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Tesis Doctoral

Combination of gentle remediation options to clean up soils simultaneously contaminated with organic and inorganic pollutants

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A mi madre

RESUMEN

La contaminación del suelo es un problema ampliamente extendido que afecta negativamente a la salud del ecosistema edáfico y, por ende, al suministro de servicios ecosistémicos. La presencia simultánea de contaminantes orgánicos e inorgánicos es una situación frecuente que agrava los efectos tóxicos de la citada contaminación y dificulta la remediación de emplazamientos contaminados.

En esta tesis, para recuperar la salud de suelos degradados por contaminación mixta, se ha evaluado la efectividad, de forma individual y combinada, de diferentes opciones de remediación biológica: (i) la **bioestimulación** mediante la aplicación de un material orgánico bioestabilizado procedente del reciclado de residuos sólidos urbanos; (ii) la **fitorremediación** mediante el cultivo de colza (*Brassica napus*); (iii) la **vermirremediación** mediante la inoculación de lombrices pertenecientes a la especie *Eisenia fetida*; y (iv) la **bioaumentación** mediante la inoculación de un consorcio de actinobacterias. Asimismo, se ha evaluado la eficacia de la **nanorremediación** a través de la aplicación de nanopartículas de hierro cero valente. Respecto a los contaminantes objeto de estudio, se ha evaluado la eficacia de las citadas técnicas para remediar un suelo contaminado con elementos traza (Zn, Cu, Cd) y gasóleo, y otro contaminado con Cr(VI) y lindano. La recuperación de la salud de suelo se midió mediante la determinación de una variedad de parámetros fisicoquímicos y biológicos con potencial indicador.

Nuestros resultados apuntan a que la inertización de los elementos traza es un primer paso esencial en la recuperación con suelos con contaminación mixta. Esta inertización puede lograrse mediante la modificación de las propiedades del suelo como consecuencia de la aplicación de una enmienda orgánica (*e.g.*, material orgánico bioestabilizado). La nanorremediación con nanopartículas de hierro cero valente, a pesar de su efectividad en la inmovilización de elementos traza como el Cr(VI), encuentra obstáculos en su aplicación como se deriva de su rápida inactivación, baja movilidad y/o toxicidad en presencia de materia orgánica. Por otra parte, el cultivo de *Brassica napus*, asistido por enmienda orgánica, posibilita la fitogestión de suelos con contaminación mixta, promoviendo la recuperación de la salud de suelo al tiempo que se obtienen beneficios socioeconómicos, por lo que se recomienda para los suelos con contaminación mixta. La efectividad de este enfoque puede incrementarse mediante su combinación con otras estrategias biológicas como la vermirremedación o la bioaumentación.

LABURPENA

Lurzoruaren kutsadura oso arazo zabaldua da, ekosistema edafikoaren osasuna kaltetzen duena, eta, beraz, zerbitzu ekosistemikoen horniketa. Kutsatzaile organiko eta inorganikoen aldibereko agerpena maizko egoera da, kutsaduraren efektu toxikoak larriagotzen dituena, eta kutsatutako inguruen erremediazioa eragozten duena.

Tesi honetan, kutsadura mistoarekin andeatutako lurzoruen osasuna berreskuratzeko asmoarekin, indibidualki eta batera aztertu da hainbat erremediazio biologikoko aukeraren eraginkortasuna: (i) **bioestimulazioa** bioegonkortutako material organiko baten aplikazioaren bidez, hiri hondakin solidoen birziklapenetik lortutakoa; (ii) **fitoerremediazioa** koltza (*Brassica napus*) labore baten bidez; (iii) **bermierremediazioa** *Eisenia fetida* espezieko zizareen inokulazioaren bidez; eta (iv) **bioaumentazioa** aktinobakterio kontsortzio baten inokulazioaren bidez. Halaber, zero balentziako burdin nanopartikulen aplikazioaren bidez **nanoerremedioazioaren** eraginkortasuna aztertu da. Ikertutako kutsatzaileei dagokienez, aipatutako tekniken eraginkortasuna ebaluatu da erremediatzeko, alde batetik, aztarna elementuekin eta gasolioarekin kutsatutako lurzoru bat, eta bestetik, Cr(VI) eta lindanorekin kutsatutako beste bat. Lurzoru osasunaren berreskurapena ahalmen adierazleko hainbat parametro fisikokimiko eta biologikoren bitartez neurtu zen.

Gure emaitzek erakusten dute aztarna elementuen inertizazioa ezinbesteko lehenengo pausu bat dela kutsadura mistoa duten lurzoruak berreskuratzeko. Inertizazio hau lortu daiteke lurzoruaren propietateen aldaketaren bidez, medeapen organiko baten aplikazioaren ondorioz (adibidez bioegonkortutako material organikoa). Nanoerremediazioa zero balentziako burdin nanopartikulak erabiltzen, nahiz eta Cr(VI) bezalako aztarna elementuen immobilizaziorako eraginkorra izan, oztopoak topatzen ditu bere aplikazioan, inaktibazio azkarra, mugikortasun txikia edo/eta materia organikoaren presentzian toxikotasuna dela eta. Bestalde, *Brassica napus* laboreak, medeapen organikoaren laguntzarekin, kutsadura mistoa duten lurzoruak fitogestionatzeko aukera ematen du, aldi berean lurzoruaren osasunaren berreskurapena sustatzen eta onura sozioekonomikoak eskuratzen. Horregatik, kutsadura mistoa duten lurzoruentzat gomendatzen da. Hurbiltze honen eraginkortasuna handitu egin daiteke beste estrategia biologikoekin konbinatuz, besteak beste bermierremediazioa edo bioaumentazioa.

SUMMARY

Soil contamination is a widespread issue that negatively affects the health of the edaphic ecosystem and, consequently, the ecosystem service supply. The simultaneous presence of organic and inorganic contaminants is a frequent situation that aggravates the toxic effects of the aforementioned contamination and hinders the remediation of polluted sites.

In this thesis, to recover soil health of soils degraded by mixed contamination, the individual and combined effectiveness of different biologic remediation options was assessed: (i) **biostimulation** through the application of an organic bio-stabilized material coming from the recycling of solid urban wastes; (ii) **phytoremediation** through the cultivation of rapeseed (*Brassica napus*); (iii) **vermiremediation** through the inoculation of *Eisenia fetida* earthworms; and (iv) **bioaugmentation** through the inoculation of an actinobacteria consortium. In addition, the effectiveness of **nanoremediation** through the application of zero valent iron nanoparticles was assessed. Regarding the studied contaminants, the effectiveness of the previously mentioned technologies was assessed for the remediation of a soil polluted with a mixture of trace elements (Zn, Cu, Cd) and diesel, and another mixture of Cr(VI) and lindane. Soil health recovery was measured by the determination of various physico-chemical and biological parameters with indicator potential.

Our results indicate that the inertization of trace elements is an essential first step for soil health recovery of soils with mixed contamination. This inertization can be achieved by changing soil properties as a consequence of the application of an organic amendment (*e.g.*, organic bio-stabilized material). Nanoremediation with zero valent iron nanoparticles, despite its effectiveness for the immobilization of trace elements like Cr(VI), finds obstacles for its application, as a consequence of their rapid inactivation, low mobility and/or toxicity in presence of organic matter. Conversely, the cultivation of *Brassica napus*, assisted by an organic amendment, makes possible the phytomanagement of soils with mixed contamination, promoting soil health recovery while obtaining socioeconomic benefits, thus it is recommended for soils with mixed contamination. The effectiveness of this approach can be enhanced by its combination with other biologic strategies such as vermiremediation or bioaugmentation.

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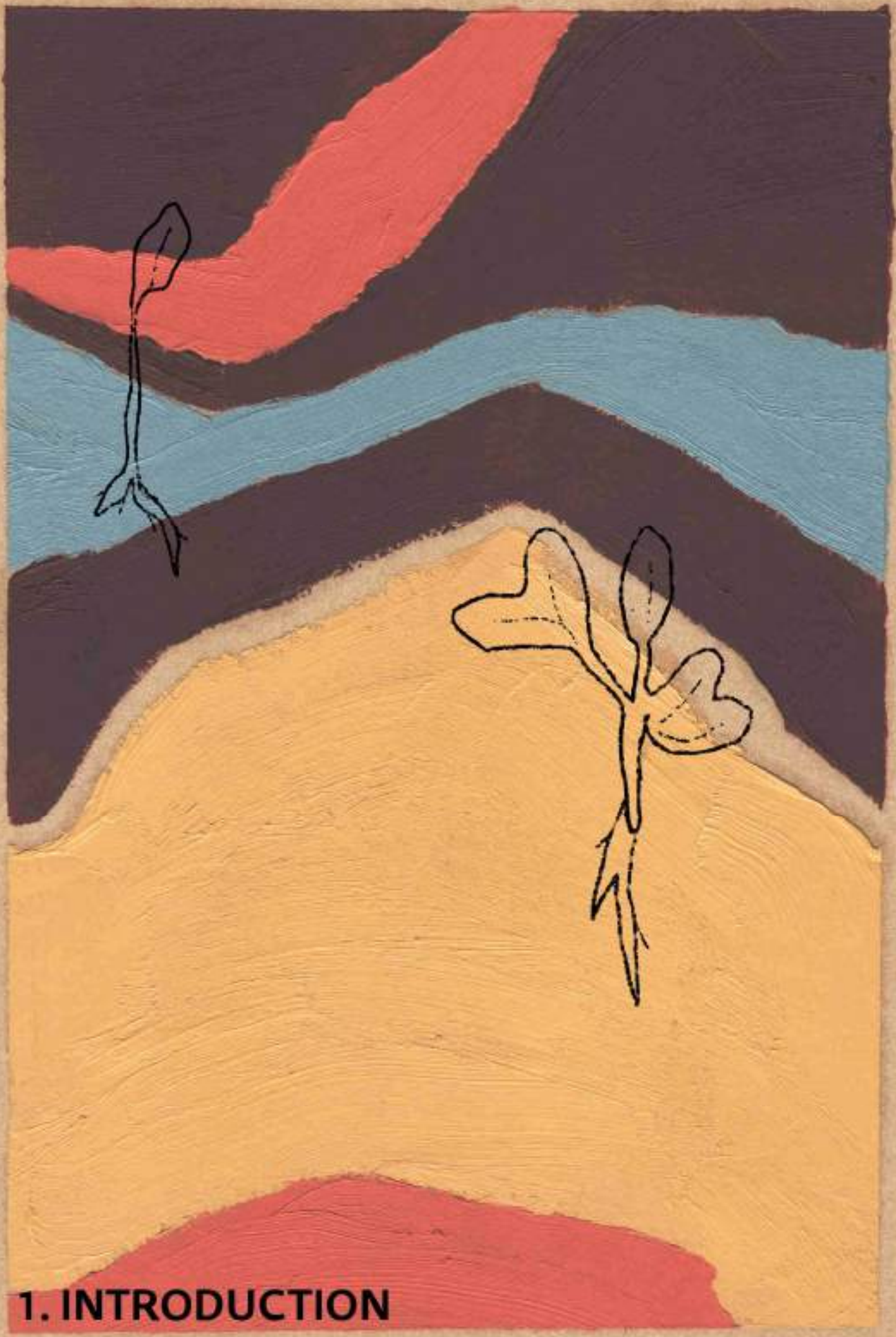


1. INTRODUCTION

1. INTRODUCTION

Rafael G. Lacalle, José M. Becerril, Carlos Garbisu, 2020. Biological methods of polluted soil remediation for an effective economically-optimal recovery of soil health and ecosystem services. **Journal of Environmental Science and Public Health**, 4: 112-133.

Abstract

Soil is one of our most important resources as it supports many critical ecological functions and ecosystem services. Nonetheless, due to a wide variety of environmentally-unsustainable anthropic activities, sadly, our soils are currently contaminated at a global scale with a myriad of potentially toxic inorganic and organic compounds. Regrettably, most, if not all, traditional physicochemical methods of soil remediation are frequently based on economically-infeasible and/or environmentally-destructive techniques. In consequence, in the last years and decades, more sustainable and innovative biological methods of soil remediation (belonging to the sometimes called "gentle remediation options") are being developed in an attempt to combine: (i) an efficient removal of soil contaminants (in terms of a decrease of total and/or bioavailable contaminant concentrations), (ii) a reduction of soil ecotoxicity, (iii) the legally- and ethically-required minimization of risk for environmental and human health, and, concomitantly, (iv) a recovery of soil health and (v) associated ecosystem services. Ideally, any soil remediation method should not only decrease the concentration of soil contaminants below regulatory limits but should also recover soil health and alongside the provision of essential ecosystem services. Unquestionably, all this must be achieved in full compliance with the binding environmental regulations and, most importantly, via the implementation of economically-feasible (preferably, profitable) strategies of soil remediation.

1.1. Soil contamination

The soil is both a highly complex ecosystem and a non-renewable resource on a human time scale, which harbors a range of physical, chemical and biological processes supporting key functions and essential ecosystem services. Likewise, soil is a dynamic living system that serves as habitat for a myriad of organisms (micro-, meso- and macrofauna) with essential roles in nutrient cycling and the mineralization of organic matter (Brevik et al., 2015). Lamentably, different anthropic activities are responsible for the current state of soil degradation through erosion, compaction, contamination, sealing, salinization and loss of organic matter and biodiversity (Epelde et al., 2009b). Soil contamination, in particular, is nowadays a serious environmental threat and challenge worldwide. In Europe, the existence of 2.5 million potentially-contaminated sites has been estimated (Van Liedekerke et al., 2014). In these sites, the most common environmental contaminants are metal(oid)s and mineral oils, affecting 35 and 24% of European contaminated soils, respectively. Chlorinated hydrocarbons appear to a lesser extent (8%) but still are a most relevant issue (Van Liedekerke et al., 2014). Frequently, contaminated sites are characterized by the simultaneous presence of different contaminants (Khan and Kathi, 2014; Mansour, 2012), thus potentially increasing their toxicity and environmental impact, and hampering the application of soil remediation technologies (Agnello et al., 2016). Soil metal(oid) contamination often results from agricultural, mining and metallurgical activities, while accidental spills and/or industrial activities are recurrently the source of soil organic contaminants. Furthermore, waste discharge and waste treatment processes are a major source of both types of soil contaminants. The U.S Environmental Protection Agency (USEPA) reported that 40% of the hazardous waste sites are contaminated with both organic and metal(oid) contaminants (USGAO, 2010).

1.2. Soil health

Soil contamination, along with other degradation processes, can negatively affect soil health (Gómez-Sagasti et al., 2012), often defined as “the capacity of a given soil to perform its functions as a living system capable of sustaining biological productivity, promoting environmental quality and maintaining plant and animal health” (Doran and Zeiss, 2000). But soil is a vastly complex environmental matrix which performs numerous, sometimes conflicting, functions from both an ecocentric and anthropocentric

perspective, and, in consequence, many different aspects must be taken into consideration in order to properly assess soil health. Most importantly, to appropriately assess soil health: (i) physical, chemical and biological properties with potential as indicators of soil functioning must always be included in the assessment (after all, physical, chemical and biological processes in the soil ecosystem are not independent but interactive processes); (ii) chemical, (eco)toxicological and ecological approaches must be incorporated to the evaluation; (iii) the intended use for the contaminated site must be taken into close consideration, as the very concept of soil health is somewhat teleological and subjective; (iv) the intrinsic temporal and spatial variability of the system (i.e., spatial heterogeneity, temporal dynamics), as well as the scale of both soil processes and the assessment itself, must be taken into account; and (v) the selection of a suitable (inevitably, often far from perfect) "healthy" reference soil, for comparison and the establishment of target purposes, should be identified.

Soil physicochemical properties such as pH, redox potential, organic matter content, texture, etc., are relevant parameters with potential as indicators of soil health which can strongly alter contaminant bioavailability and, hence, (eco)toxicity in soil. Unfortunately, for most environmental legislations, the total concentration of the contaminants is the key factor for the Environmental Risk Assessment (ERA) of contaminated soils. Nevertheless, such aspect (i.e., total concentration of soil contaminants) is not enough to properly assess or estimate the potential harmful impact of contaminants on soil functioning (Alvarenga et al., 2018). As a matter of fact, the mobility and bioavailability of soil contaminants both play a determining role in their uptake by organisms and, therefore, their (eco)toxicity (Megharaj et al., 2011; Vamerali et al., 2010). Contaminant bioavailability is possibly a much more relevant factor, compared to total contaminant concentrations, for a proper soil protection and risk assessment, as it represents the fraction that can be taken up by soil organisms and/or be leached to other environmental compartments. Specifically, metal(oild) bioavailability is mainly conditioned by soil physicochemical properties such as pH, redox potential, moisture content, organic matter content, clay content, the presence of anionic compounds, etc. (Vangronsveld and Cunningham, 1998). Regarding organic contaminants, their bioavailability and mobility depend largely on their solubility, hydrophobicity and interaction, through a variety of physicochemical processes, with the mineral and organic fraction of the soil matrix, e.g. via sorption and complexation

mechanisms (Megharaj et al., 2011). Therefore, it is recommended to always include the determination of the bioavailable fraction of the contaminants when assessing soil health and, in particular, during the selection of a soil remediation option and when monitoring the effectiveness of the chosen remediation methodology. Nonetheless, regrettably, there is no consensus about the best way to accurately estimate soil contaminant bioavailability. For metallic contaminants, the most widely accepted methodology is the use of chemical extractants like, for instance, inorganic salts, e.g. $NaNO_3$, $(NH_4)_2SO_4$ and $CaCl_2$ (Madejón et al., 2006; Menzies et al., 2007; Vázquez et al., 2008).

In any event, for a proper assessment of the impact of soil contaminants on soil health (Fig. 1.1), apart from total and bioavailable contaminant concentrations, biological indicators are required, as they directly reflect the impact of the contaminants on the soil biota (Alvarenga et al., 2018). Among them, soil microbial properties are particularly adequate for this purpose, as microorganisms play a key role in many soil functions and the provision of ecosystem services, while quickly delivering ecologically relevant information that integrates many environmental factors (Epelde et al., 2009a; Jeffery et al., 2010). Similarly, standardized (eco)toxicological bioassays with model organisms have been developed and proposed for soil (eco)toxicity studies, including, for instance, *Eisenia fetida* (Irizar et al., 2015b), *Vibrio fisheri* (Abbas et al., 2018), *Lactuca sativa* (Valerio et al., 2007) and *Cucumis sativus* (Lacalle et al., 2018a).

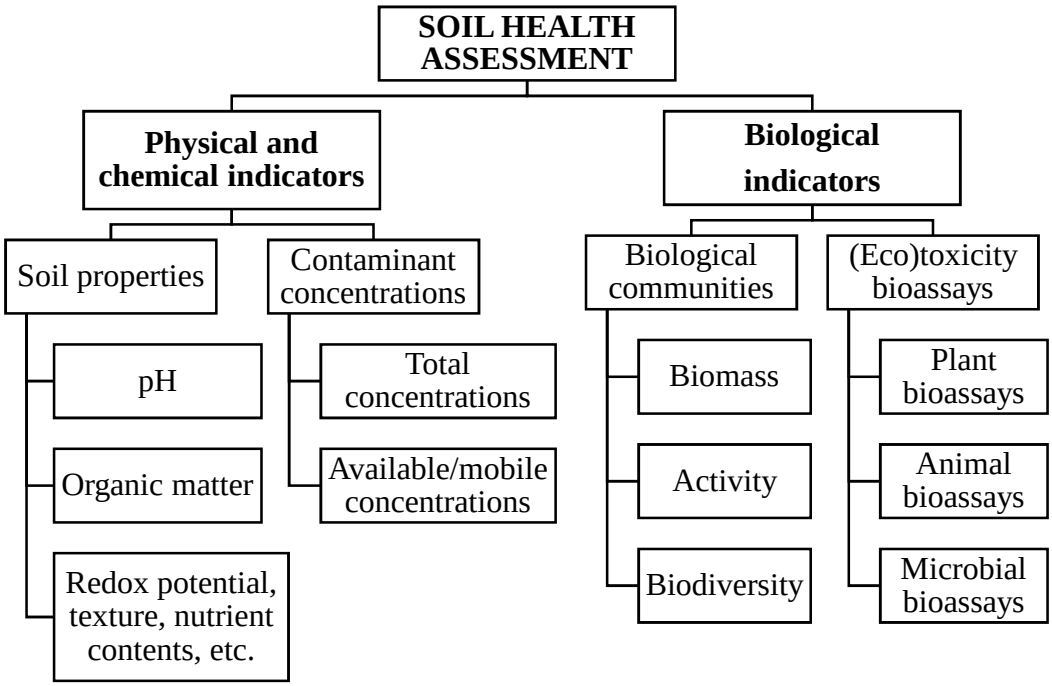


Figure 1.1: Soil health assessment.

1.3. From physicochemical techniques to gentle remediation options

Traditionally, physicochemical methods, such as excavation and transportation to a controlled landfill, incineration, chemical washing, vitrification, etc. (Ali et al., 2013), have been used to remediate contaminated soils, However, many of these physicochemical methods of soil remediation have substantial disadvantages and limitations, such as their high-cost, which frequently compromises their applicability (Houben et al., 2012), and, above all, the fact that they are often environmentally disruptive. Then, although their application can many times effectively remove and/or immobilize the target contaminants, in numerous cases the ecological status of the remediated soil is not improved during the remediation process; on the contrary, the application of these traditional physicochemical methods often leads to a partial or total destruction of the soil biota with concomitant adverse effects on soil processes, functions and health (Ali et al., 2013). On the other hand, the interaction between organic and inorganic contaminants in co-contaminated sites makes their remediation by physicochemical techniques more complex (Dubé et al., 2002).

Due to the abovementioned limitations of traditional physicochemical remediation technologies, in the last decades a variety of biological and more sustainable remediation technologies, often termed Gentle Remediation Options (GROs), have emerged (Fig. 1.2). In contraposition to conventional physicochemical remediation techniques, GROs are commonly less invasive and more respectful of the soil environment and its biota (Cundy et al., 2016). Many of these GROs aim at simultaneously (i) decrease the total and/or bioavailable concentration of soil contaminants; (ii) recover soil functionality; and, sometimes, (iii) produce renewable resources for the bio-based economy (Kidd et al., 2015; Kumpiene et al., 2014). Gentle remediation options often bring social, economic and environmental benefits by integrating sustainable remediation options (e.g., bioremediation, phytoremediation, vermiremediation) with the generation of economic revenues. In particular, the combination of phytoremediation with a profitable crop production, i.e. phytomanagement, has great potential for the recovery of contaminated sites, while providing a range of economic and other (e.g., provision of ecosystem services) benefits.

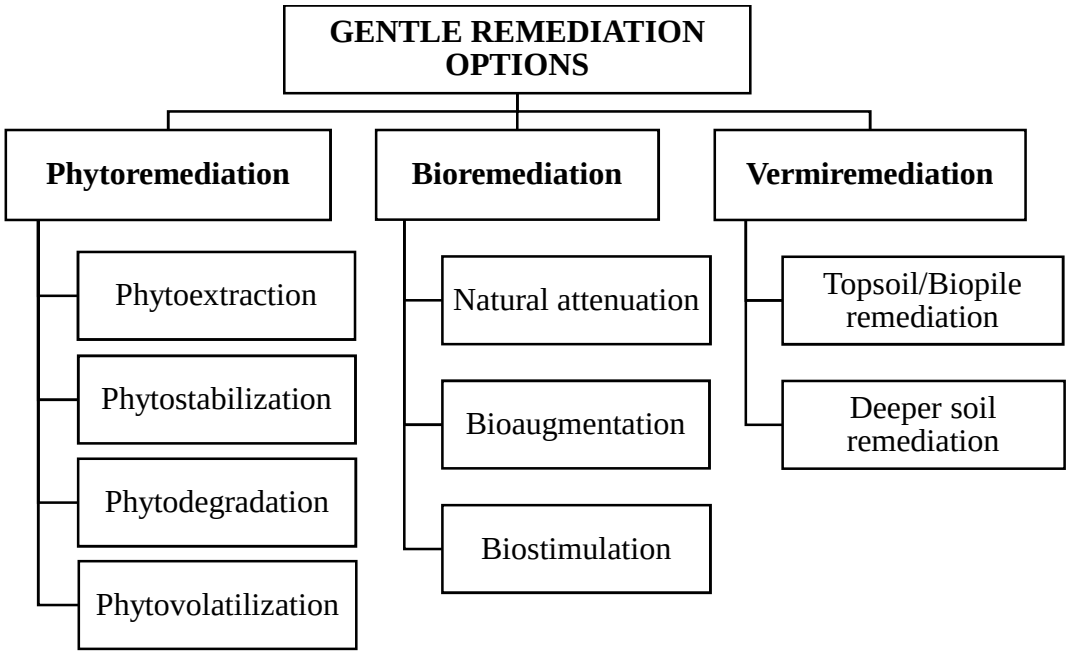


Figure 1.2: Gentle Remediation Options.

1.3.1. Phytoremediation

Phytoremediation has been defined as "the use of plants and associated microbes to reduce the concentration and/or toxic effects of contaminants in the environment" (Greipsson, 2011). Due to its low installation and maintenance costs, as well as its many environmental benefits, (Van Aken, 2009), this phytotechnology can be applied in large field sites in which other remediation options are not cost-effective or practicable (Garbisu and Alkorta, 2003). Phytoremediation techniques are suitable for the remediation of soils contaminated with both inorganic and/or organic compounds; however, they are most often applied to soils contaminated with metals. The two most common phytoremediation strategies, i.e. phytoextraction, phytostabilization, are described below.

1.3.1.1. Phytoextraction

Phytoextraction is a phytotechnology that uses the capacity of some plants to take up and translocate metal contaminants from soil to aboveground plant tissues. Subsequently, the aerial part of the plants can be harvested and, finally, incinerated, with potential benefits in terms of energy production and/or the recovery of high-added value metals (Chaney et al., 2018). For an effective phytoextraction, the selection of appropriate metal-tolerant

plant species is a crucial aspect. Plants can be classified in three categories depending on their strategy to cope with metals: (i) *excluders*, which actively limit metal uptake and can then immobilize the metal contaminants in the rhizosphere; (ii) *indicators*, which maintain a metal concentration in their tissues that reflects soil metal concentrations; and (iii) *accumulators*, which actively take up and translocate metals from soil to their shoots, thus reaching metal concentrations in their aboveground tissues higher than those present in the contaminated soil. Inside this last group, *hyperaccumulators* are extremely specialized plants that can accumulate heavy metals in their aboveground tissues at remarkably high concentrations (1-10%) (Baker, 1981; Barrutia et al., 2011a).

Accumulators and hyperaccumulators have frequently been used for phytoextraction purposes. *Noccaea caerulescens* (f.k.a. *Thlaspi caerulescens*), for instance, has been widely studied due to its remarkable capacity to accumulate zinc and/or cadmium in its shoots (Hernández-Allica et al., 2006; Jacobs et al., 2019). Some other commonly studied accumulators are *Elsholtzia splendens* (copper) (Chen et al., 2006), *Sedum plumbizincicola* (cadmium) (Cui et al., 2016) and *Chenopodium* spp. (chromium, nickel, cadmium) (Bhargava et al., 2007).

(Hyper)accumulators are certainly adapted to environments with high metal concentrations, but their growth rate and biomass are generally low. Therefore, alternatively, non-accumulator plant species but which can produce more aboveground biomass, are easier to cultivate and harvest, and show a better adaptability to prevailing environmental and climatic conditions, have also been used for phytoextraction purposes (Ali et al., 2013). After all, the effectiveness of a phytoextraction process is determined not only by contaminant concentrations in aboveground tissues, but also shoot biomass (J. T. Li et al., 2010). Due to their faster growth rate, adaptability to environmental stress and high biomass, herbaceous plants are often preferred for phytoextraction purposes, in comparison to shrubs or trees (Malik et al., 2010). Examples of plants with potential for phytoextraction strategies are: sunflower (*Helianthus annuus*), hemp (*Cannabis sativa*), and several species of the *Brassica* genus, such as Indian mustard (*B. juncea*), canola (*B. napus*) and turnip rape (*B. rapa*) (Meers et al., 2005; Solhi et al., 2005; Zalewska and Nogalska, 2014).

Unfortunately, phytoextraction has serious limitations when it comes to its practical application in the field. The major drawback for the successful application of this phytotechnology is the great amount of time required to effectively extract the metals

from the contaminated soil, particularly from those with medium and high levels of metal contamination (Zhao et al., 2003). Due to the low biomass characteristic of most hyperaccumulators, as well as the low metal uptake of non-accumulator plants that show high biomass production, a great number of harvests are required for a successful phytoextraction. Other limitations of phytoextraction are: (i) root depth, which narrows the applicability of this phytotechnology to surface soils; (ii) lack of well-known agronomic practices; and (iii) the incapability of most plants to accumulate more than one metal (Burges et al., 2018).

Another relevant aspect that cannot be neglected when applying phytoextraction strategies is the bioavailability of the metal contaminant. When applying this phytotechnology, it must always be taken into account that only a fraction of the total soil metal will be available for uptake by plants (Lasat, 2002), which *a priori* is the only fraction that can be phytoextracted. Numerous studies have explored the utilization of chelating agents to increase metal bioavailability in order to maximize phytoextraction efficiency by high biomass plants (Barrutia et al., 2010; Bian et al., 2018; Salt et al., 1995). However, this technology, known as chelator-induced phytoextraction, has raised environmental concerns derived from the risk of metal leaching to subsoil and groundwater and/or negative effects of persistent chelants on the soil biota (Burges et al., 2018; Marques et al., 2009; Yang et al., 2013). In any case, it should be noted that if the goal of a given phytoextraction initiative is only the removal of the bioavailable fraction of the metal contaminant, the time required for the process will be significantly shorter, which is, as previously mentioned, the main critique to phytoextraction (Vangronsveld et al., 2009).

1.3.1.2. Phytostabilization

Phytostabilization, focused on metal immobilization in the rhizosphere, is a GRO with great potential for those soils with moderate or high levels of metal contamination. Such immobilization can be achieved through metal precipitation or absorption/adsorption in the plant roots (Cundy et al., 2016; Hrynkiewicz et al., 2018). Indeed, besides metal absorption and/or adsorption in the root system, metal phytostabilization can also be achieved through the modification of the soil conditions: for instance, the root exudates of some plants have been reported to modify the rhizosphere pH and redox conditions, thus provoking the precipitation or complexation of potentially toxic metals (Gómez-Sagasti et al., 2012; Wu et al., 2010). Thus, phytostabilization reduces the bioavailable

fraction of metals in soil (Burges et al., 2018). By reducing the bioavailability and mobility of metals in soil, the risk of contamination of groundwater by metal leaching is reduced, as well as the entry of the metal contaminants to the food chain (Raskin and Ensley, 2000). Furthermore, a plant cover brings additional benefits to contaminated soils such as an increase in organic matter content, nutrients and soil biological activity; protection from soil erosion; improvement of soil structure; etc. (Arienzo et al., 2004; Mench et al., 2003).

Apart from being metal tolerant, suitable plants for phytostabilization should have an extensive root system, produce a large amount of biomass, and show a low root-to-shoot metal translocation rate (Alkorta et al., 2010). Many metal excluder plants, such as grasses (*Agrostis stolonifera*, *Lolium perenne*) and legumes (*Trifolium repens*, *Medicago sativa*, *Ulex europaeus*), have been effectively used to revegetate metal contaminated soils for phytostabilization purposes (Barrutia et al., 2011a, 2011b; Bidar et al., 2007; Pérez-de-Mora et al., 2006). Unlike for metal phytoextraction, shrubs and trees are commonly selected as suitable candidates for phytostabilization initiatives. Indeed, due to their capacity to stabilize metals in their massive root systems, tree and shrub species (e.g., *Populus* spp., *Salix* spp.) have been widely used for phytostabilization purposes (Pulford and Watson, 2003; Vamerali et al., 2009). Interestingly, the use of trees can lower the risk of metal leaching by reducing the downward flow of water due to their high rates of transpiration (Pulford and Watson, 2003).

The application of phytostabilization can be a challenge in highly degraded soils which, apart from metal contamination, present other problems like erosion, poor physical structure, shortage of essential nutrients and organic matter, etc. (Barrutia et al., 2011a). These problems are frequent in mine tailings and dumpsites, hampering the establishment of a healthy plant cover (Burges et al., 2016). In this respect, for an effective phytostabilization in highly degraded soils, the use of organic and/or inorganic amendments is often recommended to facilitate plant establishment and growth. This methodology is usually termed *assisted phytostabilization, aided phytostabilization* or *chemophytostabilization* (Alkorta et al., 2010). In aided phytostabilization, the promotion of plant growth can be achieved by raising soil pH, enhancing the organic matter content, providing essential nutrients, increasing the water holding capacity, reducing metal bioavailability, etc. (Alvarenga et al., 2009; Epelde et al., 2009a). Besides, the utilization of organic and inorganic amendments opens the door to the recycling of wastes, residues

and byproducts from diverse origins, in a context of Circular Economy (Míguez et al., 2020). Some amendments commonly used in aided phytostabilization studies are: animal slurry and manure, paper mill sludge, sewage sludge, urban solid wastes, litter, leonardite, lime ($CaCO_3$), etc. (Alvarenga et al., 2009; Galende et al., 2014a; Lacalle et al., 2018a; Pérez-de-Mora et al., 2006). However, prior to their use, amendments should be thoroughly analyzed. In this respect, an exhaustive physical, chemical and biological characterization of the amendments is required to minimize/avoid the risk of introducing toxic compounds or potential human pathogens into the amended soil, with concomitant hazards for environmental and human health (Goss et al., 2013).

In any event, it must be emphasized that phytostabilization does not decrease the total concentration of metals in the soil (i.e., the metal contaminants remain in the soil) but only immobilizes them, thereby reducing their mobility and bioavailability. Consequently, there is always the possibility that the metal contaminants are later mobilized due to changes in the soil conditions, with potential adverse consequences in terms of (eco)toxicity and/or metal dispersion. Therefore, phytostabilization processes must always be subjected to long-term monitoring programs regarding metal bioavailability, (eco)toxicity and soil functioning (Gómez-Sagasti et al., 2012).

The main limitation of phytostabilization for its practical application is the fact that current environmental legislations are normally based on total metal concentrations, not on bioavailable metal concentrations, and since this phytotechnology cannot reduce total metal concentrations below the reference critical values established by legislation, it is impractical from a legal point of view.

1.3.2. Phytomanagement

Despite the number of research papers on phytoremediation, its application at field scale is still limited. Some of the reasons for this phenomenon are, among others, the uncertainty around the required time-scales, the reproducibility of the results, and the current legal frameworks (Cundy et al., 2016; Mench et al., 2010). As a matter of fact, many stakeholders perceive GROs in general, and phytoremediation in particular, as slow technologies which are difficult to apply and suited only for large and marginalized areas with low value (Cundy et al., 2016; Kidd et al., 2015; Mench et al., 2010). Nonetheless, it must be emphasized that contaminated lands are an extensive and underutilized resource (Evangelou et al., 2015) which, when properly managed, can provide economic

revenues and valuable ecosystem services. In this respect, phytomanagement encourages the use of plants with phytoremediation potential as part of an integrated site management which pursues, along with the mitigation of the risks derived from the presence of the contaminants, the accomplishment of economic, social and environmental benefits (Burges et al., 2018). These benefits include the provision of green space and ecosystem services, the control of soil erosion and, above all, the generation of products and commodities (e.g., bioenergy, wood, biochar, biofortified products) (Cundy et al., 2016; Evangelou et al., 2015; Kidd et al., 2015). For that purpose, fast growing, deep rooted and easily propagated high biomass plants are often used, such as agronomical and herbaceous crop plants and trees. Phytomanagement makes site remediation an attractive option for stakeholders due to the environmental, economic and social benefits that can be obtained, while mitigating the risk resulting from the presence of the contaminants. Then, phytomanagement has been proposed as a very appealing "holding strategy" until full site regeneration is possible (Cundy et al., 2016).

1.3.3. Bioremediation

Bioremediation, or the use of microorganisms (mainly, bacteria and fungi) to clean up contaminated sites, is a sustainable option for the remediation of contaminated soils (Fingerman and Nagabhushanam, 2016). Although bioremediation can indeed be used for inorganic contaminants (Park et al., 2011), its application is more frequent for organic contaminants such as mineral oils, petroleum hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, etc. (Megharaj et al., 2011). There are three main approaches for the bioremediation of contaminated areas (Bento et al., 2005): (i) *natural attenuation*, which is naturally carried out by the native microbial populations present in the contaminated area; (ii) *bioaugmentation*, which is based on the inoculation of selected microbial strains with the capacity to degrade the target contaminants at a fast rate; and (iii) *biostimulation*, which is focused on the modification of the environmental conditions (e.g., moisture, pH, nutrients, oxygen), in order to stimulate the biodegradation of the target contaminants.

In natural attenuation processes, the degradation of the contaminants is strongly determined by the (i) metabolic capacity of the native microbial populations; (ii) the physicochemical properties of the contaminated soil; and (iii) the chemical properties of the target contaminants. Under favorable conditions, some contaminants (e.g., short-chain

petroleum hydrocarbons) show high levels of degradation by natural attenuation (Bento et al., 2005; Lacalle et al., 2018a). However, the efficiency of natural attenuation for more recalcitrant compounds is very low or null, especially for aged contaminants (Megharaj et al., 2011).

In order to maximize the efficiency of bioremediation processes, the degrading capacity of indigenous microbial populations can be stimulated, via biostimulation strategies, by adjusting the supply of essential macro- and/or micronutrients, temperature, available oxygen, soil pH, redox potential, moisture, etc. (Carberry and Wik, 2001). However, the most common practice is probably the addition of nutrients, either in inorganic form (Ramadass et al., 2018) or as organic amendments such as sewage sludge, manure, compost, etc. (Lee et al., 2008; Park et al., 2011; Ros et al., 2010). Again, it must be taken into consideration that the rate of contaminant degradation will depend on the (i) physicochemical characteristics of the soil; (ii) specific degrading microbial populations present in the contaminated soil; and (iii) chemical nature of the contaminants themselves. Therefore, it is not surprising than the type and dose of the amendments (inorganic and/or organic) must always be carefully selected considering these three aspects (Megharaj et al., 2011). Other practices include the addition of surfactants to increase contaminant availability (Zeng et al., 2018), the application of biochar (Kong et al., 2018), and the growth of plants for phytostimulation purposes (Lacalle et al., 2018a).

Bioaugmentation is focused on the inoculation of previously isolated and cultivated microbial strains, individually or as a consortium, to stimulate the biodegradation of the target organic contaminants. The inoculation of “cocktails” of degrading strains is more frequent, compared to the inoculation of individual strains, as microorganisms in consortium can combine different metabolic activities that complement each other from a bioremediation point of view (Fuentes et al., 2017; Ramadass et al., 2018; Shen et al., 2015). The microbial strains used for bioaugmentation are commonly isolated from similarly contaminated soils, sometimes from the same soil to be remediated, in order to increase the probability of their survival and their capacity to express their biodegrading activity when inoculated in the contaminated soil (Alisi et al., 2009; Tahir et al., 2016). The genetic modification of bacterial strains to improve their biodegradation performance, by means of genetically optimizing the production of enzymes and metabolic pathways relevant for the biodegradation of the target contaminants, has also been studied (Fernández-Luqueño et al., 2011; Pieper and

Reineke, 2000). However, when selecting the different strains for the bioaugmentation consortium, their compatibility with each other and their ecological fitness in the soil under remediation must be taken into consideration. Actually, bioaugmentation initiatives fail quite often, mostly due to the incapability of the inoculated strains to compete and properly develop in the contaminated soil (Megharaj et al., 2011; Polti et al., 2014).

1.3.4. Vermiremediation

Vermiremediation has been described as the use of earthworms for the removal of contaminants from soil (Sinha et al., 2008). Earthworms are known to burrow through the soil, mixing it in their guts (Eijsackers et al., 2001), and, consequently, they are capable of changing the physicochemical and biological properties of the soil, such as nutrient availability, aeration, soil structure and, hence, the activity of soil microbial communities (Rodriguez-Campos et al., 2014). In addition, earthworms can increase the interaction between soil microbial communities and contaminants, thus facilitating the biodegradation of the target contaminants (Hickman and Reid, 2008a). Some studies have investigated the interaction between earthworms and metal contaminants (Eijsackers, 2010; Kavehei et al., 2018), but vermiremediation is more commonly used for organic contaminants. Indeed, organic contaminants such as herbicides, PCBs, PAHs or, in general, petroleum-derived hydrocarbons have been successfully remediated through vermiremediation using a variety of earthworm species (Kersanté et al., 2006; Luepromchai et al., 2002; Natal-da-Luz et al., 2012; Schaefer and Juliane, 2007). A particularly interesting earthworm species, *Eisenia fetida*, has been used for vermiremediation purposes (Chachina et al., 2016), as well as bioindicator of metal ecotoxicity in soil (Irizar et al., 2015b; Shin et al., 2007).

In order to successfully apply vermiremediation, several aspects need to be considered, such as the behavior of the earthworms, their nutritional requirements, the characteristics of the soil, and the nature of the contaminants themselves. According with their location in the soil, earthworms can be cataloged as epigeic, endogeic and anecic: *epigeic earthworms*, such as *E. fetida*, require high amounts of organic matter and, then, they live at or near the soil surface where they feed on leaf litter, decaying roots and dung. In consequence, they can (i) be used to remediate topsoil; and (ii) be inoculated in biopiles employed for bioremediation purposes, where little burrowing is necessary. *Endogeic* and *anecic earthworms*, e.g. *Lumbricus terrestris*, on the other hand, are better suited for the

vermiremediation of deeper soil (Hickman and Reid, 2008a). In order to guarantee the survival of the inoculated earthworms, sometimes it is required to first ameliorate soil contamination levels (Tejada and Masciandaro, 2011). Certainly, extreme conditions (in terms of soil pH, salinity, contaminant bioavailability) or a lack of organic matter can complicate the establishment of the inoculated earthworms (Eijsackers, 2010). Organic amendments can then be used to reduce contaminant bioavailability, while adding organic matter and improving soil structure (Elliston and Oliver, 2019).

1.4. Mixed remediation technologies for mixed contamination

Mixed contamination, when inorganic and organic contaminants appear together, is present in many contaminated soils. Soils with mixed contamination combine the individual challenges from each individual class of contaminant with those derived from the combination of two or more types of contaminants with different properties. Regarding (eco)toxicity, synergistic effects on soil biota can occur from that combination. Likewise, from a remediation point of view, the efficiency of the applied techniques may be reduced by the presence of other types of contaminants or by the interaction between them (Lacalle et al., 2018a). For instance, in mixed contaminated soils, it has been reported that co-contamination with metals and organic compounds can cause metal immobilization (Galvez-Cloutier and Dubé, 2002) or, by contrast, an increase in metal mobility (Dubé et al., 2002). Therefore, the outcome of the interaction between different types of contaminants is site-specific, as it depends on the specific properties of the contaminated soil, as well as on the type and concentration of the contaminants themselves (Chirakkara et al., 2016).

Regarding the effectiveness of remediation technologies, metals can provoke toxic effects on soil microbial communities, decreasing their abundance and/or activities (Khan et al., 2010) and, consequently, reducing their capacity to degrade the target organic contaminants (Sandrin and Maier, 2003). In a phytoremediation experiment, it was observed that co-contamination with copper and pyrene decreased plant growth and the removal of pyrene, compared to the experiments performed with each contaminant individually (Chigbo et al., 2013). As a result, the application of only one remediation technology might not be effective for soils with mixed contamination. Regrettably, many remediation studies are focused on just one type of contaminant, probably due to the complexity of tackling mixed contamination cases. Nevertheless, in the last years, more

and more remediation studies have dealt with mixed-contaminated soils. For instance, bioaugmentation with a bacterial consortium has been reported to be effective for the remediation of soils simultaneously contaminated with Cr (VI) and lindane (Aparicio et al., 2018a) or pyrene (Wang et al., 2019).

The combination of plants and bacteria for phytoremediation purposes offers great potential for soils with mixed contamination (Batty and Dolan, 2013). The presence of plants can enhance the activity and functional diversity of soil microbial communities by releasing root exudates and improving the conditions for microbial growth in the rhizosphere (Balseiro-Romero et al., 2017; Barrutia et al., 2011b). Root exudates create a nutrient-rich environment which can influence the behavior of metals (Kidd et al., 2009) and enhance the biodegradation of organic contaminants (Kuiper et al., 2004).

The combination of vermiremediation with phytoremediation and bioremediation has been successfully tested for the remediation of soils contaminated with metals and organic contaminants (Elyamine et al., 2018; Fernández-Luqueño et al., 2011; Martinkosky et al., 2017; Sivaram et al., 2019). The combination of these biological remediation technologies appears promising for mixed-contaminated soils. Expectedly, factors such as type of soil, chemical properties of the contaminants, and the biological species selected for the remediation (e.g., the specific species of plants, bacteria and earthworms) will strongly modify the outcome of the remediation process.

As mentioned above, the use of amendments is very common during the implementation of GROs. In particular, organic amendments provide soil nutrients and organic matter, improve soil structure, enhance water holding capacity, alter contaminant bioavailability, etc. In consequence, organic amendment can alleviate toxicity for the species involved in the biological remediation of mixed-contaminated soils. Many studies have reported the benefits of using organic amendments during the implementation of GROs (Elyamine et al., 2018; Lacalle et al., 2018a; Marchand et al., 2018; Reddy et al., 2017). Other authors have combined the application of nanoremediation (with, for instance, nanoscale zero valent iron) with GROs with mixed results (Gómez-Sagasti et al., 2019; Huang et al., 2018, 2016; Su et al., 2016). The term *nanoremediation* refers to the application of metallic nanoparticles (<100 nm) for the remediation of contaminated sites (Gil-Díaz et al., 2017). In particular, the use of zero-valent iron nanoparticles (nZVI) has caught the attention of the scientific community for the remediation of contaminated waters and soils (Medina-Pérez et al., 2019). Zero-valent iron nanoparticles have an iron

core and a shell of iron oxide and, due to their small size, show a very high surface/volume ratio (Li et al., 2017). The iron core acts as the electron donor, while the shell plays coordination and electrostatic functions, attracting and adsorbing charged ions (Li and Zhang, 2007). Zero-valent iron nanoparticles have been applied for the remediation of both organic and inorganic contaminants. Regarding inorganic contaminants, nanoparticles can form complexes with soil metals, thus decreasing their bioavailability (Gil-Díaz et al., 2017). Besides, by changing the redox potential of the soil, nZVI can alter the speciation of the metal contaminants, decreasing their bioavailability and (eco)toxicity. For instance, nZVI can reduce Cr (VI) to Cr (III), a less toxic and bioavailable form (Singh et al., 2011a). On the other hand, nZVI have been reported to effectively remediate soils contaminated with organic compounds, especially those contaminated with organochlorinated compounds (Elliott et al., 2009; Yang et al., 2010). Nevertheless, the use of nanoparticles for soil remediation has been questioned due to their potential negative impact on soil biota. In any case, there is still a lack of information regarding the mobility, bioaccumulation, dynamics and (eco)toxicity of nZVI in the soil environment (Machado et al., 2013; Patil et al., 2016). Zero-valent iron nanoparticles have been described to provoke toxicity through two mechanisms: (i) physical damage by direct contact, disrupting cell membrane architecture and increasing permeability; and (ii) oxidative stress, leading to molecular and biochemical destruction (Xie et al., 2017). Adverse effects of nZVI have been reported in plants (Ma et al., 2013), animals (Stefaniuk et al., 2016) and microbial communities (Fajardo et al., 2012). As expected, the potential effects of nZVI on soil biota are highly conditioned by the soil type and environmental conditions (Gómez-Sagasti et al., 2019; Xie et al., 2017). Therefore, it is essential to perform an assessment of the potential effects of nZVI on soil biota prior to their application under real field conditions, in order to first establish a safe, non-toxic and effective concentration for nanoremediation purposes (Patil et al., 2016). Indeed, despite their proven effectiveness for remediation, the effect of nanoparticles on soil biota, including the biological species used for remediation, is yet full of uncertainties. Then, the potential adverse impact of nZVI on soil organisms must be tested prior to their use, alone or in combination with GROs.

In conclusion, when facing a mixed-contaminated soil, it is essential to first take into account a variety of aspects, such as soil type, nature of the contaminants,

compatibility of the remediation technologies, etc. in order to then be able to apply a tailor-made strategy for each case.

1.5. Conclusions

Economically-feasible sustainable biological methods of soil remediation (e.g., phytoremediation, phytomanagement, bioremediation, vermiremediation) are being developed to: (i) efficiently remove contaminants from soil; (ii) decrease their bioavailability, mobility, (eco)toxicity and potential risks for environmental and human health; and, simultaneously, (iii) recover soil health and the provision of ecosystem services. The remediation of mixed-contaminated soils is particularly challenging, as it combines the individual challenges for each individual contaminant with those derived from their combination. Interestingly, the combination of biological and non-biological methods offers great potential for the remediation of mixed-contaminated soils.

2. HYPOTHESIS AND OBJECTIVES

2. HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

Most of the contaminated soils in the world contain complex mixtures of trace elements and organic contaminants; however, most of the literature on soil remediation and their ecotoxicological effects is dominated by studies with one kind of contaminants. Investigation on remediation of mixed contamination needs to be addressed. The application of gentle remediation options alone or in combination other physico-chemical technologies can be a suitable strategy for the remediation of soils co-contaminated with metals and organic compounds with concomitant benefits in terms of soil health recovery.

2.2. General objective

The general objective of this work was to evaluate the effectiveness of gentle remediation options (*i.e.*, phytoremediation, vermiremediation, bioremediation via bioaugmentation or biostimulation) and/or nanoremediation with zero-valent iron nanoparticles (nZVI) for the remediation of mixed contaminated soils. This general objective was divided in five specific objectives, each one corresponding to a different chapter.

2.3. Specific objectives

1. Assess the effectiveness of phytoremediation with *Brassica napus*, combined with (i) nanoremediation with nZVI and (ii) biostimulation through the application of an organic amendment, to remediate a soil polluted with metals (Zn, Cu and Cd) and commercial diesel (Chapter 4).

2. Evaluate the effect of natural attenuation and biostimulation on the remediation of soils simultaneously contaminated with commercially available diesel/biodiesel blends and potentially toxic metals (Zn, Cu and Cd) (Chapter 5).

3. Assess the effectiveness of a combined biological remediation strategy (*i.e.*, phytoremediation with *Brassica napus* + vermiremediation with *Eisena fetida* + bioaugmentation with a bacterial consortium + biostimulation via the application of an organic amendment) to remediate a soil polluted with chromium (VI) and lindane (Chapter 6).

4. Evaluate the suitability and potential toxicity of nZVI for the nanoremediation of soils polluted with chromium (VI) and lindane, as well as its compatibility with biological remediation strategies, *i.e.* phytoremediation with *Brassica napus*, vermiremediation with *Eisena fetida*, bioaugmentation with a bacterial consortium, and biostimulation via the application of an organic amendment (Chapter 7).

5. Evaluate the effect of the application of an organic amendment and nZVI on soil Cr(VI) immobilization and soil health recovery (Chapter 8).

3. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Origin and characteristics of soils and amendments

For the experiments shown in Chapters 4 to 8, soil was collected from a peri-urban area near the city of Vitoria-Gasteiz (42º50'N; 2º40'W, northern Spain), within the Jundiz Industrial Park. This area is degraded due to the illegal discharge of mainly construction and demolition wastes. The soil (loam soil) has a very poor structure, low organic matter content and a high percentage of carbonates due to the marly deposits of the area (Martínez-Torres et al., 1985), but it is not contaminated.

Figure 3.1. Jundiz Industrial Park, where soil was collected.

In a previous project, part of this area was amended with "bio-stabilized material", a compost-like material from the "Biocompost de Álava UTE", a treatment plant for urban wastes. Prior to the addition of the amendment, the plot was cleaned of wastes, the spontaneous vegetation was removed and, finally, the soil was plowed. In September 2015, 100 t ha^{-1} of the bio-stabilized material were incorporated to the soil with a rotavator.

Soils from both unamended and amended plots were collected (topsoil: 0-15 cm) in October 2015, sieved to <6 mm, air-dried until constant weight and subjected to physicochemical characterization (Table 3.1). The soil was stored at room temperature.

Table 3.1. Physicochemical properties of the soils collected from Jundiz Industrial Park.

	Unamended soil	Amended soil
Total clay (%)	23.4	15.7
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Texture class (USDA)	Loam	Loam
pH (1:2.5)	7.9	8.0
Carbonates (%)	54.7	44.0
Organic matter (%)	1.0	19.5
C organic / N organic	6.7	8.6
Total N (% DW)	0.1	0.9
Total C organic (% DW)	0.6	7.3

3.2. Contamination of the soil

These soils were used to perform a variety of experiments to assess both the adverse impact of soil contaminants and the beneficial effects of the application of GROs. According with each experimental design, the soils were artificially contaminated as follows:

- The soil was extended in a 40 cm x 25 cm tray. Zinc, Cu and Cd were applied in the form of nitrate salts. They were dissolved in Milli-Q water and directly sprayed to the soil using an airbrush, in order to homogenize the distribution of the contaminants.

- Cr (VI) was applied in the form of $K_2Cr_2O_7$, which was dissolved in Milli-Q water and then sprayed on sand, extended in a 40 cm x 25 cm tray, using an airbrush. Afterwards, 50 g of the contaminated sand were added per kg of soil to reach the desired Cr concentration. Chromium was applied alone in Chapter 8 and together with lindane in Chapters 6 and 7.

- Lindane was dissolved in hexane and sprayed on sand already contaminated with Cr (VI) using an airbrush. Subsequently, 50 g of the co-contaminated sand were added per kg of soil to reach the desired lindane concentration (Chapters 6 and 7).

- All types of diesel were directly sprayed on sand using an airbrush. Afterwards, 50 g of the contaminated sand were added per kg of soil to reach the desired diesel concentration.

In all experiments with mixed contamination, both contaminants were added to the soil at the same time. Contaminants were applied to 0.5 kg of DW soil. Contaminated soils

were kept in 1.1 L containers and vigorously shaken for 20 min to ensure total homogeneity.

3.3. Organisms used for biological remediation experiments

3.3.1. Brassica napus

Brassica napus, commonly known as rapeseed and a member of the family Brassicaceae, is cultivated mainly for its seed, which can be used for the production of vegetable oil and biodiesel. In addition, rapeseed oil production generates rapeseed meal, a byproduct that can be used for animal feed. Seeds of *B. napus* (v. Expower) were purchased from Dekalb® (Barcelona, Spain). Thirty seeds per pot were directly sown on the soil surface.

3.3.2. Eisenia fetida

Eisenia fetida, an epigean earthworm of the family Lumbricidae, is adapted to live in organic matter like rotting vegetation, manure and compost. It is commonly used for vermicomposting. *Eisenia fetida* specimens were purchased from Lombricor S.C.A (Córdoba, Spain) and kept under controlled conditions in horse manure. Then healthy, sexually matured individuals, with a weight between 350 and 450 mg, were added per pot.

Figure 3.2: An *Eisenia fetida* specimen in the rhizosphere of *Brassica napus*.

3.3.3 Actinobacteria

Actinobacteria are a phylum of Gram-positive bacteria which play a key role in soil functioning, principally as organic matter decomposers. They can also live in water and are the source of many antibiotics. For this work, four actinobacteria strains, previously isolated from contaminated environments, were used: *Streptomyces* sp. M7, *Streptomyces* sp. MC1, *Streptomyces* sp. A5 and *Amycolatospis tucumanensis* (Aparicio et al., 2018b). Two g kg^{-1} of the bacterial consortium, containing equal proportions of each strain, was added to the experimental pots.

3.4. Application of zero-valent iron nanoparticles (nZVI)

Zero-valent iron nanoparticles, stabilized by a thin inorganic surface layer, were purchased from NanoFer Star, Nanoiron s.r.o. Nanoparticles were activated, following the manufacturer´s instructions, by making a 1:5 nZVI : Milli-Q water slurry which was homogenized in a kitchen blender at maximum power for 10 min. The slurry was stored at 4ºC for 24 h, after which it was diluted to the desired concentration and applied to the soil. In this work, two procedures of nZVI application were used:

- (i) In Chapter 7, the nZVI solution was manually mixed with the soil, up to a concentration of 5 g nZVI kg^{-1} soil.

- (ii) In Chapter 4 and 8, the nZVI solution was applied directly onto the soil. Previously, the soil had been perforated using metal sticks in order to create conducts and facilitate the dispersion of the nZVI solution through the whole pot. This technique aims to resemble a methodology that could be applied under field conditions. The final concentration was 1 g nZVI kg^{-1} soil.

3.5. Microcosm experiments

Microcosm experiments were carried out under controlled conditions. In Chapters 6, 7 and 8 a greenhouse was used, while in Chapters 4 and 5, the experiments were performed in a phytotron. In both cases, the facilities belong to the Phytotron and Greenhouse Service of the Advanced Research Facilities (SGIker) at the University of the Basque Country (UPV/EHU). The controlled conditions were as follows:

- Greenhouse: photoperiod of 14 h day / 10 h night, minimum PAR (Photosynthetically Active Radiation) intensity of 250 μmol photon m^{-2} s^{-1}, temperature of 25/18ºC, relative humidity of 50/60%.

- Phytotron: photoperiod of 14 h day / 10 h night, PAR (Photosynthetically Active Radiation) intensity of 200 μmol photon m^{-2} s^{-1}, temperature of 25/18ºC, relative humidity of 60/80%.

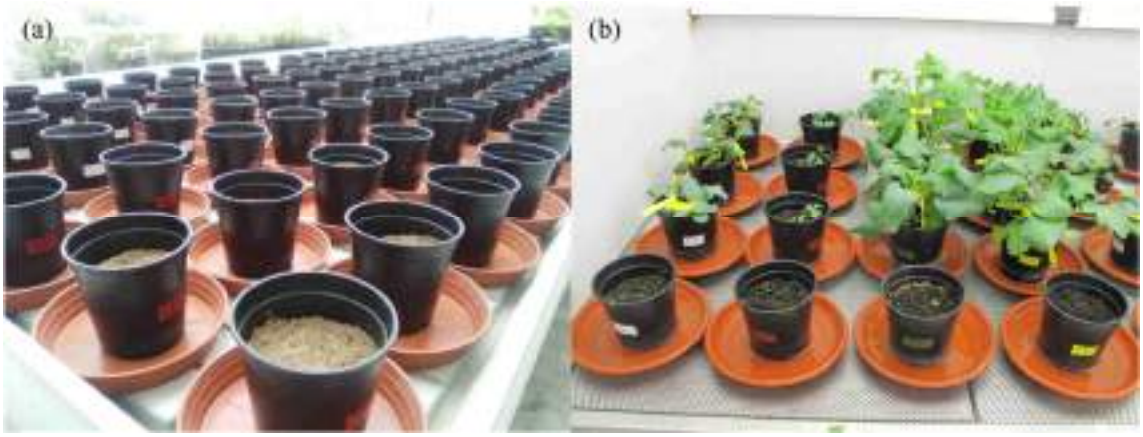


Figure 3.2. Pots in the greenhouse (a) and *Brassica napus* plants in the phytotron (b)

3.6. Soil physicochemical characterization

Oxidable organic matter, total nitrogen, total organic carbon, carbonate content and pH were determined by NEIKER-Tecnalia, the Basque Institute for Agricultural Research and Development, according to standard methods (MAPA, 1994).

3.7. Determination of contaminant concentrations

3.7.1. Extraction of pseudo-total metal fractions from soil

Extraction of metals from soils was performed by a digestion according to a standardized method (US-EPA Method 3051A, 2007). The soil was oven-dried, crushed and sieved to <0.125 mm. Approximately 0.5 g of soil was introduced into the digestion vessels, and then 10 mL concentrated nitric acid (65%) were added for Zn, Cu and Cd determination (Chapters 4 and 5). For Cr determination (Chapters 6, 7 and 8), the digestion was performed using 9 mL nitric acid and 3 mL hydrochloric acid. In both cases, the microwave digestion was carried out by a CEM Mars 5 Digestion System. Samples were maintained at 175ºC for 4.5 min, and after cooling, the digestates were diluted with Milli-Q water to 50 mL. Finally, digestates were stored at 4ºC in darkness until analysis.

3.7.2. Extraction of bioavailable metals from soil

The bioavailable fraction of metals was extracted following the method described in Houba et al. (2000) using $CaCl_2$ 0.01M. Oven-dried, crushed and sieved to <0.125 mm soil was mixed with a $CaCl_2$ solution (1:10 w/v). The solution was shaken at 221 rpm for 2 hours and filtered to <0.45 µm. Nitric acid was added to obtain a 2% concentration in order to avoid the precipitation of metals. Extracts were stored at 4ºC in darkness until analysis.

3.7.3 Extraction of soluble Cr(VI) from soil

Soil was oven-dried and grinded. The soluble fraction of Cr was extracted following the method described in Jiang et al. (2015). A 1:25 (w/v) mixture of soil and Milli-Q water was shaken in an orbital shaker at 200 rpm for 24 h and then centrifuged at 10,000 *x g* for 15 min to remove the soil from the aqueous solution. The extracts were stored at 4ºC in darkness until analysis

3.7.4. Digestion of plants and worms for metal determination

At harvest, leaves, stems and roots from *B. napus* plants were separated. Leaves and stems were surface cleaned with deionized water, while roots were also soaked with 0.01 $CaCl_2$ for 30 min to remove adsorbed metals. Plant samples were then oven-dried at 80ºC for 48 h, grinded and subjected to a digestion as described in Zhao et al. (1994). Around 200 mg of sample were placed in glass tubes, in which 5 mL of a mixture of HNO_3 and $HClO_4$ (85:15) were added. The digestion was carried out in a metallic heating block (Bloc Digest m 40, Selecta) controlled by a time/temperature process programmer (RAT-2, Selecta). The temperature curve was the following: (i) 3 h at 60ºC; (ii) 1 h at 100ºC; (iii) 1 h at 120ºC; and (iv) 5h at 195ºC. At the end of the process, the acid in the sample had completely evaporated. After cooling, 5 mL of HNO_3 were added and the tubes were re-heated at 80ºC for 30 min. Then, 15 mL of Milli-Q water were added and the samples were re-heated at 80ºC for another 30 min, after which the sample was made up to 20 mL with Milli-Q water and filtered (0.45 µm). The solutions were stored at 4ºC in darkness until analysis.

Five depurated (left on wet filter paper for 24 h to void gut content) and cleaned earthworms were dried at 120ºC for 48 h, weighted and digested in HNO_3. After acid evaporation, samples were re-suspended in 20 mL of 0.01 M HNO_3.

3.7.5. Metal determination

Soil, plant and earthworm extracts and/or digestates were analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) by the SGIker service of the UPV/EHU.

3.7.6. Diesel determination in soil

For diesel determination, soil was collected and stored at 4ºC in darkness. Prior to the analysis, the soil was oven-dried at 35ºC for 72 h, crushed and sieved to 0.125 mm. Following the method described in Bartolomé et al. (2005), after sample preparation, 0.5 g of soil was extracted in 15 mL of acetone using a CEM Mars 5 Digestion System. The filtered (0.45 µm) extract was cleaned through a solid phase extraction (SPE) with Florisil ® cartridges. The analysis was performed using Gas Chromatography-Mass Spectrometry (GC-MS). In order to study the differential degradation of total petroleum hydrocarbons (TPH), polycyclic aromatic hydrocarbons (PAH), fatty acid methyl esters (FAMEs) and n-alkanes (n-Alk) were quantified.

3.7.7. Lindane determination in soil

Lindane concentrations was quantified as described by Fuentes et al. (2011). Five grams of dried soil were mixed with 10 mL of a water-methanol-hexane (4:1:5) solution. The mixture was shaken using a vortex and centrifuged to separate the organic phase, which was then evaporated until dryness. The lindane residue was re-suspended in hexane and its quantification was performed using Gas Cromatography (Agilent 7890A).

3.7.8. Lindane determination in plants and earthworms

Lindane concentration was determined following AOAC Official Method 2007:01 (2007) and “QuEChERS” Method. Lindane was extracted from the crushed plant and worm samples using acetonitrile, magnesium sulphate and sodium acetate. The extract was cleaned by solid phase extraction with an Agilent kit (QuEChERS AOAC). Quantification was carried out by gas chromatography (Agilent 2890A).

3.8 Soil microbial properties

3.8.1. Soil basal respiration

Soil microbial activity was estimated through the quantification of soil basal respiration, which was determined as described in ISO 16072 (2002). Twenty g of fresh soil were

water-saturated and introduced in a hermetic glass jar along with a vial containing 10 mL of NaOH 0.2 M. Samples were incubated at 30ºC for 72 h in a Bench Top Incubated Shaker 3527-1 (Lab-Line), without shaking. After incubation, 4 mL of $BaCl_2$ (0.5 M) and 3-4 drops of phenolphthalein 0.1% (in ethanol) were added to the vials and a titration with HCl 0.1M was performed using a Titrette® (Brand). The same procedure was carried out using jars without the soil samples to standardize the results. These data were used to calculate the CO_2 production derived from the respiration of soil microorganisms, by applying Formula 3.1:

$$\boldsymbol{C}\ (\mu\boldsymbol{g}\ \frac{\boldsymbol{C}}{\boldsymbol{g\ soil \cdot h}}) = \frac{[\mathbf{Vc-Vm}](\mathrm{ml}) \cdot \mathbf{N}\left(\frac{\mathrm{Eq}}{\mathrm{l}}\right) \cdot \mathbf{0.5} \cdot \mathbf{12}\left(\frac{\mathrm{g}}{\mathrm{Eq}}\right).\mathbf{10^{-3}}\left(\frac{\mathrm{l}}{\mathrm{ml}}\right).\mathbf{10^{6}}\left(\frac{\mu g}{\mathrm{g}}\right)}{\mathbf{Pm}\ (\mathrm{g})\ .[\mathbf{MS}]\ .\ \mathbf{T}\ (\mathrm{h})}$$

V_c: Volume of HCl used in the titration without soil sample (mL)

V_m: Volume of HCl used in the titration with the soil sample (mL)

N: Normality of the used HCl (0.1 M)

12: Equivalent weight of C (g/Eq)

P_m: Weight of soil sample (20 g)

MS: [Dry weight / Fresh weight] ratio of the soil

T: Incubation time (h)

3.8.2. Substrate-induced respiration

Soil microbial biomass was estimated through the quantification of substrate-induced respiration, which was determined as described in ISO 17155 (2002). Two hundred mg of a mixture of glucose, KH_2PO_4, and $(NH_4)SO_4$ (31:1:5) was added to the soil previously used for the measurement of basal respiration. After homogenization, another vial containing 10 mL of NaOH 0.2 M was introduced in the jar and incubated at 30ºC for 6 h. After incubation, the remaining NaOH was subjected to titration following the same methodology as for the basal respiration. It has been reported that this short period of time allows to register the respiration of the microbial communities, without allowing them to multiply (Lin and Brookes, 1999).

3.8.3. Community-level physiological profiles (CLPPs)

Biolog EcoPlates™ (Biolog Inc., USA) were used to estimate the functional microbial diversity of the studied soils, as described in Epelde et al. (2008). The plates are composed

of 31 wells: inside of each, there is a different carbon substrate (carbohydrates, carboxylic acids, amides/amines, aminoacids, polymers) and tetrazolium, which turns purple when the substrate is catabolically degraded. Color development can be measured spectrophotometrically in order to determine community-level physiological profiles.

All the material and water used was previously sterilized in an autoclave at 121ºC for 20 min. The fresh equivalent to 1 g of dry soil was mixed in a plastic tube with 9 mL of Milli-Q water. The mixture was shaken for 1 h at 125 rpm in an orbital shaker and left 5 min to precipitate. After that, 200 µL of the supernatant were diluted with 19.8 mL of Milli-Q water in reagent reservoirs. The wells of the Biolog EcoPlate™ were inoculated with this dilution. Color development was spectrophotometrically measured at 595 nm by a PowerWave X 340 Microplate Spectrophotemeter (BioTek) at the initial time and every 24 hours during 5 days to create a growth curve. Functional microbial diversity indexes were calculated using the absorbance values at 40 h of incubation, which has been described as the time of maximal microbial growth in the Biolog Ecoplates™ (Galende et al., 2014b). That value was calculated using the data collected from the measurements performed at 24 and 48 h. The following indexes were calculated: (i) Average Well Colour Development (AWCD), which is the average absorbance value of the plate; (ii) Number of Utilized Substrates (NUS), which are the number of wells with an absorbance value >0.25; (iii) Area Under the Curve (AUC), which integrates not only the values at 40 h, but the whole activity since the beginning of the incubation. AUC is the area under the curve created from the representation of AWCD though time, from 0 to 40 h.

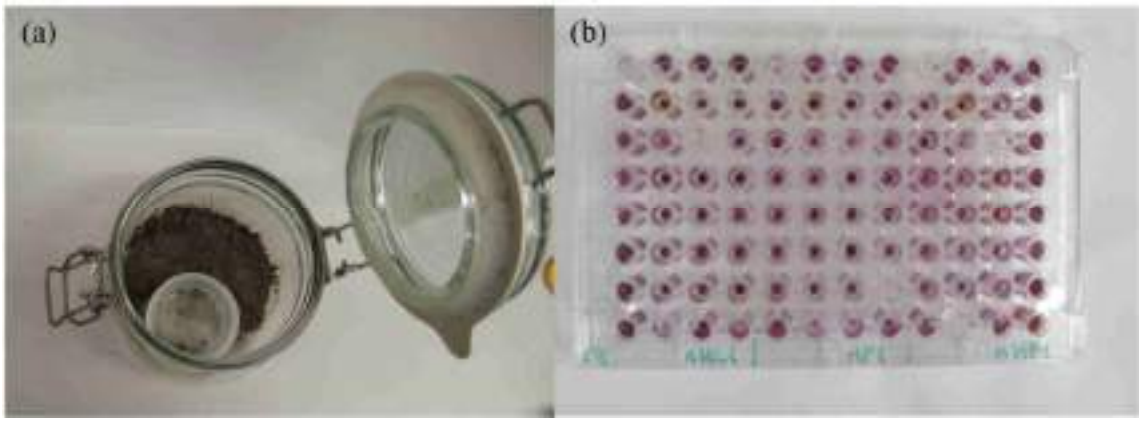


Figure 3.3. Determination of soil respiration (a) and a Biolog Ecoplate™ (b).

3.9. Toxicity bioassays

3.9.1. Phytotoxicity bioassays

Phytotoxic effects were assessed using bioassays performed with three species: *Cucumis sativus , Lactuca sativa* and *Raphanus sativus.*

Seeds of *C. sativus* (c.v. Marketmore) and *L. sativa* (c.v. May Queen) were pre-germinated in darkness on wet filter paper in 8.5 cm Petri dishes for 72 h (*C. sativus*) or 48 h (*L. sativa*) in a Sanyo incubation chamber (Sanyo Incubator) under the following conditions: 14/10 h day/night and 25/18ºC day/night. Concomitantly, 10 g of dried soil were placed on Petri dishes, saturated with deionized water, mixed vigorously and covered with filter paper. The soils were incubated in the same conditions as the seeds. After pre-germination, 7-8 (*C. sativus*) or 10-12 (*L. sativa*) seeds showing a radicle length of 5-10 mm were selected and placed in a single line over the filter paper of the dishes containing the soil. Dishes were sealed with Parafilm M™ (Bemis) wrapping film and incubated for another 72 h at the previously described conditions, with a photosynthetic photon flux density of 100 μmol photon m^{-2} s^{-1} during day-time. Pictures of the seedlings were taken right after the pre-germination and after the exposure period. Images were processed using the ImageJ software (Schneider et al., 2012) to calculate root elongation (RE) ($RE = RE_{T2} - RE_{T1}$) of each seedling.

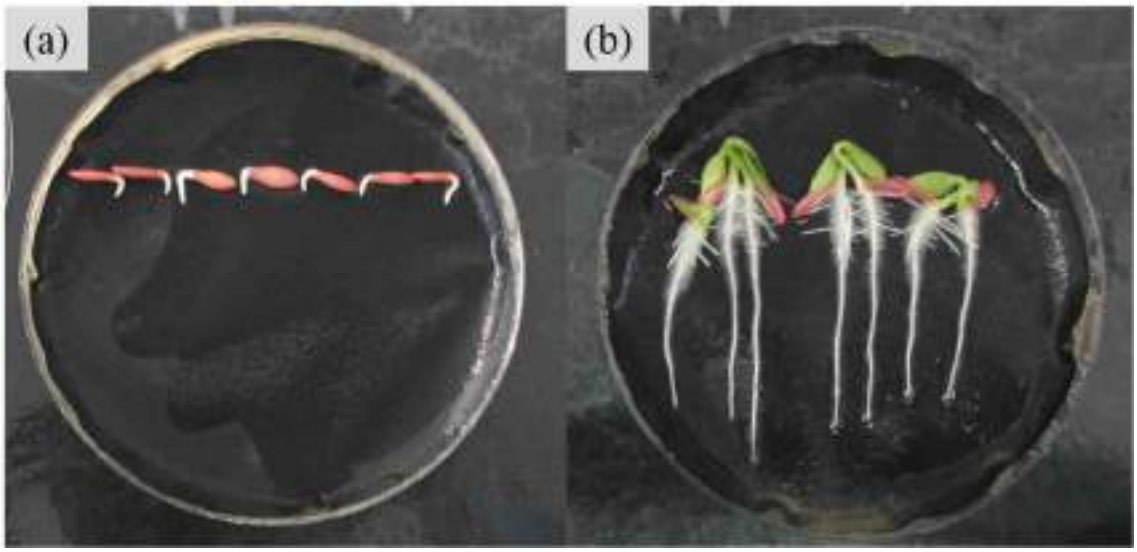


Figure 3.4. *C. sativus* seedlings at transplantation (72 h) (a) and final time (144 h) (b).

For *R. sativus*, the method described by Aparicio et al. (2019) was used. Thirty seeds were placed in petri dishes containing 15 g of soil, which had been adjusted to a humidity of 40% with sterile distilled water. Plates were sealed with Parafilm M™ (Bemis) wrapping and incubated at 22ºC for 5 days. After incubation, germination rate was calculated and the length of hypocotyls and radicles of the seedlings was measured.

3.9.2. Ecotoxicity bioassays with Eisenia fetida

Eisenia fetida was the organism selected to assess potential toxicity of the contaminants to soil invertebrates as described by Iriziar et al. (2015). The stock population was purchased from Hezieko SA (Aizarnazabal, Spain) and kept in the laboratory at 18ºC in horse manure. Healthy and clitellated earthworms of similar size (350-500 mg fresh weight) were selected and transferred to a control soil (10% sphagnum peat, 70% sand and 20% kaolin clay) to allow acclimation for 24 h. Afterwards, worms (ten individuals / 750 g soil) were placed in glass containers containing the sample soils. Containers were kept at 19ºC under controlled humidity and continuous light for 14 days. After that period, worms were collected and the following biomarkers were assessed: mortality, weight loss, coelomocyte concentration and cell viability (García-Velasco et al., 2017).

3.10. Plant physiological measurements

3.10.1. Fluorescence measurements

Prior to harvesting, plants were kept in pre-dawn conditions. Chlorophyll a fluorescence (F_0) was measured with a fluorimeter PAM 2500 (Walz, Germany). Measurements were performed on youngest fully expanded leaves. Basal (F_0) and maximal fluorescence (Fm) were measured in dark-adapted leaves with a saturation pulse of 8000 μmol photon m^{-2} s^{-1} (Fernández-Marín et al., 2015). Maximum photochemical efficiency of the PSII was estimated using the ratio $Fv/Fm = (Fm-F_0) / Fm$.

3.10.2. Tocopherol and photosynthetic pigment composition

Six discs with a diameter of 3 mm were collected from the youngest fully expanded leaf, frozen in liquid nitrogen and stored at -80ºC until processing. Samples were homogenized in acetone (100%) using a tissue-tearor (Model 395; Dremel, Mexico D.F., Mexico). After that, samples were centrifuged at 13200 *x g* for 20 min. The supernatant was collected, adjusted to a volume of 1.5 mL and filtered by a 2 μm PFTE filter

(Teknokroma, Barcelona, Spain). The samples were kept cold during the whole process and a green light was used to avoid the degradation of the pigments/tocopherols.

A new and ultra-rapid uHPLC method was developed for quantification of photosynthetic pigments and tocopherols. This method is less time- and solvent-consuming, generates less residue, and provides a higher resolution for all compounds, compared to traditional HPLC methods. Samples were injected into an Acquity™ uHPLC H-Class system (Waters®, Milford, MA, USA), using a reversed-phase column (Acquity UPLC® HSS C18 SB column, 100Å, 1.8 µm, 2.1 mm × 100 mm) and a Vanguard™ pre-column (Acquity UPLC HSS C18 SB, 1.8 µm). The mobile phase had two components: solvent A, acetonitrile: water: methanol: Tris-HCl 1 M (84:12.6:2:1.4); and solvent B, methanol: ethyl acetate (68:32). Tocopherols and pigments were eluted using a linear gradient from 100% of solvent A to 100% of solvent B for the first 2.5 min, followed by an isocratic elution of solvent B for 1 min. The initial conditions (100% solvent A) were restored with a linear gradient of 0.5 min. This isocratic elution with 100% of solvent A was maintained for 2.5 min to re-equilibrate the column prior to the next injection. The flow of the mobile phase was 0.5 mL/min, with a working pressure of around 5000 psi. The column was maintained at 45 °C in an oven. The volume of the injected sample was 2 µL. The column was preserved overnight with 100% acetonitrile at 0.02 mL/min. Pigments were analyzed with a photodiode detector (PDA uHPLC Acquity by Waters, Milford, MA) in a range of 400-700 nm for their identification, and quantification was done using the integration at 445 nm. Tocopherols were detected by fluorescence, using FLR uHPLC Acquity by Waters (Milford, MA), at an emission wavelength of 340 nm and 295 nm of excitation. Retention time and conversion factors are indicated in Table 3.2.

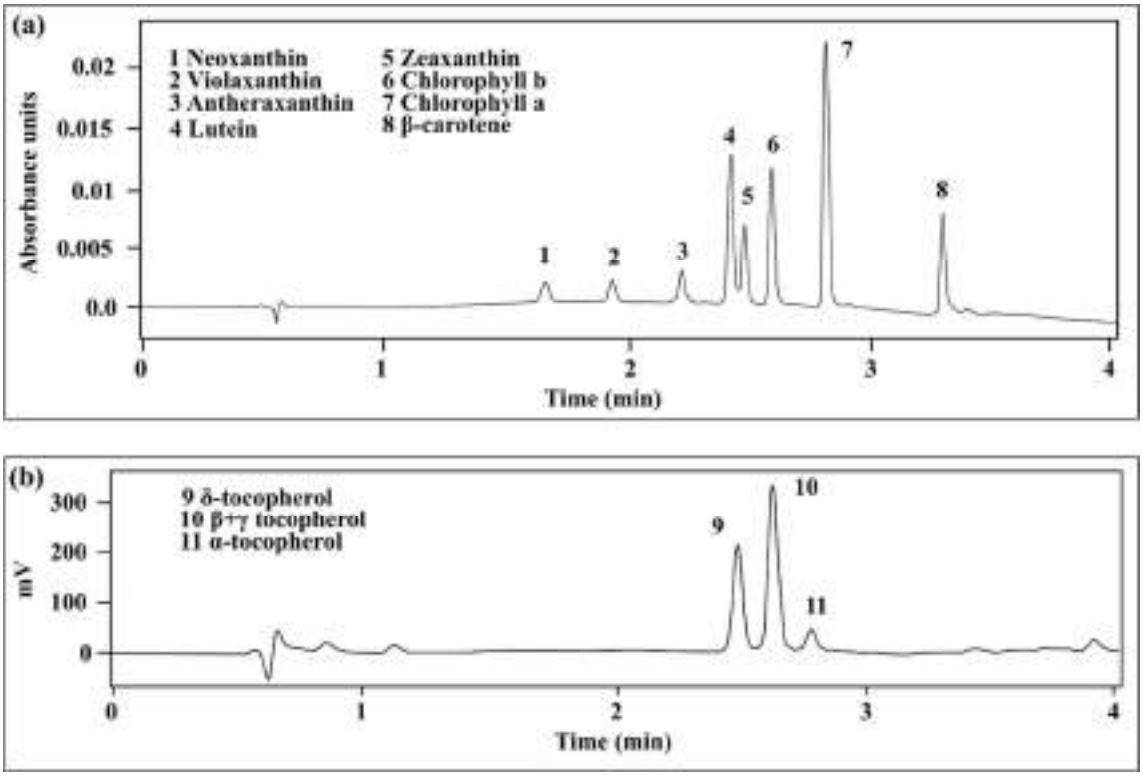


Figure 3.5. UHPLC chromatograms showing the typical pattern of pigments (a) and tocopherols (b) in *Brassica napus* leaves.

Table 3.2. Retention times (RT) and conversion factors of pigments and tocopherols analyzed with the UHPLC system.

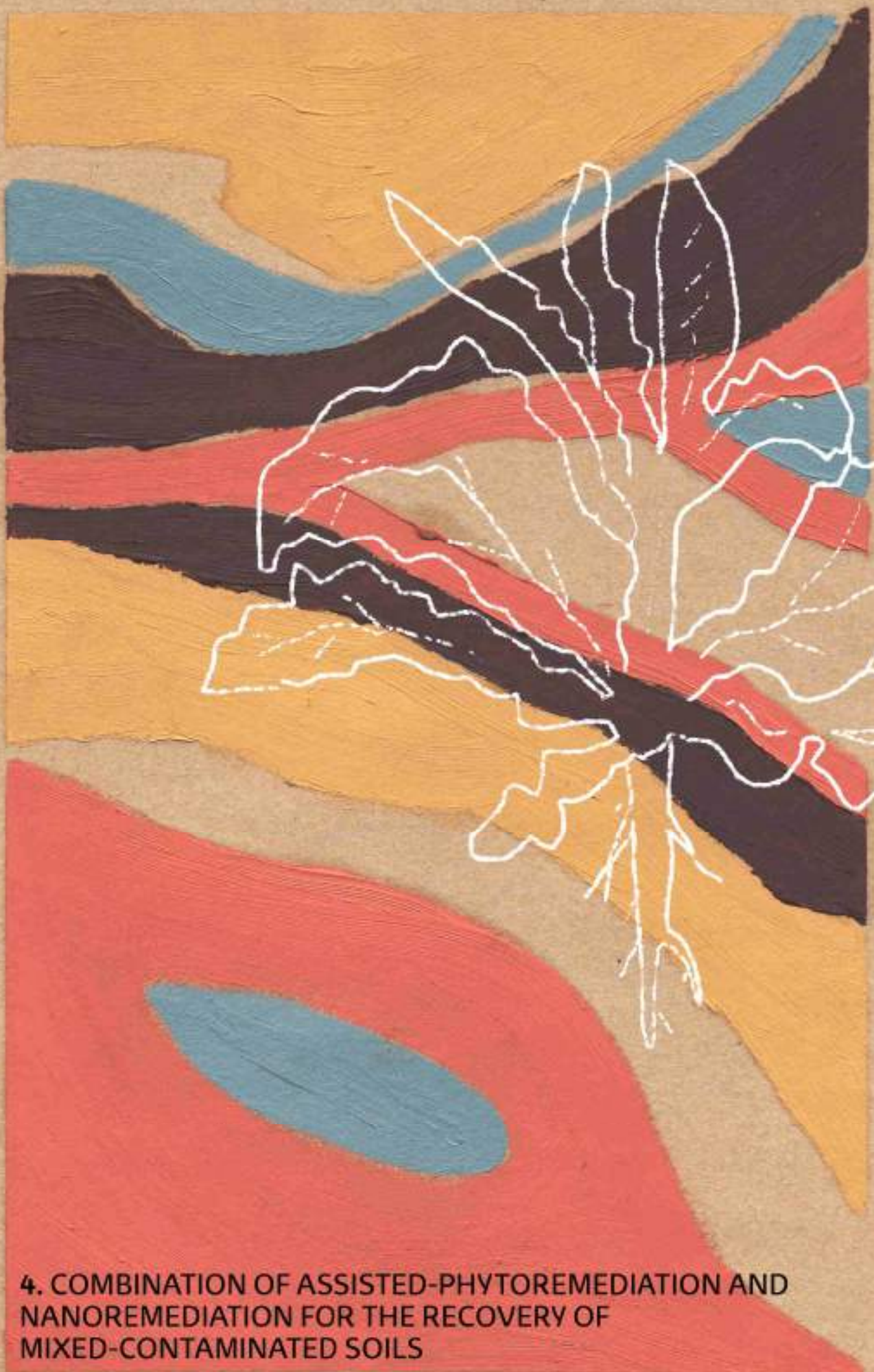
Pigment/tocopherol	RT (min)	CF (pmol mV^{-1})
Neoxanthin	1.72	1.19×10^{-4}
Violaxanthin	1.98	7.84×10^{-5}
Antheraxanthin	2.27	8.00×10^{-5}
Lutein	2.47	8.00×10^{-5}
Zeaxanthin	2.52	8.38×10^{-5}
Chlorophyll b	2.63	1.56×10^{-4}
Chlorophyll a	2.85	2.58×10^{-4}
β-carotene	3.32	8.39×10^{-5}
δ-tocopherol	2.53	7.30×10^{-7}
β+γ tocopherol	2.67	3.30×10^{-7}
α-tocopherol	2.81	2.22×10^{-6}

3.10.3. Carbon, nitrogen and hydrogen contents in plant samples

These analyses were performed by the Research Support Services of the University of A Coruña, by instant combustion, using a FlashEA1112 (ThermoFinnigan) elemental analyzer.

3.11. Data treatment

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 24. The normality of data distributions was checked performing a Kolmogorov-Smirnov and Saphiro-Wilk tests. Homocedasticity was checked using a Levene test. When the data had normal distribution, Student's t-test or one/two/three-way ANOVAs were used. Differences between groups were assessed by the post-hoc Duncan when the homocedasticity condition was met, and by the post-hoc Games-Howell when not. Data without normal distribution were subjected to non-parametric tests, such as Mann-Whitney's U test and Kruskal-Wallis test. In Chapter 7, with the purpose of integrating data from different parameters, the Integrative Biological Response (IBR) index was calculated following Beliaeff and Burgeot (2002). In Chapter 6, a Principal Component Analysis (PCA) was performed using the software The Unscrambler 9.2.



4. COMBINATION OF ASSISTED-PHYTOREMEDIATION AND NANOREMEDIATION FOR THE RECOVERY OF MIXED-CONTAMINATED SOILS

4.1. *Brassica napus* HAS A KEY ROLE IN THE RECOVERY OF THE HEALTH OF SOILS CONTAMINATED WITH METALS AND DIESEL BY RHIZOREMEDIATION

Rafael G. Lacalle, María T. Gómez-Sagasti, Unai Artetxe, Carlos Garbisu, José M. Becerril, 2018. *Brassica napus* has a key role in the recovery of the health of soils contaminated with metals and diesel by rhizoremediation. **Science of the Total Environment**, 618: 347-356.

Abstract

Contaminated soils are frequently characterized by the simultaneous presence of organic and inorganic contaminants, as well as a poor biological and nutritional status. Rhizoremediation, the combined use of phytoremediation and bioremediation, has been proposed as a Gentle Remediation Option to rehabilitate multi-contaminated soils. Recently, newer techniques, such as the application of metallic nanoparticles, are being deployed in an attempt to improve traditional remediation options. In order to implement a phytomanagement strategy on calcareous alkaline peri-urban soils simultaneously contaminated with several metals and diesel, we evaluated the effectiveness of *Brassica napus* L., a profitable crop species, assisted with organic amendment and zero-valent iron nanoparticles (nZVI). A two-month phytotron experiment was carried out using two soils, i.e. amended and unamended with organic matter. Soils were artificially contaminated with Zn, Cu and Cd (1,500, 500 and 50 mg kg^{-1}, respectively) and diesel (6,000 mg kg^{-1}). After one month of stabilization, soils were treated with nZVI and/or planted with *B. napus*. The experiment was conducted with 16 treatments resulting from the combination of the following factors: amended/unamended, contaminated/non-contaminated, planted/unplanted and nZVI/no-nZVI. Soil physicochemical characteristics and biological indicators (plant performance and soil microbial properties) were determined at several time points along the experiment. Carbonate content of soils was the crucial factor for metal immobilization and, concomitantly, reduction of metal toxicity. Organic amendment was essential to promote diesel degradation and to improve the health and biomass of *B. napus*. Soil microorganisms degraded preferably diesel hydrocarbons of biological origin (biodiesel). Plants had a remarkable positive impact on the activity and functional diversity of soil microbial communities. The nZVI were ineffective as soil remediation tools, but did not cause any toxicity. We concluded that rhizoremediation

with *B. napus* combined with an organic amendment is promising for the phytomanagement of calcareous soils with mixed (metals and diesel) contamination.

4.1.1. Introduction

In an increasingly industrialized world, soil contamination resulting from the intensification and expansion of human activities entails a serious threat to human and ecosystem health. A recent European report estimates a total of 2.5 million potentially contaminated sites in Europe, of which 340,000 are likely to require remediation (Van Liedekerke et al., 2014).

Metal(oid)s (e.g., zinc –Zn–, copper –Cu– and cadmium –Cd–) and mineral oils (e.g., diesel fuel) are among the most widely spread contaminants, currently affecting 35 and 24% of European topsoils, respectively (Van Liedekerke et al., 2014). Activities such as mining, metallurgy, agriculture and the use of fossil fuels discharge a considerable amount of metal contaminants into soils, whilst accidental spills of petroleum-based products used for transportation (typically diesel-type fuels) are the principal cause of contamination with organic compounds (Barrutia et al., 2011b). Furthermore, these contaminants frequently appear together in contaminated soils (Khan and Kathi, 2014), rendering new challenges for their remediation (Agnello et al., 2016). Until now, few studies have focused on the remediation of mixed contaminated soils (Agnello et al., 2016; Batty and Dolan, 2013), due to the higher experimental complexity and the difficulty of selecting a suitable remediation technology for the simultaneous immobilization and/or removal of metals and (bio)degradation of the organic contaminants.

Soil contamination with metals and diesel can induce unpredictable adverse effects on soils microorganisms and plants and, therefore, compromise soil health (Vamerali et al., 2010). The mobility and bioavailability of metals are, to a great extent, responsible for metal uptake and toxicity (Vamerali et al., 2010), which are in turn conditioned by soil physicochemical characteristics such as pH, redox potential, moisture content, organic matter, and clay content, etc. (Vangronsveld and Cunningham, 1998). Metal bioavailability, in combination with null biodegradability and high persistence in soils, promotes metal bioaccumulation and biomagnification along the food chain (Dar et al., 2017). Diesel fuel, unlike metals, can be biodegraded by microorganisms and, in particular, rhizosphere microbial communities (Barrutia et al., 2011b). However, its strong hydrophobic character, resulting from a low solubility and a high vapor pressure,

makes diesel rapidly associate with organic matter and minerals present in soils, rendering it less bioavailable and more recalcitrant (Megharaj et al., 2011).

In contrast to conventional physical and chemical technologies for soil remediation, which often involve high economic costs, irreversible changes in soil structure, formulation of secondary contaminants, and critical damage to soil macro- and microbiota (Gil-Díaz et al., 2016), *in situ* Gentle Remediation Options (GROs), such as phytoremediation, can provide a cost-effective, environmentally-friendly solution to soil contamination (Agnello et al., 2016). Several studies have evidenced that phytoremediation can promote not only metal phytostabilization (reduction of mobility and bioavailability) (Epelde et al., 2009a; Galende et al., 2014b, 2014c) and phytoextraction (accumulation in shoots) (Barrutia et al., 2010; Epelde et al., 2010), but also the rhizoremediation of organic contaminants (Agnello et al., 2016; Liu et al., 2017; Montpetit and Lachapelle, 2017). Plants exudate organic compounds to the rhizosphere, creating a nutrient-rich environment which influences the behavior of nutrients and metals (Kidd et al., 2009) and stimulates microbial biomass and activity, and, hence, enhances the degradation of organic contaminants (Kuiper et al., 2004) and improves soil health (Galende et al., 2014c).

The selection of plant species is a crucial aspect for phytoremediation success. There are two key criteria, often mutually exclusive, to be considered during plant selection: plant resistance to high concentrations of contaminants and high biomass production (Surriya et al., 2015). *Brassica napus* L. meets both criteria and has been recognized as suitable candidate for metal phytoremediation (Belouchrani et al., 2016). In addition, in the last decade, *B. napus* has attracted scientific and commercial attention due to its use for oil production (Cundy et al., 2016; Dhiman et al., 2016). The combination of the phytoremediation and economic potential of *B. napus* might be decisive for successful remediation of diffusely contaminated areas (Croes et al., 2013). From this perspective, the idea of “phytomanagement” arose (Cundy et al., 2016). Phytomanagement involves the use of profitable plants and the manipulation of the soil-plant system in order to control the bioavailable pool of soil contaminants, maximize economic and/or ecological revenues, and minimize environmental risks (Evangelou et al., 2015). However, the adequacy of *B. napus* to phytomanage soils simultaneously contaminated with metals and organic compounds is still largely unknown.

The success of phytoremediation also involves the recovery of soil health, defined as the ability of the soil to perform its functions (Pardo et al., 2014). Soil microbial properties can be used as ecologically relevant biological indicators of soil health, owing to their quick response, high sensitivity, and capacity to provide information that integrates many environmental factors (Gómez-Sagasti et al., 2012). Besides, biostimulation, through the application of organic and/or inorganic amendments, is a well-known strategy to enhance the success of biological remediation methods. Thus, the application of organic amendments can improve the physicochemical properties of the contaminated soil, by supplying organic matter and nutrients, affecting metal bioavailability, and stimulating the microbial degradation of organic contaminants (Galende et al., 2014c; Sandrin and Maier, 2003).

Finally, nanomaterials (diameter <100 nm) and, specifically, Zero-Valent Iron nanoparticles (nZVI), have emerged as promising tools to remediate contaminated soils and waters (Patil et al., 2016) via a strategy known as nanoremediation. nZVI particles have been used for the remediation of soils contaminated with metals (Gil-Díaz et al., 2017) and organic contaminants (Li et al., 2016). Nonetheless, there is a paucity of information on the effectiveness of nZVI for the remediation of soils simultaneously contaminated with several inorganic and organic contaminants, despite this being the most real scenario. Moreover, soil physicochemical properties can strongly influence the effectiveness and possible toxicity of the applied nanoparticles (Fujioka et al., 2016; Vítková et al., 2017). The application of nZVI to actual contaminated soil is likely to represent a beneficial practice for remediation, but, at the moment, it appears too expensive to be deployed at a large scale in contaminated field sites. Besides, their use is still surrounded by many uncertainties, including potential interference with other remediation phytotechnologies and potential risk for both human and environmental health (Patil et al., 2016).

Here, under microcosm conditions, we studied the effectiveness of nano-rhizoremediation assisted with an organic amendment for the recovery of mixed contaminated soils with organic (diesel) and inorganic contaminants (Zn, Cu, Cd). The specific objectives were as follows: (i) to evaluate the effectiveness of *B. napus* plants, and/or an organic amendment and/or nZVI, to accumulate and/or immobilize Zn, Cd, Cu and to degrade diesel; (ii) to assess the potential of these technologies for the recovery of

soil health determined by microbial and plant indicators; and, finally, (iii) to analyze the ecotoxicity of nZVI.

4.1.2. Materials and methods

4.1.2.1. Experimental design

In September 2015, a soil from a peri-urban area near the city of Vitoria-Gasteiz (42º50'N; 2º40'W, northern Spain) was amended with 100 t ha^{-1} of an organic amendment produced from the recycling of urban organic wastes. Soil from the same peri-urban area remained unamended. In July 2016, topsoil (0-15 cm) was collected from both the unamended and amended area, sieved to <6 mm, air-dried and subjected to physicochemical characterization (Table 4.1.1). In the laboratory, half of each soil was artificially contaminated with a mixture of metals and commercial diesel fuel purchased from a petrol station. Final metal concentrations were (in mg kg^{-1} DW soil): Zn (1,500), Cu (500) and Cd (50). As metals were added as nitrate salts, KNO_3 was added to non-contaminated soils (control) in order to compensate the additional content of nitrate in contaminated soils. Immediately after, diesel (6,000 mg kg^{-1} DW soil) was added to already metal contaminated soils, following ISO 15952 (2006). Then, 700 g DW of contaminated or non-contaminated soil were placed in 1 L pots to complete a total of 64 pots: 16 treatments and 4 replicates per treatment (Table 4.1.2). In order to allow contaminant stabilization, pots were kept for one month in a phytotron under the following controlled conditions: photoperiod 14/10 h day/night, temperature 25/18 ºC day/night, relative humidity 60/80% day/night, and a photosynthetic photon flux density of 200 μmol photon m^{-2} s^{-1}.

After the 1-month stabilization period (August, 2016), nZVI (NanoFer Star, Nanoiron s.r.o) were activated following the manufacturer's instructions with Milli-Q water for 24 h and then applied in aqueous solution to half of the pots (contaminated and non-contaminated) at a concentration of 1 g nZVI kg^{-1} DW soil. The nZVI treatment was identified as "n".

Table 4.1.1. Soil physicochemical characteristics.

	Unamended soil	**Amended soil**
Total clay (%)	23.4	15.7
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Texture class (USDA)	Loam	Loam
pH (1:2.5)	7.9	8.0
Carbonates (%)	54.7	44.0
Organic Matter (%)	1.0	19.5
C organic / N organic	6.7	8.6
Total N (% DW)	0.1	0.9
Total C organic (% DW)	0.6	7.3
[Zn] Tot / Bio mg kg^{-1}	41.4 / 0.0	127.8 / 0.0
[Cu] Tot / Bio mg kg^{-1}	6.9 / <0.1	73.3 / <0.1
[Cd] Tot / Bio mg kg^{-1}	0.3 / 0.0	0.5 / 0.0

Tot: Pseudo-total metal concentration; Bio: $CaCl_2$-extractable metal concentration; USDA: United States Department of Agriculture.

Soil samples for chemical analysis and biological assays were taken immediately before and after nZVI application and then stored at 4 ºC. Three days after nZVI application, half of the pots were sowed with *Brassica napus* L. (30 seeds pot^{-1}). After plant emergence (6 days), seedling number per pot was reduced to 3, by manually removing extra seedlings with their roots, and photosynthetic photon flux density was increased to 250 µmol photon m^{-2} s^{-1} to enhance plant growth. The experiment was conducted under the above-mentioned phytotron controlled conditions and pots were bottom watered periodically as needed. One month after sowing (September, 2016), plants and soils were collected for chemical analysis and biological assays.

Table 4.1.2. Experimental treatments.

	Without nZVI				With nZVI			
	Unamended		Amended		Unamended		Amended	
	Control	Mixed C	Control	Mixed C	Control	Mixed C	Control	Mixed C
Not planted	UCN	UMN	ACN	AMN	nUCN	nUMN	nACN	nAMN
Planted	UCP	UMP	ACP	AMP	nUCP	nUMP	nACP	nAMP

Control: non-contaminated soil;
Mixed C: mixed contaminated soil with several metals and diesel.

Considering the presence of amendments (A-amended/U-unamended), the presence of contaminants (C-control/M-mixed contamination), the presence of plants (P-

planted/N-non-planted) and the treatment with nZVI (n), the experiment was conducted with 64 pots belonging to 16 treatments, with 4 replicates each (Table 4.1.2).

4.1.2.2. Soil physicochemical characterization

Amended and unamended soils were collected at: (i) T0 (September, 2015), just after the addition of the organic amendment to the soil, (ii) T1 (July, 2016), immediately after the addition of the contaminants; (iii) T2 (August, 2016), just after the 1-month stabilization period; and (iv) T3 (September, 2016), at harvest time. Immediately after sampling, soils were kept at 4 ºC prior to the determination of soil microbial properties (see Section 2.3).

For the determination of soil physicochemical properties and for the root elongation phytotoxicity bioassay (see Section 2.4), soil samples were oven-dried at 35 ºC for 48 h. Soil pH was determined (1:2.5 w/v soil:water) using 10 g of 2-mm sieved dried soil and 25 mL of deionized water. Physicochemical parameters, i.e. particle size distribution, % organic matter, total organic carbon (TOC; detection of CO_2 by infrared after oxidation), total nitrogen (Kjeldhal method), and % carbonates were determined following official methods (MAPA, 1994). Dry soil samples were ground and sieved at 0.125 mm prior to the analysis of pseudo-total and $CaCl_2$-extractable fraction of metals by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700). In order to determine total concentration of Zn, Cu and Cd in soil, samples were subjected to acid digestions with HCl and HNO_3 + H_2O_2 according to US-EPA Method 3051A (2007). The $CaCl_2$-extractable fraction, an indicator of metal bioavailability, was determined according to Houba et al. (2000). Aliphatic hydrocarbon concentration in soil was determined as described by Bartolomé et al. (2005). Shortly, 15 mL of acetone (extraction solvent) was added to 0.5 g of dried soil. Extraction was performed using a MDS-2000 closed microwave solvent extraction system (CEM, Matthews, NC, USA). The filtered (0.45 µm) extract was cleaned by performing a solid phase extraction (SPE) with Florisil® cartridges. All the compounds were analyzed by Gas Chromatography–Mass Spectrometry (GC–MS). The profile of n-alkanes in the commercial diesel contained hydrocarbons from n-C9 to n-C30. In order to monitor preferential degradation of total petroleum hydrocarbons (TPH) of diesel, we identified three main groups; (i) n-Alkanes (n-Alk); (ii) polycyclic aromatic hydrocarbons (PAHs); and (iii) fatty acid methyl esters (FAMEs). We also determined n-alkane degradation according to the length of the carbon

chain, i.e. the following four fractions: (i) n-C9–C12; (ii) n-C13–C16; (iii) n-C17–C21; and (iv) n-C22–C30.

4.1.2.3. Soil microbial properties

Soil samples were used to determine the following microbial properties, as detailed in Galende et al. (2014a): (i) microbial activity was determined by basal respiration (BR) following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR) following ISO 17155 (2002); (iii) average well color development (AWCD) and (iv) number of metabolized substrates (NUS) were determined from Biolog EcoPlates™.

4.1.2.4. Root elongation phytotoxicity bioassay

A root elongation bioassay with *Cucumis sativus* was performed to evaluate soil phytotoxicity. Seeds of *C. sativus* (c.v. Marketmore) were pre-germinated on 8.5 cm diameter Petri dishes, containing wet filter paper, for 3 days under controlled conditions (14/10 h day/night; 25/18 ºC day/night; and full darkness). Concurrently, 10 g of dried soil were placed on Petri dishes, hydrated with deionized water, mixed vigorously, and covered with filter paper. After pre-germination, seven seeds of *C. sativus* showing a radicle length of 5-10 mm were placed over the filter paper of soil-containing Petri dishes. Afterwards, dishes were placed at an angle of 45º and incubated for 72 h under the following conditions: photoperiod 14/10 h day/night, temperature 25/18 ºC day/night, relative humidity 60/80 % day/night and photosynthetic photon flux density of 100 µmol photon m^{-2} s^{-1}. Three technical replicates were analyzed for each biological replicate. Images of the seedlings were taken at the beginning and after 72 h of incubation with the soil. Images were processed by ImageJ software. Root Elongation (RE) ($RE = RE_{T2} - RE_{T1}$) was calculated for each seedling. The percentage of RE was calculated considering "amended control soil, non-treated with nZVI (ACN)" as reference state (i.e., 100 %).

4.1.2.5. Plant growth, physiological status and metal concentrations

Prior to harvest, maximal photochemical efficiency of PSII (Fv/Fm) was determined in leaves of plants at predawn state using a portable modulated fluorimeter (FluorPen FP 100. Photon System Instruments) as described in García-Plazaola and Becerril (2000). Subsequently, 5 leaf discs of 6 mm diameter (40 mg FW) were collected, frozen in liquid N, and stored at -80 ºC until pigment analysis. Photosynthetic and photoprotective

pigments (chlorophylls and carotenoids) as well as lipophilic antioxidants (α-, β- and γ-tocopherol), were determined according to García-Plazaola and Becerril (2001). Finally, 1-month-old *B. napus* whole-plants were harvested. Leaves, stems and roots were manually separated, weighted (fresh weight, FW) and washed with deionized water. Roots were also soaked with 0.01 M $CaCl_2$ for 30 min to remove adsorbed metals. Plant samples were then oven-dried at 80 ºC for 48 h and their dry weights (DW) recorded and used for metal determination according to Zhao et al. (1994). Metal phytoextraction (shoot metal concentration × shoot biomass) was also calculated.

4.1.2.6. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 23. Normality was checked performing a Kolmogorov-Smirnov test. Normal data were tested using Student's t-test and ANOVA, using Duncan *post hoc* when there was homocedasticity (checked with Levene test) and Games-Howell when not. Non-normal data were analyzed by applying non-parametric tests as Mann-Whitney's U-test and Kruskal-Wallis test.

4.1.3. Results

4.1.3.1. Effect of treatments on contaminant concentrations

The soils collected for this study have a loam texture, alkaline pH (8.0) and a very high content of carbonates (55 and 44% for unamended and amended soils, respectively) (Table 4.1.1). The main difference between both soils lays in the higher values of metal (Zn, Cu and Cd) concentration (because of the concentration of these metals in the amendment, as shown in Table 4.2.1), C and N contents (both 10-fold higher), and organic matter content (19.5%) shown by the amended soil, as compared with the unamended soil.

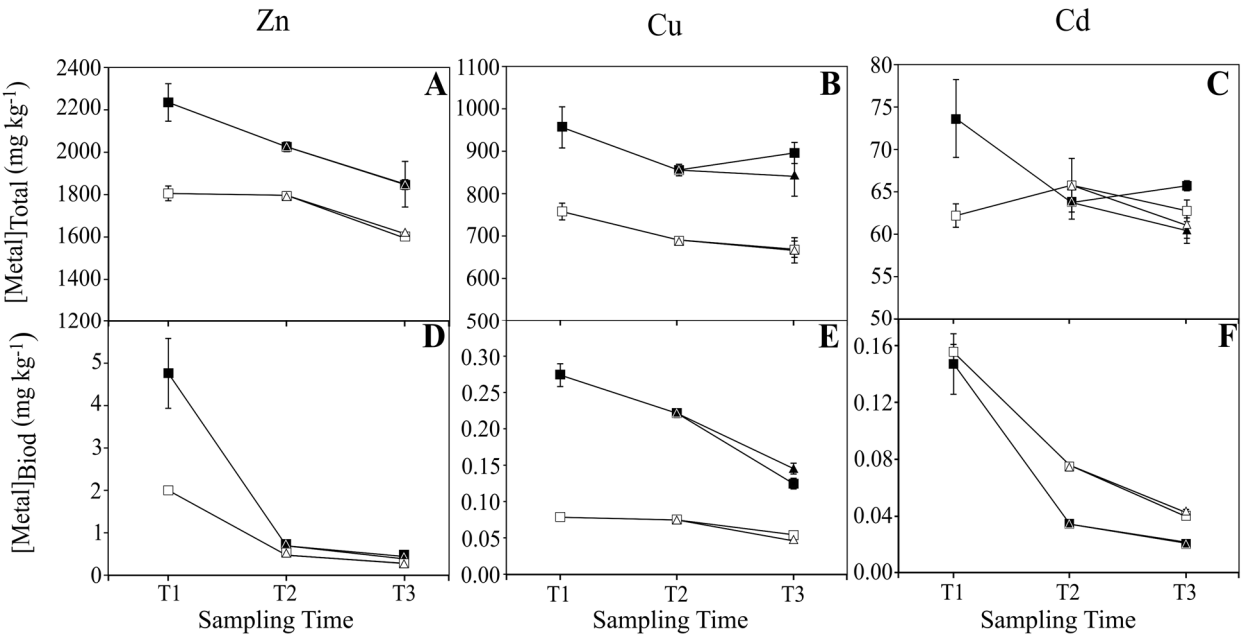


Figure 4.1.1. Pseudo-total and $CaCl_2$-extractable concentrations of Zn (A, D), Cu (B, E) and Cd (C, F) in soils. White icons represent unamended soils and black icons refer to amended soils. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

Pseudo-total Cd, Cu and Zn concentrations in soil were not lower at harvest time (T3) than immediately after the 1-month stabilization period (T2) (Fig. 4.1.1A-C). In fact, higher metal concentrations in soil were found for all metal treatments, especially in amended soils, as compared with the initial spiked concentrations of Zn, Cu and Cd (1,500, 500 and 50 mg kg^{-1} DW soil, respectively). By contrast, $CaCl_2$-extractable metal fractions decreased to a great extent as soon as metals were added to the soil (T1) (Fig. 4.1.1D-F). Immediately after metal addition (T1), $CaCl_2$-extractable metal fractions represented as low as 0.25% of the pseudo-total concentrations for all metals and treatments. Interestingly, the presence of the organic amendment increased the bioavailability of Zn and Cu (Fig. 4.1.1D, E), but reduced Cd bioavailability (Fig. 4.1.1F). Metal bioavailability progressively decreased, to a lesser extent, throughout the experiment, particularly during the stabilization period (T2). At harvest time (T3), Zn and Cu bioavailability still remained higher in the presence of the organic amendment than in its absence, while Cd bioavailability was lower. Values of pseudo-total and bioavailable metal concentrations were not significantly affected by the presence of plants (Fig. 4.1.1, Table 4.2.2) nor by the presence of nZVI (Table 4.2.2).

Immediately after diesel addition (T1), TPH concentrations in soil were 2,900 and 2,400 mg kg^{-1} DW soil for unamended (UMN) and amended (AMN) soils, respectively (Fig. 4.1.2A). At this time, the main TPH family corresponded to n-Alk (92%), followed by FAMEs (7%). The concentrations of n-Alk and FAMEs were initially lower in amended soils; soil concentration values for both families rapidly decreased after one month of stabilization (T2). This was particularly relevant for FAMEs, which almost disappeared at T2, in both amended and unamended soils. At harvest time (T3), a concentration of 900 mg TPH kg^{-1} DW soil of TPH was detected in all contaminated soils (UMN, UMP, AMN, AMP) (Fig. 4.1.2A). Neither plants (Fig. 4.1.2A) nor nZVI (Fig. 4.1.2D) affected the degradation of these compounds (Table 4.2.3).

When different n-alkanes fractions were separately analyzed (Fig. 4.1.2B), the most abundant fractions in our commercial diesel were long chain n-alkanes (n-C17–n-C21, followed by n-C22–n-C30 and n-C13–n-C16). Very low levels of n-C9–n-C12 were observed (data not shown). The degradation pattern of these longer chain n-alkanes (n-C13–to n-C30) was similar to that previously described for total n-Alk (i.e., lower concentration in amended soils and progressive degradation throughout the experiment). Concentration values detected here for the shortest n-alkanes (n-C9–n-C12) and PAHs correspond to hydrocarbons and aromatic compounds of biological origin, already present in the non-contaminated soil and in the amendment itself (data not shown).

Globally considered, at the end of the study, diesel concentration values for the main families and fractions were similar among treatments, and neither plants (Fig. 4.1.2A-C) nor nZVI (Fig. 4.1.2D-E) stimulated a preferential degradation of any of them.

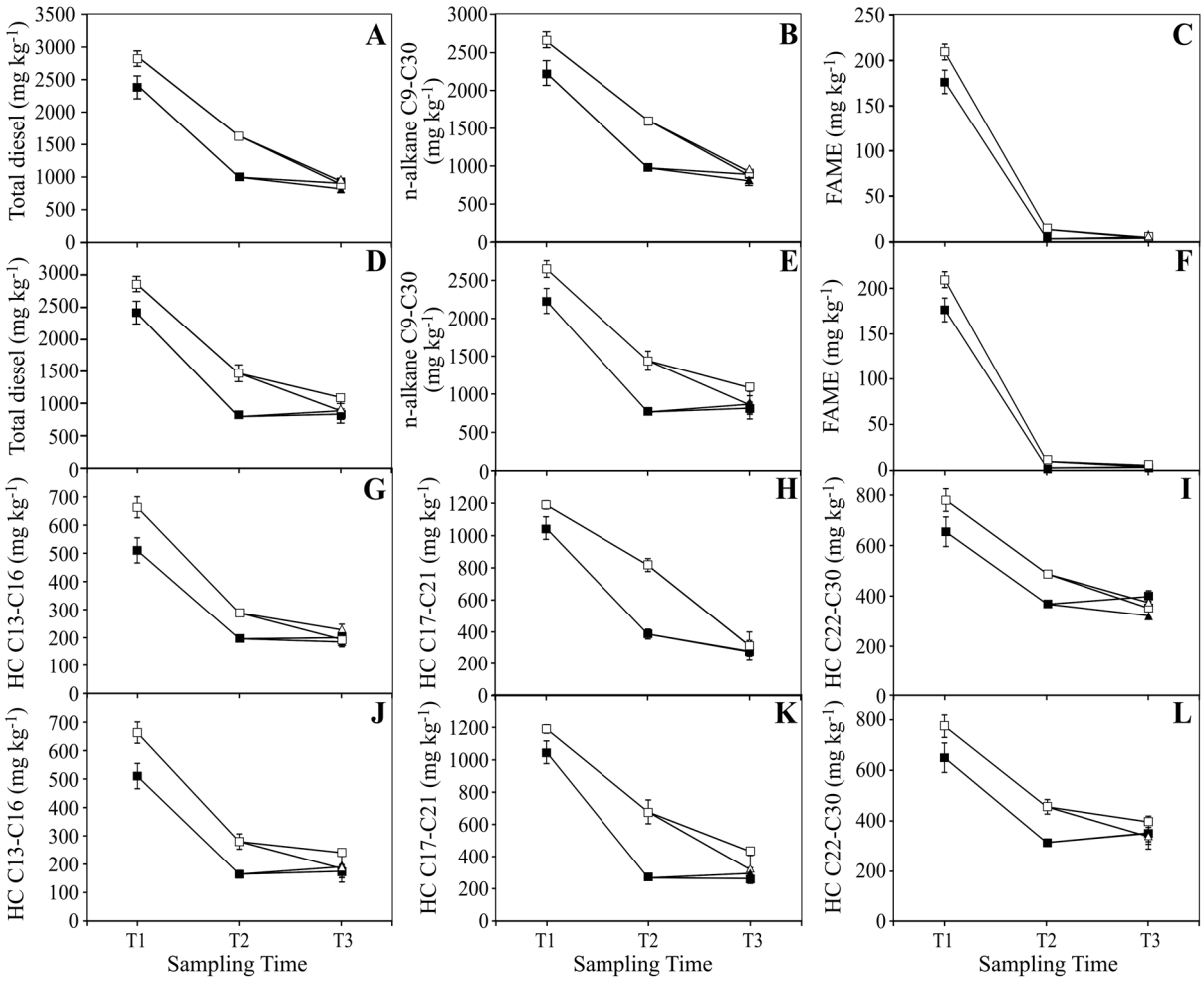


Figure 4.1.2. Total and fractioned hydrocarbon concentration in soil (without, with nZVI). Total Petroleum Hydrocarbon (TPH) (A, D); n-alkanes (n-Alk) (B, E); FAMEs (C, F); n-alkane fractions C13–C16 (G, J); C17–C21 (H, K); and C22–C30 (I, L). White icons represent unamended soils and black icons refer to amended soils. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

4.1.3.2. Effect of treatments on plant growth, physiological status and metal concentrations

As shown in Table 4.1.3, biomass of *B. napus* plants significantly increased in the presence of the organic amendment, both in control plants (ACP > 4-fold UCP) and, remarkably, in plants grown in contaminated soils (AMP >17-fold UMP). The presence of the amendment alleviated contaminant phytotoxicity, as we found no significant differences in biomass between controls (ACP) and plants grown in contaminated soils (AMP). Other plant parameters, such as photochemical efficiency, total chlorophyll, total

carotenoids and VAZ/Chl, were not affected by treatments (Table 4.1.3). However, the tocopherol/Chl index increased in plants grown in contaminated soils and in unamended soils. nZVI treatment had no significant effect on the plant parameters studied here.

Metal concentrations in plant shoots were markedly influenced by the applied metal dose, metal mobility, and the presence of the organic amendment. Plants grown in amended soils showed lower metal concentrations in their shoots than plants from unamended soils (Table 4.1.3). The highest values of metal shoot accumulation were detected for Zn, followed by Cu and Cd (Table 4.1.3). The addition of nZVI appeared to decrease shoot metal concentration, but this effect was only statistically significant for Zn and Cu in plants grown in amended soil, and for Cd in plants grown in both unamended and amended soils. Although shoot metal concentrations in plants grown in amended soils (AMP, nAMN) were lower than those in plants grown in unamended soils (UMP, nUMP), the total amount of phytoextracted metal was higher in the former, due to a higher plant biomass (Table 4.1.3). Highest values of phytoextraction were found for Zn, followed by Cu and Cd. As indicated for metal concentrations in shoots, nZVI treatment significantly decreased metal phytoextraction in contaminated amended soils (AMP *vs.* nAMP, Table 4.1.3). In any case, phytoextraction values were very low for all treatments, most likely due to the low values of metal bioavailability.

Table 4.1.3. Plant parameters in Brassica napus at harvest time (T3). Biomass (g); [Metal]Shoot (mg kg^{-1}); Phytoextraction (µg); Fv/Fm; Chl a+b (pmol mm-2). CART, VAZ and TOCT were expressed in pmol pmol Chl^{-1}.

	UCP	UMP	ACP	AMP	nUCP	nUMP	nACP	nAMP
BShoot	1.1 ± 0.2 b	0.3 ± <0.1 c	4.8 ± 0.8 a	5.3 ± 0.5 a	1.2 ± 0.2 b	0.4 ± <0.1 c	4.3 ± 0.8 a	5.9 ± 0.4 a
ZnShoot	-	1305.2 ± 85.6 a		497.3 ± 54.8 b	-	1162.7 ± 137.3 a		361.3 ± 8.2 c
CuShoot	-	51.9 ± 3.5 a	-	27.8 ± 2.88 a	-	41.4 ± 7.1 a	-	19.0 ± 0.4 b
CdShoot	-	29.7 ± 4.8 a	-	14.1 ± 1.09 b	-	18.7 ± 2.1 b	-	8.7 ± 0.2 c
PZn	-	383.0 ± 36.3 b	-	2549.4 ± 144.6 a		512.3 ± 86.8 b	-	2129.0 ± 165.1 a
PCu	-	15.2 ± 1.4 c	-	143.1 ± 10.8 a	-	18.5 ± 4.1 c	-	112.0 ± 9.1 b
PCd	-	8.5 ± 0.8 c	-	72.6 ± 4.5 a	-	8.2 ± 1.2 c	-	51.2 ± 4.1 b
Fv/Fm	0.78 ± 0.01 ab	0.78 ± <0.1 ab	0.79 ± 0.1 ab	0.76 ± <0.1 b	0.77 ± <0.1 ab	0.78 ± <0.1 ab	0.79 ± <0.1 a	0.78 ± <0.1 a
Chl a+b	548.4 ± 27.0 a	515.1 ± 9.4 a	598.7 ± 26.9 a	539.8 ± 47.5 a	601.8 ± 31.3 a	600.4 ± 47.3 a	514.8 ± 20.3 a	477.6 ± 18.3 a
CART	319.6 ± 6.6 a	306.8 ± 5.5 a	306.2 ± 3.5 a	314.4 ± 4.9 a	317.9 ± 7.0 a	312.9 ± 8.0 a	301.9 ± 2.7 a	308.6 ± 4.1 a
VAZ	66.4 ± 5.1 a	62.1 ± 1.1 ab	57.7 ± 1.3 ab	60.6 ± 3.1 ab	66.5 ± 3.4 a	68.1 ± 3.0 a	55.5 ± 2.3 b	52.2± 1.9 b
TOCT		28.8 ± 1.6 bc	18.7 ± 1.5 cd	38.2 ± 3.3 b	29.3 ± 4.7 bc	32.8 ± 2.7 b	16.6 ± 1.0 d	52.4 ± 5.4 a

B: Biomass; P: Phytoextraction; CART: Total Carotenoids; VAZ: Total violaxanthin, antherxanthin and zeaxanthin; TOCT : Total Tocopherols

4.1.3.3. Effects of treatments on biological indicators of soil health

4.1.3.3.1. Soil microbial properties

At T0 and T1, amended and unamended soils showed similar SIR values (Fig. 4.1.3C, D); in contrast, higher BR values were found in amended soils (Fig. 4.1.3A, B). At the end of the experiment (T3), control (non-contaminated) amended soils presented higher values of both BR and SIR than unamended soils. The addition of contaminants (T2) had no effect on SIR values (Fig. 4.1.3C, D), but greatly increased BR values (Fig. 4.1.3A, B). Indeed, BR was the most increased microbial parameter, not only as a result of the application of the amendment and contaminants but also due to the presence of plants.

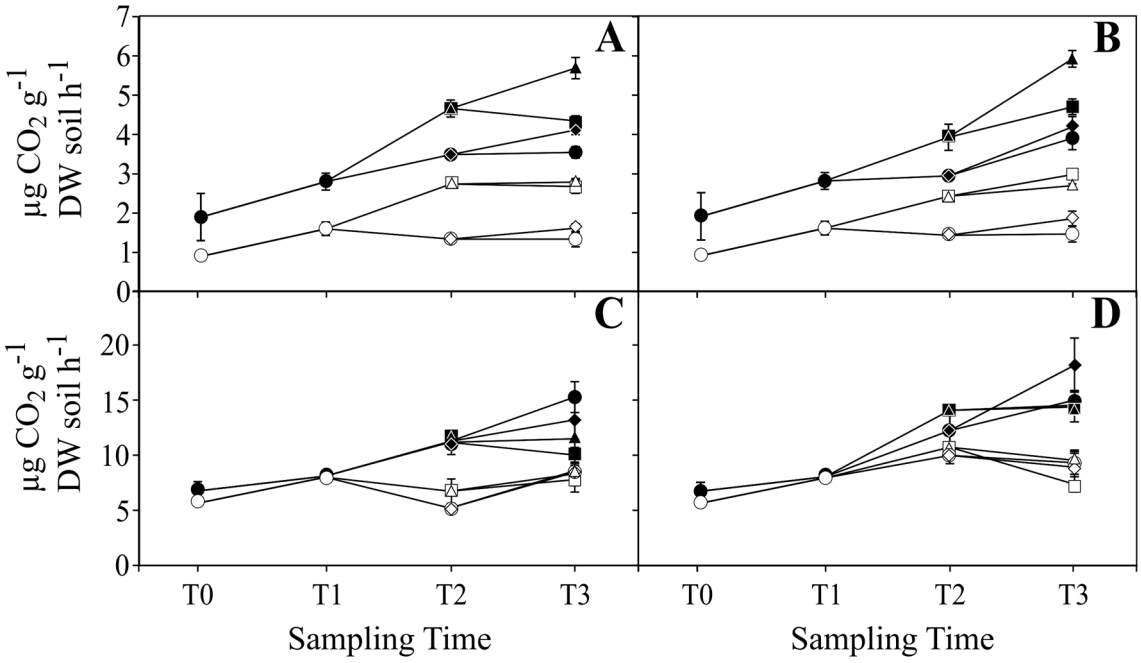


Figure 4.1.3. Soil microbial activity determined by Basal Respiration (BR) (A, B) and microbial biomass determined by Substrate-Induced Respiration (SIR) (C, D) in soil without and with nZVI. White icons represent unamended soils and black icons refer to amended soils. Circles: non-contaminated, unplanted. Diamonds: non-contaminated, planted. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T0: Time of soil collection, immediately after amendment application. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

Consequently, at the end of the study (T3), AMP and n-AMP treatments showed the highest BR values. This fact could be related to plant wellness, as we did not detect such an effect on unamended planted soils (UCP). At T2, AWCD and NUS values

decreased in all treatments, but without significant differences among them (Fig. 4.1.4). At harvest time (T3), highest AWCD and NUS values (Fig. 4.1.4A, C) were observed for treatments with amendment, plants and contaminants (AMP, nAMP). nZVI treatment did not have any significant effect on microbial activity and biomass (Fig. 4.1.3A-D), nor on microbial functional diversity (Fig. 4.1.4A-D). The results of the statistical tests are shown in Tables 4.2.4 and 4.2.5.

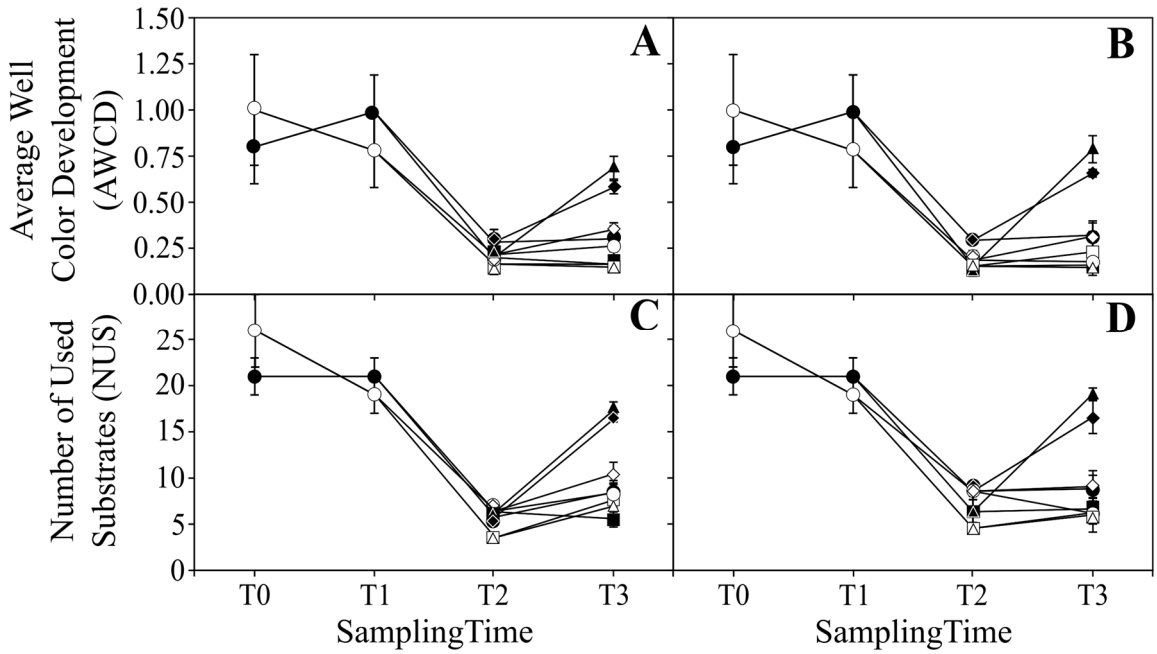


Figure 4.1.4. Soil microbial functional diversity measured with Biolog™ Ecoplates (without, with nZVI). Average Well Color Development (AWCD) (A, B), Number of Used Substrates (C, D). White icons represent unamended soils and black icons refer to amended soils. Circles: Non-contaminated, non-planted. Diamonds: Non-contaminated, planted. Squares: mix contaminated, non-planted. Triangles: mix contaminated, planted. T0: Time of soil collection, immediately after amendment application. T1: Time immediately after the artificial contamination of the soil. T2: Time one month after soil contamination (sowing time). T3: Time two months after soil contamination (harvesting time).

4.1.3.3.2. Effect of treatments on soil phytotoxicity

At T2, *C. sativus* seedlings exposed to the mixed contaminated soil were notably affected by the presence of the amendment. A significant decrease of root elongation (RE) was obtained in contaminated unamended soils (Fig. 4.1.5A, Table 4.2.6), whereas a significant increase in RE was observed in corresponding amended soils (AMN, nAMN; Fig. 4.1.5A). At harvest time (T3), RE % of *C. sativus* seedlings was significantly higher than in T2 in almost all treatments (Fig. 4.1.5B). The negative effect of the mixed

contamination was only observed in unamended soils, while there were no differences between control (non-contaminated) and contaminated amended soils. Neither the presence of *B. napus* plants nor the nZVI treatment had a clear impact on the RE of *C. sativus* seedlings.

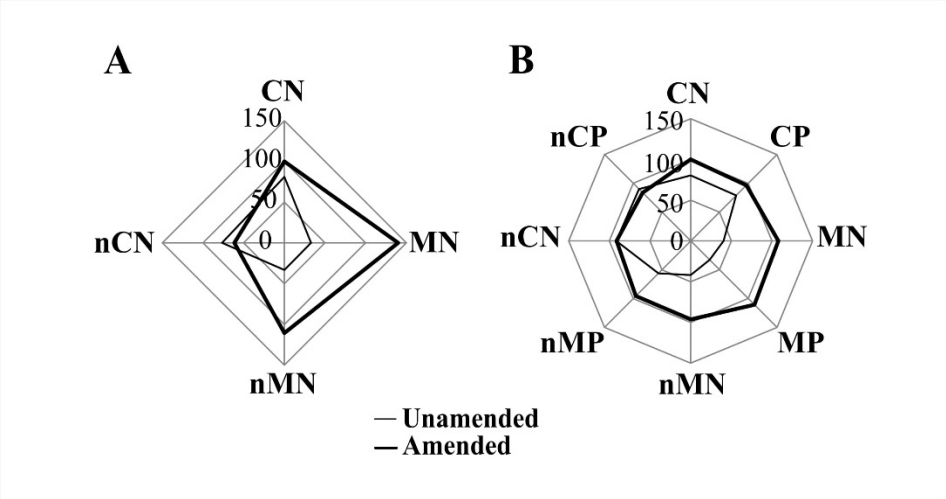


Figure 4.1.5. Variation of Root Elongation percentage (RE %) of *Cucumis sativus* seedlings in T2 (sowing time) (A) and T3 (harvesting time) (B) time-points. The thin line represents unamended soils, whilst the bold line represents amended ones.

4.1.4. Discussion

Phytomanagement focuses on the growth of profitable crops on contaminated vacant sites, in order to simultaneously maximize both economic profit and the provision of ecosystem services, while reducing contaminant mobility and bioavailability, and, hence, adverse ecological impact (Cundy et al., 2016). However, few studies have been conducted to establish the suitability of GROs and associated strategies for mixed contaminated (with inorganic and organic compounds) soils, such as those often found in industrial and urban brownfields. In this study, in order to explore feasible strategies to reduce environmental risk and increase soil functions, we studied soils from a peri-urban vacant area. Here, soils were artificially contaminated with Zn, Cu, Cd and diesel, as these contaminants are commonly found in mixed contaminated soils. Our experimental approach allows to (i) specifically select any combination of contaminants and contaminant concentrations, (i) remove or immobilize such contaminants during the timeframe of the experiment, and (iii) compare the values of soil health parameters between contaminated/remediated soil and uncontaminated (control) soil. However, due

to some key factors such as the aging of contaminants and the complexity of natural field conditions, our results cannot be directly related to actual contaminated soils.

4.1.4.1. Contaminant concentrations and physicochemical parameters

The most relevant result obtained from the addition of metals to this peri-urban soil was the large and rapid immobilization of metals observed in both amended and unamended soils prior to the stabilization period (Fig. 4.1.1D-F). Precipitation and adsorption are two key processes affecting soil metal bioavailability, and both processes are greatly dependent on soil pH (Adriano, 2001). Alkaline pH values, such as those observed here, can enhance precipitation and adsorption processes (Adriano, 2001) and, therefore, contribute to the low values of metal bioavailability observed in this study. However, the soil factor that can better explain these low bioavailability values is most likely the large carbonate content of our soils (44 and 55% for amended and unamended soils, respectively) (Table 4.1.1). Other soil components, such as sulfates, hydroxides, phosphates, silicate clay (Adriano, 2001) and organic matter (Alvarenga et al., 2009), as well as plant growth (Galende et al., 2014c) can secondarily account for a reduction in metal bioavailability. The continuous interaction of metal contaminants with all these soil components could explain the progressive reduction of metal bioavailability observed along the study, after T1. Organic amendments can decrease metal bioavailability (Park et al., 2011), not only due to the interaction between metals and organic components, but also due to the increase in pH (Galende et al., 2014b). Conversely, Cu and Zn bioavailability was increased in amended soils. This contradictory effect could be explained by the lower carbonate content of amended soils, as well as the fact that the organic amendment itself adds Zn and Cu to the soil (Table 4.1.1).

The presence of plants can also decrease metal bioavailability through plant metal uptake and/or metal immobilization in the rhizosphere (Park et al., 2011). However, under our experimental conditions, metal bioavailability in soil was not influenced by *B. napus* growth. The low bioavailability of metals in soil (Fig. 4.1.1D-F) is probably responsible for the severely limited metal accumulation in shoots and phytoextraction (Table 4.1.3). Accordingly, Zn showed highest values of bioavailable concentration and, concomitantly, highest values of metal accumulation and amount of metal phytoextracted. Regarding shoot metal accumulation, the most relevant difference refers to the lower shoot metal concentrations found in amended soils, compared with unamended ones, owing to the

growth dilution factor (Galende et al., 2014c; Hill and Larsen, 2005). Actually, AMP shoot biomass was 17-fold higher than UMP. Due to this growth dilution factor, plants grown in amended soils extracted more Zn (6.6-fold), Cu (9.5-fold) and Cd (10-fold) than those grown in unamended soils. In any event, the total amount of metal phytoextracted was very low for all cases and, then, pseudo-total metal concentrations in soils did not decrease to a significant extent. The higher pseudo-total metal concentrations detected in our study, compared to the spiked doses, can be explained by the sample processing. Thus, soil samples were ground, sieved and, then, fine particles were collected. Metals are usually associated to the fine particle-size fractions of the soil (Xu et al., 2014). This is an important methodological issue that should be taken into account to avoid a possible overestimation or underestimation of total metal concentrations in soil studies.

Under our experimental conditions, nZVI are expected to oxidize very fast, thus forming iron oxides that might then adsorb heavy metals (Komárek et al., 2013; Tiberg et al., 2016). However, considering the high level of metal immobilization in our soil due to its physicochemical properties, together with the fact that the values of Zn, Cu and Cd bioavailability in soil were not significantly different in the absence *versus* the presence of nZVI, it is most likely that, under our experimental conditions, nZVI did not interact with metals. Nevertheless, a statistically significant reduction of metal concentrations in shoots was observed in plants grown in the presence of nZVI and, as a result, a lower metal phytoextraction was found. Martínez-Fernández et al. (2016) reported that nanoparticles can interfere with root hydraulic conductivity, thus affecting the uptake and translocation of some elements and nutrients, but not generating stress to plants. Similarly, in our study, nZVI nanoparticles did not affect plant physiological status and health. The low levels of tocopherol indicated that ACP and nACP treatments had the lowest levels of oxidative stress.

Brassica napus is a good candidate for phytomanagement, as it is a profitable crop currently used for biodiesel production and, besides, it can efficiently accumulate metals in its shoots (Van Ginneken et al., 2007). So far, most of the studies on metal accumulation by *B. napus* have been performed dealing with only one metal, but some problems might arise when this species is exposed to a polymetallic contamination (Cojocaru et al., 2016; Mourato et al., 2015). Likewise, the presence of organic contaminants can decrease metal phytoextraction (Batty and Dolan, 2013). To our knowledge, this is the first study that addresses the co-contamination of soils with several

metals and an organic compound (diesel) through the implementation of a GRO using *B. napus*, an organic amendment and nZVI. Selection of a diesel-tolerant plant species is essential for successful rhizoremediation of this organic contaminant (Balseiro-Romero et al., 2016; Barrutia et al., 2011b). *Brassica napus* has been reported as a diesel-tolerant species with potential for phytoremediation (Wojtera-Kwiczor et al., 2014). Dissipation of diesel can occur via a rapid non-biodegradative process (T0) caused by evaporation of low molecular weight compounds (Barrutia et al., 2011b), followed by a second slower phase associated to biodegradative processes by indigenous or inoculated microorganisms (Balseiro-Romero et al., 2016). The faster degradation of some hydrocarbon families (i.e., n-Alk, FAMEs) in our amended soils (Fig. 4.1.2B-C) is probably due to the higher values of microbial activity present in those soils (Fig. 4.1.3). According to Balseiro-Romero et al. (2016), diesel degradation can be stimulated in soils with high organic matter content due to a better supply of nutrients and reduced toxicity by the adsorption of toxic compounds to the organic matrix. Interestingly, FAMEs (i.e., hexadecanoic acid, methyl ester; heptadecanoic acid, 16-methyl-, methyl ester; and 8-Octadecenoic acid, methyl ester) showed faster degradation rates: in fact, only 5% of their initial concentration remained after the stabilization period. FAMEs, derived from trans-esterification of animal fats or vegetable oils, are components of biodiesel and are also added to conventional diesel at low concentrations (ca. 7%). This preferential degradation of FAMEs over other diesel components has also been described for marine microorganisms (DeMello et al., 2007), and indicates that the metabolism of soil microorganisms could be more adapted for fatty acid catabolism, favouring the degradation of biodiesel components. Finally, time attenuated the differences between unamended/amended, planted/unplanted and nZVI/no-nZVI soils in terms of TPH content, thereby observing no significant differences between them at harvest time. The use of nZVI to promote the degradation of recalcitrant organic contaminants, such as polycyclic or chlorinated hydrocarbons, has been reported by several authors (Chang and Kang, 2009; San Román et al., 2013; Sunkara et al., 2010). Under our experimental conditions, however, nZVI had no clear effect on diesel degradation in soil.

4.1.4.2. Biological indicators of soil health

As pointed out above, the mitigation of potential risks to ecological receptors and the improvement of soil functions are key aspects of phytomanagement initiatives. Microbial activity, biomass and functional diversity parameters (Epelde et al., 2014), as well as soil

phytotoxicity bioassays (Quintela-Sabarís et al., 2017), are frequently used as bioindicators of soil health (Galende et al., 2014c). According to our results (Fig. 4.1.4), amended soils had higher values of microbial activity and biomass, most likely due to the input of labile organic carbon easily metabolized by soil microbial communities (Galende et al., 2014b; Ros et al., 2003). After soil contamination with metals and diesel (T2), microbial activity was greatly stimulated in both amended and unamended soils. This could be understood as a consequence of: (i) a lethal effect of the added contaminants on some soil microbial populations, leading to the active growth of oportunistic populations from the labile C release associated to microbial death (Balseiro-Romero et al., 2016); (ii) the metabolic utilization of hydrocarbons present in the diesel formulation as substrates for microbial growth and activity (Siddiqui and Adams, 2002); and (iii) a requirement of more energy (microbial activity) for survival under the stressing environmental conditions characteristic of contaminated soils (Zhou et al., 2013). The fact that microbial biomass was not affected in the presence of a higher microbial activity (Fig. 4.1.3A) can be interpreted as a need to deviate energy from growth to maintain essential cell processes, in order to cope with contamination-induced stress, as microorganisms often require more energy to survive under unfavourable conditions.

Brassica napus growth also stimulated soil microbial activity (Fig. 4.1.3A). The presence of plants can help increase microbial activity in contaminated soils by releasing root exudates and creating suitable conditions for microbial growth in the rhizosphere (Balseiro-Romero et al., 2017; Barrutia et al., 2011b). *Brassica napus* plants not only increased the activity of microbial communities in contaminated soils, but also its functional diversity (Fig. 4.1.4). These observations are in agreement with results by Barrutia et al. (2011), who reported a stimulatory effect of plants on microbial functional diversity, reflected by Biolog^{TM} data and values of enzyme activities. These effects were not observed in contaminated unamended soils with *B. napus*, due to the low performance and biomass of plants under these conditions (Table 4.1.3).

Root elongation of *C. sativus* seedlings were also used to assess soil health, as this species has been described as sensitive to metal contamination (Baderna et al., 2015; Visioli et al., 2014). As shown in Fig. 4.1.5A, the organic amendment had a positive effect on root elongation at T2, in agreement with the increased wellness of *B. napus* plants and the stimulation of microbial communities observed in amended soils at T2. On the contrary, at this time-point, we detected a phytotoxic effect (decrease of root elongation)

in unamended soils. This can be explained by the lower levels of diesel present at that time in the amended soils, as a result of microbial degradation. In a bioassay performed with red clover exposed to hydrocarbon contamination, Juvonen et al. (2000) found that compost addition reduced hydrocarbon-induced phytotoxicity. At the end of our study (T3, Fig. 4.1.5B), root elongation was near the optimum value (score value close to 100%) in all amended soils, without any significant differences among treatments. However, a severe inhibition of root elongation was observed in contaminated unamended soil (Fig. 4.1.5B), in agreement with the results of soil microbial parameters observed in these soils. These findings highlight the importance of amendments to stimulate plants and soil microbial communities, and to improve soil health, while reducing total and bioavailable contaminant concentrations and, hence, ecological risk.

Finally, under our experimental conditions, the application of nZVI did not decrease soil contaminant concentrations (Fig. 4.1.1 and 4.1.2), but it decreased plant metal accumulation (Table 4.1.3). This can be taken into consideration to improve phytostabilization initiatives and reduce the entry of metals to the food web. Moreover, nZVI had no toxic effect on soil microbial communities (Fig. 4.1.3 and 4.1.4), although they initially caused inhibitory effects on *C. sativus* seedlings root elongation (Fig. 4.1.5A), possibly due to some interaction with organic matter. This phytotoxic effect was temporary and disappeared after one month (Fig. 4.1.5B), which could explain the contrasting observations reported by other authors (Stefaniuk et al., 2016).

4.1.5. Conclusions

This study highlights the importance of soil components (e.g., pH, carbonates and organic matter content) and organisms (microorganisms and *B. napus*) as essential tools for the design of phytomanagement strategies aimed at mixed contaminated (Zn, Cu, Cd and diesel) soils. Our calcareous soils presented low values of metal bioavailability, preventing metal entry in the food web and, thus, reducing metal ecotoxicity. Poor performance of diesel-tolerant profitable crops, such as *B. napus*, and native soil microbial populations during rhizoremediation was overcome by the application of organic amendments, which increased soil microbial activity and improved plant physiological status and growth. Under these circumstances, soil microbial communities were able to degrade diesel components, preferably fatty acids methyl esters. The presence of *B. napus* increased soil microbial activity and functional diversity. nZVI

reduced shoot metal concentrations and phytoextraction performance, whilst they were not effective as remediation tools for diesel contaminated soil. At the applied doses, nZVI did not cause toxicity symptoms on soil health bioindicators, other than a reduction in root elongation possibly mediated by an indirect effect of nZVI with organic matter. To our knowledge, this is the first study that reports the usefulness of the combination of *B. napus* plants, organic amendment and nZVI for the nano-rhizoremediation of soils simultaneously contaminated with several metals and diesel, and as a suitable strategy for the phytomanagement of very poor alkaline soils.

4.2. EFFECTIVENESS AND ECOTOXICITY OF ZERO-VALENT IRON NANOPARTICLES DURING RHIZOREMEDIATION OF SOIL CONTAMINATED WITH Zn, Cu, Cd AND DIESEL

Rafael G. Lacalle, María T. Gómez-Sagasti, Unai Artetxe, Carlos Garbisu, José M. Becerril, 2018. Effectiveness and ecotoxicity of zero-valent iron nanoparticles during rhizoremediation of soil contaminated with Zn, Cu, Cd and diesel. **Data in Brief**, 17: 47-56.

Abstract

The remediation of soils simultaneously contaminated with organic and inorganic compounds is still a challenging task. The application of metallic nanoparticles, such as zero-valent iron nanoparticles (nZVI), for soil remediation is highly promising, but their effectiveness and potential ecotoxicity must be further investigated. In addition, the performance of nZVI when combined with other remediation strategies is a topic of great interest. Here, we present data on soil chemical (pseudo-total and $CaCl_2$-extractable metal concentrations; petroleum hydrocarbon concentrations) and biological properties (microbial properties and phytotoxicity) after the application of nZVI to soil simultaneously contaminated with Zn, Cu, Cd and diesel, in the absence and presence of other remediation treatments such as the application of an organic amendment and the growth of *Brassica napus* plants. Soils were artificially contaminated with the abovementioned contaminants. Then, after an aging period of one month, nZVI were applied to the soil and, subsequently, *B. napus* seeds were sown. Plants were left to grow for one month. Soil samples were collected immediately after artificially contaminating the soil (T1), at sowing (T2) and at harvesting (T3). Overall, the application of nZVI had no effect on contaminant removal, nor on soil microbial parameters. In contrast, it did cause an indirect toxic effect on plant root elongation due to the interaction of nZVI with soil organic matter. These data are useful for researchers and companies interested in the effectiveness and ecotoxicity of zero-valent iron nanoparticles during the remediation of soil contaminated with metals and hydrocarbons, especially when combined with Gentle Remediation Options.

Specifications Table

Subject area	*Environmental Sciences, Plant Sciences*
More specific subject area	*Soil ecotoxicity, nanoparticles, bioremediation*
Type of data	*Tables*
How data was acquired	*Data collected from an experiment on nZVI-assisted rhizoremediation of mixed contaminated soil*
Data format	*Analyzed*
Experimental factors	*nZVI were applied to soils with combinations of the following factors: mixed contamination (metals and diesel), organic amendment, Brassica napus plants*
Experimental features	*Analysis of pseudo-total and extractable Zn, Cu and Cd concentrations, petroleum hydrocarbon concentrations; biomass, activity and functional diversity of soil microbial communities; soil phytotoxicity*
Data source location	*Leioa, Spain (43.329456, -2.969329)*
Data accessibility	*Data are available in the article*
Related research article	*Lacalle, R.G., Gómez-Sagasti, M.T., Artetxe, U., Garbisu, C., Becerril, J.M., 2018. Brassica napus has a key role in the recovery of the health of soils contaminated with metals and diesel by rhizoremediation. Sci. Total Environ. 618, 347–356.*

Value of the Data

- Data show the lack of effectiveness of nZVI for the assisted rhizoremediation of soils contaminated with metals and diesel.
- Data reveal the ecotoxicity of nZVI to plants, mediated by their interaction with soil organic matter.
- Data are useful for the design of soil remediation strategies using nZVI nanoparticles.

4.2.1. Data

Data provided here were generated during an experiment carried out to study the effectiveness of nZVI for the remediation of mixed contaminated soils. Besides, their potential toxicity for plants and soil microbial communities was investigated. Finally,

these data supplement our study on the recovery of the health of soils contaminated with metals and diesel by rhizoremediation (Lacalle et al., 2018a).

4.2.1.1. Chemical parameters

Table 4.2.1 shows the characterization of the organic amendment used in this experiment. This characterization showed a high content of total organic matter, a high C/N ratio, moderate levels of some metals (Cu, Zn), and the absence of *Salmonella* spp. and *Escherichia coli*. Table 4.2.2 shows pseudo-total (A-C) and $CaCl_2$-extractable (D-F) metal concentrations in soil. Pseudo-total concentrations decreased along the experiment. Zinc and Cu concentrations in amended soil were higher, compared to non-amended controls, due to the presence of these metals in the amendment itself. nZVI application had no effect on pseudo-total metal concentrations. $CaCl_2$-extractable metal concentrations meant a very small fraction of pseudo-total metal concentrations, and decayed over the experimental period, especially for Zn. Similarly, the presence of Cu and Zn in the amendment itself contributed to the higher values of $CaCl_2$-extractable metal concentrations observed in amended soils. $CaCl_2$-extractable metal concentrations were similar in the absence *versus* presence of nZVI. Table 4.2.3 shows the concentration of total petroleum hydrocarbons-TPH (A), as well as that of the different fractions: n-alkanes (B); fatty acid methyl esters-FAME (C); alkane fraction n-C13-n-C16 (D); alkane fraction n-C17-n-C21 (D); alkane fraction n-C22-n-C30 (D), for treatments with and without nZVI. Degradation was more accentuated for the FAME fraction (90%) than for n-alkanes (60%). Degradation was faster in the amended soil. The n-C17-C21 fraction was the most abundant and most easily degraded fraction. On the other hand, n-C22-C30 was the most recalcitrant fraction. The application of nZVI had no effect on the degradation of petroleum hydrocarbons. This lack of effectivity of nZVI on metal and hydrocarbon remediation could be partially due to the application of uncoated nanoparticles.

4.2.1.2. Biological parameters

Values of soil microbial properties (BS-basal respiration, SIR-substrate induced respiration, AWCD-average well color development, NUS-number of used substrates) in the absence and presence of nZVI are shown in Table 4.2.4 and Table 4.2.5, respectively. Overall, values of BR and SIR were higher in the presence of the amendment. Basal respiration increased in soils exposed to the mixed contamination, while SIR values were

similar to those observed in control soil. nZVI application did not have any significant effect on these respiratory parameters. Regarding soil microbial functional diversity, AWCD and NUS values were not affected by the application of the amendment nor by the presence of the contaminants, but they were highly stimulated by the presence of *B. napus* plants. The application of nZVI did not affect the soil microbial functional diversity. Table 4.2.6 shows data from the root elongation bioassay performed with *Cucumis sativus* in soils treated with (A) and without (B) nZVI. A general trend towards decreased root elongation values in the presence of contaminants and increased values in the presence of the amendment was identified. The application of nZVI caused an indirect toxic effect on plant root elongation due to the interaction of nZVI with soil organic matter. This interaction of nZVI and soil organic matter needs further investigation.

Table 4.2.1. Characterization of the organic amendment used in this experiment.

Agronomic parameters	
Organic matter (%)	29.6
Humidity (%)	22.6
Organic C / Organic N	13.2
Sanitary parameters	
Salmonella spp.	Absent
Escherichia coli	Absent
Metal concentrations	
Cd (mg kg^{-1})	1.3
Cu (mg kg^{-1})	241
Ni (mg kg^{-1})	25.2
Pb (mg kg^{-1})	57.2
Zn (mg kg^{-1})	368
Hg (mg kg^{-1})	0.6
Cr (mg kg^{-1})	32

Table 4.2.2. Pseudo-total (A-C) and $CaCl_2$-extractable (D-F) metal concentrations (mg kg^{-1}) in soil for the different sampling times (T1-T3). Different letters indicate statistical significance ($P < 0.05$) between treatments, and numbers indicate statistical significance ($P < 0.05$) between sampling times. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments treated with and without nZVI.

	UMN	UMP	AMN	AMP	nUMN	nUMP	nAMN	nAMP
(A) [Zn]T								
T1	1805.4 ± 34.7 b1		2234.6 ± 88.3 a12		1805.4 ± 34.7 b1		2234.6 ± 88.3 a1	
T2	1796.2 ± 16.9 b1		2025.1 ± 22.5a1		1878.5 ± 28.9 a1		1925.2 ± 18.8 a1*	
T3	1593.8 ± 9.44 b2	1615.1 ± 3.9 b	1846.7 ± 23.4 a2	1849.0 ± 107.6 ab	1406.4 ± 23.3 c2*	1479.2 ± 28.8 c*	1893.0 ± 48.6 b1	2073.4 ± 43.0 a
(B) [Cu]T								
T1	757.6 ± 19.6 b1		956.1 ± 48.5 a1		757.6 ± 19.6 b1		956.1 ± 48.5 a1	
T2	690.8 ± 5.9 b1		855.5 ± 13.6 a1		771.8 ± 55.8 a1		802.6 ± 14.5 a1	
T3	666.1 ± 29.9 b1	668.6 ± 19.2 b	895.7 ± 24.8 a1	840.5 ± 46.7 a	610.6 ± 13.9 b1	605.8 ± 19.9 b	894.4 ± 23.7 a1	946.4 ± 35.5 a
(C) [Cd]T								
T1	62.2 ± 1.4 a1		73.6 ± 4.6 a1		62.2 ± 1.45 a1		73.6 ± 4.6 a1	
T2	65.8 ± 3.2 a1		63.7 ± 2.0 a1		67.5 ± 2.0 a1		59.0 ± 1.2 b2	
T3	62.8 ± 1.3 a1	61.0 ± 1.5 a1	65.7 ± 0.6 a1	60.4 ± 1.5 a1	61.4 ± 2.6 a1	58.1 ± 1.7 a	63.1 ± 3.1 a12	64.8 ± 1.7 a
(D) [Zn]E								
T1	2.0 ± 0.1 b1		4.8 ± 0.8 a1		2.0 ± 0.1 b1		4.8 ± 0.8 a1	
T2	0.5 ± <0.1 ab2		0.7 ± <0.1 a2		0.4 ± <0.1 b2		0.5 ± <0.1 ab2	
T3	0.3 ± <0.1 b3	0.3 ± <0.1 b	0.4 ± 0.1 a2	0.4 ± <0.1 a	0.3 ± <0.1 b3	0.3 ± <0.1 a*	0.3 ± <0.1 a2	0.3 ± <0.1 a*
(E) [Cu]E								
T1	0.08 ± <0.1 b1		0.3 ± <0.1 a1		0.1 ± <0.1 b1		0.3 ± <0.1 a1	
T2	0.1 ± <0.1 b1		0.2 ± <0.1 a2		<0.1 ± <0.1 b1		0.3 ± <0.1 a1	
T3	0.1 ± <0.1 b2	0.1 ± <0.1 b	0.1 ± <0.1 a3	0.2 ± <0.1 a	0.1 ± <0.1 c1*	0.1 ± <0.1 c*	0.2 ± <0.1 b2*	0.2 ± <0.1 a*
(F) [Cd]E								
T1	0.2 ± <0.1 a1		0.15 ± <0.1 a		0.2 ± <0.1 a1		0.2 ± <0.1 a1	
T2	0.1 ± <0.1 a2		<0.1 ± <0.1 b2		0.1 ± <0.1 a2		<0.1 ± <0.1 a2	
T3	<0.1 ± <0.1 a3	<0.1 ± <0.1 a	<0.1 ± <0.1 b12	<0.1 ± <0.1 b	<0.1 ± <0.1 a2	<0.1 ± <0.1 a	<0.1 ± <0.1 b2	<0.1 ± <0.1 b

Table 4.2.3. Concentrations (mg kg^{-1}) of TPH (Total Petroleum Hydrocarbons, A); n-Alk (n-alkanes, B); FAMEs (Fatty Acid Methyl Esters, C); and n-alkane fractions (D-F) in soil for the different sampling times (T1-T3). Different letters indicate statistical significance ($P < 0.05$) between treatments, and numbers indicate statistical significance ($P < 0.05$) between sampling times. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments treated with and without nZVI.

	UMN	UMP	AMN	AMP	nUMN	nUMP	nAMN	nAMP
(A) TPH								
T1	2860.5 ± 117.7 a1		2417.3 ± 175.6 a1		2860.5 ± 117.7 a1		2417.3 ± 175.6 a1	
T2	1631.5 ± 46.1 a2		997.4 ± 25.6 b2		1467.5 ± 131.8 a2		793.5 ± 32.4 b2*	
T3	888.6 ± 15.1 a3	933.8 ± 23.3 a	905.5 ± 21.2 a2	816.7 ± 59.5 a	1089.8 ± 49.7 a3*	883.9 ± 189.4 a	831.9 ± 81.3 a2	883.9 ± 113.1 a
(B) n-Alk								
T1	2648.2 ± 109.3 a1		2231.0 ± 163.2		2648.2 ± 109.3 a1		2230.9 ± 163.2	
T2	608.4 ± 47.3 a2		975.0 ± 33.4 b2		1440.9 ± 126.0 a2		766.8 ± 29.3 b2*	
T3	874.9 ± 15.8 a3	924.6 ± 23.0 a	891.0 ± 20.2 a2	804.4 ± 60.6 a	1082.9 ± 49.3 a3*	858.9 ± 185.5 a	815.3 ± 81.2 a2	867.0 ± 111.6 a
(C) FAME								
T1	209.5 ± 8.6 a1		176.5 ± 12.9 a1		209.5 ± 8.6 a1		176.5 ± 12.9 a1	
T2	13.5 ± 0.2 a2		3.3 ± 0.4 b2		9.7 ± 1.2 a2*		2.7 ± 0.2 b2	
T3	3.6 ± 0.4 a3	4.5 ± 0.2 a	3.9 ± 0.6 a2	5.2 ± 1.6 a	5.5 ± 0.4 a2	3.6 ± 1.3 a	3.3 ± 0.1 a2	3.7 ± 0.4 a
(D) n-C13–n-C16								
T1	662.8 ± 37.3 a1		510.4 ± 44.7 a1		662.8 ± 37.3 a1		510.4 ± 44.7 a1	
T2	288.0 ± 4.1 a2		196.1 ± 6.2 b2		280.0 ± 26.9 a2		164.5 ± 13.1 b2	
T3	194.0 ± 21.3 a3	227.6 ± 20.3 a	200.1 ± 9.8 a2	184.5 ± 17.6 a	240.9 ± 9.0 a2	184.7 ± 48.3 a	175.1 ± 23.7 a2	190.5 ± 37.5 a
(E) n-C17–n-C21								
T1	1190.2 ± 26.2		1046.2 ± 70.0 a1		1190.2 ± 26.2 a1		1046.2 ± 70.0 a1	
T2	816.6 ± 40.2 a2		386.7 ± 30.7 b2		677.7 ± 73.5 a2		266.9 ± 7.0 b2	
T3	314.2 ± 33.0 a3	312.7 ± 9.0 a	275.7 ± 6.9 a2	278.8 ± 30.3 a	431.6 ± 18.7 a3*	319.5 ± 87.4 a	262.6 ± 17.2 a2	295.1 ± 35.7 a
(F) n-C22–n-C30								
T1	783.3 ± 44.8 a1		657.2 ± 58.9 a1		783.3 ± 44.8 a1		657.2 ± 58.9 a1	
T2	488.1 ± 14.5 a2		367.6 ± 7.7 b2		460.1 ± 29.5 a2		316.3 ± 15.0 b2*	
T3	351.2 ± 36.3 a3	374.1 ± 5.9 a	398.3 ± 23.6 a2	321.9 ± 16.1 a	399.7 ± 22.9 a2	340.0 ± 50.0 a	353.4 ± 43.7 a2	355.1 ± 34.9 a

Table 4.2.4. Soil microbial properties in the absence of nZVI. BR: Basal Respiration (µg CO_2 g^{-1} DW soil h^{-1}); SIR: Substrate Induced Respiration (µg CO_2 g^{-1} DW soil h^{-1}); AWCD: Average Well Color Development; NUS: Number of Used Substrates. Sampling times = T0-T3. Different letters indicate statistical significance ($P < 0.05$) between treatments, and numbers indicate statistical significance ($P < 0.05$) between sampling times.

	UCN	UCP	UMN	UMP	ACN	ACP	AMN	AMP
BR								
T0	0.9 ± 0.1 b1				1.9 ± 0.3 a1			
T1	1.6 ± 0.2 b1				2.8 ± 0.4 a2			
T2	1.3 ± 0.1 d1		2.7 ± 0.1 c1		3.5 ± <0.1 b2		4.7 ± 0.2 a1	
T3	1.3 ± 0.2 e1	1.6 ± 0.1 e	2.7 ± 0.2 d1	2.8 ± 0.1 cd	3.5 ± 0.1 bc2	4.1 ± 0.1 b	4.4 ± 0.1 b1	5.7 ± 0.3 a
SIR								
T0	5.7 ± 0.2 a1				6.8 ± 0.5 a1			
T1	8 ± 0 a2				8.1 ± 0.1 a12			
T2	5.2 ± <0.1 b1		6.7 ± 1.1 b1		11.3 ± 0.4 a3		11.2 ± 1.1 a1	
T3	8.6 ± 0.7 b2	8.4 ± 0.7 b	7.8 ± 1.1 b1	8.3 ± 1.1 b	15.3 ± 1.4 a23	13.2 ± 1.8 ab	10.0 ± 0.7 ab1	11.5 ± 1.8 ab
AWCD								
T0	1.0 ± 0.2 a1				0.8 ± 0.1 a1			
T1	0.8 ± 0.1 a2				0.9 ± 0.2 a1			
T2	0.2 ± <0.1 a1		0.2 ± 0.1 a1		0.3 ± 0.1 a2		0.2 ± <0.1 a1	
T3	0.3 ± <0.1 bcd2	0.4 ± <0.1 b	0.2 ± <0.1 cd1	0.2 ± <0.1 d	0.3 ± 0.1 bc2	0.6 ± <0.1 a	0.2 ± <0.1 cd1	0.7 ± <0.1 a
NUS								
T0	25.8 ± 2.3 a1				21.1 ± 1.2 a1			
T1	19.0 ± 1.2 a2				20.3 ± 1.3 a1			
T2	6.4 ± 0.3 a3		3.6 ± 0.4 b1		5.8 ± 0.4 a2		6.3 ± 0.2 a1	
T3	8.4 ± 1.3 bc3	10.5 ± 1.2 b	7.7 ± 0.8 bc2	7.0 ± 0.7 bc	8.5 ± 1.0 bc2	16.5 ± 0.4 a	5.6 ± 0.9 c1	17.5 ± 0.7 a

Table 4.2.5. Soil microbial properties in the presence of nZVI. BR: Basal Respiration (µg CO_2 g^{-1} DW soil h^{-1}); SIR: Substrate Induced Respiration (µg CO_2 g^{-1} DW soil h^{-1}); AWCD: Average Well Color Development; NUS: Number of Used Substrates. Sampling times = T0-T3. Different letters indicate statistical significance ($P < 0.05$) between treatments, and numbers indicate statistical significance ($P < 0.05$) between sampling times (T0-T3). Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments treated with and without nZVI.

	nUCN	nUCP	nUMN	nUMP	nACN	nACP	nAMN	nAMP
BR								
T0	0.9 ± <0.1 b1				1.9 ± 0.3 a1			
T1	1.6 ± 0.2 b2				2.8 ± 0.4 a12			
T2	1.4 ± 0.1 c2		2.4 ± 0.1 b1		2.9 ± 0.1 b2*		3.9 ± 0.3 a1	
T3	1.5 ± 0.2 e2	1.8 ± 0.2 e	3.0 ± 0.1 d2	2.7 ± <0.1 d	3.9 ± 0.3 c3	4.2 ± 0.3 bc	4.7 ± 0.2 b1	5.9 ± 0.2 a
SIR								
T0	5.7 ± 0.2 a1				6.8 ± 0.5 a1			
T1	8.0 ± <0.1 a2				8.1 ± 0.1 a1			
T2	10.0 ± 0.7 a12*		10.8 ± 0.4 a1*		12.3 ± 1.7 a12		14.12 ± 0.4 a1	
T3	9.4 ± 1.0 b12	9.0 ± 1.3 b	7.4 ± 0.7 b2	9.6 ± 1.0 b	15.1 ± 0.9 a2	18.2 ± 2.5 a	14.4 ± 1.4 a1*	14.6 ± 0.7 a
AWCD								
T0	1.0 ± 0.2 a1				0.8 ± 0.1 a1			
T1	0.8± 0.1 a1				1.0 ± 0.2 a1			
T2	0.2 ± 0.1 a2		0.2 ± <0.1 a1		0.3 ± <0.1 a2		0.2 ± 0.1 a1	
T3	0.2 ± <0.1 c2	0.3 ± <0.1 b	0.1 ± <0.1 c1	0.1 ± <0.1 c	0.3 ± <0.1 b2	0.7 ± <0.1 a	0.1 ± <0.1 c1	0.8 ± <0.1 a
NUS								
T0	25.8 ± 2.3 a1				21.1 ± 1.2 a1			
T1	19.0 ± 1.2 a2				20.3 ± 1.3 a1			
T2	8.6 ± 1.0 a3		4.6 ± 0.6 b1		8.6 ± 1.0 a2*		6.3 ± 1.7 ab1	
T3	6.2 ± 1.0 b3	9.1 ± 1.7 b	6.3 ± 1.3 b1	6.0 ± 1.9 b	8.8 ± 1.5 b2	16.6 ± 0.8 a	6.7 ± 1.1 b1	19.1 ± 0.6 a

Table 4.2.6. Root Elongation (RE, mm) of *Cucumis sativus* seedlings exposed to treatments without nZVI (A) and with nZVI (B) at T2 and T3 sampling times. Different letters indicate statistical significance ($P < 0.05$) between treatments, and numbers indicate statistical significance ($P < 0.05$) between sampling times. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments treated with and without nZVI.

	UCN	UCP	UMN	UMP	ACN	ACP	AMN	AMP
(A) RE without nZVI								
T2	20.1 ± 0.9 a1		8.2 ± 0.6 b1		24.8 ± 1.1 b1		34.7 ± 2.6 a1	
T3	41.1 ± 0.6 a2	40.4 ± 0.8 a	20.6 ± 2.9 c2	16.8 ± 1.3 c	51.1 ± 1.6 bc2	49.5 ± 2.3 c	55.1 ± 0.5 ab2	56.7 ± 1.6 a

	nUCN	nUCP	nUMN	nUMP	nACN	nACP	nAMN	nAMP
(B) RE with nZVI								
T2	19.0 ± 3.2 a1		8.2 ± 0.3 b1		15.2 ± 2.1 c1*		27.5 ± 1.8 b1*	
T3	46.8 ± 0.5 a2	46.0 ± 1.9 a2	21.5 ± 2.1 c2	28.8 ± 4.5 b2	46.7 ± 1.4 cd2	42.9 ± 3.0 d*	49.2 ± 0.7 c2*	48.8 ± 1.0 c

4.2.2. Experimental design, materials and methods

Two topsoils were collected (Time = T0) from a peri-urban area: one amended with 100 t ha^{-1} of an organic material produced from the recycling of urban organic wastes, and the other without such amendment. The organic amendment was obtained from the "BIOCOMPOST DE ALAVA" company, an urban waste treatment plant. After selective separation and sieving, organic matter from domestic waste of the city of Vitoria-Gasteiz (Spain) was stored for 6 months before use. Soil was sieved to <6 mm, air-dried, and half of each soil was artificially contaminated with a mixture of metals and commercial diesel fuel purchased from a petrol station (T1). Experimental metal concentrations were (in mg kg^{-1} DW soil): Zn (1,500), Cu (500) and Cd (50). Immediately after, diesel (6,000 mg kg^{-1} DW soil) was added to already metal contaminated soils, following ISO 15952 (2006). Then, 700 g DW of contaminated or non-contaminated soil were placed in 1 L pots. In order to allow contaminant stabilization, pots were kept for one month in a phytotron under the following controlled conditions: photoperiod 14/10 h day/night, temperature 25/18 ºC day/night, relative humidity 60/80% day/night, and a photosynthetic photon flux density of 200 µmol photon m^{-2} s^{-1}. After the 1-month stabilization period (T2), nZVI (NanoFer Star, Nanoiron s.r.o) were activated following manufacturer's instructions with Milli-Q water for 24 h and then applied in aqueous solution to half of the pots (contaminated and non-contaminated) at a concentration of 1 g nZVI kg^{-1} DW soil. Three days later, *Brassica napus* seeds were sown on half of the pots, and plants were harvested a month later (T3). Soil samples were collected at spiking (T1), sowing (T2) and harvesting time (T3).

Contaminant concentrations were measured in the collected soil samples. In order to measure pseudo-total Zn, Cu and Cd concentrations, samples were digested according to US-EPA Method 3051A (2007). For extractable metal concentrations, an extraction was performed following Houba et al. (Houba et al., 2000). Metal concentrations were quantified by Inductively-Coupled Plasma Mass Spectrometry (Agilent 7700). Total petroleum hydrocarbon and fatty acid methyl ester concentrations in soil were measured by Gas Chromatography–Mass Spectrometry (GC–MS), as described in Bartolomé et al. (2005). Soil samples were used to determine the following microbial properties, as detailed in Galende et al. (2014): (i) microbial activity was determined by basal respiration (BR) following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR) following ISO 17155 (2002); (iii)

average well color development (AWCD) and (iv) number of metabolized substrates (NUS) were determined from Biolog EcoPlates™ following Epelde et al. (2014). A root elongation bioassay with *Cucumis sativus* was performed to determine soil phytotoxicity. Seeds of *C. sativus* (c.v. Marketmore) were pre-germinated on Petri dishes, containing wet filter paper, for 3 days under controlled conditions (14/10 h day/night; 25/18 ºC day/night; and full darkness). Concurrently, 10 g of dried soil were placed on Petri dishes, hydrated with deionized water, mixed vigorously, and covered with filter paper. After pre-germination, seven seeds of *C. sativus* showing a radicle length of 5-10 mm were placed over the filter paper of soil-containing Petri dishes. Afterwards, dishes were placed for 72 h under the following conditions: photoperiod 14/10 h day/night, temperature 25/18 ºC day/night, relative humidity 60/80 % day/night, and photosynthetic photon flux density of 100 µmol photon m^{-2} s^{-1}. Images of the seedlings were taken at the beginning and after 72 h of incubation with the soil. Images were processed by ImageJ Software. Root Elongation (RE) ($RE = RE_{T2} - RE_{T1}$) was calculated for each seedling. Data were statistically analyzed using ANOVA-test when data were normally distributed and Kruskal-Wallis test when they were not. Kolmogorov-Smirnov was used as normality test.

Samples were identified according to the following codes:

Table 4.2.7. Codes of the samples according to their treatments.

	Unamended (U)				Amended (A)			
	Control (C)		Mixed contamination (M)		Control (C)		Mixed contamination (M)	
	Without nZVI	With nZVI (n)	Without nZVI	With nZVI (n)	Without nZVI	With nZVI (n)	Without nZVI	With nZVI (n)
Not planted	UCN	nUCN	UMN	nUMN	ACN	nACN	AMN	nAMN
Planted	UCP	nUCP	UMP	nUMP	ACP	nACP	AMP	nAMP

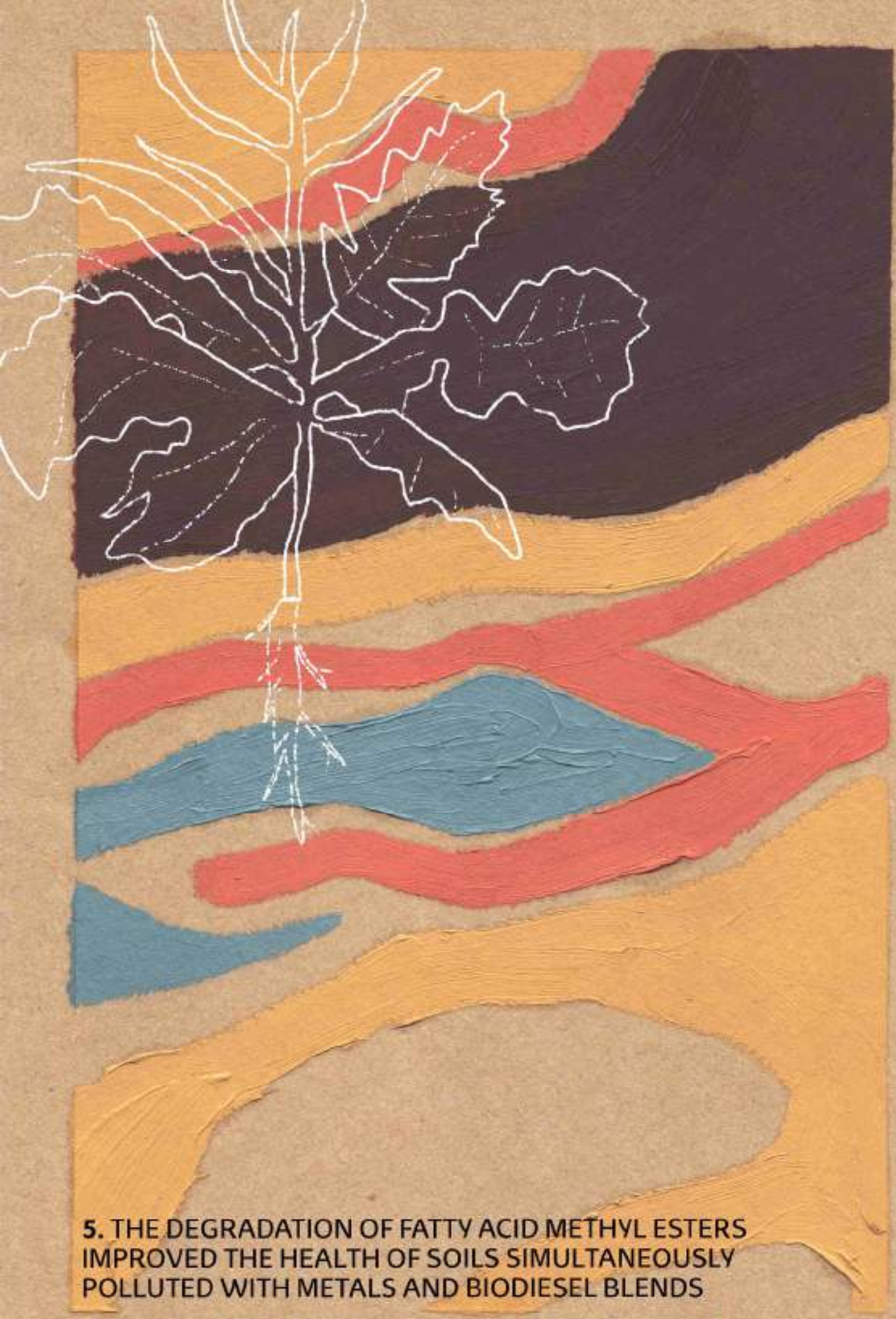
4.3 Conclusiones del capítulo

- Las propiedades químicas del suelo como el pH moderadamente alcalino y alto contenido en carbonatos son las responables de la baja disponibilidad de metales y las bajas tasas de fitoextracción.

- Las comunidades microbianas nativas del suelo fueron capaces de degradar parcialmente gasóleo comercial, preferentemente los metil ésteres de ácidos grasos de origen biológico y las fracciones ligeras de origen fósil.

- La enmienda orgánica procedente de material bioestabilizado es esencial para estimular el crecimiento de *Brassica napus* y la actividad y biomasa de las comunidades microbianas en los suelos con contaminación mixta por Zn, Cd, Cu y gasóleo.

- La aplicación dea aplicación de nanopartículas de hierro cero valente no es una tecnología efectiva para la remediación de suelos suelos con contaminación mixta por Zn, Cd, Cu y gasóleo, aunque no son tóxicas para la biota.



5. THE DEGRADATION OF FATTY ACID METHYL ESTERS IMPROVED THE HEALTH OF SOILS SIMULTANEOUSLY POLLUTED WITH METALS AND BIODIESEL BLENDS

5. THE DEGRADATION OF FATTY ACID METHYL ESTERS IMPROVED THE HEALTH OF SOILS SIMULTANEOUSLY POLLUTED WITH METALS AND BIODIESEL BLENDS

Rafael G. Lacalle, María T. Gómez-Sagasti, Unai Artetxe, Carlos Garbisu, José M. Becerril, 2020. The degradation of fatty acid methyl esters improved the health of soils simultaneously polluted with metals and biodiesel blends. **Fuel** (submitted)

Abstract

Fuels from renewable biological resources are currently being used as an alternative or complement to petroleum-derived fossil fuels. The objective of this work was to investigate the degradation dynamics of commercially-available biodiesel blends (containing 1, 5 or 16% biodiesel, i.e. B1, B5 and B16) in soil simultaneously polluted with metals and biodiesel blends. To this purpose, soil was artificially polluted with 6,000 mg biodiesel blend per kg DW soil (B1, B5 or B16) and a mixture of metals: 1,500 mg Zn kg^{-1} DW soil + 500 mg Cu kg^{-1} DW soil + 50 mg Cd kg^{-1} DW soil. Artificially-polluted soils were then arranged in a phytotron under controlled conditions and monitored for 30 days. The bioremediation capacity of a bio-stabilized municipal solid waste, as organic amendment for biostimulation purposes, was evaluated. Soil health was monitored by measuring soil microbial indicators (biomass, activity and diversity parameters) and performing phytotoxicity bioassays with *Cucumis sativus*. The degradation of the fatty acid methyl esters (FAME) present in biodiesel was higher than that of the corresponding alkane homologues from diesel. The addition of the bio-stabilized municipal solid waste increased FAME degradation and microbial activity, and alleviated phytotoxicity. *Cucumis sativus* was more sensitive to pollution-induced effects than soil microbial communities. Our data showed that, after 30 days, organically-amended soils polluted with B16 experienced the greatest improvement in soil health (in the presence of the abovementioned metal mixture). It was concluded that biodiesel-containing fuel might cause a lower impact on soil health than diesel of fossil origin.

5.1. Introduction

Global oil demand grew by 1.3 million barrels per day in 2018 (IEA, 2019). Besides posing energetic and climatic concerns, the extensive use of fuels (mainly, fossil fuels) has led to a worrying increase in environmental pollution. Indeed, our environment is, at this time, polluted with large amounts of petroleum hydrocarbons (Safdari et al., 2018). Petroleum-based hydrocarbons are known to cause adverse impacts on humans and the environment (Tahhan et al., 2011). In fact, some hydrocarbons are considered high-priority environmental pollutants (Safdari et al., 2018; Wu et al., 2017, 2016). Petroleum-based fuels (e.g., diesel) are composed of complex mixtures of straight- and branched-chain aliphatic hydrocarbons (alkanes) and aromatic hydrocarbons. In addition, co-pollution with petroleum hydrocarbons and metal(oid)s is a widely reported issue (Khudur et al., 2018) which increases the complexity of the potential environmental problems and, concomitantly, their possible solutions.

In the past decade, biodiesel, derived from renewable biological resources, has acquired more space in the world energy matrix (Knothe, 2010; Wakil et al., 2015; Wieczorek et al., 2015). Biodiesel mainly consists of Fatty Acid Methyl Esters (FAME) resulting from the transesterification of vegetable oils, animal fats or waste oils and fats (CONCAWE, 2009; Ginn et al., 2009). Commercially-available biodiesels are commonly identified by the percentage of biodiesel in the fuel blend, using the letter B followed by the corresponding percentage. Although B100 (100% biodiesel) can indeed be directly used as fuel, biodiesel is usually blended with diesel from fossil origin to improve the properties of the fuel without requiring modifications to the engines (Silitonga et al., 2013). Commercially-available biodiesel blends usually range from 5 to 20% (v/v), referred to as B5 and B20, respectively.

Compared to conventional diesel from fossil origin, apart from being a sulfur-free fuel, biodiesel stands out for its higher degradation rates (Lacalle et al., 2018a; Lisiecki et al., 2014), lower acute toxicity to organisms, higher combustion efficiency, and reduced emissions of greenhouse gases and particulate matter (Meyer et al., 2018; Wakil et al., 2015). Moreover, the use of biodiesel can bring social benefits, such as the revitalization of rural areas and the creation of new jobs (Kiss et al., 2008). However, the production of biodiesel is surrounded by much controversy due to competition with food crops for the use of arable lands. To address this food-energy-environment trilemma (Tilman et al.,

2009), it has been proposed (Atabani et al., 2013) to cultivate oil crops only in degraded soils not suitable for food-agricultural purposes.

Since most hydrocarbons are known to be susceptible to biodegradation by microbial populations, bioremediation is considered a suitable technology for the remediation of soils polluted with biodiesel blends. Bioremediation has gained popularity during the last years and decades due to its being a more cost-effective and environmentally-friendly strategy than many traditional physicochemical techniques of remediation (Lisiecki et al., 2014). Importantly, bioremediation must pursue not only the removal of pollutants, but also the recovery of soil health (i.e., the ability of a given soil to perform its functions as a living system) (Gómez-Sagasti et al., 2012). Several studies (Thomas et al., 2017) have reported the biodegradation of biodiesels under both aerobic and anaerobic conditions but much more information is still required regarding the rate and extent of their degradation, as well as the preferential degradation of specific FAME (Thomas et al., 2017). In particular, there are not many studies on the degradation of commercially-available biodiesel blends in soil (particularly, in the presence of metals), as well as on their impact on soil health, with the reported results being often inconsistent (Wieczorek et al., 2015). As aforementioned, mixed-pollution scenarios present further challenges, because the presence of, for instance, potentially toxic metals as co-pollutants can hinder the microbial degradation of hydrocarbons (Khudur et al., 2019; Olaniran et al., 2013).

The effectiveness of biodegradation processes (bioremediation) can be enhanced by the addition of organic amendments for biostimulation purposes, which can boost native soil microbial biomass and activity and, consequently, increase hydrocarbon degradation (Ros et al., 2010). The application of organic amendments can be of special utility for mixed-polluted soils, since they can simultaneously decrease the bioavailability and, consequently, toxicity of metals (Park et al., 2011). The use of organic amendments produced out of agro-industrial (Galende et al., 2014c) or urban solid wastes (Lacalle et al., 2018a; Míguez et al., 2020) is a frequent practice in soil remediation. The reutilization of organic wastes contributes toward the objectives of the EU's Zero Waste Policy, End-Of-Waste Policy, and Circular Economy Strategy (Gómez-Sagasti et al., 2018).

Set against this background, this study aimed to evaluate the degradation dynamics of commercially-available biodiesel blends (i.e., B1, B5 and B16) in mixed-polluted soils (with Zn, Cu and Cd) amended with a bio-stabilized organic urban waste

for biostimulation purposes. Soil microbial properties (microbial biomass, activity and diversity) were used as indicators of soil health. Root elongation bioassay tests with *Cucumis sativus* were performed to assess soil phytotoxicity. The effects of the organic amendment on hydrocarbon degradation, metal stabilization and soil health were evaluated. We hypothesized that FAME (from biodiesel of biological origin) would suffer a faster and more effective degradation than their corresponding alkanes homologues (from diesel of fossil origin). Moreover, we speculated that the presence of the organic amendment (bio-stabilized organic urban waste) would stimulate soil microbial activity, alleviate soil phytotoxicity and, hence, improve soil health.

5.2. Materials and methods

5.2.1. Experimental design

The soil used for this microcosm experiment was collected from the peri-urban area of Vitoria-Gasteiz (Northern Spain, 42º50'N; 2º40'W). An organic amendment, i.e. a bio-stabilized material produced from the recycling of urban organic wastes (Míguez et al., 2020), was incorporated (100 t ha^{-1}) to half of the collected soil by manual mixing (*amended* soil, "A"), while the other half remained unamended (*unamended* soil, "U"). Both A and U soils were sieved to less than 6 mm, air-dried and subjected to physicochemical characterization (Table 5.1).

Both soils were spiked with a mixture of metals (1,500 mg Zn kg^{-1} DW soil + Cu (500 mg Cu kg^{-1} DW soil + 50 mg Cd kg^{-1} DW soil; as nitrate salts) and then artificially polluted (6,000 mg kg^{-1} DW soil) with three commercially-available biodiesel blends: B1 (1% biodiesel), B5 (5% biodiesel) and B16 (16% biodiesel). Finally, soils were thoroughly homogenized by manual mixing. Non-polluted soils were also included in the experiment as controls.

Experimental pots were filled with 0.2 kg DW soil, establishing four replicates for each treatment. Soils were monitored at the following sampling times: 1, 4, 8, 16 and 30 days after pollution. Pots were placed in a phytotron where they were kept during 30 days under controlled conditions (photoperiod = 14/10 h day/night; temperature = 25/18 °C day/night; relative humidity = 60/80% day/night; photosynthetic photon flux density = 200 µmol photon m^{-2} s^{-1}). Soils were watered periodically to maintain constant soil moisture.

5.2.2. Determination of pollutant concentrations

At each sampling time, metal and hydrocarbon concentrations in soil were monitored. For pseudo-total and bioavailable metal concentrations, soil samples were oven-dried at 80 ºC, grinded and sieved to less than 0.125 mm. For the determination of pseudo-total metal concentrations, an acid digestion with HNO_3 was carried out as described by the US-EPA Method 3051A (US-EPA Method 3051A, 2007). For bioavailable metal concentrations, an extraction with $CaCl_2$ was performed as described by Houba et al. (2000). Total and bioavailable metal concentrations were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Agilent 7700).

For the determination of hydrocarbon concentrations, soils were frozen in liquid nitrogen, kept at -80 ºC and, finally, lyophilized. As described by Bartolomé et al. (2005), the extraction of hydrocarbons was performed in acetone, filtered and cleaned by solid phase extraction (SPE) with Florisil® cartridges. The quantification of hydrocarbons was carried out by Gas Chromatography-Mass Spectrometry (GC-MS) (Agilent 7890).

5.2.3 Determination of biological parameters

For the determination of biological parameters, soil samples were stored fresh at 4 ºC and then analyzed within two months of collection. Microbial activity was estimated by basal respiration (BR), as described in ISO 16072 (2002). Microbial biomass was estimated by substrate-induced respiration (SIR), as described in ISO 17155 (2002). Functional microbial diversity was determined as the number of utilized substrates (NUS) in Biolog EcoPlates™, as described by Galende et al. (2014b). The substrate consumption activity (SCA, also known as area under the curve or AUC) and carbon substrate utilization profiles were also determined from Biolog EcoPlates™. Root elongation phytotoxicity bioassays were performed with *Cucumis sativus* following Lacalle et al. (2018a).

5.2.4 Statistical analysis

Statistical analyses were performed using the software package IBM SPSS Statistics for Windows, Version 24. Normality was checked with the Kolmogorov-Smirnov test. ANOVA was performed on normally distributed data, using Duncan *post hoc* when there was homocedasticity (checked with Levene test) and Games-Howell when there was not. Kruskal-Wallis test was performed on non-normally distributed data. For a clearer

visualization of the figures, the results from the statistical analyses are presented in Supplementary Material (Tables S5.1-S5.5).

5.3. Results and Discussion

5.3.1 Pseudo-total and bioavailable metal concentrations

Mixed-polluted soils present particular difficulties that go beyond the challenges presented by individual pollutants. The ecotoxic effects of metals may not only decrease soil health (Epelde et al., 2009a) but also reduce the effectiveness of bioremediation strategies by inhibiting the biodegradation of organic pollutants (Sandrin and Maier, 2003). As expected, in our study, pseudo-total metal concentrations did not vary throughout the experimental period (Table 5.2). Significantly higher Zn and Cu concentrations were found in organically-amended than in unamended soils, due to the presence of Zn and Cu in the amendment itself (Table 5.1).

Table 5.1. Physicochemical characteristics of unamended and amended soils.

Parameter	Unamended	Amended
Total clay (%)	23.4	15.7
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Texture class (USDA)	Loam	Loam
pH (1:2.5)	7.9	8.0
Carbonates (%)	54.7	44.0
Organic matter (% W)	1.0	19.5
C organic / N organic	6.7	8.6
Total N (% DW)	0.1	0.9
Total C organic (% DW)	0.6	7.3
[Zn] Tot/Bio (mg kg^{-1} DW)	41.4 / 0.0	127.8 / 0.0
[Cu] Tot/Bio (mg kg^{-1} DW)	6.9 / <0.1	73.3 / <0.1
[Cd] Tot/Bio (mg kg^{-1} DW)	0.3 / 0.0	0.5 / 0.0

Table 5.2. Pseudo-total metal concentrations at Day 1 and Day 30. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended (U) and amended (A) soils for each sampling time and treatment.

	[Zn] (mg kg^{-1})		[Cu] (mg kg^{-1})		[Cd] (mg kg^{-1})	
Treatment	Day 1	Day 30	Day 1	Day 30	Day 1	Day 30
UB16	1236±31 a2	1396±30 a1	421±21 a1	438±16 a1	41.5±1.6 a1	44.3±1.1 a1
AB16	1397±34 a2*	1599±43 a1*	493±21 a1	560±26 a1*	43.6±1.4 a1	47.8±1.2 a1
UB5	1283±57 a1	1312±76 a1	434±27 a1	415±24 a1	42.7±2.3 a1	41.6±2.0 a1
AB5	1291±25 a2	1525±62 a1	444±14 a2	522±22 a1*	39.7±0.9 a2	45.9±1.9 a1
UB1	1260±27 a2	1454±18 a1	413±13 a2	469±4 a1	41.5±1.0 a2	46.7±0.2 a1
AB1	1349±33 a2	1680±52 a1*	470±19 a2*	571±16 a1*	41.8±1.2 a2	50.2±1.4 a1

Bioavailable ($CaCl_2$-extractable) metal concentrations in soil are frequently used to estimate metal mobility and toxicity (Vamerali et al., 2010). In our study, in general, bioavailable metal concentrations were very low, representing around 1% of their corresponding pseudo-total metal concentrations (Table 5.3). No significant differences were observed between the soils artificially polluted with the biodiesel blends (i.e., B1, B5 and B16). Higher bioavailable Zn and Cu concentrations were found in organically-amended *vs.* unamended soils, presumably due to their higher pseudo-total metal concentrations. Metal bioavailability remained stable throughout the experimental period. Metal bioavailability in soil is governed by several factors, most relevantly, soil pH, organic matter content and precipitation with anions (Adriano, 2001; Alvarenga et al., 2009; Rieuwerts et al., 1998). In our study, the slightly alkaline soil pH (8.0 and 7.9 for A and U soils, respectively) and the high carbonate content (44 and 55% for A and U soils, respectively) were most likely responsible for metal precipitation and, consequently, low bioavailability values, as previously reported (Lacalle et al., 2018a).

Table 5.3. Bioavailable metal concentrations at Day 1, 4, 8, 16 and 30. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended (U) and amended (A) soils for each sampling time and treatment.

	Treatment	Day 1	Day 4	Day 8	Day 16	Day 30
[Zn] (mg kg^{-1})	UB16	4.01±0.16 a1	4.77±0.25 a12	4.74±0.65 ab12	3.24±0.16 a23	2.99±0.30 a3
	AB16	7.70±0.58 a12*	6.80±0.39 a12*	7.19±0.12 a2*	6.13±0.26 a12*	6.09±0.18 a1*
	UB5	3.74±0.27 a12	4.49±0.30 a1	3.98±0.09 a12	3.16±0.06 a23	2.81±0.17 a3
	AB5	5.90±0.32 a12*	6.74±0.32 a12*	7.37±0.41 a2*	6.31±0.27 a1*	5.49±0.25 ab2*
	UB1	3.92±0.22 a13	5.37±0.17 a1	4.55±0.14 b12	3.05±0.16 a4	3.14±0.23 a34
	AB1	6.28±0.38 a12*	6.30±0.55 a12	7.10±0.17 a2*	6.55±0.31 a1*	5.24±0.10 b1*
[Cu] (mg kg^{-1})	UB16	1.94±0.15 a123	1.87±0.06 a2	1.85±0.21 a123	1.44±0.05 a3	1.24±0.11 a3
	AB16	3.32±0.11 a1*	2.88±0.29 a12*	2.16±0.14 a2*	2.66±0.22 a12*	2.90±0.21 a12*
	UB5	1.61±0.05 a1	1.62±0.20 a1	1.25±0.06 b2	1.14±0.07 b2	0.95±0.11 a2
	AB5	2.49±0.12 b1*	2.73±0.08 ab1*	4.47±0.30 a1*	3.15±0.23 a1*	2.65±0.26 a1*
	UB1	1.60±0.05 a1	1.51±0.19 a12	1.20±0.05 b123	0.90±0.02 c3	0.96±0.12 a23
	AB1	2.60±0.11 b1*	2.10±0.04 b1*	2.87±0.18 b1*	3.06±0.46 a1*	2.25±0.17 a1*
[Cd] (mg kg^{-1})	UB16	0.81±0.03 a1	0.61±0.02 a23	0.72±0.05 a2	0.50±0.02 a3	0.63±0.07 a23
	AB16	0.54±0.03 a1*	0.42±0.01 a12*	0.43±0.00 a12*	0.39±0.01 a2*	0.42±0.01 a12*
	UB5	0.75±0.04 a1	0.62±0.04 a12	0.66±0.01 a1	0.52±0.01 a2	0.57±0.03 a12
	AB5	0.42±0.02 b1*	0.42±0.00 ab1*	0.44±0.01 a1*	0.40±0.01 a1*	0.38±0.01 ab1*
	UB1	0.75±0.04 a1	0.67±0.08 a12	0.68±0.01 a12	0.57±0.02 a2	0.66±0.01 a12
	AB1	0.44±0.02 ab1*	0.39±0.03 b1*	0.43±0.01 a1*	0.40±0.01 a1*	0.38±0.01 b1*

5.3.2. Hydrocarbon concentrations

Diesel fuels are complex mixtures of chemically stable hydrocarbons, such as *n*-alkanes and polycyclic aromatic hydrocarbons (PAHs), among others. Fatty acid methyl esters are derived from the transesterification of animal fats, vegetable oils (from canola, sunflower, soy, etc.) or recycled fats. The minimum percentage of FAME for a fuel to be considered biodiesel is 8% (Demirbas, 2009).

Due to their very low presence in diesel (≈1%), PAH concentrations in our study soils were very low (data not shown). One day after the application of biodiesel blends to our experimental soil, more than 50% of the total *n*-alkanes had been dissipated (Fig. 5.1A,B,C). Other authors (Barrutia et al., 2011b; Saviozzi et al., 2009; Serrano et al., 2008) have also reported an initial very fast dissipation of diesel fuel, particularly of those fractions with a lower molecular weight. In our case, 98% of the light-weight fractions (i.e., C9-C12) was dissipated during the first day of incubation (Fig. 5.1D, E, F). By contrast, after rapid dissipation at the beginning of the experiment, C13-C16 and C17-

C21 fractions suffered a slow but progressive dissipation until the end of the study in both A and U soils, as reported by other authors (Balseiro-Romero et al., 2017). Soils polluted with B16 (Fig. 5.1G, J) and B5 (Fig. 5.1H, K) showed similar trends: after one day of incubation, degradation values for the C13-C16 fraction were between 64 and 72% in both A and U soils (corresponding values at the end of the experiment were between 80 and 85%) (Fig. 5.1G, H); similarly, after one day of incubation, degradation values for the C17-C21 fraction were between 39 and 50% in both A and U soils (corresponding values at the end of the experiment were between 61 and 69%) (Fig. 5.1J, K). In soils polluted with B1, for the C13-C16 fraction, 72 and 76% degradation values were observed after one and 30 days of incubation, respectively (Fig. 5.1I); for the C17-C21 fraction, degradation values after one and 30 days of incubation were very similar (≈50%) (Fig. 5.1L). The presence of FAME can increase the degradation of *n*-alkanes by means of co-metabolism (Pasqualino et al., 2006; Silva et al., 2012). In our study, one would expect to observe this phenomenon with more probability in B5 and B16 soils, compared to B1 soils, which could explain the higher *n*-alkanes degradation values observed in B5 and B16 soils. In any case, the low bioavailability of *n*-alkanes in soil (Balseiro-Romero et al., 2018; Saviozzi et al., 2009), together with pollutant ecotoxicity (Jørgensen et al., 2000), can hinder the biodegradation of alkanes, which could explain the relatively low rates of degradation after the fast initial dissipation. Expectedly, the heavier fraction (C22-C30) remained stable after the initial dissipation and was the most recalcitrant fraction (Fig. 5.1M, N, O).

Certainly, many authors (Barrutia et al., 2011b; Fernández et al., 2011) have studied the remediation of diesel-polluted soils, but comparisons among different biodiesel blends are scarce in the literature. Moreover, most of those studies do not use commercially-available blends and/or are performed in simpler matrices, such as sand (Lisiecki et al., 2014; Meyer et al., 2018; Woźniak-Karczewska et al., 2019). The studies that deal with co-contamination of soil with biodiesel blends and metals are even scarcer. Under our experimental conditions, the application of the bio-stabilized organic amendment did not cause a clear impact on the degradation of *n*-alkanes, in contrast to previous works (Lacalle et al., 2018a; Ros et al., 2010; Saviozzi et al., 2009) which reported a stimulatory effect of organic amendments on diesel degradation in soil.

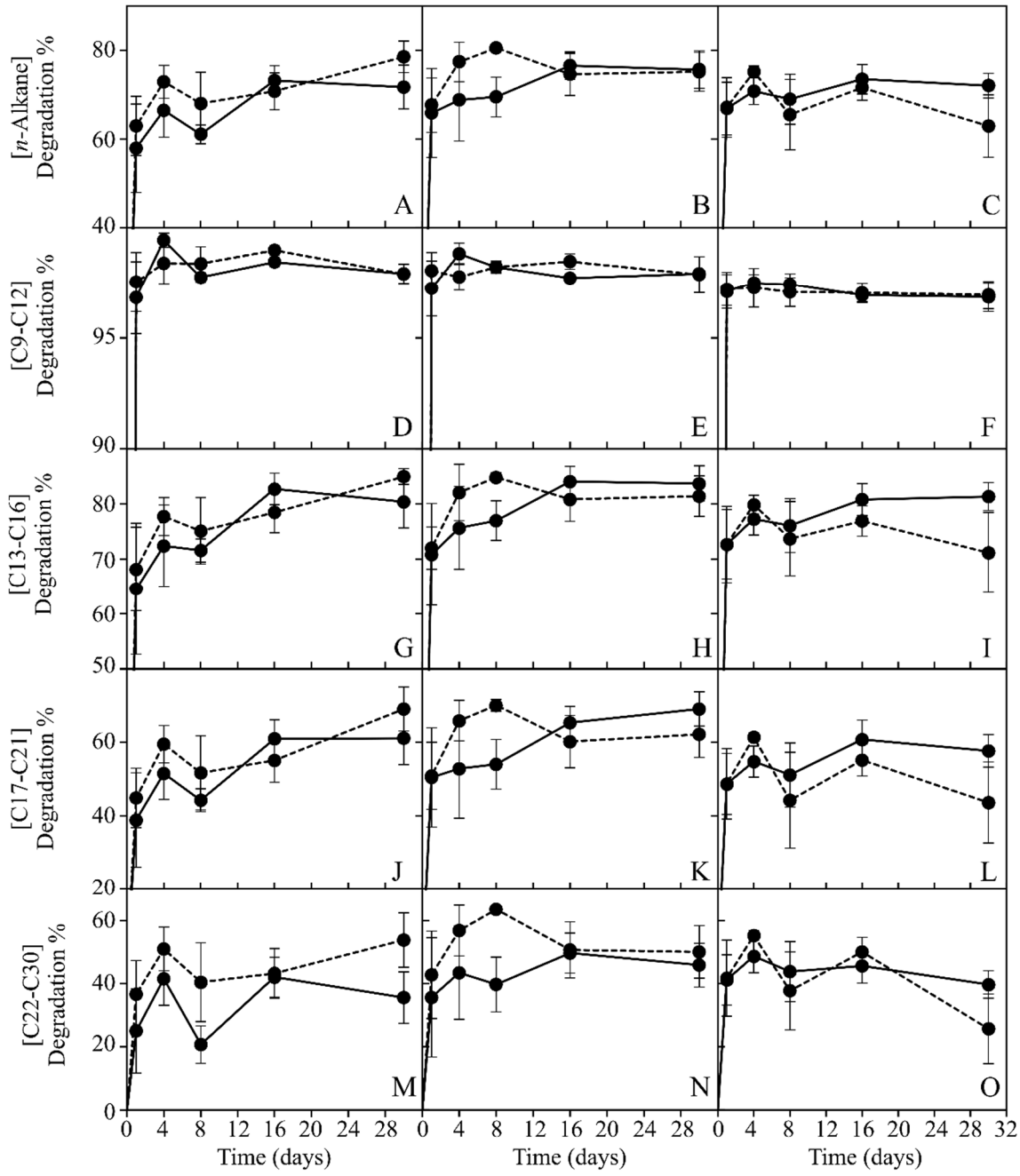


Figure 5.1. Degradation (%) of total *n*-alkanes (A, B, C) and alkane fractions, i.e. C9-C12 (D, E, F), C13-C16 (G, H, I), C17-C21 (J, K, L), C22-C30 (M, N, O) (mg kg^{-1}soil). Discontinuous line refers to unamended soils; continuous line to amended soils.

In our commercial blends, the two dominant FAME were hexadecanoic acid, methyl ester (C17) and 9,12-octadecanoic acid (C18). As previously reported (Lacalle et al., 2018a, 2018b), the degradation of FAME (Fig. 5.2A, B, C) was faster and more accentuated than that observed for *n*-alkanes (Fig. 5.1A, B, C). This was particularly

notorious in soils polluted with B16 (Fig. 5.2A). During the first 4 days, 91 and 80% of the spiked FAME was dissipated in A and U soils, respectively. In this case, the application of the organic amendment did stimulate FAME degradation. Balseiro-Romero et al. (Balseiro-Romero et al., 2016) reported that diesel degradation in soil can be enhanced by the addition of organic matter through better nutrient supply and toxicity alleviation. Furthermore, FAME might be easier to degrade by native microbial communities, since they can directly metabolize them (Thomas et al., 2017). In order to identify the main factor determining hydrocarbon degradability, i.e. chain length or fuel source (fossil origin *vs.* renewable biological origin), we compared hydrocarbons of the same chain length but different origin: (i) C17 alkane –heptadecane– (fossil origin) *vs.* hexadecanoic acid, methyl ester (biological origin) (Fig. 5.2D, E, F); and (ii) C18 alkane –octadecane– (fossil origin) *vs.* 9,12-octadecanoic acid (biological origin) (Fig. 5.2 G, H, I), finding out that hydrocarbons of biological origin showed greater degradation values than those of fossil origin. The preferential degradation of FAME over other diesel compounds has been studied in the marine environment (DeMello et al., 2007), but studies in soil are scarce (Lacalle et al., 2018a). Silva et al. (2019) reported high degradation rates of pure biodiesels (B100) of different origins, mainly composed of FAME of 16 and 18 carbon atoms. However, as abovementioned, pure biodiesels are not used as commercial fuels.

Native microorganisms are better prepared for the degradation of partially oxidized hydrocarbons, such as FAME, as they can be directly catabolized via β-oxidation (Fathepure, 2014). The *n*-alkanes, however, require to be previously oxidized by monooxygenase to secondary alcohols, then to ketones, and finally to fatty acids (Van Hamme et al., 2003). Two different monooxygenases are required depending on *n*-alkane chain length: (i) monooxygenase AlkB, along with cytochrome P450, is necessary for short-chain alkanes (C8-C16) (Van Beilen and Funhoff, 2007), while monooxygenase AlmA is responsible for the oxidation of long-chain alkanes (Fathepure, 2014). These metabolic requirements make fossil fuels more difficult to degrade than those of renewable biological origin (derived from vegetable oils or animal fats). From an ecotoxicological and remediation perspective, this is an advantage of biodiesels compared to 100% fossil diesels.

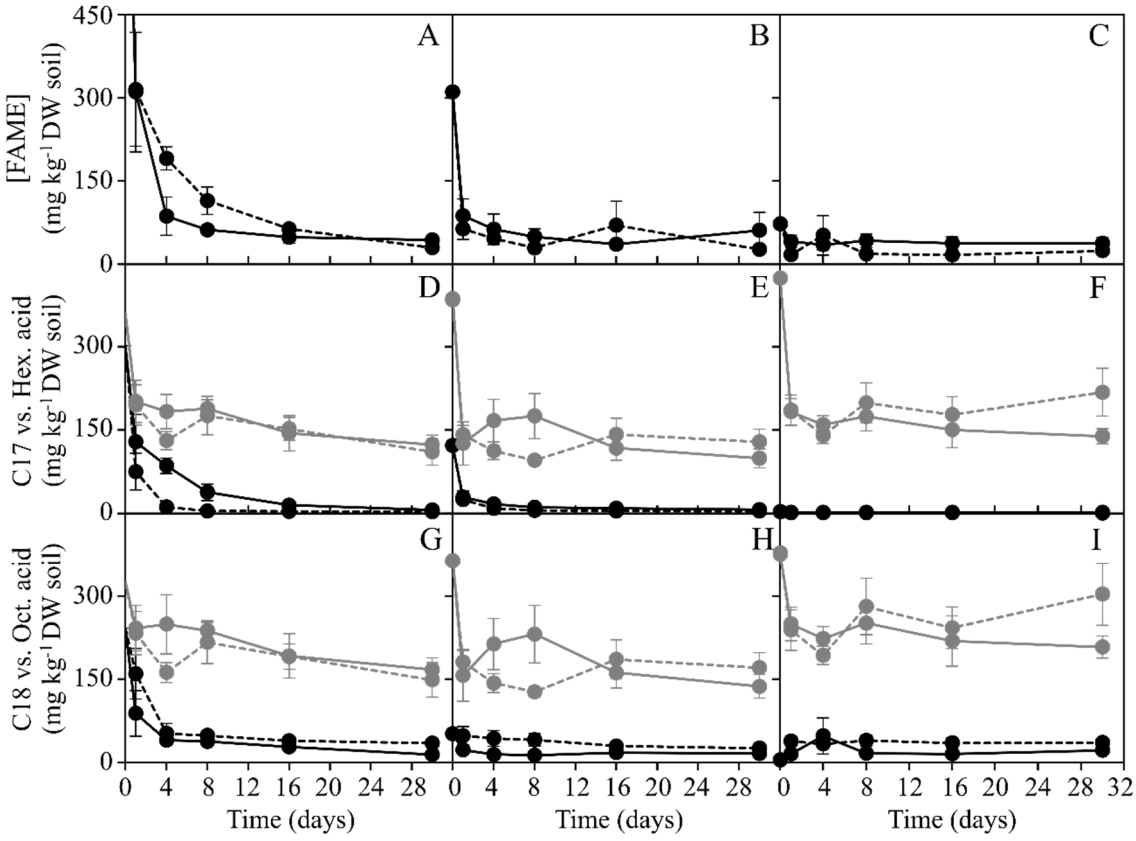


Figure 5.2. Concentration of total FAME (A, B, C). Comparison of the degradation of two FAME of biological origin (hexadecanoic acid, 9,12-octadecanoic acid; in black) *vs.* two alkanes of fossil origin (C17, C18; in grey) (mg kg^{-1}soil) (D-I). Discontinuous line refers to unamended soils; continuous line to amended soils.

5.3.3. Soil microbial properties

In order to estimate the impact of pollutants and treatments on soil health, a variety of soil microbial parameters (reflecting the activity, biomass and diversity of soil microbial communities) were measured as indicators of soil functioning.

Microbial activity, estimated as soil basal respiration, was significantly higher in control (non-polluted) A soils (Fig. 5.3B) than in control U soils (Fig. 5.3A). The presence of the organic amendment increased basal respiration in all treatments (control, B1, B5, B16) and at all sampling times (1, 4, 8, 16 and 30 days), compared to their unamended counterparts. The labile, easily degradable organic matter provided by the amendment enhanced not only microbial activity (basal respiration) but also microbial biomass (substrate-induced respiration; see below) (Fig. 5.3C, D), as previously reported (Galende et al., 2014c; Ros et al., 2003). The same bio-stabilized organic amendment was used by

Míguez et al. (2020) for the restoration of an urban vacant land, observing a stimulatory effect on soil microbial activity. Respiration measurements can provide information on pollutant-induced stress on soil microbial communities, as well as on biodegradation rates, since the aerobic biodegradation of hydrocarbons is translated into higher CO_2 production rates (Silva et al., 2012). The presence of pollutants (metals and biodiesel blends) did not provoke a clear effect on soil microbial activity. Other studies, by contrast, reported an increase of microbial activity in soils polluted with fuels, arguing that hydrocarbons were being used as an energy source (Siddiqui and Adams, 2002). Here it must be taken into consideration that the presence of metals can hamper the degradation of organic pollutants.

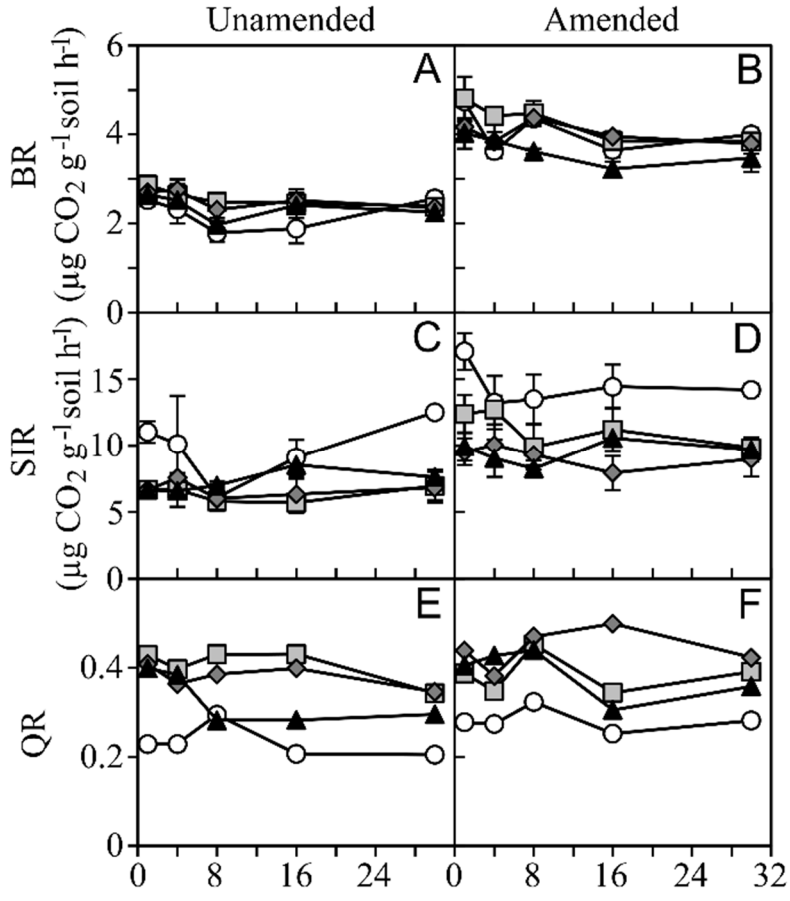


Figure 5.3. Basal respiration (A, B), substrate-induced respiration-SIR (C, D) and respiratory quotient-QR (E, F) (µg CO_2 g^{-1} dry soil h^{-1}). White circles represent non-polluted soils, light gray squares represent B16, dark gray diamonds represent B5, and black triangles represent B1.

Both control (non-polluted) and polluted A soils showed (Fig. 5.3D) higher values of microbial biomass than U soils (Fig. 5.3C). The presence of pollutants caused a slight reduction of SIR values, compared to non-polluted controls, in both A and U soils. This reduction in microbial biomass, with similar values of microbial activity in the absence and presence of pollutants, suggests an activation of the metabolic activity of soil microbial communities in the presence of pollutants as a stress response (Lacalle et al., 2018a; Zhou et al., 2013). The respiratory quotient (QR), or the relation between microbial activity and biomass, was higher in polluted *vs.* control soils, suggesting a stress response by soil microbial communities (Galende et al., 2014b). In any case, the negative effect of pollutants on soil microbial communities was moderate, suggesting their good adaptation capacity to the mixed contamination studied here.

Regarding functional microbial diversity estimated with Biolog Ecoplates^{TM} data, at the beginning of the experiment, significantly higher values of both NUS and SCA were observed in control (non-polluted) than in polluted soils (Fig. 5.4B, D). Amended soils (Fig. 5.4D) showed, in general, higher values of SCA than U soils (Fig. 5.4C), in agreement with the higher values of soil microbial activity (Fig. 5.3). This higher carbon substrate utilization is probably due to the variety and abundance of easily-degradable organic substances present in the amendment (Frąc et al., 2012; Jones et al., 2010). When carbon substrates were analyzed individually (Tables S5.6 and S5.7), we observed that, in both U and A control soils, the most utilized substrates were nitrogenated compounds (L-arginine, L-asparagine), organic acids (D-galactonic acid, D-galacturonic acid, hydroxybutyric acid) and carbohydrates (D-Manitol). The functional diversity of control soils (Fig. 5.4) decreased as time passed, being this decrease more notorious in A than in U soils. At the beginning of the experiment, organically-amended soils had an abundance of labile, easily-metabolized substrates, available for fast consumption by soil microorganisms. As shown in Table S5.7, during the first days of incubation (from Day 1 to Day 8), a higher utilization of different carbon substrates (carbohydrates, organic acids) was observed in A soils. Afterwards, such utilization was stabilized or even disappeared. The depletion of easily available nutrients, along with the acclimation of the microbial communities to the incubation conditions, might have contributed to the observed reduction of functional microbial diversity (Galende et al., 2014b).

The presence of biodiesel blends initially had a drastic deleterious effect on the capacity of microbial communities to use carbon substrates in both U (Fig. 5.4A) and A

(Fig. 5.4B) soils. After one day of incubation, in polluted soils, no amendment-induced stimulatory effect on soil functional microbial diversity was observed. However, microbial communities seemed to adapt quickly to the pollution: from Day 4 onwards, NUS and SCA values increased in polluted soils (Fig. 5.4). The acclimation to the experimental conditions and the progressive decrease of the lighter fraction of alkanes (Fig. 5.1G-L) might explain such recovery. By the end of the experiment, NUS and SCA values in polluted U soils almost reached the values shown by control soil (Fig. 5.4A,C; Table S5.4). In polluted A soils, however, although a certain recovery was observed, easily-degradable carbon sources, such as organic acids and carbohydrates (Borowik et al., 2018; Galende et al., 2014b), were barely consumed in comparison to control A soils. At the end of the experiment, A soils polluted with B16 showed similar SCA values and higher NUS values than controls (by contrast, values shown by B1 and B5 soils were lower, being this difference statistically significant for B1) (Fig. 5.4B,D; Table S5.4). This suggests a lower toxicity of B16 compared to B1 and B5.

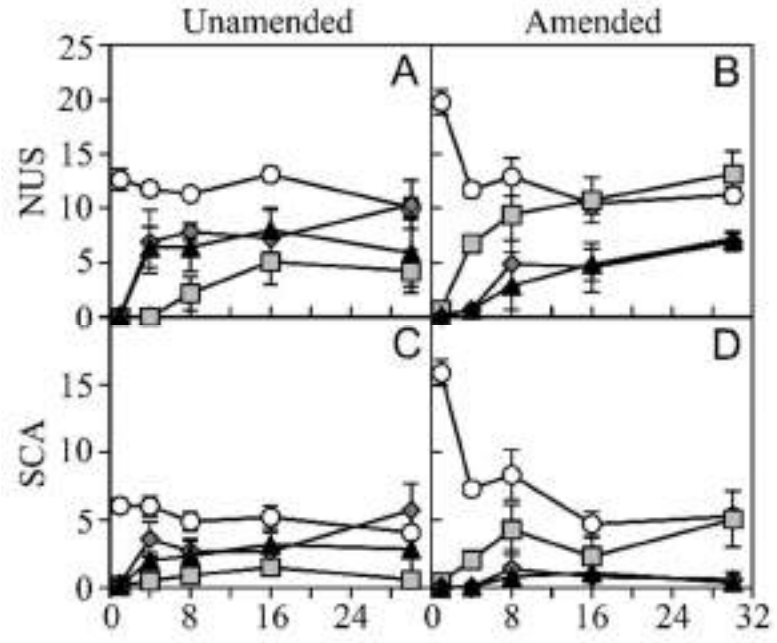


Figure 5.4. Number of utilized substrates-NUS (A, B) and substrate consumption activity-SCA (C, D). White circles represent non-polluted soils, light gray squares represent B16, dark gray diamonds represent B5, and black triangles represent B1.

There are evidences pointing to structural and functional changes in microbial diversity in soils polluted with hydrocarbons (Ros et al., 2014). However, further studies

on the impact of biodiesel blends on soil microbial communities, specifically during soil remediation processes (e.g., by natural attenuation) are required.

5.3.4. Phytotoxicity

Cucumis sativus, a sensitive plant species suitable for soil toxicity bioassays (OECD, 2006; US-EPA OPPTS 850.4200, 1996), was used here to estimate soil phytotoxicity. The application of the organic amendment had a positive effect on root elongation in all treatments (Table S5.5). On the contrary, the presence of pollutants had a negative effect on root elongation in both A and U soils, as previously observed (Lacalle et al., 2018a, 2018b). Consequently, root elongation inhibition rates were calculated using the values shown by the A control soil as reference, as this amended non-polluted soil represented the best scenario for plant growth. No significant differences were observed between the three biodiesel blends. Unamended polluted soils showed a 60-70% inhibition of root elongation at the beginning of the experiment, which increased to around 80% by the end of the experiment. Their organically-amended counterparts showed a lower inhibition at the beginning of the experiment (around 40%) which later increased to around 60% (Fig. 5.5). Therefore, the pollutants, presumably the biodiesel blends (since metals were highly immobilized), did cause phytotoxicity. This phytotoxicity endured over time until the end of the experiment, despite the progressive degradation of alkanes. Compared to soil microbial indicators, *C. sativus* plants were more sensitive to the presence of pollutants.

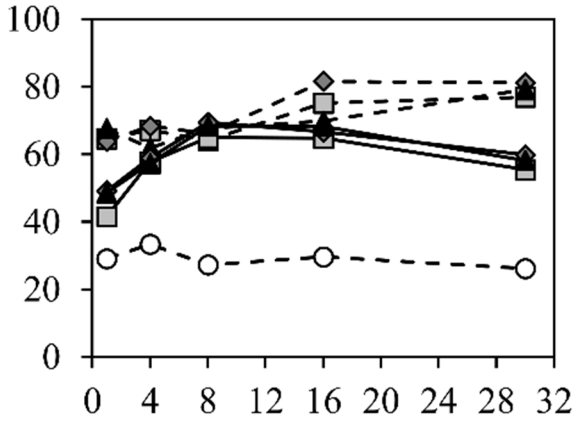


Figure 5.5. Root elongation inhibition rate (%) of *Cucumis sativus*. White circles represent non-polluted soils, light gray squares represent B16, dark gray diamonds represent B5, and black triangles represent B1.

When assessing soil health, it is important to use bioindicators that include different taxa, since different organisms may respond differently to pollutants and play different roles in the soil ecosystem. The inhibition of root length is an easily measurable indicator of phytotoxicity (Visioli et al., 2014). Regarding the effect of fuel origin (fossil *vs.* renewable biological) on phytotoxicity, there is no consensus: biodiesel has been reported to be less (Cruz et al., 2014) and more (Hawrot-Paw et al., 2015) phytotoxic than petroleum-derived diesel, depending on the specific plant species (Hawrot-Paw et al., 2015). However, in our study, their phytotoxicity was similar.

5.4. Conclusions

Degradation dynamics of hydrocarbons were origin-dependent: the diesel fraction (fossil origin) suffered a very high dissipation of its lighter chains in a very short period of time, while longer chains were more recalcitrant; in turn, the biodiesel fraction (renewable biological origin) was degraded fast and almost completely, probably because soil microbial communities were better suited to metabolize fatty acids via β-oxidation. The biodiesel blends with higher content of FAME (B16 and B5) showed progressive and sustained alkane degradation, unlike B1, which could be due to co-metabolism. The application of bio-stabilized material as organic amendment for biostimulation purposes increased soil microbial activity and FAME degradation, and partially alleviated phytotoxicity. While soil microbial communities were relatively tolerant to the presence of pollutants, the root elongation of *C. sativus* in those soils was severely affected, highlighting the importance of using different taxa as bioindicators of soil health. It was concluded that biodiesel-containing fuel might cause a lower impact on soil health than diesel of fossil origin. Nonetheless, a deeper understanding of biodiesel degradation is needed to adequately implement effective bioremediation strategies.

5.5. Supplementary material

Table S5.1. [*n*-Alk]: total *n*-alkanes. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended and amended soils for each sampling time and treatment.

		Unamended			Amended		
Parameter	**Day**	**B16**	**B5**	**B1**	**B16**	**B5**	**B1**
[*n*-Alk]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1
[C9-C12]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1
[C13-C16]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1
[C17-C21]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1
[C22-C30]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1

Table S5.2. Statistical results for FAME degradation and C17 and C18 alkanes. [FAME]: total fatty acid methyl esters; [Hexadec.]: hexadecanoic acid; [9,12-Octod.]: 9,12-octodecanoic acid; [C17]: heptadecane and [C18]: octodecane. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended and amended soils for each sampling time and treatment.

		Unamended			Amended		
Parameter	**Day**	**B16**	**B5**	**B1**	**B16**	**B5**	**B1**
[FAME]	**1**	a12	a1	b1	a1	ab1	b1
	4	a1	b1	b1	b1	ab1	a1
	8	a12	b1	b1	a2*	a1	a1
	16	a2	a1	a1	a2	a1	a1
	30	a2	a1	a1	a2	a1	a1
[Hexadec.]	**1**	a12	a1	b1	a1	ab1	b1
	4	a1	b1	b1	a1	a1	a1
	8	a12	ab1	b1	a	a1	a1
	16	a12	ab1	b1	a1	a1*	a1
	30	a2	a1	a1	ab1*	a1	b1
[9,12-Octod.]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a2	a1	a1
	8	a1	a1	a1	a2	a1	a1
	16	a1	a1	a1	a2	a1	a1
	30	a1	a1	a1	a2	a1	a1
[C17]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1*	a1
	8	a1	a1	a1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	a1	a1	a1	a1	a1	a1*
[C18]	**1**	a1	a1	a1	a1	a1	a1
	4	a1	a1	a1	a1	a1*	a1
	8	a1	ab1	b1	a1	a1	a1
	16	a1	a1	a1	a1	a1	a1
	30	b1	b1	a1	a1	a1	a1*

Table S5.3: Statistical results for soil respiration. BR: Basal respiration; SIR: Substrate-induced respiration. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended and amended soils for each sampling time and treatment.

		Unamended				Amended			
Parameter	**Day**	**C**	**B16**	**B5**	**B1**	**C**	**B16**	**B5**	**B1**
	1	a1	a1	a1	a1	a1*	a12*	a1*	a1*
	4	a1	a1	a1	a12	a3*	a1*	a1*	a1*
BR	8	b1	a1	ab1	ab2	a12	a12*	a1*	b1*
	16	a1	a1	a1	a12	ab3*	a12*	a1*	b1
	30	a1	a1	a1	a12	a23*	a2*	a1*	a1*
	1	a12	a1	a1	a1	a1*	a1*	a1	a1*
	4	a12	a1	a1	a1	a1	a1*	a1	a1
SIR	8	a2	a1	a1	a1	a1*	ab1	ab1*	b1
	16	a12	a1	a1	a1	a1	ab1*	b1	ab1
	30	a1	b1	b1	b1	a1*	b1	b1	b1

Table S5.4. Statistical results for soil functional microbial diversity. NUS: number of utilized substrates; SCA: Substrate consumption activity. Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended and amended soils for each sampling time and treatment.

		Unamended				Amended			
Parameter	**Day**	**C**	**B16**	**B5**	**B1**	**C**	**B16**	**B5**	**B1**
	1	a1	b1	b2	b1	a1	b2*	b2	b3
	4	a1	a12	a1	b1	a1	b1*	c1*	c23*
NUS	**8**	a1	b1	b1	ab1	a1	ab1	ab1	b123
	16	a1	b1	ab1	ab1	a1	a1	a1	a12
	30	a1	a1	a1	a1	ab1	a1*	bc1	c1
	1	a1	a1	a1	a1	a1	b1	bc1	c1
	4	a1	ab1	ab1	b1	a12	b1*	c1*	c1*
SCA	**8**	a1	b1	ab1	ab1	a123	ab1	b1	b1
	16	a1	a1	a1	a1	a3	a1	a1	a1
	30	a1	a1	a1	a1	a23	ab1	ab1	b1

Table S5.5. Root elongation (mm). Letters represent significant differences among treatments. Numbers represent significant differences among sampling times within the same treatment. Asterisks (*) represent significant differences among unamended and amended soils for each sampling time and treatment.

	Unamended				Amended			
Day	**C**	**B16**	**B5**	**B1**	**C**	**B16**	**B5**	**B1**
1	34.9±2.2 a1	17.4±1.4 a1	17.8±1.7 a1	15.9±0.7 a12	49.3±5.2 a1	28.7±1.5 b1*	25.0±1.6 b1	25.3±2.4 b1*
4	37.8±0.7 a1	18.6±0.3 b1	18.0±1.5 b1	21.6±2.3 b1	56.8±4.8 a1*	24.0±4.7 b1	23.3±1.2 b12	24.3±1.1 b1
8	36.7±5.4 a1	18.1±1.4 a1	17.1±1.5 a1	16.3±2.3 a12	50.4±1.6 a1	17.7±2.5 a1	15.4±1.4 a3	16.0±3.5 a1
16	35.9±2.5 a1	12.7±1.0 b2	9.4±0.6 b2	15.4±3.0 b12	51.1±3.0 a1	18.0±1.2 a1*	17.0±3.0 a12	16.2±2.5 a1
30	38.9±2.1 a1	12.1±1.2 a2	9.9±0.7 a2	11.0±1.6 a2	52.7±0.4 a1	23.5±2.5 a1*	21.2±1.0 a123	22.1±3.3 a1

Table S5.6. Substrate consumption activity for the most utilized carbon substrates in unamended soils after 1, 4, 8, 16 and 30 days of incubation. Light grey indicates low activity (SCA<10), dark grey indicates medium activity (SCA=10-20), and black indicates high activity (SCA>20).

		Control					**B16**					**B5**					**B1**				
	Day	1	4	8	16	30	1	4	8	16	30	1	4	8	16	30	1	4	8	16	30
Nitrogenated compounds	Phenylethylamine		6	3	4											8					
	Putrescine	5	9	9	8	6							14	4		18					8
	L-arginine	9	8	9	6	8							4	6	4	11	4		7		4
	L-asparagine	20	23	17	20	21				10	4		23	16	9	30	9	10	12		9
	L-Phenylalanine			3	6	7										6					
	L-serine		12	10	11	8							10	6	5	11	5	5	7		4
	L-Threonine																				
	Glycyl-L-Glutamic Acid																				
Carbohydrates	C-Cellobiose	16			4						4										
	D-Lactose																				
	Methyl-D-Glucoside	13	6																		
	D-Xylose	14																			
	i-Erythritol																				
	D-Mannitol	27	14	13	12	11				4	6		5	9	6	3	8	7	7		4
	N-Acetyl-D-glucosamine	15	9	7	5								6		6		5	3	5		
Organic acids	D-Glucosaminic acid	7	6	4	5	3													5		
	D-Galactonic acid lactone	15	15	10	11	12							4	7	4		6	6	7		5
	D-Galacturonic acid	22	15	6	6	4							9	6	6	14	6	4	10		9
	2-Hydroxy benzoic acid																				
	4-Hydroxy benzoic acid	9	9	4	5	4							7	4		10		3	4		4
	Hydroxy butyric Acid	19	13	7	11	7										14					9
	Itaconic acid		4		4	4									5	5	3	6	3		
	Keto butyric acid	4									3					4					
	D-Malic acid				4						3					4					
Miscellaneous	Pyrubic Acid Methyl Ester	13	10	12	15	11					4		6	4	7	12		7	7		5
	Glucose-1-Phosphate	5																			
	D,L-Glycerol Phosphate																				
	Tween 40	11	7	12	9	7							5	5	6	14	4	4	7		5
	Tween 80	10	7	12	8	7							5	5	7	13	6	4	8		5
	Cyclodextrin																				
	Glycogen										4										

Table S5.7. Substrate consumption activity for the most utilized carbon substrates in amended soils after 1, 4, 8, 16 and 30 days of incubation. Light grey indicates low activity (SCA<10), dark grey indicates medium activity (SCA=10-20), and black indicates high activity (SCA>20).

		Control					**B16**					**B5**					**B1**				
	Day	1	4	8	16	30	1	4	8	16	30	1	4	8	16	30	1	4	8	16	30
Nitrogenated compounds	Phenylethylamine	3				4					5										
	Putrescine	6	6	6	6	5			9		10										
	L-arginine	13	15	13	9	9	4	8	4	4				4							
	L-asparagine	30	28	26	24	20	6	13	15	30				11	5				7	6	
	L-Phenylalanine	5								3											4
	L-serine	11	8	10	4	4	4	8	3	8				7							
	L-Threonine																				
	Glycyl-L-Glutamic Acid									3						5					4
Carbohydrates	C-Cellobiose	43		9		3				4	4										
	D-Lactose	14																			
	Methyl-D-Glucoside	37		11						4	6				3	5					5
	D-Xylose	24																			
	i-Erythritol	7																			
	D-Mannitol	37	21	23	16	13	6	9	5	6				7					4	5	
	N-Acetyl-D-glucosamine	51	7	12		4	4	6						3							
Organic acids	D-Glucosaminic acid	11	11	9	5	8		4													
	D-Galactonic acid lactone	32	16	16	12	11	8	6	5					6							
	D-Galacturonic acid	35	21	24	17	16	4	5	8	15				6		6					6
	2-Hydroxy benzoic acid																				
	4-Hydroxy benzoic acid	12	10	10	7	7		6	3	15											
	Hydroxy butyric Acid	27	24	23	13	19				13											
	Itaconic acid	11	6	9	4	7	5	5	4					4					4		
	Keto butyric acid								4	7											5
	D-Malic acid				5			8		3											
Miscellaneous	Pyrubic Acid Methyl Ester	23	18	16	10	8	4	7	4	13		4	7			7			8	3	4
	Glucose-1-Phosphate	21																			
	D,L-Glycerol Phosphate	7																			
	Tween 40	12	10	11	6	6	6	6		9				5							
	Tween 80	13	10	11	9	8	7	8		9				5					4		
	Cyclodextrin								4							3					3
	Glycogen		4	4					10	10						10				5	10

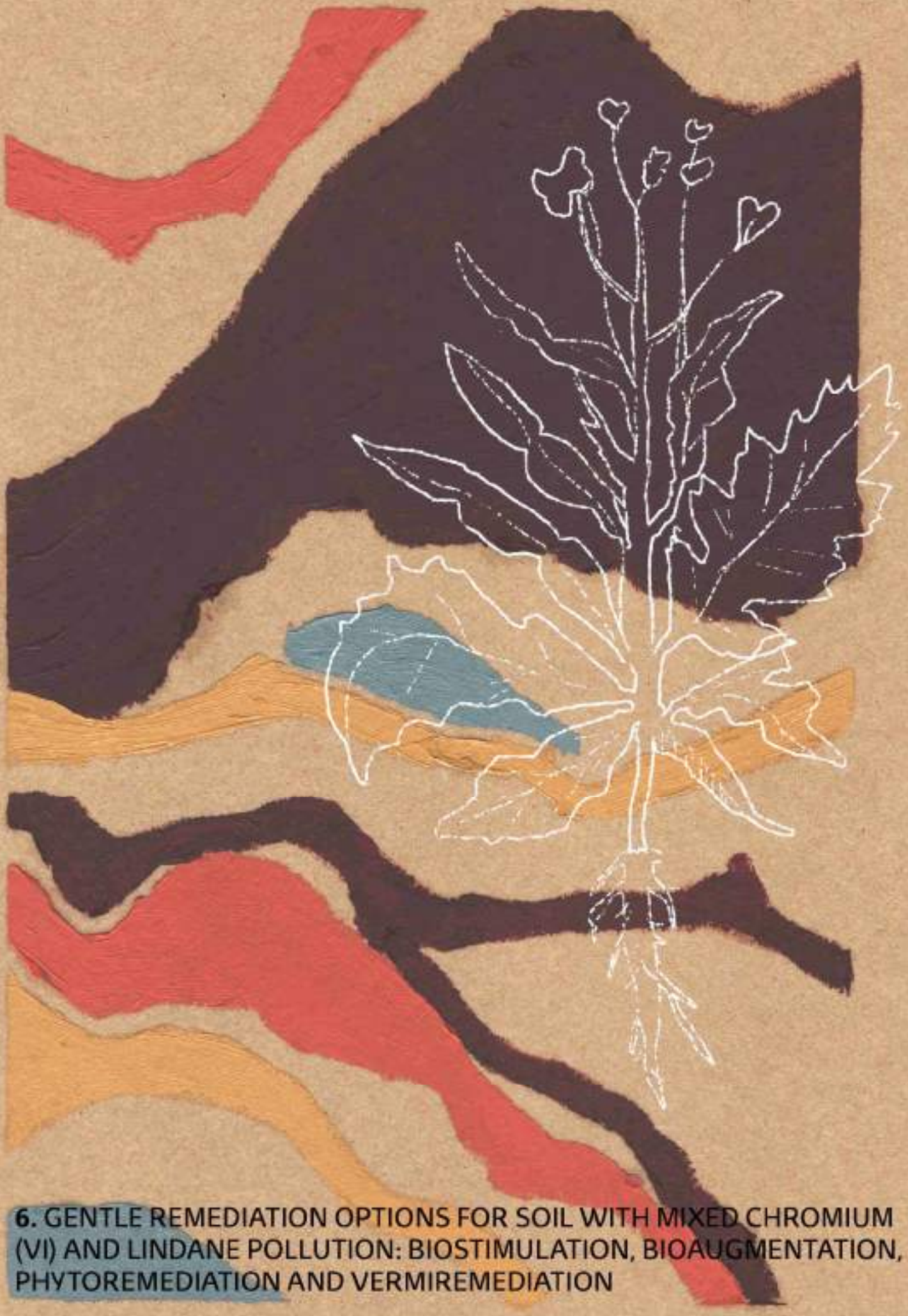
5.6 Conclusiones del capítulo

- Las comunidades microbianas naturales del suelo, estimuladas por la materia orgánica del suelo, degradan de forma muy rápida y total los metil ésteres de ácidos grasos presentes en la fracción biodiesel de los gasóleos comerciales.

- La degradación de los alcanos de origen fósil son degradados por los microorganimos del suelo, siendo esta degradación más rápida y efectiva en los compuestos de cadena más corta (C9-C16)

- En los gasóleos comerciales con mayor contenido en biodiesel (5 y 16%) la degradación de alcanos es mayor y continua mientras que en que en gasóleos sin biodiesel su degradación fue menor y se frenó tras las primeras semanas.

- Las comunidades microbianas presentes en el suelo se adaptan bien a la contaminación mixta por metales y gasóleo, ya que no muestran toxicidad y son capaces de degradar parcialmente el carburante. Por el contrario, en esos suelos los bioensayos con *Cucumis sativus* indican que mantienen una elevada fitotoxicidad.



6. GENTLE REMEDIATION OPTIONS FOR SOIL WITH MIXED CHROMIUM (VI) AND LINDANE POLLUTION: BIOSTIMULATION, BIOAUGMENTATION, PHYTOREMEDIATION AND VERMIREMEDIATION

6. GENTLE REMEDIATION OPTIONS FOR SOIL WITH MIXED CHROMIUM (VI) AND LINDANE POLLUTION: BIOSTIMULATION, BIOAUGMENTATION, PHYTOREMEDIATION AND VERMIREMEDIATION

Rafael G. Lacalle, Juan D. Aparicio, Unai Artetxe, Erik Urionabarrenetxea, Marta A. Polti, Manuel Soto, Carlos Garbisu, José M. Becerril, 2020. Gentle remediation options for soil with mixed chromium (vi) and lindane pollution: biostimulation, bioaugmentation, phytoremediation and vermiremediation. **Heliyon** (Submitted)

Abstract

The combination of organic and inorganic pollutants in soil intensifies toxicity and impedes remediation compared to soils with only one kind of pollutant. Gentle Remediation Options (GROs) such as bioaugmentation, phytoremediation, vermiremediation, and biostimulation may be cost-effective and environmentally friendly solutions. However, their compatibility and effectiveness in remediating soils that contain a mixture of pollutants have not been widely explored. This study assessed the individual and combined effectiveness of these GROs in enhancing or recovering the health of soil containing two pollutants, hexavalent chromium [Cr(VI)] and the gamma isomer of hexachlorocyclohexane (lindane). A greenhouse experiment was performed, using soil amended or not with organic matter. The amended and non-amended soils were artificially polluted with lindane and two concentrations of Cr(VI), 100 or 300 mg kg^{-1}. All types of soils received the following treatments: (i) no treatment; (ii) bioaugmentation with an actinobacteria consortium; (iii) vermiremediation with *Eisenia fetida*; (iv) phytoremediation with *Brassica napus*; (v) Bioaugmentation + vermiremediation; (vi) Bioaugmentation + phytoremediation; or (vii) Bioaugmentation + vermiremediation + phytoremediation. After two months of treatment, the health status of the remediator organisms was assessed, the plants were harvested, and worms and samples were collected. Soil health was determined based on decreases of soluble Cr and lindane, microbial properties, and toxicity bioassays using both plants and worms. Cr(VI) caused high toxicity, but some GROs were efficient in recovering soil health: (i) Organic matter decreased soluble Cr in the soil, alleviating toxicity; (ii) The actinobacteria consortium was effective in removing both Cr(VI) and lindane; (iii) *B. napus* and *E. fetida* had a positive effect on the removal of the pollutants, and improved microbial parameters. The

combination of all the techniques was, overall, more effective than the individual applications. We concluded that this is a promising approach for the phytomanagement of soils polluted with Cr(VI) and lindane.

6.1. Introduction

The intensification and expansion of human activity caused by industrial growth has increased environmental pollution, threatening human and ecosystem health. Pollution and its negative effects are enhanced when organic pollutants (herbicides, pesticides, petroleum hydrocarbons, etc.) and inorganic compounds (metals, metalloids, etc.) coexist, a phenomenon known as mixed pollution or co-pollution. This leads to dangerous and unpredictable situations resulting from the toxicity of each compound and the interactions among compounds and with soil organisms (Batty and Dolan, 2013).

The presence of both organic and inorganic pollutants in soil is a widespread problem, since more than a third of polluted sites contain more than one type of pollutant (Polti et al., 2014). In particular, mixed pollution with the metal hexavalent chromium [Cr(VI)] and the pesticide lindane has been detected recently in different parts of the world, where both compounds have been reported in concentrations that exceeded the allowed maxima (Aparicio et al., 2018a, 2018b; Arienzo et al., 2013).

Cr(VI) is found in a wide variety of sites, due to its use in many industries such as metallurgy or tanning (Bankar et al., 2009). Cr(VI) has been reported to be 1000-fold more cytotoxic and mutagenic than Cr(III) (Biedermann and Landolph, 1990). Moreover, Cr(III) tends to precipitate, while Cr(VI) is more soluble (Zayed and Terry, 2003). The gamma isomer of hexachlorocyclohexane (γ-HCH), commercially known as lindane, is a highly chlorinated, recalcitrant organochlorine pesticide with toxic effects on animals, including humans. Lindane is accumulated in biological tissues and biomagnified through the food chain, and has been reported in soil, water, air, plants, animals, food, and humans, among others (Fuentes et al., 2011).

Gentle Remediation Options (GROs) such as bioaugmentation, phytoremediation, vermiremediation, and biostimulation have received considerable attention in recent years as effective risk-management strategies to reduce the transfer of contaminants to local receptors, through *in-situ* stabilization or extraction of pollutants (Cundy et al., 2013). These biological treatments can provide a cost-effective, environmentally friendly solution to soil co-pollution (Agnello et al., 2016), and are increasingly employed in place of the traditional remediation technologies.

Bioaugmentation attempts to improve the degradation capacity in polluted areas by introducing into the soil microorganisms capable of degrading pollutants or

transforming pollutants into non-toxic or less-toxic species, and has been used for both chromium and lindane remediation (Alvarez et al., 2012; Bajaj et al., 2017; Gutiérrez-Corona et al., 2016). While several bacterial strains have been identified as Cr(VI) or lindane bioremediators, few studies have examined their effects on mixtures of these pollutants. Recently, Aparicio et al. (2018b) found that an actinobacteria consortium was effective in reducing high concentrations of Cr(VI) and lindane from polluted soils. Actinobacteria are a group of bacteria commonly found in soil, and their physiological diversity allows them to degrade a wide variety of substances and to play an important role in recycling (Goodfellow et al., 1988; Kieser et al., 2000). However, bioaugmentation has limitations, since the survival of inoculated bacteria is affected by soil characteristics and the existing microbial communities (Cycoń et al., 2017).

Metal phytoremediation includes phytostabilization (reduction of pollutant mobility and bioavailability) (Epelde et al., 2009a; Galende et al., 2014b, 2014c) and phytoextraction (metal accumulation in plant shoots) (Barrutia et al., 2010; Epelde et al., 2010). Phytoremediation may also be suitable for the rhizoremediation of organic pollutants (Liu et al., 2017; Montpetit and Lachapelle, 2017), and organic compounds that roots exude to the rhizosphere create a nutrient-rich environment that stimulates microbial communities, enhancing the degradation of organic pollutants (Kuiper et al., 2004). Canola or oilseed rape (*Brassica napus* L.) has been reported to be a suitable candidate for metal phytoremediation (Belouchrani et al., 2016), rhizoremediation of organic pollutants such as diesel fuel (Lacalle et al., 2018a), and for polychlorinated compounds (Javorská et al., 2009). *B. napus* has attracted attention from scientists and industries, due to its potential for oil production from polluted soils (Cundy et al., 2016; Dhiman et al., 2016). These characteristics make *B. napus* a good candidate for phytomanagement, which envisages remediation of the soil while also generating social, environmental and economic benefits (Burges et al., 2018; Evangelou et al., 2015). Ontañón et al. (2014), in a rhizoremediation study using *B. napus*, found a reduction of Cr(VI) to Cr(III) and phenol degradation in a co-polluted hydroponic system. To date, no studies have examined remediation of soils co-polluted with lindane and chromium. Previous studies have shown that *B. napus* is moderately tolerant to mixed metal and organic pollution (Lacalle et al., 2018a, 2018b). Nevertheless, the capacity of *B. napus* to reduce the toxicity of this kind of mixed pollution has not been tested.

Another biological-remediation technology that has recently attracted attention

from the scientific community is vermiremediation (Sinha et al., 2008). This technology uses earthworms to remediate soils containing metals (Suthar, 2008) and organic pollutants, including some chlorinated compounds (Shi et al., 2020). Earthworms burrow through the soil, mixing it, affecting its structure, and altering its nutritional profile and bacterial and fungal communities (Rodriguez-Campos et al., 2014). Vermiremediation has been used in combination with other GROs, such as bioremediation and phytoremediation (Ekperusi and Aigbodion, 2015; Lemtiri et al., 2016). These combined technologies open new possibilities for soil remediation in a holistic approach, considering soil-earthworm-plant-microbial interactions in the ecological context of the polluted soil. *Eisenia fetida* is a good candidate as a vermiremediator (Chachina et al., 2016; Suthar, 2008) and has also been widely used as an indicator of soil health (Irizar et al., 2015a; Shin et al., 2007).

Applications of GROs commonly include modification of polluted-soil conditions and/or application of amendments that enhance the biological activity of soil organisms, a process known as biostimulation. Organic amendments are a good choice for this purpose and have been widely used (Kästner and Miltner, 2016), as they add nutrients and carbon sources to the soil, promoting plant growth and microbial activity (Galende et al., 2014c) as well as the soil fauna (Dubey et al., 2019). They can also impact the oxidation status of metals and their bioavailability (Park et al., 2011).

Each biological technology for soil remediation has certain limitations, and the simultaneous presence of inorganic and organic pollutants poses its own particular problems. These restrictions could be counteracted by a combination of technologies to remediate soil pollution, together with recovery of soil health. Accordingly, the aim of this study was to assess the individual and combined effectiveness of *B. napus* plants, and/or an actinobacteria consortium, and/or *E. fetida* earthworms as remediation strategies for soil polluted with Cr(VI) and lindane, in the presence or absence of an organic amendment.

6.2. Materials and methods

6.2.1. Experimental design

For this study, two soil samples were collected from a peri-urban area near the city of Vitoria-Gasteiz (42°50′N; 02°40′W, northern Spain). One sample was taken from soil

amended four months previously with 100 t ha^{-1} of an organic amendment consisting of recycled urban organic wastes from the city (A). The other sample was taken from unamended (U) soil near the amended plot. Both samples were collected from the topsoil (0–15 cm), sieved to < 2 mm, and air-dried prior to physicochemical characterization (Table 6.1).

In order to artificially contaminate the soils, a stock solution of 5 g L^{-1} of Cr(VI) was prepared as $K_2Cr_2O_7$. The solution was sterilized by filtration, using Millipore filters with 0.22 µm pore size. A lindane stock solution was prepared at 5 g L^{-1} using acetone as the solvent. The soils were artificially polluted with both Cr(VI) and lindane solutions and mixed to homogenize them, establishing three conditions for the experiment: (i) control (C), with no pollution; (ii) moderate pollution (M), with 100 ppm of Cr(VI) and 15 ppm of lindane; and (iii) high pollution (H), with 300 ppm of Cr(VI) and 15 ppm lindane. All assays were carried out in pots with 1 kg of soil and kept in a greenhouse to allow the pollutants to interact with the soil components. Samples were taken weekly to monitor the concentration of Cr(VI), which stabilized one month after it was added to the soil (data not shown). The greenhouse conditions were: photoperiod 14/10 h day/night, temperature 25/18 °C day/night, relative humidity 60/70% day/night.

After the one-month stabilization period, each soil sample was homogenized and the following treatments were applied: (i) no treatment (NT); (ii) inoculation of actinobacteria consortium (Ac); (iii) addition of 10 *Eisenia fetida* adult individuals (Ef); (iv) sowing of 20 *Brassica napus* seeds (Bn); (v) *E. fetida* + actinobacteria consortium (Ac+Ef); (vi) *B. napus* + actinobacteria consortium (Ac+Bn); and (vii) *E. fetida* + *B. napus* + actinobacteria consortium (Ac+Ef+Bn). The actinobacteria consortium was applied first, to allow the bacteria to colonize the soil, and the other biological treatments were applied 14 days later. After the *B. napus* seeds germinated, the seedlings were thinned to leave 5 seedlings per pot. The pots were kept in the greenhouse under the above environmental conditions for two months after the treatments were applied.

Inoculation was carried out according to Aparicio et al. (2018b). The actinobacteria *Streptomyces* sp. M7, *Streptomyces* sp. A5, *Streptomyces* sp. MC1, and *Amycolaptosis tucumanensis* DSM 45259 were used. They had been isolated from environments polluted with pesticides and metals, and were selected for their compatibility and effectiveness in reducing Cr(VI) and lindane concentrations simultaneously in soil in a previous study (Polti et al., 2014). The bacterial inoculum was

prepared as follows: spores of the four actinobacteria species were inoculated individually in flasks with 30 mL of Tryptic Soy Broth (TSB) and then incubated in an orbital shaker at 30 °C, 180 rpm. After 72 h, microbial biomass was recovered by centrifugation (8385 × *g*), washed twice, and resuspended in sterile distilled water to a final concentration of 100 g L^{-1}. After the stabilization period, soils were inoculated with 2 g kg^{-1} of the quadruple actinobacteria consortium. Equal proportions of biomass of each strain were added in order to reach the desired final concentration.

Seeds of *B. napus* (v. Expower) were provided by the commercial supplier Dekalb® (Barcelona, Spain). Specimens of *E. fetida* were provided by the commercial supplier Lombricor S.C.A. (Córdoba, Spain). The earthworms were kept in the laboratory under controlled conditions (19 °C and 60% relative humidity) with weekly addition of horse manure as a nutrient source. In order to guarantee the homogeneity of the earthworms used in the assays, healthy, sexually mature (clitellated) individuals weighing between 350–450 mg were selected.

During the course of the experiment, the soil was maintained at water-holding capacity (WHC) by irrigating the pots when necessary. Fifty-six days after the plants and worms were introduced, the plants were harvested, the worms were collected and counted, and soil samples were taken and immediately stored at 4 °C for preservation until analysis.

6.2.2. Physicochemical determinations

6.2.2.1. Total Cr and soluble Cr(VI) determination in soil

The soil was oven-dried at 35 °C for 72 h and sieved to < 0.125 mm before analysis. In order to determine the total Cr content in the soil, samples were acid-digested (HCl and HNO_3), according to the method recommended by the US Environmental Protection Agency (US-EPA Method 3051A, 2007), using a Mars V microwave digestion oven. The total concentration of Cr was determined by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) with a limit of quantification (LOQ) of 0.03 μg L^{-1}. Accuracy was ensured using NIST Standard Reference Material 1640. The soluble fraction of Cr, composed of Cr(VI), was extracted following the method described by Jiang et al. (2015). Briefly, a 1:25 (p/v) mixture of soil and Milli-Q water was shaken at 200 rpm for 24 h, centrifuged at 10,000 × *g* for 15 min, and then filtered (0.45 μm) to remove the soil from the aqueous solution. The extract was analyzed using the same method as for total chromium.

6.2.2.2. Cr concentration in plants

Plants were oven-dried at 35 °C for 72 h, milled, and digested in a mixture of HNO_3 and $HClO_4$ (Zhao et al., 1994). Cr concentration was analyzed by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) (LOQ: 0.03 μg L^{-1}), with accuracy ensured using NIST Standard Reference Material 1640.

6.2.2.3. Lindane concentration in soil

Lindane concentration was determined according to Fuentes et al. (2011). Ten mL of a water-methanol-hexane (4:1:5) solution was added to 5 g of soil. The mixture was agitated using a vortex mixer and centrifuged to separate the organic phase, which was removed and evaporated to dryness. The lindane residue was resuspended in n-hexane and quantified as in Fuentes et al. (2011), using a gas chromatograph (Agilent 7890A; LOQ: 170 μg L^{-1}).

6.2.2.4. Lindane determination in plants

Lindane concentration in plants was determined using the "QuEChERS" method for extraction and analysis of pesticides, as described by Quintero et al. (2005). This method has two phases: i) lindane extraction from dry, milled plant samples with acetonitrile, magnesium sulfate and sodium acetate; ii) cleansing of the extract by solid-phase extraction with an Agilent kit (QuEChERS AOAC). Lindane was quantified as in Fuentes et al. (2011), by gas chromatography (Agilent 7890A; LOQ: 170 μg L^{-1}).

6.2.3. Soil microbial properties

Soil microbial properties were determined as described by Galende et al. (2014a): (i) microbial activity was determined by basal respiration (BR), following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR), following ISO 17155 (2002); and (iii) number of metabolized substrates (NUS) was determined using Biolog EcoPlates™.

6.2.4. Phytotoxicity bioassay with Cucumis sativus

In order to evaluate soil phytotoxicity, a root-elongation bioassay was performed. Briefly, pre-germinated seeds of *Cucumis sativus* were exposed for 72 h to 10 g of the soil under controlled conditions, and root elongation was measured after the exposure time,

following the method described by Lacalle et al. (2018a). Roots were measured using the software ImageJ (Schneider et al., 2012).

6.2.5. Toxicity bioassay with Eisenia fetida

Soil toxicity was assessed using a bioassay with *Eisenia fetida* worms, as described by Irizar et al. (2015b). Healthy clitellated earthworms of similar size, kept at 19 °C in constant humidity, were transferred to a non-polluted soil for 24 h for acclimation. Then, the earthworms were cleaned, and 10 individuals were weighed and placed in each jar containing the soils collected from the experiment, at 40% of the soil water-holding capacity. Three replicates per soil were established. After 14 days, the worms were removed from the jars and their mortality and weight were recorded.

6.2.6. Photosynthetic pigment profile and tocopherols

Prior to plant harvesting, six discs, each with a diameter of 3 mm, were collected from the youngest fully expanded leaf, frozen in liquid nitrogen, and stored at –80 °C until analysis. Leaf discs were homogenized using a tissue-tearor (Model 395; Dremel, México, D.F., Mexico) in 1 mL cold acetone. Then, samples were centrifuged at 13200 × *g* for 20 min at 4 °C; the supernatant was collected, adjusted to a volume of 1.5 mL and filtered through a 2-µm PFTE filter (Teknokroma, Barcelona, Spain) and refrigerated until analysis.

A new and ultra-rapid uHPLC method was developed for quantification of photosynthetic pigments and tocopherols. This method is less time- and solvent-consuming, generates less residue, and provides a higher resolution for all compounds than traditional HPLC methods. Samples were injected into an Acquity™ uHPLC H-Class system (Waters®, Milford, MA, USA), using a reversed-phase column (Acquity UPLC® HSS C18 SB column, 100Å, 1.8 µm, 2.1 mm × 100 mm) and a Vanguard™ pre-column (Acquity UPLC HSS C18 SB, 1.8 µm). The mobile phase had two components: solvent A, acetonitrile: water: methanol: Tris-HCl 1 M (84:12.6:2:1.4); and solvent B, methanol: ethyl acetate (68:32). Tocopherols and pigments were eluted using a linear gradient from 100% of solvent A to 100% of solvent B for the first 2.5 min, followed by an isocratic elution of solvent B for 1 min, and the initial conditions (100% solvent A) were restored with a linear gradient of 0.5 min. This isocratic elution with 100% of solvent A was maintained for 2.5 min to re-equilibrate the column prior to the next injection. The flow of the mobile phase was 0.5 mL/min, with a working pressure of around 5000 psi.

The column was maintained at 45 °C, in an oven. The volume of the injected sample was 2 µL. The column was preserved overnight with 100% acetonitrile at 0.02 mL/min.

Photosynthetic pigments were detected with a photodiode detector (Acquity PDA uHPLC; Waters) in a range of 400–700 nm for their identification, and were quantified by (usually) integration at 445 nm. Tocopherols were detected by fluorescence, using FLR uHPLC Acquity (Waters), setting an excitation wavelength of 295 nm and an emission wavelength of 340 nm.

Pigments and tocopherols were identified and quantified by spectral characteristic and retention time (RT), using known concentrations of standards as described by García-Plazaola and Becerril (1999). Under our experimental conditions, photosynthetic pigments were detected and integrated at 445 nm and showed the following RT (in min): neoxanthin (1.72), violaxanthin (1.98), antheraxanthin (2.27), lutein (2.47), zeaxanthin (2.52), chlorophyll b (2.63), chlorophyll a (2.85), α-carotene (3.29), and β-carotene (3.32). For tocopherols under the fluorimetric conditions described above, the RT (in min) were: δ-tocopherol (2.53), β+γ tocopherol (2.67), and α-tocopherol (2.81). Under the chromatographic characteristics described above, the conversion factors (pmol per injection/area unit) were: neoxanthin (1.19×10^{-4}), violaxanthin (7.84×10^{-5}), antheraxanthin (8.00×10^{-5}), lutein (8.00×10^{-5}), zeaxanthin (8.38×10^{-5}), chlorophyll b (1.56×10^{-4}), chlorophyll a (2.58×10^{-4}), α–carotene (6.95×10^{-5}), and β-carotene (8.39×10^{-5}). For tocopherols under the fluorimetric conditions described above the RT (in min) were: δ-tocopherol (7.30×10^{-7}), β+γ tocopherol (3.30×10^{-7}), and α-tocopherol (2.22×10^{-6}).

6.2.7. Statistical analysis

The statistical analyses used IBM SPSS Statistics for Windows, Version 24. Normality was checked with a Shapiro-Wilk test. Data were tested by means of a 2-way ANOVA (post-hoc: Tukey/Duncan). A Principal Components Analysis was performed, using The Unscrambler Version 9.2.

6.3. Results and Discussion

6.3.1. Soil physicochemical properties

Table 6.1 lists the physicochemical properties of both the unamended and amended soils. Both are loamy soils with alkaline pH and a high carbonate content. The unamended soil was poor in quality, with very low organic-matter content (1%) compared to the amended soil (2.6%), and also contained low levels of other nutrients such as N and total organic carbon, which can reduce plant and microorganism growth.

At the end of the experiment, the total chromium content remained unaltered in all treatments (data not shown), indicating that there were no leaching processes or significant Cr extraction by *B. napus* plants. Soluble chromium [Cr(VI)] showed very different concentrations, depending on the treatment; however, in all test conditions, the final soluble-chromium concentration was lower than the initial concentration (Fig. 6.1A, B). This could be due to the reactivity of Cr(VI), which reacts with organic matter or inorganic minerals present in the soil and is reduced to Cr(III). The resulting Cr(III) could be precipitated as hydroxides or interact with clay minerals, which have a high metal-binding capacity (Sandrin and Maier, 2003). Here, the largest effect was due to the organic amendment, which significantly decreased the concentration of soluble chromium (Fig. 6.1B). Organic matter plays an important role in the bioavailability of Cr in soil, through its potential to reduce Cr(VI) to Cr(III). Addition of amendments rich in organic matter presumably accelerates the reduction of Cr(VI) to inert chromite [Cr(III)] (Antoniadis et al., 2018). In the polluted soils without amendment and with no biological treatment (NT), soluble Cr(VI) was 15.7% and 37.8% of total Cr for moderate and high pollution levels, respectively; while in soils with the organic amendment, the soluble fraction of chromium was less than 1% of the total Cr in both cases.

Table 6.1. Soil physicochemical properties. (USDA: United States Department of Agriculture)

	Unamended soil	**Amended soil**
Texture class (USDA)	Loam	Loam
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Total clay (%)	23.4	15.7
Carbonates (%)	54.7	44.0
Organic Matter (%)	1.0	19.5
Total C organic (% DW)	0.6	7.3
Total N (% DW)	0.1	0.9
C organic / N organic	6.7	8.6
Total S (% DW)	< 0.05	< 0.05
pH (1:2.5)	7.9	8.0
[Cr] (C) (mg kg^{-1})	25.2	25.5
[Cr] (M) (mg kg^{-1})	125.2	124.9
[Cr] (H) (mg kg^{-1})	325.9	324.9
[Lindane] (C) (mg kg^{-1})	0	0
[Lindane] (M) (mg kg^{-1})	13.6	15.3
[Lindane] (H) (mg kg^{-1})	14.0	13.3

Pollution level: control (C), moderate (M), high (H)

The actinobacteria consortium (Ac) was highly effective in reducing Cr(VI) to Cr(III), as it significantly decreased the soluble Cr concentration in non-amended polluted soils in comparison with non-bioaugmented non-amended soils (Fig. 6.1A). In fact, in amended soils, none of the biological treatments applied had any effect in decreasing soluble chromium concentration (Fig. 6.1B). The reason may be that an equilibrium concentration (threshold) was reached, and/or the concentration was so low (1 mg kg^{-1}) that it is very difficult to stimulate biological reduction of the metal (Simón Solá et al., 2019). These findings agree with those of previous studies (Aparicio et al., 2018a, 2018b; Marta A. Polti et al., 2009; Polti et al., 2014).

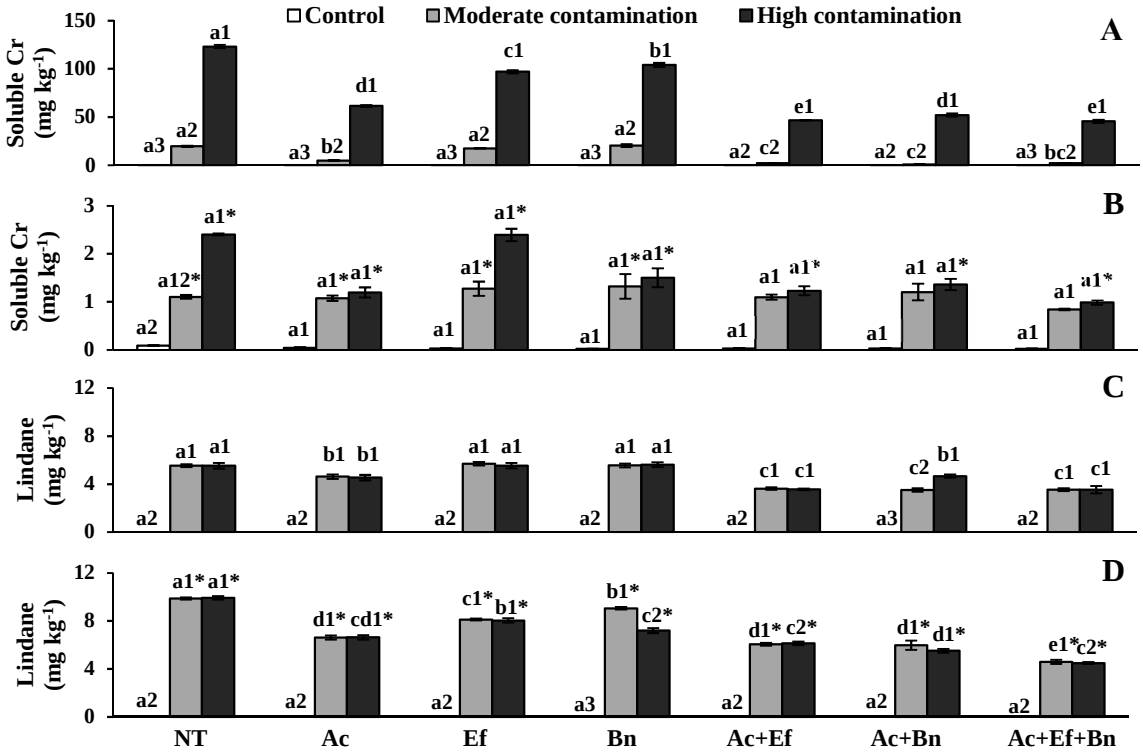


Figure 6.1. Pollutant concentrations. Soluble Cr concentration in unamended (A) and amended (B) soils. Lindane concentration in unamended (C) and amended (D) soils. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

The other biological treatments with *E. fetida* and *B. napus* significantly reduced the concentration of soluble Cr in non-amended soils (Fig. 6.1A), but less than the reduction caused by the consortium (Fig. 6.1A). Nevertheless, in these soils the combination of the three biological treatments resulted in the lowest Cr(VI) levels (Ac+Bn+Ef). In any case, the concentration of Cr(VI) in the Ac+Bn+Ef treatment in the unamended soil was higher than the concentration of Cr(VI) produced by any treatment in amended soil (Fig. 6.1B), which demonstrates the high effectiveness of the organic amendment in reducing the most toxic species of Cr.

Lindane residual concentrations are shown in Figs. 6.1C and 6.1D. Natural attenuation occurred; in both soils with no biological treatment, the concentration decreased compared to the initial values. Organic matter inhibited the natural attenuation of lindane, as the concentration in amended soils (Fig. 6.1D) remained significantly higher than in the unamended ones (Fig. 6.1C). To our knowledge, this effect has not been previously reported; it could be explained by the bacteria metabolizing the more easily

degradable organic compounds provided by the organic matter instead of the lindane. The actinobacteria consortium (Ac) was the most effective individual biological treatment in degrading lindane, since the concentration in bioaugmented soils was significantly lower than in the non-bioaugmented ones (Figs. 6.1C, D). Degradation by actinobacteria was more pronounced in the amended soils, which had a higher concentration in the untreated soils at the end of the experiment. *B. napus* (Bn) significantly increased lindane degradation, probably by stimulating the microorganisms through exudates from the plant roots (Simon Sola et al., 2017). Concomitantly, *E. fetida* (Ef) also stimulated degradation, probably by improving the aeration of the soil (Rodriguez-Campos et al., 2014). The combination of the actinobacteria consortium with *E. fetida* (Ac+Ef) or with *B. napus* (Ac+Bn) increased the degradation more than when applied individually, and the effect on degradation of the three combined (Ac+Ef+Bn) was significantly higher than the binary treatments (Figs. 6.1C, D). Our results indicate that organic matter reduced soluble Cr, but it may have interfered with the optimal degradation of lindane. When the organic-matter content in the soil was low, the microorganisms metabolized lindane more efficiently, showing a synergistic effect in the presence of plants and worms.

6.3.2. Status of plants and worms

As mentioned before, the unamended soil was a low-quality soil for plant and worm growth, since in the absence of contaminants the shoot biomass was very low, but improved after amendment with organic matter and actinobacteria (Table 6.2). In addition, the pollutants present in the soil highly impacted the plants and worms. None of them survived in the unamended soils spiked with the highest concentrations of pollutants (Tables 6.2 and 6.3). At moderate pollution levels, the plants survived only in the treatment with the lowest soluble Cr(VI) concentration, reached in the treatment Ac+Bn (Table 6.2). Soluble chromium, rather than total chromium, was toxic to the plants and worms. In contrast, in amended soils, with lower concentrations of Cr(VI), both types of organisms survived in all treatments. The importance of organic matter should be highlighted, since the plants and worms survived in all the treatments, which the toxicity reduction by the actinobacteria consortium (Ac+Bn, Ac+Ef, Ac+Ef+Bn) did not achieve in unamended soils (Tables 6.2 and 6.3). The plants and worms benefited not only from the reduction of soluble Cr, but also from their better performance in the enriched soil.

Chromium phytotoxicity has been widely studied (Han et al., 2004; Samantaray et al., 1998b), and its impact on *B. napus* was clearly revealed through the plant mortality (Table 6.2). In unamended soils, the surviving plants reached a significantly lower biomass compared to controls, and only the presence of actinobacteria made the survival of plants possible (Table 6.2). The organic amendment highly stimulated plant development in the control soils, but even more in the polluted ones. This indicates that levels of Cr(VI) as low as 1–3 mg kg^{-1} were not phytotoxic and might even have had a certain hormetic effect on plant growth, as reported for several species, including some members of Brassicaceae (Morkunas et al., 2018). On the other hand, the level of lindane used in our study was far below phytotoxic levels, and the tolerance of species of *Brassica* to lindane makes these species suitable for phytoremediation. Regarding the photosynthetic pigment content of the plants, the organic amendment also mimicked the observed stimulation of biomass in the presence of Cr, significantly increasing the content of Chl a+b and carotenoids (Table 6.2). The plants that survived in the moderately polluted unamended soils (only in those combined with the actinobacteria consortium) showed similar values to those in amended soils which indicates that the addition of actinobacteria allowed the plants to maintain normal physiological activity. Conversely, the presence of the organic amendment had a larger positive impact on photoprotective mechanisms, as the de-epoxidation ratio seemed to be similar to or lower than control values. The proportions of individual carotenoids (neoxanthin, violaxanthin, lutein, anteraxanthin, zeaxanthin, and β-carotene) to total chlorophyll did not change, as shown in Table 6.2. Total carotenoid content was not affected, following the same pattern as other photosynthetic pigments such as chlorophyll.

As mentioned above, *E. fetida* was also affected by the presence of Cr(VI) and lindane levels in the soils. Although toxic effects of lindane on *E. fetida* have been reported, most were at concentrations much higher than that used in our experiment (Lock et al., 2002; Shi et al., 2007). Therefore, we presume that the observed toxicity was due mainly to the effect of chromium. The earthworms in our experiment survived in all types of soils except in unamended soil with a high pollution level. In these conditions, not even the beneficial effects of the actinobacteria consortium were enough to make the soil survivable, although they positively affected mortality and weight loss (%) of the worms in the other soils (Table 6.3). In the Ac+Ef treatment, weight loss of *E. fetida* in soils with moderate pollution was significantly lower than in the treatment without actinobacteria,

and the beneficial effect of actinobacteria was marked in the control amended soils. As described by Irizar et al. (2015b), organic matter significantly reduced metal toxicity to *E. fetida*, through an improvement of nutritional status, which is essential to trigger protective mechanisms. This improvement, along with the reduction of Cr(VI) caused by the organic amendment, positively affected worm health, significantly reducing weight loss in all treatments, especially when combined with actinobacteria (Table 6.3).

Table 6.2. Plant parameters. Shoot dry biomass (DW), total chlorophyll (Chl a+b), ratio of antheraxanthin + zeaxanthin : violaxanthin + antheraxantin + zeaxanthin (AZ:VAZ), and total carotenoid content (Carot).

	Soil type	Treatment	C	M	H
Shoot DW (g)	U	Bn	1.2 ± 0.1 b	Ø	Ø
		Ac+Bn	1.4 ± 0.1 b1	0.61 ± 0.03 2	Ø
		Ac+Bn+Ef	2.1 ± 0.2 a	Ø	Ø
	A	Bn	3.2 ± 0.2 c3*	7.4 ± 0.2 a1	5.29 ± 0.23 a2
		Ac+Bn	6.1 ± 0.2 a2*	8.0 ± 0.1 a1*	5.31 ± 0.06 a3
		Ac+Bn+Ef	4.5 ± 0.2 b1*	4.7 ± 0.2 b1	4.64 ± 0.02 b1
Chl a+b (pmol mm^{-2})	U	Bn	103.9 ± 11.8 a	Ø	Ø
		Ac+Bn	104.2 ± 11.5 a2	344.4 ± 20.8 1	Ø
		Ac+Bn+Ef	157.7 ± 3.13 a	Ø	Ø
	A	Bn	196.6 ± 9.8 a2*	301.8 ± 35.9 a1	311.09 ± 15.89 a1
		Ac+Bn	220.2 ± 27.1 a1*	258.3 ± 60.8 a1*	169.63 ± 6.24 b1
		Ac+Bn+Ef	258.2 ± 20.2 a1*	300.5 ± 23.5 a1	296.63 ± 43.41 a1
AZ:VAZ	U	Bn	0.37 ± 0.03 a	Ø	Ø
		Ac+Bn	0.25 ± 0.03 b1	0.04 ± 0.00 2	Ø
		Ac+Bn+Ef	0.20 ± 0.04 b	Ø	Ø
	A	Bn	0.15 ± 0.04 a*	0.13 ± 0.04 a	0.11 ± 0.03 b
		Ac+Bn	0.10 ± 0.01 a2*	0.08 ± 0.03 a2	0.27 ± 0.02 a1
		Ac+Bn+Ef	0.10 ± 0.01 a*	0.09 ± 0.02 a	0.11 ± 0.01 b
Carot (pmol mm^{-2})	U	Bn	35.1 ± 3.9 a	Ø	Ø
		Ac+Bn	36.3 ±2.1 a2	101.6 ± 6.7 1	Ø
		Ac+Bn+Ef	51.5 ± 8.1 a	Ø	Ø
	A	Bn	63.03 ± 2.6 a2*	92.9 ± 9.8 a1	93.9 ±5.8 a1
		Ac+Bn	72.2 ± a12*	82.7 ±17.1 a1	53.2 ±1.8 b2
		Ac+Bn+Ef	78.6 ± 5.7 a1*	90.2 ±6.9 a1	91.8 ±12.3 a1

Soil types: unamended (U), amended with organic matter (A).
Pollution level: control (C), moderate (M), high (H)
Treatments: *Brassica napus* (Bn), actinobacteria + *B. napus* (Ac+Bn), actinobacteria + *B. napus* + *Eisenia fetida* (Ac+Bn+Ef).
Ø indicates that no specimen survived the treatment.
Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels.
* indicates statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

Table 6.3. Weight loss of *Eisenia fetida* worms in the pots during the experiment.

Soil type	Treatment	C	M	H
	Ef	44.2 ± 0.7 b2	77.2 ± 0.9 a1	Ø
U	Ac+EF	42.5 ± 2.8 b2	61.4 ± 2.3 b1	Ø
	Ac+Bn+Ef	54.8 ± 1.6 a2	63.3 ± 2.5 b1	Ø
	Ef	34.5 ± 0.9 a2*	34.4 ± 2.1 a2*	52.6 ± 2.0 b1*
A	Ac+Ef	8.35 ± 2.0 b3*	19.4 ± 2.2 b2*	38.2 ± 1.5 c1*
	Ac+Bn+Ef	38.8 ± 0.6 a2*	38.9 ± 3.7 a2*	64.9 ± 1.9 a1*

Soil types: unamended (U), amended with organic matter (A).
Pollution level: control (C), moderate (M), high (H)
Treatments: *Eisenia fetida* (Ef), actinobacteria + *E. fetida* (Ac+Ef), actinobacteria + *Brassica napus* + *E. fetida* (Ac+Bn+Ef).
Ø indicates that no specimen survived that treatment.
Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels.
* indicates statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

However, a slight negative effect on mortality and weight loss was found when actinobacteria, *B. napus* and *E. fetida* were combined (Ac+Ef+Bn). The presence of *B. napus* resulted in a significant weight loss by the worms, especially in the amended soils (Table 6.3). The reason could be that the plants were able to survive and develop a larger biomass (Table 6.2). Similarly, the biomass of plant shoots was significantly smaller when they were sharing the pot with the *E. fetida* worms. This antagonistic effect was not observed by other authors (Ghavidel et al., 2018; Wen et al., 2004). However, Lemtiri et al. (2016) found that *E. fetida* worms weighed less when growing in planted pots. This weight loss might be due to competition for space in the pot.

The concentrations of Cr and lindane (data not shown) in plant shoots and worms were very low. Although the concentration of Cr in *E. fetida* individuals collected from unamended soils was higher (30–80 mg kg^{-1}), the total concentration in the biomass of all worms was still low. In any case, the benefits of vermiremediation are not the extraction of pollutants through the worms, but rather the reduction of pollutant ecotoxicity and improvement of soil health.

The ability of the *B. napus* plants to develop a high biomass in amended polluted soils, combined with the low accumulation of Cr in their shoots, indicates the possibility of phytomanagement of co-polluted soils with chromium and lindane. It may be possible to obtain economic benefits from highly polluted soils during their remediation, and there is no risk that the pollutants might enter the food chain through the cultivation of rapeseed.

6.3.3. Microbial parameters

Microbial communities are excellent indicators of soil health, and parameters such as microbial activity, biomass and functional diversity have been used as bioindicators in studies of polluted soil (Epelde et al., 2010; Galende et al., 2014c). In this study, basal respiration, which is a good indicator of microbial activity in soil, decreased in the presence of the pollutants in both the unamended (Fig. 6.2A) and amended soils (Fig. 6.2B). The organic amendment significantly increased this metabolic activity in all soils, including controls, due to the input of easily degradable nutrient sources (Galende et al., 2014b; Lacalle et al., 2018a). As shown in Table 6.1, total organic carbon was 12-fold higher in amended soils. This effect also contributed to the reduction of Cr(VI) due to the organic matter, as discussed above. Consequently, basal respiration levels in soils with moderate pollution were similar to the control in most cases (Fig. 6.2B). Moreover, in the highly polluted soils, basal respiration levels in amended soils (Fig. 6.2A) increased, compared to the unamended soils. Biological treatments were not as effective as the organic amendment, but overall, the best treatments were Ac+Bn and Ac+Ef+Bn. In conclusion, *B. napus* plants and the inoculation of the actinobacteria consortium seemed to play a crucial role in reinforcing soil microbial activity.

Soil microbial biomass, as assessed by the substrate-induced respiration (SIR), increased in the presence of organic matter (Fig. 6.2D), compared with the biomass in unamended soils (Fig. 6.2C). Organic matter had a slight effect on the controls, but significantly increased SIR in almost all the polluted soils, which indicated that the alleviation of soil toxicity allowed the microbial biomass to increase. The biological treatments resulted in no significant differences in SIR . In any case, the most successful treatments were the combination of the actinobacteria consortium and *B. napus*, with or without *E. fetida*.

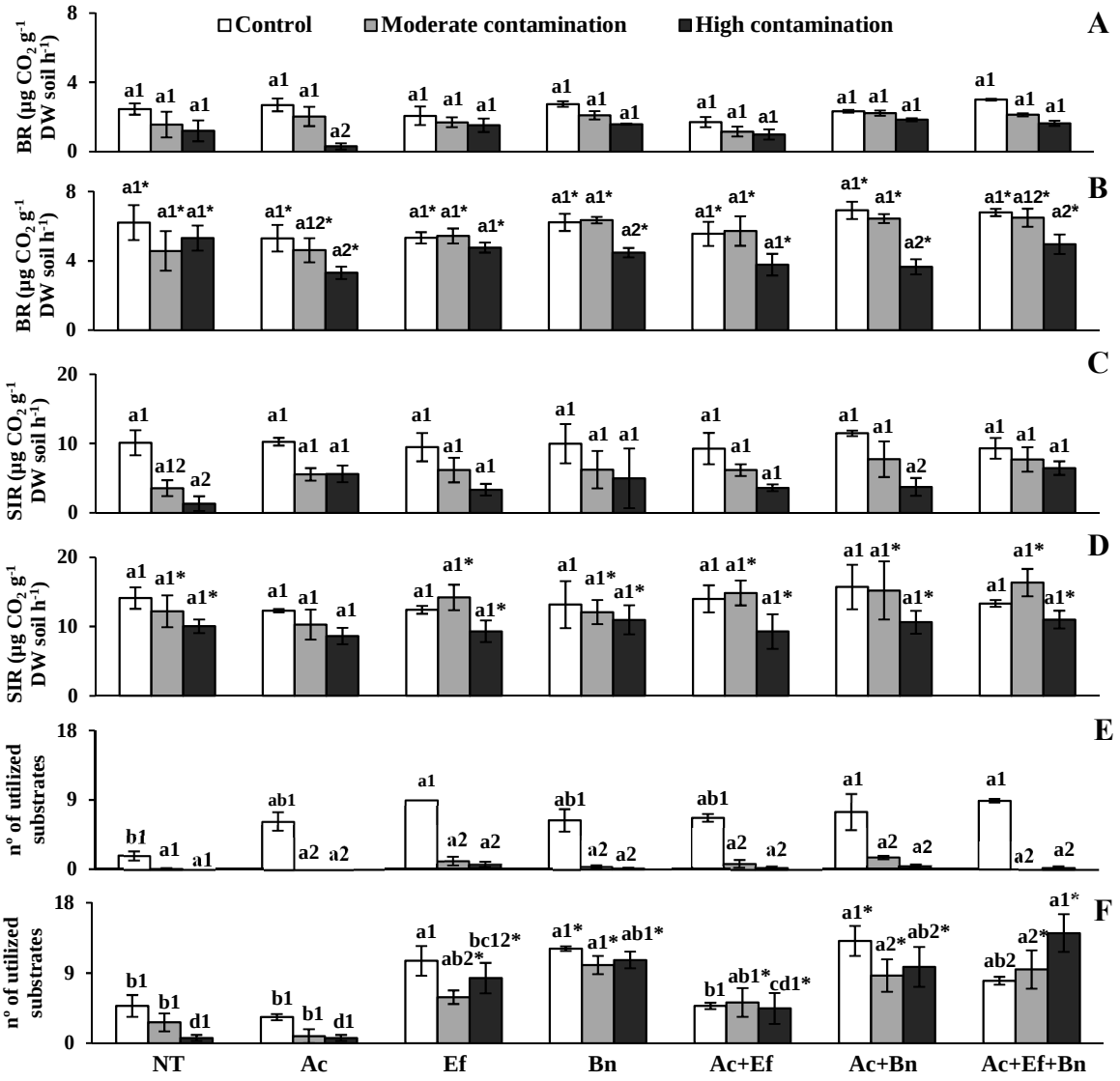


Figure 6.2. Microbial properties. Soil functional microbial diversity expressed by Number of Utilized Substrates (NUS) in the Biolog EcoPlates (A, B); soil basal respiration (BR) (C, D); and soil substrate-induced respiration (SIR) (E, F) in unamended and amended soils. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

The selected indicator of the functional microbial diversity of the soils was the number of utilized substrates (NUS) of the Biolog Ecoplates. Figure 6.2E shows that in unamended soils, the pollutants had a strong negative impact on functional microbial diversity. The biological treatments increased the NUS of the unamended control soils, but were not very successful in increasing the number of substrates utilized in the polluted soils. This result may be due to the death or poor performance of most of the remediator organisms in the polluted soils (Tables 6.2, 6.3). The situation was very different in the

amended soils, which showed higher values of NUS overall, although the pollutants still had a negative effect (Fig. 6.2F). Moreover, some of the biological treatments were effective in stimulating the functional diversity of the amended polluted soils. So, soils treated with *E. fetida* worms, and especially *B. napus* plants, increased the NUS values in polluted soils, reaching values similar to the control. The addition of organic matter and stimulation by plants increase functional microbial diversity, improving the health of polluted soils (Burges et al., 2016). The actinobacteria consortium, conversely, did not increase this parameter, or even seemed to reduce it, as in the Ac and Ac+Ef treatments (Fig. 6.2F). This could indicate that the actinobacteria consortium might be more competitive than the autochthonous microbial communities, reducing the microbial functional diversity. Actinobacteria usually can compete and produce antibiotics with antagonistic effects on other microorganisms (Polti et al., 2014). As mentioned for other microbial, plant and worm parameters, the consortium of the three organisms was the best treatment to improve microbial functional diversity.

6.3.4. Ecotoxicity bioassays with E. fetida

The ecotoxicity bioassays with *E. fetida* showed high toxicity in terms of mortality (Fig. 6.3A, B). For worms in the untreated (NT) unamended soil, exposed to the moderate level of pollution, survival was reduced to 53%, and to 0% at the high pollution level (Fig. 6.3A). The phytoremediation and vermiremediation treatments did not improve these mortality levels. In contrast, bioremediation by the actinobacteria consortium (Ac, Ac+Ef, Ac+Bn, Ac+Ef+Bn) significantly alleviated the soil toxicity to the worms, increasing their survival rates at the moderate pollution level to 97% and to 30–40% at the high pollution level. This effectiveness was directly related to the levels of Cr(VI) in the soils, more than to the levels of lindane. It appears that the toxicity in these soils was due to Cr, which agrees with the results for toxicity to the worms used as remediator organisms (Table 6.3). In any case, the addition of organic matter was the most effective treatment in reducing the toxicity in earthworms, since their survival rates in all the amended soils were close to 100%, even in the highly polluted soils (Fig. 6.3B). Therefore, differences between biological treatments were not observed in the case of amended soils (Fig. 6.3B). These results agree with observations in other studies with earthworms (Irizar et al., 2015b; Rüdel et al., 2001) and are congruent with the present observations on the earthworms in the pots (Table 6.3), whose status was significantly improved by the higher concentration of organic matter and lower levels of Cr(VI).

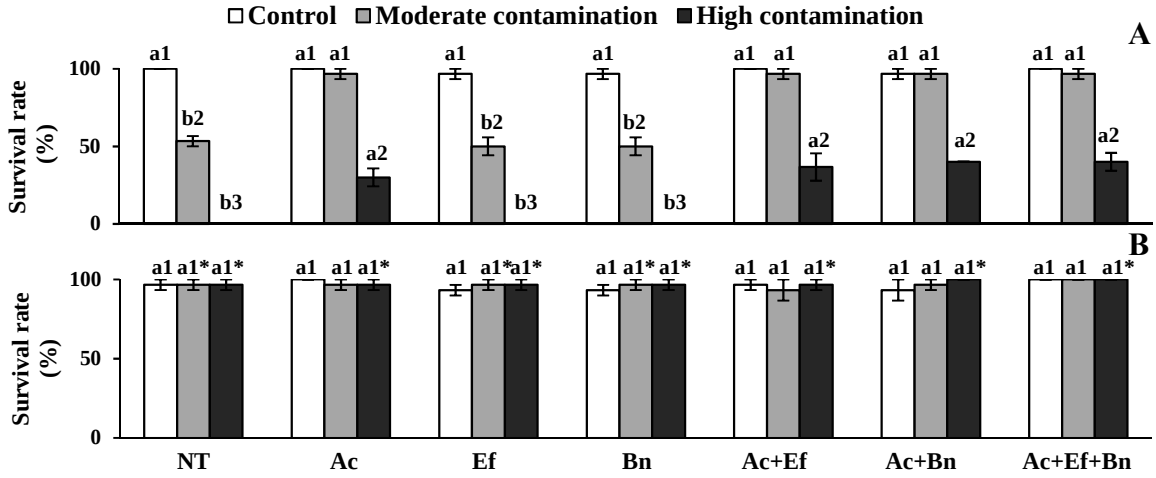


Figure 6.3. Survival rate of *Eisenia fetida* individuals in the ecotoxicity bioassays, in soils without (A) and with (B) organic amendment. Different letters indicate statistical significance ($P < 0.05$) between biological treatments, and numbers indicate statistical significance ($P < 0.05$) between pollution levels. Asterisks refer to statistical significance ($P < 0.05$) between homologous treatments with and without organic amendment.

6.3.5 Principal Components Analysis

In the Principal Components Analysis (PCA), the first two principal components explained 66% of the variance, and the samples were clearly segregated in the bi-plot (Fig. 6.6.4). The first principal component accounted for 44% of the variance and segregated the soils across the x axis by the toxicity of hexavalent chromium. Hence, the higher the pollution, the higher the impact on the indicators of soil health. The second principal component accounted for 22% of the variance and separated the soils by the presence or absence of the organic amendment, which, as mentioned above, was key for reducing chromium toxicity in this experiment and is related to many biological indicators. The results indicated that the presence of organic matter attenuates or nullifies the differences caused by the biological treatments, due to its capacity to reduce Cr(VI) to Cr(III) almost completely, and therefore alleviating the ecotoxicological effects of Cr.

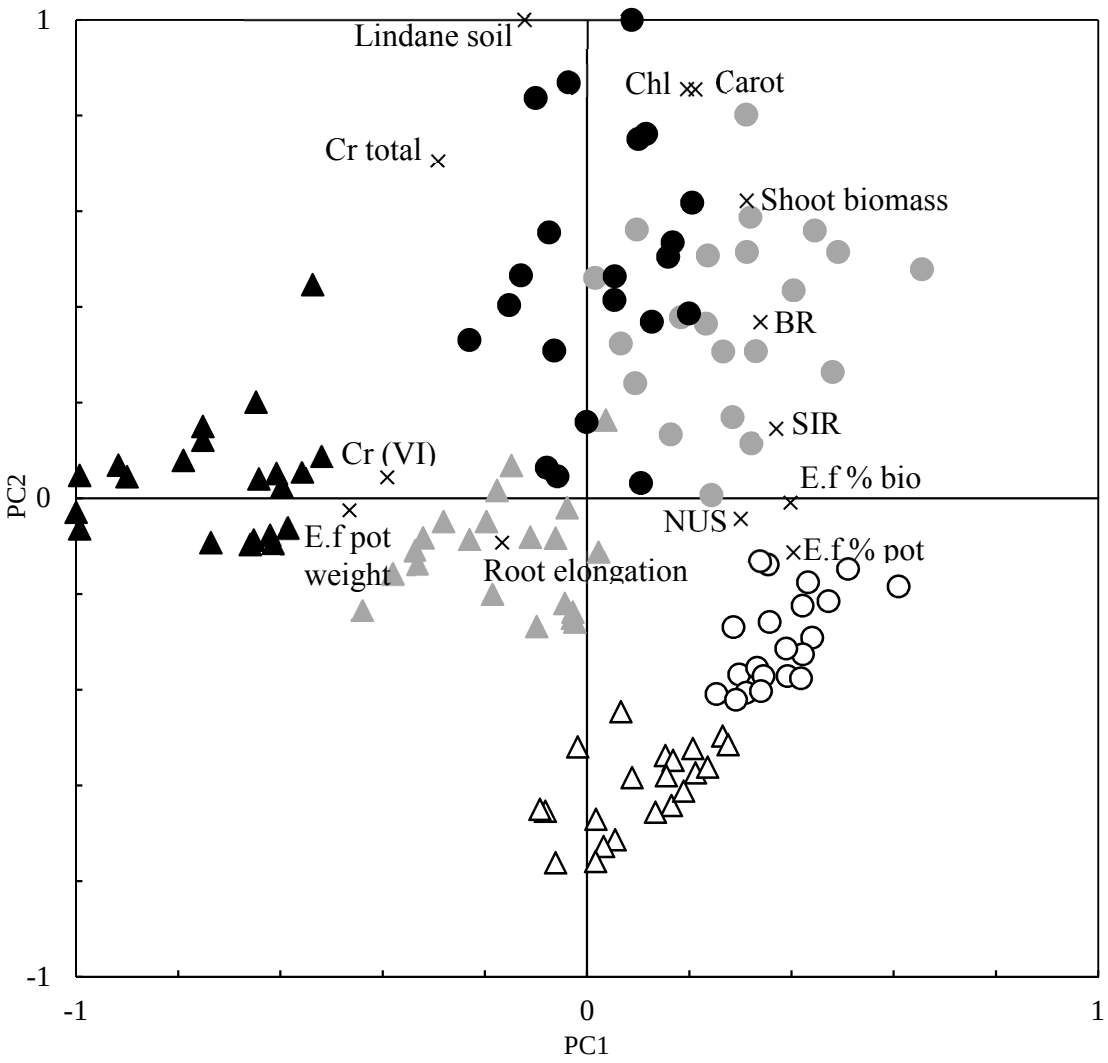


Fig. 6.4. Principal Components Analysis, including lindane concentration in soil (Lindane soil), total (Cr total) and soluble [Cr(VI)] chromium in soil, soil basal respiration (BR), substrate-induced respiration (SIR), Number of Utilized Substrates, the survival rate of *Eisenia fetida* worms in both the pots (E.f pot survival) and the bioassay (E.f bio), weight loss of *E. fetida* individuals in the pots (E.f pot weight) and *B. napus* shoot biomass (Shoot biomass), total chlorophyll content (Chl) and carotenoid content (Carot). White, gray and black icons indicate control, moderate and high pollution, respectively. Triangles indicate unamended soils and circles indicate amended soils. Crosses indicate the position of variables.

Most of the parameters determined in the soil (BR, SIR, NUS) or in the remediator plants or worms are closely related to each other and to the organic amendment, and opposed to the toxic soluble Cr(VI). Weight loss of *E. fetida* individuals in the pots was highly correlated with soluble Cr(VI), due to the toxicity of hexavalent chromium. Root elongation in the *C. sativus* bioassay (data not shown), on the other hand, appeared around the middle of the PCA. This was due to the lack of acute phytotoxicity to the seedlings in the bioassay. Regarding the parameters measured for the potted plants, shoot biomass,

total chlorophyll, and carotenoid content were closely related to each other as plant wellness indicators. Biological parameters, especially those relative to the *B. napus* plants, were strongly influenced by the organic amendment.

6.4. Conclusions

In the soils spiked with both Cr(VI) and lindane, the concentration of hexavalent chromium was the main component of toxicity. After application of the organic-matter amendment and/or several bioremediation techniques (alone or in combination), most of the hexavalent chromium was reduced to its less-toxic form, trivalent chromium. The most effective treatment was the addition of organic matter, followed by the bioaugmentation treatment with the actinobacteria consortium, which was composed of species that were originally isolated in a medium with Cr and lindane. The consortium was able both to degrade part of the lindane and to reduce the levels of Cr(VI). This reduction of Cr(VI) lowered the toxicity of the soils, as reflected in many biological indicators of soil health, such as the improvement in the growth and health of *Brassica napus* and in the survival of *Eisenia fetida* individuals. Combined with the organic amendment, especially with the added actinobacteria and *E. fetida*, *B. napus* proved to be suitable for phytomanagement of soils with this kind of pollution. To our knowledge, the combination of organic matter with the actinobacteria consortium, *B. napus* and *E. fetida* has not been reported previously. Our results showed that this was the most successful treatment overall and would be a suitable strategy to reduce contamination and improve the health of soils co-polluted with hexavalent chromium and lindane.

6.5 Conclusiones del capítulo

- El cromo (VI) es una especie altamente biodisponible de los suelos produciendo una fuerte toxicidad en los organismos (plantas, lombrices y microorganimos).

- Las enmiendas orgánicas son una estrategia muy efectiva para reducir los niveles de la forma tóxica Cr(VI) transformándolo a Cr(III), pero, en cambio, interfiere limitando la degradación de lindano.

- Las estrategias de bioremediación que utilizan actinobacterias procedentes de suelos contaminados con Cr y lindano son más efectivas para degradar el insecticida y reducir el Cr biodisponible que la fitorremediación o la vermirremediación.

- La combinación de una enmienda orgánica, bioaumentación, fitorremediación y vermirremediación es la estrategia más efectiva para mejorar la salud de los suelos contaminados con cromo y lindano, considerando la eliminación de los contaminantes y la reducción de la ecotoxicidad.

7. SUCCESSFUL REMEDIATION OF SOILS WITH MIXED CONTAMINATION OF CHROMIUM AND LINDANE: INTEGRATION OF BIOLOGICAL AND PHYSICO-CHEMICAL STRATEGIES

7. SUCCESSFUL REMEDIATION OF SOILS WITH MIXED CONTAMINATION OF CHROMIUM AND LINDANE: INTEGRATION OF BIOLOGICAL AND PHYSICO-CHEMICAL STRATEGIES

Juan Daniel Aparicio Rafael G. Lacalle, Unai Artetxe, Erik Urionabarrenetxea, José María Becerril, Marta Alejandra Polti, Carlos Garbisu, Manuel Soto, 2020. Successful remediation of soils with mixed contamination of chromium and lindane: Integration of biological and physico-chemical strategies. **Journal of Environmental Management** (Submitted)

Abstract

Soil mixed contamination with metals and organic pollutants, like Cr(VI) and lindane, is currently a main environmental challenge. Biological strategies, such as biostimulation, bioaugmentation, phytoremediation and vermiremediation, and nanoremediation with nanoscale zero-valent iron (nZVI) are promising approaches for polluted soil health recovery. The combination of different remediation strategies might be key for addressing this problem. For this reason, a greenhouse experiment was performed using soil without or with organic amendment. Both soils were contaminated with lindane (15 mg kg^{-1}) and Cr(VI) (100 or 300 mg kg^{-1}). After one month of aging, the following treatments were applied: (i) combination of bioaugmentation (actinobacteria), phytoremediation (*Brassica napus*) and vermiremediation (*Eisenia fetida*), or (ii) nanoremediation with nZVI or (iii) combination of biological treatments and nanoremediation. After 60 days, wellness of plants and earthworms was assessed, also, soil health was evaluated trough physicochemical parameters and biological indicators. Cr(VI) was more toxic and decreased soil health, however, it was reduced to Cr(III) by the amendment and nZVI and, to a lesser extent, by the biological treatment. Lindane was more effectively degraded through bioremediation. In non-polluted soils, nZVI had strong deleterious effects on soil biota when combined with the organic matter, but this effect was reverted in soils with high concentration of Cr(VI). Therefore, under our experimental conditions bioremediation might be the best for soils with moderate concentration of Cr(VI) and organic matter. The application of nZVI in soils with high content of organic matter should be avoided except for soils with very high concentrations of Cr(VI).

7.1. Introduction

Mixed contamination of soils with organic and inorganic pollutants is a widespread issue, and one of the main current environmental challenges. In particular, co-contamination with Cr(VI) and lindane (γ-1,2,3,4,5,6-hexachlorocyclohexene), an organochlorine pesticide, is still reported in concentrations exceeding the maximum permitted all over the world (Aparicio et al., 2018a). Despite there have been advances in both physico-chemical and biological remediation techniques, there are still no cost-effective, sustainable and environmentally friendly solutions for this problem (Batty and Dolan, 2013).

Several studies have demonstrated the effectiveness of nZVI in soil remediation toward Cr(VI) reduction to Cr(III) . The mechanism of Cr(VI) remediation by nZVI mainly involves adsorption, reduction and co-precipitation (Peng et al., 2017). Furthermore, nZVI technology demonstrated utility for lindane removal in soil, and, in general, these processes occurred through reductive dehalogenation (Anza et al., 2019; Elliott et al., 2009). On the other hand, it must be considered that the physicochemical transformations of nZVI in the environmental matrices are extremely complicated, presenting both beneficial and harmful impacts (Dwivedi et al., 2015). For instance, soil type modifies the effect of nZVI on bacterial communities; furthermore, observed phylogenetic changes in communities were more important than functional changes (Fajardo et al., 2019), and thus, the overall effort of the soil ecosystem might involve the maintenance of functionality following nZVI exposure. Bruton et al. (2015) reported that the corrosion of ZVI stimulates the activity of dehalorespiring bacteria, and consequently, the reduction of chlorinated contaminants, since the H_2 produced would serve as an electron donor. In contrast, under laboratory conditions, nZVI can inhibit bacterial development, however, this effect can be compensated by coating the nZVI with polyelectrolytes or organic matter (Bruton et al., 2015; Fajardo et al., 2019).

On the other hand, biological treatments are effective biotechnological tools for environmental remediation by degrading/transforming various contaminants using biological activity.

Bioaugmentation is a technology which is being widely studied, generating promising results. This consists of the inoculation of the environmental matrix with microorganisms with the ability to reduce the toxicity of pollutants (Thapa et al., 2012).

Microorganisms can break down organic pollutants by using them as a source of carbon and energy, or by co-metabolism. Moreover, metals can be transformed from one oxidation state to another or form an organic complex, changing its water solubility and decreasing its toxicity (Ayangbenro and Babalola, 2017). In this sense, Aparicio et al. (2018a) reported that an actinobacterial consortium was able to survive under high concentrations of Cr(VI) and lindane, besides to remove both contaminants from soils under anthropogenic contamination. Nevertheless, bioaugmentation has some limitations, as the bioremediation effectiveness and survival of inoculated bacteria is conditioned by the contamination levels, characteristics of the receptor soil and the nature of its microbial communities (Cycoń et al., 2017).

Phytoremediation is another alternative remediation biotechnology to recover contaminated soils (Cristaldi et al., 2017). Recently, *Vigna mungo* was successfully used to remove Cr(VI) from contaminated soil through absorption and accumulation (Saravanan et al., 2019). Depending on the type of removal mechanism involved, phytoremediation can be classified as phytoextraction (absorption and accumulation), rhizofiltration (extraction from an aqueous matrix), phytostabilization (immobilization into the soil matrix), phytovolatilization (extraction of volatile compounds from soil and volatilize them from foliage), phytodegradation and rhizodegradation (pollutant degradation by microorganisms from the rhizosphere) among others (Chandra and Kumar, 2017). Species as *Brassica napus* was previously demonstrated effective for the concomitant removal of Cr and phenol from soils (Ontañon et al., 2014). However, the disadvantages of phytoremediation include prolonged treatment time, dependence on climatic and seasonal conditions, sensitivity to diseases and pests, among others (Koptsik, 2014).

Vermiremediation using the earthworm *Eisenia fetida* is one of the most recent technologies for the restoration of contaminated environments. Earthworms significantly change the soil physicochemical properties, altering the availability of organic and inorganic compounds (Hickman and Reid, 2008b). Earthworms absorb metals from contaminated soil through direct contact of the skin with the soil solution or through intestinal uptake of water, contaminated food and/or soil particles. Moreover, earthworms increase the contact between organic contaminants and the soil microorganisms, accelerating the removal of contaminant from soil (Rodriguez-Campos et al., 2014). However, *E. fetida* earthworms are greatly sensitive to heavy metals and organic

pollutants, specially to Cr(VI) and lindane (Aparicio et al., 2019).

The application of substances that enhance the biological activity of the organisms of the soil, known as bioestimulation, usually accompanies the biological treatments described above. Organic amendments are a good choice for this purpose and have been widely used (Kästner and Miltner, 2016), as they add nutrients and carbon sources to the soil, promoting plant growth and microbial activity, as well as the soil fauna. They can also affect the oxidation status of metals and their bioavailability (Park et al., 2011). In addition, the application of organic amendments in degraded soils can be an opportunity for the valorization of wastes and byproducts that are currently stored in landfills, which is aligned with the principles of circular economy (Míguez et al., 2020).

As previously seen, each technology used for soil remediation, including physicochemical or biological treatments, have certain limitations, in addition to the fact that each contaminated site has unique edapho-climatic characteristics. These restrictions could be counteracted by the combination of technologies; however, there are no systematic studies that allow evaluating the performance of the different combinations of technologies to optimize remediation, considering the prevailing conditions in each case. Therefore, the objective of this study was to evaluate the effectiveness of the combination of a series of biological (biostimulation, bioaugmentation, phytoremediation, vermiremediation) and physicochemical (nanoremediation) strategies for the remediation of soils contaminated with a mixture of Cr(VI) and lindane, as organic and inorganic contaminant models.

7.2. Materials and methods

7.2.1. Experimental design

Two soils were collected form a peri-urban area near the city of Vitoria-Gasteiz (42°50′N; 2°40′W, Northern Spain), being one of them (A) amended with 100 t ha^{-1} of organic amendment derived from the recycling of urban organic wastes from the city, and the other one kept unamended (U). Both soils were collected from near the surface (5-15 cm deep), transported to the laboratory, and they were air dried and sieved to <2 mm in order to obtain homogenized soil samples. The physicochemical characteristics of the soils are listed in Table S7.1. Hexavalent chromium and lindane contamination was not detected in both soils (Table 7.1).

In order to artificially contaminate the soils, a stock aqueous solution of 5 g L^{-1} of Cr(VI) was prepared as $K_2Cr_2O_7$ (Cicarelli, Argentina). The solution was sterilized by filtration, using Millipore 0.22 µm pore size filters. Lindane (Sigma-Aldrich, United States) stock solution was prepared at 5 g L^{-1} using acetone as a solvent. Soils were artificially contaminated with both Cr(VI) and lindane solutions, stablishing three conditions for the experiment: (1) non-contaminated soil (NCS); (2) soil co-contaminated with 100 mg kg^{-1} of Cr(VI) and 15 mg kg^{-1} of lindane (Cr100 + Lin15); or (3) soil co-contaminated with 300 mg kg^{-1} of Cr(VI) and 15 mg kg^{-1} lindane (Cr300 + Lin15). All assays were carried out in pots containing 1 kg of soil and kept in a greenhouse to let the pollutants stabilize. The conditions at the greenhouse were the following: photoperiod 14/10 h day/night, temperature 25/18 °C day/night, relative humidity 60/70% day/night.

After one month of stabilization, soil was homogenized and the following treatments were applied: (i) biological treatment (BT): actinobacteria consortium + *Eisenia fetida* adult individuals + *Brassica napus* plants; (ii) physicochemical treatment: nZVI; (iii) physicochemical and biological treatment: nZVI + BT; and soil where none of these treatments were applied (Non-treatment). Pots were kept at the previously mentioned conditions for 60 days.

nZVI were provided by NANO IRON S.R.O. (Rajhrad-Czech Republic). According manufacturer recommendations, a stock nZVI suspension was prepared (250 g L^{-1}): 100 g were suspended in 400 mL of water and mixed 10 min in blender machine, minimizing fresh air intake. nZVI was added to the soil and mixed vigorously. It was left to act three days before applying any other biological treatment.

The preparation of the bacterial inoculum was carried out according to Aparicio et al.(2018b). Briefly, spores of *Streptomyces* sp. M7, *Streptomyces* sp. MC1, *Streptomyces* sp. A5 and *Amycolatospis tucumanensis* were individually inoculated into flasks with 150 ml of tryptic soy broth (TSB) and incubated at 30 °C, 180 rpm. After 72 h, actinobacterial biomass was recovered by centrifugation (8385 ×*g*), washed and resuspended in distilled water. The soils were inoculated with 2 g kg^{-1} of the consortium (0.5 g kg^{-1} of each strain). The soils were left 14 days for the establishment and stabilization of the consortium. After that period, the other biological treatments were applied.

E. fetida specimens (Lombricor S.C.A., Córdoba-Spain) were kept in the laboratory under controlled conditions (19 ºC and 60% relative humidity) with a weekly contribution of horse manure. All the specimens employed during the assays were healthy, sexually mature (clitellated) with a weight between 350-450 mg.

Brassica napus seeds were sowed in each pot. The germination (%) was registered after 5 days, and survival (%) after 2 weeks. After plant establishment, with the aim of having the same number of plants, only five plants per pot were preserved.

During the whole experiment, pots were kept at water holding capacity (WHC) by irrigating when necessary. At the end of the experiment period, plants were harvested, earthworms were collected, counted and weighted, and soil samples were taken, which were immediately stored at 4ºC for its conservation until analyses.

7.2.2. Plant biomass and pigment composition determinations

Prior to harvesting, 6 discs with a diameter of 3 mm were collected from the youngest fully expanded leaf, frozen in liquid nitrogen and stored at −80ºC until processing. Photosynthetic and photoprotective pigments (chlorophylls and carotenoids) were determined according to García-Plazaola and Becerril (2001). Plants were harvested, washed thoroughly with deionized water separated leaves, stems + petioles and roots. These tissues were oven-dried at 35ºC for 72 h to calculate dry weights (DW) and determinate Cr and lindane concentration.

7.2.3. Physico-chemical determinations

7.2.3.1. Total Cr and soluble Cr(VI) determination in soil

Soil was oven-dried at 35ºC for 72 h and sieved to <0.125 mm. In order to determine total Cr, an acidic digestion (HCl and HNO_3) was carried out according to the Method 3051A (US-EPA Method 3051A, 2007), using a microwave MARS V. After digestion, all extracts were filtered and transferred to 50 mL volumetric flasks.

The soluble Cr(VI) was extracted according to Jiang et al. (2015). A 1:25 (p/v) mixture of soil and Milli-Q water was shaken at 200 rpm for 24 h and centrifuged at 10,000 × *g* for 15 min to remove the soil from the aqueous solution and filtered to <0.45 μm. The content of Cr was determined by Inductively-Coupled Plasma-Mass Spectroscopy (ICP-MS) (Agilent 7700), with a limit of quantification (LOQ) of 0.03 μg L^{-1}. Accuracy was ensured using NIST Standard Reference Material 1640.

7.2.3.2. Cr concentration in plant and earthworms

As indicated above, oven dried tissues of roots, stem + petioles, and leaves were milled and 0.200 g were placed in Pyrex tubes, and 5 mL of HNO_3-HCl_1O_4 (85:15) were added. The tubes were mixed and left to stand for two hours at room temperature. Digestion was carried out using a Bloc Digest m 40 Selecta, connected to a microprocessor-controlled RAT-2 Selecta) and following the protocol optimized by Zhao et al. (1994). After digestion and cooling, 5 mL of HCl $20\%_{v/v}$ was added, the tubes were mixed and rewarmed at 80°C for 30 min, and after cooling, the solution was diluted to 20 mL.

Depurated (left on wet filter paper for 24 h to void gut content) and cleaned earthworms (n = 5) were dried in pools at 120 ºC for 48 h, weighted and digested in HNO_3 Tracepur® 69%. Once the concentrated acid was evaporated, pellets were resuspended in 20 mL of 0.01 M HNO_3 Tracepur®.

The plants and earthworm extracts were analyzed using the same methodology as for the total chromium.

7.2.3.3. Lindane concentration in soil

Lindane extraction and quantification was done according to Aparicio et al. (2018a). Briefly, n-hexane, water, and methanol (5:4:1) were added to 5 g of soil, mixed and centrifuged at 8385 × *g* for 10 min. The extract was evaporated and finally suspended in 1 mL of n-hexane. Quantification was performed using a gas chromatograph (Agilent 7890A; LOQ: 170 μg L^{-1}) (Aparicio et al., 2018a).

7.2.3.4. Lindane determination in plants and earthworms

Lindane concentration in plants and earthworms was determined according to the method described by Quintero et al. (2005) for extraction and analysis of pesticides, the "QuEChERS" method. This method has two phases: i) lindane extraction from the crushed plant and earthworm samples with acetonitrile, magnesium sulphate and sodium acetate; ii) cleanse of the extract by solid phase extraction with an Agilent kit (QuEChERS AOAC). Quantification was carried out by gas chromatography following the same methodology as for lindane concentration in soil.

7.2.4. Ecotoxicological bioassays

7.2.4.1. Phytotoxicity bioassay with Raphanus sativus

The biomarkers evaluated on *R. sativus* were germination (%) and hypocotyl and root elongation (cm), according to Aparicio et al. (2019). Briefly, thirty seeds were placed in Petri dishes with 15 g of soil and incubated at 22 °C for 5 days. The number of germinated seeds was recorded and the hypocotyl and seedling roots were measured.

7.2.4.2. Toxicity bioassay with E. fetida

In the case of *E. fetida* earthworms mortality and weight loss were assessed, as well as cell level biomarkers such as number of coelomocyte and cell viability (García-Velasco et al., 2017).

The earthworm immune cells or coelomocytes were obtained according to Irizar et al. (2014). Briefly, after the exposure to soil samples, each earthworm was submerged in extraction solution [1 mL of Ca and Mg free phosphate buffered saline (PBS)-EDTA 0.02 %] and coelomocytes were obtained by electrical stimulation (9V). The obtained solution was centrifuged at 1000 × *g* for 10 minutes at 10 °C. Then, the supernatant was removed and cells were washed twice and resuspended in PBS to obtain a stock solution of 10^6 cells mL^{-1} of coelomocytes.

Evaluation of the number of coelomocytes and their viability was carried out through the Neutral Red Uptake (NRU) assay (Irizar et al., 2014).

7.2.4.3. Soil microbial properties

The following soil microbial properties were determined according to Galende et al. (2014): (i) microbial activity was determined by basal respiration (BR) following ISO 16072 (2002); (ii) potentially active microbial biomass was determined by substrate-induced respiration (SIR) following ISO 17155 (2002); (iii) average well color development (AWCD); (iv) general bacterial activity calculated as the area under the curve (AUC); and (v) number of metabolized substrates (NUS) were determined from Biolog EcoPlates™.

7.2.4.4. Integrated Biomarker Response/n (IBR/n)

Integrative Biomarker Response (IBR) index was calculated for each bioindicator with the aim of integrating alterations at different biomarkers, following the procedure described by Beliaeff and Burgeot (2002). The calculation method is based on relative

differences between the biomarkers in each given data set. Thus, the IBR index is computed by summing-up triangular star plot areas (multivariate graphic method) for each two neighboring biomarkers in a given data set, according to the following procedure: (1) calculation of the mean and standard deviation for each sample; (2) standardization of data for each sample: $x_i' = (x_i - x) / s$; where, x_i' = standardized value of the biomarker; x_i = mean value of a biomarker from each sample; x = general mean value of x_i calculated from all compared samples (data set); s = standard deviation of x_i calculated from all samples; (3) addition of the standardized value obtained for each sample to the absolute standardized value of the minimum value in the data set: $y_i = x_i' + |x_{min}'|$; (4) calculation of the Star Plot triangular areas as $A_i = (y_i \times y_{i+1} \times \sin\alpha) / 2$, where y_i and y_{i+1} are the standardized values of each biomarker and its next biomarker in the star plot, respectively, and α is the angle (in radians) formed by each two consecutive axis where the biomarkers are represented in the Start Plot ($\alpha = 2\pi / n$; where n is the number of biomarkers); and (5) calculation of the IBR index which is the summing-up of all the Star Plot triangular areas ($IBR = \sum A_i$). Then, IBR/n was calculated (Marigómez et al., 2013).

7.2.5. Statistical analysis

All the assays and their respective controls were performed at least as three biologically independent replicates. For the statistical analysis of data, the Infostat software was used (version: 2018, Argentina). After checking the normality and homogeneity of the data, these were subject to one-way variance analysis (One-way ANOVA), considering a probability level of $p < 0.05$ as significant. They were also analyzed using the Tukey post-test ($p < 0.05$) in order to identify significant differences between treatments.

7.3. Results and Discussion

7.3.1. Pollutant content in the soil

Cr(VI) is highly reactive; when it spills on the ground, it immediately reacts with the organic matter and clay minerals present in the soil. From spiking with initial Cr concentration of 100 and 300 mg/kg (nominal concentrations), the soluble metal fraction was reduced until 19.6 and 123.1 mg kg^{-1}, respectively, in unamended soils (Table 7.1). In amended soils, the soluble Cr(VI) was greatly reduced from 100 and 300 mg/kg until 1.1 and 2.4 mg kg^{-1}, respectively (Table 7.1). Likewise, it has been widely reported that

the presence of amendment in soils significantly decreases the concentration of Cr(VI) due to their reactivity with the organic matter (Choppala et al., 2018).

Table 7.1: Hexavalent chromium and lindane concentration in soil. Treatments: Non-treatment: co-contaminated soil without treatments; Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15).

		Cr(VI) (mg kg^{-1})		Lindane (mg kg^{-1})	
		U	**A**	**U**	**A**
Non-treatment	**NCS**	ND	ND	ND	ND
	Cr100 + Lin15	19.6 ± 1.1^{c}	1.1 ± 0.1^{d}	$5.6 \pm 0.2^{c'}$	$9.9 \pm 0.2^{a'}$
	Cr300 + Lin15	123.1 ± 4.0^{a}	2.4 ± 0.03^{d}	$5.5 \pm 0.4^{c'}$	$9.9 \pm 0.2^{a'}$
BT	**NCS**	ND	ND	ND	ND
	Cr100 + Lin15	2.3 ± 0.2^{d}	0.8 ± 0.03^{d}	$3.5 \pm 0.2^{f'}$	$4.6 \pm 0.3^{d'}$
	Cr300 + Lin15	45.4 ± 2.1^{b}	1.0 ± 0.1^{d}	$3.5 \pm 2.3^{ef'}$	$4.5 \pm 0.1d^{e'}$
nZVI	**NCS**	ND	ND	ND	ND
	Cr100 + Lin15	0.3 ± 0.1^{d}	0.9 ± 0.1^{d}	$5.6 \pm 0.1^{c'}$	$9.4 \pm 0.5^{ab'}$
	Cr300 + Lin15	0.9 ± 0.1^{d}	1.8 ± 0.1^{d}	$5.5 \pm 0.6^{c'}$	$9.5 \pm 0.5^{a'}$
nZVI + BT	**NCS**	ND	ND	ND	ND
	Cr100 + Lin15	0.3 ± 0.1^{d}	0.9 ± 0.0^{d}	$1.3 \pm 0.1^{g'}$	$8.6 \pm 0.1^{b'}$
	Cr300 + Lin15	0.4 ± 0.0^{d}	1.10 ± 0.1^{d}	$1.38 \pm 0.2^{g'}$	$8.6 \pm 0.1^{b'}$

ND: non-detected. Values sharing the same letter were not significantly different (p <0.05).

On the other hand, native microbial communities could have the ability to degrade, remove, and/or transform contaminating chemical products (Miao et al., 2019). In this sense, a natural attenuation process has been confirmed in the present study. The initial lindane concentration (nominal concentration: 15 mg kg^{-1}) was reduced to 5.6 mg kg^{-1} in unamended soils, and 9.9 mg kg^{-1} in amended soils without the application of any treatment (Table 7.1). Soils rich in organic matter show a greater microbial population, which could be tolerant to contaminants but unable to eliminate them (Albarracín et al., 2005). Furthermore, the lower decrease in the amended soils in comparison with unamended ones could be also attributed to the protective effect exerted by organic matter by decreasing the bioavailability of the pesticide (Hofman et al., 2014).

The treatment with nZVI and the combined treatment of nZVI with the three biological agents (nZVI + BT), were very effective to decrease levels of Cr(VI) below 2 mg kg^{-1} in both, amended and unamended soils (Table 7.1). In recent years, remediation

of Cr(VI) by nZVI has been developed and proved to be one of the most promising technologies for the immobilization of Cr(VI) (Mitra et al., 2017). Fe^0 gradually oxidized to Fe^{2+}/Fe^{3+} concomitantly with a reduction of Cr(VI) to harmless Cr(III), followed by co-precipitation as Fe-Cr (oxy)hydroxides. Several authors reported that nZVI could achieve a nearly complete Cr(VI) reduction (Dong et al., 2017). However, the performance, migration, and transformation of nZVI disturb the physico-chemical conditions of the soil and inevitably disrupts the soil ecosystem affecting finally to soil organisms (Jiang et al., 2018), as will be discussed below. Although have been proven very effective, the use of nZVI as a remediation strategy can still be considered as controversial due to potential collateral negative effect to the ecosystem.

The most accepted mechanism of bacterial Cr(VI) removal comprises the Cr(VI) reduction by extracellular enzymes, and the intracellular Cr(VI) reduction that occurs in four steps: biosorption, transport into cells, cytosolic Cr(VI) reduction, and Cr(III) accumulation (Karthik et al., 2017). However, the fraction immobilized inside the cell is usually minimal (around 10% of the Cr total present in the media) (Marta Alejandra Polti et al., 2009). Even so, when the cells die, the lysate is released into the media, including the Cr(III) previously immobilized. In this sense, Cr(VI) reduction is the most suitable measure for its remediation. In the treatment of the unamended soils with the biological agents (BT), Cr(VI) reduction was significantly high. The soluble Cr(VI) was reduced 63% compared to the non-treated soil (until 45.4 mg kg^{-1}) in the soil initially contaminated with 300 mg kg^{-1}, and 88% (until 2.3 mg kg^{-1}) in the soil with an initial Cr(VI) concentration of 100 mg kg^{-1} (Table 7.1). Importantly, the *B. napus* plants did not survive in any of the unamended BT soils (Fig. 7.1). In addition, the mortality of *E. fetida* was 100% in the soil spiked with 300 mg kg^{-1} of Cr(VI), and 50% in the soil with an initial Cr(VI) concentration of 100 mg kg^{-1} (Fig. 7.2). Therefore, the additional removal of Cr(VI) by BT, as compared to values of non-treatment (Table 7.1), could mainly be attributed to the actinobacterial consortium. In fact, this quadruple consortium had already proven to be able to eliminate significant levels of Cr(VI) in liquid media, artificially contaminated soils and even in anthropogenically contaminated soils (Aparicio et al., 2018b, 2018a).

On the other hand, Cr(VI) concentrations remained low in every amended soil, regardless the initial chromium concentration or treatment applied (Table 7.1). These concentrations probably correspond to the chemical equilibrium of this metallic species

in the conditions modified by the organic matter (Shahid et al., 2017), making the presence of the organic matter the most effective factor decreasing Cr(VI) concentration.

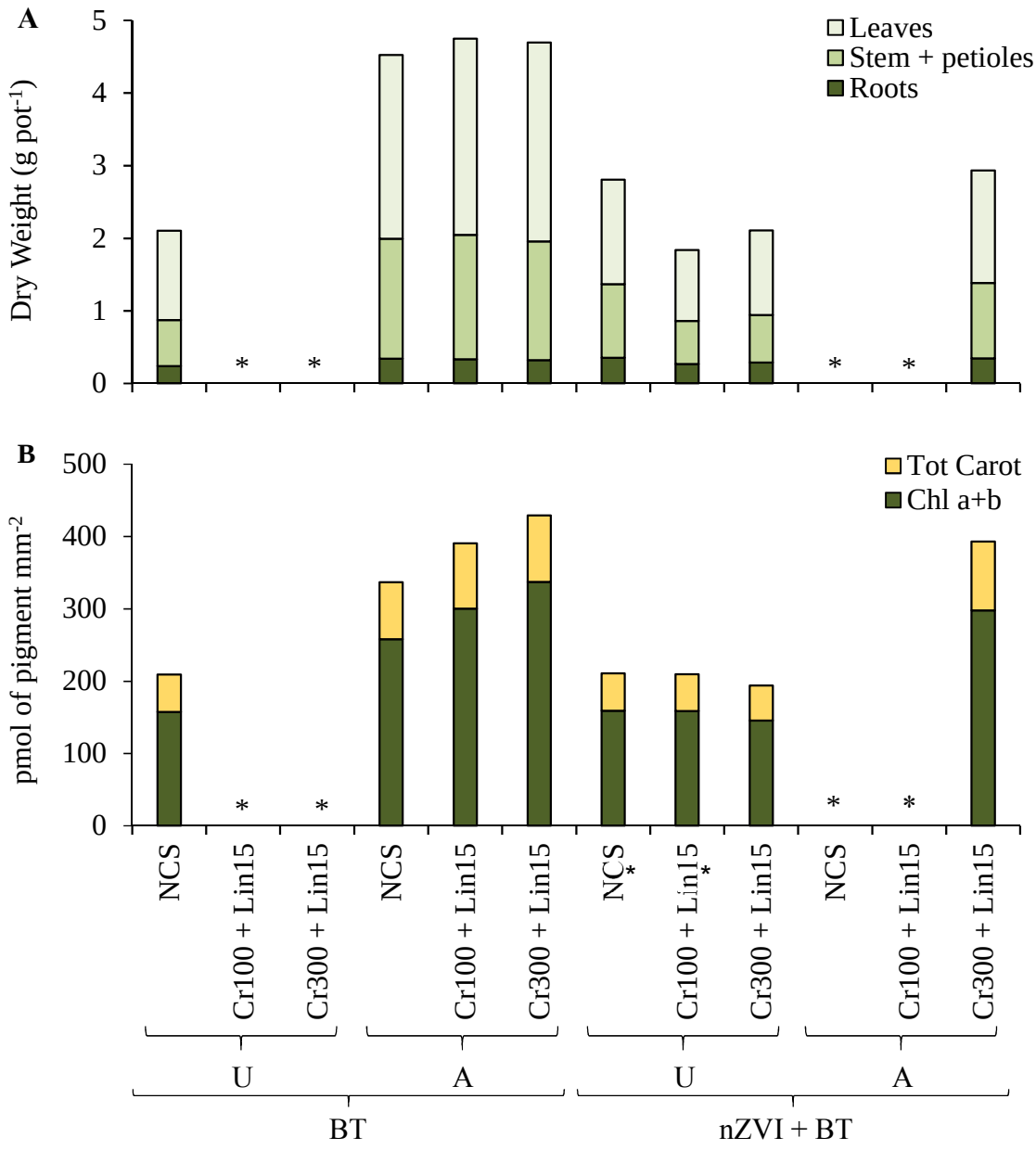


Figure 7.1. (A) Biomass dry weight of *Brassica napus* plant parts and (B) Pigment composition of *Brassica napus* leaves. Chl a+b: Total chlorophyll concentration. Tot Carot: Total carotenoid content. Treatments: Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15). Values sharing the same letter were not significantly different (p <0.05). * non-survival.

Regarding the dissipation of lindane, the maximal degradation in amended soils was achieved through the biological treatment (BT), where the concentration of the pesticide was reduced from 9.9 mg kg^{-1} to 4.6 mg kg^{-1} (>56%) (Table 7.1), presumably due to microbial degradation. The aerobic lindane biodegradation by actinobacteria was already demonstrated by Sineli et al. (2018), it consists in the progressive elimination of the chlorine and hydrogen atoms and the subsequent formation of double bonds; the chlorine atoms are possibly replaced by hydroxyls. In addition, the mineralization of lindane is feasible since proteins from the down-stream degradation pathway were identified. Earthworms have strong interactions with microorganisms to mineralize organic matter, increasing lindane bioavailability, and facilitating the pesticide degradation (Rodriguez-Campos et al., 2019). Plant cooperation in the process is also essential. Plants release root exudates containing different substances and nutrients such as proteins and complex carbohydrates which attract the microorganisms, inducing specific gene expression, and promoting detoxification processes in the rhizosphere (Gkorezis et al., 2016). Furthermore, earthworms have a beneficial effect on plant growth due to all the changes they produce in the soil, such as macro aggregation, increase in nutrient availability (N, P, K), and changes in soil bulk density (Taheri et al., 2018).

Although in unamended soil the initial concentration of lindane was quite lower than that of amended soil (5.6 and 9.9 mg kg^{-1}, respectively), its concentration after the biological treatment was not so different situations (3.5 and 4.6 mg kg^{-1}, respectively) (Table 7.1). The relative removal of the pesticide by indigenous microbial populations was lower in unamended soil, where the high concentration of Cr(VI) did not allow the survival of any of the *B. napus* plants (Fig. 7.1) and the conditions for the development of *E. fetida* were adverse (Fig. 7.2). However, the activity of actinobacteria of BT increased lindane degradation in both amended an non amended soils and reduce toxicity of Cr (VI) and lindane to plants (Fig. 7.1) and earthworms (Fig. 7.2).

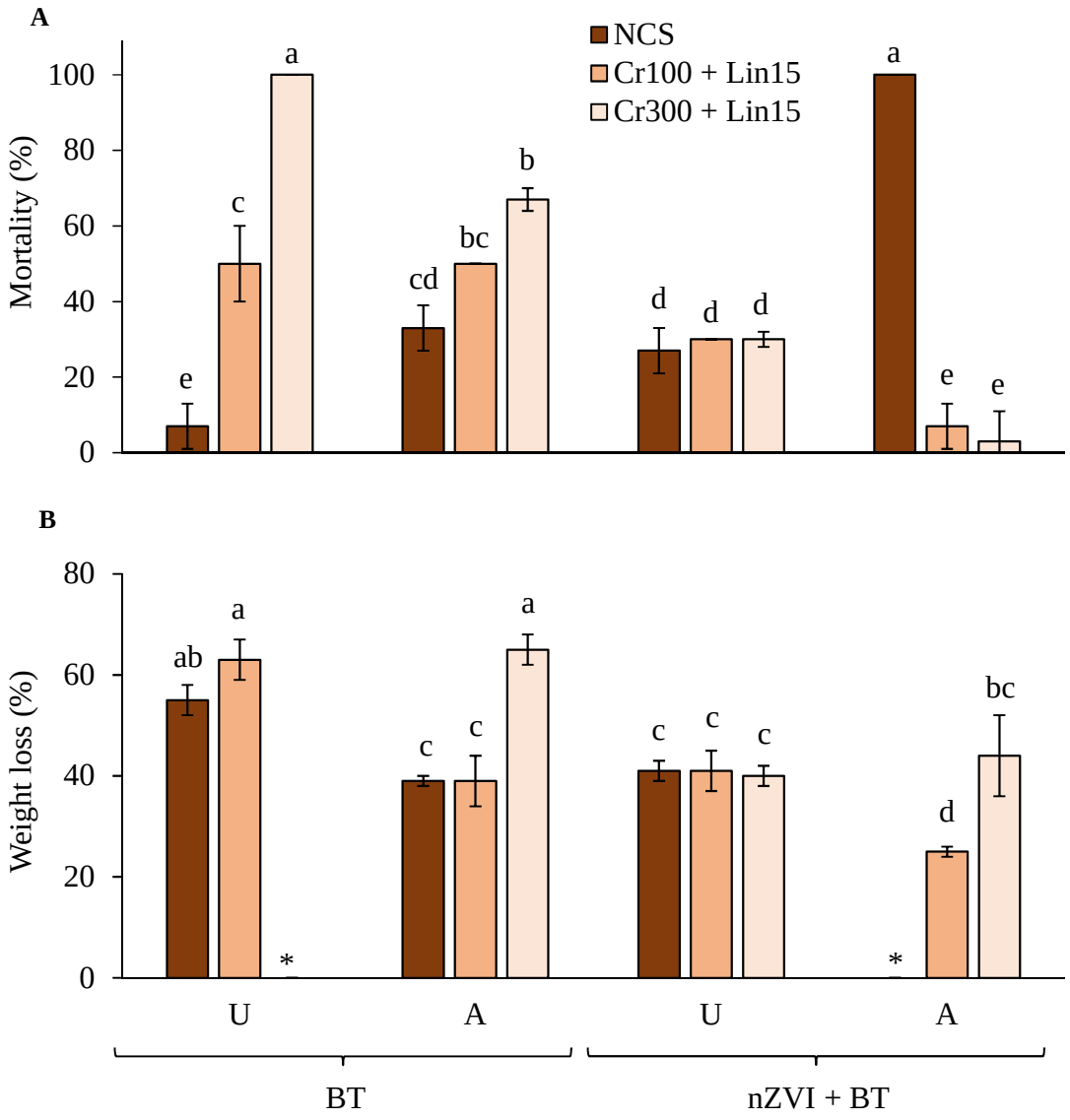


Figure 7.2. (A) Mortality and (B) Weight loss of the earthworms. Treatments: Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15). Values sharing the same letter were not significantly different (p <0.05). * non-survival.

Recently, a large volume of research work has been conducted to explore nZVI for cost-effective decontamination of organic pollutants, including lindane, demonstrating great potential for soil remediation (Li et al., 2016). However, a single contaminant was tested in these cases. Concerning to metals, nZVI can react with both cations and oxyanions, including Cr(VI), through direct or coordinative reactions, becoming to biological-friendly iron oxide or hydroxide (Su et al., 2016). Nevertheless, the large-scale application of nZVI is still challenged by severe particle aggregation and

surface passivation. The former is driven by the tendency of nZVI to reduce system surface energy, while the latter becomes more severe during the reaction as the precipitations of ferrous (hydro)oxides encapsulate nZVI, impeding its contact with other contaminants (Chen et al., 2008). This could explain why Cr(VI) removal achieved in both amended and unamended soils treated with nZVI was high, while the lindane removal was less than 14% (Table 7.1), compared to the soil without biological treatment nor nZVI.

In unamended soil, the maximal lindane removal was reached with the combined treatment nZVI + BT (80% compared to untreated soil) (Table 7.1). In contrast, low pesticide removal was achieved with the same treatment in amended soil (Table 7.1). Organic matter plays important role in both adsorption and electron transfer processes. It is adsorbed on the nZVI surface and forms complex with iron species (Xu et al., 2013), and suffer a rapid aggregation and agglomeration often forming micro-sized fractal aggregates, which subsequently lead to a significant loss in reactivity and decreased environmental mobility and affect pesticide degradation, through an alteration of its bioavailability (D. Jiang et al., 2018). According with our results, the interaction between organic matter and nZVI on amended soils caused dying of plants and earthworm (Fig. 7.1, 7.2), and this effect drastically reduced the effectiveness of lindane degradation of the combined biological treatment (Table 7.1).

7.3.2. Status of remediator organisms

7.3.2.1. E. fetida earthworms

Although it was demonstrated that plants improve soil conditions leading to develop soil organisms (Rodriguez-Campos et al., 2019), the results of the combination of the three biological technologies suggest that space limitation caused by high root density affected the normal growth and behavior of earthworms in the soil. In soils unamended and non-contaminated submitted to the biological treatment, where plants had low biomass (Fig. 7.1A) the mortality of earthworms was 7% and the weight loss was 55% (Fig. 7.2), while in amended soil, which allowed higher plant biomass (Fig. 7.1A) the percentages of earthworm mortality and weight loss were 33% and 39%, respectively (Fig. 7.2).

Despite the mortality rate of earthworms can be affected by organochlorine pesticides and metals, Kokta (1992) showed that this kind of pesticides presents elevated values of LC_{50} (concentration that causes mortality to half of tested organisms in a single

exposure), revealing a lack of toxicity to earthworms. On the other hand, Sivakumar and Subbhuraam (2005) demonstrate high toxic of Cr(VI) to *E. fetida*, affecting critical factors, including survival and growth. In this sense, in the co-contaminated soils treated with the biological agents, the increase of mortality and weight loss could be mainly attributed to Cr(VI) concentration. In the non-treated soils with an initial Cr(VI) concentration of 100 mg kg^{-1}, the earthworm mortality was 50% in both unamended and amended soils, and the weight loss was 63% and 41%, respectively (Fig. 7.2). The presence of higher Cr(VI) concentration (Cr300 + Lin15) in unamended soils had a noticeable impact on the earthworms, causing total mortality of specimens. On the other hand, in amended soils mortality was 67%, and weight loss was 65% (Fig. 7.2). In all cases, mortality was preceded by morphological changes (oozing of coelomic fluid and cliteral bulge) and behavioral changes included slow movements and the formation of a structure similar to a knot in the anterior end of the earthworms.

Interestingly, in amended and non-contaminated soils under the combined treatment (nZVI + BT), the mortality of *E. fetida* was 100%. In contrast, in unamended soils the mortality of specimens was 27% and the weight loss was 41% (Fig. 7.2). The nZVI released into the uncontaminated environments is added spontaneously with itself (homoaggregation), and this aggregation increases in the presence of natural minerals, organic colloids and organic wastes (heteroaggregation) (Dwivedi et al., 2015). The interaction of nZVI with both, organic and inorganic ligands, results in an excessive aggregation with changes at the physicochemical, macromolecular and biological levels. Surface coatings (including ions, polysaccharide/protein, and organic matter) and macroaggregates disturb the physiochemical conditions of the soil, modifying the environment (Dwivedi et al., 2015). These conditions may be less favorable for the development of the ecosystem. This phenomenon greatly hinders the environmental applications of nZVI. In unamended contaminated soils under nZVI treatment, the earthworm's mortality was 30%, and the weight loss was around 40%, regardless of the initial metal concentration, which is a substantial improvement, compared to the high values in unamended soils without nZVI. No significant differences were observed in comparison with the non-contaminated soil (Fig. 7.2).

In amended contaminated soils, the mortality was less than 10% while weight loss was 25% and 44%, in treatments Cr100 + Lin15 and Cr300 + Lin15, respectively (Fig. 7.2). The rapid reaction of Cr(VI) with the nZVI could prevent the formation of surface

coatings and macroaggregates with the organic matter, reducing the physiochemical alterations and ecosystem disturb (Dwivedi et al., 2015; Peng et al., 2017).

Chromium accumulation in the earthworms was higher at the highest initial concentration (Cr300 + Lin15) (Table 7.2), moreover, it was higher in unamended soils than in amended soils (Table 7.2). Both results were predictable, due to the metal accumulation in earthworms is related to their bioavailability (Demuynck et al., 2014).

Table 7.2. Total chromium and lindane concentration in earthworms. Treatments: Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15).

		Cr (mg/kg)		Lindane (mg/kg)	
		U	**A**	**U**	**A**
BT	**NCS**	ND	ND	ND	ND
	Cr100 + Lin15	30.5 ± 1.4^{a}	3.3 ± 0.5^{de}	$0.09 \pm 0.01^{a'}$	$0.08 \pm 0.01^{a'}$
	Cr300 + Lin15	*	12.6 ± 0.3^{c}	*	$0.09 \pm 0.01^{a'}$
nZVI + BT	**NCS**	ND	*	ND	*
	Cr100 + Lin15	12.7 ± 0.6^{c}	2.2 ± 0.5^{e}	$0.09 \pm 0.01^{a'}$	$0.10 \pm 0.02^{a'}$
	Cr300 + Lin15	21.6 ± 1.7^{b}	5.5 ± 1.2^{d}	$0.09 \pm 0.01^{a'}$	$0.09 \pm 0.01^{a'}$

ND: non-detected. * non-survival. Values sharing the same letter were not significantly different (p <0.05).

Very low concentrations of lindane were quantified in *E. fetida*, regardless of the presence or absence of the amendment or the treatments applied (Table 7.2). The low amount of lindane accumulated in earthworms could be attributed to its degradation in the soil and binding to organic matrix, which render a subsequent lowered bioavailability. Additionally, the free lindane also could decrease over time because desorption of bound compounds from the soil was not fast enough to provide the equilibrium in the soil-pore water system. It is important to highlight that soil invertebrates are mostly exposed by pesticides via pore water (Jager et al., 2003). In this sense, the bioavailable fractions were predominantly depleted/degraded, and in addition earthworms eliminate part of the accumulated compounds via sequestration in cellular ligands/compartments producing non-bioavailable residues despite of remaining in soil. As a result, the rate of mass transfer to pore water is limited for persistent organic pollutant uptake (Šmídová and Hofman, 2014) as occurred under present exposure conditions.

However, although pollutant accumulation was detected inside individuals collected from contaminated soils, the levels were negligible considering the initial concentrations of pollutants. Furthermore, the objective of vermiremediation is not to extract contaminants through accumulation/sequestration in cell or tissue compartments of earthworms. Indeed, the objective is to favor the development of the microbiota and decrease the bioavailability of pollutants (Rodriguez-Campos et al., 2019).

7.3.2.2. B. napus plants

In unamended biologically treated soils, the Cr(VI) had a notorious negative impact on *B. napus*. Although germination was higher than 80%, none of the plants survived at the end of the study but in non-contaminated soil, germination and plant survival was 96% (Table 7.3). In all amended soils, treated with the biological agents, the germination and survival were 95% or higher (Table 7.3). These results are in accordance with the Cr(VI) concentration detected in those soils (Table 7.1), since the higher the Cr(VI) concentration, the greater the negative effect on germination and survival (Aparicio et al., 2019). Previous studies have shown the toxic effects of Cr(VI) on the physiological processes of plants, such as photosynthesis, water relations and mineral nutrition, seed germination, seedling growth and chlorophyll content, among other effects (Shanker et al., 2005).

Table 7.3. Seed germination (G) and survival (S) of *Brassica napus* plants. Treatments: Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15). Values sharing the same letter were not significantly different (p <0.05). G: seed germination. S: survival.

		G (% ± SD)		S (% ± SD)	
		U	**A**	**U**	**A**
BT	**NCS**	96 ± 3^{ab}	95 ± 3^{ab}	$96 \pm 3^{a'}$	$95 \pm 3^{a'}$
	Cr100 + Lin15	99 ± 1^{a}	95 ± 3^{ab}	-	$95 \pm 3^{a'}$
	Cr300 + Lin15	81 ± 5^{bc}	98 ± 4^{a}	-	$98 \pm 4^{a'}$
nZVI + BT	**NCS**	97 ± 3^{a}	37 ± 8^{d}	$97 \pm 3^{a'}$	-
	Cr100 + Lin15	99 ± 1^{a}	35 ± 7^{d}	$99 \pm 1^{a'}$	$4 \pm 1^{c'}$
	Cr300 + Lin15	67 ± 7^{c}	73 ± 8^{c}	$67 \pm 7^{b'}$	$73 \pm 8^{b'}$

Values sharing the same letter were not significantly different (p <0.05).

Under our experimental conditions, nZVI did not have a direct negative effect on the germination and survival of plants in unamended soils, since these parameters were 97% in non-contaminated soils, and 99% in those contaminated with 100 mg kg^{-1} of Cr(VI). On the other hand, both parameters were less than 70% in unamended soil contaminated with the highest metal concentration (Table 7.3). These phytotoxic effect could be related to the higher concentration of Cr(VI) detected in those soils (Table 7.1).

Previous studies of the effects of nZVI on plants are inconclusive (D. Jiang et al., 2018). Plants in soils treated with nZVI show in some cases stimulation of seed germination, growth and increase in biomass and chlorophyll content (Libralato et al., 2016). In other cases, direct deposition of nZVI in the seeds surface slows down their germination and development, shorts the root elongation by blocking transshipment of water and nutrient element by the membrane pores, and visible micro and macronutrients deficiency symptoms are observed (Rede et al., 2016). Nevertheless, it should be considered a negative indirect effect on plants rather than a direct effect. In our study, as observed with the earthworms, the combination of nZVI and organic matter was deleterious for the plants, as none of them survived in soils without contamination (NCS). We found that germination and survival in amended soils were higher (73%) only in those with the highest Cr concentration (Table 7.3). An excess of iron content in the soil may lead to the interaction between nZVI-organic and inorganic ligands, increasing seed surface coatings formation and soil aggregation and compaction, which could have had a negative impact in the plant growth (Mu et al., 2017). In the soil with greater contamination of Cr(VI), the remaining fraction of unreacted nZVI was much smaller, so the effect of organic matter could be counteracted.

Organic amendment of nutrient-deficient soils has been reported to improve plant growth (Yu et al., 2019). In the present work, increases in plant biomass and content of Chl a+b and carotenoids were observed in the amended soils (Fig. 7.1). The improved plant growth could be attributed to the following factors: 1) nutrients provided by the amendment; 2) greater efficiency in the use of nutrients; and 3) favorable rhizosphere environment. Organic amendments contain mineral nutrients including N, P, K, Ca, Mg, S, Mn, Cu, Zn, and B. Also, the interactions among plant roots, amended soils, and microbe would create a healthy rhizosphere for plant growth (Yu et al., 2019).

In amended and contaminated soils submitted to the biological treatment, there was a tendency to increase plant biomass (Fig. 7.1A) and Chl a+b and carotenoids

contents (Fig. 7.1B) together which stimulation metal concentration, especially in leaves (Fig. 7.3). In contrast, the effect was inverse on unamended and contaminated soils (Fig. 7.1B). The concentration of Cr in plant tissues was low, in according to the values of soluble Cr(VI) (Table 7.1). In fact, the increase on photosynthetic pigments observed (Fig. 7.1A) could be explained by the hormesis phenomenon. This is an adaptive response of organisms to moderate stress, consisting of a biphasic response to an environmental agent characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect (Mattson, 2008). Low doses of chromium have been reported to provoke hormetic effects in some *Brassicaceae* species (Morkunas et al., 2018).

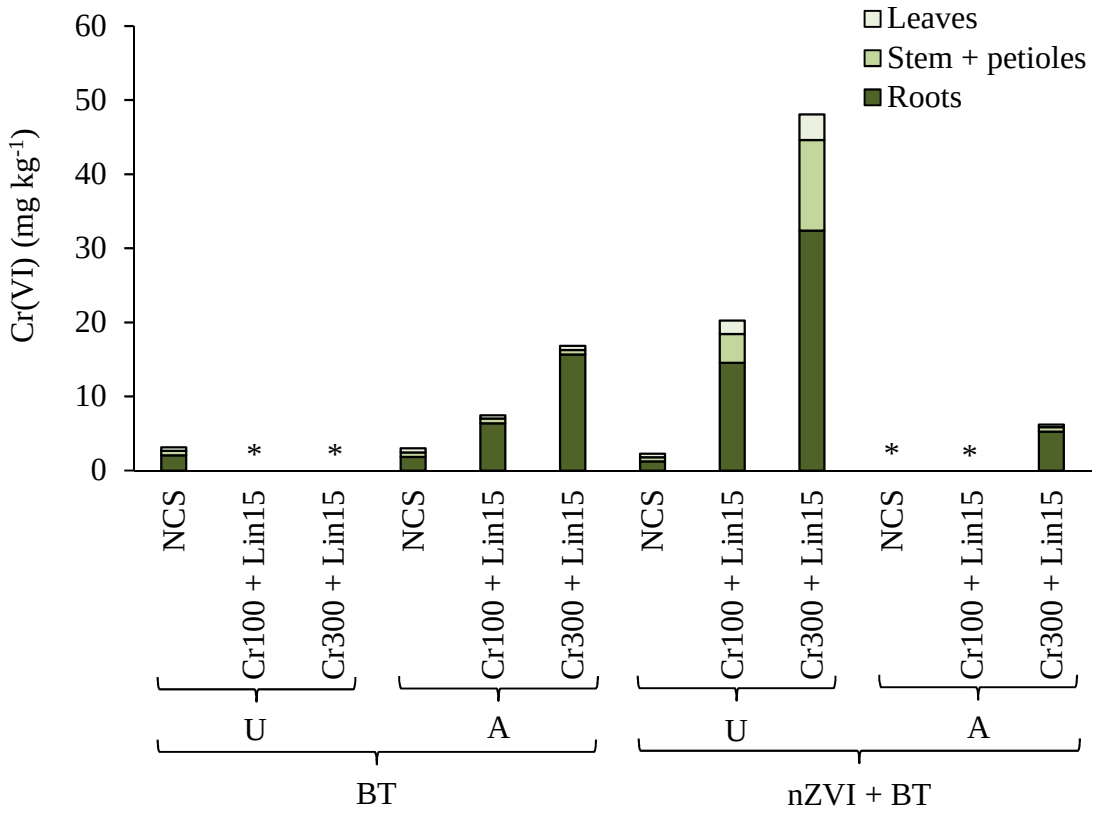


Figure 7.3. Chromium contained in *Brassica napus* tissues. Treatments: Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15). Values sharing the same letter were not significantly different (p <0.05). * non-survival.

Lindane content in *B. napus* plant parts was also determined (data not shown), the concentrations being very low in roots (≈ 0.6 mg kg^{-1}) and stems + petioles (≈ 0.1 mg kg^{-}

^{1}), and undetectable in leaves. In addition, no statistic differences were observed among treatments These results again highlight the hydrophobicity of lindane and therefore its low absorption and translocation to shoots. These results point out a low toxicity of this pesticide for *B. napus* and indicate that the elimination of the pesticide in the soil was due to microbial degradation in soil.

The chromium accumulation by *B. napus* occurred mainly at the root, with very low translocation to the shoots, and it was higher in soils with the highest initial Cr concentration (Fig. 7.3). The accumulation in the root can happen by phytochemical complexation in the root zone, which could precipitate or immobilize chromium in the root and store such complexes in the vacuolar space of plant cells (phytosequestration) (Sinha et al., 2018).

In plants growing in contaminated and amended soils under BT, concentrations of chromium in leaves and stems + petioles were similar to those detected in plants grown in non-contaminated (Fig. 7.3). Similar results of absorption and accumulation of chromium were informed to *Citrus sinensis* (0.2 to 0.3 mg kg^{-1}), *Pyrus commuhis* (0.03 to 0.9 mg kg^{-1}), *Triticum* spp. (10.2 to 14.8 mg kg^{-1}) and *Zea mays* (0.2 to 0.7 mg kg^{-1}) (Samantaray et al., 1998a). Several studies reported that chromium phytotoxicity, accumulation rate and translocation to shoots and leaves not only depend on plant species, Cr speciation, and bioavailability (Yu et al., 2007). Also, organic matter content and chelating agents play an important role in Cr absorption and translocation (Zhang et al., 2010). Organic matter reduces Cr(VI) transport through the soil by its reduction to Cr(III). Once reduced, negatively charged functional groups associated with organic constituents adsorb cationic chromium, irreversibly retaining it in the soil matrix (Kim et al., 2015).

Nevertheless, in plants from unamended soils treated with nZVI + BT, a higher translocation of the metal to the aerial part was observed (Fig. 7.3). The mechanisms of Cr(VI) removal by nZVI is based on their reduction to Cr(III), subsequently followed by precipitation of Cr(III) on the surface of nZVI in the form of a layer of chromium-iron oxides/hydroxides/oxyhydroxides (Franco et al., 2009). In these cases, a slight increase of chromium in the labile form use was observed mainly due to the partial transformation of Cr(III) to its mobile forms due to complex formation with humus substances present in the soils immediately after reduction, and to the presence of precipitated products only slightly adsorbed to the soil matrix (Di Palma et al., 2015). This could explain the

increased uptake of chromium by plants in co-contaminated and unamended soils treated with nZVI + BT.

Phytoremediation of Cr contaminated soil is primarily based on phytoextraction process, where a specific hyperaccumulator is used to extract the pollutant through its roots which are then translocated to other plant parts (Hsiao et al., 2007). Cr hyperaccumulator plants can accumulate more than 1000 mg Cr per kg dry weight in their tissues (Zhang et al., 2007). This is not the case of *B. napus*, since the chromium extraction by the plants was very low, discarding phytoextraction as a suitable technique. However, the lack of translocation of chromium to the shoots of the plant would allow the use of *B. napus* for phytomanagement strategies of soils polluted with chromium. In this sense, there is no risk of chromium entering the food chain through the production of the rapeseed. Also, as an agronomic species for biofuel production it would be suitable for the remediation of polluted marginal lands while obtaining economic and social revenues (Cundy et al., 2016).

7.3.3. Effectiveness evaluation of the remediation process

In order to apply a soil restoration technology in the field, it is first necessary to assess their effectiveness, in terms of safety for living organisms. For instance, after the treatment, not only the contaminant concentration should decrease, but also the toxicity has to be significantly reduced (Aparicio et al., 2019). Due to their high sensitivity to fluctuations in the system, ecotoxicity tests have overcome limitations, such as non-quantitative recovery of the analyte and lack of accuracy or reproducibility leading to better evaluation of soil health and quality (Hirano and Tamae, 2011). Also, it is important to evaluate organisms, belonging to relevant taxons of the soil ecosystems, and thus obtain a comprehensive assessment of the soil health and their impact on flora, fauna and microbiota (Moradas et al., 2008). Bioindicators are organisms with high sensitivity and measurable responses to these toxic compounds (Aparicio et al., 2019; García-Velasco et al., 2017). These responses are called biomarkers, and can be recorded at different levels of biological complexity (Sobrero and Ronco, 2004).

Aparicio et al. (2019) reported that *R. sativus* and *E. fetida*, and their respective biomarkers, were suitable to evaluate the effectiveness of the restoration process of soils co-contaminated with Cr(VI) and lindane, evidencing the effect on fauna and flora of soil, respectively. On the other hand, microbial communities are good indicators of soil health

too, and parameters such as microbial activity, biomass and functional diversity have been used as biomarkers in studies of contaminated soil (Epelde et al., 2010).

Integrative Biological Response (IBR) index were calculated for each species with the aim of integrating alterations at different levels of biological complexity, following the procedure described by Beliaeff and Burgeot (2002). The most representative parameters and biomarkers in *E. fetida* (exhibiting significant differences between exposure groups) were used for this purpose: mortality, weight loss, and concentration and cell viability of extruded coelomocytes. For *R. sativus*, the biomarkers included in IBR index calculation were germination and the length of hypocotyls and roots of the seedling. Basal respiration, induced respiration, and AWCD (40 h), AUC (40h), and NUS (40 h) from Biolog EcoPlates™ were considered for the calculation of the IBR index on microbiota.

The IBR index for *R. sativus* (Fig. 7.4A), *E. fetida* (Fig. 7.4B), and microbiota (Fig. 7.4C) exposed to the co-contaminated and unamended soils without treatments exhibited the highest values with respect to treated soils in an increasing dose-effect pattern, indicating that these bioindicators were highly affected and reflect the toxicity imposed by pollutants. In non-contaminated and unamended soils, none of the three treatments produced an alteration in the bioindicators compared to the untreated soil (Fig. 7.4), except for the microbiota from the soil treated with nZVI and the biological agents (nZVI+BT), where the IBR index was significantly reduced, indicating an improvement in the measured biomarkers (Fig. 7.4C). Root exudates released by plants, and humus produced by earthworms could also improve microbial development. These substances contain small proteins and carbohydrates, which are adsorbed onto nZVI particles, hindering direct contact between nZVI and microbial cells, which could contribute to a bactericidal effect (Chen et al., 2011).

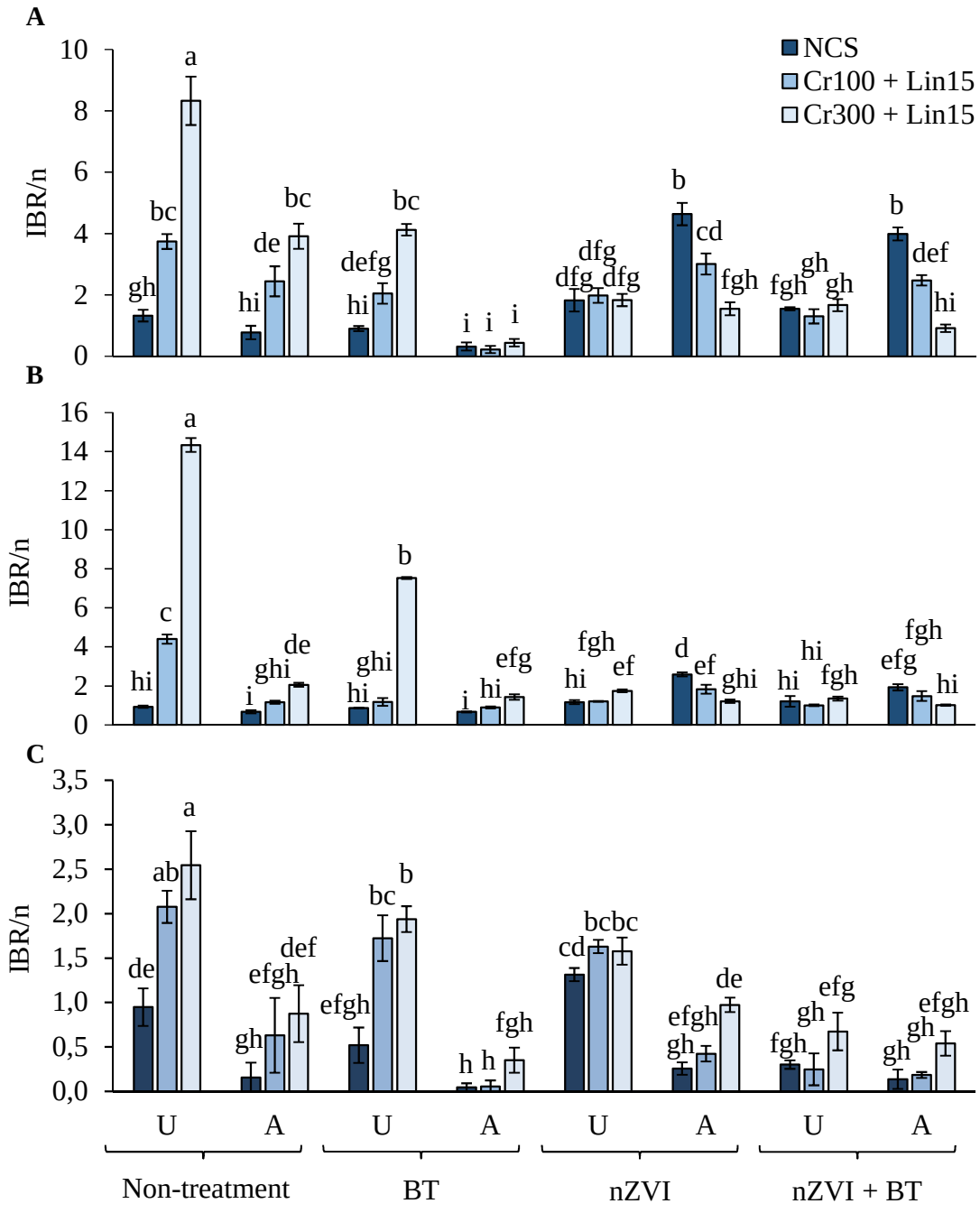


Figure 7.4: Integrated Biomarker Response/n (IBR/n) calculated for (A) *Raphanus sativus*, (B) *Eisenia fetida* and (C) soil microbial properties. Treatments: Non-treatment: co-contaminated soil without treatments; Biological treatment (BT); Nanoremediation (nZVI); Nanoremediation + Biological treatment (nZVI + BT); Unamended soil (U); Amended soil (A). Conditions: Non-contaminated soil (NCS); Soil co-contaminated with 100 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr100 + Lin15); Soil co-contaminated with 300 mg kg^{-1} of chromium and 15 mg kg^{-1} of lindane (Cr300 + Lin15). Values sharing the same letter were not significantly different (p <0.05).

The biological treatment significantly reduced all three IBR index in contaminated and unamended soils (Fig. 7.4). This reduction was even greater with the combined treatment (nZVI + BT) for microbial IBR (Fig. 7.4C), but for *R. sativus* (Fig. 7.4A) and

E. fetida (Fig. 7.B) the presence of nZVI along with the biological treatment did not have a positive effect. The improvement of the microbial IBR under nZVI + BT could be attributed to two factors. On the one hand, a rapid decrease of Cr(VI) concentration together with hydrogen evolution and redox potential shifts caused by nZVI can finally lead to favorable conditions for microbiota development, since it is described that chromium is toxic to microorganisms (Pradhan et al., 2019). On the other hand, higher eukaryotic organisms, such as plants and earthworms, produce beneficial effects in microbial communities (Lacalle et al., 2018a; Rodriguez-Campos et al., 2019).

In non-contaminated and amended soils, the treatment with nZVI + BT increased the IBR index in *R. sativus* and *E. fetida* compared to non-treated soils (Fig. 7.4A, 7.4B), indicating a loss in soil health. Meanwhile, no effect was observed in soil treated with the biological agents (Fig. 7.4A, 7.4B). On the other hand, the microbiota IBR was not altered by the application of any of the treatments. (Fig. 7.4C). As explained above, macroaggregation and compaction caused by the addition of the nZVI may disturb the physiochemical conditions of the soil, modifying the ecosystem (Dwivedi et al., 2015). This new environment could have hindered the development of plants and earthworms without significantly affecting the microbiota.

Except for the previously mentioned negative interaction between nZVI and amendment, the presence of higher organic matter content caused overall the improvement of IBR of the three taxa, compared to the unamended soils (Fig. 7.4). In amended contaminated soils, the biological treatment was able to reduce IBR index of *R. sativus*, while for *E. fetida* and the microbiota it remained at similar values (Fig. 7.4). The application of nZVI (both alone or combined with the biological treatments) did not have effect on the IBR indexes (Fig. 7.4) of soils with moderate contamination (Cr100 + Lin15), except for a slightly deleterious effect on *R. sativus* (Fig. 7.4A) and *E. fetida* (Fig. 7.4B) caused by the nZVI treatment, presumably due to the same reasons as in the NCS amended soil. Conversely, at high initial metal concentration (Cr300 + Lin15), both nZVI and nZVI + BT treatments decreased the IBR indexes for both bioindicators (Fig. 7.4A, 7.4B), even though this reduction was overall not higher than in the biological treatment alone. In contrast, the microbiota IBR was not altered by the application of treatments that included nZVI (Fig. 7.4C).

7.4. Conclusions

In the present study, a substantial natural attenuation of lindane in unamended soil without any treatment was observed, while chromium (VI) remained highly available, causing high toxicity. The biological, amendment and nanoparticle treatments applied, were very effective reducing Cr(VI) to Cr(III), therefore its availability and soil toxicity. The biological treatment decreased Cr(VI) concentration, but not as much as the organic amendment or the application of nZVI. On the other hand, the biological treatment was able to stimulate lindane degradation to a great extent, while the organic amendment and nZVI limited such degradation. The reduction of Cr(VI) to Cr (III) had a beneficial effect on soil health bioindicators. The decrease of mobility and soil toxicity by the organic matter allowed the survival and better performance of the *B. napus*, *E. fetida*, and soil microbes, and opens the door to phytomanagement strategies of soils polluted with chromium and lindane. We reported that nZVI did not have toxic effect on the organisms of the experiment when applied in unamended soils, but a significant deleterious effect was observed mainly on earthworms and plants when the content of organic matter in the soil is high, due to their reactivity with other components of the soil in the absence of pollutants. Therefore, the combination of the organic amendment, the triple biological treatment plus nZVI may be the best strategy to remediate soils with high concentrations of Cr(VI) and lindane, while for moderate levels of chromium the application of an organic amendment plus the biological treatment is probably the most cost-effective treatment from the point of view of improvement in remediation and sustainability.

7.5 Supplementary material

Table S7.1. Physicochemical characterization of unamended (U) and amended (A) soil.

Parameters	U	A
Texture class (USDA)	Loam	Loam
Coarse sand (%)	17.9	14.5
Fine sand (%)	21.3	25.1
Total silt (%)	37.5	44.0
Total clay (%)	23.4	15.7
Carbonates (%)	54.7	44.0
Organic matter (%)	1.0	19.5
Total C organic (% DW)	0.6	7.3
Total N (% DW)	0.1	0.9
C organic / N organic	6.7	8.6
Total S (% DW)	< 0.05	< 0.05
pH (1:2.5)	7.9	8.0
Total Cr (mg kg^{-1})	25.23	25.52

7.6. Conclusiones del capítulo

- Tanto la aplicación de nanopartículas de hierro cero valente (nZVI) como de materia orgánica al suelo son estrategias de remediación muy efectivas para reducir la especie más soluble y toxica del Cr (Cr VI).

- La aplicación de nZVI no afecta directamente la degradación de lindano, pero estimula su degradación en combinación con los tratamientos biológicos al reducir la toxicidad del cromo en los organismos.

- Las nZVI, en ausencia de Cr, reaccionan con la materia orgánica del suelo y causando toxicidad a plantas y lombrices, posiblemente por cambios en la estructura del suelo.

- La combinación de tratamientos biológicos (biorremediación, fitorremediación y vermiremediación) asistidos por una enmienda orgánica se postula como el mejor tratamiento para suelos contaminados con Cr(VI) y lindano, frente a la nanorremediación con nanopartículas de hierro cero valente.

8. EFFECTS OF THE APPLICATION OF AN ORGANIC AMENDMENT AND NANO-SCALE ZERO-VALENT IRON PARTICLES ON SOIL Cr(VI) REMEDIATION

8. EFFECTS OF THE APPLICATION OF AN ORGANIC AMENDMENT AND NANO-SCALE ZERO-VALENT IRON PARTICLES ON SOIL Cr(VI) REMEDIATION

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Abstract

Chromium is considered an environmental pollutant of much concern whose toxicity depends, to a great extent, on its valence state, with Cr(VI) being more soluble, bioavailable and toxic, compared to Cr(III). Nanoremediation is a promising strategy for the remediation of metal pollutants by changing their valence state. However, among other aspects, its effectiveness for soil remediation is seriously hampered by the interaction of nanoparticles with soil organic matter. In this study, soil was (i) amended with two doses of a municipal solid organic waste and (ii) artificially polluted with 300 mg Cr(VI) kg^{-1} DW soil. After a period of aging, a nanoremediation treatment with nano-scale zero-valent iron particles (1 g nZVI kg^{-1} DW soil) was applied. The efficiency of the remediation treatment was assessed in terms of Cr(VI) immobilization and recovery of soil health. The presence of the organic amendment caused (i) a decrease of redox potential, (ii) Cr(VI) immobilization via its reduction to Cr(III), (iii) a stimulation of soil microbial communities and (iv) an improvement of soil health, compared to unamended soil. By contrast, nZVI did not have any impact on Cr(VI) immobilization nor on soil health. It was concluded that, unlike the presence of the organic amendment, nanoremediation with nZVI was not a valid option for soils polluted with Cr(VI) under our experimental conditions.

8.1. Introduction

Soil is a non-renewable resource at human scale which provides a great number of soil functions and crucial ecosystem services (Brevik et al., 2015). In consequence, we must preserve soil health broadly defined as “the capacity of a living soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and promote plant and animal health” (Doran et al., 1996).

In Europe, it was estimated that there are 2.5 million potentially-contaminated sites, with metals being the most abundant contaminants in European topsoils (Van Liedekerke et al., 2014). Importantly, metals cannot be degraded and are subject to their bioaccumulation and biomagnification through the food chain (Dar et al., 2017).

Due to its wide industrial use, chromium is nowadays considered an environmental pollutant of great concern. Its bioavailability is determined by the oxidation state: Cr(VI) is highly soluble and, therefore, available and potentially (eco)toxic; on the contrary, Cr(III) has low solubility and is easily adsorbed by soil minerals (Aldmour et al., 2019; Polti et al., 2007). Soil physicochemical properties, such as clay content, organic matter (OM), pH, redox potential, moisture content, etc. strongly influence metal mobility and bioavailability in soil (Vangronsveld and Cunningham, 1998). Soil OM plays a key role in Cr(VI) immobilization by means of reduction and/or sorption (Banks et al., 2006; Choppala et al., 2018).

When assessing the impact of contamination on soil health, it is advisable to measure not only total metal concentrations, but also bioavailable fractions, which are more responsible for their mobility, availability, (eco)toxicity and, concomitantly, negative effects on soil health (Alkorta et al., 2010; Megharaj et al., 2011; Vamerali et al., 2010). In any case, the assessment of soil health is a highly complex issue which, among other aspects, requires the simultaneous determination of a wide variety of physical, chemical and biological properties as indicators of soil functioning. Specifically, (micro)biological indicators of soil health are most effective due to their quick response, sensitivity, ecological relevance and capacity to provide information that integrates many environmental factors (Garbisu et al., 2011; Mijangos et al., 2010). In consequence, microbial biomass, activity and diversity parameters have frequently been used as indicators of the impact of contaminants on soil health (Burges et al., 2017;

Galende et al., 2014b). Likewise, standardized (eco)toxicological bioassays with model organisms (*e.g.*, *Eisenia fetida*, *Vibrio fisheri*, *Lactuca sativa*) are commonly used as bioindicators of soil health (Gómez-Sagasti et al., 2012).

Traditionally, soil remediation has been addressed by the application of physicochemical techniques which are usually economically-costly, environmentally-disruptive and focused only on contaminant removal and not on the recovery of soil health (Gil-Díaz et al., 2016). But, ideally, the ultimate goal of a sound remediation initiative should be to efficiently remove contaminants (total and/or bioavailable), decrease ecotoxicity, minimize risk for environmental and human health, and recover soil health and associated ecosystem services. Consequently, in the last years and decades, there has been an increasing interest in the development of more sustainable, cost-effective soil remediation alternatives, such as the so-called Gentle Remediation Options-GROs (*e.g.*, phytoremediation, bioremediation, amendment-aided remediation) (Agnello et al., 2016).

The application of biological methods for soil chromium remediation often encounters difficulties due to its high toxicity (Han et al., 2004; Samantaray et al., 1998b). Nanoremediation strategies, like the application of nano-scale zero-valent iron, have been effectively used for the remediation of soils contaminated with Cr(VI) (Singh et al., 2012). Nevertheless, its effectiveness, as well as the potential negative effects of nZVI, are strongly influenced by the soil´s physicochemical properties (Fujioka et al., 2016; Vítková et al., 2017). The interaction of nZVI with inorganic and, especially, organic ligands in the soil can provoke macroaggregation and compaction (Dwivedi et al., 2015). Therefore, the positive and negative effects of nZVI can differ considerably depending on soil OM quantity and quality.

Organic wastes are increasingly being used as amendments during soil remediation initiatives. Contaminated soils, which often lack nutrients and OM and have a poor structure, can greatly benefit from the input of suitable organic wastes, with the additional advantage of reusing an otherwise discarded material. Thus, organic amendments from various origins have commonly been used for the remediation of metal contaminated soils (Antoniadis et al., 2018; Míguez et al., 2020; Park et al., 2011).

The aim of this study was to evaluate (i) the influence of the presence of an organic amendment (a bio-stabilized municipal solid waste); and (ii) the effect of nanoremediation with nZVI on soil Cr(VI) immobilization and, hence, soil health

recovery, as reflected by the values of soil microbial indicators (biomass, activity and diversity parameters) and ecotoxicological data from phytotoxicity bioassays with *Lactuca sativa*. We hypothesized that both nZVI (via reduction) and the organic amendment (via reduction and/or sorption) would result in soil Cr(VI) immobilization and, hence, soil health recovery. Nonetheless, we anticipated that the beneficial nanoremediation effects of nZVI could be hampered by their inactivation via interaction with soil OM.

8.2. Materials and methods

8.2.1. Experimental design

A microcosm experiment was carried out using soil from the peri-urban area of the city of Vitoria-Gasteiz (42°50′N; 2°40′W, Northern Spain). Soil was collected from the topsoil (0-15 cm), sieved to < 6 mm, and air-dried until constant weight. The soil was amended (see below) with the bio-stabilized organic fraction of solid urban wastes from the city of Vitoria-Gasteiz. This bio-stabilized organic amendment was acquired from BIOCOMPOST DE ÁLAVA, UTE, an urban waste treatment plant in Vitoria-Gasteiz. The bio-stabilized organic amendment had an OM content of 37.0% (on a dry weight-DW basis), with 23.3% of total organic carbon, an organic carbon / organic nitrogen ratio of 12.9, and the following nutrient contents (on a DW basis): 2.2% for nitrogen, 19.8 g kg^{-1} for phosphorus and 12.0 g kg^{-1} for potassium.

Three doses of this organic amendment were applied, in triplicate, to our experimental soil: (i) unamended soil, U = 0% of organic amendment, as control; (ii) medium dose of amendment, M = 10% w/w of organic amendment; and (iii) high dose of amendment, H = 20% w/w of organic amendment. The amended soils were then subjected to physicochemical characterization (Table 8.1).

Half of the U, M and H soil was artificially polluted (P) with a K_2CrO_7 solution to reach a final concentration of 300 mg Cr(VI) kg^{-1} DW soil. The artificially-polluted soil was thoroughly homogenized with a laboratory mixer. The remaining half was left unpolluted as control (C).

Subsequently, 0.4 kg of each soil (unamended, amended, polluted and unpolluted soils) was placed in 0.5 L pots. A sample of each soil was taken at this time (time = day 0). Soils were then placed in a greenhouse under controlled conditions (temperature =

25/18 °C day/night; relative humidity = 60/70% day/night; photoperiod = 14/10 h day/night) for one month for stabilization/ageing purposes. Soils were watered periodically to keep them near water holding capacity.

After the abovementioned 30-day stabilization period (time = day 30), nZVI (NanoFer Star, Nanoiron s.r.o) were applied to half of the soils (the other half was left untreated for comparison purposes: nanoremediated *vs.* non-nanoremediated soil) following the manufacturer´s instructions. Nano-scale zero-valent iron particles (n) were added in aqueous solution to reach a final concentration of 1 g nZVI kg^{-1} DW soil. The nZVI-treated soil was thoroughly homogenized with a laboratory mixer.

The experiment was conducted with 36 pots from 12 treatments (in triplicate): UC, nUC, MC, nMC, HC, nHC, UP, nUP, MP, nMP, HP and nHP (U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment; P: polluted with Cr(VI); C: non-polluted control; n: ZVI-treated). Soil samples were collected two months after nZVI application (time = day 90) to assess medium-term effects of the applied treatments.

8.2.2. Soil physicochemical characterization

Soil was oven-dried at 70 ºC for 72 h and crushed to < 0.125 mm particle size. Oxidizable OM, total nitrogen, C/N ratio and carbonate content were determined following official methods (MAPA, 1994). For the determination of soil pH, electrical conductivity (EC) and redox potential, a 1:25 (w/v) mixture of soil and deionized water was shaken at 200 rpm for 1 h and then centrifuged at 10,000 x g for 15 min. The same solution was used for the determination of these three parameters using a pH-meter, a conductivity meter and a redox-meter, respectively.

For the quantification of total chromium [Cr(III) + Cr(VI)], soils were subjected to an acid digestion (HNO_3+HCl) (US-EPA Method 3051A, 2007) and then analyzed by Inductively-Coupled Plasma-Mass Spectrometry (ICP-MS) (Agilent 7700) with a limit of quantification of 0.03 μg L^{-1}. The soluble fraction of chromium [Cr(VI)] was determined following Jiang et al. (2015): soluble chromium was extracted by shaking a 1:25 (w/v) mixture of soil and Milli-Q water at 200 rpm for 1 h, followed by centrifugation at 10,000 x g for 15 min. The extract was analyzed by ICP-MS.

8.2.3. Soil microbial parameters

Soil basal respiration (BR) was measured following ISO 16072 (2002). Substrate-induced respiration (SIR) was measured following ISO 17155 (2002). Biolog Ecoplates™ were used to determine community-level physiological profiles as estimates of microbial functional diversity (Galende et al., 2014b). Both the number of utilized carbon substrates (NUS) and substrate-consumption activity (SCA, also called Area Under the Curve-AUC) were calculated from Biolog Ecoplates™ data (Galende et al., 2014a).

8.2.4. Soil phytotoxicity

Soil phytotoxicity was measured using a root elongation bioassay with *Lactuca sativa* (c.v. May Queen), following the methodology described for *Cucumis sativus* by Lacalle et al. (2018a) but adapted to *Lactuca sativa*. Seeds were pre-germinated in wet filter paper for 48 h until emerged seeds showed a radicle length of 5 mm. Subsequently, emerged seeds were placed in Petri dishes containing 10 g of previously hydrated soil and, then, covered with black filter paper. Dishes were placed at an angle of 45º and incubated for 72 h under controlled conditions (photoperiod = 14/10 h day/night; temperature = 25/18 ºC day/night; relative humidity = 60/80 % day/night; photosynthetic photon flux density = 100 µmol photon m^{-2} s^{-1}). Root elongation was measured at the beginning and after 72 h by taking a photograph and then analyzing the image using software ImageJ (Schneider et al., 2012).

8.2.5. Statistical analysis

The software package IBM SPSS Statistics for Windows, Version 24 was used for all the statistical tests. Normality was checked using Kolmogorov-Smirnov test and homocedasticity was checked using Levene test. Two-way ANOVA was performed, applying Duncan *post hoc*.

8.3. Results and discussion

8.3.1 Soil physicochemical properties

The soil used in this study was a loam soil with high carbonate content (40-50%) and a slightly alkaline pH (8.0-8.3) which remained stable for all treatments and sampling times (data not shown). Conversely, other soil physicochemical parameters were notoriously modified by the addition of the bio-stabilized organic amendment. Electrical conductivity

(Table 8.3, Table 8.4) was significantly higher in organically-amended soils, most likely due to the solutes present in the amendment itself: this increase was persistent in time with highest values being found in H soils (Table 8.1). In agreement with other authors (Dhaliwal et al., 2019), redox potential, on the other hand, was significantly (Table 8.3, Table 8.4) decreased by the application of the organic amendment, with lowest values being observed in H soils (Table 8.1). Redox potential was not significantly affected by the application of nZVI, despite their well-known reductive capacity (Vítková et al., 2017). Values of oxidizable OM and total N were significantly (Table 8.3, Table 8.4) increased by the application of the bio-stabilized organic amendment (Table 8.1), owing to the previously mentioned high contents of OM and nitrogen present in the amendment. Actually, by the end of the study (at day 90), organically-amended soils still had higher values of oxidizable OM than unamended ones (Table 8.1). Finally, values of the C/N ratio in soil, which had been also moderately increased by the addition of the organic amendment, were slightly lower at day 90 (Table 8.1).

8.3.2. Soil chromium

As expected, total chromium concentrations in soil did not suffer statistically (Table 8.3) significant changes throughout the experimental period (Table 8.2). Concentrations of Cr(VI) in soil at day 0 were considerably high (ca. 30% of soil total chromium concentrations) (Table 8.2). Over time, soil contaminants can interact with the soil´s organic and inorganic fractions, with concomitant changes in their speciation, solubility and availability, until they finally reach an equilibrium, in a process often called “aging” (Adriano, 2001; Park et al., 2011). The soil used in this study has a high content of carbonates which can immobilize soil chromium as Cr(III) (Bolan and Duraisamy, 2003). The presence of manganese oxide (MnO_2) has been reported to play a significant role in chromium speciation, favouring the oxidation of Cr(III) to Cr(VI) (Di Palma et al., 2018). Nevertheless, in our study, soil Mn concentrations were <10 mg kg^{-1} DW soil, which strongly reduces the possibility of Mn having any effect on chromium speciation.

Table 8.1. Effect of treatments on soil physicochemical properties. OOM: oxidizable organic matter; E_h: redox potential; EC: electrical conductivity; U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment; n: ZVI-treated.

		Day 0		Day 90	
		Control	Polluted	Control	Polluted
E_h (mV)	U	163.47 ± 0.81	171.80 ± 2.17	167.50 ± 0.90	179.00 ± 2.97
	M	114.03 ± 1.43	110.83 ± 1.31	121.37 ± 3.06	116.93 ± 1.08
	H	86.83 ± 1.43	90.97 ± 1.48	86.27 ± 1.86	96.23 ± 2.92
	nU			169.33 ± 3.84	173.10 ± 3.45
	nM			126.70 ± 2.21	119.97 ± 2.14
	nH			89.70 ± 2.99	100.23 ± 1.00
EC (mS cm^{-1})	U	0.32 ± 0.04	0.39 ± 0.02	0.65 ± 0.01	0.59 ± 0.02
	M	1.31 ± 0.16	1.62 ± 0.09	1.82 ± 0.28	1.97 ± 0.44
	H	2.56 ± 0.06	2.87 ± 0.35	2.58 ± 0.47	2.55 ± 0.09
	nU			0.69 ± 0.03	0.58 ± 0.09
	nM			1.39 ± 0.43	1.73 ± 0.06
	nH			3.85 ± 0.89	2.59 ± 0.23
OOM (%)	U	1.84 ± 0.07	1.93 ± 0.13	1.80 ± 0.04	1.80 ± 0.05
	M	4.97 ± 0.10	5.06 ± 0.15	3.75 ± 0.06	4.00 ± 0.12
	H	8.26 ± 0.55	10.05 ± 1.92	5.63 ± 0.11	6.05 ± 0.46
	nU			1.83 ± 0.11	1.79 ± 0.06
	nM			3.57 ± 0.13	3.75 ± 0.04
	nH			6.50 ± 0.47	5.84 ± 0.54
Total N (%)	U	0.14 ± 0.00	0.13 ± 0.01	0.13 ± 0.00	0.12 ± 0.00
	M	0.32 ± 0.02	0.33 ± 0.04	0.29 ± 0.00	0.28 ± 0.01
	H	0.56 ± 0.05	0.58 ± 0.08	0.43 ± 0.01	0.47 ± 0.01
	nU			0.13 ± 0.00	0.12 ± 0.00
	nM			0.25 ± 0.01	0.27 ± 0.01
	nH			0.46 ± 0.01	0.42 ± 0.03
C/N ratio	U	8.50 ± 0.13	8.66 ± 0.68	8.04 ± 0.16	8.49 ± 0.41
	M	9.35 ± 0.69	9.11 ± 1.23	7.60 ± 0.17	8.23 ± 0.27
	H	8.07 ± 0.41	9.94 ± 0.55	7.61 ± 0.13	7.50 ± 0.63
	nU			8.00 ± 0.65	8.47 ± 0.40
	nM			8.21 ± 0.23	8.09 ± 0.23
	nH			8.25 ± 0.37	8.00 ± 0.24

At day 90, the presence of the organic amendment caused a significant reduction (Table 8.3) of Cr(VI) concentrations in chromium-polluted soils (Table 8.2), as previously reported by other authors (Antoniadis et al., 2018; Banks et al., 2006). The reduction of the redox potential caused by the bio-stabilized organic amendment (179 mV

in U soils; 117 mV in M soils; 96 mV in H soils) in chromium-polluted soils at day 90 (Table 8.1) is most likely, at least partly, responsible for the observed reduction of Cr(VI) concentrations under our soil pH conditions (Xia et al., 2019). Apart from this shift in redox potential, the addition of the organic amendment could have favoured Cr(VI) reduction to Cr(III) by incorporating humic substances to the soil. More precisely, carboxylic acids have been reported to enhance electron transmission from metals such as As, Mn, Fe and Ti to Cr(VI), causing its reduction (B. Jiang et al., 2018; Jiang et al., 2019). However, these reactions were reported to take place mainly under acidic conditions (B. Jiang et al., 2018; Jiang et al., 2019). In general, no statistically significant differences (Table 8.4) in Cr(VI) concentrations were observed between M and H soils (Table 8.2). It was concluded that the presence of the bio-stabilized organic amendment led to Cr(VI) immobilization.

Table 8.2. Effect of treatments on total and soluble chromium [Cr(VI)] concentrations in soil. U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment; n: ZVI-treated.

		Day 0		**Day 90**	
		Control	Polluted	Control	Polluted
Total Cr (mg kg^{-1})	U	40.2 ± 2.2	224.1 ± 36.4	38.1 ± 0.5	291.2 ± 89.9
	M	38.4 ± 0.5	255.4 ± 10.2	34.1 ± 1.0	236.7 ± 30.5
	H	37.9 ± 0.3	325.6 ± 14.5	34.9 ± 2.0	240.2 ± 6.7
	nU			40.9 ± 0.9	238.8 ± 31.5
	nM			37.3 ± 1.5	232.9 ± 20.8
	nH			36.8 ± 1.1	335.7 ± 35.3
Cr(VI) (mg kg^{-1})	U	0.16 ± 0.08	73.9 ± 3.5	0.02 ± 0.01	88.95 ± 24.0
	M	0.11 ± 0.02	65.9 ± 2.5	0.14 ± 0.08	15.3 ± 3.6
	H	0.22 ± 0.00	96.3 ± 6.0	0.09 ± 0.01	21.2 ± 3.1
	nU			0.02 ± 0.00	91.2 ± 16.4
	nM			0.07 ± 0.01	12.2 ± 2.8
	nH			0.11 ± 0.01	23.1 ± 6.0

Regarding nanoremediation, nZVI have been described (Singh et al., 2012) to provoke the reduction of Cr(VI) to Cr(III), which precipitates on the surface of the nanoparticles forming a layer of chromium-iron oxides/hydroxides/oxy-hydroxides. The effectiveness of nZVI for soil remediation is dependent upon multiple soil factors and interactions which are not fully understood yet (Galdames et al., 2017). The effectiveness

of nZVI for soil Cr(VI) remediation can be decreased in the presence of high amounts of OM, whose components (*e.g.*, humic acids) can interact with the nZVI, thus avoiding their desired interaction with the target contaminants (Giasuddin et al., 2007; Gueye et al., 2016). Under our experimental conditions, nZVI had no significant effect on Cr(VI) immobilization via its reduction to Cr(III). The nZVI-induced immobilization of metals is largely mediated by alteration of the redox conditions (Vítková et al., 2017). In our study, no significant differences in redox potential were observed between nZVI-treated and untreated soils, which could explain their lack of efficiency in terms of Cr(VI) immobilization. Most nanoremediation studies have been carried out in aqueous media (freshwater, groundwater, residual water, etc.). Soil nanoremediation studies are less common, especially under real field conditions (Patil et al., 2016). Furthermore, soil nanoremediation studies are usually performed under laboratory microcosms conditions, in which homogenization and contact between nanoparticles and contaminants is greatly facilitated (Alidokht et al., 2011; Gil-Díaz et al., 2017; Singh et al., 2012). One possible explanation for the lack of effect of nZVI observed here is the low dose of nZVI applied in our study (1 g nZVI kg^{-1} DW soil), compared to that used by other authors (Galdames et al., 2017). In any case, the high cost of nZVI for remediation purposes is a major obstacle for their application under real field conditions. A lot of research is currently being conducted to enhance the suitability of nZVI for soil nanoremediation, including the exploration of strategies to increase nZVI distribution in soil and avoid their aggregation (Su et al., 2016).

Table 8.3. *p*-values from repeated measures two-way ANOVA. OOM: oxidizable organic matter. BR: basal respiration. SIR: substrate-induced respiration. SCA: substrate-consumption activity. NUS: number of utilized substrates. RE: root elongation of *Lactuca sativa*.

	Time	**Amendment**	**Pollution**	**nZVI**
E_h	0.000	0.000	0.010	0.197
EC	0.043	0.000	0.195	0.598
OOM	0.000	0.000	0.779	0.771
Total N	0.000	0.000	0.723	0.129
C/N ratio	0.001	0.669	0.096	0.234
Total Cr	0.635	0.84	0.000	0.668
Soluble Cr	0.193	0.009	0.000	0.372
BR	0.000	0.000	0.011	0.722
SIR	0.000	0.000	0.000	0.831
SCA	0.000	0.000	0.000	0.977
NUS	0.000	0.000	0.000	0.345
RE	0.000	0.003	0.000	0.253

Table 8.4. Statistically significant differences among the three organic amendment doses (U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment), according to two-way ANOVA and Duncan *post hoc*. Different letters indicate significant differences. EC: electrical conductivity; OOM: oxidizable organic matter; BR: basal respiration; SIR: substrate-induced respiration; SCA: substrate-consumption activity; NUS: number of utilized substrates; RE: Root elongation of *Lactuca sativa*. Cr tot: soil total chromium concentration.

	E_h	EC	OOM	Total N	C/N	Cr tot	Cr(VI)	BR	SIR	SCA	NUS	RE
U	c	c	c	c	a	a	a	c	c	c	c	a
M	b	b	b	b	a	a	b	b	b	b	b	b
H	a	a	a	a	a	a	b	a	a	a	a	b

8.3.3. Microbial indicators of soil health

Microbial properties are excellent indicators of soil health and, in consequence, they have been frequently used to assess the impact of contamination, as well as the effectiveness of remediation processes, on soil health (Epelde et al., 2014). The presence of the organic amendment significantly (Table 8.3, Table 8.4) increased soil basal (Fig. 8.1A) and substrate-induced respiration (Fig. 8.1B) at day 0, in both control and polluted soils. Soils artificially polluted with chromium showed significantly lower values of both BR (Fig. 8.1A) and SIR (Fig. 8.1B), compared to unpolluted controls, showing the well-known toxicity provoked by Cr(VI) on microbial populations (Speir et al., 1995). Organic amendments provide the soil with easily degradable OM and viable active microorganisms, thus usually resulting in increased microbial activity and biomass (Lacalle et al., 2018a). The reduction in BR and SIR values provoked by Cr(VI) was around 50 and 30% in U and H soils, respectively, pointing out to the fact that the presence of the organic amendment partially alleviated Cr(VI) toxicity for soil microbial communities (Table 8.2).

By the end of the experiment (at day 90), values of microbial biomass (Fig. 8.1B) and, especially, microbial activity (Fig. 8.1A) were lower in both control and polluted soils, compared to day 0. This decrease was more notorious in amended soils (especially in H soils). The degradation of the most labile, easily degradable OM over time (Table 8.1) could explain this reduction of BR and SIR values from day 0 to day 90 (Bernal et al., 1998; Galende et al., 2014b).

The Cr(VI)-induced toxicity observed at day 0 in microbial activity and biomass values was significantly reduced by day 90: for organically-amended soils, the Cr(VI)-

induced toxicity observed at day 0 had almost disappeared at day 90 (<5%) for BR values (Fig. 8.1A) and had been considerably reduced (<15%) for SIR values (Fig. 8.1B). The Cr(VI) reduction mediated by the presence of the organic amendment (Table 8.2), along with its capacity to biostimulate microbial communities, might have alleviated the Cr(VI)-induced toxic effects (Antoniadis et al., 2018; Park et al., 2011). Likewise, the organic amendment-induced biostimulation of soil microbial communities might have promoted the bacterial reduction of Cr(VI) to Cr(III) (Banks et al., 2006). In U soils, higher Cr(VI) concentrations were observed, resulting in a 23 and 60% inhibition of BR and SIR values, respectively.

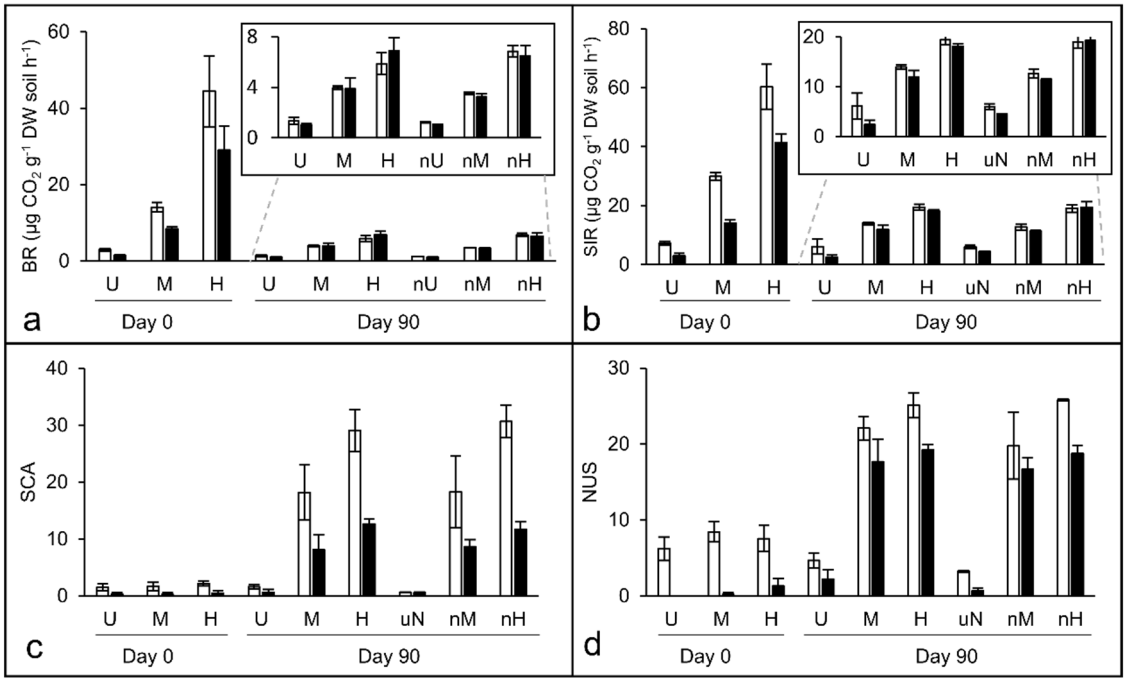

Figure 8.1. Effect of treatments on soil microbial parameters: basal respiration-BR (A), substrate-induced respiration-SIR (B), substrate-consumption activity-SCA (C), and number of utilized substrates-NUS (D). White bars represent control non-polluted soils. Black bars represent polluted soils. BR and SIR graphs feature a secondary graph with a more adequate scale to observe values at day 90. U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment; n: ZVI-treated.

The addition of nZVI did not have any clear beneficial or harmful effects on soil microbial activity (Fig. 8.1A) or biomass (Fig. 8.1B). Metallic nanoparticles can have deleterious effects on microorganisms (Lefevre et al., 2015). However, their inactivation via contact with soil OM, along with a decrease in their ability to interact due to aggregation, might turn them less reactive and, therefore, non-toxic (Chen et al., 2008; Z.

Li et al., 2010). In our study, this lack of effect of nZVI on soil microbial activity and biomass was also observed for soil physicochemical properties (Table 8.1), as previously reported (Lacalle et al., 2018b). Unfortunately, studies on the impact of nZVI on soil microbial activity and biomass are insufficient (Anza et al., 2019) with the results being strongly dependent on the specific pollutant and soil properties (Gómez-Sagasti et al., 2019).

At day 0, SCA values obtained from Biolog Ecoplates™ data were low in both polluted and non-polluted control soils (Fig. 8.1C). In turn, NUS values were lower in polluted *vs.* non-polluted soils (Fig. 8.1D). Microbial communities often require an acclimation period to be able to metabolize carbon substrates (Steven, 2017). Metals, such as chromium, can affect soil microbial functional diversity and composition (Pradhan et al., 2019). This Cr(VI)-induced inhibitory effect was less noticeable for SCA values (Fig. 8.1C), which suggests that Cr(VI) has a greater effect on the capacity of microbial communities to utilize different carbon substrates than on the overall capacity of those communities to metabolize carbon sources. Organic amendments provide a source of different and abundant easily-degradable substrates (Jones et al., 2010). In consequence, in our study, soil microbial activity and biomass were significantly (Table 8.3) increased by the application of the bio-stabilized organic amendment. However, at day 0, no effect of the organic amendment was detected in terms of microbial functional diversity, which could be explained by the lack of enough time for the microorganisms to synthesize the enzymes required for the metabolism of some of the carbon substrates present in Biolog Ecoplates™.

At day 90, unamended non-polluted control soils showed similar, or even slightly lower, SCA (Fig. 8.1C) and NUS (Fig. 8.1D) values, as compared to day 0. The stabilization of environmental conditions during the incubation period, as well as the consumption of labile compounds, can lead to a reduction of microbial functional diversity (Galende et al., 2014c). Similarly to BR and SIR, in our study, the presence of the organic amendment (M and H soils) greatly increased SCA (Fig. 8.1C) and NUS (Fig. 8.1D) values, with highest values being found in H soils. This is consistent with the response of BR and SIR values, as well as with the lower levels of Cr(VI) present at day 90 (Table 8.2). In polluted soils, the presence of the organic amendment prevented the negative effect of Cr(VI) on NUS values (Fig. 8.1D). In turn, SCA values in polluted amended soils suffered a drastic reduction (<50% with respect to controls).

As previously described for soil microbial biomass and activity, the addition of nZVI did not provoke any significant effects on soil microbial functional diversity (Table 8.3), in agreement with Fajardo et al. (2019) who reported a low impact of nZVI on microbial biodiversity and functionality (however, nZVI effects varied depending on soil type).

Our microbial data point out to (i) an stimulatory effect of the presence of the bio-stabilized organic amendment in terms of microbial biomass, microbial activity and the capacity of the soil bacterial heterotrophic cultivable communities to utilize different carbon substrates; and (ii) an inhibitory effect of Cr(VI) on SCA values (this parameter integrates the degradation of all carbon substrates along the incubation time), even at the low concentrations found in M and H soils (Table 8.2). Finally, the application of nZVI did not cause any beneficial or harmful effect on soil parameters.

8.3.4 Soil phytotoxicity

Phytotoxicity bioassays are commonly used as monitoring tools for the assessment of soil health (Quintela-Sabarís et al., 2017). *Lactuca sativa* bioassays are commonly used in metal contaminated soils, and have proven their sensitivity to Cr(VI) contamination (Martí et al., 2007). In our study, at day 0, the highest value of root elongation was recorded, as expected, in unamended non-polluted control soils (Fig. 8.2). The addition of the organic amendment caused a significant reduction of root elongation in control non-polluted soils (this reduction was higher in H soils *vs.* M soils). This reduction of root elongation may be a plant response to an increase of salinity (Hamdi et al., 2006), since the presence of the organic amendment resulted in significantly higher EC values (Table 8.1). In any case, it can also be due to an acclimation response to higher nutrient availability in amended soils, as fertilization can induce a reduction of root elongation (Fageria and Moreira, 2011; Zhao et al., 2014). In unamended soils, at day 0, Cr(VI) caused a marked inhibitory effect on *L. sativa* root elongation (*i.e.*, a 98% reduction, compared to controls). However, at day 0, the presence of the organic amendment (M and H soils) partly alleviated this Cr(VI)-induced inhibitory effect on root elongation, presumably due to Cr(VI) immobilization via its reduction to Cr(III). Organic matter can play a key role in decreasing Cr(VI) phytotoxicity (Bolan et al., 2003).

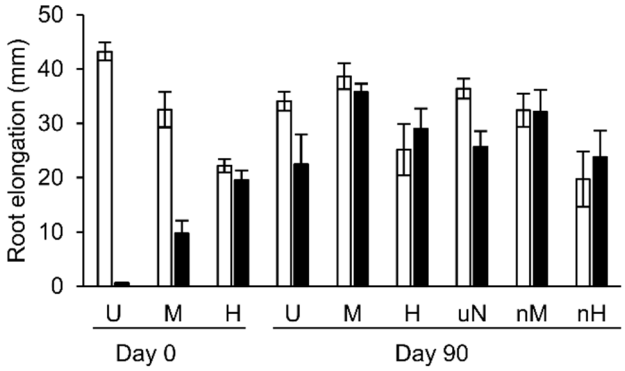

Figure 8.2. Root elongation values from the *Lactuca sativa* bioassay. White bars represent control non-polluted soils. Black bars represent polluted soils. U: unamended; M: medium dose of organic amendment; H: high dose of organic amendment; n: ZVI-treated.

At day 90, a significant reduction of Cr(VI) toxicity was observed in all treatments (Fig. 8.2). However, in unamended soils, root elongation values were still lower in polluted *vs.* non-polluted control soils. Therefore, at day 90, M soils were the most favorable from a phytotoxicity point of view, as this dose appears to alleviate Cr(VI) toxicity while causing less root elongation inhibition due to an excess of organic matter and nutrients. This response is in agreement with Cr(VI) concentrations (Table 8.2): M soils showed the lowest values of Cr(VI).

Regarding the effects of nZVI on root elongation, no beneficial or harmful effects were observed. As abovementioned, Cr(VI) concentrations were not altered by the application of nZVI. Not surprisingly, different studies have reported mixed results regarding the effect of nZVI on plant growth and physiology, since, as previously stated, the impact of nZVI on soil biota and growing plants depends on multiple factors. Thus, a recent study with tomato plants (Brasili et al., 2020) has reported beneficial effects of nZVI treatment on seed germination and seedling development, presumably by increasing water uptake and alleviating Cr(VI) toxicity. Conversely, it has been reported (Mokarram-Kashtiban et al., 2019) that, depending on the dose, nanoparticles can negatively affect plant growth by their aggregation on the root surface and penetration into epidermal cells (Ma et al., 2013). Precisely, nZVI have been reported to negatively affect *L. sativus* germination and root elongation (Rede et al., 2016). In any event, there are not many works that use root elongation bioassays in which seedlings are not in direct contact with the soil but separated by a filter paper, like it is our case. As described above for soil microbial properties, it is likely that the aggregation and inactivation of nZVI had minimized their potential impact (beneficial or deleterious) on root elongation. Organic

matter has been described to modify the aggregation and deposition properties of nanoparticles (Navarro et al., 2008). Nonetheless, in our study, the effect (better, the lack of effect) of nZVI was not dependent on soil OM content.

8.4. Conclusions

This study highlights the importance of soil properties, such as pH, redox potential, OM and carbonate content, for Cr(VI) immobilization via its reduction to Cr(III) and, hence, its toxicity. The presence of the bio-stabilized organic amendment increased oxidizable OM content and decreased soil redox potential, key factors for the reduction of Cr(VI) to Cr(III). The immobilization of Cr(VI) was accompanied by an improvement of soil health, as reflected by the values of soil microbial biomass, activity and functional diversity, as well as *L. sativa* root elongation. Regarding the dose of the organic amendment (medium *vs.* high), no significant differences were observed. Under our experimental conditions, the application of nZVI for nanoremediation purposes did not have any impact on soil properties and, concomitantly, soil health. Then, since the presence of the organic amendment did cause Cr(VI) immobilization and an improvement of soil health, while nZVI had no impact on any of the studied parameters, it was concluded that, unlike the presence of the organic amendment, nanoremediation with nZVI was not a valid option for soils polluted with Cr(VI) under our experimental conditions.

8.5. Conclusiones del capítulo

- La presencia de materia orgánica por encima del 5% en los suelos reduce el potencial redox del suelo, contribuyendo a la transformación de Cr(VI) en Cr(III).

- La reducción de la concentración de Cr(VI), que es la fracción soluble y tóxica, se traduce en una importante mejora de los bioindicadores microbianos y vegetales.

- La aplicación superficial de nanopartículas de hierro cero valente (nZVI) en el suelo no fue efectiva para reducir la toxicidad del Cr (VI), posiblemente debido a su limitada dispersión en el suelo. Determinar la forma de aplicación de este tipo de compuestos es esencial para su efectividad y para minimizar sus posibles efectos secundarios en la biota.

9. DISCUSIÓN GENERAL

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9.1. El problema de la contaminación mixta

9.1.1. ¿Cuál es el problema?

El suelo es, al mismo tiempo, un recurso natural no renovable a escala humana, y un ecosistema complejo y dinámico. Es un sistema que forma parte esencial de los ciclos biogeoquímicos, que presta varios servicios ecosistémicos, y de sus funciones depende la supervivencia de buena parte de las especies de la Tierra, incluido el ser humano. Lamentablemente, la actividad antrópica y/o el manejo de prácticas de gestión no sostenibles ha provocado una importante degradación de los suelos a nivel mundial causadas por varios procesos siendo las principales amenazas en Europa: la erosión y deslizamientos, la disminución de la materia orgánica, la compactación, el sellado, la salinización, la desertificación, el encharcamiento, pérdida de biodiversidad y contaminación (Van-Camp et al., 2004).

Actividades como la minería, la industria, la agricultura o el vertido incontrolado de residuos ha provocado la contaminación del suelo con múltiples sustancias tanto orgánicas como inorgánicas, las cuales aparecen combinadas con frecuencia, dando lugar a un tipo de contaminación mixta del suelo o co-contaminación, que hacen muy difícil no sólo su detección o identificación sino también la evaluación su ecotoxicidad e impacto ambiental (Olaniran et al., 2013). Este tipo de contaminación es muy frecuente y la Agencia de Protección del Medio Ambiente (EPA) de los Estados Unidos indicó que el 40% de los vertederos peligrosos presenta contaminación mixta (USGAO, 2010). El problema de la contaminación mixta se agrava ya que tiene una gran distribución geográfica y comprende una amplia gama de posibles mezclas de contaminantes orgánicos e inorgánicos (Agnello et al., 2016; Arienzo et al., 2013; Chirakkara et al., 2016; Polti et al., 2014).

La contaminación del suelo provoca daños ambientales, sociales y económicos. Por un lado, causa un severo daño al ecosistema edáfico, y puede llegar a otros medios, como el aire o el agua, y con posterioridad a los organismos, incorporándose a cadena trófica. Finalmente, la salud humana puede verse afectada, ya sea por contacto directo y/o a través del consumo de productos alimentarios contaminados. La contaminación limita o imposibilita los diferentes usos del suelo, lo cual conlleva un efecto negativo sobre el

desarrollo social de la comunidad y genera pérdidas económicas tanto directas como indirectas. Por tanto, la remediación de los suelos contaminados es una cuestión ambiental, social y económica que debe ser realizada con una prioridad alta.

Sin embargo, la remediación de la contaminación mixta en los suelos presenta complicaciones técnicas adicionales a los desafíos que presentan la descontaminación de los contaminantes por separado. La toxicidad de mezclas de contaminantes orgánicos e inorgánicos es mayor que la de dichos contaminantes individualmente (Khudur et al., 2019), y, de hecho, se ha observado un efecto sinérgico en los daños causados sobre la biota (Lin et al., 2006). Propiedades tan importantes que determinan la ecotoxicidad, como son la biodisponibilidad y movilidad de metales y contaminantes orgánicos, pueden ser verse alteradas por la presencia conjunta de ambos tipos de contaminantes (Batty and Dolan, 2013; Olaniran et al., 2013), haciendo de la contaminación mixta un problema muy complejo e impredecible. Por otra parte, la remediación de cada tipo de contaminante (orgánico o inorgánico) requiere soluciones técnicas diferentes. Por un lado, los compuestos orgánicos pueden ser transformados, degradados incluso hasta la mineralización, mientras que los inorgánicos no pueden ser degradados y deben ser extraídos del suelo, aunque en algunos casos su cambio en el estado redox permite su inertización y/o cambios en sus propiedades de movilidad y toxicidad. Esto hace muy difícil la descontaminación simultánea de ambos tipos de contaminantes y explica los escasos estudios y tecnologías desarrolladas para este tipo de contaminación, ya que cada tipo de contaminante requiere el uso de tecnologías de remediación específicas que interfieren entre sí e incluso son incompatibles.

9.1.2. ¿Qué soluciones existen y qué limitaciones encuentran con la contaminación mixta?

La contaminación de un sistema tan complejo como el suelo es un problema que lleva estudiándose décadas. Sin embargo, todavía no se ha encontrado una solución completamente satisfactoria para el mismo, ni siquiera para suelos con un solo tipo de contaminante, mucho menos para suelos con contaminación mixta.

Por su efectividad y rapidez en la eliminación de los contaminantes, Tradicionalmente, las tecnologías fisicoquímicas (TFQ) han sido las empleadas en la remediación de los suelos contaminados. Estas técnicas se enfocan principalmente en eliminar o inertizar los contaminantes, ya sea evaporándolos, incinerándolos (en el caso

de los contaminantes orgánicos), extrayéndolos mediante aireación, lavados químicos, conteniéndolos mediante técnicas como el sellado, la vitrificación, etc., o directamente excavando el suelo para su traslado a un vertedero Además de su alto coste económico, desde el punto de vista técnico, algunas de estas tecnologías son de difícil aplicación *in situ* y/o a gran escala en campo, limitando su aplicación generalizada. En los casos de contaminación mixta, las TFQ de remediación encuentran problemas adicionales, ya que las técnicas que se emplean para la eliminación de un tipo de contaminante pueden ver reducida su efectividad por la presencia del otro. Por ejemplo, la efectividad de los lavados químicos de contaminantes inorgánicos puede verse reducida por la presencia de contaminantes orgánicos viscosos, ya que altera la hidrodinámica del suelo (Poly y Sreedeep, 2011).

En los últimos años la aplicación de nanopartículas de distintos metales, como el hierro cero valente, ha captado la atención científica como una tecnología fisicoquímica eficaz tanto para la eliminación de los contaminantes inorgánicos, como orgánicos. Es necesario realizar aún estudios del efecto e impacto de esta tecnología como herramienta eficaz en la remediación de la contaminación del suelo, por las incertidumbres acumuladas hasta la fecha sobre su efectividad y potenciales efectos ecotóxicos (Anza et al., 2019; San Román et al., 2013).

Pese a la gran efectividad de las TFQ en la eliminación de los contaminantes, recientemente se van imponiendo restricciones a su uso indiscriminado en la remediación de los suelos debido al impacto en la salud de suelo. Como se ha comentado anteriormente, el suelo es un sistema complejo y vivo, y la interacción entre sus componentes físicos, químicos y biológicos es esencial para el desarrollo de sus funciones y servicios ecosistémicos. Gran parte de las técnicas fisicoquímicas, si bien pueden eliminar el contaminante, no sólo no recuperan, sino que incluso alteran o destruyen la estructura, la composición química, y biota del suelo, modificando de forma irreversible algunas las propiedades físicas, químicas y biológicas cosustanciales al suelo. En definitiva, no recuperan la salud del suelo, que podría definirse como la capacidad del suelo para funcionar como un sistema vivo para sustentar la productividad biológica, promover la calidad ambiental y mantener la salud de plantas y animales (Doran y Zeiss, 2000). La concentración de la fracción biodisponible de los contaminantes, que es la determinante para la toxicidad, debe ser un indicador esencial a tener en cuenta, así como

los efectos tóxicos sobre la biota mediante la evaluación de bioindicadores en organismos representativos de los taxones relevantes del suelo.

Como una alternativa más sostenible a las TFQ, hace algunas décadas que se aplican tecnologías menos intrusivas que tienen en cuanta los procesos y funciones del suelo, denominadas Opciones Suaves de Remediación (GRO: Gentle Remediation Options), que consisten en el uso de organismos (fitorremediación, biorremediación, vermirremediación, etc.) y agentes que mejoran la propiedades físicas, químicas o biológicas del suelo (enmiendas orgánicas o inorgánicas, biocarbones, etc.), contribuyendo no solo a la disminución de los contaminantes y su toxicidad, sino también a promover la recuperación de la salud de suelo con criterios de sostenibilidad, recuperando su funcionalidad y servicios ecosistémicos.

La fitorremediación, tiene cierta eficacia con la eliminación de los contaminantes orgánicos, generalmente se utiliza para contaminantes metálicos, aprovechando la capacidad de las plantas ya sea para extraerlos a su parte aérea (fitoextracción) o para inmovilizarlos en su rizosfera (fitoestabilización) (Ali et al., 2013). Recientemente se ha propuesto la fitogestión como una estrategia de remediación de suelos contaminados que combina los beneficios ambientales de la fitorremediación con la utilización de especies vegetales que proporcionen un beneficio económico (biomasa, industrial, etc.). Así, el tiempo de remediación deja de ser una limitación en la aplicación de esta GRO, debido al retorno económico, la integración paisajística y el beneficio social durante la remediación del suelo contaminado (Evangelou et al., 2015). La biorremediación es de utilidad fundamentalmente para contaminantes orgánicos, ya que pueden ser degradados por las comunidades microbianas (Megharaj et al., 2011), aunque también puede ser efectiva para la inertización de algunos metales (Verma and Kuila, 2019). La vermirremediación se emplea fundamentalmente con compuestos orgánicos, ya que las lombrices en el suelo favorecen la estructura del suelo promoviendo su degradación (Shi et al., 2020).

Una limitación en el uso de las GRO es su menor efectividad y lentitud al compararlas con las TFQ. Sin embargo, la gestión sostenible de un recurso como son los suelos, aunque estén contaminados, requiere estrategias de remediación como las GRO que son alternativas baratas, ambientalmente amigables y pueden resultar eficaces en suelos con contaminación mixta. No obstante, aunque cada una de las GRO tiene sus propias ventajas, aplicadas de forma aislada pueden encontrar dificultades para hacer frente a la contaminación mixta, como que los organismos sean resistentes sólo a un tipo

de contaminante, efectos sinérgicos de la toxicidad de la mezcla o por interacciones entre los propios contaminantes (Liu et al., 2017). Existen todavía pocos estudios que hayan investigado las posibles aplicaciones de las GRO en suelos con mezclas de contaminantes orgánicos e inorgánicos, y es necesario investigar sus bondades, sus limitaciones y las compatibilidades entre las GRO y con otras TFQ como nuevas estrategias de gestión sostenible para la remediación de los suelos.

9.1.3. Oportunidades: Identificando la solución

Para afrontar el complejo desafío de la contaminación mixta del suelo es necesario comprobar cuál es la efectividad de las distintas tecnologías de remediación aplicadas a cada tipo de escenario, ya que la elección de la/s estrategia/s más adecuada/s dependerá de los contaminantes tipo, concentración, y otros factores climáticos y del propio suelo que se pretende descontaminar. Puesto que la mayoría de estrategias están pensadas para la eliminación de un contaminante, la aplicación conjunta de dos tecnologías complementarias podría resultar eficaz para la remediación de suelos con contaminación mixta. Ya existen algunos estudios en los que la combinación de fitorremediación y biorremediación ha sido eficaz en suelos con metales y compuestos orgánicos (Agnello et al., 2016; Ontañon et al., 2014), pero, dada la multiplicidad de posibles combinaciones entre contaminantes, condiciones edáficas y ambientales, para la mayoría de los casos hay un desconocimiento sobre los posibles antagonismos y sinergias entre tecnologías, así como su complementariedad y efectividad para su aplicación *in situ*. Además, estas técnicas biológicas pueden y deben combinarse también con tratamientos como la adición de enmiendas (orgánicas, inorgánicas) al suelo y/o con la aplicación de nanopartículas, promoviendo la mejora de las condiciones de suelo, reduciendo la ecotoxicidad, y aumentando la efectividad de las tecnologías biológicas.

Para que una estrategia de remediación se convierta en una estrategia rentable y efectiva debe contribuir a eliminar el contaminante total o biodisponible, reduciendo la ecotoxicidad y permitir la recuperación de la salud de suelo, además de ser económicamente viable y compatible con una gestión sostenible del terreno que aporte beneficios ambientales, sociales y económicos. Como ya se ha indicado, éstos serían los objetivos de la fitogestión, que aboga por el uso de las GRO compatibilizado con la obtención de bienes y servicios ambientales y socioeconómicos, como puede ser, por ejemplo, la mitigación de los gases de efecto invernadero, la provisión de espacios verdes,

mejora del paisaje, producción de materias, o energías renovables como biocombustibles (Cundy et al., 2016). Los cultivos energéticos han recibido críticas por su competencia con los cultivos alimentarios, pero la producción de biocombustibles empleando emplazamientos degradados/contaminados brindaría beneficios socioeconómicos evitando el conflicto con la producción de alimentos.

Partiendo de este escenario, esta tesis se ha llevado a cabo con el objetivo de evaluar la efectividad de diferentes estrategias (GRO, TFQ) evaluando sus posibles sinergias y/o antagonismos que permitan identificar su aplicabilidad *in situ* como una estrategia para la remediación de suelos con contaminación mixta (contaminantes orgánicos e inorgánicos). Como modelo de aplicabilidad en esta tesis se han investigado dos tipos de contaminación mixta, combinando dos contaminantes orgánicos (gasóleo o lindano) con diferentes metales (Zn, Cu, Cd, o Cr). Puesto que los dos tipos de contaminantes del suelo más abundantes en suelos europeos son los metales y los aceites minerales (Van Liedekerke et al., 2014), el primer tipo de contaminación mixta, empleada en el Capítulo 4, consistió en tres metales relativamente frecuentes en suelos contaminados, como el Zn, el Cu y el Cd, y un combustible (gasóleo comercial). En la época actual, en la que se prevé un agotamiento de los combustibles fósiles y se persigue la transición energética a fuentes de energía renovables como los biocombustibles, y será interesante determinar su impacto y degradabilidad en suelos en comparación con sus homólogos de origen fósil. Para ello, en el Capítulo 5 se ha realizado un estudio de contaminación mixta para determinar el impacto de tipos de gasóleo (con distinta proporción de biodiesel) y metales (Zn, Cu, y Cd) y su degradabilidad en el suelo.

El otro tipo de contaminación mixta empleada en la tesis fue cromo (VI) y lindano, una mezcla menos frecuente, pero con efectos toxicológicos muy graves (Aparicio et al., 2018b; Arienzo et al., 2013). Al contrario que los metales Zn, Cu y el Cd que en disolución se encuentran en forma catiónica, el cromo se encuentra en forma de anión cuando está en su forma hexavalente, mientras que en su forma trivalente puede estar tanto en forma de anión como de catión, siendo esta última la más frecuente (Zayed y Terry, 2003). Su solubilidad y toxicidad dependen en gran medida de su especiación, siendo la forma hexavalente, Cr(VI), mucho más tóxica que la trivalente, Cr(III). El lindano, por su parte, se trata de un pesticida organoclorado, recalcitrante, de toxicidad y carcinogenicidad muy superiores a la del gasóleo. Por tanto, tanto por su composición y

estructura química como por su potencial toxicidad) esta mezcla presentaría una mayor ecotoxicidad y, presumiblemente, una mayor dificultad en su eliminación.

El suelo empleado en esta tesis proviene de un área periurbana degradada en la zona industrial de Júndiz, y por tanto es un suelo con muy bajo contenido en materia orgánica y nutrientes (algo frecuente en los suelos degradados y contaminados). Aunque el suelo recogido con el que hemos realizado el estudio no estaba contaminado, y es un buen modelo de estudio porque presenta las características típicas de los suelos periurbanos de la ciudad de Vitoria-Gasteiz con suelos degradados, pobres en nutrientes, con alto contenido en carbonatos, es frecuente que estas áreas periurbanas hayan sufrido vertidos de origen industrial con contaminantes orgánicos e inorgánicos. Por ello, en los estudios realizados fue necesario añadir una enmienda y los componentes de la contaminación mixta que se ha indicado.

Dado el bajo nivel de nutrientes y materia orgánica para nuestros estudios se optó por incorporar a modo de pretratamiento una enmienda orgánica a partir de material bioestabilizado, procedente del reciclado de la fracción orgánica de los residuos sólidos urbanos. El uso de este tipo de enmiendas es una alternativa de bajo coste económico y una forma de dar uso a un subproducto, lo cual se adecúa al paradigma de la Economía Circular. La aplicación de una enmienda de materia orgánica puede aumentar la fertilidad del suelo, incrementando su contenido en materia orgánica y nutrientes, lo cual facilita el desarrollo de cultivos y la biota del suelo en general (Míguez et al., 2020). Además del aporte de nutrientes, las enmiendas orgánicas pueden ser un tratamiento efectivo en sí mismo para la contaminación mixta, reduciendo la disponibilidad de metales (Galende et al., 2014a) y estimulando la degradación de compuestos orgánicos (Kästner and Miltner, 2016). Por tanto, la adición de enmiendas podría ser una estrategia adecuada para mejorar las condiciones del suelo, favoreciendo una mayor efectividad de las GRO o TFQ que se apliquen después. Por su doble capacidad para inmovilizar determinados metales y para degradar diversos compuestos orgánicos (Xue et al., 2018), las nanopartículas de hierro cero valente se seleccionaron como otra posible tecnología para los suelos con contaminación mixta. Concretamente, se ha observado su eficacia reduciendo Cr(VI) a su forma trivalente (Di Palma et al., 2015) y degradando lindano (Anza et al., 2019). Su aplicación podría reducir la concentración y/o toxicidad de los contaminantes, mejorando la salud de suelo, y facilitando también la acción de otras estrategias de remediación aplicadas *a posteriori*. Además, es necesario acotar las incertidumbres sobre sus

potenciales efectos adversos y su aplicabilidad y efectividad con las GRO, La biorremediación es sin duda una de las tecnologías de remediación biológicas más eficaces para la descontaminación de contaminantes orgánicos. Varias son las estrategias que se pueden evaluar. Por un lado, es necesario evaluar los fenómenos de atenuación natural, para conocer la respuesta de las comunidades microbianas nativas del suelo ante la contaminación, y hasta dónde llega su capacidad natural de remediación. La bioestimulación mediante enmiendas como las previamente comentadas podría ser una opción para aumentar la degradación de contaminantes orgánicos en las adversas condiciones para los microorganismos causadas por la contaminación mixta. Como se ha comentado, estimulan a la microbiota, pudiendo favorecer la degradación de contaminantes (Kästner and Miltner, 2016), además del efecto beneficioso al reducir la toxicidad de los metales. Por otro lado, la bioaumentación del suelo inoculando bacterias con capacidad para degradar/transformar los contaminantes es una estrategia prometedora, pero que encuentra sus principales limitaciones en la capacidad de adaptación y supervivencia del inóculo en el suelo. El consorcio de actinobacterias seleccionado en esta tesis ha demostrado en estudios anteriores su efectividad reduciendo las concentraciones de cromo (VI) y lindano simultáneamente (Aparicio et al., 2018a; Polti et al., 2014).

Para poder llevar a cabo un manejo sostenible del terreno contaminado a través de la fitogestión, es necesario emplear una especie vegetal que, además de ser de interés agronómico, sea tolerante a la contaminación mixta. Entre las Brassicáceas, la colza (*Brassica napus*) es un cultivo energético cuyo aceite ha sido utilizado para la producción de biodiesel y es tolerante a distintos tipos de contaminantes. Concretamente, *B. napus* ha sido utilizada para la fitorremediación de suelos contaminados con metales como el Zn y el Cd (Cojocaru et al., 2016; Dhiman et al., 2016), e incluso estudios de remediación de suelos con contaminación mixta de cromo y un contaminante orgánico (Ontañon et al., 2014). Además, la salud de suelos similares a los utilizados en este estudio mejoró tras el cultivo de colza, respondiendo muy bien a la aplicación de enmienda orgánica (Míguez et al., 2020). Por las características anteriores *B. napus*, fue seleccionada como especie fitorremediadora para los experimentos de esta tesis.

La utilización de lombrices para compostaje de residuos y mejorar las condiciones del suelo ha ampliamente estudiado (Lim et al., 2016). Sin embargo, su utilización en procesos de remediación de suelos contaminados es poco conocido, aunque la

vermirremediación es una tecnología que puede contribuir a mejorar las condiciones edáficas que potencien la eliminación de contaminantes por plantas (Lemtiri et al., 2016) o microorganismos (Martinkosky et al., 2017). Las lombrices pueden modificar la biodisponibilidad de los contaminantes y favorecer la degradación de contaminantes orgánicos (Elyamine et al., 2018; Hickman and Reid, 2008b). En este estudio se ha seleccionado la especie *Eisenia fetida*, especie de fácil cultivo y manejo y que ya ha probado su eficacia en la remediación tanto de hidrocarburos (Chachina et al., 2016) como de metales (Elyamine et al., 2018). Además, se ha observado que la combinación de *E. fetida* y una brasicácea (*Brassica juncea*) puede incrementar la fitoextracción de metales como el cadmio (Kaur et al., 2018).

9.1.4. Indicadores para seguir el proceso remediador

La monitorización de la descontaminación y la recuperación de la salud del suelo es una fase esencial que debe contemplarse en cualquier proceso remediador. La concentración total de contaminantes es el indicador más utilizado en la legislación para determinar el análisis de riesgo ambiental y la declaración de suelos contaminados (Antoniadis et al., 2019). Sin embargo, existen otros indicadores como la fracción biodisponible, que está directamente relacionada con la ecotoxicidad y es un aspecto crucial que debe ser valorado, ya que es la que determina su potencial movilidad y toxicidad. Aunque no hay un consenso que determine la mejor metodología para la determinación de la disponibilidad, siendo lo más habitual la determinación de la fracción y/o la fracción extraída con una amplia gama de extractantes químicos (Madejón et al., 2006; Menzies et al., 2007; Vázquez et al., 2008).

Para poder evaluar adecuadamente la salud de suelo, además de los indicadores químicos indicados (concentración total y disponible), es necesario conocer el impacto de los contaminantes y de los tratamientos de remediación en la biota, atendiendo a los diferentes taxones relevantes del suelo. En esta tesis se ha seleccionado la evaluación del estado de las comunidades microbianas del suelo a través de parámetros tales como la actividad, biomasa y diversidad funcional de los microorganismos, los cuales, aunque no representan a la totalidad de las comunidades microbianas del suelo, se han empleado de forma eficaz como bioindicadores en otros estudios de remediación (Galende et al., 2014c). Asimismo, se han seleccionado los bioensayos de toxicidad con organismos modelo de invertebrados (*Eisenia fetida*) (Irizar et al., 2015a) y bioensayos de

germinación y elongación radical con diferentes especies de plantas, como *Cucumis sativus* (Lacalle et al., 2018a), *Lactuca sativa* (Płaza et al., 2005) y *Raphanus sativus* (Aparicio et al., 2019). Si bien los organismos modelo a menudo no son especies representativas del ecosistema, sí permiten la evaluar y comparar la ecotoxicidad entre unos suelos y otros de manera estandarizada. Asimismo, la evaluación del estado de salud (peso, supervivencia, procesos fisiológicos, etc) de los propios organismos remediadores aplicados en el suelo es un indicador complementario de la efectividad de los tratamientos aplicados. En el caso de las plantas, la composición de pigmentos fotosintéticos y tocoferoles, y la eficiencia fotoquímica (Fv/Fm), son indicadores de la salud de la planta.

La combinación de indicadores fisicoquímicos, como la concentración total y (bio)disponible de los contaminantes, e indicadores biológicos en los taxones relevantes de la biota del suelo (microorganismos, plantas y lombrices) han sido seleccionado en esta tesis para la evaluación y monitorización de la salud de suelo y, por tanto, para determinar la eficacia de las técnicas de remediación empleadas.

9.2. Las soluciones

9.2.1. Bioestimulación mediante enmiendas orgánicas

Con frecuencia, los suelos contaminados, además de la presencia de los contaminantes, muestran otros problemas adicionales que alteran sus características físicas y químicas, las cuales, incluso en ausencia de los contaminantes, pueden impedir su normal funcionalidad y el suministro de servicios ecosistémicos (Epelde et al., 2009b; Pereira et al., 2018). Esta degradación adicional, muy común en los suelos contaminados, es un obstáculo que debe ser convenientemente evaluado y gestionado para poder acometer con éxito cualquier proceso de remediación. Entre otras, las características físico-químicas que deben ser evaluadas en suelos contaminados son su textura y estructura, su composición nutricional, el contenido en materia orgánica, el pH, la capacidad de intercambio catiónico, la conductividad hidráulica y la retención hídrica. Entre las estrategias sostenibles para la remediación de suelos contaminados se encuentran la adición de enmiendas inorgánicas y/u orgánicas, que corrigen deficiencias y modifican características esenciales para la funcionalidad de los suelos. La adición de materia orgánica (MO) a los suelos contaminados puede traer grandes beneficios para la biota edáfica a través de procesos de bioestimulación, ya que es una fuente de nutrientes,

incrementa la capacidad de intercambio catiónico, mejora la estructura y la retención hídrica del suelo (Garcia et al., 2017). Además, la adición de MO puede tener un efecto sobre los contaminantes del suelo, ya que promueve su inertización y/o degradación, que se traduce, en definitiva, en la disminución de sus concentraciones totales y/o disponibles, estimulando el mejor desarrollo de la biota del suelo y favoreciendo la mejora de la salud del suelo.

En esta tesis se ha estudiado la efectividad del material bioestabilizado como enmienda orgánica para su uso en suelos con contaminación mixta. Esta enmienda es un material obtenido del reciclaje de residuos orgánicos de la ciudad de Vitoria-Gasteiz. Actualmente, este material no tiene un uso definido y se almacena en un vertedero, por lo que se está tratando de encontrar alguna utilidad al mismo. Nuestro estudio evalúa la idoneidad de este residuo orgánico para su uso en procesos de bio/fitorremediación de suelos con bajos niveles de materia orgánica, como son los suelos de la zona periurbana de Vitoria-Gasteiz objeto de estudio. Sin embargo, el uso de este tipo de material puede entrañar algunos potenciales riesgos que deben ser evaluados antes de su aplicación de forma generalizada. Al proceder de residuos orgánicos urbanos, la calidad y los componentes de esta enmienda pueden ser variables en función de la estacionalidad y la zona de recogida de basuras. Por tanto, es necesario realizar un pretratamiento de cribado que descarte la ausencia de material particulado artificial, así como un análisis de los materiales aportados al suelo antes de su aplicación, de cara a garantizar cierta homogeneidad y la ausencia de contaminantes químicos y/o biológicos. En nuestro caso, el material bioestabilizado contenía concentraciones moderadas de Zn y Cu, que incrementaron ligeramente la concentración total de ambos metales en los suelos enmendados, pero su efecto en las concentraciones biodisponibles no fue significativo (Capítulos 4 y 5), ya que los metales fueron rápidamente inmovilizados en el suelo a causa de su alto contenido en carbonatos y su pH moderadamente alcalino (Adriano, 2001). La aplicación del material bioestabilizado (Capítulos 4-8) incrementa el contenido en materia orgánica total y carbono orgánico oxidable en el suelo, lo cual se traduce en efectos beneficiosos en la reducción de la biodisponibilidad de metales y la mejora de las propiedades físico-químicas y biológicas del suelo. A este efecto también contribuyeron otros componentes inorgánicos del suelo, tales como el alto contenido en carbonatos de los suelos. De hecho, la baja disponibilidad de los metales en los suelos donde se añadieron Zn, Cd y Cu (capítulos 4y 5) se debe precisamente al alto contenido en

carbonatos y al pH que caracterizan estos suelos y provocan la citada inertización, justificando su baja toxicidad para los microorganismos y las plantas. De acuerdo con nuestros resultados, la aplicación de enmiendas inorgánicas con alto contenido en carbonatos sería recomendable en la remediación de suelos contaminados con metales (Zn, Cd, Cu y Cr) y bajos contenidos de carbonatos y pH ácido. En estos mismos suelos, en ausencia de la enmienda orgánica, la concentración de Cr(VI) se mantiene en niveles altos (capítulos 6-8), ya que las condiciones no son lo suficientemente reductoras y el pH elevado no favorece la adsorción del Cr(VI) (Adriano, 2001). Sin embargo, la aplicación de la enmienda orgánica tiene un efecto drástico en la reducción de los niveles de Cr(VI). Esto se debe a que la materia orgánica causó el descenso del potencial redox del suelo y, en consecuencia, la reducción de Cr(VI) a Cr(III) (Xia et al., 2019), lo cual coincide con las observaciones de otros autores (Antoniadis et al., 2018; Choppala et al., 2018). El Cr(III), en las condiciones alcalinas propiciadas por los carbonatos, sería rápidamente inmovilizado (Zayed and Terry, 2003). Las enmiendas inorgánicas con alto contenido en carbonatos y enmiendas orgánicas podrían aplicarse de forma complementaria para suelos contaminados con metales (Zn, Cu, Cd, Cr).

La disminución de la biodisponibilidad de los metales debido a componentes del suelo (carbonatos, materia orgánica), si bien reduce la ecotoxicidad de los suelos, puede suponer una limitación importante para estrategias como la fitoextracción, donde se busca la disminución de la concentración total de metales.

Los materiales orgánicos presentes en el material bioestabilizado fueron fácilmente degradados por las comunidades microbianas edáficas, aumentando, en términos generales, la biomasa y actividad microbiana en todos los tratamientos en los que se aplicó, aunque esto no se tradujo en un aumento significativo de la diversidad funcional de dichas comunidades. El efecto de la materia orgánica en la degradación de los contaminantes orgánicos no fue tan pronunciado como en la inmovilización de metales. Existe cierta controversia sobre el efecto de la MO en la degradación de contaminantes. Se ha indicado que la estimulación de la microbiota mediante el aporte de nutrientes y la adsorción de los compuestos tóxicos puede aumentar la degradación de diversos contaminantes orgánicos (Balseiro-Romero et al., 2016; Megharaj et al., 2011). Si bien hemos comprobado (Capítulos 4 y 5) un aumento en las tasas de degradación de hidrocarburos, con el tiempo este efecto se ve amortiguado y la concentración recalcitrante final, compuesta mayoritariamente por los hidrocarburos de cadena más

larga, fue similar. Incluso la degradación de lindano (Capítulos 6 y 7) se ve perjudicada por un mayor contenido en materia orgánica, lo cual podría deberse a que los microorganismos limitan la degradación de lindano cuando hay otras fuentes alternativas de carbono en el suelo y/o a una reducción de la disponibilidad del lindano debido a su adsorción a la materia orgánica (Hofman et al., 2014).

Factores como los propios componentes del suelo, la composición de las comunidades microbianas del suelo, la propia composición química de la enmienda o la naturaleza de los contaminantes orgánicos pueden influir en la degradación de estos contaminantes. La bioestimulación mediante la aplicación de enmiendas orgánicas es una estrategia recomendable para la remediación de suelos con contaminación mixta, ya que promueven la inertización de metales, reduciendo la toxicidad para la biota y favoreciendo la degradación de contaminantes orgánicos.

9.2.2. Aplicación de estrategias biológicas

Como se observó en los Capítulos 4 y 5, entre los hidrocarburos presentes en los combustibles objeto de estudio, los *n*-alcanos de origen fósil fueron degradados por los microorganismos del suelo en función de la longitud de cadena, siendo las cadenas cortas más fácilmente degradables, mientras que la fracción más pesada fue más recalcitrante. Los metil ésteres de ácidos grasos (FAME: Fatty Acid Methyl Esther) que conforman el biodiesel, en cambio, sí pueden ser completamente degradados mediante procesos de atenuación natural, gracias a su metabolización más sencilla (Thomas et al., 2017), e incluso favorecer la degradación de los n-alcanos por co-metabolismo (Pasqualino et al., 2006), tal y como se observó en esta tesis. Una mejor degradación, combinada con un menor impacto en la salud de la biota edáfica, haría de los combustibles con mayores contenidos en biodiesel una opción más recomendable desde el punto de vista ambiental. Las comunidades microbianas indígenas del suelo también pueden degradar contaminantes orgánicos organoclorados como el lindano (Capítulos 6 y 7). Algunos autores (Balseiro-Romero et al., 2018; Langenhoff et al., 2002) han señalado que la capacidad natural de los microorganismos nativos del suelo para degradar estos contaminantes, aunque existe, es insuficiente para eliminarlos en su totalidad. También nuestros estudios apuntan en esta dirección, ya que la atenuación natural no es suficiente para eliminar por completo los *n*-alcanos ni el lindano, por lo que es necesario implementar estrategias de remediación más directas.

Las GRO son estrategias prometedoras para la recuperación sostenible de emplazamientos con contaminación mixta, por lo que en esta tesis se ha estudiado la efectividad individual y combinada de diversas estrategias biológicas de remediación. Para poder llevar a cabo esta recuperación mediante un proceso de fitogestión, se seleccionó la colza como especie fitorremediadora. Esta especie ha mostrado cierta tolerancia a la contaminación mixta de nuestro estudio, pero su desarrollo se ha visto dificultado en presencia de mezclas de contaminantes (Capítulos 4, 6 y 7), una afección que se ha visto acrecentada por la deficiencia de nutrientes del suelo contaminado. Por ello, la aplicación previa de enmiendas que reduzcan la ecotoxicidad y aporten materia orgánica y nutrientes al suelo es imprescindible para llevar a cabo una adecuada fitogestión con este cultivo en un suelo con contaminación mixta como el de nuestro estudio.

Aunque otros autores han señalado a *B. napus* como una especie adecuada para la fitoextracción de metales como Zn, Cu, Cd y Cr (Brunetti et al., 2011; Cojocaru et al., 2016), en esta tesis la fitoextracción de estos metales fue baja y no afectó a la concentración total de metales del suelo. Esto en parte se debe a que, al ser sensible a los metales, cuando la concentración biodisponible de éstos es lo bastante alta como para entrar en los tejidos de la planta, la fitotoxicidad que provoca impide el desarrollo de las plantas; por el contrario, cuando la biodisponibilidad es baja, la planta desarrolla biomasa, pero la concentración de metales en sus tejidos es insuficiente. Esta baja disponibilidad debido a los componentes del suelo fue patente para el Zn, Cu y Cd en los suelos, como se ha discutido en el apartado 9.2.1. Algunas especies de plantas, como las plantas hiperacumuladoras, disponen de mecanismos fisiológicos para acumular metales incluso con bajos niveles de biodisponibilidad, pero estas especies suelen desarrollar poca biomasa. La incapacidad de producir grandes cantidades de biomasa en presencia de altas concentraciones de metales en el suelo es una de las más importantes limitaciones de la fitoextracción, que se ha intentado solucionar, con éxito limitado, mediante ingeniería genética, prácticas agronómicas y/o la aplicación de quelantes (Sheoran et al., 2016). La estimulación de la fitorremediación, en general, y de la fitoextracción, en particular, con bacterias promotoras del crecimiento vegetal (Wood et al., 2016) o con lombrices (Kaur et al., 2018) también se han propuesto para la mejora de los procesos de remediación. En cualquier caso, para que la fitoextracción sea efectiva es necesario que la disponibilidad de metales sea moderada o alta, como en las tecnologías de fitoextracción asistida con

agentes extractantes, que incrementan la concentración de los metales en la disolución del suelo, y finalmente favorecen su acumulación en las partes aéreas de la planta. Esta efectividad no está exenta de riesgo por la toxicidad para la biota y la posibilidad de lixiviación y contaminación de otros compartimentos ambientales. Por otra parte, en suelos donde el nivel de metales es muy elevado, el tiempo necesario para la fitoextracción de metales sería largo, lo cual limita su aplicabilidad en estos suelos para disminuir sus niveles a los valores de referencia para admitirlos como suelos no contaminados. Lo cierto es que apenas existen aplicaciones comerciales de la fitoextracción, o incluso ensayos de campo con éxito (Robinson et al., 2015), lo cual genera incertidumbres sobre la aplicabilidad real de estas tecnologías, al menos de la forma en la que se ha llevado a cabo hasta el momento. Asimismo, estrategias que funcionan a escala microcosmos suelen fracasar en condiciones de campo (Brunetti et al., 2011). La faceta positiva de una baja fitoextracción sería la limitación de riesgo de transmisión de los metales a la cadena trófica, minimizando el riesgo en la fitogestión de emplazamientos con suelos contaminados. Como una alternativa a la fitoextracción, la fitoestabilización persigue la contención de los metales en la rizosfera de las plantas, ya sea secuestrándolos en su raíz o alterando las condiciones del suelo rizosférico de tal forma que se reduzca su disponibilidad (Bolan et al., 2011). Así, la fitoestabilización disminuye la movilidad, la biodisponibilidad y, consecuentemente, la ecotoxicidad de los metales, al tiempo que se provee al suelo de los beneficios de una cubierta vegetal, tales como la protección frente a la erosión, la estimulación de las comunidades biológicas edáficas, la fijación de carbono, etc. En esta tesis, la especie *B. napus* no incrementó ni redujo la disponibilidad de los metales (Zn, Cu, Cd, Cr), presumiblemente por su ya de por sí baja concentración biodisponible debido a su interacción con la materia orgánica, y los componentes y factores edáficos, como el contenido en carbonatos. Por tanto, bajo nuestras condiciones experimentales la fitoestabilización es una tecnología que tiene poco impacto en el suelo, cuando se aplica en combinación con enmiendas orgánicas y/o inorgánicas, sin perjuicio de que bajo otras circunstancias y/o suelos diferentes sus efectos pudieran variar.

Con respecto a los otros contaminantes presentes en la contaminación mixta, los contaminantes orgánicos, la utilización de la fitorremediación para estimular la degradación de estos compuestos es un tema de creciente interés. Las plantas podrían intervenir en la remediación de contaminantes orgánicos de varias formas: i)

fitodegradación, degradando directamente los compuestos orgánicos dentro de sus tejidos; ii) rizodegradación, estimulando a las comunidades microbianas del suelo mediante exudados radicales; iii) fitovolatilización, absorbiendo y volatilizando los compuestos orgánicos con la corriente transpiratoria; iv) fitoextracción, acumulándolos en sus tejidos (Abdullah et al., 2020). Este incremento en la degradación de hidrocarburos en presencia de *B. napus* no se observó en los suelos contaminados con gasóleo y metales (Capítulo 4). Sin embargo *B. napus* sí provocó una mayor degradación del lindano en los suelos contaminados con cromo y lindano (Capítulo 6). Las plantas podrían aumentar la degradación de lindano mediante su interacción con la microbiota de su rizosfera, aunque se ha indicado que este efecto puede variar en función de la especie vegetal (Feng et al., 2020). La combinación del cultivo de colza y la enmienda orgánica causó un aumento sinérgico en la diversidad funcional microbiana, indicando que en presencia de *B. napus* y de la enmienda orgánica aportada al suelo cambia la composición de las comunidades bacterianas para la utilización de más sustratos, y este efecto promueve la degradación de lindano. Otros autores también han asociado una mayor diversidad microbiana con la degradación de compuestos orgánicos (Segura and Ramos, 2013).

Es destacable que un contaminante tan tóxico y recalcitrante como el lindano, compuesto organoclorado, sea degradado por las bacterias nativas del suelo en presencia de un cultivo de interés industrial como la colza, ya que abre la posibilidad de su aplicabilidad en proyectos de fitogestión. El binomio enmienda-cultivo es esencial para la recuperación sostenible de suelos degradados, inertizando los contaminantes inorgánicos, favoreciendo la degradación de los orgánicos, y generando beneficios ambientales, sociales y económicos derivados de la fitogestión. No obstante, la efectividad de esta estrategia se puede mejorar mediante su combinación con otras biotecnologías, como puede ser la vermirremediación, una estrategia relativamente poco estudiada para la recuperación de suelos contaminados, pero que puede tener efectos significativos en las condiciones del suelo. Como sucede con el cultivo de *B. napus*, para su desarrollo en suelos pobres con contaminación mixta, *E. fetida* requiere de la presencia de una enmienda orgánica que alivie la toxicidad provocada por los metales y aporte nutrientes y materia orgánica (Irizar et al., 2015a, 2015b). Nuestros resultados indican que la utilización de lombrices de la especie *E. fetida* causó un aumento de la diversidad funcional microbiana en combinación con la materia orgánica (Capítulo 6), acompañada de una mayor degradación de lindano, lo cual apuntaría, como ya habíamos observado

con las plantas, a que un factor clave para estimular la degradación de lindano es el incremento en la biodiversidad funcional de las comunidades microbianas del suelo. Parte del efecto de la vermirremediación puede deberse al aumento la disponibilidad de los contaminantes orgánicos al mineralizar la MO (Rodriguez-Campos et al., 2014) e incrementar el contacto entre los contaminantes y las comunidades microbianas (Hickman and Reid, 2008b), lo cual también favorecería su degradación. Un problema relacionado con esta actividad es que la mineralización de la MO y el aumento de la disponibilidad de algunos contaminantes (orgánicos e inorgánicos) podría conducir a un aumento de su toxicidad y movilidad, aspecto a considerar antes de la aplicación de esta tecnología. No obstante, nuestros resultados indican que la presencia de *E. fetida* no contribuyó a una removilización del Cr(VI) soluble, por lo que su utilización sería segura bajo unas condiciones experimentales similares. Algunos autores han descrito una interacción sinérgica entre la aplicación de tecnologías de fitorremediación y vermirremediación, pero en este estudio no se ha detectado que sus beneficios al aplicarlas en conjunto vayan más allá de la suma de sus beneficios por separado.

Ya hemos indicado el efecto beneficioso de la aplicación de las tecnologías de bioestimulación con enmiendas orgánicas, fitorremediación y/o vermirremediación para estimular el efecto degradador de las comunidades nativas del suelo, identificando como factor clave el incremento de la diversidad catabólica de los sustratos presentes. Sin embargo, otra estrategia que podría combinarse con la fitorremediación asistida por enmienda es la bioaumentación (adición de microorganismos con características especiales para promover la descontaminación del suelo). Esta técnica es habitualmente empleada para degradar contaminantes orgánicos, pero también puede ser de utilidad para la transformación de algunos metales a formas menos tóxicas (Alvarez et al., 2017) y para aumentar la eficacia de la fitoextracción (Almeida et al., 2017). A pesar de sus prometedores resultados en experimentos de laboratorio bajo condiciones controladas, la aplicación en campo de esta estrategia resulta mucho menos eficaz de lo esperable y está rodeada de incertidumbres. La bioaumentación fracasa en muchas ocasiones a causa de la incapacidad del consorcio inoculado para sobrevivir en el suelo, ya sea por las propias condiciones edafoclimáticas del mismo, por no tolerar la toxicidad de los contaminantes, especialmente cuando hay contaminación mixta, por no ser capaz de competir con los microorganismos autóctonos del suelo, o por perder efectividad al verse expuesto a compuestos intermedios derivados de la degradación del contaminante parental (Cycoń

et al., 2017). Además, cada suelo es diferente, por lo que la efectividad de una cepa o consorcio concretos en un suelo puede que no se traslade a otro. Un grupo de bacterias que han destacado como efectivas en procesos de bioaumentación ha sido el de las actinobacterias, como se deriva de su capacidad para degradar y/o transformar los contaminantes (Alvarez et al., 2017). En esta tesis se ha utilizado un consorcio de actinobacterias, aisladas de medios contaminados con cromo y compuestos organoclorados, formado por varias cepas de *Streptomyces* sp. y *Amycolaptosis tucumanensis*. En consonancia con estudios previos (Aparicio et al., 2019; Polti et al., 2014), en los suelos de esta tesis (Capítulos 6 y 7) el consorcio inoculado logró una eliminación simultánea de lindano y Cr(VI), transformando este último a su forma trivalente (Karthik et al., 2017). Su efecto se tradujo en una menor ecotoxicidad en el suelo, un mejor desarrollo de plantas y lombrices y, por tanto, potenció la efectividad de la fitorremediación y vermirremediación. Al contrario que *B. napus* o *E. fetida*, el consorcio fue efectivo incluso sin la ayuda de la enmienda orgánica, aunque sí se observó que la degradación de lindano es mayor cuando la toxicidad del cromo se reduce.

La aplicación combinada de la bioaumentación, fitorremediación y vermiremediación ha resultado ser la tecnología más efectiva en la descontaminación del suelo en presencia de Cr y lindano y en la recuperación de la salud del suelo. Este resultado abre la puerta a nuevas estrategias en la utilización conjunta de tecnologías biológicas complementarias como las ensayadas en este proyecto. Si bien su eficacia debe ser testada en condiciones de campo, la efectividad de un mismo consorcio en distintos experimentos en microcosmos con suelos diferentes, así como su complementariedad con otras estrategias biológicas, suponen un buen punto de partida de cara a su futura aplicación en campo.

A pesar de las prometedoras aplicaciones de las estrategias biológicas, existen algunas cuestiones sin resolver en torno a su aplicabilidad y efectividad. Con respecto a la aplicación en campo de estas tecnologías, surgen cuestiones de difícil respuesta que podrían comprometer su viabilidad. En el caso de la colza, existen prácticas agronómicas establecidas para su cultivo que pueden seguirse en los procesos de fitogestión. En el caso de la vermirremediación y la bioaumentación, resulta difícil calcular qué cantidad de lombrices/inóculo es la adecuada en ensayos de campo, un factor del que dependerá el coste de su aplicación y que, por tanto, debe precisarse. En el caso de las lombrices, su supervivencia podría verse comprometida por su sensibilidad a las variables ambientales

(Butt and Lowe, 2011) y además podrían migrar a zonas menos estresantes, por lo que el número final que quedaría en el suelo a remediar podría variar. En el caso de la bioaumentación, durante los primeros días tras la inoculación el número de bacterias suele reducirse antes de estabilizarse, por lo que no inocular lo suficiente puede comprometer la supervivencia del consorcio (Hong et al., 2007; Singh et al., 2006). Otra cuestión que limita la efectividad de esta estrategia es la profundidad que alcanzan las tecnologías aplicadas. En el caso de *B. napus*, la profundidad de su sistema radicular ronda los 150 cm, en función de la variedad (Johnston et al., 2002), mientras que para *E. fetida* se ha observado una profundidad de excavado de hasta 30 cm (Li et al., 2015). En el caso de la bioaumentación, si se aplica el inóculo en superficie, su penetración variará en función la permeabilidad del suelo y la dispersión posterior de los microorganismos. Por tanto, es necesario hacer una buena evaluación de la profundidad de la contaminación en el suelo y, en caso de que supere el área de efecto de las estrategias biológicas, buscar alternativas que solucionen el problema, como el uso de otras especies con mayor profundidad (como árboles en el caso de la fitorremediación o especies anécicas/endógeas en el caso de la vermirremediación) o trasladar a la superficie las capas profundas del suelo. Por último, hay que tener en cuenta que la inertización de los metales en el suelo, si bien reduce la toxicidad de los mismos y mejora la salud de suelo, no los extrae. La biodisponibilidad es un parámetro variable, por lo que existe la posibilidad de que la inertización conseguida no sea permanente, y se revierta con el tiempo y los cambios en las condiciones del suelo. Además, la legislación por lo general contempla la concentración total de los contaminantes, sin atender a su disponibilidad.

Por tanto, la bioestimulación mediante enmiendas orgánicas y/o inorgánicas que reduzcan la toxicidad de los metales, seguida de una combinación de estrategias biológicas, como son la fitorremediación, vermirremediación y bioaumentación, es una estrategia de remediación suave que se postula como adecuada para la recuperación sostenible de suelos con mezclas de contaminantes orgánicos e inorgánicos. Combinadas, estas estrategias inertizan los metales, degradan los contaminantes orgánicos y mejoran la salud de la biota del suelo. No en vano, cada vez más estudios empiezan a explorar las posibilidades de combinar estas estrategias. No obstante, este enfoque presenta todavía algunas incertidumbres como las apuntadas que deben ser investigadas antes de su aplicación generalizada en condiciones de campo.

9.2.3. Nanorremediación con nanopartículas de hierro cero valente

La aplicación de nanopartículas de hierro cero valente (nZVI) se utiliza actualmente tanto para la degradación de contaminantes orgánicos como para la inmovilización de metales (Zhao et al., 2016). Su uso en el medio acuoso está ampliamente investigado, pero hay menos conocimiento sobre su efectividad en el suelo, particularmente en condiciones de campo (Patil et al., 2016). Una cuestión que dificulta la aplicación de las nanopartículas en el suelo es su escasa movilidad y su rápida inactivación (Mandal et al., 2020; Su et al., 2016), lo cual puede conducir a una falta de efectividad al no distribuirse adecuadamente por el suelo ni interactuar con los contaminantes. Nuestros resultados indican que uno de los principales factores limitantes de esta tecnología es precisamente que su efectividad está condicionada por la metodología de aplicación de las nanopartículas. Efectivamente, en dos de los tres experimentos llevados a cabo con nanopartículas en esta tesis (Capítulos 4 y 8), la irrigación de la suspensión acuosa de éstas en la superficie de los tiestos no tuvo efectos reseñables en los contaminantes del suelo ni en la biota, presumiblemente a causa de su escasa dispersión en la columna del suelo. Sin embargo, cuando las nanopartículas de hierro cero valente logran una adecuada homogenización con el suelo, facilitando su mezcla, su efectividad en la descontaminación es muy elevada, como se observó en el Capítulo 7. En dicho capítulo, una dosis mayor de nZVI (5 mg kg^{-1} frente a 1 mg kg^{-1}), mezclada exhaustivamente con el suelo de forma manual, logró una alta reducción de la concentración soluble de Cr(VI), reduciéndolo a Cr(III) (Di Palma et al., 2015). Esta reducción se tradujo en una menor ecotoxicidad en el suelo y una estimulación de las estrategias biológicas (bioaumentación, fitorremediación y vermirremediación) en la reducción de lindano. En contra de los hallazgos de otros autores, las nanopartículas no tuvieron en ningún caso un efecto directo por si mismas en la degradación del contaminante orgánico (San Román et al., 2013; Singh et al., 2011b), por lo que sería necesaria su combinación con otras estrategias de remediación, preferiblemente biológicas.

La potencial toxicidad de las nZVI en la biota edáfica es una cuestión rodeada de incertidumbres. Hay todavía un gran desconocimiento sobre las complejas interacciones de las nanopartículas con los diferentes elementos del suelo (Galdames et al., 2017), y se ha observado que su toxicidad varía en función del mismo (Gómez-Sagasti et al., 2019). En esta tesis, la toxicidad de las nanopartículas estaba más relacionado con presencia o ausencia de MO en el suelo que con cualquier otro factor. En los suelos no enmendados

no se detectó un efecto tóxico por parte de las nanopartículas, mientras que en los suelos enmendados con alto porcentaje de MO la aplicación de nanopartículas causó graves efectos tóxicos (Capítulo 7). Estos efectos fueron especialmente dramáticos para el cultivo de colza y las lombrices, reduciendo drásticamente la efectividad de la vermirrediación y fitorremediación. Esta toxicidad, podría deberse a interacciones fisico-químicas entre la materia orgánica y las nanopartículas, provocando cambios de agregación y compactación del suelo (Dwivedi et al., 2015). Esta interacción se vería reducida en presencia de altas cantidades de contaminante, al reaccionar las nanopartículas con éste en lugar de con la MO.

Por tanto, la nanorremediación se presenta como una tecnología con numerosos obstáculos para su aplicación en suelos con contaminación mixta, a pesar de su efectividad en la reducción del Cr(VI). Por un lado, su baja movilidad limita fuertemente su efectividad en el suelo, y en condiciones de campo es difícil garantizar la adecuada distribución de las nZVI. Por otro lado, su interacción con la MO hace desaconsejable su aplicación en suelos con alto contenido en MO o en combinación con enmiendas orgánica, salvo quizá en suelos con muy alta concentración de contaminantes en los que las enmiendas no fueran suficientemente efectivas. Además, el coste de aplicar esta tecnología es elevado en comparación con las GRO.

9.3. Síntesis y próximos pasos

A lo largo del desarrollo de esta tesis se ha evaluado la efectividad de diferentes estrategias, individualmente y en conjunto, para la recuperación de la salud de suelos con mezclas de contaminantes orgánicos e inorgánicos. Para su evaluación se ha tenido en cuenta la concentración total y biodisponible de los contaminantes, su impacto en la biota y la viabilidad de las diferentes estrategias para ser aplicadas en un modelo de gestión sostenible.

Los componentes y propiedades del suelo, como el pH, el potencial redox o el contenido en MO y carbonatos, entre otros, son factores claves para la salud de suelo en general, y en particular para la dinámica de los contaminantes. En esta tesis se subraya el papel de las enmiendas orgánicas e inorgánicas para la inertización de los contaminantes y el aporte de nutrientes y MO, esenciales para el desarrollo de la biota edáfica. En concreto, se ha validado el uso de una enmienda de material bioestabilizado a partir de la fracción orgánica de los residuos sólidos urbanos. Su uso para la recuperación de suelos

contaminados permitiría la revalorización de un material actualmente considerado como residuo pero que, como se ha comprobado en todos los capítulos de esta tesis, puede mejorar sustancialmente la salud de suelos con contaminación mixta. Las nanopartículas de hierro cero valente son otra estrategia que podría ser efectiva para la inertización de metales como el cromo, cuya toxicidad y movilidad depende directamente de su especiación. No obstante, las nanopartículas encuentran numerosos obstáculos en su aplicación, como su rápida inactivación y su escasa movilidad, así como su alta reactividad con componentes de la MO del suelo. Teniendo en cuenta los múltiples beneficios aportados al suelo por la MO, así como el bajo coste de las enmiendas orgánicas en comparación con la aplicación de nanopartículas, la adición de MO al suelo sería preferible a la aplicación de nZVI en la mayoría de los casos. El uso de una enmienda orgánica sería, en términos generales, más económico, más efectivo en términos de recuperación de la salud de suelo, y entrañaría menos riesgos potenciales que el uso de nanopartículas de hierro cero valente. En casos concretos, en los que la concentración de metal fuera demasiado alta como para ser inertizada por las enmiendas orgánicas y/o inorgánicas, las nZVI podrían ser de utilidad, aunque sería recomendable aplicarlas antes que cualquier enmienda orgánica, para evitar su interacción con la MO, y habría que procurar su adecuada dispersión por el suelo.

La inertización de los metales es un primer paso fundamental para la recuperación de la salud de los suelos con contaminación mixta, ya que éstos pueden ejercer una muy importante toxicidad sobre la biota edáfica, imposibilitando el manejo sostenible de estos suelos mediante procesos como la fitogestión, e impidiendo la degradación biológica de los contaminantes orgánicos. Si bien las enmiendas son efectivas en la inertización de metales, para la degradación de los contaminantes orgánicos es necesaria su combinación con otras opciones suaves de remediación. La aplicación conjunta de un cultivo de *B. napus* (fitorremediación), de *E. fetida* (vermirremediación) y la inoculación de un consorcio de actinobacterias (bioaumentación) es efectiva para la degradación de contaminantes orgánicos como el lindano, mejora la salud del suelo y puede tener un efecto adicional en la inmovilización de los metales. Las plantas y las lombrices se benefician especialmente de la presencia de enmiendas orgánicas, actuando de forma sinérgica en el aumento de la diversidad funcional microbiana, un factor que podría ser clave en la degradación de los contaminantes orgánicos. De entre estas tres tecnologías biológicas, el cultivo con *B. napus* cobra especial relevancia ya que, además de sus efectos

positivos sobre la salud de suelo, favorece la fitogestión del emplazamiento contaminado, obteniendo beneficios ambientales, sociales y económicos durante el proceso de recuperación del suelo. El binomio formado por las enmiendas y el cultivo sería básico para la recuperación sostenible de los suelos con contaminación mixta, pudiendo aumentarse la efectividad con otras tecnologías. Las complementariedades y sinergias entre las estrategias biológicas y las enmiendas hacen que los beneficios de su combinación superen con creces los obtenidos individualmente por las mismas, postulándose como un enfoque adecuado para los suelos con contaminación mixta.

No obstante, las bondades de estas tecnologías deben ser probadas primero en ensayos de campo, en los cuales su efectividad podría variar con respecto a los experimentos en condiciones controladas. En el campo, la capacidad de adaptación de los organismos y, por tanto, su efectividad en la recuperación del suelo puede verse sensiblemente mermada por aspectos como las propiedades del suelo, las condiciones ambientales o la competición con otros organismos. Existen todavía incertidumbres sobre la metodología y las dosis de aplicación óptimas en algunas de estas tecnologías. Además, en este estudio ya se observan algunas debilidades. Como se ha comentado previamente, la efectividad de estas tecnologías está limitada a la profundidad que son capaces de alcanzar, siendo necesaria la búsqueda de alternativas para aquellos casos en que los contaminantes están localizados a más profundidad. Por otro lado, la degradación completa de los contaminantes orgánicos es un desafío, ya que es habitual que las fracciones recalcitrantes, como las cadenas más pesadas de los *n*-alcanos presentes en el gasóleo, apenas se degraden.

Sin embargo, la principal limitación de este tipo de gestión de los suelos contaminados es que, aunque su toxicidad y movilidad se reduce, los contaminantes metálicos siguen en el suelo. La biodisponibilidad de los metales puede variar con el paso del tiempo y/o cambios en las condiciones del suelo (pH, potencial redox, mineralización de la MO, etc.). En consecuencia, la fitogestión de emplazamientos contaminados implica necesariamente la puesta en marcha de un sistema de monitorización de parámetros clave de la salud de suelo, como la concentración biodisponible y la ecotoxicidad, que permiten controlar la evolución de la salud de suelo a lo largo del tiempo. Por otro lado, la legislación, por lo general, no contempla las concentraciones disponibles, y mucho menos los bioindicadores de toxicidad o salud del suelo, sino que se basa en las concentraciones totales de los contaminantes. Es de imperiosa necesidad que se produzca un cambio de

paradigma en el enfoque de la administración hacia la contaminación del suelo, de tal modo que aspectos como la disponibilidad o la ecotoxicidad sean tenidos en cuenta en la legislación y la gestión de los emplazamientos contaminados. Para ello, es necesaria la selección de una serie de parámetros e indicadores estandarizados en torno a los cuales sea posible legislar. Este cambio de paradigma facilitaría la implementación de las GRO, las cuales a día de hoy son vistas con reticencia por los principales actores (gestores, administración, etc.) a causa del largo periodo requerido para cumplir con la normativa. Las GRO son lentas en la reducción de la concentración total de contaminante, pero relativamente rápidas recuperando la salud del suelo. Un menor periodo de tiempo requerido para alcanzar los objetivos fijados por la normativa, combinado con los beneficios intrínsecos que aportan, harían de las GRO estrategias más atractivas.

El objetivo último debería ser la eliminación de los contaminantes del suelo, por lo que es necesario seguir investigando en la consecución de este objetivo, pero en el proceso, la fitogestión ofrecería una solución sostenible para la recuperación de funciones y servicios ecosistémicos del suelo al tiempo que se obtiene una regeneración socioeconómica de la comunidad.

10. CONCLUSIONS

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1. The organic bio-stabilized material studied here is a suitable amendment for the remediation of mixed contaminated soils, as it promotes metal inertization, stimulates soil microbial communities and enhances plant growth.
2. Metal inertization can be an essential first step for the degradation of organic compounds in mixed contaminated soils, since metals can cause significant toxicity to soil degrading microorganisms.
3. Hydrocarbons of biological origin, like fatty acid methyl esters, are more easily degraded by indigenous soil microbial communities than those from fossil origin.
4. Phytoremediation with *Brassica napus* assisted by an organic amendment is recommended for mixed contaminated soils, as it promotes both the removal of soil contaminants and the recovery of soil health. The effectiveness of this approach can be enhanced by its combination with other GRO strategies as vermiremediation and/or bioaugmentation.
5. An increase in soil microbial functional diversity is a key factor for the degradation of soil lindane, which can be achieved through the combination of *Brassica napus* growth, the inoculation of *Eisenia fetida* worms, and the application of an organic amendment.
6. Bioaugmentation with a bacterial consortium can promote the recovery of soils simultaneously polluted with chromium (VI) and lindane, by reducing Cr(VI) to Cr(III), decreasing lindane concentrations, and enhancing the effectiveness of phytoremediation and vermiremediation strategies.
7. Nanoremediation with zero-valent iron nanoparticles can be effective for the reduction of Cr(VI) to Cr(III), decreasing its toxicity and facilitating the biological remediation of mixed contaminated soils. However, the capacity of nZVI for soil remediation purposes is strongly conditioned by the application method, since their rapid inactivation and low mobility hinder their interaction with soil contaminants. Besides, the potential toxicity of nZVI is conditioned, at least partially, by the presence of soil organic matter.
8. Considering effectiveness, cost and potential risk, the application of organic and/or inorganic amendments appears preferable for the remediation of mixed contaminated soils, compared to nanoremediation with nZVI.

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