C control sample; B2.5, B5, B10 bentonite 2.5, 5 and 10%; Z2.5, Z5.0, Z10 zeolite 2.5, 5 and 10%)

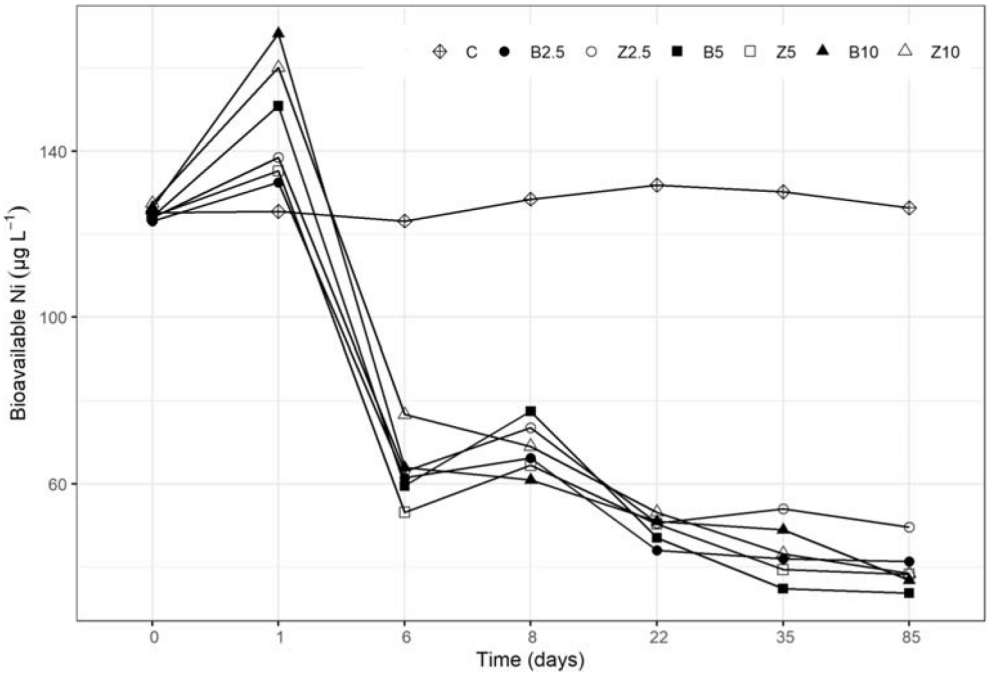

Figure 1.2 Trend of the bioavailable Ni fraction during the 85-day experiment for the different treatments (C control sample; B2.5, B5, B10 bentonite 2.5, 5 and 10%; Z2.5, Z5.0, Z10 zeolite 2.5, 5 and 10%).

Table 1.1 Soluble Ni concentration (μg L^{-1}) measured in the collected lime samples at each sampling time (n =6± s.d.).

Subsamples	Time (days)						
	0	**1**	**6**	**8**	**22**	**35**	**85**
C	29.0 ± 2.9^{a}	26.4 ± 2.2^{a}	29.5 ± 1.2^{a}	28.5 ± 1.8^{a}	29.3 ± 2.2^{a}	26.3 ± 3.4^{a}	26.1 ± 2.3^{a}
B2.5	27.3 ± 2.9^{a}	11.5 ± 1.1^{e}	13.0 ± 1.4^{d}	12.5 ± 2.1^{d}	11.5 ± 1.5^{bc}	14.0 ± 2.5^{cd}	13.7 ± 1.3^{b}
Z2.5	31.5 ± 1.6^{a}	17.5 ± 1.7^{cd}	17.5 ± 1.9^{c}	17.5 ± 3.0^{c}	13.5 ± 1.9^{bc}	13.8 ± 2.7^{cd}	13.6 ± 1.1^{b}
B5	27.5 ± 4.8^{a}	19.2 ± 2.8^{bcd}	18.0 ± 2.5^{c}	12.5 ± 0.9^{d}	9.5 ± 1.4^{c}	10.0 ± 1.1^{d}	9.0 ± 0.6^{c}
Z5	29.8 ± 3.9^{a}	16.0 ± 3.6^{de}	12.5 ± 1.5^{d}	11.1 ± 1.4^{d}	12.5 ± 1.2^{bc}	14.1 ± 0.8^{cd}	10.6 ± 1.0^{c}
B10	32.6 ± 1.3^{a}	24.1 ± 1.7^{ab}	25.1 ± 1.9^{b}	23.7 ± 3.4^{b}	15.6 ± 4.4^{b}	17.4 ± 2.0^{cd}	14.7 ± 1.5^{b}
Z10	31.0 ± 2.4^{a}	22.0 ± 5.5^{abc}	23.7 ± 2.7^{b}	21.9 ± 3.0^{b}	15.1 ± 3.5^{b}	19.5 ± 2.7^{b}	14.4 ± 1.4^{b}

Lower case letters indicate the significant differences in each sampling time (values in the same vertical column followed by the same letter are not significantly different according to the Tukey test at $p < 0.05$)

C control sample; *B2.5*, *B5*, *B10* bentonite 2.5, 5 and 10%; *Z2.5*, *Z5.0*, *Z10* zeolite 2.5, 5 and 10%

Table 1.2 Bioavailable Ni concentration (μg L^{-1}) measured in the collected lime samples at each sampling time (n =6± s.d.).

Subsamples	Time (days)						
	0	1	6	8	22	35	85
C	125.2 ± 4.3^{a}	125.5 ± 3.7^{f}	123.2 ± 4.1^{a}	128.4 ± 3.1^{a}	131.8 ± 3.3^{a}	130.3 ± 3.7^{a}	126.4 ± 3.8^{a}
B2.5	123.1 ± 1.9^{a}	132.5 ± 2.7^{e}	61.5 ± 5.0^{cd}	66.2 ± 2.2^{de}	44.0 ± 1.4^{c}	42.0 ± 5.7^{cd}	41.3 ± 3.0^{c}
Z2.5	124.0 ± 1.4^{a}	138.5 ± 3.0^{d}	63.0 ± 3.3^{c}	73.5 ± 2.6^{bc}	50.5 ± 2.4^{bc}	54.0 ± 4.4^{b}	49.6 ± 2.7^{b}
B5	124.2 ± 1.7^{a}	150.9 ± 2.8^{c}	59.5 ± 1.7^{cd}	77.5 ± 4.3^{b}	47.0 ± 2.6^{bc}	34.8 ± 3.0^{d}	33.7 ± 6.1^{d}
Z5	124.3 ± 3.4^{a}	135.3 ± 2.7^{de}	53.2 ± 4.0^{d}	64.5 ± 2.4^{de}	50.4 ± 2.1^{bc}	39.3 ± 7.1^{cd}	38.2 ± 5.1^{cd}
B10	126.4 ± 1.0^{a}	168.3 ± 1.2^{a}	63.9 ± 5.4^{c}	60.9 ± 4.1^{e}	51.0 ± 9.80^{bc}	48.9 ± 6.7^{bc}	36.8 ± 2.9^{cd}
Z10	127.4 ± 2.1^{a}	161.3 ± 3.6^{b}	76.7 ± 7.8^{b}	69.0 ± 2.1^{cd}	53.2 ± 3.6^{b}	43.2 ± 7.2^{cd}	38.4 ± 4.2^{cd}

Lower case letters indicate the significant differences in each sampling time (values in the same vertical column followed by the same letter are not significantly different according to the Tukey test at $p < 0.05$)

C control sample; *B2.5*, *B5*, *B10* bentonite 2.5, 5 and 10%; *Z2.5*, *Z5.0*, *Z10* zeolite 2.5, 5 and 10%

Table 1.3 Percent reduction of water-soluble and bioavailable Ni in carbonation lime treated with sequestrants at three concentrations with respect to the initial concentration of the control at time zero.

	Subsamples	Time (days)					
		1	6	8	22	35	85
Water-soluble Ni (%)	B2.5	60.3	55.1	56.8	60.4	51.7	52.8
	Z2.5	39.6	39.6	39.6	53.3	52.3	53.1
	B5	33.7	37.9	56.8	67.2	65.4	69.0
	Z5	44.9	56.9	61.8	57.0	51.4	63.4
	B10	16.9	13.4	18.4	46.1	40.1	49.4
	Z10	24.0	18.2	24.3	47.8	32.7	50.3
Bioavailable Ni (%)	B2.5	−5.9	50.9	47.0	64.8	66.4	67.0
	Z2.5	−10.6	49.6	41.3	59.7	56.9	60.4
	B5	−20.6	52.4	38.1	62.5	72.2	73.1
	Z5	−8.1	57.5	48.5	59.8	68.6	69.5
	B10	−34.5	48.9	51.3	59.3	60.9	70.6
	Z10	−28.9	38.7	44.9	57.5	65.5	69.3

B2.5, B5, B10 bentonite 2.5, 5 and 10%; Z2.5, Z5.0, Z10 zeolite 2.5, 5 and 0%

CHAPTER 2: Phytoremediation potential of crop plants in countering Nickel contamination in carbonation lime coming from the sugar industry

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Abstract

The phytoremediation potential of four crop species cultivated on carbonation lime coming from the sugar industry with water-soluble nickel (Ni) exceeding the Italian legal limit of 10 μg L^{-1} was assessed. Two autumn–winter species (spinach and canola) were tested with and without the addition of bentonite in a greenhouse experiment in order to overcome prolonged unfavourable weather conditions. Two spring-summer species (sunflower and sorghum) were grown in outdoor boxes. Plant species were selected among crops of interest for phytoremediation and their rotation throughout the year enable to maintain a permanent vegetation cover. Nickel concentration in different plant tissues and the concentrations of soluble and bioavailable Ni in lime were measured. In the greenhouse study, soluble Ni decreased below the legal limit in all the tests, and the combined effect of bentonite and plants reduced Ni in lime mainly in the bioavailable fraction. Spinach and sunflower emerged to be more suitable for phytoextraction than canola and sorghum, because of the higher concentration of the metal in the epigeal portions. The results from the outdoor experiment highlighted that sorghum has a good phytostabilisation potential since its ability to accumulate Ni mainly at the root level and to attract a significant amount of bioavailable Ni in the rhizosphere. This study arose from a real scenario of environmental contamination and investigated the potential of different approaches on the bioremediation of a specific industrial waste product.

Keywords: phytoremediation; phytoextraction; phytostabilisation; nickel; carbonation lime; canola; spinach; sunflower; sorghum; rhizosphere.

2.1 Introduction

Environmental pollution by Potentially Toxic Elements (PTE) has become a serious problem since the 1940s, when rapid industrialisation and urbanisation started to cause contamination (Merian, 1984). PTE cannot be biodegraded and therefore accumulate in the ecosystem and enter the food chain (Ali et al., 2019; Bian et al., 2014; Lv et al., 2019).

Several physical and chemical methods have been used for removing potentially toxic elements from contaminated matrices (Brusseau et al., 2019); nowadays, these traditional remediation technologies are being replaced by biological ones, usually known as bioremediation technologies (Rodríguez-Eugenio et al., 2018; Sharma et al., 2018). Compared with conventional clean-up methods, bioremediation has lower capital costs, and it is considered to be aesthetically pleasing (Vidali, 2009). Among bioremediation technologies, phytoremediation exploits plants to remove pollutants from the environment, or to render them harmless by degradation or immobilisation (Nathanail and Bardos, 2004; Raskin et al., 1997; Salt et al., 1995; Wan et al., 2015). Since the nineties (Baker et al., 1994, 1991), a growing scientific and commercial interest has developed regarding this promising technology as a more eco-friendly, non-intrusive and cost-effective remediation method for the reclamation of polluted sites (Clayton, 2007; Koptsik, 2014). However, some limitations should be taken into account when applying phytoremediation: long-term efficiency, the depth of action limited to the root zone, the need for proper handling and disposal of the biomass produced and a careful evaluation of the specific site scenario (i.e., plant requirements-pollution level) (Chen et al., 2015; Eevers et al., 2017; Farraji et al., 2016; Syam et al., 2016). Depending on the nature of the pollutants and their concentration, the choice for the optimal species is a function of the encroachment capability given by tolerance mechanisms (exclusion and detoxification) that allow for maintaining a low concentration of the potentially toxic element in the cytoplasm and the most sensitive compartments of plant cells (Ernst et al., 1992). Likewise, information regarding the substrate and climate of the place where the remediation

is requested, and the site accessibility for the cultivation machines (Kokyo et al., 2014) should be compared with the needs of the crop to be used (i.e., temperatures required in the development phases, water and nutritional needs) (Sarma, 2011). In more detail, substrate aeration, pH, cation exchange capacity (CEC) and the presence of organic matter, clay components and the elements available affects the bioavailability of potentially toxic element and thus their accumulation in plant tissues (Bhardwaj et al., 2014; Bolan et al., 2003; Liu et al., 2010; Paz-Alberto and Sigua, 2013).

According to the different mechanisms involved, there are several phytoremediation approaches for the PTE decontamination of solid matrices: phytoextraction (pollutants taken up into the plant biomass), phytostabilisation (limiting the mobility and bioavailability in soil by plant roots), and phytovolatilisation (conversion of pollutants to a volatile form and their subsequent release into the atmosphere) (Ali et al., 2013; Alkorta et al., 2004). Depending on the phytoremediation method, the specific features of many plant species can prove useful (Radziemska, 2018). Usually, hyperaccumulator plants that can accumulate high amounts of PTE in their above-ground tissues without adverse effects (Baker et al., 1994; Brooks, 1998; Chaney et al., 1997; Raskin and Ensley, 2000) are chosen for phytoextraction, but such plants typically produce small amounts of biomass and have no economic value (Pulford et al., 2002). The species used for phytostabilisation must grow rapidly with a well-developed root system but do not need particular habitat requirements (Mendez and Maier, 2008). The translocation factor (TF) given by the ratio between the concentration of PTE in the aerial part of the plants and the concentration of the same in the roots is one of the key factors in the evaluation of plants for phytoextraction processes (Mattina et al., 2003). A TF higher than 1 signifies tolerance of the plant towards the PTE and its ability to move it in the epigean portions, and, according to the Baker theory, this plant is considered a good accumulator usable for phytoextraction (Baker and Brooks, 1989; Bedabati Chanu and Gupta, 2016).

Combining two or three different remediation approaches can lead to more effective results (Armishaw et al., 1992). Aided phytostabilisation (Radziemska et al., 2018), or chemophytostabilisation (Grobelak and Napora, 2015), is an interesting example of a co-remediation technique in which amendments such as clay minerals, organic compost, phosphates, lime and zero-valent iron are applied in the polluted substrate to help the plants to deactivate or immobilise PTE (Derakhshan Nejad et al., 2018; Houben et al., 2012; Stefaniuk et al., 2015; Xu et al., 2017).

Among PTE, mercury (Hg), copper (Cu), silver (Ag), cadmium (Cd), zinc (Zn), lead (Pb), chrome (Cr), cobalt (Cb) and nickel (Ni) are considered the most toxic (Bryan, 1980), but all elements in high concentrations are potentially harmful (Adiloğlu et al., 2016). The sources of PTE in the environment may be natural (geogenic origin) or anthropogenic from activities such as mining, burning of fossil fuel, fertiliser application, disposal of household debris, municipal and industrial wastes (Alloway, (Alloway, 2013; Bradl, 2005). As described by Spellman (Spellman, 2017), certain recyclable wastes like biosolids from sewage treatment plants, food processing companies, and other sources are commonly reused as fertilisers. However, some careful testing is needed in order to control problems concerning the eventual release of water-soluble, mobile pollutants.

This research came from a collaboration with Sadam Spa, an Italian company in the sugar industry, involved in an environmental recovery project of a disused quarry. According to the Italian Legislative Decree n.186 (Italian Official Gazzetta, 2006), the recovery project was aimed at remodelling the morphology of the quarry using lime from sugar refining as a filling material. The subsequent assessment of chemical pollutants in lime, conducted by Marche Regional Environmental Protection Agency (ARPAM), highlighted a contaminated area exceeding the legal limit of soluble Ni concentration (10 μg L^{-1}).

The aim of this study was to assess the phytoremediation capacity of four plant species - spinach (*Spinacia oleracea*) and canola (*Brassica napus*) in a greenhouse study, and sorghum (*Sorghum vulgare*) and sunflower (*Helianthus annuus*) in an

outdoor experiment - in order to decrease the concentration of the soluble Ni below the legal limit set by the Italian Legislative Decree n.186 (Italian Official Gazzetta, 2006). This study was performed to support the application of phytoremediation techniques required for the remediation of a real contaminated site. Crop plants have most of the properties required by real-case applications of phytoremediation techniques, such as wide availability, cheapness, ease of growing and consistent biomass production (Ciura et al., 2005; Keller et al., 2003; Meers et al., 2005). Nevertheless, since the ability to tolerate and accumulate potentially toxic element is also a fundamental requirement, crop species already known in the scientific field for their metal storage capacity have been selected (Adesodun et al., 2010; Chaturvedi et al., 2019; Dell'Amico et al., 2008; Giordani et al., 2005; Gunduz et al., 2012; Revathi et al., 2011; Salt et al., 1995; Sarma, 2011; Solhi et al., 2005; Turan and Esringü, 2007; Van Ginneken et al., 2007; Verdiloo and Homaee, 2008; Wilson-Corral et al., 2011; Zhuang et al., 2009). Moreover, as these species have either an autumn–winter cycle (canola and spinach) or a spring-summer cycle (sorghum and sunflower), their rotation throughout the year permit to maintain a permanent vegetation cover in the contaminated site.

Aided phytoremediation was tested using bentonite in the greenhouse experiment since a previous laboratory study on the same substrate showed that this clay mineral is a good sequestrant of Ni when it is added at doses of 5% (w/w) (Casucci et al., 2020).

The results highlight that spinach and sunflower should be preferred for phytoextraction over canola and sorghum, given their ability to store Ni in the epigeal portions. In the greenhouse study, soluble Ni decreased below the legal limit in all the tests, and the combined effect of bentonite and plants reduced Ni in lime primarily in the bioavailable fraction. İn the outdoor experiment, the metal was accumulated mainly in sorghum roots, and a significant amount of bioavailable Ni was found in the rhizosphere, indicating sorghum as a good option for the phytostabilisation technique.

2.2 Results

2.2.1 Greenhouse Experiment

2.2.1.1 Nickel in Lime

Figure 2.1 reports the concentration of soluble and bioavailable nickel in lime before and after the plant harvest. Before sowing, Ni concentration was similar among samples since the inter quartile range values were 3.0 (median 15.0) and 21.7 (median 331.0) for soluble and bioavailable fractions, respectively. A considerable decrease in soluble Ni concentration was observed following the harvest compared to the initial concentration (Figure 2.1a), and differences between trials were not significant, even when bentonite was added to contaminated lime. Bioavailable Ni was significantly reduced in all treatments (Figure 2.1b). Regardless of the species, the addition of bentonite further decreased bioavailable Ni in lime. The median concentration of Ni after treatments was slightly lower using canola compared to spinach, both in soluble and bioavailable fractions (Figure 2.1).

2.2.1.2 Nickel in Plants

Figure 2.2 reports nickel concentrations in plants. Ni concentration in roots (Figure 2.2a) was significantly higher in canola than in spinach, and, for both species, the addition of bentonite led to a higher accumulation of the metal that differed significantly from the control. Concerning the epigeal portion (Figure 2.2b), Ni accumulation was significantly higher in spinach than in canola and considerably higher than the control, while, in canola, no significant differences were found between treated and control plants. The addition of bentonite does not seem to significantly affect metal accumulation in either hypogeal or epigeal portions.

The translocation factor of the plants grown in the greenhouse experiment, calculated as the ratio between the concentration of Ni in epigeal and hypogeal tissues, is shown in Table 2.1. Between the two tested species, spinach is the one that presents in all trials a translocation factor higher than one.

The biomass production of plants grown on contaminated lime was similar to that measured for control plants within the same species (Table S2.1); in particular, the average weights of the hypogeal and epigeal portions of canola plants were 4.48 ± 0.75 and 14.97 ± 1.16 g, respectively, while, for spinach plants, these weights were 0.57 ± 0.21 and 5.54 ± 0.74 g, respectively. Considering the metal concentrations in the various plant tissues and the biomass produced, the mean values of treated and control plants. The addition of bentonite does not seem to significantly affect metal phytoextracted Ni per plant were 30.9 ± 6.4 (B), 34.3 ± 4.6 (B+), 28.3 ± 3.5 (BC), 12.5 ± 0.8 (S), 10.7 ± 2.1 (S+) and 5.9 ± 0.2 µg (SC).

2.2.2 Outdoor Experiment

2.2.2.1 Nickel in Lime

Figure 2.3 reports the concentration of soluble and bioavailable nickel in the four boxes used for the outdoor experiment, before and after the plant harvest.

The initial concentration of soluble Ni was similar for the four boxes, with medians varying from 7.9 µg L^{-1} (Box 3) to 9.1 µg L^{-1} (Box 1) and no significant differences were highlighted using the Kruskal–Wallis test ($p > 0.05$) (Figure S2.1a). On the contrary, regarding the initial concentration of bioavailable Ni, box 1 had the highest median (570.7 µg kg^{-1}), while box 4 had the lowest (309.9 µg kg^{-1}), and significant pairwise differences (Dunn's test) were found (Figure S2.1b). Boxes 2 and 3 were the only ones with similar initial soluble and bioavailable Ni concentrations (Figure S2.1).

A similar Ni distribution pattern between pre- and post-harvest concentrations was observed in all boxes (Figure 2.3). After the harvest, Ni concentrations were significantly higher at 0–30 cm of depth respect to 30–60 cm of depth for both fractions, and this behaviour was particularly evident for the bioavailable Ni (Figure 2.3b).

After the harvest, a comparison of the boxes showed no significant differences in soluble and bioavailable Ni for box 2 cultivated with sorghum and box 3 cultivated with sunflower, at both sampling depths (Figure S2.2).

Table 2.2 shows the concentration of soluble and bioavailable Ni in the rhizosphere of the plants at the end of the experiment. No significant differences between plants and boxes were found for soluble Ni, while the bioavailable fraction was high in all cases the highest being for sorghum in box 4. A lower pH value (7.84 ± 0.04) was measured on the rhizosphere samples with respect to all the other samples.

2.2.2.2 Nickel in Plants

Figure 2.4 shows the Ni accumulation in different parts of the plants grown either in the four boxes or in the uncontaminated substrate. Ni was accumulated more in the roots than in the other parts by both plants species (Figure 2.4a). In roots and stems, Ni accumulation was higher in sorghum than in sunflower (Figure 2.4a, b). Sorghum roots accumulated a higher concentration of Ni than the control, even if the differences are not statistically significant, while this was not true for sunflower plants and for both species in stems. Similar Ni concentrations were found in leaves and infructescences, regardless of the plant species (Figure 2.4c, d). Significant differences with respect to the controls were found for sunflower in leaves and for sorghum in infructescences.

The translocation factor regarding the plants in the outdoor experiment is shown in Table 2.3. In contrast to sorghum, sunflowers grown in both boxes 1 and 3 showed a TF greater than one.

The biomass produced by both sorghum and sunflower plants grown in the contaminated boxes was similar to that measured in the respective controls (Table S2.2). The average dry plant weights (g) recorded for roots, stems, leaves and infructescences were 25.55 ± 3.31, 85.75 ± 4.86, 37.66 ± 8.44 and 35.86 ± 3.25 for sunflower, and 15.74 ± 2.16, 38.57 ± 2.34, 21.37 ± 2.38 and 14.16 ± 2.64g for sorghum, respectively. The mean values of phytoextracted Ni per plant were as

follows: 230.2 11.3 (1H), 234.9 ± 21.4 (3H), 223.2 ± 9.3 (H), 231.1 ± 27.0 (2S), 246.3 ± 10.2 (4S) and 165.3 ± 27.8 μg (S).

2.3 Discussion

2.3.1 Greenhouse Experiment

In the greenhouse experiment, the phytoremediation capacity of canola (*Brassica napus*) and spinach (*Spinacia oleracea*) was tested with and without the addition of 5% of bentonite.

Many authors have studied the phytoextraction capacity of *Brassica napus* (Çakmakci and Ucar, 2014; Marchiol et al., 2004; Purakayastha et al., 2008) and Spinacia oleracea (Alia et al., 2015; Pathak et al., 2013; Salaskar et al., 2011) and the effect of the addition of sequestrants such as bentonite in decreasing the available form of potentially toxic element (Akpomie and Dawodu, 2015; Casucci et al., 2020; Chaves et al., 2017; Ling et al., 2007).

In the present experiment, the Ni soluble fraction following the harvest was considerably reduced, and a concentration under the legal limit of 10 μg L^{-1} was achieved in all trials (Figure 2.1a).

Canola showed a higher concentration of the metal in the hypogeal portion than in the epigeal portion, and similar patterns of Ni accumulation have been found in previous studies (Adiloğlu et al., 2016; Brunetti et al., 2011). In agreement with other authors (Farraji et al., 2014; Panwar et al., 2001; Pathak et al., 2013; Salaskar et al., 2011), the opposite behaviour was observed for spinach, which accumulated Ni mostly in the aerial tissues.

As highlighted by the results, spinach seems suitable for the phytoextraction of Ni in lime, while the low translocation factor of canola makes it appropriate for phytostabilisation techniques. With a view to a practical application of phytoremediation techniques, the differences in biomass production between species should be taken into account, as the biomass of canola is considerably higher than that of spinach, as reported above.

The addition of bentonite may improve phytoremediation outcomes since it reduces both the soluble and bioavailable fractions of Ni due to the capacity of this mineral to adsorb the metal in its external and internal sites, as described by other authors (Akpomie and Dawodu, 2015; Nachtegaal and Sparks, 2003; Wasilkowski et al., 2017). As reported in previous work (Casucci et al., 2020), the competition of bentonite adsorption sites with Ni adsorbed on organic matter and the stabilisation of the metal in internal adsorption sites of bentonite could reduce the available concentration of Ni in lime (Figure 2.1b). The above findings support the use of a combination of sequestrants and plants in a practical case of bioremediation.

2.3.2 Outdoor Experiment

Before sowing, the concentrations of soluble and bioavailable Ni measured in the four boxes were different with respect to the values observed for lime collected in a previous field survey and used for the greenhouse experiment. This different starting condition was not surprising, considering that six months had elapsed between the two sampling campaigns, during which the site was subject to weather events, speciations and mobilizations of the metal may have occurred. Nevertheless, the low soluble Ni concentrations detected in May 2018 provided a real case for a typical phytoremediation trial, since high concentrations and lack of time represent the main limitations for the application of this technique (Marchiol et al., 2007).

After harvesting, there was a clear tendency of the soluble and available forms of the metal to move towards the first 0–30 cm in depth both in the sunflower and the sorghum boxes (Figure 2.3); this depth represents the layer where the root system is developed, indicating that the metal distribution in the boxes could be due to the attraction exerted by the roots and by their radical exudates. The function of roots in attracting and sequestering potentially toxic element has been highlighted by many authors who all agree that the presence of metals induces a

higher production of root exudates, such as low molecular weight organic acids (Y. T. Chen et al., 2017; Luo et al., 2014; Montiel-Rozas et al., 2016), which play an important role in the immobilisation of the available form of metals.

Boxes 2 and 3, which had similar initial Ni concentrations, showed no significant differences in the levels of soluble and bioavailable Ni measured after the harvest of sorghum and sunflower, respectively (Figure S2.1). Despite these results, in sorghum stems and root tissues, the Ni concentration was significantly higher than in sunflower plants (Figure 2.4a, b), but at the end of the trials, this difference in extraction capacity was compensated by greater production of biomass by the sunflower compared to the sorghum.

The noticeable capability of sorghum to accumulate metals in roots was also reported in other studies (Angelova et al., 2011; Naeini et al., 2018; Soudek et al., 2014) in which the authors suggest to use sorghum spp. in phytostabilisation rather than in phytoextraction experiments.

A further demonstration of the ability of root apparatus to attract the available form of Ni is reported in Table 2.2, where the concentration of bioavailable Ni in the rhizosphere of the plants always proved to be higher than in the 0–30 cm layer after harvest.

Although sunflower plants accumulated a considerable amount of Ni in the root portion, their translocation factor showed values higher than 1; therefore, these plants could be used for phytoextraction purposes on this lime. Finally, the presence of a high content of organic matter (OM) in the substrate (Table 2.4) might have helped in metal sequestration, because OM promotes the formation of OM-Ni complexes and exudate production by the roots of the plants (Montiel-Rozas et al., 2016).

2.4 Materials and Methods

The experimental design was performed with two different case studies: a greenhouse experiment with autumn–winter plants and an outdoor experiment with summer–spring plants.

In both tests, the substrate used to grow the plants was carbonation lime, a residue of the industrial process of sugar juice purification, made up of 90% calcium carbonate (Casucci et al., 2020). This substrate was sampled where the lime was used as the filling material for a disused quarry in a recovery project (municipality of Monte Roberto, Marche, Italy). The samples were collected from four square plots (2 m side) equally spaced along a transect covering the whole length of the contaminated area mapped by ARPAM. The main properties of the substrate were determined from samples collected during two different field surveys, one in autumn 2017, and one in spring 2018. A clearly alkaline reaction and a high organic matter content were found (Table 2.4). The substrate pH was determined by a glass electrode in distilled water (pH H_2O) suspensions at a 1:2.5 soil to liquid ratio. Total Ni concentration was determined by the acid digestion method; the dry and 2-mm sieved substrates were digested in HNO_3, 65% v/v, (28 L kg^{-1}) overnight, 2 mL of H_2O_2, 30% v/v, was then added, and, after 6 h, the blend was heated to 90 °C for 90 min and filtered. Analyses of the eluates were performed using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Agilent 5100 VDV). The organic matter was determined according to Walkley and Black (Walkley and Black, 1934).

An uncontaminated carbonation lime (soluble Ni concentration under 2 µg L^{-1}), collected from the same site but outside of the contaminated area, was used to cultivate plants as a reference control.

2.4.1 Greenhouse Experiment

Because of prolonged unfavourable weather conditions that occurred at the contaminated site during autumn–winter months, the experiment was conducted in the experimental greenhouse at Marche Polytechnic University, Ancona, Italy, with a daytime temperature of 24 ± 3 °C and a night temperature of 20 ± 3 °C. Plants of *Spinacia oleracea L.* (spinach) and *Brassica napus L.* (canola) were grown separately in plastic jars having a volume of 3 litres. The plants were grown under natural photoperiod and with a relative humidity of 60% ± 2%.

Lime samples were collected from each of the four square plots delineated in the contaminated area in November 2017, and they were mixed carefully, air-dried and 2-mm sieved. After processing, the concentrations of soluble and bioavailable Ni in nine sub-samples were measured to ensure that both Ni fractions were homogeneously distributed in the substrate.

Six trials were set up in eighteen jars (three replicates per trial) as follows: *Brassica* without bentonite (B), *Brassica* with bentonite added at 5% (*w*/*w*) (B+), *Brassica* control (BC), *Spinacia* without bentonite (S), *Spinacia* with bentonite added at 5% (*w*/*w*) (S+) and *Spinacia* control (SC). Commercial activated bentonite with a mesh size of 100–500 μm, composed of 85%–90% montmorillonite and 1%–4% inert silica was used. The main physico-chemical characteristics of the bentonite used were: a surface area of 88.0 $m^2\ g^{-1}$; a CEC of 150 meq/100 g; and a pH of 9.5 (sol. 5%).

To prevent emergence failures, ten seeds were initially sown in each jar. Subsequently, only four seedlings per pot for canola and six for spinach were allowed to grow.

Manual irrigation was provided to avoid water stress from the time of sowing up to the harvesting at the end of the vegetative cycle of the crops (approximately 110 days).

After the harvest, all the plants from each jar were sampled, gently washed with deionised water, separated into the epigeal and hypogeal portion, oven-dried (70 °C until the plant tissue was completely dry), weighed, milled and analysed to assess the concentration of Ni in the tissues.

At the end of the experiment, three samples of substrate were collected from each jar, obtaining nine replicates per trial. After re-drying, the concentration of soluble and bioavailable fraction of Ni was measured.

2.4.2 Outdoor Experiment

Lime samples were collected from each of the four square plots delineated in the contaminated area in May 2018. Four boxes were placed directly in the study area, exposed to weather conditions, and each was filled with samples coming from a different square plot, in order to represent the spatial heterogeneity of the Ni distribution in the site.

Nine sub-samples were collected from each 1 m^3 box, along independent zig-zag paths at different depths to achieve randomness and to measure the soluble and bioavailable Ni before sowing.

Boxes 1 and 3 were sown with *Helianthus annuus L.* (sunflower), while boxes 2 and 4 were cultivated with *Sorghum vulgare L*. Only nine sunflower plants and fifteen plants of sorghum were allowed to grow per box. Single manual irrigation was provided immediately after sowing.

At the end of the biological cycle (around 90 days), a third of the plants grown in each box were harvested whole and then carefully separated into roots, stems, leaves and infructescences, oven-dried (70 °C until the plant tissue were completely dry), weighed, milled and analysed to determine the Ni contents.

After plant eradication, both the substrate intimately adhering to the root surface (rhizosphere) and the lime from each box at two different depths (0–30 and 30–60 cm) were collected in order to assess the distribution of soluble and bioavailable Ni fractions following cultivation.

2.4.3 Ni Extraction and Analysis

2.4.3.1 Substrate

The soluble Ni extraction was carried out with distilled water (1 g/10 mL) according to the cession-test reported in the Italian Legislative Decree n. 186 (UNI 10802, 2013). The duly sealed samples were subjected to agitation and the liquid/solid separation was then reached by centrifugation for 5 min and filtering. The bioavailable fraction was extracted with a solution of Diethylenetriaminepentaacetic acid (DTPA), $CaCl_2 \cdot 2H_2O$ (0.01 M) and triethanolamine (0.1 M) at pH 7.3 (1 g/2 mL), following the indications in the Italian O*ffi*cial Gazette n. 248 (Italian Official Gazette, 1999).

Analyses of the eluates were performed using a plasma emission spectrometer ICP-OES Agilent 5100 VDV. The operating analysis of the system was as follows: radio frequency power 1.4 kW, plasma gas flow 12 L min^{-1}, auxiliary gas flow 1.0 L/min, nebulizer flow 0.7 L min^{-1}, observation view axial, replicate readings 3, selected Ni wavelengths 231.604 nm.

2.4.3.2 Plants

The dry milled plant tissues were digested in HNO_3 (28 L kg^{-1}) overnight, 2 mL of H_2O_2 was then added (30%), and, after 6 h, the blend was heated to 90 °C for 90 min and filtered (Huang et al., 2004).

Analyses of the eluates were performed using a plasma emission spectrometer ICP-OES Agilent 5100 VDV. The operating analysis of the system was as follows: radio frequency power 1.4 kW, plasma gas flow 12 L min^{-1}, auxiliary gas flow 1.0 L/min, nebulizer flow 0.7 L min^{-1}, observation view axial, replicate readings 3, selected Ni wavelengths 231.604 nm.

2.4.4 Statistical Analysis

Nonparametric tests were used because of non-normal distributions. The significance of the differences in the content of Ni in different morphological parts of the plants and in lime samples was calculated using the Kruskal–Wallis test and Dunn's post-hoc test. Either Benjamini–Hochberg or Hochberg *p*-value adjustments were used depending on the number of tests performed, as suggested in literature (Bender and Lange, 2001; S. Y. Chen et al., 2017). Statistical analyses were performed in R software (R Development Core Team ersion 3.5.2) using 'dunn.test' package version 1.3.5.

2.5 Conclusions

Spinach and sunflower emerged to be suitable for the phytoextraction of Ni in lime, while the low translocation factor of canola and sorghum makes them more appropriate for phytostabilisation techniques. The addition of bentonite may improve phytoremediation outcomes since this mineral reduces both the soluble and, to a greater extent, the bioavailable fractions of Ni due to its capacity to adsorb the metal. The above findings support the use of a combination of sequestrants and plants in a practical case of bioremediation.

The results from the outdoor experiment highlight that sorghum has a good phytostabilisation potential due to its ability to accumulate Ni mainly at the root level and to attract a significant amount of bioavailable Ni in the rhizosphere. Moreover, the response of plants to the presence of potentially toxic elements may vary according to the elements and the different concentrations of their available forms. For both experiments, limited differences in biomass production between plants grown on contaminated lime and those grown on control were observed, this is probably due to the Ni concentrations in the tissues, which were in all cases lower than the phytotoxicity threshold of 50 mg Kg^{-1} indicated for moderately tolerant species to this metal (Sengar et al., 2008).

The focus of future research could take in account several unresolved issues concerning the use of bioremediation for sites contaminated with PTE, studying the possibility of using plants that are even more efficient in the hyper-accumulation of metals and are able to develop a wide and deep root apparatus. Studies on the possibility to solve practical problems of contamination with high metal concentrations should be performed, combining them with the use of different sequestering minerals that are able to immobilise the metal in the plant root layers. Other interesting proposals to enhance the phytoremediation performance could be the use of plant growth-promoting bacteria (PGPB) and melatonin, which biostimulate the plant growth and improve the plant tolerance against PTE (Arnao and Hernández-Ruiz, 2019; Asif et al., 2019; Seleiman et al., 2020).

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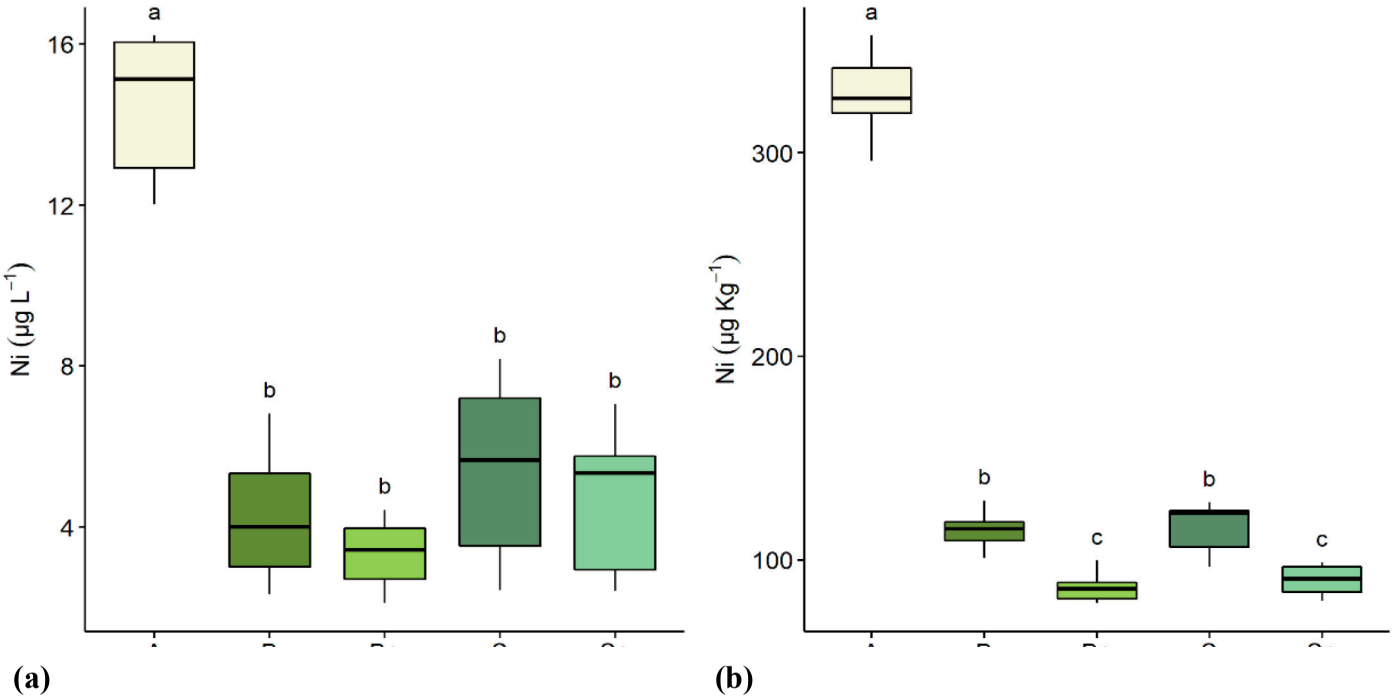


Figure 2.1 Concentration of nickel in lime before and after plant harvest. (a) Soluble Ni (µg L^{-1}) in limesamples; (b) bioavailable Ni (µg Kg^{-1}) in lime samples. Letters on the x-axis refer to concentration before sowing (A) and after the harvest of: Brassica napus without bentonite (B) and with bentonite (B+); Spinacia oleracea without bentonite (S) and with bentonite (S+). Lower case letters refer to Dunn'sKruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05).

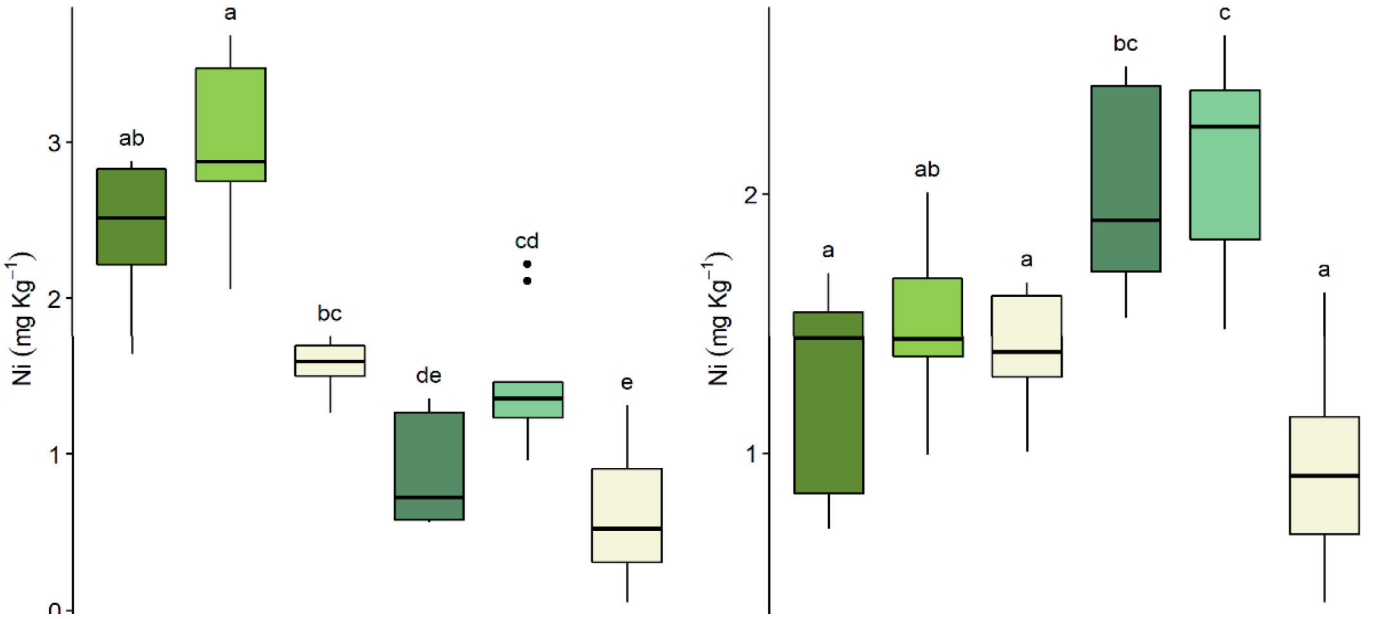


Figure 2.2 Nickel concentration (mg kg^{-1} dry weight) in plants. (a) Hypogeal portion; (b) epigeal portion. Letters on the x-axis refer to concentration in: (B) Brassica napus plants grown on contaminated lime without bentonite (B), with bentonite (B+) and Brassica napus plants grown on uncontaminated lime as a control (BC); Spinacia oleracea plants grown on contaminated lime without bentonite (S) and with bentonite (S+) and Spinacia oleracea plants grown on uncontaminated lime as a control (SC). Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05).

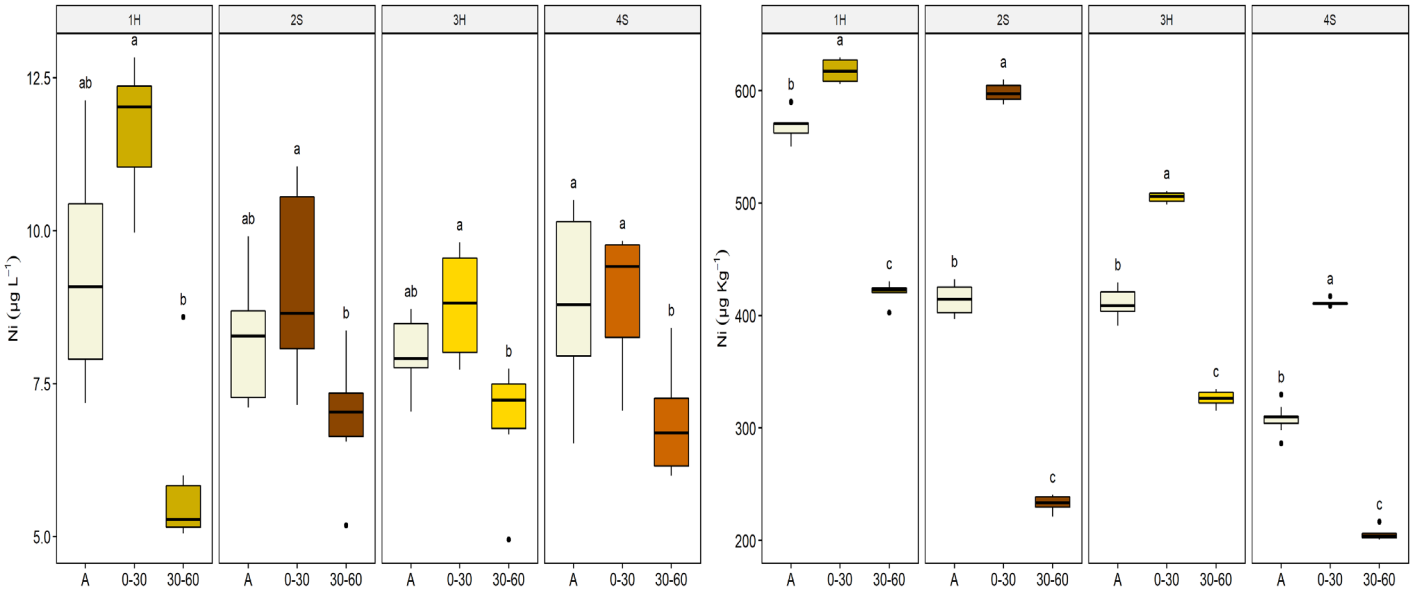


Figure 2.3 Concentration of nickel in lime in boxes 1–4 before and after plant harvest. (**a**) Soluble Ni (µg L^{-1}); (**b**) bioavailable Ni (µg Kg^{-1}). Codes on the x-axis refer to initial concentration (A), and concentration after the plant harvest grouped by sampling depth (0–30 cm, 30–60 cm). Boxes 1 and 3 cultivated with *Helianthus annuus* (1H; 3H); boxes 2 and 4 cultivated with *Sorghum vulgare* (2S; 4S). Lower case letters refer to Multiple comparisons in each box (Dunn's Kruskal–Wallis, Hochberg *p*-value adjustment, α-level = 0.05).

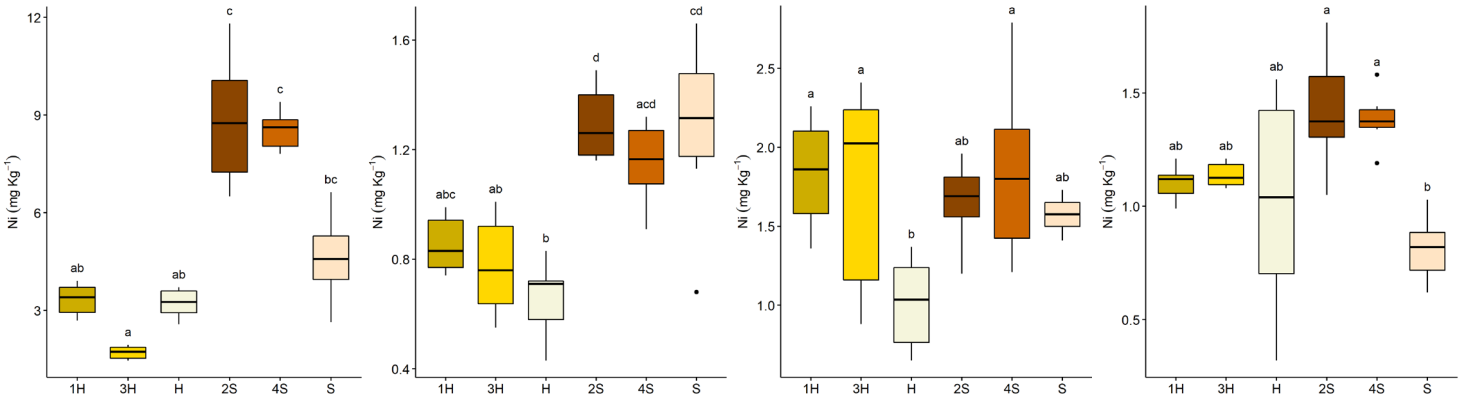


Figure 2.4 Ni concentration (mg kg^{-1} dry weight) in the different portions of the plants. (**a**) Roots, (**b**) stems, (**c**) leaves, (**d**) infructescences. Codes on the x-axis refer to concentration in: (1H) *Helianthus* in box 1; (3H) *Helianthus* in box 3; (H) *Helianthus* control in uncontaminated lime; (2S) *Sorghum* in box 2; (4S) *Sorghum* in box 4; (S) *Sorghum* control in uncontaminated lime. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05).

Table 2.1 Translocation factor for Ni in plants growing in the greenhouse experiment (reported values are the medians of nine replicates and interquartile range).

Trial	TF
B	0.57 (0.14)
B+	0.51 (0.12)
BC	0.83 (0.07)
S	2.70 (0.99)
S+	1.67 (0.70)
SC	1.52 (0.54)

Table 2.2 Soluble ($\mu g\ L^{-1}$) and bioavailable ($\mu g\ Kg^{-1}$) Ni concentration in the rhizosphere (reported values are the median of nine replicates and interquartile range). Different letters indicate significant differences (ns: not significant differences), Dunn's Kruskal–Wallis multiple comparisons test (Benjamini–Hochberg p-value adjustment, α-level = 0.05).

Box	Plant	Soluble Ni	Bioavailable Ni
1	*Helianthus annuus*	10.2 (0.7) ns	681.2 (74.4) ab
2	*Sorghum vulgare*	8.0 (0.6) ns	637.9 (10.3) a
3	*Helianthus annuus*	10.3 (1.7) ns	692.2 (78.9) ab
4	*Sorghum vulgare*	8.4 (1.5) ns	763.1 (15.7) b

Table 2.3 Translocation factor for Ni in plants growing in the outdoor experiment (reported values are the medians of six replicates and interquartile range).

Box	TF
1H	1.07 (0.26)
3H	2.04 (0.30)
H	0.84 (0.23)
2S	0.52 (0.13)
4S	0.51 (0.05)
S	0.83 (0.31)

Table 2.4 Basic properties of the substrate used for the experiments.

Parameter	Values
pH	8.36 ± 0.10 [1]
Organic matter (%)	7.2
Total Ni (mg Kg^{-1})	2.62 ± 0.63 [1]

[1] Reported pH and Ni concentrations are means of sixteen replicates ± standard deviation.

Supplementary materials for Chapter 2

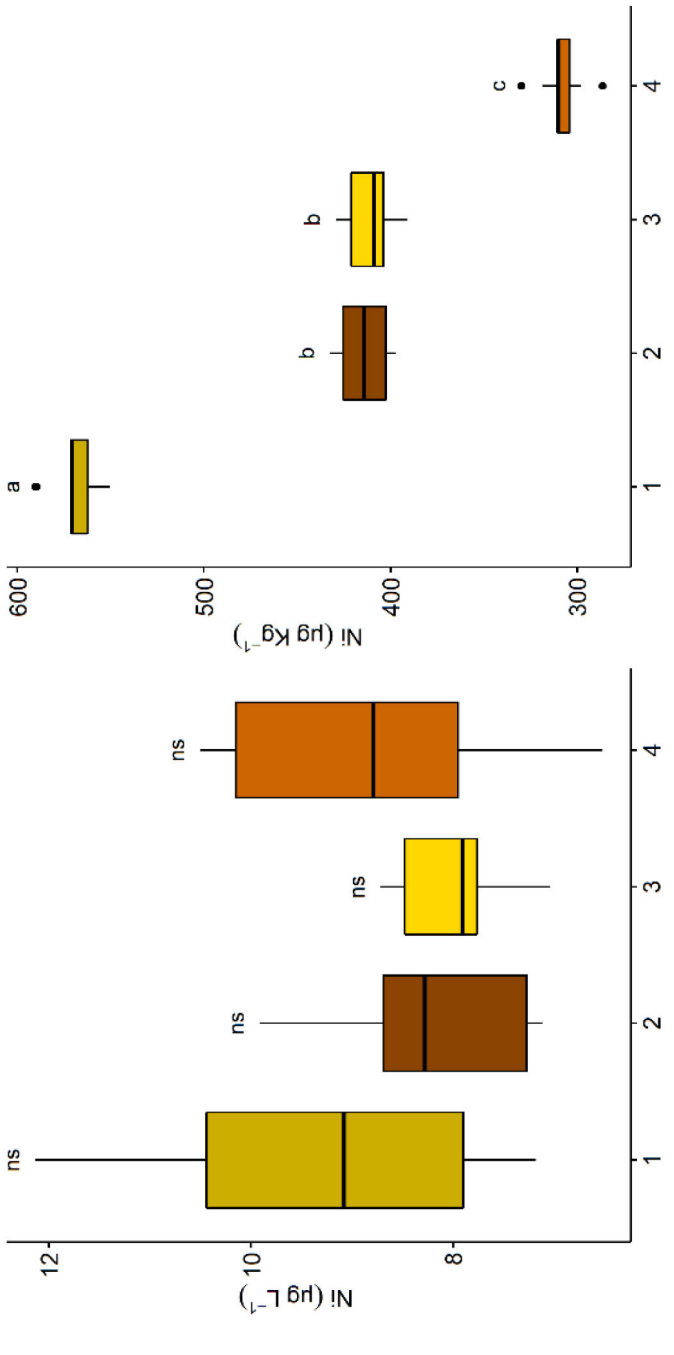
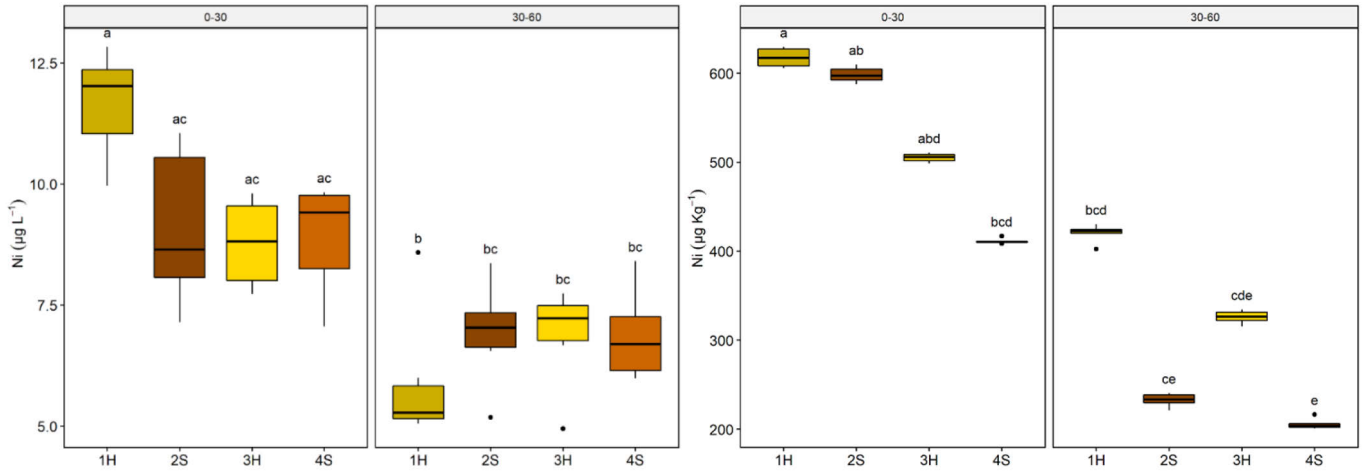


Figure S2.1 (**a**) Pre-sowing concentration of soluble Ni (µg L^{-1}) in lime within boxes 1-4; (**b**) Pre-sowing concentration of biovaiable Ni (µg Kg^{-1}) in lime within boxes 1-4. Number in the x-axis refer to the four boxes. Dunn's Kruskal-Wallis multiple comparisons (Benjamini-Hochberg p-value adjustment, α-level=0.05).



(a) **(b)**

Figure S2.2 (a) Post-harvest concentration of soluble Ni (µg L^{-1}) in lime within boxes 1-4. (b) Post-harvest concentration of bioavailable Ni (µg Kg^{-1}) in lime within boxes 1-4. Box 1 and 3 after *Helianthus annuus* (1H, 3H); box 2 and 4 after *Sorghum vulgare* (2S, 4S). Ni concentration was grouped by depth of sampling (0-30 cm, 30-60 cm). Dunn's Kruskal-Wallis multiple comparisons (Benjamini-Hochberg p-value adjustment, α-level=0.05) was performed using data from both sampling depths.

Table S2.1 Dry weight (g) of biomass per plant in the greenhouse experiment (reported values are mean ± standard deviation).

Trial	Hypogeal	Epigeal
B	4.52±0.64	15.16±0.87
B+	4.40±0.81	14.71±1.22
BC	4.50±0.80	14.58±1.07
S	0.79±0.11	5.91±0.39
S+	0.37±0.10	4.73±0.51
SC	0.56±0.14	5.97±0.49

Table S2.2 Dry weight (g) of biomass per plant in the outdoor experiment (reported values are mean ± standard deviation).

Box	Root	Stem	Leaf	Infructescence
1H	22.37±2.38	81.16±3.89	27.30±2.77	33.58±2.51
3H	27.21±2.19	87.66±3.27	44.50±3.72	37.00±2.27
H	27.09±1.89	88.43±2.32	41.17±1.67	37.00±3.53
2S	14.59±1.19	38.86±1.87	20.76±1.50	13.98±2.13
4S	16.98±2.00	39.54±1.46	21.60±2.20	14.19±2.43
S	15.66±2.20	37.32±2.66	21.75±2.87	14.13±3.00

CHAPTER 3: Sub-Lethal effects of pesticides on the DNA of soil organisms as early ecotoxicological biomarkers

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Abstract

This review describes the researches performed in the last years to assess the impact of pesticide sub-lethal doses on soil microorganisms and non-target organisms in agricultural soil ecosystems. The overview was developed through the careful description and a critical analysis of three methodologies based on culture-independent approaches involving DNA extraction and sequencing (denaturing gradient gel electrophoresis, DGGE; next-generation sequencing, NGS) to characterize the microbial population and DNA damage assessment (comet assay) to determine the effect on soil invertebrates. The examination of the related published articles showed a continuous improvement of the possibility to detect the detrimental effect of the pesticides on soil microorganisms and non-target organisms at sub-lethal doses, i.e., doses which have no lethal effect on the organisms. Considering the overall critical discussion on microbial soil monitoring in the function of pesticide treatments, we can confirm the usefulness of PCR-DGGE as a screening technique to assess the genetic diversity of microbial communities. Nowadays, DGGE remains a preliminary technique to highlight rapidly the main differences in microbial community composition, which is able to give further information if coupled with culture-dependent microbiological approaches, while thorough assessments must be gained by high-throughput techniques such as NGS. The comet assay represents an elective technique for assessing genotoxicity in environmental biomonitoring, being mature after decades of implementation and widely used worldwide for its direct, simple, and affordable implementation. Nonetheless, in order to promote the consistency and reliability of results, regulatory bodies should provide guidelines on the optimal use of this tool, strongly indicating

the most reliable indicators of DNA damage. This review may help the European Regulation Authority in deriving new ecotoxicological endpoints to be included in the Registration Procedure of new pesticides.

Keywords: pesticides, DNA, soil microorganisms, earthworms, ecotoxicological biomarkers, denaturing gradient gel electrophoresis, comet assay, next-generation sequencing.

3.1 Introduction

Anthropogenic activities are associated with the massive disposal of contaminants that, in many cases, could be potentially genotoxic and carcinogenic. This poses a major challenge for regulatory authorities and environmental managers to protect the quality and the services provided by natural resources. Simple detection/quantification of xenobiotics in abiotic and biotic compartments has limited relevance, particularly when they occur as complex mixtures, unless their biological or ecological effects are properly evaluated. Biological systems indeed provide important information, which is not readily available from direct chemical analyses of the environmental samples and, thus, are increasingly used as diagnostic tools for integrated environmental management (Sarkar et al., 2006).

The effects of agrochemicals on the soil ecosystem components represented by microorganisms and macroinvertebrates deserve particular attention. The relationship between plant root apparatus and soil microbial communities is strictly linked with soil fertility at a chemical, biochemical, and microbiological level, and several studies on the interactions between root exudates and soil microbial biomass growth, structure, and activity were carried out in detail during the last 20 years (Mergel et al., 1998; Nannipieri et al., 2008; Steinauer et al., 2016; de Vries et al., 2019). Rhizobacteria and fungi can stimulate plant growth through the production of phytostimulators (auxins, gibberellins), increase nutrient uptake, and induce tolerance to plants against abiotic stress or by suppressing biotic stressors like plant diseases or pests (Vryzas, 2016): any change and/or disturbance to the

delicate regulation of these interactions can thus result in an impairment of soil fertility.

The use of pesticides against plants pests, weeds, and pathogens was proven to affect the chemical and biological fertility of soils in several cases, including a number of potential adverse effects versus soil microorganisms and/or non-target organisms. During the past, classical studies were performed evidencing the effects of pesticides on the whole soil microbial biomass and/or on the soil biochemical and enzymatic activities (Perucci et al., 2000; Vischetti et al., 2000, 2002; Puglisi et al., 2005,

2012; Nannipieri et al., 2012; Sofo et al., 2012; Suciu et al., 2019), and contrasting results were derived, highlighting the detrimental effect in the major part of the studies but, in some cases, also stimulating effects due to pesticides acting as a carbon source (Puglisi, 2012).

Pesticide ecotoxicology is a relatively new branch of toxicology, being the study of the adverse effect of pesticides versus non-target organisms, including different species living in the ecosystems. Ecotoxicological indexes relative to different commercial pesticides are included in the Dossier for the Registration Procedure. To authorize a pesticide, risk assessment in the EU, the United States of America (USA), and most other Countries requires that the predicted environmental exposure concentration is below a concentration considered safe for non- target organisms (Boivin and Poulsen, 2017). In the EU, in a first tier of risk assessment, this safe concentration is established by the European Food Safety Authority (EFSA) in cooperation

with national agencies of the EU member states through a combination of standard toxicity tests, i.e., tests performed with single chemicals and single species under laboratory conditions without additional stressors, and safety factors that account for uncertainties in the extrapolation to real ecosystems [European Parliament, 2009; European Union [EU] (2011)]. The current methodologies to assess the toxicity of pesticides for terrestrial biota by the European Chemical Agency (ECHA) take

into account the mortality, reproduction activity, and morphological and behavioural changes of earthworms, collembola and predatory mites (OECD, 2004, 2016a,b). At the same time, for microorganisms, ECHA evaluates nitrogen and carbon transformation activity (OECD, 2000a,b). In the last years, unexpected negative effects of pesticide residues on non-target organisms were detected in different ecosystems and under different pesticide exposure levels (Desneux et al., 2007; Beketov et al., 2013; Brühl et al., 2013; Wood and Goulson, 2017), and the assumptions of the Regulation Authorities have been often contradicted. In this context, the novel introduction of proper methodologies to ascertain the damage effect of pesticides on the soil living organisms is a decisive step to ascertain any undesired effect at a high tier of safety (Schäfer et al., 2019). Recently, EFSA has issued a scientific opinion on the risk assessment of plant protection products on soil organisms (Ockleford et al., 2017), where microorganisms are also included. While acknowledging the limitations of assays based on single microbial species, EFSA recommends the use of molecular methodologies addressing whole communities but highlights the difficulties of interpreting complex outcomes for regulatory purposes.

Additional endpoints were introduced in the last years as improved estimators of the sub-lethal effects of pesticides, and in this respect, genotoxicity represents a critical marker of xenobiotic exposure having important repercussions not only on the viability of non-target species but also on the ecological fitness of the organisms. Indeed, a clear link between genotoxicity and lowered reproductive performance and embryotoxicity has been highlighted. Therefore, new methodologies have been introduced to study the effects of sub-lethal doses, i.e., doses that have no lethal effects on the organisms, versus soil biota: comet assay, denaturing gradient gel electrophoresis (DGGE), and more recently next-generation sequencing (NGS). The comet assay addresses DNA damage on invertebrates, while DGGE and NGS use DNA and RNA biomarker genes as molecular tools to evaluate changes in the microbial community.

The review describes the researches performed in the last years on the effect of pesticides on soil microorganisms and non-target organisms, using new approaches involving DNA extraction and sequencing (DGGE; NGS) to characterize the microbial population and DNA damage assessment (comet assay) to determine the effect on soil invertebrates.

The aim was to detect the impact of pesticide sub-lethal doses in agricultural soil ecosystems and help European Registration Authorities to derive ecotoxicological parameters useful for the pesticide Registration Procedure at a high tier of risk assessment, taking into account the difficulties of interpreting complex outcomes for regulatory purposes.

3.2 Critical analysis of the scientific literature

3.2.1 Denaturing Gradient Gel Electrophoresis

Over the last decade, PCR-based molecular fingerprinting techniques, giving a direct comparative overview of the composition and diversity of soil microbiota, replaced most other post-PCR analytical methods (van Elsas and Boersma, 2011).

Specific examples of direct molecular monitoring approaches in soil microbiology are represented by DGGE, temperature gradient gel electrophoresis (TGGE) (Heuer and Smalla, 1997; Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism (T-RFLP) (Kuske et al., 2002), single-strand conformational polymorphism (SSCP) (Schwieger and Tebbe, 1998), ribosomal internal spacer analysis (RISA) (Ranjard et al., 2000), and length heterogeneity-PCR (LH-PCR) (Ritchie et al., 2000). Among culture-independent fingerprinting methods, the DGGE, firstly theoretically described in the early 80s by Fischer and Lerman (1979), has been widely applied (Hoshino and Matsumoto, 2007; Coppola et al., 2011; Umar et al., 2017). To obtain optimal results in DGGE analysis, the first practical aspect concerns the high-quality extraction of total DNA from samples and then the control of amplification via PCR selecting universal primers targeting part of the 16S or 18S rRNA sequences for bacteria and fungi, respectively. Subsequently, the separation

is based on the differences in the mobility of partially melted DNA (with the same length but different sequences) in polyacrylamide gels containing a linear urea and formamide gradient of DNA, properly set (Fakruddin and Mannan, 2013). The identification of the microbial species can be obtained by sequencing the band excised by the polyacrylamide gel (Salles et al., 2004).

Denaturing gradient gel electrophoresis was originally developed to detect small mutation changes in DNA sequences, but quickly the technique has been applied for transversal microbial analyses, thanks to evident advantages such as rapidness, high reproducibility, and low costs (Fakruddin and Mannan, 2013). Currently, PCR-DGGE is a routine and widely used method in soil ecosystem studies. Nevertheless, the technique has some limitations, such as PCR biases, variable DNA extraction efficiency, difficult sample handing (Fakruddin and Mannan, 2013), and limited sensitivity (Nocker et al., 2007).

Other problems in DGGE analysis could be the formation of heteroduplex molecules, which can alter the distribution of the bands in the acrylamide gel (Ercolini, 2004). As discussed below, DGGE is also suffering from a very limited resolution power as compared to NGS, with the latter replacing it in several studies. Nevertheless, DGGE is still commonly employed for soil microbial ecology studies in order to monitor population structure and dynamics during that time, especially because of its ability to provide a representative profile of the dominant microbial diversity from specific environments such as soils (Ercolini, 2004; Munaut et al., 2011). In particular, DGGE is a useful tool for the rapid evaluation of microbial profiles in complex ecosystems (Kurtzman et al., 2011; Ng et al., 2014; Di Lenola et al., 2017; Zhang C. et al., 2017), allowing a rapid and efficient separation of the DNA fragments (Umar et al., 2017).

In common with the NGS methods discussed below, different primer sets can be used in PCR-DGGE, thus addressing microbial communities at the phylogenetic level (e.g., 16S primers for bacteria and archaea, ITS for fungi) or at the functional level, depending on functional selected genes. PCR-DGGE protocols optimized for

use with soil DNA constitute a consolidated and reliable method that can be used in addition to culture-dependent methods to obtain a complete picture of microbial diversity and dynamics. It can also be as a possible alternative to the most modern NGS techniques since it does not require complex bioinformatics for the analyses of results (Shokralla et al., 2012; da Silva Barros et al., 2019; Hemmat-Jou et al., 2019; Ruanpanun and Nimnoi, 2020).

The analysis of the relevant articles published in the last years on the effects of pesticides upon the soil microorganisms, determined through the DGGE technique, is summarized in Table 3.1.

Recently, Di Lenola et al. (2017) compared different molecular methods to assess soil microbial diversity considered as a complex habitat. Delgado-Baquerizo et al. (2016) already established that anthropogenic pressures such as chemical pollution or agriculture practices strongly affect microbial composition and identified that PCR-DGGE as a rapid method to highlight this shift is promising.

Pesticides are defined as bioactive and toxic substances that can influence, directly or indirectly, soil productivity, and agroecosystem quality (Joergensen and Emmerling, 2006). Their principal function is the growth inhibition of target organisms even if their effects can be extended to non-target microorganisms, causing an alteration in the microbial community structure (Tortella et al., 2013a,b). The evaluation of pesticide impact on non-target organisms in soils, including microorganisms, could be a useful tool to monitor soil health and evaluating their quality. Some years ago, Imfeld and Vuilleumier (2012) already made an extensive investigation on industrially produced pesticides in agriculture in relation to the contamination of soil ecosystems. The authors considered a large variety of cultivation-dependent and -independent methods potentially applied to measure and interpret the effects of pesticide exposure, expanding the study in the specific context of the responses of the soil microflora to pesticide exposure. They established a systematic combination of microbial culture-based and molecular culture-independent methods that will comprehensive contribution to this field.

Gao et al. (2012) compared DGGE and T-RFLP techniques for monitoring the effect of a natural pesticide, Pseudomonas fluorescens 2P24, on the soil fungal community in the cucumber rhizosphere. In this case, DGGE results indicated that the fungal community was shocked at the beginning of the trial, but it improved gradually after 1 month, with the decline of P. fluorescens 2P24. This study revealed the transient effect of biological control agents against microbial populations.

Analogously, Chen et al. (2013) studied the effect of Bacillus subtilis B579, another natural biological pesticide, on rhizobacteria community structure, using cultivation-based analysis coupled with DGGE to profile the changes of bacterial community structure. As expected, also in this case, the analysis revealed a minimal and transitory effect on microbial populations.

Gupta et al. (2013) applied a DGGE approach to compare the effects of two chemical pesticides (chlorpyrifos and endosulfan) and a biopesticide (azadirachtin) on the bacterial community in rhizospheric soil. In this case, results showed that high doses of azadirachtin simulated the effects of chemical pesticides on bacterial communities showing a significant dose dependent effect.

The efficacy of natural biopesticides emphasizes the need to widely investigate their effect in agriculture before accepting them as safe alternatives to chemical pesticides.

Tortella et al. (2013a; 2013b), studied the effects of chemical pesticides (atrazine and carbendazim) on the microbial community of a biopurification system, revealing a transient reduction of the microbial population after each treatment. Indeed, the DGGE analysis confirmed that microbial structure remained stable for a long time.

More recently, Diez et al. (2017) evaluated the role of the rhizosphere in pesticide dissipation and consequential microbial community changes in a biopurification system. Analogous to the results obtained by Coppola et al. (2011) and Marinozzi et al. (2013), they found that microbial communities were immediately modified 1 day after the fungicides treatments, but the community structure was recovered at the end of the experiment. The shift in the composition was thus only transitory and, at the end of the trial, the populations returned to their initial robustness.

Umar et al. (2017) recently applied DGGE fingerprinting of 16S rDNA to study the bacterial profile of Nigerian agricultural soil. This study confirmed the complex nature of this matrix, and the PCR-DGGE approach gave the advantage of not requiring previous knowledge on this habitat, providing an immediate picture of specific microbial population constituents in both a qualitative and a semi-quantitative way.

In contrast with these critically discussed results, other researchers demonstrate the irreversible effect of chemical pesticides on non-target microbial soil populations. Among them, Hoshino and Matsumoto (2007) revealed that the fumigants chloropicrin and 1,3-dichloropropene (1,3-D) have an adverse effect on fungal community structure. They showed that the chloropicrin treatment changed soil DGGE profiles significantly after 2 months of treatment. The DGGE profiles were not completely recovered even after a period of 12 months. On the other hand, the DGGE profiles of 1,3-D-treated soils showed a small change after 2 months of fumigation, but then after 6 months the treated DGGE profiles became indistinguishable from the controls.

The effects of chlorpyrifos on fungal abundance and community structure as revealed by DGGE were assayed by Huang et al. (2016). Significant inhibition of fungal abundance was induced by chlorpyrifos, a persistent insecticide that is widely used in agriculture despite being very potentially dangerous to non-target environmental organisms. Analogously, Chu et al. (2008) observed the inhibitory effect on the fungal community by chlorpyrifos.

Wang et al. (2019) studied the ecological toxicity of metalaxyl applications on soil microorganisms; the study of T-RFLP and DGGE revealed that metalaxyl inhibits the growth of fungi.

Lin et al. (2016) studied the impact on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol-contaminated soils, evaluating the community structure on day 42 by DGGE with the 16S rRNA amplification of the V3 region. This study addressed the roles and

mechanisms by which two earthworm species (epigeic *Eisenia fetida* and endogeic Amynthas robustus) affect the soil microbial community and enzyme activity during the bioremediation of PCP-contaminated soils. The results obtained confirmed that the soil microbial community was changed by the earthworm treatments.

Considering the overall critical discussion on microbial soil monitoring in the function of pesticide treatments, we can confirm the usefulness of PCR-DGGE as a screening technique to assess the genetic diversity of microbial communities. Nowadays, DGGE remains a preliminary technique to fast highlight the main differences in microbial community composition and is able to give further information if coupled with culture-dependent microbiological approaches, while thorough assessments can be gained by high-throughput techniques such as NGS.

3.2.2 Next-Generation Sequencing

Next-generation sequencing is an advanced sequencing method that allows the high-throughput nucleotide sequencing of millions of DNA strands in parallel, resulting in reads that are analyzed through bioinformatics, thanks to the availability of reference sequence libraries. Among the available NGS technologies, Illumina is nowadays mostly applied because of throughput, data quality, and costs per sequenced nucleotide. The use of NGS in recent years has increased in many scientific fields (e.g., Clinical Research, Food Science, Agricultural Science, Toxicology) as it became cheaper and therefore accessible to most labs all over the world (Beedanagari and John, 2014; Rockmann et al., 2019; Wiedmann and Carroll, 2019). The technology provides a thorough depiction of soil microbiology, paving the way for the discovery of previously unexplored compositions and diversities of both culturable and unculturable soil microorganisms (Simon and Daniel, 2011; Thompson et al., 2017). Current approaches used to study the diversity of soil microorganisms by NGS are based on phylogenetic and functional marker genes to address the two main questions: “who is there?” and “what are they doing?” This PCR-based approach often uses the amplification of targeted genomic regions of the

16S rRNA marker gene for bacterial diversity and 18S or ITS (internal transcribed spacer) for fungal diversity to answer the first question of "who is there?" (Vasileiadis et al., 2013). Despite the fact that the method itself is highly sensitive and is often the representative of microbial diversity, the presence of relic DNA (DNA of extracellular origin/from the cells that are no longer intact) is its main drawback (Carini et al., 2016). Moreover, the use of metagenomic analysis in combination with other methods (e.g., metatranscriptomics, metabolomics) to reveal the impact of active members on the results, thus answering the latter question of "what are they doing?" has been recently adopted by many (Jansson and Baker, 2016; Jansson and Hofmockel, 2018; Shakya et al., 2019). The method is thus a useful approach to understand the impacts of pesticides on soil microorganisms from rhizosphere to bulk soil, both in the short and long term. A review by Imfeld and Vuilleumier (2012) suggested that up until that year of publication, the use of NGS to investigate the impact of pesticides on the soil bacterial populations has not been reported. However, our current review shows that it is almost of common use nowadays, making precedent approaches almost obsolete, mainly because of its unprecedented sensitivity. Indeed, NGS, besides revealing the effectiveness of the applied pesticide on its target, may also reveal the unintended impact of pesticide through screening of non-target groups present in soil (e.g., the impact of insecticide on bacteria, herbicide, on fungi) (Kollah et al., 2019; Mallet et al., 2019). In addition, it also has the potential to reveal crucial information regarding the presence and activity of microbial degraders of pesticides and other contaminants in the soil for remediation purposes (Jeffries et al., 2018).

The studies evaluated in this review include works that focus on the impact of various types of pesticides (i.e., herbicides, fungicides, insecticides, fumigants, nematicide, and one novel bacteriocide) either separately on bacteria and fungi or on bacteria and fungi all together. Interestingly, as shown in Table 3.2, most of the works focused only on the impact of one to three pesticides on either bacterial or fungal communities. Present literature review shows that, while some of the earlier

works investigated biodiversity of soil microbial communities often focusing on commonly used regions of the DNA, some of the most recent works, on the other hand, focused on specific communities focusing on various roles and specific genes to correctly evaluate the impact of the treatments on the functionality of soil fertility's key players (Gallego et al., 2019; Tang et al., 2019; Fang et al., 2020). Only two studies, Armalyte et al. (2019) and Panelli et al. (2017), focused on the overall impact of the specialized farming systems and their relative pesticides' overall impact on either bacterial or fungal communities.

Particularly, in the work of Armalyte et al. (2019), the comparison of soil bacterial communities revealed no major differences among the main phyla of bacteria between the two farming systems with similar soil structure and pH, suggesting the important role played by these factors. However, slight differences and minor shifts of lower taxa were observed following the treatments and fertilization regimes, and these were interpreted as minor shifts in which the soil community is responding and adjusting itself to handle these treatments and restabilize its balance. The study of Panelli et al. (2017), on the other hand, revealed the alteration of fungal consortia starting from the first year of the study, due to various management conditions. It was found that Ascomycota always predominated, with the exception of conventional farming in which high abundance of Basidiomycete species was detected.

The field study of Storck et al. (2018) was the only one in which an insecticide, a herbicide, and a fungicide (namely chlorpyrifos, isoproturon, and tebuconazole) were evaluated altogether. The authors found that the a-diversity of soil bacteria varied more in microcosms as compared to fields. In the field conditions, significant differences in OTUs (operational taxonomic units) were observed for all pesticides by the end of a 70-day-long experiment. Pesticides, regardless of doses implemented, failed to have an impact on the a-diversity indices analyzed in this study. Similarly, also b-diversity was not affected by any of the pesticides nor the doses as the composition of soil bacterial community did not change significantly over time.

However, the insecticide chlorpyrifos caused a slight but significant temporary impact at the microcosm level, whereas the fungicide tebuconazole caused a slight but significant temporary impact at the field level only. The herbicide isoproturon, on the other hand, did not cause significant changes in the b-diversity of soil bacteria neither in microcosms nor in the field. These small and transient changes were mainly attributed to various degradation dynamics and transformation products of pesticides. However, as the b-diversity of soil bacteria eventually recovered, all of the pesticides were conclusively referred to as low risk pesticides.

3.2.2.1Fungicides

Wang et al. (2020) assessed the impact of the fungicide azoxystrobin on soil microbial communities, revealing a significant dose-dependent impact. Particularly, the OTUs richness and biodiversity, according to the Shannon index, decreased. Furthermore, *Streptomyces* and *Sphingomonas* were found to be dominating genera in most of the treated soils. This particular result was attributed to the use of the pesticide as a carbon source for growth because both *Streptomyces* and *Sphingomonas* hold a potential as bioremediation agents in the soils contaminated with pesticides. Furthermore, in a study carried out by Zhang C. et al. (2019), it was found that a commonly used fungicide pyraclostrobin also caused a significant impact in the abundances of genera with important roles in soil fertility and pollutant biodegradation. However, this time in contrast to azoxystrobin, the fungicide pyraclostrobin decreased the relative abundance of the *Sphingomonas* genus regardless of exposure levels. *Cupriavidus*, *Methylobacillus*, and *Methylophilus*, genera that include degraders of pollutants and methane oxidators with some roles in salt stress tolerance and denitrification, were also affected negatively.

3.2.2.2 Insecticides

Feng et al. (2019) studied the impact of lindane on root-associated microbiomes of rice and found that root-associated bacteria were more sensitive to the presence of

this insecticide as compared to fungi. The a-diversity analyzed through the Shannon index revealed that the higher insecticide levels had significantly decreased the bacterial and fungal diversity of both rhizosphere and bulk soils. It was also found that regardless of rice cultivars used, lindane significantly decreased the a-diversity also in the endosphere. Furthermore, their results showed that bacteria and fungi were both affected by lindane, respectively, in the phylum and class levels. Chloroflexi, Proteobacteria, and Actinobacteria were found to be dominant phyla, while contrasting responses to lindane from Acidobacteria and Firmicutes were obtained. In the case of fungal community, Basidiomycota and Ascomycota were found to be dominant phyla with several classes of both dominating most of the rhizosphere. However, lindane at various doses did not significantly change the composition of the fungal community.

The longest-term field study considered in this review was that of Jeffries et al. (2018), in which the legacy effect of the Chlorpyrifos insecticide was revealed. It was found that the degradation of pesticide was maintained even after its discontinued use 13 years ago, and the soil microbial community was able to adapt and degrade the pesticide but was still reflected in the latest samplings. Authors found that α-diversity was negatively related to degradation rates, which explained the slower degradation of chlorpyrifos in highly diverse soils. Microbial abundance, on the other hand, together with metabolic function abundances, was found to be positively related to degradation. Results related to a higher abundance of both microbes and pathway mechanisms employed by these microbes were fur her explained by taxonomic analysis and degradation assays.

Furthermore, Chen et al. (2017) investigated the impact of a novel neonicotinoid Paichongding (IPP) on soil bacterial community in a study in which pyrosequencing was used as one of the early studies of NGS in the present review. Degradation of IPP and its four variants were also investigated. A significant impact of IPP in this study on soil bacterial community, both positive and negative, was found to be closely connected to soil type. During the course of the experiment, α-diversity was

found to be gradually increasing in the control group while it was decreasing in the IPP group. This contrast eventually caused significant differences between control and IPP-treated soils. Chen et al. (2017) further investigated the impact of the inoculation of an IPP degrading strain into soil in their experiment and found that the inclusion of IPP degrading strain positively affected the microbial species diversity in contaminated soil. Furthermore, IPP also caused significant changes in the composition of the microbial communities after spraying. Differences were found both at the phylum and genus levels in both inoculated a d non-inoculated soils due to accumulation of IPP metabolites.

3.2.2.3 Herbicides

Farthing et al. (2020) found that commonly used weed suppression techniques of repeated glyphosate application, repeated glyphosate application + imidazolinone herbicide use, and repeated glyphosate application mechanical above-ground biomass removal had only little impact on bacteria and archaea. In particular, none of these weed-suppressing techniques had a significant impact over controls in terms of α-diversity indexes (species richness and Shannon diversity). The only significant difference was observed between communities of different locations, but this difference was found to be related only to the field differences rather than treatment impact. Although overall changes and compositions generally suggested the absence of overlap following treatments with herbicides, Farthing et al. (2020) found that changes in a number of OTUs were quite similar regardless of the experimental site. This phenomenon was still observed a year following the treatment. The authors also highlighted the necessity of different field site inclusions in the trials, as various responses eventually led to minimal changes that were attributed to re-existing local microbial communities of soils in these fields.

Tang et al. (2019) investigated the use of glyphosate in the cultivation of transgenic herbicide-resistant plants and found that there was no significant impact on the diversity and structures of rhizosphere bacterial communities in one growing season.

The differences in rhizosphere bacteria were only caused by plant growth stages. These results indicated that the growth stage was the most important factor influencing rhizosphere bacterial communities' diversity, in contrast to the hypothesized influence of cultivar and herbicide application.

Mallet et al. (2019) studied the impact of leptospermone, a natural b-triketone weed killer, on the fungal community in a microcosms study. It was found that leptospermone caused significant shifts in community structure and diversity in fungal communities of soils used in their experiment. Starting from the beginning of the experiments, significant differences were found in the α-diversity of the fungal community according to Chao1, Shannon, and Simpson between soil types and controls. During the experiments, the impact of herbicide treatment resulted in further significant differences both between soil types and controls. Authors found significantly decreased observed richness even after only 4 days together with changes in fungal community composition in one of the soils used. Even though the impact of the herbicide on α-diversity was different on different soils used in this experiment, fungal community β-diversity changed regardless of soil type. Mallet et al. (2019) also showed that even after the complete degradation of the used herbicide, the recovery of the fungal community was possible. However, recovery was only possible for the soil in which indigenous fungal community prior to experiments was already diverse and rich. The findings indicate how important it is to assess the impact of the various soil types not only from the physicochemical point of view but also from the indigenous community point of view for their resilience.

Fomesafen, a diphenyl ether broadleaf weed killer widely used in soybeans and other legumes, was tested for its impact on the microbial community composition of rhizosphere bacteria by Hu et al. (2019). Depending on the dose of its application, fomesafen had an impact on both α and β diversity. Particularly for α-diversity, according to Shannon index, negative impacts on rhizosphere bacteria were proportional to the application dose, regardless of sampling times. This impact was related to the direct toxicity of fomesafen and competitive changes adapted by some

taxa in community. The authors concluded that functional impacts were long-lasting on the soil microbial community despite the rapid degradation rate of this herbicide in the rhizosphere. Finally, Jiang et al. (2017) revealed that the long- term application of acetochlor (2-chloro-N-(ethoxymethyl)-N- (2-ethyl-6-methylphenyl) acetomide) had no significant impact on overall soil microbial community composition after 9 years of treatment. Particularly for α-diversity, observed species, the Chao1, Shannon, and Simpson indexes indicated various alternation between manual weeding and pesticides. This result eventually interpreted as a reduction on the biodiversity of soil bacteria caused by manual weeding was more significant than the one caused by herbicide application.

3.2.2.4 Fumigants

The impact of fumigants chloropicrin (CP), dazomet (DZ), dimethyl disulfide (DMDS), allyl isothiocyanate (AITC), and 1,3- D on the total bacterial community and on denitrifiers were studied by Fang et al. (2020). According to the diversity indexes of Shannon, Chao1, and ACE, the α-diversity of soil bacteria significantly decreased for the whole experiment following the fumigation with CP. However, 24 days after fumigation, diversity in the soils of the remaining fumigants were found to be significantly higher than the controls. Later, sampling revealed that biodiversity of the bacterial community in the soils of DZ, DMDS, AITC, and 1,3-D eventually were not significantly different from controls. The stimulation effect of this fumigant, therefore, remained only as a short-term and transient one. Fang et al. (2020) also found that although initially affected negatively by fumigation, denitrifiers eventually recovered. Relative abundances eventually increased significantly in relation with stimulated microbial denitrification caused by fumigation of soil.

Zhang D. L. et al. (2019) also investigated the impact of 1,3- D fumigation on soil bacterial community. The authors also paid particular attention to the abundance of ammonia oxidation genes of the bacterial community in an attempt to evaluate again

the unintended impact that a fumigant could have on soil fertility. It was found that soil fumigation with 1,3-D reduced the abundance of total bacteria and AOA-amoA and AOB-amoA genes. It was found that 1,3-D fumigation significantly reduced the total number of species according to the Ace and Chao diversity indexes. The bacterial community recovered from this reduction as the experiment continued.

Finally, in a multi-year field study by Zhang S. T. et al. (2017), the impact of chloropicrin fumigation on the bacterial community of soil was investigated for a 3-year-long continuous fumigation. Continuous fumigation for 3 consecutive years was found to be detrimental for the soil bacterial community in terms of microbial richness and diversity. In detail, α-diversity, as measured by the Chao and Shannon Index, was found to be significantly lower after 3 years of fumigation when compared to control and year-long fumigation. Although slightly to a lesser extent than 3 years, fumigation after 1 year was still detrimental to both microbiota and species richness. Differences were further investigated in the phyla and genus levels to evaluate the impact the duration of fumigation had on the bacterial community. Differences between a year-long and 3-year-long fumigation significantly increased only at the genus level. Authors further implemented the least discriminant effect size analysis to be able to identify the main phylotypes behind the differences obtained. It was found that Nitrospirae and Saccharibacteria were two most prominent phyla in, respectively, no-fumigation and 3-year- long fumigation samples. However, identifying a biomarker for a year-long fumigation at the phylum level was not possible. Therefore, the authors identified *Nocardiopsis* at the genus level as a biomarker for a year-long fumigation sample. Authors also found that increased fumigation years reduced the incidences of bacterial wilt disease.

3.2.2.5 Nematicides

The study of Gallego et al. (2019) was the only one dealing with the impact of a nematicide on soil bacteria: the molecule studied was oxamyl, and particular attention was also devoted to degradation kinetics. Soil bacterial community was not

significantly affected by oxamyl in terms of α- and β-diversity. However, the abundance of a specialized fraction of oxamyl degraders increased in agreement with mineralization of the nematicide. The nematicide use also increased the abundance of the specialized fraction of the soil bacterial community carrying the *cehA* gene, which means that oxamyl induced hanges in the abundance of oxamyl-degrading microorganisms.

3.2.3 Comet Assay

Detection of DNA damage and its extent is of paramount importance in different fields of basic and applied medical and health sciences, including environmental studies for verifying the toxicity of xenobiotics or other chemicals released in the environment. Among the different methods developed to quantify DNA damage, the single-cell electrophoresis or comet assay is prominent, being a simple, rapid, and sensitive method for measuring DNA breaks in small numbers of cells (Glei et al., 2016). When standardized and validated, the comet assay can provide valuable information for hazard identification and risk assessment of environmental exposure to environmental pollution in humans, in sentinel organisms, or *in vitro* toxicity studies (de Lapuente et al., 2015).

The method was originally Id in the 80s by Ostling and Johanson (1984) who used the technique to quantify DNA damage in mammalian cells exposed to gamma rays. The technique was further improved by Singh et al. (1988), who developed an alkaline version of the assay, the most commonly used, that enables the detection of alkali labile sites in addition to single- and double-strand breaks visualized using the original neutral version. The assay involves very limited processing steps, not requiring DNA isolation and purification. On the contrary, cells are directly embedded in a low melting agarose matrix at temperatures compatible with cellular viability and stratified on a microscopy slide. After gel solidification microgels on slides are subjected to a lysis step in a saline solution containing detergents that promote cellular membrane degradation and protein precipitation leaving on the

slide nucleoids constituted simply by DNA. Subsequently, slides are transferred into an electrophoretic chamber, and DNA is allowed to unwind in electrophoresis buffer, that in the version developed by Singh is at pH > 13, and electrophoresed in the same buffer. Finally, cells are neutralized and either stained with DNA intercalating dyes, mainly fluorescent such as DAPI, ethidium bromide or SYBR Gold and analyzed directly or dehydrated for further analysis. Analysis is conducted using a fluorescent microscope; the image of the comet produced by electrophoresis is represented by a head of intact DNA and a tail of damaged DNA streaming away from it in the direction of electrophoresis. Classification of comet according to their DNA damage can be either done using visual scores but more commonly is supported by an image analysis software that is capable of extrapolating numerical indexes of damage, the most common of them being the length (Tail Length) of the comet and the percentage of DNA present in the comet (Tail Intensity%). A third index of reference widely used is a product of the previously mentioned indexes and is known as Tail Moment. New promising techniques in the classification of comet cells is provided by artificial intelligence algorithms applied to image analysis that will improve and accelerate the classification process in the near future (Bernardini et al., 2019). Despite its popularity, the comet assay still has some shortcomings mainly due to a high inter-operator as well as inter-laboratory variability and the limited use of calibrators. Indeed, although the process is very straightforward, limited variations in each one of the methodological steps described may complicate the comparison of data from different laboratories, in particular when the starting material is not constituted by cultured cell lines as in the case of reference organisms used in the ecotoxicological assessment. One of the purposes of the present review is to summarize not only the application but also some critical methodological details in order to describe the most suitable methodological approaches in line with the paper published in the past 5 years in the field of soil environmental ecotoxicology. The analysis of the relevant articles published in the last years on the effects of pesticides upon the soil earthworms, determined through comet assay, is summarized in Table 3.3.

Among the techniques developed to assess DNA damage, the comet assay has received remarkable interest for its easy and cost-effective implementation. Although it was originally designed mainly for human and cultured cells, ecotoxicological applications widened the analysis to a broad set of invertebrate species. Within ecotoxicological applications, the technique was primarily used for genotoxicity assessment in marine and freshwater invertebrates, and subsequently, it was extended to terrestrial invertebrates. Soil invertebrates are known to be efficient accumulators of xenobiotics and to respond to their exposure in a sensitive and measurable manner, hence their popular use as bioindicators of soil contamination. The comet assay has been applied to various annelids including polychaetes, oligochaetes, and leeches, although the majority of studies were carried out on selected species of earthworms (*Eisenia* spp.) that are recognized models to assess soil quality and environmental impacts of cropping systems and pollutants. In fact, among soil organisms, earthworms deserve particular interest because of their ecological role in soil biocenosis representing 60–80% of the total biomass; by ingesting soil particles, they represent extremely pertinent bio-indicators (Sanchez-Hernandez, 2006).

Sub-lethal markers such as DNA damage are pivotal to identify potential environmental risks, taking into account the chronicity of exposure of non-target organisms to pollutants widely used in agricultural practices (e.g., insecticides, fungicides, and herbicides) (Di Marzio et al., 2005).

The bioavailability of pesticides in soils is affected by the amount and quality of pesticide-absorbing soil colloids and microbial growth and activity, which results in a different extent of pesticide biodegradation (Castillo et al., 2008; Katagi, 2013).

According to the relevance of earthworms in soil ecotoxicology studies, in the present review, we revised the application of the comet assay in the investigation of environmental pesticide sub-lethal effects in these non-target species. The literature of the last 5 years on the topic consists of 16 articles: only one among them was conducted in field conditions related to the effects of persistent organic pollutants

(POPs), including those introduced as pesticides in the environment (Espinosa-Reyes et al., 2019). In particular, in this study, genotoxicity was tested in wild earthworms in soils at different levels of urbanization (industrial, urban, and rural areas). The analysis unambiguously identified the highest concentration of POPs and the highest rate of DNA damage in industrial areas. Urban and rural areas had a different composition of POPs with the more prominent influence of agrochemistry in rural areas; however, the latter seemed to have a lower impact, compared to urban organic pollutants in triggering DNA damage in earthworm coelomocytes.

With the exception of this paper, all other screened articles were conducted in standardized laboratory conditions through the application of OECD guidelines. Moreover, these studies did not use wild earthworms; on the contrary, they mainly used as a reference organism *E. fetida*, with the exception of three articles (Fouché et al., 2016; Chevillot et al., 2017; Mincarelli et al., 2019) that analyzed the effects of pesticides on the other commonly used earthworm reference species *89ndreia andrei*. As reported in Table 3.3, most of the studies used three organisms as an experimental unit for experimental conditions, and exceptionally few studies increased the observational sample to up to 10 or 18 samples (Wang et al., 2015; Chen et al., 2018; Espinosa- Reyes et al., 2019). In all studies, DNA damage assessment was conducted on coelomocytes – hemocytes of the annelids that can be recovered from the coelomic fluid with non-invasive techniques by submerging the organism in ethanol 5% saline solution that might be integrated with the chelating agent EDTA and the mucolytic agent guaiacol or similar. The studies explored the potential genotoxic effect of a broad set of chemicals including as insecticides Cyantraniliprole (Qiao et al., 2019), Acetamiprid (Li B. et al., 2018), Neonicotionoids (Chevillot et al., 2017), Imidacloprid (Wang et al., 2016), Spirotetramat (Zhang et al., 2015), Thiacloprid (Feng et al., 2015), and Guadipyr (Wang et al., 2015) that represented the most numerous, followed by fungicides Pyraclostrobin (Ma et al., 2019), Fluoxastrobin (Zhang et al., 2018), Tebuconazole (Chen et al., 2018), and a smaller set referred to herbicides Tribenuron (Chen et al.,

2018) and Mesotrione (Li X. et al., 2018) or general pesticides including Polychlorinated biphenyls (Duan et al., 2017) and natural toxins used as biofumigants (Fouché et al., 2016) and copper sulfide (Mincarelli et al., 2019) that is one of the few chemical treatments allowed in organic farming. In all studies, synthetic compounds were studies at increasing doses of exposure normally below 10 mg/kg of soil, with the exception of the insecticide Guadipyr (Wang et al., 2015) that was used between 10 and 100 mg/kg of soil. Furthermore, typically, DNA damage was evaluated at specific time points, most commonly once a week up to 1 month. Studies by Shen et al. (2015) and Feng et al. (2015) reported in Table 3.2 investigated the effect of pollutant exposure up to 6 weeks or 2 months. Interestingly in both studies, it was observed that prolonged exposure led to a significant decrease in the rate of DNA damage highlighting a potential adaptation to the environmental stress that is compatible with the ecology of *Eisenia* spp. that is quite resistant and therefore also useful as a sentinel organism for sub-lethal toxicity.

Most of the studies screened used as a reference index Olive Tail Moment (OTM), which is a widely used index of DNA damage that essentially represents the product of the percentage of total DNA in the tail and the distance between the centers of the mass of head and tail regions [Olive moment = (tail mean- head mean) X % of DNA in the tail]. This is quite surprising considering that tail intensity DNA% has been mainly used in recent investigations due to its robustness (Collins, 2004; Kumaravel and Jha, 2006).

DNA damage was often related to markers of oxidative stress that exhibited a similar trend in response to pollutants. This is central in describing the mechanism of genotoxicity of pesticides that often is mediated by an increase in cellular reactive oxygen species formation due to cellular impairment (Zhang et al., 2015, 2018; Duan et al., 2017; Li B. et al., 2018; Li X. et al., 2018; Qiao et al., 2019). Interestingly despite the different nature and target of the pesticides tested, a common pattern of response is often evincible in the biological response of *Eisenia* spp. with a dose-dependent response to the chemical at each time point analyzed; however, when they

are observed on a longitudinal time scale during the typical month of exposure (28 days) at the lowest dose of chemicals used, often a decrease in DNA damage is observed from the second week of exposure highlighting an adaptive response; at intermediate dosage, DNA damage tends to stabilize during the last 2 weeks of exposure while at the highest concentration DNA damage is able to increase in comparison to the previous time points even after 1 month of exposure (Li X. et al., 2018; Zhang et al., 2018; Ma et al., 2019). Mincarelli et al. (2019) also showed an adaptive response at the level of gene expression of metallothionein as well as proteins involved in the immunological response (antimicrobial peptide fetidin and toll-like receptors).

The decrease in the extent of DNA damage might be the result of an adaptive response to low doses of chemicals but is also known to be potentially due to the artifactual decrease in the mobility of damaged DNA associated to the DNA-DNA or DNA-protein crosslink. This seemed to be the case of the insecticide Imidacloprid (Wang et al., 2016) that differs from the other pesticides that showed a dose-dependent decrease of DNA damage already after 1 week of exposure despite a significant increase in markers of oxidative stress and damage. Also, the data relative to the genotoxicity of the neonicotinoid insecticide Guadipyr presented by Wang et al. (2015) are in antithesis with other reports showing very low toxicity in general, and also at the DNA integrity level, following exposure at a concentration 10 times higher compared to other pesticides considered in this review. The compatibility of the high doses of chemicals nominally used with the reproduction and viability of earthworms seems to suggest that Guadipyr is remarkably safer compared to other insecticides; however, the lack of positive control of toxicity in the same set of experiments and bioaccumulation data at the organism level might suggest the need for further investigation to better classify the toxicity of this compound.

Finally, a particular mention should be devoted to innovative approaches of the use of the comet assay among the selected articles. Chevillot et al. (2017) studied the biological exposure of earthworms to complex chemical mixture exposure that

mimics environmental complex matrixes. Despite the exciting experimental design, however, the section on DNA damage is quite limited and indicates a slight but significant increase in DNA damage of earthworm coelomocytes following exposure to neonicotinoids.

Another application of the comet assay for ecotoxicological soil studies is to investigate the use of particular amendments, such as organic material, and verify whether they could affect the pesticide concentrations and its toxic potential. The study by Shen et al. (2015) developed along this line demonstrating that humic acid alleviated the damage to DNA, proteins, and lipid membranes caused by nickel and deltamethrin spiked in the soil. Despite the shortcomings mainly associated with operator dependent and interlaboratory variability, the comet assay remains an elective technique for assessing genotoxicity in environmental biomonitoring, being mature after decades of implementation and widely used worldwide for its direct, simple, and affordable implementation. Nonetheless, in order to promote the consistency and reliability of results, regulatory bodies should provide guidelines on the optimal use of this tool, strongly indicating the most reliable indicators of DNA damage. In this respect, most of the articles reported in the review use OTM, while in the last decades, many evidences seem to highlight DNA tail intensity% as the most informative index characterized by a broader dynamic range. Moreover, the use of computer-aided image analysis software rather than visual scoring should be strongly suggested as well as the use of appropriate reference of damage such as negative and positive controls treated with DNA damaging agents or gamma radiation, ideally at least at 2 doses. Reference cells should be used as standard and to verify the reproducibility of the techniques and do not necessarily have to be of the same type of analytical samples; indeed an optimal reproducibility could be achieved with cultured cells.

3.3 Conclusion

The examination of the published articles showed a continuous improvement of the possibility to detect the detrimental effect of the pesticides on soil microorganisms and non- target organisms at a sub-lethal level. In the present review, we considered the methodological aspects considering the most promising DNA-based methodologies used to generate data for biodiversity and biomonitoring studies; moreover, we described the specific outcomes of research papers using these techniques in describing the effect of pesticides on soil. The critical analysis of the papers examined can help registration authorities to derive ecotoxicological parameters useful for a high-tier risk assessment. Regarding microbial communities, soil microorganisms play key roles in significant ecological processes such as bioremediation, recycling of elements, soil structure establishment, and degradation of organic matter and chemical xenobiotics (Umar et al., 2017; Walvekar et al., 2017). Among them, pesticides are the most common contaminants in the agricultural field (Wołejko et al., 2020), and their persistence in the soil can alter the physico-chemical structure and microbiota composition causing a negative impact on soil biodiversity (Diez et al., 2017). Indeed, the extensive use of pesticides has gradually led to soil contamination with proven damages to environmental health (Fernandes et al., 2020; Kafaei et al., 2020). Reported data using DGGE-PCR studies confirmed that the chemical nature and the doses used play a role in the disturbance of microbiological complexity of the soil.

The use of biopesticides, differently from synthetic ones, does not affect the microbial community reversibly (Reali and Fiuza, 2016; Umar et al., 2017). In this respect, several studies have shown that the pesticide-induced microbial changes are mainly transient and the structure of the microbial population can improve gradually (Gao et al., 2012; Chen et al., 2013). However, this advantage is lost at high concentrations. Gupta et al. (2013) found that the treatments of rhizosphere with high doses of biopesticide azadirachtin had an adverse effect on the microbial population.

They were suggesting that concerning the environmental impact, the lower toxicity of biopesticide is lost at high concentrations.

Regarding synthetic pesticide impact on microbial soil, some PCR-DGGE studies suggest that also in this case in the long term, they seem not to affect the rhizosphere population negatively.

For example, Coppola et al. (2011); Marinozzi et al. (2013), and more recently Diez et al. (2017) show that the acute soil population modification is reversible since over a short time the soil community was able to return to the initial composition as reported by Tortella et al. (2013b).

However, these studies are in contrast with previous evidence (Hoshino and Matsumoto, 2007; Chu et al., 2008; Huang et al., 2016; Wang et al., 2019) showing that synthetic pesticides significantly affect the abundance of soil microorganisms, causing irreparable damage. The evidence shows the ecological toxicity of several pesticides such as metalaxil and chloropicrin on the fungal community (Hoshino and Matsumoto, 2007; Wang et al., 2019).

Further studies using novel high-throughput techniques such as NGS has shown that in laboratory experiments the majority of the pesticides used had a significant impact on the microbial communities with the sole exception of the observations by Tang et al. (2019) in which the changes were attributed to plant growth stages rather than the Glyphosate. In the field experiments, on the other hand, significant changes were mostly depending on the dose, period, and frequency of application, and the characteristics of the soil, including its indigenous microbial community properties. It is therefore not possible to pinpoint exactly one type of pesticide or substrate as the source of major impacts. Several studies indicated that the rapid shift of communities in favor of pesticide-degrading groups already presents in the microbial community. The presence and activity of pesticide-degrading groups before the application of tested pesticides may be considered as preliminary indicators of synergies among molecules and of the possible outcome of pesticide application. Pesticides used had relatively lower impact, as expected, in the experiments where

soil microbial communities were already highly diverse and rich from the beginning. Several studies cited in this work underlined the importance of carrying out various samplings throughout the cropping season. This was because changes in microbial composition happen during the growing season via plant–microbes interaction, and some transient changes may also be explained by the presence of the crops rather than pesticides. Furthermore, the replication of the experiments in different fields is also necessary in order to comprehend the real impact of pesticide(s), as different fields have different physical characteristics and microbial diversity. These factors can indeed affect the response and recovery of the microbial communities. Using the comet assay, the reported study analyzed the toxicity of pesticides in non-target organisms, in this case, referring to soil macroinvertebrates, in terms of DNA damage. Remarkably the main effect on soil biocenosis is also confirmed in this studies that support a high level of adaptation and overtime to the pesticides, suggesting that dose and time of observation are critical aspects to take into consideration in environmental risk assessment. Although this aspect may seem quite obvious, this consideration should be addressed in particular in relation to natural compounds that have limited constraint in terms of dosage to be used, whereas the discussed data suggests that their toxicity at high concentrations should be carefully evaluated. In the light of the above cited findings, we can suggest that the way forward for studies on the impact of pesticides on soil ecosystem lies in the inclusion of more than one soil type/field in the studies; samplings at various intervals during the experiment in order to better assess the shifts and recovery of the microbial community; and coupling the overall biodiversity and functionality with that of key microorganisms of agricultural and soil fertility relevance such as denitrifiers, saprophytic fungi, and archaea. The inclusion of these factors into studies would, therefore, pave the way to understanding that also the unintended impact of a secondary nature on the terrestrial ecosystems besides the main purpose of the pesticide should be considered, which is a very important point for both regulatory authorities and researchers. DNA-based methodological tools provide a

robust and efficient technique for the evaluation of microbiological biodiversity as well as early biomarkers of genomic damage in soil organisms that represent an early reporter of stress and potential risk for biota survival and reproduction. The major advantage of these techniques is related to the uniformity of protocols and high-throughput capabilities that enable rapid and comprehensive assessment, addressing major challenges in ecological risk assessment (i.e., provide information at higher levels of biological organization moving from individual organisms to communities and population) and the ability to consider multiple stressors and context dependencies, allowing recovery analysis. On the other hand, at the interlaboratory level, care should be taken in order to standardize the methodologies and gain reproducible results using appropriate reference markers. Moreover, information should be handed in an integrated manner with other biological and environmental indicators using multivariate predictive models and multimetric approaches.

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Table 3.1 Summary of the relevant articles on the application of denaturing gradient gel electrophoresis technique to the pesticide effect on soil microorganisms.

References	Substance	Type- Study	Comments
Wang et al.(2020)	Azoxystrobin	Fungicide- Lab	Significant dose dependent impact of the substance on the bacterial community diversity and changes in its composition following exposure to specialized degraders.
Farthing et al.(2020)	Glyphosate and imidazolinone	Herbicide- Field	Transient significant and overall little impact following treatments. Changes mostly attributed to the field properties.
Fang et al.(2020)	Chloropicrin, dazomet, dimethyl disulfide, allyl isothiocyanate and 1,3-dichloropropene	Fumigant-Lab	Transient significant changes with initial diversity decline and brief stimulation. Fumigant type dependent various responses from soil bacterial community and its denitrifiers.
Zhang D. L. et al. (2019)	1,3-dichloropropene	Fumigant-Field	Bacterial community composition remained unaffected by 1,3-D fumigation whereas its impact was detrimental to biodiversity of bacteria, AOA-amoA and AOB-amoA genes.
Zhang C. et al.(2019)	Pyraclostrobin	Fungicide-Lab	Significant impact in the abundances of genera with important roles in soil fertility and pollutant biodegradation.
Tang et al.(2019)	Glufosinate (glyphosate)	Herbicide-Lab	Some variations in bacterial diversity of rhizosphere caused only by plant growth stages. These changes were not attributed to treatments.
Mallet et al.(2019)	Leptospermone	Herbicide-Lab	Significant shifts in community structure and diversity in fungal communities of soils caused by natural weed killer leptospermone. Recovery was only possible for the soil in which indigenous fungal community prior to experiments was already diverse and rich.
Hu et al. (2019)	Fomesafen	Herbicide-Field	A dose dependent effect impact on diversity. Long-lasting significant impact on the soil microbial community and changes toward specialized microorganisms that are able to degrade Fomesafen.
Gallego et al.(2019)	Oxamyl	Nematicide-Field	Increased abundance of the specialized fraction and transient changes in the composition total bacterial community.
Feng et al.(2019)	Lindane	Insecticide-Field	Bacteria were than fungi but stable community structure exhibited in the hybrid rice under lindane stress.
Storck et al.(2018)	Chlorpyrifos, isoproturon, and tebuconazole	Insecticide; herbicide; fungicide-Field	Diversity and composition varied over time more in mesocosms than field. Overall, all pesticides referred as low-risk.
Jeffries et al.(2018)	Chlorpyrifos	Insecticide-Field	Legacy effect after 13 years, community that is able to adapt and degrade OP is still reflected.
Zhang S. T. et al. (2017)	Chloropicrin	Fumigant-Field	Richness and diversity after 3 years continuous fumigation were the lowest. Increase of fumigation years reduced the incidence of bacterial wilt.
Jiang et al.(2017)	Acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetomide)	Herbicide-Field	No significant impact on soil microbial community composition after 9 years of treatments. Changes between 8th and 9th year were found to be not related to herbicide but to a seasonality.
Chen et al.(2017)	Paichongding neonicotinoid	Insecticide-Lab	Significant, both positive and negative, soil type dependent impact on soil bacterial community. Diversity was found to be gradually increasing in control group while it was decreasing in the IPP group. The inoculation of an IPP degrading strain positively affected the microbial species diversity in contaminated soil.
Panelli et al.(2017)	Farming systems analysis	Various-Field	Fungal and bacterial community alterations caused by various treatments, exact comparison of two systems were not possible.

Table 3.2 Summary of the relevant articles on the application of next-generation sequencing to the pesticide effect on soil microorganisms.

References	Substance	Type	Study	Organism	Comment
Ma et al. (2019)	Pyraclostrobin	Fungicide	Lab	*E. fetida*	DNA damage increases at 7 and 14 days.
Espinosa-Reyes et al. (2019)	Persistent organic pollutants	"Pesticide"	Field	Not specified	DNA damage correlated to POP accumulation in soil.
Qiao et al. (2019)	Cyantraniliprole	Insecticide	Lab	*E. fetida*	Increase oxidative stress damage alters antioxidant cellular status inducing DNA damage.
Zhang et al. (2018)	Fluoxastrobin	Fungicide	Lab	*E. fetida*	Oxidative stress parameters correlated to DNA damage.
Li B. et al. (2018)	Acetamiprid	Insecticide	Lab	*E. fetida*	Maximum of DNA damage after 14 days. Recovery phase started after 21 days.
Chen et al. (2018)	Tribenuron methyl Tebuconazole	Herbicide and fungicide	Lab	*E. fetida*	Not significance changes in OTM and TL at all pesticides concentrations alone and combined.
Li X. et al. (2018)	Mesotrione	Herbicide	Lab	*E. fetida*	Maximal OTM values at highest dose at day 28.
Chevillot et al. (2017)	Neonicotionoids	Insecticide	Lab	*E. Andrei*	The low NEOs concentrations were not lethal but induce significant increase in class 4 (extremely DNA damage).
Duan et al. (2017)	Polychlorinated biphenyls	Pesticide	Lab	*E. fetida*	PCB treatment increase DNA damage in both type of soil, even at lowest concentration tested.
Wang et al. (2016)	Imidacloprid	Insecticide	Lab	*E. fetida*	OTM and tail DNA% increased at 7 days increasing doses. At 21 and 28 days DNA cross- links were observed.
Fouché et al. (2016)	Biofumigants, glucosinolates	Natural toxins	Lab	*E. Andrei*	Broccoli and oilseed radish DNA damage level similar to ctrl. Only Mustard significantly different to ctrl.
Shen et al. (2015)	Deltamethrin	Pesticide	Lab	*E. fetida*	Nickel produces more DNA damage compared to Deltamethrin. Synergistic effect in increasing DNA damage.
Zhang et al. (2015)	Spirotetramat	Insecticide	Lab	*E. fetida*	Extent of DNA damage reveal 90% worms have low DNA damage at 0.25 mg/kg.
Feng et al. (2015)	Thiacloprid	Insecticide	Lab	*E. fetida*	1 and 3 mg/kg reached higher DNA damage at 28 and 35 days.
Mincarelli et al. (2019)	Copper sulfide	Pesticide	Lab	E. andrei	DNA damage at concentrations lower than those found in most agricultural soils worldwide after 9 days of exposure.
Wang et al. (2015)	Guadipyr	Insecticide	Lab	*E. fetida*	Guadipyr has no effect on OTM, TM, Tail DNA%.

Table 3.3 Summary of the relevant articles on the application of comet assay to the pesticide effect on soil earthworms.

References	Substance	Type	Study	Structure impact	Comments
Hoshino and Matsumoto (2007)	Chloropicrin and 1,3-Dichloropropene	Fumigants	Field	Significant effect	Chloropicrin changed DGGE profiles radically and no recovery was found after 1 year.
Coppola et al. (2011)	Penconazole, Dimethomorph, Metalaxyl, Azoxystrobin, Cyprodinil, and Fludioxonil	Fungicides	Lab	Transitory effects	Evident variation of microbial population after pesticides treatments; no significant differences at the end of the experiment.
Gao et al. (2012)	*Pseudomonas fluorescens* 2P24	Biological control	Field	Transitory effects	Fungal community was significantly shocked at first, but it improved gradually after 1 month.
Gupta et al. (2013)	Chlorpyrifos, endosulfan and azadirachtin	Chemical and natural insecticides	Lab	Dose- dependent significant	High doses of azadirachtin simulated the effects of chemical pesticides
Chen et al. (2013)	*B. subtilis* B579	Biological control	Lab	Minimal and transient effects	Only minimal and temporary changes in rhizobacterial population structure were detected.
Tortella et al. (2013a)	Atrazine	Herbicide	Lab	Transitory effects	Robustness of microbial community toward the treatments.
Tortella et al. (2013b)	Carbendazim	Fungicide	Lab	Transitory effects	Microbial population remained stable over the time when compared to the untreated control.
Lin et al. (2016)	Pentachlorophenol	Herbicide	Lab	Positive effects vs. earthworms	The microbial population was changed by the earthworm treatments.
Huang et al. (2016)	Chlorpyrifos	Insecticides	Lab	Significant effects	The insecticide inhibits the fungal abundance significantly.
Diez et al. (2017)	Atrazine, chlorpyrifos, and iprodione	Herbicide, insecticide, and fungicide	Lab	Transitory effects	Evident variation of microbial community detected only at first treatment.
Wang et al. (2019)	Metalaxyl	Fungicides	Field	Significant effects	Metalaxyl inhibits the growth of fungi.

CHAPTER 4: Adsorption and degradation of three pesticides in a vineyard soil and in an organic biomix

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Abstract

A soil and an organic biomix (soil/vine branch/garden compost 20/40/40) were used in this lab experiment to evaluate adsorption and degradation parameters for three pesticides (chlorpyrifos, metalaxyl and cymoxanil) used in a vineyard. Adsorption in the biomix material was higher than in the soil for the three pesticides and chlorpyrifos was the most adsorbed pesticide. The role of the organic carbon is essential for enhancing the adsortion of the three pesticides, especially for the most apolar chlorpyrifos. Degradation was generally faster in the biomix material than in the soil although the process was slower in the case of chlorpyrifos if compared with the other two chemicals, due to a more toxic e*ff*ect of this pesticide on soil microflora and a larger adsorption of this pesticide on the organic biomix that reduces its availability for dissipation. Amendment with cheap and available organic wastes or a grass-covered management of soil in the vineyard could reduce the impact of pesticides in the vineyard ecosystem and contribute to the sustainable management of chemicals in the environment.

Keywords: pesticides; adsorption; degradation; organic biomix

4.1 Introduction

Recent research has shown that only a small percentage of the pesticides used in vineyards reaches its target; most of the chemicals reach surface and ground water as a result of di*ff*use contamination from percolation, run-o*ff*, drainage and drift (Gil et al., 2007; Monaci et al., 2011; Vischetti et al., 2008). Another form of environmental contamination due to pesticides is from point sources, including tank filling, washing and waste disposal, spillages and leaks from faulty equipment

(De Wilde et al., 2007; Karanasios et al., 2012; Torstensson and Castillo, 1997; Vischetti et al., 2007).

The use of pesticides against plant pests, weeds and pathogens has been shown to a*ff*ect the chemical and biological fertility of soils in several cases, and includes a number of potential adverse e*ff*ects on soil microorganisms and/or non-target organisms (Vischetti et al., 2020). Long-time exposure of non-target organisms for long time to high concentration of pesticide residues in soils could have several harmful e*ff*ects including genotoxic and carcinogenic risks and may even lead to death in the most serious cases.

Hence, it is of fundamental importance to have a thorough knowledge of the behavior of pesticides in soils in di*ff*erent situations and with the influence of external and internal factors and environmental conditions so as to avoid the long persistence and high bioavailability of these chemicals. The new Registration Procedure allows only "safe" molecules to be included as active ingredients in Annex 1 of EU Regulation 1108 (2009). These molecules are easily adsorbed and rapidly degraded in soils after their use against crop pests and diseases. The most important chemo-dynamic processes for understanding the fate and behaviour of pesticides in soils are adsorption and degradation, whose parameters are generally derived in laboratory experiments and under standard conditions.

The main factors influencing the fate of pesticides in soils and organic substrates are the amount and quality of organic matter, and, generally, pesticide adsorption and degradation was found more e*ff*ective in organic wastes than in soils (Coppola et al., 2011b; Cox et al., 2000; Karanasios et al., 2010). For this reason, together with many others, soil amendment with organic wastes coming from di*ff*erent sites and activities has become a widespread agricultural practice, involving a number of activities to recycle organic wastes even in the perspective of circular economy (Breure et al., 2018). In this context, some research groups in Italy have started to conduct research in vineyards, testing the possible use of various cheap organic residues as amendments to reduce the negative impact of xenobiotics (Fait et al.,

2007; Vischetti et al., 2004). Further studies were pointed out to test various agricultural organic wastes as amendment in soil and/or as organic biofilters to adsorb and degrade pesticides in agricultural waters coming from washing of agricultural equipment's (Coppola et al., 2011a; De Wilde et al., 2009; Karanasios et al., 2010). The present paper describes a laboratory trial to test the e*ffi*ciency of a biomix composed of soil, green compost and vine branches in adsorbing and degrading three of the most widely used pesticides in vineyards in Italy, chlorpyrifos (insecticide), metalaxyl (fungicide) and cymoxanil (fungicide), chosen for their di*ff*erent characteristics with regards to soil adsorption and degradation processes. Adsorption and degradation parameters were derived in the laboratory both for the soil and the biomix and the influence of di*ff*erent organic carbon fractions on the two chemo-dynamic processes was assessed.

4.2 Materials and Methods

4.2.1 Soil, Biomix and Pesticides

The soil used was a topsoil (mixed calcic Haploxcrept) supplied by the Experimental Farm of the Marche Polytechnic University, Ancona, Italy, while the biomix was based on 20% soil, 40% garden compost composted for five years and 40% vine branches. The biomix composition was chosen for the tentative to use it as biofilter for water decontamination by pesticides in vineyard. The organic wastes were thoroughly chopped (0.2–0.5 cm) before mixing them with the 2 mm soil sample. The main characteristics of the soil, the organic wastes and the biomix are reported in Table 4.1. All the forms of organic carbon were determined in order to evaluate their influence on adsorption and degradation of the pesticides used in the experiment.

The tested pesticides were supplied by Labor Dr. Ehrenstorfer-Schäfers (Augsburg, D); the chemicals had purities of 98.4% (chlorpyrifos, CH), 99.5% (metalaxyl, ME) and 96% (cymoxanil, CY). Their main characteristics are reported in Table 4.2.

4.2.2 Determination of Organic Carbon Fractions

Substrates were thoroughly homogenized by hand kneading before analyses. Different fractions of organic carbon were determined in triplicate on air-dried samples in all the organic substrates and in soil. Total organic carbon (TOC) was determined using the Walkley–Black method without external heating and slightly modified to avoid underestimation of total organic carbon. Briefly, samples were passed through a 0.5 mm sieve, and 0.2 g of each organic substrate were used. 20 mL of potassium dichromate ($K_2Cr_2O_7$ 1/6M) and 40 mL of concentrated H_2SO_4 (96%) were added to the samples. After half an hour, the excess of dichromate was titrated with ammonium ferrous sulfate $(NH_4)_2Fe(SO_4)\cdot 6H_2O$ (0.5 M) to calculate the amount of total organic carbon.

Chemical analyses of organic carbon fractions were performed by extracting total organic carbon (TEC) in an alkaline solution as reported by Schnitzer (1982) with slight modifications. Briefly, 10 g of each organic substrate were treated with a sodium hydroxide and sodium pyrophosphate 0.1 M solution (10:1 liquid:solid ratio) and shaken in the dark on an orbital shaker at 160 rpm at room temperature overnight. The mixture was centrifuged at 13,000 rpm for 15 min, and the supernatant was collected. The insoluble residue of the extraction was washed three times using 50 mL of deionized water. The rinsed solution was then centrifuged (14,000 rpm for 15 min) and added to the alkaline extract. The alkaline extract was subdivided into two aliquots. One aliquot was analyzed by using the Walkley Black (WB) method to determine the total extractable organic carbon (TEC) which represents the humified fraction. The other aliquot was subjected to fractionation to obtain the humic and fulvic acid aliquots. Concentrated H_2SO_4 was added to reach pH < 2 in order to determine the precipitation of the humic acid (HA) fraction, which was allowed to coagulate for 24 h at 4 °C. The HA fraction was then separated from the soluble fulvic acid (FA) fraction by centrifugation for 15 min at 13,000 rpm. The supernatant containing only the FA fraction was then

collected, and the relative organic carbon content was determined using the WB method. The organic carbon concentration relative to the HA fraction was then estimated by subtracting the thus determined amount of FA fraction from the determined amount of TEC.

4.2.3 Adsorption Studies

Soil: 20 g of 2 mm air-dried sieved samples in centrifuge tubes were added to 100 mL of pesticide solution in $CaCl_2$ 0.02 M at pH 7.0 with an initial concentration of 0.1, 0.5, 1, 2 and 4 mg/L. The experiment was performed with three replicates for each concentration and separately for each pesticide. After 16 h on the shaker at 20 °C, the time necessary to reach adsorption equilibrium, tubes were centrifuged for 20 min at 6000 rotations. The solution was filtered, separated with $CHCl_3$ (50 mL × 3) and evaporated in a rotary evaporator at 40 °C, rinsed with 1 mL of CH_3OH and aliquots (20 mL) were injected into the HPLC.

Biomix: the procedure adopted was similar to the one used for the soil; however, the initial concentration of the three pesticides in the $CaCl_2$ was higher than in soil due to the greater adsorption capacity of the organic materials. Initial concentrations were: 1, 2, 4 and 8 and 16 mg/L for ME and CY and 2, 5, 10, 50 and 100 mg H.

The adsorption parameters k_F (Freundlich constant) and n (power coefficient) were derived using the Freundlich Equation:

in logarithmic form:

$$C_s = k_F C_e^n \qquad (1)$$

$$\log C_s = \log k_F + n \log C_e \qquad (2)$$

where C_s is the ratio of pesticide to adsorbent mass (mg/kg) and C_e is the equilibrium concentration of the adsorbate (mg/L). The Freundlich constant k_F allows to calculate k_{Foc} (organic carbon partition coefficient), which is defined by

$$k_{Foc} = (k_F/\%\text{oc})100 \qquad (3)$$

where oc is the organic carbon content of soil or biomix.

4.2.4 Degradation Studies

Degradation studies were performed on the same soil and biomix used for the adsorption studies. Initial doses of the three pesticides were calculated according to the vineyard experiment carried out by Fait et al. (2007), hypothesizing that with repeated treatments in vineyard, high doses of pesticide can be concentrated in a limited area on the ground. The doses were 16.6 ppm, 13.3 ppm and 14.2 ppm, respectively, for ME, CH and CY in the soil and 131.6 ppm, 26.31 ppm and 32.9 ppm in the biomix. The experiment was performed separately for each pesticide both in soil and biomix, using containers of 1 kg of air-dried sieved soil and 1 kg of homogenized biomix for each pesticide and each concentration. All the samples were at 60% of their water holding capacity and incubated at 20 °C in the dark. Samples were weighed every day in order to maintain the correct humidity. At di*ff*erent intervals of time after treatment, 40 g subsamples in triplicate were extracted from each container and analyzed for pesticide residues. The subsamples were collected after 0, 3, 7, 15, 30, 60, 90 and 150 days for CH and ME in the soil and in the biomix and after 0, 3, 7, 15 days for CY in the soil and 0, 3, 7, 10 days for CY in the biomix. Soil and biomix subsamples were weighed in centrifuge tubes, 80 mL methanol/water solution was added (80:20), the mixture was shaken for one hour, centrifuged at 6000 rotations for 15 min and then filtered. All extracted samples were added to 80 mL of H_2O, separated using chloroform (80 mL × 2), evaporated, dissolved in 1 mL methanol and analyzed. Adsorption

samples followed the same procedure from the chloroform separation step onwards.

Degradation parameters were derived by applying the first order kinetics to degradation data, as a natural logarithm of the residual concentration versus time. From the rate constant k of the Equations, it was possible to derive the half-life value ($t_{1/2}$, days):

$$t_{1/2} = 0.693/k \qquad (4)$$

4.2.5 Analyses

The samples coming from adsorption and degradation studies were analyzed by HPLC, using a Spectra SYSTEM P 4000 equipped with UV detector (wavelength 242 nm for CY and 230 nm for CH and ME) and Supelcosil C18 column, 25 cm × 4.6 mm i.d.; analysis conditions for CH and ME were: flow rate of 1 mL/min, mobile phase of water/CH_3CN 30/70, while for CY, the flow rate, this was 0.8 mL/min and the mobile phase consisted of water/CH_3CN 60/40. Under these conditions, retention times were 3.7 min for ME, 5.3 min for CY and 10.7 min for CH. Limit Of Detection (LOD) was 10 ng for CY and ME and 12 ng for CH. Recoveries were between 90% and 98% in soil and between 87% and 96% in biomix.

4.2.6 Statistical Analysis

Student's t Least Significant Differences (LSD) tests were performed at significance level $p < 0.05$ for the mean separation between soil and biomix samples.

4.3 Results and Discussion

4.3.1 Adsorption

The adsorption isotherms for the three pesticides in the soil and in the biomix are reported in Figure 4.1, while Table 4.3 reports the derived values of the adsorption parameters based on the Freundlich model.

The Freundlich model fitted the adsorption data with values of R^2 always being significant at $p < 0.001$ level.

The k_F values obtained for the soil showed that CH ($k_F = 31.2$) was better adsorbed than ME (0.84) and CY (0.33). The K_{Foc} value of 2836.6 was close to the lower limit of the range of PPDB [18] and was somewhat lower than those found by Huang and Lee (2001), who reported results from 3900 to 6100 for two soil types containing 1.3% organic carbon.

The k_F value found in soil for ME is in the same order as the ones found by Andrades et al. (2001) who reported k_F values between 0.01 and 0.64 in sixteen Spanish vineyard soils with an organic matter content between 0.31% and 1.37%; the same authors reported k_F values between 1.05 and 2.83 for natural soils with an organic matter percentage between 3.3% and 8.2%. The k_F value of 0.33 found for CY in soil is in agreement with EPA values (USEPA, 1988), which ranged between 0.29 and 0.79 in soil with 1.3% of organic matter. ME and CY are much more soluble in water than CH and much more polar, according to the octanol–water partition (k_{ow}) values. In fact, the adsorption values, k_F, are ranged according k_{ow}, rather than according their solubility values, and the k_{oc} values in soil reported in the Pesticide Properties Data Base (PPDB) were considerably lower than the results for CH. The k_{oc} values found in soil were 76.6 for ME and 30.0 for CY, falling just in the range of the PPDB report and that of CH was very close to the lower value reported by PPDB.

The biomix showed a good adsorption efficiency for the three pesticides, increasing the values of the adsorption parameters in all cases respect to the soil, albeit to a different extent: k_F for CH was about 29-fold higher in the biomix than in the soil, for ME about 9-fold and for CY about 6-fold higher, indicating that the most apolar compound CH is adsorbed much better in a substrate, which is rich in organic matter compared with ME and CY which are more polar and water soluble pesticides (Table 4.2), even if the k_F values of the last two pesticides increased in

any case, indicating the positive effect of organic matter also in the adsorption of soluble pesticides.

The Freundlich exponent *n* increased in all cases from soil to biomix, indicating a higher affinity of the three pesticides for the substrate with a high organic carbon content.

Several authors (De Wilde et al., 2009; Karanasios et al., 2010; Vischetti et al., 2013) studied the adsorption of some pesticides in different organic substrates and soils, with the main aim to find a substrate suitable to be used in organic biofilters, especially for southern European conditions, due to the unavailability of organic natural substrates such as peat. The values of adsorption parameters found in the present experiment are in agreement with those found by the above authors, which showed increasing in k_F values in the same order of those of the present experiment and positively correlated to the organic carbon content of the adsorbing substrate. CH maintained a value of k_{Foc} in the biomix similar to that in soil, while ME and CY showed a k_{Foc} value in the biomix which was lower than in soil, indicating a decrease in the adsorption power of the unit of the organic carbon in the biomix, probably due to the different fraction of organic carbon reported in Table 4.1; the humified fraction is mainly responsible for pesticide adsorption in the organic matter of soils and humic acids are more effective than fulvic acids in adsorbing apolar compounds. The humified fraction of biomix is about 76% of total organic carbon (21.8%, with respect to a total of 28.7%) with a slight prevalence of humic acids and this is reflected in a preferential adsorption of CH compared with ME and CY. In a previous paper, Vischetti et al. (2013) reported a similar behavior for ME in different organic substrates and soils with an increase in organic substrates with respect to soils, especially in more humified substrates which are rich in humic acids. However, the relative ability (relative to OC units) was lower for the more polar ME and CY. This would mean that the biomix was relatively less efficient in retaining those polar pesticides.

4.3.2 Degradation

The degradation trends of the three pesticides in soil and biomix are reported in Figure 4.2, while Table 4.4 reports the degradation parameters derived by applying the first order kinetics to the degradation data.

The first-order model was well fitted with the degradation data, being significant at $p < 0.001$ level in all cases. The half-life values in soil, shown in Table 4.4, were higher for CH than ME and CY, in line with the half-life range reported in the PPDB (Table 4.2) and in agreement with other authors: Reeves (2003) reported CH $t_{1/2}$ in di*ff*erent types of soils between 43 and 126 days, whilst Sukul and Spiteller (2001) found ME $t_{1/2}$ in soil between 36 and 301 days.

The degradation for the three pesticides was found to be faster in the biomix than in the soil, indicating an important role of organic carbon and microbial population in promoting pesticide degradation, as reported by other authors (Coppola et al., 2007; Marinozzi et al., 2013). Half-live values in the biomix decreased by 32.2%, 47.0% and 62.2% for CH, ME and CY, respectively. The half-life value for CH decreased less than those of ME and CY. Some authors (Coppola et al., 2007) found that this behavior is caused by the main metabolite TCP (3,5,6-trichloro-2-pyridinol) which shows antimicrobial properties. On the contrary, the degradation of ME and CY in the biomix is faster than that of CH; the presence of organic carbon and a sound microbial population in the biomix could promote the faster degradation of the two pesticides, as reported by er authors (Coppola et al., 2011a; Karanasios et al., 2010).

4.4 Conclusions

This experiment has shown that the addition of organic substrates to soils improves pesticide adsorption and degradation, since organic matter is mainly responsible for pesticide–soil interactions. The most efficient fraction in pesticide adsorption is the humic carbon and, above all, the humic acid fraction. Pesticide degradation was enhanced by the addition of organic substrates to soils, even if the process was

more evident for those pesticides which are less toxic or nontoxic for soil microflora.

The soil amendment with cheap and available organic wastes in the vineyard could reduce the impact of pesticides in this ecosystem and contribute to a sustainable management of chemicals in the environment.

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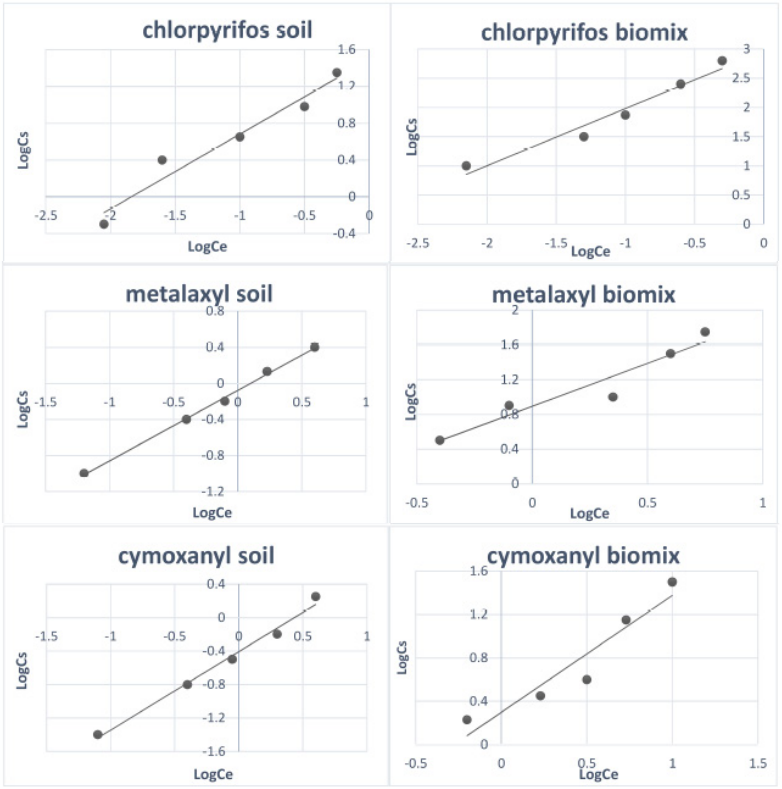

Figure 4.1 Adsorption isotherms for the three pesticides derived from the batch equilibrium experiment (data are the means of three replicates; standard error even <1.8% in soil samples and <3.1% in biomix samples). R^2 values always significant at $p < 0.001$ level.

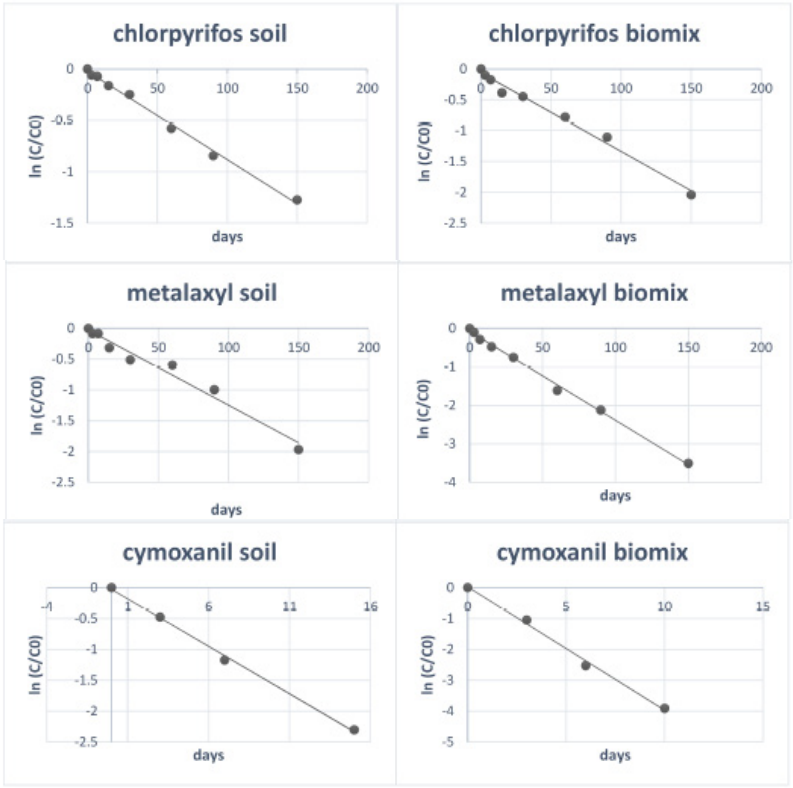

Figure 4.2 Degradation patterns of the three pesticides in the soil and in the biomix derived by applying first order kinetics to the data from the degradation laboratory experiment (data are the mean of three replicates; standard error even <2.1% both in soil and biomix samples); R^2 values always significant at $p < 0.001$ level.

Table 4.1 pH (H_2O) and organic carbon content (%) of soil, organic wastes and biomix used in the experiment (mean of three replicates ± standard deviation).

	Soil	Compost	Vine Branches	Biomix
pH	8.2	5.1	7.9	7.9
TOC	1.1 ± 0.1	31.2 ± 1.4	32.5 ± 1.1	28.7 ± 0.9
TEC	0.8 ± 0.1	21.2 ± 1.2	4.5 ± 0.4	21.8 ± 0.9
HAC	0.6 ± 0.0	16.5 ± 0.8	1.1 ± 0.1	6.8 ± 0.3
FAC	0.2 ± 0.0	4.7 ± 0.4	3.4 ± 0.2	6.0 ± 0.4

TOC Total Organic Carbon; TEC = Total Extractable Carbon; HAC = Humic Acid Carbon; FAC = Fulvic Carbon.

Table 4.2 Main physico-chemical characteristics of the pesticides used in the experiment.

	Chlorpyrifos	Metalaxyl	Cymoxanyl
Water solubility (mg/L)	1.05	8400	780
Vapour pressure (mPa)	1.43	0.75	0.15
$t_{1/2}$ soil lab 20 °C (days)	386	36	1.5
DT50 field (days)	27.6	38.7	3.5
Koc range (L/kg)	3187–7733	29.6–283.8	15.1–87.1

Data derived from Pesticide Properties DataBase (PPDB).

Table 4.3 Adsorption parameters for the three pesticides in soil and biomix (data are the mean of three replicates; standard error even <1.8% in soil samples and <3.1% in biomix samples; low case letters represent Least Significant Differences at $p < 0.05$ in soil and biomix, separately for each pesticides.

	kp		*n*		R^2		*kFoc*	
	Soil	**Biomix**	**Soil**	**Biomix**	**Soil**	**Biomix**	**Soil**	**Biomix**
chlorpyrifos	31.2[b]	909 [a]	0.81	0.98	0.952	0.957	2836[a]	3168[a]
metalaxyl	0.84[b]	7.81[a]	0.78	0.99	0.997	0.917	76.6[a]	28.2[b]
cymoxanil	0.33[b]	1.99[a]	0.94	1.1	0.988	0.903	30.0[a]	6.9[b]

Table 4.4 Degradation parameters of the three pesticides in soil and biomix derived by applying first order kinetics model to the degradation data (data are the mean of three replicates; standard error even <2.1% both in soil and biomix samples; low case letters represent least significant differences at $p < 0.05$ in soil and biomix, separately for each pesticides).

	k_{deg} (Days^{-1})		$t_{1/2}$ (Days)		R^2	
	Soil	**Biomix**	**Soil**	**Biomix**	**Soil**	**Biomix**
chlorpyrifos	0.0086	0.0127	80.6 [a]	54.6 [b]	0.994	0.988
metalaxyl	0.0122	0.0230	56.8 [a]	30.1 [b]	0.972	0.996
cymoxanil	0.1536	0.3992	4.5 [a]	1.7 [b]	0.998	0.995

CHAPTER 5: Ecotoxicological effects of a synthetic and a natural insecticide on earthworms and soil bacterial community

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Abstract

Earthworms and microbial communities are essential non-target soil organisms that are useful to assess the collateral impact of pesticides. The present paper reports three laboratory experiments performed to investigate the effects of sub-lethal doses of two insecticides, a biologically-derived (spinosad) and a synthetic organophosphate (chlorpyrifos), on earthworm *Eisenia fetida* and microorganisms in organic soil. The effects were studied in terms of behaviour, reproduction, survival, and DNA damage (comet assay) in earthworms, and Next Generation Sequencing-Illumina was employed to detect the changes in the microbial community. In addition, the influence of earthworms on the degradation kinetics of insecticides and on microbial diversity was evaluated. The weights, reproductive activity and behaviour of earthworms were particularly compromised and followed a dose-dependent trend in chlorpyrifos trials, where the insecticide's degradation wasn't affected by the presence of *Eisenia fetida*. However, earthworms contributed to spinosad's metabolisation without significantly impacting their health. Early DNA damage was estimated in earthworms exposed to chlorpyrifos, while the impact of spinosad was significant only at the end of the toxicity test. The analysis on the microbial community indicated the buffering effect earthworms had on the bacterial communities starting from earliest sampling until the end of the trial, as well as bacterial community members' degradation response to pesticides over time.

Keywords: microbial community, earthworm, spinosad, chlorpyrifos, Comet assay, Next-generation sequencing.

5.1 Introduction

The intensive use of pesticides for crop protection against diseases led to the widespread presence of these compounds in soils (Hussain et al., 2009; Zhou et al., 2011; Stepić et al., 2013). Pesticide use may affect soil fertility and non-target organisms such as microorganisms and macroinvertebrates (Puglisi, 2012; Mincarelli et al., 2019; Vischetti et al., 2020). A broad toxicity spectrum was reported in ecosystems in correlation to pesticide exposure levels (Desneux, Decourtye and Delpuech, 2007; Beketov et al., 2013; Brühl et al., 2013; Wood and Goulson, 2017) and evaluating non-specific impact on soil-biota could help Regulatory Authorities to avoid underestimating the effect of pesticides (Schäfer et al., 2019). Therefore, using molecular methods and genotoxicity assays, sub-lethal dose impact evaluation is often recommended. However, data interpretation for regulatory purposes still debated (Ockleford et al., 2017; Vischetti et al., 2020).

Earthworms and microbial communities are commonly used in ecotoxicological studies because they represent a large fraction of soil living biomass and are essential in soil functioning (Pelosi et al., 2014; Delgado-Baquerizo et al., 2016; Umar, Tahir and Agbo, 2017). *Eisenia fetida* (*E.fetida*) is most commonly earthworm as exposure to pesticide-contaminated soil is almost direct due to its simple digestive system and limited tegumentary system (Sanchez-Hernandez, Ríos and Attademo, 2018; Svobodová et al., 2018; Zhu et al., 2020). Pesticides can also affect soil microbial communities that are important to earthworms due to the enzymatic support from microbial symbionts that inhabit their gastrointestinal lumen, and at the same time, the mucilaginous secretions of earthworms usually increase exogenous microorganisms activity (Zapata et al., 2017; Gonzales-Condori, Choquenaira-Quispe and Ramírez-Revilla, 2020). Aktar et al. (2009) reported that, among pesticides, insecticides caused the highest acute toxicity. Nonetheless, their comprehensive impact remains poorly investigated as leaf applied insecticides are scarcely investigated compared to herbicides. However, run-off to soil may occur at excess doses (Gil et al., 2007; Monaci et al., 2011; Cesco et al., 2021). Chlorpyrifos,

an organophosphate insecticide, is extensively used worldwide on a range of economically important crops (Thengodkar and Sivakami, 2010; Sud et al., 2020). Its toxicity on earthworms was previously investigated by avoidance, behaviour, survival and reproduction assays (Zhou et al., 2007; Yasmin and D'Souza, 2010; Hundal et al., 2016); recently, an additive and synergic toxicity effect was ascertained for chlorpyrifos mixed to other pesticides on *E. fetida* acetylcholinesterase levels and cellulose activity (Teng et al., 2022), while an effect on mortality and on the gut microbiome of the earthworm *Eudrilus euginae* was observed after exposure to chlorpyrifos at a Lethal Concentration 50 dose, i.e. the concentration expected to kill 50% of a group of test animals when administered as a single exposure (LC_{50}) (Krishnaswamy et al., 2021). Moreover a number of studies demonstrated its ecotoxicity versus other non-target organisms, such as rainbow trout larvae (Weeks Santos et al., 2021) and *Danio rerio* (Mena et al., 2022) and toxicity versus humans, such as pregnant women (Taheri et al., 2022) and human brain (Miller et al., 2021). Over the past 20 years, "natural" insecticides have become increasingly adopted as an environmentally safe alternative to synthetic insecticides (Williams, Valle and Viñuela, 2003; Biondi et al., 2012; Tamez-Guerra et al., 2017). Among those, spinosad claims lower environmental toxicity due to its natural origin; a mixture of spynosins by soil actinomycete *Saccharopolyspora spinose.* Apart some paper reporting the effects of spinosad on non-target organisms such as beneficial arthropods (Biondi *et al.,* 2012) or beneficial insects (Martelli *et al.,* 2022) , very little is known about the impact of these insecticides' effect on earthworms and the soil microbial community (Badawy et al., 2016; Sekulić et al., 2020; Sparks et al., 2020) while evaluating early damage to soil organisms using DNA-based methodological tools might improve the global understanding of the stresses induced by the pesticides on the agro-ecosystems.

The present study aims to ascertain the effect of sub-lethal doses of the two insecticides mentioned above on earthworm *E. fetida* and the soil microbial community with the hypothesis that the different nature of studied insecticides could

affect the functionality and health of earthworms and microbial communities differently and that the presence of earthworms favours the degradation of pesticides. Therefore we conducted; (i) avoidance and reproduction tests to investigate insecticides' harmful effects on earthworms in terms of fertility and behaviour, (ii) a genotoxicity test using the comet assay to detect the DNA damage in earthworm coelomocytes, and finally (iii) Next Generation Sequencing (NGS)-Illumina sequencing to investigate changes in the microbial community. Parallel experiments were also performed without earthworms to evaluate their influence on insecticide degradation kinetics and microbial diversity.

5.2 Materials and methods

5.2.1 Earthworms

E. fetida were supplied by the Lombricoltura Bella Farnia (Sabaudia, Italy). They were reared at 20 ± 1 °C in organic compost and fed with organic oats and vegetables. Adults with a well-developed clitellum (300-600 mg wet mass) were selected and acclimatised in the same substrate used in tests (Li et al., 2018; Zou et al., 2018; Zhang, Saleem and Wang, 2019). Ten adults were used in each replicate in experiments.

5.2.2 Soil

Topsoil from an orchard managed with organic agricultural practices was collected from the experimental farm of the Polytechnic University of Marche (Agugliano, Italy). This Vertic Eutrodept, clay loam agricultural soil with the following properties was used across the experiments: pH 8.2; organic matter 8%; cation exchange capacity 28.3 meq 100 g^{-1}, air dried and sieved at < 2mm.

5.2.3 Insecticides and contamination

Commercial formulation Laser 480 (Spinosad, g 44.2 corresponding to 480g L^{-1}) and Dursban 75 WG (chlorpyrifos g 75.0 corresponding to 489 g kg^{-1}) were supplied by Dow AgroSciences (Milano, Italy), while analytical standards of spinosad (CAS 168316-95-8, purity ≥ 95.0 %) and chlorpyrifos (CAS 2921-88-2, purity ≥ 98.0 %) were obtained from Sigma-Aldrich (Milano, Italy) and their proprieties are

summarised in Supplementary Table S5.1. Solutions of these pesticides were freshly prepared in deionised water to adjust the soil moisture content before experiments (40±10 dry mass) in chemically inert plastic containers (18 x 9 x 9 cm) with a lid that permits gaseous exchange. Two concentrations were employed for each insecticide for the avoidance and reproduction test. More in detail, Dursban 75 WG, was added at the doses of 50 % in the trials with earthworms ($C_{50}E$) and 70 % ($C_{70}E$) of the LC_{50} indicated for the pesticide formulation corresponding to 340,5 mg kg^{-1} and 476,7 mg kg^{-1} of Dursban 75 WG respectively. Laser 480 was tested at the doses of 70 % in the trials with earthworms ($S_{70}E$) and 150 % ($S_{150}E$) of the LC_{50} indicated for its pesticide formulation corresponding to 735 mg kg^{-1} and 1575 mg kg^{-1} , respectively. The toxicity test was conducted with a working concentration of 70 % of the LC_{50} for both insecticide formulations.

5.2.4 Avoidance test

A "dual-control" test was run to assess that earthworms do not tend to aggregate and have a random distribution between the two sections (Yeardley, Lazorchak and Gast, 1996; Hund-Rinke and Wiechering, 2001). The avoidance test was then conducted to evaluate the ability of earthworms to detect and avoid the contaminated substrate (García-Santos and Keller-Forrer, 2011; Jordaan, Reinecke and Reinecke, 2012; Martínez Morcillo et al., 2013) in five replicates with the two-chamber design, as described by ISO 17512-1 (2008). One-half of the box was filled with 250 grams dry weight of the contaminated substrate, the other half was filled with the same quantity of the substrate without the insecticide, and the earthworms were placed onto the separating line. After 2 days, a divider was inserted, and the earthworms on both chambers were counted. The results of the avoidance test are expressed as the net response (NR) in percentage according to ISO (2008):

$$NR = \lfloor (C - T) \div N \rfloor \times 100$$

where C and T are the numbers of worms in the control substrate and the treated substrate, respectively, N is the total number of worms in each box.

A positive NR indicates an avoidance of the contaminated substrate, whereas a negative value indicates an attraction to the pesticide tested (Xu et al., 2020).

5.2.5 Reproduction test

Insecticides' impact on earthworms' reproductive output (and other sub-lethal endpoints) was assessed through a reproduction test following the OECD guideline (OECD, 2016).

All trials and an uncontaminated control with earthworms (ctrlE), in three replicates, were kept under a controlled temperature (20 ± 1 °C) for 56 days. Adult earthworms in each replicate have been weighed and observed weekly: any unusual behaviour and morphology anomalies were recorded. After 28 days, adults were removed from the containers while substrate containing juveniles and cocoons were left for another 4 weeks-incubation. On day 56, the number of juveniles and the cocoons in each replicate were recorded. The growth rate (GR, %) was calculated as follows:

$$GR = \lfloor (W_t - W_0) \div W_0 \rfloor \times 100\%$$

where W_0 is the initial average weight of earthworms, and W_t is the average weight of earthworms on day 28. A positive rate means the growth stimulation, while a negative rate indicates growth inhibition (Xie et al. 2013).

5.2.6 Toxicity test

The toxicity test was conducted to investigate the effects of the insecticides on earthworms' DNA and microbial communities with three replicates for every insecticide and control with earthworms (ctrlE). A gram of the substrate was taken at each sampling for the soil bacterial community and insecticide residues analysis, and an earthworm in each container (three earthworms for trial) was randomly collected at 1, 21 and 28 days for DNA damage analysis through the comet assay. A parallel test was conducted without adding earthworms to evaluate differences in the trend of insecticide residues and the evolution of the soil bacterial community according to the presence or absence of *E. fetida*. Specifically, the parallel test consisted of chlorpyrifos at the dose of 70 % without earthworms (C_{70}), spinosad at

the dose of 70 % without earthworms (S_{70}) and an uncontaminated control without earthworms (ctrl).

5.2.6.1 Insecticides extraction and analysis

The extraction and analysis of chlorpyrifos followed the protocol described by Vischetti et al. (2008); spinosad was extracted and analysed following the method described by Sharma et al. (2007), and its residues were reported as a sum of spinosyn A and spinosyn D (Telesiński et al., 2015). Analyses were performed by HPLC using a Spectra SYSTEM P 4000, equipped with a Supelcosil C18 column (25 cm x 4.6 mm i.d.) and a UV-detector as in Akbar et al. (2010). Flow rate was 1 mL min−1 and the eluent was acetonitrile:water 70:30. Under these condi- tions, retention time was 6 min for chlorpyrifos and 3 and 5 min for spinosyn A and D, respectively, and the Limit of Detection was 0.67 mg L^{-1} for chlorpyrifos and 0.59 mg L^{-1} for spinosad.

5.2.6.2 Comet assay

Coelomocytes were collected as described in Eyambe *et al.* (1991) with slight modifications. Each earthworm was immersed for 4 minutes in an extrusion buffer of 5 % ethanol, 95 % PBS, 2.5 mg mL^{-1} Na_2-EDTA, and 10 mg mL^{-1} guaiacol glyceryl ether (pH 7.3). Coelomocytes were washed and collected by centrifugation (300 g, 10 min, 4°C). The washed cells were counted, resuspended in Low melting agarose (LMA 1 %, 37°C) and stratified on HT Trevigen slides pre-coated with Normal Melting Agarose (NMA 1 %). Each spot was produced by layering LMA containing 3000 cells; each sample was stratified in triplicate. The solidification, lysis and unwinding phases were carried out following Mincarelli et al. (2016). Electrophoresis was conducted at 11 V cm^{-1} for 20 min at 4ºC. Slides were washed in H_2O, neutralised in buffer (0.4 M Tris-HCl pH 7.5), dehydrated in 75 % methanol (Valverde *et al.* 1999; Mincarelli *et al.* 2016), stained with Sybr Gold and then imaged using Lionheart FX Automated Microscope (Biotek, U.S.A.) at 200 × 200 magnification. Comet images were acquired in triplicate and processed to calculate

the major DNA damage index: Tail length (TL), Tail moment (TM), and Tail intensity (TI) (Tiano et al. 2005; Orlando et al. 2018).

5.2.6.3 Analysis of soil bacterial community diversity

Biodiversity analysis of soil bacterial community was based on High Throughput Sequencing (HTS) of 16S rDNA amplicons. Total genomic DNA was isolated using Soil DNA Isolation Kit (NORGEN Biotek, Canada) following the manufacturer's protocol, and V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using the universal primers 343F (5′-TACGGRAGGCAGCAG-3′) and 802R (5′-TACNVGGGTWTCTAATCC-3′), as previously described in detail (Vasileiadis *et al.* 2012, 2015; Bandini *et al.* 2021). Thermal cycling conditions, primer concentrations and volumes are provided in Supplementary Table S5.2.

5.2.7 Statistical analysis and bioinformatics

Statistical analyses were performed in R software (R Core Team 2018, version 3.5.2) with linear mixed-effect models and pairwise significance between groups when the data respected the assumptions. Where the assumptions were not respected, the non-parametric Kruskal-Wallis test and Dunn's post hoc test were used (Bonferroni *p*-value adjustment, $\alpha = 0.05$). Statistical analyses of soil bacterial community diversity were carried out as previously detailed (Vasileiadis et al. 2013; Połka et al. 2015; Cesco et al. 2021). Sequence data is available through Sequence Read Archive (NCBI-SRA) BioProject ID PRJNAXXXXXX.

5.3 Results

5.3.1 Avoidance response

The results of the preliminary dual-control test showed that both validity criteria were achieved for the avoidance tests considering that no earthworms died or escaped and there was no significant preference or aggregation to one section when the same substrate was placed on each side.

The effects of the two insecticides on the avoidance behaviour are given in Figure 5.1; no earthworm escaped or died during the exposure period.

All trials had a positive NR value. Only in the trial with chlorpyrifos at the upper dose ($C_{70}E$), the NR value exceeds 80% (dotted line).

5.3.2 Reproduction responses

The trend of the mean weight of earthworms in each trial is reported in Figure 5.2. Significant differences between the treatments were observed starting from day 14, where the earthworm weight in chlorpyrifos trials resulted significantly lower than the control. In contrast, less marked differences with respect to the control were observed for the spinosad trials. Chlorpyrifos at the highest dose ($C_{70}E$) showed a significant loss of earthworm weight from day 21. At 21 days, the weight measured in the spinosad trial at the highest dose ($S_{150}E$) resulted lower than the control. At the end of the experiment (28 days), also the trial with spinosad at the lowest dose ($S_{70}E$) recorded a significant weight loss. A summary of the observations on the health status of earthworms during this test is reported in Table 5.1.

No mortality was observed in the control and spinosad trial at the lowest dose ($S_{70}E$), while 3.33% was recorded in spinosad at the highest dose ($S_{150}E$) from 21 days. Mortality occurred from the 21 days on at the lowest concentration ($C_{50}E$) of chlorpyrifos, and increased by 10% was recorded at 28 days. Mortality at the highest dose of chlorpyrifos ($C_{70}E$) started on the 14 days differed significantly from other treatments on 21 days (23 %) until it reached a percentage of 40 % on 28 days. Unusual behaviours were absent in control and spinosad, while started on 7 days in the chlorpyrifos treatments. Morphological anomalies were observed only in chlorpyrifos treatments from day 21 (Table 5.1). The production of cocoons in spinosad treatments did not differ significantly from the control. In contrast, there was an evident low production in treatments with chlorpyrifos most significant at the highest dose ($C_{70}E$). Similarly, minimal production of juveniles was recorded at the lowest dose ($C_{50}E$), and no young were counted at the highest ($C_{70}E$).

5.3.3 Toxicity test responses

5.3.3.1 Insecticide residues

The residues of the two insecticides found in the soil during the experiment, which represent the real exposition of the soil organisms to the toxic effect, are shown in Figure 5.3.

Insecticides degradation in soil proceeded with almost the same rate and half-life values, calculated applying the first-order kinetics to the degradation data, resulted in 29.6 days for $C_{70}E$, 26.5 for C_{70}, 19.3 for $S_{70}E$, and 28.9 for S_{70}, showing a slightly faster degradation for spinosad in soil with earthworms respect to the soil alone, while the presence of earthworms did not influence the degradation of chlorpyrifos. Significant differences between the recovery rate of spinosad in the presence of earthworms ($S_{70}E$) and without earthworms (S_{70}) were found.

5.3.3.2 Effect on DNA of living cells

Figure 5.4 shows TM, TL, and TI indexes measured during the toxicity test. Due to their not Gaussian distribution, comet assay data are rep- resented as box plots where the box represent 50 % of the data contained between the 25th and the 75th percentile, and the bars represent the upper and the lower quartiles of distribution. The median value is re- ported in the box by the line that divides the box into two parts.

A significant increase in DNA damage indexes was detected in chlorpyrifos treatment after one day of exposure. On day 21, an increase in all three DNA damage indexes was confirmed; nonetheless, significant differences in the distributions were recorded only for TL. At 28 days, it was impossible to analyse the test's genotoxic damage due to the absence of surviving earthworms. In contrast, spinosad appears to have less impact on coelomocytes. In fact, DNA damage indexes became significant only at the end of the experiment.

5.3.3.3 Impact on soil bacterial community diversity

Hierarchical clustering of bacterial communities at the family level across all samples in this study is presented in Figure 5.5 indicate the formation of clusters

mainly as a function of time. Dynamic response of bacterial communities starting from the first day with the most significant impact on 7 and 14 days was observed. Community composition stabilised after the 14 days, and the group of taxa that contributed less than a 5% "other" was predominant in all samples. On the 7 days, the impact of Bacillaceae was found to be highly pronounced within all the samples almost regardless of treatments and clusters were mostly driven by the families of; *Microbacteriace, Bacillaceae, Nocardioidaceae, Cytophagaceae* and unclassified Solirubrobacterales together with Bacteroidetes. Bacteria from *Chitinophagaceae, Sphingobacteriaceae, Rhodospirillaceae, Erythrobacteraceae, Sphingomonadaceae, Xanthomonadaceae*, families were significant in the formation of the clusters observed in the final samplings.

The impact of pesticides, earthworm's presence and their combination at the beginning (1d) and the end of the experiments (28d) is shown by multivariate canonical correspondence analysis (CCA) in Figure 5.6. These findings confirmed what was observed with the taxonomical clustering of bacterial communities; marked differences caused by various treatments at the beginning of the experiments and fade-out phenomenon as time passed by. Earthworms presence was of utmost importance (p=0.007) for the clustering of bacterial communities in the presence of pesticides (Figure 5.6 c).

At 28 days, the impact of pesticides alone (Figure 5.6 d) has become insignificant (p=0.248), while the effect of the earthworms was still significant (p=0.035*)* (Figure 5.6 e).

The Metastats analysis results singled out several bacterial OTUs of which abundances were significantly affected by the presence of the pesticides in this experiment at the beginning (Figure 5.7 Left) and at the end of experiments (Figure 5.7 Right). Significantly affected OTUs are indicated with significance letters in Figure 5.7.

5.4 Discussion

In the present study, earthworms showed a tendency to avoid contaminated soils. However, only chlorpyrifos at the highest dose resulted in a net response rate above the 80% threshold, indicating a harmful environment (ISO, 2008; Li et al., 2015). A concentration-dependent significant weight loss in chlorpyrifos treatments is in agreement with De Silva et al. (2010). The biomass also decreased in spinosad presence, but it was still less than in chlorpyrifos. Weight loss, a physiological stress index according to Van Gestel et al. (1995) and Frampton et al. (2006), is a dose and time dependent factor, which our results are in agreement with Zhou et al. (2007) were unable to assess the earthworms reproductive activity due to deficient cocoon production at much lower doses of chlorpyrifos. Our results confirm the toxicity of chlorpyrifos on earthworm fertility. On the contrary, in spinosad treatments, reproduction output was not disturbed in agreement with Sekulić et al. (2020), although they worked with lower doses. The hatchability values further confirmed the less impact of spinosad compared to chlorpyrifos. In general the health status of earthworms and their reproductive capacity were closely correlated as reported by Robidoux et al. (2001).

Residual levels of chlorpyrifos were not affected by earthworms, agreeing with Sanchez-Hernandez et al. (2018a). Probably its high Koc cause chlorpyrifos absorption on soil organic matter easily (Mackay, Shiu and Lee, 2006), making it poorly bioavailable (Megharaj et al., 2011). Chlorpyrifos was toxic to the soil bacterial community, and its persistence in the soil is also related to its limited biodegradation (Singh, Walker and Wright, 2002). Several authors (Racke, Laskowski and Schultz, 1990; Coppola et al., 2007) found that antimicrobial properties of its metabolite TCP (3,5,6-trichloro-2-pyridinol) to be the main reason. Chlorpyrifos half-life in the present experiment was lower than those reported by Pesticide Properties Data Base, where it is classified between very persistent/persistent pesticides with a typical half-life value in the soil of 386 days and a range of 19.9-1000 days for a different type of soil. This difference could be

due to the good organic carbon content, which contributed to adsorption on soil colloids and efficient microbial activity. The degradation of spinosad in its natural components occurs through a combination of processes, above all by photodegradation and microbial degradation (Tamez-Guerra et al., 2017). The half-life of this natural insecticide was measured between 9-10 days in case of soil photolysis or between 9-17 days in the absence of light (Thompson, Dutton and Sparks, 2000). The values found in the present experiment are in accordance with those reported in the bibliography, considering that the spinosad experiment was conducted in the dark and demonstrated its low persistence in soil with recovery values that declined consistently with time (Barden 1998; Mandal and Singh, 2013; Telesiński et al., 2015). The presence of earthworms contributes to metabolising spinosad; probably, their digging activity permits more excellent aeration, a condition that allows a faster degradation of spinosad, according to Thompson et al. (2002).

Comet assay is a rapid and sensitive for the detection of DNA damage on the cell level and an essential biomarker in earthworm ecotoxicology (Fourie, Reinecke and Reinecke, 2007; Mincarelli et al., 2019), but only a few papers worked on terrestrial habitat (Martin et al., 2005; Xiao et al., 2006). In the present study, chlorpyrifos compromised the DNA integrity of the coelomocytes immediately for all three indexes analysed (TM, TL and TI). Looking at TL, which is a better indicator of toxicity at low levels of DNA damage (Collins, 2004; Kumaravel et al., 2009), the negative effect of chlorpyrifos is confirmed at 21 days. Despite this, we observed an adaptive mechanism or a selection of resistant coelomocytes for TM and TI, leading to a lower level of toxicity, while at 28 days, a toxic effect with a dramatic decrease in viability was observed. Our data are in agreement with the limited set of studies on genotoxicity induced by chlorpyrifos on earthworms (Casabé et al., 2007; Piola et al., 2009; Curieses et al., 2018), where a significant increase in DNA damage in *E.fetida* coelomocytes treated with chlorpyrifos occurs. The data regarding the

spinosad trial point to a lower and less acute toxicity in terms of organism viability and sub-lethal coelomocyte genotoxicity.

Community composition and relative abundances of soil bacteria in our study are in agreement with Liao et al. (2018), and the presence of *Cytophagaceae*, *Microbacteriace*, *Nocardioidaceae*, too, confirms the findings of the Schlatter et al. (2019) on the potential of earthworms as ecosystem engineers also affecting microbial communities. Some *Microbacteriaceae* and *Bacillaceae* are symbionts of earthworms (Tang et al., 2012; Aira, Pérez-Losada and Domínguez, 2018), and the differences in abundances of these may indicate the passage from soil to the service of the earthworms. In contrast, the negative impact of chlorpyrifos on *Sphingomonas* sp was reported by Medo et al. (2015), but *Bacteroides* sp. and *Bacillus* sp. abundances were similarly lower in chlorpyrifos contaminated soil (Wang et al., 2019). These changes could be related to the fact that exposure to pesticides influences soil bacterial diversity and the gut community composition of earthworms by reducing energy resources and activating the antioxidant systems (Chang et al., 2021) and the immediate impact of the presence of spinosad and chlorpyrifos had on some of the OTUs may be related to pesticide degradation activities of; *Bacillus* sp. (Zeilinger et al., 2010; Oladipo, Burt and Maboeta, 2019; Narayanan et al., 2020; Zhang et al., 2020), *Sphingomonas* sp. (Kumar et al., 2021), *Pseudomonas* sp (Kumar *et al.* 2021), *Luteimonas* sp. (Liu et al., 2019; Elyamine and Hu, 2020; You et al., 2021). Furthermore, the omnipresent Sphingobacteriaceae, such as *Pedobacter composti/luteus* is a resource of secondary microbial metabolites without known chemistries, bioactivities and ecological roles (Figueiredo et al., 2021) in support of its important presence in Metastats. *Caulobacteraceae* sp. abundance at the 28d is in accordance with Schlatter (2019), in which earthworm presence in the soil was found to be beneficial. Our findings also agree with the only study in the literature on *Ilumatobacter* sp. by Vasileiandis *et al*. (2018) for the initial negative but transient impact of these insecticides. Overall, the effect of these insecticides at sub-lethal doses are in accordance with the recent review by Vischetti *et al*. (2020) regarding

its dependence on time scale and adaptability of the microorganisms. To the best of our knowledge, the present study is the first to report the impact of spinosad and chlorpyrifos on *Intrasporangium* sp., *Saccharibacteria* sp. and *Phenylobacterium* sp.

5.5 Conclusions

The present study found that earthworms' behaviour, state of health and reproduction align with the damages at the DNA level. Chlorpyrifos caused a substantial morphological impairment and functional anomalies from the earliest samplings, while the impact of spinosad remained minimal. Former also negatively affected DNA integrity at early stages, and the degenerative trend led to the death of the later samplings. Whereas the latter's impact was significant only after 28 days of exposure. Time was the main factor for bacterial community changes, and then, treatments and earthworms' presence were important factors too, indicating the crucial role of *E. fetida* on the toxicity of the insecticides. The present work, by multi-technique approach, successfully identified the non-target impact of these insecticides at an early stage, reflecting the ecosystem's health status and therefore sets an example to future studies on how to estimate the potential real environmental impact of pesticides.

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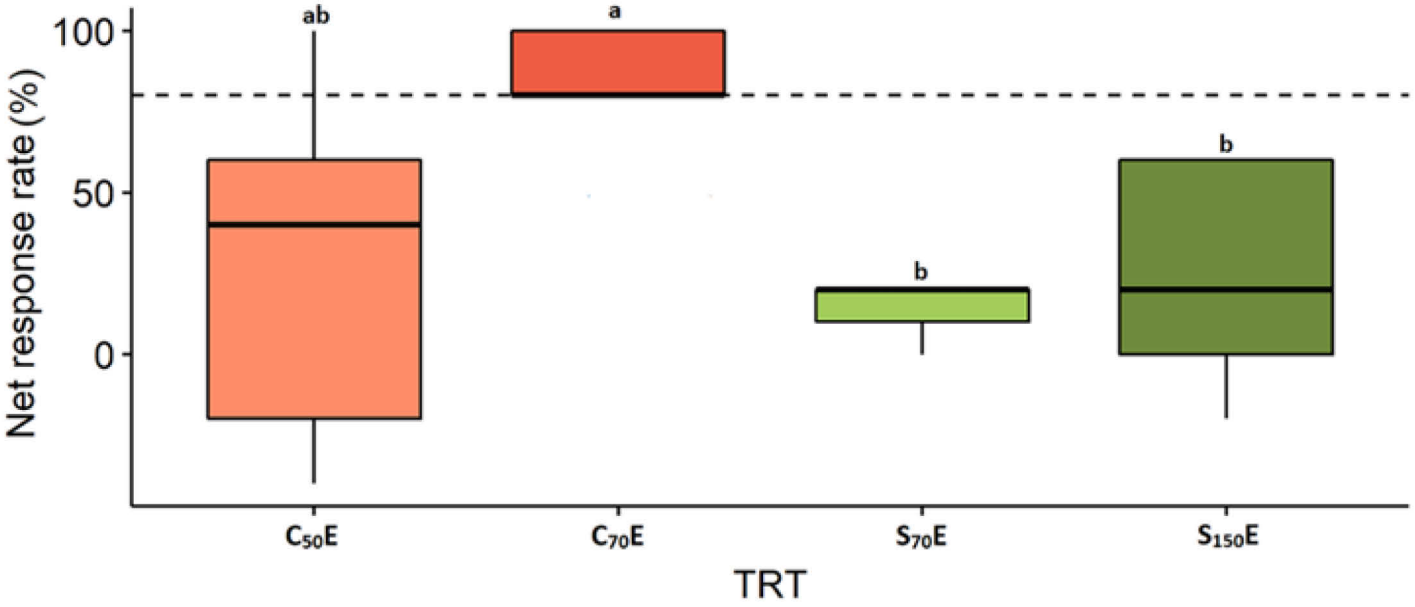

Figure 5.1 Avoidance behaviour in *E. fetida* expressed in Net Response rate. Treatments (TRT): $C_{50}E$ (chlorpyrifos up to 50 % of the LC_{50}), $C_{70}E$ (chlorpyrifos up to 70 % of the LC_{50}), $S_{70}E$ (spinosad up to 70 % of the LC_{50}), and $S_{150}E$ (spinosad up to 150 % of the LC_{50}). According to Dunn's Kruskal-Wallis multiple comparisons, treatments with different lowercase letters were significantly different.

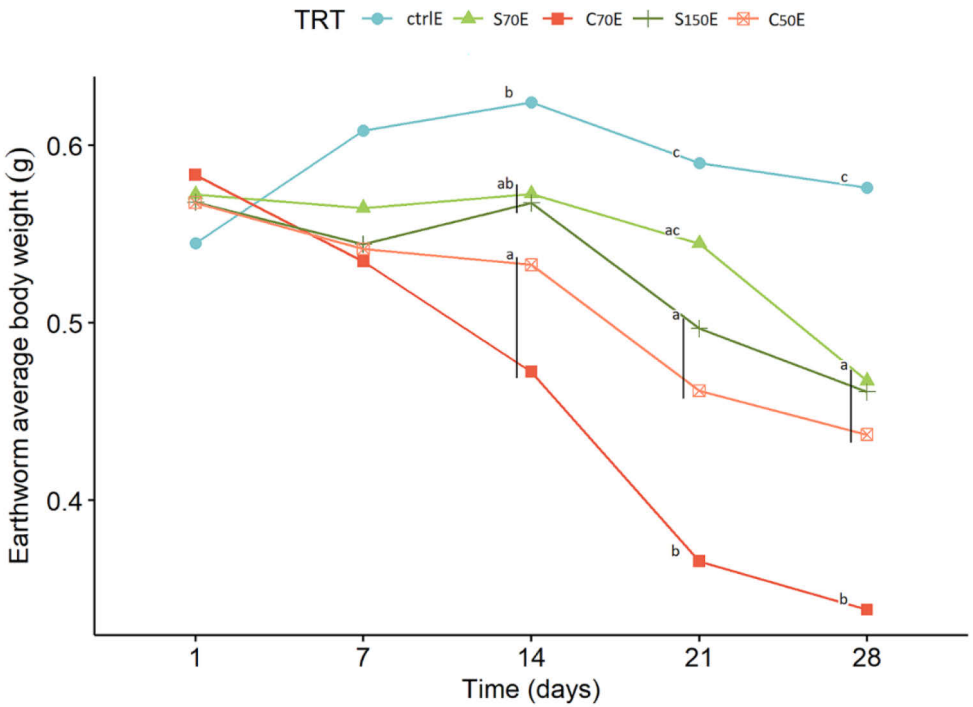

Figure 5.2 Weight trend during the reproduction test. Treatments (TRT): ctrlE (negative control), $S_{70}E$ (spinosad up to 70 % of the LC_{50}), $S_{150}E$ (spinosad up to 150 % of theLC_{50}), $C_{50}E$ (chlorpyrifos up to 50 % of the LC_{50}) and $C_{70}E$ (chlorpyrifos up to 70 % of the LC_{50}). According to Dunn's Kruskal–Wallis multiple comparisons, treatments at the same time with different lowercase letters were significantly different.

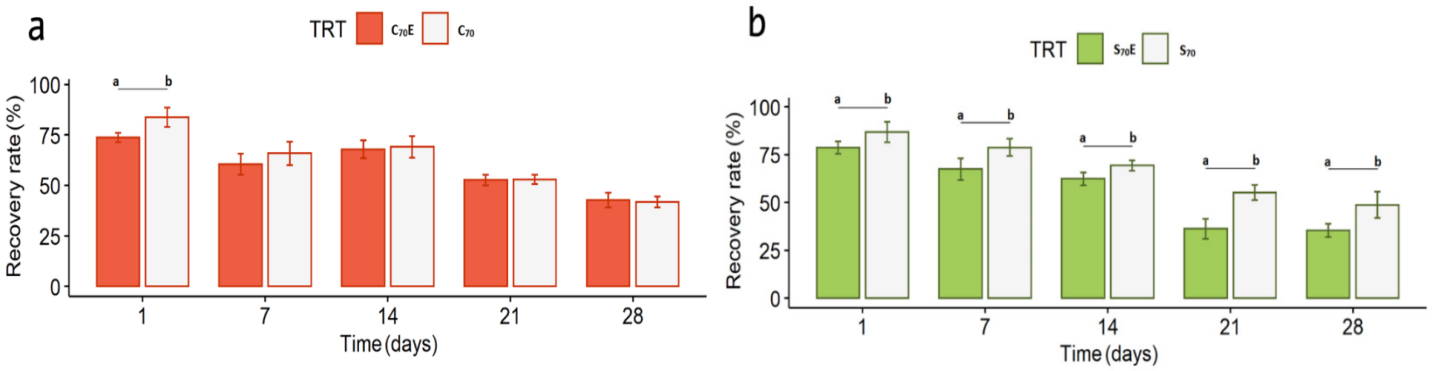

Figure 5.3 Insecticides recovery from the soil. **a)** Chlorpyrifos. Treatments (TRT) of chlorpyrifos up to 70 % of the LC_{50} with earthworms ($C_{70}E$) and without earthworms (C_{70}). **b)** Spinosad. Treatments (TRT) of spinosad up to 70 % of the LC_{50} with earthworms ($S_{70}E$) and without earthworms (S_{70}). Data were present as means ± RSD (n=3). Lowercase letters refer to significant differences between treatments at each sampling time according to Linear mixed-effects models multiple comparisons.

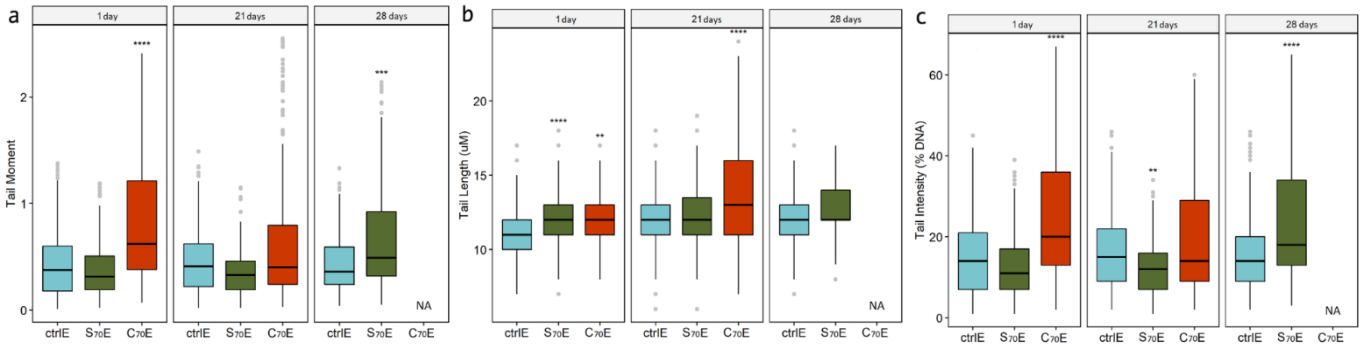

Figure 5.4 Tail moment **(a)**, Tail Length **(b)** and Tail Intensity **(c)** in *E.fetida* coelomocytes. Treatments (TRT): ctrlE (negative control), $S_{70}E$ (spinosad up to 70 % of the LC_{50}), $C_{70}E$ (chlorpyrifos up to 70 % of the LC_{50}). Significance of variation was calculated versus unexposed control according to Dunn's Kruskal–Wallis multiple comparisons (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

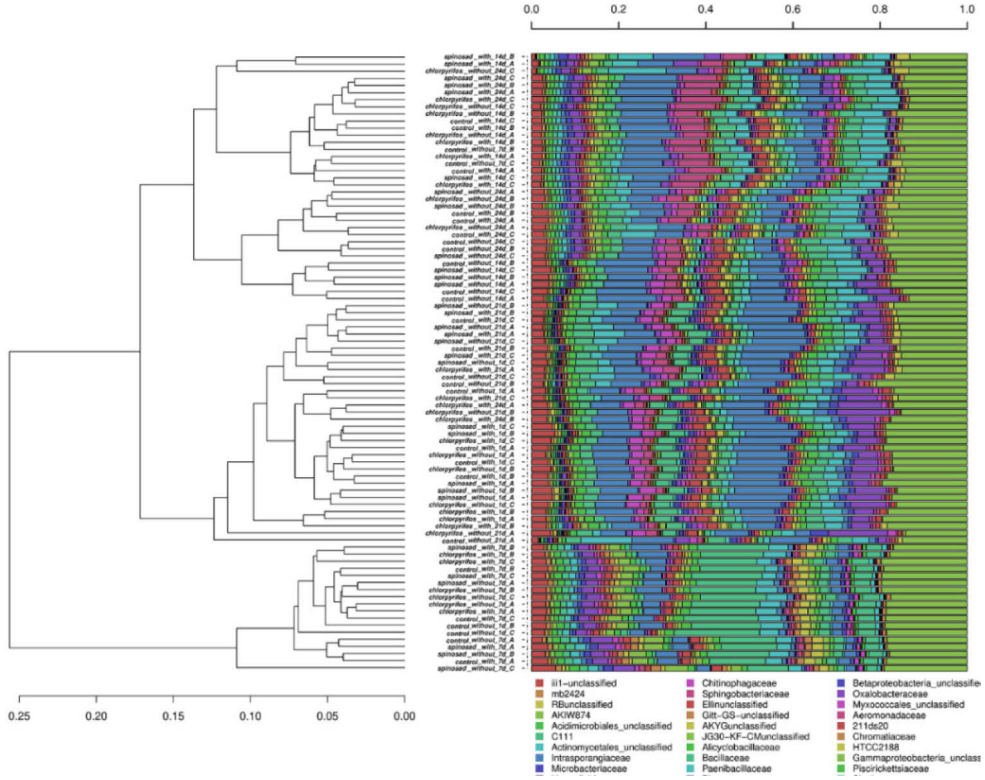

Figure 5.5 Taxonomic comparison of soil bacterial communities at the family level through hierarchical clustering across all samples used in this study. Clusters were identified with the average linkage algorithm for taxa that contributed at least 5 % to a single sample. Taxa that contributed less than this threshold were added to the sequence group denoted "other."

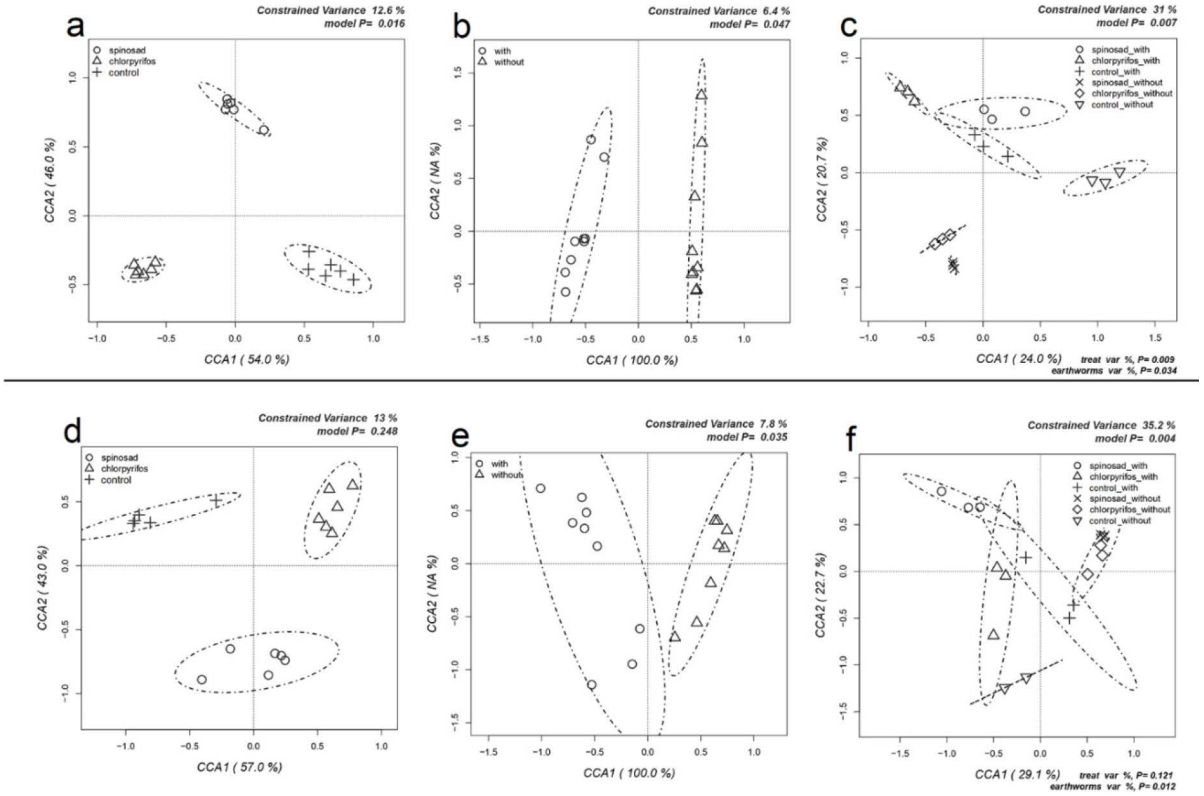

Figure 5.6 Canonical correspondence analyses (CCAs) on the impact of the various factors on the structure of soil bacterial communities; pesticides **(a,d)**, earthworms **(b,e)**, pesticides and earthworms together **(c,f)** by days after treatment (**1d:** upper half, **28d:** lower half). These were determined by the relative abundances of all the OTUs obtained by Illumina sequencing of bacterial 16S amplicons.

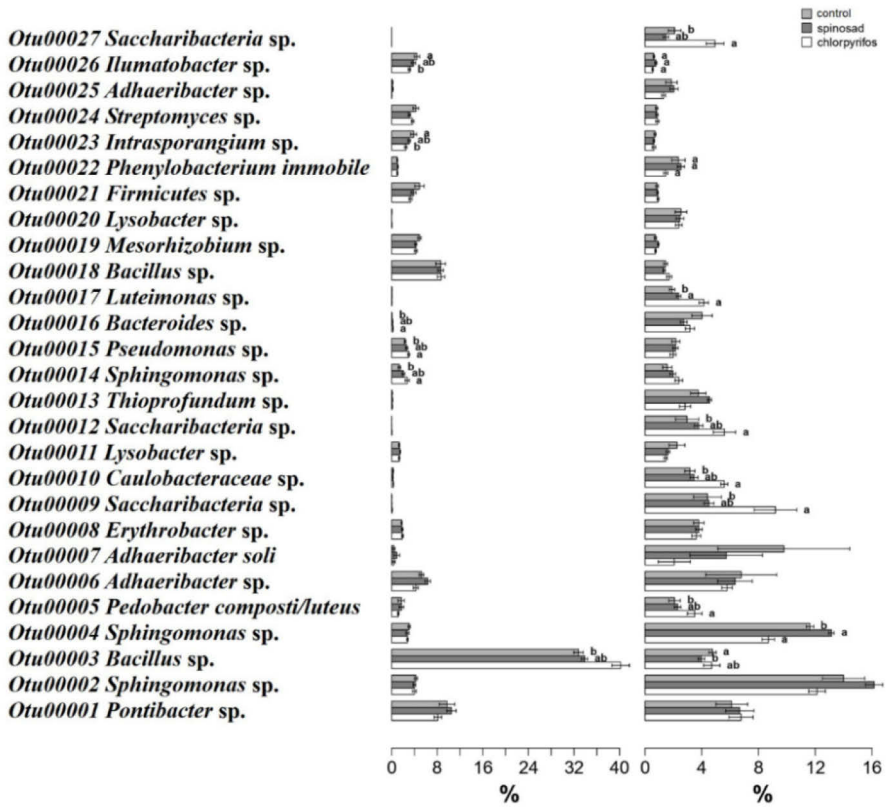

Figure 5.7 Bacterial OTUs as analysed by a Metastats aimed at identifying the ones with significant differences (letters or *, $p \leq 0.05$) between pesticides treatments (Left: First sampling at 1d, Right: Final sampling at 28d).

Table 5.1 Observations on earthworms in the reproduction test.

PARAMETERS	TRT ctrlE	$S_{70}E$	$S_{150}E$	$C_{50}E$	$C_{70}E$
NO. COCOONS/ REPLICATE (± SD; 56 DAYS)	72.67 ±11.24[b]	70.00 ±9.00[b]	59.00 ±2.00[ab]	19.67 ±7.23[ab]	4.67 ±8.08[a]
NO. JUVENILES/ REPLICATE (± SD; 56 DAYS)	159.00 ±20.07[b]	138.33 ±13.01[ab]	68.00 ±37.27[ab]	3.33 ±3.06[a]	**NA**
HATCHABILITY (% ±RSD;56 DAYS)	2.19 ±0.05[b]	1.98 ±0.03[ab]	1.14 ±0.52[ab]	0.15 ±0.87[a]	**NA**
GROWTH RATE (% ±RSD; 28 DAYS)	5.63 ±2.76[b]	-18.79 ±0.82[ab]	-19.95 ±0.52[ab]	-23.96 ±0.07 [ab]	-42.40 ±0.08[a]
MORTALITY (% ±RSD; 28 DAYS)	**NA**	**NA**	3.33 ±1.73	10.00 ±1.00	40.00 ±0.25
UNUSUAL BEHAVIOUR	**NA**	**NA**	**NA**	✓	✓
MORPHOLOGICAL ANOMALIES	**NA**	**NA**	**NA**	✓	✓

According to Dunn's Kruskal–Wallis multiple comparisons, treatments with different lowercase letters were significantly different.
NA: not available✓: presence of the parameter
Unusual behaviour: low reactivity, less digging activity, compulsive movements
Morphological anomalies: injuries, miniaturisation, abnormal colouring

Table S5.1 Physical and chemical properties of chlorpyrifos and spinosad.

	Active ingredient proprieties	
	Chlorpyrifos	**Spinosad**
Pesticide type	Organophosphorus insecticide	Natural insecticide, microorganism derived
Mode of action	Acetylcholinesterase inhibitor	Acetylcholinesterase inhibitor
Molecular mass (g/mol)	350.58	731.98+745.98
Solubility in water (mg/L)	1.05	7.6
Kow (Log P)	4.7	4.1
Koc (L/kg)	5509	34600
Laboratory soil DT50 (20°)	386	15
Vapour pressure at 20 °C (mPa)	1.43	1.00×10^{-05}
	Insecticides commercial formulations	
	Dursban 75 WG	**Laser 480**
Active ingredient (%)	75	44
Formulation form	Powder	Liquid
Purchased from	Dow AgroSciences	Dow AgroSciences
LC_{50}, Eisenia fetida, 14 d (mg/kg)	681	>1000
Exposure concentrations		
Reproduction tests (mg/kg d.w.)	340,5 ; 476,7	735 ; 1575
Avoidance tests (mg/kg d.w.)	340,5 ; 476,7	735 ;1575
Toxicity tests (mg/kg d. w.)	476,7	735

Pesticide properties obtained from the PPDB database http://sitem.herts.ac.uk/aeru/ppdb/.

Table S5.2 PCR reaction mixtures and thermal profiles for different target genes used.

Target Gene	Reaction Mix	Volume (µL)	Step 1
16s rRNA 1st step	Phusion Flash High-Fidelity Master Mix	12.5	94 °C - 5 min 25x {94°C 30s; 50°C 30s; 72°C 30s} 72 °C 10 min.
	Nuclease free water	8	
	DNA template (1ng/µL)	2	
	Primer 343F (10µM) (5′-TACGGRAGGCAGCAG-3′)	1.25	
	Primer 802R (10µM) (5′-TACNVGGGTWTCTAATCC-3′)	1.25	
16s rRNA 2nd step	Phusion Flash High-Fidelity Master Mix	12.5	94 °C - 5min 10x {95°C 30s; 50°C 30s; 30°C 30s} 72 °C 10min.
	Nuclease free water	8	
	1st Step Amplicons	1.25	
	Primer 343F (10µM) (5′-TACGGRAGGCAGCAG-3′)	1.25	
	Primer 802R (10µM) (5′-TACNVGGGTWTCTAATCC-3′)	1.25	

CHAPTER 6: Copper monitoring in vineyard soils of central Italy subjected to three antifungal treatments, and effects of copper sub-lethal doses on the earthworm *Eisenia fetida*

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Abstract

The extensive employment of copper-based fungicides has increased copper concentration in vineyard soils. The present study's objectives were to monitor copper concentration in two vineyard soils during two cropping seasons, and study the ecotoxicological effects on the earthworm *Eisenia fetida*. Total, soluble and bioavailable copper fractions were measured at the end of two cropping seasons and at different depths in two vineyards of central Italy, characterised by three anticryptogamic control methods: copper compounds, chitosan, and combined treatments of them. A laboratory experiment to assess the effects on *Eisenia fetida* was conducted with soil samples collected in the vineyards with a mean copper concentration of 60 mg/kg and two higher concentrations of 90 and 150 mg/kg. Results showed low levels of total copper concentration in the first 20 cm of soils, regardless of antifungal treatment, highlighting a prudent management of the vineyards under study, but the soluble fractions showed a significant increase in all samples during the two cropping seasons. At the dose of 150 mg/kg, earthworms suffer during the first two days, showing weight loss and DNA-damage, but they are able to recover until day 28, showing no permanent harm at this copper concentration in soil.

Keywords: copper fungicide; soil contamination; ecotoxicology; earthworms; *Eisenia fetida*; Comet Assay; vineyard.

6.1 Introduction

Copper (Cu) is an essential micronutrient, but when in excess, can be harmful to biological systems. Due to anthropogenic activities, the concentration of Cu has increased in the environment and can affect human health, considering the possibility of a dietary intake associated with contaminated crops (Rai et al. 2019; Kumar et al. 2021). If ingested in large quantities, Cu can cause hepatic and neurological diseases (Gaetke, Chow-Johnson and Chow 2014).

One of the major anthropogenic sources of copper contamination in soils is the long-term use of Cu-based compounds in agrochemistry which contributed to a significant increase in soil Cu concentrations over the last decades (Merrington, Rogers and Van Zwieten 2002; Wightwick et al. 2008; Komárek et al. 2010). Azimi et al. estimate an annual copper deposition for agricultural inputs that varies between 100 and 800 g Cu/km^2 depending on fertiliser and type of crop (Azimi et al. 2004).

A recent large-scale study showed the highest Cu concentrations in vineyard soils, among other land uses, with a mean concentration of 49.3 mg/kg, which is three times higher than the average general concentration in European soils, and copper contamination occurs mainly in wet areas due to the frequent fungicide treatments. Copper values reported by this survey show high variability between samples, of which a considerable percentage (14.6%) exceeds 100 mg/kg (Ballabio et al. 2018). Many worrying cases regarding copper in vineyard soils were detected at the European level (Droz et al. 2021). For instance, concentrations around 200-400 mg/kg were found in French soils (Jacobson et al. 2005; Chopin et al. 2008; Probst, Schüler and Joergensen 2008), while in Spain, values ranging between 500 and 600 mg/kg were measured (Nóvoa-Muñoz et al. 2007; Pateiro-Moure et al. 2007); furthermore, in some Croatian vineyards, Cu reached 700 mg/kg (Romić et al. 2004).

In Italy, several studies and regional surveys report highly variable concentrations of copper among vineyards, ranging from 30 to over 300 mg/kg, so there are cases in which copper exceeds the national law threshold indicated for land for agricultural

use of 200 mg/kg (Bretzel and Calderisi 2006; Cattani et al. 2006; Dell'Amico et al. 2008; ARPA Piemonte 2014; ARPAV 2019).

The biological nature of metal bioavailability requires biological assays to understand its mechanism of toxicity(Lanno et al. 2004). Among soil organisms, earthworms are more susceptible to metal pollution than many other soil invertebrates and could be used as biological tools in ecotoxicological studies (Puglisi et al. 2009; Bari et al. 2010; Spagnuolo et al. 2010; Wang et al. 2018). Several earthworm toxicity assays relative to different thresholds of toxicity can be studied to evaluate the potential adverse effects of xenobiotics on the environment (Vischetti et al. 2020). While chemical analytical methods alone are not adequate for a comprehensive quantification of metal bioavailability and both Cu speciation in soils, biological parameters can provide a useful complementary approach to the evaluation of Cu bioavailability in soil (Wang, Zhou and Cang 2014).

As copper-based compounds used in agriculture meet the criteria of persistence and toxicity, Commission Implementing Regulation 2015/408 (European Commission 2015) included these substances (copper hydroxide, copper oxychloride, Bordeaux mixture, tribasic copper sulphate and copper oxide) in the list of chemical candidates for substitution. Currently, the European Commission concluded that further ecotoxicological studies, specifically on soil organisms, are needed for updated risk assessment (European Commission 2018).

Karimi et al. (2020) reported that the primary consumers of copper-based fungicides, organic viticulture, presented better soil biological quality than conventional viticulture, but the relative contribution of each viticultural practice could not be established, and no data on the effect of Cu application was provided. A No Observed Adverse Effect concentration (NOAEC) of 4 kg Cu/ha/year on average was reported from a study considering the transient effects on abundance and biomass of earthworms (Strumpf et al. 2015).

From an exhaustive meta-analysis on Cu ecotoxicology relative to 19 articles out of 300 papers relevant to copper and soil biological quality performed by Karimi et al.

(2021) it was assessed that Cu accumulated during years in the soil started to be deleterious for earthworms biomass at concentrations above 200 kg Cu/ha/year. Moreover, literature analysis shows a substantial gap in relation to ecotoxicological effects at doses below 4 kg Cu/ha/year chronic contamination, in particular in relation to long-term effects on soil biodiversity (Karimi et al. 2021). This data highlights the need of studies that address the environmental risk linked to historical use of copper in European vineyard soils.

Moreover, at the European level, research has been promoted on alternative products to supplement or substitute cupric fungicides, focusing on natural compounds registered as plant protection products (PPP) or plant biostimulants (Romanazzi et al. 2021). Among the substitutes for copper in the antifungal fight in the vineyard, growing interest is observed for chitosan as it is a biocompatible, biodegradable and non-toxic polymer molecule (Romanazzi, Feliziani and Sivakumar 2018).

The main objective of the present study was to verify the effects on copper concentrations in the soil due to the application of three different antifungal control strategies: i) cupric compounds, ii) chitosan, iii) alternation of cupric compounds and chitosan.

The three copper fractions (total, bioavailable and soluble) were monitored in two commercial organic vineyards conducting experiments for two years to test the long-term effects of natural alternative treatments to copper.

It has been hypothesised that the careful management of treatments, following national guidelines and using new generation antifungals as partial or total substitutes for cupric compounds may not cause concern in the accumulation of this element in the soil.

Furthermore, a second objective was to better assess the impact of copper, at three sub-lethal doses, on the soil ecosystem by conducting an ecotoxicological test with earthworms on mesocosms set up with natural soil and which provided early damage detection through the comet assay technique.

6.2 Materials and Methods

6.2.1 Monitoring campaign

6.2.1.1 Study areas

This study was carried out in two pilot vineyards in the Ancona province (Marche Region, Central Italy), distant about 37 km from each other. Specifically, one vineyard (V) is in the Varano hamlet of Ancona (centroid coordinates 43°33'11.42"N; 13°32'10.85"E), while the other (C) is located in Castelplanio municipality (centroid coordinates 43°30'16.03"N; 13° 4'54.31"E). As stated in the only complete Italian map that takes into account the Worldwide Bioclimatic Classification System according to Rivas-Martínez (1993), both vineyards fall into the macrobioclimate Temperate and the Submediterranean bioclimatic variant (Pesaresi, Biondi and Casavecchia 2017). The following bioclimatic units (in top-down hierarchical order) are bioclimate Oceanic Temperate, thermotype Mesotemperate (Lower and Upper respectively for V and C vineyard) and ombrotype Subhumid (Lower and Upper respectively for V and C vineyard).

According to what is indicated in Italy's soil map and the Typological Units database, the types of soil can be catalogued in *Haplic Calcisol* and *Calcaric Cambisol*, respectively, for Castelplanio and Varano.

In both organic vineyards, experiments are underway on different antifungal treatments. For this reason, each vineyard is interested in several treatments, and each antifungal treatment is conducted in groups of three consecutive rows. The length of the rows in the two vineyards is not regular; in the Varano vineyard, the average length of a row is 150 m, while in the case of Castelplanio of 100 m, the distance between the rows is fixed at 2.2 m and 2.5 m for the Varano and Castelplanio vineyards respectively.

6.2.1.2 Sampling

Two monitoring campaigns were carried out at the end of the harvest (October 2020 / October 2021) in the two vineyards C and V.

In both vineyards, the sampling regarded the rows in which the following antifungal treatments are applied:

A) Copper treatments (use for the entire growing season of copper-based products);

B) Alternate treatments (use copper in the first part of the season and after flowering use of chitosan at 0.5% of p.a.);

C) Chitosan treatments (use of 0.5% p.a. chitosan for the entire growing season).

In "A", an average of 2.5 kg/ha of copper metal (Cu^{2+}) was used, mainly distributed in the form of copper sulphate tribasic, neutralised copper sulphate, copper hydroxide and cuprous oxide, while for "B", the use of an average of 1 kg/ha of Cu metal distributed with the same products was estimated. The number of Cu treatments is summarised in Table 6.1, for the two years 2020 and 2021.

For each plot consisting of three contiguous rows and relating to one of the three theses previously indicated (A, B and C), the central row was selected (to avoid drift contamination), and five equidistant sampling points were spaced along the entire length of the row as reported in Figure 6.1a and c. In each of these five points, two soil samples were taken at the two depths of 0-20 cm (Z) and 20-40 cm (Q).

This sampling design was used in both vineyards in 2020 and was repeated in the same way in 2021.

Each sample was air-dried, ground manually, and sieved at 2 mm to obtain about 3 kg of fine earth for each sample.

To obtain three homogeneous samples for each depth (Z and Q) and thesis (A, B and C), the same soil portions of each of the five sampling points were suitably mixed for a total of 12 macro-samples (3 theses X 2 vineyards x 2 depths), each analysed for physico-chemical characteristics (Table 6.2).

To get a picture of the two vineyards' climatic situation and the two years of sampling (Figure 6.2), data relating to the rainfall regimes and median temperatures measured in weather stations closest to the two vineyards from the Marche Civil Protection database (www.protezionecivile.regione.marche.it) were analysed and processed.

For the Varano vineyard (V), station 613-Baraccola (sensor code 2854) was selected, while for the Castelplanio (C), station 506-Moie (sensor code 3021) was used.

6.2.2 Ecotoxicological study on earthworm *Eisenia fetida*

6.2.2.2 Experimental design

The evaluation of different Cu concentrations effects on *Eisenia fetida* was carried out using the Varano soil collected in 2020 at a depth of 0-20 cm and belonging to thesis A (Copper treatments: VAZ); mean total Cu content of 60 mg/kg. To test other concentrations, this soil was further contaminated with the commercial product Siaram 20 WG (Isagro), containing copper sulphate. Specifically, fresh solutions of Siaram 20 WG, have been prepared and added to the starting soil (VAZ) to reach the final total copper concentrations of 90 mg/kg (VAZ90) and 150 mg/kg (VAZ150). Before starting the ecotoxicological test, all three treatments were incubated for 2 days for equilibration.

In the laboratory*, E.fetida* earthworms were reared at 20±1°C in organic compost and fed with organic vegetables.

Each treatment was conducted in three replicates consisting of 600 grams of dry soil, 12 purged earthworms and 27% of soil moisture.

Five sampling times (2, 7, 14, 21, 28 days from contamination) were established to follow the evolution of morphological and behavioural changes in earthworms. All earthworms per replicate were carefully removed from the substrate and observed at each sampling time. Then, one random earthworm per replicate (three earthworms per treatment) was taken for the Comet assay, while the others were returned to the respective vessel.

Any visible change in morphology (narrowing, miniaturisation, lesions, etc.), behaviour (spasms, disability to dig, etc.), and mortality (calculated as percentage variation compared to the initial number of earthworms) were recorded following the OECD guidelines (OECD 1984, 2016).

6.2.2.3 Comet assay

Earthworms coelomocytes were collected following the Eyambe protocol (1991) with slight modifications. Briefly, each earthworm was immersed for 4 minutes at room temperature in an extrusion buffer of 5% ethanol, 95% PBS, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glyceryl ether (pH 7.3). Coelomocytes were washed and collected by centrifugation (300g, 10 min, 4°C). The washed cells were counted, resuspended in Low melting agarose (LMA 1%) at 37 °C, and stratified on HT Trevigen slides pre-coated with 1% Normal Melting Agarose (NMA 1%). Each spot was produced by layering LMA containing 3000 cells; each sample was stratified in triplicate. The solidification, lysis and unwinding phases were carried out following Mincarelli et al. (2016). Electrophoresis was conducted at 11 V/cm for 20 min in a refrigerated room at 4 ◦C. Slides were washed in H_2O, neutralised in buffer (0.4 M Tris-HCl buffer adjusted to pH 7.5), and finally dehydrated in 75% methanol (Valverde et al. 1999; Mincarelli et al. 2016). Once dried, slides were stained with Sybr Gold, and images were automatically collected using Lionheart FX Automated Microscope (Biotek, Winooski, Vermont, U.S.A.). Observations were performed at a magnification of 200×. 200 Comet images for each treatment at each time point were acquired in triplicate and subsequently image processed using a custom made analytical software able automatically detect comet and calculate the major DNA damage index: Tail length (TL), Tail moment (TM), and Tail intensity (TI) (Tiano et al. 2005; Orlando et al. 2018).

6.2.3 Copper analyses

The soluble Cu extraction was carried out with distilled water (1g/10mL) accord-ing to the cession-test reported in the UNI EN 12457-Part 2 (2004) while the bioavailable fraction was extracted with a solution of Diethylenetriaminepentaacetic acid (DTPA), $CaCl_2 \cdot 2H_2O$ (0.01 M) and triethanolamine (0.1 M) at pH 7.3 (1g/2mL), following the indications in the Italian Official Gazette n. 248 (1999). For the total Cu extraction, the protocol in aqua regia by Kasassi et al. (2008) was employed, with some modifications: 0.5 grams of dry soil and 2 mL of hydrogen peroxide (H_2O_2) at

30% are left overnight; after 12 hours, the acid attack is carried out by adding 7 mL of HNO_3; the test tubes are placed with a float in a preheated water bath (> 85 ° C) for 15 hours; at the end of 15 hours, the samples are filtered. According to EPA 6010D (2014), the Cu analysis was carried out by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent mod. 5800).

In the monitoring study, for each of the 12 groups (3 theses X 2 vineyards X 2 depths), the measurements were carried out in five replicates, each corresponding to five sampling points. Instead, the copper measurements were carried out in three replicates for each treatment in the ecotoxicological study.

6.2.4 Statistical analysis

Differences in copper concentrations between groups were assessed using a non-parametric test because of non-normal distributions of the data. Statistical analyses were performed in R software version 4.1.3 (R Core Team 2022).

Specifically, the Kruskal-Wallis test was employed to evaluate the presence of significant differences. When this test reported such presence, in order to determine which groups differed from the others, post-hoc pairwise Dunn test was conducted (Dunn 1964). A compact letter display was constructed using the cld function of the rcompanion package of R (Mangiafico 2017) to show significant differences (α = 0.05) between the least-squares means. Groups not sharing any letters are significantly different.

Statistical analysis of Tail intensity data comes from the comet assay was performed using the GraphPad Prism version 5 software. Kolmogov–Smirnov test has been used to check data distribution, and significant differences among groups have been verified using ANOVA one way test and Dunn's post hoc test.

6.3 Results

6.3.1 Monitoring study

It can be observed from Figure 6.2 that the temperature trend was very similar for the two sites, with the highest average monthly temperatures in August in 2020

(25.12 and 24.92 ° C for C and V, respectively) and July in 2021 (26.24 and 25.46 ° C for C and V, respectively).

The lower average monthly temperatures were measured in January in both vineyards (in 2020, values of 6.09 and 4.98 ° C, while in 2021, 5.73 and 4.55 ° C were recorded for C and V, respectively).

The rainfall recorded in 2020 and 2021 was more conspicuous in the Castelplanio vineyard than for the Varano vineyard, except in sporadic cases (January, May, June, July 2021 and March 2020).

For vineyard C the wettest months were December (110.8 mm), June (110.6 mm) and May (102.6 mm) in 2020 and November (179.0 mm), December (139.8 mm) and October (138.0 mm) in 2021.

Near the V vineyard, December (92,4 mm), March (84.6 mm) and October (84.2 mm) were the wettest months in 2020, while for 2021, there was greater cumulative rainfall in November (169.4 mm), December (97.0 mm) and January (93.8 mm).

The sampling campaigns conducted during the two years allowed to perform an accurate analysis of Cu concentrations in the two soil layers of the experimental vineyards.

The results for the year 2020 at the two depths of 0-20 and 20-40 cm are reported in Figures 6.3, 6.4 and 6.5, respectively, for the three Cu fractions; total, bioavailable and soluble.

As shown in Figure 6.3, the metal is mainly accumulated in the first 20 cm of soil; differences between the two depths are significant in all three theses for the Varano vineyard, while for Castelplanio vineyard, the trend was confirmed but with no statistically significant differences. It does not seem that the different thesis (A, B and C) have led to significant differences in the concentrations of total copper; however, higher values were found in the thesis in which the Cu dose distributed was the highest (A).

The values of bioavailable copper ranged between 6.59% and 16.96% with respect to total Cu, among vineyards, theses and depth. The Varano vineyard, thesis A,

where the greatest amount (2.5 kg/ha) of copper was added to the soil, showed a significantly higher concentration of bioavailable than the other two theses in the first 20 cm of soil. The same trend was also found in Castelplanio vineyard but with values not statistically different.

Observing the values of soluble copper in Figure 6.5 (in µg/kg), they result in very low percentages compared to the other fractions, with a greater amount of the metal in the thesis A in both vineyards.

In analogy with the data reported for 2020, the data relative to the 2021 monitoring campaign are reported in Figures 6.6, 6.7 and 6.8, respectively, for total, bioavailable and soluble Cu. Even if the trend for the three Cu species in the three theses A, B and C is similar to the year 2020, no significant differences were found between the three theses. A decreasing trend was observed between the two depths in both vineyards, even if not statistically significant, indicating lower mobility of copper in this second year of experimentation.

Table 6.3 shows the two-year variations of the three copper fractions for each sample. The variation of Cu concentration was calculated as a percentage of the initial concentration measured in the 2020 monitoring campaign.

In the case of total Cu, a generalised decrease in Cu concentration was observed, but only in the case of the greater depth of the Castelplanio vineyard, thesis with the Cu-treatment (CAQ) the variation between 2020 and 2021 is statistically significant.

Regarding bioavailable Cu, significant changes were recorded in two samples, VBZ and CAQ.

On the contrary, the growth trend of soluble copper is evident and almost always statistically significant. However, the changes in concentration during the two years is always referred to a very little percentage of the total.

6.3.2 Ecotoxicological study on earthworm *Eisenia fetida*

6.3.2.1 Cu concentrations in soil

The Cu concentration within the three treatments resulted stable during the 28 days of the test and the fluctuations in concentrations are not to be considered significant

between the beginning and the end of the test. Consistently with the fortifications, during the entire trial, we see the significant differences between the concentrations of the three highest Cu fractions (soluble, bioavailable and total) measured in the VAZ150 samples as compared to the smaller ones in VAZ (Table S6.1).

As shown in Figure 6.9, a decrease in mean weight was measured in all three concentrations on day 2, markedly for the 150 mg/kg dose (VAZ150). The weights at the field concentration (VAZ) and 90 mg/kg (VAZ90), after recovery on day 7, remained almost constant, while the weights of VAZ150 had a recovery trend higher than the other two doses at days 7, and continues to increase until day 28.

The observations conducted on earthworms during the test seem to follow a dose-response trend. The first appearance of morphological anomalies is recorded at day 14 only at the copper dose of 150 mg/kg, while in the case of 90 mg/kg, it was observed from day 21. Unusual behaviour such as lying motionless on the surface thus inability to dig was found at the end of the test (day 28) at the two fortified doses (VAZ90 and VAZ150).

Suffering observations were completely absent at the field dose (VAZ) for the entire test period. Mortality was revealed in VAZ150 from day 14 (2.8 %), and it has increased till the end with a percentage of 5.6 and 8.3 respectively at 21 and 28 days.

Regarding DNA damage of earthworms, the results in terms of Tail Intensity (TI) are shown in Figure 6.10.

At the field concentration (VAZ), the DNA damage showed a trend of decrease over time with respect to the damage at day 2 (TI values of 83%), reaching TI values significantly different of 72% and 78% at days 21 and 28, respectively. In the VAZ90 treatment, no significant differences were found between the damage at days 7, 14, 21 and 28 and the initial damage at day 2.

An evident and significant trend of decrease in DNA damage indexes was observed in the thesis with the highest Cu concentration (VAZ150), indeed despite the fluctuation measured at 14 and 21 days, the DNA damage remains always significantly lower respect to that observed at day 2.

Table 6.4 reports the median Tail Intensity values and the differences in DNA damage at the five sampling times between the test at field Cu concentration (VAZ) and those at 90 and 150 mg/kg (VAZ90 and VAZ150). The comparison showed that at the higher dose of copper, from 7 days onwards, significantly lower DNA damage indexes were recorded except for day 21. On the contrary, the damage levels measured in VAZ90 are similar to those measured in the coelomocytes analysed in the earthworms maintained at the field dose (VAZ).

6.4 Discussion

The monitoring campaign conducted in the two years 2020 and 2021, showed a concentration of copper in the first 20 cm of soil higher than that measured in the 20-40 cm at all sampling points. The accumulation of copper in vineyards soil, especially in the first 15 cm of depth, is widespread (Lamichhane et al. 2018).

This is quite expected considering that the first layers of soil are those most exposed to the residues of the spraying of copper-based fungicides and that they are also the richest in organic matter (Table 6.2), which is the main responsible for the adsorption of Cu (Romić et al. 2014).

The values of total copper measured ranged between 36.64 mg/kg and 63.82 mg/kg, among vineyards, theses and depth, showing that the cupric treatments carried out over the years of cultivation have scrupulously followed the European and national laws regulating Cu amount to be used in organic farming setting at 4 kg/ha/year (U.E Commission 2018). Values found are lower for both depths than the contamination threshold concentration for agricultural areas set at 200 mg/kg by the Italian Decree n ° 46 (Italian Official Gazette 2019), and still, they are always below 100 mg/kg, which is the most stringent limit in Italy for the copper content in agricultural soils destined for fertilisation with sewage sludge (Italian Official Gazette 1992).

At the European level, there is a lot of variability between Nations regarding the limits of metals in agricultural soils. For example, the values indicated are lower than the Italian ones in Germany, Belgium, the Czech Republic (Reimann et al. 2014) and Poland (Polish Ministry of the Environment 2002), which provide thresholds of 20-

60, 50-72, 100 and 150 mg/kg of copper. On the other hand, some Nations indicate higher levels of copper, i.e. Denmark (Danish Environmental Protection Agency 2015) and Portugal (Decreto-Lei N.276/2009 2009), with 500 and 1000 mg/kg, respectively. At a European level, therefore, there is no univocal standard regarding the concentrations of copper allowed in agricultural soils. Still, there is a guideline relating to agricultural soils in which sewage sludge is applied, establishing reference concentrations for some metals. For copper, these values are included in the range from 50 to 140 mg/kg and can be considered a reference range for environmental risk (European Commission 1986; Albanese et al. 2015).

The concentrations found in the present work and the high variability of the data obtained are similar or lower if compared with the copper accumulation values in vineyard soils found in reports of various Italian Regions (ARPA Piemonte 2014; ARPAV 2019). The mean Cu concentration measured by the LUCAS topsoil survey in 25 European Nations was 16.86 mg/kg, with high variability; vineyards are the land cover class with the highest percentage of soil samples displaying higher than 100 mg/kg (Ballabio et al. 2018).

From the ecotoxicological point of view, it is mainly important to determine the bioavailable Cu because it represents the fraction of the total metal content in the soil that biota can utilise (Lanno et al. 2004). The values of bioavailable copper as a percentage of the total Cu obtained in this monitoring are certainly lower than the average value of 32% found in the measurement campaign carried out by the Region Veneto, Italy, in 2019 (Garlato 2019).

Observing the values of soluble copper, always in the order of ppb, these are a minimum percentage if compared to the other forms. Therefore, it suggests that the risks of copper diffusion by leaching towards the deep layers or flowing towards surface waters or erosive transport with meteoric waters are minimal in the conditions tested.

The high pH values of these soils can also contribute to insolubilize Cu in the form of hydroxide (Kabata-Pendias and Pendias 2001), whereby the bioavailable and

soluble forms can be limited to very lower percentages of the total than other soils, with values ranging from 7.24 to 16.96% of bioavailable copper and from 0.26 to 1.33% of soluble copper respect the total copper. Finally, organic cultivation allows the soils to retain metals much more than the soils conventionally cultivated and often transforms them into insoluble or unavailable chemical species (Mackie et al. 2013). The characteristics of high CSC, pH and organic substance of the tested soils favour the metal's immobilisation and reduce its absorption by plants and microorganisms, preventing them from uptake it at toxic levels as reported elsewhere (Duan et al. 2016). The reduction in the availability of copper can positively influences bacterial biodiversity, which has been proven to be sensitive to this metal in the soil (Singh et al. 2014). On the other hand, in terms of edaphic biota, it seems that fungal richness is resilient to copper, in fact, according to Keiblinger et al. (2018), the effects on the number of taxa are transitory.

The changes in the total copper concentration between the two years under study were not significant, and in any case, they decreased in most of the samples. This could be considered as a positive aspect from an environmental point of view, even further investigation on the fate of Cu, including plant uptake, leaching in the deeper layers and runoff and erosion are necessary. The high number of no significant changes in bioavailable Cu concentration, joined with similar behaviour of the total Cu species, allow us to affirm that the management of Cu treatments in the two vineyards tested can be considered appropriate and do not represent a significant impact from an environmental point of view.

Only the soluble copper significantly increased in both vineyards in 2021. The temperatures observed at the two sampling sites followed a very similar trend, with 2021 with higher monthly average summer temperatures than in 2020. In terms of rainfall, on the other hand, the Castelplanio vineyard received higher water supplies than Varano, both in 2020 and 2021, which may have influenced greater leaching of copper in the Castelplanio vineyard compared to that of Varano; the soluble copper increase was less evident (range of +13.80 to +50.97 %) than the percentage increase

recorded in the samples of Varano (range of + 48.26 to +76.05 %). In any case, it should be borne in mind that we are referring to very low concentrations with respect to the total Cu; thus, it is challenging to establish the climatic impact on the behaviour of this fraction in the soil.

Regarding the ecotoxicological study conducted with earthworms, in all treatments (VAZ, VAZ90 and VAZ150), the three copper fractions in the soil do not change significantly over time. The treatments at field concentration (60 mg/kg) and 90 mg/kg seem to generate the same effect in terms of the values of the three metal fractions over time and are not associated with any sign of harm to earthworms such as abnormal behaviour or death. Comet assay results confirm what has been observed in the weight trend: it seems that in the case of the higher dose (VAZ150), an acute insult was observed in the first 2 days of exposure followed by a recovery in the subsequent days, with an increase of the average weight correlated to the noticeable decrease in DNA-damage. Duan et al. (2016) found weight gain in earthworms subjected to sub-lethal copper concentrations with ranges similar to those tested in the present study (below 320 mg/kg) and assumed that biomass and survival alone are not parameters that give a complete view on toxic effects.

The results found in the present study are in agreement with those described in a review by La Torre et al. (2018) on Cu use in agriculture; even though earthworms may avoid toxic Cu levels by migrating to uncontaminated soil, they may also adapt to certain levels of contamination with the development of biochemical mechanisms of detoxification (Strumpf et al. 2015; Wang et al. 2018).

Some authors confirm the concepts of adaptation to copper doses comparable with the highest amount present in this experiment; Mincarelli et al. (2019) found that nine days after the treatment, TI was significantly lower than in the previous days; authors ascribed this behaviour to the increasing level of metallothioneins at a dose of 120 mg/kg, which are responsible for copper detoxification.

Nonetheless, mortality of 8.3% was recorded at the end of the experiment in the treatment with 150 mg/kg of copper, suggesting a toxic effect of Cu on earthworms in these experimental conditions.

6.5 Conclusions

The monitoring campaigns in the two years of experimentation highlight that the vineyard management regarding Cu-treatments in the two farms involved in the investigation was prudent. The concentrations of the three forms of copper did not undergo significant variations. In the two-year monitoring, an overall decrease in total Cu concentration in soil occurred, and this can be considered a positive factor from an environmental point of view. However, frequent leaching and/or runoff events could be associated with increases in the concentration of Cu in surface and deep waters, which could cause concern. Particular attention should be paid to the types of copper-based fungicides used, possibly preferring slow-release ones to avoid environmental dispersion as much as possible.

In the ecotoxicological experiment, the highest dose of 150 mg/kg caused an initial toxic effect, higher than the other two doses, that was followed by adaptive responses in the earthworms that lead to DNA repair overall weight increases. It can be stated that at the field concentration (60 Cu mg/kg), the ecotoxicological effects on non-target species worm Eisenia fetida are negligible. Even at artificially increased doses of 90 and 150 mg/kg, the adverse effects regarding the earthworm weight and DNA damage are transient and not worrying. Further research is needed about ecotoxicological effects of Cu on soil nontarget organisms, by testing a wider range of copper concentrations and involving novel type of biomarker to be analysed.

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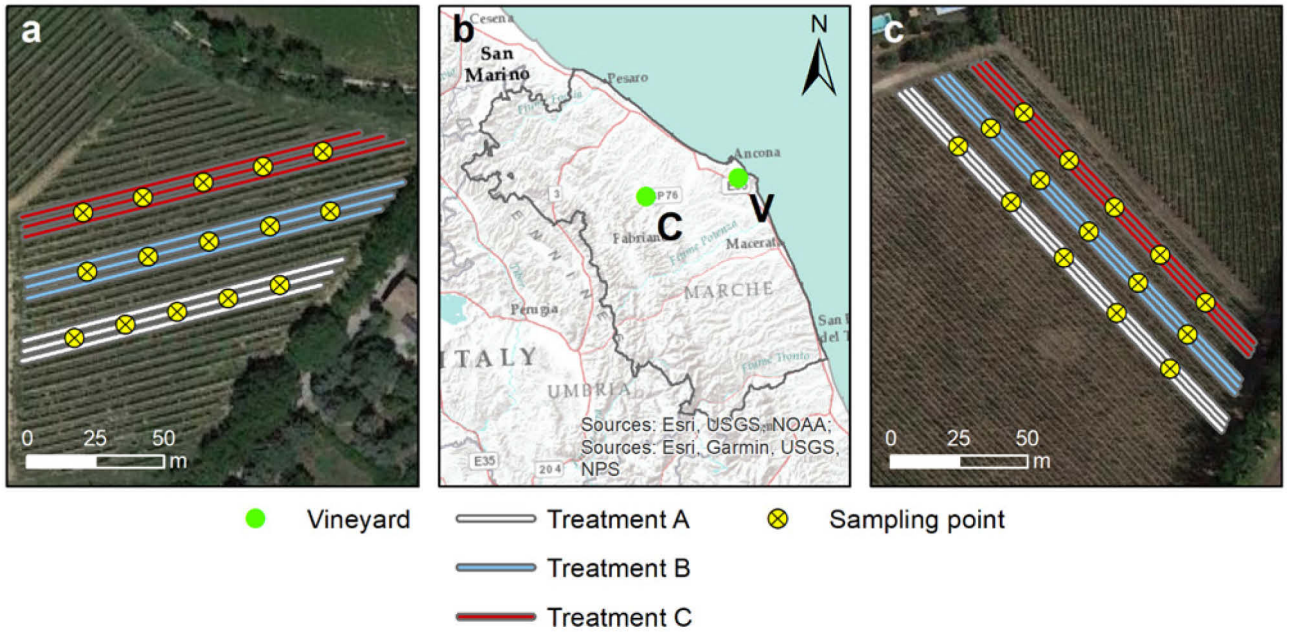

Figure 6.1 Sampling map in the two vineyards under study. a) Sampling in Castelplanio vineyard, b) Location of the two vineyards Castelplanio (C) and Varano (V) in the Marche region, c) Sampling in Varano vineyard. Treatments refer to the antifungal strategies applied on the rows: A) Cu treatments, B) Alternating treatments with Cu and chitosan, and C) Chitosan treatments.

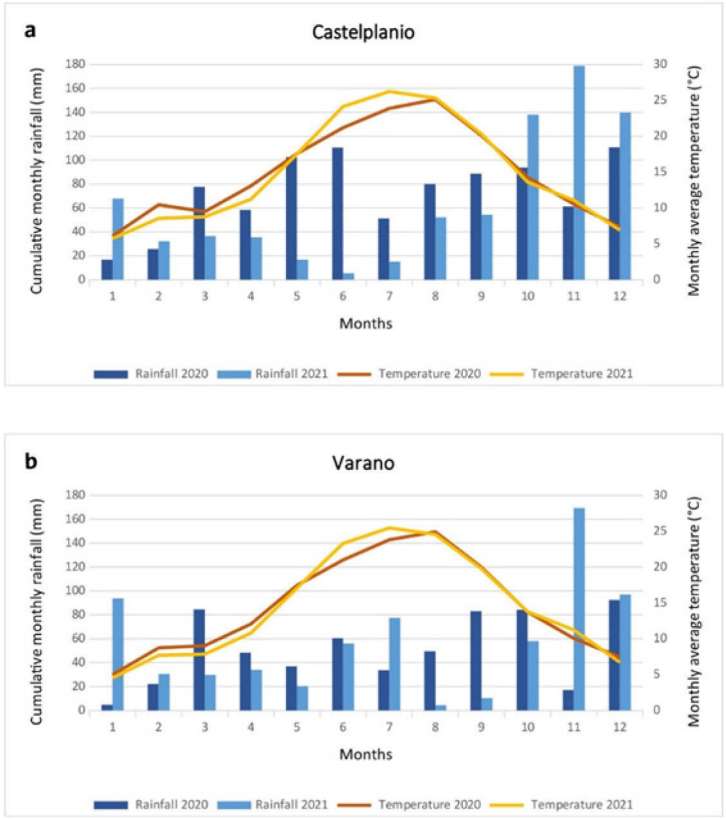

Figure 6.2 Climatic conditions in the two vineyards under study in 2020 and 2021. a) Castelplanio vineyard, b) Varano vineyard.

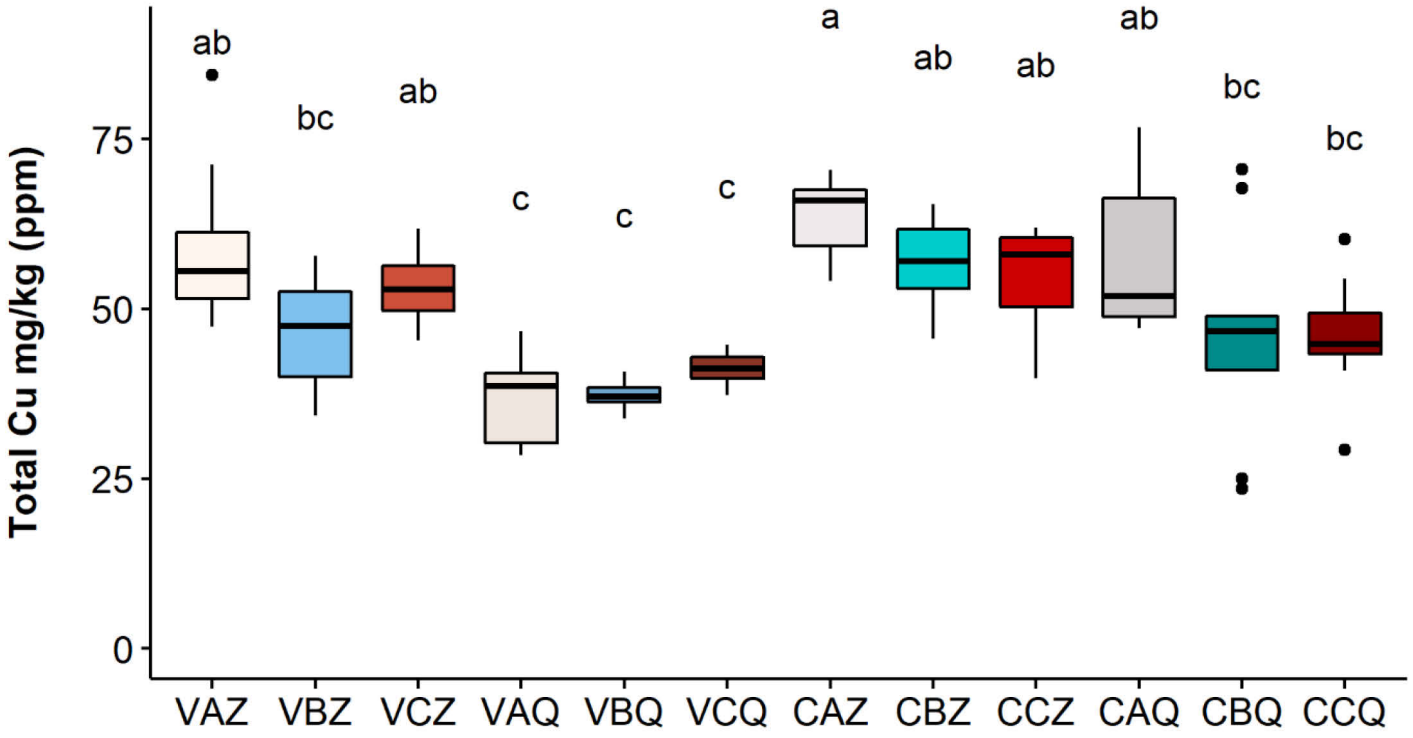

Figure 6.3 Total Cu concentration in each sample in 2020. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

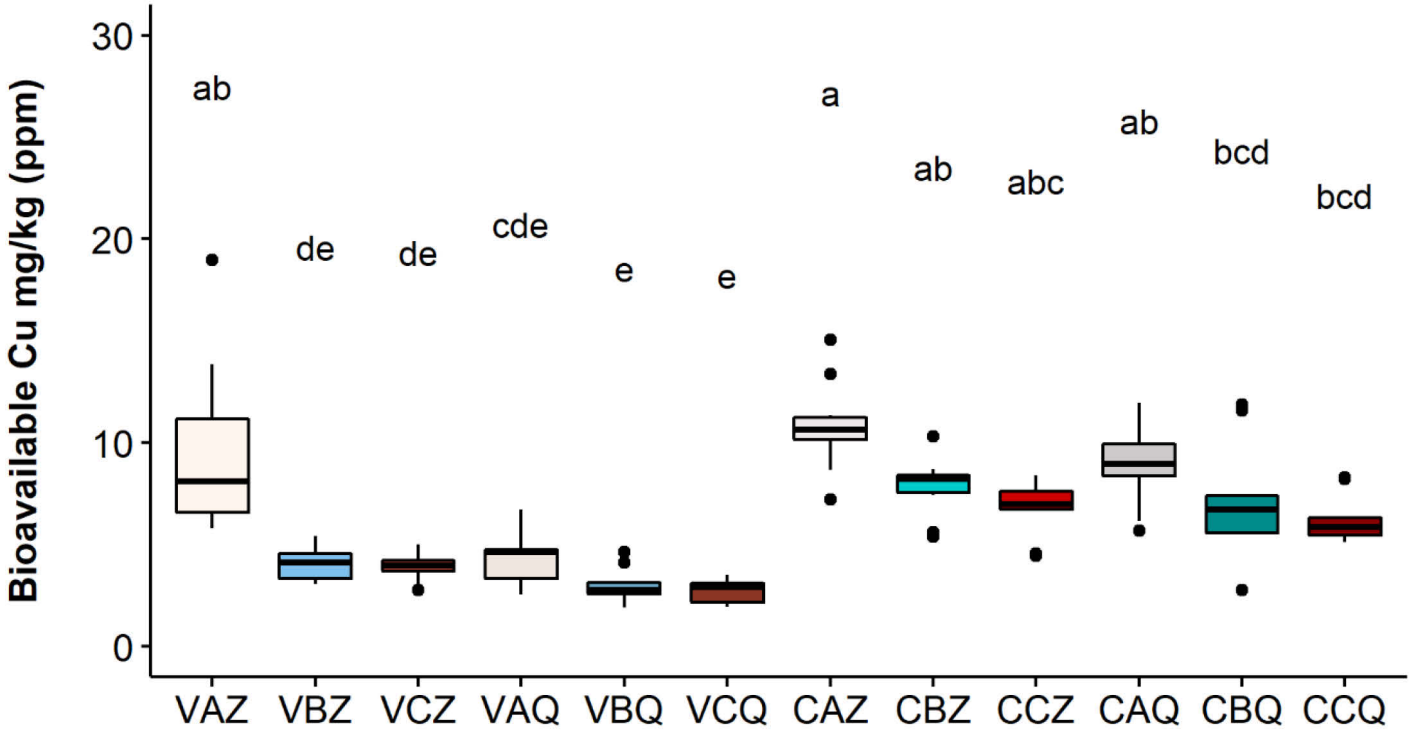

Figure 6.4 Bioavailable Cu concentration in each sample in 2020. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

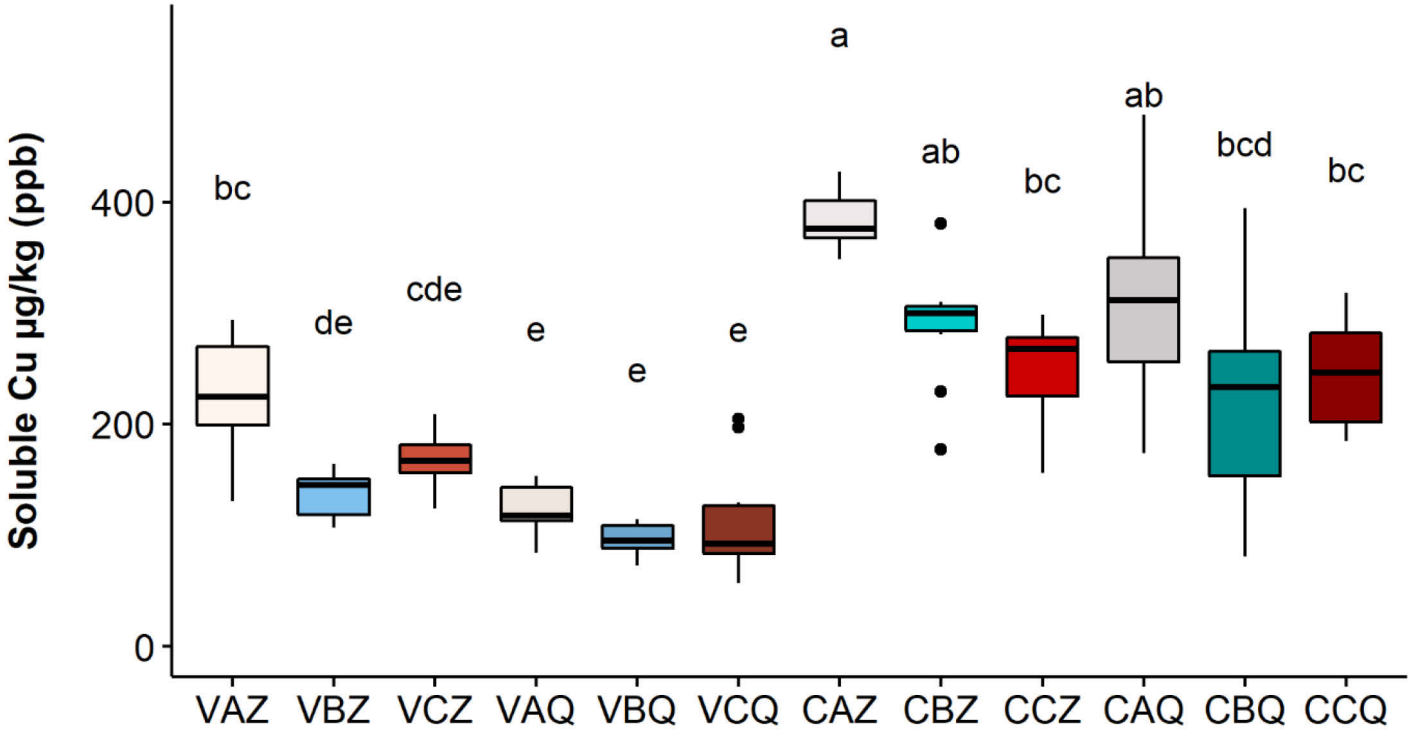

Figure 6.5 Soluble Cu concentration in each sample in 2020. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

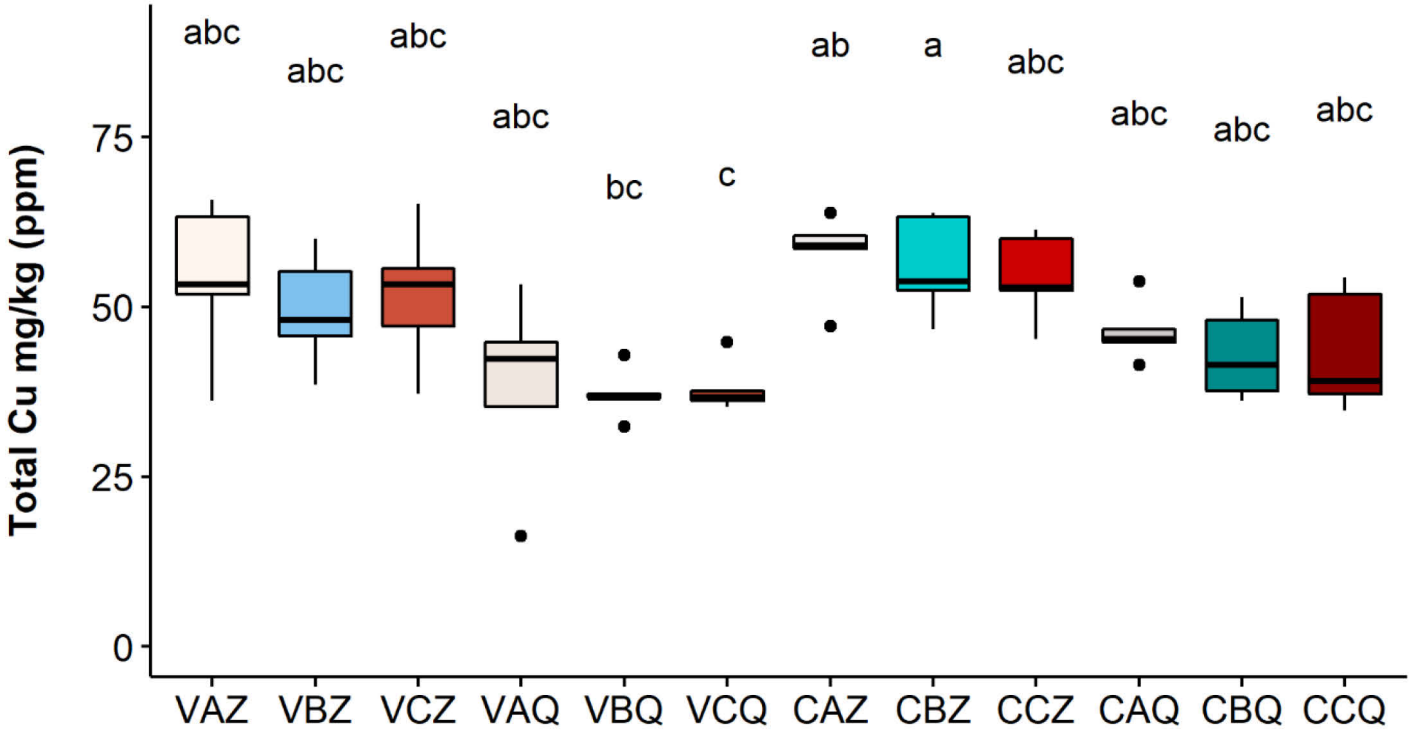

Figure 6.6 Total Cu concentration in each sample in 2021. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

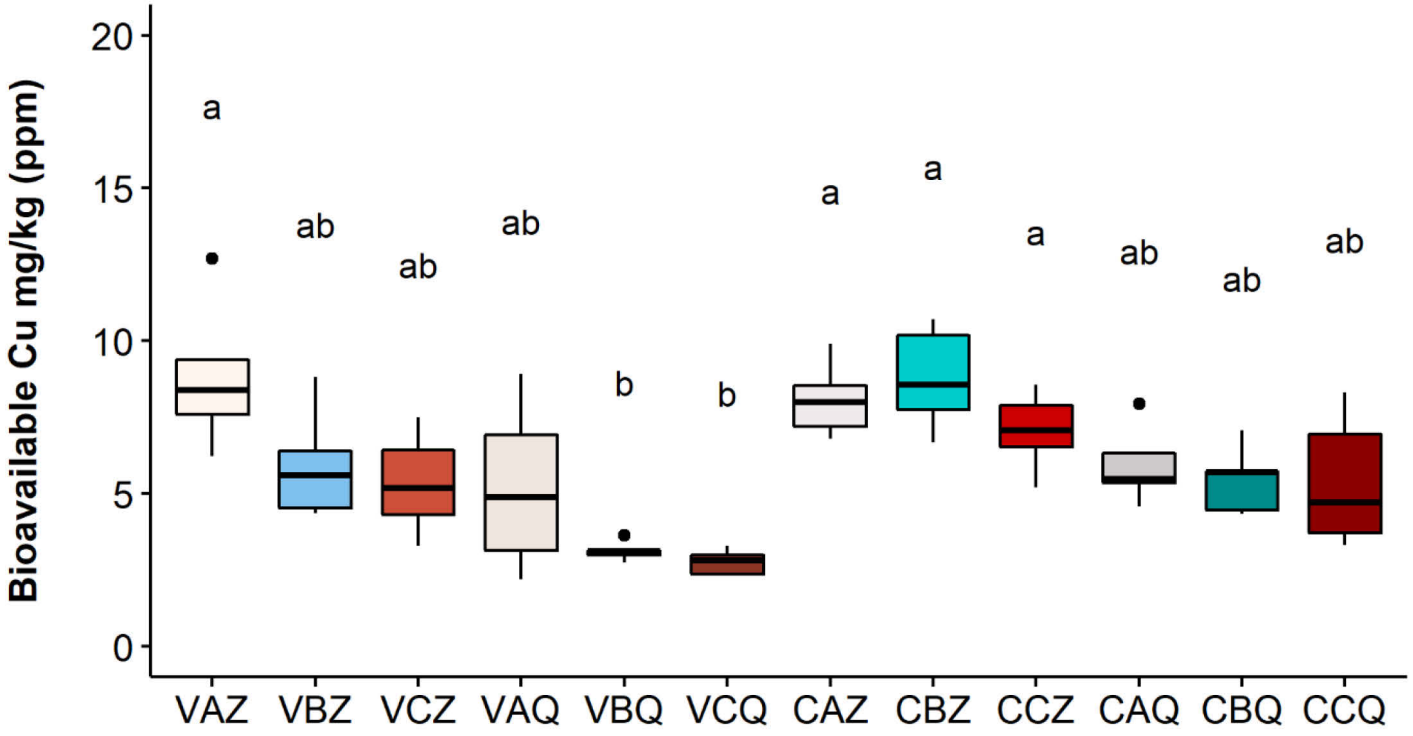

Figure 6.7 Bioavailable Cu concentration in each sample in 2021. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

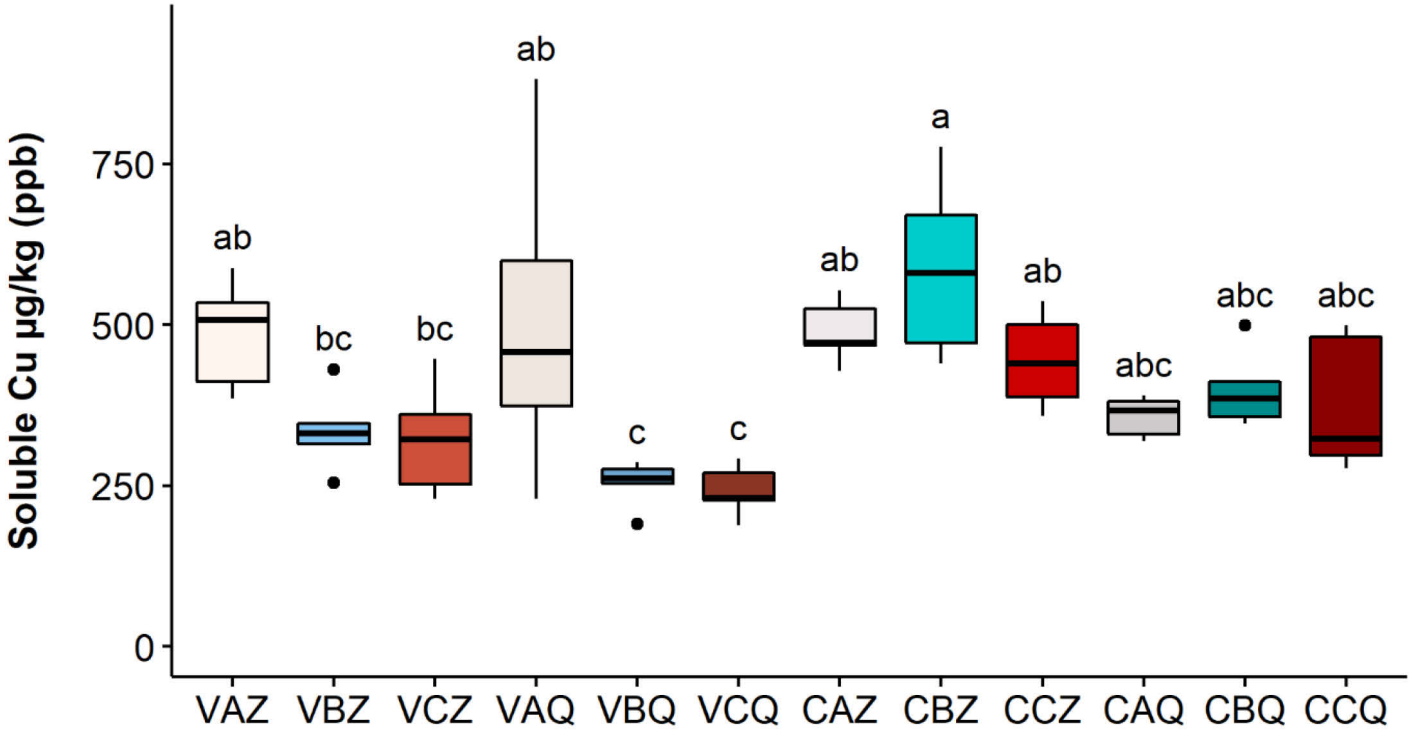

Figure 6.8 Soluble Cu concentration in each sample in 2021. Lower case letters refer to Dunn's Kruskal–Wallis multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05). Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

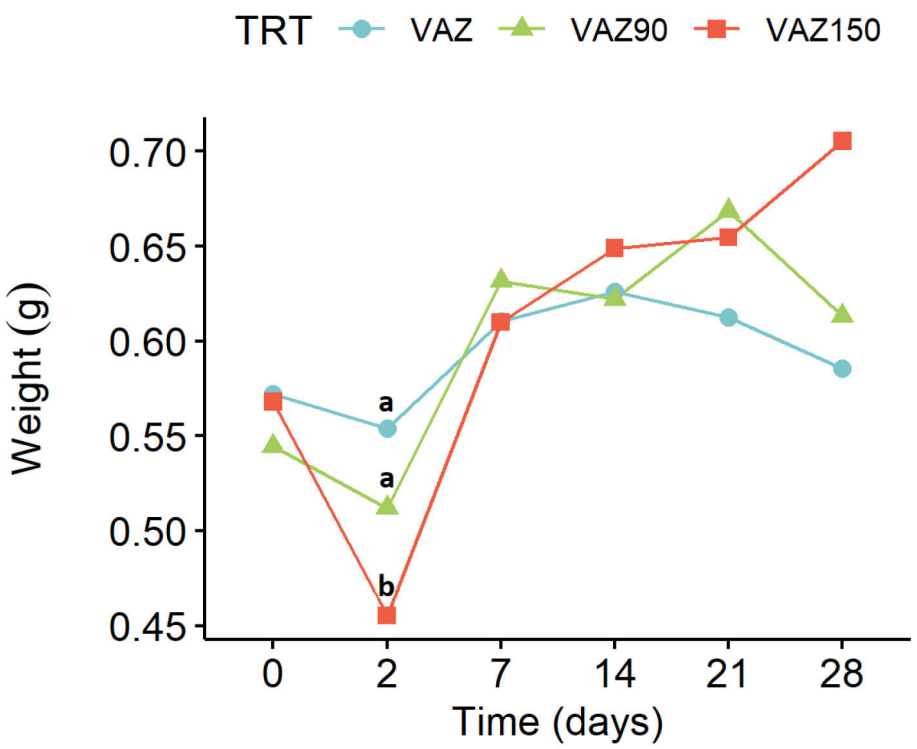

Figure 6.9 Earthworms mean weight trend during the ecotoxicology test. According to Dunn's multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05), different letters in the same time indicate significant differences between the treatments. When no letter is shown, there are no significant differences between treatments.

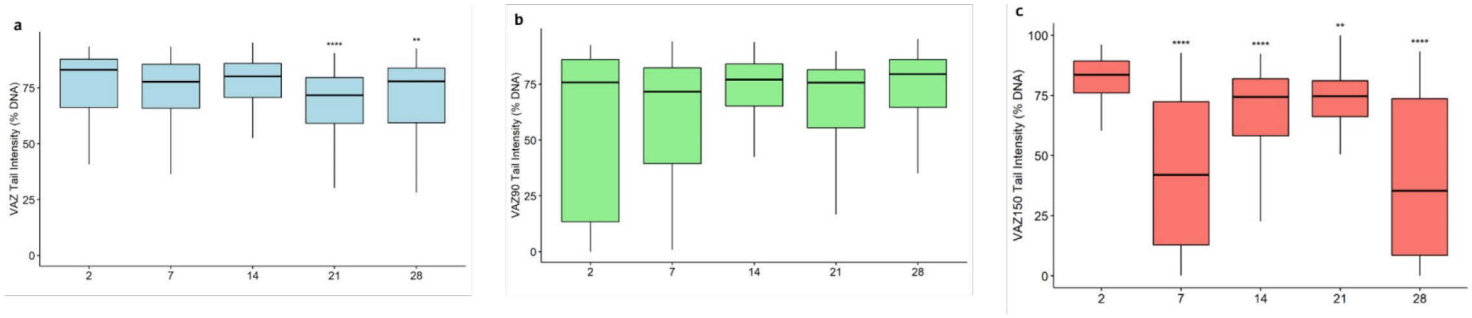

Figure 6.10 Tail Intensity in earthworms coelomocytes. a) trial at the field copper concentration (VAZ), b) trial at the fortified copper concentration of 90 mg/kg (VAZ90), and c) trial at the fortified copper concentration of 150 mg/kg (VAZ150). According to Dunn's test, asterisks relate to significant differences of each time compared to day 2 from contamination (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$).

Table 6.1 Summary of the number of Cu-treatments carried out during the last two years in the vineyards under study.

Vineyards	Thesis	N° of Cu treatments/year	Year
Varano (V)	A	9	2020
		7	2021
	B	4	2020
		5	2021
Castelplanio (C)	A	9	2020
		5	2021
	B	4	2020
		4	2021

Table 6.2 Summary of main physico-chemical characteristics of the soils under experiment, analysis protocols by Italian Gazzetta Ufficiale n° 248 (Italian Official Gazette 1999).

Sample code[1]	pH (H2O)	Texture	Sand	Silt	Clay	Conductivity	OM	CEC
			%			dS/m	%	Meq/100g
VAZ	8.1	Silty Clay Loam	19.5	51.0	29.5	0.644	2.53	24.5
VAQ	8.2		20.8	49.0	30.2	0.587	1.26	25.6
VBZ	8.2	Clay Loam	21.5	45.6	32.9	0.61	2.05	30.9
VBQ	8.2		20.4	47.3	32.3	0.536	1.50	30.6
VCZ	8.2	Silty Clay	19.9	48.5	31.6	0.627	2.02	29.1
VCQ	8.2	Loam	15.4	50.6	34.0	0.516	1.69	29.6
CAZ	8.1	Loam	44.3	29.8	25.9	0.638	2.22	25.4
CAQ	8.2	Sandy Clay Loam	47.6	27.1	25.3	0.597	1.18	24.5
CBZ	8.1		41.0	33.6	25.4	0.869	2.28	22.4
CBQ	8.2	Loam	42.4	31.5	26.1	0.731	1.33	22.0
CCZ	8.1		43.3	31.5	25.2	0.614	2.02	24.1
CCQ	8.1		41.3	33.4	25.3	0.717	1.38	21.9

[1] Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis, A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth; Z) 0-20 cm, Q) 20-40 cm.

Table 6.3 Percent of variation in copper concentration in the two years of experimentation.

Sample codes[1]	Cu Percentage variation between two years (%)		
	Tot	Bio	Sol
VAZ	-8.64	-8.61	+53.27**
VBZ	+5.68	+32.15*	+59.12**
VCZ	-3.07	+26.71	+48.24**
VAQ	+4.54	+15.59	+76.05**
VBQ	-0.14	+4.87	+59.12**
VCQ	-8.59	+1.19	+52.66**
CAZ	-10.40	-33.75	+21.31**
CBZ	-0.66	+10.40	+50.97**
CCZ	-0.26	+3.22	+44.13**
CAQ	-24.44*	-51.51*	+13.80
CBQ	-7.11	-25.03	+43.73*
CCQ	-5.47	-15.04	+34.56*

Significance of Cu variation between 2020 and 2021 was calculated in each sample according to Dunn's test (*$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, Benjamini–Hochberg p-value adjustment, α-level = 0.05). Abbreviations Legend: Tot refer to total Cu, Bio refer to bioavailable Cu, and Sol refer to soluble Cu. [1]Codes legend: the first letter refers to the vineyard; C) Castelplanio, V) Varano. The second letter refers to the thesis: A) Cu treatments, B) Alternate treatments with Cu and chitosan, C) Chitosan treatments. The third letter refers to the depth: Z) 0-20 cm, Q) 20-40 cm. + indicates an increase in Cu concentration, -indicates a decrease in Cu concentration.

Table 6.4 Tail intensity (TI) median values during the preliminary study.

Treatments	Time (Days)				
	2	**7**	**14**	**21**	**28**
VAZ	83%	78%	80%	72%	78%
VAZ90	78%*	72%*	77%	76%	80%
VAZ150	83%	42%****	74%***	75%	35%****

According to Dunn's test (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001), asterisks relate to significant differences in each time between fortified treatments (VAZ90 and VAZ150) and the field concentration (VAZ).

Table S 6.1 Copper concentrations (mg/kg) during the ecotoxicological test.

Time (days)	VAZ Tot	VAZ Bio	VAZ Sol	VAZ90 Tot	VAZ90 Bio	VAZ90 Sol	VAZ150 Tot	VAZ150 Bio	VAZ150 Sol
2	62.30 ± 1.71	9.64^{b} ± 0.13	0.49^{ab} ± 0.03	104.80 ± 9.19	25.33^{ab} ± 0.60	0.60 ± 0.01	*136.10 ± 0.20	*55.08 ± 0.71	*0.70^{b} ± 0.03
7	-	13.00^{ab} ± 0.24	0.50^{ab} ± 0.03	-	23.92^{ab} ± 1.03	1.08 ± 0.12	-	*53.10 ± 2.83	1.02^{ab} ± 0.12
14	59.50 ± 0.53	13.78^{a} ± 0.10	0.58^{a} ± 0.04	87.73 ± 1.61	26.60^{a} ± 0.57	0.77 ± 0.03	*138.70 ± 7.77	*43.38 ± 1.42	*1.22^{a} ± 0.09
21	-	13.90^{a} ± 0.37	0.57^{ab} ± 0.06	-	17.37^{b} ± 0.89	0.60 ± 0.39	-	*49.65 ± 4.93	1.08^{ab} ± 0.03
28	60.23 ± 0.22	11.95^{ab} ± 1.34	0.45^{b} ± 0.01	96.88 ± 2.47	19.73^{b} ± 3.59	0.60 ± 0.03	*134.78 ± 3.29	*35.93 ± 5.83	*1.01^{ab} ± 0.07

Different letters in the same column (same treatment) indicate significant differences between the times; Asterisks on the same line (same time of sampling) indicate significant differences of fortified treatments (VAZ90 and VAZ150) respect the baseline soil (VAZ).
Dunn's test for multiple comparisons following the Kruskall-wallis test significant result, Benjamini–Hochberg p-value adjustment, α-level = 0.05; Abbreviations Legend:Tot refer to total Cu, Bio refer to bioavailable Cu and Sol refer to soluble Cu.

CHAPTER 7: Copper toxicity on *Eisenia Fetida* and microbial community in vineyard soil

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Abstract

The paper reports three ecotoxicological tests on earthworms *Eisenia fetida* of four copper sub-lethal concentrations; 55, 110, 165 and 220 mg/kg. In addition to the standard avoidance and reproduction tests, a toxicity test was set up to evaluate; early damage to earthworm DNA, bioaccumulation factor and effects on the structure of the bacterial community within the earthworm (gut microbiota) and in the soil in the presence or absence of *Eisenia fetida*. Furthermore, copper speciation kinetics in soil was assessed.

The results showed a dose-dependent avoidance response by earthworms and exceeded the habitat threshold limit at the two highest doses of 165 and 220 ppm. From the reproduction test, an inverse proportionality was observed between reproductive output and copper concentration in the soil, with evident adverse effects starting from the dose of 110 ppm and significantly evident at 220 ppm.

The toxicity test is in progress, and the results will be available within a few months. The present research will provide new information on the ecotoxicity of sub-lethal doses of copper to earthworms, on their cellular response and the identification of the effects of this metal in terms of microbial composition.

Keywords: copper; bacterial community; ecotoxicology; earthworms; *Eisenia fetida*; Comet Assay; gut microbiota.

7.1 Introduction

Italy, France and Spain provide around 60% of the global wine production; thus, viticulture is considered a crucial agricultural sector in the Mediterranean region (Hall and Mitchell, 2000). In vineyards, the use of copper (Cu) compounds against fungal disease has a history dating back more than a hundred years (Merry, Tiller and Alston, 1983). These long-term distributions have caused many cases of copper accumulation in soils and adverse effects on non-target species (Helling, Reinecke

and Reinecke, 2000; Maboeta, Reinecke and Reinecke, 2002; Van Zwieten et al., 2004). Although Cu is an essential micronutrient for soil biota and other organisms, an excess can generate toxicity (Tarradellas, Bitton and Rossel, 1996). Metals in the soil can change the microbial communities that populate this environmental compart and those that colonise the intestines of organisms that live there (Šrut et al., 2019). The impact of copper-based fungicides on earthworms and soil microbial community and the consequential detriment of soil function is recognised (Merrington, Rogers and Van Zwieten, 2002; FAO and ITPS, 2017).

Among soil biota, earthworms have a fundamental role in guaranteeing the fertility and health of this environmental compart (Edwards and Bohlen, 1996). These annelids interact and modify the bacterial communities and the speciation and availability of metals throughout soil ingestion and digging activity (Sizmur and Hodson, 2009). Earthworms are considered good indicators of inorganic and organic pollutants (Jager et al., 2003; Vijver et al., 2003), and *Eisenia fetida* (*E.fetida*) is the common species employed for standardised ecotoxicity tests (OECD 1984, 2016; ISO-17512-1, 2008). Because environmental pollutants such as metals and pesticides can alter earthworms directly and their gut microbiome, the study of these modifications deserve particular attention to evaluate pollutants biological effects as critical indicators of soil status (Yausheva et al., 2016; Jin et al., 2017; Šrut et al., 2019; Vischetti et al. 2020). Indeed soil quality is a key lever of sustainable viticulture (Karimi et al., 2020). Furthermore, changes in gut microbiota and pollutants themself can lead to a disequilibrium in the host immune system that, in the case of earthworms, is provided by immune cells called coelomocytes (Jin et al., 2017; Swart et al., 2020). For these reasons, genotoxicity analysis throughout Comet assay can be an essential tool to evaluate stress-induced in ecotoxicological studies (Tice et al., 2000; Li et al., 2009; Lourenço et al., 2011; Mincarelli et al., 2016b).

In the present paper, the standardised avoidance and reproduction tests on earthworms were conducted together with a new toxicity test for the evaluation of changes in microbial community composition, earthworms bioaccumulation and

early DNA damage as an implementation tool for the risk assessment of natural soil collected in vineyards and contaminated with several copper concentrations. Fortified copper concentrations were selected remaining near the contamination threshold concentration of 200 mg/kg indicated for agricultural soils by the Decree No. 46 in Annex 2 (Italian Official Gazette, 2019) and of the LC_{50} (Lethal Concentration 50) referring to copper sulphate for earthworm Eisenia fetida fixed at values higher than 155 mg/kg, as indicated in the Pesticides Properties DataBase (PPDB).

7.2 Materials and methods

7.2.1 Earthworms

E. fetida earthworms were reared at 20 ± 1 °C in organic compost in the laboratory and fed with organic oats and vegetables. Ten adults with a well-developed clitellum (300-600 mg wet mass) were used in each replicate in all the experiments after acclimatisation in the same substrate used in tests (Li et al., 2018; Zou et al., 2018; Zhang, Saleem and Wang, 2019).

7.2.2 Soils

The top-soil (first 0-20 cm of depth) collected from a vineyard managed with organic agricultural practices with the following properties was used across the experiments as the starting soil after it was air-dried and sieved at 2 mm: pH 7.79; organic matter 0,8%; conductivity 530 nS/cm, total copper field concentration of 55 mg/kg (FC).

To test other copper concentrations in a geometric series, FC soil was further contaminated with the commercial product Siaram 20 WG, which derives from copper sulphate by reaching the final Cu concentrations of 110 mg/kg (110), 165 mg/kg (165) and 220 mg/kg (220). In addition, an artificial soil (ART) was prepared following the standardised guideline (OECD, 1984) and used as a negative control with the following features: baseline total copper concentration of 7 mg/kg, pH 6.5; organic matter 2,0%; conductivity 530 nS/cm.

Each treatment (FC, 110, 165, 220, ART) was conducted in three replicates consisting of 500 grams of dry soil, 10 earthworms and water until a humidity of 27%.

7.2.3 Avoidance experiment

Initially a "dual control" test was performed to assess that earthworms do not tend to prefer one of the two sections if they are filled with the same substrate (Yeardley, Lazorchak and Gast, 1996; Hund-Rinke and Wiechering, 2001). The avoidance test was then conducted to find whether the earthworm *E.fetida* avoids contaminated soils (García-Santos and Keller-Forrer, 2011; Jordaan, Reinecke and Reinecke, 2012; Martínez Morcillo et al., 2013) in five replicates with the two-chamber design, as described by ISO 17512-1 (2008). One-half of the box was filled with 250 grams dry weight of the copper contaminated substrate, and the other half was filled with the same quantity of the artificial uncontaminated soil (ART); ten earthworms were placed in the middle of the box. After 2 days, the earthworms on both chambers were counted. The results of the avoidance test are expressed as the net response (NR) in percentage according to ISO (2008):

$$NR = \lfloor (C - T) \div N \rfloor \times 100$$

where C and T are the numbers of worms in the artificial soil and the contaminated soil, respectively, N is the total number of worms in each box.

7.2.5 Reproduction experiment

Copper impact on earthworms' reproductive output (and other sub-lethal endpoints) was assessed through a reproduction test following the OECD guideline (OECD, 2016).

All treatments, in three replicates, were kept under a controlled temperature (20 ±1°C) for 56 days. Adult earthworms in each replicate have been weighed and observed weekly: any unusual behaviour and morphology anomalies were recorded. After 28 days, adults were removed from the containers while substrate containing juveniles and cocoons were left for another 4 weeks-incubation. On day 56, the

number of juveniles and the cocoons in each replicate were recorded. The growth rate (GR, %) was calculated as follows:

$$GR = \lfloor (W_t - W_0) \div W_0 \rfloor \times 100\%$$

where W_0 is the initial average weight of earthworms, and W_t is the average weight of earthworms on day 28. A positive rate means the growth stimulation, while a negative rate indicates growth inhibition (Xie et al., 2013).

7.2.6 Toxicity experiment

The toxicity test was conducted to investigate the effects of copper on earthworms' DNA and microbial communities with three replicates. At 2, 14 and 28 days, analysis of the soil and earthworms' gut bacterial community, available and total copper concentrations in soil. Furthermore, earthworm copper bioaccumulation and DNA-damage were assessed at the three sampling times. A parallel test was conducted without adding earthworms to evaluate any differences in the trend of copper concentration in soil and the evolution of the soil bacterial community according to the presence or absence of *E.fetida*.

7.2.6.1 Copper extraction and analysis

Total Cu concentration in soils was determined by the acid digestion method described by Kasassi et al. (2008) with some modification; 0.5 dry grams of soil were digested for 15 hours in a floating water bath at temperatures over 85°C with 7 ml of HNO_3 (65% v/v) after they were left overnight with 2 mL of H_2O_2 (30% v/v). The bioavailable Cu fraction was extracted with a solution of Diethylenetriaminepentaacetic acid (DTPA), $CaCl_2 \cdot 2H_2O$ (0.01 M) and triethanolamine (0.1 M) at pH 7.3 (1g/2mL), following the indications in the Italian Official Gazette n. 248 (1999). Total Cu concentration in earthworms was detected as follows: after leaving the earthworms overnight to purge, they were euthanatized with 70% ethanol and dried and ground as described by Wang et al. (2018) then each earthworm was digested as described by Tang et al. (2019).

Analyses of the eluates were performed using an ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometer, Agilent mod. 5800) according to EPA 6010D (2014).

7.2.6.2 Comet assay

Coelomocytes were collected as described in Eyambe protocol (1991) with slight modifications. Each earthworm was immersed for 4 minutes in an extrusion buffer of 5% ethanol, 95% PBS, 2.5 mg/mL Na_2-EDTA, and 10 mg/mL guaiacol glyceryl ether (pH 7.3). Coelomocytes were washed and collected by centrifugation (300g, 10 min, 4°C). The washed cells were counted, resuspended in Low melting agarose (LMA 1%, 37 °C) and stratified on HT Trevigen slides pre-coated with Normal Melting Agarose (NMA 1%). Each spot was produced by layering LMA containing 3000 cells; each sample was stratified in triplicate. The solidification, lysis and unwinding phases were carried out following Mincarelli et al. (2016). Electrophoresis was conducted at 11 V/cm for 20 min at 4ºC. Slides were washed in H_2O, neutralised in buffer (0.4 M Tris-HCl pH 7.5), dehydrated in 75% methanol (Valverde *et al.* 1999; Mincarelli *et al.* 2016), stained with Sybr Gold and then imaged using Lionheart FX Automated Microscope (Biotek, U.S.A.) at 200×200 magnification. Comet images were acquired in triplicate and processed to calculate the major DNA damage index: Tail length (TL), Tail moment (TM), and Tail intensity (TI) (Tiano et al. 2005; Orlando et al. 2018).

7.2.6.3 Analysis of bacterial community diversity

Biodiversity analysis of soil and gut bacterial community was based on High Throughput Sequencing (HTS) of 16S rDNA amplicons.

For the gut bacterial DNA collecion each extruded earthworm was euthanized in ethanol (70%) and the midgut (spanning 20 segments posterior to the clitellum) was dissected using sterile equipment and stored at −20 ◦C until DNA analysis.

Total genomic DNA of soil and gut was isolated using Soil DNA Isolation Kit (NORGEN Biotek, Canada) following the manufacturer's protocol, and V3-V4 region of 16S ribosomal RNA (rRNA) gene was amplified using the universal

primers 343F (5′-TACGGRAGGCAGCAG-3′) and 802R (5′-TACNVGGGTWTCTAATCC-3′), as previously described in detail (Vasileiadis et al. 2012, 2015; Bandini et al. 2021).

7.2.7 Satistical analysis

The significance of the differences in the avoidance and reproduction responses between treatments were assessed using the Kruskal–Wallis test and Dunn's post-hoc test performed in R software (R Core Team 2022 version 4.1.3) using 'dunn.test' package version 1.3.5. Non-parametric tests were used because of non-normal distributions also in the evaluation of the significant differences ($\alpha = 0.05$) among copper concentrations in earthworms and soils in the toxicity experiment.

Some statistical analyses on the toxicity experiment data are currently underway.

7.3. Results and discussions

7.3.1 Avoidance feedback

The effects of the four Cu concentrations on the avoidance behaviour tests are reported in Figure 1; no earthworm escaped or died during the exposure period. A positive NR indicates an avoidance of the contaminated substrate, whereas a negative value indicates an attraction to the tested contaminant (Xu et al., 2020; Gainer et al., 2022). Following the ISO (2008), when more than 80% of earthworms avoid the treated substrate, the soil has limited habitat function, and in terms of NR, this occurs with values from 70%.

It was measured a positive Net Response (NR) value in all trials. It can be seen that the earthworms followed a dose-response behaviour; as contamination increased, a more significant number of earthworms preferred artificial soil. At the two highest copper concentrations tested (165 and 220 mg/kg), more than 80% of earthworms (respectively 97 and 98%) prefer the uncontaminated soil indicating that these two Cu doses represent a limiting habitat. In Xing et al. (2017), the habitat limit was exceeded by 128 mg/kg of copper in the soil and likewise to the present study, an NR of 100% is measured at the dose of 160 mg/kg (in our case the median NR values are 100% for both 165 and 220 mg/kg).

Similar results were also obtained with other earthworm species. Lukkari et al. (2005) found that *Aporrectodea tuberculata* begins to avoid soil contaminated by copper at around 50 mg/kg and from 79 mg/kg onwards, the habitation threshold was exceeded. In the most recent work by Renaud et al. (2022), avoidance test results are expressed in avoidance concentration (AC); it was found that the 50% of *Eisenia andrei* individuals avoided soil contaminated by 49.5 mg/kg (AC50) and an AC80 about 112 mg/kg of Cu in the tested soil. In the present study, at 55 mg/kg already, 65% of earthworms avoid contaminated soil, while 80% of earthworms avoid contaminated substrate with 110 mg/kg of copper (i.e. a median NR of 40%).

7.3.2 Reproduction feedback

The trend of the earthworm weights during the reproduction test is reported in Figure 7.2. An evident weight decrease in the treatment at 220 mg/kg on day 7 was recovered in the following days up to values similar to those measured in the other theses, except for the one at 165 mg/kg, which showed a trend of weight gain until 28 days.

It seems that at the copper concentrations ranging from 110 and 165 mg/kg, earthworms should be able to adapt and increase their weight. The dose of 220 ppm, after an initial recovery, undergoes a toxic effect of the high Cu concentration, leading to a strong weight loss of the individuals, while the average weights of earthworms in soil with the lowest doses of copper (55 and 110 ppm) are not significantly different from those of the uncontaminated soil (ART).

We can hypothesise that, in this type of soil, earthworms do not have adverse effects on the growth up to concentrations of 110 ppm. Around 165 mg/kg of Cu in soil, earthworms seem to adapt and respond with an increase in weight. In contrast, from 220 onwards, copper involves visible toxicity from substantial weight losses that are not recovered over time. Perhaps earthworms reduce their trophic activity because they are bothered by the high presence of copper, as already stated by other authors (Tatsi et al., 2018). This weight trend in which, at some concentrations of copper, there is an increase in biomass while beyond certain thresholds, this significantly

decreases is reported in previous studies conducted on similar soils (Chapter 6) or different from the one being studied (Owojori et al., 2010; Clasen et al., 2021).
Also, in the reproduction test by Lukkari et al. (2005) conducted on a substrate with a more consistent organic substance content (around 7%) than that of the present study, the biomass of earthworms (Aporrectodea tuberculata) increases at intermediate copper doses (from 53 to 79 mg/kg) while it decreases at the highest concentrations (from 119, 178 and 268 mg/kg).
Some variability in copper thresholds where adverse effects on earthworms occur due to various factors such as substrate properties, earthworm species (Calisi et al., 2011; Duan et al., 2016; Karimi et al., 2021) and the formulation of administered copper should be considered (Wang et al., 2018; Gainer et al., 2022).
In other studies, copper concentrations causing decreases in earthworm biomass are 100 to 300 mg/kg of copper in soil (Malecki et al., 1982; Svendsen and Weeks, 1997).
On the contrary, in the paper by Helling et al. (2000), there are adverse effects on the growth of earthworms subjected to soils treated with Cu already at 8.92 mg/kg, in the case of the highest tested dose (346.85 mg/kg) a growth stasis, but it should be noted that freshly hatched earthworms were used in this paper instead of clitellate earthworms as in the present study. It is known that young earthworms are more sensitive to xenobiotics than adult ones (Spurgeon and Hopkin, 1996).
In terms of Growth rate (GR) reported with the other parameters measured in the reproduction test in table 7.1, significant differences are noted between the growth measured at the end of 28 days of earthworms subjected to the highest concentration of copper compared to the control.
The dose-dependent trend is evident for all the reproduction outputs, but the true detachment is observed at the higher dose, which causes phenomena of suffering in earthworms' growth and reproductive outcomes compared to the uncontaminated trial (ART).

The number of cocoons is considered one of the most sensible reproduction parameters counted; it was observed a cocoon production is inversely proportional to the concentration of copper in the soil, as reported in the literature (Duan et al., 2016; Tatsi et al., 2018; Clasen et al., 2021). In Owojori et al. (2010), the threshold copper concentration that determines a significant decrease in cocoon production compared to the control is above 80 mg/kg. For a natural soil, the copper EC50 for cocoon production in *E. fetida* was 210 mg/kg in a laboratory study by Scott-Fordsmand et al. (2000); in the present study, at a similar dose (220 mg/kg), it was observed a presence of cocoons halved compared to that measured in ART.

Generally, the reproduction results agree with what has been previously studied by other authors, where the negative effects on the reproductive activity of earthworms increase as the concentration of copper in the soil increases; these effects became evident starting from doses of around 120 ppm (Gao et al., 2016).

7.3.3 Toxicity experiment feedback

7.3.3.1 Copper measurements

Copper concentrations in earthworms are reported in figure 7.3. At the end of the experiment, Cu concentrations in earthworms from trials 110 and 165 were significantly higher than the copper found in individuals from the artificial soil without copper (ART) and soil with the lowest levels of this metal (FC, i.e. 55 mg/kg). As the copper in the soil increases to 220 mg/kg, no respective increase in the metal is observed within the organisms analyzed; this may indicate that earthworms, beyond a certain threshold of copper in the soil, implement an internal regulation of this element, therefore they stop accumulating it in their own tissues, this behaviour has also been observed in other studies (Spurgeon and Hopkin, 1999; Nahmani et al., 2007; Natal-da-Luz et al., 2011; Gao et al., 2016; Richardson et al., 2020).

Statistical analysis had shown no significant differences between the copper values (both total and bioavailable) measured in the tests with or without earthworms or in

the same treatment between the first (2 days) and last (28 days) time of sampling (Table 7.2). Also, in the paper by Fujii and Kaneko (2009), the earthworm's activities did not significantly influence the values of copper extracted with DTPA in a 28 days-test conducted on aged-contaminated or fresh spiked copper soils. In contrast, according to Dandan et al. (2007), when the soil contaminated with copper is hosted by earthworms of different species such as Metaphire guillelmi, these cause an increase in the fraction extracted with DTPA.

Considering the literature, 28 days are until the metal equilibrium is reached inside the earthworms (Spurgeon and Hopkin, 1999; Kennette et al., 2002). The bioaccumulation factor (BAF) was calculated by relating the earthworm's concentration to the total copper in the soil, as suggested by the review of Richardson et al. (2020).

BAFs always lower than one were obtained (0.2, 0.4, 0.2, and 0.1, respectively, for the FC, 110, 165 and 220 tests), indicating that copper is not bioaccumulated in earthworms; this consideration was also reported in a study regarding several forest soil and earthworms species conducted by Richardson et al. (2015).

The analysis regarding the copper effect on the earthworm's DNA through comet assay and the evaluation of the changes in microbial structure in gut and soil is still in progress.

7.4 Conclusions

At the European level, the candidacy of copper compounds for substitution is currently under discussion (EC, 2018). The studies behind this debate date back to more than 20 years ago, when the rule of the maximum dose of 4 kg/ha/year in organic viticulture (EFSA et al., 2018) was not yet in force, thus new experiments about the impact of copper in organic viticulture, especially on soil organisms, are highly recommended (Karimi et al., 2021). To date, there are not many studies regarding the effects that this metal can have on earthworms reproduction and development (Clasen et al., 2021).

In this context, the research conducted could improve the understanding of the ecotoxicity of this metal in soil. The avoidance endpoint showed the highest sensitivity of earthworms to copper concentrations in soil but alone could give controversial responses because it is related only to a soil habitat function (Pelosi et al., 2014). For these reasons conducting a battery of different ecotoxicological tests can lead to a more accurate assessment of the real impacts of xenobiotics in the soil ecosystem.

Although earthworms can avoid soil contaminated with copper only above certain concentrations, which in this study are above 110 mg/kg, there are significant responses in terms of biomass change. Specifically, earthworms can regain their weight as long as the dose of 165 mg/kg is not exceeded, over 220 mg/kg of copper is administered to soil weight loss, and the decrease of reproductive output were irreversible.

To date, we cannot exhaustively conclude this study because we are processing the data relating to the toxicity test, which will also take into account the earthworms responses in terms of genotoxicity, as well as variations in the intestinal and soil microbiome, highly sensitive parameter to soil pollution.

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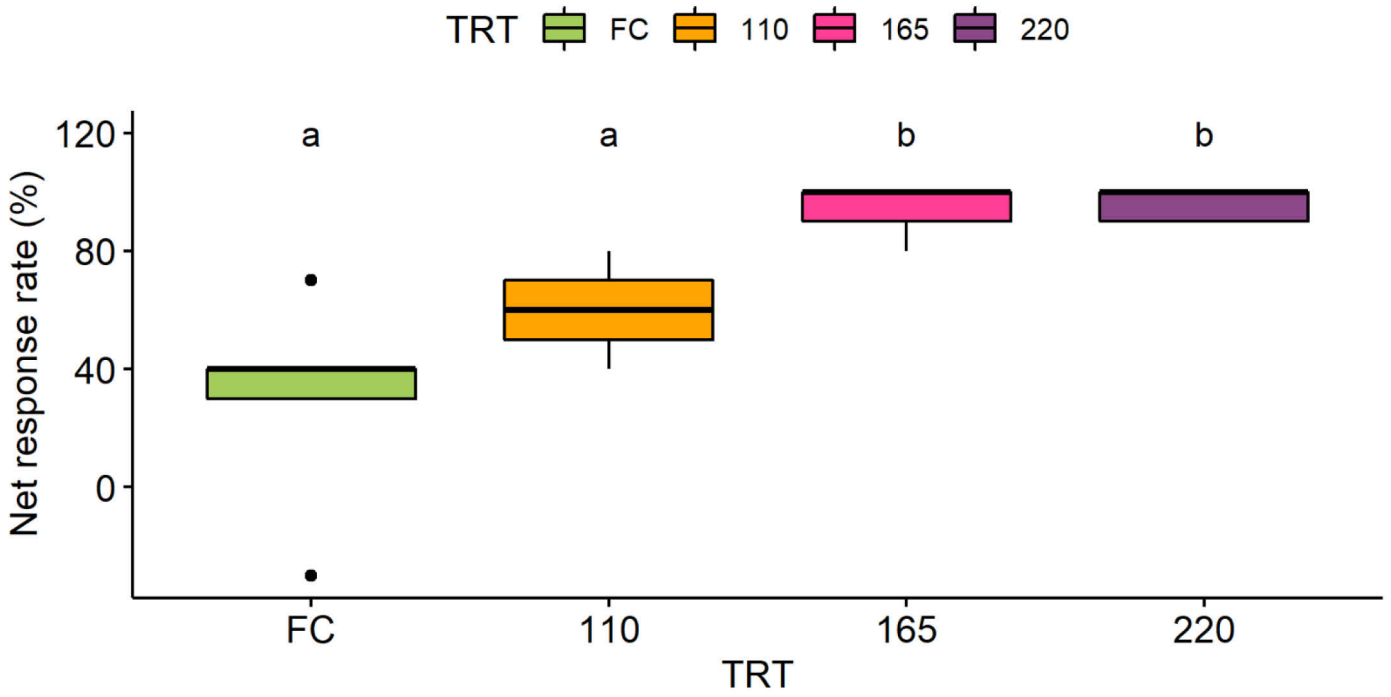

Figure 7.1 Avoidance behaviour in *Eisenia fetida* expressed as Net Response rate. According to Dunn's Kruskal-Wallis multiple comparisons, treatments with different lowercase letters were significantly different. Treatments codes: FC, field concentration of 55 mg/kg; 110 Cu concentration of 110 mg/kg, 165, Cu concentration of 165 mg/kg and 220, Cu concentration of 220 mg/kg.

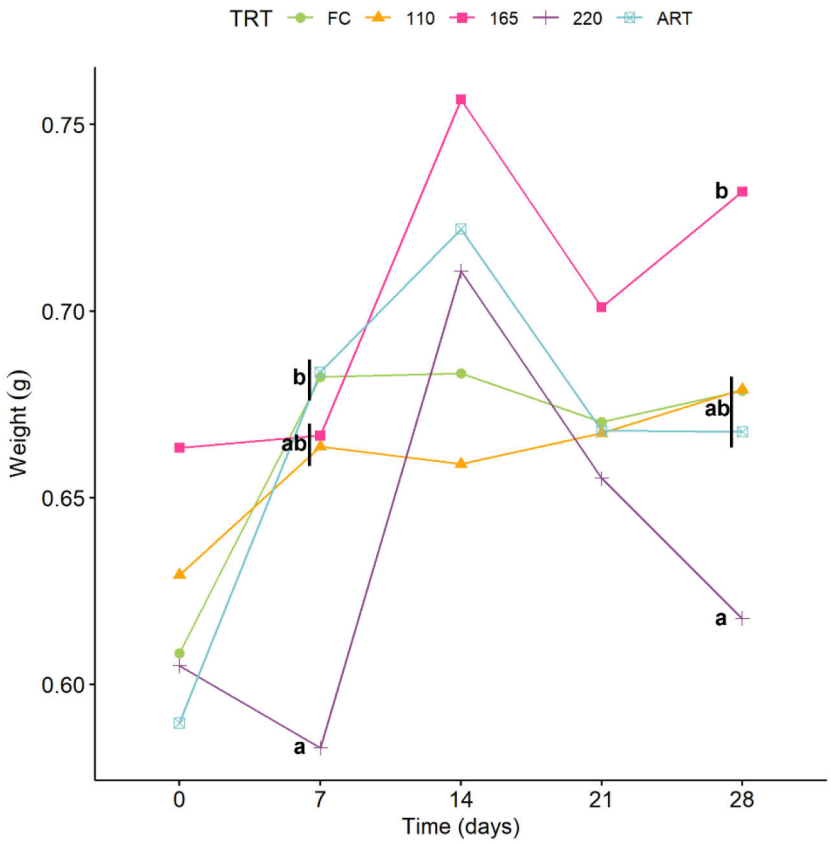

Figure 7.2 Earthworms mean weight during the reproduction test. According to Dunn's multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05), different letters in the same time indicate significant differences between the treatments. When no letter is shown, there are no significant differences between treatments.

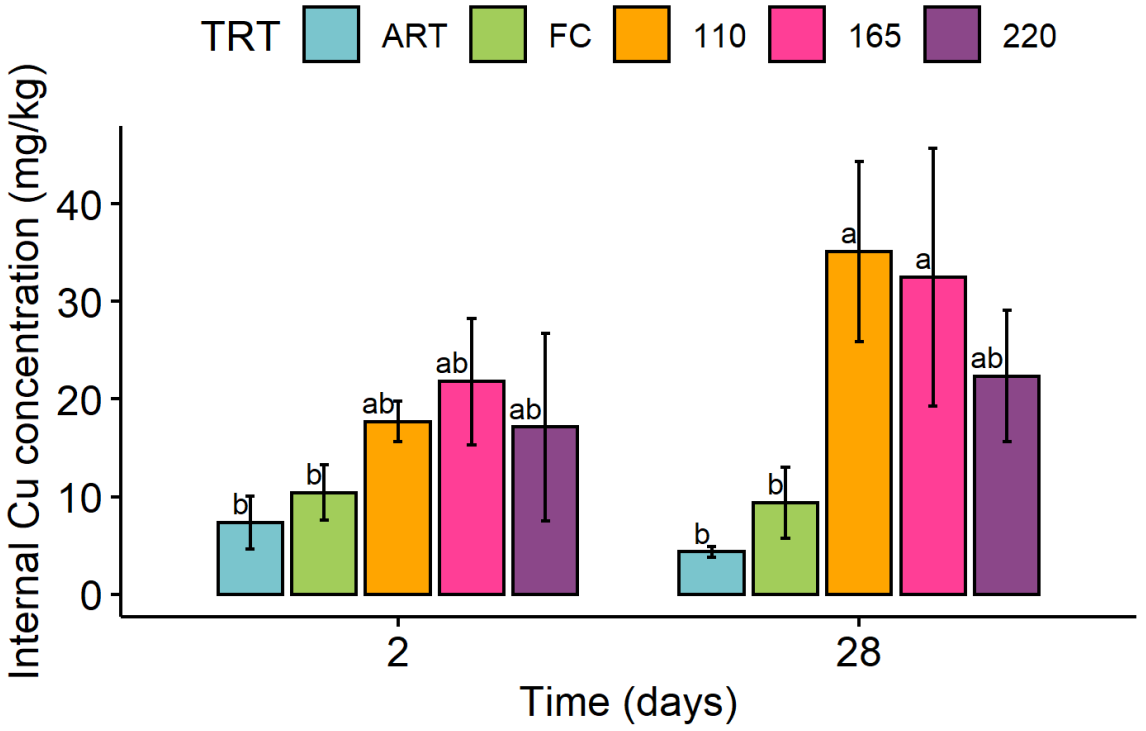

Figure 7.3 Copper earthworms concentrations at the first (2 days) and last (28 days) times of sampling of the toxicity experiment. According to Dunn's multiple comparisons (Benjamini–Hochberg p-value adjustment, α-level = 0.05), different letters in the same time indicate significant differences between the treatments.

Table 7.1 Observations on earthworms reproduction output in the reproduction experiment.

PARAMETERS	TRT				
	ART	**FC**	**110**	**165**	**220**
N° Cocoons/Replicate	52.00±7.55^{b}	51.33±2.52^{b}	42.33±7.64^{ab}	35.33±7.64^{ab}	21.33±4.62^{a}
N° Juveniles/Replicate	115.00±9.85^{b}	109.67±9.50^{b}	61.33±8.96^{ab}	64.00±9.00^{ab}	34.33±10.10^{a}
Growth Rate (%)	13.20±4.06^{b}	11.5±4.96^{ab}	7.59±1.53^{ab}	10.30±3.22^{ab}	2.57±1.20^{a}
Mortality (%)	NA	NA	NA	NA	13.33±9.43

According to Dunn's Kruskal–Wallis multiple comparisons, treatments with different lowercase letters were significantly different.

Table 7.2 Total and bioavailable copper concentration (mg/kg) in the toxicity experiment.

	Total Cu		**Bioavailable Cu**	
	2 Days	28 Days	2 Days	28 Days
ART	3.09±0.37	1.27±0.54	0.14±0.09	0.10±0.03
ART_E	3.46±0.40	3.99±0.57	0.11±0.01	0.11±0.03
FC	53.12±3.03	48.40±3.52	3.82±0.23	4.56±0.42
FC_E	61.51±0.68	57.02±0.97	4.46±0.29	3.79±0.30
110	104.13±8.44	95.96±4.93	24.4±0.75	20±1.73
110_E	101.05±6.46	90.71±4.61	23.1±1.25	15.9±1.49
165	143.48±3.53	129.09±5.50	50.4±7.15	34.9±2.15
165_E	149.66±10.00	132.91±11.00	45.8±2.11	25.2±2.85
220	191.35±4.85	180.82±13.9	53.0±9.97	52.5±4.52
220_E	178.68±11.0	186.04±11.50	66.5±2.70	38.6±8.72

Concluding remarks

The present work has been carried out to respond to specific case studies all relating to the issue of soil pollution, but which move at different levels of interest such as; remedying an ongoing case of environmental contamination (Chapters 1 and 2), understanding the kinetics of pesticides in various substrates (Chapter 4) and their ecotoxicity in the soil ecosystem (Chapters 3, 5, 6 and 7).

The research conducted on remediation techniques confirmed the immobilising and adsorbing potential of zeolite and bentonite compared to the metal nickel in the lime substrate. Among the various doses tested, the 5% one gave the best results in sequestration of the metal that was also favoured by the physical and chemical properties of the substrate, i.e. alkaline pH and high percentage of organic matter (Chapter 1). Spinach and sunflowers are interesting species for phytoextraction of nickel, while sorghum and canola are more attractive in terms of phytostabilisation of the metal in the underground portions and the rhizosphere. The combined use of phytoremediation and sequestering materials such as bentonite can be a winning strategy in solving real cases of nickel contamination in lime (Chapter 2).

Regarding the techniques for assessing the harmful effects of sub-lethal doses of pesticides on the soil ecosystem, numerous examples in the bibliography carry out studies on non-target organisms such as earthworms and microorganisms that intimately inhabit this fundamental environmental sector. From the critical analysis of the papers, promising techniques emerge that work at the level of damage to earthworms' DNA and the microbiota's biodiversity. Many studies report that the most influencing factors on the

adverse effects of pesticides are their chemical structure, dosage, and the initial state of health of the soil. In general, the application of natural or synthetic pesticides leads to effects that may be transitory as long as they are not high doses and repeatedly administered over time. In contrast, several studies on synthetic pesticides have shown irreversible modifications to the abundance of soil microorganisms. The review shows the need to include in the studies more than a variety of soils and collection times to understand better the impacts of pesticides on the soil ecosystem over time, also choosing to integrate more biological indicators with early biomarkers of genomic damage in organisms of the soil (chapter 3).

Once in the soil, the pesticides' environmental fate is dictated by numerous factors, mainly the abundance of organic matter, which is also brought to the soil to reduce its harmful effects. The research conducted on the kinetics of adsorption and desorption of the pesticides chlorpyrifos, metalaxyl and cymoxanil has shown that the administration of organic matter favours their degradation and that the humic acid fraction is the most efficient in terms of adsorption (chapter 4).

The high organic matter content seems to have also influenced the strong adsorption of chlorpyrifos and spinosad in the study in which these two insecticides were compared in terms of ecotoxicological effects on soil microbiota and earthworms. The research showed that earthworms could promote the metabolisation of the natural insecticide spinosad and do not present significant damage in terms of reproductive activity or biomass. On the contrary, the persistence of chlorpyrifos is not affected by the presence of earthworms, whose health is strongly compromised by the insecticide and affects the level of early damage to the DNA analysed through the comet

assay technique. The bacterial community changes mainly as a function of time and the absence of earthworms in contaminated soils, and the species *Eisenia fetida* seems to have a buffer effect on the impact of insecticides on the microbial composition in the soil. The work confirms how the multi-technique approach successfully identifies damage to the soil ecosystem by pesticides (Chapter 5).

In addition to insecticides, copper-based fungicides widely used in viticulture can also affect the soil biome, especially when metal accumulation occurs. In the monitoring study conducted on two vineyards during two vegetative seasons, it was seen how careful management of copper-based antifungal treatments does not necessarily determine an accumulation of the total fraction of this element. Despite this, particular attention must be paid to the trend of the soluble fraction, which, following repeated treatments, can lead to the leaching of the metal into groundwater. Analyses on the effects of different sub-lethal copper concentrations conducted on earthworms showed that, although the death of individuals does not always occur, they develop within certain doses, which on the natural soil under study are around 150 mg/kg, answers adaptation with the recovery of weights and DNA damage (chapter 6).

Considering the need also requested at the European level of further studies on the adverse effects of copper compounds on the integrity of the soil system, other concentrations and analyses have shown that *Eisenia fetida* can recognise and avoid soils contaminated with copper and the avoidance of these substrates as well as the reproductive activity of earthworms appear to follow a dose-dependent response. At the concentration of 220 mg/kg of copper in the soil, the harmful effects of the metal on earthworms are

irreversible. Despite the presence of earthworms, it does not seem that these affect the concentrations of total and bioavailable copper at the tested doses; in fact, the analysis of the metal inside them did not show bioaccumulation of copper in any of the tests conducted.

It will be interesting to finish the study by evaluating the response to these further concentrations at a genotoxic level and analysing the bacterial community both in the tested soils and inside the earthworm's gut. Experimentation in this regard is still in progress, and the results processed will be disclosed as soon as they are available (Chapter 7).