Potential of alfalfa for use in chemically and biologically assisted phytoremediation of soil co-contaminated with petroleum hydrocarbons and metals

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POTENTIAL OF ALFALFA FOR USE IN CHEMICALLY AND BIOLOGICALLY ASSISTED PHYTOREMEDIATION OF SOIL CO-CONTAMINATED WITH PETROLEUM HYDROCARBONS AND METALS

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List of Abbreviations

ACC: 1-aminocyclopropane-1-carboxylate
Alf: Alfalfa
ANOVA: One-way analysis of variance
ARE: Artificial Root Exudates
AS: Agricultural soil
BA: Bioaugmentation
B[a]P: Benzo[a]pyrene
BCF: Bioconcentration factor
BTEX: Benzene, toluene, ethyl benzene, and xylenes
CA: Citric acid
CEC: Cation exchange capacity
CFU: Colony forming unit
CMC: Critical micelle cocentration
CTMAB: Cetyltrimethylammonium bromide
DDPB: Dodecylpyridinium bromide
DOM: Dissolved organic matter
DDE: Dichlorodiphenyldichloroethylene
DDT: Dichlorodiphenyltrichloroethane
DTPA: Diethylendiaminepentacetic acid
DW: Dry weight
EDTA: Ethylenediaminetetraacetic acid
EGTA: Ethylenegluatarotriacetic acid
EPS: Exopolisaccharide
F_0: Minimal fluorescence yield of a dark-adapted sample.
F_m: Maximum fluorescence yield of a dark-adapted sample.
F_v/F_m: Maximum PSII photochemical efficiency
FISH: Fluorescence *in situ* hybridization
GC-FID: Gas chromatography with flame ionization detector
HM: Heavy metal
IAA: Indol acetic acid
ICP-OES: Inductively coupled plasma-optical emission spectrometry
INT: Iodonitrotetrazolium violet
K_{ow}: Octanol-water partition coefficient
LMWOAs: Low molecular weight organic acids
MiA: Malic acid
MoA: Malonic acid
MDA: Malondialdehyde
MPN: Most-probable-number
NA: Natural attenuation
NTA: Nitrilotriacetate
NRE: Natural root exudates

OA: Organic acid
OFMSW: Organicfraction of municipal solid waste
Ox: Oxalate
Pa: *Pseudomonas aeruginosa*
PAHs: Polycyclic aromatic hydrocarbons
PCB-PCT: Polychlorinated bi and terphenyls
PCNB: Pentachloronitrobenzene
PGPR: Plant growth promoting rhizobacteria
Phe: Phenanthrene
*p*NPB: p-nitrophenyl butyrate
*p*NP: p-nitrophenol
PPFD: Photosynthesis photon flux density
PR: Phytoremediation
PS: Polluted soil
PSII: Photosystem II
Pyr: Pyrene
ROS: Reactive oxygen species
SA: Succinic acid
SDS: Sodium dodecyl sulfate
SEPR: Surfactant enhanced phytoremediation
SOM: Soil organic matter
TA: Tartaric acid
TBA: Thiobarbituric acid
TPH: Total petroleum hydrocarbons
TCE: Trichloethylene
TF: Translocation factor
THM: Total heterotrophic microflora
Tw-80: Tween® 80 (polyethylene glycol sorbitan monooleate)
USEPA: United States Environmental Protection Agency

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Abstract

Potential of alfalfa for use in chemically and biologically assisted phytoremediation of soil co-contaminated with petroleum hydrocarbons and metals

General background: As a result of human activity, soil resources have been contaminated with heavy metals and petroleum hydrocarbons. The great number of co-contaminated soils in the environment shows just how important it is to find remediation solutions adequate in such complex scenarios, which had seldom been studied before. Phytoremediation is a biological remediation technology, which takes advantage of the intrinsic physiological abilities of plants to remediate contaminated media. Plants and their associated microorganisms perform phytoremediation processes (*e.g.* phytoextraction and rhizodegradation), which can bring about the clean-up of co-contaminated soils. However, a major constraint which hinders the success of phytotechnologies is low bioavailability of pollutants in soil. As a result, chemically and biologically assisted phytoremediation are possible strategies used to overcome this limitation and enhance the efficiency of remediation. The chemical approach presented in this study involves adding biodegradable soil amendments to increase the ability of contaminants for being transferred from soil to plants and microorganisms. The biological strategy explored herein consists of inoculating contaminated soils with bacteria (bioaugmentation) able to improve remediation of pollutants and/or promote plant features.

Main objectives: a) investigating the phytoremediation potential of alfalfa (*Medicago sativa* L.) in co-contaminated soils b) studying the effects of the low molecular weight organic acid citric acid and the surfactant Tween® 80 on the phytoremediation process c) assisting phytoremediation with a bioaugmentation approach using *Pseudomonas aeruginosa* bacteria.

Methodologies: Determining germination and mortality rates, assessing plant physiological parameters. Quantifying plant biomass, heavy metals in plants, total petroleum hydrocarbons (TPH) in soil, soil microbiological indicators. Calculating phytoremediation parameters.

Remarkable results: Alfalfa presented low tolerance to TPH contaminated soil at 8400 mg kg^{-1} soil, which was improved when TPH were present at a lower rate of concentration (3600 mg kg^{-1} soil). Alfalfa was able to take up limited quantities of metals (<100 mg kg^{-1} dry matter), while it had a positive effect on promoting the microbial number of alkane degraders and lipase activity in the rhizosphere. Moreover, the combined application of citric acid and Tween® 80 resulted in a greater improvement of these parameters. Bioaugmentation with *P. aeruginosa* had a promoting effect on alfalfa biomass (71% increase of plant total dry biomass). In

addition, the highest TPH removal rates (68%, after 90 days of experiment) were obtained in soils vegetated with alfalfa and bioaugmented with *P. aeruginosa*.

Overall conclusion: Alfalfa can tolerate a heavy metal and petroleum hydrocarbon co-contaminated soil (subject to TPH levels), which is an essential characteristic of any plant species used in phytoremediation. Alfalfa could not be considered as an active heavy metal removal species as it was not able to phytoextract significant amounts of heavy metals (still in the presence of soil amendments or bioaugmentation). Nevertheless, the enhancement of microbial number and activity in the rhizosphere encouraged the potential of this plant species to be successfully used in the remediation of petroleum hydrocarbons. These effects were further enhanced by the joint application of soil amendments. Finally, the combination of phytoremediation and bioaugmentation seems to be a promising approach to remediate petroleum hydrocarbons, when present in co-contaminated soils.

Key words: Bioaugmentation, Contaminated Soils, Heavy metals, Organic Acids, Petroleum Hydrocarbons, Phytoremediation, Surfactants.

Résumé

Utilisation de la luzerne pour le traitement par phytoremédiation assistée chimiquement et biologiquement de sols co-contaminés par des métaux lourds et des hydrobarbures pétroliers

Contexte general: En raison des activités anthropiques, les sols sont souvent contaminés par des métaux lourds et des hydrocarbures pétroliers. Le nombre important de sites co-contaminés dans l'environnement met en lumière la nécessité de trouver des solutions adéquates à ces scénarios complexes d'assainissement, qui, de plus, sont rarement étudiés. Parmi les techniques d'assainissement biologique, la phytoremédiation est une technique qui se base sur les propriétés naturelles des plantes pour assainir les sols. L'utilisation conjointe des plantes et des microorganismes pour dépolluer les sols co-contaminés est une stratégie de traitement en plein essor. Cependant, l'obstacle majeur qui entrave la réussite de tels traitements est la faible biodisponibilité des polluants dans le sol. Par conséquent, la phytoremédiation peut être assistée par des traitements chimiques et/ou biologiques afin de surmonter cette limitation et d'améliorer l'efficacité de l'assainissement. Dans cette étude, l'approche chimique implique l'ajout d'amendements biodégradables. Enfin, la stratégie biologique retenue dans ce travail est la bioaugmentation qui consiste à ajouter dans le sol des bactéries capables d'améliorer l'assainissement des polluants et/ou favoriser la croissance des plantes.

Principaux objectifs: a) Étudier le potentiel de la luzerne pour la phytoremédiation des sols co-contaminés, b) Étudier les effets de l'acide organique de faible poids moléculaire acide citrique et le tensioactif Tween® 80 sur le processus de phytoremédiation et c) Étudier l'effet de la bioaugmentation avec la bactérie *Pseudomonas aeruginosa* sur le processus de phytoremédiation.

Méthodes: Détermination des taux de germination et de mortalité, évaluation des paramètres physiologiques des plantes. Quantification de la biomasse végétale, des métaux lourds dans les plantes, hydrocarbures pétroliers totaux (HCT) dans le sol, et indicateurs microbiologiques du sol. Calcul des paramètres de phytoremediation.

Résultats remarquables: La luzerne a présenté une faible tolérance aux HCT du sol à 8400 mg kg^{-1} de matière sèche (MS). Celle-ci qui a été améliorée lorsque les HCT étaient présents à plus faible concentration (3600 mg kg^{-1} MS). La luzerne a été en mesure de prendre les métaux dans une proportion limitée (<100 mg kg^{-1} MS), tandis qu'elle a eu un effet positif sur le nombre de microorganismes du sol capables de dégrader les alcanes et sur l'activité de la lipase dans la rhizosphère. En outre, l'application combinée de l'acide citrique et du Tween® 80 a donné lieu à une amélioration plus importante de nombre et de l'activité microbienne dans la rhizosphère.

La bioaugmentation avec *P. aeruginosa* a eu un effet sur l'amélioration de la biomasse de luzerne (augmentation de la biomasse végétale sèche totale de 71%). En outre, les taux les plus élevés d'élimination des HCT (68%, après 90 jours d'expérience) ont été obtenues dans les sols plantés avec la luzerne et bioaugmentées par *P. aeruginosa*.

Conclusion générale: La luzerne pourrait tolérer le sol co-contaminé par des métaux lourds et des hydrocarbures pétroliers, ce qui est une caractéristique essentielle en phytoremédiation. La luzerne ne peut cependant pas être considérée comme une espèce capable d'extraire activement les métaux lourds, même en présence d'amendements chimiques ou par bioaugmentation. Néanmoins, l'augmentation du nombre et de l'activité microbienne dans la rhizosphère a confirmé le potentiel de cette plante à être utilisée avec succès dans le traitement des hydrocarbures pétroliers. Ces effets ont été par ailleurs renforcés par l'application conjointe d'acide citrique et de Tween® 80. Enfin, la combinaison de la phytoremédiation et de la bioaugmentation semble une approche prometteuse pour réaliser l'assainissement des hydrocarbures pétroliers, lorsqu'ils sont présents dans des sols co-contaminés.

Mots clés: acides organiques, bioaugmentation, hydrocarbures pétroliers, métaux lourds, phytoremédiation, sols contaminés, tensioactifs.

Sintesi

Uso di erba medica per il fitorimedio di suoli co-contaminati da metalli pesanti e idrocarburi petroliferi, assistito da trattamenti chimici e biologici

Contesto generale: A seguito delle attività antropiche, i suoli restano spesso contaminati da metalli pesanti e idrocarburi petroliferi. L'alta frequenza di occorrenza di suoli co-contaminati nell'ambiente mette in luce la necessità di trovare metodi di bonifica adeguati a tali scenari complessi, che, inoltre, sono scarsamente studiati. Il fitorimedio è una tecnologia di bonifica biologica, che sfrutta le capacità fisiologiche intrinseche delle piante per bonificare i mezzi contaminati. Le piante e i loro microrganismi associati eseguono processi di fitorimedio (tra i quali ricordiamo la fitoestrazione e rizodegradazione), che possono risanare i suoli co-contaminati. Tuttavia, un vincolo importante che ostacola il successo di queste fitotecnologie è la bassa biodisponibilità degli inquinanti nel suolo. Per questo, i processi di fitorimediazione possono essere assistiti da trattamenti chimici e biologici che superano questo limite e migliorano l'efficienza della bonifica. L'approccio chimico presentato in questo studio comporta l'aggiunta di ammendanti biodegradabili che aumentano la capacità dei contaminanti di essere trasferiti dal suolo alle piante e ai microrganismi. Inoltre, la strategia biologica qui esplorata prevede l'inoculazione di batteri nel suolo (bioaugmentation) in grado di migliorare la bonifica degli inquinanti e/o di promuovere le caratteristiche della pianta.

Obiettivi principali: a) Indagare il potenziale di fitorimedio dell' erba medica (*Medicago sativa* L.) in suoli co-contaminati b) Studiare gli effetti di due ammendanti chimici (l'acido organico di basso peso molecolare acido citrico e il tensioattivo Tween® 80) sul processo di fitorimedio c) Assistere la fitorimediazione con un approccio di bioaugmentation utilizzando il batterio *Pseudomonas aeruginosa*.

Metodologie: Determinazione dei tassi di germinazione e di mortalità delle piante, valutazione dei parametri fisiologici della pianta. Quantificazione della biomassa vegetale, dei metalli pesanti nelle piante, degli idrocarburi totali (IT) nel suolo e d'indicatori microbiologici del suolo. Calcolo dei parametri di fitorimedio.

Risultati notevoli: L'erba medica presenta bassa tolleranza al suolo contaminato con concentrazioni di IT di 8400 mg kg^{-1} peso secco (PS); la tolleranza è migliorata abbassando la concentrazione di IT (3600 mg kg^{-1} PS). L'erba medica è stata in grado di assorbire i metalli in misura limitata (<100 mg kg^{-1} PS), mentre ha avuto un effetto positivo nella promozione del numero di microrganismi degradatori di alcani e nell'attività della lipasi nella rizosfera. Inoltre, l'applicazione combinata di acido citrico e Tween® 80 ha determinato un miglioramento maggiore di questi parametri microbiologici. La bioaugmentation con *P. aeruginosa* ha promosso la biomassa

dell’erba medica (aumento del 71% nella biomassa totale). Inoltre, i più alti tassi di rimozione di IT (68%, dopo 90 giorni di esperimento) sono stati ottenuti in terreni vegetati con l’erba medica e inoculati con *P. aeruginosa*.

Conclusione generale: L’erba medica può tollerare metalli pesanti e idrocarburi petroliferi in suoli co-contaminati. Questa è una caratteristica essenziale per tutte le specie vegetali da utilizzare in fitorimedio. L’erba medica non può essere considerata come una specie attiva nella rimozione di metalli pesanti, in quanto non é stata in grado di fitoestrarre notevoli quantità di metalli (addirittura in presenza di ammendanti chimici o bioaugmentation). Tuttavia, l’aumento del numero e dell'attività dei batteri nella rizosfera ha confermato il potenziale di questa specie vegetale da utilizzare con successo nel trattamento degli idrocarburi petroliferi. Questi effetti sono stati ulteriormente migliorati attraverso l'applicazione congiunta degli ammendanti acido citrico e Tween® 80. Infine, la combinazione di fitorimedio e bioaugmentation sembra un approccio promettente per realizzare la bonifica di idrocarburi petroliferi, quando sono presenti in terreni co-contaminati.

Parole chiave: acidi organici, bioaugmentation, fitorimedio, idrocarburi petroliferi, metalli pesanti, suoli inquinati, tensioattivi.

Samenvatting

Potentieel van alfalfa voor gebruik in chemische en biologische fytoremediatie van door petroleumkoolwaterstoffen en zware metalen verontreinigde bodems

Achtergrond: Als gevolg van menselijke activiteit zijn bodemrijkdommen verontreinigd met zware metalen en petroleumkoolwaterstoffen. Het groot aantal meervoudig verontreinigde bodems in het milieu laat zien hoe belangrijk het is om saneringsoplossingen te vinden die afdoende zijn in dergelijke complexe scenario's, die zelden eerder bestudeerd zijn. Fytoremediatie is een biologische saneringstechnologie die gebruik maakt van de intrinsieke fysiologische capaciteiten van planten om verontreinigde materie te saneren. Planten en de aan hen verbonden micro-organismen voeren fytoremediatieprocessen uit (bijvoorbeeld fytoextractie en rhizodegradatie), die kunnen leiden tot de opschoning van meervoudig verontreinigde bodems. Echter, een belangrijke beperking die het succes van fytotechnologieën belemmert is de lage biobeschikbaarheid van verontreinigingen in de bodem. Hierdoor zijn chemische en biologische fytoremediatie mogelijke strategieën om deze beperking te overwinnen en de efficiëntie van sanering te verhogen. De chemische benadering die in deze studie gepresenteerd wordt, omvat het toevoegen van biologisch afbreekbare bodemverbeteraars om het waarschijnlijker te maken dat de verontreinigingen vanuit de bodemdeeltjes worden overgeheveld naar planten en micro-organismen. De biologische strategie die hierin wordt onderzocht bestaat uit het aan verontreinigde bodems toevoegen van bacteriën die de sanering van verontreinigende stoffen kunnen bevorderen en/of planteigenschappen kunnen verbeteren (bioaugmentatie).

Hoofddoelstellingen: a) Het onderzoeken van het fytoremediatie-potentieel van alfalfa (*Medicago sativa* L.) in meervoudig verontreinigde bodems. b) Het bestuderen van de effecten van het laagmoleculaire organische zuur citroenzuur en de surfactant Tween® 80 op het fytoremediatieproces. c) Het bevorderen van fytoremediatie met een bioaugmentatiebenadering die gebruik maakt van *Pseudomonas aeruginosa* bacteriën.

Methodologieën: Het bepalen van de ontkieming en afstervingsratio, het beoordelen van de fysiologische parameters van de planten. Kwantificering plantaardige biomassa, zware metalen in planten, totaal aan petroleumkoolwaterstoffen (KWS) in de bodem, microbiologische indicatoren in de bodem. Het berekenen van fytoremediatie-parameters.

Opmerkelijke resultaten: Alfalfa toonde een lage tolerantie voor met KWS verontreinigde grond bij 8400 mg/kg^{1} aarde, maar dit verbeterde wanneer een lagere concentratie KWS aanwezig was (3600 mg /kg^{1} aarde). Alfalfa kon een beperkte hoeveelheid metalen opnemen (<100 mg /kg^{1} droge stof), terwijl het een positief effect

had op de bevordering van het aantal microben die zorgen voor degradatie van alkanen en tevens op de lipase-activiteit in de rhizosfeer. Bovendien leidde de gecombineerde toepassing van citroenzuur en Tween® 80 tot een grotere verbetering van deze parameters. Bioaugmentatie met *P. aeruginosa* had een bevorderend effect op alfalfa-biomassa (71% toename van totale plantaardige droge biomassa). Bovendien werden de hoogste KWS afnamewaarden (68%, na 90 dagen van het experiment) verkregen in bodems begroeid met alfalfa en waarop bioaugmentatie was toegepast met *P. aeruginosa*.

Algemene conclusie: Alfalfa kan een meervoudig verontreinigde bodem verdragen die vervuild is met zware metalen en petroleumkoolwaterstof (afhankelijk van KWS niveaus), wat een essentieel kenmerk is van elke plantensoort die gebruikt zou kunnen worden in fytoremediatie. Alfalfa kan niet worden beschouwd als een soort die zware metalen actief verwijdert, aangezien het niet in staat was om significante hoeveelheden zware metalen te verwijderen door middel van fytoextractie (met gebruikmaking van bodemverbeteraars of bioaugmentatie). Niettemin moedigde de verbetering van het aantal microben en van de activiteit in de rhizosfeer aan tot het nader bekijken van het potentieel van deze plantensoort om met succes te worden gebruikt bij de sanering van petroleumkoolwaterstoffen. Deze effecten werden nog versterkt door de gecombineerde toepassing van bodemverbeteraars.Tenslotte lijkt de combinatie van fytosanering en bioaugmentatie een veelbelovende benadering voor de sanering van petroleumkoolwaterstoffen, wanneer deze aanwezig zijn in meervoudig verontreinigde bodems.

Trefwoorden: bioaugmentatie, bodemsanering, oppervlakteactieve stoffen, organische zuren, petroleumkoolwaterstoffen, verontreinigde grond, zware metalen.

Chapter 1

Introduction

1.Introduction

1.1. Environmental problem: soil pollution

1.1.1. Definition of contamination and pollution

The term contamination refers to the presence of a substance where it should not be or at concentrations above natural (baseline) levels. Generally, a contaminant is an undesired material although it does not have to be necessarily harmful. Thus, a contaminated soil is a soil whose chemical state deviates from the normal composition but does not have a detrimental effect on organisms (Kabata-Pendias, 2011). By contrast, pollution occurs when an element or a substance is present in greater amounts than background concentrations, generally as a result of human activity, and has a net detrimental effect on the environment and its components, principally affecting biological processes in living organisms (plants, animals, humans) (Kabata-Pendias, 2011). In consequence, all pollutants are contaminants, but not all contaminants are pollutants (Chapman, 2007). In spite of these semantic differences, it is not uncommon that both terms are considered as synonyms by many communities and even scientific journals. Although in the present manuscript the terms contaminant/contamination and pollutant/pollution may be used interchangeably, it was considered opportune to clarify the difference between them.

1.1.2. Generalities of soil pollution

Soil pollution arises in the environment principally as the result of anthropogenic activities. The direct discharge of industrial wastes to soil, the accidental spillages of chemicals, the application of agricultural chemicals (pesticides) to soils, the percolation of contaminated surface water to subsurface stratum or improper disposal of wastes (*e.g.* leaching of wastes from landfills) are just a few examples causing soil pollution with a variety of inorganic and organic pollutants (Mirsal, 2004). Generally, two main types of sources of environmental pollution can be distinguished. If the origin of the pollution can be traced to a single point, it is called point source pollution, which is usually present in a concentrated nature (namely high levels and often on a small area). On the contrary, if the pollutants are spread in the environment, or the pollution is of a general nature and cannot be traced to a single source, it is called diffuse pollution or non-point-source pollution (Mirsal, 2004). The nature and degree of pollution for each polluted site vary widely, but in most cases, polluted sites do not create immediate dangers and serious risks to the surrounding population. Instead, associated risks to polluted sites are generally those resulting from exposure to pollutants at low doses over a long period of time, which may even correspond to a lifetime. It is also frequent that a polluted site becomes a threat to groundwater or surface water putting drinking water resources in jeopardy. In any case, damage to a given target is not possible unless the risk source and the target are in contact (direct or indirectly) allowing a transfer of pollutants from the source to the target (Wilson, 1991). Only when these three

parameters (source of pollution, transfer and target) occur, risk does exist. When this arises, the application of suitable risk assessment methodologies is essential, in order to identify the issues of concern and define the suitable actions to be implemented (BASOL, 2014). Treatment of soils may be applied in situ (without removing the bulk soil) or ex situ, which involves the removal of contaminated media, either for off-site disposal or for on-site treatment and subsequent return to the subsurface. Existing remediation technologies can be classified in four major types: a) chemical and physical methods, b) biological methods, c) fixation methods and d) thermal destruction methods. The choice of one or another remediation technology is the result of a cost-benefit assessment that evaluates many aspects such as the concentration of pollutants, the risk engendered by the pollution, the available financial resources and time restrictions (Mirsal, 2004). It is beyond the scope of this study to examine the distinctive features of each different type of remediation technology. Moreover, this thesis will focus on one single remediation technology: phytoremediation, which belongs to biological methods.

1.1.3. Overview of polluted sites and soils in France

In France, the extent of contaminated soils is well known and there is a legal framework to identify and deal with each environmental problem. The French approach is to set the objectives of rehabilitation according to the intended use of the site (*e.g.* agricultural, industrial, forestry, residential use). An implication of this is that the treatment of the site will be accomplished only when its future purpose is established. According to this approach, it is not so much pollution that is problematic but its impact (potential or actual) on the environment, which must be accurately addressed. This strategy is termed *treat according to use* and it is now used by almost all countries of the European Union. Another feature of the French approach is not to establish generic values defining soil quality, but to perform specific site studies, which determine the aims of the rehabilitation for each particular site (MEDDE, 2007; BASOL, 2014).

The French Ministry of Ecology, Sustainable Development and Energy created BASOL, a database of polluted or potentially polluted sites and soils calling a preventive or remedial government action. The information gathered in this database covers the key aspects of soil management, which can be listed as follows: a) location of the site, b) technical situation, c) nature of pollutants, d) impact of polluted sites, e) origin of the government action, and f) monitoring of groundwater quality. Each one of the mentioned aspects will be briefly described below.

At the present time, France presents 5759 polluted or potentially polluted sites, which are broadly distributed in the country, but in an uneven way. In fact, 72 % of the polluted sites are spread on only 25% of the French territory. The three most affected regions are Rhône-Alpes, Nord-Pas-de-Calais and Aquitaine, which concentrate 17.62, 11.51 and 9.83% of polluted sites, respectively. All identified sites are grouped in five categories according to their technical situation: a) treated site free of restrictions (646 sites), b) site under work in progress (864 sites), c) site set to safety and/or to be the subject of a diagnosis (358 sites), d) site under evaluation (1052) and e) treated site with

monitoring and/or usage restrictions (2839 sites). Numerous types of pollutants are present in the sites of concern. Table 1.1 summarizes the type of pollutants found (alone or in combination) in the affected sites, in terms of occurrence. As can be seen from the table, the most prevalent pollutants are metals and hydrocarbons, which affect 60.13% and 23.53% of soils, respectively. It is also important to highlight that contamination of groundwater is found in 70.07% of the cases. Through the effect of different mechanisms (*e.g.* runoff, volatilization, plant uptake) pollutants in the soil can become mobile and impact the man, an ecosystem or a water resource. Among the sites in the inventory, 2938 (51.02%) have been found to have an impact (*e.g.* on surface water, on groundwater, on sediments, on plants for human and animal consumption, or on animals for human consumption), 559 (9.71%) have demonstrated no impact and the rest remains indeterminate. With respect to the origin of the government action on polluted sites, three possibilities can be distinguished: it may be the result of a presumption of pollution, it may be the consequence of finding an impact or it may be spontaneously reported by site managers. Finally, management of groundwater quality requires either detecting or monitoring actions depending on whether the pollution of groundwater is known or not. Since 2000, the sites listed within BASOL must implement a quality monitoring of groundwater or have a technical justification for lack of supervision.

Table 1.1 Types of pollutants affecting French contaminated sites

Pollutant	Occurrence in polluted soils (%)
Arsenic (As)	8.39
Barium (Ba)	1.84
Cadmium (Cd)	3.91
Cobalt (Co)	0.45
Chrome (Cr)	9.05
Copper (Cu)	8.79
Mercury (Hg)	3.25
Molybdenum (Mb)	0.35
Nickel (Ni)	6.27
Lead (Pb)	11.3
Selenium (Se)	0.30
Zinc (Zn)	6.23
Sulphates	0.17
Chlorides	0.10
Ammonium	0.38
BTEX	1.81
TCE	0.47
Hydrocarbons	23.5
PAHs	10.3
Cyanides	3.72
PCB-PCT	3.72
Halogenated solvents	9.05
Non-halogenated solvents	2.29
Pesticides	0.87

(BASOL, 2014). BTEX: benzene, toluene, ethyl benzene, and xylenes. TCE: trichlorethylene. PAHs: polycyclic aromatic hydrocarbons. PCB-PCT: Polychlorinated bi and terphenyls.

1.1.4. Pollutants of concern

Since the presence of petroleum hydrocarbons and heavy metals is so diffuse in French polluted sites, the present thesis is centered on both types of pollutants. Moreover, is not uncommon that pollutants of different types are present simultaneously in polluted soils intensifying the threat that they represent. As a result, the problem of co-contaminated soils is particularly addressed.

1.1.4.1. Heavy metals

There is no whole consensus on the definition of the term *heavy metal*. Criteria that have been used with the aim to define this term included atomic weight, atomic number density or chemical properties. Besides, in the scientific literature heavy metal has been

generally employed to refer to metals and semimetals (metalloids) associated with toxicity effects or chemical hazards rather than other intrinsic physicochemical properties (Kabata-Pendias, 2011).

Heavy metals originate from various sources. The input of heavy metals in the environment is the result of anthropogenic activities, mainly related to energy and mineral consumption. Common sources of heavy metals include mining, industrial and municipal wastes, motor vehicle emissions, lead-acid batteries, fertilizers, pesticides, and all sewage-derived materials (Kabata-Pendias, 2011).

Trace element speciation refers to the distribution between the various chemical species in which metals can be found (Tessier et al., 1979). In soils, metals are distributed mainly in two phases: the soil solution and the soil solid phases. Metals in the soil solution phase can exist as free ions, inorganic and organic complexes and suspended colloids of clay, organic matter and sesquioxides (Gobran et al., 2000). Conversely, the soil solid phases contain metals exchangeably bound to charged surfaces, complexed with organic matter, in hydrated oxides of Fe and Mn, as precipitates (carbonates, phosphates, sulfides) or as structural components in minerals (Gobran et al., 2000). The behavior and fate of heavy metals in soils depends on numerous physicochemical processes: a) dissolution, b) sorption, c) complexation, d) migration, e) precipitation, f) occlusion, g) diffusion into minerals, h) binding by organic substances, i) absorption and sorption by microbiota and j) volatilization. These processes are certainly affected by soil properties, such as cation exchange capacity (CEC), pH, redox potential and texture. Moreover, the fate of metals accumulated in soils is subjected to a number of mechanisms: leaching, plant uptake, erosion, or deflation, which would conduct to metal depletion. However these processes are very slow and thus the persistence of trace metals in soil appears to be practically permanent. Calculated half-lives of trace metals in soils are in the order of several hundred years, indicating that the complete removal of metallic contaminants from soils is nearly impossible. Long persistence together with toxicity and bioaccumulation make heavy metals a threat for the environment and living organisms (Kabata-Pendias, 2011).

Exposition to heavy metals may occur in several ways by oral, dermal or inhalation route. For instance, drinking water sources can be polluted by heavy metals. Moreover, plants growing on heavy metal polluted soil or exposed to heavy metals through the uptake of polluted water may result contaminated, endangering the food chain. Likewise, absorption through skin owing to direct contact with polluted soil is another potential source of heavy metal exposition. Motor vehicle emissions are a major source of airborne contaminants as well. As heavy metals are hard to metabolize they accumulate in living organisms causing detrimental effects. The toxicity exerted by heavy metals is mostly the result of the interaction with biomolecules (*e.g.* proteins, enzymes, nucleic acids) interfering with their normal functioning. Exposure to heavy metals can have carcinogenic, nervous system, immune system and circulatory effects (Kabata-Pendias, 2011). Table 1.2 and Table 1.3 sum up further characteristics of Cu, Pb, Zn, which are the representative heavy metals subject of the present thesis.

Table 1.2 Selected properties of heavy metals of concern

Parameter	Copper (Cu)	Lead (Pb)	Zinc (Zn)
Atomic number	29	82	30
Atomic weight	63.54	207.20	65.38
Atomic radius [a] (pm)	60-91	181	153
Oxidation state [b]	+1, +2	+2, +4	+2
Density (kg m^{-3})	8920	1135	7130
Mean Background on Surface Soils [c] (mg kg^{-1} soil)	39	27	70
Maximum Allowable Concentration [d] (mg kg^{-1} soil)	60-150	20-300	100-300
Trigger Action Value [e] (mg kg^{-1} soil)	60-500	50-300	200-1500

[a] Approximate average values for the main oxidation states.
[b] Valence values in bold are for main oxidation states.
[c] World soil average calculated as the mean values for various soils of different countries.
[d] Values most commonly reported in the literature, compiled by Kabata-Pendias (2011).
[e] Values proposed in some European countries, compiled from various sources by Kabata-Pendias (2011).
Adapted from Kabata-Pendias (2011).

Table 1.3 Selected properties of heavy metals of concern, related to plant physiology and phytotoxicity

Parameter	Copper (Cu)	Lead (Pb)	Zinc (Zn)
Deficient (mg kg^{-1}) [a]	2-5	-	-
Sufficient or Normal (mg kg^{-1}) [a]	5-30	5-10	27-150
Excessive or Toxic (mg kg^{-1}) [a]	20-100	30-300	100-400
Function in plants	Essential element. Constituent of oxidases, plastocyanins. Possesses a role in: Cell wall metabolism Photosynthesis and respiration Carbohydrate and nitrate metabolisms Water permeability Reproduction Disease resistance	Non-essential element for plants	Essential element. Constituent of anhydrases, dehydrogenases, proteinases, peptidases, and phosphohydrolases. Possesses a role in: Metabolism of carbohydrates, proteins, phosphates, auxins, RNA, and ribosome formations. Membrane permeability Cellular components stabilization Dry and hot weather resistance Bacterial and fungal disease resistance.

(Continued on next page)

Table 1.3 Selected properties of heavy metals of concern, related to plant physiology and phytotoxicity (continued)

Parameter	Copper (Cu)	Lead (Pb)	Zinc (Zn)
Reported mechanisms responsible of metal phytotoxicity	Tissue damage and elongation of root cells Alteration of membrane permeability, causing root leakage of ions (*e.g.*, K^+, PO_4^{3-}) and solutes Peroxidation of chloroplast membrane lipids and inhibition of photosynthetic electron transport Immobilization of Cu in cell walls, in cell vacuoles, and in non-diffusible Cu-protein complexes Damage to DNA, and in consequence, inhibition of photosynthetic processes	Inhibition of respiration and photosynthesis due to the disturbance of the electron transfer reaction. Destruction of the plasmalemma, which, in effect, disturbs the permeability for water and leads to impaired plant growth.	They are likely to be similar to those reported for other trace metals. However, Zn is not considered to be highly phytotoxic
Symptoms of metal phytotoxicity	Dark green leaves followed by induced Fe chlorosis Thick, short, or barbed-wire roots Depressed tillering Changes in lipid content Losses of polypeptides involved in photochemical activities	Dark green leaves Wilting of older leaves Stunted foliage Brown short roots	Chlorotic and necrotic leaf tips Interveinal chlorosis in new leaves Retarded growth of entire plant Injured roots resemble barbed wire

[a] Approximate concentrations of trace elements in mature leaf tissue generalized for various species (mg kg^{-1}, on fresh weight basis)
Adapted from Kabata-Pendias (2011)

1.1.4.2. Total petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) is the term used to describe a large family of heterogeneous compounds that are found in crude oil and whose main chemical constituents are carbon and hydrogen atoms. As they exist as a mixture of so many different compounds is more practical to quantify them in environmental samples as a group of congeners rather than separately. TPH can be divided into groups (fractions) of petroleum hydrocarbons that act alike in the soil or water. It can be distinguished two main fractions: aromatics and aliphatics, which in turn, can be subdivided in additional groups containing individual compounds with carbon chains of different length (Todd et al., 1999).

The use of petroleum-based products (*e.g.* gasoline, kerosene, fuel oil, mineral oil, and asphalt) for human purposes, which is mainly related to the use of fuels for transportation, heating and power-generation, proves indispensable in modern life. However, as the number of facilities, individuals, and processes as well as the various ways in which the products are stored and handled is so diffuse, contamination of the environment by them is not uncommon (Osuji and Onojake, 2006; Russell et al., 2009). For instance, TPH can enter the environment from industrial releases, through accidental spills or leaks from containers, or as byproducts from commercial or private uses. TPH entering the environment can affect all environmental compartments: water, air, and soil (Wang et al., 2014b). When TPH is released to water, light TPH fractions will float forming thin surface films, while heavier TPH fractions will accumulate in the sediment at the bottom of the water (Ou et al., 2004). In addition, some TPH compounds released to the soil may evaporate into the air while others may move downwards, dissolve into the groundwater and move away from the release area (Teng et al., 2013). Other TPH compounds may attach to particles in the soil staying for a long period of time.

TPH are organic compounds susceptible to biodegradation. They are used as a source of energy for microorganisms obtaining carbon dioxide, water, and microbial biomass as final products. TPH metabolism by soil and water microorganisms (bacteria and fungi) represents one of the primary mechanisms that allows TPH dissipation from the environment. TPH compounds exhibit different susceptibility to microbial degradation, but in general it occurs in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. The n-alkanes, n-alkyl aromatics, and the aromatics in the C_{10}-C_{22} range are the most readily biodegradable; n-alkanes, n-alkyl aromatics, and aromatics in the C_5-C_9 range are biodegradable at low concentrations by some microorganisms, but are generally volatilized; n-alkanes in the C_1-C_4 ranges are biodegradable only by a narrow range of specialized hydrocarbon degraders; and n-alkanes, n-alkyl aromatics, and aromatics above C_{22} are generally not available to degrading microorganisms. Hydrocarbons with condensed ring structures, such as polycyclic aromatic hydrocarbons (PAHs) with four or more rings, have been shown to be relatively resistant to biodegradation, while PAHs with only two or three rings (*e.g.*, naphthalene, anthracene) are more easily biodegraded

(Atlas, 1981; Leahy and Colwell, 1990). Environmental factors such as oxygen content, pH, temperature, water activity and nutrient concentrations, affect the rate of biodegradation of petroleum hydrocarbons by bacteria and fungi. Optimal parameters for biodegradation are summarized in Table 1.4.

Table 1.4 Optimal conditions for petroleum hydrocarbon biodegradation

Parameter	Optimal conditions
O_2 content	O_2 is essential for the oxidation catalyzed by oxygenase enzymes. (Anaerobic conditions lead to extremely low rates of biodegradation)
pH	Close to neutrality
Temperature	From 18 ºC to 30 ºC
H_2O activity	Within 50-70% of the water holding capacity. Excessive moisture will limit the gaseous supply of oxygen needed for the aerobic biodegradation.
Nutrients	Suitable supply of nutrients, mainly nitrogen and phosphorus.

(Atlas, 1981; Leahy and Colwell, 1990).

The release of TPH to the environment does not always lead to exposure and toxicity to human beings. This only occurs if coming in contact with the substance of concern. Moreover, and as for any toxic substance, toxicity effects on the individual depend on several aspects: (1) pathway of exposure (*i.e.* by oral, dermal or inhalation exposure), (2) time and number of exposures (acute, chronic), (3) dose and physical form of the substance and (4) individual factors (*e.g.* genetic background, sex, age, diet, lifestyle, overall health state). TPH exposition may arise from many sources. The general population may be exposed to gasoline fumes at the pump, spilled crankcase oil on pavement, chemicals used at home or work, or certain pesticides that contain TPH components as solvents. Other circumstances that may lead to a TPH exposition include breathing TPH compounds evaporating from a spill or leak, drinking contaminated water, children playing in contaminated soil (Edwards, 2014; Smargiassi et al., 2014). Moreover, occupations related to the extraction and refine of crude oil or to the manufacture of petroleum and other hydrocarbon products, result in an increased TPH exposition for the employees (Sahmel et al., 2013; Rushton et al., 2014).

The toxicity effects of TPH compounds will vary according the different compounds present in TPH fractions. For instance, n-hexane can cause a nerve disorder called *peripheral neuropathy* (Wang et al., 2014a). Similarly, compounds such as benzene, toluene, and xylene, can affect the human central nervous system (Proctor et al., 2014). Moreover it has been determined that benzene is carcinogenic to humans and other TPH compounds or petroleum products, such as benzo(a)pyrene and gasoline, are considered to be probably and possibly carcinogenic to humans (Rushton et al., 2014). It has been reported that swallowing some petroleum products such as gasoline and kerosene causes irritation of the throat and stomach, central nervous system depression, difficulty

breathing, and pneumonia from breathing liquid into the lungs (Gonullu et al., 2013). Certain TPH compounds can be irritating to the skin and eyes. Effects on blood, immune system, liver, spleen, kidneys, developing foetus, and lungs, have also been reported for particular TPH compounds (Bahadar et al., 2014).

1.2. Remediation technology: phytoremediation

1.2.1. Generalities about phytoremediation

Phytoremediation comprises a group of emerging biological remediation technologies that use plants to remove pollutants from the environment or to make them harmless (Salt et al., 1998). Plants can be used to partially or substantially remediate different media, such as soil, sludge, sediment, groundwater, surface water and waste water contaminated with a wide variety of inorganic and organic contaminants. Phytoremediation removal technologies imply the cleaning-up of the contaminated media, while phytoremediation containment technologies entail a reduction in the mobility, bioavailability and/or toxicity of the pollutant in the environment (Gobran et al., 2000).

Phytoremediation is based on natural physiological processes of plants that include water and nutrient uptake, translocation, accumulation, transpiration, gas exchange, photosynthetic metabolism and exudate release; which in turn, lead to different types of phytoremediation mechanisms that conduct contaminant remediation or containment (Tsao, 2003). These main phytoremediation technologies are phytostabilization, phytoextraction, phytodegradation, rhizodegradation, phytovolatilization and evapotranspiration, each of which are exploited in specific design applications to treat a certain environmental issue depending on the goal to be achieved, the type of contaminated media and pollutants of concern (Tsao, 2003). Table 1.5 summarizes the main characteristics of each phytoremediation technology. Major advantages reported for phytotechnologies, as compared to traditional chemical and physical remediation technologies (*e.g.* soil washing, chemical oxidation, air venting and sparging, electrokinetics, etc.), include relatively low cost, low maintenance, applicable to simultaneously remediate sites with mixed contaminants, less environmental impact, possible reuse of the treated soil and high public acceptance due to the inherently esthetic nature of planted sites. On the other hand, the main drawback is the longer restoration time that may be required to achieve cleanup goals (Susarla et al., 2002). Other limitations of phytotechnologies are related to the plant tolerance of contaminants, the disposal of plant wastes and the low bioavailability of pollutants to plants (Peralta-Videa et al., 2004; Sas-Nowosielska et al., 2004; Evangelou et al., 2007). In spite of these limitations, phytoremediation is a promising remediation technology, whose development is increasing since its emergence.

Table 1.5 Summary of phytoremediation technologies

Phytotechnology	Clean-up Goal	Mechanism of remediation	Type of contaminants
Phytostabilization	Containment	Contaminants are immobilized in the root zone through adsorption, absorption and precipitation processes.	Inorganic and organic
Phytoextraction	Remediation	Extraction of contaminants by plant roots and translocation to the above ground tissues.	Inorganic
Phytodegradation	Remediation	Uptake and transformation of contaminants by plant enzymes.	Organic (moderately hydrophobic compounds)
Rhizodegradation	Remediation	Metabolism of contaminants by rhizosphere microorganisms, whose growth and activity are supported by the release of plant root exudates.	Organic
Phytovolatilization	Remediation	Plants transform contaminants into more volatile and less polluting substances that are released to the atmosphere through transpiration.	Inorganic and organic (moderately hydrophobic compounds)
Evapotranspiration	Containment	Rain water interception, evaporation and plant transpiration that reduces contaminant infiltration.	Inorganic and organic (water soluble organics)

Adapted from Interstate Technology and Regulatory Cooperation (ITRC) Work Group, (2001).

1.2.2. Overview of phytoextraction and rhizodegradation

Phytoextraction and rhizodegradation are two types of phytoremediation technologies that can be used to clean-up contaminated soils with inorganic contaminants like heavy metals and organic pollutants such as TPH (Tsao, 2003). In phytoextraction, plants have a central role as heavy metals are taken up by plant roots and translocated to the above ground tissues (Salt et al., 1995). To enable heavy metal uptake it is necessary that the heavy metal is located at the vicinity of the roots or at the boundary between soil and root (Clemens et al., 2002). This contact is accomplished when the inorganic compound is dissolved in the transpirational stream that is then carried into the root zone and into the plant (Clemens et al., 2002). As a consequence of the extraction and storage of heavy metals by plants, soils could be remediated (Chaney et al., 1997). Differently to phytoextraction, in rhizodegradation plants have a secondary role in the dissipation of organic contaminants. The plant roots, through the release of root exudates, provide energy sources that support the growth of microorganisms in the rhizosphere *i.e.* the volume of soil influenced by the root and the colonizing microorganisms (Hiltner, 1904). The role of the rhizosphere is essential toward remediation purposes (Kuiper et al., 2004) and strongly depends on the processes occurring in this particular volume of soil (Hinsinger et al., 2006). The rhizosphere represents about 1-3 mm around the root surface and in this area plants, microorganisms, other soil organisms, soil structure and chemistry, all interact in a complex way (Lynch, 1990). Thus, in rhizodegradation, the clean-up goal is the remediation of soils through the degradation of organic contaminants by rhizosphere soil microorganisms, whose growth is enhanced by plants (Kuiper et al., 2004; Fan et al., 2008).

One of the limiting factors in both phytoremediation processes is the low bioavailability of pollutants in soils. Bioavailability is defined as the proportion of a chemical compound that is freely available to living organisms, thus able to cross the cellular membrane of the organism from the medium where the organism lives at a given time (Semple et al., 2004). In the context of phytoextraction heavy metals need to be bioavailable in order to be able to be taken up by plants. Similarly, in rhizodegradation organic pollutants must be bioavailable to soil microorganisms so that they can be metabolized. Chemical and biological strategies to increase bioavailability of pollutants with the aim to assist and improve the phytoremedial process are one of the key aspects of the present thesis.

1.2.3. Use of alfalfa in phytoremediation

Alfalfa (*Medicago sativa* L.) is a flowering perennial plant that belongs to the Fabaceae family. Its flowers vary in color from purple to yellow and are borne in loose clusters. Pods of alfalfa range from the sickle type to those that are twisted into spirals. Each pod contains several small kidney shaped seeds. Stems of alfalfa plants are erect and grow from a woody crown to about 1 m tall. New growth occurs from buds in the crown. The plant has a tap root system (it has a dominant central root from which other roots sprout laterally) which may penetrate deep (4-5 m.) into the soil. Leaves are alternately

arranged on the stem and are normally trifoliate (USDA, 2002). Figure 1.1 shows different parts of alfalfa plants.

Alfalfa grows best on deep, well drained, friable soils. Lands subject to frequent overflows or high water tables are unfavorable for alfalfa. The pH of the soil should be close to neutrality (6.8-7.5), as alfalfa is sensitive to acidic conditions. It is extensively grown throughout the world (mainly in United States, Canada, Argentina, Australia, Southern Europe, South Africa and the Middle East), being used principally as forage for cattle (USDA, 2002).

Alfalfa presents a number of remarkable characteristics for phytoremediation: 1) is a perennial plant with fast growth rates; 2) produces large biomass above and below ground (Coburn, 1912); 3) develops an extensive tap root system with considerable soil deep exploration ability; 4) establishes a vast niche for the development of rhizosphere microorganisms (Kirk et al., 2005); 5) associates with symbiotic Rhizobium bacteria allowing nitrogen fixation and letting alfalfa grow in soils with high C/N ratios (Truchet et al., 1991); 6) is a phreatophyte species, *i.e.* can draw water from a deep water table, which is especially useful for groundwater remediation through hydraulic control and 7) is widely distributed, well adapting to different climatic conditions. Over the past decade, there has been a widespread use of alfalfa in phytoremediation. Heavy metals like Cd, Cr, Cu, Ni and Zn (Peralta-Videa et al., 2002; Peralta-Videa et al., 2004; Bonfranceschi et al., 2009), petroleum hydrocarbons (Wiltse et al., 1998; Kirk et al., 2002), PAHs (Fan et al., 2008) or organochlorines (Li and Yang, 2013) have all been targeted by phytoremediation with this species. Moreover, recent findings have shown promising results for alfalfa phytoremediation of co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013). Table 1.6 reviews a number of phytoremediation experiments where alfalfa plants were used to deal with pollutants of different kinds in soils.

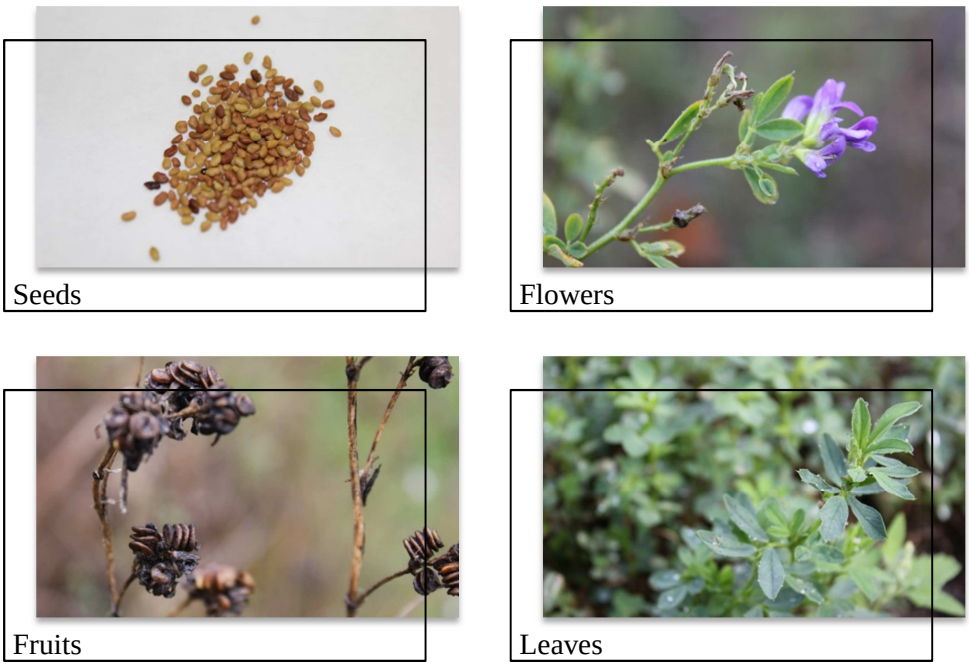


Figure 1.1 Alfalfa (*Medicago sativa* L.) plants

Table 1.6 Phytoremediation experiments with alfalfa

Soil Contaminants (mg kg^{-1})	Type and duration	Remarkable results	Reference
Cd: 50 Cu: 50 Ni: 50 Zn: 50	Photoperiod controlled conditions, 15 days	Alfalfa was able to take up elements from multi-metal contaminated soils following the sequence: Ni>Cd>Zn>Cu. Maximum shoot concentrations were 437, 202, 160, 105 mg kg^{-1} dry weight, respectively.	(Peralta-Videa et al., 2002)
Total petroleum Hydrocarbons: 31000	Growth room, 56 days	In the presence of alfalfa the number of total petroleum degraders and alkane degraders were increased (5 and 15-fold increase, respectively).	(Kirk et al., 2005)
Pyrene: 9.7, 49, 102, 199, 493	Greenhouse, 63 days	Bacterial and fungi counts were 5.0–7.5 and 1.8–2.3 times higher in alfalfa rhizosphere than in non-rhizosphere soil, respectively. The average removal of pyrene in the rhizosphere soil of alfalfa was 6% higher than that in the non-rhizosphere soil.	(Fan et al., 2008)
16 polycyclic aromatic hydrocarbons (PAHs): 1924, 106 Zn: 2086, 2745 Cd: 2.66, 2.14 Pb: 482, 673 Ni: 97.3, 102.3	Field, 4 years	Alfalfa cover alone did not affect total contaminant concentrations in soil. However, it was most efficient in improving the contamination impact on the environment (limiting water fluxed) and in increasing the biological diversity and abundance (microbial, fauna).	(Ouvrard et al., 2011)
Cu: not available Benzo[a]pyrene (B[a]P): 1, 10, 100	Greenhouse, 60 days	Microbial biomass and the degradation rate of B[a]P were enhanced in the presence of alfalfa. Degradation rates ranged from 39.8% to 86.0%.	(Ding and Luo, 2005)

(Continued on next page)

Table 1.6 Phytoremediation experiments with alfalfa (continued)

Soil Contaminants (mg kg^{-1})	Type and duration	Remarkable results	Reference
Pentachloronitrobenzene (PCNB): 10	Growth chamber, 20 days	Alfalfa was able to accumulate PCNB. PCNB degradation rates were 17.84-29.26% higher in the presence of alfalfa. The process of PCNB degradation was mainly through the biodegradation, which occurred concomitantly with phytoextraction in the presence of alfalfa plants. Several soil enzyme activities were increased following the planting of alfalfa.	(Li and Yang, 2013)
Hg: 10, 20, 30, 40 Trichloroethylene (TCE): 100, 200, 300, 400	Greenhouse, 21 days	Transgenic alfalfa expressing glutathione S-transferase and human P450 genes were more resistant to the toxic effects of Hg and TCE than nontransgenic plants.	(Zhang et al., 2013)

1.2.4. Chemically-assisted phytoremediation

Chemically-assisted phytoremediation refers to the addition of chemical amendments with the aim to improve the phytoremediation process. In the present thesis two particular types of soil amendments will be addressed: low molecular weight organic acids (LMWOAs) and surfactants. They will be succinctly introduced in the following section. Chapter 2 presents a more detailed description of chemically-assisted phytoremediation with such type of soil amendments.

1.2.4.1. Low molecular weight organic acids

LMWOAs are organic compounds containing a chain of a few carbon atoms and at least one acid functional group (–COOH, carboxylic group). They are weak acids presenting different acidic behaviors and as the carboxylic groups dissociate, the organic acid can carry one or more negative charges (McMurry, 2009). As a result of their acidic properties, organic acids can act as ligands binding metals and forming organometallic complexes. In LMWOA-assisted phytoextraction metal binding capacity of chelates is used to increase heavy metal uptake by plants.

Among LMWOAs citric acid (Table 1.7) is of particular interest. It has been reported to increase soil desorption of heavy metals like Cu, Cd and Pb as well as to enhance their uptake by several plant species (Chen et al., 2003; Gao et al., 2003; Quartacci et al., 2005; do Nascimento et al., 2006; Qu et al., 2011). Furthermore, citric acid enhanced soil desorption of organics like PAHs and organochlorine pesticides, and even their plant uptake (White et al., 2003; An et al., 2010; Gao et al., 2010a; Gao et al., 2010b;

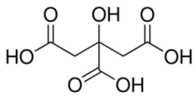
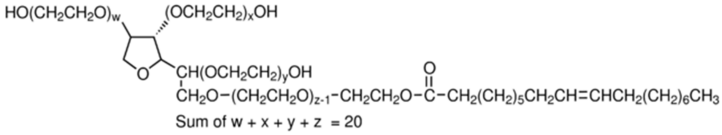
Mitton et al., 2012). Citric acid is the LMWOA that was used as representative compound in LMWOA-assisted phytoremediation experiments of the present thesis.

1.2.4.2. Surfactants

Surfactants are amphiphilic compounds that have both hydrophobic and hydrophilic groups in their molecular structure (Pletnev, 2001). One of the central characteristics of surfactants is their property to aggregate forming micelles in aqueous solution when the critical micelle concentration (CMC) is exceeded (McNaught and Wilkinson, 1997). This particular arrangement creates a spherical structure in which the hydrophilic part of the surfactant is in contact with the polar solvent, while the hydrophobic region of the molecule remains sequestered in the center avoiding the contact with the hydrophilic medium. A distinctive feature of surfactants when arranged in these clusters is that the non-polar central part of the micelle can interact with hydrophobic organic compounds increasing their water solubility. As a result, surfactants can increase the bioavailability of hydrophobic compounds, property that has been used in surfactant-enhanced phytoremediation (Gao et al., 2007).

Tween® 80 (polyethylene glycol sorbitan monooleate, Table 1.7) is a non-ionic surfactant that has been shown to increase soil desorption of organochloride pesticides (Gonzalez et al., 2010), as well as to enhance plant uptake (Gao et al., 2008) and removal of PAHs (Cheng et al., 2008) and petroleum hydrocarbons (Adetutu et al., 2012) from soils. Moreover, Tween® 80 has been recently used to assist the phytoremediation of soils co-contaminated with Cd and benzo[a]pyrene (Sun et al., 2013). Tween® 80 is the model surfactant that was used in surfactant-enhanced phytoremediation experiments of the current thesis.

Table 1.7 Chemical characteristics of citric acid and Tween® 80

Soil amendment	Citric Acid	Tween® 80
Amendment type	Low molecular weight organic acid	Non-ionic surfactant
Molecular formula	$C_6H_8O_7$	$C_{64}H_{124}O_{26}$
Molecular weight (g mol^{-1})	192	1310
CMC (mM)	-	0.010
Chemical Structure		

Tween® 80: polyethylene glycol sorbitan monooleate
CMC: critical micelle concentration at 25°C
(Mukerjee and Mysels, 1971; Morrison and Boyd, 1983)

1.2.5. Biologically-assisted phytoremediation

Another strategy that can be used alone or in combination with phytoremediation relies on the introduction of microorganisms to polluted soils. Bioaugmentation improves the biodegradative capacities of contaminated sites by the introduction of single strains or consortia of microorganisms with desired catalytic capabilities, and thus competent for the degradation of the pollutants of concern (Mrozik and Piotrowska-Seget, 2010).
Bioaugmentation can be done through various alternatives: a) addition of exogenous microorganisms, b) reinoculation of soil with indigenous microorganisms and c) selection of appropriate microorganisms from sites with similar contaminants (Lebeau, 2011). In addition to bioaugmentation with single strains it is also possible to use a consortium of microorganisms. This strategy may be more effective than the application of particular individual strains by the fact that intermediates of a catabolic pathway of one strain may be further degraded by other strains possessing suitable catabolic pathways (Bois et al., 2013; Huguenot et al., In Press).
The success of bioaugmentation depends on several biotic and abiotic factors which determine the possibility of maintaining a proper number and biomass of the introduced strains. Major factors affecting bioaugmentation are enumerated in Table 1.8.

Table 1.8 Factors influencing bioaugmentation

Biotic Factors	Abiotic Factors
Survival and growth	Temperature
Microbial interactions (competition, mutualism, symbiosis, predation) with indigenous microorganisms	Moisture content
Enzyme induction and activity	pH
Metabolic activity	Eh
Production of toxic metabolites from degradation compounds	Aeration
	Organic matter
	Availability of nutrients
	Availability of electron acceptors
	Amount and bioavailability of substrates and contaminants
	Soil type

(Lebeau, 2011)

Another crucial aspect in bioaugmentation is the method to deliver inoculants into soil. Inoculants can be relatively easily dispersed into surface soil introduced in liquid culture. However, it is difficult to ensure the delivery of the inoculants to subsurface environments as microorganisms may adhere to soil organic matter limiting an homogeneous distribution. To improve the delivery of inoculants several technologies with encapsulated or immobilized cells using carrier materials have been developed (Braud et al., 2006; Jézéquel and Lebeau, 2008).

Desired characteristics for microorganisms to be used in bioaugmentation include: a) fast growth, b) easy culturable, c) ability to tolerate high concentrations of pollutants and d) ability to survive in different environmental conditions (Mrozik and Piotrowska-Seget, 2010).

In a previous study, Bento et al. (2005) have reported that bioaugmentation was the most effective method, as compared with biostimulation and bioattenuation, in the removal of light fraction (C_{12}-C_{23}) of petroleum hydrocarbons. Among microorganisms used in bioaugmentation, *Pseudomonas aeruginosa* is a gram negative bacteria that has been used to assist the remediation of diesel oil and crude petroleum-oil hydrocarbon contaminated soils (Ueno et al., 2006; Das and Mukherjee, 2007). This bacteria exhibits several characteristics that make it suitable for bioaugmentation. One of the main features of this strain is its ability to produce surfactants, which render organic pollutants more accessible and more easily degradable as a result (Zhang et al., 2012). Moreover, it is widely found in contaminated environments, can be easily isolated and cultured and it shows rapid growth as well (Zhang et al., 2012). Because of all the above mentioned attributes, *P. aeruginosa* was chosen to be employed in biologically-assisted phytoremediation experiments of the present thesis.

1.3. Objectives

The major objectives of this research project are: a) to investigate the potential of alfalfa plants for the phytoremediation of soils co-contaminated by heavy metals and petroleum

hydrocarbons and b) to study chemical and biological strategies to assist the phytoremediation process.

Particular objectives are to determine the extent to which alfalfa can tolerate a co-contaminated soil and whether it contributes to the remediation of pollutants through the phytoextraction of heavy metals and the rhizodegradation of petroleum hydrocarbons.

In the context of chemically-assisted phytoremediation, this thesis examines alfalfa tolerance to two types of soil amendments, namely citric acid and Tween® 80, as well as the way in which they influence the phytoremediation process, when applied individually and in combination.

Finally, an approach of biologically-assisted phytoremediation is also assessed. This study seeks to ascertain the role of bioaugmentation with *Pseudomonas aeruginosa* in the remediation process, with and without the presence of alfalfa vegetation.

1.4. Novelty of the project

Although it is not uncommon that metallic and organic contaminants are present together in polluted sites, environmental research has tended to focus on the remediation of single pollutants rather than tackling multiple contaminants. The high occurrence of co-contamination in soils highlights the need to develop adapted remediation strategies. In this context, phytoremediation is not only an environmentally friendly alternative to traditional remediation technologies, but also a feasible strategy for the remediation of multiple pollutants when present simultaneously. Although in the past years the study of phytoremediation in heavy metal or organic contaminated soil has been widely studied (Salt et al., 1995; Cavallini et al., 1999; Gao and Zhu, 2004; Kim et al., 2004; Kathi and Khan, 2011), less information is available regarding phytoremediation of sites co-contaminated with metal and organic pollutants. Furthermore, there is currently a lack of evidence on using the combination of phytoextraction and rhizodegradation to treat soils both contaminated with heavy metals and petroleum hydrocarbons.

There are several important areas where this study makes an original contribution to phytoremediation with alfalfa species. In the past years, alfalfa has been used to target multiple pollutants in phytoremediation (Peralta-Videa et al., 2002; Kirk et al., 2005; Fan et al., 2008; Li and Yang, 2013). However, only a few studies have focused on co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013), while no previous study has specifically targeted heavy metal and petroleum hydrocarbon phytoremediation with alfalfa. In addition, no research has been found that investigated the phytotoxicity of different levels of citric acid and Tween® 80 on alfalfa species, nor their role in assisting alfalfa phytoremediation of co-contaminated soils. Moreover, there is a lack of information in what respects to comparative studies contemplating bioattenuation, bioaugmentation and phytoremediation.

As a result, this study aims to contribute to the knowledge of phytoremediation of co-contaminated soils, which is a growing area of research, by exploring the potential of alfalfa species as well as the possibilities of chemically and biologically assisted phytoremediation.

1.5. Structure of the thesis

The overall structure of the present thesis takes the form of eight chapters and one appendix.

The first chapter begins by laying out the research context. A brief review on contaminated soils with a special focus on the French situation is described. Heavy metals and TPH are presented as the pollutants of concern and phytoremediation technologies are proposed as a biological remediation approach, with a particular interest on alfalfa species. Chemically- and biologically-assisted phytoremediation are introduced as strategies to improve the phytoremediation process. At the end of the chapter the objectives as well as the original aspects of the thesis are stated.

Chapter two presents a bibliographic research focused on two types of biodegradable soil amendments: low molecular weight organic acids and surfactants, evaluating the feasibility of their application in the frame of assisted phytoremediation.

The following four chapters of the thesis comprise the findings of the research through experiments at laboratory scale.

Chapter three examines the potential of alfalfa for the phytoremediation of a soil co-contaminated by heavy metals (Cu, Pb and Zn at 76, 100 and 98 mg kg^{-1} soil dry weight (DW), respectively) and petroleum hydrocarbons (TPH at 8400 mg kg^{-1} DW). The results of this experiment reveal low tolerance of alfalfa towards this soil, limited phytoextraction ability and only an initial enhancement of rhizosphere microbiological indicators, favorable for rhizodegradation. With the aim to improve the phytoremediation process by alfalfa, two approaches are adopted, namely chemically and biologically assisted phytoremediation. These findings are presented in the subsequent chapters.

The fourth and fifth chapter deal with chemically assisted phytoremediation. Chapter four presents a preliminary study that evaluates the effects of citric acid and Tween® 80 on the development of alfalfa plants growing in a non-contaminated soil. This study supports the feasibility of using these chemical amendments in assisted phytoremediation with alfalfa, which is assessed afterwards in chapter five. In the study presented in this chapter, citric acid and Tween® 80 are applied (individually and in combination) to a soil co-contaminated by heavy metals (Cu, Pb and Zn at 87, 100 and 110 mg kg^{-1} soil dry weight (DW), respectively) and petroleum hydrocarbons (TPH at 3600 mg kg^{-1} DW), vegetated with alfalfa. This experiment demonstrates an improved tolerance of alfalfa plants towards this soil. Although the application of soil amendments appears not to improve metal phytoextraction, it further promotes microbial number and activity in the rhizosphere of alfalfa indicating a potential for rhizodegradation.

The sixth chapter presents the findings of biologically-assisted phytoremediation, comparing several biological strategies (*i.e.* natural attenuation, phytoremediation, bioaugmentation and the combination of phytoremediation and bioaugmentation) for the remediation of a co-contaminated soil (Cu, Pb, Zn and TPH at 87, 100, 110 and 3600 mg kg^{-1} DW, respectively). Soil bioaugmentation demonstrates to have a growth promoting effect on alfalfa, while in general, it does not improve total uptake of heavy

metals by plant shoots. The highest soil TPH removal rates are obtained through the joint action of bacteria and plants in the treatment that combines phytoremediation and bioaugmentation. The findings presented in chapter seven complement chapter six, reporting the results of several parameters (*i.e.* biomass, maximum quantum yield of photosystem II (PSII) and plant content of chlorophyll, flavonols and malondialdehyde) to evaluate physiology of alfalfa growing in a bioaugmented co-contaminated soil. In addition, these parameters are also studied in a non-contaminated agricultural soil.

The final chapter draws upon the entire thesis, principally overviewing and comparing the findings obtained from chapter three to seven. The implications of such findings are discussed and an overall conclusion is presented. This chapter concludes with final considerations (*i.e.* phytoremediation at different scales, phytomanagement of contaminated soils, legislative issues and exposure risk in relation with phytoremediation) and future perspectives.

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Chapter 2

Enhanced phytoremediation: a review of low molecular weight organic acids and surfactants used as amendments

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Abstract

The contamination of soils with inorganic and organic pollutants is a diffuse environmental issue of significant relevance. Phytoremediation has been proposed as an economically feasible and sustainable remediation technology even if low bioavailability of contaminants constitutes one of the main limitations restricting the success of phytotechnologies. To overcome this constraint the addition of biodegradable amendments has been recently proposed in alternative to synthetic ones. This paper presents an overview of two types of biodegradable soil amendments: low molecular weight organic acids and surfactants, evaluating the feasibility of their application in the frame of soil remediation throughout enhanced phytoremediation.

Keywords

Phytoextraction, rhizodegradation, soil remediation, heavy metals, polycyclic aromatic hydrocarbons, organic amendments.

2.Enhanced phytoremediation: a review of low molecular weight organic acids and surfactants used as amendments

2.1. Introduction

The contamination of soil resources with heavy metals and organic contaminants originates either from natural and anthropogenic sources representing a global environmental issue of great concern.

Heavy metal is the generic term to refer to a group of metal and metalloids with atomic density greater than 4000 kg m^{-3}. Even though some of them are essential micronutrients for both animal and vegetal, at higher concentrations they can lead to severe poisoning. For instance, Co, Cu, Mo, Ni and Zn, are essential trace elements for plant growth, while other elements such as Cd, Hg and Pb demonstrate no apparent function for plants (Cavallini et al., 1999; Lasat, 2002; Ait Ali et al., 2004). However, all of them exhibit toxicity to living organisms above a threshold concentration which depends on the metal, the living organism and the physicochemical properties of the considered soil. Moreover, metal speciation which refers to the distribution between the various chemical species in which metals can be found (Tessier et al., 1979; National Research Council, 2003), determines metal bioavailability, which in turn influences the toxic effects on biological systems (van Hullebusch et al., 2005). Speciation affects the mobilization pattern of trace elements in the environment as well (Alloway, 1995). Many remediation technologies have been developed to treat heavy metal contaminated media (Hashim et al., 2011). Although heavy metals are persistent contaminants which cannot be biodegraded, they can be treated by phytoremediation technologies such as phytoextraction or phytostabilization (Lasat, 2002).

Among organic contaminants, polycyclic aromatic hydrocarbons (PAHs) are critical pollutants. PAHs are chemical compounds made up of more than two fused aromatic rings in a linear or clustered arrangement, usually containing only carbon and hydrogen atoms (Agency for Toxic Substances & Disease Registry, 1996). These compounds have low water solubility, high melting and boiling points and low vapor pressure (Clar, 1964). PAHs arise in the environment from natural (*e.g.* forest fires and volcanic eruptions) and anthropogenic (*e.g.* vehicular emissions, residential wood burning, petroleum catalytic cracking, and industrial combustion of fossil fuel) sources (Medeiros et al., 2005; Wilcke, 2007; Boitsov et al., 2009). These pollutants are of great significance because of their adverse health effects *i.e.* toxicity, mutagenicity and carcinogenicity (Mumtaz and George, 1995). Although PAHs in soil may undergo volatilization, photolysis, plant uptake and soil sorption processes, microbial degradation constitutes their major dissipation pathway as they can be used as a carbon source by microorganisms (Joner et al., 2001; Haritash and Kaushik, 2009). Thus, fungi and bacteria can metabolize hydrocarbons and complete their mineralization to carbon dioxide and water or at least transform these pollutants into harmless products (Atlas, 1981). The ability of microorganisms to degrade hydrocarbons leads to the possibility

of using biological methods (*e.g.* bioremediation, phytoremediation) to remediate hydrocarbon contaminated media (Thapa et al., 2012).

Even though heavy metals are often associated with organic pollutants in contaminated soils, these multiple pollution situations and its remediation have been poorly studied. Phytoremediation is one of the remediation technologies that could be used to deal with these contaminants when they are present individually or collectively in co-contaminated sites (Roy et al., 2005; Ouvrard et al., 2011; Chigbo et al., 2013; Hechmi et al., 2013; Sung et al., 2013). The basic definition of phytoremediation is the use of plants to partially or substantially remediate contaminated media (Salt et al., 1998). Phytoremediation removal technologies imply the cleaning-up of the contaminated media, while phytoremediation containment technologies entail a reduction in the mobility, bioavailability and/or toxicity of the pollutant in the environment. Phytoremediation is based on natural physiological properties of plants that include: water and nutrient uptake, translocation, accumulation, transpiration, gas exchange, photosynthetic metabolism and exudate release, which in turn lead to different types of phytoremediation mechanisms that conduct contaminant remediation or containment (Tsao, 2003). These main phytoremediation technologies are: phytostabilization, phytoextraction, phytotransformation, rhizodegradation, phytovolatilization and evapotranspiration, each of which are exploited in specific design applications to treat a certain environmental issue depending on the goal to be achieved, the type of contaminated media and pollutants of concern (Tsao, 2003). In particular, phytoextraction and rhizodegradation can be used to clean-up contaminated soils with inorganic and organic contaminants, respectively. In phytoextraction, plants have a central role as heavy metals are taken up by the roots, translocated and accumulated in the above ground tissues (Salt *et al.*, 1995; Marques *et al.*, 2009). Several processes are involved during heavy metal phytoextraction, including: mobilization and uptake from the soil, compartmentation and sequestration within the root, xylem loading and transport, distribution between metal sinks in the aerial parts, and finally sequestration and storage in leaf cells (Clemens et al., 2002). As a consequence of these processes carried out by plants, heavy metal contaminated media could be remediated (Chaney et al., 1997). In contrast to phytoextraction, in rhizodegradation, plants have a secondary role in the dissipation of organic contaminants (Gerhardt et al., 2009). The plant roots, through the release of root exudates, provide energy sources that support the growth of microorganisms in the rhizosphere *i.e.* the volume of soil influenced by the root and the colonizing microorganisms (Hiltner, 1904). The rhizosphere represents about 1-3 mm around the root surface and in this area plants, microorganisms, other soil organisms, soil structure and chemistry, all interact in a complex way (Lynch, 1990). Thus, in rhizodegradation, the clean-up goal is the remediation of soils through the degradation of organic contaminants by rhizospheric microorganisms, whose growth is enhanced by plants (Kuiper et al., 2004; Fan et al., 2008).

Major advantages reported for phytotechnologies, as compared to traditional chemical and physical remediation technologies (*e.g.* soil washing, *in situ* chemical oxidation, air venting and sparging, electrokinetics, etc.), include: relatively low cost, low

maintenance, aptness to remediate extended areas of moderately contaminated soil, suitability to simultaneously remediate sites with mixed contaminants, low environmental impact, possibility to reuse the treated soil and high public acceptance due to the inherently esthetic nature of planted sites. On the other hand, the main drawback is the longer restoration time that may be required to achieve cleanup goals (Susarla et al., 2002). Other limitations of phytotechnologies are related to the plant tolerance to contaminants (Peralta-Videa et al., 2004), the disposal of plant wastes (Sas-Nowosielska et al., 2004) and the low bioavailability of pollutants (Evangelou et al., 2007).

Semple et al. (2004) define bioavailability as the proportion of a chemical compound that is freely available to living organisms, thus able to cross the cellular membrane of the organism from the medium where the organism lives at a given time. These authors also make the distinction between this term and the related one of bioaccessibility which encompasses not only what is actually bioavailable but also what would potentially be if the organism had access to the chemical. Bioavailability is influenced by many factors, such as contaminant type and concentration, the soil physicochemical characteristics and plant and microorganisms involved (National Research Council, 2003). Low bioavailability of contaminants in soils may restrict the success of the mentioned phytoremediation technologies and, as a result, many research attempts have been done in order to increase the ability of pollutants to be transferred from a soil compartment to plants or microorganisms to accomplish its accumulation and/or degradation.

One of the most diffused approaches to increase the bioavailability of heavy metals to plants and as a consequence to improve the phytoextraction efficiency, has been the application of synthetic chelating agents that render metals soluble in soil solution so that they can be uptaken by plants, *i.e.* chelate-assisted phytoextraction (Evangelou *et al.*, 2007; Meers *et al.*, 2008; Marques *et al.*, 2009). Nevertheless, the use of synthetic aminopolycarboxylic acids like ethylene diamine tetraacetic acid (EDTA), which has been widely used to assist phytoextraction of heavy metals (Epelde et al., 2008; Labanowski et al., 2008), is currently falling into disuse due to the poor biodegradability, leaching risks and high toxicity of such compounds (Evangelou et al., 2007). For these reasons, research on chelate-assisted phytoextraction tends to look for alternative compounds that combine high biodegradability, low phytotoxicity and chelating strength. In this context, natural low molecular weight organic acids (LMWOAs) were recently used to enhance phytoremediation of heavy metals (Chen et al., 2003; Quartacci et al., 2005; Evangelou et al., 2006; Han et al., 2006; Duquène et al., 2009; Qu et al., 2011). Another approach that has been used with the aim to increase the bioavailability of pollutants during phytoremediation is surfactant enhanced phytoremediation (SEPR) (Di Gregorio et al., 2006; Cheng et al., 2008; Wu et al., 2008a; Almeida et al., 2009; Gunawardana et al., 2010; Zhang et al., 2010). This strategy consists in the use of surfactants to increase the water solubility of organic contaminants and thus improve the mobility and biodegradation of pollutants throughout phytoremediation (Gao et al., 2007).

This article reviews concisely the main characteristics of LMWOAs and surfactants as well as their behavior and fate in the soil environment. Several experiments that

assessed desorption of contaminants from soil in the presence of LMWOAs and surfactants are pondered. Furthermore, recent studies of LMWOA- and surfactant-enhanced phytoremediation are reviewed and compared. Finally, toxicity effects of LMWOAs and surfactants towards plants within phytoremediation are considered as well. This review is mostly focused on the remediation of soils polluted with heavy metals and/or PAHs.

2.2. Low molecular weight organic acids

2.2.1. Organic acids at the soil-plant interface

LMWOAs are organic compounds containing a chain of a few carbon atoms and at least one acid functional group (-COOH, carboxylic group). They are weak acids presenting different acidic behaviors and as the carboxylic groups dissociate, the organic acids (OAs) can carry one or more negative charges. OAs are commonly found in all living organisms playing important roles not only in the energy production metabolism as intermediates in the tricarboxylic cycle but also in most cell metabolic pathways (McMurry, 2009).

At the soil-plant interface, the existence of OAs is the result of the balance of multiple processes that principally include the production and release by plants and microorganisms, the uptake and mineralization by soil microorganisms and the sorption-desorption to soil particles (Jones, 1998). Thus, soils represent a complex environment where OAs interact with plants, microorganisms and organo-mineral particles in an intricate way.

Although microbes are known to produce OAs, especially in situations where nutrients may be limiting (Takao, 1965; Carson et al., 1992), plant root exudates constitute the predominant input of OAs in rhizosphere soils. In this context, OAs are, with sugars and aminoacids, among the soluble compounds exuded by plant roots in the rhizosphere. Pinton et al. (2007) compiled a wide list of OAs released by plant roots, which included: acetic, aconitic, aldonic, ascorbic, benzoic, butyric, caffeic, citric, erythonic, ferulic, formic, fumaric, glutaric, glycolic, glyoxilic, lactic, malic, malonic, oxalacetic, oxalic, p-coumaric, p-hydroxybenzoic, piscidic, propionic, pyruvic, succinic, syringic, tartaric, tetronic, valeric and vanillic acids. Table 2.1 shows the chemical characteristics of some of these common aliphatic acids. Amounts as well as relative proportions of OAs released by plants are variable depending on plant species and physiological status (age, nutritional condition, stress factors) and influenced by the soil environment. Jones (1998) reviewed the soil solution concentration of OAs reported in the literature for different plant species finding in general, OA concentrations in the order of 0.5-10 μM. This author also pointed out that the experimental methodology used for the quantification of OAs may limit the understanding about the rhizosphere exudates released by plant roots. Many studies of root exudates are made from solution culture studies *i.e.* synthetic liquid culture medium, as it is easier to collect the root exudates in these conditions. However, roots grown in hydroponics may be morphologically and physiologically different from plants grown in natural soil. Moreover, the aeration,

microbial and nutrient statuses are also different in artificial aqueous media from real soil environments. For these reasons, many difficulties may arise when extrapolating results from this sort of studies to soils.

As a result of pKa values of OAs and the pH of the cytosol, which is close to neutrality, OAs exist in their dissociated form and are released in the rhizosphere mainly as organic anions. Thus, the exudation of OAs has little effect in the acidification of the rhizosphere (Gobran et al., 2000). The OA efflux occurs across the plasma membrane of root cells both by passive diffusion and membrane channel proteins following a favorable electrochemical potential gradient (Jones, 1998; Ryan et al., 2001). Moreover, the release of OAs as organic anions requires the presence of an accompanying counter ion to maintain the electrical neutrality. This can be achieved through the release of a cation or through the uptake of an anion together with the OA release. For instance, when malate is released from wheat (*Triticum aestivum*) roots K^+ is the accompaniment cation (Ryan et al., 1995). In the same way, K^+ has been shown to be the counter ion released with citrate by arabidopsis (*Arabidopsis thaliana*) (Murphy et al., 1999).

The exudation of OA by plant roots has been related to three main functions: nutrient deficiency, metal toxicity and anoxia (Ryan et al., 2001). It has been observed that plant roots release OAs under nutrient (*e.g.* Fe and P) deficiencies to improve the mobilization and uptake of nutrients. The release of OAs in these conditions increases the availability of nutrients for root uptake through the chelation of cations. For instance, citrate is released by roots of dicotyledonous plants grown in calcareous soils where Fe is in its insoluble form: ferric oxyhydroxides ($Fe(OH)_3$). Citrate can form a complex with Fe^{3+}, which is then reduced and uptaken by plants (Fox et al., 1996). As well, OA release (mainly citrate and malate) is one of the mechanisms used by plants to mobilize unavailable P. Although the total amount of P may be high in soils, only a little part of it is in a soluble form accessible to plants. For this reason, the release of OAs is crucial in the plant acquisition of P increasing its concentration in the soil solution by solubilizing minerals and desorbing P from mineral surfaces (Randall et al., 2001). Besides, OAs participate in the detoxification of metals like Al, which is known to inhibit the root growth of some plant species. For example, aconitic, citric, malic and oxalic acids are released by plant roots forming Al-OA complexes that prevent Al^{3+} rhizotoxicity through the chelation of Al ions in the rhizosphere, thus increasing root tolerance to Al (Delhaize et al., 1993; Pellet et al., 1995; Ma, 2000). Likewise, it has been observed that oxalic and malic acids could be important in alleviating phytotoxicity of rice plants under Cr stress (Zeng et al., 2008). In addition, it has been demonstrated that OAs also play a role in case of anaerobic stress. Under anoxia, roots change their metabolism from aerobic to fermentative. The lactic acid formed by this process is released to the rhizosphere avoiding its accumulation in the cellular metabolic pool as it may be toxic to cellular metabolism (Xia and Saglio, 1992).

Plants are known to have a positive effect on the survival of microorganisms (Bashan et al., 1995). The release of OAs into the root zone is known to enhance the development of rhizosphere bacteria, which can use these organic compounds as source of energy. OA uptake by microorganisms occurs via specific transporter proteins that are selective for either dicarboxylic or tricarboxylic acids. The decomposition of OAs follows

Michaelis-Menten kinetics with typically 60% of the OA mineralized to CO_2 and 40% incorporated into new cell biomass (Jones et al., 1996). OAs biodegradation in soils takes place at rates that may vary according to the OA type and soil environment. Ström et al. (2001) reported 33% and 30% biodegradation rates for malate and citrate respectively, after 24 h in a calcareous soil. In contrast, oxalate seemed to be resistant to microbial degradation (7% biodegradation rate) probably because of the formation of Ca-oxalate precipitates that limited its metabolism. Wen et al. (2009) studied the degradation rates of citric acid in soils with different physicochemical characteristics and contaminated with heavy metals, reporting, on average, a cumulative degradation of 69% for citric acid after 20 days. Soil properties played an important role in the degradation of citric acid: organic matter content, cation exchange capacity and pH were found to be positively associated with biomass carbon and thus citric acid degradation. This study also showed that the presence of Cd-contaminated soil inhibited citric acid degradation, and this effect was more pronounced in case of Cd and Zn co-contamination. Similarly, Brynhildsen and Rosswall (1997) observed that metal complexation affected the mineralization rates of citrate by mixed microbial communities from soil extracts. Interestingly, after 14 days about 80% of the free citric acid was degraded, while the degradation of citrate complexed with Zn, Cu or Co was almost totally inhibited, suggesting that the formation of complexes with metals exerted a protective effect on the mineralization of citric acid. These authors also reported that free citric acid mineralization rates in the soils under study varied between 51 and 67% after 36 days. In contrast, malate showed faster degradation rates as reported by Jones et al. (1996), who studied the kinetics and characteristics of malate degradation in four acidic soils. They reported a rapid breakdown of malate in all soils, with a half-life of approximately 1.7 h. Similarly, predicted half-life of malate in calcareous soils is approximately 3 h (Ström et al., 2001). Regarding soil properties influencing the interaction with OAs, it has been observed that OA sorption to the solid phase of soils is particularly high in surface horizons that are rich in Fe and Al oxyhydroxides and, as a result, this process may affect the degree of OA biodegradation (van Hees et al., 2003). Van Hees et al. (2002) studied the mineralization kinetics of citrate, oxalate and acetate in different soil horizons finding greater biodegradation rates in the surface organic horizons than in the deeper mineral ones. These differences were attributed to stronger sorption processes rather than lower microbial activity in the deeper horizons.

Apart from enhancing the development of bacteria already present in the rhizosphere, OAs can act as chemical signals that induce the movement of motile microorganisms towards the plant roots. For instance, it has been demonstrated that to form the symbiotic association between the legume soybean (*Glycine max*) and the soil bacteria *Bradyrhizobium japonicum*, dicarboxylic acids released by *G. max* roots play a key role acting as natural chemoattractants (Barbour et al., 1991).

As a result of the enhanced microbial activity in the rhizosphere it could be expected that the decomposition of OAs in the rhizosphere soil is faster than in the bulk soil. In this sense, Ström et al. (2001) found that malate decomposition rates in rhizosphere soil are 0.25-1-fold faster than in bulk soil. On the contrary, citrate and oxalate were

consumed at similar rates in rhizosphere and bulk soils. These differences may reflect the adaptation of rhizospheric microorganisms to the plant exudation pattern, since the uptake and mineralization of OAs by microorganisms is correlated with root exudation (Jones et al., 1996). In addition, a spatially and temporally heterogeneous pattern of OA turnover could be correlated with the oxygen release in the rhizosphere. In this sense, a recent study performed by Blossfeld et al. (2011) revealed that there are changing zones of production and consumption of OAs by anaerobic and aerobic microflora due to changes from hypoxic to oxic conditions on a micro-scale level within the rhizosphere of *Juncus* species.

Due to the central roles of plant exudates as suppliers of OAs in the rhizosphere, usually higher concentrations of OAs are found in the rhizosphere compared to those present in the bulk soil. Cieśliński et al. (1998), reported water extractable LMWOAs concentrations up to 953.6 μmol kg^{-1} in the rhizosphere of two cultivars of durum wheat (*Triticum turgidum* var. *durum*) grown in three different soils while no water extractable OAs were found in the bulk soil. Acetic and succinic acids were the predominant OAs among oxalic, fumaric, L-malic, tartaric, citric, propionic and butyric acids that were also found. Likewise, Ström et al. (2001) reported that the amounts of extractable OAs (aconitate, citrate, isocitrate, malate, oxalate) were significantly greater in the rhizosphere soil of maize (*Zea Mays*) relative to those in the bulk soil. In addition, Cieśliński et al. (1998) showed that LMWOAs quantity and composition varied with soil type, highlighting the great influence of chemical and biological properties of soils. According to these authors the differences in OA exudation found in different soils could be the result of different soil fertility levels which affect root growth, as well as of different rhizosphere soil microbe-root interactions.

Another aspect of OAs in the soil environment is their interaction with the soil solid phase, which is influenced by OA and soil chemical properties. The degree of association between OAs and soils solid phase relies on the charge of the OA, increasing with its valence. As a result the adsorption degree follows the sequence: monovalent < divalent < trivalent OAs (Jones and Brassington, 1998; Jones et al., 2003).

Table 2.1 Chemical characteristics of common aliphatic organic acids

Organic acid	Molecular Formula	Carbon chain length	Number of carboxylic groups	pK_{a1}	pK_{a2}	pK_{a3}
Formic	CH_2O_2	1	1	3.74	-	-
Acetic	$C_2H_4O_2$	2	1	4.74	-	-
Pyruvic	$C_3H_4O_3$	3	1	2.39	-	-
Lactic	$C_3H_6O_3$	3	1	3.86	-	-
Butyric	$C_4H_8O_2$	4	1	4.82	-	-
Oxalic	$C_2H_2O_4$	2	2	1.27	4.28	-
Succinic	$C_4H_6O_4$	4	2	4.19	5.64	-
Fumaric	$C_4H_4O_4$	4	2	2.02	4.39	-
Malic	$C_4H_6O_5$	4	2	3.40	5.11	-
Tartaric	$C_4H_6O_6$	4	2	3.03	4.37	-
Citric	$C_6H_8O_7$	5	3	3.13	4.76	6.40
Isocitric	$C_6H_8O_7$	5	3	3.29	4.71	6.40

(Kortüm et al., 1961; Serjeant and Dempsey, 1979; Morrison and Boyd, 1983)
pKa: negative logarithm of the acid dissociation constant, Ka for dilute aqueous organic acid solutions at 25°C

2.2.2. LMWOA-enhanced desorption of contaminants from soil

As a result of their acidic properties, OAs can act as ligands binding metals and forming organometallic complexes. OAs are able to form complexes with metals in various stoichiometric ways and structures. Those OAs that have more than one electron donor group, such as oxalic and citric acids, can form one or more rings when complexed with metals (Basolo and Johnson, 1964; Martell and Hancock, 1996). In these cases, the OAs can be termed chelating agents and the resulting complexes as metal chelates (Martell and Calvin, 1952). The main factors that influence the complexation process are the relative concentrations of OAs and metals, the pH and the stability constant of each metal-OA complex, as well as ionic strength and the presence of competing ions (Devêvre *et al.*, 1996).

In soils, chelating agents initially act complexing the metals that are solubilized in the soil solution. Therefore, the free-metal activity decreases causing a shift in equilibrium according to Le Chatelier's principle (Le Chatelier, 1884), which results in the dissolution of previously unavailable metals (Gobran et al., 2000). The process stops when the chelating agent is saturated, when there is no more metal in the solid phase or when the equilibrium solubility of the metal is achieved. In this way, chelating agents can increase the concentration of metals in the soil solution (Gobran et al., 2000).

Due to the chelating ability of LMWOAs, which can form soluble complexes with metal cations, it could be expected that LMWOAs affect the interaction of metals with soils, reducing their soil adsorption as a result (He *et al.*, 2005). In this way, LMWOAs have been tested in desorption experiments with heavy metal contaminated soils (Table 2.2). The influence of citrate and tartrate on Cu and Cd desorption from naturally and artificially contaminated soils was studied by Gao et al. (2003), finding that these OAs may have a dual behavior on metal desorption depending on its concentration. At low

concentrations, citrate and tartrate inhibited Cu and Cd desorption while this effect was reverted at higher concentration, which is in accordance with the behaviour of these two metals in acidic condition. These authors also demonstrated that metal desorption was affected by the pH and electrolyte condition. In the same way, Yuan et al. (2007) observed an enhanced desorption of Cu and Cd in the presence of OAs. Cu desorption was enhanced by citric, oxalic and tartaric acids while the desorption of Cd was only enhanced by oxalic acid. In all cases, the desorption effect was highly influenced by pH. The formation of ligand-metal complexes was one of the proposed mechanisms that contributed to the desorption of heavy metals from soils. Similarly, Chen et al. (2003) demonstrated that citric acid decreased Cd, and to a less extent Pb, adsorption to soils. This effect was attributed to a decrease of pH in the presence of citric acid. In other desorption experiment with OAs, Qin et al. (2004) studied the effect of citric, malic and acetic acids on Cd, Cu and Pb desorption from soils. However, in this study the pH appeared not to be the dominant factor governing the release of metals while LMWOAs demonstrated to play a dominant role. These authors found that metal desorption behavior was consistent with the stability constants of metal-LMWOA complexes and also related to the chemical structures (number of carboxylic groups) and acidic properties (pK_a) of LMWOAs. Finally, this study also highlighted the influence of soil properties such as pH, cation exchange capacity, organic matter and manganese oxide content on the amount of desorbed metals. Quartacci et al. (2005), found citric acid able to desorb Cd from soils as well. Although citric acid was less effective than other chelates, it showed a 3-fold increase in comparison to water. Similarly, Krishnamurti et al. (1997) studied the kinetics of Cd release from soils in the presence of various OAs (*i.e.* acetic, citric, oxalic, fumaric and succinic acids) showing that LMWOAs can influence the rate of Cd release from different soils increasing the solubility of Cd through the formation of soluble Cd-LMWOAs. Further desorption studies with citric acid demonstrated a 200-fold increase in U desorption from contaminated soils (Huang et al., 1998).

In addition to the role of promoting heavy metal desorption from soils, LMWOAs have been used in desorption studies with organic contaminants as well (Table 2.2). An et al. (2010) assessed the effect of OAs on the adsorption-desorption behavior of PAHs. Acetic, citric, lactic, oxalic and tartaric acids inhibited pyrene adsorption to soils while promoted its desorption to different extents and the most significant effects were observed for citric and oxalic acids. Moreover, recent experiments demonstrated that the addition of artificial root exudates has a positive effect on PAH desorption from spiked soils, and this effect may be mainly due to the presence of OAs in the exudates. Zhu et al. (2009) showed that when microorganisms are present, natural root exudates collected from the culture solution of *Z. mays* can promote the desorption of phenanthrene. Likewise, Gao et al. (2010a) studied the influence of artificial root exudates on phenanthrene and pyrene desorption finding differences according to the concentration of root exudates, the content of organic matter in soils and the ageing time. Similar results were obtained when testing the direct addition of citric, oxalic and malic acids, achieving the most significant results for citric acid on the phenanthrene desorption from soils with low organic matter contents and less aged soil (Gao et al., 2010b). To

explain the enhanced desorption of organic contaminants in the presence of LMWOAs the following mechanism has been proposed. Metal cations can act as "bridges" in organic matter-soil mineral complexes. However, the addition of LMWOAs can dissolve the metallic cations breaking the so called "bridges" and resulting in the release of soil organic matter (SOM) into solution phase (correlating with an increase in the dissolved organic matter (DOM) content) and also promoting the desorption of hydrophobic organic compounds like hydrocarbons (Gao et al., 2003; White et al., 2003; Ling et al., 2009; Gao et al., 2010b). To explain the different influences on hydrocarbon desorption among OAs, An et al. (2010) highlighted the importance of the chemical structure of OAs. In this sense, ternary OAs (*i.e.* citric acid) could provide more anions for complexing than other acids (*i.e.* acetic acid). As a result, ternary acids could be more efficient in desorbing metals and SOM from soils carrying more adsorbed hydrocarbons with them. The observation done by Gao et al. (2010a) supports this hypothesis, as they demonstrated that citric acid (ternary OA) had a stronger effect than oxalic acid (binary OA) on PAHs desorption from soil.

Other type of organic contaminant whose desorption from soil has been increased by LMWOAs are organochlorine pesticides. In this sense, White et al. (2003) tested six different LMWOAs at various concentrations for their ability to increase *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) desorption. Best results were obtained for citric acid at 2.75 mol kg^{-1} soil with a 58% desorption increase compared to water. Similarly, Luo et al. (2006) addressed the effects of oxalate and plant root exudates of *Z. mays*, *T. aestivum* and ryegrass (*Lolium rigidum*) on the desorption of *p,p'*-dichlorodiphenyltrichloroethane (*p,p'*-DDT) in soils. Oxalate increased *p,p'*-DDT desorption even at very low concentrations and similar increased desorption effects were observed in the presence of plant root exudates. These authors also reported that soil properties influenced the degree of desorption. A negative correlation was found between the amounts of *p,p'*-DDT desorption and the soil organic carbon content, thus suggesting that hydrophobic compounds like *p,p'*-DDT are likely to bind with soil organic carbon becoming less mobile. Oxalate and root exudates disrupted the soil structure, altering the organo-mineral linkages and resulting in the release of organic carbon and metal ions into the aqueous phase. As *p,p'*-DDT was complexed with this fraction of organic carbon, its desorption was enhanced. The same process was proposed by Gonzalez et al. (2010) to explain the increased desorption of the highly hydrophobic pesticides *p,p'*-DDT, *p,p'*-DDE and α-cypermethrin in the presence of citrate and oxalate in aged and freshly spiked soils.

As final point and in the context of the impact of antibiotics in the environment, Zhang and Dong (2008) studied the soil adsorption of norfloxacin in the presence of OAs. Increasing concentrations of citric, malic and salicylic acids (0-2.5 mM) decreased the adsorption of norfloxacin to soils. The authors proposed that the formation of soil-organic anion-Al complexes could inhibit the competitive adsorption of norfloxacin. In addition, the formation of complexes between organic anions and norfloxacin cations may also be a mechanism involved to explain the decreased soil adsorption of norfloxacin in these conditions.

For further information regarding the retention processes affecting organic contaminants in the soil environment, the reader can refer to Schwarzenbach et al. (2003) and Calvet et al. (2005).

Table 2.2 Relevant experiments of LMWOA enhanced desorption of contaminants from soils

Contaminant class	Contaminant (mg kg^{-1} soil)	LMWOA (mM)	Major desorption fold increases	Reference
Heavy Metals	Cd: 281 Pb: 518	CA: 1, 3	CA at 3 mM: 0.4 for Cd and 0.04 for Pb	(Chen et al., 2003)
	Cd: 197, 233, 258 Cu: 168, 245, 357	CA: 0,1-20 TA: 0,1-20	CA: 14.0 for Cd (at 197 mg kg^{-1}) and 34.7 for Cu (at 168 mg kg^{-1}) TA: 1.6 for Cd (at 197 mg kg^{-1}) and 6.4 for Cu (at 168 mg kg^{-1}) CA and TA at 20 mM	(Gao et al., 2003)
	Cd: 50, 100, 150, 200	CA: 1, 2	Average 3.0-fold increase.	(Quartacci et al., 2005)
Polycyclic Aromatic Hydrocarbons	Phe: 100 Pyr: 30	ARE[1]: 0-1000 CA: 0-1000 OA: 0-1000	ARE at 1000 mM: 2.2 for Phe and 1.6 for Pyr CA: 0.7 for Phe and 0.1 for Pyr [#] OA: 0.4 for Phe and 0.1 for Pyr [#] CA and OA at 100 mM. [#] Fold increases with respect to ARE at 100 mM	(Gao et al., 2010a)
	Phe: 100 Pyr: 30	CA: 0-1000 OA: 0-1000	CA: 0.9 for Phe and 0.5 for Pyr. OA: 0.7 for Phe and 0.3 for Pyr. CA and OA at 1000 mM	(Ling et al., 2009)

(Continued on next page)

Table 2.2 Relevant experiments of LMWOA enhanced desorption of contaminants from soils (continued)

Contaminant class	Contaminant (mg kg^{-1} soil)	LMWOA (mM)	Major desorption fold increases	Reference
Organo-chloride pesticides	*p,p'*-DDE: 0.3	CA: 1-100 MiA: 1-100 MoA: 1-100 OA: 1-100 SA: 1-100 TA: 1-100	CA: 0.6 MiA: 0.3 MoA: 0.4 OA: 0.5 SA: 0.2 TA: 0.3 LMWOAs at 50 mM	(White et al., 2003)
	p,p'-DDT: 2	NRE[2]: n.s Ox: 0.5-100	NRE from maize: 3.3, from wheat: 2.8 and from ryegrass: 6.7 Ox at 10 mM or higher: 3.9	(Luo et al., 2006)

Contaminants: *p,p'*-Dichlorodiphenyldichloroethylene (*p,p'*-DDE), *p,p'*-Dichloro diphenyl trichloroethane (*p,p'*-DDT), Phenanthrene (Phe), Pyrene (Pyr). LMWOAs: Acetic acid (AA), Citrate (Ci), Citric acid (CA), Malic acid (MiA), Malonic acid (MoA), Oxalate (Ox), Oxalic acid (OA), Succinic acid (SA), Tartaric acid (TA).
[1]ARE: Artificial Root Exudates. Solutions of 50 mM glucose, fructose and sucrose; 25 mM SA and Mia; 12.5 mM serine, arginine and cysteine.
[2]NRE: Natural Root Exudates collected from maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) or ryegrass (*Lolium rigidum* L.).
n.s.: concentration not specified.

2.2.3. LMWOAs enhanced phytoremediation

The ability of LMWOAs to form soluble compounds with metal cations can be used in phytoremediation to increase metal bioavailability and, as a result, improve phytoextraction rates. Plant metal uptake is more efficient when metals are in their soluble form in order to maximize the contact with the root cells so that they can be dissolved in the transpirational stream and carried into the plant (Clemens et al., 2002). Following the formation of the chelate-metal complex, metal uptake by plants may be achieved by different mechanisms that consist of: absorption of the free metal after its release from the chelating agent, absorption of intact chelate-metal complex, or exchange of metal between the chelating agent and a plant metabolic ligand (Gobran et al., 2000).

In the last years, there were several reports that studied the effect of LMWOAs on phytoremediation with the aim of using OAs to enhance the phytoextraction of heavy metals (Table 2.3). In this context, some authors have assessed the role of OAs in comparison to classic synthetic chelates. Evangelou et al. (2006) investigated the application of citric, oxalic and tartaric acids as an alternative to EDTA. Positive results were obtained for citric acid (62.5 mmol kg^{-1} soil), which showed a better performance

than EDTA (0.125 mmol kg^{-1} soil) in enhancing Cu uptake by tobacco (*Nicotiana tabacum*). Similarly, Quartacci et al. (2005) made a comparative study testing nitrilotriacetate (NTA) and citric acid to enhance Cd uptake by Indian mustard (*Brassica juncea*). The addition of amendments at 20 mmol kg^{-1} soil produced average increases of Cd shoot accumulation of 31% for NTA and 57% for citric acid. Moreover, do Nascimento et al. (2006) compared the addition of citric, gallic, oxalic and vanillic acids with the synthetic chelates EDTA and diethylendiaminepentacetic acid (DTPA) to enhance the phytoextraction of heavy metals by *B. juncea*. Citric and gallic acids enhanced the net removal of Cd, Ni and Zn at a similar rate than the synthetic chelates when applied at the same concentrations (10 mmol kg^{-1} soil). Thus, these authors concluded that OAs could be as efficient as synthetic chelates to enhance the phytoextraction of multi-metal contaminated soils. Conversely, some tested OAs seemed to be unsuitable to enhance phytoextraction of heavy metals from soils compared to synthetic chelates. For instance, citric, oxalic and tartaric acids (62.5 mmol kg^{-1} soil) did not increase Pb uptake by *N. tabacum* in contrast to EDTA treatment (0.125 mmol kg^{-1} soil), which enhanced Pb shoot concentration by more than 2-fold (Evangelou et al., 2006). Wu et al. (2003) found that in contaminated soils vegetated with *B. juncea* and amended with citric, malic and oxalic acids (3 mmol kg^{-1} soil) there was a negligible increase in soil solution concentrations of Cd, Cu, Pb and Zn compared to EDTA (3 mmol kg^{-1} soil), thus limiting the potential use of this kind of amendment to increase phytoextraction of heavy metals from soil.

An indirect role in enhancing heavy metal phytoextraction was attributed to LMWOAs as well. The ability of the organic fraction of municipal solid wastes (OFMSW) to enhance heavy metal (Cr, Cu, Ni, Pb, Zn) uptake by *Z. mays* was examined by Salati et al. (2010), reporting an increase in heavy metal shoot concentration from 23% to 302% depending on the heavy metal considered. Concomitantly, in the presence of OFMSW it was detected a 41.6-fold increase in soil DOM. Although the mechanism by which plants could uptake heavy metals bound to DOM is not fully understood, it is suggested that LMWOAs, which comprise DOM, can be taken up by plant roots along with the metals they have bound resulting in increased phytoextraction rates.

Some studies have investigated the influence of OAs on Cd uptake by plants. Interestingly, Cieśliński et al. (1998) studied the relationship between rhizosphere LMWOAs and Cd accumulation by two cultivars of *T. turgidum* that varied in their Cd accumulating ability. They found that Cd accumulation by high and low Cd accumulation cultivars of *T. turgidum* was correlated to the amounts of LMWOAs found in the rhizosphere soil of each cultivar. They proposed that the levels of LMWOAs influence the solubilization of particulate-bound Cd into soil solution and determine Cd phytoaccumulation as a result. Likewise, Han et al. (2006) verified that acetic and malic acids increased the uptake of Cd by *Z. mays* plants grown in hydroponics. In addition, these authors also studied the mechanisms underlying OA enhanced Cd uptake. It was hypothesized that Cd formed complexes with OAs in the root zone that could subsequently decompose and liberate Cd. Cd root uptake across the plasma membrane occurred probably mediated by Zn transporters. Moreover, plant response to elevated Cd levels involved the release of OAs by *Z. mays* roots, which

could complex Cd and act as a resistance mechanism to alleviate Cd toxicity. In addition, a similar mechanism for Cd uptake implicating the formation of Cd-citrate complexes followed by its dissociation within the diffusion layer and/or at the root surface was proposed by Panfili et al. (2009) to explain Cd uptake by *T. turgidum* var. *durum*. To explain the differences among OAs in enhancing heavy metal uptake by plants it is necessary to consider the binding constants of the OA-metal complexes. Han et al. (2006) found differences in the ability of acetic and malic acids to enhance Cd uptake by *Z. mays*, being acetic acid more effective. In this study, Cd uptake by plants appeared to occur after the dissociation of OA-metal complexes, which allowed the liberation of Cd. As the complex capacity of acetic acid with Cd is lower than that of malic acid, Cd release from labile Cd-acetic acid complexes was easier and could lead to a higher uptake of Cd than Cd-malic acid complexes.

In some cases, it has been observed that the main effect of OAs was not on the metal uptake but in the metal translocation from plant roots to shoots. Chen et al. (2003) demonstrated that citric acid increased the root to shoot translocation of Cd and Pb (1.4 and 1.9-fold increase in the translocation factors respectively) in radish (*Raphanus sativus*) plants grown in hydroponics, whilst their uptake rates decreased. Similarly, an hydroponic experiment with barley (*Hordeum vulgare*) conducted by Wu and Zhang (2002) showed that ascorbic acid increased shoot accumulation of Cd by enhancing its translocation from roots to the above ground tissues.

OAs have also been used as amendments in phytoremediation experiments dealing with U contaminated soils. Citric acid has shown to be efficient in enhancing plant uptake of U as demonstrated by Huang et al. (1998) who reported more than 1000-fold increase in U shoot accumulation by four plant species (*B. juncea, Brassica chinensis, Brassica narinosa*, and *Amaranth cruentus*) when soils were amended with citric acid at 20 mmol kg^{-1} soil. In addition, Duquène et al. (2009) reported, in the presence of citric acid at 5 mmol kg^{-1} soil, an increase in ^{238}U shoot concentrations of 3 and 5-fold for ryegrass (*Lolium perenne*) and *B. juncea* respectively. However, the increase in plant uptake has not always been found to be directly proportional to the increase in soil solution concentrations. In general, the increase in soil solution concentrations was higher than the increase in plant uptake in the presence of amendments.

Biodegradation rates of LMWOAs may limit the effectiveness of these compounds in assisting phytoextraction. Krishnamurti et al. (1997) analyzed Cd release from soils, noticing that Cd formerly forming part of Cd-LMWOA (*i.e.* acetic, citric, fumaric, oxalic and succinic) complexes was adsorbed onto negatively charged soil particles after LMWOA biodegradation. In addition they showed an increase of Cd release from the soils to the soil solution with the renewal of LMWOA application after every 2 hours. Meers et al. (2004) studied the timing of LMWOA application in a calcareous clayey soil vegetated with *Z. mays*. They tested the effects of several OAs (*i.e.* ascorbic, citric, oxalic and salicylic acids, and NH_4 acetate) on heavy metalphytoextraction at a dose of 2 mmol kg^{-1} soil, applying them to soils 1 day before sowing. In these conditions they observed no significant increase in Cd, Cu, Pb and Zn shoot uptake. As a result, they concluded that they would rather apply OAs soon before harvesting than

near the sowing time in order to overcome the biodegradation of OAs. According to Meers et al. (2008) the selection of the most suitable time to amend soils should be made taking into consideration phytotoxicity, plant uptake dynamics and attenuation of induced effects in the soil.

Apart from considering the exogenous application of OAs, it is also promising the direct role of plant-associated microbes because through the production and release of OAs they may increase heavy metal mobility for plant uptake and thus, improve phytoremediation. Through this bioaugmentation approach, microorganisms can improve phytoremediation not only directly acting as sources of OAs, which influences the metal uptake by plants but also indirectly by promoting shoot and root biomass, which influences total metal removal (Lebeau et al., 2008; Marques et al., 2009; Rajkumar et al., 2012; Bois et al., 2013; Yang et al., 2013). This approach was carried out by Chen et al. (2005) who determined the influence of bacteria inoculation on Cu uptake by *Elsholtzia splendens*. The addition of bacterial strains isolated from the rhizosphere of *E. splendens* enhanced Cu accumulation in plant shoots (up to 2.2-fold increase) and roots (up to 2.5-fold increase), and it was hypothesized that OAs excreted by bacteria could facilitate this process.

The potential of OAs in enhancing phytoremediation has been recently broaden to other pollutants rather than heavy metals. Mitton et al. (2012) studied the effect of carboxylic acids on the phytoremediation potential of organochlorine pesticides by willow (*Salix humboldtiana*). The combined addition of citrate and oxalate enhanced the bioavailability and hence plant uptake and translocation of *p,p'*-DDT, *p,p'*-DDE to the aerial tissues. It is hypothesized that LMWOA may cause an alteration of the soil matrix that may subsequently increase pollutant availability (White and Kottler, 2002). As a result, in the presence of these amendments, *S. humboldtiana* could be consider as medium-high accumulator of *p,p'*-DDT.

Regarding the phytotoxicity of OAs when used as amendments during phytoextraction protocols, different effects on biomass and visible toxicity symptoms, which varied according to plant type, OA and concentration used, have been reported. Certain adverse toxicity effects have been observed in the presence of LMWOAs. For instance, Evangelou et al. (2006) reported lower shoot dry weight and chlorosis in *N. tabacum* treated with citric, oxalic or tartaric acids at concentrations above 62.5 mmol kg^{-1} soil. In turn, do Nascimento et al. (2006) noticed visual symptoms of toxicity such as chlorosis and necrosis on *B. juncea* leaves when applying citric, gallic, oxalic or vanillic acids at 10 mmol kg^{-1} soil. Other deleterious effects include, as observed by Duquène et al. (2009), 45% reduction in water consumption rates and even *B. juncea* death after the addition of citric acid at 5 mmol kg^{-1}. On the contrary, it has also been reported neither phytotoxicity, nor decrease and still slight increase in biomass production in the presence of LMWOAs. For example, do Nascimento et al. (2006) observed no significant difference in the dry matter yield of *B. juncea* in the presence of citric, gallic, oxalic or vanillic acids at 10 mmol kg^{-1} soil. In the same way, Luo et al. (2005) observed no significant effects on *Z. mays* and white bean (*Phaseolus vulgaris*) biomass when treated with citric acid at 5 mmol kg^{-1} soil. Moreover, Qu et al. (2011) reported an

increase in the biomass of alfalfa (*Medicago sativa* L.) treated with sodium hydrogen phosphate/citric acid mixtures.

Table 2.3 Relevant experiments of LMWOA enhanced phytoremediation of heavy metals

LMWOA (mmol kg^{-1} soil)	Contaminant (mg kg^{-1} soil)	Media	Plant species	Duration/ Experiment type	Phytotoxicity/ Effect on plant biomass	Major fold increases in plant HM uptake	Reference
CA: 10 GA: 10 OA: 10 VA: 10	Cd: 50 Zn: 300 Cu: 200 Ni: 200 Pb: 500	Spiked soil	Indian mustard (*B. juncea*)	42 days/ Greenhouse	Chlorosis and necrosis on leaves. No significant difference in plant biomass.	CA: 9.3 for Cu in shoots. GA: 1.61 for Ni in roots.	(do Nascimento et al., 2006)
CA: 62.5 OA: 62.5 TA: 62.5	Cu: 225, 450 Pb: 300, 600	Spiked soil	Tobacco (*N. tabacum*)	21 days/ Greenhouse	Above 62.5 mmol kg^{-1}: chlorosis. Decrease in shoot biomass.	CA: 3.5 for Cu (at 450 mg kg^{-1}) in shoots	(Evangelou et al., 2006)
CA (0.05 - 0.37)/ Na_2HPO_4 mixtures	Cd: 75.3 Zn: 290.7 Cu: 31.8 Ni: 47.4 Pb: 398.3 As: 150.4 Cr: 182.7 Hg: 4.6 Mo: 711.0	Soil from Mo mine	Alfalfa (*M. sativa)*	30 days/ Greenhouse Outdoors	No phytotoxicity. Increase in plant biomass.	CA/ Na_2HPO_4 mixtures: 4.3 for Mo in shoots and 2.2 for Mo in roots (average results).	(Qu et al., 2011)

(Continued on next page)

Table 2.3 Relevant experiments of LMWOA enhanced phytoremediation of heavy metals (continued)

LMWOA (mmol kg^{-1} soil)	Contaminant (mg kg^{-1} soil)	Media	Plant species	Duration/ Experiment type	Phytotoxicity/ Effect on plant biomass	Major fold increases in plant HM uptake	Reference
CA: 5 NH_4-Citrate/CA: 2.5/2.5 OA: 5	Cd: 1, 2 Zn: 704, 151 Cu: 467, 209 Pb: 254, 35 Cr: 467, 209 U: 14, 41	Soils with industrial or natural contamination history	Indian mustard (*B. juncea*) Ryegrass (*L. perenne*)	44 days/ Greenhouse	For Indian mustard treated with CA: 45% reduction in water consumption, plant death. 27% decrease in shoot biomass.	CA: 4.5 for U in shoots of Indian mustard and ryegrass	(Duquène et al., 2009)
AA: 20 CA: 5, 10, 15, 20 MA: 20	U: 280, 750	Soil from industrial site	Indian mustard (*B. juncea*) Chinese cabbage (*B. chinensis*)	28 days/ Growth chamber	No data available.	CA (at 20 mmol kg^{-1}): 100 for U (at 750 mg kg^{-1}) in shoots of Indian mustard and Chinese cabbage.	(Huang et al., 1998)
CA: 10, 20	Cd: 50, 100, 150, 200	Spiked soils	Indian mustard (*B. juncea*)	37 days/ Growth chamber	No significant difference in shoot biomass.	CA (at 20 mmol kg^{-1}): 2 for Cd (at 200 mg kg^{-1}) in shoots of Indian mustard.	(Quartacci et al., 2005)

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Table 2.3 Relevant experiments of LMWOA enhanced phytoremediation of heavy metals (continued)

LMWOA (mmol kg^{-1} soil)	Contaminant (mg kg^{-1} soil)	Media	Plant species	Duration/ Experiment type	Phytotoxicity/ Effect on plant biomass	Major fold increases in plant HM uptake	Reference
CA: 0.5	Cd: 2 Pb: 10	Spiked water	Radish (*R. sativus*)	14 days/ Hydroponics	Plant growth improvement.	CA: 0.4 for Cd in shoots.	(Chen et al., 2003)
AA: 0.005-0.5 MA: 0.005-0.5	Cd: 0.6	Spiked water	Maize (*Z. Mays*)	2 days/ Hydroponics	No data available.	AA: 1.1 in roots, 0.85 in stems and 0.7 in leaves.	(Han et al., 2006)

HM: Heavy metal, LMWOAs: Acetic acid (AA), Citric acid (CA), Gallic acid (GA): Malic acid (MA), Oxalic acid (OA), Tartaric acid (TA), Vanillic acid (VA).

2.3. Surfactants

2.3.1. Generalities

Surfactants are amphiphilic compounds that have both hydrophobic and hydrophilic groups in their molecular structure (Pletnev, 2001). Depending on the chemical nature of the hydrophilic part, surfactants can be classified as non-ionics (with no charge) and ionics, which in turn can be cationic, anionic or amphoteric differing if they have positive, negative or both charges, respectively (Pletnev, 2001). One of the central characteristics of surfactants is their property to aggregate forming micelles in aqueous solution. This particular arrangement creates a spherical structure in which the hydrophilic part of the surfactant is in contact with the polar solvent, while the hydrophobic region of the molecule remains sequestered in the center avoiding the contact with the hydrophilic medium. The formation of micelles depends on the concentration of the surfactant. At low concentrations, surfactants exist as monomers. However, above a certain concentration *i.e.* critical micelle concentration (CMC), the thermodynamics of the system enables the formation of micelles (McNaught and Wilkinson, 1997). The CMC is a characteristic of each surfactant and depends on the chemical structure, *i.e.* the hydrophilic and hydrophobic parts of the molecule. Non-ionic surfactants have lower CMC levels than anionic and cationic surfactants (Ying, 2006) and in general, the CMC decreases with increases in the hydrophobic character of the molecule (Haigh, 1996). Other factors such as the temperature of the solution and the presence of electrolytes, affect the CMC as well (Haigh, 1996). A distinctive feature of surfactants when arranged in these clusters is that the non-polar central part of the micelle can interact with hydrophobic organic compounds increasing their water solubility. As a result, surfactants can increase the bioavailability of hydrophobic compounds, property that has been used for environmental applications in many remediation technologies, including phytoremediation (Gao et al., 2007).

Apart from surfactants of synthetic origin (Table 2.4a), an additional class of surfactants are biosurfactants (Table 2.4b), which are defined as low molecular weight microbial surface-active compounds (Neu, 1996). These surface-active metabolites are naturally produced by both prokaryotic (bacteria) and eukaryotic (yeasts) microorganisms. Regarding their chemical structure, these amphiphilic compounds are made up of combinations of saccharides and lipids (glycolipids) or peptides and lipids (lipopeptides) and they have molecular weights between 500 and 1500 Da (Van Hamme et al., 2006). Due to their amphiphilic chemical nature, biosurfactants as well as their synthetic counterparts, can form micelles in aqueous solution and the CMC values typically range from 1 to 200 mg l^{-1} (Ward, 2010). The natural production of biosurfactants by microorganisms has been involved in several functions and related to many processes, such as antimicrobial activity, microbial growth enhancement by increasing the bioavailability of hydrophobic substrates, attachment of microorganisms to surfaces, bacterial pathogenesis and biofilm formation (Ron and Rosenberg, 2001). There are three principal types of biosurfactants: sophorolipids, surfactins and

rhamnolipids. Sophorolipids are glycolipids produced by yeasts of the genus *Candida*. The hydrophilic portion is constituted by sophorose (disaccharide of glucose) while the hydrophobic part is a fatty acid chain of 16 or 18 carbon atoms with different degrees of saturation (Van Bogaert and Soetaert, 2010). Surfactins are cyclic lipopeptides mainly synthesized by *Bacillus subtilis* species and formed by a polar part of seven aminoacids in a looped structure and a hydrophobic fatty acid chain of 13 to 15 carbons (Jacques, 2010). Rhamnolipids are glycolipids made up of one or two units of the sugar rhamnose (leading to mono and di-rhamnolipids, respectively) and a non-polar part of β-hydroxy-decanoic acid chains. They are mainly produced by the bacteria *Pseudomonas aeruginosa* (Abdel-Mawgoud et al., 2010). In general, biosurfactants production by microorganisms does not occur as a single unique component. Indeed, most times biosurfactants are produced as a mixture of congener molecules with a range of different related structures varying, for instance, in the length of the fatty acid chain, degree of saturation or configuration of the molecular structure (Haigh, 1996). As biosurfactants may facilitate the release of contaminants from soils, they have been used with different aims in the context of environmental applications (Mulligan, 2005). Several environmental remediation techniques have been described for biosurfactants, including their use in protocols of soil washing, bio and phytoremediation (Pacwa-Plociniczak et al., 2011). With the aim to maintain the environmental sustainability, in the last years there is a tendency to move towards biosurfactants as an alternative to chemically-synthesized surfactants (Marchant and Banat, 2012a, b). Due to the fact that biosurfactants can be readily biodegraded they may produce less toxicity to the environment than other more recalcitrant chemical surfactants, rendering their use an environmentally-friendly choice (Mulligan, 2005).

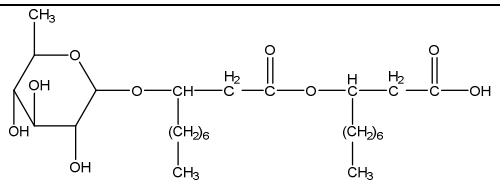
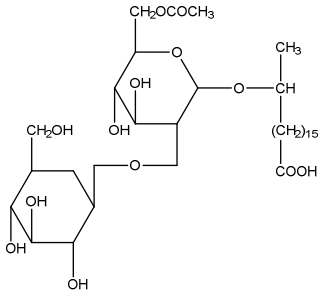
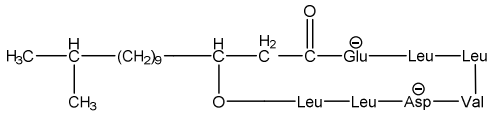
Table 2.4 Chemical characteristics of common surfactants

a) Surfactants of synthetic origin

Type	Surfactant	Molecular formula	Molecular weight (g mol^{-1})	CMC (mM)
Anionic	SDS	$C_{12}H_{25}O_4SNa$	288	8.1
Non-ionic	Brij® 35	$C_{58}H_{118}O_{24}$	1198	0.060
	Triton™ X-100	$C_{14}H_{22}O(C_2H_4O)_n$ (n= 9, 10)	625	0.240
	Tween® 80	$C_{64}H_{124}O_{26}$	1310	0.010

(Mukerjee and Mysels, 1971; Hait and Moulik, 2001)
CMC: critical micelle concentration at 25°C
Surfactants: polyoxyethylene (23) lauryl ether (Brij® 35), polyoxyethylene (20) sorbitanmonooleate (Tween® 80), sodium dodecyl sulfate (SDS), t-octylphenoxypolyethoxyethanol (Triton™ X-100)

b) Surfactants of synthetic origin

Type	Surfactant	Producer microorganism	Chemical structure of a representative congener
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*	
	Sophorolipids	*Candida bombicola*	
Lipopeptids	Surfactins	*Bacillus subtilis*	

(Mulligan, 2005)
The CMCs (critical micelle concentrations) of biosurfactants generally range from 1 to 200 mg l^{-1} and their molecular mass is from 500 to 1500 Da (Lang and Wagner, 1987)

2.3.2. Surfactants in soil

When surfactants are present in the soil system, sorption interaction processes take place. A two phase mechanism has been proposed to explain the sorption process, which relies on surfactant concentration. In the first step, at low concentrations, surfactant molecules are adsorbed to the soil solid surface interacting through electrostatic attractions. Afterwards, when increasing surfactant concentration, further molecules are adsorbed interacting through their hydrocarbon chains by hydrophobic interactions with the formerly adsorbed species (Gu and Rupprecht, 1990; Ying, 2006). Surfactant molecules attached to the soil and forming these particular aggregates are termed hemimicelles and admicelles (Behrends, 1999). Apart from the surfactant concentration, sorption effects are also influenced by the surfactant type and soil characteristics. For instance, cationic surfactants tend to adsorb to negatively charged soil components such as clay and organic matter through electrostatic interactions. By contrast, anionic and non-ionic surfactant sorption to soil relies mainly in hydrophobic interaction processes between the surfactant and the SOM content (Haigh, 1996). In the same way, Ying (2006) reviewed the sorption coefficients of several types of surfactants to different media (sediments, sludge and soil), reporting a general sorption trend in the order: cationic > non-ionic > anionic. The sorption of surfactants to soils also influences its distribution in this media. Surfactants are distributed between the soil surface and the water that fills the pore space. However, this allocation is not equal and a greater part of

the surfactant is adsorbed onto the soil rather than remaining in solution. Furthermore, sorption of surfactants to soils significantly influences its degradation in the environment. Surfactants, as biodegradable compounds can be metabolized by microorganisms when utilized as substrates for energy in aerobic and/or anaerobic conditions (Ying, 2006). Biodegradation rates vary, but in general terms reported surfactant half-lives are in the order of days. For instance, Knaebel et al. (1994) studied the mineralization of two different types of surfactants in soil: linear alkylbenzene sulphonate (anionic) and linear alcohol ethoxylate (non-ionic) finding for both a mean half-life of 2 days. Similarly, Pawar et al. (2009) studied the biodegradation of sodium dodecyl sulfate (SDS, anionic) and polyoxyethylene (20) sorbitanmonooleate (Tween® 80, non-ionic) by microorganisms isolated from a river bank. They reported more than 90% reduction for both surfactants after 6 days. One of the factors that affect the biodegradation of surfactants is its concentration. It has been demonstrated that the degradation rates of surfactants decreases above the CMC, and this effect may be due to a lower bioavailability of the surfactant when it is in the micellar arrangement in contrast with the monomeric form (Zhang et al., 1999). In addition, the chemical structure of the surfactant also determines its degradation. As a general rule, the presence of chain branching in the alkyl chain results in more recalcitrance (Scott and Jones, 2000). Lastly, other environmental effects that might also influence the biodegradation degree of surfactants were reviewed by Scott and Jones (2000) and include the content of dissolved oxygen, the pH, the presence of complexing compounds and the formation of salts. It is worth to point out that to achieve the complete degradation of a surfactant it may be needed a consortium of bacteria rather than unique species because the metabolic faculties of individual microorganisms may be restricted (van Ginkel, 1996).

2.3.3. Surfactant enhanced desorption of contaminants from soil

Surfactants may affect the mobility of organic compounds in soil through micellar solubilization. For this reason, they have been used in desorption experiments to test if in the presence of surfactants organic compounds are desorbed (Table 2.5). Improvement in the desorption efficiency and, as a consequence in the mobility and bioavailability of organic compounds in aqueous phase is central to remediate organic contaminated soils by bio/phytoremediation.

Synthetic surfactants were tested in desorption experiments with soils contaminated with organic or inorganic pollutants. Alcántara et al. (2009) studied the desorption of PAHs from soil, testing the potential of five non-ionic surfactants to enhance the solubility of benzanthracene, fluoranthene and pyrene as individual and mixed contaminants. Tween® 80 removed more than 80% of the three PAHs tested as individual contaminants. Similar results were found for PAHs in binary and ternary combinations; even though the single-component level of desorption could not be used to predict the binary or ternary mixture level of removal, probably because of variations in the solubility properties of contaminants when present in mixtures. Pesticides have been tested for their desorption behavior from soil in the presence of surfactants as well.

Gonzalez et al. (2010) observed that the non-ionic surfactant Tween® 80 effectively enhanced the desorption of *p,p'*-DDT, *p,p'*-DDE and α-cypermethrin. Furthermore, the anionic surfactant SDS enhanced the desorption of *p,p'*-DDT, *p,p'*-DDE, α-cypermethrin, α-endosulfan and endosulfan sulfate. Finally, Ramamurthy et al. (2008) investigated the sorption/desorption behavior of heavy metals from an artificially contaminated sandy soil in the presence of surfactants. Surfactants such as SDS, alpha-olefin sulfonate (AOT) and Triton™ X-100 demonstrated to be effective in enhancing the removal of both Cu and Zn from soil, although Zn removal was greater than Cu removal, probably because Cu binds more strongly to the soil matrix than Zn. The effectiveness of surfactants followed the order: Triton™ X-100 > SDS > AOT for Cu, and SDS > AOT > Triton™ X-100 for Zn. Best performance was obtained at surfactant concentrations slightly above the CMC, suggesting that the micelles indirectly caused the mobilization and removal of these metals.

Apart from synthetic surfactants, biosurfactants have also been studied for their desorption properties. For example, An et al. (2011) assessed the effect of rhamnolipids on the desorption of phenanthrene from a spiked soil. They tested rhamnolipids at various concentrations above the CMC, observing an enhancement in phenanthrene desorption with increased rhamnolipids concentration. The desorption effect was also affected by the soil texture and organic content, being greater for a clay loam soil with low organic matter. Biosurfactants have been tested to enhance the removal of total petroleum hydrocarbons (TPH) from contaminated soils as well. Lai et al. (2009) performed a comparative study in which synthetic and biosurfactants were evaluated for their action in TPH removal. Biosurfactants showed a superior performance than synthetic ones, following the order: rhamnolipids > surfactin > Triton™ X-100 > Tween® 80. Likewise, a batch experiment with TPH contaminated soil conducted by Liu et al. (2012) demonstrated that the addition of rhamnolipids at 100 mg kg^{-1} soil, improved TPH degradation and this effect varied according to the SOM. TPH degradation in the presence of rhamnolipids was higher in the soil with higher SOM. The authors hypothesized that high SOM improved water holding capacity of the soils, permitting the formation of a better emulsion between the diesel oil and the rhamnolipids. As a consequence, the bioavailability of TPH to microorganisms was enhanced, and their biodegradation increased.

In addition to single-surfactant studies, some authors have recently reported the effect of the application of mixtures of surfactants on the solubilization of organic compounds. This strategy has been applied with the aim to increase contaminant remediation rates without rising surfactant concentrations, as this latter case would lead to increased remediation costs and potential surfactant contamination. In this sense, Zhu and Feng (2003) evaluated the capabilities of mixed anionic-nonionic surfactants in enhancing the water solubility of PAHs. They tested surfactant mixes of SDS with *t*-octylphenoxypolyethoxyethanol (Triton™ X-100), octylphenol ethoxylate (Triton™ X-305) or polyoxyethylene (23) lauryl ether (Brij® 35), finding the greatest synergistic power for the mix SDS-Triton™ X-305. The synergistic solubilization of PAHs by mixed-surfactants was attributed to the formation of mixed-micelles, the lower CMC of

the mixed-surfactant solutions and the increase of the solute partition coefficient between micelle and aqueous phase. In another similar study, Alcántara et al. (2009) performed an experiment with a combination of two non-ionic surfactants: polyoxyethylene (20) sorbitanmonolaurate (Tween® 20) and Tween® 80. They observed that the maximum level of fluoranthene and pyrene removal (more than 90% and 80% respectively) occurred in the presence of combined surfactants, while for benzanthracene the removal obtained in the presence of the combination of surfactants was similar to that obtained with Tween® 80 alone (around 88%). Similarly, Sales et al. (2011) observed that the solubility of naphthalene was enhanced by a mixture of Tween® 80 and fatty acids (sodium laurate), also demonstrating a synergistic effect for this combination of surfactants. Another study with mixed amendments was performed by Cheng and Wong (2006) to evaluate the combined effect of Tween® 80 and DOM on the desorption of PAHs in a soil-water system. The addition of DOM (from pig manure or pig manure compost) concomitantly with Tween® 80 caused an average of 1.8 and 3.1-fold desorption increase for phenanthrene and pyrene respectively, compared to the sole use of Tween® 80. This desorption enhancement was attributed to the formation of DOM-Tween® 80 complexes with a stronger desorbing capacity to mobilize PAHs from soil into the aqueous phase. The use of composed amendment solutions was employed to cope with the problem of co-contaminated soils as well. Fonseca et al. (2011) assessed the desorption of Pb and phenanthrene co-contaminated soils in the presence of combined solutions of the synthetic chelate EDTA and the non-ionic surfactants Brij® 35 or Tween® 80. Extractions of 48% and 55% were obtained for Pb and phenanthrene, respectively, with EDTA-Brij® 35 composed solution. The authors conclude that this kind of composed solutions could be used to enhance the remediation of co-contaminated soils for example, in the context of phytoremediation techniques. Furthermore, the use of biosurfactants in combined amendments has been recently studied by An et al. (2011). These authors assessed the desorption characteristics of phenanthrene in the presence of combinations of rhamnolipids and OAs, *i.e.* acetic, citric, oxalic or tartaric acids. The best desorption result was obtained for the mix rhamnolipids-citric acid, which reached an average 0.3-fold increase compared to the single use of rhamnolipids. According to the authors, phenanthrene desorption enhancement could be attributed to the synergistic actions of rhamnolipids and OAs through potentially different modes of action. Among them, they propose that OAs disrupt the linkage between the organic matter and the soil matrix. As a result, phenanthrene is released from the soil along with the desorbed organic matter. Rhamnolipids, in turn, facilitate the solubilization of phenanthrene molecules bound and unbound to the released organic matter.

Table 2.5 Relevant experiments of surfactant enhanced desorption of contaminants from soils

Contaminant class	Contaminants (mg kg^{-1} soil)	Surfactant (mM)	Major desorption fold increases	Reference
Heavy Metals	Cu: 1.216 Zn: 1.152	AOT: 0-20 SDS: 0-32 Tx-100: 0-8	AOT at 1.25 mM: 5.0 for Cu and 0.4 for Zn SDS at 10 mM: 5.3 for Cu and 0.4 for Zn Tx-100 at 0.50 mM: 7.0 for Cu and 0.3 for Zn	(Ramamurthy et al., 2008)
Hydrocarbons	Phe: 100	RhL: 0.087, 0.17, 0.35	RhL at 200 mg kg^{-1}: 2.9	(An et al., 2011)
	TPH: 3000, 9000	Sfc: 0.48, 0.97, 1.93 RhL: 0.87, 1.73, 3.47	Sfc at 0.48 mM: 0.5 for TPH at 9000 mg kg^{-1} RhL at 0.87 mM: 0.9 for at 3000 mg kg^{-1}	(Lai et al., 2009)
Organo-chloride pesticides	*p,p'*-DDT: 0.05, 4.2 *p,p'*-DDE: 0.55, 5.8	SDS: 16.2, 81.0 Tw-80: 0.024, 0.12	SDS at 81.0 mM: up to 45 Tw-80 at 0.024: up to 5	(Gonzalez et al., 2010)

Contaminants: *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), *p,p'*-dichlorodiphenyltrichloroethane (*p,p'*-DDT), phenanthrene (Phe), pyrene (Pyr), total petroleum hydrocarbons (TPH). Surfactants: dioctyl sulfosuccinate (AOT), rhamnolipids (RhL), sodium dodecyl sulfate (SDS), surfactin (Sfc), Triton™ X-100: t-octylphenoxypolyethoxyethanol (Tx-100), Tween® 80: polyoxyethylene (20) sorbitanmonooleate (Tw-80)

2.3.4. Surfactant enhanced phytoremediation

2.3.4.1. Synthetic surfactants

SEPR is a remediation strategy consisting in the use of surfactants to improve the mobility and biodegradation of pollutants throughout phytoremediation. The addition of surfactants as amendments to organic polluted media has been primarily used with the aim to increase the bioavailability of hydrophobic compounds by enhancing the mass transfer from the soil solid to aqueous liquid phase (Gao et al., 2007). The main implication of this is to facilitate the degradation of pollutants principally by microorganisms at the rhizosphere level (rhizodegradation) and potentially by plants that could take up and metabolize moderately hydrophobic organic contaminants (phytotransformation) (Dietz and Schnoor, 2001).

Several SEPR experiments were conducted in the last years testing different types of synthetic surfactants, plants and contaminated media (Table 2.6). For instance, experimental studies performed in hydroponic conditions evaluated the plant uptake of PAHs in the presence of non-ionic surfactants at concentrations above and below the CMC (Gao et al., 2006; Gao et al., 2008). These authors demonstrated that the use of Tween® 80 and Brij® 35, when present at low concentrations, could enhance pyrene and phenanthrene uptake by red clover (*Trifolium pretense*) and ryegrass (*Lolium multiflorum*), respectively. On the contrary, cationic surfactants appeared to be effective in enhancing the soil retention of PAHs. Lu and Zhu (2009) tested two synthetic surfactants: cetyltrimethylammonium bromide (CTMAB) and dodecylpyridinium bromide (DDPB), which led to a reduction of phenanthrene and pyrene uptake by common vegetables such as chrysanthemum (*Chrysanthemum coronarium*), cabbage (*Brassica campestris*) and lettuce (*Lactuca sativa*), due to an enhancement in PAH soil sorption in the presence of surfactants. This property of cationic surfactants could be eventually utilized to produce safe agricultural food products from plants grown on contaminated soils. Even if this study supports the fact that PAHs could be uptaken by plants, in most cases plant uptake or accumulation of such hydrophobic compounds are considered negligible and degradation by soil microorganisms is found as the main pathway for the removal of PAHs (Haritash and Kaushik, 2009). Cheng et al. (2008) studied the effect of Tween® 80 on PAH removal from a spiked soil vegetated with tall wheatgrass (*Agropyron elongatum*). Tween® 80 had a positive effect on pyrene removal (0.3-fold increase) probably due to its solubilizing and desorbing capacity, which improved the bioavailability of the contaminant. Biodegradation was established as the key mechanism for PAH dissipation, while plant uptake was insignificant. In a different experiment, phenanthrene dissipation in spiked soils vegetated with *M. sativa* and amended with Triton™ X-100 was studied by Wu et al. (2008a). Triton™ X-100 had a particular effect on phenanthrene remaining soil concentration: it decreased the residual concentrations of phenanthrene in the bulk soils while increased it in the rhizosphere soil. The authors hypothesized that the surfactant was likely to change the desorption behavior of phenanthrene enhancing its mobility and transportation from bulk soil to the rhizosphere, which seemed to act as a sink for phenanthrene. In addition it could also be possible that Triton™ X-100 causes a decrease in rhizosphere phenanthrene degradation due to toxic effects on arbuscular mycorrhizal fungi or the associated microflora. Nevertheless, this study also demonstrates that arbuscular mycorrhizal inoculation in combination with Triton™ X-100 had a positive effect on phenanthrene dissipation. Similarly, the association of arbuscular mycorrizal fungal colonization of *M. sativa* roots and the treatment with Triton™ X-100 resulted in a successful remediation of soils affected by organochloride pesticides. Wu et al. (2008b) showed that this combined treatment improved *M. sativa* root and shoot accumulation of *p,p'*-DDT, mainly due to an increase in the adsorption of *p,p'*-DDT on colonized roots, which resulted in *p,p'*-DDT dissipation from soil. Once more, it was observed a distinct distribution of the contaminant between rhizosphere and bulk soils, in which *p,p'*-DDT was sequestered in the rhizosphere zone due to its increased mobility in the presence of Triton™ X-100.

In addition to SEPR of organic contaminants, surfactants may also have a part in removing heavy metals from soils, probably through the formation of complexes, micelles and ion exchange processes (Pacwa-Plociniczak et al., 2011). A few phytoremediation studies have reported that surfactants can improve metal availability to plants influencing metal phytostabilization and/or phytoextraction potential of plants. Among them, Almeida et al. (2009) demonstrated that Triton™ X-100 and, to a less extent SDS, could favor Cu sorption by the salt marsh plant *Halimione portulacoides.* Triton™ X-100 promoted the adsorption/absorption of Cu by the plant root, while not improving its translocation. In turn, Liu et al. (2009) studied the effects of SDS on Cd phytoremediation by the ornamental plant *Althaea rosea*. SDS could not only increase the dry biomass of the plants (up to 28% increase), but also promote Cd accumulation in shoots (up to 2.1 times) and roots, as well as increasing the Cd translocation factor of this species. Thus, SDS was effective in enhancing phytoremediation with *A. rosea*, which could be regarded as a potential Cd-hyperaccumulator with chemical enhancement. To cope with heavy metal contaminated media, mixtures of surfactants with chelating agents have been assessed as well. For example, satisfactory results were obtained for Pb phytoextraction from contaminated soils vegetated with *B. juncea*, where the surfactant Triton™ X-100 stimulated the plant uptake of EDTA-Pb complexes. However, to overcome the phytotoxic effect that this represented, it was necessary to implement a bioaugmentation treatment consisting in the inoculation with an autochthonous plant growth-promoting rhizobacterium, which protected *B. juncea* possibly by lowering plant ethylene synthesis (Di Gregorio et al., 2006). Similarly, the combined effect of SDS and the synthetic chelate ethylenegluatarotriacetic acid (EGTA) was effective in enhancing *A. rosea* biomass of roots and shoots, Cd uptake from soil and Cd translocation from shoots to roots (Liu et al., 2009).

The presence of surfactants when used as amendments in the frame of SEPR could cause stress and toxicity to plants as well as affect the biomass yield. Various effects on biomass and visible toxicity symptoms, which varied principally according to plant species, surfactant type and concentration used, have been reported. In general terms, among non-ionic surfactants, Triton™ X-100 showed more negative effects than Tween® 80. In the presence of Tween® 80 at 8 times the CMC, no significant difference in *T. Pretense* biomass or phytotoxicity effects were observed after 12 days of growth in hydroponics (Gao et al., 2008). Likewise, the application of Tween® 80 at concentrations up to 100 mg kg^{-1} soil did not affect the germination rates of *A. elongatum* or cause any significant effect on the biomass yields (Cheng et al., 2008). Other type of surfactant from the group of anionic ones, which showed no apparent damage to plants, is SDS. The photosynthetic efficiency of *H. portulacoides* was not affected by the addition of SDS at 8 mM (CMC) (Almeida et al., 2009). By contrast, the treatment with Triton™ X-100, in most cases demonstrated some plant damage. Triton™ X-100 application (0.1% w/w in the soil) generally decreased *M. sativa* shoot and root biomass as well as the percentage of mycorrhizal root colonization. (Wu et al., 2008b). Phytotoxic effects of Triton™ X-100 (at concentrations 5-10 times higher than its CMC) were also reported for *B. juncea* as well as a 72% decrease in plant biomass

production (Di Gregorio et al., 2006). These effects were attributed to a direct damage effect of the surfactant on the cell phospholipid plasma membranes of plant roots (Cserháti, 1995). Adverse effects were also reported for the non-ionic surfactant Brij® 35. In the presence of this surfactant, *L. multiflorum* biomass grown in hydroponics was generally significantly smaller. Moreover, at concentrations of 148 mg l^{-1} and above it caused toxicity symptoms that included interruption of plant growth, leaves turning to brown and roots becoming gray (Gao et al., 2006).

2.3.4.2. Biosurfactants

Biologically-produced surfactants (biosurfactants) have certain particular features that could improve SEPR. Main advantages of biosurfactants rely on their greater biodegradability and lower toxicity. Therefore, in the last years, some experiments have been carried out to assess the role of biosurfactants within the context of phytoremediation (Table 2.6). The inclusion of biosurfactants into phytoremediation systems can be done by two main strategies: applying biosurfactants as solutions obtained from the culture of biosurfactant producing microorganisms or by inoculation of the contaminated media with microorganisms able to produce biosurfactants (bioaugmentation). The second approach is particularly interesting because the production of biosurfactants can occur *in situ* at soils, but typically most SEPR are performed according to the first approach. For instance, hydroponic experiments with species of ryegrass (*Lolium*) demonstrated that rhamnolipids could be used to improve the remediation efficiency of organic and inorganic contaminants. Zhu and Zhang (2008) evaluated the effect of rhamnolipids on the uptake of PAHs by *L. multiflorum*. They observed that within a certain range of concentrations the root uptake of phenanthrene and pyrene could increase, with the maximum uptake at 0.5 times the CMC. Likewise, Gunawardana et al. (2010) tested rhamnolipids alone and in combination with other natural amendments for their effect on heavy metal uptake by *L. perenne*. A combined treatment of rhamnolipids (at 1.7 times the CMC) and ethylenediamine-N,N'-disuccinic acid (EDDS) produced a 22, 8 and 2-fold increase in the shoot concentrations of Cu, Cd and Pb, respectively. In addition, mixed treatment of rhamnolipids, citric acid and EDDS resulted in higher improvements (38, 9 and 3-fold increases for Cu, Cd and Pb shoot uptake, respectively). In spite of this, rhamnolipids applied alone had little effect, possibly due to the high molecular mass of the metal-rhamnolipid complexes, which can limit its uptake through the roots. Although both combined treatments (rhamnolipids+EDDS and rhamnolipids+EDDS+citric acid) considerably increased heavy metal translocation, this led to plant toxicity symptoms, that theoretically could be overcome if amendments are applied shortly before harvesting. Gunawardana et al. (2010) also conclude that the combination of rhamnolipids and citric acid could be an alternative to the individual application of citric acid or rhamnolipids for Cu and Pb phytoextraction or Cd phytoextraction, respectively, as these combined treatments had no significant effects on biomass yield, while enhanced shoot metal accumulation. Similarly, a different experiment with combined amendments showed that the synergistic use of rhamnolipids, arbuscular mycorrizal

fungi and aromatic hydrocarbon degrading bacteria increased the removal of PAHs in contaminated soils vegetated with *M. sativa* (Zhang et al., 2010).

Concerning the bioaugmentation strategy during SEPR, Sheng et al. (2008) assessed the effect of a biosurfactant-producing and heavy metal resistant strain of *Bacillus* sp. on plant growth and Cd uptake from contaminated soils. The inoculation with *Bacillus* strain significantly enhanced both biomass of tomato (*Lycopersicon esculentum*) and its Cd uptake. The stimulation of plant growth was attributed to the production of indole acetic acid and siderophores by the tested bacterial strain, while its production of biosurfactants could cause the increased solubilization and Cd accumulation by plants.

Regarding the effects of biosurfactants on plants during SEPR, rhamnolipids have demonstrated both favorable and detrimental influences on plants. Single application of rhamnolipids (150 mg kg^{-1} soil) slightly increased *M. sativa* dry weight, but it was observed that if rhamnolipids were applied in combination with PAHs-degrading bacteria and arbuscular mycorrhizal fungi, they could significantly improve shoot and root biomass of *M. sativa* (Zhang et al., 2010). In addition, rhamnolipids (at 0.5 CMC or below) could stimulate the growth of *L. multiflorum* shoots (Zhu and Zhang, 2008). These authors proposed that the increased root permeability in the presence of surfactants may lead to a more efficient uptake of nutrients, which could be one of the mechanisms involved to explain such enhancement in plant biomass yield. Conversely, *L. perenne* treated with rhamnolipids (individually and in combination) displayed symptoms of toxicity, (*e.g.* necrosis of leaf tips) which became more serious during the course of the experiment. In addition, rhamnolipids (at 1.7 times the CMC) combined treatment with EDDS and citric acid resulted in significant shoot and root biomass decrease (Gunawardana et al., 2010). According to these authors, the observed negative effects may be due to the damage caused in cell membranes by rhamnolipids (specially being detrimental for metal exclusion mechanisms), which in turn could allow greater uptake of higher bioavailable metal-amendment complexes from heavy metal contaminated soils.

Table 2.6 Relevant experiments of surfactant enhanced phytoremediation of inorganic and organic pollutants

a) Heavy Metals

Surfactant (mM)	HM (mg kg^{-1} soil)	Media	Plant species	Duration/ Experiment type	Phytotoxicity/ Impact on plant biomass	Major fold increases in plant HM uptake	Reference
Tx-100: 1.6, 3.1	Pb: 465	Soil from industrial area	Indian Mustard *(B. juncea)*	42 days Greenhouse	72% decrease in plant biomass.	0.48 for EDTA-Pb complexes.	(Di Gregorio et al., 2006)
Tx-100: 0.25 SDS: 0.25, 8	Cu: 10.2	Spiked sediments and water	Sea Purslane (*H. portulacoi-des)*	6 days Outdoors	No decrease in photosynthetic efficiency.	Triton^{TM} X-100: 2 in roots.	(Almeida et al., 2009)
SDS: 0.5, 1, 2 mmol kg^{-1}	Cd: 30, 100	Spiked soil	*A. rosea*	4 months Outdoors	Up to 28% increase in shoot biomass.	SDS at 2 mmol kg^{-1}: 2.1 for Cd at 30 mg kg^{-1} in shoots	(Liu et al., 2009)
RhL: 0.15	Cd: 1 Cu: 10 Pb: 5	Spiked water	Ryegrass (*L. perenne*)	30 days Hydroponics	Necrosis of leaf tips. No significant effect on plant biomass.	Approximately 0.3 for Cu in roots and 2 for Cd in shoots.	(Gunawardana et al., 2010)

HM: Heavy metal. Surfactants: rhamnolipids (RhL), sodium dodecyl sulfate (SDS), Triton^{TM} X-100: t-octylphenoxypolyethoxyethanol (Tx-100)

b) Polycyclic aromatic hydrocarbons

Surfactant (mg kg^{-1} soil)	PAH (mg kg^{-1} soil)	Media	Plant species	Duration / Experiment type/	Phytotoxicity / Impact on plant biomass	Major fold increases in plant PAH uptake	Major fold increase in PAH dissipation	Reference
Tw-80: 0-105.6	Phe: 1.0 Pyr: 0.12	Spiked water	Red clover *(T. pretense)*	12 days Greenhouse	No significant increase in plant biomass.	Tw-80 at 6.6 mg kg^{-1}: 0.18-1.15	.	(Gao et al., 2008)
RhL: 6.4, 12.9, 25.8, 51.5	Phe: 1.0 Pyr: 0.12	Spiked water	Ryegrass (*L. multiflorum*)	17 days Greenhouse	Approximately up to 50% increase in shoot and root biomass.	RhL at 25.8 mg kg^{-1}: 4.6 for Phe and 0.8 for Pyr		(Zhu and Zhang, 2008)
Bj-35: 18.5, 37.0, 74.0, 148, 296	Phe: 0.52 Pyr: 0.12	Spiked water	Ryegrass (*L. multiflorum*)	10 days Greenhouse	Above 148 mg kg^{-1}: brown leaves, gray roots, growth stunt. Up to 44% decrease in plant biomass.	Bj-35 at 37.0 mg kg^{-1}: 1.04	Bj-35 at 296 mg kg^{-1}: -0.7 for Phe and -0.6 for Pyr.	(Gao et al., 2006)
RhL: 150	15 PAHs. Total: 12.9	Soil from sewage irriga-ted farm-land	Alfalfa (*M. sativa*)	90 days Greenhouse	No significant increase of shoot (12%) and root (7%) biomass.		0.06 for total PAHs	(Zhang et al., 2010)

(Continued on next page)

b) Polycyclic aromatic hydrocarbons (continued)

Surfactant (mg kg^{-1} soil)	PAH (mg kg^{-1} soil)	Media	Plant species	Duration / Experiment type/	Phytotoxicity / Impact on plant biomass	Major fold increases in plant PAH uptake	Major fold increase in PAH dissipation	Reference
Tw-80: 20, 100	Phe: 294 Pyr: 296	Spiked soil	Tall wheatgrass *(A. elongatum)*	60 days Greenhouse	No negative effect on germination rates or plant biomass.	.	Tw-80 at 100 mmol: 0.3 for Pyr	(Cheng et al., 2008)
Tx-100: 1000	Phe: 2.5, 5, 10	Spiked soil	Alfalfa (*M. sativa*)	60 days Growth chamber	24% average increase in root biomass. 9% average decrease in shoot biomass.		0.7 for Phe at 2.5 mg kg^{-1}	(Wu et al., 2008a)

Contaminants: phenanthrene (Phe), pyrene (Pyr). Surfactants: Brij® 35: polyoxyethylene (23) lauryl ether (Bj-35), rhamnolipids (RhL), Triton™ X-100: t-octylphenoxypolyethoxyethanol (Tx-100), Tween® 80: polyoxyethylene (20) sorbitanmonooleate (Tw-80)

2.4. Conclusions

Desorption experiments can be a first step to assess the interaction between amendments and contaminants in soils. This review shows that LMWOAs and surfactants have a significant potential to increase the bioavailability of contaminants in soil, feature that can result in the application of these compounds during enhanced phytoremediation.

One of the benefits of using LMWOAs as amendments in phytoremediation is a reduction in excessive mobilizing effects and leaching risks with respect to synthetic chelates. This is due to their higher biodegradation rates, which leads to a lower persistence in the soils. However, high biodegradation rates of LMWOAs may also be one of the principal reasons for the low effectiveness of these compounds in assisting phytoextraction. It is likely that in the first step LMWOAs solubilize metals in the soil solution, but as LMWOAs are degraded by soil microorganisms, their action is limited. For this reason, a single application of LMWOAs may not be sufficient to enhance metal accumulation in plants up to levels adequate for an efficient use in phytoextraction technology. Another benefit of LMWOAs is that, in general terms, they have less toxicity towards plants than synthetic chelates, allowing higher plant biomass production. Total removal of metals during phytoextraction depends both on plant metal concentration and biomass production. In this sense, as observed by do Nascimento et al. (2006), higher biomass production due to less phytotoxicity when applying OAs could compensate for lower metal concentrations in plant shoots with respect to synthetic chelates resulting in similar phytoextraction rates for OAs than for synthetic chelates. The main challenge of LMWOA enhanced phytoremediation remains to find an ideal amendment tolerated by plants and able to keep metals soluble as long as necessary to enhance phytoextraction but without persisting excessively in the soil because that would lead to increased leaching risks. Though, this leaching effect could be potentially reduced by increasing plant density and taking advantage of the evapotranspiration process carried out by plants.

As for LMWOAs, when adding surfactants in the context of SEPR it is important to estimate multiple aspects to select the most suitable one. In some cases, the introduction of surfactants (especially synthetic ones) may lead to contamination concerns. Although the potential toxicity of the surfactant itself is important, surfactant enhanced desorption of organic contaminants from soils may increase pollutant availability, also producing phytotoxic effects (Zhang *et al.*, 2010). Moreover, it has been observed that the introduction of surfactants may modify soil physics, chemistry and biology (Kuhnt, 1993). The concentration of surfactants is also a parameter to consider. As a consequence of surfactant sorption to soils, the effective concentration of surfactant in soil solution able to form micelles and hence solubilize contaminants may be limited. As a result, more surfactant than expected may be needed to achieve the CMC in the soil (Haigh, 1996).

Although the mentioned limitations, the range of possibilities to use LMWOAs and surfactants has been demonstrated to be broadened, which is encouraging. Increased

desorption and thus bioavailability of heavy metals and organic contaminants could be achieved either by the use of LMWOAs or surfactants. Hence, this would lead to the possibility of using these compounds to deal with the problem of co-contamination. Combinations of LMWOAs and surfactants could be a strategy with auspicious potential too. Moreover, LMWOAs and surfactants could be used with biostimulation purposes as additional carbon sources for microorganisms, resulting in higher bacterial growth and increased organic contaminant biodegradation rates (Bautista et al., 2009).

Regarding the perspectives of chelate-assisted phytoextraction, Evangelou et al. (2007) expressed a doubtful point of view, questioning if further research would really lead to progress in this field, based on the fact that more studies would lead to different observations, ambiguity of which relies on the specific experimental conditions of each study. These authors believed that chelate-assisted phytoextraction had reached a turning point, and instead supported the strategy of classic phytoextraction coupled to the obtaining of bioenergy to compensate for the longer restoration times that may be needed in the absence of chelates.

As shown above, LMWOA and surfactant-enhanced phytoremediation experiments resulted in different observations, which may be related to heterogeneous experimental conditions (*e.g.* different concentrations and classes of contaminants, concentrations, nature, biodegradation rates and strategy of application of chemical treatments, as well as soil characteristics and plants involved). Although enhanced phytoremediation by LMWOAs and surfactants is definitely not a fully developed technique, extensive progress has been made in characterizing the potential of such amendments during phytoremediation. This review highlights that appropriate amendment types, concentrations and exposure times are key concepts to be considered in order to make this technology feasible. Future research should focus on kinetics and timing application of LMWOA, surfactants and their combinations to achieve the optimization at lab scale before it can be effectively applied in pilot/field experiments. Finally, an exhaustive understanding of each particular situation is necessary in order to adapt and use the best strategy to a cost effective approach together with a reduction of the operating time.

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Chapter 3

Phytoremediation potential of alfalfa in co-contaminated soil

This chapter has been submitted to Water, Air & Soil Pollution journal under the form of an original research paper entitled: Phytoremediation potential of alfalfa (*Medicago sativa* L.) in heavy metal and hydrocarbon co-contaminated soil. Agnello A.C., Huguenot D., van Hullebusch E.D., and Esposito G.

Abstract

A pot experiment was conducted to evaluate the phytoremediation potential of alfalfa (*Medicago sativa* L.) in soil contaminated by petroleum hydrocarbons and heavy metals. Germination rates, biomass yield and plant mortality over 150 days of experiment were assessed in order to evaluate alfalfa tolerance to the co-contaminated soil. Heavy metal concentration in plant parts was determined so as to assess phytoextraction capacity. Microbial counts of alkane degraders and lipase activity were studied as soil bioindicators of rhizodegradation potential. The results showed that alfalfa could germinate in the co-contaminated soil (germination rates 66%) but plant growth was stunted after 60 days. Shoot and root biomass were scarce and after 150 days of experiment 100% plant mortality was observed. Alfalfa plants were able to uptake heavy metals, while poor metal translocation took place. The microbial number of alkane degraders and lipase activity were enhanced in the rhizosphere of alfalfa, particularly after 60 days (rhizosphere effect values of 3.3 and 1.4, respectively), but these effects gradually diminished as plants deteriorated. The findings of this research suggest a limited potential to use alfalfa for phytoremediation of co-contaminated soil used in this study.

Keywords

Phytoremediation, heavy metals, hydrocarbons, alfalfa (*Medicago sativa* L.), co-contaminated soil

3. Phytoremediation potential of alfalfa in co-contaminated soil

3.1. Introduction

Human activity, directly or indirectly, leads to a deterioration of the environment. Industry (*e.g.* metal mining) and agriculture (*e.g.* use of chemical fertilizers) result in heavy metal pollution of the biosphere (Kabata-Pendias, 2011). Likewise, crude oil production and the use of petroleum products give rise to major concerns given the high incidence of accidental spillages, industrial releases, or discharges as byproducts of commercial and private uses in urban areas. These result in widespread pollution of the environment (Russell et al., 2009). Heavy metals and petroleum hydrocarbons, due to their toxicological characteristics, are a serious risk to human health and the environment (Agency for Toxic Substances & Disease Registry, 1998). Although it is not uncommon that metal and organic contaminants are both present in polluted sites together (Obiajunwa et al., 2002), environmental research has tended to focus on the remediation of single pollutants instead of tackling multiple contaminants. Frequent occurrence of co-contaminated soils in the environment reveals just how important it is to find adequate remediation solutions (Sandrin and Maier, 2003).

Phytoremediation is a green remediation approach based on the use of plants to remove pollutants from the environment or to render them harmless (McCutcheon and Schnoor, 2004). Phytoremediation can be a feasible cost-effective remediation strategy if contaminants are present up to levels that can be tolerated by plants and if the time to achieve remedial goals is not a priority. Extensive research has been carried out on phytoremediation of inorganic or organic contaminated media, and recently more studies have targeted co-contaminated soils (Ouvrard et al., 2011; Chigbo et al., 2013; Hechmi et al., 2013; Sung et al., 2013).

Remediation of heavy metals involves phytoremediation processes such as phytoextraction and phytostabilization. Although heavy metals are inorganic compounds that cannot be degraded by plants any further, they can be removed from the contaminated soil through phytoextraction. Heavy metals can be uptaken by plant roots, translocated and accumulated in the aboveground tissues of the plant. To complete heavy metal removal, plants must be harvested and their biomass must finally be disposed of (Salt et al., 1995). In addition, in situ containment of heavy metals in soils can be achieved through phytostabilization, which is caused by the plant altering the biological, chemical or physical characteristics of the soil. Processes such as root-mediated precipitation and root surface sorption can stabilize metals within the root area, decreasing their mobility. Phytostabilization is not a clean-up technique but a containment measure, which reduces contaminant leaching and runoff and also minimizes erosion and dispersion of the contaminated soil (Mendez and Maier, 2008). In contrast, organic contaminants like petroleum hydrocarbons are susceptible to biodegradation, and therefore can be targeted by another phytoremediation process: rhizodegradation, which entails the enhancement of biodegradation within the root zone by rhizosphere microorganisms. This process is driven by stimulation of the suitable

microbial populations, *i.e.* able to degrade the pollutant in question. Increasing the microbial number and activity under the influence of plant roots (*i.e.* the rhizosphere effect) relies on the release of root exudates. In order to ensure the rhizosphere effect close contact between plant roots and contaminated soil is essential. As a result, the depth of root penetration and its density determines the process (White and Newman, 2011).

Alfalfa (*Medicago sativa* L.) presents a number of remarkable characteristics for phytoremediation: 1) it is a perennial plant with fast growth rates; 2) it produces large biomass (Coburn, 1912); 3) develops an extensive tap root system establishing a vast niche for the development of rhizosphere microorganisms (Kirk et al., 2005); 4) it associates with symbiotic Rhizobium bacteria allowing nitrogen fixation and letting alfalfa grow in soils with high C:N ratios (Truchet et al., 1991); and 5) it is diffusely distributed worldwide, well adapting to different climatic conditions. Over the past decade, there has been widespread use of alfalfa in phytoremediation. Heavy metals like Cd, Cr, Cu, Ni and Zn (Peralta-Videa et al., 2002; Peralta-Videa et al., 2004; Bonfranceschi et al., 2009), petroleum hydrocarbons (Wiltse et al., 1998; Kirk et al., 2002), polycyclic aromatic hydrocarbons (PAHs) (Fan et al., 2008) or organochlorines (Li and Yang, 2013) have all been targeted by phytoremediation with this species. Moreover, recent findings have shown promising results for alfalfa phytoremediation of co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013). However, no previous study has addressed the subject of phytoremediation potential of alfalfa in heavy metal and petroleum hydrocarbon co-contaminated soils.

The aims of the present study were to investigate alfalfa tolerance to a co-contaminated soil and if the presence of alfalfa vegetation could contribute to heavy metal remediation and/or indicate petroleum hydrocarbon removal combining different forms of phytoremediation.

3.2. Materials and methods

3.2.1. Soil origin and properties

Soil samples were collected from a French urban area close to a fuel station with a history of contamination by heavy metals and petroleum hydrocarbons, mostly diesel. Samples were taken with a drill auger, which allowed collecting soil from different depths between 0 and 100 cm. The different soil fractions were mixed unequally as it was technically not possible to ensure the mixing of soils from different depths in equivalent proportions. The soil was homogenized, sieved to pass through a 6 mm mesh and used for the pot experiment. Selected chemical and physical properties of this soil (*sondage 4*) are presented in Table 3.1. Physicochemical characterization of soil samples was performed by an external laboratory: ALcontrol Laboratories. ALcontrol is accredited by the Cofrac (Comité français d'accréditation) and by the RvA (Raad voor Accreditatie) under number L028, in accordance with the criteria of laboratory analysis: ISO / IEC 17025:2005. All their services are performed in accordance with their general conditions, registered under KVK number 24265286 at the Rotterdam Chamber of

Commerce, Netherlands. Analysis are performed in accordance with French standards (NF: Norme française), the Dutch Standards Institute (NEN: Nederlands Normalisatie-instituut) and the International Organization for Standardization (ISO). The following analyses were performed: actual soil pH (NF ISO 10693), cation exchange capacity (NF X 31-130), organic carbon and organic matter (NF ISO 14235), total nitrogen (sum of N Kjeldahl, NO_2^- and NO_3^- internal method, NEN 6604), C/N ratio (calculated as the ratio between the content of organic carbon and total nitrogen), P_2O_5 (Joret-Hebert method, NF X 31-161), K_2O, MgO and CaO (NF X 31-108), DTPA (diethylene triamine pentaacetic acid) available fraction of Fe and Mn (NF X 31-121), water available fraction of B (NF X 31-122), soil texture (NF X 31-107), content of As, Cd, Cr, Cu, Pb, Ni and Zn (internal method: destruction in accordance with NEN 6961, analysis in accordance with ISO 22036), content of Hg (NEN 6950, destruction in accordance with NEN 6961, analysis in accordance with NEN-ISO 16772), petroleum hydrocarbon fractions: C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{21} and C_{21}-C_{40} (internal method: acetone, hexane extraction, purification and analysis by GC-FID) and Total C_{10}-C_{40} (Equivalent to NEN-EN-ISO 16703).

Table 3.1 Chemical and physical properties of the soil (*sondage 4*)

Agronomic Parameters	
pH (H_2O)	8.1
Cation Exchange Capacity at soil pH ($cmol^+ kg^{-1}$ DW)	15.7
Organic Matter ($g\ kg^{-1}$ DW)	44.6
Organic Carbon ($g\ kg^{-1}$ DW)	25.8
Total Nitrogen ($mg\ kg^{-1}$ DW)	610
C/N ratio	42
P_2O_5 ($g\ kg^{-1}$ DW)	0.12
K_2O ($g\ kg^{-1}$ DW)	0.16
MgO ($g\ kg^{-1}$ DW)	0.12
CaO ($g\ kg^{-1}$ DW)	9.45
Fe* ($mg\ kg^{-1}$ DW)	140
Mn* ($mg\ kg^{-1}$ DW)	20.4
B* ($mg\ kg^{-1}$ DW)	0.94
Sand (%)	67.8
Silt (%)	25.1
Clay (%)	7.1
Heavy Metals ($mg\ kg^{-1}$ DW)	
As	7.4
Cd	<0.4
Cr	<15
Cu	76
Hg	3.5
Pb	100
Ni	8.1
Zn	98
Hydrocarbons ($mg\ kg^{-1}$ DW)	
C_{10}-C_{12}	640
C_{12}-C_{16}	3000
C_{16}-C_{21}	3400
C_{21}-C_{40}	1400
Total C_{10}-C_{40}	8400

DW: dry weight
* DTPA (diethylenetriaminepentaacetic acid) extraction

3.2.2. Plants

Alfalfa seeds (*Medicago sativa* L. v. La Bella Campagnola, purity: 99%, germinability: 85%) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min, thoroughly rinsed three times with sterile water and used for the pot experiment (Qu et al., 2011).

3.2.3. Pot experiment

Disinfected alfalfa seeds were sown in plastic pots (7×7×6.7 cm) filled with 200 g of contaminated soil (10 seeds per pot). Number of seedlings emerging daily were counted from day of planting in order to determine germination rates. Nine days after sowing,

seedlings were tined to six per pot in order to obtain uniform plant size and characteristics. Non-vegetated pots were used as controls. The experiment was performed in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352. Growth conditions: photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C, photosynthesis photon flux density (PPFD) of 130 μmol m^{-2} s^{-1}) and plants received water daily gently spraying with tap water. The location of pots was randomly changed daily (within the same shelf and also between different shelves in the growth chamber).
Plant mortality rates over the experimental time were recorded. Plant mortality was determined after counting live and dead plants, which were differentiated by visual inspection. Plants were considered as dead when no new leaves were growing anymore and old leaves were dried.
Plants were harvested after 60, 90, 120 and 150 days (plants were grown in parallel). At harvesting times, plants were removed from pots, and roots and shoots were separated. Roots were washed with distilled water to remove soil particles and blotted with tissue paper. The plant material was put in the oven at 70°C for 3 days (Campbell and Plank, 1998) and dry weights of shoots and roots were recorded. Soil was sampled at the same harvesting times and kept at 4 °C until further analyses. In the case of vegetated pots, rhizosphere soil samples were taken. In order to collect rhizosphere soil, plant roots were vigorously shaken by hand, taking care of the roots integrity. The external soil not attached to roots was removed, while the soil in the immediate vicinity of roots was kept for the analyses.

3.2.4. Analysis of heavy metal content in plants

Dried plant material was wet digested with 65% nitric acid and 30% hydrogen peroxide in a digestion block (LabTech DigiBlock Digester ED16S) at 125 °C for 1h (Campbell and Plank, 1998). Heating cycles and hydrogen peroxide addition were repeated to obtain a clear digest. To remove residual particles, mineralized samples were filtered, brought to final volume and stored at 4 °C until heavy metal analysis by Inductively Coupled Plasma-Optical Emission Spectrometry (PerkinElmer Optima 8300 ICP-OES Spectrometer). Cu, Pb and Zn were analyzed at the respective wavelengths of 324.752 nm, 220.353 nm and 213.857 nm.

3.2.5. Soil microbiology

3.2.5.1. Number of aliphatic hydrocarbon degraders

Aliphatic hydrocarbon degraders were counted by the most-probable-number (MPN) method described by Wrenn and Venosa (1996), using 96-well microtiter plates. Briefly, Bushnell-Haas medium supplemented with 2% NaCl was used as the growth medium and n-hexadecane as the selective growth substrate. 10-fold serial dilutions were performed from a suspension of fresh soil and buffer (0.1 % sodium pyrophosphate and 2% NaCl, pH 7.5). Plates were inoculated with the appropriate dilutions, in 5 replicates. Microplates were incubated for 2 weeks at room temperature. To identify positive wells, plates were incubated overnight with iodonitrotetrazolium

violet (3 g l^{-1}). MPN of alkane degraders per g of soil was calculated according to Briones Jr. and Reichardt (1999).

3.2.5.2. Soil lipase activity

Soil lipase activity was measured through the colorimetric method described by Margesin et al. (2002). In brief, fresh soil sample was mixed with phosphate buffer (100 mM NaH_2PO_4/NaOH buffer, pH 7.25), and pre-warmed at 30°C for 10 min. Substrate (100 mM p-nitrophenyl butyrate (*p*NPB) in 2-propanol) was added and tubes were incubated at 30°C for 10 min. To stop the reaction, the tubes were cooled for 10 min on ice. Tubes were centrifuged at 2000 *g* for 5 min and the absorbance of the released p-nitrophenol (*p*NP) in the supernatants was measured spectrophotometrically (PerkinElmer LAMBDA 10 UV/Vis Spectrophotometer) at 400 nm against the reagent blank. A standard solution of *p*NP (100 μg ml^{-1} in phosphate buffer) was used to prepare a calibration curve in the presence of soil. Lipase activity was expressed as μg *p*NP (g soil × 10 min)$^{-1}$.

3.2.6. Phytoremediation parameters

To evaluate the ability of metal phytoextraction by alfalfa, the following parameters were considered: a) plant biomass, b) root:shoot ratio (R:S), calculated as the ratio between the dry weight of roots and the dry weight of shoots, c) metal concentration in plant tissues, d) translocation factors (TFs) calculated as the metal in shoots to the metal in roots ratio and e) bioconcentration factors (BCFs) calculated as the ratio between metal concentration in plant tissues and total metal initial soil concentration. Concerning the potential of rhizodegradation, rhizosphere effect values were calculated as the ratios: MPN of rhizosphere soil/MPN of non-planted soil and soil lipase activity of rhizosphere soil/ soil lipase activity of non-planted soil.

3.2.7. Statistical analysis

Unless stated to the contrary, all data reported are averaged values of three independent replicates. For the quantification of heavy metals in plant tissues, due to scarce plant biomass, plants from three replicates were pooled together to make one single sample. When possible, treatment effects were statistically evaluated by one-way analysis of variance (ANOVA) and multiple comparisons of means by Tukey contrasts. Differences were considered significant at $p<0.05$. The statistical analysis was accomplished with R software, version 3.0.2 (R Core Team, 2014).

3.3. Results and discussion

3.3.1. Plant tolerance to co-contaminated soil

After sowing alfalfa seeds, germination rates in the co-contaminated soil were scored. In the following days, germination rates gradually increased, until the maximum was reached: 66% of alfalfa seeds germinated by day nine (data not shown). Figure 3.1

presents the experimental data on plant biomass as a function of experimental time. As shown in the figure, the alfalfa biomass yield obtained in this experiment was limited. The biomass levels of shoots reached after 60 days of growth were maintained from that time on and no significant differences between harvesting times were observed. As a result, it is apparent that shoot development was stunted. In addition, root growth was not only hindered but inhibited throughout the experiment. This effect was reflected in a continuous reduction of R:S ratios: 1.5, 1.0, 0.9 and 0.6 after 60, 90, 120 and 150 days of experiment, respectively. After 150 days, a significant reduction (46%) in root dry matter was observed, with respect to the first harvesting time. Moreover, alfalfa survival was severely affected in the co-contaminated soil and premature plant death was manifest. As shown in Figure 3.2, mortality rates constantly increased over time, reaching absolute plant mortality at 150 days of experiment.

The polluted soil used in this study appeared to be a harmful environment for alfalfa plants, leading to serious adverse effects on alfalfa germination and growth. Seed germination is known to be a sensitive process affected by environmental factors like the presence of soil pollutants (Moosavi et al., 2012). Heavy metals are known to inhibit water uptake by the embryo (Kranner and Colville, 2011) and to cause oxidative stress after permeation through the seed coat, disrupting the respiration process (Ko et al., 2012). Likewise, oil coating the seed may prevent oxygen and water uptake and oil penetrating seed coats may result in the embryo death (Baker, 1970). Therefore, these deleterious processes may explain the observed reduction in alfalfa germination rates in the present co-contaminated soil. The inhibition of alfalfa germination rates in the presence of hydrocarbons has already been reported at lower threshold levels by Al-Ghazawi et al. (2005), who observed a decline (15-30%) in alfalfa seeds germination at 500 mg kg^{-1} diesel or higher, in a filter paper media germination test. Similarly, Cr has been identified as a heavy metal responsible for alfalfa germination inhibition (Peralta-Videa et al., 2002).

Furthermore, alfalfa biomass yield was severely impacted by the presence of pollutants. The present findings seem to be consistent with previous research which found that the simultaneous presence of Cd, Cu, Ni, and Zn, at 50 mg kg^{-1} dry weight (DW) each, significantly reduced the shoot length of alfalfa, possibly due to a combined stress exerted by the heavy metal mixture, as this effect did not take place in soils individually contaminated with the heavy metals with more than 50 mg kg^{-1} DW (Peralta-Videa et al., 2002). Concerning alfalfa sensitivity to petroleum hydrocarbons, a previous study reported that growth of alfalfa seedlings was stressed and stunted in a soil contaminated with total petroleum hydrocarbons (TPH) at high levels (31000 mg kg^{-1} DW) (Kirk et al., 2005). However, the current study demonstrates that alfalfa tolerance to hydrocarbons is substantially lower since phytotoxicity was manifest at a TPH soil concentration of 8400 mg kg^{-1} DW. This discrepancy may be related to the fact that contaminant concentration alone is not sufficient to predict phytotoxicity and other factors such as the composition of heterogeneous petroleum hydrocarbon fractions and soil-hydrocarbon interactions must also be considered (Salanitro et al., 1997). Moreover, the simultaneous presence of heavy metals together with petroleum hydrocarbons may add a further contribution to plant toxicity.

The calculation of R:S ratios is one indicator that allows to assess the overall plant health. It appears that alfalfa roots were more sensitive than shoots to the toxic effect exerted by the co-contaminated soil as demonstrated by the greater negative impact on root biomass than on shoot biomass. As a result, the decrease in R:S ratios stemmed from a decrease in root biomass and not from an increase in shoot weight. It seems possible that the direct contact between polluted soil and the root surface may have contributed to the root sensitivity (Kummerová et al., 2013). Mechanisms underlying heavy metal and petroleum hydrocarbon phytotoxicity may be related both to direct effects on plant physiology (*e.g.* cell membrane disruption, damage of photosynthetic apparatus) or indirect ones such as, altering the biological, chemical and physical properties of the soil in which plants grow (Baker, 1970; Kabata-Pendias, 2011). Apart from the primary mechanisms that occurred, it is evident that they were intense enough to produce absolutely lethal effects on alfalfa plants after 150 days.

High above ground biomass yield is a requisite for phytoextraction purposes; while the establishment of a rich root system creates a favorable niche for rhizosphere microorganisms involved in rhizodegradation. The fact that in this study low biomass yield and high mortality rates were verified, severely limits the use of alfalfa for phytoremediation purposes of the present soil.

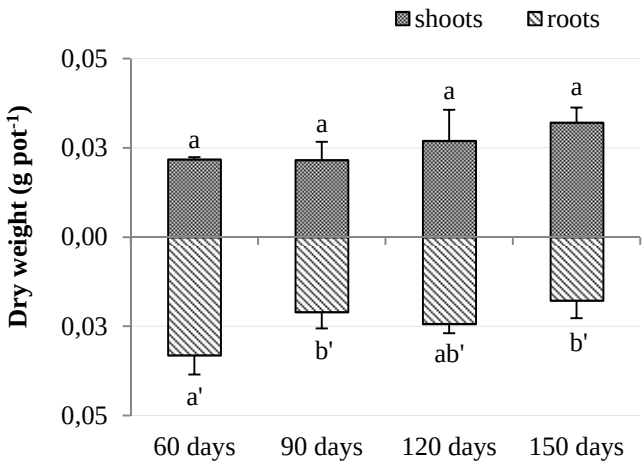

Figure 3.1 Biomass of alfalfa

Dry weight (g pot^{-1}) of shoots and roots. Values are expressed as means ± standard deviations of triplicate measurements. Different letters above the columns indicate statistically significant differences between the data sets (p<0.05).

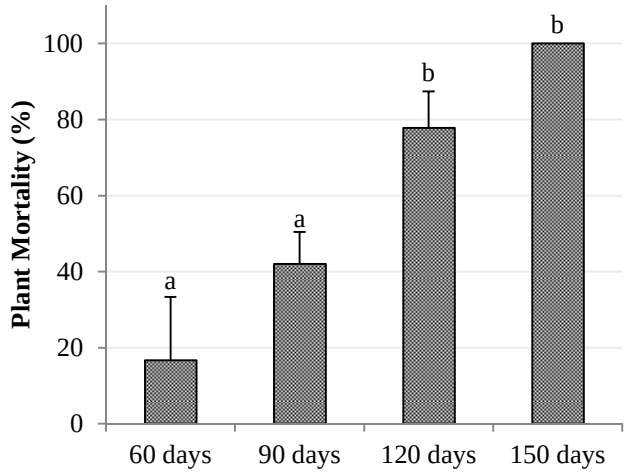

Figure 3.2 Mortality rates of alfalfa
Plant mortality (%) values are expressed as means ± standard deviations of triplicate measurements. Different letters above the columns indicate statistically significant differences between the data sets (p<0.05).

3.3.2. Heavy metals in plant tissues

Table 3.2 shows the data of Cu, Pb and Zn concentrations in alfalfa tissues depending on the experimental time. As can be seen from the table, the extent of plant uptake varied with metal type. Heavy metal concentrations in shoots and roots of alfalfa after 150 days of experiment were in the following order: Zn > Pb > Cu and Zn > Cu > Pb, respectively. Metal contents of all elements were substantially higher in roots than in shoots. Maximum metal concentration in roots reached 333.2, 245.8 and 231,7 mg kg^{-1} DW for Zn, Cu and Pb, respectively. In shoots, concentrations did not exceed 219.8, 110.6 and 74.8 mg kg^{-1} DW for Zn, Pb, and Cu, respectively. These results show that heavy metals were mainly accumulated in root tissues, revealing, in general, poor metal translocation from roots to shoots. As shown by TF data, Cu and Pb were slightly translocated (average TFs ranged from 0.31 to 0.38) while Zn was the most translocated element, presenting an averaged TF value of 0.64. As demonstrated by the BCF values of shoots, Cu and Pb were accumulated in alfalfa aerial parts to equivalent extents. Average BCF values for these metals ranged from 0.75 to 0.81. In contrast, Zn was accumulated to a greater extent (mainly in the first 90 days of experiment) presenting an average BCF of 1.97. BCF values of roots were considerably higher than those of shoots. Average BCF values were: 2.03 for Pb, 2.63 for Cu and 3.09 for Zn.
The uptake of heavy metals by plants varies according to plant species, but soil characteristics and metal speciation also determine the process (Kabata-Pendias, 2011). Soil properties like a high pH value or elevated organic matter content decrease metal mobility in soils and as a result their plant uptake (Gobran et al., 2000). Moreover, antagonistic effects between metals in multi-metal contaminated soils (Flogeac et al., 2007) as well as the simultaneous presence of organic pollutants can decrease plant uptake of heavy metals in co-contaminated soils (Lin et al., 2008).

TF and BCF were calculated to better evaluate the potential of alfalfa for phytoremediation purposes. The TF represents the ability of the plant to transfer the metals from roots to shoots. In the present study TF values were low (always <1), revealing low mobility of metals towards aboveground tissues, while immobilization of heavy metals in roots was favored. Qu et al. (2011) have previously reported a similar pattern of limited heavy metal translocation by alfalfa, with TF values of 0.40 for Cu, 0.61 for Zn and 0.79 for Pb. Results of the present study are also similar to those previously reported by Qu et al. (2011), although the latter study was conducted in different conditions *i.e.* a multi-metal contaminated soil, without organic pollutants. In addition, BCFs were calculated as indicators of the ability of the plant to accumulate metals in plant tissues from soils. BCF values of shoots calculated in this study are comparable to those previously reported by Qu et al. (2011), who found BCFs of shoots of 0.81 for Pb, 1.42 for Cu and 1.81 for Zn. Likewise, they reported higher BCFs of roots than shoots. The fact that in the present study heavy metals were poorly translocated and preferentially accumulated in alfalfa roots may further support the idea of an association between high concentration of heavy metals in roots and increased phytoxicity affecting plant root biomass, as discussed in the previous section.

The phytoextraction ability of a plant relies on the total amount of metal that can be uptaken, which depends on both, metal concentration in plant harvestable tissues and plant biomass yield. The fact that heavy metals were mainly accumulated in roots and the scarce plant biomass obtained, result in negligible total heavy metal uptake by alfalfa, hence low phytoextraction ability. However, the fact that heavy metals were accumulated to a certain extent in plant roots (low TF but high BCF of roots) could lead to the possibility of phytostabilization of heavy metals, provided that plants were able to tolerate their presence. Moreover, a vegetative cover with alfalfa species could improve ecosystem functioning and physicochemical properties of the contaminated soil (Ouvrard et al., 2011; Hamdi et al., 2012).

Table 3.2 Heavy metal phytoextraction parameters

	Cu				Pb				Zn			
	60 d	90 d	120 d	150 d	60 d	90 d	120 d	150 d	60 d	90 d	120 d	150 d
Shoots (mg kg^{-1} DW)	57.4	65.0	48.2	74.8	52.8	71.2	65.5	110.6	212.1	219.8	175.5	164.1
Roots (mg kg^{-1} DW)	152.3	183.0	218.2	245.8	230.2	137.9	210.7	231.7	333.2	263.9	298.9	315.2
TF	0.38	0.36	0.22	0.30	0.23	0.52	0.31	0.48	0.64	0.83	0.59	0.52
BCF of Shoots	0.76	0.86	0.63	0.98	0.53	0.71	0.66	1.11	2.16	2.24	1.79	1.67
BCF of Roots	2.00	2.41	2.87	3.23	2.30	1.38	2.11	2.32	3.40	2.69	3.05	3.22

DW: dry weight, TF: translocation factors, BCF: bioconcentration factors

3.3.3. Effect of plants on soil microbiology

Table 3.3 shows the experimental data on MPN of aliphatic hydrocarbon degraders and soil lipase activity. Although fluctuating, the general trend showed an increase in the two parameters over the 150-day experimental period, both in planted and unplanted conditions. The presence of alfalfa plants stimulated microbial number and activity in the rhizosphere, as demonstrated by the higher values obtained in vegetated pots relative to the unplanted control. However, as the experiment continued the plant promoting effect became less pronounced, as can be corroborated by decreasing rhizosphere effect values for MPN enhancement: 3.3, 1.1, 1.6 and 0.6 after 60, 90, 120 and 150 days of experiment, respectively. A similar tendency was observed for lipase enzyme, with rhizosphere effect values of 1.4, 1.3, 1.1 and 1.0 after 60, 90, 120 and 150 days of experiment, respectively.

Microbial counts of alkane degraders and lipase activity are soil bioindicators of hydrocarbon rhizodegradation potential. The MPN of soil aliphatic hydrocarbon degrading bacteria is a quantitative marker of the population of microorganisms able to metabolize aliphatic hydrocarbons (Wrenn and Venosa, 1996). In addition, soil lipase activity can be a suitable parameter to monitor oil biodegradation in soil, as microbial enzymatic systems responsible for lipid degradation may be similar to those involved in oil decomposition (Margesin et al., 1999). Both bioindicators can be related to the potential of a soil for hydrocarbon dissipation, as revealed by the positive correlation between soil hydrocarbon removal and the mentioned bioindicators (Wrenn and Venosa, 1996; Margesin et al., 1999).

Rhizosphere effect values were calculated to adjudge the magnitude of plant root influence over the non-planted soil on soil microbial number and activity. In accordance with the present results, a former study has reported a rhizosphere effect value of 5 in the MPN of hexadecane degrading microorganisms for alfalfa growing in a hydrocarbon contaminated soil (31000 mg kg^{-1} DW), after 49 days of experiment (Kirk et al., 2005). They also reported a positive effect of alfalfa on rhizospheric total heterotrophic bacteria and total petroleum degrading bacteria. Similarly, a study performed with alfalfa growing in soils co-contaminated by heavy metals and PAHs showed an increase in both total and PAH-degrading bacteria populations in the rhizosphere of alfalfa (Ouvrard et al., 2011). Enhancement of lipase activity in the presence of plants has been previously reported as well. Gaskin and Bentham (2010) observed a significant stimulation of soil lipase activity in the rhizosphere of Australian grasses growing in hydrocarbon-contaminated soil, relative to non-vegetated control.

The rhizosphere effect refers to the positive influence of plant roots on microbial population and activity in the rhizosphere (Manoharachary and Mukerji, 2006). This effect is mainly the result of rhizodeposition, *i.e.* the release of organic compounds by plants, which supplies microorganisms with nutrients (Nguyen, 2009). In addition, roots offer mechanical support for the attachment of microorganisms as well as an improvement of soil physicochemical properties (*e.g.* aeration), which further benefit the development of microorganisms in the rhizosphere (Lynch, 1990). The current study found that as the experiment advanced the rhizosphere effect declined. This result may

be explained by the fact that as time passed plant physiology was gradually deteriorated. This fact was also reflected in a reduction of root biomass and possibly related to the accumulation of metals in these tissues. It can thus be hypothesized that alfalfa plants were able to create a proper niche for the development of rhizosphere microorganisms during the first 60 days of experiment. However, from then on phytotoxicity was manifest and the rhizosphere effect was hindered. Therefore it seems that the potential of alfalfa to enhance rhizodegradation remains limited to the initial phase of plant development when the rhizosphere effect is evident and before root phytotoxicity takes place. Still, these results need to be interpreted with caution because in the present study the residual soil petroleum hydrocarbon concentration was not measured and as a result the correlation between bioindicators and pollutant removal was not established.

Table 3.3 Soil microbial number of alkane degraders and lipase activity

Treatment	Time	MPN of soil aliphatic degraders (MPN (g soil)$^{-1}$)	Soil lipase activity (µg *p*NP (g soil × 10 min)$^{-1}$)
Soil	60 d	$(4.3 \pm 1.9) \times 10^6$	182 ± 3
	90 d	$(1.3 \pm 0.2) \times 10^7$	505 ± 11
	120 d	$(7.1 \pm 0.1) \times 10^6$	470 ± 34
	150 d	$(1.2 \pm 0.6) \times 10^8$	610 ± 16
Soil + Alfalfa	60 d	$(1.4 \pm 0.1) \times 10^7$	248 ± 6
	90 d	$(1.4 \pm 0.01) \times 10^7$	661 ± 15
	120 d	$(1.2 \pm 0.1) \times 10^7$	515 ± 43
	150 d	$(6.6 \pm 9.7) \times 10^7$	633 ± 57

Values are expressed as means ± standard deviations of duplicate measurements.
Initial (prior to planting) MPN of alkane degraders (g soil)$^{-1}$: $(2.7 \pm 0.7) \times 10^6$ and soil lipase activity: 399 ± 3 (µg *p*NP (g soil × 10 min)$^{-1}$).

3.4. Conclusions

On the whole, these results suggest that the presence of heavy metals and petroleum hydrocarbons, at the studied concentrations in the present soil, are probably above the phytotoxicity threshold for alfalfa restricting plant growth and survival. Although it seems possible that the presence of pollutants was a key factor affecting plant performance, other causes cannot be excluded. The soil nutrient state is of significant relevance for plants to grow healthy. As a result nutrient deficiencies (*e.g.* nitrogen, phosphorus) may have also resulted in a significant reduction of plant yield and shorten stand life.

The findings of this study do not support strong recommendations to use alfalfa for metal phytoextraction. In spite of this, the accumulation of heavy metals in plant roots could lead to the possibility of phytostabilization of metals in the root zone. Moreover, the initial concomitant increase in alkane-degrading microbial numbers and lipase activity in the rhizosphere of alfalfa plants could potentially result in enhanced rhizodegradation of hydrocarbons. In any case and in order to make these approaches feasible, alfalfa tolerance to contaminants will have to be improved. Future studies

could assess further amendments to improve the soil structure or the use of fertilisers to provide essential nutrients to plants. Other strategies that could result in increased plant tolerance are a reduction in the level of soil pollutants, bioaugmentation of soil with plant growth promoting rhizobacteria or even the use of genetically modified plants.

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Chapter 4

Phytotoxicity of citric acid and Tween® 80 for potential use as soil amendments in enhanced phytoremediation

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Abstract

Enhanced phytoremediation adding biodegradable amendments like low molecular weight organic acids and surfactants is an interesting area of current research to overcome the limitation that represents low bioavailability of pollutants in soils. However, prior to their use in assisted phytoremediation, it is necessary to test if amendments *per se* exert any toxic effect to plants and to optimize their application mode. In this context, the present study assessed the effects of citric acid and Tween® 80 (polyethylene glycol sorbitan monooleate) on the development of alfalfa (*Medicago sativa* L.) plants, as influenced by their concentration and frequency of application to evaluate the feasibility for their future use in enhanced phytoremediation of co-contaminated soils. The results showed that citric acid negatively affected plant germination, while it did not have any significant effect on biomass or chlorophyll content. In turn, Tween® 80 did not affect plant germination and showed a trend to increase biomass, as well as it did not have any significant effect on chlorophyll levels. alfalfa appeared to tolerate citric acid and Tween® 80 at the tested concentrations, applied weekly. Consequently, citric acid and Tween® 80 could be potentially utilized to assist phytoremediation of contaminated soils vegetated with alfalfa.

Keywords

Soil remediation, heavy metals, organic contaminants, alfalfa (*Medicago sativa* L.), citric acid, Tween® 80.

4. Phytotoxicity of citric acid and Tween® 80 for potential use as soil amendments in enhanced phytoremediation

4.1. Introduction

Phytoremediation is one of the remediation approaches which can be used to deal with inorganic and organic contaminants when they are present individually or collectively in co-contaminated sites (Ouvrard et al., 2011). In particular, phytoextraction and rhizodegradation are two types of phytoremediation technologies that can be employed together to clean-up contaminated soils with inorganic contaminants like heavy metals and organic pollutants such as hydrocarbons (Tsao, 2003).

When dealing with phytoextraction, plants have a central role as heavy metals are taken up by the roots, translocated and accumulated in the above ground tissues (Salt et al., 1995). Several processes are involved during metal phytoextraction including mobilization and uptake from the soil, compartmentalization and sequestration within the root, xylem loading and transport, distribution between metal sinks in the aerial parts, and finally sequestration and storage in leaf cells (Clemens et al., 2002). As a consequence, heavy metal contaminated media potentially could be remediated by cultivation of plants and harvesting the metal containing biomass.

When compared to phytoextraction, plants used in rhizodegradation have a secondary role in the dissipation of organic contaminants. The plant roots, through the release of root exudates, provide energy sources that support the growth of microorganisms in the rhizosphere *i.e.* the volume of soil influenced by the root and the colonizing microorganisms (Hiltner, 1904). Thus, in rhizodegradation, the clean-up goal is the remediation of soils through the degradation of organic contaminants by rhizospheric microorganisms, whose growth is enhanced by plants exudates (Kuiper et al., 2004).

Alfalfa (*Medicago sativa* L.) exhibits interesting characteristics to be used in phytoremediation. It is a fast growing perennial plant, which leads to high biomass harvests (Coburn, 1912); it can develop an extensive root system that provides a large surface for the support of rhizosphere microorganisms (Kirk et al., 2005), and presents root nodules with bacteria able to fix nitrogen (Truchet et al., 1991) allowing alfalfa to grow in soils with high C/N ratios. Alfalfa has been demonstrated to be able to grow in contaminated media and has been used for the phytoremediation of heavy metals and organic contaminants. Previous studies have shown that alfalfa can phytoextract heavy metals such as Cd, Cr, Cu, Ni and Zn (Peralta-Videa et al., 2002). In addition, alfalfa has been demonstrated to have a role in the remediation of organic contaminants like polycyclic aromatic hydrocarbons (PAHs) (Fan et al., 2008) or organochlorines (Li and Yang, 2013). Moreover, it has been also studied for the phytoremediation of co-contaminated soils in a short-term greenhouse experiment (Ding and Luo, 2005), in a long-term field experiment (Ouvrard et al., 2011) and more recently, through the improvement of genetic engineering techniques (Zhang et al., 2013).

Still, one of the limitations that may restrict the success of phytoremediation technologies is the low bioavailability of contaminants in soils (Evangelou et al., 2007; Meers et al., 2008). As a result, many research attempts have been done in order to increase the ability of pollutants to be transferred from a soil compartment to plants or microorganisms to accomplish its accumulation and/or degradation pathway.

One of the most diffused approaches to increase the bioavailability of heavy metals to non-hyperaccumulator plants has been the application of chelating agents that increase metal availability in soil solution to be finally uptaken by plants, *i.e.* chelate-assisted phytoextraction (Evangelou et al., 2007; Meers et al., 2008). Nevertheless, the use of synthetic aminopolycarboxylic acids like ethylene diamine tetraacetic acid (EDTA), which has been widely used to assist phytoextraction of heavy metals (López et al., 2005), poses adverse effects due to the poor biodegradability, leaching risks and high toxicity of such compounds (Evangelou et al., 2007). For these reasons, research on chelate-assisted phytoextraction tends to look for alternative compounds that combine high biodegradability, low phytotoxicity and chelating strength. In this context, there is a renovate interest on low molecular weight organic acids (LMWOAs), whose use as soil amendments to enhance phytoremediation of heavy metals has already been reported for many years (Huang et al., 1998). Among LMWOAs, citric acid is a tricarboxylic acid which has been reported to increase both heavy metal desorption from soils (Gao et al., 2003) and uptake by several plant species (Chen et al., 2003; do Nascimento et al., 2006; Evangelou et al., 2006; Duquène et al., 2009). However, few studies assessed the phytotoxic effects of citric acid on alfalfa and the influence of citric acid on heavy metal uptake by alfalfa (Qu et al., 2011).

Additionally, surfactant-enhanced phytoremediation is a remediation strategy consisting in the use of surface active compounds with amphiphilic chemical structure to increase the water solubility of organic contaminants and thus improve the mobility and biodegradation of pollutants throughout phytoremediation (Gao et al., 2007). The addition of surfactants as amendments to an organic polluted media has been primarily used to increase bioavailability of hydrophobic compounds by enhancing the mass transfer from the soil solid to aqueous liquid phase. The main implication of this is to facilitate the degradation of pollutants principally by microorganisms at the rhizosphere level (rhizodegradation) and potentially by plants that could take up and metabolize moderately hydrophobic organic contaminants (phytotransformation) (Dietz and Schnoor, 2001). Surfactant-enhanced phytoremediation has been primarily used to deal with organic contaminants like PAHs (Wu et al., 2008), but also to remediate heavy metal contaminated media (Almeida et al., 2009). In particular, Tween® 80 is a non-ionic surfactant that has been shown to increase the desorption of organochloride pesticides from soils (Gonzalez et al., 2010), as well us plant uptake (Gao et al., 2008) and removal (Cheng et al., 2008) of PAHs. Though, little information is available regarding the phytotoxicity of Tween® 80 on alfalfa and if its application affects the dissipation of contaminants in soils vegetated with alfalfa.

Evidence from LMWOA and surfactant-assisted phytoremediation experiments has shown different effectiveness depending on the type and concentration of amendments, strategy of application, type and concentration of pollutants, plant species and soil

characteristics (Agnello et al., 2014). In addition, when using such kind of amendments it is essential to know if these compounds themselves exert any toxicity effect toward the plant species in order to value if they can be used for phytoremediation purposes. One of the mechanisms by which chelating agents and surfactants may increase metal uptake is through root membrane disruption, which facilitates plant-metal accumulation as a result (Nowack et al., 2006; Evangelou et al., 2007). This fact reveals that certain degree of phytotoxicity at the root level may be required for soil amendments to be effective in this way. However, elevated doses of amendments may also produce other adverse phytotoxic effects (*i.e.* inhibition of seed germination, hindering of plant growth or alteration of plant physiology), making hard to establish the limit between desirable and detrimental phytotoxicity.

In this sense, the aim of the present study was to assess the effects of the LMWOA citric acid and the surfactant Tween® 80 on the development of alfalfa plants, as affected by their concentration and mode of application. The outcome of this work is expected to provide insights regarding the application strategy of citric acid and Tween® 80 to successively use them as amendments to enhance the phytoremediation of soils contaminated with heavy metals and petroleum hydrocarbons, and vegetated with alfalfa.

4.2. Materials and methods

4.2.1. Chemicals

The tricarboxylic acid citric acid ($C_6H_8O_7$, molecular weight: 192 g mol^{-1}, pKa_1: 3.13, pKa_2: 4.76, pKa_3: 6.40) used in this experiment was purchased from Carlo Erba Reagents Group ($C_6H_8O_7.H_2O$, purity >99.5%). The anionic surfactant Tween® 80 (polyethylene glycol sorbitan monooleate, $C_{64}H_{124}O_{26}$, molecular weight: 1310 g mol^{-1}, critical micelle concentration (CMC): 0.012 mM) was obtained from Sigma-Aldrich Chemical Co. All the other chemicals used (*i.e.* acetone, hydrogen peroxide) were of analytical grade.

4.2.2. Plants

Alfalfa seeds (*Medicago sativa* L. v. La Bella Campagnola, purity: 99%, germinability: 85%) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min (Qu et al., 2011). After that, seeds were washed three times with sterile water and used for the pot experiment or for the germination test.

4.2.3. Pot experiment

Disinfected seeds were germinated and seedlings grown for 14 days in Styrofoam trays. Subsequently, three seedlings of uniform size were selected and transplanted in each plastic pot (10 cm diameter, 8 cm height) filled with 100 g of commercial soil (organic carbon: 20%, organic nitrogen: 0.4%, organic matter: 40%, undefined mineral fraction) and formerly lined with a plastic bag to prevent liquid loss. Pots were put outdoors and

received water daily by gentle spraying with tap water. The experiment was performed from May 28th to July 27th and diurnal temperatures varied between 16-30°C (data not shown). The experimental design included the weekly treatment of pots with a range of concentrations of citric acid (5, 15, 45 and 90 mmol kg^{-1} dry soil) or Tween® 80 (at 0.25, 0.5, 1 and 3 times the CMC). A revision of citric acid-enhanced phytoremediation experiments showed citric acid concentrations varying from 0.5 (Chen et al., 2003) to 62.5 mmol kg^{-1} dry soil (Evangelou et al., 2006). Although higher concentrations could not be used during phytoremediation due to phytotoxicity, practical or economic reasons; a wider range of concentrations was chosen to compensate for the lower bulk density of the used organic commercial soil with respect to a mineral soil, plausible target of future phytoremediation experiments. Selected concentration for Tween® 80 encompassed several values above and below the CMC, which were related to doses used in previous phytoremediation experiences with this surfactant (Cheng et al., 2008). There were chosen weekly applications of amendments with the aim to keep elevated, stable and effective soil concentrations when assessing amendment impact on alfalfa, but considering that such frequency of application should be decreased to make feasible a phytoremediation approach. The control treatment received the same amount of distilled water instead of the amendments solutions. Each condition was replicated three times for statistical purposes. Plants were harvested after 1, 4, 6 and 8 weeks growth (the different treatments were grown in parallel) and every time three days after the amendment application. Plant parts were separated into roots and shoots. Afterwards, roots were washed with tap water to remove soil particles and blotted with tissue paper. Subsequently, the vegetal material was put in the oven at 70°C for 3 days (Campbell and Plank, 1998). Finally, dry weights of shoots and roots were recorded. One day before the mentioned harvesting times, one plant in each pot was removed and its leaves were used for chlorophyll determination.

4.2.4. Chlorophyll determination

Chlorophyll was extracted from plants and analyzed using the Arnon method (Pocock et al., 2004). Briefly, fresh leaf tissue was grind in a chilled mortar and pestled in acetone. The extract was centrifuged to clarify and afterwards diluted in 80% acetone. Absorbance of the extract was measured in the spectrometer (Perkin Elmer Lambda 10 UV/VIS Spectrometer) at 663 (Chlorophyll a) and 645 nm (Chlorophyll b). Total chlorophyll concentration was calculated according to the equations of Arnon (Arnon, 1949; Porra, 2002) and expressed as mg of total chlorophyll per g of fresh leaf weight.

4.2.5. Germination test

12 disinfected alfalfa seeds were put in Petri dishes (10 cm diameter) covered with filter paper (Whatman N°42, 90 mm diameter). The experimental design included the treatment of Petri dishes with solutions of citric acid at 5, 15, 45 and 90 mM or Tween® 80 at 0.25, 0.5, 1 and 3 times the CMC. Control treatment consisted of the addition of distilled water instead of the amendments solutions. Each condition was replicated three times for statistical purposes. All the material and solutions used in the case of citric

acid treatment were sterile in order to avoid microorganism contamination, which was previously observed. Petri dishes were left in the darkness at room temperature (23°C) and germinated seeds were quantified after 24, 48 and 72 hours.

4.2.6. Statistical analysis

The experiment was arranged in a completely randomized design. All data reported are averaged values of three independent replicates. Data were statistically evaluated by one-way analysis of variance (ANOVA) and significantly different means were assessed by the Tukey's test. Differences between treatments were considered significant at $p<0.05$. The statistical analysis was accomplished with R software, version 3.0.2 (R Core Team, 2014).

4.3. Results and discussion

4.3.1. Effects on germination rates

Germination rates of alfalfa in the presence of citric acid at the different tested concentrations differed significantly from the control (Figure 4.1a) and decreased with increasing concentrations of citric acid. After 3 days, the germination rate obtained for control was 92%, while in the presence of citric acid at 5 mM it was 56% and only 6% at 15 mM. When citric acid was applied at 45 mM or higher concentrations the germination was totally inhibited. These results indicate that citric acid hinders the germination of alfalfa in a concentration-dependent manner. Similar observations were done by Wu et al. (2006), who observed that acetic, citric and oxalic acids were toxic to the germination of cress (*Lepidium sativum*) seeds. These authors assumed a seed germination index of 100% in distilled water and observed that citric acid at 1.7 mM reduced the germination index to 37%. Moreover, oxalic (2.5 mM) and acetic (5 mM) acids were highly toxic, resulting in a germination index of 8% and 0% respectively. Likewise, Eşen et al. (2006) observed that citric acid (5.7 mM) exposures longer than 48 hours substantially decreased black cherry (*Prunus avium*) seeds germination, possibly due to embryo damage. As well, Lynch (1980), observed that certain organic acids had inhibitory effects on seed germination: benzoic acid and salicylic acids (5 mM) decreased germination rates of barley (*Hordeum vulgare*) to 60% and acetic acid (30 mM) to 77%. In addition, organic acids have shown a negative effect on the early development of seedlings. Cui et al. (2007) performed a seed germination test of zinnia (*Zinnia elegans* Jacq.) in presence of citric, oxalic and tartaric acids at various concentrations (1.2-9.6 mM). After 7 days, these organic acids negatively affected the root length of seedlings and tartaric and oxalic acids decreased their shoot length as well.

Citric acid solutions used in the present germination test covered a range of pH between 2.1 and 2.8 (n.b.: pH calculation was done by assuming that citric acid is deprotonated once, thus only using Ka_1 and considering as negligible Ka_2 and Ka_3). It is likely that the adverse effect of citric acid on alfalfa germination is due to an increase in H^+ ions concentration. This hypothesis is consistent with previous results obtained by Ryan et

al. (1975), who observed a significant decrease in alfalfa germination rates when pH was below 4.0 using sulfuric acid as acidulant. At pH 3.0, they reported 30% inhibition, magnitude which is comparable to that reported here: 44 % inhibition for citric acid at 5 mM, *i.e.* pH 2.8. Although pH decrease could be a major factor causing the inhibition of alfalfa germination, a direct effect of citric acid cannot be excluded.

By contrast, germination rates of alfalfa in the presence of Tween® 80 at the different tested concentrations did not differ significantly from the control (Figure 4.1b). After 3 days, germination rates were more than 90%, indicating that Tween® 80 did not affect the germination of alfalfa. Comparable results were obtained by Cheng et al. (2008), who evaluated the germination rates of tall wheatgrass (*Agropyron elongatum*) in a PAH-contaminated soil amended with Tween® 80 at concentrations up to 100 mg kg^{-1} soil, observing no difference with the control and obtaining germination rates in the range of 84% to 87%. Moreover, it has been observed that Tween® 80 may exhibit morphogenic properties. Parr and Norman (1964) studied the effect of Tween® 80 (0.01% v/v) on organ development of 4-day-old barley (*Hordeum vulgare*) seedlings, finding that Tween® 80 enhanced the length of coleoptiles, roots and leaves by 6%, 30%, and 59% respectively.

To select the most suitable moment to amend soils, the effect of amendments on plant germination should be considered, which is especially important in the case of annual crops. In a typical phytoremediation application with alfalfa, plants would be planted and allowed to grow promoting the initial establishment of the root system. Subsequently soil amendments would be applied. If amendments were applied before certain plant establishment occurred, they could be degraded before producing any effect. After amendment addition plants would be harvested and allowed to regrow without any need for replanting, due to the perennial nature of alfalfa species. In this context, the relevance of amendment application impact on alfalfa germination rates could be questionable. Hence, it could be conceivably that during the extended period that a phytoremediation approach would require, replanting may be needed, for instance in case of mortality of established plants. Although secondary, the effect of amendments on germination rates has also certain implications concerning the inherent reproduction of alfalfa by seed production. Results from this experiment show that, although not recommended, Tween® 80 could potentially be applied near the sowing time while for citric acid, which inhibited the germination of alfalfa, it could be necessary to supply it after the germination of plants. Moreover, this approach could be convenient to overcome limitations in the effectiveness of LMWOAs due to their rapid biodegradation in soils. This is consistent with earlier observations made by Meers et al. (2004), who studied the timing application of LMWOA in a calcareous clayey soil vegetated with maize (*Zea mays*). They tested the effects of several organic acids (*i.e.* ascorbic, citric, oxalic and salicylic acid acids, and NH_4 acetate) on heavy metal phytoextraction at a dose of 2 mmol kg^{-1} soil, applying them to soils 1 day before sowing. In these conditions they observed no significant increase in Cd, Cu, Pb and Zn shoot uptake. As a result, Meers et al. (2004) concluded that it would be better to apply organic acids soon before harvesting than near the sowing time in order to overcome the biodegradation of organic acids.

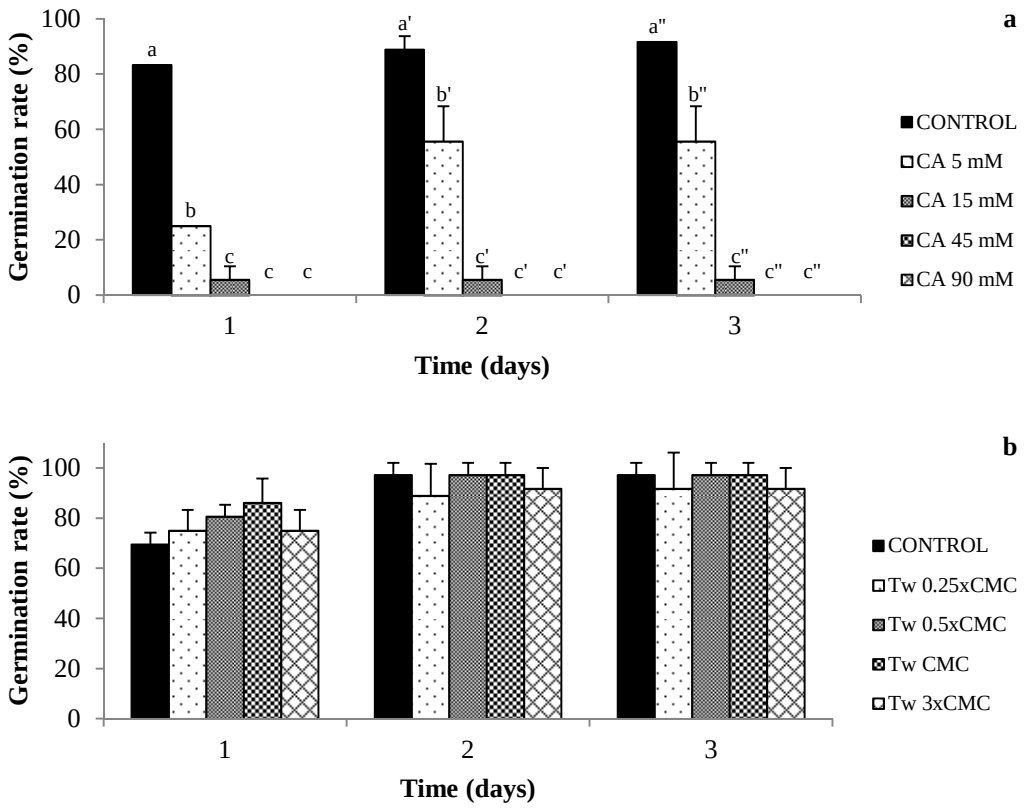

Figure 4.1 Germination rates of alfalfa
Alfalfa seeds germinating in the presence of: (a) citric acid (CA) and (b) Tween® 80 (Tw) as a function of time. Values are average ± S.D. (n=3). Values followed by different letters are significantly different (p<0.05). Lack of letter means no significant difference (p<0.05) between treatments and controls.

4.3.2. Effects on biomass production

Biomass of alfalfa shoots and roots increased throughout the 8 weeks that the experiment lasted, for all the conditions evaluated, indicating that the amendments did not hinder plant growth.

There was no evident effect on alfalfa biomass by increasing concentrations of citric acid: the application of citric acid at the tested concentrations did not affect significantly the biomass of shoots (Figure 4.2a) and roots (Figure 4.3a) of alfalfa with respect to controls at any of the harvesting times. Similarly, it was observed no significant difference in the dry matter yield of Indian mustard (*Brassica juncea*) in the presence of citric acid at 10 mmol kg^{-1} soil (do Nascimento et al., 2006) as well as no significant effects on *Z. mays* and white bean (*Phaseolus vulgaris*) biomass when treated with citric acid at 5 mmol kg^{-1} soil (Luo et al., 2005). Furthermore, there are several reports in the literature which support that citric acid could contribute to alleviate heavy metal stress on plants, preventing negative effects on plant biomass and growth. In this sense, Gao et al. (2010) observed that the application of citric acid (20 mmol kg^{-1}) significantly improved the biomass of black nightshade (*Solanum nigrum*) growing in Cd-

contaminated soils and Qu et al. (2011) reported an increase in the biomass of alfalfa treating the heavy metal polluted soil with sodium hydrogen phosphate/citric acid mixtures. Similarly, Najeeb et al. (2011) found that citric acid (2.5 and 5 mM) improved root dry weight and root morphological characters (*e.g.* root diameter, surface area and volume) of mat rush (*Juncus effuses*) growth in Cd-contaminated soils. In turn, Jean et al. (2008) showed that a single or double application of 5 and 10 mmol kg^{-1} citric acid to a vegetated soil contaminated with Cr and Ni resulted in a decrease in root and leaf biomass of *Datura innoxia*, while at a lower concentration (1 mmol kg^{-1}) there were no significant differences when compared to controls. Moreover, whatever the concentration and the application mode to the contaminated soil, citric acid did not significantly deteriorate the plant net photosynthetic rate. In contrast, other reports demonstrated that citric acid may produce negative effects on plant biomass and physiology (do Nascimento et al., 2006; Evangelou et al., 2006; Duquène et al., 2009). It is evident that the effects of citric acid on plant biomass may vary according to citric acid concentration and mode of application, plant species and soil characteristics. As a result, it is hard to generalize and predict the effects of citric acid on plants and thus, it is essential to study the conditions of every particular situation (nature, concentration and supply frequency of organic acid and also plant species and its nutritional status).

alfalfa is known to be sensitive to soil pH. Thus, the consequences of citric acid on plant biomass and physiology can not only be due to the effect of citric acid *per se*, but also because of its influence on soil pH. One of the mechanisms determining alfalfa intolerance to soil acidity is mediated by ions such as Al and Mn. Solubility of these ions is enhanced as soil acidic conditions increase, attaining phytotoxic levels (*e.g.* root extension inhibition) when soil pH is below 5.5 (Haby et al., 1992). Although soil pH measurements were not performed throughout the experiment, it is likely that the transient pH decrease subsequent to citric acid addition was not sufficient to substantially affect alfalfa yield, as no significant decrease in alfalfa biomass, with respect to non-amended control, was observed. Additionally, alfalfa nodulation is a process susceptible to soil pH, in which acidic conditions may alter the symbiotic interaction between alfalfa and rhizobia bacteria (Segundo et al., 1998). Even though the study of the effects of citric acid on plant-bacteria symbiosis was beyond the scopes of the present study, it was possible to verify that when citric acid was applied to the soil (even at the highest concentration) root nodulation still occurred, as evidenced by visual inspection of the root system when plants were harvested.

Concerning Tween® 80, in general terms its application increased alfalfa shoot (Figure 4.2b) and root (Figure 4.3b) biomass compared to controls. This difference was statistically significant for shoots when Tween® 80 was applied at 0.5×CMC and at CMC for plants harvested after 1 and 4 weeks. In the case of plant roots, differences were significant at concentrations above 0.5×CMC for plants harvested after 1 week and at all the experimental concentrations for plants harvested after 4 weeks. After 6 weeks of experiment, only the application of Tween® 80 at the CMC resulted in a significant increase of root biomass. It has been previously demonstrated that in the presence of Tween® 80 at 8 times the CMC, no significant difference in red clover (*Trifolium pretense*) biomass or phytotoxicity effects were observed after 12 days of growth in a

hydroponics study with phenanthrene and pyrene spiked water (Gao et al., 2008). Likewise, the application of Tween® 80 at concentrations up to 100 mg kg^{-1} soil did not cause any significant effect on the biomass yields of *A. elongatum* (Cheng et al., 2008). Concerning the improvement of plant biomass by surfactants, Zhu and Zhang (2008) reported that biologically produced surfactants such as rhamnolipids (at 0.5×CMC or below) could stimulate the growth of ryegrass (*Lolium multiflorum*) shoots growing in phenanthrene and pyrene spiked water. These authors proposed that the increased root permeability in the presence of biosurfactants may lead to a more efficient uptake of nutrients, which could be one of the mechanisms involved to explain such enhancement in plant biomass yield.

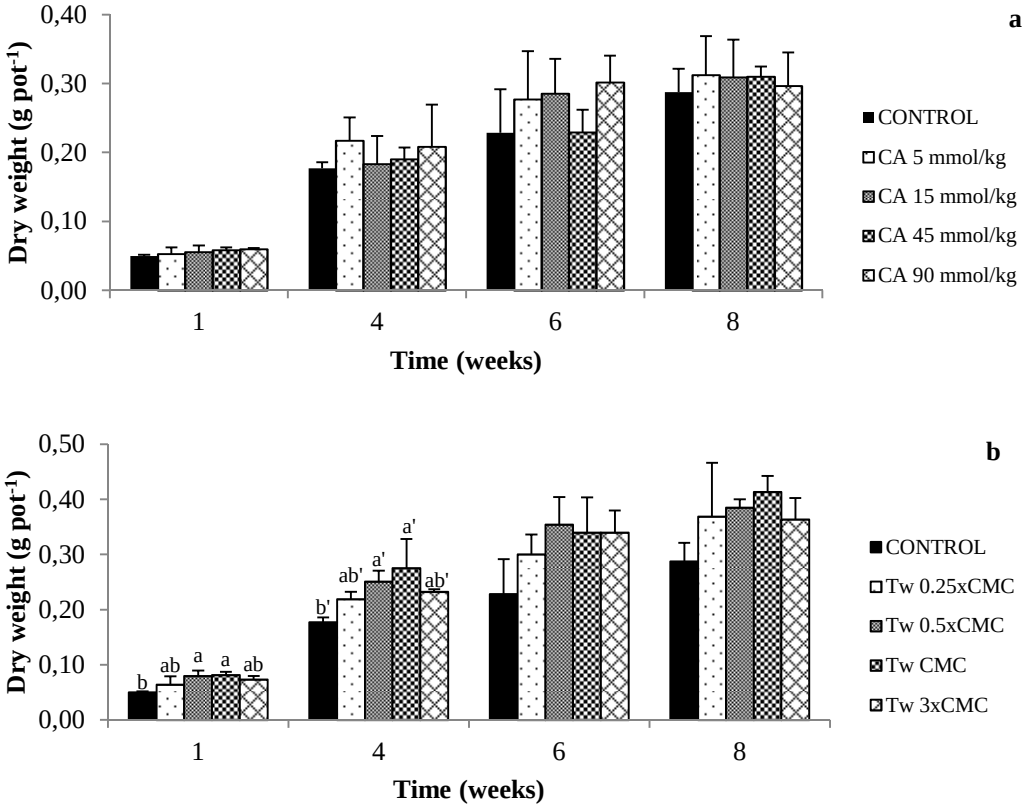

Figure 4.2 Biomass of alfalfa shoots

Dry weight (g pot^{-1}) of alfalfa shoots treated with (a) citric acid (CA) and (b) Tween® 80 (Tw) as a function of time. Values are average ± S.D. (n=3). Values followed by different letters are significantly different (p<0.05). Lack of letter means no significant difference (p<0.05) between treatments and controls.

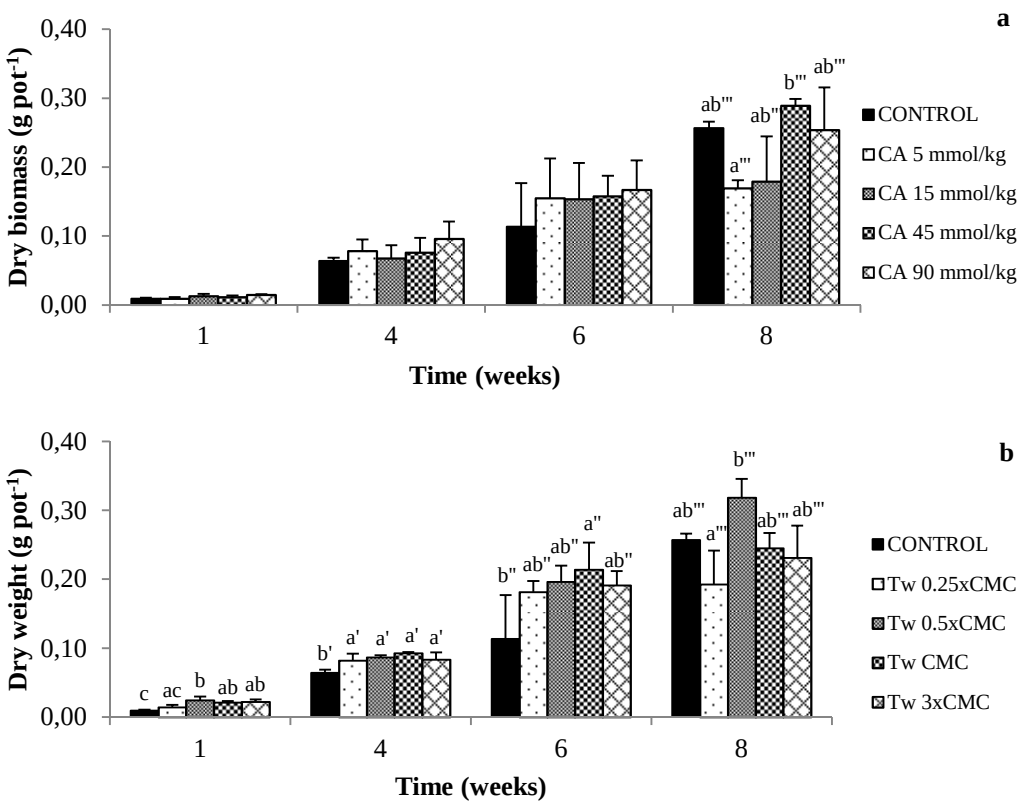

Figure 4.3 Biomass of alfalfa roots
Dry weight (g pot^{-1}) of alfalfa roots treated with (a) citric acid (CA) and (b) Tween® 80 (Tw) as a function of time. Values are average ± S.D. (n=3). Values followed by different letters are significantly different (p<0.05). Lack of letter means no significant difference (p<0.05) between treatments and controls.

4.3.3. Effects on chlorophyll content

Leaf chlorophyll content was studied as a parameter to assess plant health in the presence of the amendments. During the first four weeks, total chlorophyll content in. alfalfa leaves experienced, on average, 1.24-fold increase from weeks 1 to 4. However, after one month total chlorophyll content tended to diminish and by week 8 it was observed a 33% decrease relative to the chlorophyll content at week 4. This was the general behaviour observed, with no distinction between control, citric acid and Tween® 80 treatments. A previous study demonstrated that alfalfa plants under drought stress suffered a considerable reduction in their chlorophyll content (Antolín et al., 1995). The present experiment was performed between May-July, with increasing temperatures as the study progressed. As a result, the decrease in chlorophyll content that affected all the plants after week 4, whatever the treatment was, could be the result of a moderate water restriction due to higher ambient temperatures.

Although certain visual toxicity symptoms, *i.e.* foliar chlorosis in a mottled pattern, were manifested during the experiment for citric acid treated plants (mainly at the highest concentration and from week 4 on), this was not reflected in any significant

difference in the chlorophyll content between citric acid and control plants (Table 4.1). The observed chlorosis could be the expression of solute uptake disequilibrium due to root structure alteration mediated by high levels of citric acid (Evangelou et al., 2006).
It has been demonstrated that surfactants may have a negative impact on chlorophyll content. Lewis (1990) reviewed the chronic toxicities levels of different surfactants to algae, which are known to affect not only their chlorophyll content but also other parameters such as growth, protein synthesis, and photosynthesis. Although not fully understood, the mechanism underlying surfactant toxicity was in general attributed to the alteration of membrane permeability to nutrients and chemicals. Similarly, Kráľová et al. (1992) demonstrated that surfactants of the type 1-alkyl-1-ethylpiperidinium bromides and 1-alkylpiperidine-N-oxides inhibited chlorophyll synthesis in the green algae *Chlorella vulgaris*. In turn, Triton (alkyl aryl polyether alcohols) surfactants decreased duckweed (*Lemna minor*) chlorophyll content to different degrees depending on surfactant structure (Caux et al., 1988). In spite of this previous experimental evidence, in the present study Tween® 80 did not cause any significant effect on the chlorophyll content of alfalfa (Table 4.1). This result could be in accordance with the observations of Neumann and Prinz (1974), who performed a bioassay with beet roots showing that, differently from other surfactants, Tween® 80 appeared not to damage cell membranes, at least at the tested doses (up to 0.1 % w/v).

Table 4.1 Chlorophyll content in alfalfa

Treatment	Total Chlorophyll (mg g^{-1} fresh weight)			
	1 week	4 weeks	6 weeks	8 weeks
Control	1.40 ± 0.25	3.36 ± 1.00	2.78 ± 0.62	2.28 ± 0.77
CA 5 mmol kg^{-1}	1.59 ± 0.50	4.11 ± 0.98	2.87 ± 0.61	2.25 ± 0.81
CA 15 mmol kg^{-1}	1.73 ± 0.30	4.05 ± 0.72	2.92 ± 0.35	2.59 ± 0.82
CA 45 mmol kg^{-1}	1.47 ± 0.37	2.74 ± 0.57	3.43 ± 0.48	2.24 ± 0.52
CA 90 mmol kg^{-1}	1.67 ± 0.38	3.08 ± 1.63	2.71 ± 0.74	1.66 ± 0.32
Tw 0.25×CMC	1.53 ± 0.20	3.38 ± 0.54	2.95 ± 0.25	2.31 ± 0.58
Tw 0.5×CMC	1.50 ± 0.21	3.66 ± 0.65	3.43 ± 0.35	2.35 ± 0.23
Tw CMC	1.21 ± 0.25	2.78 ± 0.58	3.02 ± 0.98	2.45 ± 0.71
Tw 3×CMC	1.38 ± 0.20	3.19 ± 0.54	2.90 ± 0.66	2.23 ± 0.56

Values are average ± S.D. (n=3). There were no significant differences (p<0.05) between treatments and controls.

4.4. Conclusions

The present study was designed to determine the effect of the LMWOA citric acid and the surfactant Tween® 80 on alfalfa germination rates, plant biomass production and chlorophyll content, using weekly applications of soil amendments and varying their concentrations.
The results of this study, while preliminary, suggest that alfalfa can tolerate citric acid and Tween® 80 at the tested concentrations, applied once a week. Consequently, citric

acid and Tween® 80 could be potentially utilized as soil amendments to assist phytoremediation. In particular, this initial work established the base to successively use these amendments in future experiments of assisted phytoremediation of soils co-contaminated with heavy metals (*i.e.* Cd, Cr, Cu, Hg, Ni, Pb, Zn) and petroleum hydrocarbons, and vegetated with alfalfa. Though, feasibility to apply these soil amendments in future experiments of assisted phytoremediation should be interpreted with caution because the behavior (*e.g.* sorption, half-life) of citric acid and Tween® 80 may be considerably different according to soil types. The findings reported here could be extrapolated to soils with analogous properties, principally soils with important proportions of organic matter (Histosols), whose restoration could be targeted by assisted phytoremediation. Conversely, extending the present findings to soils with diverse characteristics will be difficult and further studies, which take these variables into account, will need to be undertaken. For instance, in future investigations it might be possible to use distinct soil matrices to test phytotoxicity of soil amendments.

The present findings may be helpful to understand the impact of the LMWOA citric acid and the surfactant Tween® 80 on alfalfa, in terms of phytotoxicity. Nevertheless, defining the most effective combination of dose and frequency of amendment application that takes full advantage of phytoremediation processes but minimize undesirable phytotoxicity still represents a great challenge.

4.5. Acknowledgements

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Chapter 5

Citric acid- and Tween® 80-assisted phytoremediation of co-contaminated soil vegetated with alfalfa

This chapter has been submitted to *Environmental Science and Pollution Research* journal for publication as an original research paper:
Agnello, A.C., Huguenot, D., van Hullebusch, E.D., Esposito. Citric acid- and Tween® 80-assisted phytoremediation of multi-contaminated soils vegetated with alfalfa (*Medicago sativa* L.)

Abstract

The present study assessed the phytoremediation potential of alfalfa (*Medicago sativa* L.) in a co-contaminated (*i.e.* heavy metals and petroleum hydrocarbons) soil and the effects of citric acid and Tween® 80 (polyethylene glycol sorbitan monooleate), applied individually and combined together, in the phytoremediation process. The results showed that alfalfa plants could tolerate and grow in a co-contaminated soil. Over a 90-day experimental time, shoot and root biomass increased and negligible plant mortality occurred. Heavy metals were uptaken by alfalfa to a limited extent, mostly by plant roots and their concentration in plant tissues were in the following order: Zn > Cu > Pb. The alfalfa rhizosphere effect was manifest, enhancing both the microbial population (alkane degraders) and activity (lipase enzyme), with rhizosphere effects of 28.1 and 2.0, respectively, after 90 days. Soil amendments did not significantly enhance plant metal concentration or total uptake. By contrast, the combination of citric acid and Tween® 80 significantly improved alkane degraders (5.3-fold increase) and lipase activity (1.0-fold increase) in the rhizosphere of amended plants, after 30 days of experiment. This evidence supports the phytoremediation potential of alfalfa species to promote the remediation of heavy metal and hydrocarbon co-contaminated soils and the possibility to enhance the phytoremediation process through the joint application of citric acid and Tween® 80.

Keywords

Soil remediation, heavy metals, hydrocarbons, alfalfa (*Medicago sativa* L.), citric acid, Tween® 80.

5. Citric acid- and Tween® 80-assisted phytoremediation of co-contaminated soil vegetated with alfalfa

5.1. Introduction

In France, major pollutants in terms of occurrence (found individually or in combination) are heavy metals (*e.g.* As, Cd, Co, Cr, Cu, Hg, Ni, Pb or Zn) and petroleum hydrocarbons, impacting 63% and 26% of French affected sites, respectively (BASOL, 2014). Such pollutants pose serious risks both to human health and the environment and it is not uncommon that they are present as mixtures of inorganic and organic contaminants in co-contaminated soils. Phytoremediation is a green remediation approach based on the use of plants to remove pollutants from the environment or to render them harmless (Salt et al., 1998; McCutcheon and Schnoor, 2004). Plant-based technologies can target both inorganic and organic pollutants and in recent years, an increasing interest to study the phytoremediation of co-contaminated soils emerged (Ouvrard et al., 2011; Chigbo et al., 2013; Hechmi et al., 2013; Sung et al., 2013). In particular, the combination of phytoextraction and rhizodegradation processes can be employed together to clean-up co-contaminated soils. Phytoextraction is among the phytotechnologies used for heavy metal remediation and involves: metal uptake by plant roots, translocation of metals from roots to shoots and finally metal accumulation in the above ground tissues (Salt et al., 1995). In addition, rhizodegradation is one of the mechanisms involved in organic contaminant phytoremediation, through the so-called *rhizosphere effect*, in which plant root exudates enhance rhizosphere microbial population and activity, thereby improving the metabolism of organic pollutants (White and Newman, 2011). One of the main constraints hindering the success of phytoextraction and rhizodegradation is low bioavailability of pollutants. To overcome this limitation, amendment-enhanced phytoremediation is one of the strategies that has been used (Evangelou et al., 2007; Meers et al., 2008) consisting in the addition of appropriate amendments to vegetated soils. This study will principally focus on two types of soil amendments: low molecular weight organic acids (LMWOAs) and surfactants.

LMWOAs are biodegradable compounds that possess carboxylic functional groups with chelating ability, which increases the bioavailability of heavy metals (Huang et al., 1998). In addition, LMWOAs have been described to increase the bioavailability of organic compounds as well (White et al., 2003; Gao et al., 2010c). Among LMWOAs, citric acid has been particularly studied. It has been reported to increase soil desorption of heavy metals like Cu, Cd and Pb as well as to enhance their uptake by several plant species (Chen et al., 2003; Gao et al., 2003; Quartacci et al., 2005; do Nascimento et al., 2006; Qu et al., 2011). Similarly, citric acid enhanced soil desorption of organics like PAHs and organochlorine pesticides, and even their plant uptake (White et al., 2003; An et al., 2010; Gao et al., 2010b; Gao et al., 2010c; Mitton et al., 2012).

Surfactant-enhanced phytoremediation consists in the application of surfactants as amendments. Surfactants present an amphiphilic chemical structure, which can increase the water solubility and bioavailability of hydrophobic compounds improving their phytoremediation (Pletnev, 2001; Gao et al., 2007). Although surfactants have been mostly used to increase desorption and bioavailability of organic contaminants (Gao et al., 2006; Wu et al., 2008), they may also influence heavy metal bioavailability through the formation of complexes, micelles and ion exchange processes (Mulligan et al., 2001; Pacwa-Plociniczak et al., 2011). In particular, Tween® 80 (polyethylene glycol sorbitan monooleate) is a non-ionic surfactant that has been shown to increase soil desorption of organochloride pesticides (Gonzalez et al., 2010), as well as to enhance plant uptake (Gao et al., 2008) and removal (Cheng et al., 2008) of PAHs, and facilitate removal of hydrocarbons from soils (Adetutu et al., 2012). Moreover, Tween® 80 has been recently used to assist the phytoremediation of soils co-contaminated with Cd and benzo[a]pyrene (Sun et al., 2013).

LMWOA and surfactant-assisted phytoremediation experiments have demonstrated a variable effectiveness, showing the importance to study each particular system, as it is difficult to generalize and predict results (Agnello et al., 2014). Phytotoxity of citric acid and Tween® 80 in a non-contaminated soil has been previously assessed demonstrating that these amendments could be potentially used to assist phytoeremediation (Agnello et al., In Press). However, no previous research has studied the influence of citric acid and/or Tween® 80 on the phytoremediation of co-contaminated soils vegetated with alfalfa (*Medicago sativa* L.). This species has been subject to phytoremediation studies because it presents several favourable traits. It is a fast growing perennial plant, able to develop high above ground biomass and an extensive root system that serves as a niche for the development of rhizosphere microorganisms (Coburn, 1912; Kirk et al., 2005). Moreover, the presence of root nodules with nitrogen fixing bacteria allows alfalfa to grow in soils with high C/N ratios (Truchet et al., 1991). Several studies have reported the potential use of alfalfa species in the phytoremediation of heavy metals such as Cd, Cr, Cu, Ni and Zn (Peralta-Videa et al., 2002; Peralta-Videa et al., 2004; Bonfranceschi et al., 2009) and organic contaminants like petroleum hydrocarbons (Wiltse et al., 1998; Kirk et al., 2002), polycyclic aromatic hydrocarbons (PAHs) (Fan et al., 2008) or organochlorines (Li and Yang, 2013). However, only few studies have investigated the use of alfalfa in the phytoremediation of co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013).

The present study has two primary aims. Firstly, to assess the phytoremediation potential of alfalfa to remediate soils contaminated with heavy metals and petroleum hydrocarbons. Secondly, to evaluate the effects of individual and combined applications of citric acid and Tween® 80 on the phytoremediation process. Different parameters were examined such as plant biomass and heavy metal concentration to evaluate phytoextraction, and the number of alkane degraders and soil lipase activity, to indirectly assess rhizodegradation. Phytoremediation parameters were also calculated.

5.2. Materials and methods

5.2.1. Chemicals

The tricarboxylic acid, citric acid ($C_6H_8O_7$, molecular weight: 192 g mol^{-1}), used in this experiment was purchased from Carlo Erba Reagents Group ($C_6H_8O_7.H_2O$, purity >99.5%). The anionic surfactant Tween® 80 (polyethylene glycol sorbitan monooleate, $C_{64}H_{124}O_{26}$, molecular weight: 1310 g mol^{-1}, critical micelle concentration (CMC): 0.012 mM) was obtained from Sigma-Aldrich Chemical Co. All the other chemicals used were of analytical grade.

5.2.2. Soil origin and properties

Soil samples were collected from a French urban area close to a fuel station with a history of contamination by heavy metals and petroleum hydrocarbons, mostly diesel. Samples were taken with a drill auger, which allowed collecting soil from different depths between 0 and 100 cm. The different soil fractions were mixed unequally as it was technically not possible to ensure the mixing of soils from different depths in equivalent proportions. For this study, this soil (*sondage 4*) was sieved to pass through a 6 mm mesh and homogenized. To limit the level of pollutants in order to improve alfalfa performance, the contaminated soil was mixed (1:1 w/w) with soil from the same site but characterized by negligible hydrocarbon contamination (*sondage 3*). Before mixing, this soil was sieved through a 2 mm mesh. Selected chemical and physical properties of the 1:1 w/w mix of both soils (*sondage 3/4*) are presented in Table 5.1. Physicochemical characterization of soil samples was performed by an external laboratory: ALcontrol Laboratories. ALcontrol is accredited by the Cofrac (Comité français d'accréditation) and by the RvA (Raad voor Accreditatie) under number L028, in accordance with the criteria of laboratory analysis: ISO / IEC 17025:2005. All their services are performed in accordance with their general conditions, registered under KVK number 24265286 at the Rotterdam Chamber of Commerce, Netherlands. Analysis are performed in accordance with French standards (NF: Norme française), the Dutch Standards Institute (NEN: Nederlands Normalisatie-instituut) and the International Organization for Standardization (ISO). The following analyses were performed: actual soil pH (NF ISO 10693), cation exchange capacity (NF X 31-130), organic carbon and organic matter (NF ISO 14235), total nitrogen (sum of N Kjeldahl, NO_2^- and NO_3^- internal method, NEN 6604), C/N ratio (calculated as the ratio between the content of organic carbon and total nitrogen), P_2O_5 (Joret-Hebert method, NF X 31-161), K_2O, MgO and CaO (NF X 31-108), DTPA (diethylene triamine pentaacetic acid) available fraction of Fe and Mn (NF X 31-121), water available fraction of B (NF X 31-122), soil texture (NF X 31-107), content of As, Cd, Cr, Cu, Pb, Ni and Zn (internal method: destruction in accordance with NEN 6961, analysis in accordance with ISO 22036), content of Hg (NEN 6950, destruction in accordance with NEN 6961, analysis in accordance with NEN-ISO 16772), petroleum hydrocarbon fractions: C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{21} and C_{21}-C_{40} (internal method: acetone, hexane extraction, purification and analysis by GC-FID) and Total C_{10}-C_{40} (Equivalent to NEN-EN-ISO 16703).

This mix was used for the pot experiment as alfalfa still exhibited high germination rates in the 1:1 w/w mix (data not shown).

Table 5.1 Chemical and physical properties of the soil (*sondage 3/4*)

Agronomic Parameters	
pH (H_2O)	8.1
Cation Exchange Capacity at soil pH ($cmol^+ kg^{-1}$ DW)	10.7
Organic Matter ($g\ kg^{-1}$ DW)	49
Organic Carbon ($g\ kg^{-1}$ DW)	28.3
Total Nitrogen ($mg\ kg^{-1}$ DW)	640
C/N ratio	44
P_2O_5 ($g\ kg^{-1}$ DW)	0.10
K_2O ($g\ kg^{-1}$ DW)	0.09
MgO ($g\ kg^{-1}$ DW)	0.12
CaO ($g\ kg^{-1}$ DW)	9.63
Fe* ($mg\ kg^{-1}$ DW)	116
Mn* ($mg\ kg^{-1}$ DW)	19.5
B* ($mg\ kg^{-1}$ DW)	0.71
Sand (%)	82.6
Silt (%)	12.5
Clay (%)	4.9
Heavy Metals ($mg\ kg^{-1}$ DW)	
As	7.4
Cd	0.36
Cr	<10
Cu	87
Hg	1.0
Pb	100
Ni	8.7
Zn	110
Hydrocarbons ($mg\ kg^{-1}$ DW)	
C_{10}-C_{12}	130
C_{12}-C_{16}	1100
C_{16}-C_{21}	1600
C_{21}-C_{40}	830
Total C_{10}-C_{40}	3600

DW: dry weight
* DTPA (diethylenetriaminepentaacetic acid) extraction

5.2.3. Plants

Alfalfa seeds (*Medicago sativa* L. v. La Bella Campagnola, purity: 99%, germinability: 85%) were surface disinfected by immersion in 2% (v/v) hydrogen peroxide for 8 min (Qu et al., 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

5.2.4. Growth chamber experiment

Disinfected alfalfa seeds were sown in a commercial soil (organic carbon: 20%, organic nitrogen: 0.4%, organic matter: 40%, dry matter content: 58%), where seedlings grew for 30 days in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352. Growth conditions: photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C, photosynthesis photon flux density (PPFD) of 130 μmol m^{-2} s^{-1}). Subsequently, six seedlings of uniform size were selected and transplanted in plastic pots (10 cm diameter, 8 cm height) filled with 200 g of the soil under study. Previous research showed that tolerance of alfalfa plants towards heavy metals is positively correlated with the plant age (Peralta-Videa et al., 2004). For this reason, in the present study, seedlings where transplanted to the polluted soils just after growing for 30 days in the commercial soil.

Pots containing the transplants were put in the growth chamber (same conditions as stated above) and received water daily by gentle spraying with tap water. The experimental design included the fortnightly treatment of pots with citric acid (CA: 15 mmol kg^{-1} dry soil), Tween® 80 (Tw-80: 0.036 mmol kg^{-1} dry soil), or the combination of citric acid and Tween® 80 (CA+Tw-80: 15 and 0.036 mmol kg^{-1} dry soil, respectively). Selected concentrations have been demonstrated to be well tolerated by alfalfa (Agnello et al., In Press) and they are related to those found in the literature (Quartacci et al., 2005; Gonzalez et al., 2010). Amendments were applied fortnightly in order to keep the concentrations in soil stable and effective while minimizing plant damage or environmental impact. Controls of unplanted and planted (C) soil received the same amount of distilled water instead of the amendments solutions. The location of pots was randomly changed daily (within the same shelf and also between different shelves in the growth chamber). Each condition for each harvesting time was performed in triplicate pots. Plants in every single pot for the corresponding condition (vegetated pots used as control or amended with citric acid, Tween® 80, or the combination of citric acid and Tween® 80) were harvested after 30, 60 and 90 days of growth in the polluted soil (the different treatments were grown in parallel), every time three days after amendment application. Plants were removed from pots, and roots and shoots were separated. Roots were washed with distilled water to remove soil particles and blotted with tissue paper. The plant material was put in the oven at 70°C for 3 days (Campbell and Plank, 1998) and dry weights of shoots and roots were recorded. Soil was sampled at the same harvesting times and kept at 4 °C until further analyses. In the case of vegetated pots, rhizosphere soil samples were taken. In order to collect rhizosphere soil, plant roots were vigorously shaken by hand, taking care of the roots integrity. The external soil not attached to roots was removed, while the soil in the immediate vicinity of roots was kept for the analyses.

5.2.5. Analyses of heavy metal content in plants

Prior to elemental analyses, dried plant material was wet digested as described by Campbell and Plank (1998). Briefly, plant material was digested with 5 ml concentrated

nitric acid and 2 ml 30% hydrogen peroxide in a digestion block (LabTech DigiBlock Digester ED16S) at 125 °C for 1h. Heating cycles and hydrogen peroxide addition were repeated three times to obtain a clear digest. To remove residual particles, mineralized samples were filtered through cellulose filters (pore size 2.5 µm) and brought to a final volume of 50 ml. Samples were additionally filtered through nitrocellulose syringe filters (pore size 0.45 µm) and stored at 4 °C until heavy metals were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (PerkinElmer Optima 8300 ICP-OES Spectrometer). Cu, Pb and Zn were analyzed at the respective wavelengths of 324.752 nm, 220.353 nm and 213.857 nm.

5.2.6. Soil microbiology

5.2.5.1. Number of aliphatic hydrocarbon degraders

Aliphatic hydrocarbon degraders were counted by the most-probable-number (MPN) method described by Wrenn and Venosa (1996), using 96-well microtiter plates. Bushnell-Haas medium supplemented with 2% NaCl was used as the growth medium (180 µl per well) and n-hexadecane (5 µl per well) was added as the selective growth substrate. 10-fold serial dilutions were performed from a suspension of 1 g of fresh soil and 10 ml of 0.1 % sodium pyrophosphate (pH 7.5) and 2% NaCl. Plates were inoculated by adding 20 µl of the dilutions from 10^{-2} to 10^{-7}, in 5 replicates. Microplates were incubated for 2 weeks at room temperature. Afterwards, 50 µl of iodonitrotetrazolium violet (INT, 3 g l^{-1}) were added to identify positive wells in which, INT is reduced to an insoluble formazan that deposits intracellularly as a red precipitate. The scoring was done after incubating overnight with INT at room temperature. MPN of alkane degraders per g of soil was calculated according to Briones Jr. and Reichardt (1999).

5.2.5.2. Soil lipase activity

Soil lipase activity was measured through the colorimetric method described by Margesin et al. (2002). 0.1 g of fresh soil was mixed with 5 ml 100 mM NaH_2PO_4/NaOH buffer, pH 7.25, and pre-warmed at 30°C for 10 min. 50 µl of substrate solution (100 mM p-nitrophenyl butyrate (*p*NPB) in 2-propanol) were added and tubes were incubated at 30°C for 10 min. To stop the reaction, the tubes were cooled for 10 min on ice. Tubes were centrifuged at 2000 g for 5 min and the absorbance of the released p-nitrophenol (*p*NP) in the supernatants was measured spectrophotometrically (PerkinElmer LAMBDA 10 UV/Vis Spectrophotometer) at 400 nm against the reagent blank. A standard solution of *p*NP (100 µg *p*NP ml^{-1} phosphate buffer) was used to prepare a calibration curve in the presence of soil. In order to measure the *p*NP released from the substrate, a control was prepared without soil. After subtracting the control reading (hydrolysis in absence of soil) from the sample reading (hydrolysis in presence of soil), soil lipase activity was calculated and expressed as µg *p*NP (g soil × 10 min)$^{-1}$.

5.2.7. Phytoremediation parameters

To evaluate the performance of metal phytoextraction the following parameters were considered: a) plant biomass, b) metal concentration in plant tissues, c) translocation factors (TF) calculated as the metal in shoots to the metal in roots ratio and d) total metal uptake per pot, calculated as the product of the metal concentration in plant parts by the plant biomass per pot.
Concerning the potential of rhizodegradation, the rhizosphere effect *i.e.* the influence of the plant over the non-planted soil, was evaluated by calculating the ratios: MPN of rhizosphere soil/MPN of non-planted soil and soil lipase activity of rhizosphere soil/soil lipase activity of non-planted soil.

5.2.8. Statistical analysis

The experiment was arranged in a completely randomized design. All data reported were averaged values of three independent replicates. Treatment effects were statistically evaluated by one-way analysis of variance (ANOVA) and multiple comparisons of means by Tukey contrasts. Differences were considered significant at $p<0.05$. The statistical analysis was accomplished with R software, version 3.0.2 (R Core Team, 2014).

5.3. Results and discussion

5.3.1. Plant growth and response to soil amendments

Immediately after transplanting alfalfa seedlings in the contaminated soil, all plants survived and at 90 days plant mortality was only 0.5 % (data not shown). This is in accordance with previous evidence, where tolerance of alfalfa plants to Cd, Cu and Zn (at 480, 575, 775 mg kg^{-1} dry soil, respectively) was demonstrated to be positively correlated with the growth stage (Peralta-Videa et al., 2004).
As shown in Figure 5.1, from 30 to 90 days there was a significant increase in plant biomass for all the experimental conditions, and this enhancement was greater for roots than for shoots. There was a 1.5, 1.1, 0.8 and 1.1-fold increase in shoot dry weight for control, citric acid, Tween® 80 and the combined treatment, respectively, whereas for roots the fold increases were: 3.2, 2.1, 2.1 and 2.6, respectively. Throughout the 90 days of the experiment, alfalfa growth was not hindered and both above and below ground biomass progressively increased.
Previous studies have demonstrated that alfalfa can grow in soils individually contaminated with heavy metals at more than 50 mg kg^{-1} dry soil (Peralta et al., 2001a; Peralta et al., 2001b). One of the mechanisms involved in alfalfa heavy metal tolerance may be related to the expression of metallothioneins, *i.e.* metal-binding ligands in plant cells that prevent metals from binding to physiologically important functional groups (Wang et al., 2011). In contrast, it has been observed that when heavy metals (Cd, Cu, Ni, and Zn) were present in a mixture (at 50 mg kg^{-1} each) they exerted combined stress and affected the shoot length of alfalfa (Peralta-Videa et al., 2002).

Regarding alfalfa tolerance towards petroleum hydrocarbons, Kirk et al. (2002) documented an hormesis response (*i.e.* the stimulation of plant growth at low levels of contamination) in alfalfa grown in 10000 and 15000 mg kg^{-1} hydrocarbon-contaminated soils. However, at higher levels of soil petroleum hydrocarbon contamination (31000 mg kg^{-1}) alfalfa could still germinate but growth of seedlings was stressed and stunted (Kirk et al., 2005). It seems possible that high levels of petroleum hydrocarbons exert a negative effect on alfalfa, directly affecting plant physiology or indirectly, altering the physical and chemical properties of the soil where plants are developing.

Alfalfa tolerance to pollutants is currently well documented in the literature for a remarkable variety of inorganic and/or organic contaminants (Wiltse et al., 1998; Peralta-Videa et al., 2002; Fan et al., 2008; Li and Yang, 2013). Likewise, the present results provide further support for the phytoremediation potential of alfalfa, extending the ability of this species to grow in the simultaneous presence of heavy metals and petroleum hydrocarbons at the studied concentrations, which seem to be below the phytotoxicity threshold for alfalfa. In addition, high above ground biomass is an important feature for phytoextraction purposes; while an abundant root system creates a rich environment for the development of microorganisms involved in rhizodegradation.

The effect of amendments on alfalfa biomass, with respect to the control, varied with time. After 30 days, no treatment influenced plant biomass. However, from 60 days on, it was observed that Tween® 80 negatively affected plant biomass; and this effect was significant for both shoots and roots at 60 days and only for roots at 90 days. Interestingly, this negative effect on plant biomass appeared to be counteracted by the joint application of Tween® 80 and citric acid. In the presence of the combined treatment (or citric acid alone) there was no significant decrease in plant biomass. In a previous short study in non-contaminated soils (Agnello et al., In Press), it was observed that Tween® 80 did not negatively affect alfalfa biomass and there was even a trend to increase it. It was hypothesized that surfactants could increase root permeability resulting in a more efficient uptake of nutrients, which would explain the positive effect on plant biomass (Zhu and Zhang, 2008). However, in contaminated soils, the increase in root permeability mediated by Tween® 80 could lead contaminants to exert plant toxicity, negatively affecting plant growth as a result. Nevertheless, the chelating properties of citric acid could prevent the toxicity of such contaminants, as reflected by the non-negative impact on plant biomass, in the presence of the organic acid. This observation is supported by previous studies, which showed that citric acid could have a role in alleviating heavy metal stress on plants (Gao et al., 2010a; Najeeb et al., 2011; Qu et al., 2011).

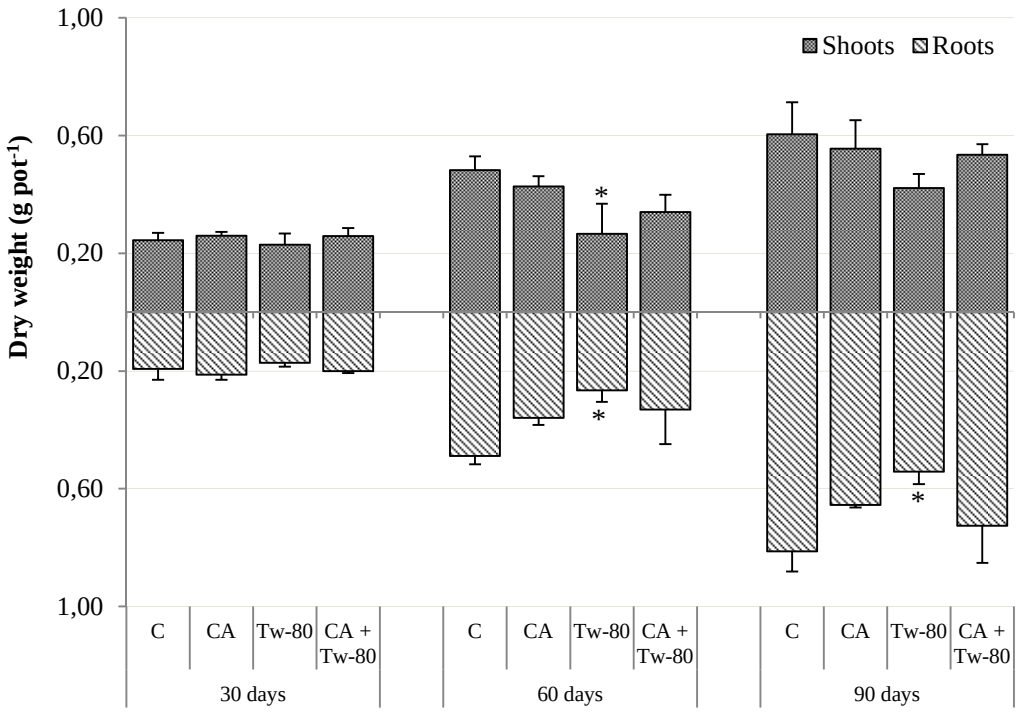

Figure 5.1 Biomass of alfalfa

Dry weight (g pot^{-1}) of shoots and roots in not amended pots (C) and in pots treated with citric acid (CA), Tween® 80 (Tw-80) or citric acid and Tween® 80 (CA + Tw-80). Values are expressed as means ± standard deviations of triplicate measurements. The symbol * indicates that mean values are significantly different between control and amended treatment at a definite time (p<0.05).

5.3.2. Phytoextraction performance

Figure 5.2 shows the data of Cu, Pb and Zn concentrations in alfalfa tissues depending on the treatment and experimental time. Heavy metal concentrations in shoots and roots of control alfalfa after 90 days of experiment were below 100 mg kg^{-1} DW (dry weight) and in the following order: Zn > Cu > Pb. Plant metal concentrations obtained in the current study were considerably lower than those reported formerly by Peralta-Videa et al. (2002), who observed a lack of specificity for metal uptake by alfalfa and found that at least 100 mg kg^{-1} DW of Cu and Zn were present in the shoot tissues of alfalfa plants growing in a multi-metal freshly spiked soil. There are several possible explanations for the lower plant metal concentration reported here, mainly related to the soil used in this study. Firstly, the ageing effect due to chronic pollution may lead to poor bioavailability of heavy metals to plants in aged soils with respect to a freshly spiked one (Bruus Pedersen et al., 2000; Chigbo and Batty, 2013). Another possible explanation is that the presence of hydrocarbons impairs the mobility of metals limiting their bioavailability in soils. Simultaneous occurrence of hydrocarbons and heavy metals in soil can negatively affect metal uptake and accumulation by plants, as supported by a previous study which showed that the ability of Cu phytoextraction by maize was inhibited under co-contamination of pyrene (Lin et al., 2008). Finally, multi-metal contaminated soils

consists of a complex matrix where metals interact with each other influencing, in turn plant metal uptake. These metal interactions may result in additive or synergistic effects if the sorption capacity of metals in the mixture decreases due to a competitive process. However, antagonistic effects between metals have also been described (Alloway, 1995; Luo and Rimmer, 1995; Flogeac et al., 2007; Branzini et al., 2012).

Among the amendments, citric acid did not have a significant effect on heavy metal plant concentration relative to control. Although prior evidence demonstrated that sodium hydrogen phosphate/citric acid mixtures could enhance the phytoextraction efficiency of heavy metals (As, Cd, Cr, Cu, Hg, Mo, Ni, Pb, Zn) by alfalfa (Qu et al., 2011), in the present study such an effect was not observed in the presence of citric acid alone. In contrast, Tween® 80 significantly increased heavy metal concentration in roots with respect to alfalfa controls, producing increases of 0.74-fold for Pb (at 90 days) and 0.79-fold for Zn (at 60 days). A significant increase in Zn root concentration at 60 days was also observed when Tween® 80 was applied in combination with citric acid. These findings support the fact that Tween® 80 can influence heavy metal uptake by alfalfa. However, it is conceivable that a higher accumulation of heavy metals in plant tissues mediated by Tween® 80 exerted toxicity effects on alfalfa, justifying the observed reduction in plant biomass when the surfactant was present.

Sun et al. (2013) recently reported a similar positive effect of Tween® 80 on heavy metal accumulation by plants. They found an increase in Cd concentration in the tissues (roots, stems, leaves and shoots) of *Tagetes patula* growing in a soil contaminated with Cd and benzo[a]pyrene. The observed increase in metal accumulation in the presence of Tween® 80 could be attributed to a direct effect on plants, *i.e.* an increase of root permeability due to biological membrane disruption mediated by surfactants (Jones, 1992). Moreover, it is also possible that surfactants act indirectly through the formation of complexes, micelles and ion exchange processes with metals in soils (Mulligan et al., 2001; Pacwa-Plociniczak et al., 2011).

None of the amendments significantly influenced shoot concentration of heavy metals, with respect to non-amended control, implying no positive influence of tested amendments on metal phytotextraction by alfalfa, at least at the present doses and application rates. This fact was supported by the calculation of the translocation factors (TF). Independently of the tested condition, contents of heavy metals in the roots compared with the shoots were higher, revealing poor metal translocation from roots to shoots (TF < 1, data not shown) and limited phytoextraction potential as a consequence. Interestingly, an increase in the TF during time of all metals was observed in control alfalfa, suggesting no saturation of aerial parts by metals. Calculated TF at 90 days for control alfalfa were: 0.84, 0.49 and 0.30 for Zn, Pb and Cu, respectively. These results are in accordance with a previous phytoremediation experiment in multi-metal contaminated soil, which showed more accumulation of heavy metals in alfalfa roots than in aerial parts, with TF of 0.61, 0.79and 0.40 for Zn, Pb and Cu, respectively, after 30 days of trial (Qu et al., 2011).

Figure 5.3 presents the total amount of metals extracted by plant parts. Heavy metal uptake by alfalfa increased with time, both in control and amended pots. After 90 days, Cu, Pb and Zn uptake by roots significantly increased, relative to the uptake found at 30

days, for all the conditions. For heavy metal shoot uptake, the trend was the same. A significant enhancement at 90 days relative to 30 days for all the conditions was observed, except for Cu in citric acid amended pots and for Pb in the pots which received Tween® 80 and the combined treatment. Total heavy metal uptake by shoots and roots of control alfalfa after 90 days of experiment was in the following order: Zn > Cu > Pb. Total metal uptake by alfalfa was not significantly increased by any amendment with respect to control. This outcome is the result of the insufficient plant biomass, which cannot compensate for a deficient enhancement in metal concentrati

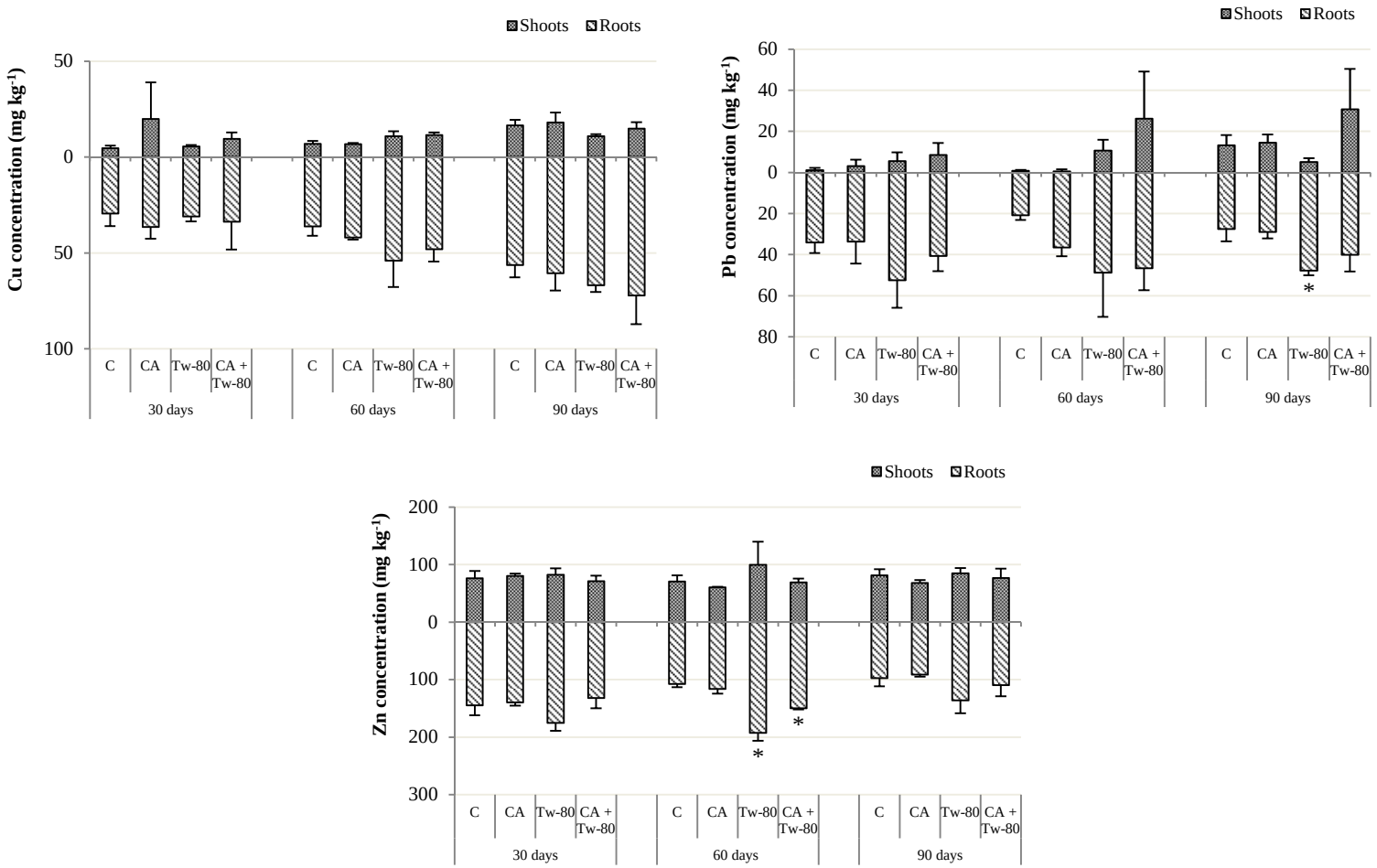

Figure 5.2 Metal concentration in alfalfa

Concentration (mg kg^{-1} dry weight) in alfalfa shoots and roots in not amended pots (C) and in pots treated with citric acid (CA), Tween® 80 (Tw-80) or citric acid and Tween® 80 (CA + Tw-80). * indicates that mean values are significantly different between control and amended treatment at a definite time (p<0.05).

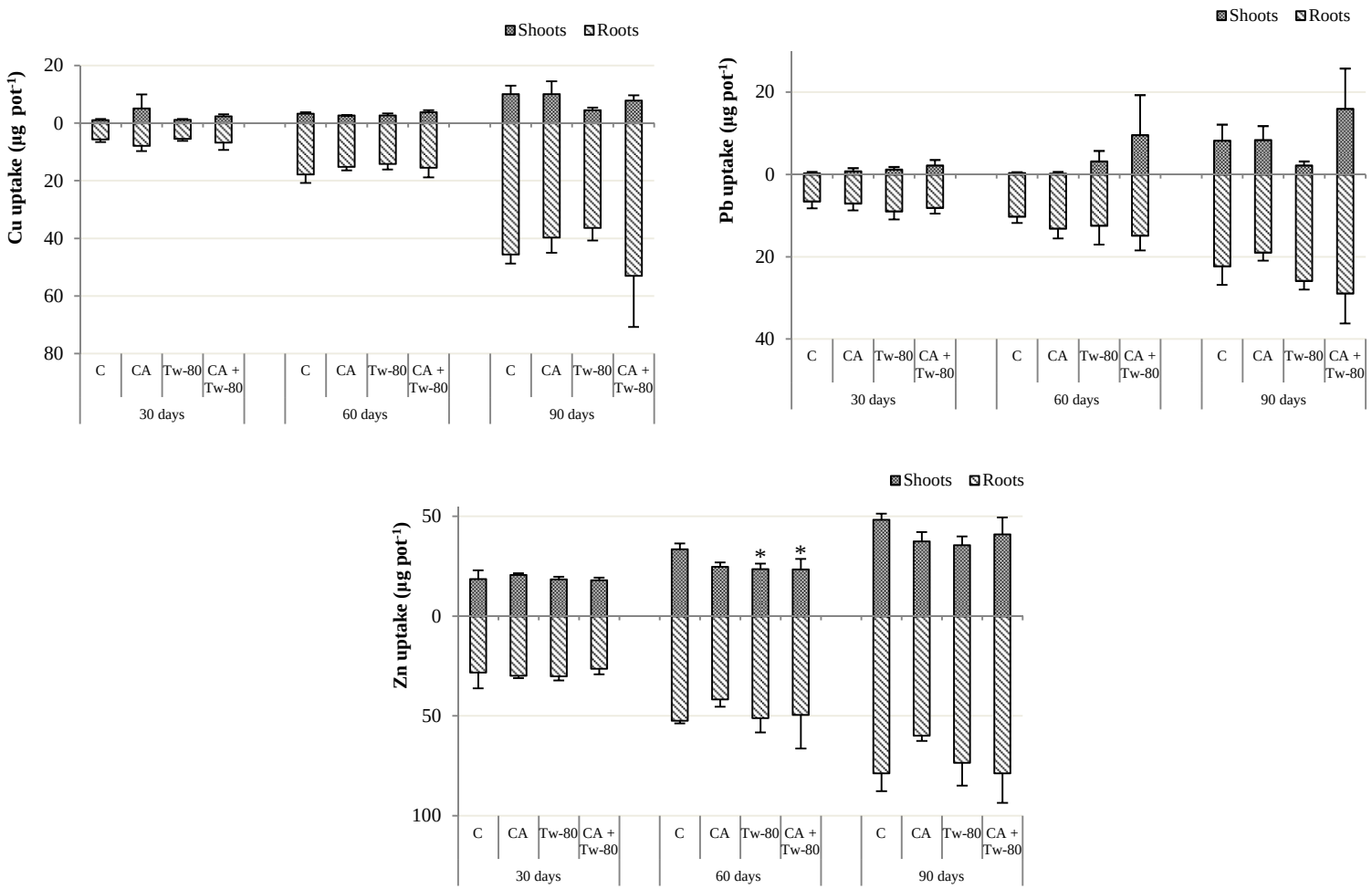

Figure 5.3 Metal uptake by alfalfa

Metal uptake (µg pot^{-1}) by shoots and roots in not amended pots (C) and in pots treated with citric acid (CA), Tween® 80 (Tw-80) or citric acid and Tween® 80 (CA + Tw-80). * indicates that mean values are significantly different between control and amended treatment at a definite time (p<0.05).

5.3.3. Effect of plants and soil amendments on soil microbiology

5.3.3.1. Effect on aliphatic hydrocarbon degraders

Table 5.2 shows the results of the MPN of aliphatic hydrocarbon degraders. Soil alkane degraders were found to increase over the 90-day experimental period for all the conditions tested. Microbial abundance in the unplanted control soil tended to increase (81-fold increase relative to initial value), although MPN values were comparable over the experimental period. In contrast, in the presence of alfalfa there was a significant enhancement of the initial number of soil alkane degraders after 90 days (2294-fold increase). Although not significant, the abundance of soil hydrocarbon-degrading microbial community in the rhizosphere of alfalfa was higher than in the unplanted control soil. Rhizosphere effects of 1.8, 8.5 and 28.1 were obtained after 30, 60 and 90 days of experiment, respectively.

The MPN of soil aliphatic hydrocarbon degrading bacteria is a quantitative indicator of the population of microorganisms able to metabolize aliphatic hydrocarbons. Therefore, it can be expected that there is a positive correlation between the number of soil aliphatic hydrocarbon degrading bacteria and the dissipation of pollutants such as petroleum hydrocarbons in the soil (Wrenn and Venosa, 1996). The findings of the current study are consistent with those previously presented by Kirk et al. (2005), who reported a rhizosphere effect value of 5 for alfalfa growing in an hydrocarbon contaminated soil, after 49 days of experiment. Likewise, Nichols et al. (1997) reported an enhancement (rhizosphere effect of 2.8) in the MPN of microorganisms capable of using a mixture of organic chemicals, in the rhizosphere of alfalfa grown in soil spiked with six compounds that are found in crude oil, after 63 days. The enhancement of total microbial biomass in the rhizosphere of alfalfa plants had already been demonstrated in soils contaminated by PAHs (Fan et al., 2008). Moreover, in petroleum-contaminated soils, rhizospheric total heterotrophic bacterial and petroleum degrading bacterial numbers were enhanced by alfalfa plants (Kirk et al., 2005). In soils co-contaminated by heavy metals and PAHs a positive effect on both total and PAH-degrading bacteria populations was noted in the rhizosphere of alfalfa (Ouvrard et al., 2011). An analogous positive effect in the total abundance of hydrocarbon-degrading microorganisms relative to non-vegetated control was demonstrated in other plant species such as Australian grasses (Gaskin and Bentham, 2010). The positive effect of plants to support and enhance rhizosphere microbial community is thoroughly documented in the literature, and many physicochemical mechanisms are supposed to be involved: *e.g.* the release of root exudates, which create a nutrient-rich environment favorable for microbial development; the physical effect of root growth improving aeration and the mechanical support provided by roots delivering a suitable surface for microbial colonization (Lynch, 1990).

Concerning amended treatments, the MPN of alkane degraders increased by 724-, 2017- and 4993-fold for Tween® 80, citric acid and the combined treatment, respectively, over the 90 day experiment. Irrespective of the experimental time, the general trend for MPN

counts was in the following order: combined treatment > citric acid > Tween® 80. Citric acid alone and in combination with Tween® 80 positively influenced the population of alkane degraders relative to the vegetated control. The joint application of citric acid and Tween® 80 significantly increased the MPN of alkane degraders by 5.3-fold increase at 30 days, while it improved by 2.7- and 1.2-fold increase at 60 and 90 days, respectively. In the latter cases variability was too high for these differences to be statistically significant. The enhancement of alkane degraders in the presence of citric acid can be the result of its use as a source of energy by microorganisms (Ström et al., 2001). In addition, previous studies have reported the enhancement of organic pollutants desorption from soil in the presence of citric acid (An et al., 2010; Gao et al., 2010c). As a result it could be possible that alkane degraders are enriched due to an increase in hydrocarbon bioavailability facilitated by citric acid. This effect on pollutant bioavailability could be enhanced when Tween® 80 is also present. This hypothesis is supported by a former study in which the application of rhamnolipid biosurfactant with citric acid produced a higher desorption of phenanthrene compared to single rhamnolipid application (An et al., 2011). In contrast, the single application of Tween® 80 did not have a significant effect on alkane degraders, and the tendency was a decrease of the microbial population with respect to non-amended plants. Analogous results showed that the addition of Tween® 80 had no significant effect on the population size of both total heterotrophic bacteria and PAH degraders in vegetated (*Agropyron elongatum*) soil spiked with phenanthrene and pyrene (Cheng et al., 2008).

Table 5.2 Soil microbial number of alkane degraders

Treatment	Number of alkane degraders (MPN (g soil)$^{-1}$)		
	30 days	60 days	90 days
Soil	4.7 (±2.3) × 10^6 aA	1.5 (±0.3) × 10^6 aA	1.8 (±1.7) × 10^7 aA
Soil + Alfalfa	8.3 (±4.0) × 10^6 aA	1.2 (±0.8) × 10^7 abA	5.0 (±1.7) × 10^8 aB
Soil + Alfalfa + CA	2.3 (±0.9) × 10^7 abA	2.2 (±2.0) × 10^7 abA	4.4 (±2.6) × 10^8 aB
Soil + Alfalfa + Tw-80	9.7 (±5.2) × 10^6 aA	7.6 (±5.8) × 10^6 aA	1.6 (±1.9) × 10^8 aA
Soil + Alfalfa + CA + Tw-80	5.2 (± 2.2) × 10^7 bA	4.5 (±2.1) × 10^7 bA	1.1 (±1.2) × 10^9 aA

Citric acid (CA), Tween® 80 (Tw-80). Initial (prior to planting) MPN of alkane degraders (g soil)$^{-1}$: 2.2 (±1.2) × 10^5. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case and upper case letters following the data in a column and in a row, respectively, means significant differences among the data (p<0.05).

5.3.3.2. Effect on soil lipase activity and relation with aliphatic hydrocarbon degraders

Table 5.3 shows the experimental data on soil lipase activity. Although fluctuating, the general trend showed an increase in lipase activity over time. In the vegetated control a significant enhancement (1.9-fold increase) of lipase activity was observed after 90 days relative to the initial value, while for the unplanted control soil only a 0.40-fold increase was found. The presence of alfalfa plants stimulated lipase activity in the rhizosphere, as demonstrated by the higher values obtained in vegetated pots relative to the unplanted control. This effect was significant after 90 days, with a 1.0-fold increase. Rhizosphere effects of 2.0, 1.2 and 2.0 were obtained after 30, 60 and 90 days of experiment, respectively.

Soil lipase activity can be a suitable bioindicator to monitor oil biodegradation in soil, based on the assumption that microbial enzymatic systems responsible for lipid degradation may be similar to those involved in oil decomposition (Margesin et al., 1999). Increased lipase activity implies an increase in the general soil biogeochemical activity, where hydrocarbons are used as substrates and metabolized by soil microorganisms. Soil lipase activity can be related to the potential of a soil for hydrocarbon dissipation, as demonstrated by the negative correlation between residual soil hydrocarbon content and soil lipase activity (Margesin et al., 1999). Previous studies have demonstrated that lipase activity can be enhanced in the presence of plants. For instance, a significant stimulation of soil lipase activity has been reported in the rhizosphere of Australian grasses growing in hydrocarbon-contaminated soil, relative to non-vegetated control (Gaskin and Bentham, 2010). The higher enzyme activity in the rhizosphere can be explained by different mechanisms. Firstly, the stimulation of microbial activity mediated by rhizodeposition of organic carbon by plants, which creates an environment rich in organic substrates for microorganisms. Secondly, a direct contribution by plants releasing enzymes by roots or by lysis of root cells (Nannipieri et al., 2012).

Regarding the amendments, soil lipase activity constantly increased over time in the presence of citric acid alone and in combination with Tween® 80. After 90 days a significant enhancement of 3.7- and 2.6-fold increase was observed for citric acid and for the combination of citric acid and Tween® 80, respectively and relative to the initial value. In addition, these treatments exhibited an enhanced lipase activity with respect to vegetated controls, which was significant for the combined treatment at 30 days (1.0-fold increase) and for citric acid at 90 days (0.65-fold increase). The positive effect of citric acid on lipase activity may be explained by the mobilization of metal ions in the presence of the organic acid. In the review by Sharma et al. (2001) the positive effects of metal ions (*e.g.* Ca, Co, Cu, Fe, Mg) on lipase production by microorganisms were reported, but also inhibition of lipase activity was described by metals (*e.g.* Ag, Fe, Hg, Zn), possibly as a result of enzyme conformation alteration.

Conversely, in the presence of Tween® 80 alone lipase activity reached the maximum after 60 days (2.0-fold increase relative to the initial value), and decreased afterwards to a value comparable with that found in the non-planted control soil. Lipase activity was

still lower in the presence of Tween® 80 than in the planted control, showing the negative impact of the surfactant. This inhibiting effect is in accordance with a previous study that evaluated the effect of the non-ionic surfactant Triton X-100 on the lipase activity of chronically and freshly oil-polluted soils, demonstrating that Triton X-100 severely inhibited (84% inhibition) enzyme activity (Margesin et al., 2002). The mechanisms underlying the inhibitory effects on enzyme activity by surfactants may be related to the denaturing properties of these agents (Kanwar et al., 2005). Furthermore, it has been hypothesized that surfactants may adsorb to the lipid surface of the substrate, altering the interaction with lipase enzyme as a result (Gargouri et al., 1983).

Squared correlation (r^2) coefficients between soil lipase activity and number of alkane degraders were calculated to estimate the strength of the relationship between both variables. Interestingly, the highest correlation was obtained when the combined treatment of citric acid and Tween® 80 was applied, (r^2: 0.772). In all other conditions r^2 obtained values were: 0.0054, 0.3180, 0.1243 and 0.0138 for non-vegetated control, vegetated control, citric acid and Tween® 80 amended alfalfa pots, respectively. It is likely that a connection between soil lipase activity and number of alkane degraders does exist. Although the quantity of microorganisms able to degrade alkanes could be a major factor determining soil lipase activity it is certainly not the only one. Global enzyme activity in the soil is the result of the contribution of a multitude of microbial species, plants and microfauna (Nannipieri et al., 2012).

Table 5.3 Soil lipase activity

Treatment	Lipase activity (μg *p*NP (g soil × 10 min)$^{-1}$)		
	30 days	60 days	90 days
Soil	66 ± 40 aA	426 ± 15 aC	188 ± 36 aB
Soil + Alfalfa	132 ± 3 abA	522 ± 55 aC	384 ± 56 bB
Soil + Alfalfa + CA	190 ± 7 bcB	510 ± 17 aC	632 ± 26 cD
Soil + Alfalfa + Tw-80	102 ± 15 aA	407 ± 52 aB	175 ± 16 aA
Soil + Alfalfa + CA + Tw-80	266 ± 15 cB	449 ± 23 aC	484 ± 3 bC

Citric acid (CA), Tween® 80 (Tw-80). Initial (prior to planting) soil lipase activity: 135 ± 11 μg *p*NP (g soil × 10 min)$^{-1}$). Values are expressed as means ± standard deviations of triplicate measurements. Different lower case and upper case letters following the data in a column and in a row, respectively, means significant differences among the data (p<0.05).

5.4. Conclusions

The present study assessed the phytoremediation potential of alfalfa in a co-contaminated soil and the effects of citric acid and Tween® 80, applied individually and combined together, in the phytoremediation process.

Under the experimental conditions presented here, alfalfa cannot be considered as an actively heavy metal removal species. Although alfalfa was not able to phytoextract significant amounts of heavy metals, still in the presence of the tested soil amendments, it could tolerate a co-contaminated soil, which is an essential characteristic for any plant species to be used in phytoremediation. Moreover, this is the first study reporting an

enhancement of alkane degrader population and lipase activity in the rhizosphere of alfalfa growing in a co-contaminated soil encouraging the potential of this plant species to be successfully used in the remediation of petroleum hydrocarbons. The joint application of citric acid and Tween® 80 further stimulated the quantity and metabolism of the rhizosphere community able to degrade hydrocarbons, supporting a promising use of such soil amendments in assisted phytoremediation, to trigger the cleaning up of petroleum hydrocarbons. Nevertheless, these data need to be interpreted cautiously because the present study is limited by the lack of information on the residual soil petroleum hydrocarbon concentration.

In future investigations it might be possible to test if different soil amendments or application doses could successfully enhance metal phytoextraction rates by alfalfa. In addition, the possible use of biologically-produced metabolites as amendments could be assessed to go further in the field of biological soil remediation. Finally, additional work is required to better establish the link and correlation between soil lipase activity increase, alkane degraders' enhancement and petroleum hydrocarbon dissipation, in the presence of plants and soil amendments.

5.5. Acknowledgements

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Chapter 6

Comparative bioremediation of co-contaminated soil by natural attenuation, bioaugmentation and phytoremediation

Abstract

Biological remediation technologies are an environmentally friendly approach for the treatment of polluted soils. This study evaluated four bioremediation strategies: a) natural attenuation, b) bioaugmentation with *P. aeruginosa*, c) phytoremediation with alfalfa (*Medicago sativa* L.) and d) bioaugmentation-assisted phytoremediation, for the treatment of a heavy metal and petroleum hydrocarbon co-contaminated soil. The results showed that alfalfa plants were able to tolerate and grow in the co-contaminated soil. In addition, bioaugmentation treatment enhanced shoot and root biomass by 56 % and 105 %, respectively after and 90 days of experiment. The content of heavy metals in alfalfa plants was limited and following the order: Zn > Cu > Pb. Heavy metals were mainly concentrated in plant roots and were poorly translocated. Bioaugmentation-assisted phytoremediation generally decreased metal concentration in plant organs as well as metal translocation, but increased the total uptake of Cu by alfalfa roots and that of Zn by shoots. Bioaugmentation-assisted phytoremediation treatment showed the highest removal rates of petroleum hydrocarbons (68 %), followed by bioaugmentation (59 %), phytoremediation (47 %) and natural attenuation (37 %). Although soil lipase activity and the number of alkane degraders tended to be higher when alfalfa and/or *P. aeruginosa* were present in the system, there was not an absolute correlation between these parameters and petroleum hydrocarbon removal.

The findings of this study suggest that bioaugmentation-assisted phytoremediation could be a promising bioremediation option for the treatment of co-contaminated soils.

Keywords

Co-contamination, heavy metals, petroleum hydrocarbons, natural attenuation, bioaugmentation, phytoremediation

6. Comparative bioremediation of co-contaminated soil by natural attenuation, bioaugmentation and phytoremediation

6.1. Introduction

The most prevalent pollutants in French polluted sites are heavy metals and petroleum hydrocarbons, which affect 60 % and 23 % of soils, respectively (BASOL, 2014). They arise in the environment from various sources deriving from anthropogenic activities. Heavy metals originate mainly from human activities related to energy and mineral consumption (Kabata-Pendias, 2011), while petroleum hydrocarbons usually come from accidental spills (Russell et al., 2009). Both types of pollutants entail a danger for the environment and living organisms. Moreover, it is not uncommon that such pollutants are present simultaneously in polluted soils strengthening the threat that they pose.

Among existing soil remediation technologies biological methods are environmentally friendly and particularly attractive because of their low cost and relatively simple maintenance (Mirsal, 2004). Natural attenuation, bioaugmentation and phytoremediation are examples of biological remediation strategies and can be used for the remediation of soils affected by different types of pollutants. Natural (phyto)attenuation consists in the *in situ* metabolism of target compounds by indigenous microbial communities, which, through microbial reactions, drive the natural attenuation of both organic and inorganic contaminants. In order to make natural attenuation a feasible strategy, the postulated microbial metabolic transformations must be not only possible but also ongoing and sustainable (Smets and Pritchard, 2003). Bioaugmentation consents an increase of intrinsic biodegradative capacities of contaminated sites by the introduction of single strains or consortia of microorganisms with the desired catalytic capabilities (Mrozik and Piotrowska-Seget, 2010; Lebeau, 2011). Finally, phytoremediation comprises a group of technologies that use plants and their associated microorganisms to remove pollutants from the environment or to make them harmless (Salt et al., 1998). The uptake and accumulation of heavy metals by plants (phytoextraction) and the metabolism of organic pollutants by rhizosphere microorganisms (rhizodegradation) are examples of phytoremediation processes. Natural attenuation, bioaugmentation and phytoremediation approaches can be used not only as remediation technologies in themselves but also in combination. For instance, bioaugmentation can be coupled with phytoremediation to intensify clean-up processes (White, 2001; Glick, 2003). In particular, bioaugmentation-assisted phytoextraction optimizes the synergistic effect of plants and microorganisms and has been used for the cleaning-up of soils contaminated by metals (Lebeau et al., 2008; Huguenot et al., In Press). This enhanced trace element uptake in the presence of microorganisms can be attributed to beneficial effects on plant growth and/or by increasing the plant availability of trace elements in the rhizosphere (Sessitch et al., 2013). Moreover, plant-microorganism associations can also be used to facilitate the removal of organic contaminants (Glick, 2010). In particular, some studies have addressed the combined

use of plants and biodegradative bacteria with the aim to remove petroleum products (Lin et al., 2008b), which seems to be a promising remediation strategy.

A key aspect in biological remediation methods is the selection of appropriate plant-bacteria partnerships for the remediation of polluted soils (Khan et al., 2013). Among plants used in phytoremediation, alfalfa (*Medicago sativa* L.) is of particular relevance. It is a fast growing species that produces large biomass (Coburn, 1912), develops an extensive tap root system favourable for the establishment of rhizosphere microorganisms (Kirk et al., 2005) and can associate with symbiotic nitrogen fixing bacteria (Truchet et al., 1991). Alfalfa has been used to remediate several types of pollutants: heavy metals like Cd, Cr, Cu, Ni and Zn (Peralta-Videa et al., 2002; Peralta-Videa et al., 2004; Bonfranceschi et al., 2009), petroleum hydrocarbons (Wiltse et al., 1998; Kirk et al., 2002), polycyclic aromatic hydrocarbons (PAHs) (Fan et al., 2008) or organochlorines (Li and Yang, 2013). Moreover, recent findings have shown promising results for alfalfa phytoremediation of co-contaminated soils (Ding and Luo, 2005; Ouvrard et al., 2011; Zhang et al., 2013).

Among bacteria strains used for bioremediation, *Pseudomonas aeruginosa* is especially interesting because it can improve pollutant remediation through various mechanisms. Firstly, *P. aeruginosa* has been described to produce metal chelating siderophores, which could improve metal bioavailability (Visca et al., 2006). Secondly it can produce biosurfactants (rhamnolipids) that enhance the solubilization of poor water soluble organic compounds and the mobility of heavy metals (Mulligan, 2005; Zhang et al., 2012) improving their bioavailability. As a result, *P. aeruginosa* has been tested for bioremediation of metals (Singh et al., 2013) and hydrocarbons (Das and Mukherjee, 2007). Finally, a role as plant growth promoting rhizobacteria (PGPR) has been described for *P. aeruginosa*, which leads to improved plant growth, and possibly enhanced phytoremediation rates (Wang et al., 2011).

The aim of this study was to perform a comparative assessment of four bioremediation strategies: a) natural attenuation, b) bioaugmentation with *P. aeruginosa*, c) phytoremediation with alfalfa and d) bioaugmentation-assisted phytoremediation, for the treatment of a heavy metal and petroleum hydrocarbon co-contaminated soil.

6.2. Materials and methods

6.2.1. Soils samples, plants and bacteria

Soil samples were collected from a French urban area close to a fuel station with a history of contamination by heavy metals and petroleum hydrocarbons, mostly diesel. Samples were taken with a drill auger, which allowed collecting soil from different depths between 0 and 100 cm. The different soil fractions were mixed unequally as it was technically not possible to ensure the mixing of soils from different depths in equivalent proportions. This soil (*sondage 4*) was sieved to pass through a 6 mm mesh and homogenized. To limit the level of pollutants in order to improve alfalfa performance, the contaminated soil was mixed (1:1 w/w) with soil from the same site but characterized by negligible hydrocarbon contamination (*sondage 3*). Before mixing,

this soil was sieved through a 2 mm mesh. Selected chemical and physical properties of the 1:1 w/w mix of both soils (*sondage 3/4*) are presented in Table 6.1. Physicochemical characterization of soil samples was performed by an external laboratory: ALcontrol Laboratories. ALcontrol is accredited by the Cofrac (Comité français d'accréditation) and by the RvA (Raad voor Accreditatie) under number L028, in accordance with the criteria of laboratory analysis: ISO / IEC 17025:2005. All their services are performed in accordance with their general conditions, registered under KVK number 24265286 at the Rotterdam Chamber of Commerce, Netherlands. Analysis are performed in accordance with French standards (NF: Norme française), the Dutch Standards Institute (NEN: Nederlands Normalisatie-instituut) and the International Organization for Standardization (ISO). The following analyses were performed: actual soil pH (NF ISO 10693), cation exchange capacity (NF X 31-130), organic carbon and organic matter (NF ISO 14235), total nitrogen (sum of N Kjeldahl, NO_2^- and NO_3^- internal method, NEN 6604), C/N ratio (calculated as the ratio between the content of organic carbon and total nitrogen), P_2O_5 (Joret-Hebert method, NF X 31-161), K_2O, MgO and CaO (NF X 31-108), DTPA (diethylene triamine pentaacetic acid) available fraction of Fe and Mn (NF X 31-121), water available fraction of B (NF X 31-122), soil texture (NF X 31-107), content of As, Cd, Cr, Cu, Pb, Ni and Zn (internal method: destruction in accordance with NEN 6961, analysis in accordance with ISO 22036), content of Hg (NEN 6950, destruction in accordance with NEN 6961, analysis in accordance with NEN-ISO 16772), petroleum hydrocarbon fractions: C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{21} and C_{21}-C_{40} (internal method: acetone, hexane extraction, purification and analysis by GC-FID) and Total C_{10}-C_{40} (Equivalent to NEN-EN-ISO 16703).

Alfalfa seeds (*Medicago sativa* L. v. La Bella Campagnola, purity: 99 %, germinability: 85 %) were surface disinfected by immersion in 2 % (v/v) hydrogen peroxide for 8 min (Qu et al., 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

The bacterial strain *Pseudomonas aeruginosa* ATCC® 9027 was used as inoculum for the bioaugmentation treatments. This strain was bought as Vitroids™ discs (Sigma-Aldrich) of bacteria (1000 CFU).

Table 6.1 Chemical and physical properties of the soil (*sondage 3/4*)

Agronomic Parameters	
pH (H_2O)	8.1
Cation Exchange Capacity at soil pH ($cmol^+ kg^{-1}$ DW)	10.7
Organic Matter (g kg^{-1} DW)	49
Organic Carbon (g kg^{-1} DW)	28.3
Total Nitrogen (mg kg^{-1} DW)	640
C/N ratio	44
P_2O_5 (g kg^{-1} DW)	0.10
K_2O (g kg^{-1} DW)	0.09
MgO (g kg^{-1} DW)	0.12
CaO (g kg^{-1} DW)	9.63
Fe* (mg kg^{-1} DW)	116
Mn* (mg kg^{-1} DW)	19.5
B* (mg kg^{-1} DW)	0.71
Sand (%)	82.6
Silt (%)	12.5
Clay (%)	4.9
Heavy Metals (mg kg^{-1} DW)	
As	7.4
Cd	0.36
Cr	<10
Cu	87
Hg	1.0
Pb	100
Ni	8.7
Zn	110
Hydrocarbons (mg kg^{-1} DW)	
C_{10}-C_{12}	130
C_{12}-C_{16}	1100
C_{16}-C_{21}	1600
C_{21}-C_{40}	830
Total C_{10}-C_{40}	3600

DW: dry weight
* DTPA (diethylenetriaminepentaacetic acid) extraction

6.2.2. Pot experiment

Disinfected alfalfa seeds were sown in a commercial soil (organic carbon: 20 %, organic nitrogen: 0.4 %, organic matter: 40 %, dry matter content: 58 %), where seedlings grew for 21 days in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352). Growth conditions were as following: photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C, photosynthesis photon flux density (PPFD) of 130 µmol m^{-2} s^{-1}. Subsequently, ten seedlings of uniform size were selected and transplanted in plastic pots (7×7×6.7 cm) filled with 200 g of fresh soil. Transplantation of alfalfa seedlings was the strategy chosen because it had been previously demonstrated that heavy metal tolerance is positively correlated with the age of alfalfa plants (Peralta-

Videa et al., 2004). Pots containing the transplants were put in the growth chamber (same conditions as stated above) and received water daily. The location of pots was randomly changed daily (within the same shelf and also between different shelves in the growth chamber).

The experimental design included four conditions to evaluate heavy metal and petroleum hydrocarbon remediation. The treatments were: (a) natural attenuation (NA, intrinsic clean up ability of the soil), (b) bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), (c) phytoremediation (PR, soil vegetated with alfalfa), and (d) bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain). Bioaugmentation was applied every 15 days, *i.e.*, up to six times during the experiment. *P. aeruginosa* was added to pots as 5 ml of cell suspension (4.0×10^{11}-1.0×10^{12} cells ml^{-1}). Non-bioaugmented pots received the same amount of sterile distilled water. Each condition was performed in triplicates.

Plants were harvested after 30, 60 and 90 days of growth in the polluted soil (the different treatments were grown in parallel), every time three days after bioaugmentation. Plants were removed from pots, and roots and shoots were separated. Roots were washed with distilled water to remove attached soil particles and with ethylenediaminetetraacetic acid (EDTA, 10 mM) to remove adsorbed metals. Roots were further rinsed with distilled water and blotted with tissue paper. The plant material was put in the oven at 70°C for 3 days (Campbell and Plank, 1998) and dry weights of shoots and roots were recorded.

Soil samples were taken at 0, 30, 60 and 90 days and kept at 4°C until further soil analyses (number of aliphatic hydrocarbon degraders and soil lipase activity). Moreover, the number of soil total heterotrophs was determined every 7 days and total petroleum hydrocarbons (TPH) were quantified at 0 and 90 days. In the case of vegetated pots, rhizosphere soil samples were taken. In order to collect rhizosphere soil, plant roots were vigorously shaken by hand, taking care of the roots integrity. The external soil not attached to roots was removed, while the soil in the immediate vicinity of roots was kept for the above mentioned analyses.

6.2.3. Analysis of heavy metal content in plants

Prior to elemental analyses, dried plant material was wet digested as described by Campbell and Plank (1998). Briefly, plant material was digested with 5 ml concentrated nitric acid and 2 ml 30 % hydrogen peroxide in a digestion block (LabTech DigiBlock Digester ED16S) at 125 °C for 1h. Heating cycles and hydrogen peroxide additions were repeated three times to obtain a clear digest. To remove residual particles, mineralized samples were filtered through cellulose filters (pore size 2.5 μm) and brought to a final volume of 20 ml. Samples were additionally filtered through nitrocellulose syringe filters (pore size 0.45 μm) and stored at 4 °C until heavy metals were analyzed by Inductively Coupled Plasma-Optical Emission Spectrometry (PerkinElmer Optima 8300 ICP-OES Spectrometer). Cu, Pb and Zn were analyzed at the respective wavelengths of 324.752 nm, 220.353 nm and 213.857 nm.

6.2.4. Soil analyses

6.2.4.1. Number of total heterotrophs

A soil suspension was prepared mixing 1 g of fresh soil and 10 ml of KCl solution (9 g l^{-1}, pH 7.0). Total heterotrophic microflora (THM) was counted in microwell plates, filled with 200 µl of Luria-Bertani broth and inoculated with 20 µl of appropriate dilutions of the soil suspension. Plates were incubated 5 d at 25 °C. The number of positive wells (visible turbidity) was scored and the microbial concentrations in soil were calculated by using the most probable number method designed by Briones Jr. and Reichardt (1999).

6.2.4.2. Number of aliphatic hydrocarbon degraders

Aliphatic hydrocarbon degraders were counted by the most-probable-number (MPN) method described by Wrenn and Venosa (1996), using 96-well microtiter plates. Bushnell-Haas medium supplemented with 2 % NaCl was used as the growth medium (180 µl per well) and n-hexadecane (5 µl per well) was added as the selective growth substrate. 10-fold serial dilutions were performed from a suspension of 1 g of fresh soil and 10 ml of 0.1 % sodium pyrophosphate (pH 7.5) and 2 % NaCl. Plates were inoculated by adding 20 µl of the dilutions from 10^{-2} to 10^{-7}, in 5 replicates. Microplates were incubated for 2 weeks at room temperature. Afterwards, 50 µl of iodonitrotetrazolium violet (INT, 3 g l^{-1}) were added to identify positive wells in which, INT is reduced to an insoluble formazan that deposits intracellularly as a red precipitate. The scoring was done after incubating overnight with INT at room temperature. MPN of alkane degraders per g of soil was calculated according to Briones Jr. and Reichardt (1999).

6.2.4.3. Soil lipase activity

Soil lipase activity was measured through the colorimetric method described by Margesin et al. (2002). 0.1 g of fresh soil was mixed with 5 ml 100 mM NaH_2PO_4/NaOH buffer, pH 7.25, and pre-warmed at 30°C for 10 min. 50 µl of substrate solution (100 mM p-nitrophenyl butyrate (*p*NPB) in 2-propanol) were added and tubes were incubated at 30°C for 10 min. The tubes were cooled for 10 min on ice to stop the reaction. The tubes were centrifuged at 2000 g for 5 min and the absorbance of the released p-nitrophenol (*p*NP) in the supernatants was measured spectrophotometrically (PerkinElmer LAMBDA 10 UV/Vis Spectrophotometer) at 400 nm against the reagent blank. A standard solution of *p*NP (100 µg *p*NP ml^{-1} phosphate buffer) was used to prepare a calibration curve in the presence of soil. In order to measure the *p*NP released from the substrate, a control was prepared without soil. After subtracting the control reading (hydrolysis in absence of soil) from the sample reading (hydrolysis in presence of soil), soil lipase activity was calculated and expressed as µg *p*NP (g soil × 10 min)$^{-1}$.

6.2.4.4. Total petroleum hydrocarbons

Total petroleum hydrocarbons (TPH) and their fractions (C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{21}, C_{21}-C_{40}) were quantified in soil samples by an external laboratory: ALcontrol Laboratories, which performed and internal method consisting on acetone-hexane extraction followed by purification and analysis by gas chromatography with flame ionization detector (GC-FID).

6.2.5. Phytoremediation parameters

To evaluate the ability of metal phytoextraction by alfalfa, the following parameters were considered: a) plant biomass (dry weight of roots and shoots), b) metal concentration in plant tissues (roots and shoots), c) translocation factors (TFs) calculated as the metal in shoots to the metal in roots ratio and d) bioconcentration factors (BCFs) of shoots and roots calculated as the ratio between metal concentration in plant tissues (shoots or roots, respectively) and total metal initial soil concentration.
Plant contribution to rhizodegradation potential was evaluated by calculating the following ratios: MPN of rhizosphere soil (in phytoremediation or bioaugmentation-assisted remediation treatments) / MPN of non-planted soil (in natural attenuation or bioaugmentation treatments) and soil lipase activity of rhizosphere soil (in phytoremediation or bioaugmentation-assisted remediation treatments) / soil lipase activity of non-planted soil (in natural attenuation or bioaugmentation treatments).

6.2.6. Statistical analysis

The experiment was arranged in a completely randomized design. All data reported were averaged values of three independent replicates. Treatment effects were statistically evaluated by one-way analysis of variance (ANOVA) and multiple comparisons of means by Tukey contrasts. Differences were considered significant at $p<0.05$. The statistical analysis was accomplished with R software, version 3.0.2 (R Core Team, 2014).

6.3. Results and discussion

6.3.1. Plant biomass

After transplanting alfalfa seedlings in the contaminated soil, all plants survived and no plant mortality was evidenced throughout the 90-day experiment. Figure 6.1 presents the experimental data on plant biomass for alfalfa growing in bioaugmented and non-bioaugmented soil as a function of experimental time. Alfalfa growth was not hindered and both above and below ground biomass continuously increased for both treatments. After 90 days there was a significant enhancement in plant biomass, with respect to that at the moment of transplanting. This enhancement of shoot biomass was of 24 and 38-fold for alfalfa growing in non-bioaugmented and bioaugmented soil, respectively. For root biomass, 167 and 341-fold increase was observed for alfalfa growing in non-bioaugmented and bioaugmented soil, respectively. Bioaugmentation with *P.*

aeruginosa had a positive effect on plant biomass production. There was an initial trend to improve plant biomass, which became significant for shoots and roots after 60 and 90 days, respectively. Soil inoculation with *P. aeruginosa* enhanced shoot biomass by 15, 33 and 56 % at 30, 60 and 90 days, respectively. Similarly, root biomass was also increased by 13, 19 and 105 % at 30, 60 and 90 days, respectively.

The results of this study indicate that alfalfa was able to grow in the simultaneous presence of heavy metals (*i.e.* Cu, Pb and Zn at 87, 100 and 110 mg kg^{-1} soil DW, respectively) and petroleum hydrocarbons (total C_{10}-C_{40} at 3600 mg kg^{-1} soil DW). High above ground biomass yield is a requisite for phytoextraction purposes. In addition, the establishment of a rich root system creates a favorable niche for rhizosphere microorganisms involved in rhizodegradation. As alfalfa combines this two features in the present experimental conditions, alfalfa could be a promising plant model for the phytoremediation of the present co-contaminated soil.

Several factors influence plant tolerance/sensitivity towards heavy metals and petroleum hydrocarbons. Although pollutant concentration is certainly a key factor determining plant phytotoxicity, is not sufficient to predict it. Other factors such as metal speciation, composition of heterogeneous petroleum hydrocarbon fractions, soil-pollutant and heavy metal-petroleum hydrocarbon interactions must also be considered (Salanitro et al., 1997). For instance, previous studies reported that heavy metals had a distinct effect on alfalfa when present individually or in a mix. It was observed that alfalfa could grow in soils individually contaminated with heavy metals at more than 50 mg kg^{-1} DW (Peralta et al., 2001) but if heavy metals were present in a mixture (Cd, Cu, Ni, and Zn at 50 mg kg^{-1} DW each) they exerted combined stress affecting the shoot length of alfalfa (Peralta-Videa et al., 2002). Concerning alfalfa tolerance towards petroleum hydrocarbons, Kirk et al. (2005) observed differences according to total petroleum hydrocarbon (TPH) soil concentration. They reported no phytotoxicity up to 15000 mg kg^{-1} DW, while they observed that growth of alfalfa seedlings was stressed and stunted at higher TPH levels (31000 mg kg^{-1} DW).

Mechanisms underlying heavy metal and petroleum hydrocarbon phytotoxicity may be related both to direct effects on plant physiology (*e.g.* cell membrane disruption, damage of photosynthetic apparatus) or indirectly, altering the physical and chemical properties of the soil where plants are growing (Baker, 1970; Kabata-Pendias, 2011).

In this study, the fact that alfalfa biomass continuously increased and no plant mortality was observed consent to assume that alfalfa was able to tolerate the polluted soil, at least to a certain extent compatible with phytoremediation purposes. Nevertheless biomass data were also recorded in the same growing conditions but in a non-contaminated agricultural soil attaining considerably higher biomass yields (data available in chapter 7). Therefore, the presence of soil pollutants could be a major factor, if not the only one, causing biomass reduction.

Another important finding of this study was that bioaugmentation with *P. aeruginosa* had a promoting effect on alfalfa growing in the co-contaminated soil, which further encourages the application of bioaugmentation-assisted phytoremediation.

These results are in accordance with the findings of other studies, in which plant growth promoting ability of *P. aeruginosa* was studied. For instance, it has been demonstrated

that *P. aeruginosa* promoted not only dry matter accumulation but also symbiotic attributes (*e.g.* nodule numbers and leghemoglobin content), grain yield and protein of chickpea (*Cicer arietinum* L.) growing in a soil contaminated with Cr (Oves et al., 2013). The growth promoting ability (under both normal and stress conditions) of PGPR could be attributed to several mechanisms. PGPR may facilitate the plant growth: a) directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or b) indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents (Ahemad and Kibret, 2014). In particular, several plant growth promoting traits have been described for *P. aeruginosa*. For instance, increase of P solubilization, indole acetic acid (IAA) and exopolysaccharide (EPS) production have been reported for *P. aeruginosa* growing in the presence and absence of Cr (Oves et al., 2013). Likewise, *P. aeruginosa* has been found to produce secondary metabolites with antibiotic activity useful for the control of plant diseases caused by pathogenic *Xanthomonas* species (Spago et al., 2014). Moreover, PGPR able to metabolize pollutants may also improve plant growth and development indirectly, as a result of pollutant reduction in the media where plants are growing (Khan et al., 2013). In this sense, *P. aeruginosa* has been reported to promote green pea (*Pisum sativum* L.) growth and alleviate lead toxicity through the production of metallothioneins (Naik et al., 2012). Similarly, inoculation with *P. aeruginosa* in a phenol-spiked soil vegetated with corn (*Zea mays*) resulted in plant growth promotion, which correlated with the decrease in soil phenol content (Wang et al., 2011).

Although the scope of this study was limited in terms of establishing which are the mechanisms responsible for alfalfa growth promotion by *P. aeruginosa* in the present co-contaminated soil, one or more of the above mentioned direct and indirect mechanisms might be involved.

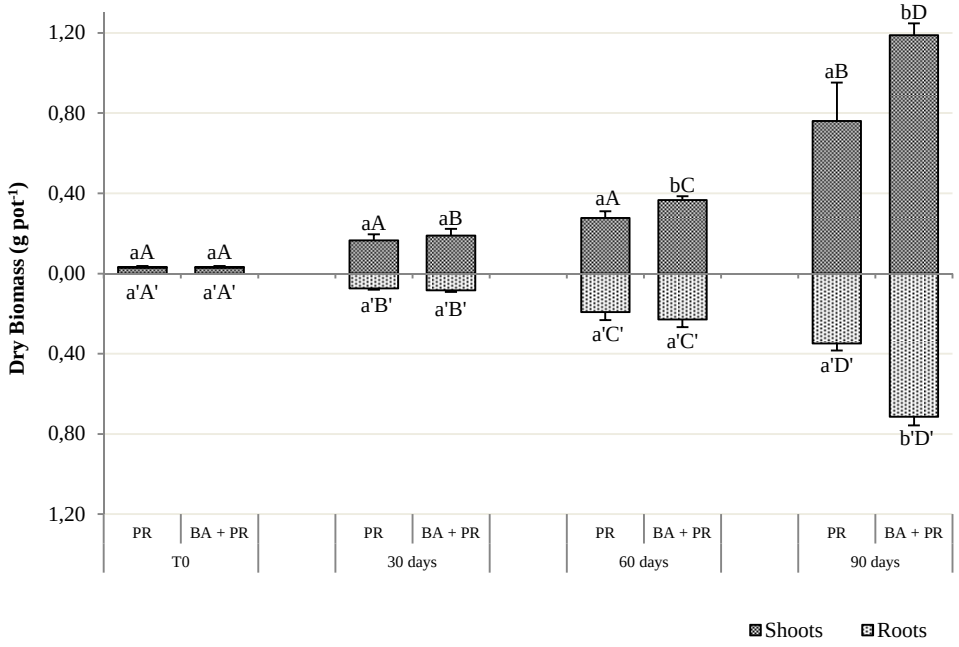

Figure 6.1 Plant Biomass

Dry biomass (g pot^{-1}) for two treatments: phytoremediation (PR, soil vegetated with alfalfa), and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) at initial time (T_0) and after 30, 60 and 90 days of experiment. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter means significant differences between treatments, at a definite time. Different upper case letter means significant differences between experimental times, for a definite treatment ($p<0.05$). The symbol ’ distinguishes root from shoot statiscal analysis.

6.3.2. Phytoremediation treatments and uptake of heavy metals

Figure 6.2 and Table 6.2 show the data of Cu, Pb and Zn concentrations in alfalfa tissues depending on treatment and experimental time. Heavy metal concentrations in shoots and roots of alfalfa growing in non-bioaugmented soil were, in decreasing order: Zn > Cu > Pb. Metal contents of all elements were substantially higher in roots than in shoots. Maximum metal concentration in roots reached 168.5, 70.8 and 22.7 mg kg^{-1} DW while in shoots they did not exceed 77.8, 20.5 and 17.2 mg kg^{-1} DW for Zn, Cu and Pb, respectively. As shown by TF data (Table 6.2), Pb was the most translocated element (average TF value of 0.76) while Cu was the least translocated element (average TF value of 0.37). As demonstrated by the BCF values, BCF of roots were higher than for shoots. Average BCF of shoots and roots were in the following decreasing order: Zn > Cu > Pb.

The extent of metal accumulation in alfalfa tissues was influenced by the bioaugmentation treatment. Heavy metal concentrations in shoots and roots of plants growing in bioaugmented soil were, in decreasing order: Zn > Cu > Pb. The general trend was that Pb and Zn concentrations in alfalfa tissues tended to be lower when

bioaugmentation was performed. In contrast, Cu concentrations in plant roots were higher in the bioaugmentation treatment. Likewise, TF values were always lower in bioaugmented treatment, except for Zn at 60 and 90 days. Averaged BCF values of shoots and roots were also lower in bioaugmented plants, except for Cu BCF of roots.

Although alfalfa is not an hyperaccumulator, previous studies have demonstrated certain accumulation of metals in alfalfa harvestable tissues (Peralta-Videa et al., 2002; Qu et al., 2011). When the heavy metals were at trace levels in the soil, the amounts of Zn in the shoot plant tissues (160 mg kg^{-1} soil DW) were significantly higher than Cd, Cu, and Ni. By contrast, when the soil was artificially contaminated with a soil mixture of Cd, Cu, Ni, and Zn (each one at 50 mg kg^{-1} soil DW), maximum concentrations reported in alfalfa shoots were 437, 202, 160, 105 mg kg^{-1} DW for Ni, Cd, Zn and Cu, respectively (Peralta-Videa et al., 2002). Apart from the intrinsic ability of plant species to uptake metals, also soil characteristics shape the process and may negatively influence plant uptake of metals (Kabata-Pendias, 2011). Soil alkaline pH and sorption to organic matter may decrease metal mobility in soils (Gobran et al., 2000). Moreover, antagonistic effects between metals in multi-metal contaminated soils (Flogeac et al., 2007) as well as the simultaneous presence of organic pollutants and the ageing time can also decrease plant uptake of heavy metals in co-contaminated soils (Lin et al., 2008a). TF were determined to evaluate the ability of the plant to transfer the metals from roots to shoots. In this study, heavy metals uptaken by alfalfa mainly accumulated in root tissues, revealing poor metal translocation from roots to shoots and preferential accumulation in alfalfa roots (TF<1). This is in agreement with Qu et al. (2011), who have reported a similar pattern of limited heavy metal translocation by alfalfa, with comparable TF values of 0.40 for Cu, 0.61 for Zn and 0.79 for Pb. In addition, BCFs were calculated as indicators of the ability of the plant to accumulate metals in plant tissues from soils. The results of this study indicate that Zn is the metal that can be accumulated the most by alfalfa roots (BCF >1).

In this study, bioaugmentation with *P. aeruginosa* was generally found to cause a decrease in Pb and Zn accumulation by alfalfa tissues. This result may be explained by the fact that bacteria can biosorb metals (Lebeau et al., 2008). In particular, *P. aeruginosa* has been reported to biosorb metals like Cu, Ni, Pb and Zn (Gabr et al., 2008; Pérez Silva et al., 2009). It could be hypothesised that metal immobilization onto bacteria due to biosorption processes may contribute to alleviate metal phytotoxicity by lowering their accumulation in the plant. As a result, bioaugmentation may reduce the stress caused by metal toxicity and allow more plant growth, which is in accordance with the biomass data presented in the previous section. Interestingly, soil bioaugmentation had a different effect on Cu accumulation, increasing its concentration in alfalfa roots. This finding is supported by previous research which showed an increase of metal concentration in plants when soil was bioaugmented (Dupponois et al., 2006; Braud et al., 2009; Płociniczak et al., 2013). *P. aeruginosa* is known to synthetize metabolites (*e.g.* biosurfactants, siderophores, organic acids) that can enhance metal bioavailability (Braud et al., 2006), which justifies how microorganisms may increase plant trace element uptake (Sessitch et al., 2013). Nevertheless, in the present study the production of such metabolites was not tested *in situ*. As a result it is

not possible to ensure that the synthesis of such molecules is taking place and data should be interpreted with caution. The distinct impact of bioaugmentation on heavy metal accumulation by alfalfa (increased accumulation for Cu and decreased accumulation for Pb and Zn) may be explained by specific coordination properties of chelating molecules produced by bacteria towards particular metals. This hypothesis is supported by a recent study of Cornu et al. (2013), who found contrasting effects of one siderophore produced by *P. aeruginosa* (pyoverdine) on the bioavailability of Cu and Cd in a calcareous soil. They observed that the application of pyoverdine, enhanced the mobility, the phytoavailability and the phytoextraction of Cu while the fate of Cd was not affected. This effect was the result of different coordination properties of pyoverdine towards Cd and Cu: the stability constant of pyoverdine-Cu complexes was found much higher than that of pyoverdine-Cd complexes.

Metal translocation in alfalfa plants decreased in bioaugmented soils. Although it is not clear the cause of such effect, the present findings seem to be consistent with Lebeau et al (2008), who reviewed several experiments of phytoextraction-assisted bioaugmentation with bacteria and found that PGPR always decrease TF. They also found that BCF vary irrespective of bioaugmentation. In the present study, bioaugmentation tended to cause a decrease in BCF values (except for Cu BCF of roots) as a result of the effect of bioaugmentation on plant metal concentration.

The total amount of metals uptaken by alfalfa plants (Figure 6.3) depends both on plant biomass and metal concentration in plant tissues and it is an essential parameter to evaluate the significance of the remediation process. In the present study, bioaugmentation resulted in an increase of plant biomass simultaneously to a) a decrease of metal concentration accumulated by alfalfa (the case of Cu in shoots at any time, Pb in shoots and roots at any time and Zn in roots and shoots at 30 and 90 days) or b) an increase of metal concentration accumulated by alfalfa (the case of Cu in roots at any time and Zn in shoots and roots at 60 days). In the first case (a), the net effect on the total amount extracted by plants will depend on the variable (biomass increase or plant metal concentration decrease) of greater magnitude. For instance, total uptake of Pb by alfalfa shoots was significantly reduced at 90 days as the enhancement of plant biomass was not enough to compensate for a decrease in shoot Pb concentration. The second case (b) is the most favorable scenario (increase in plant biomass and metal concentration) that will indeed result in enhanced metal extraction by plants. In the present study that was the case for Cu, whose total uptake by alfalfa roots was significantly enhanced at 60 and 90 days and for Zn, whose total uptake by alfalfa shoots was significantly enhanced at 60 days.

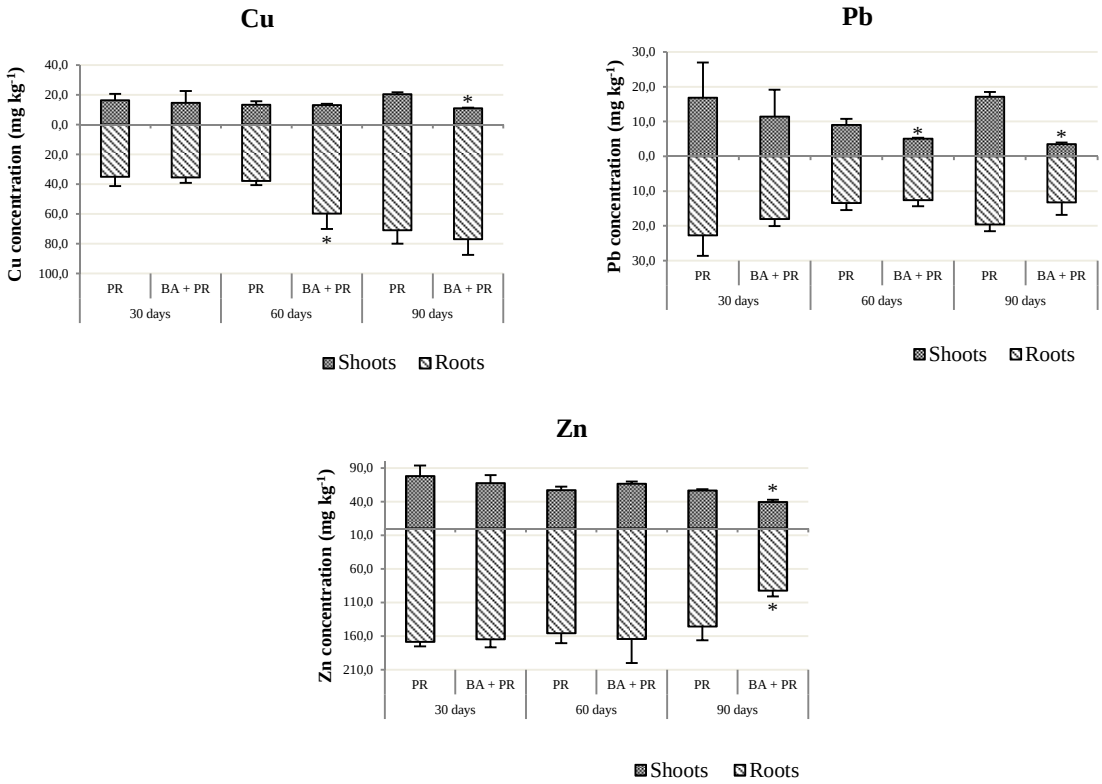

Figure 6.2 Metal concentration in alfalfa

Concentration (mg kg^{-1} DW) in alfalfa shoots and roots for two treatments: phytoremediation (PR, soil vegetated with alfalfa), and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 30, 60 and 90 days of experiment. Values are expressed as means ± standard deviations of triplicate measurements. * indicates that mean values are significantly different between treatments at a definite time ($p<0.05$).

Table 6.2 Heavy metal phytoextraction parameters

a) Phytoremediation treatment (PR, soil vegetated with alfalfa)

	Cu			Pb			Zn		
	30 d	60 d	90 d	30 d	60 d	90 d	30 d	60 d	90 d
Shoots	16,2	13,4	20,5	16,9	9,0	17,2	77,8	57,2	56,7
(mg kg^{-1} DW)	± 4,4	± 2,2	± 1,2	±10,2	±1,7	±1,3	± 15,7	±5,2	±1,6
Roots	35,0	37,9	70,8	22,7	13,5	19,6	168,5	155,7	145,6
(mg kg^{-1} DW)	± 6,2	± 2,7	± 9,1	± 6,0	± 2,0	± 1,9	± 6,6	± 14,6	± 20,6
TF	0,46	0,35	0,29	0,74	0,67	0,87	0,46	0,37	0,39
BCF of Shoots	0,19	0,15	0,24	0,17	0,09	0,17	0,71	0,52	0,52
BCF of Roots	0,40	0,44	0,81	0,23	0,13	0,20	1,53	1,42	1.32

Metal concentration values are expressed as means ± standard deviations of triplicate measurements.
DW: dry weight, TF: translocation factors, BCF: bioconcentration factors

b) Bioaugmentation-assisted phytoremediation treatment (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain)

	Cu			Pb			Zn		
	30	60	90	30	60	90	30	60	90
Shoots	14,7	13,1	10,9	11,4	5,1	3,5	67,4	66,6	39,4
(mg kg^{-1} DW)	± 7,9	± 0,8	± 0,5	± 7,7	±0,2	±0,4	± 12,0	±3,2	±3,4
Roots	35,5	59,8	76,9	18,1	12,6	13,2	164,7	164,4	92,4
(mg kg^{-1} DW)	± 3,6	± 10,1	± 10,5	± 2,0	± 1,8	± 3,6	± 11,8	± 3,4	±8,7
TF	0,42	0,22	0,14	0,63	0,40	0,27	0,41	0,40	0,43
BCF of Shoots	0,17	0,15	0,13	0,11	0,05	0,04	0,61	0,61	0,36
BCF of Roots	0,41	0,69	0,88	0,18	0,13	0,13	1,50	1,49	0,84

Metal concentration values are expressed as means ± standard deviations of triplicate measurements.
DW: dry weight, TF: translocation factors, BCF: bioconcentration factors

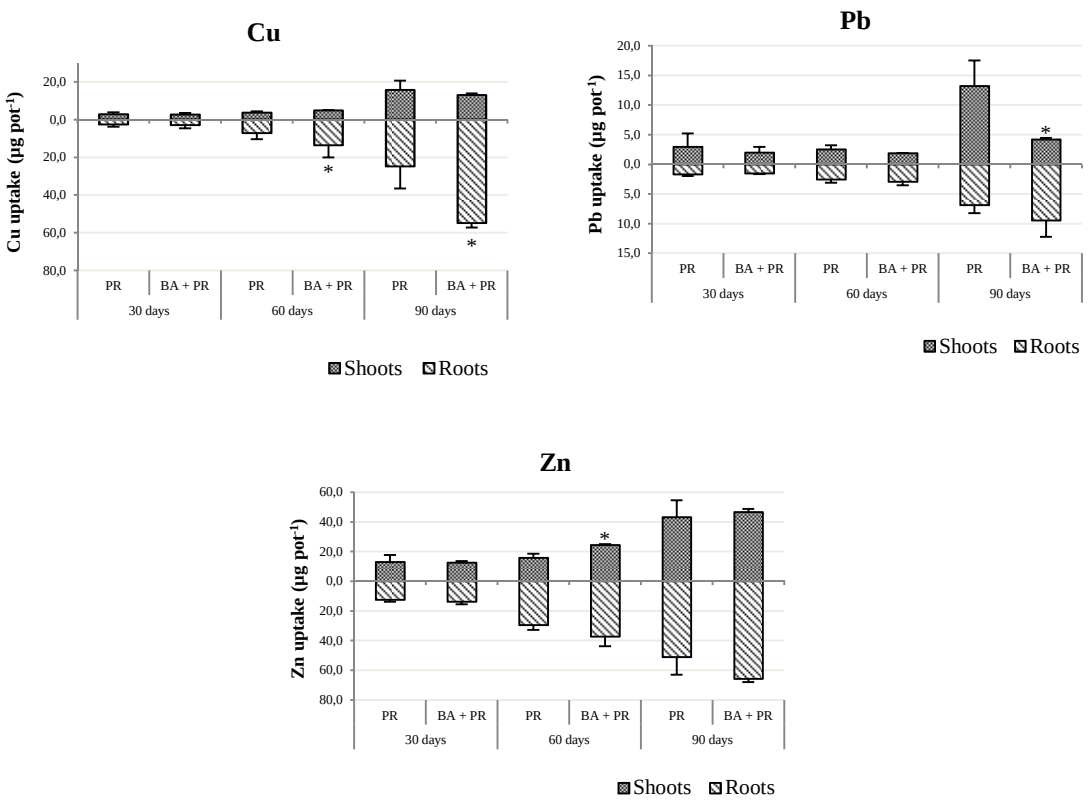

Figure 6.3 Metal uptake by alfalfa

Uptake (µg pot^{-1}) by shoots and roots for two treatments: phytoremediation (PR, soil vegetated with alfalfa), and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 30, 60 and 90 days of experiment. Values are expressed as means ± standard deviations of triplicate measurements. * indicates that mean values are significantly different between treatments at a definite time ($p<0.05$).

6.3.3. Soil microbial number and activity

Figure 6.4 shows the microbial number of total heterotrophs for the bioremediation treatments throughout the experiment. Although fluctuating, countings of total heterotrophs tended to be higher in vegetated treatments (phytoremediation and bioaugmentation-assisted phytoremediation) than in non-vegetated ones (natural attenuation and bioaugmentation), confirming the role of plants to enhance microbial populations in the rhizosphere (Pinton et al., 2007). The rhizosphere is known to sustain a great number of diverse microorganisms because it constitutes a rich environment where the supply of water, oxygen and nutrients is strongly influenced by plant activity (Hawkes et al., 2011). Plant stimulates the growth of rhizospheric microorganisms by releasing high amounts and different types of C from the roots, mainly organic acids and sugars (Haichar et al., 2014).

As expected, soon after every inoculation time it was observed a rise in the number of total heterotrophs population, in the case of bioaugmented treatments. This was always the case except soon after the fifth inoculation (day 66), where the counts of total heterotrophs were not as high as expected based on the previously obtained values. It is difficult to explain this discrepant result, but an experimental error cannot be excluded. Over time, there was an increasing tendency in the number of total heterotrophs in bioaugmented treatments. Maximum counts reached up to 1.35×10^{12} (bioaugmentation treatment) and 2.34×10^{12} (bioaugmentation-assisted phytoremediation treatment) at 80 days of experiment, after inoculating for sixth time. It was also observed that, on average, bioaugmented and non-bioaugmented treatments differed by four orders of magnitude. Every rise in microbial population was followed by a falling-off in the successive seven days. A decline of the inoculated bacterial populations in a few days after bioaugmentation has been often reported in bioaugmentation experiments (Bois et al., 2013). It seems possible that this is due to biotic (*e.g.* competition with indigenous microorganisms) and abiotic (*e.g.* availability of nutrients) factors that affect survival of the inoculum, which is one of the key aspects limiting the success of bioaugmentation (Lebeau et al., 2008). Therefore, in the present study, it was adopted the strategy to apply several consecutive inoculations with the aim to maintain an elevated number of microorganisms throughout the experiment, as already performed by Huguenot et al. (In Press). The pronounced difference in microbial counts of total heterotrophs between bioaugmented and non-bioaugmented treatments may indicate that the inoculated microorganisms were competitive towards the native indigenous bacteria. However, in order to be conclusive in that hypothesis particular analyses (*e.g.* fluorescence *in situ* hybridization, FISH) that specifically follow the fate of the inoculated strain are needed.

Table 6.3 shows the results of soil microbial number of alkane degraders, which varied depending on time and bioremediation treatment. Despite the fact that variability was too high to make differences significant, it can be seen from the data a tendency to increase microbial number of alkane degraders by day 90, with respect to the initial value, for all the treatments. The greatest enhancement was observed for the bioaugmentation treatment, with a difference of three orders of magnitude between the end and the beginning of the experiment. In general, number of alkane degraders was

found in the following decreasing order between treatments: bioaugmentation > bioaugmentation and phytoremediation > phytoremediation > natural attenuation. However, differences among treatments were only significant between bioaugmentation and natural attenuation at 30 days and between bioaugmentation and phytoremediation at 60 days. The contribution of plants to enhance the MPN of alkane degraders is presented in Table 6.5, where it can be observed rhizosphere effect values on MPN of alkane degraders greater than one only for phytoremediation treatment, with a maximum rhizosphere effect value of 11.9 at 90 days.

Table 6.4 presents the experimental data on lipase activity in soil. As can be seen from the table soil lipase activity was very fluctuating and, irrespective of the treatment rose at 30 days, fell at 60 days and rose again at 90 days. Relative to the initial value, it was observed a significant enhancement of lipase activity by the end of the experiment for all the treatments. The greatest enhancement was verified for bioaugmentation treatment with a 7.8-fold increase. Although the pattern of lipase activity varied among bioremediation treatments, natural attenuation showed generally the least microbial activity compared with the other treatments. Table 6.5 presents rhizosphere effect values on soil lipase activity greater than 1 mainly for the phytoremediation treatment, with a maximum rhizosphere effect value of 2.8 at 60 days.

The number of alkane degraders and lipase activity are soil bioindicators of hydrocarbon biodegradation potential. The number of soil aliphatic hydrocarbon degrading bacteria is a quantitative marker of the population of microorganisms able to metabolize aliphatic hydrocarbons (Wrenn and Venosa, 1996). In addition; soil lipase activity can be a suitable parameter to monitor oil biodegradation in soil, as microbial enzymatic systems responsible for lipid degradation may be similar to those involved in oil decomposition (Margesin et al., 1999). The current study found that bioremediation treatments in which inoculation with *P. aeruginosa* was done presented higher levels of soil microbial number of alkane degraders and soil lipase activity. This finding is in agreement with the ability of *P. aeruginosa* to produce and secrete extracellular lipases (Gilbert, 1993; Jaeger et al., 1994) as well as the faculty of this species to degrade petroleum hydrocarbons by means of the suitable enzyme pathways (Ji et al., 2013).

Rhizosphere effect values were calculated to evaluate the plant root influence over the non-planted soil on soil microbial number and activity. The rhizosphere effect refers to the positive influence of plant roots on microbial population and activity in the rhizosphere (Manoharachary and Mukerji, 2006). This effect is principally the result of rhizodeposition, *i.e.* the release of organic compounds by plants, which supplies microorganisms with nutrients (Nguyen, 2009). In addition, roots offer mechanical support for the attachment of microorganisms as well as an improvement of soil physicochemical properties (*e.g.* aeration), which further benefit the development of microorganisms in the rhizosphere (Lynch, 1990). In this study, plant contribution to enhance microbial number and activity appeared to be limited, as the presence of vegetation (phytoremediation and bioaugmentation-assisted phytoremediation treatments) did not always result in a greater improvement, with respect to unvegetated soil (natural attenuation and bioaugmentation treatments). The positive effect of plants on microbial number and activity in the rhizosphere seemed to be particularly diluted

when bioaugmentation was performed, as shown by rhizosphere effect values below 1 in the case of bioaugmentation-assisted phytoremediation treatments.

The number of alkane degraders and soil lipase activity did not exhibit the same behaviour throughout the experiment. As a result, it is not possible to establish a well-defined relationship between soil lipase activity and number of alkane degraders. Despite the fact that the population of alkane degraders may contribute to soil lipase activity, another microbial species, plants and microfauna are also an input of soil lipase activity (Nannipieri et al., 2012).

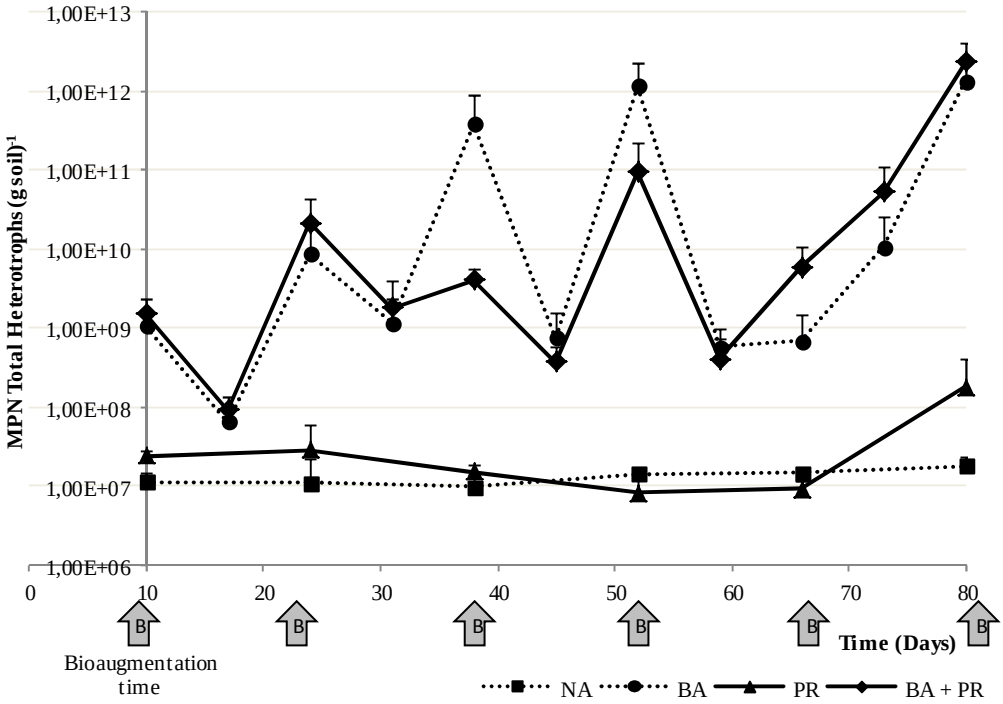

Figure 6.4 Soil microbial number of total heterotrophs

Most probable number (MPN g^{-1} soil) for the treatments: natural attenuation (NA, intrinsic clean up ability of the soil), bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), phytoremediation (PR, soil vegetated with alfalfa) and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain), throughout the 90-day experiment. Values are expressed as means ± standard deviations of triplicate measurements.

Table 6.3 Soil microbial number of alkane degraders

Day	MPN of soil aliphatic degraders (MPN (g soil)$^{-1}$)			
	NA	BA	PR	BA + PR
0	(1,3 ± 0,6) × 10^5 aA	(1,3 ± 0,6) × 10^5 aA	(1,3 ± 0,6) × 10^5 aA	(1,3 ± 0,6) × 10^5 aA
30	(6,2 ± 4,1) × 10^5 aA	(9,7 ± 3,1) × 10^7 aB	(1,6 ± 0,4) × 10^6 aA	(4,1 ± 1,8) × 10^6 aA
60	(1,5 ± 0,2) × 10^6 aAB	(1,2 ± 0,7) × 10^7 aB	(1,0 ± 0,4) × 10^6 aA	(1,1 ± 0,5) × 10^7 aAB
90	(2,0 ± 1,6) × 10^6 aA	(2,2 ± 2,6) × 10^8 aA	(2,4 ± 3,2) × 10^7 aA	(9,8 ± 9,6) × 10^7 aA

NA: natural attenuation (intrinsic clean up ability of the soil), BA: bioaugmentation (soil inoculated with *P. aeruginosa* strain), PR: phytoremediation (soil vegetated with alfalfa) and BA+PR: bioaugmentation-assisted phytoremediation (soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain).
Values are expressed as means ± standard deviations of triplicate measurements. Different lower case and upper case letters following the data in a column and in a row, respectively, mean significant differences among the data ($p<0.05$).

Table 6.4 Soil lipase activity

Day	Lipase activity in soil (μg *p*NP (g soil × 10 min)$^{-1}$)			
	NA	BA	PR	BA + PR
0	116 ± 2 aA	116 ± 2 aA	116 ± 2 aA	116 ± 2 aA
30	530 ± 43 bA	653 ± 18 cAB	697 ± 54 cB	529 ± 3 bA
60	70 ± 18 aA	198 ± 3 bAB	196 ± 55 aAB	277 ± 94 aB
90	522 ± 36 bB	1024 ± 11 dD	394 ± 16 bA	882 ± 28 cC

NA: natural attenuation (intrinsic clean up ability of the soil), BA: bioaugmentation (soil inoculated with *P. aeruginosa* strain), PR: phytoremediation (soil vegetated with alfalfa) and BA+PR: bioaugmentation-assisted phytoremediation (soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain).
Values are expressed as means ± standard deviations of duplicate or triplicate measurements. Different lower case and upper case letters following the data in a column and in a row, respectively, mean significant differences among the data ($p<0.05$).

Table 6.5 Rhizosphere effect values

Day	Rhizosphere effect on MPN of alkane degraders		Rhizosphere effect on soil lipase activity	
	PR	BA + PR	PR	BA + PR
30	2,5	0,0	1,3	0,8
60	0,7	0,9	2,8	1,4
90	11,9	0,5	0,8	0,9

PR: phytoremediation (soil vegetated with alfalfa), BA+PR bioaugmentation-assisted phytoremediation (soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain)

6.3.4. Bioremediation treatments and removal of petroleum hydrocarbons

The effect of bioremediation treatments on soil concentration of TPH fractions is shown in Figure 6.5. All bioremediation treatments affected the concentration of TPH fractions after 90 days of experiment. Natural attenuation and phytoremediation treatments significantly reduced all TPH fractions except the C_{10}-C_{21} fraction, relative to the initial concentration. In contrast, bioaugmentation and bioaugmentation-assisted phytoremediation significantly reduce all TPH fractions between C_{10} and C_{40}.

Removal rates of different TPH fractions after 90 days of experiment were also calculated for the different bioremediation treatments (Figure 6.6). Irrespective of the bioremediation treatment, the pattern of removal of the light TPH fractions was higher than that of the heavy TPH fractions and removal rates were in the following decreasing order: C_{10}-C_{12} > C_{12}-C_{16} > C_{16}-C_{21} > C_{21}-C_{40}. There are several possible explanations for this observed pattern of removal, which is possibly related to the chemical structure of n-alkanes that determines their physico-chemical properties. Firstly, it is possible that hydrocarbons with shorter carbon chain length are more susceptible to microbial attack and more readily biodegradable as a consequence (Ji et al., 2013). Secondly, the hydrophobicity of *n*-alkanes increases with an increase in molecular mass. As a result, it is possible that the fractions with longer C chains (and higher octanol–water partition coefficient, K_{ow}) are less bioavailable for biodegradation due to higher sorption onto organic matter (Guo et al., 2010). Finally, the boiling points of alkanes increases with their number of carbons, and thus their chain length and molecular mass (Mehta and Mehta, 2005). Therefore, a higher dissipation through evaporation could be expected for the fractions of shorter C length.

The extent of TPH removal varied among bioremediation treatments. Bioaugmentation-assisted phytoremediation treatment showed the highest removal rates of TPH (68 % for total TPH), followed by bioaugmentation (59 %), phytoremediation (47 %) and natural attenuation (37 %). The results of this study showed that natural attenuation significantly reduced TPH levels in the present polluted soil. This implies that indigenous microorganisms of this soil were not only adapted to the conditions of their habitat, but also functional and able to degrade TPH. In accordance with the present results, Serrano et al. (2008) demonstrated that natural attenuation of diesel aliphatic hydrocarbons can occur to substantial extents. They observed that aliphatic hydrocarbons were used as sources of carbon and energy by soil microorganisms and that soil quality indicators and microbiological parameters regained their original levels about 200 days after the spill. Although evaluating the impact of metals on the biodegradation of petroleum hydrocarbons was not within the scopes of this study, it is important to highlight that TPH removal rates obtained in a co-contaminated soil are possibly lower than those obtained in the absence of metals. These differences can be explained by the fact that metals have been demonstrated to affect the biodegradation of organic pollutants as a result of their negative impact on the physiology and ecology of organic degrading microorganisms (Sandrin and Maier, 2003).

Although natural attenuation is the simplest approach among bioremediation technologies, it can be improved through the association with other biological strategies

to achieve reduced clean-up times. This experiment confirmed that vegetation with alfalfa species (phytoremediation treatment) led to higher removal rates. These findings support the idea of plant contribution to pollutant dissipation possibly through an enhancement of microbial number and/or activity in the rhizosphere (Pinton et al., 2007; Segura and Ramos, 2013). As shown by rhizosphere effect values, it seems that plants had a greater contribution by enhancing microbial number of alkane degraders than by stimulating lipase activity. This is possibly the result of plant-bacteria interactions mediated by root exudates rich in organic compounds, such as amino acids, organic acids, sugars, enzyme and complex carbohydrates, which provide carbon source and energy for the growth of rhizosphere microorganisms (Haichar et al., 2014). An increased number of bacteria capable of petroleum degradation in an hydrocarbon-contaminated soil vegetated with alfalfa has already been reported in the literature (Kirk et al., 2005), as well as enhanced pyrene degradation in the rhizosphere of alfalfa (Fan et al., 2008). Moreover, recent studies support the idea of an active role of alfalfa plants in the rhizospheric degradation of hydrocarbons as the result of the action of plant enzymes released in root exudates (Muratova et al., 2014). Thus, the increased degradation of pollutants in the rhizosphere could be the result of a combined action of plants and rhizospheric microorganisms. Finally, another possible explanation for the enhanced pollutant removal rates in the presence of vegetation may be related to an abiotic contribution of root exudates to the rhizosphere effect. Root exudates have been demonstrated to enhance soil desorption of pollutants, improving bioavailability and subsequent biodegradation potential as a result (LeFevre et al., 2013; Sun et al., 2013).

In turn, bioaugmentation with *P. aeruginosa* resulted in even greater remediation efficiency. The present findings seem to be consistent with a previous comparative study which demonstrated that bioaugmentation was more effective than natural attenuation on the degradation of light (C_{12}–C_{23}) and heavy (C_{23}–C_{40}) fractions of TPH in soil samples (Bento et al., 2005)**.** It can be hypothesised that the observed increase in TPH removal rates when soil inoculation was performed are due to *P. aeruginosa* hydrocarbon-degrading ability (Ueno et al., 2006; Liu et al., 2012; Ji et al., 2013). Nevertheless, specific techniques (*e.g.* FISH) are required to attribute petroleum hydrocarbon degradation to *P. aeruginosa*. The observed increase in TPH removal rates when bioaugmentation was performed could be further facilitated by the production of biosurfactants that increase organic pollutant bioavailability (Zhang et al., 2012). Relative to natural attenuation, a 10 and 22 % increase in removal rates was obtained for phytoremediation and bioaugmentation, respectively. It is apparent from this data that the contribution of TPH removal of bacteria (bioaugmentation treatment) was greater than that of plants (phytoremediation treatment). However, the effect of plants is not only limited to the enhancement of pollutant dissipation in the rhizosphere. The presence of plants makes several noteworthy additional contributions, which renders advisable the vegetation of a contaminated site. Plants have a role in sequestering CO_2 and greenhouse gas emissions. In addition, vegetation improves control of soil erosion, surface water runoff and infiltration. Moreover, the presence of plants improves physico-chemical properties of the soil as well as ecosystem functioning and landscape aesthetics.

The present study also demonstrated that the combination of plants and microorganisms in bioaugmentation-assisted phytoremediation treatment gave rise to the best performance in TPH removal, among the bioremediation treatments tested. The effects of bacteria and plant contribution seemed to be additive rather than synergic, as the bioaugmentation-assisted phytoremediation treatment resulted in a 31 % increase of TPH removal rates, relative to natural attenuation. After 90 days, the content of total TPH in bioaugmentation-assisted phytoremediation treatment was 39 % and 20 % lower than that of phytoremediation and bioaugmentation treatments, respectively. It is possible to hypothesise that this reduction in the content of TPH pollutants when soils were inoculated with *P. aeruginosa* may result in a higher biomass production of alfalfa plants as a consequence of decreased toxicity exerted by TPH pollutants. This hypothesis is consistent with the obtained results of higher biomass of alfalfa when bioaugmentation was performed. The association between alfalfa and *P. aeruginosa* appeared to be particularly effective in terms of TPH removal, probably due to the fact that *P. aeruginosa* combines petroleum hydrocarbon biodegradative ability and plant growth-promoting activity at the root level. It is likely therefore that the reduction of TPH in the rhizosphere of alfalfa resulted both from the ability to degrade TPH of a) inoculated microorganisms and b) rhizosphere-associated microorganisms growing in the surroundings of alfalfa roots. The results of this study demonstrate that the combined use of plant and bacteria is a more promising strategy for the remediation of petroleum hydrocarbons, as compared to bioaugmentation or phytoremediation applied alone.

Although this study has successfully demonstrated a positive contribution of bioaugmentation (alone and in combination with plants), the application of bacteria to soils may have certain limitations in terms of implementing such strategy at higher scale being thus more feasible the application of bioaugmentation in confined systems, which are easier to control. In any case, careful cost-effectiveness of the process should be contemplated, considering not only economic aspects (*e.g.* costs of design, engineering of the site and monitoring) but also safety issues in order to determine whether it is safe to introduce a new species to an environment that it is not native to.

Contrary to expectations, this study did not find evident consistency between the number of alkane degraders, soil lipase activity and TPH removal rates. At 90 days the following divergent trends were obtained in 1) Number of alkane degraders: NA < PR < BA+PR < BA, 2) Soil lipase activity: PR < NA < BA+PR < BA and 3) TPH removal: NA < PR < BA < BA+PR. The three parameters were higher in bioaugmented treatments that in non-bioaugmented treatments. However, the presence of plants leads to a more variable behavior. The lack of a clear correlation indicates that the population of alkane degraders and the soil lipase activity were possibly not the only factors determining the removal of TPH.

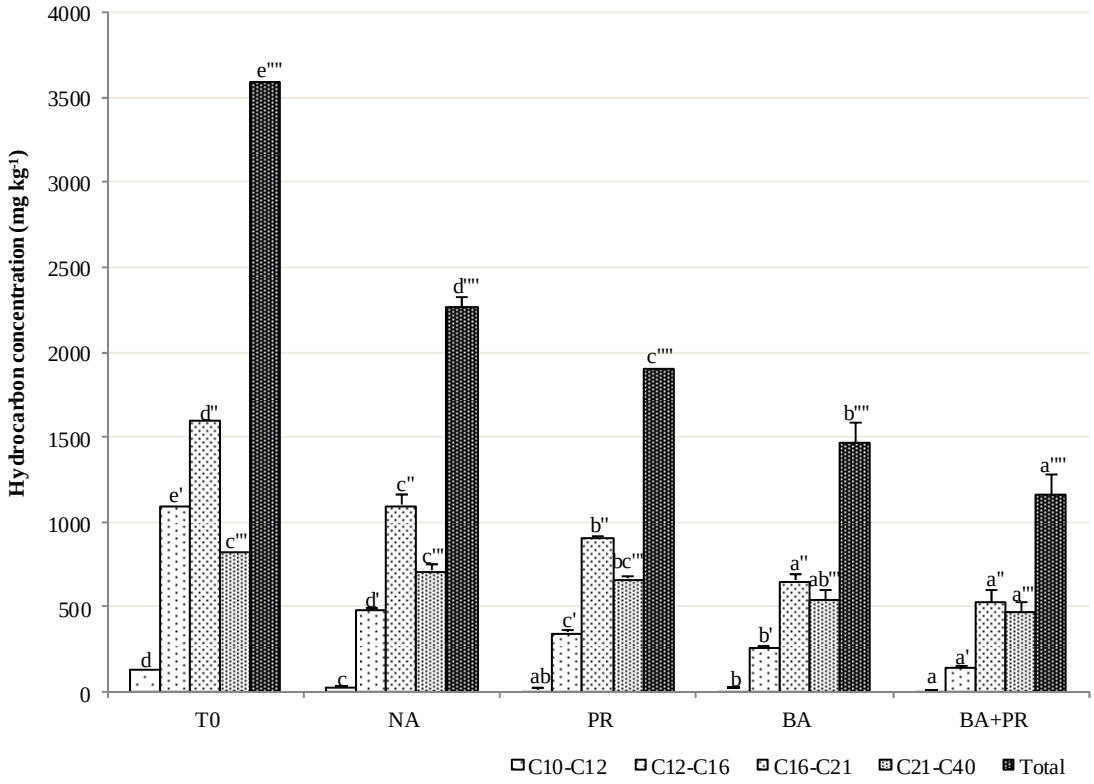

Figure 6.5 Soil concentration of petroleum hydrocarbons
Concentration (mg kg^{-1} soil DW) of petroleum hydrocarbon fractions for the treatments: natural attenuation (NA, intrinsic clean up ability of the soil), phytoremediation (PR, soil vegetated with alfalfa), bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 90 days of experiment and at initial time (T_0). Values are expressed as means ± standard deviations of triplicate measurements (except at T_0). Different letters indicates that mean values are significantly different between treatments ($p<0.05$).

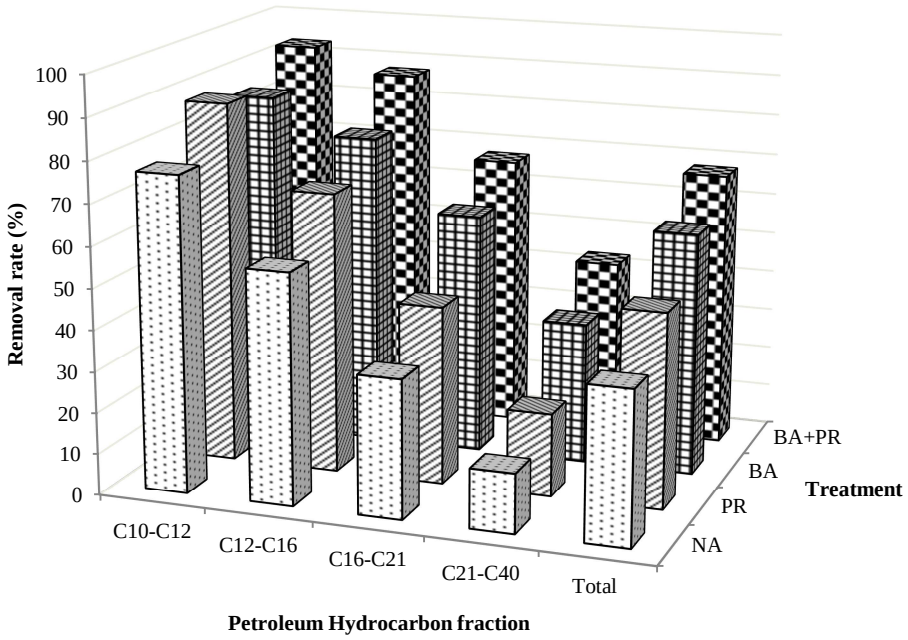

Figure 6.6 Removal rates of petroleum hydrocarbons
Removal rates (%) of petroleum hydrocarbon fractions for the treatments: natural attenuation (NA, intrinsic clean up ability of the soil), phytoremediation (PR, soil vegetated with alfalfa), bioaugmentation (BA, soil inoculated with *P. aeruginosa* strain), and bioaugmentation-assisted phytoremediation (BA+PR, soil vegetated with alfalfa and inoculated with *P. aeruginosa* strain) after 90 days of experiment

6.4. Conclusion

The present study was designed to compare four bioremediation strategies: a) natural attenuation, b) bioaugmentation with *P. aeruginosa*, c) phytoremediation with alfalfa and d) bioaugmentation-assisted phytoremediation, for the treatment of a heavy metal and petroleum hydrocarbon co-contaminated soil.

This study has shown that alfalfa was able to tolerate and grow in the co-contaminated soil. In addition, the bioaugmentation treatment had a promoting effect on alfalfa biomass. The content of heavy metals in alfalfa plants was limited, mainly concentrated in plant roots and poorly translocated. Bioaugmentation-assisted phytoremediation generally decreased metal concentration in plant parts as well as metal translocation, but increased the total uptake of Cu by plant roots and that of Zn by shoots. Bioaugmentation-assisted phytoremediation treatment showed the highest removal rates of TPH, followed by bioaugmentation, phytoremediation and natural attenuation. Soil lipase activity and the number of alkane degraders tended to be higher when alfalfa and/or *P. aeruginosa* were present in the system, but a definite correlation between these parameters and TPH removal could not be found.

Taken together, these results support the idea that alfalfa-*P. aeruginosa* could be an effective partnership for the remediation of co-contaminated soils. Bioaugmentation had a significant effect as PGPR, alleviating the phytotoxicity caused by soil pollutants. In

contrast, bacteria effect to enhance heavy metal uptake by shoots tended to be more limited. As a result, this system seemed to be more suitable for metal stabilization at the root level rather than for metal phytoextraction. The combined effects of alfalfa and *P. aeruginosa* were particularly relevant for TPH removal, principally as the result of bacteria contribution. The present study provides additional evidence with respect to bioaugmentation-assisted phytoremediation of co-contaminated soils and demonstrates that it could be a suitable approach to reduce clean-up time and improve natural attenuation.

For a better understanding of bioaugmentation-assisted phytoremediation further research might explore the mechanisms responsible for alfalfa growth promoting effects of *P. aeruginosa*. More research is also required to determine if the production of metabolites (*e.g.* siderophores, organic acids, biosurfactants) that could enhance pollutant bioavailabilty by *P. aeruginosa* is taking place *in situ* after bioaugmentation. In any case, a close monitoring after inoculation is critical in order to ensure succesful inoculum survival, colonization and metabolic activity.

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Chapter 7

Physiological impacts of co-contaminated soil and bioaugmentation on alfalfa plants

7. Physiological impacts of co-contaminated soil and bioaugmentation on alfalfa plants

7.1. Introduction

This chapter presents supplementary data that completes the results presented in chapter 6. This study had two primary aims: 1) to evaluate the development and physiology of alfalfa plants growing in a non-contaminated agricultural soil and in a polluted soil and 2) to ascertain the influence of soil bioaugmentation with *Pseudomonas aeruginosa* on alfalfa development and physiology when plants are growing in a non-contaminated agricultural soil and in a polluted soil. Several parameters (biomass, maximum quantum yield of photosystem II (PSII) and plant content of chlorophyll, flavonols and malondialdehyde) to evaluate plant physiology were followed in alfalfa plants growing in an agricultural and a co-contaminated soil bioaugmented or not with *P. aeruginosa*, over a 90-day experimental time.

7.2. Materials and methods

7.2.1. Soils samples, plants and bacteria

Two types of soil samples were used: a) agricultural soil (AS) and b) polluted soil (PS). Samples of AS were collected in an agricultural area. This soil was used to test the performance of plants when pollutants were not present.

Samples for PS were collected from a French urban area close to a fuel station with a history of contamination by heavy metals and petroleum hydrocarbons, mostly diesel. Samples were taken with a drill auger, which allowed collecting soil from different depths between 0 and 100 cm. The different soil fractions were mixed unequally as it was technically not possible to ensure the mixing of soils from different depths in equivalent proportions. This soil (*sondage 4*) was sieved to pass through a 6 mm mesh and homogenized. To limit the level of pollutants in order to improve alfalfa performance, the contaminated soil was mixed (1:1 w/w) with soil from the same site but characterized by negligible hydrocarbon contamination (*sondage 3*). Before mixing, this soil was sieved through a 2 mm mesh.

Selected chemical and physical properties of AS and PS (soil mix *sondage 3/4*) soils are presented in Table 7.1 and Table 7.2, respectively. Physicochemical characterization of soil *sondage 3/4* samples was performed by an external laboratory: ALcontrol Laboratories. ALcontrol is accredited by the Cofrac (Comité français d'accréditation) and by the RvA (Raad voor Accreditatie) under number L028, in accordance with the criteria of laboratory analysis: ISO / IEC 17025:2005. All their services are performed in accordance with their general conditions, registered under KVK number 24265286 at the Rotterdam Chamber of Commerce, Netherlands. Analysis are performed in accordance with French standards (NF: Norme française), the Dutch Standards Institute (NEN: Nederlands Normalisatie-instituut) and the International Organization for Standardization (ISO). The following analyses were performed: actual soil pH (NF ISO

10693), cation exchange capacity (NF X 31-130), organic carbon and organic matter (NF ISO 14235), total nitrogen (sum of N Kjeldahl, NO_2^- and NO_3^- internal method, NEN 6604), C/N ratio (calculated as the ratio between the content of organic carbon and total nitrogen), P_2O_5 (Joret-Hebert method, NF X 31-161), K_2O, MgO and CaO (NF X 31-108), DTPA (diethylene triamine pentaacetic acid) available fraction of Fe and Mn (NF X 31-121), water available fraction of B (NF X 31-122), soil texture (NF X 31-107), content of As, Cd, Cr, Cu, Pb, Ni and Zn (internal method: destruction in accordance with NEN 6961, analysis in accordance with ISO 22036), content of Hg (NEN 6950, destruction in accordance with NEN 6961, analysis in accordance with NEN-ISO 16772), petroleum hydrocarbon fractions: C_{10}-C_{12}, C_{12}-C_{16}, C_{16}-C_{21} and C_{21}-C_{40} (internal method: acetone, hexane extraction, purification and analysis by GC-FID) and Total C_{10}-C_{40} (Equivalent to NEN-EN-ISO 16703).

Alfalfa seeds (*Medicago sativa* L. v. La Bella Campagnola, purity: 99 %, germinability: 85 %) were surface disinfected by immersion in 2 % (v/v) hydrogen peroxide for 8 min (Qu et al., 2011), in order to avoid the addition of non-indigenous microorganisms to the system. Then, seeds were thoroughly rinsed three times with sterile water and used for the pot experiment.

The bacterial strain *Pseudomonas aeruginosa* ATCC® 9027 (Sigma-Aldrich) was used as inoculum for the bioaugmentation treatments.

Table 7.1 Chemical and physical properties of the agricultural soil (AS)

Agronomic Parameters	
pH (H_2O)	8.0
Cation Exchange Capacity at soil pH ($cmol^+ kg^{-1}$ DW)	32.0
Organic Matter ($g\ kg^{-1}$ DW)	31.7
Organic Carbon ($g\ kg^{-1}$ DW)	18.4
Total Nitrogen ($mg\ kg^{-1}$ DW)	885
C/N ratio	21
Sand (%)	11.3
Silt (%)	32.7
Clay (%)	56.0

Table 7.2 Chemical and physical properties of the polluted soil (PS, *sondage 3/4*)

Agronomic Parameters	
pH (H_2O)	8.1
Cation Exchange Capacity at soil pH ($cmol^+ kg^{-1}$ DW)	10.7
Organic Matter ($g\ kg^{-1}$ DW)	49
Organic Carbon ($g\ kg^{-1}$ DW)	28.3
Total Nitrogen ($mg\ kg^{-1}$ DW)	640
C/N ratio	44
P_2O_5 ($g\ kg^{-1}$ DW)	0.10
K_2O ($g\ kg^{-1}$ DW)	0.09
MgO ($g\ kg^{-1}$ DW)	0.12
CaO ($g\ kg^{-1}$ DW)	9.63
Fe* ($mg\ kg^{-1}$ DW)	116
Mn* ($mg\ kg^{-1}$ DW)	19.5
B* ($mg\ kg^{-1}$ DW)	0.71
Sand (%)	82.6
Silt (%)	12.5
Clay (%)	4.9
Heavy Metals ($mg\ kg^{-1}$ DW)	
As	7.4
Cd	0.36
Cr	<10
Cu	87
Hg	1.0
Pb	100
Ni	8.7
Zn	110
Hydrocarbons ($mg\ kg^{-1}$ DW)	
C_{10}-C_{12}	130
C_{12}-C_{16}	1100
C_{16}-C_{21}	1600
C_{21}-C_{40}	830
Total C_{10}-C_{40}	3600

DW: dry weight

* DTPA (diethylenetriaminepentaacetic acid) extraction

7.2.2. Pot experiment

Disinfected alfalfa seeds were sown in a potting soil (organic carbon: 20 %, organic nitrogen: 0.4 %, organic matter: 40 %, dry matter content: 58 %), where seedlings grew for 21 days in a growth chamber (Sanyo Versatile Environmental Test Chamber MLR-352). Growth conditions were as following: photoperiod of 16 h light at 22 °C and 8 h dark at 18 °C, photosynthesis photon flux density (PPFD) of 130 μmol m^{-2} s^{-1}. Subsequently, 10 seedlings of uniform size were selected and transplanted in plastic pots (7×7×6.7 cm) filled with 200 g of fresh soil (AS or PS). Pots containing the transplants were put in the growth chamber (same conditions as stated above) and received water daily. The location of pots was randomly changed daily (within the same shelf and also between different shelves in the growth chamber).
The experimental design included four treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* strain (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* strain (PS + Alf + Pa). All treatments were performed in triplicates. Bioaugmentation was done every 15 days, for a total of six bioaugmentation events during the experiment. *P. aeruginosa* was added to pots as 5 ml of cell suspension (4.0×10^{11}-1.0×10^{12} cells ml^{-1}). Non-bioaugmented pots received the same amount of sterile distilled water. Each condition was performed in triplicates.

7.2.3. Plant biomass

Plants were harvested 30, 60 and 90 days after transplanting (the different treatments were grown in parallel), every time three days after bioaugmentation. Plants were removed from pots, and roots and shoots were separated. Roots were washed with distilled water to remove attached soil particles and with ethylenediaminetetraacetic acid (EDTA, 10 mM) to remove adsorbed metals. Roots were further rinsed with distilled water and blotted with tissue paper. The plant material was put in the oven at 70 °C for 3 days (Campbell and Plank, 1998) and dry weights of shoots and roots were recorded.

7.2.4. Maximum quantum yield of photosystem (PS) II (F_v/F_m)

The maximum quantum yield was measured with a portable pulse modulated fluorimeter (Hansatech Fluorescence Monitoring System, FMS1) able to detect chlorophyll fluorescence emissions. Leaves were dark adapted for at least 15 min using leaf clips designed for use with the FMS1. Following dark adaptation the modulated light was turned on, the minimal fluorescence (F_0, with all PSII reaction centres fully open) signal recorded and then a saturating pulse applied to measure the maximal fluorescence (F_m, with all PSII reaction centres fully closed). The maximum quantum yield of PSII, which quantifies the maximal efficiency of photon capture by open PSII reaction centers, was calculated as $F_v/F_m = (F_m-F_0)/F_m$ (Papageorgiou and Govindjee, 2007).

7.2.5. Chlorophyll and flavonols content

The chlorophyll index and the flavonols index were measured using the sensor DUALEX SCIENTIFIC+™. This portable battery-powered fluorimeter with a light-emitting diode possesses a leaf-clip to measure in an instantaneous and non-destructive way chlorophyll content in plant leaves and flavonols content in plant epidermis. Each measurement was performed on two leaves per pot in order to have a representative sample. Chlorophyll and flavonols indices were calculated as described by (Cerovic et al., 2012) and results were expressed in arbitrary units (a.u.).

7.2.6. Malondialdehyde content

Malondialdehyde (MDA) was measured according to the colorimetric method in which MDA contained in fresh plant tissue extracts reacts with thiobarbituric acid (TBA) at 95 °C during 25 min to form a coloured product whose absorbance is recorded at 532 nm. The modified method described by (Hodges et al., 1999) allows to correct for plant interfering compounds that also absorb at 532 nm. The effect of these interferences is avoided by subtracting the absorbance at 532 nm of a solution containing plant extract incubated without TBA from an identical solution containing TBA. Results of MDA concentration are expressed as nmol MDA equivalents per g of fresh plant tissue.

7.2.7. Statistical analysis

The experiment was arranged in a completely randomized design. All data reported were averaged values of three independent replicates. Data were statistically evaluated by one-way analysis of variance (ANOVA) and multiple comparisons of means by Tukey contrasts. Differences were considered significant at $p<0.05$. The statistical analysis was accomplished with R software, version 3.0.2 (R Core Team, 2014).

7.3. Results

7.3.1. Plant biomass

Figure 7.1 shows the experimental data on plant biomass for the four treatments and as a function of time. Alfalfa growth was not hindered and both above and below ground biomass continuously increased for all the treatments. After 90 days there was always a significant enhancement in plant biomass, with respect to that at the moment of transplanting. For plants growing in the agricultural soil there was an 85 and 89-fold increase for shoots and 961 and 998-fold increase for roots of alfalfa growing in non-bioaugmented and bioaugmented soil, respectively. Although of less magnitude, for plants growing in the polluted soil the enhancement was of 24 and 38-fold increase for shoots and 167 and 341-fold increase for roots of alfalfa growing in non-bioaugmented and bioaugmented soil, respectively.

The comparison between biomass yield in polluted and agricultural soil revealed a significant shoot biomass reduction up to 71 and 57 % for alfalfa growing in non-bioaugmented and bioaugmented soils, respectively at 90 days. For roots, it was

observed a significant reduction of 83 and 66 % for alfalfa growing in non-bioaugmented and bioaugmented soils, respectively, at 90 days.

Bioaugmentation with *P. aeruginosa* had a positive effect on plant yield principally in the polluted soil. Soil inoculation with *P. aeruginosa* enhanced shoot biomass by 15, 33 and 56 % at 30, 60 and 90 days, respectively. Similarly, root biomass was also increased by 13, 19 and 105 % at 30, 60 and 90 days, respectively. In contrast the improvement was considerably less pronounced in the agricultural soil and only from 60 days on. There was just a 2 and 5 % enhancement for shoots and 8 and 4 % enhancement for roots after 60 and 90 days, respectively.

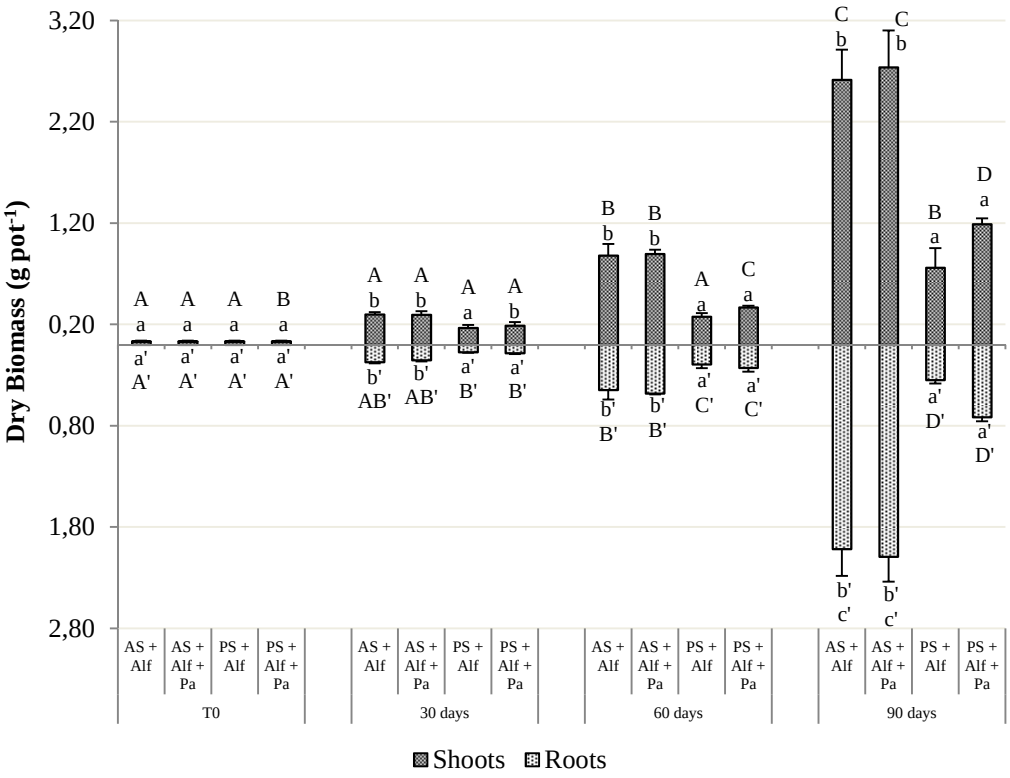

Figure 7.1 Biomass of alfalfa

Dry biomass (g pot^{-1}) for four treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (PS + Alf + Pa) at initial time (T0) and after 30, 60 and 90 days of experiment. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter above (for shoots) or below (for roots (‘)) a column indicates that mean values are significantly different between treatments at a definite time (p<0.05). Different upper case letter above (for shoots) or below (for roots (‘)) a column indicates that mean values are significantly different throughout time for a single treatment (p<0.05).

7.3.2. Maximum Quantum Yield of photosystem (PS) II (F_v/F_m)

The results of maximum quantum yield of PSII (F_v/F_m) are presented in Figure 7.2. Fv/F_m values were constant over time for alfalfa plants growing in the agricultural soil

with comparable values for plants growing in bioaugmented or non-bioaugmented soil (Average F_v/F_m: 0.84). On the contrary, there was a significant reduction in F_v/F_m values 15 days after transplanting alfalfa seedlings to the polluted soil in both bioaugmented and non-bioaugmented treatments ($F_v/F_m < 0.75$), compared to the initial F_v/F_m value (0.83). As time passed, these values tend to improve mirroring those obtained in the agricultural soil. Interestingly when bioaugmentation was performed the recovery of F_v/F_m values was favoured.

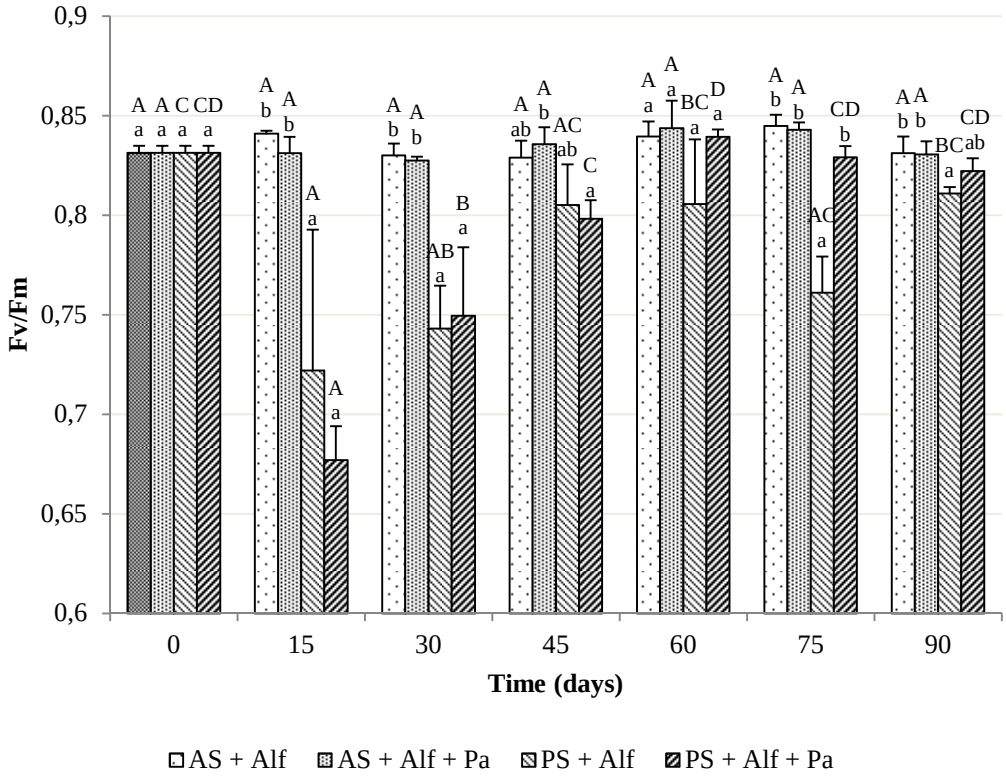

Figure 7.2 Maximum quantum yield of photosystem II (F_v/F_m) in alfalfa
Treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (PS + Alf + Pa) as a function of experimental time. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter above a column indicates that mean values are significantly different between treatments at a definite time ($p<0.05$). Different upper case letter above a column indicates that mean values are significantly different throughout time for a single treatment ($p<0.05$).

7.3.3. Chlorophyll content

The results of chlorophyll content in alfalfa leaves are provided in Figure 7.3. For alfalfa plants growing in the agricultural soil, chlorophyll content continuously and significantly increased as experimental time passed, attaining quite constant values by the end of the experiment. On the other hand, chlorophyll content in alfalfa leaves suffered a significant fall (almost 50 % decrease) 15 days after transplanting seedlings to the polluted soil. Afterwards, chlorophyll content gradually increased for both biougmentated and non-bioaugmented treatments, but in the presence of bacteria this improvement was additionally facilitated. At 90 days it was observed a significant 1.04 and 1.57-fold increase for non-bioaugmented and bioaugmented plants, respectively and relative to the chlorophyll content at 15 days.

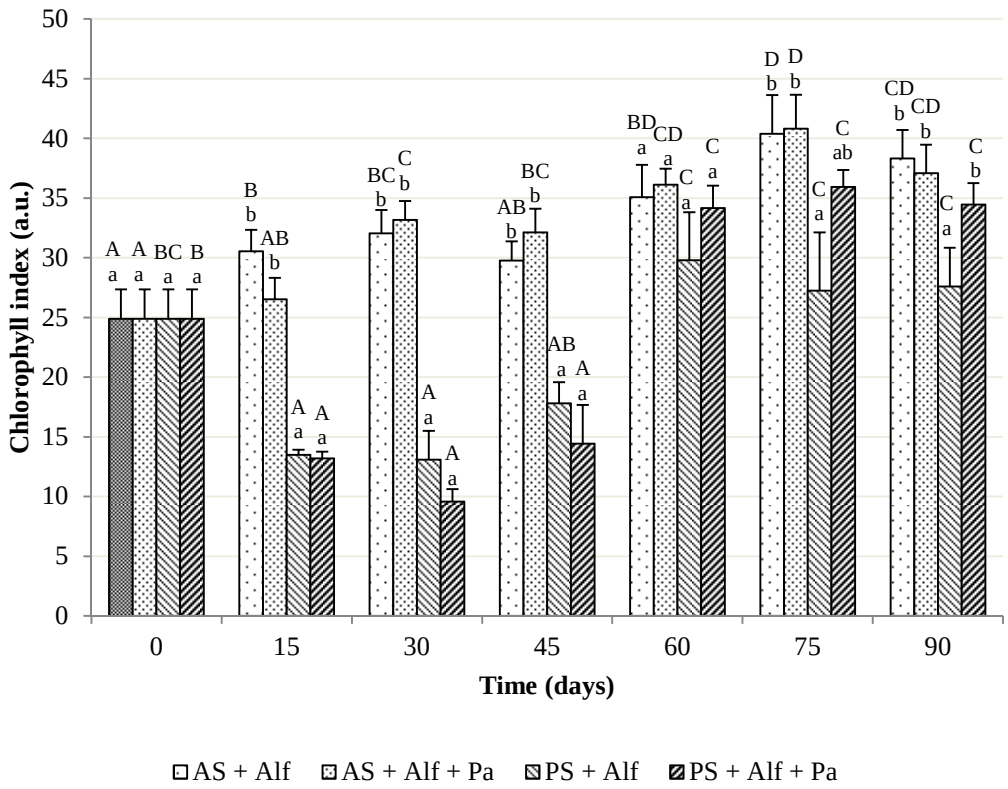

Figure 7.3 Chlorophyll content in alfalfa
Chlorophyll index (arbitrary units, a.u.) in alfalfa leaves for four treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b)) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (PS + Alf + Pa) as a function of experimental time. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter above a column indicates that mean values are significantly different between treatments at a definite time (p<0.05). Different upper case letter above a column indicates that mean values are significantly different throughout time for a single treatment (p<0.05).

7.3.4. Flavonols content

Figure 7.4 presents the results of flavonols content in alfalfa. As shown in the figure, flavonols content significantly increased over time, independently of the type of soil and bioaugmentation treatment. From 45 days on, and specially at 75 days, the highest flavonols contents were found in plants growing in the polluted soil that was not inoculated with *P. aeruginosa*. The remaining treatments presented comparable flavonols contents.

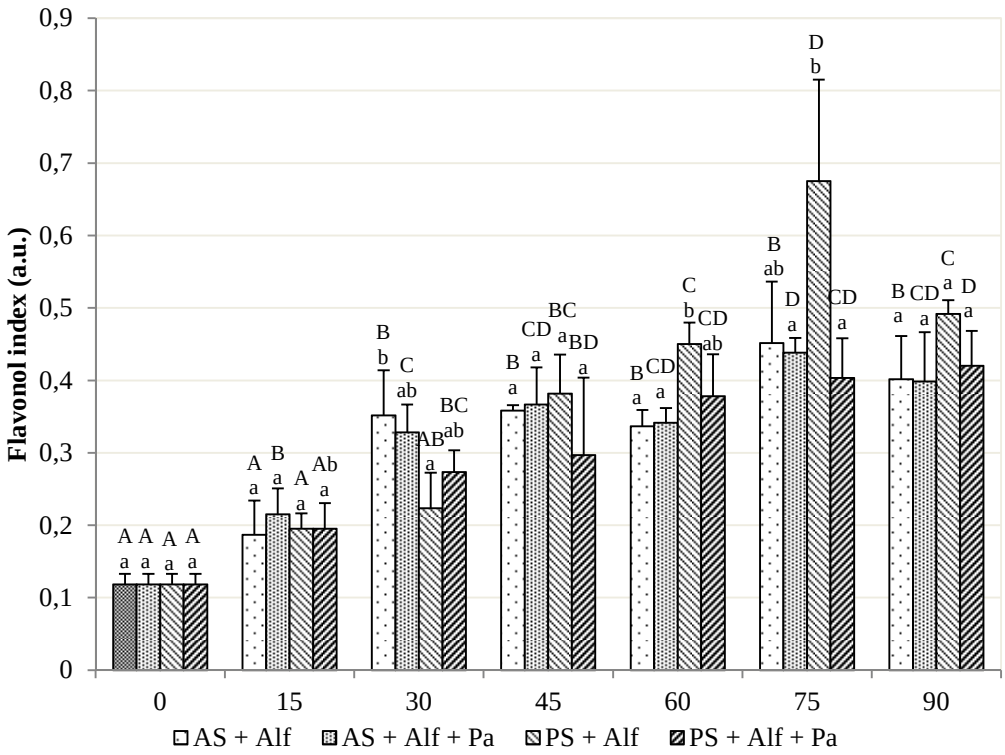

Figure 7.4 Flavonols content in alfalfa

Flavonol index (arbitrary units, a.u.) in alfalfa leaves for four treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (PS + Alf + Pa) as a function of experimental time. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter above a column indicates that mean values are significantly different between treatments at a definite time ($p<0.05$). Different upper case letter above a column indicates that mean values are significantly different throughout time for a single treatment ($p<0.05$).

7.3.5. Malondialdehyde (MDA) content

The results obtained from MDA analysis are shown in Figure 7.5. The highest levels in MDA content of plant tissues were found at the moment of transplanting seedlings and after 30 days of experiment. Subsequently MDA content tended to decrease. Among treatments, at 30 days the greatest MDA levels were found in plants growing in the polluted and non-bioaugmented soil (23 % higher than plants growing in the polluted but bioaugmented soil and 48 % higher than plants growing in the non-polluted and non-bioaugmented soil).

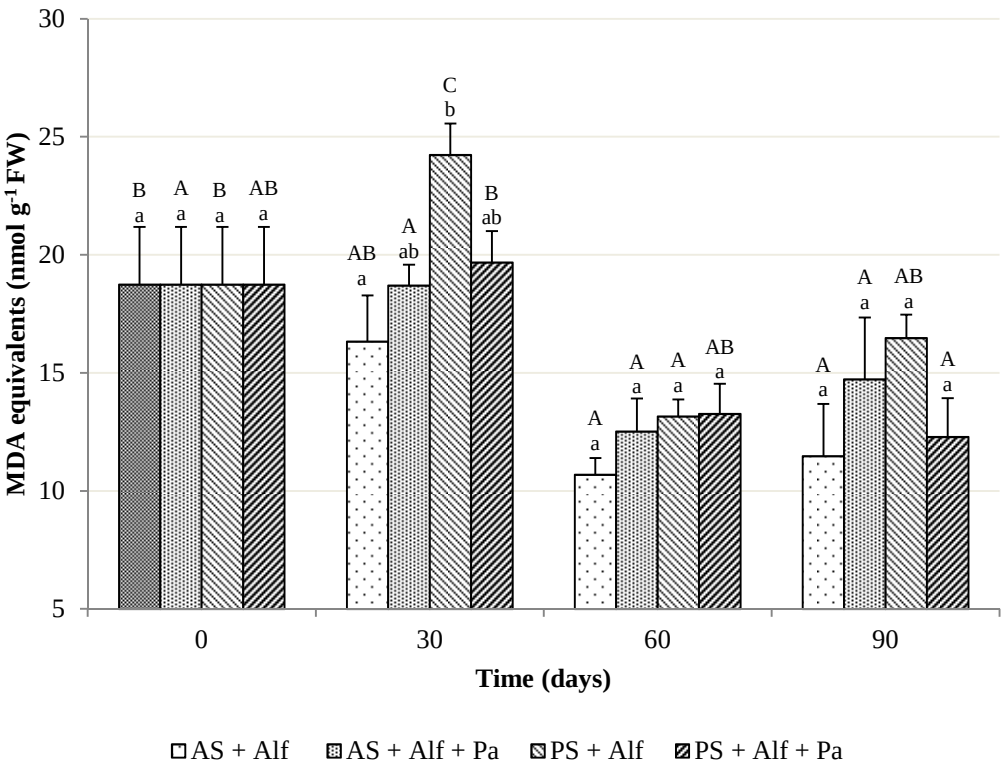

Figure 7.5 Malondialdehyde (MDA) content in alfalfa
MDA (nmol g^{-1} fresh weight, FW) content in alfalfa leaves for four treatments: (a) agricultural soil vegetated with alfalfa (AS + Alf), (b) agricultural soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (AS + Alf + Pa), (c) polluted soil vegetated with alfalfa (PS + Alf), and (d) polluted soil vegetated with alfalfa and bioaugmented with *P. aeruginosa* (PS + Alf + Pa) at initial time (T0) and after 30, 60 and 90 days of experiment. Values are expressed as means ± standard deviations of triplicate measurements. Different lower case letter above a column indicates that mean values are significantly different between treatments at a definite time (p<0.05). Different upper case letter above a column indicates that mean values are significantly different throughout time for a single treatment (p<0.05).

7.4. Discussion

This study evaluated alfalfa growth and physiology in a non-contaminated agricultural soil and in a polluted soil. Plant biomass of both roots and shoots was lower in the polluted soil, although root biomass was comparatively more strongly reduced. Roots might have been more sensitive than shoots to the effects of soil pollutants as a result of a direct contact with them (Kummerová et al., 2013). In accordance with the present results, inhibition of plant growth in the presence of hydrocarbons and/or heavy metals has already been reported for alfalfa species (Peralta-Videa et al., 2002; Fan et al., 2008). Mechanisms underlying heavy metal and petroleum hydrocarbon phytotoxicity may be related both to direct effects on plant physiology (*e.g.* cell membrane disruption, damage of photosynthetic apparatus) or indirectly, altering the biological, chemical and physical properties of the soil where plants are growing (Baker, 1970; Kabata-Pendias, 2011). The study of physiological parameters showed symptoms of phytotoxicity in plants growing in the polluted soil. Chlorophyll fluorescence measurements are useful to estimate the photochemical activity of PSII. The ratio F_v/F_m represents the maximum potential quantum efficiency of PSII if all capable reaction centers were open. In general, F_v/F_m is about 0.8 in healthy leaves and a decrease in F_v/F_m (fewer open reaction centers available) is indicative of plant stress and dysfunction of PSII (Pessarakli, 2005). In the present study, photosynthesis process carried out by alfalfa plants was strongly affected as observed by the decrease in F_v/F_m ratio and by the decrease in leaf chlorophyll content in alfalfa plants growing in the polluted soil. In addition, high levels of MDA were also found. MDA is a secondary end product of the oxidation of polyunsaturated fatty acids (Del Rio et al., 2005), used as an index of general lipid peroxidation (Hodges et al., 1999). Oxidative processes are responsible of cell membranes damage, which may modify membrane fluidity and permeability. These modifications can result in the alteration of electron transfer in photosystems and the reduction of photosynthetic efficiency (Del Rio et al., 2005; Demidchik, In Press). The increase of lipid peroxidation, as observed by an increase in MDA content, is indicative of toxicity that resulted in oxidative stress, possibly responsible of physiological perturbations on alfalfa plants. In response to oxidative stress plants are able to develop antioxidant defense systems, which comprises the synthesis of protective compounds with antioxidant activity (Gill and Tuteja, 2010). Among them, flavonols are a class of flavonoids, which are plant secondary metabolites able to inhibit the generation of reactive oxygen species (ROS) and reduce the levels of ROS once they are formed (Agati et al., 2012). The findings of the current study suggest that the synthesis of flavonols occurred in alfalfa plants growing in the polluted soil, possibly in response to oxidative stress.

After transplanting alfalfa plants to the polluted soil it was observed a negative impact on plant biomass (reduction of shoot and root yield) and photosynthetic machinery (reduction in F_v/F_m ratio and chlorophyll content), which was evident at 30 days of experiment. It is likely that these effects were the result of oxidative processes taking place in stressed plants, which exhibited an increase in MDA content at 30 days. Subsequently alfalfa plants started to adapt to the unfavourable environment triggering a

plant defense system. It could be hypothesized that the synthesis of molecules with antoxidative activity (flavonols) helped to counteract the oxidative events taking place. This hypothesis could be supported by the decrease in MDA content at 60 and 90 days. As a consequence of plant acclimatization to the polluted soil, physiological parameters (F_v/F_m ratio and chlorophyll content) returned to the levels found in plants grown in the agricultural soil and plant growth in the co-contaminated soil was not hindered.

The presence of petroleum hydrocarbons and heavy metals appeared to be a determining factor affecting the growth and physiology of plants growing in the polluted soil. However, these results need to be interpreted with caution because soil properties differed between the polluted soil and the non-contaminated agricultural soil. Soil characteristics such as the nutrient state, organic matter content or texture may have also influenced plant performance. As a result, it is not possible to attribute the observed differences exclusively to the presence of co-contamination.

The present study also assessed the influence of soil bioaugmentation with *Pseudomonas aeruginosa* on alfalfa growth and physiology for plants growing in a non-contaminated agricultural and a polluted soil. Bioaugmentation treatment seemed somehow to counteract the negative impact of soil pollutants on plant biomass and physiology parameters. *Pseudomonas aeruginosa* is a plant growth promoting rhizobacteria (PGPR) able to promote plant growth through several mechanisms: a) directly by either assisting in resource acquisition or modulating plant hormone levels, or b) indirectly by decreasing the inhibitory effects of plant pathogens (Ahemad and Kibret, 2014). Moreover, PGPR able to metabolize pollutants may also improve plant growth, as a result of organic pollutant biodegradation in the media where plants are growing (Khan et al., 2013). Moreover, bacteria can biosorb metals, which would result in a decrease of their mobility and their toxicity towards plants (Lebeau et al., 2008). This study showed that plant growth promoting ability of *P. aeruginosa* was mainly observed in the polluted soil. Therefore, it is likely that alfalfa growth promotion by *P. aeruginosa* is the result of decreased pollutant phytotoxicity and/or the influence on plant phytohormones in stressed plants. Furthermore, it has been demonstrated that plant growth promoting bacteria enhance plant tolerance to biotic and abiotic stress, mitigating the levels of ROS (Jebara et al., 2005; Cerqueira Rodrigues et al., 2013). Hence, it could be hypothesised that soil inoculation with *P. aeruginosa* alleviated oxidative stress in alfalfa plants, which is in accordance with the observed decrease in MDA content and the increase in F_v/F_m values and chlorophyll content, relative to those found in alfalfa growing in the non-bioaugmented polluted soil.

7.5. Conclusion

This work analysed the behaviour of alfalfa plants growing in a polluted soil and in a non-contaminated agricultural soil. The measurements of plant biomass and selected physiological parameters showed a negative influence on alfalfa growing in the polluted soil. Heavy metals and petroleum hydrocarbons might have been at the origin of bad plant performance although other factors cannot be excluded. Alfalfa plants were able to tolerate the presence of pollutants, and to develop an adaptative response to the hostile

soil environment. As plant tolerance to pollutants is one of the crucial characteristics for plant species to be used in phytoremediation, it could be predicted a viable application of alfalfa species with this purpose. Moreover, the impact of soil bioaugmentation with *P. aeruginosa* on alfalfa was addressed. Soil inoculation with *P. aeruginosa* promoted plant growth and appeared to alleviate plant toxicity towards the co-contaminated soil. Therefore, suitable plant-bacteria associations could represent a promising solution to improve the clean-up of soils through a bacteria-assisted phytoremediation approach.

7.6. Acknowledgements

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Chapter 8

General experimental overview, final considerations and future perspectives

8. General experimental overview, final considerations and future perspectives.

8.1. Overview relating the experiments performed

This research project was undertaken to investigate the potential of alfalfa (*Medicago sativa* L.) plants for the phytoremediation of co-contaminated soils and to study chemical and biological strategies to assist the phytoremediation process. To accomplish these aims a set of five experiments were conducted. A summary of the whole experimental design, highlighting the main characteristics of each experiment is presented in Table 8.1.

The starting point of this research was the biological treatment of a real co-contaminated soil (*sondage 4*). This soil was affected by the simultaneous presence of inorganic and organic pollutants. Among inorganics, the main heavy metals present were Cu, Pb and Zn (at 76, 100 and 98 mg kg^{-1} soil dry weight (DW)), while petroleum hydrocarbons were the organic pollutants of concern (total petroleum hydrocarbon (TPH) concentration: 8400 mg kg^{-1} DW). A first preliminary experiment was performed to assess the behaviour of alfalfa plants in this co-contaminated soil over a relatively long timeframe of five months (Chapter 3). The results of this investigation showed important plant growth restriction and elevated plant mortality. These effects could be attributed to the presence of heavy metals and petroleum hydrocarbons in the soil, which were probably above the phytotoxicity threshold for alfalfa. However, other causes such as soil nutrient deficiencies cannot be excluded. Heavy metals were uptaken by plants to a limited extent but microbiological indicators were enhanced in the rhizosphere, which could be promising for rhizodegradation of hydrocarbons. However, it was apparent from this study that in order to make this approach feasible, alfalfa tolerance to contaminants had to be improved and probably phytoremediation needed to be assisted by means of supplementary treatments.

In order to reduce phytotoxicity of the co-contaminated soil towards alfalfa three strategies were adopted for the subsequent phytoremediation studies. Firstly, the soil concentration of TPHs was reduced by mixing (1:1 w/w) the soil *sondage 4* with soil from the same site but characterized by negligible hydrocarbon contamination (soil *sondage 3*). The resulting soil mix (soil *sondage 3/4*), contained heavy metals at similar levels (Cu: 87, Pb: 100 and Zn: 110 mg kg^{-1} DW), while TPH content was comparatively reduced (3600 mg kg^{-1} DW). Secondly, germination of alfalfa seedlings was performed in a non-polluted soil. Just after a short growth phase in a separate substrate, seedlings were transplanted to the polluted soils subject to phytoremediation. Finally, it was proposed that phytoremediation by alfalfa could be assisted by chemical and biological strategies to improve the efficiency of the remediation process.

The method adopted for chemical assistance of phytoremediation involved the use of two types of soil amendments: namely the low molecular weight organic acid citric acid and the non-ionic surfactant Tween® 80 (polyethylene glycol sorbitan monooleate). Next, a second preliminary study was performed to test if the selected amendments *per*

se could exert any toxic effect on alfalfa plants growing in a non-polluted soil, thus not affected by pollutant stress (Chapter 4). A set of four levels of concentration of each amendment were analyzed and an intensive frequency of weekly applications to vegetated soils was implemented. This study found that alfalfa could tolerate citric acid and Tween® 80 (significant inhibition of plant biomass or decrease in chlorophyll content were not observed), thus supporting the upcoming use of such amendments in future experiments of chemically assisted phytoremediation. The following step was to perform a phytoremediation experiment in the soil *sondage 3/4* and in the presence of individual and combined applications of citric acid and Tween® 80 (Chapter 5). This experiment demonstrated that alfalfa could better tolerate the levels of pollutants present in soil *sondage 3/4*. Plant biomass increased in the course of the experiment and negligible plant mortality occurred. One difference between soils *sondage 4* and *sondage 3/4* was related to TPH content. Thus, it is possible to hypothesise that the presence of petroleum hydrocarbons could be a key factor determining phytotoxicity rather than heavy metals such as Cu, Pb or Zn, whose content was similar in both soils. Nonetheless, it is not possible to exclude the deleterious effects of other metals like Hg, whose concentration was higher in soil *sondage 4* than in *sondage 3/4* and whose role was not addressed in the present study. In addition, there is also another possible factor influencing the distinctive plant performance in soils *sondage 4* and *sondage 3/4*, related to the fact that plants where not germinated in soil *sondage 3/4* but transplanted after a pre-growth phase in a non-polluted soil.

The complementary data presented in chapter 7 demonstrated that biomass yield and parameters to assess plant physiology were affected after transplanting alfalfa plants in the polluted soil (*sondage 3/4*), relative to the non-polluted agricultural soil. Yet again, petroleum hydrocarbons and heavy metals could be a determining factor affecting the growth and physiology of plants. However, this role could not unambiguously be attributed to pollutants as other soil properties (nutrient state, organic matter content or texture) differed between the polluted soil and the non-contaminated agricultural soil. Interestingly, 30 days after transplanting, alfalfa plants appeared to develop an adaptative response to the co-contaminated soil, as demonstrated by the restitution of plant physiological parameters and the increasing plant growth.

The phytoremediation experiment presented in chapter 5 (*i.e.* in soil *sondage 3/4*) also demonstrated that heavy metal content in plant parts was lower than in the previous phytoremediation experiment (*i.e.* in soil *sondage 4*), while alfalfa rhizosphere effect was yet again present, enhancing both the microbial population and activity. The second major finding of this experiment was that the application of soil amendments was not effective at enhancing metal uptake by alfalfa shoots, limiting the phytoextraction potential of this strategy. In contrast, the joint application of citric acid and Tween® 80 further promoted microbial number and activity in the rhizosphere, stressing the potential improvement in hydrocarbon biodegradation that could be attained in the presence of the combined treatment.

As a final point, biologically assisted phytoremediation was considered, performing an experiment in the soil *sondage 3/4*, vegetated with alfalfa and bioaugmented with *Pseudomonas aeruginosa* (Chapter 6). Soil inoculation with this strain demonstrated to

have a growth promoting effect on alfalfa, while in general, it did not improve total uptake of heavy metals by plant shoots, thus restricting the feasibility of phytoextraction. Conversely, the highest TPH removal rates were obtained through the joint action of inoculated bacteria and plants in the bacteria-assisted phytoremediation treatment. Interestingly, inoculation with *P. aeruginosa* seemed somehow to counteract the negative impact of soil pollutants on plant physiology (Chapter 7). These findings provide further evidence to support the fact that the combined action of suitable plant-bacteria partnerships can be effective to treat hydrocarbon-polluted soils, even in the presence of heavy metals.

Some of the results obtained from chemical- and biological-assisted phytoremediation experiments are compared in Table 8.2. In terms of plant biomass, the highest yields of shoots were obtained for the biological treatment, while the highest yields of roots were attained with the joint application of chemical amendments. Concerning heavy metals, none of the treatments resulted in a considerable enhancement of metal concentration in plant harvestable tissues. As a result, assisted phytoextraction appears not to be practicable. The highest rhizodegradation potential appeared to occur in the presence of the combined chemical treatment (citric acid and Tween® 80) and when soil was bioaugmented, as could be observed by the highest improvements in the number of alkane degraders and soil lipase activity, respectively. Nevertheless, the information provided by these microbiological indicators needs to be corroborated by the quantification of the remaining TPH content in soil. The most important limitation lied in the fact that TPH data was not available for chemical-assisted treatments. As a result, it was not possible to resolve which one of the approaches (chemical or biological-assisted remediation) was superior in relation to TPH removal efficiency.

In conclusion, alfalfa growing in the co-contaminated soil studied herein demonstrated a promising potential for rhizodegradation of petroleum hydrocarbons, enhanced by the simultaneous presence of citric acid and Tween® 80 or by bioaugmentation with *P. aeruginosa*. In contrast, poor removal ability limited the achievement of heavy metal phytoextraction, even with the assistance of chemical or biological treatments. In spite of this, the fact that heavy metals concentrated mainly at the root zone consents to the possibility of heavy metal containment through phytostabilization.

A natural progression of this work is to analyse this approach in terms of phytomanagement. At present, the principal use of alfalfa is its cultivation as a forage crop. As a result, it could be proposed the coupling of phytoremediation (*i.e.* phytostabilization of metals in the root zone and rhizodegradation of petroleum hydrocarbons) with the economical valorization of alfalfa as a forage crop. This suggestion is supported by the fact that heavy metal concentrations found in above-ground tissues of alfalfa appeared to be below the tolerable concentrations of trace elements in agronomic crops (*i.e.* Zn: 50-100, Cu: 5-20 and Pb: 0.5-10 mg kg^{-1} DW of mature leaf tissue (Kabata-Pendias, 2011)).

A number of possible future studies are proposed in section 8.5.

Table 8.1 Summary of the experiments

General description	Thesis chapter and Experiment title	Experimental design	Main objectives	Most important results	Major conclusions
Phytoremediation	*Chapter 3* Phytoremediation potential of alfalfa (*Medicago sativa* L.) in heavy metal and hydrocarbon co-contaminated soil.	Duration: 150 days Setting: growth chamber Soil: *Sondage 4* Treatments: Soil Soil + Alfalfa	To investigate the potential of alfalfa plants for the phytoremediation of soils co-contaminated by heavy metals and petroleum hydrocarbons	Alfalfa could germinate but plant biomass was scarce and growth was stunted after 60 days. After 150 days 100% plant mortality was observed. Alfalfa plants were able to uptake heavy metals, while poor metal translocation took place. Microbial number and activity were enhanced in the rhizosphere, but these effects were reverted as plant deterioration progressed.	The co-contaminated soil was not tolerated by alfalfa. Therefore, alfalfa would not be recommended for phytoremediation of this soil. Otherwise, lower levels of pollutants and/or assisted phytoremediation strategies should be taken into consideration.

(Continued on next page)

Table 8.1 Summary of the experiments (continued)

General description	Thesis chapter and Experiment title	Experimental design	Main objectives	Most important results	Major conclusions
Phytotoxicity	*Chapter 4* Phytotoxicity of citric acid and Tween® 80 for potential use as soil amendments in enhanced phytoremediation	Duration: 56 days Setting: outdoors Soil: Commercial soil Treatments: Soil + Alfalfa Soil + Alfalfa + CA[a] Soil + Alfalfa + Tw-80[b]	To assess alfalfa tolerance to two types of soil amendments: citric acid and Tween® 80.	CA negatively affected plant germination, while it did not have any significant effect on biomass or chlorophyll content. Tw-80 did not affect plant germination and showed a trend to increase biomass, as well as it did not have any significant effect on chlorophyll levels.	Alfalfa appeared to tolerate CA and Tw-80 at the tested concentrations, applied weekly. Consequently, CA and Tw-80 could be potentially utilized to assist phytoremediation of contaminated soils vegetated with alfalfa.

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Table 8.1 Summary of the experiments (continued)

General description	Thesis chapter and Experiment title	Experimental design	Main objectives	Most important results	Major conclusions
Chemically-assisted Phytoremediation	*Chapter 5* Citric acid- and Tween® 80-assisted phytoremediation of co-contaminated soils vegetated with alfalfa (*Medicago sativa* L.)	Duration: 90 days Setting: growth chamber Soil: *Sondage 3/4* Treatments: Soil Soil + Alfalfa Soil + Alfalfa + CA[c] Soil + Alfalfa + Tw-80[d] Soil + Alfalfa + CA[c] + Tw-80[d]	a) To investigate the potential of alfalfa plants for the phytoremediation of soils co-contaminated by heavy metals and petroleum hydrocarbons b) To study the influence of citric acid and Tween® 80 on the phytoremediation process, when applied individually and in combination.	a) Alfalfa could grow and negligible plant mortality occurred. Heavy metals were uptaken to a limited extent, mostly by plant roots. Microbial number and activity were enhanced in the rhizosphere. b) Soil amendments did not significantly enhance plant metal concentration or total uptake. The combination of CA and Tw-80 significantly improved microbial number and activity in the rhizosphere.	This evidence supports the phytoremediation potential of alfalfa species to promote the remediation of heavy metal and hydrocarbon co-contaminated soils and the possibility to enhance the phytoremediation process through the joint application of CA and Tw-80.

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Table 8.1 Summary of the experiments (continued)

General description	Thesis chapter and Experiment title	Experimental design	Main objectives	Most important results	Major conclusions
Biologically-assisted Phytoremediation	*Chapter 6* Comparative bioremediation of co-contaminated soils by natural attenuation, bioaugmentation and phytoremediation	Duration: 90 days Setting: growth chamber Soil: *Sondage 3/4* Treatments: Soil Soil + Alfalfa Soil + Pa^e Soil + Alfalfa + Pa^e	To perform a comparative evaluation of four bioremediation strategies: a) natural attenuation (NA), b) bioaugmentation with *P. aeruginosa* (BA), c) phytoremediation with alfalfa (PR) and d) bioaugmentation-assisted phytoremediation (BA+ PR), for the treatment of a co-contaminated soil.	The content of heavy metals in alfalfa plants was limited, they mainly concentrated in plant roots and they were poorly translocated. Bioaugmentation enhanced plant biomass, decreased the concentration of most metals in plant parts as well as metal translocation, but increased the total uptake of Cu by alfalfa roots and that of Zn by shoots. Removal rates of TPH were 68%, 59%, 47% and 37% for BA+ PR, BA, PR and NA, respectively.	The findings of this study suggest that bioaugmentation-assisted phytoremediation could be a promising bioremediation option for the removal of soil petroleum hydrocarbons, even when they are present simultaneously with heavy metals.

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Table 8.1 Summary of the experiments (continued)

General description	Thesis chapter and Experiment title	Experimental design	Main objectives	Most important results	Major conclusions
Phytotoxicity	*Chapter 7* Physiological impacts of co-contaminated soil and bioaugmentation on alfalfa (*Medicago sativa* L.) plants Phytoremediation	Duration: 90 days Setting: growth chamber Soil: *Sondage 3/4* and an agricultural soil Treatments: Soil Soil + Alfalfa Soil + Pa[e] Soil + Alfalfa + Pa	To assess the impact of a co-contaminated soil and bioaugmentation with *Pseudomonas aeruginosa* on alfalfa development and physiology.	A reduction in biomass yield, F_v/F_m values and chlorophyll content as well as an increase in flavonol and MDA content was observed in plants growing in the co-contaminated soil relative to the agricultural soil. Bioaugmentation promoted plant growth and seemed to counteract the negative impact of soil pollutants on plant physiological parameters.	The co-contaminated soil affected plant growth and physiology. As time passed alfalfa plants developed an adaptative response to the co-contaminated soil. Bioaugmentation treatment appeared to alleviate plant toxicity towards the co-contaminated soil.

CA: citric acid, Tw-80: Tween® 80, Pa: *Pseudomonas aeruginosa*, TPH: Total Petroleum Hydrocarbons, F_v/F_m: Maximum Quantum Yield of photosystem II, MDA: malondialdehyde

[a] Concentrations tested: 5, 15, 45 and 90 mmol kg^{-1} dry soil, applied every 7 days

[b] Concentrations tested: 0.003, 0.006, 0.012 and 0.036 mmol kg^{-1} dry soil, applied every 7 days

[c] Applied at 15 mmol kg^{-1} dry soil, every 15 days

[d] Applied at 0.036 mmol kg^{-1} dry soil, every 15 days

[e] Bioaugmentation was done every 15 days. Bacteria concentrations ranged between 4.0×10^{11} and 1.0×10^{12} CFU kg^{-1} dry soil

Table 8.2 Effect of chemical and biological treatments on some phytoremediation parameters

Parameter		Soil + Alfalfa*	Soil + Alfalfa + CA	Soil + Alfalfa + Tw-80	Soil + Alfalfa + CA + Tw-80	Soil + Alfalfa + Pa
Biomass (g DW plant^{-1})	Shoots	0.0884 ± 0.0214	0.0926 ± 0.0161	0,0703 ± 0.0080	0.0891 ± 0.0059	0.1189 ± 0.0059
	Roots	0.0852 ± 0.0556	0,1091 ± 0.0015	0,0902 ± 0.0072	0.1209 ± 0.0212	0.0714 ± 0.0042
Shoot Metal concentration (mg kg^{-1} DW)	Cu	18.6 ± 2.9	18.0 ± 5.3	10.9 ± 1.0	14.9 ± 3.4	10,9 ± 0,5
	Pb	15.2 ± 3.9	14.6 ± 4.0	5.1 ± 2.0	30.7 ± 19.8	3,5 ± 0,4
	Zn	69.0 ± 15.1	68.2 ± 5.0	85.0 ± 8.9	76.9 ± 16.1	39,4 ± 3,4
Root Metal concentration (mg kg^{-1} DW)	Cu	63.5 ± 10.6	60.6 ± 8.9	66.9 ± 3.5	72.2 ± 14.9	76,9 ± 10,5
	Pb	23.6 ± 5.8	29.0 ± 3.1	47.8 ± 2.3	40.0 ± 8.2	13,2 ± 3,6
	Zn	121.5 ± 30.8	91.3 ± 3.4	136.2 ± 22.4	109.4 ± 19.2	92,4 ± 8,7
MPN alkane degraders (MPN (g soil)$^{-1}$)		(2.1±2.8) × 10^8	4.4 (± 2.6) × 10^8	1.6 (± 1.9) × 10^8	1.1 (± 1.2) × 10^9	9,8 (± 9,6) × 10^7
Lipase activity (µg *p*NP (g soil ×10 min)$^{-1}$)		388 ± 41	632 ± 26	175 ± 16	484 ± 3	882 ± 28
TPH removal (%)		47 ± 0	n.a.	n.a.	n.a.	68 ± 3

* The values presented here are an average from the data obtained in two independent phytoremediation experiments, after 90 days of experiment

CA: citric acid, Tw-80: Tween® 80, Pa: *Pseudomonas aeruginosa*, DW: dry weight, MPN: Most Probable Number, *p*NP: *p*-Nitrophenol, TPH: Total Petroleum Hydrocarbons, n.a.: data not available.

8.2. Phytoremediation at different scales: from lab experiments to field studies

It is well known that phytoremediation experiments should be performed at different scales because the information obtained at each level is complementary. As a result research studies from lab experiments of short duration to long term in the field, passing through in-between experiments of moderate length in small field plots are recommended. The body of literature concerning phytoremediation experiments at the lab scale is considerably vaster than the number of field studies. Lab studies are an essential requirement for fundamental research as basic mechanisms can be elucidated when variables are controlled. However, obtaining successful results in lab phytoremediation experiments does not guarantee reproducibility at the field level. These differences can be explained by the fact that the real field situation implies a multitude of possibilities with highly variable physical, chemical and biological uncontrollable conditions. As a result, the general rule is never directly upscale from short term pot experiments to the whole field site without making tests in an intermediate set up of moderate length in field plots. To elucidate this problematic, Reinhold et al. (2014) assessed the applicability of results obtained from laboratory studies to real systems. They made a direct comparative study between small scale (lab) and real scale (field) experiments in order to compare and contrast the conclusions that can be drawn from both types of experiments. They established that conclusions were applicable to both situations most of the time (66%), but not always and that the experiments performed in columns tended to over-predict the benefits of phytotechnologies. In order to minimize this effect, the authors suggested increasing the scale and length of the experiment to allow for a steady state and account for temporal variability. Finally, they also recommended increasing the number of replicates to improve the power of statistical tests.

The lack of reproducibility between lab and field studies is one of the reasons that hinders plant-based approaches and restricts the wide use of such technologies as practical site solutions. Moving from the lab to the field is critical, and this can only be accomplished through field projects involving multidisciplinary teams of work. The EU FP7 GREENLAND Project is an example of this kind of holistic approach (Puschenreiter et al., 2014). GREENLAND is a European project managed by a consortium of specialists of various disciplines working together on the subject of gentle remediation options using plants, microbes and soil amendments, for the treatment of trace element contaminated land at low cost and on an environmentally friendly basis (Cundy et al., 2013). The creation of such a network of long-term case studies in Europe allows the comparison of remediation efficiency under different conditions of soil characteristics, climate, pollution levels, etc. Moreover, different valorization options are tested in order to assess the potential of using the biomass as a profitable raw material (Bert et al., 2014). The generation of this kind of projects appears to be central to bring gentle remediation options into wide-spread practical applications. In this sense, it seems optimistic the fact that in the recent years the number of field studies has increased. Moreover, it is encouraging the fact that several

field applications employing related strategies as those studied here have demonstrated to be effective. For instance, chemically-assisted phytoextraction has been shown to be successful in a recent field study performed by Freitas et al. (2013). They demonstrated that the application of citric acid at a rate of 40 mmol kg^{-1} soil promoted a 14-fold increase in the Pb concentration of maize (*Zea mays*) shoots. According to authors' estimations, Pb clean-up would be feasible in a frame time of about 20 years with the assistance of citric acid, while without the assistance of chelates it would take more than 150 years. Moreover, the possibility of coupling phytoextraction with bioenergy production could result in an extra economical profit. In addition, bioaugmentation strategy has also been demonstrated to be feasible at large scale. Szulc et al. (2014) assessed the influence of bioaugmentation on diesel oil biodegradation efficiency during a one-year field study. They observed that bioaugmentation with an hydrocarbon degrading consortium (including *Pseudomonas* bacterial taxa) notably improved the biodegradation efficiency compared to natural attenuation. Concerning the use of alfalfa in field studies, Tu et al. (2014) demonstrated that the removal of polychlorinated biphenyls (PCBs) was more efficient in the presence of alfalfa vegetation, probably due to the association of alfalfa plants and *Sinorhizobium meliloti* bacteria, as demonstrated by the co-localization of PCBs and *S. meliloti* in the nodules of alfalfa plants. Besides soil clean-up, vegetation covers in contaminated land would have additional benefits such as erosion control, improving soil quality and functionality, and providing wildlife habitat. This is in agreement with the observations of Ouvrard et al. (2011), who performed an interesting long-term field study in a co-contaminated soil (heavy metals and polycyclic aromatic hydrocarbons) vegetated with alfalfa. They observed that the presence of the plant cover alone did not affect total contaminant concentrations in soil. However, they observed that the presence of plants was efficient in improving the contamination impact on the environment and in increasing the soil biological diversity. For instance, higher densities of total and PAH-degrading bacteria, increase of soil fauna biodiversity (mesofauna and macrofauna) and decrease in leaching water volume, were observed in the presence of plants.

8.3. Phytomanagement of contaminated sites

Considering that contaminated land is an extensive but usually under-utilized resource, the possibility to use contaminated areas with economic purposes is a field of remarkable relevance. In this context, the concept of *phytomanagement* involves the practices that combine profitable crop production with the gradual reduction of soil contamination by phytoremediation. Successful phytomanagement should be a profitable operation, by producing valuable plant biomass products (Robinson et al., 2009). This means that remediation phytotechnologies could be coupled with the economical valorization of the plant biomass, rather than just generating plant wastes to be finally disposed at hazardous waste sites.

The widest use for phytoremediation crops has been the production of renewable energy (Witters et al., 2012). Obtaining of different forms of bioenergy have been described, including not only the combustion of plant biomass for energy production and heating

but also alternative forms such as biofuels and biogas (Gomes, 2012). The classical approach consists on growing willows and poplars under short rotation coppice (SRC), *i.e.* intensive cultivations characterized by high density plantations of fast-growing trees for short rotation (1–4 years) and plant cycles (less than 20 years). SRC on metal contaminated soil allows combining soil remediation by phytoextraction on one hand, and production of biomass for energy purposes on the other (Laureysens et al., 2004; Dickinson and Pulford, 2005; Ruttens et al., 2011). Post-harvest processing generally requires a pre-treatment (*e.g.* compaction, composting, pyrolysis) in order to decrease biomass water content and as a result reduce its volume and weight (Sas-Nowosielska et al., 2004). Subsequently, plant biomass is burnt in boilers equipped with efficient filters to minimize air pollution. In order to couple the remediation of contaminated soils with an economic benefit, another novel approach that has been proposed consists in the use of biofuel plants for phytoremediation (Pandey et al., 2012; Oh et al., 2013). A recent study performed by Zhao et al. (2014) demonstrated that marginal urban land could be used for biofuel production. Sunflowers (*Helianthus annuus*) growing in an urban marginal soil contaminated by low levels of Pb and As took up heavy metals to a limited extent, indicating that sunflowers produced on this land could be a safe biofuel feedstock able to generate an energy gain. Moreover, biogas production from anaerobic digestion of contaminated maize (*Zea mays* L.) has recently been demonstrated to be feasible (Witters et al., 2014b).

Besides bioenergy production from plant biomass, some researchers have explored the possibility to recover and recycle metals from metal-rich biomass. Recent experiments have demonstrated that metallic cations contained in Ni hyperaccumulators can be chemically recovered and serve the preparation of heterogeneous catalysts used in synthetic transformations (Losfeld et al., 2012). This interesting approach allows transforming contaminated biomass into novel catalysts for modern organic chemistry, in line with the principles of green chemistry.

In turn, other applications have been explored for plant species accumulating low levels of heavy metals. Fässler et al. (2010) performed a 6-year field experiment with maize, sunflower and tobacco (*Nicotiana tabacum* L.) in crop rotation. Low levels of metal accumulation by plants hindered the cleaning-up of the site by means of phytoextraction. However, the authors proposed that such land could be used to generate profitable crops, including the production of safe (low Cd) stock fodder fortified with Zn, green manure for micronutrient-deficient soils, or also bioenergy, as described previously. Finally, Evangelou et al. (2014) have recently proposed an original approach that consists on obtaining biochar from the pyrolysis of birch trees (*Betula pendula*) growing in polluted soils but taking up metals to a limited extent. Successively, they demonstrated that trace element-contaminated biochar from such phytomanaged sites could be used as fertilizer for biofortification of crops growing on low-fertility soils (*e.g.* low Zn concentrations).

Lastly, the integration of phytoremedation crops in an agricultural system, is currently a noteworthy area under development (Witters et al., 2014a).

The examples presented in this section support the fact that suitable phytomanagement practices can make of phytoremediation a sustainable remediation technology with an added economic value.

8.4. Phytoremediation and exposure risk

Phytoremediation meets the requirements for sustainable development. However, like any other human activity it is not a risk-free practice. Potential adverse effects of phytoremediation may include: a) the introduction and dissemination of alien plant species, b) the potential transfer of contaminants to the food chain, c) the toxicity of non-biodegradable soil amendments, d) the use of detrimental soil cultivation practices and e) the generation of plant contaminated material. As a result, the potential impact of phytoremediation needs to be assessed. Marmiroli et al. (2014) have recently proposed a specific methodology to evaluate such risks. This methodology takes into consideration not only scientific knowledge, but also each particular scenario (implied plants, pollutants, environment, phytotechnology, etc). The model they developed is formed by two components: a conceptual model represented as a flowchart decision tree and an electronic questionnaire of about 300 questions. The output information consists on a report containing all the information and data inserted as well as a list of the identified potential adverse effects.

Another aspect that raises many questions concerns the legislative issues applicable to phytotechnologies, in which both European and Member States legislation are involved. In the case of phytotechnologies applied in metal-contaminated land, a number of steps have been identified to define the legislation aspects that should be taken into account. The following six steps with the corresponding legal considerations have been established: 1) the status and use of the land (soil threshold values, use of crops), 2) planting/sowing (use of invasive or exotic plant species, use of genetically modified organisms, soil management practices), 3) growing (principles of good agricultural practices, use of pesticides), 4) harvesting (classification of the harvested material), 5) processing (input and output threshold values during energy conversion of plant biomass) and 6) using the remainders (further use or final disposal) (Hoppenbrouwers et al., 2014).

Progress has been make but still further work in the legal aspects related to the entire phytoremediation cycle is crucial and is an area of current and intensive labor, for instance in the frame of the EU FP7 GREENLAND Project.

8.5. Future work

The results presented in this thesis, from studies performed at laboratory scale, have gone some way towards enhancing our understanding of assisted phytoremediation processes in co-contaminated soils. However, more research is needed to fill remaining knowledge gaps. Therefore, a number of possible studies are proposed to be addressed in future experiments.

Concerning phytoremediation with alfalfa species, future studies investigating the mechanisms by which alfalfa plants enhance petroleum hydrocarbon dissipation in the rhizosphere would be very interesting. Assessing the effect of alfalfa root exudates on promoting the activity and number of alkane-degraders as well as the role of root exudates in enhancing petroleum hydrocarbon desorption from soils would be valuable. Additionally, it would be worth to identify the root release of plant enzymes with degrading hydrocarbon function.

Regarding chemically assisted phytoremediation, it is central determining TPH removal rates in the presence of alfalfa, citric acid and Tween® 80. It would also be of interest testing if the application of citric acid and Tween® 80 at a range of broad concentrations could enhance the bioavailable fraction of heavy metals and petroleum hydrocarbons in co-contaminated soil. In addition, further work needs to be done to establish whether exists a correlation between soil lipase activity, number of alkane degraders and petroleum hydrocarbon dissipation, in the presence of alfalfa, citric acid and Tween® 80.

With reference to biologically-assisted phytoremediation, it is recommended to monitor the survival of bacteria in bioaugmented pots throughout the experiment. Further research may explore the mechanisms by which *P. aeruginosa* promotes alfalfa growth (*e.g.* production of organic acids, siderophores, indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and promotion of phosphorus solubilization). Another possible area of future research would be to investigate the soil *in situ* production of metabolites by *P. aeruginosa* (*e.g.* siderophores, organic acids, biosurfactants) that could increase pollutant bioavailability after bioaugmentation is done. Finally, it could also be suggested to investigate the association between chemical and biological treatments to assist phytoremediation.

As highlighted in section 8.2 the findings obtained from experiments at laboratory scale might not be transferable straightforward to real scale applications. Therefore, further trials performed in greenhouse, field plots and increasing the experimental time should also be assessed and are strongly recommended.

To conclude, further research should be done to investigate if the concentration of heavy metals in plant harvestable tissues of alfalfa growing in a co-contaminated soil in real field is compatible with the agronomic use of the biomass. This is needed to put into effect a phytomanagement practice aiming to integrate phytoremediation crops in an agricultural system. Finally, in order to assess the impact of phytoremediation and identify the associated potential risks, specific risk assessment methods designed to evaluate phytoremediation technologies should be applied.

8.6. References

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Appendix

Supplementary data: images

Flowers

Leaves

Flowers and leaves

Fruits

Alfalfa (*Medicago sativa* L.) plants

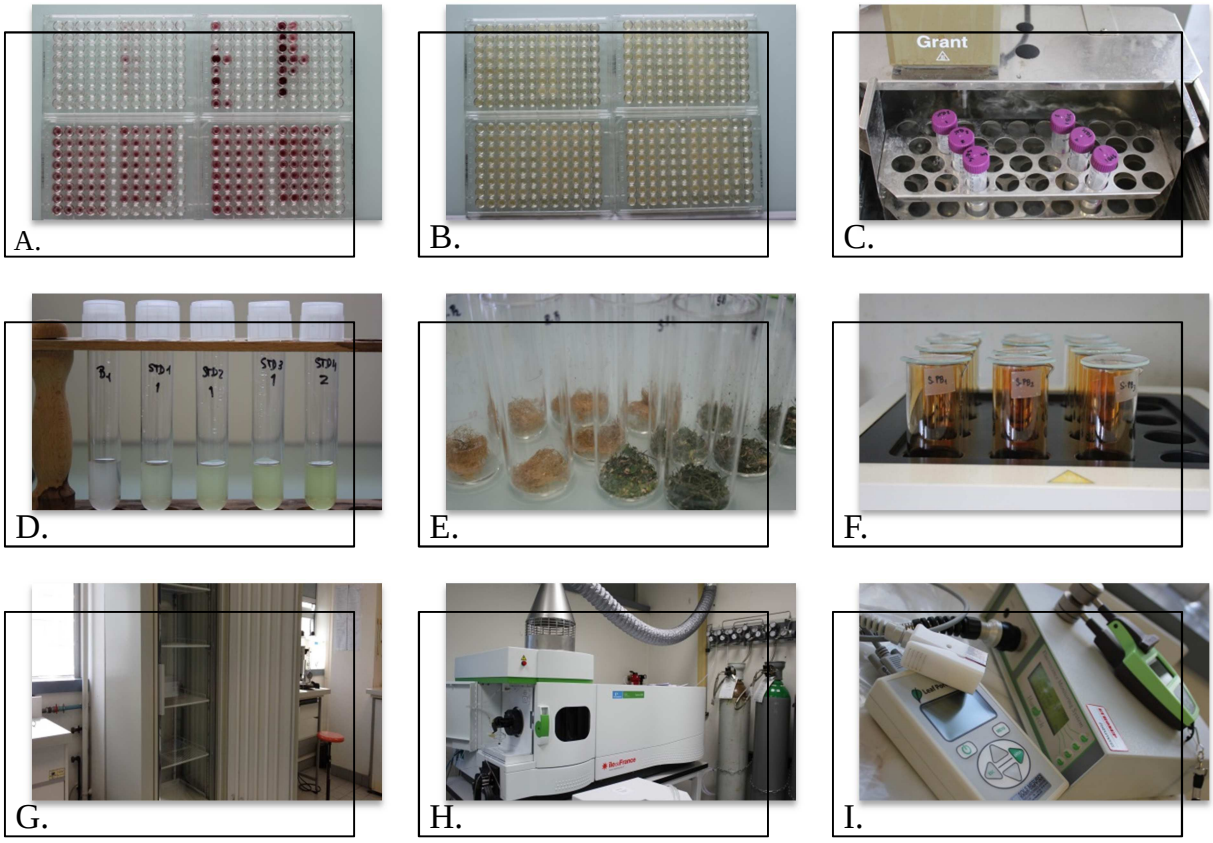
Soil *sondage 3*

Soil *sondage 4*

Soils *sondage 3* and *4*

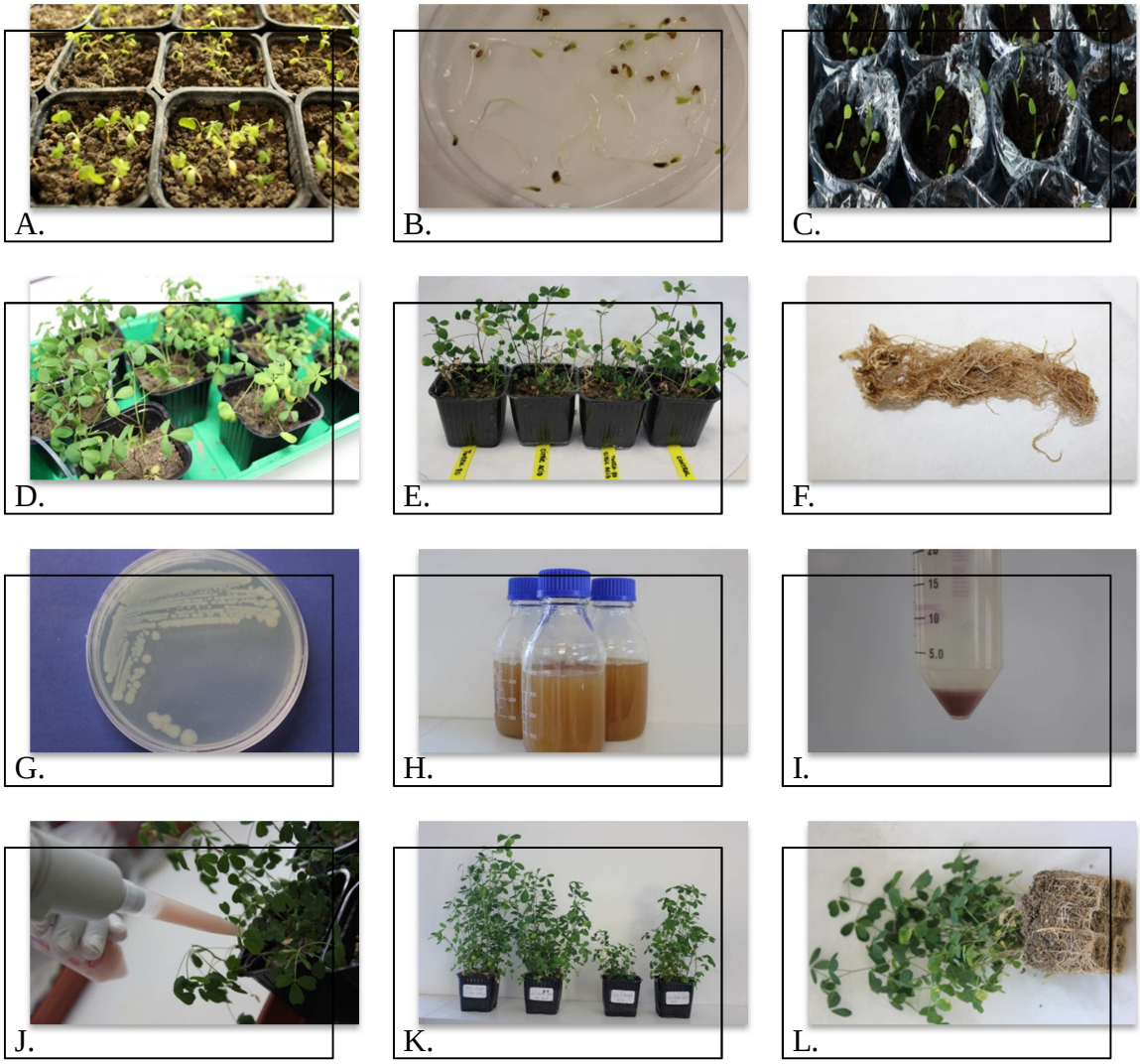
Soil mix: *sondage 3/4*

Soil samples



Lab materials and equipment.

A. Microplates used for the determination of most probable number of alkane degraders. B. Microplates used for the determination of most probable of total heterotrophs. C. Water bath incubator where takes place the reaction for soil lipase activity determination. D. Set of tubes for calibration curve during soil lipase activity essay. E. Dry plant tissue in digestion tubes before wet digestion. F. Mineralization of plant tissue by wet digestion in the digestion block. G. Plant growth chamber. H. Inductively Coupled Plasma-Optical Emission Spectrometer. I. Fluorimeters and porometer.



Experiments of phytoremediation and bioaugmentation

Alfalfa plants after 30 days of growth in soil *sondage 4*. B. Alfalfa seedlings in Petri dishes after 3 days (Germination test). C. Alfalfa plants after 14 days in commercial soil growing outdoors. D. Alfalfa plants just transplanted to soil *sondage 3/4*. E. Alfalfa plants after 90 days of growth in soil *sondage 3/4* (from right to left: control, citric acid + Tween 80, citric acid and Tween 80 amended soil). F. Washed roots of alfalfa plants after 90 days of growth in soil *sondage 3/4*. G. *Pseudomonas aeruginosa* strain growing in lysogeny broth (LB) agar-plates. H. Culture of *Pseudomonas aeruginosa* in lysogeny broth (LB) liquid medium. I. Pellet of *Pseudomonas aeruginosa* sedimentated after centrifugation. J. Inoculation with *Pseudomonas aeruginosa* in soil *sondage 3/4* vegetated with alfalfa. K. Alfalfa plants after 90 days of growth in soil, from right to left: *sondage 3/4* or agricultural soil, with or without the inoculation with *Pseudomonas aeruginosa.* (from right to left: control, citric acid + Tween® 80, citric acid and Tween® 80 amended soil). L. Alfalfa plants after 90 days of growth in soil *sondage 3/4* inoculated with *Pseudomonas aeruginosa*.

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