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Impacts of Trace Element Pollution on Sewage Sludge and Soil-Plant Systems: Developing Remediation Techniques and Assessing Plant Uptake Prevention Measures

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A Gianni

You know the kind of life that I live
When your smile meets my smile
A something's got to give
I ain't no false prophet
No, I'm nobody's bride
Can't remember, when I was born
And I forgot when I died

Bob Dylan

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Abstract

Trace Elements (TEs) pollution is a significant environmental concern due to its toxic effects on human and ecosystem health and its potential to bioaccumulate in the food chain and to threaten species survival, leading to a decline in biodiversity. Urban areas, industrial and mining activities, agricultural practices, all contribute to the release of TEs into the environment posing a significant risk to human health and ecosystems. Several techniques have been developed to control TEs into the environment. This work presents the findings of three-year PhD program that focused on research on TEs pollution. The study discusses three fundamental aspects related to this topic from the perspective of sustainable development, environmental and human health. (1) High levels of TEs contamination prevent the use of sewage sludge (SS) as a fertilizer in agriculture, despite its potential as a soil amendment. Developing effective techniques to manage TEs contamination in SS is critical to ensure its safe use in agriculture and promote resource efficiency through sludge reuse. The first goal of the study was to investigate the potential of a low-impact washing treatment to reduce TEs contamination in sewage sludge (SS) and make it suitable for use as a sustainable organic fertilizer in agriculture. The treatment uses a neutral saline solution to remove metal cations adsorbed to the solid phase of the sludge, and its efficacy was tested at various pH levels and with different types of sludge. The results showed a good performance in TEs removal of the washing treatment on dehydrated sludges, specifically for toxic elements such as Ni and Cd. (2) Another purpose of the study was to evaluate different strategies to limit the TEs uptake by horticultural crops (specifically, *Cucumis Melo L.*). This study addressed the effect of seasonality, *Trichoderma* inoculation and clinoptilolite application on chromium (Cr), copper (Cu) and lead (Pb) content of early- and late-ripening cultivars of *Cucumis Melo L.*. The use of *Trichoderma* and clinoptilolite did not have a noticeable impact on the uptake of TEs by plants. However, early season cultivation was strongly linked to a decrease in the uptake of Cu and Pb. The correlation between the Cu and Pb content and the calcium (Ca) content in stems and leaves (which serves as an indicator of varying transpiration rates during different growing seasons) suggests that these metals may be taken up through non-selective cation channels used as a drought stress defence mechanism. (3). Finally, the accumulation of copper and the effect of its bioavailable fraction on bacterial and fungal communities in the rhizosphere soil of two vineyards, featuring two different varieties of *Vitis vinifera* grown for varying lengths of time, were evaluated. Results indicated that there was a notable variation in microbial (alpha) diversity between the vineyards studied, but no variation between the two grape varieties. However, beta diversity, which pertains to the diversity of microbes and fungi, was found to be significantly different among vineyards for both. Although there was a significant difference in microbiome diversity and Cu accumulation between the grape varieties, no correlation was observed between Cu levels and the diversity of rhizosphere microbiota.

Key words: trace elements; sewage sludge; washing treatment; plant uptake; Trichoderma; clinoptilolite; rhizosphere; bioavailable copper; alpha & beta diversity.

General introduction

Trace Elements (TEs) pollution is a major environmental concern due to its toxic effects on both human and ecosystem health. Heavy metals, such as lead, arsenic, and cadmium, are toxic even at low concentrations and can accumulate in the body over time, leading to severe health issues such as neurological disorders, cancer, and kidney damage. Elements such as copper and zinc are essential for the metabolic activity of living things, yet overuse and subsequent accumulation in the environment becomes a potential health and ecosystem risk. Indeed, TEs can bioaccumulate in the food chain and pose a threat to the survival of various species, leading to a decline in biodiversity (Briffa et al., 2020; Nriagu, 1989).

Urban areas are a major source of TEs pollution due to the high density of population and the associated activities. The burning of fossil fuels, such as coal and oil, releases TEs into the air, which can then be deposited on the ground and water bodies through precipitation (Nriagu, 1989).

Industrial activities also contribute significantly to the release of TEs into the environment. Mining, smelting, and refining of metal ores are major sources of TEs pollution. Burning of fossil fuels, the use of chemicals, and the improper disposal of waste can also contribute to the release of TEs into the environment (Gupta et al., 2019).

Agricultural activities also contribute to the release of TEs into the environment. The use of pesticides, fertilizers, and waste irrigation water can lead to the contamination of soil and water bodies. In addition, the application of sewage sludge (SS) as a fertilizing agent can also contribute to the contamination of soil with TEs (Gupta et al., 2019).

Overall, the release of TEs into the environment from these urban, industrial, and agricultural activities poses a major risk to human health and the environment. It is important to mitigate and control these sources of TEs pollution to protect the health and well-being of both humans and ecosystems (Azhar et al., 2022).

Therefore, the environmental contamination of TEs has attracted significant attention in recent years, and various methods have been developed to remove them from the environment. These methods include physical, chemical, and biological treatment methods, each with their own advantages and limitation (Azhar et al., 2022; Briffa et al., 2020).

From an agronomical perspective, it is currently impossible to avoid introducing trace elements (TEs) into soil through the use of fertilizers and pesticides. For example, copper has been used as a fungicide since the 1880s and remains the primary element used to combat major fungal diseases in all agricultural production guidelines. This has led to a general accumulation of copper in agricultural soils (Mackie et al., 2012).

However, remediation techniques to remove TEs in agricultural soils is in most cases limited by the operational costs and the incompatibility of treatments (i.e. ex-situ washing treatment) with farm production.

Therefore, the research in this field is mainly focused on development of strategies to stabilized TEs in soil and make them unavailable for plant uptake (Azhar et al., 2022).

The high levels of TEs contamination are also the primary reason that makes not possible to use SS as a fertilizer for agricultural purposes. SS, a by-product of wastewater treatment, has the potential to be used as a soil amendment in agriculture as part of the circular economy, in line with the principle advocated by the EC (2020). Utilization of sludge in agriculture can help reduce the amount of sludge disposed in landfills, decrease the demand for synthetic fertilizers, and improve soil quality. According to the European Commission Report (EC, 2008), more than 10 million tons of dry solids of sludge were produced in Member States (26 EU) in 2008 (Gherghel et al., 2019), and production increases year by year with increasing population and industrial activities (di Giacomo & Romano, 2022). Therefore, it is crucial to develop effective techniques for sludge treatment to reduce its heavy metal contamination and ensure its safe use in agriculture. In addition, sludge reuse represents an opportunity to promote the circular economy by reducing waste and increasing resource efficiency.

Overview and aim of the research

This paper presents the findings of three-year PhD program that focused on research on TEs pollution. The following chapters discuss three fundamental aspects related to this topic from the perspective of sustainable development, environmental and human health.

The importance of sustainable sewage sludge (SS) management has been well-established. In line with the principles of the circular economy (Korhonen et al., 2018; Veyssière et al., 2022), it is crucial to adopt management practices that prioritize resource efficiency. From a waste management perspective, the most sustainable option for SS is its use as an organic fertilizer in agriculture (B. Sharma et al., 2017, 2019). However, the presence of TEs in SS often prevents its use in agriculture due to the potential risk of contaminating soil and crops (Gherghel et al., 2019).

The first chapter aims to explore the potential of a low-impact washing treatment to reduce the heavy metal contamination of sewage sludge (SS) and enable its use as a sustainable organic fertilizer in agriculture. The treatment involved a neutral saline solution ($CaCl_2$ 0.5M) to induce the detachment of metal cations adsorbed to the solid phase of the sludge (Buscaroli et al., 2019a). The washing efficacy was tested at different pH levels, from natural sludge pH value to 0, and treating different sludge matrices corresponding to the principal topologies of sludge:

- *Surplus sludge*
- *Stabilised sludge*
- *Dehydrated sludge*
- *Freshwater clarification sludge.*

The second aspect of TEs contamination approached in this thesis work was the uptake and translocation of TEs in plants and the potential risk to human health through the food chain. Specifically, the second chapter reports a summarize of the study, recently published (Brecchia et al., 2022) and reported as an attachment, that addresses different agricultural strategies to limit TEs uptake and translocation by melon plants (*Cucumis melo* L.), one of the most crops cultivated in Italy [Italian Institute of Statistics (ISTAT) 2021]. This field study experiments the effect of seasonality, *Trichoderma* inoculation and clinoptilolite application on the PTEs content of early- and late-ripening cultivars of *Cucumis melo* L. The use of adsorbent minerals (i.e., Clinoptilolites) or competing organisms has already been extensively tested for their agronomic effects (Berg, 2009; Nakhli et al., 2017a), but their effects on retention and reduced availability of elements harmful to plants is not entirely understood at a field scale (Leggo et al., 2006; Leggo & Ledésert, 2001; Oste et al., 2002a; Téllez Vargas et al., 2017a).

Finally, the third chapter reports a study conducted in collaboration with Genetics area of the Department of Agricultural and Food Sciences (DISTAL) that aims to evaluate the differences in copper (Cu) accumulation in two different vineyards, and two Grape varieties, located in the Po valley (Italy), with diverse planting ages. *Vitis vinifera* is one of the most cultivated crops in Mediterranean climate countries, such as Italy, with a great economic impact specifically in the wine industry. The use of Cu as a plant protection product against pathogenic diseases is one of the important topics in organic farming management. Specifically, Cu is used to control fungal and bacterial pathogens, such as grapes downy mildew *(Plasmopara viticola*), potato late blight (*Phytophthora infestans*), and apple scab (*Venturia spp.*) (Brun et al., 2001; Mackie et al., 2012). As a result, the widespread application of copper-based fungicides has resulted in an accumulation of copper in certain agricultural soils that significantly exceeds the trace amounts necessary for healthy plant growth (Mackie et al., 2012). Although the total copper content in soils is a useful indicator of soil deficiency and/or contamination, it does not provide enough information about its environmental impact. (Brun et al., 2001). Copper availability to biota (as a nutrient or toxin) and its mobility are the most important factors to be considered when assessing its effect on the soil environment (Pietrzak & McPhail, 2004).

In this study, were analysed the total Cu content and the bioavailable Cu in rhizosphere soil (the portion of soil explored by the root system, where the pH may be more acidic and more Cu may be dissolved and potentially taken up by plants). Were also correlated the data on bioavailable Cu content with the alpha and beta diversity of the soil microbiome to evaluate the effect of different Cu concentrations on soil biodiversity.

Chapter 1

Remediation of polluted sewage sludges from wastewater treatment plants through low environmental impact technologies for the reuse: $CaCl_2$ washing treatment

Nomenclature

WWTPs: Wastewater Treatment Plants
PTEs: Potentially Toxic Elements
TEs: Trace elements
SS: Sewage Sludge
RS: Raw Sludge
SUS: Surplus Sludge
STS: Stabilized Sludge
DS: Dehydrated Sludge
CS: Clarification Sludge

1.1 Introduction

1.1.1 Sewage sludge

The sustainable management of sewage sludge deriving from wastewater treatment plants (WWTPs) represents an important critical issue worldwide and a fundamental challenge in terms of society development and the circular economy (Nguyen et al., 2022).

Sewage sludge (SS) can be defined as the solid residue derived from industrial and urban wastewater treatment process. Commonly, the raw materials are constituted by 92-96% of water (Hoang et al., 2022), which makes difficult its manage and disposal. Additionally, as absorbent material in aquatic environment, SS is one of the main accumulation sites for potential toxic elements (PTEs) found in liquid phase, especially trace elements (TEs). On dry weight basis, the 20-40% is composed by organic substances rich in nutrient elements [e.g., nitrogen (N), phosphorous (P) and potassium (K)] and live organisms which makes necessary treatments to improve the its biological quality limiting putrefactive phenomena (Collivignarelli et al., 2019). The SS production is constantly increasing due to the rapid rise of global population and the intensification of industrial activities (Nguyen et al., 2022). In Europe, the directive 86/278/EEC encouraged the commitment of member states on the reuse of sludge in agriculture dictating guidelines to prevent harmful effects on environment by limiting the possible transfer of trace elements (TEs) and pathogens.

From environmental point of view, land farming of SS is preferable due to the positive contribution to soil system as a conditioner and to recover organic and inorganic nutrients (Bertoncini et al., 2008; Elmi et al., 2020; Fijalkowski et al., 2017; Poulsen et al., 2013; Roig et al., 2012).

The last European Report (European Commission, 2014) highlighted the effectiveness of the directive and the increasing of the use of SS in agriculture up to 48% of the total produced.

According with the latest report of Higher Institute for Environmental Protection and Research (ISPRA, 2021), in Italy 4.1 million tons of SS in 2019 were produced, 7.4% more respect to 2018. Of these wastes, approximately 58% was disposed of in landfills after treatments (physical and biological treatments, incineration), the 39% was reused (i) for the benefit of farming and for recovery of organic and inorganic substances (75%), (ii) for waste trade in construction market (22%) and (iii) for energy production (2%). The SS management is regulated by D.Lgs 152/2006 that implements the discipline of Legislative Decree 99/1992 (transposition of Directive 86/278/EEC) imposing limits on contamination values for sludges reuse in agriculture. However, the high sludge TEs contamination level often makes the land spreading illegal (Geng et al., 2020). Therefore, less virtuous manage methods such as incineration and disposal still remain the most used methodologies due to the high costs of treatments aimed at decontamination .

1.1.2 WWTPs management of Sewage Sludge

The treatments performed on SS in WWTPs aim to decrease of volume of manage material and his biological stabilization (**Figure 1**).

- *Biological stabilization*

Raw Sludge (RS) entering in WWTPs undergoes an initial thickening phase in settling tanks to separate surplus water (Hyrycz et al., 2022; Vaxelaire et al., 1999). The resulting material, named Surplus Sludge (SUS), is loaded with high OM content and active microorganisms. If not treated, SUS will be naturally subjected to anaerobic digestion and generate an unpleasant smell due to the release of volatile organic and inorganic compounds such as NH_3 and H_2S(Zhu et al., 2016). Moreover, the direct application of SUS to the soil could cause the leaching of OM and pathogens (i.e. *E. coli, Salmonella* spp. and *Enterococcus*) to the groundwater (Al-Gheethi et al., 2018).

Based on the size and the modernity of the WWTPs, biological stability of SS can be achieved mainly using chemical stabilization, anaerobic digestion and aerobic composting.

<u>Chemical stabilization</u> is rarely used due to temporary nature of the treatment. It consists in adding hydrate lime [$Ca(OH)_2$] to SS. In this way, pH increasing take to the deactivation of bacteria activity and, consequently, the putrefactive phenomena (Hoang et al., 2022). However, the carbonatation reaction between lime and atmospheric CO_2 returns pH levels to useful values for bacteria activity, over time:

$Ca(OH)_2 + CO_2 \rightarrow CaCO_3 + H_2O$

<u>Anaerobic digestion</u> is widely used in modern plants. It is a biological process that uses microbes to convert biodegradable organic matters into biogas ($CH_4 + CO_2$) under oxygen-free conditions (Hoang et al., 2022; Khanh Nguyen et al., 2021; Raheem et al., 2018). In Italy, it is used to process around 380 m^3/d of SS, while generating about 6100 m^3/d of biogas (Rittmann et al., 2008). The main advantage of this technology consists in "*total energy recovery*" through the co-generation of electricity, used to directly cover plant self-consumption, and thermal energy used to heat digesters and/or to feed networks of WWTPs district heating.

<u>Aerobic digestion</u> is applied in medium-sized and small-sized WWTPs for economic considerations. In this process, organic matter is oxidized and products such as carbon dioxide, nitrate and phosphate are generated with release of heat. At the same way is curried out the stabilization process in which biologically stable products are produced and both the sludge mass and volume are reduced (Z. Zhang et al., 2016).

<u>Composting</u> is an aerobic process in which microbes naturally present in sludge decompose biodegradable organic matter to form a stable solid product (de Bertoldi et al., 1983; Lv et al., 2018). The process includes three steps: mesophilic (low temperature), thermophilic (high temperature, 60-70°C), and maturation (cooling down). The high temperature achieved during the second step ensures disinfection from pathogens (de Bertoldi et al., 1983). Normally, the high water-content and low C/N ratio of sludge make necessary adding amendment materials such as straw, ash, or sawdust in a co-composting process to improve the nutritional balance for microorganisms and porosity (Hoang et al., 2022) and to decrease the sludge humidity

(from 70-80% to 55-50%). Aerobic composting represents an optimal methodology to treat SS for landfarming by converting the sludge into more uniform source of organic carbon and nutrients (Margesin et al., 2006). Therefore, composting remains an underutilized strategy within WWTPs for the problems related to TEs sludge contamination, which affects the possibility of the use in agriculture. In fact, aerobic composting is not useful to mitigate TEs contamination and, additionally, the process can increase their availability to plant uptake (Ingelmo et al., 2012; Zhang et al., 2017).

- *Dewatering*

The volume of raw sludge is chiefly composed by water (~96%) that can be divided into three types: free water, interstitial water, and bound water (Möller, 1983; Vesilind, 1994). Free (or inter-particulate water) is not combined to sludge solid matrix and it can move freely within the sludge system. It has the highest content (60-70%) and represents the easiest water to separate from the sludge. The interstitial water accounts approximately 25% of the total water volume. It exists as water combined with colloids surface through electrochemical interactions and not free-flowing in sludge suspension system (Hyrycz et al., 2022; Vaxelaire et al., 1999; Vaxelaire & Cézac, 2004; X. Zhang et al., 2022). Colloids particles represent the most part of sludge dry weight. Water molecules absorbed by the large specific surface area forms a stable suspension phase. The bound water has the higher solid-water potential because it is part of the sludge particle structure and microorganisms. Normally, bound water is difficult to be separate from the solid matrix. However, the total amount is usually small (~3%).

The SS deep dewatering can be achieved turning the interstitial water into free water which can be removed more easily following mechanical treatments (i.e. decanter centrifuge or a high-pressure filtration process) (B. Tang & Zhang, 2014). The conditioning processes are curried out within the WWTPs commonly using inorganic or organic compounds to favour the flocculation of sludge colloids. In this way, the charge structure on the surface is changed and the retention power on water molecules is reduced. As inorganic compounds, iron and aluminium salts are the most used. Iron salts include ferric chloride ($FeCl_3$), ferrous chloride ($FeCl_2$), ferric sulphate ($Fe_2(SO_4)_3 \cdot 4H_2O$); aluminium salts include aluminium sulphate ($Al_2(SO_4)_3 \cdot 18H_2O$), aluminium chloride ($AlCl_3$), etc. (X. Zhang et al., 2022). Trivalent metal ions initiate hydrolysis reactions in water forming metal hydroxides (Duan & Gregory, 2003), as per following equation:

$$M^{3+} + H_2O \rightarrow M(OH)^{2+} + H^+$$
$$M(OH)^{2+} + H_2O \rightarrow M(OH)_2^+ + H^+$$
$$M(OH)_2^+ + H_2O \rightarrow M(OH)_3 + H^+$$
$$M(OH)_3 + H_2O \rightarrow M(OH)_4^- + H^+$$

These molecules absorb sludge particles to form larger and more weighty flocs. The sludge structure is compressed by the action of electric neutralization and adsorption, resulting in a decrease in its water holding capacity and an increase in the sludge dewatering (Niu et al., 2013).

Among organic compounds, cationic polyelectrolytes as i.e. polyacrylamide are widely used as sludge flocculant. These polymers are soluble in water and easily absorbed by electronegative particles surface forming stable aggregates (X. Zhang et al., 2022). Respect to Fe and Al-salts, the optimum dosage of cationic polyelectrolytes is considerably less but this kind of reagent is more expensive than chemicals (Lee et al., 2014). On the other hand, higher salts dosage increases the conditioning effectiveness but can increasing also secondary pollution phenomena (X. Zhang et al., 2022).

Ultimately, the chois of the flocculant compounds is normally liked to the final course of treated SS. Iron and aluminium salts will be increasing the ashes amount, therefore, conditioning treatment with organic compounds is preferable when the sludge will be incinerated. On the contrary, salts compounds are recommendable when the sludge will be reused in farmland due to the apport of useful elements to the soil.

Anyway, the combination of organic polyelectrolytes and Fe or Al salts is preferable to treat SS. Indeed, inorganic salts are also used to decrease P content in wastewater through precipitation process as follow equations:

$Al^{3+} + H_nPO_4^{3-n} \rightleftarrows AlPO_4 + nH^+$ (Kirk et al., 1988; Yeoman et al., 1988)

$Fe^{3+} + H_nPO_4^{3-n} \rightleftarrows FePO_4 + nH^+$ (Jenkins et al., 1971; Yeoman et al., 1988)

It is widely known that phosphorus, in the form of phosphate, is a major uncontrolled pollutant in wastewater resulting from sludge treatment, and it is responsible for the degradation of water quality (eutrophication phenomena) (Jenkins et al., 1971; Kirkham, 1982).

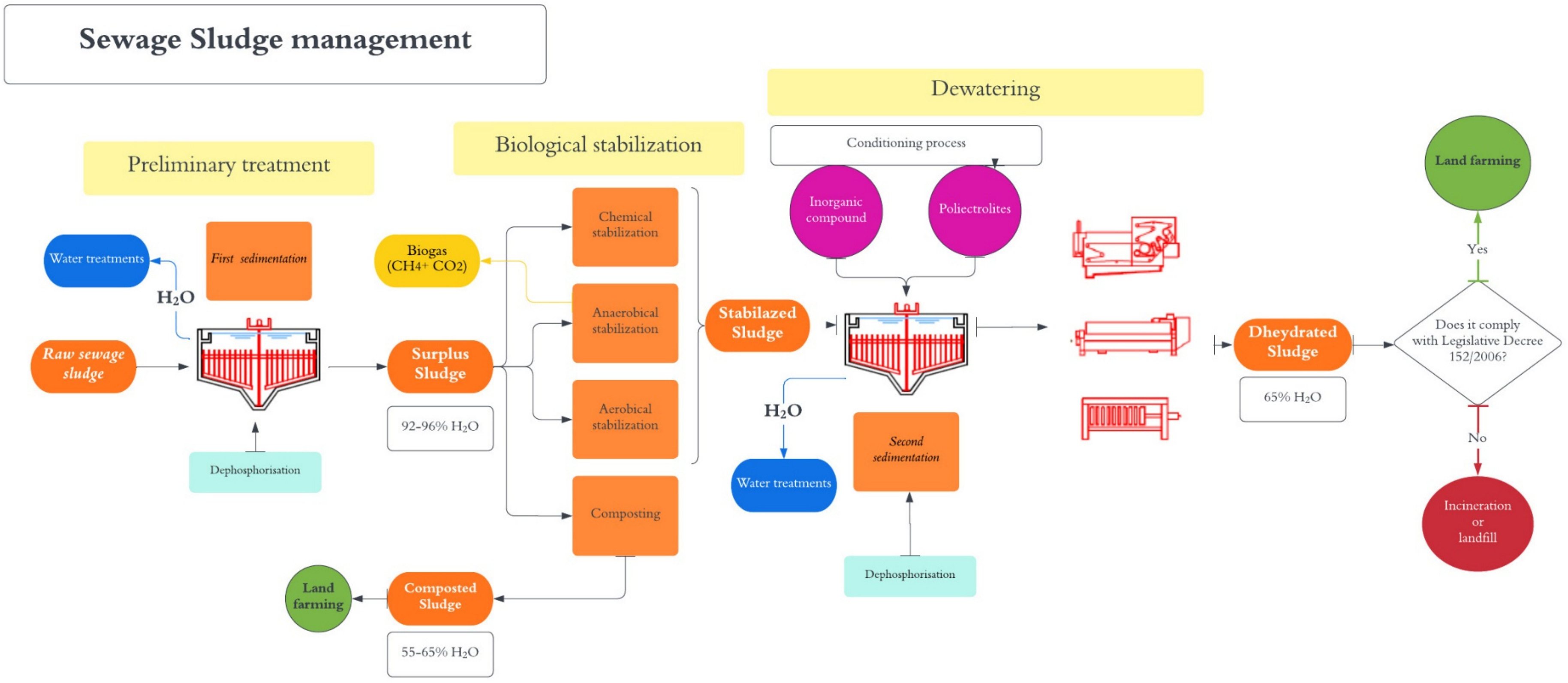

Figure 1 Sewage sludge management scheme in wastewater treatment plants

1.1.3 Treatments to TEs removal strategies: state of art

1.1.3.1 TEs contamination

Recently, the removal strategies of TEs from SS has become an important research focus due to the problems related to environmental management of the waste treated in WWTPs. TEs contamination is the primary obstacle that forbids the virtuous reuse of SS as an amendment in land farming. The spreading of polluted SS in soils can generate an accumulation of TEs (i.e. Cr, Cu, Ni, Pb and Zn) potentially available to plant with phytotoxic effects (Fang et al., 2019; Memoli et al., 2019). In addition, TEs leaching phenomena from polluted SS can result in contamination of ground/surface water and, overall, pose a risk to human health by entering the soil-crop-food chain (Geng et al., 2020; Singh & Agrawal, 2008). However, these critical issues are related to the bound typology that characterize the TE-solid interactions, which reveal the TEs availability to be absorb by living beings (Fijalkowski et al., 2017).

It is well known that the most toxic TE fraction exists in exchangeable form. Ion exchange normally occurs among divalent ions and the active groups on organic colloid surfaces (i.e. carboxylates [$-COO^-$], alcohols [-OH], amines [$-NH_2$] and amides [$-CONH_2$]) (Escudero et al., 2016; Pagnanelli et al., 2009). This fraction is the most available because the ions can easily pass into solution in soluble form. However, many research had shown that in SS more stable adsorption mechanism, such as OM complexation and inorganic precipitation, are predominant (Y. Li et al., 2020; Xu et al., 2020).

Sludge OM are composed from 60 to 90% by extracellular polymeric substances (polysaccharides, proteins, lipids and humic substance) which are largely cross-linked by multivalent metal ions. Indeed, the large number and nature of functional groups (carboxylates, phosphates, sulphates, amines, amides) may be combined with TEs leading to formation of stable complexes (Escudero et al., 2016; Geng et al., 2020; Y. Li et al., 2020). Additionally, inorganic salts (i.e. sulphates and phosphates) with part of TEs tend to form insoluble precipitates, such as sulphides, oxyhydroxides and phosphonates (Geng et al., 2020).

In complexed or precipitated form, TEs are not leachable or readily available to living being absorption but can become as a result of a change in environmental conditions (i.e. pH, redox potential). Consequently, TEs stabilization/immobilization treatment such as lime addition may be not sufficient to limit environmental risk when it comes to SS use in land farming (Burton et al., 2006; Eggleton & Thomas, 2004). For these considerations, it is necessary to improve effective strategies to removing TEs from SS. In addition, the removal good results must be couple with attention to maintaining material qualities and economic/ecological feasibility for WWTPs.

1.1.3.2 TEs removal treatments

During the past years, several methods have been developed for removal TEs from SS. It is well known that the availability, as well as, the toxicity of TEs depends on their chemical speciation. Such an assessment can be made by evaluating the amount of TEs bound to each fraction (adsorbed > precipitated > complexed > residual) using sequence analysis (Davidson et al., 2003; Rauret et al., 1999). Many authors agree that elements such as Cu and Cr exhibit low mobility in the SS system due to high affinity for humic acids and thus to easy complexation (Lake et al., 1984; Lasheen & Ammar, 2009; Tytła et al., 2016). Instead, Cd, Zn and Ni are frequently present in the exchangeable, carbonate and Fe/Mn-oxide forms as the most mobile fractions (Lasheen & Ammar, 2009). TEs removal efficiency increases with their mobility in SS system, because TEs with high mobility are easier to solubilize in the sludge liquid phase. The transition of TEs from more stable chemical forms to soluble forms is the basis of major decontamination methods such as ion-exchange treatment, chemical agent treatment, electrokinetic process and bioleaching (Geng et al., 2020).

- *Chemical agent treatment*

Chemical removal methods imply the use of chemical extractant such as organic and inorganic acid and chelating agents (CA).

Acid extraction or acid leaching involves the dissolution of TEs in SS (Geng et al., 2020). The removal efficacy depending on many parameters such as type of acid, pH, agitation time and temperature. Several authors had conducted studies on the best extraction condition in SS with inorganic and organic acids (A.H.M.Veeken, 1999; Gheju et al., 2011; Kuan et al., 2010; Stylianou et al., 2007; Yoshizaki & Tomida, 2000). Overall, the results reported the positive correlation between the effectiveness of TEs removal and increasing the amount of acid used as of contact time and high operating temperature.

CA have been extensively studied as an experimental methodology for TEs removal from soil and SS. Operationally, CA bind TEs to form metal-chelating complexes that modify metal speciation. About SS, the most recent researches have produced interested results with the utilization of EDTA (ethylene diamine tetraacetic acid) and GLDA [N, N-bis (carboxymethyl) glutamic acid]. Gheju et al. (2011) mixed EDTA with biosolids for an extraction process of 6 h, found that the removal efficiencies of Zn, Pb, Ni, Cd, Cr and Cu were approximately 34%, 20%, 16%, 10%, 7% and 5%, respectively (Gheju et al., 2011). However, better results have been achieved with the use of GLDA that under optimal operational conditions, 89%, 82% and 84% of Cd, Ni and Cu content in the sludge was removed, approximately (Suanon et al., 2016; Wu et al., 2015).

At the large-scale, the application of chemical agent treatments is underused due to the high costs of plant processes and the large amounts of materials (Geng et al., 2020). The good effectiveness of acid extraction and metal chelation is contrasted with the environmental impact due to the non-biodegradability of the extractive agents and the risk of secondary pollution such as the production of toxic gases and solid residues.

- *Electrokinetic process*

Through the inserting electrodes in the SS system, electrokinetic technique (EK) uses the direct electrical current to change TEs chemical form (Acar & Alshawabkeh, 1993; Geng et al., 2020). At the cathode, oxygen, hydrogen and metal ions are reduced, while oxidation reactions occur at the anode . These processes induce species transport by coupled conduction phenomena such as electrophoresis, electroosmosis and electromigration (Acar & Alshawabkeh, 1993). The transition and removal of TEs can be accomplished by electrodeposition, precipitation, or ion exchange (Li et al., 1998).

The SS water is used as a conductive medium undergoing electrolysis reaction at the anode and at the cathode as follows:

Anode: $2H_2O - 4e^- \rightarrow O_2 + 4H^+$

Cathode: $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$

In this way, pH levels at the electrodes change from values ~2 (anode) to values ~11 (cathode) (Li et al., 1998). High pH levels near the cathode inhibit TEs solubilization due to the presence of hydroxides that combining with the metal cations shifting to more stable forms (Geng et al., 2020; Li et al., 1998; Tang et al., 2020; Weng & Yuan, 2001). This phenomenon reduces the efficacy of EK that, usually, is coupled to other technics such as acid treatments or adding chelating agent in order to improve the TEs removal process.

Peng et al. (2011) studied the electrokinetic remediation after bioleaching, the contents of Cu and Zn in sewage sludge decreased from 296.4 and 3756.2 mg kg^{-1} to 63.4 and 33.3 mg kg^{-1}, respectively, which could meet the Chinese standard for land application of the heavy metals in sewage sludge. During bioleaching, the change into soluble and ionic fractions of organic/sulphide-Cu and the carbonate-bound and organic-sulphide fractions of Zn were observed. Thus, soluble TEs could easily migrate to the electrode zone (Peng et al., 2011).

Good performance was obtained by Pei et al. (2016) who used the EK method for removal TEs from SS by adding ethylenediamine, a chelating agent, to the anode. The addition of the chelating agent had a significant effect on the change in speciation and migration of TEs. A decrease in the amount of TEs in their stable species and an increase in the exchangeable form state were observed. The removal rates of Zn, Cu and Pb were 87.51%, 67.64% and 57.79% respectively (Pei et al., 2016).

EK process has been also studied by Wang et al. (2022) in SS to TEs removal end nutrient recover assisted by biodegradable agent such as citric acid, $FeCl_3$, ammonium hydroxide, tetrasodium iminodisuccinate (IDS), and tea saponin. The results obtained shown a decrease of TEs risk potential linked to plant uptake and leaching into water system. Additionally, low nutrient loss in SS was observed (X. Wang et al., 2022).

Overall, EK technology can obtain high removal efficacy of TEs, especially if assisted with other processes or additive agents. However, the combination with other removal methods lead to an increase in cost of an already prohibitively expensive technology on a large scale. In addition, the additives such as acids and chelating agents may cause secondary pollution in the environment (Geng et al., 2020).

- *Bioleaching*

Bioleaching process uses microorganisms to convert insoluble TEs bound to SS (usually metal sulphide, e.g., CuS, NiS, ZnS) into soluble form (usually metal sulphate, e.g., $CuSO_4$, $NiSO_4$, $ZnSO_4$) (Fig.) (Gu et al., 2017). Consequently, metals are extracted into aqueous solution. Respect to chemicals methods, this technology implies mild operational capabilities reducing the chemical consumption by 80%, and is generally preferred due to environmental friendliness (Geng et al., 2020; Gu et al., 2017). Bioleaching microorganisms are mostly acidophilic and chemolithoautotrophic iron- and sulfur-oxidizing bacteria that are commonly belonging to the following genera: *Thiobacillus*, *Acidithiobacillus, Halothiobacillus, Thermithiobacillus, Leptospirillum,* and *Sulfolobus*. Bioleaching mechanisms can be direct or indirect. In direct bioleaching, insoluble metal sulphides are directly oxidised by *At. Thiooxidans* (1)/ *At. Ferrooxidans* (2) to soluble metal sulphates:

$$MS + 2O_2 \xrightarrow{\textit{At. Thiooxidans}} M^{2+} + SO_4 \ (1)$$

$$MS + 2O_2 \xrightarrow{\textit{At. Ferrooxidans}} M^{2+} + SO_4 \ (2)$$

In indirect sulfur-based bioleaching reduced elemental sulfur (S^0) are added as an external energy source. These species are oxidised by *At. thiooxidans* to sulfuric acid, resulting in a decrease in SS pH, which will further increase the solubilization of metals:

$$2S_0 + 3O_2 + 2H_2O \xrightarrow{\textit{At. Thiooxidans}} 2H_2SO_4$$

$$2H_2SO_4 + \text{sludge-M} \rightarrow \text{Sludge-2H} + M^{2+}\ SO_4$$

In indirect iron-based bioleaching metal sulphides are oxidised by ferric ions. First Fe^{2+} is oxidised to Fe^{+3} by *At. Ferrooxidans:*

$$4FeSO_4 + O_2 + 2H_2SO_4 \xrightarrow{\textit{At. Ferrooxidans}} 2Fe_2(SO_4)_3 + 2H_2O$$

then, Fe^3, as an electron acceptor, oxidises metal sulphides to metal sulphates:

$$Fe_2(SO_4)_3 + 2MS + 4H_2O + 2O_2 \rightarrow 2MSO_4 + 8FeSO_4 + 4H_2SO_4$$

sulfuric acid in turn oxidises metal sulphides to metal sulphates:

$$2MS + O_2 + 2H_2SO_4 \rightarrow 2MSO_4 + 2S + 2H_2O$$

elemental sulfur is oxidated again by *At. Ferrooxidans* generating sulphuric acid, which will further increase the solubilization of metals:

$$2S + 3O_2 + 2H_2O \xrightarrow{\textit{At. Ferrooxidans}} 2H_2SO_4$$

During last decades several studies have been conducted on bioleaching technology to improve the TEs removal efficacy from SS (Blais et al., 1992; Gu et al., 2017; Wen et al., 2012; J. W. C. Wong & Gu, 2004). Under optimal condition, Wong and Henry (1985) observed a removal a rate of 79-85% for Cd; 66-80% for Cu; 70-78% for Ni, and 81-90% for Zn with bioleaching time of 8 days using *At. ferrooxidans* to bioleach TEs from anaerobically digested SS (L. Wong & Henry, 1985). Zhou Q. et al. (2017) obtained a removal efficacy of 89.6% (Cu), 72.8% (Zn) and 39.4% (Pb), in 6 days bioleaching using co-inoculation of *At.*

Ferrooxidans and *At. Thiooxidans* (Zhou et al., 2017). Besides the removal efficacy, the time of leaching process is the main critical issue of this technique. In fact, bioleaching process requires, generally, a long operation time (15 days) to achieve the optimal pH level for TEs solubilization. For this reason, integration approaches with other methods are been developed. For example, Fontmorin & Sillanpää (2015) combined bioleaching/Fenton-like process reducing the leaching time from 18 days (bioleaching alone) to 6 days. The removal of Cd, Cr, Cu, Pb and Zn reached 34.8%, 14.5%, 86.3%, 30.9% and 97.9%, respectively (Fontmorin & Sillanpää, 2015).

Many strategies to treat SS for TEs removal have been well-developed and good results have been achieved. However, each method has its deficiencies, for example, chemical agents are expensive and not eco-friendly, EK processes alone cannot solubilised TEs and bioleaching require a long operation time, often non-compatible with the large volumes to be processed. Therefore, the main decontamination treatments cannot be used extensively. At present, precise knowledge of the dynamics of chemical change and TE distribution in the SS system has not yet been reached. Consequently, removal mechanism must be optimised following a research guideline that equally considers the treatment reaction mechanism and the chemical form of metals involved.

1.1.3 Aim of the work

The purpose of this work was to develop an effective and environmentally friendly decontamination method for the removal of TE from SS that is compatible with WWTPs normal operations. The methodology involves the ion-exchange mechanism induced by the washing with low-impact $CaCl_2$ salt solution. The experimentation started with the research of Buscaroli E. et al. (2019) that use $CaCl_2$ washing treatment to remove TEs from fresh-water channel sediments. As first test, the research obtained good results working on not pre-treat samples and reducing organic and sulphide-Cu and carbonate-Zn concentration of 26% and 10%, respectively. The opportunity to work with not pre-treat material, taking advantage from the sample water naturally content, would make the washing operation easier within a WWTP. The removal of TEs from environmental matrices is achieved by the combined effect of metal displacement by calcium divalent ions and TEs complexation by chloride anions. The effect of $CaCl_2$ on the restoration of soils polluted with metals has been thoroughly examined (Maejima et al., 2007; Makino et al., 2006). Regarding SS decontamination, chlorinating agents (NaCl, $MgCl_2$, $CaCl_2$) have been used to extract TEs from SS ash during incineration (Fraissler et al., 2009; Nowak et al., 2012). Xia Y. et al. (2020) used chlorinating agents during the pyrolysis of sludge with a TEs removal efficacy >86% for Zn, Mn, Cu and Pb, respectively (Xia et al., 2020).

In this work, washing treatment with $CaCl_2$ was tested on different types of sludge matrices corresponding to different steps of SS management and different products used for sludge dewatering. Each sample was tested at different pH levels in order to find the optimal treatment condition. In addition, the results of TEs removal were supported by the metal speciation (assessed by sequential extraction analysis, BCR) in order to evaluate the TEs behaviours within SS system during the washing treatment.

1.2 Materials and methods

1.2.1 SS sampling

Four sludge typologies were selected in order to test the $CaCl_2$ washing efficacy on different polluted solid matrices.

- Surplus sludge (SUS)
- Stabilized sludge (STS)
- Dehydrated sludge (DS)
- Freshwater clarification sludge (CS)

First sampling campaign (Jul.2020)

Surplus sludge (SUS) and Stabilized sludge (STS) were collected from waste treatment plant of San Matteo della Decima (Bologna, Italy) using one 10 L pail. SUS represents the first waste deriving from water separation by gravitational sedimentation. At this level the sludge moist percentage is 98%, approximately. Then, the surplus sludge is stabilized through an aerobic digestion and thickening process which improve the sludge biological characteristics (pathogen reduction, smell abatement, reduction of putrefactive phenomena) and increase the percentage of dry matter by 4%.

Dehydrated sludge (DS) was collected from Bologna WWTP (IDAR) using a 10 L container. The sludge dehydration is carried out through dynamic thickening with rotating drum following anaerobic stabilization. Before the physical dehydration process, polyelectrolytes (DRYFLOC® EM 2050, SNF Italia and TILLFLOCK® CL 1465, Tillmans) and ferric chloride ($FeCl_3$) are added to facilitate the water molecules detachment from solid surface.

The last sludge sample (CS) was collected in Cavanella d'Adige (VE) from a landfill authorized for the accumulation of fresh-water clarification sludge using a 10 L container. CSs are subjected to physical dehydration through belt press after the field spreading.

Second sampling campaign (Jul.2022)

Two years after the first sampled campaign, other two samples of DS were collected from IDAR [DS (II)] and Castenaso WWTP (BO) [DS (III)] using two 10 L containers. DS (II) sample was physically dehydrated with dynamic thickening with rotating drum following anaerobic stabilization. Before the physical dehydration process, polyelectrolytes (DRYFLOC® EM 2050, SNF Italia and TILLFLOCK® CL 1465, Tillmans) and aluminium sulphate [$Al_2(SO_4)_3$] are added to facilitate the water molecules detachment from solid surface. The DS (III) sample was physically dehydrated with dynamic thickening with rotating drum following anaerobic stabilization. Before the physical dehydration process, polyelectrolytes (DRYFLOC® EM 2050, SNF Italia and TILLFLOCK® CL 1465, Tillmans) and ferric chloride ($FeCl_3$) are added to facilitate the water molecules detachment from solid surface.

1.2.2 SS characterization

The physico-chemical characterizations were performed on air-dried SS samples, grinded through a ball mill (Retsch, Haan, Germany), and sieved at 2 mm. All the samples were analysed for pH [International Organization for Standardization (ISO, 2021)], electrical conductivity (EC) (ISO, 1994), total organic carbon (TOC) (ISO, 1995b), total nitrogen (TN) (ISO, 1995c) and total carbonates (ISO, 1995a). Particle size distribution was determined by the pipette method (Gee and Bauder, 1986) using sodium hexametaphosphate as a dispersant.

The exchanges bases and cation exchange capacity (CEC) were determinate through the solid matrix saturation with ammonium cation (NH_4^+) in two steps, following the methodology proposed by United States Department of Agriculture Natural Resources Conservation Service (Soil Conservation Service (SCS), 1972).

The total content of Al and Fe amorphous was determinate with a solution of oxalic acid and ammonium oxalate according to the method proposed by Schwertmann U. & Taylor R. M. in 1977 (Schwertmann. U. & Taylor. R. M., 1977).

Pseudo-total metal content of soil samples was determined through microwave acid digestion (ISO, 2012). Digested extracts were filtered through Whatman no. 42 filter paper and analysed by inductively coupled plasma optical emission spectroscopy (ISO, 2008), using an ICP-OES Spectro Arcos (Ametek, Germany).

1.2.3 $CaCl_2$ washing at different pH levels

The washing treatment with $CaCl_2$ was performed on fresh SS samples at the original pH and an acid pH (by HCl Fluka, 37%) at intervals of 1.0 pH value, down to 0.

Calcium chloride ($CaCl_2 \cdot 2H_2O$; Merck) and Milli-Q water were used to prepare a 0.5 M $CaCl_2$ washing solution taking to account the sample moist level to obtain a ratio w (mg dw): v (mL $CaCl_2$ 0.5 M) of 1:2.5, following the method used by Buscaroli et al. (2019). Aliquots of fresh samples, equivalent of 100 g of dry mass, were placed with 250 mL of washing solution in 500 mL centrifuge tubes. Washing with titration at pH values less than the original were carried out using an automatic titrator (HI931 Potentiometric Titration System Manual-HI932, Hana Instrument). Then, the suspensions were shacked for 6 hours at room temperature. The washing solutions were separated from solid samples by centrifugation at 8000 rpm for 10 minutes. The solid residues were washed with Milli-Q water until the original EC values were restored. Control trials were conducted following the same procedures described above in the presence of 25 mL of Milli-Q water in place of $CaCl_2$ solution.

1.2.4 Sequential extraction BCR

In this study, modified three-step BCR method, proposed by Rauret G. et al (1999) and Mossop and Davidson (2003), was used. Accordingly, 1.0 g of dry sludge was extracted in a 50-mL borosilicate centrifuge tube with different extracting solution. The first fraction (exchangeable fraction) was considered the most soluble and 0.11 M acetic acid was used as the extractant. The second extraction (reducible fraction) was performed using 0.1 M hydroxylamine hydrochloride at pH 2.0. Then, in the third extraction (oxidable fraction), 1 M ammonium acetate (pH 2) was used following oxidation with 8.8 M H_2O_2. All the samples were stirred at room temperature overnight. Finally, residual fraction was estimated as the difference between the total metal concentration and the sum of those obtained in the three fractions.

1.2.5 Statistical analysis

Multivariate Principal Component Analysis (PCA) analysis was performed for the statistical analysis of SSs characterization data using a covariance matrix to build the PCA plot. The software Minitab® 15 (Minitab, 2020) was used for chemometrics analysis.

The effects of $CaCl_2$ treatment on SD was assessed using the analysis of variance (ANOVA) ($P < 0.05$), followed by the Tuckey post hoc test ($P < 0.05$). The analysis was conducted in the R environment (R Core Team, 2020).

Correlation analysis were performed using Microsoft Excel software (Microsoft Corporation, 2018). Pearson's correlation coefficients were calculated to assess the relationship between variables.

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1.3 Results and discussion

1.3.1 SS physico-chemical characterization

In **Table 1** the results of SS physico-chemical characterization are reported. As expected, sludge moisture appreciably changes based on the sludge typology. SUS and STS samples present the higher moisture levels (91.0 and 90.0%, respectively) than the DS and CS samples (75.2 and 65.7%, respectively). SUS, STS, and DS samples present moisture levels typically found in the respective operational stages of WWTPs, i.e., first sedimentation, anaerobic digestion, and dewatering. On the other hands, CS, that was sampled in controlled landfill, is characterized by a lower retention power in virtu of the texture class (sandy loam) and the low content of organic matter (4.3 %).

The pH levels of SUS, STS and DS present a slight variation remaining around neutrality (6.7, 6.8, 6.4, respectively). Instead, pH value of CS is higher (8.2), presumably due to the higher concentration of carbonates (9.4%) than the SUS, STS and DS (2.3%, 2.4%, <DL, respectively). In addition, DS shows a 0.8-unit difference between pH measured in H_2O versus pH measured in saline solution ($CaCl_2$) compared with the 0.2-unit change in the other samples. This value indicates the higher exchangeable acidity and lower capacity to buffer changes in pH of the dehydrated sample respect to SUS, STS and CS. This is consistent with the lack of carbonates in DS.

The CEC values are higher in SUS and STS samples (354.45 and 261.33 $cmol_{(+)}$ kg^{-1}, respectively) than DS (97.38 $cmol_{(+)}$ kg^{-1}). With similar TOC content (SUS: 28.63%, STS:34.38% and DS: 31.00%), the CEC differences can be explained by the texture class, having a higher content of fine mineral matter in SUS (silt loam) and STS (sandy clay loam) than DS (sandy loam), and by the dewatering treatment of DS with polyelectrolytes and ferric chloride that result in microaggregate flocculation. Instead, in CS sample the CEC is very low (14.28 $cmol_{(+)}$ kg^{-1}). This is due to the low organic matter content and to sandy texture.

The use of $FeCl_3$ as a flocculating agent for the dewatering process results in a significant increase in Fe and P content on the dry weight of DS due to the precipitation of insoluble iron phosphates. In fact, P content in DS (33420 mg kg^{-1}) increases until three times than SUS and STS (13663 and 11938 mg kg^{-1}, respectively). While CS presents a very low content (1197 mg kg^{-1}) as expected by low endowment of organic matter. Similarly, amorphous Fe content in DS (43287 mg kg^{-1}) greatly exceeds its concentration in the non-dewater sludge samples (SUS: 5795 mg kg^{-1}; STS: 5668 mg kg^{-1}; CS: 8682 mg kg^{-1}). On the other hand, CS and DS present the higher Al concentration (22851 and 9356 mg kg^{-1}, respectively) respect to SUS and STS (1937 and 1915 mg kg^{-1}, respectively) as expected by the high mineral component.

Based on characterization data, in **Figure 2** the multivariate PCA analysis are reported. The cluster separation among samples not dehydrated (SUS and STS), DS and CS is evident.

Table 1 Physical and chemical characterization of SS samples (SUS: surplus sludge; STS: stabilized sludge; DS: dehydrated sludge; CS: freshwater clarification sludge). Errors are expressed as a standard deviation.

		Samples			
Parameters	Unit	**SUS**	**STS**	**DS**	**CS**
Humidity (U)	%	91.04 ± 0.30	90.00 ± 0.10	75.20 ± 0.22	65.7 ± 1.9
Dry matter (Dm)	%	8.96 ± 0.30	10.00 ± 0.10	24.80 ± 0.22	34.3 ± 1.9
pH (H_2O)	log[H^+]	6.7 ± 0.1	6.8 ± 0.1	6.4 ± 0.1	8.2 ± 0.1
pH ($CaCl_2$)	log[H^+]	6.5 ± 0.1	6.6 ± 0.1	5.8 ± 0.1	8.0 ± 0.1
Electrical conductivity (EC)	mS/cm	0.445 ± 0.10	0.931 ± 0.10	0.556 ± 0.025	0.447 ± 0.10
Cation exchange capacity (CEC)	$cmol_{(+)}$ kg^{-1}	354.45 ± 86.08	261.33 ± 18.20	97.38 ± 1.12	14.28 ± 0.16
Total organic carbon (TOC)	% dw	28.63 ± 0.27	34.38 ± 0.17	31.00 ± 0.75	4.63 ± 0.05
Total nitrogen (TN)	% dw	5.17 ± 0.01	6.24 ± 0.08	5.57 ± 0.13	0.19 ± 0.001
Total phosphorous (P)	mg kg^{-1} dw	13663 ± 193.2	11938 ± 104.3	33420 ± 69.10	1197 ± 8.898
Amorphous iron (Fe)	mg kg^{-1} dw	5795 ± 467.2	5668 ± 543.7	43287 ± 2198	8682 ± 259
Amorphous aluminium (Al)	mg kg^{-1} dw	1937 ± 56.7	1915 ± 172.4	9356 ± 487.4	22851 ± 830.2
Carbonates	% dw	2.3 ± 0.07	2.4 ± 0.28	n.a. (<DL)	9.4 ± 0.03
Texture Class	USDA	Silt loam	Sandy clay loam	Sandy loam	Sandy loam

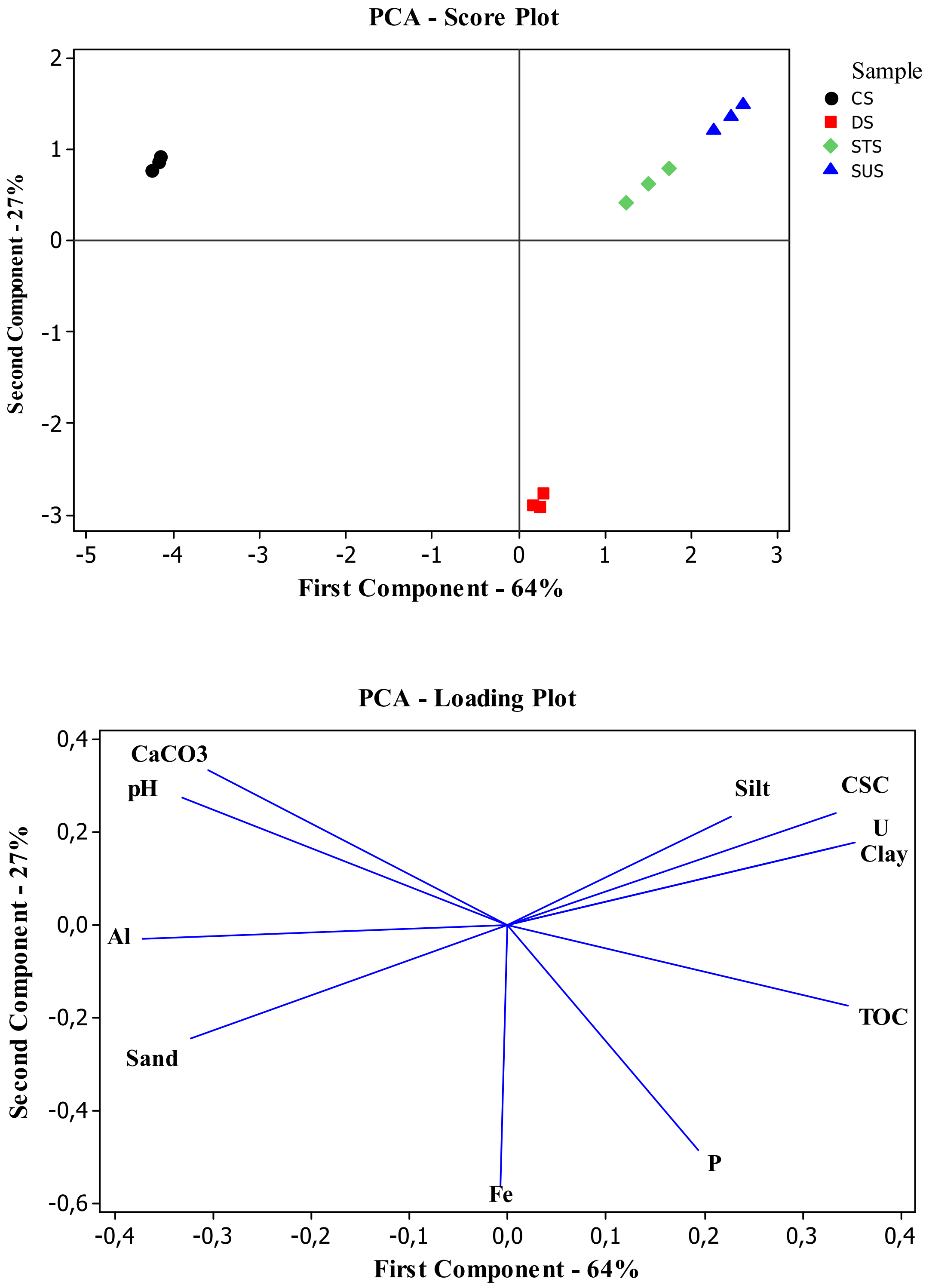


Figure 2 Score plot and loading plot defined by first two components of the physico-chemical characterization of SS samples.

1.3.2 Pseudo-total content and partitioning of TEs in SS

In **Table 2** the pseudo-total TEs concentration in SS samples are reported. The table also shows the concentration thresholds imposed by Italian legislation. As can be seen, SUS, STS, and DS do not meet the requirements for agricultural beneficial reuse because Cu concentrations (299.7, 327.6 and 503.0 mg kg^{-1}, respectively) and Zn concentrations (844.0, 915.4 and 997 mg kg^{-1}, respectively) exceed the legal thresholds for the farm land application (Cu: 200 mg kg^{-1} and Zn: 300 mg kg^{-1}; Ministerial Degree of 1/Mar/2019, nr. 46 - Attach. 2). On the other hand, all the analysed SS could be used for environmental restoration operas in industrial and commercial sites (Legislative Degree nr. 152/06 Attach. 5, Part IV, Table 1). The CS sample presents lower TEs concentrations than the other samples, except in the case of Cr (97. 35 mg kg^{-1}), compared to SUS, STS and DS (63.77, 61.45, 84.74 mg kg^{-1}, respectively), and Ni (70.83 mg kg^{-1}) with respect to SUS and STS (24.22 and 25.06 mg kg^{-1}, respectively). DS has the higher Ni concentration.

The lower concentration of TEs in CS can be explained by the lower content of colloids (organic matter and clay) of the solid matrix and therefore by the lower capacity to bind TEs through adsorption phenomena.

The metal partitioning of unaltered SS samples, as determined by the sequential chemical extraction (BCR), is shown in **Table 3**. In SUS, STS, and DS samples, it can be observed that most of the content of Cu and Cd shared between reducible and oxidable fraction, while As and Zn is manly found in exchangeable fraction. Ni is equally distributed in each fraction and Pb is mainly bound to the reducible. As expected, most of the Cr is found in residual fraction and strongly complexed by organic matter in oxidable fraction. More specifically TEs partitioning in SUS, STS and DS can be simplified as follows:

As: Exchangeable > Residual > Reducible > Oxidable
Cd: Oxidable > Reducible > Exchangeable > Residual
Cr: Residual > Oxidable > Reducible > Exchangeable
Cu: Oxidable > Reducible > Residual > Exchangeable
Ni: Oxidable > Residual > Exchangeable > Reducible (in DS: Exc. > Red. > Res. > Oxi.)
Pb: Reducible > Residual > Oxidable > Exchangeable
Zn: Exchangeable > Reducible > Residual > Oxidable (in STS: Red. > Exc. > Res.> Oxi.)

It can be observed that the exchangeable fraction represents the dominant part for both As and Zn. This fraction is very unstable and sensitive to the environmental conditions. On the contrary, Cd, Cr, Cu, Ni and Pb mobilize much harder because are found precipitate as oxyhydroxides, complexed to organic matter or in the crystal lattice.

In CS, all TEs are found in the residual fraction, except for Cd that follows the same behaviour outlined above.

Table 2 Pseudo-total content of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Nickel (Ni) and Zinc (Zn), expressed in mg kg^{-1}, of sludges samples (SUS: surplus sludge; STS: stabilized sludge; DS: dehydrated sludge; CS: freshwater clarification sludge) compared with Italian legal thresholds. Errors are expressed as a standard deviation.

	TEs						
Samples	**As**	**Cd**	**Cr**	**Cu**	**Ni**	**Pb**	**Zn**
SUS	6.388 ± 0.077	0.885 ± 0.007	63.77 ± 1.756	299.7 ± 5.304	24.22 ± 0.420	49.84 ± 0.365	844.0 ± 5.023
STS	9.655 ± 0.108	0.929 ± 0.016	61.45 ± 0.507	327.6 ± 3.167	25.06 ± 0.256	53.08 ± 1.437	915.4 ± 6.595
DS	4.167 ± 0.049	2.222 ± 0.105	84.74 ± 2.208	503.0 ± 18.77	82.97 ± 2.811	77.83 ± 2.841	997.2 ± 28.84
CS	0.366 ± 0.034	0.178 ± 0.012	97.35 ± 1.376	49.39 ± 0.306	70.83 ± 0.752	32.55 ± 0.244	115.6 ± 0.627
	LT						
Residential and public use*	20	2	150	120	120	100	150
Farm Land**	30	5	150	200	120	100	300
Industrial use*	50	15	800	600	500	1000	1500

* Legislative Degree nr. 152/06 Attach. 5, Part IV, Table 1

**Ministerial Degree of 1/Mar/2019, nr. 46 - Attach. 2

Table 3 Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Nickel (Ni) and Zinc (Zn), partitioning in the unaltered SS samples (SUS: surplus sludge; STS: stabilized sludge; DS: dehydrated sludge; CS: freshwater clarification sludge) expressed in percentage on total amount.

	Fractions	**As**	**Cd**	**Cr**	**Cu**	**Ni**	**Pb**	**Zn**
SUS	*Exchangeable %*	80.9	7.0	3.3	10.7	24.7	0.1	61.3
	Reducible %	10.6	22.0	12.5	38.1	21.0	79.7	29.3
	Oxidable %	0.0	71.0	35.2	46.2	27.9	1.5	3.4
	Residual %	8.6	0.0	49.1	5.0	26.4	18.7	6.0
STS	*Exchangeable %*	54.1	0.0	5.0	1.0	21.9	0.8	25.9
	Reducible %	17.3	25.9	12.7	29.0	21.4	67.9	43.6
	Oxidable %	2.8	74.1	35.5	55.4	33.1	9.2	13.8
	Residual %	25.8	0.0	46.8	14.7	23.6	22.1	16.7
DS	*Exchangeable %*	25.3	0.0	1.4	4.9	43.0	0.0	56.3
	Reducible %	0.0	68.0	15.5	33.8	22.4	29.3	31.9
	Oxidable %	0.0	26.7	31.5	39.6	15.5	3.2	1.5
	Residual %	74.7	5.2	51.6	21.7	19.2	67.5	10.4
CS	*Exchangeable %*	0.0	0.0	0.9	0.0	1.1	0.0	0.7
	Reducible %	0.0	85.5	1.8	2.7	5.1	17.6	15.9
	Oxidable %	0.0	14.5	7.5	35.1	10.2	17.2	12.4
	Residual %	100.0	0.0	89.9	62.2	83.7	65.2	70.9

1.3.3 SS treatments efficacy

The effect of washing treatments with $CaCl_2$ for TEs removal on SS samples is shown in **Table 4**. For each sample, washing treatments were carried out at the natural pH value of SS and at all values from natural to 0. In not-dehydrated samples (SUS and STS) and in CS sample, $CaCl_2$ washing had no effect on TEs removal. The decrease in TEs concentrations is only visible at very low pH levels. In SUS sample, at pH 1, Cu, Pb an Zn concentrations decrease by 40.6%, 70.0% and 87.0%, respectively. At the same, in STS sample Zn and Pb concentrations decrease by 78.5% and 77.35%, respectively. In these cases the process is brought about by the exchange of protons (from the acid) through the solubilization of heavy metals in sludge as shown in follow equation:

Sludge-M + Acid → Sludge + M^{n+} + Acid (Geng et al., 2020; Stylianou et al., 2007)

These results are attributed to an increase of acid concentration resulting an increase in protonation and of the energy available for breaking chemical bonds of the metals in the sludge (Naoum et al., 2001; Stylianou et al., 2007).

In the CS sample, none of the treatments were effective in reducing the concentrations of TEs, likely due to the geochemical nature of the contamination (as suggested by their high residual fraction, **Table 3**).

In contrast, $CaCl_2$ washing treatment had a consistent effect on DS sample. Specifically, As concentration decreased by 41.0%, Cd by 84.7%, Cr by 42,4%, Cu by 47.8%, Ni by 70.0%, Pb by 44.1% and Zn by 25.0%. Instead, in DS samples treated with acid (from pH 4 to 2), the decreasing of TEs concentration is not visible. However, like in the case of non-dehydrated SS samples, at pH values lower than 2, a significant reduction in TEs concentrations was observed. Specifically, As by 33.0%, Cd by 94.0%, Cr by 14%, Cu by 82%, Ni by 70.0%, Pb by 80.0% and Zn by 94.2%.

In **Figure 3**, the partitioning of TEs in DS sample for each treatment are reported. As shown, $CaCl_2$ washing treatment is effective in reducing the concentrations of TEs in all bound fractions. Despite the reduced concentrations, the percentage distribution of Cr, Cu, and Pb, that are bound to more stable fractions (as shown in **Table 3**), does not change significantly. For Cd, the $CaCl_2$ washing treatment is effective in reducing the concentrations in the reducible and oxidable fractions. On the other hand, the treatment mainly reduced the concentrations in the exchangeable fractions of Ni and Zn. For As, residual fraction results in the main fraction removed.

To confirm the treatment efficacy, $CaCl_2$ washing was performed on two DS samples [DS (II) and DS (III)] collected in the second sampling campaign, two years after the first trial. **Table 5** shows the results of second trial on DS (II) and DS (III). As reported, in DS (II) and DS (III) samples, As concentration decreases by 39.1 and 37.4%, Cd by 73.6 and 71.2%, Cr by 61.2 and 59.5%, Cu by 37.0 and 32.7%, Ni by 76.0 and 74.1%, Pb by 31.85 and 27.7% and Zn by 31.9 and 28.4%, respectively. Therefore, the reduction of TEs concentration confirms the effectiveness of the treatment as already seen in the first trial.

Additionally, in **Figure 4** the means of TEs concentration in DS samples, unaltered and treated with $CaCl_2$, are reported. As shown, statistically significant differences in TEs concentrations between not-treated sample and the samples washed with $CaCl_2$ solution, at natural pH value, has been observed for all TEs analysed under ANOVA.

The variables that determine the difference among samples could be the cause of the different treatment outcome in the four SS typologies.

Respect to not-dehydrated samples (SUS and STS) and CS, DS was subjected to conditioning treatments aimed at coagulation of suspended solids and precipitation of phosphorus (P). The chemical agents used for P removal by wastewater were $FeCl_3$ [in DS and DS(III)] and $Al_2(SO_4)_3$ in DS(II). This explains the higher content of amorphous Fe and total P found in dry matter of DS compared with the other samples (**Table 1**).

The clusters shown in **Figure 2** (multivariate PCA analysis), splits the samples, in the first component, based on pH, TOC, and texture composition, separating SUS, STS and DS by CS. In the second component, DS is distinguished from SUS and STS by its Fe and P content.

The addition of Ca^{2+} in not-dehydrated fresh samples (SUS and STS) may have led to P precipitation as Calcium Hydroxyapatite accordingly with following equation:

$10Ca^{2+} + 6PO_4^{3-} + 2OH^- \rightleftarrows Ca_{10}(PO_4)_6(OH)_2$ (Jenkins et al., 1971)

Therefore, the effect of TEs displacement by calcium ions is inhibited by the presence of phosphate ions in the liquid phase of SS.

All things considered, the good effect of $CaCl_2$ washing treatment on DS compared with other tested samples can be attributed to the pre-treatment performed during dehydration process.

As a demonstration, in **Figure 5** the correlations between total P content (in dry matter) and the percentage of exchanged TEs in not-dehydrated SS (SUS and STS), CS and DS sample are reported. Positive correlations for all TEs are observed (R^2: 0.981, 0.737, 0.945, 0.949, 0.992, 0.934, 0.906, for As, Cd, Cr, Cu, Ni, Pb and Zn, respectively), thus supporting the figure that phosphates ions in the liquid phase inhibit the efficacy of $CaCl_2$ washing treatment.

Table 4. Pseudo-total content of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Nickel (Ni) and Zinc (Zn), expressed in mg kg^{-1}, in sludge samples (SUS: surplus sludge; STS: stabilized sludge; DS: dehydrated sludge; CS: freshwater clarification sludge). TEs content are reported for unaltered sludge samples and for the samples treated with $CaCl_2$ saline solution from natural pH value down to 0. Errors are expressed as a standard deviation.

Treatment	**As**	**Cd**	**Cr**	**Cu**	**Ni**	**Pb**	**Zn**
	SUS						
Unaltered	6.388 ± 0.077	0.885 ± 0.007	63.77 ± 1.756	299.7 ± 5.304	24.22 ± 0.420	49.84 ± 0.365	844.0 ± 5.023
$CaCl_2$	6.850 ± 0.020	0.989 ± 0.005	62.26 ± 0.121	345.1 ± 1.298	24.61 ± 0.201	56.67 ± 1.039	954.0 ± 2.392
$CaCl_2$ - pH 5	6.870 ± 0.019	1.046 ± 0.025	62.94 ± 0.494	344.7 ± 1.523	24.55 ± 0.053	56.92 ± 1.030	961.7 ± 3.445
$CaCl_2$ - pH 4	6.698 ± 0.061	1.101 ± 0.024	66.14 ± 0.947	380.3 ± 1.910	24.07 ± 0.381	63.87 ± 1.182	988.3 ± 7.808
$CaCl_2$ - pH 3	6.817 ± 0.081	1.130 ± 0.034	71.23 ± 0.713	403.6 ± 2.105	22.45 ± 0.303	66.92 ± 0.492	844.3 ± 8.951
$CaCl_2$ - pH 2	6.521 ± 0.155	0.857 ± 0.012	66.33 ± 0.381	373.5 ± 3.407	18.58 ± 0.057	55.96 ± 1.122	520.9 ± 4.617
$CaCl_2$ - pH 1	5.271 ± 0.113	0.288 ± 0.009	69.38 ± 0.848	178.4 ± 1.010	17.05 ± 0.119	14.84 ± 0.744	109.6 ± 2.503
$CaCl_2$ - pH 0	5.998 ± 0.208	0.286 ± 0.009	67.59 ± 0.557	178.0 ± 0.986	16.92 ± 0.146	11.72 ± 0.526	120.0 ± 1.171
	STS						
Unaltered	9.655 ± 0.108	0.929 ± 0.016	61.45 ± 0.507	327.6 ± 3.167	25.06 ± 0.256	53.08 ± 1.437	915.4 ± 6.595
$CaCl_2$	2.023 ± 0.063	0.450 ± 0.012	60.57 ± 1.994	297.8 ± 2.257	24.46 ± 0.648	50.33 ± 0.967	805.7 ± 13.55
$CaCl_2$ - pH 5	8.814 ± 0.118	0.950 ± 0.016	64.84 ± 0.904	333.1 ± 4.797	25.02 ± 0.145	54.36 ± 1.468	932.0 ± 9.628
$CaCl_2$ - pH 4	9.189 ± 0.131	1.012 ± 0.015	68.98 ± 0.732	369.3 ± 4.779	23.49 ± 0.253	62.65 ± 0.286	972.9 ± 5.612
$CaCl_2$ - pH 3	9.844 ± 0.121	1.053 ± 0.012	67.31 ± 0.269	379.3 ± 2.081	22.27 ± 0.480	64.67 ± 0.641	964.1 ± 7.791
$CaCl_2$ - pH 2	10.46 ± 0.173	1.031 ± 0.017	65.84 ± 0.744	398.7 ± 3.799	20.69 ± 0.182	65.63 ± 0.566	663.8 ± 1.637
$CaCl_2$ - pH 1	7.832 ± 0.053	0.301 ± 0.018	62.67 ± 0.732	304.6 ± 6.427	16.28 ± 0.174	12.99 ± 0.685	197.2 ± 1.517
$CaCl_2$ - pH 0	10.36 ± 0.112	0.642 ± 0.078	69.38 ± 1.060	394.7 ± 2.190	18.16 ± 0.257	35.00 ± 0.368	246.9 ± 9.413
	DS						
Unaltered	4.167 ± 0.049	2.222 ± 0.105	84.74 ± 2.208	503.0 ± 18.77	82.97 ± 2.811	77.83 ± 2.841	997.2 ± 28.84
$CaCl_2$	2.460 ± 0.363	0.340 ± 0.034	48.85 ± 2.515	262.4 ± 10.40	25.35 ± 0.611	43.55 ± 1.909	753.7 ± 16.30
$CaCl_2$ - pH 4	3.702 ± 0.082	1.452 ± 0.034	89.92 ± 0.861	510.6 ± 10.51	62.52 ± 1.065	74.94 ± 1.550	842.7 ± 15.70
$CaCl_2$ - pH 3	3.906 ± 0.110	0.938 ± 0.052	82.50 ± 5.362	511.1 ± 22.03	46.77 ± 2.409	79.00 ± 4.170	454.8 ± 19.63
$CaCl_2$ - pH 2	3.614 ± 0.011	0.734 ± 0.047	89.90 ± 1.027	365.6 ± 7.660	36.14 ± 0.585	74.11 ± 1.657	133.6 ± 2.426
$CaCl_2$ - pH 1	3.558 ± 0.432	0.159 ± 0.011	77.65 ± 1.880	141.8 ± 3.148	24.07 ± 0.787	44.58 ± 0.879	58.47 ± 1.546

$CaCl_2$ - pH 0	2.801 ± 0.614	0.135 ± 0.019	72.85 ± 0.297	91.16 ± 0.302	25.01 ± 0.379	16.35 ± 0.222	57.78 ± 0.144
	CS						
Unaltered	0.366 ± 0.034	0.178 ± 0.012	97.35 ± 1.376	49.39 ± 0.306	70.83 ± 0.752	32.55 ± 0.244	115.6 ± 0.627
$CaCl_2$	0.392 ± 0.181	0.153 ± 0.002	105.6 ± 3.812	54.37 ± 3.686	74.73 ± 1.283	35.80 ± 1.581	121.3 ± 2.833
$CaCl_2$ - pH 6	0.301 ± 0.021	0.152 ± 0.016	106.8 ± 1.745	54.68 ± 1.382	76.42 ± 1.717	38.09 ± 1.487	125.0 ± 3.279
$CaCl_2$ - pH 5	0.301 ± 0.007	0.126 ± 0.007	121.9 ± 2.016	59.77 ± 0.129	84.50 ± 0.171	45.48 ± 0.455	137.4 ± 0.085
$CaCl_2$ - pH 4	0.278 ± 0.024	0.098 ± 0.015	136.2 ± 2.291	71.55 ± 1.272	99.75 ± 1.874	50.36 ± 2.087	154.3 ± 4.066
$CaCl_2$ - pH 3	0.240 ± 0.038	<DL	133.3 ± 4.318	65.07 ± 4.083	94.89 ± 4.856	41.03 ± 2.508	145.6 ± 6.884
$CaCl_2$ - pH 2	0.239 ± 0.015	<DL	141.0 ± 2.301	57.73 ± 0.538	98.12 ± 0.422	23.65 ± 0.800	127.1 ± 1.685
$CaCl_2$ - pH 1	0.184 ± 0.009	<DL	149.9 ± 7.217	38.96 ± 7.193	97.01 ± 1.305	16.28 ± 3.135	108.2 ± 11.75
$CaCl_2$ - pH 0	0.300 ± 0.111	<DL	153.3 ± 4.773	30.56 ± 0.610	94.48 ± 1.531	12.21 ± 1.065	93.53 ± 1.084

Table 5 Pseudo-total content of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Lead (Pb), Nickel (Ni) and Zinc (Zn), expressed in mg kg^{-1}, in DS (II) and DS (III). TEs content are reported for unaltered sludge samples and for the samples treated with $CaCl_2$ saline solution. Errors are expressed as a standard deviation.

		As	**Cd**	**Cr**	**Cu**	**Ni**	**Pb**	**Zn**
DS (II)	Unaltered	4.141 ± 0.210	2.605 ± 0.025	108.1 ± 0.733	651.3 ± 16.98	131.4 ± 0.762	88.78 ± 1.378	1422 ± 15.81
	$CaCl_2$	2.519 ± 0.212	0.688 ± 0.012	41.92 ± 1.659	410.2 ± 7.899	31.55 ± 0.805	60.50 ± 1.148	968.7 ± 20.02
DS (III)	Unaltered	3.962 ± 0.149	2.500 ± 0.023	103.2 ± 1.892	622.2 ± 4.674	119.5 ± 0.94	85.46 ± 0.799	1384 ± 12.34
	$CaCl_2$	2.477 ± 0.083	0.720 ± 0.042	41.76 ± 0.595	419.1 ± 6.894	30.85 ± 0.362	61.72 ± 0.905	990.0 ± 35.42

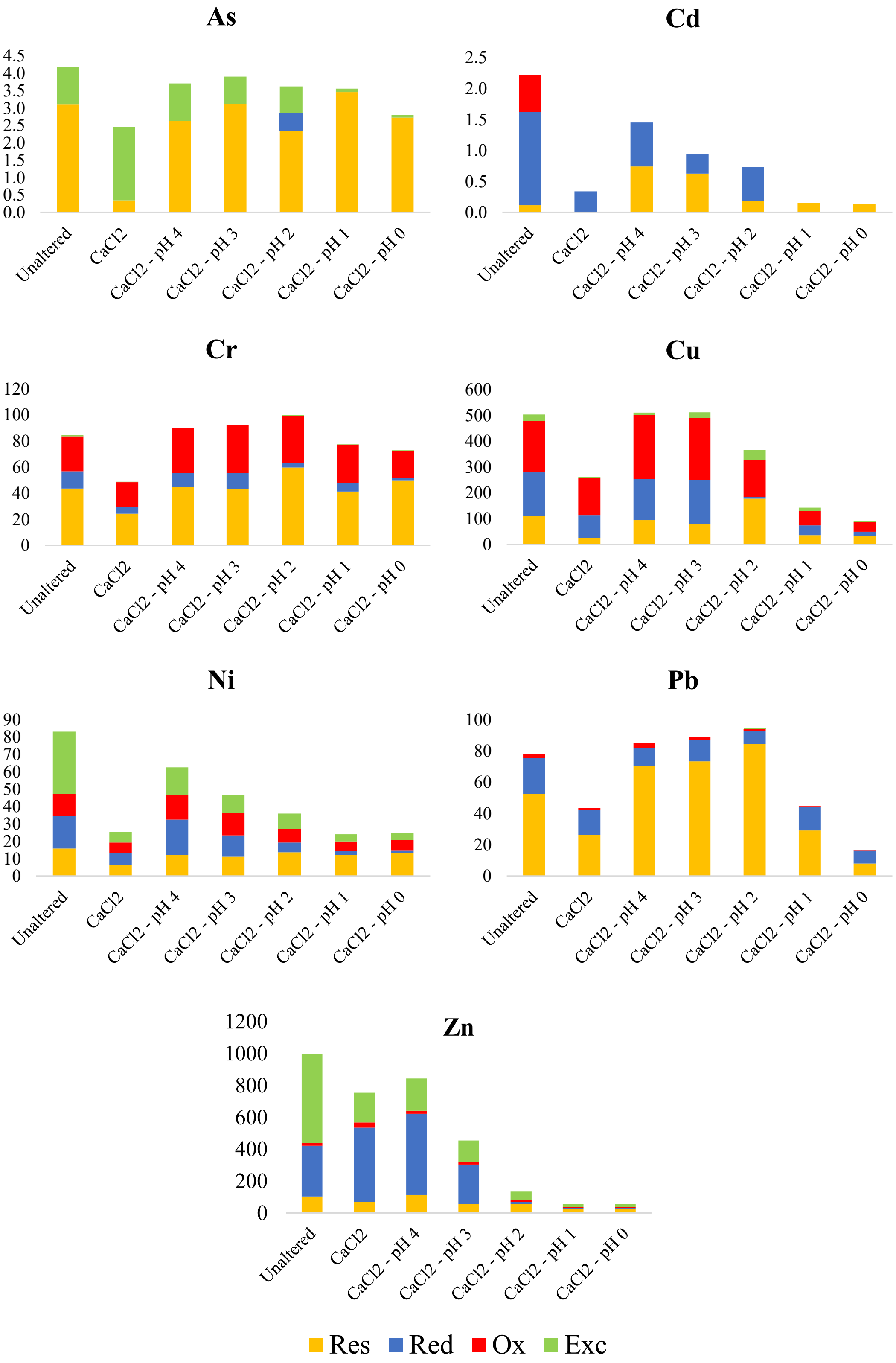


Figure 3 Three-steps BCR sequential extraction of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni), Lead (Pb) and Zinc (Zn) in DS sample. Concentration values of exchangeable, oxidable, reducible and residual fractions are expressed in mg kg^{-1}.

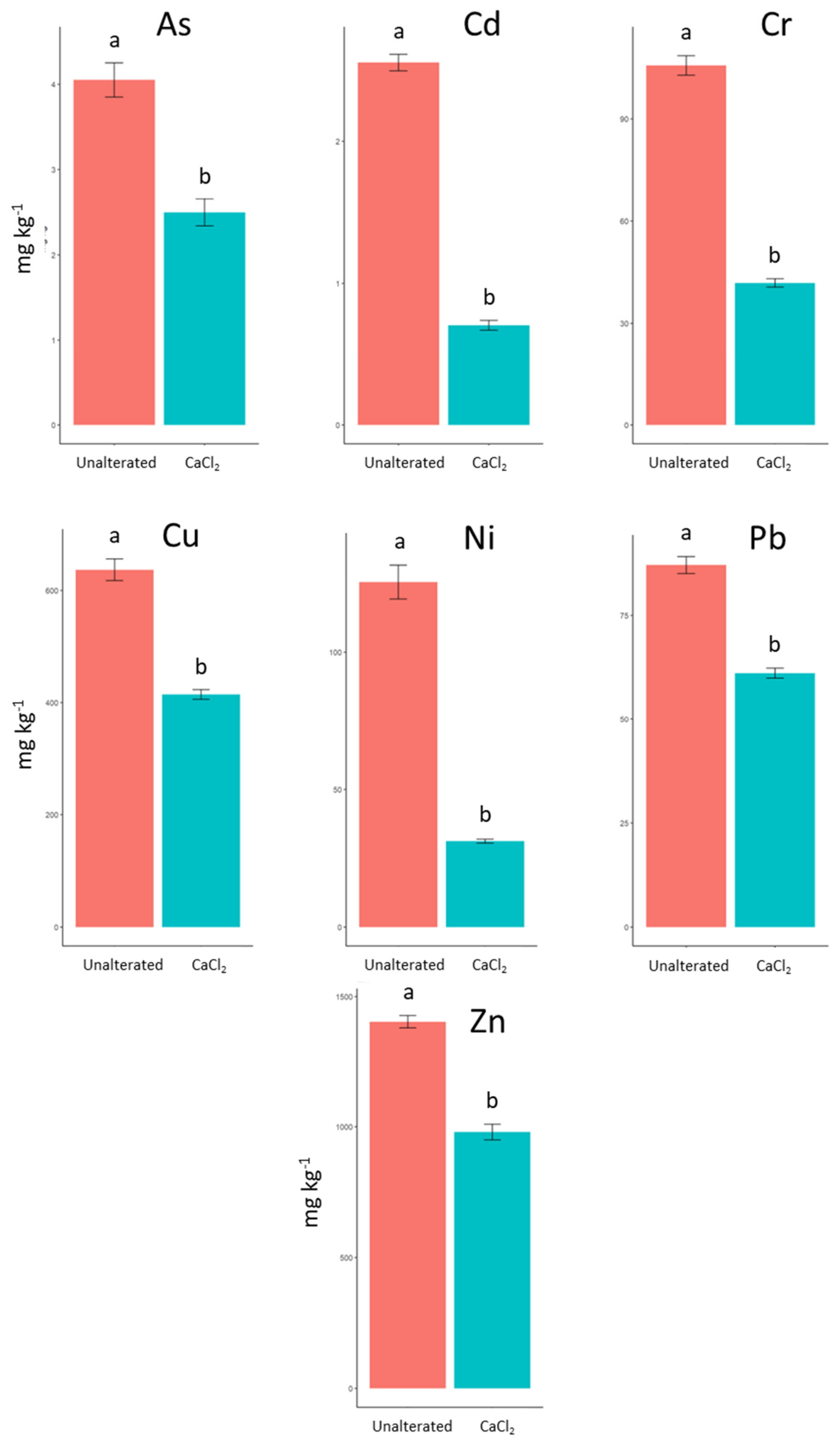


Figure 4 Means of Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni), Lead (Pb) and Zinc (Zn) in DS sample. Error bars represent the standard errors and different lower-case letters indicate significant differences ($P < 0.05$) between unaltered sample and treated samples determined by Tuckey post hoc test.

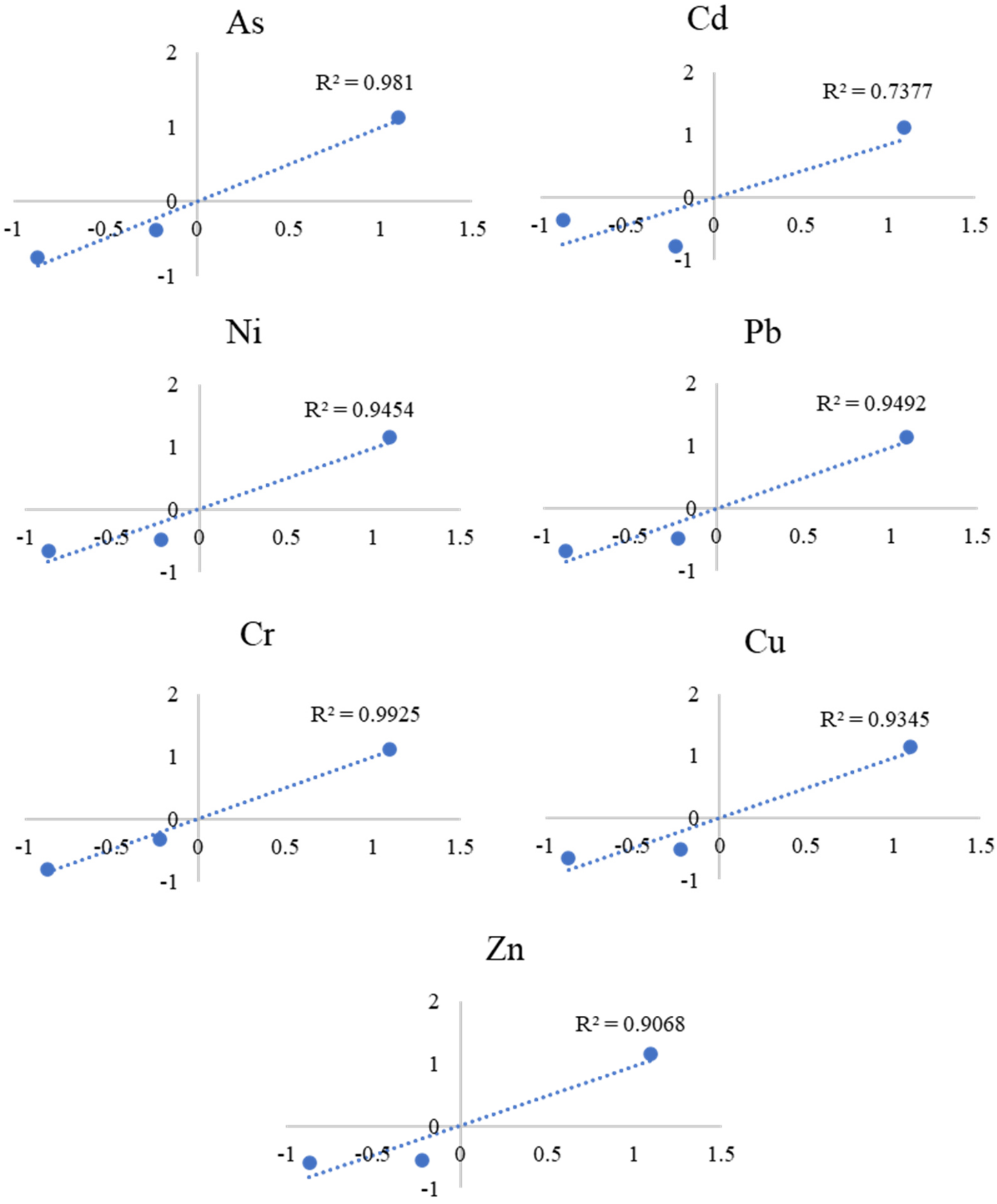


Figure 5 Correlations between P content (x-axis) and percentage of exchanged Arsenic (As), Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni), Lead (Pb) and Zinc (Zn) (y-axis) after $CaCl_2$ washing treatment in not-dehydrated SS (SUS and STS), CS and DS sample. The matrices were normalized using the root mean square deviation.

1.4 Conclusions

The $CaCl_2$ washing treatments were conducted on different types of SS. The samples were chosen based on the main processes to which the sludge is subjected before its final destination. The aim of this experimentation was to use an eco-friendly method to make it possible to reuse sludge sustainably for agricultural purposes, reducing the trace elements (TEs) contamination below acceptable levels.

Overall, the $CaCl_2$ washing treatment had no effect in reducing the concentration of TEs in sludge samples that were not subjected to dewatering pre-treatment. However, in the case of a sludge sample that had undergone dewatering pre-treatment, the washing treatment reduced the concentration of arsenic (As) by 39%, cadmium (Cd) by 76%, chromium (Cr) by 54%, copper (Cu) by 38%, nickel (Ni) by 73%, lead (Pb) by 34%, and zinc (Zn) by 28% on average. Moreover, through chemical speciation of TEs, it has been observed that the treatment is effective in all phases of element binding.

Dewatering pre-treatment was performed through the use of organic flocculant agents such as cationic polyelectrolytes (i.e. polyacrylamide) and inorganic salts such as iron chloride and aluminium sulphate. The goal of the combination of both organic and inorganic agents is the flocculation of dispersed solids and the precipitation of dissolved phosphorus to prevent eutrophication phenomena. Phosphate ions in liquid phase of SS inhibit the efficacy of $CaCl_2$ washing likely because phosphorous and calcium ions form calcium hydroxyapatite, which reduces the effectiveness of the $CaCl_2$ washing.

In this case, the addition of acid to decrease the pH level and induce TE solubilization did not increase the treatment efficacy for all SS samples.

Though the washing conditions operated on the SS did not succeed in lowering the Cu and Zi content below the Italian threshold limits to agricultural reuse, these preliminary findings indicate the $CaCl_2$ washing as a promising method abating most of the concentration of toxic elements such as Ni and Cd.

Chapter 2

Effect of the growing season, Trichoderma, and clinoptilolite application on potentially toxic elements (PTEs) uptake by Cucumis melo L.

2.1 Introduction

Human activities such as industrial processes and the use of amendments, fertilizers, and untreated water can contribute to the accumulation of Potentially Toxic Elements (PTEs) in cultivated soils (He et al., 2005). While elements like Cu and Zn are essential micronutrients that plants actively absorb, non-essential elements like Pb, As, and Cd can also be taken up by plant roots and transported into edible tissues (Lasat, 2002; Uchimiya et al., 2020).

Excessive levels of certain PTEs, such as copper (Cu) and lead (Pb), in the edible parts of food in the European Union are considered a cause of noncompliance, and regulations set maximum allowable levels within the internal market (European Commission, 2006; European Commission, 2008). The use of Cu salts, such as sulphates, commonly known as Bordeaux mixture, is allowed with some limitations on several crops (including melon) in integrated pest management (European Parliament, 2009) and in organic crop production. Copper-based products have been widely used in the past century due to their fungicidal properties (Lamichhane et al., 2018a), leading to an accumulation of Cu in cultivated soils. The intensification of agriculture and the reduction of crop diversity have also contributed to the accumulation of Cu in soils (Lamichhane et al., 2018a).

One way to decrease heavy metal content in contaminated soils is through phytoremediation techniques (Ali et al., 2013), soil washing (e.g., Buscaroli et al., 2019), and soil dilution (Dermont et al., 2008). Agricultural strategies can also be implemented to mitigate the risk of PTEs entering the food chain by preventing their uptake and translocation in plants. This can be achieved through soil conditioning or adjusting the pH to reduce the mobility and availability of metal ions (Uchimiya et al., 2020).

To this end, researchers and professional horticulture companies have explored alternative solutions, such as the use of natural zeolite-based products like clinoptilolite. These products have been suggested for use in agriculture due to their high cation exchange capacity and nutrient content (Nakhli et al., 2017b). However, their effectiveness as PTEs immobilizers is controversial, as there are few studies on clinoptilolite's ability to stabilize PTEs (Oste et al., 2002b).

One potential strategy being evaluated for preventing the translocation of PTEs to crops is soil treatment using *Trichoderma* sp. fungi. Some recent studies have shown that inoculation of *Trichoderma* sp. can reduce the assimilation of micronutrients by plants, especially copper (Cu) (de Santiago et al., 2011; Téllez

Vargas et al., 2017b). The mechanism behind this effect is unclear, but it is speculated that competition between *Trichoderma* and the inoculated crop for micronutrients may be involved. If this strategy is effective in multiple tests and its mechanisms are fully understood, association of *Trichoderma* with crops grown in Cu-rich soil could be a valuable way to reduce excess Cu accumulation.

Italy is the third largest cultivator of melons (*Cucumis melo L.*) and the second largest producer in Europe, according to the Italian Institute of Statistics (ISTAT) and the Food and Agriculture Organization of the United Nations (FAO) respectively. Melons, like other plants in the Cucurbitaceae family, have the ability to absorb and transport organic and inorganic contaminants (Campbell et al., 2009; Mattina et al., 2006, 2004).

This study aimed at examining the effectiveness of various agricultural techniques on the uptake and transport of PTEs (Cu, Cr, and Pb) in three commercially significant melon cultivars in Italy. The techniques included the use of commercial grade clinoptilolites, Trichoderma inoculation, and variations in cultivation season. In addition, a detailed mass balance of PTEs introduced into the soil-crop system through irrigation water, fertilization and pest management was assessed.

2.2 Materials and methods

The experimental field was in the municipality of Viadana, MN, Italy (44°58'21"N, 10°35'11"E) within the production area of Agricola Don Camillo S.C.a.R.L. (**Figure 1**), one of the largest melon producers in Italy. The plot used for the trials had an extension of 0.5 ha.

Three commercial melon hybrids were selected for experimental cultivation: (i) Django F1, an early-ripening cultivar, (ii) Costantino F1, and (iii) EXPE504 F1, both late-ripening cultivar.

The first experimental field was set with the three varieties during spring (or Time 1) from April 2 to June 25, 2020. The second one was set in summer or (Time 2) from June 1 to August 24, 2020.

At both growing periods (Time 1 and 2) and on all three crop varieties, different treatments were evaluated: *Trichoderma* inoculation alone or combined with the most common clinoptilolite-based commercial products.

The acronyms of the different treatments are reported as follows:

- C: control not treated;
- T: inoculation with *Trichoderma*;
- TFA: inoculation with *Trichoderma* and foliar application of clinoptilolite powder ("foliar action");
- TFT: inoculation with *Trichoderma* and application of clinoptilolite powder via fertigation;
- TP: inoculation with *Trichoderma* and soil application of clinoptilolite pellets.

The initial pool of TE in the soil was evaluated through analysis on the total and potentially available to plant content. Also was evaluated the TE input to soil crop system through irrigation water, analysing the liquid phase and suspended solids, fertilizers, pesticides and the products used for the tests to understand the entity of the soil enrichment respect to the initial pool.

The plants were sampled at the end of each growing period, when the fruit were ripe for the market. Each plant was divided in three sub-sample corresponding to stem, leaves and fruit. Then, the tissues were analysed for TE content through microwave acid digestion with nitric acid and hydrogen peroxide. Then, the solutions were analyzed by ICP-OES.

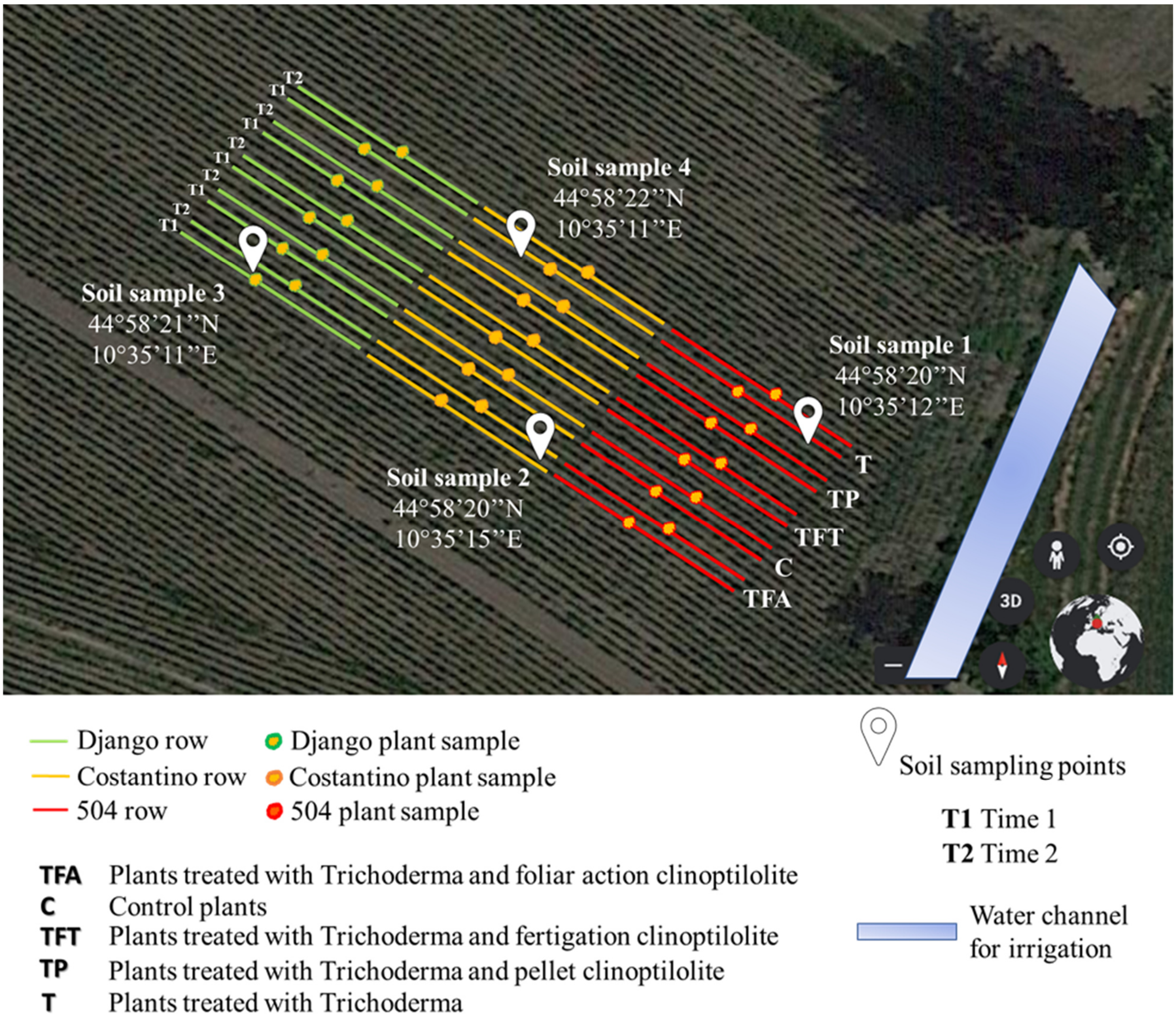


Figure 1 Experimental field. Soil and plant sampling positions are indicated. Plants were sampled from the inner part of each cultivar row to avoid, as much as possible, side effects from the different vicinal varieties.

2.2.1 Statistical analysis

Statistical analysis of the data on metals content in the epigeal biomass of plants (stem, leaves, and fruit) was conducted in R environment (R Core Team 2020). The effects of variety, treatment, and time were assessed using a split-split plot ANOVA ($P < 0{,}05$), followed by an LSD post hoc test ($P < 0.05$) with Bonferroni adjustment. The differences in metal content in the different epigeal fractions (stem, leaves, fruit) for the two times of transplanting considered were tested with a two-way ANOVA ($P < 0{,}05$) followed by an LSD post hoc test ($P < 0,05$) with Bonferroni adjustment. The correlation matrix of the metal concentration in plant parts was computed using Pearson's correlation coefficient.

2.3 Results and discussion

2.3.1 PTEs pool in soil

According to the results shown in **Table 1**, only a few elements were found in significant amounts in the soil: chromium, copper, and lead. While chromium was present in large quantities, it was in an insoluble form and therefore not available to plants. On the other hand, the available portions of copper and lead were 11.0% and 7.5%, respectively. These values were expected, as it is common for copper and lead to be more bioavailable in alkaline soils due to their presence in the form of carbonates or as complexed with negatively charged soil organic matter (Shahid et al., 2017). These forms allow PTEs to be complexed by plant-derived carboxylates and absorbed by roots. However, chromium is typically not available to plants due to its ability to form stable, insoluble species in soils with pH values greater than 5.5 (Shahid et al., 2017).

Table 1 TEs content of 0-30 cm topsoil samples. In brackets, DTPA extractable metal content expressed as a percentage of the total amount. Errors are expressed as a standard deviation.

TEs (mg kg^{-1})	**Soil samples**				
	1	**2**	**3**	**4**	**Average**
Cr	208.2 ± 2.9	212.1 ± 2.7	197.0 ± 4.2	205.6 ± 2.0	**205.0 ± 2.9**
	(0.01%)	(0.01%)	(0.01%)	(0.01%)	**(0.01%)**
Cu	114.1 ± 1.4	91.3 ± 0.5	99.2 ± 3.9	72.4 ± 0.8	**87.6 ± 1.8**
	(11.84%)	(10.20%)	(12.25%)	(10.05%)	**(11.08%)**
Pb	33.8 ± 4.6	33.4 ± 6.2	35.4 ± 1.8	36.1 ± 0.8	**35.0 ± 3.0**
	(6.88%)	(7.93%)	(7.48%)	(7.73%)	**(7.51%)**

2.3.2 Incidence of PTE input

Table 2 presents the total PTE input to the soil-plant system from all agricultural practices (irrigation, fertilization, pest control, and product use for testing). Copper input was evenly distributed among treatments due to the use of the same main input (copper salts). On the other hand, the input of Cr and Pb varied slightly among treatments within the same growing period and increased from the first to the second period, largely because of the differing volume and composition of irrigation water. However, the input of each element was small compared to the pre-existing pool in the soil, so it is unlikely that these slight variations in PTE input impacted metal assimilation.

Table 2 Total and potentially bioavailable-to-plant input (irrigation water, fertilizers, pesticides and treatments products) of Cr, Cu, Pb, bioavailability was assessed as a DTPA extractable fraction.

Time 1		**Cr**	**Cu**	**Pb**
	total input (g ha^{-1})	12.82	810.3	4.145
	bioavailable fraction (%)	3.694	38.51	0.281
Time 2				
	total input (g ha^{-1})	18.09	814.7	6.457
	bioavailable fraction (%)	3.520	38.67	0.186

2.3.3 Effect of variety, treatments, and seasonality on PTEs uptake

Figure 2 (a) shows the average Cr, Cu, and Pb contents in the aerial part of plant biomass (stem, leaves, and fruit) for each treatment in both growing periods

Cr levels in plants did not show statistically different concentrations either among treatments or between growing periods. For Cu and Pb, no differences among treatments were observed for plants within the same growing period. In contrast, statistically significant differences resulted between Time 1 and Time 2. Cu and Pb were, on average, about three (69.25 – 187.0 mg kg^{-1}) and four (0.214 – 0.885 mg kg^{-1}) times higher at Time 2 with respect to Time 1, respectively.

In **Figure 2 (b)**, the marginal means of the metal uptake of each cultivar (namely, 504, Costantino, and Django) in the two growing periods were reported. The three cultivars did not uptake statistically different amounts of Cr, neither within the same growing period nor between the two growing periods. Moreover, the Cu and Pb uptake of the cultivars was not statistically different within the same growing period.

The trend in metals uptake by melon plants shown in **Figures 2 a** and **b** highlighted the lack of significant variability among both treatments and melon cultivars.

Figure 2 (c) shows the marginal means of metal content in the stem, leaves, and fruit of plants grown at Time 1 and Time 2. Statistically significant differences in Cr, Cu, and Pb accumulation among the plant parts were observed. The higher plant uptake of Pb and Cu at Time 2 with respect to Time 1 seems to affect leaves and stem (Pb) mostly or leaves only (Cu). The fruit was the least accumulating plant part for Cu and Pb. In fruit, Cu content did not seem to be affected by the seasonality of the growing period, while Pb content seemed to be partially affected.

As far as the quality of melon fruit was concerned, the effect of variety and growing seasonality on Cu and Pb content of fruit was reconsidered on a fresh weight basis.

In **Table 3**, the marginal means of Cu and Pb concentration in the fresh fruit are reported. In all cases, the fruit was safely edible for the EU market according to the current maximum concentration of Pb and Cu permitted in melon fruit of 0.1 and 5 mg kg^{-1} fresh weight, respectively (European Commission, 2006; European Commission, 2008).

Cu concentration in fresh fruit ranged from 1.88 mg kg^{-1} in ‘Django’ to 2.22 mg kg^{-1} in ‘Costantino’. Despite a slight tendency to absorb lower Cu quantities by early-ripening variety, no significant effect of variety on Cu concentration was assessed. Concerning Pb, its concentration in fresh fruit spaced from a value of 0.01 mg kg^{-1} in ‘Costantino’ to 0.027 mg kg^{-1} in ‘Django’. Again, no significant effect of the variety, as well as of variety:time interaction, was found under the two-way ANOVA. The only significant factor was the growing period: Cu and Pb concentration in fresh fruit cultivated at Time 2 increased by 25% ($p < 0,001$) and 312% ($p = 0,044$), respectively, respect to Time 1.

As big differences in Cu, Pb, and Ca concentration were observed from Time 1 and Time 2 in plant parts **(Figure 5)**, the Pearson correlation (*r*) matrix for the metals in the plant parts was calculated (**Table 4)**. As a result, positive correlations were observed both between Ca and Cu or Pb in leaves (r = 0,84 and 0,76, respectively) and stem (r = 0,89 and 0,74, respectively), thus supporting the figure that absorption and translocation of Pb and Cu are correlated with Ca uptake.

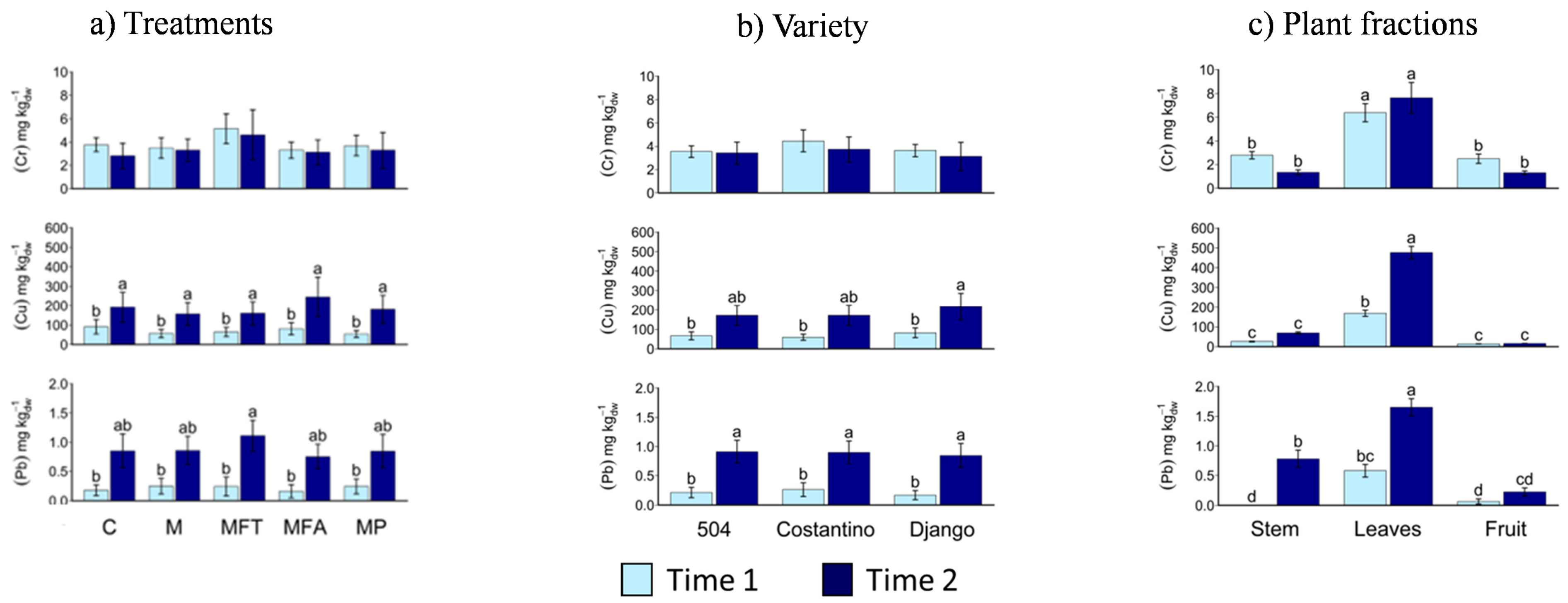


Figure 2. Marginal means of chromium (Cr), copper (Cu), lead (Pb) content in melon plants (overall PTE concentration in aerial biomass), expressed as dry weight (DW) for the tested treatments (a), variety (b) and for plant fractions (c). Error bars represent standard errors. Different lower-case letters indicate significant differences ($P < 0,05$) between tretments:time, variety:time and plant fractions:time interaction as determined by the Fisher's LSD test.

(C: control not treated; T: *Trichoderma* inoculation; TFA: *Trichoderma* inoculation and clinoptilolite for foliar action; TFT: *Trichoderma* inoculation and clinoptilolite in fertigation; TP: *Trichoderma* inoculation and clinoptilolite pellet)

Table 3. Marginal means of copper (Cu) and lead (Pb) content in the fresh fruit (mg kg^{-1} fresh weight). Different lower case letters indicate significant differences ($P < 0,05$) as determined by the Fisher's least significant difference (LSD) test.

	Cu	**Pb**
Variety		
Costantino	2.22	0.010
Django	**1.88**	0.027
504	1.91	0.025
p-value	*0.044*	*0.462*
Quality value (EC)	**5.0**	**0.1**
Time		
Time 1	**1.77**^{b}	**0.008**^{b}
Time 2	**2.23**^{a}	**0.033**^{a}
p-value	*<0.001*	*0.044*
Variety*Time		
P-value	*0.738*	*0.959*

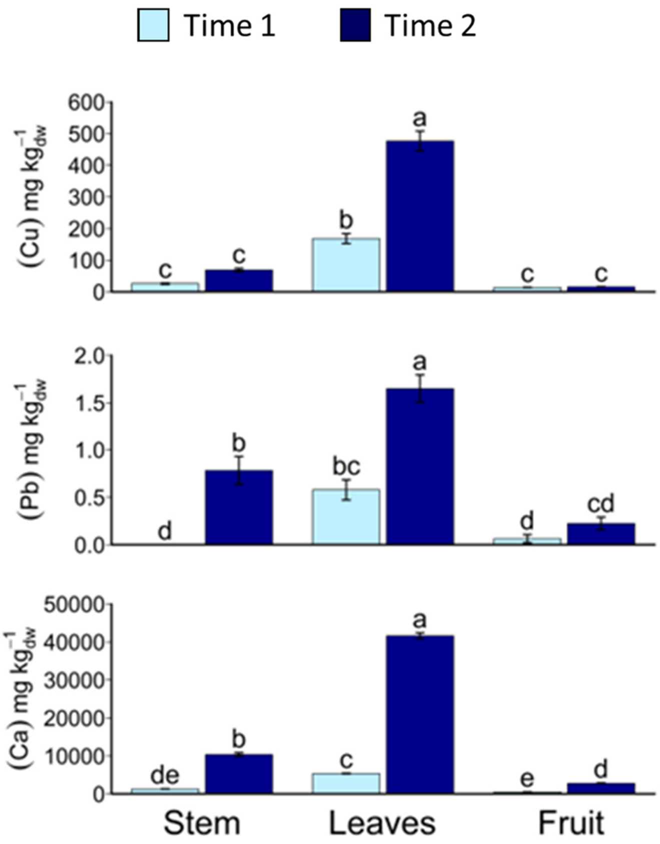
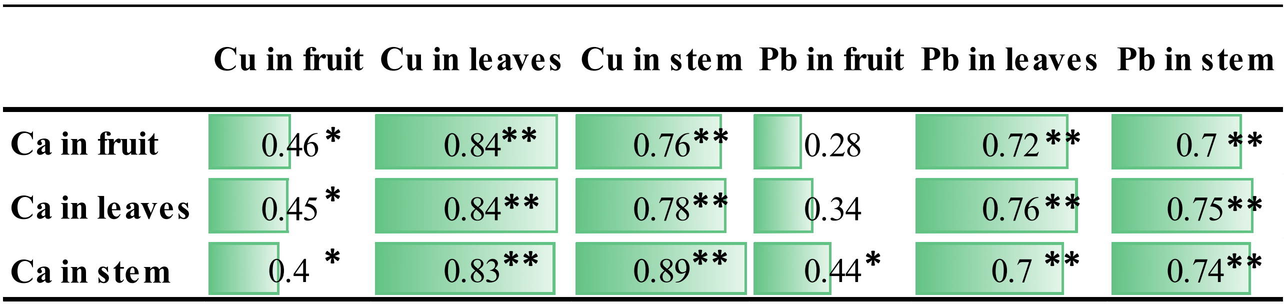


Figure 3 Marginal means of copper (Cu), lead (Pb) and calcium (Ca) content in melon plants (overall PTE concentration in aerial biomass), expressed as dry weight (DW) for plant fractions. Error bars represent standard errors. Different lower-case letters indicate significant differences (P < 0,05) between fractions:time interaction as determined by the Fisher's LSD test.

Table 4 Pearson's correlation coefficients matrix of copper (Cu), lead (Pb), and calcium (Ca) content of fruit, leaves, and stem.



	Cu in fruit	Cu in leaves	Cu in stem	Pb in fruit	Pb in leaves	Pb in stem
Ca in fruit	0.46 *	0.84**	0.76**	0.28	0.72 **	0.7 **
Ca in leaves	0.45 *	0.84**	0.78**	0.34	0.76 **	0.75**
Ca in stem	0.4 *	0.83**	0.89**	0.44*	0.7 **	0.74**

* Significant at P < 0.05; ** Significant at P < 0.01.

The higher Ca uptake was likely due to a higher nutrient flux through the soil-root-shoot system following the climate variation between the two growing periods, i.e., a higher transpiration rate. In the plants, Ca content was within the typical range of 1 – 50 mg g^{-1} DW (Kirkby & Pilbeam, 1984) for higher vascular plants.

Largely, Ca is absorbed by roots through active diffusion following water influx (Kirkby & Pilbeam, 1984). Diffusion is mediated by Ca nonselective cation channels (Ca-NSCCs), specialized transmembrane pores permeable to different cations, which are activated/deactivated by ion flux or different stimuli (e.g., environmental stress) (Demidchik et al., 2002). These channels are involved in several defense processes against heat stresses (Naeem et al., 2020) and act as a heat sensor that causes hyperaccumulation of Ca and other compatible solutes. Moreover, Ca signal transduction has a role in leaf surface temperature regulation by regulating stomatal conductance (Demidchik & Maathuis, 2007). Several studies indicated Ca-NSCCSs as a possible pathway for heavy metals uptake (Clemens et al., 2002; Demidchik et al., 2018; Pourrut et al., 2008; Sanz et al., 2019; H. Wang et al., 2007) owing to the significant permeability for both mono- and divalent-cations (Demidchik & Maathuis, 2007). Moreover, positive correlation between transpiration flux increase and Cu and Zn uptake is commonly found (Tani & Barrington, 2005a, 2005b).

In our plants, most of the considered metals accumulated in leaves. As defined by several studies on plant metal uptake and translocation mechanisms, the vacuole of leaf cells represents the main storage site for both excess nutrients and PTEs (Krämer, 2010; Pilon-Smits, 2005; S. S. Sharma et al., 2016). The metals translocation to above-ground tissues plays an ecological role as a detoxification and a herbivores defense strategy (Martinoia et al., 2012; Rascio & Navari-Izzo, 2011; S. S. Sharma et al., 2016).

Because of these observations, it was reasonable to suppose that, in our study, the increase in Cu, Pb and Ca uptake during the second growing season could depend mainly on the signal activation of Ca channels in response to the higher evapotranspiration rate typical of the summer growing period as shown by the higher (+14%) irrigation volume used.

2.4 Conclusions

Under integrated pest management, Cr uptake by Cucumis melo L. was not affected by seasonality, early or late-ripening varieties, or Trichoderma inoculation alone or combined with pellet, foliar action, and fertilization with clinoptilolite-based treatments. This confirms its known low availability to plants. On the other hand, the leaf translocation of Cu was significantly limited by the early cultivation period and, therefore, by early-ripening varieties. A similar pattern was observed with Pb. This result is particularly relevant for reducing the overall uptake of Cu and Pb, which are regulated within the EU in food products, in the case of melon crops grown in soils with high levels of these PTEs. The strong correlation between Ca uptake, used as a proxy for transpiration rate, and Cu and Pb accumulation, which mainly affected leaves, may indicate the involvement of Ca non-selective cation channels as a possible main entry point for these PTEs in the aboveground biomass.

In terms of market quality of fresh fruit, the lowest Cu concentration was found in the early-ripening 'Django' variety. Tendentially, lower average Pb concentrations in fruit were found in the early cultivation season, but no significant effects of variety or growing period were observed. These results suggest that a possible strategy for mitigating Cu and Pb uptake by melon plants, as well as the Cu and Pb concentration of fresh fruit, is to anticipate the growing period of melons. Under field conditions, the cultivation of early-ripening cultivars had a significant impact on reducing metal translocation and the Cu concentration in the fruit.

In the context of PTE risk management, the results of this study support the use of early-ripening cultivars in place of late-ripening ones in cases where significant PTE contamination is expected (e.g., soils with critical or high PTE levels). Additionally, as most Cu and Pb are accumulated in leaf tissues, it is important to minimize plowing of crop residues. Furthermore, it is advisable to manage Cu-based pesticides to avoid long-term accumulation in soil.

This study has been already published. For more detailed information, the publication is suitable as an attachment of this thesis.

Chapter 3

Correlation between bioavailable copper levels and microbiome diversity in grapevine rhizosphere

3.1 Introduction

In organic farming management, the use of trace elements (TEs) such as copper (Cu) as plant protection products (PPP) against pathogenic diseases is a topic of environmental relevance (Genova et al., 2022; Roviello et al., 2021). Copper is particularly effective in controlling fungal and bacterial pathogens, such as grapes downy mildew (*Plasmopara viticola*), potato late blight (*Phytophthora infestans*), and apple scab (*Venturia* spp) (Capinera & Dickens, 2016; Dorn et al., 2007; la Torre et al., 2011). Cu-based fungicides, known as Bordeaux mixture, has been used in defined amounts in integrated pest management and organic crop production, according to European Parliament regulations since 2009. However, excessive use of these products in the past century has led to increased Cu levels in cultivated soils, as a result of agricultural intensification and reduced crop diversification, according to studies by La Torre et al. 2011 and Lamichhane et al. 2018. Additionally, high levels of Cu can lead to low crop yields, environmental issues and toxicity in living organisms and human health. TEs are non-degradable pollutants and, therefore, their correct management becomes crucial in a sustainable agriculture approach (Briffa et al., 2020).

The use of TEs can also harm the soil microbiome by altering prokaryotic and eukaryotic communities and changing their diversity. Microbial communities, particularly bacteria and fungi, are essential to the sustainable functioning of natural and managed ecosystems. These communities play a crucial role in nutrient cycling and organic matter degradation, and can greatly affect the productivity and biodiversity of aboveground ecosystems (Fagnano et al., 2020; Nunes et al., 2016; Rocca et al., 2019).

Grape (*Vitis vinifera* L.) is one of the most widely cultivated fruit crops with a great economic impact. The interaction between microorganism and grapevine is an important aspect in sustainable agriculture and it could affect plant yield, fruit/wane taste and human health (Guarino et al., 2020). These interactions could be beneficial, phytopathogenic, or even neutral for the plant (Compant et al., 2011; Schulz et al., 1999) and consequently would alter the fruit yield, quality, and fermentation for wine production (Belda et al., 2017; Pinto, Pinho, Sousa, Pinheiro, Egas, & Gomes, 2014).

The rhizosphere is a narrow zone of soil surrounding and influenced by the roots and heavily populated by microorganisms (Reinhold-Hurek et al., 2015). As this environment is characterized by high root and microbial activity which can influence the chemical form and the bioavailability of TEs, the aim of this study was to delve deeper into the topic of soil microbiota diversity in relation to copper (Cu) accumulation in vineyard rhizosphere. The variation in total Cu content and its bioavailable portion in the rhizosphere soil of two vineyards with different planting age were assessed. The correlation between bioavailable Cu levels in soil and microbiota diversity was assessed implementing next-generation sequencing (NGS) of targeted amplicons protocol (reviewed in Franzosa et al., 2015).

The text part reported in this thesis is related to the contribution I personally gave to a more extensive study conducted in collaboration with Dot. Armin Shapari, PhD student of Genetics area of the department of agricultural and food sciences (DISTAL).

3.2 Materials and methods

3.2.1 Soil sampling

Two different northern Italian agricultural vineyards, namely Molinari and Picozzi, were considered in this study. Molinari vineyard is located in Valsamoggia municipality (44°33’43’’N; 11°07’23’’E) and has an extension of 5.5 hectares (ha) located entirely on the plain. In this vineyard, two grapevine varieties namely Alionza and Pignoletto were selected. Alionza variety, which has been planted in 1980, developed along a 100-m long row. Pignoletto variety, which has been planted since 1997, has an extension of 3.0 ha. Both varieties, grafted on local ancient different rootstocks, have been treated according to the Integrated Plant Production Guidelines*. Picozzi vineyard, on the other hand, is located in Ozzano dell’Emilia municipality (44°23’46’’N; 11°26’02’’E) with an extension of 12.5 ha, extending into hilly areas with slopes ranging from 10 to 20%. In this vineyard, Pignoletto grapevine variety, with an extension of 1.5 ha, was selected. This variety, grafted on SO4 rootstock, has been growing since 2016 and has been treated according to the Integrated Plant Production Guidelines*.

Rhizosphere soil samples were collected in February 2018 from Molinari and Picozzi vineyards.

In Molinari vineyard, 20 soil cores were collected at 10-cm soil depth from the soil surface at both the eastern and western side of the stem (because of the row extension along the N-S direction) of 10 plants according to a nonsystematic W-like pattern sampling within Pignoletto variety. Similarly, in Alionza grapevine variety, 8 soil cores were collected at eastern and western side of 4 plants selected according to a nonsystematic W-like pattern sampling.

In Picozzi vineyard, 20 soil cores were collected at 10-cm soil depth from the soil surface at both the southern and northern side of the stem (because of the row extension along the W-E direction) of 10 plants according to a nonsystematic W-like pattern sampling within Pignoletto variety.

*https://agricoltura.regione.emilia-romagna.it/produzioni-agroalimentari/temi/bio-agro-climambiente/agricoltura-integrata/disciplinari-produzione-integrata-vegetale

3.2.2 Analysis of soil proprieties and elemental characterization

All soil samples were air-dried, grinded and sieved at 2 mm. Sieved samples were analyzed for pH (ISO, 2021), electrical conductivity – EC (ISO, 1994), total organic carbon – TOC (ISO, 1995b), total nitrogen - TN (ISO, 1995c), and total carbonates (ISO, 1995a). Particle size distribution was determined by the pipette method (Gee & Bauder, 2018) using sodium hexametaphosphate as a dispersant. The soil samples were analyzed for pseudo-total and potentially available-to-plant (bioavailable) metal content as well as metagenomics analysis. Pseudo-total concentration of trace metals was assessed on air-dried samples by nitric acid/chloric acid (1/3 v/v) digestion and analyzed by ICP-OES (ISO, 2012). Bioavailable content of trace metals was assessed on air-dried samples by diethylenetriaminepentaacetic acid (DTPA) extraction, adapting the method from Lindsay and Norvell (1978). The DTPA extractable metal fraction is considered a valuable proxy for the determination of element potential bioavailability for plants in alkaline soils (Lindsay & Norvell, 1978). Briefly, soil samples were added to a DTPA solution (1.97 gL^{-1} DTPA, 1.46 gL^{-1} $CaCl_2$ $2H_2O$, 14.92 gL^{-1} triethanolamine) with 1:2 w:v ratio, at pH 7.3, shaken for 2 h, filtered through Whatman no. 42 paper, and analysed by ICP-OES (ISO, 2012). The t-test has been performed to show the differences between parameters in the two vineyards and cultivars.

3.2.3 Genomic DNA extraction

The following part has been developed by Genetics area of the department of agricultural and food sciences (DISTAL).

The process of obtaining genomic DNA from samples of soil found in the rhizosphere was completed using the QIAGEN D Neasy Power Soil kit. After verifying the quality and quantity, the genomic DNA samples were amplified using PCR to identify taxonomic characteristics in the form of gene loci (16S locus for archaea and bacteria, ITS region for fungi). The V3-V4 region of the bacterial 16S rRNA gene was amplified with the primer set of 341F and 805R, while the ITS1 and ITS4 regions of fungi were amplified using the primer set of ITS1 and ITS4 (White, 1990).

3.2.4 Processing of DNA sequence data

The following part has been developed by Genetics area of DISTAL.

Analyses of diversity (alpha and beta) were conducted using QIIME2 plugins. For alpha diversity, the Shannon index was used to estimate both the abundance and evenness of the taxa present for bacterial and fungal communities (Anderson, 2001; Callahan et al., 2016). The Faith's PD index was used to measure biodiversity that incorporates phylogenetic differences between species (Faith, 2018), and the Evenness index was applied to measure relative evenness of species richness (Jost, 2010). Beta diversity between samples was evaluated by computing the Unweighted UniFrac distances, for both 16S and ITS datasets. Principal Coordinate Analysis (PCoA) was also applied to Unweighted UniFrac distances, and that the analysis was carried out in R environment. Correlation analysis was performed between bioavailable Cu, total Cu, pH and EC with microbiota diversity based on the Shannon index for both 16S and ITS using non-parametric Spearman correlation in the QIIME2 plugin (Zhang et al., 2017).

3.3 Results and discussion

3.3.1 Soil analysis

A total of 48 soil samples were collected and analyzed for chemical-physical properties (Table 1). The range of variation of soil pH (7.3 – 8.2), electrical conductivity (0.22 – 0.25 mS cm^{-1}), TOC (11.52 – 18.90 mg kg^{-1}), TN content (1.47 – 2.36 mg kg^{-1}) and total carbonates (8.04 – 16% dm) was typical of the geographical area under investigation (https://ambiente.regione.emilia-romagna.it/en/).

As shown in **Figure 1- a, b, c**, the results revealed a relatively strong difference in total and bioavailable Cu between the two vineyards. The bioavailable Cu levels observed in the Molinari and Picozzi vineyards were found within the range of 28.5 to 64 mg/kg and 1.5 to 4.5 mg/kg, respectively. Furthermore, a strong correlation between total and bioavailable Cu was observed (R^2: 0.98, **Figure 1-d).** The values of total content of copper for both vineyards were within the typical range observed in Italian soils (https://ambiente.regione.emilia-romagna.it/en/).

The potentially bioavailable-to-plant levels of copper in the soil samples from the Molinari and Picozzi vineyards were found, on average, to be significantly different (46.6 vs 2.77 mg kg^{-1}, on average, respectively, with a P value of <0.05, according to t-test analysis, **Table 1**). However, when looking at the bioavailable copper levels within the Molinari vineyard, there was no significant difference between Pignoletto and Alionza cultivars (42.5 vs 50.7 mg kg^{-1} respectively, **Table 1**).

The difference in total Cu accumulation in soil is thought to be due to factors such as the age of the grapevines (Mol: Alionza and Pignoletto 1980 and 1996, respectively; Pic: Pignoletto, 2016), and therefore to the number of years of Cu-based treatment within the Molinari farm, and the topological differences between the two farms. The greater slope of the land in Picozzi farm facilitates surface runoff and percolation of water and elements, thereby decreasing stagnation and accumulation phenomena.

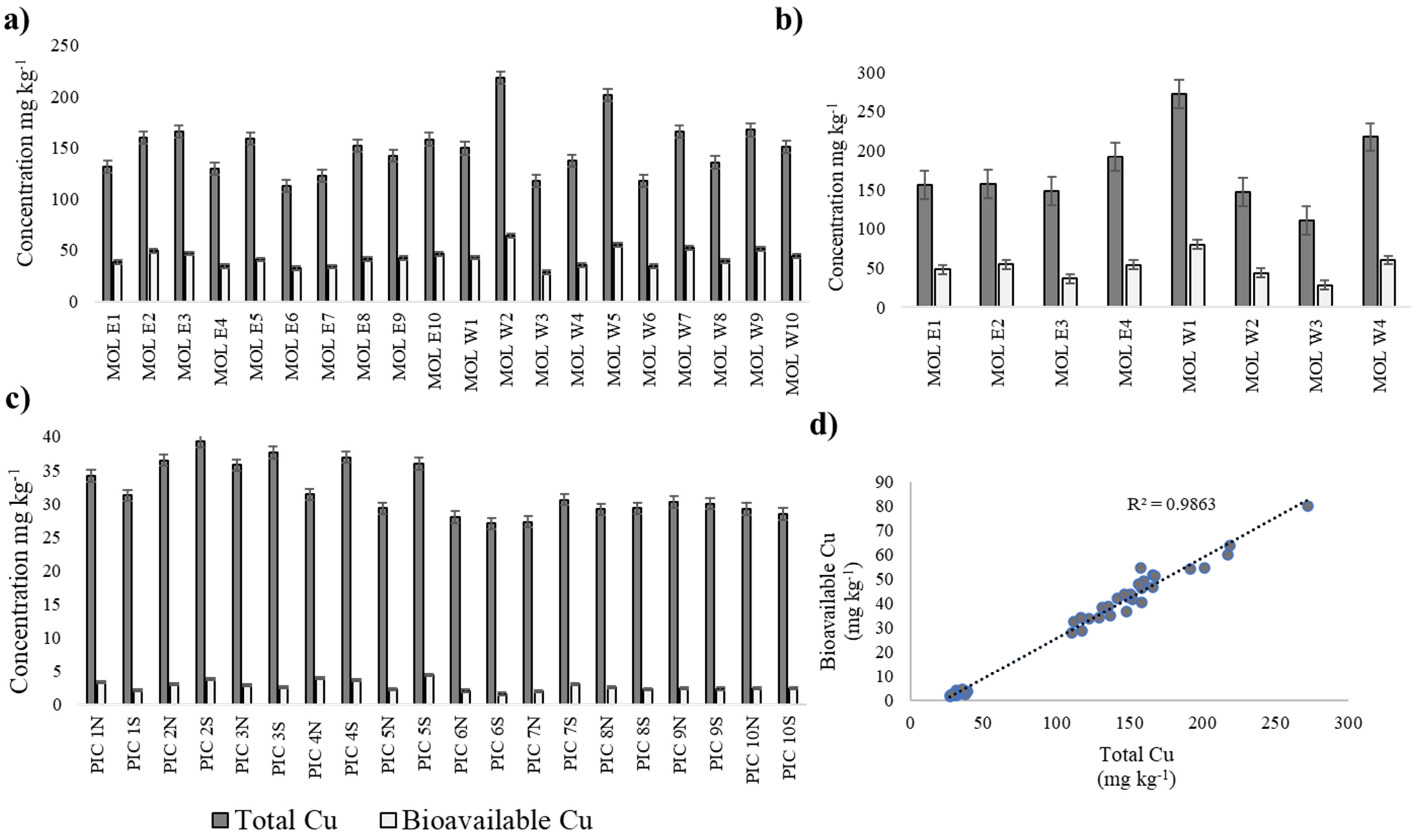


Figure 1 Total and potentially bioavailable-to-plant copper (Cu) levels in soil samples at Molinari (MOL) vineyard (E & W = sampled at eastern and western part of row, respectively): Pignoletto (a) and Alionza (b) and total and potentially bioavailable-to-plant Cu levels in soil samples at Picozzi (PIC) vineyard (N & S = sampled at north and south part of row, respectively): Pignoletto (c). Correlation between total and bioavailable Cu content for all samples (d).

Table 1 Results of chemical-physical analysis of soil samples from Molinari and Picozzi farms. t-Test was used to calculate the statistical differences between the two farms and between the two varieties.

Parameter (unit)	**Molinari (Pignoletto)**	**Molinari (Alionza)**	**Pignoletto vs. Alionza *P-value* (t-test)**	**Picozzi (Pignoletto)**	**Molinari vs. Picozzi *P-value* (t-test)**
pH-H_2O	7.9 ± 0.0	7.3 ± 0.3	n.s	8.2 ± 0.3	< 0.001
pH-$CaCl_2$	7.5 ± 0.1	7.3 ± 0.1	-	7.6 ± 0.1	-
EC (mS cm^{-1})	0.23 ± 0.05	0.25 ± 0.03	< 0.01	0.22 ± 0.02	< 0.001
TOC (mg kg^{-1})	11.52 ± 0.74	18.90 ± 1.60	-	15.35 ± 0.12	-
TN (mg kg^{-1})	1.47 ± 0.06	2.36 ± 0.22	-	1.76 ± 0.41	-
Total carbonates (% dm)	8.04 ± 0.30	11.49 ± 0.05	-	16.00 ± 0.93	-
Total copper (Cu)	149.59 ± 27.12	175.07 ± 50.50	n.s	31.863 ± 3.847	< 0.001
Bioavailable copper (Cu)	42.52 ± 8.695	50.73 ± 15.81	n.s	2.77 ± 0.747	< 0.001
Textural class	silty clay loam	silty clay loam	-	silty clay	-

n.s. not significant
“-“ data not available

3.3.2 Rhizosphere microbial diversity in grapevine

The Shannon (H) index was used to evaluate the alpha diversity of soil samples from the Molinari and Picozzi vineyards, as well as of the two cultivars in the Molinari vineyard (**Figure 2**). The microbial diversity did not differ between vineyards for both bacteria and fungi (Kruskal-Wallis test. **Figure 2 a-b**). Within the Molinari vineyard, the two cultivars Alionza and Pignoletto did not show differences in both bacterial and fungal diversity (Kruskal-Wallis test. **Figure 2 c-d**). However, the overall Shannon index of bacterial communities was significantly higher than fungal communities (8.1 vs 6.3. Kruskal-Wallis test, $P < 0.05$. **Figure 2**).

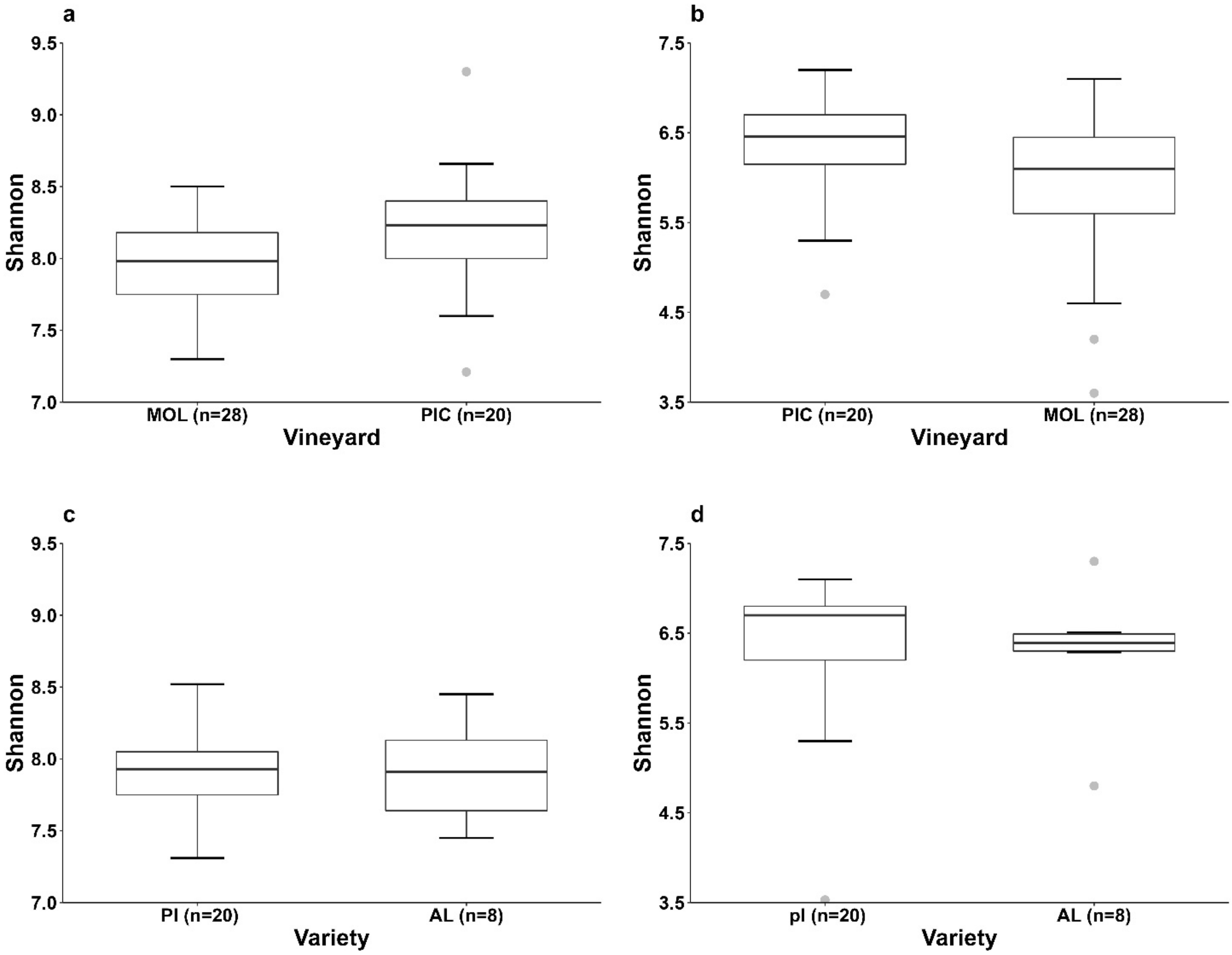


Figure 2. Alpha diversity based on Shannon index for: a, b) 16S rRNA genes and ITS loci for Molinari (MOL) and Picozzi (PIC) vineyards; c, d) 16S rRNA genes and ITS loci for Pignoletto (PI) and Alionza (AL) varieties in Molinari vineyard.

To evaluate the overall differences in microbiome diversity (beta diversity) between the samples for both 16S and ITS, principal coordinates analysis (PCoA) plots was constructed by computing the Unweighted UniFrac dissimilarity index in R environment (**Figure 3**). The PCoA plots showed a clear separation between samples from the Molinari and Picozzi vineyards for both 16S and ITS samples. However, no such separation was observed between the Pignoletto and Alionza varieties for 16S and ITS samples. To statistically evaluate the Unweighted UniFrac dissimilarity index and assess the differences between the Molinari and Picozzi vineyards, as well as the Pignoletto and Alionza varieties, in both 16S and ITS samples pairwise PERMANOVA was used. The Pseudo-F values of pairwise PERMANOVA for the two vineyards were found to be 23.65 and 61.24 for 16S and ITS, respectively and were found to be statistically significant at a p-value < 0.001. In contrast, the results of PERMANOVA between the two varieties for 16S (F = 2.74) and ITS (F = 6.34) samples were not found to be statistically significant (**Figure 3**).

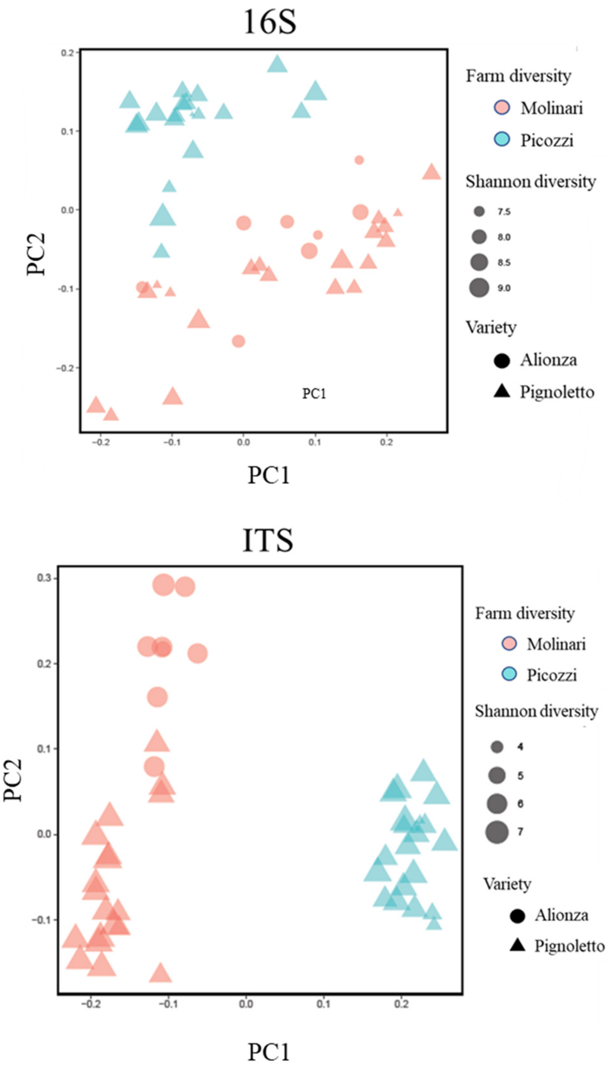


Figure 3 Principal coordinates analysis (PCoA) plots evaluated by computing the Unweighted UniFrac dissimilarity index for 16S and ITS. Molinari and Picozzi indicate the two different vineyards, Alionza and Pignoletto indicate the two different grape cultivars sampled within the Molinari vineyard.

3.3.3 Correlation between copper levels and composition of grapevine microbiota

Using a QIIME 2 plugin, the non-parametric spearman correlation based on Shannon diversity was performed in Molinari and Picozzi vineyards separately (**Figure 4**). The results demonstrated that there was no correlation between bioavailable Cu and bacterial and fungal microbiota diversities in none of the two vineyards (**Figure 4**). The correlation between bacterial diversity and bioavailable Cu in Molinari (test statistic = 0.25, p-value = 0.27) and Picozzi (test statistic = 0.15, p-value = 0.43) vineyards using non-parametric Spearman's test evaluation was not significant in neither of the vineyards. Similarly, the correlation test for fungal diversity and bioavailable Cu assessed using Spearman's test did not show significant pattern in Molinari (test statistic = 0.11, p-value = 0.63) and Picozzi (test statistic = 0.13, p-value = 0.85) vineyards (**Figure 4**).

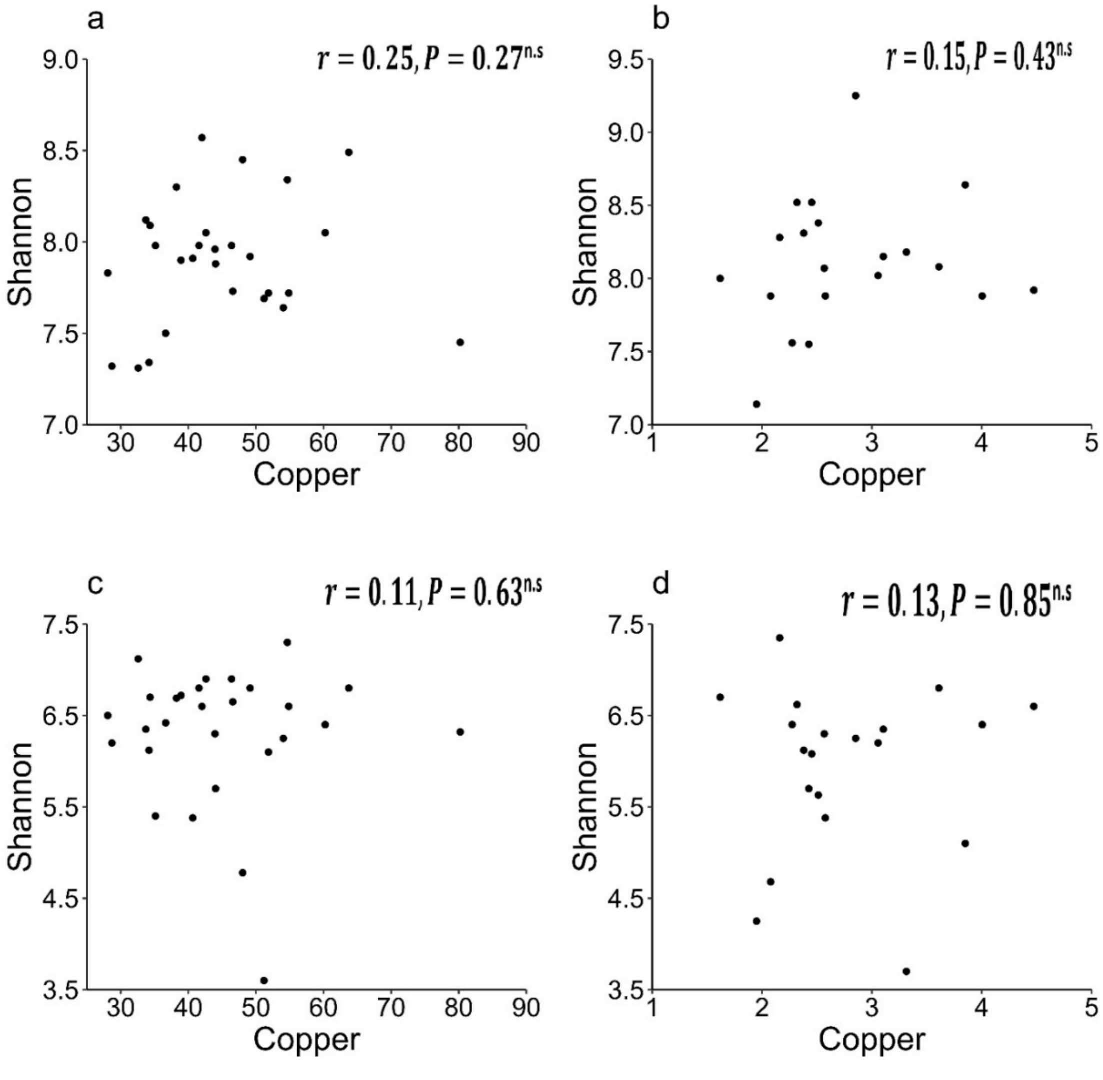


Figure 4. Scatter plots showing the correlation (Spearman) between the bioavailable Copper (express as mg kg^{-1} of soil) and microbiota diversities using Shannon index in: a) Molinari vineyard, 16S; b) Picozzi vineyard, 16S; c) Molinari vineyard, ITS; and d) Picozzi vineyard, ITS.

The Cu levels found in the Molinari and Picozzi vineyards ranged between 28.5 and 64 mg/kg and 1.5 to 4.5 mg/kg, respectively, and were within the typical range for Italian vineyards. In each vineyard, the results of non-parametric Spearman correlation test between Cu levels and overall microbial diversity (Shannon index) did not show any significant relationship for either the bacterial or fungal communities. Additionally, there were significant differences in microbial diversity between the Molinari and Picozzi vineyards, but not between the Pignoletto and Alionza grape varieties in the Molinari vineyard. However, this diversity was not found to be significant between the two vineyards when looking at the 16S and ITS samples. These differences may be attributed to a variety of factors, including geographical location and other environmental factors.

3.4 Conclusions

The European Commission (EU 2021/2115, http://data.europa.eu/eli/reg/2021/2115/oj) recently proposed binding targets to reduce by 50% the use of chemical pesticides by 2030. Therefore, there is increasing pressure on agriculture to limit the use of copper in organic and integrated pest management of farming systems. Several studies have shown that copper long-term overuse can have negative effect on the environment, human health, and farm production (Coelho et al., 2020; Guarino et al., 2020; Lamichhane et al., 2018; Pinto, Pinho, Sousa, Pinheiro, Egas, & C. Gomes, 2014; Steffan et al., 2018).

In this study, we found a copper accumulation in soils that have been cultivated for many years (Molinari farm). The strong correlation observed between total and bioavailable copper observed suggests that a soil Cu accumulation could expose biota to the risks of an excessive Cu assimilation. Nevertheless, surprisingly, no significant effect has been found between the bioavailable-to-plant copper amount in the rhizosphere soil of each vineyard and the microbial (alpha) diversity.

General conclusions

The results of this study emphasized the significance of evaluating various aspects of TEs contamination. Urban, industrial, and agricultural activities are the main sources of anthropogenic environmental diffusion of these pollutants, which can pose a risk to both human health and ecosystems. Indeed, TEs can be absorbed by crops and macro and microorganisms then entering in the trophic chain. To date, some elements such as copper are very important resources because they are globally used as pest controller to fight the major crop disease. However, prolonged overuse can result in a build-up of these pollutants that is challenging to remediate. Concurrently, markets and institutions are taking steps to curb the uncontrolled spread and accumulation of these pollutants. They are doing so by implementing increasingly stringent limits on the concentration of certain elements in products intended for a more informed and discerning public. Additionally, they are setting targets to decrease the use of products that contain potentially harmful elements.

In this field, research is being conducted to find strategies to limit contamination from toxic elements and reduce the danger from their accumulation. However, the dynamics of toxic elements in the environment are not fully understood at the field scale due to the many factors that depend on specific sites. Therefore, it is difficult to find universal solutions. The literature is often contradictory, and strategies that work in one environment may not be effective in others.

This study reports three researches conducted on this topic from different perspectives.

A remediation strategy involving a saline washing of polluted sewage sludge was evaluated. The sludge management represent a crucial issue due to the large amount produced annually and due to the difficulties encountered in virtuously reusing these materials. Indeed, sewage sludge must be treated at authorized plants to prevent biological risks and decrease the amount of material to manage. However, their reuse in agriculture is often prevented by the concentration levels of TEs, which do not comply with regulations, and incineration or disposal remain the only solutions.
As a first step of research, the good results obtained by the treatment tested in this study on dehydrated sludge place the $CaCl_2$ washing as a possible remediation methodology for TEs abatement. Other aspects will be evaluated to optimize the process, such as the minimum contact time and the possibility of reusing the extracting solution multiple times.

The study of TEs uptake by melon plants found that climate, seasonality and the cultivar choice greatly affected the plant's absorption of potential toxic elements. However, the results also showed that products such as clinoptilolites and *Trichoderma*, which had previously been tested with positive effects, did not limit TEs uptake in this field study. Another important aspect was the marked TEs accumulation in leaves which requires careful plant waste management to limit accumulation in the soil.

Finally, the copper accumulation in two vineyards with different planting ages, both cultivated under organic farming protocols, was evaluated. The correlation between the portion of the total amount of bioavailable copper and the microbiome in the rhizosphere soil was also assessed. The aim was to understand how copper accumulation, a common figure in cultivated soil, affects microbial communities (bacteria and fungi), which are essential for sustainable ecosystem functioning.

This field study revealed significant differences in copper accumulation between young, growing soils and soils that have received treatment for several years. Similarly, the portion of copper that is able to be utilized by living organisms increases with the total concentration, increasing the risk of excessive exposure. However, no correlation was found between bioavailable copper levels and microbial diversity in this study.

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Attachments

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Effect of the Growing Season, *Trichoderma*, and Clinoptilolite Application on Potentially Toxic Elements Uptake by *Cucumis melo* L.

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Keywords. clinoptilolite, food safety, heavy metals, plant uptake, *Trichoderma*

***Abstract.* The extent to which different agricultural strategies may affect the uptake of potentially toxic elements (PTEs) by cropped plants is not entirely understood at a field scale. This study addresses the effect of seasonality, *Trichoderma* inoculation alone, or combined with different applications of commercial-grade clinoptilolite (i.e., foliar action, fertigation, and pellet) on the PTE content of early- and late-ripening cultivars of *Cucumis melo* L. Two similar field experiments were performed in spring and summer. For each cultivar/treatment combination, the input of PTEs [namely, chromium (Cr), copper (Cu), and lead (Pb)] into the soil-crop system through irrigation water, fertilizers, pesticides, and treatment products (i.e., *Trichoderma* and clinoptilolite products), as well as the PTE content of melon stem, leaves, and fruit, were measured through inductively coupled plasma-optic emission spectrometry (ICP-OES). Neither *Trichoderma* alone nor with clinoptilolite had a visible effect on PTE uptake by plants, whereas early season cultivation was strongly associated with reduced uptake of Cu and Pb. The high correlation of Cu and Pb content with stem and leaf calcium (Ca) content (used as a proxy for different transpiration rates under different growing seasons) indicated a possible uptake of these metals through Ca nonselective cation channels as a defense against drought stress. Reduced Cu and Pb concentrations were found in early-ripening fruit cultivated in spring. Concerning Cu and Pb risk management, in case of significant contamination in Mediterranean calcareous soils, early-ripening *Cucumis melo* L. cultivars are suggested instead of late-ripening ones.**

In cultivated soils, high PTE concentration of allochthonous origin are commonly attributed to anthropogenic industrial activities and the utilization of amendments, fertilizers, and poorly treated water (He et al. 2005; Thornton 1981). High levels of copper (Cu) and zinc (Zn) can accumulate in plants, as they are actively assimilated as essential micronutrients (Clemens et al. 2002). Similarly, nonessential elements, such as lead (Pb), arsenic (As), and cadmium (Cd), can be taken up by the crop root system and translocated into edible tissues (Lasat 2002; Sekara et al. 2005; Uchimiya et al. 2020).

In the European Union, excessive concentration of some PTEs (such as Cu and Pb) in food edible parts are considered a cause of food noncompliance, and regulation set a maximum amount tolerated within the inner market (European Commission 2006, 2008). Nevertheless, lower maximum PTEs concentration may be set by member states or within certain market segments (Buscaroli et al. 2021). The use of Cu salts as sulfates, commonly known as Bordeaux mixture, is permitted with some limitations on several crops (including melon) in integrated pest management (European Parliament 2009) and in organic crop production. Cu-based products have been massively used in the past century due to their fungicidal properties (Lamichhane et al. 2018), thus increasing the Cu pool in cultivated soils (La Torre et al. 2018). Agriculture intensification and reduction of crop diversification favored Cu accumulation in soils (Lamichhane et al. 2018). Moreover, the increasingly adopted practice of plowing crop residues (green manure) reduces Cu and other nutrition elements removal by harvest.

From an environmental point of view, the reduction of heavy metal content in polluted soils is generally achieved through phytoremediation techniques (Ali et al. 2013), soil washing (Buscaroli et al. 2019), and soil dilution (Dermont et al. 2008). Otherwise, agricultural strategies aimed at mitigating the risk of PTEs entering the food chain prevent their plant uptake and translocation through soil conditioning or pH corrections to reduce metal ion mobility and availability (Uchimiya et al. 2020).

Alternative solutions have been investigated by academia or proposed by professional horticulture companies. This is the case of all-purpose natural zeolite-based products, such as clinoptilolite, whose use in agriculture was proposed in past years owing to zeolite's high cation exchange capacity and nutrient content (Nakhli et al. 2017). More controversial is their use as PTE immobilizers, as studies on clinoptilolite's ability to stabilize PTEs are scarce and limited to particular applications (Leggo and Ledésert 2001; Leggo et al. 2006; Oste et al. 2002).

Another proposed strategy currently evaluated to prevent PTEs translocation to crops is soil treatment based on *Trichoderma* sp. fungi inoculation. *Trichoderma* sp. is a competitor of pathogenic fungi and can establish a mutualistic symbiotic endophytic association with certain crops (Berg 2009; Woo et al. 2006). Recent studies pointed out the capacity of symbiotic *Trichoderma* sp. to reduce micronutrient plant assimilation (de Santiago et al. 2011; Téllez Vargas et al. 2017), especially Cu. This mechanism is unclear, but it has been hypothesized that competition between *Trichoderma* and the inoculated crop for micronutrients is involved. If confirmed in multiple tests and fully understood, *Trichoderma* association with crops cultivated in Cu-rich substrates could be a valuable strategy to reduce excessive Cu accumulation.

Melon (*Cucumis melo* L.) is the third most largely cultivated horticultural crop in Italy [Italian Institute of Statistics (ISTAT) 2021]. The consumption of melon fruit peaked at 9.5 annual kilograms per capita in 2020 [Services Institute for Agri-food Market (ISMEA) 2020]. Italy is also the second largest European producer of this crop [Food and Agriculture Organization of United Nations (FAO) 2021]. Like other genera of the Cucurbitaceae family, melon may mobilize, uptake, and translocate organic and inorganic contaminants (Campbell et al. 2009; Mattina et al. 2004, 2006).

The aim of this study was to evaluate different agricultural strategies to elucidate the uptake and translocation of PTEs (i.e., Cu, Cr, and Pb) to three different melon cultivars of relevant commercial interest in the Italian market. Specifically, the effect of different applications of commercial-grade clinoptilolites, *Trichoderma* inoculation, and seasonality on PTE content of three different melon cultivars were evaluated. In parallel, a detailed mass

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balance of PTEs introduced into the soil-crop system through irrigation water, fertilization, and pest management was assessed. Results indicated that *Trichoderma* alone or combined with clinoptilolite treatments did not affect PTE uptake, whereas early season cultivation was strongly associated with lower Cu and Pb uptake.

Materials and Methods

Experimental field design and bulk soil analysis. The experimental field was in the municipality of Viadana, MN, Italy (44°58′21″N, 10°35′11″E) within the production area of Agricola Don Camillo S.C.a.R.L. (Fig. 1), one of the largest melon producers in Italy. The plot used for the trials had an extension of 0.5 ha.

Before the beginning of the experimental trial, on 1 Jun 2020, a bulk soil sampling was performed. The soil was sampled according to nonsystematic "W" pattern sampling. Four soil core samples of 30 cm in depth were collected at positions shown in Fig. 1. The soil sampling was established at the expected melon roots development depth. Single soil core samples were homogenized, air-dried, manually ground, and sieved at 2 mm. Samples were analyzed for pH [International Organization for Standardization (ISO) 2021], electrical conductivity (EC) (ISO 1994), total organic carbon (TOC) (ISO 1995a), total nitrogen (TN) (ISO 1995b), and total carbonates (ISO 1995c). Particle size distribution was determined by the pipette method (Gee and Bauder 1986) using sodium hexametaphosphate as a dispersant.

Pseudo-total metal content of soil samples was determined through microwave acid digestion in *aqua regia* (ISO 2012). Digested extracts were filtered through Whatman no. 42 filter paper and analyzed by ICP-OES (ISO 2008a), using an ICP-OES Spectro Arcos (Ametek, Germany).

The fraction of potentially available-to-plant metal content was assessed by diethylentriaminopentaacetic acid (DTPA) extraction, adapting the method from Lindsay and Norvell (1978). The DTPA extractable metal fraction is considered a valuable proxy for the determination of element potential bioavailability for plants in alkaline soils (Lindsay and Norvell 1978). Briefly, soil samples were added to a DTPA solution (1.97 $g \cdot L^{-1}$ DTPA, 1.46 $g \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 14.92 $g \cdot L^{-1}$ triethanolamine) with 1:2 w:v ratio, at pH 7.3, shaken for 2 h, filtered through Whatman no. 42 paper, and analyzed by ICP-OES.

All analyses were performed in triplicate.

Field trials. Three commercial melon hybrids were selected for experimental cultivation: 1) Django F1, an early-ripening cultivar, 2) Costantino F1 and 3) EXPE504 F1, both late-ripening. All cultivars (from now on, called Django, Costantino, and 504, respectively, for the sake of brevity) were chosen for their commercial value, and provided by HM.Clause. No data were available beforehand on their specific capacity to mobilize or uptake PTEs differently. To even out the differences in PTEs uptake between early and late-ripening cultivars, Django, Costantino, and 504 were cultivated in both periods.

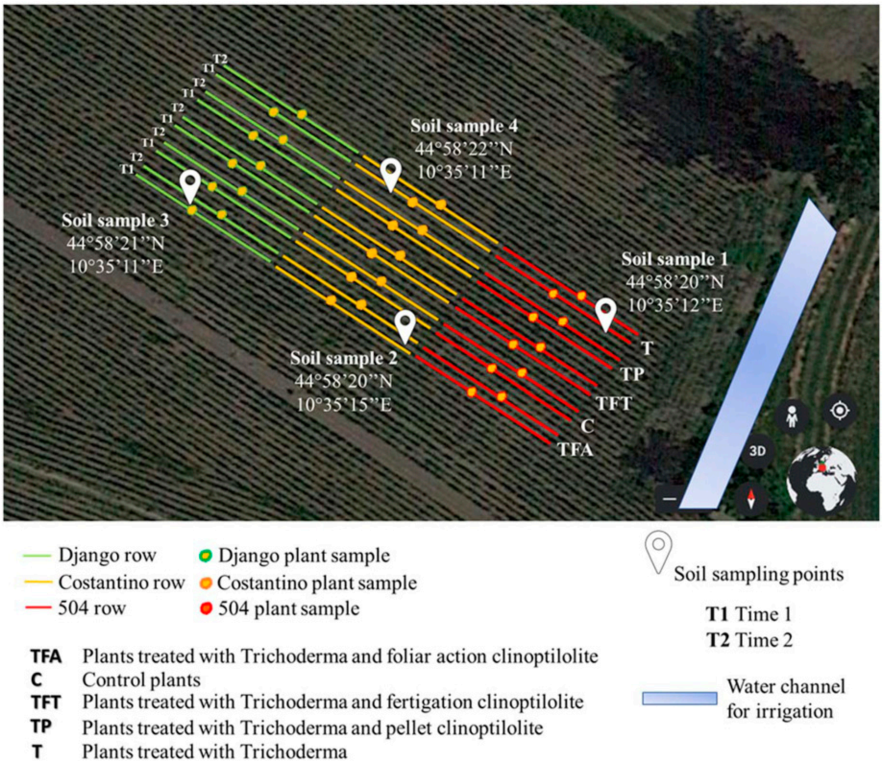

Fig. 1. Experimental field. Soil and plant sampling positions are indicated. Plants were sampled from the inner part of each cultivar row to avoid, as much as possible, side effects from the different vicinal varieties.

In 2020, two field trials with a duration of 84 d each were performed during spring (Time 1: planted on 2 Apr and harvested on 25 Jun) and summer (Time 2: planted on 1 Jun and harvested on 24 Aug). The planting layout provided for the rows to be laid in line on growing beds of 20 cm high from ground level. The distance between rows was 2 m and, along the row, the distance between plants was 1 m. The cultivars were planted according to the scheme indicated in Fig. 1. All three cultivars were fertilized and protected according to the integrated pest management protocol given as supporting information in Supplemental Table 1.

At both growing periods (Times 1 and 2) and on all three crop varieties, different treatments were evaluated: *Trichoderma* inoculation alone or combined with the most common clinoptilolite-based commercial products.

The acronyms of the different treatments are reported as follows:

- C: control not treated
- T: inoculation with *Trichoderma*
- TFA: inoculation with *Trichoderma* and foliar application of powdered clinoptilolite ("foliar action")
- TFT: inoculation with *Trichoderma* and application of powdered clinoptilolite via fertigation
- TP: inoculation with *Trichoderma* and soil application of clinoptilolite pellets

The products used, the dosage, and the application conditions for *Trichoderma* inoculation and clinoptilolite treatments are reported as supporting information in Supplemental Table 1.

A weather station ECO 4M (DigitEco Srl., Bologna, Italy) was installed at 20-m height from ground level close to the experimental plot (44°58′17″N, 10°35′03″E) for meteorological data collection. The average daily temperature, rainfall, and relative humidity of the period corresponding to the two experimental trials are reported as a supporting information in Supplemental Fig. 1. The average daily temperature showed a trend to increase from 13 °C on the first day of the experimentation (2 Apr) to 22.5 °C on the last one (20 Aug). During the first growing season, the total amount of rainfall was 126 mm spread over 31 d, whereas, during the second growing season, it was 197 mm spread over 26 d. The average relative humidity curve shows a variable trend with peaks of 90% corresponding to rainy days.

Trace elements analysis of fertilizers, pesticides, and products used for treatments. Pseudo-total metal content of the fertilizers, the pesticides, and the inoculation product used in the trials were determined through a wet acid attack on flame. Briefly, 5 g of each sample was placed in a 250-mL wide-neck flask. In general, 21 mL of hydrochloric acid (37% HCl for trace analysis; Honeywell Fluka, Muskegon, MI, USA) and 7 mL of nitric acid (65% HNO_3 for trace analysis;

Table 1. Physical and chemical characterization of 0 to 30-cm topsoil samples. Errors are expressed as a standard deviation.

	Soil samples					
Parameter	1	2	3	4	Avg	
pH H_2O	8.80 ± 0.01	8.80 ± 0.01	8.80 ± 0.01	8.80 ± 0.01	8.80 ± 0.01	
pH $CaCl_2$	7.90 ± 0.01	8.00 ± 0.01	8.10 ± 0.01	8.10 ± 0.01	8.00 ± 0.01	
Electrical conductivity (ds·m^{-1})	0.151 ± 0.004	0.143 ± 0.003	0.145 ± 0.006	0.154 ± 0.001	0.148 ± 0.002	
Total organic carbon (%)	1.329 ± 0.008	1.429 ± 0.029	1.412 ± 0.117	1.535 ± 0.019	1.426 ± 0.018	
Total nitrogen (%)	0.193 ± 0.003	0.210 ± 0.008	0.205 ± 0.003	0.226 ± 0.004	0.229 ± 0.005	
Carbonates ($CaCO_3$%)	7.952 ± 0.420	7.251 ± 1.055	8.210 ± 1.087	7.798 ± 0.776	7.803 ± 0.834	
Textural class (USDA[i] class)	Silty Clay Loam	Silty Clay Loam	Silty Clay Loam	Silty Clay Loam		
PTEs content (mg·kg^{-1})						Italian threshold concentration in soils[ii]
Chromium[iii]	208.2 ± 2.9 (0.01%)	212.1 ± 2.7 (0.01%)	197.0 ± 4.2 (0.01%)	205.6 ± 2.0 (0.01%)	205.0 ± 2.9 (0.01%)	150
Copper[iii]	114.1 ± 1.4 (11.84%)	91.3 ± 0.5 (10.20%)	99.2 ± 3.9 (12.25%)	72.4 ± 0.8 (10.05%)	87.6 ± 1.8 (11.08%)	100
Lead[iii]	33.8 ± 4.6 (6.88%)	33.4 ± 6.2 (7.93%)	35.4 ± 1.8 (7.48%)	36.1 ± 0.8 (7.73%)	35.0 ± 3.0 (7.51%)	200

[i] U.S. Department of Agriculture (Soil Survey Staff 1999).

[ii] D.Lgs. N°46 01/03/2019.

[iii] Diethylentriaminopentaacetic acid extractable potentially toxic elements (PTEs) content expressed in brackets as a percentage of the total PTEs amount.

Honeywell, Fluka) were added to the sample, and the mixture was heated on a Bunsen flame and brought to boiling. Only for organic fertilizers (namely, Lieta Veg, Agriges and Examine L®, K&A), digestion was completed by adding a further volume of 65% nitric acid and a few milliliters of hydrogen peroxide (30% H_2O_2 for electronic use; Honeywell, Fluka) until complete dissolution of the solid matrix. Pseudo-total metal content of the clinoptilolite-based products were determined through microwave acid digestion by adding to 0.250 g of each sample, 6 mL HCl (37%), 2 mL HNO_3 (65%), 2 mL HF (40% for trace analysis; Honeywell, Fluka), and 0.5 mL H_2O_2 (30%).

The digestates obtained were filtered through Whatman no. 42 filter paper, diluted to 20 mL with milliQ® water and analyzed by ICP-OES.

To evaluate to what extent any product contained PTEs readily available to plants, the metal fraction potentially available to plants of fertilizers, pesticides, and products used for treatments was assessed on DTPA extracts as already described for soil.

All analyses were performed in triplicate.

Irrigation protocol and water analysis. The irrigation was performed with water from a freshwater canal that is adjacent to the experimental field (see Fig. 1) through a drop-by-drop system equipped with a unit control that allowed us to measure the water daily distributed to each experimental field rows. The total irrigation water used separately for crops during the first and second growing periods was 192 and 218 m^3, respectively.

The irrigation water was collected monthly and characterized. On 15 Apr, 15 May, 15 Jun, 15 Jul, and 15 Aug 2020, a sample (10 L) of water was collected from the suction pipe connected to the freshwater canal. Water samples were filtered by Whatman 0.45-μm pore size nylon membrane filter to separate suspended solids from the liquid phase (ISO 1997). Both liquid and solid fractions were analyzed.

Filtered water was characterized for pH (ISO 2008b), EC (ISO 1985), and trace elements content. Trace elements analysis was carried out by adding 0.15 mL of HNO_3 (1% v/v) to 15 mL of each filtered water sample and analyzed by ICP-OES.

Suspended solids were air-dried, ground, and analyzed for pseudo-total metals content through microwave acid digestion (ISO 2012). Digested samples were filtered through Whatman no. 42 filter paper and analyzed by ICP-OES (ISO 2008a).

All analyses were performed in triplicate.

Table 2. Physical and chemical characterization of irrigation water. Error expressed as a standard deviation. In brackets, the detection limit (DL) value of the analytical method.

		Water samples				
Parameter	Sampling date	15 Apr 2020	15 May 2020	15 Jun 2020	15 Jul 2020	15 Aug 2020
pH		9.5 ± 0.1	8.0 ± 0.1	8.2 ± 0.1	8.4 ± 0.1	8.2 ± 0.1
Electrical conductivity (dS·m^{-1})		0.479 ± 0.040	0.286 ± 0.008	0.279 ± 0.007	0.271 ± 0.010	0.258 ± 0.005
Suspended solids (mg·L^{-1})		81 ± 1.0	83 ± 1.0	83 ± 1.0	93 ± 1.0	90 ± 1.0
Chromium (mg·L^{-1})	Filtered water	<DL (0.001)	<DL (0.001)	<DL (0.001)	<DL (0.001)	<DL (0.001)
	Suspended solids	0.012 ± 0.015	0.049 ± 0.001	0.049 ± 0.001	0.030 ± 0.001	0.034 ± 0.001
	Total	0.012	0.049	0.049	0.03	0.034
Copper (mg·L^{-1})	Filtered water	0.006 ± 0.001	0.003 ± 0.001	0.003 ± 0.001	0.016 ± 0.001	0.016 ± 0.001
	Suspended solids	0.008 ± 0.001	0.024 ± 0.001	0.024 ± 0.001	0.010 ± 0.001	0.010 ± 0.001
	Total	0.014	0.027	0.027	0.026	0.026
Lead (mg·L^{-1})	Filtered water	<DL (0.003)	<DL (0.003)	<DL (0.003)	<DL (0.003)	<DL (0.003)
	Suspended solids	0.003 ± 0.001	0.010 ± 0.001	0.010 ± 0.001	0.010 ± 0.001	0.016 ± 0.001
	Total	0.003	0.010	0.010	0.010	0.016
Calcium (mg·L^{-1})	Filtered water	12.72 ± 0.290	35.69 ± 0.17	35.69 ± 0.17	17.35 ± 0.001	16.19 ± 0.406
	Suspended solids	17.72 ± 1.000	18.79 ± 1.000	18.79 ± 1.000	31.10 ± 1.000	32.43 ± 1.000
	Total	30.44	54.48	54.48	48.45	48.62
Silicon (mg·L^{-1})	Filtered water	0.156 ± 0.010	0.643 ± 0.010	0.643 ± 0.010	<DL (0.001)	<DL (0.001)
	Suspended solids	0.471 ± 0.001	0.524 ± 0.001	0.524 ± 0.001	0.491 ± 0.001	0.481 ± 0.001
	Total	0.627	1.167	1.167	0.491	0.481

Table 3. Total calcium, silicon, and potentially toxic elements (PTEs) content of fertilizers, pesticides, and tested products used during both growing seasons, expressed in mg·kg^{-1}. Errors are expressed as a standard deviation. Diethylentriaminopentaacetic acid (DTPA) extractable PTEs content expressed in brackets as a percentage of the total PTEs amount.

Fertilizer commercial name	Element				
	Chromium	Copper	Lead	Calcium	Silicon
Lieta Veg	1.180 ± 0.271 (64.41%)	4.904 ± 1.263 (38.15%)	<DL	425.5 ± 32.45 (n.a.)	2.448 ± 0.052 (n.a.)
Examine L	1.470 ± 0.111 (67.81%)	2.079 ± 0.000 (10.08%)	0.510 ± 0.053 (0.00%)	8545 ± 361.2 (n.a.)	17.74 ± 0.122 (n.a.)
New Ferstim Idro	1.769 ± 0.266 (40.58%)	0.295 ± 0.107 (0.00%)	<DL	472.2 ± 20.53 (n.a.)	34.61 ± 1.660 (n.a.)
Idrocomplex New Blu	1.851 ± 0.012 (92.83%)	<DL	<DL	123.0 ± 19.85 (n.a.)	36.60 ± 0.212 (n.a.)
Pesticide commercial name					
Presidium One	5.45 ± 1.887 (2.31%)	2.639 ± 0.763 (48.16%)	<DL	314.7 ± 98.67 (n.a.)	10,750 ± 2.3000 (n.a.)
Volare	0.474 ± 0.002 (19.31%)	1.428 ± 0.084 (48.16%)	<DL	262.8 ± 86.82 (n.a.)	8351 ± 34.70 (n.a.)
Zoxium	7.57 ± 0.422 (0.86%)	1.343 ± 0.361 (100.0%)	<DL	2212 ± 128.2 (n.a.)	11,433 ± 203.2 (n.a.)
Thiopron	0.427 ± 0.142 (0.00%)	4.996 ± 1.597 (4.00%)	<DL	409.1 ± 2.183 (n.a.)	12,966 ± 800.4 (n.a.)
Poltiglia Disperss	9.33 ± 0.216 (2.39%)	19,099 ± 19.30 (38.66%)	4.283 ± 1.110 (0.00%)	74,722 ± 943.3 (n.a.)	1424 ± 660.4 (n.a.)
Clinoptilolite commercial name					
Clinogold (pellet)	6.879 ± 0.248 (1.72%)	3.846 ± 1.181 (1.76%)	38.62 ± 1.578 (0.00%)	1990 ± 68.05 (n.a.)	31,557 ± 825.8 (n.a.)
Clinogold (fertigation)	10.12 ± 0.432 (0.00%)	8.350 ± 1.209 (3.95%)	20.65 ± 1.520 (16.7%)	1877 ± 8.143 (n.a.)	30,968 ± 965.0 (n.a.)
Rock Powder (foliar action)	13.76 ± 0.000 (0.00%)	10.29 ± 0.000 (14.3%)	30.92 ± 1.050 (13.0%)	1825 ± 180.0 (n.a.)	31,363 ± 762.6 (n.a.)
Tusal (Inoculant)	4.318 ± 0.374 (0.00%)	7.603 ± 0.873 (19.93%)	47.62 ± 0.429 (0.00%)	843.6 ± 1.810 (n.a.)	1054 ± 67.71 (n.a.)
Detection limit	0.001	0.001	0.010	0.010	0.030

n.a. = not available (no official procedure to assess the potentially bioavailable-to-plant fraction is suitable).

Plant sample preparation and trace element analysis. At the end of each trial period, when most melon fruits were considered ripe for the market (i.e., 25 Jul and 24 Aug 2020, for Time 1 and Time 2, respectively), the epigeal portion of one single plant per variety per treatment per growing period (for a total of 30 plants) was collected at the positions shown in Fig. 1. The plants collected had a variation of the epigeal biomass within ± 10%. Nonripe fruit was discarded. Each plant was divided into three subsamples: stem, leaves, and fruit. Plant parts were thoroughly washed with tap water and then rinsed with deionized water. The parts were then dried in a ventilated oven at 60 °C for 72 h and finely ground with a food blender. The variation of the dry mass of all parts for each plant was < 10%. The water content of fruit samples was determined as a ponderal loss.

Trace element analysis was performed for each plant part (stem, leaves, and fruit). The metals content of the plant parts was determined through microwave acid digestion using 3:1 v/v ratio of 65% nitric acid and 30% hydrogen peroxide. After digestion, the solutions were filtered and analyzed by ICP-OES. Analyses were performed in triplicate.

Statistical analysis. Statistical analysis of the data on metals content in the epigeal plant's biomass (stem, leaves, and fruit) was conducted in the R environment (R Core Team 2020). The effects of variety, treatment, and time were assessed using a split-split plot analysis of variance (ANOVA) ($P < 0.05$), followed by an least significant difference (LSD) post hoc test ($P < 0.05$) with Bonferroni adjustment. The differences in metal content in the different epigeal fractions of plants (stem, leaves, fruit) for the two times of transplanting considered were tested with a two-way ANOVA ($P < 0.05$) followed by an LSD post hoc test ($P < 0.05$) with Bonferroni adjustment. The correlation matrix of the metal concentration in plant parts was computed using Pearson's correlation coefficient.

Results

Characterization of soil, irrigation water, fertilizers, pesticides, and treatment products. Characteristics of soil, irrigation water, and all products used for crop production are presented in Tables 1, 2, and 3, respectively.

The soil was silty clay loam, according to the U.S. Department of Agriculture classification system (Soil Survey Staff 1999), with an average alkaline pH ($\mathrm{pH_{H2O}}$ = 8.8; $\mathrm{pH_{CaCl2}}$ = 8.0) that was in line with its total carbonate content (7.8%) (Table 1).

Table 4. Calcium, silicon, and potentially toxic elements (PTEs) input (g·ha^{-1}) to the soil/crop system due to irrigation water, fertilizers, pesticides, and trial products. Errors are expressed as a standard deviation.

Element	Chromium	Copper	Lead	Calcium	Silicon
Time 1					
Irrigation water	12.14 ± 3.552	6.964 ± 3.648	2.908 ± 1.228	16,018 ± 2298	247.4 ± 112.4
Fertilizers	0.226 ± 0.022	0.300 ± 0.036	0.040 ± 0.004	708.4 ± 30.62	3.044 ± 0.056
Pesticides	0.466 ± 0.022	802.4 ± 0.844	0.180 ± 0.044	3164 ± 39.04	704.0 ± 60.46
of which Poltiglia Disperss	*0.392 ± 0.010*	*802.2 ± 0.810*	*0.180 ± 0.046*	*3138 ± 39.60*	*59.80 ± 27.72*
Treatments					
Clinogold Clinoptilolite (pellet)	*1.030 ± 0.040*	*0.580 ± 0.180*	*5.800 ± 0.240*	*298.5 ± 10.20*	*4733 ± 123.8*
Clinogold Clinoptilolite (fertigation)	*0.100 ± 0.040*	*0.080 ± 0.010*	*0.210 ± 0.020*	*18.77 ± 0.080*	*309.6 ± 9.650*
Rock Powder Clinoptilolite (foliar action)	*0.100 ± 0.010*	*0.070 ± 0.010*	*0.220 ± 0.010*	*12.77 ± 1.260*	*219.5 ± 5.330*
Tusal (fertigation)	*0.005 ± 0.001*	*0.008 ± 0.001*	*0.045 ± 0.001*	*0.803 ± 0.003*	*1.003 ± 0.063*
Time 2					
Irrigation water	17.58 ± 1.206	11.63 ± 0.382	5.380 ± 0.956	22,234 ± 414.0	340.0 ± 15.19
Fertilizers	0.266 ± 0.030	0.550 ± 0.088	0.052 ± 0.003	886.8 ± 38.80	2.676 ± 0.010
Pesticides	0.466 ± 0.022	802.4 ± 0.844	0.180 ± 0.044	3164 ± 39.04	704.0 ± 60.46
of which Poltiglia Disperss	*0.392 ± 0.010*	*802.2 ± 0.810*	*0.180 ± 0.046*	*3138 ± 39.60*	*59.80 ± 27.72*
Treatments					
Clinogold Clinoptilolite (pellet)	*1.030 ± 0.040*	*0.580 ± 0.180*	*5.800 ± 0.240*	*298.5 ± 10.20*	*4733 ± 123.8*
Clinogold Clinoptilolite (fertigation)	*0.100 ± 0.040*	*0.080 ± 0.010*	*0.210 ± 0.020*	*18.77 ± 0.080*	*309.6 ± 9.650*
Rock Powder Clinoptilolite (foliar action)	*0.100 ± 0.010*	*0.070 ± 0.010*	*0.220 ± 0.010*	*12.77 ± 1.260*	*219.5 ± 5.330*
Tusal (fertigation)	*0.005 ± 0.001*	*0.008 ± 0.001*	*0.045 ± 0.001*	*0.803 ± 0.003*	*1.003 ± 0.063*

Data in italics report the specific name of products used for the tests and the pesticide of major relevance.

Table 5. Total and diethylentriaminopentaacetic acid (DTPA) extractable input of chromium (Cr), copper (Cu), lead (Pb), calcium (Ca), and silicon (Si) per single treatment (C = control not treated; T = *Trichoderma* inoculation; TFA = *Trichoderma* inoculation and clinoptilolite for foliar action; TFT = *Trichoderma* inoculation and clinoptilolite in fertigation; TP = *Trichoderma* inoculation and clinoptilolite pellet). Bioavailability of Cr, Cu, and Pb was assessed as a DTPA extractable fraction. Ca and Si bioavailability was assessed as a water-soluble fraction.

Treatment		Cr	Cu	Pb	Ca	Si
		Time 1				
C	Total input (g·ha^{-1})	12.57	810.1	2.863	24,154	1,031
	Bioavailable content (g·ha^{-1})	0.168	312.0	0.000	10,368	142.30
	Bioavailable (green) fraction (%)					
T	Total input (g·ha^{-1})	12.57	810.1	2.911	24,155	1,032
	Bioavailable content (g·ha^{-1})	0.546	312.1	0.000	10,369	142.39
	Bioavailable (green) fraction (%)					
TFA	Total input (g·ha^{-1})	12.67	810.2	3.127	24,168	1,252
	Bioavailable content (g·ha^{-1})	0.546	312.1	0.028	10,369	142.4
	Bioavailable (green) fraction (%)					
TFT	Total input (g·ha^{-1})	12.68	810.2	3.117	24,174	1,342
	Bioavailable content (g·ha^{-1})	0.546	312.1	0.030	10,369	142.4
	Bioavailable (green) fraction (%)					
TP	Total input (g·ha^{-1})	13.61	810.7	8.704	24,454	5,765.8
	Bioavailable content (g·ha^{-1})	0.563	312.1	0.000	10,369	142.4
	Bioavailable (green) fraction (%)					
		Time 2				
C	Total input (g·ha^{-1})	17.88	814.6	5.176	26,286	1,046
	Bioavailable content (g·ha^{-1})	0.633	315.04	0.000	10,876	120.7
	Bioavailable (green) fraction (%)					
T	Total input (g·ha^{-1})	17.88	814.6	5.224	26,287	1,047
	Bioavailable content (g·ha^{-1})	0.633	315.0	0.000	10,876	120.7
	Bioavailable (green) fraction (%)					
TFA	Total input (g·ha^{-1})	17.88	814.6	5.440	26,299	1,267
	Bioavailable content (g·ha^{-1})	0.633	315.0	0.030	10,876	120.7
	Bioavailable (green) fraction (%)					
TFT	Total input (g·ha^{-1})	17.88	814.6	5.430	26,305	1,357
	Bioavailable content (g·ha^{-1})	0.633	315.0	0.030	10,876	120.7
	Bioavailable (green) fraction (%)					
TP	Total input (g·ha^{-1})	18.915	815.2	11.017	26,585	5,781
	Bioavailable content (g·ha^{-1})	0.651	315.1	0.000	10,876	120.7
	Bioavailable (green) fraction (%)					

The soil had low salinity (EC = 0.15 ds·m^{-1}) and a well-endowment of TOC and TN (1.43 and 0.23%, respectively), according to quality criteria defined for surrounding soils (ARPAV 2007).

Among PTEs, the average total content of Cr (205.0 ± 2.9 mg·kg^{-1}) was found to exceed the legal threshold of 150 mg·kg^{-1} [Legislative Decree (D. Lgs.) N°46 01/03/2019] (Table 1). Moreover, the total Cu content at sampling positions 1 (114.1 ± 1.4 mg·kg^{-1}) and 3 (99.2 ± 3.9 mg·kg^{-1}) slightly exceeded the threshold of 100 mg·kg^{-1}. The average total content of Pb was safely far from the legal threshold. Nevertheless, Pb was reported in the table because of its peculiar accumulation profile in the melon crop, as is discussed further on.

The potential bioavailability-to-plant content, expressed as a percentage of the total content, was in the order: Cu > Pb >> Cr (11.1%, 7.5%, and 0.01% of the corresponding average total content, respectively, Table 1).

Regarding the characterization of irrigation water samples that were collected monthly from Apr to Aug 2020 (Table 2), the samples were similar for pH (8.0–8.4) and EC (from 0.258 to 0.271 dS·m^{-1}), except for that collected on 15 Apr (pH = 9.5; EC = 0.479 dS·m^{-1}). The total metal amount of irrigation water is given as a sum of the amount contained in the filtered water and the suspended solids contained within. Cr and Pb were found only in the suspended solids of the water samples with concentrations ≤ 0.049 and ≤ 0.016 mg·L^{-1}, respectively. Cu was found in filtered water and suspended solids with total content of ≤ 0.027 mg·L^{-1}. In general, all the water parameters were compliant with international water quality standards for irrigation water (Pescod 1992).

The total content and the bioavailable fraction of PTEs of all the products used for crop production (namely, fertilizers, pesticides, clinoptilolite, and *Trichoderma*-based products) are reported in Table 3. Among the products, Poltiglia Disperss contained the highest amount of Cu (19,099 mg·kg^{-1}), 38.66% of which was assessed as potentially bioavailable-to-plant (DTPA extractable). Appreciable levels of Pb were found in the *Trichoderma*-based product Tusal (47.62 mg·kg^{-1}) and in the three clinoptilolite-based products (20.65–38.62 mg·kg^{-1}) with measurable bioavailable fractions in the formulates for fertigation and foliar action only (16.7% and 13.0% of the total, respectively). Low levels of Cr were found in pesticides (≤ 9.33 mg·kg^{-1}) and in clinoptilolite samples (≤ 13.76 mg·kg^{-1}) with bioavailable percentages low and highly variable among the products.

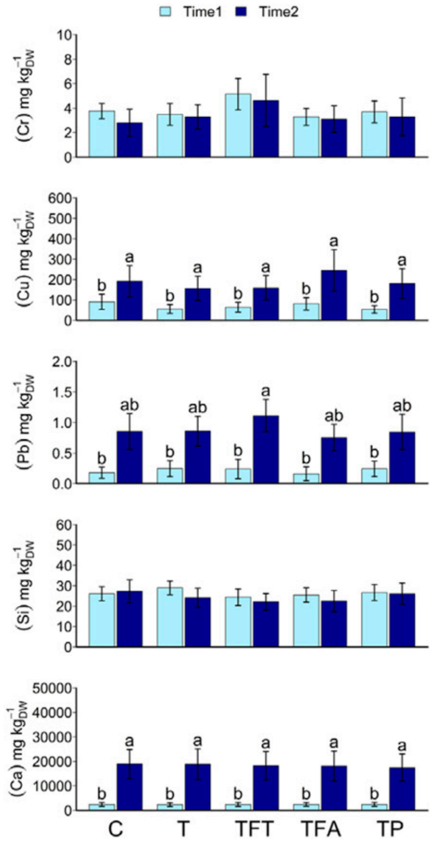

Fig. 2. Marginal means of chromium (Cr), copper (Cu), lead (Pb), silicon (Si), and calcium (Ca) content in melon plants (overall potentially toxic element concentration in aerial biomass), expressed as dry weight (DW) for the tested treatments. Error bars represent the standard errors and different lower-case letters indicate significant differences ($P < 0.05$) between treatment:time interaction as determined by the Fisher's least significant difference test. C = control not treated; T = *Trichoderma* inoculation; TFA = *Trichoderma* inoculation and clinoptilolite for foliar action; TFT = *Trichoderma* inoculation and clinoptilolite in fertigation; TP = *Trichoderma* inoculation and clinoptilolite pellet.

Total and bioavailable PTEs input. The metal input from irrigation water to each crop-soil system corresponding to a given treatment was obtained by multiplying the total metal content of the water collected on the 15th day of every month by the volume of irrigation water distributed to the corresponding field plot during that month.

As detailed in Tables 4 and 5, Cr input at both growing periods was mainly due to irrigation water (12.14–17.58 g·ha^{-1}) and, to a minor extent, to clinoptilolite pellet in TP treatment (1.03 g·ha^{-1}). Total Cr amounts received by different treatments (i.e., irrigation, fertilization, application of pesticides, and treatment products) were limited and in the range of 12.57 to 13.61 g·ha^{-1} at Time 1 and of 17.88 to 18.91 g·ha^{-1} at Time 2. Nevertheless, the bioavailable fraction that entered the soil-crop system was negligible in all treatments with respect to the total amount. Similar observations could be extended to the total input of Pb that was low and in the range of 2.86 to 8.70 g·ha^{-1} at Time 1 and of 5.18 to 11.02 at Time 2 (Table 5). The primary Pb sources were clinoptilolite pellet (TP treatment) and irrigation water (5.80 g·ha^{-1} and in the range 2.91–5.38 g·ha^{-1}, respectively) (Table 4) with no effect on the bioavailable portion (Table 5). On the contrary, for all treatments, the total input of Cu was high (≥ 810.1 g·ha^{-1}) and mainly due to Poltiglia Disperss (802.2 g·ha^{-1}, Supplemental Table 1) with a consistent bioavailable fraction (38.7%, Table 3).

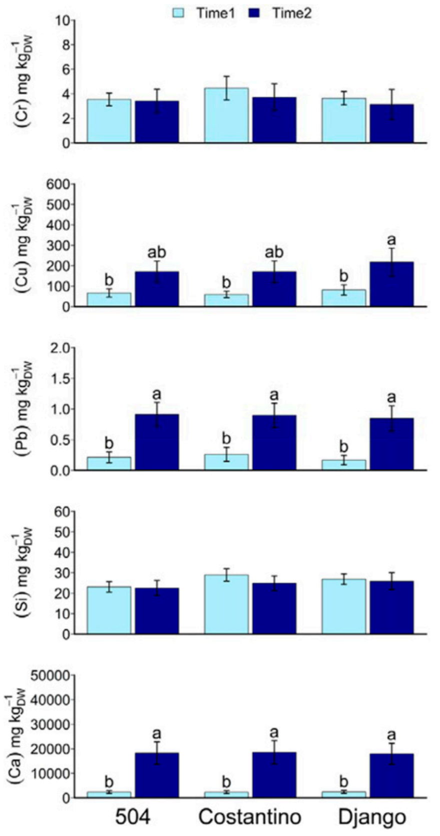

Fig. 3. Marginal means of chromium (Cr), copper (Cu), lead (Pb), silicon (Si), and calcium (Ca) content in melon plants (overall potentially toxic element concentration in aerial biomass), expressed as dry weight (DW), for variety (namely, '504', 'Costantino', and 'Django'). Error bars represent standard errors. Different lower-case letters indicate significant differences ($P < 0.05$) between crop variety:time interaction as determined by the Fisher's LSD test.

Effect of variety, treatments, and seasonality on PTEs uptake by crop. Figure 2 shows the average Cr, Cu, and Pb contents in the aerial part of plant biomass (stem, leaves, and fruit) for each treatment in both growing periods. Plant Ca content was reported as a proxy for verifying a differential transpiration rate (Marschner 2011; McLaughlin and Wimmer 1999; White and Broadley 2003) under different growing seasons. Si content was reported as well due to the claim reported in De Smedt et al. (2015) that some clinoptilolite-based products, foliar action especially, can create a silica-film coating to protect the leaves from external pathogens.

Cr levels in plants did not show statistically different concentrations either among treatments or between growing periods (Fig. 2). For Cu, Pb, and Ca, no differences among treatments were observed for plants within the same growing period. In contrast, statistically significant differences resulted between Time 1 and Time 2. Cu, Pb, and Ca uptake resulted, on average, approximately three (69.25–187.0 mg·kg^{-1}), four (0.214–0.885 mg·kg^{-1}), and seven (2384–18,254 mg·kg^{-1}), respectively, times higher at Time 2 with respect to Time 1 (Fig. 2).

In Fig. 3, the marginal means of the metal uptake of each cultivar (namely, 504, Costantino, and Django) in the two growing periods were reported. The three cultivars did not uptake statistically different amounts of Cr and Si, neither within the same growing period nor between the two growing periods. Moreover, the Cu, Pb, and Ca uptake of the cultivars was not statistically different within the same growing period.

The trend in metals uptake by melon plants shown in Figs. 2 and 3 highlighted the lack of significant variability among both treatments and melon cultivars.

Figure 4 shows the marginal means of metal content in the stem, leaves, and fruit of plants grown at Time 1 and Time 2. Statistically significant differences in Cr, Cu, and Pb accumulation among the plant parts were observed. The higher plant uptake of Pb and Cu at Time 2 with respect to Time 1 seems to affect leaves and stem (Pb) mostly or leaves only (Cu). The fruit was the least accumulating plant part for Cu and Pb. In fruit, Cu content did not seem to be affected by the seasonality of the growing period, whereas Pb content seemed to be only partially affected (Fig. 4). On a dry weight (DW) basis, the increase of Cu average concentration in fruit at Time 2 with respect to Time 1 resulted moderate (15.83 and 13.68 mg·kg^{-1} DW, respectively) whereas the increase of Pb, on average, was more consistent (0.224 and 0.064 mg·kg^{-1} DW, respectively).

As big differences in Cu, Pb, and Ca concentration were observed from Time 1 and Time 2 in plant parts, the Pearson correlation (r) matrix for the metals in the plant parts was calculated (Table 6). As a result, positive correlations were observed both between Ca and Cu or Pb in leaves ($r = 0.84$ and 0.76, respectively) and stem ($r = 0.89$ and 0.74, respectively), thus supporting the figure that absorption and translocation of Pb and Cu are correlated with Ca uptake.

As far as the quality of melon fruit was concerned, the effect of variety and growing seasonality on Cu and Pb content of fruit was reconsidered on a fresh weight basis. Cu concentration in fresh fruit ranged from 1.88 mg·kg^{-1} in 'Django' to 2.22 mg·kg^{-1} in 'Costantino'. Despite a slight tendency, no significant effect of variety on Cu concentration was assessed. Concerning Pb, its concentration in fresh fruit spaced from a value of 0.01 mg·kg^{-1} in 'Costantino' to 0.03 mg·kg^{-1} in 'Django'.

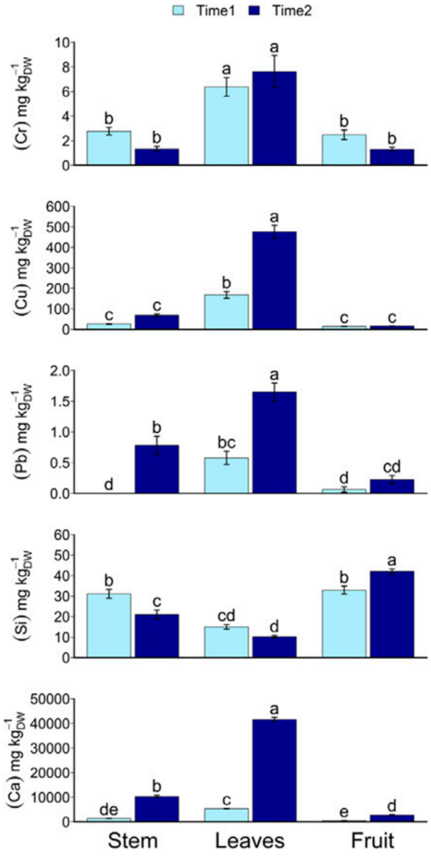

Fig. 4. Marginal means of chromium (Cr), copper (Cu), lead (Pb), silicon (Si), and calcium (Ca) content of plant fractions (stem, leaves, and fruit) expressed as dry weight (DW). Error bars represent standard errors. Different lower-case letters indicate significant differences ($P < 0.05$) between plant fractions:time interaction as determined by the Fisher's least significant difference test.

Again, no significant effect of the variety, as well as of variety:time interaction, was found under the two-way ANOVA. The only significant factor was the growing period. Cu and Pb concentration in fresh fruit cultivated at Time 2 increased by 25% ($P < 0.001$) and 312% ($P = 0.044$), respectively, with respect to Time 1.

In Table 7, the marginal means of Cu and Pb concentration in the fresh fruit are reported. In all cases, the fruit was safely edible for the EU market according to the current maximum concentration of Pb and Cu permitted in melon fruit of 0.1 and 5.0 $mg \cdot kg^{-1}$ fresh weight, respectively (European Commission, 2006, 2008).

Discussion

PTEs pool in soil. In the studied plot, soil Cr and Cu measured content was slightly higher than legal thresholds (Table 1). Nevertheless, this farmland is still suitable for food production, as Italian regulation allows cultivation of areas whose natural background PTE levels are slightly higher than the thresholds. In fact, Cr and Cu background values for Viadana municipality, as reported in ARPAE (2020) ($151\ mg \cdot kg^{-1} < Cr < 225\ mg \cdot kg^{-1}$; $61\ mg \cdot kg^{-1} < Cu < 120\ mg \cdot kg^{-1}$), exceed the legal thresholds. Such Cr and Cu levels are rather typical in Po alluvial valley, especially in the immediate vicinity of Po river, as in this case. Indeed, contamination levels were mild, and of no serious concern from an environmental safety standpoint.

When potentially available-to-plants soil PTEs fraction was considered, Cu and Pb showed a considerably higher availability with respect to Cr (Table 1). These figures were expected in that, generally, in alkaline soils the bioavailability of Cu and Pb is mainly due to their forms as carbonates or complexed by the negatively charged moieties of soil organic matter (Alloway 2012; Shahid et al. 2012). In these forms, PTEs are suitable to be complexed by plant-derived carboxylates and then to be absorbed by roots. On the contrary, the unavailability of Cr(III) for plants is mainly due to its ability to form stable and insoluble species in soils at pH values > 5.5 (Alloway 2012; Shahid et al. 2017).

Incidence of PTEs input due to irrigation, fertilization, and pest control. The physical and chemical characteristics of the monthly sampled irrigation water was rather homogeneous, with the sole exception of the sample collected on 15 Apr (Table 2). The sample showed the highest pH value and EC with respect to the others. Such a high pH could be reasonably explained by larger input to the water canal of ammonia coming from surrounding cropped fields owing to the use of animal-based fertilizers/amendments locally produced by intensive animal farming, which is very extended in the area. The interruption of animal-based fertilizer distribution in later periods, and in summer, brought the pH to more neutral values in the water samples that were subsequently collected (pH $\leq$ 8.4). Moreover, the high value of suspended solids in water lastly sampled (15 Jul and 15 Aug) could be reasonably explained by the drier conditions of the summer period. Despite the larger amount (+56%) of rainfall at Time 2 with respect to Time 1, in the last 2 months of Time 2 only a few rainy days and four heavy rainfalls occurred (Supplemental Fig. 1). The drier summer conditions imposed an irrigation volume for the crop at Time 2 larger (+14%) than that used at Time 1 (218 and 192 m^3, respectively).

Considering the overall PTEs total input to the soil-plant system due to all the agricultural practices, Cu input resulted homogeneously distributed among treatments because of the same primary input (copper salts). On the other hand, Cr and Pb input slightly varied among treatments within the same growing periods and increased from the first to the second growing period mainly because of the different volumes and composition of irrigation water.

Nevertheless, each element input was negligible compared with the soil pool. Therefore, it is reasonable to assume that such slight variations of PTEs input unlikely influenced metal assimilation.

Effect of seasonality on PTEs translocation to plants. Likely, Cr uptake by plants produced under different treatments and growing seasons (Fig. 2) was flattened by its unavailability either in soil (Table 1) and/or in its input to the soil-crop system through irrigation water and products for crop production (Table 5). Similarly, plant Si content did not statistically differ among treatments and between the growing periods, thus ruling out any detectable accumulation in plant trials treated with clinoptilolite-based products (namely, pellet, fertigation product, and foliar action product in TP, TFT, and TFA treatments, respectively).

For Ca, Cu, and Pb, their considerably higher average uptake assessed during Time 2 with respect to Time 1 was not supported by a proportional increase of their total and potentially bioavailable input to the soil-crop system through irrigation water and products for crop production at Time 2 (Table 5). More likely, the higher uptake was due to a higher nutrient flux through the soil-root-shoot system following the climate variation between the two growing periods (Supplemental Fig. 1), that is, a higher transpiration rate. In the plants, Ca content was within the

Table 6. Pearson's correlation coefficients matrix of copper (Cu), lead (Pb), and calcium (Ca) content of fruit, leaves, and stem.

	Cu in fruit	Cu in leaves	Cu in stem	Cr in fruit	Cr in leaves	Cr in stem	Pb in fruit	Pb in leaves	Pb in stem	Ca in fruit	Ca in leaves	Ca in stem
Cu in fruit	1											
Cu in leaves	0.46^{i}	1										
Cu in stem	0.38^{i}	0.84^{ii}	1									
Cr in fruit	0.07	−0.31	−0.35	1								
Cr in leaves	0.3	−0.03	−0.07	−0.09	1							
Cr in stem	−0.04	-0.59^{ii}	-0.53^{ii}	0.63^{ii}	0.29	1						
Pb in fruit	0.25	0.43^{i}	0.42^{i}	0	0.32	0.03	1					
Pb in leaves	0.34	0.47^{ii}	0.47^{ii}	−0.3	0.59^{ii}	−0.23	0.26	1				
Pb in stem	0.5^{ii}	0.53^{ii}	0.58^{ii}	−0.28	0.45^{i}	−0.24	0.25	0.63^{ii}	1			
Ca in fruit	0.46^{ii}	0.84^{ii}	0.76^{ii}	-0.44^{i}	0.13	-0.59^{ii}	0.28	0.72^{ii}	0.7^{ii}	1		
Ca in leaves	0.45^{i}	0.84^{ii}	0.78^{ii}	-0.46^{i}	0.21	-0.55^{ii}	0.34	0.76^{ii}	0.75^{ii}	0.97^{ii}	1	
Ca in stem	0.4^{i}	0.83^{ii}	0.89^{ii}	-0.43^{i}	0.14	-0.52^{ii}	0.44^{i}	0.7^{ii}	0.74^{ii}	0.92^{ii}	0.95^{ii}	1

[i] Significant at $P < 0.05$.

[ii] Significant at $P < 0.01$.

Table 7. Marginal means of copper (Cu) and lead (Pb) content in the fresh fruit ($mg\cdot kg^{-1}$ fresh weight). Different lower-case letters indicate significant differences ($P < 0.05$) as determined by the Fisher's least significant difference test.

	Cu	Pb
Variety		
Costantino	2.22	0.010
Django	1.88	0.027
504	1.91	0.025
P	0.044	0.462
Time		
Time 1	1.77 b	0.008 b
Time 2	2.23 a	0.033 a
P	<0.001	0.044
Variety × Time		
P	0.738	0.959

typical range of 1 to 50 $mg\cdot g^{-1}$ DW (Kirkby and Pilbeam 1984) for higher vascular plants.

Largely, Ca is transported within roots through water mass flux (Kirkby and Pilbeam 1984). Absorption is mediated by Ca nonselective cation channels (Ca-NSCCs), specialized transmembrane pores permeable to different cations, which are activated/deactivated by ion flux or different stimuli (e.g., environmental stress) (Demidchik et al. 2002). These channels are involved in several defense processes against heat stresses (Naeem et al. 2020) and act as a heat sensor that causes hyperaccumulation of Ca and other compatible solutes. Moreover, Ca signal transduction has a role in leaf surface temperature regulation by regulating stomatal conductance (Demidchik and Maathuis 2007). Several studies indicated Ca-NSCCSs as a possible pathway for heavy metals uptake (Clemens 2006; Demidchik et al. 2018; Gallego et al. 2012; Pourrut et al. 2008; Robinson et al. 2009; Sanz et al. 2019; Wang et al. 2007) owing to the significant permeability for both mono and divalent cations (Demidchik and Maathuis 2007). Moreover, positive correlation between transpiration flux increase and Cu and Zn uptake is commonly found (Tani and Barrington 2005a, 2005b).

In our plants, most of the considered metals accumulated in leaves, except for Si. As defined by several studies on plant metal uptake and translocation mechanisms, the vacuole of leaf cells represents the main storage site for both excess nutrients and PTEs (Krämer 2010; Pilon-Smits 2005; Sharma et al. 2016). The metals translocation to above-ground tissues plays an ecological role as a detoxification and a herbivores defense strategy (Boyd 2007; Martinoia et al. 2012; Rascio and Navari-Izzo 2011; Sharma et al. 2016).

Because of these observations, it was reasonable to suppose that, in our study, the increase in Cu, Pb, and Ca uptake during the second growing season could depend mainly on the signal activation of Ca channels in response to the higher evapotranspiration rate typical of the summer growing period and the higher (+14%) irrigation volume used.

Conclusions

The field study was conducted on calcareous agricultural soil whose Cr and Cu contents were lower than the background levels but higher than legal threshold limits: a typical figure of intensively cropped soils that have been regularly fertilized, amended with composts, and treated with copper salts.

Under integrated pest management, Cr uptake by *Cucumis melo* L. was unaffected by seasonality, early- or late-ripening varieties, and *Trichoderma* inoculation alone or combined with pellet, foliar action, and fertigation clinoptilolite-based treatments, thus confirming its well-known low availability to plants.

On the contrary, the leaf translocation of Cu was significantly limited by the early cultivation period and, thus, by early-ripening varieties. A similar figure was observed with Pb, although its soil content was abundantly lower than the Italian threshold concentration. This result is of particular interest in reducing the overall uptake of Cu and Pb, whose presence in food is regulated within the European Union, in case the melon crop is cultivated in soils with sensible levels of these PTEs. The strong correlation between Ca uptake, used as a proxy of transpiration rate, and Cu and Pb accumulation, that affected leaves mainly, may indicate the involvement of Ca nonselective cation channels as a possible main entry for the PTEs in the epigeal biomass.

Concerning the market quality of fresh fruit, the lowest Cu concentration was found in the early-ripening 'Django'. Tendentially, lower average Pb concentration in fruit was found in the early cultivation season, but no significant effects of variety and growing period were observed.

These results indicate that a possible strategy to mitigate Cu and Pb uptake by melon plants, as well as Cu and Pb concentration of fresh fruit, can be achieved by anticipating the growing period of melons. Under field conditions, the cultivation of early-ripening cultivars had a significant impact on reducing the metal translocation and the Cu concentration in the fruit.

To the scope of PTEs risk management, the results of the present study support the strategies aimed to use early-ripening cultivars in place of late-ripening ones in case a significant PTEs contamination is expected (i.e., soils with critical PTEs levels). Moreover, as most Cu and Pb are accumulated in leave tissues, plowing of crop residues should be limited as much as possible. More generally, manage Cu-based pesticides to avoid long-term accumulation in soil.

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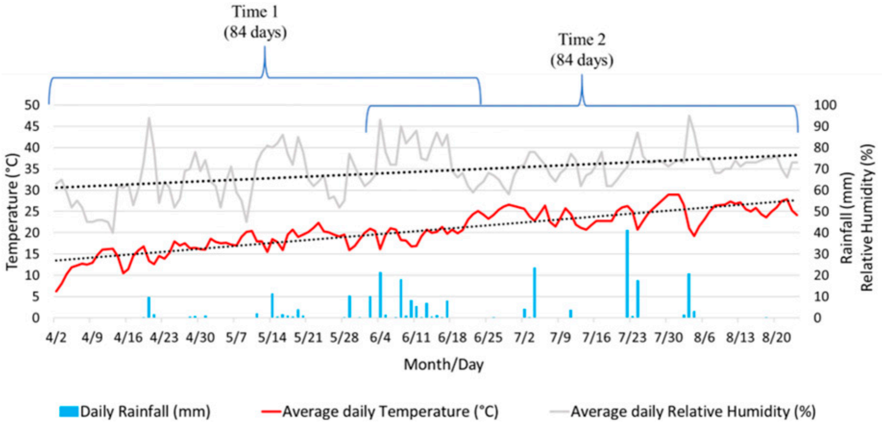

Supplemental Fig. 1. Graph of average temperature, average relative humidity, and daily rainfall from 2 Apr to 24 Aug 2020. The duration of the two growing periods (Time 1 and 2) is indicated.

Supplemental Table 1. Fertilizers, pesticides, and products used for treatments.

Products used in all five treatments			
		Dosage ($kg·ha^{-1}$ or $L·ha^{-1}$)	
Trade name	Composition or active ingredient	Time 2	Time 1
Fertilizers			
Lieta Veg, Agriges	N = 2.5%	20	70
Examine L®, K&A	N = 3%	80	100
New Ferstim Idro, Scam	NPK = 6–12–0	24	—
Idrocomplex New Blu, Scam	NPK = 20–20–20	20	20
Pesticides			
Volare® Bayer	fluopicolide + propamocarb (62.5 + 625 $g·L^{-1}$)	3.2	3.2
Presidium One®, Gowan Italia	zoxamide + dimethomorph (180 + 180 $g·L^{-1}$)	6	6
Zoxium® 240 SC, Gowan Italia	zoxamide (240 $g·L^{-1}$)	3	3
Poltiglia Disperss®, UPL Italia	copper sulfate (Cu = 20%)	42	42
Thiopron®, UPL Italia	sulfur (825 $g·L^{-1}$)	40	0
Treatment specific products			
Product name (description) *treatment*	Composition	Dosage ($kg·ha^{-1}$or $L·ha^{-1}$)	
		Time 1	Time 1
Tusal® (Liquid for fertigation), *T, TFA, TFT, TP*	*Trichoderma atroviride* T11 + *Trichoderma asperellum* T25 (0.5 + 0.5%)	1	1
Clinogold, BHYP® (pellet - soil conditioner) *TP*	Clinoptilolite (95%)	150	150
Clinogold, BHYP® (powder for fertigation) *TFT*	Clinoptilolite (95%)	10	10
Rock powder, Midori® (powder for foliar action) *TFA*	Clinoptilolite (83% to 94%)	8	8

T = inoculation with *Trichoderma;* TFA = inoculation with *Trichoderma* and foliar application of powdered clinoptilolite ("foliar action"); TFT = inoculation with *Trichoderma* and application of powdered clinoptilolite via fertigation; TP = inoculation with *Trichoderma* and soil application of clinoptilolite pellets.