n° ordre 3151

THESE

PRESENTEE A

L'UNIVERSITÉ BORDEAUX I

& UNIVERSITÀ DEGLI STUDI DI FIRENZE

ECOLE DOCTORALE SCIENCES DE LA VIE, GÉOSCIENCES, SCIENCES DE L'ENVIRONNEMENT

Par Federico VALORI

pour obtenir le grade de

DOCTEUR

Spécialité: Biogéochimie de l'Environnement

Dottore di ricerca in Scienza del Suolo e Climatologia

Effects of root exudates on cadmium bioavailability in the rhizosphere. Assessment of cadmium toxicity to plants and microorganisms, and soil remediation.

Directeurs de Recherche: Pr. L. Landi & Dr. M. Mench

Soutenue le 11 avril 2006

Après avis de:

M. J. VANGRONSVELD (Professeur, Université de Hasselt, Belgique) **Rapporteur**

M. C. ROBIN (Chargé de Recherche, HdR, INRA, Nancy, France) **Rapporteur**

Devant la commission d'examen formée de :

Mme L. LANDI (Professeur, Université de Florence, Italie) **Président**

M. S. GREGO (Professeur, Université de Tuscia di Viterbo, Italie) **Examinateurs**

M. M. MENCH (Directeur de Recherches, HdR, INRA/Université de Bordeaux 1, France)

M. R. MICHALET (Professeur, Université de Bordeaux 1, France)

-2006-

ACKNOWLEDGMENTS

I am grateful to Prof. Richard Michalet and the whole Community Ecology Group at the UMR BIOGECO INRA 1202 of the University of Bordeaux 1 for their hospitality during my experience in France.

To Estelle, Emmanuel, Sylvain, and Blaise go my particular thanks for their patience in dealing with my moods each day.

Dr. Michel Mench was an excellent teacher and tutor, and to him goes all my appreciation for having been a master of life, both during my visit in Bordeaux and, despite the distance, later. Thank you, Michel.

I am grateful to Prof. Ludo Diels, Karolien.Vanbroekhoven and Albert.Bossus at the VITO (Flemish Institute for Technological Research) Mol, Belgium for their hospitality.

The research was financially supported by the French-Italian VINCI project.

Volevo anche ringraziare i miei tutori in Italia, la Professoressa Loretta Landi ed il Professor Paolo Nannipieri, per avermi sempre seguito e consigliato. Un grazie anche al Dott. Giancarlo Renella per i suoi continui suggerimenti e stimoli professionali. A tutto il Dipartimento di Scienze del Suolo e Nutrizione della Pianta prima di tutto devo le mie scuse per aver stressato tutti in questi ultimi mesi con le mie paturnie da tesi di dottorato. A Luca Calamai e Ottorino Luca Pantani un grazie per aver sempre creduto a scatola chiusa nelle mie capacità e per tutti i consigli. Un grazie a Judith per il suo incondizionato sostegno morale di questi anni. A Maria Teresa, Giacomo Certini, Giacomo Pietramellara, a Paola, Irene, Samuele, Tommaso e Laura; alle nuove entrate, a cui cedo volentieri il testimone, Caterina e Giulia; a Paola Ricci che ha impedito che mi chiudessero il conto in banca mentre ero all'estero, a Fabrizio, insomma non voglio dimenticare nessuno e abbraccio tutti. Grazie di cuore.

A mia madre e mio padre un profondo grazie per continuare a sostenermi in tutto e per tutto e per essere sempre stati, anche nei periodi peggiori che abbiamo attraversato, un caldo rifugio dove tornare nei momenti difficili. A mia nonna che ha lasciato un profondo ed incolmabile vuoto. A mio fratello, alla mia meravigliosa cognata Ricarda, ed ovviante ai bellissimi nipoti Leandro e Fedra. A Orlando, Mirella ed Andrea per la forza che la certezza della loro presenza mi ha sempre dato. Al resto della mia famiglia che porto sempre con me, grazie.

Vorrei ringraziare anche il Dott. Alberto e sua moglie Enrica, nonché la nonna Giselda e tutta la famiglia di Barbara per avermi accolto fra loro, e per il loro impegno a farmi sentire sempre ben accetto.

Un grazie a Tommaso con la speranza di vedersi più spesso; a Pablo e Corrado che ormai sono molto di più che amici da quasi un quarto di secolo (anche questa tesi conferma che …"fAries si fa tutto…"). Al mio "faro illuminante " Alessandro dico che gli voglio bene ed a sua moglie Raffaella mando un bacio (F.a.P ora e sempre). A Danilo, a Sara e al Biondo un grazie per l'amicizia. A Tino, Chino Opolino e Cocco grazie per il calore.

Barbara, oggi sei già il mondo, domani sarai anche la mia famiglia. Ci aspettano momenti duri e momenti in cui vacilleremo ma ci saranno tanti e tanti giorni meravigliosi e indimenticabili.

Come alla notte segue sempre il giorno noi percorreremo questa vita fianco a fianco, per sempre.

A te che sei la mia donna, amante, amica e futura moglie voglio dedicare questo mio lavoro che per tanti versi e più di tutti, hai contribuito a realizzare.

Ti amo Barbara, sempre tuo Federico.

CONTENTS: 1

The thesis was prepared at:

Università degli Studi di Firenze, Dipartimento di Scienze del Suolo e Nutrizione della Pianta – Piazzale delle Cascine 28, 50144, Firenze, Italia.

UMR BIOGECO INRA 1202, Ecologie des Communautés, Université Bordeaux 1, avenue des Facultés F-33405, Talence, France.

Abstract

Effects of root exudates on cadmium bioavailability in the rhizosphere. Assessment of cadmium toxicity to plants and microorganisms, and soil remediation.

Long-term Cd contaminated soils were used to assess the effects of low molecular weight root exudates, released in a model system by a filter placed on the soil surface, on microbial C and N biomass, microbial respiration, inorganic N pools, and cadmium bioavailability in the rhizosphere. Cadmium was added in field plots (AGIR experiment, Bordeaux, France) between 1988 and 1990 as $Cd(NO_3)_2$ to reach concentrations ranging from 0.7 to 38.7 mg Cd kg^{-1} soil d.w. in the 0-0.3 m depth layer of sandy soils. Soils were sampled in 2003.

200 mg C kg^{-1} of model root exudates (MRE) glucose, citric acid and glutamic acid were added at 0, 2, 4 and 7d. Periodically, at 0, 4, 7, and 14d, the soil was sampled from two layers, i.e. 0–2 and 6–14 mm below the filter's surface, which were indicated as rhizosphere and bulk soils, respectively.

Cadmium concentration in soil did not affect the MRE mineralization, N inorganic content and microbial C biomass were not reduced. N microbial biomass showed a slightly reduction. The soil respiration increased in higher cadmium contaminated soils. Labile fraction and cadmium bioavailability were measured by single step chemical extractions and by the BIOMET® bacterial biosensor system. Cadmium bioavailability was detectable only in soils contaminated with 38.7 mg Cd kg^{-1}. The Cd bioavailability was affected mostly by citric and glutamic acids. The value of Cd-labile fraction measured with 1M NH_4NO_3 extraction was similar in the rhizosphere and bulk soils and generally the Cd mobility was not affected by the MRE. The Cd solubility was low even in the higher contaminated soils, and this may explain the lack of negative effects of Cd on biochemical parameters. The low Cd mobility would be imputed to the buffer properties of AGIR soils. In fact no pH changes were measured, even with citric acid treatments. The results of biosensor in higher contaminated soils were in agreement with the results from chemical extraction showing low exposure to Cd and Cd uptake for bacteria. The potential use of *Holcus lanatus* L. as a plant species usable in aided-phytoremediation, was preliminary assess in hydroponic culture for 4w. Plants were precultivated in the AGIR soils of highest contaminated plots (38.7 mg Cd kg^{-1} d.w.) and control plots (0.7 mg Cd kg^{-1} d.w.), and then transplants were collected.

Contaminated nutrient solutions were used to mimic the extractable Cd contents and pH of original plots (pH 7 and pH 5.5, respectively). Cadmium uptake and storage was measured in shoots, leaves, and roots. Guaiacol peroxidase activity (GuPx) in leaves, total chlorophyll and carotenoid contents were quantified to monitor the effects of cadmium exposure on plants. Plants grown in control nutrient solution had the highest yields and lowest Cd concentration. The leaf GuPx activity was higher in plants grown in Cd contaminated nutrient solution in response to the oxidative stress condition induced by Cd exposure and Cd accumulation in plant tissues. Chlorophyll and carotenoid contents were clearly higher in leaves of plants cultivated in control nutrient solution. No effect of solution pH was noticed. Cadmium accumulated in plants was mostly localized in roots with a low percentage of translocation in shoots. It was therefore relevant to use *H. lanatus* plants in aided-phytoremediation experiments carried out both in laboratory and greenhouse conditions.

Cadmium-contaminated soils were remediated according to the *in situ* stabilization technique using three inorganic amendments: beringite+iron grit (5+1% w/w, pH 8.0), sepiolite (6% w/w, pH 8.5), bentonite (6% w/w, pH 3.5). Mobility of cadmium was monitored in soil pore water in the first days after the soil amendment (0, 2, 4, 7, and 14d) and after 6 months using a soil moisture sampler (Rhizon MOM). Cadmium concentration in soil pore water was reduced by sepiolite (reduction ranged between 50%-98.7%) and beringite+iron grit (reduction between 30.4% and 84.6%) but bentonite, due to its low pH, initially increased the Cd availability.

The effectiveness of *in situ* soil remediation through stabilisation was assessed in the amended soils with three different cadmium tolerant plant species (non-tolerant: *Phaseolus vulgaris* L., medium tolerant: *Holcus lanatus,* tolerant: *Lactuca sativa* L. var ”smeraldo”). Visual stress symptoms and biometrical parameters were monitored, and total chlorophyll content was measured. No visual symptoms were detected on aerial plant parts but despite soil amendment reduced the Cd availability, attention is required on the growth conditions created by the minerals that may have higher negative effects (water deficiency and soil compression) than those due to cadmium exposure. Lettuce grown in soils treated with bentonite presented low biomass independently of cadmium concentration. Based on shoot yield, *H. lanatus* grown on amended Cd-contaminated soils did not experience negative impacts and confirmed its potential role of plant species that can potentially be employed in aided phytostabilization.

Keywords: bentonite, beringite, microbial biomass, biosensor, cadmium, selective extraction, root exudates, *Holcus lanatus* L., *Lactuca sativa* L., *Phaseolus vulgaris* L.,

phytoremediation, sepiolite, *in situ* stabilization, soil remediation, rhizosphere, contaminated soil, soil pore water.

Résumé

Effets des exsudats racinaires sur la biodisponibilité du cadmium dans la rhizosphère. Evaluation de la toxicité du cadmium pour les plantes et les microorganismes, et remédiation du sol.

Des sols contaminés uniquement en cadmium (Cd), depuis une longue période, ont d'abord été utilisés pour évaluer les effets d'exsudats racinaires de faible masse moléculaire, libérés dans un système modèle par un filtre placé à la surface du sol, sur les biomasses microbiennes carbonées et azotées, la respiration microbienne, le pool inorganique d'azote et la biodisponibilité de Cd dans la rhizosphère. Le cadmium a été apporté dans les parcelles (6 m X 3 m) d'un essai de longue durée (essai de longue durée AGIR, Bordeaux, France), sur la période 1988-1990, sous forme de $Cd(NO_3)_2$ et les concentrations en Cd variaient en 2003 de 0,7 à 38,7 mg Cd kg^{-1} sol d.w. dans l'horizon de surface (0-0.3 m) de ces sols sableux (Arenic Udifluvent). L'équivalent de 200 mg C kg^{-1} sol sous forme d'une solution modèle d'exsudats racinaires (MRE), de glucose, d'acide citrique ou d'acide glutamique ont été apportés à la surface du sol. Périodiquement, i.e. à t = 0, 4, 7, et 14 j, le sol a été échantillonné dans deux couches de sol distantes respectivement de 0–2 mm et 6–14 mm par rapport au-dessous de la surface du filtre, correspondant de façon arbitraire à la rhizosphère et au sol distant non rhizosphérique.

Les concentrations totales croissantes en Cd du sol n'ont pas affecté la minéralisation des exsudats racinaires modèles (MRE), et la biomasse microbienne en C n'est pas diminuée. La biomasse microbienne en N est diminuée. La respiration microbienne a augmenté dans les sols plus contaminés. La fraction labile et la biodisponibilité du Cd des sols ont été ensuite déterminées par une extraction sélective et le biosenseur bactérien BIOMET®. La biodisponibilité de Cd, révélée par la bioluminescence des bactéries, n'a été détectée que dans les sols contaminés contenant 38,7 mg Cd kg^{-1}. La biodisponibilité de Cd a été modifiée essentiellement en présence des acides citrique et glutamique. La quantité de Cd dans la fraction labile extraite par une solution 1M NH_4NO_3 1M était similaire dans les sols rhizosphériques et non-rhizosphériques. En général, la mobilité de Cd n'a pas été modifiée par la présence de la solution MRE. La

solubilité de Cd était faible même dans les sols les plus contaminés, et ceci expliquerait l'absence d'effet négatif sur les paramètres biochimiques. La mobilité réduite du Cd serait attribuable aux propriétés tampons des sols de l'essai AGIR. Aucune modification du pH du sol n'a été mesurée même dans les sols des traitements à l'acide citrique. Les résultats des biosenseurs dans les sols les plus contaminés en Cd ont été en accord avec ceux de l'extraction sélective, indiquant à la fois une faible exposition au Cd et une faible bioaccumulation de Cd dans les bactéries.

La possibilité d'utiliser une espèce végétale, *Holcus lanatus* L., pour la phytoremédiation aidée de sols contaminés en Cd a été étudiée de façon préliminaire en solution hydroponique sur une période de 4 semaines. Les plantes ont été pré-cultivées dans les parcelles AGIR contrôles (fonds pédogéochimique 0,7 mg Cd kg^{-1} d.w.) et fortement contaminées (38,7 mg Cd kg^{-1} d.w.), puis des transplants ont été prélevés. Les plantes, dont le système racinaire a été lavé, ont été placées dans une solution nutritive simulant la concentration en Cd extrait et le pH des sols (pH 7 et 5,5, respectivement) des parcelles d'origine. La concentration et la quantité de Cd ont été quantifiées dans les parties aériennes, les feuilles et les racines. On a mesuré les activités enzymatiques Guaiacol peroxidase (GuPx) dans les feuilles, ainsi que leur densité en chlorophylle et en caroténoïdes totaux afin de déterminer l'effet de l'exposition des plantes au Cd. Les plantes de *H. lanatus* cultivées sur les solutions de contrôle, non contaminées, ont donné les plus forts rendements en biomasse et les plus faibles concentrations en Cd. L'activité GuPx dans les feuilles était la plus élevée dans les plantes exposées aux solutions nutritives contaminées, en réponse aux conditions de stress oxydant induites par l'exposition au Cd et son accumulation dans les tissus des végétaux. Les densités de chlorophylle et de caroténoïdes totaux étaient clairement plus élevées dans les feuilles des plantes cultivées dans les solutions nutritives contrôles, non contaminées. Au même niveau d'exposition en Cd, la valeur du pH de la solution n'a pas eu d'effet sur la réponse des végétaux. Le cadmium s'est accumulé essentiellement dans les racines de *H. lanatus*, avec un faible pourcentage de translocation vers les parties aériennes. Il était donc probant d'utiliser cette espèce végétale pour des études de phytostabilisation aidée en laboratoire et en serre.

L'assainissement des sols contaminés en Cd a été étudié en utilisant une technique de stabilisation *in situ* basée sur l'apport au sol de l'un des 3 composés inorganiques suivants: beringite+grenailles d'acier (5+1% w/w, pH 8.0), sépiolite (6% w/w, pH 8.5), et bentonite (6% w/w, pH 3.5). La concentration en Cd a été mesurée dans la solution du sol (prélevée par bougie poreuse de type Rhizon MOM) au cours des premiers jours

suivants l'amendement du sol (t = 0, 2, 4, 7, et 14 j) et après 6 mois de réaction. La concentration en Cd dans la solution du sol a été diminuée dans les traitements par la sépiolite (réduction entre 50%-98,7% selon le niveau de contamination en Cd des sols) et par la combinaison beringite+grenailles d'acier (réduction entre 30,4% et 84,6%). Le traitement par la bentonite, à cause de son faible pH, a initialement augmenté la concentration en Cd dans la solution du sol.

L'efficacité de la remédiation des sols contaminés en Cd par stabilisation *in situ* a été évaluée en vase de végétation placés sous serre, avec 3 végétaux de sensibilité différente à l'exposition au Cd (sensible: *Phaseolus vulgaris* L., moyennement tolérante: *Holcus lanatus*, et tolérante: *Lactuca sativa* L. var "smeraldo"). Les symptômes visibles de stress sur les parties aériennes et les paramètres biométriques des 3 végétaux ont été déterminés. La densité de chlorophylle totale a été mesurée. Aucun symptôme visible n'a été détecté sur les parties aériennes des végétaux. Bien que l'amendement du sol ait diminué la concentration en Cd dans la solution du sol, une attention doit être portée sur les conditions de sol crées par les amendements. Certains d'entre eux augmentent la rétention de l'eau ou favorisent la compaction du sol. Ceci a eu des répercussions négatives sur la croissance des végétaux, plus importantes d'ailleurs que les perturbations générées par la présence du Cd dans le sol. Il s'agit en particulier de la croissance de la laitue dans les sols amendés avec la bentonite, sa croissance étant limitée quel que soit le niveau de contamination en Cd du sol. D'après la biomasse de parties aériennes produites, *H. lanatus*, cultivée sur les sols contaminés en Cd et amendés, n'indiquait pas d'effet négatif. Ceci a confirmé cette espèce végétale comme un candidat possible pouvant être employé pour une remédiation de sols contaminés en Cd utilisant la phytostabilisation aidée.

Mots-clés: bentonite, beringite, biomasse microbienne, biosenseur, cadmium, extraction sélective, exsudat racinaire, fer zéro-valent, *Holcus lanatus* L. , *Lactuca sativa* L., *Phaseolus vulgaris* L., phytoremediation, sépiolite, stabilisation *in situ*, remédiation du sol, rhizosphère, sol contaminé, solution du sol.

Riassunto

Effetti degli essudati radicali sulla biodisponibilitá del cadmio nella rizosfera. Valutazione della tossicitá del cadmio per piante e microrganismi, e soil remediation.

Da un esperimento di lungo termine condotto a Bordeaux (France), e denominato AGIR, nel 2003 sono stati prelevati i suoli utilizzati in questo esperimento. La prerogativa di questi suoli è quella di contenere cadmio come unico inquinante a diverse concentrazioni e a diversi pH. Per ottenere i suoli AGIR, il cadmio è stato aggiunto come $Cd(NO_3)_2$ nel periodo fra 1988 e 1990, in modo da raggiungere una concentrazione che oscilla fra 0.7 e 38.7 mg Cd totale kg^{-1} p.s. nei primi 30 centimetri di profondità.

Questi suoli sono stati utilizzati in una serie di esperimenti volti a valutare l'effetto di acidi organici a basso peso molecolare, sulla biomassa carbonio ed azoto, respirazione microbica, il contenuto d'azoto inorganico e per valutare la biodisponibilità di cadmio nel suolo rizosferico a seguito del rilascio di essudati radicali.

Per simulare il rilascio degli essudati radicali nel terreno è stato utilizzato un sistema modello della rizosfera (MRS) dotato di un filtro in cellulosa, perfettamente aderente alla superficie del suolo, sul quale sono state aggiunte le soluzioni contententi gli essudati radicali modello (MRE, 800 mg C kg^{-1} in totale) che lentamente vengono rilasciati nel terreno sottostante.

Gli MRE utilizzati sono stati: glucosio, acido citrico e acido glutammico, acqua come controllo. Gli essudati sono stati aggiunti dopo 0, 2, 4 e 7 giorni.

Dopo 0, 4, 7, 14 giorni di incubazione, i suoli sono stati campionati in due strati 0–2 e 6–14 mm al di sotto della superficie del filtro. Questi strati sono stati considerati rispettivamente rizosfera e terreno non rizosferico (bulk).

Dai risultati ottenuti il cadmio presente nel suolo sembra non influenzare la mineralizzazione dei composti aggiunti. La respirazione microbica mostra valori più elevati nei suoli maggiormente contaminati, mentre la biomassa carbonio non mostra riduzioni legate alla presenza di cadmio alle diverse concentrazioni. La biomassa azoto invece viene ridotta nei suoli fortemente contaminati. Infine, l'azoto inorganico non subisce l'effetto della presenza del cadmio.

I pools di cadmio chimicamente disponibile e biodisponibile, sono stati misurati rispettivamente mediante estrazione con singolo solvente e mediante il biosensore

batterico BIOMET®. La biodisponibilità di cadmio è stata rilevata solo nei suoli più contaminati con una concentrazione totale di 38.7 mg Cd totale kg^{-1}p.s. I risultati del biosensore inoltre evidenziano come la biodisponibilità di Cd nel terreno rizosferico, sia stata influenzata maggiormente dall'acido citrico ed in parte dall'acido glutammico. I valori di cadmio disponibile ottenuti dall'estrazione con NH_4NO_3 1M risultano simili sia nel suolo rizosferico che nel bulk, ed in generale la disponibilità di cadmio non viene influenzata dagli MRE. Il contenuto di cadmio si è rivelato molto basso (nell'ordine dei μg Cd kg^{-1}) anche nei suoli fortemente contaminati e questo risultato contribuisce a spiegare il motivo della sua poca influenza sui parametri biochimici.

La bassa mobilità del cadmio può essere imputata alle capacità tampone dei suoli AGIR in relazione alla quantità di essudati radicali aggiunti. Infatti non sono riportate variazioni significative di pH persino nei suoli trattati con acido citrico.

I risultati del biosensore, ottenuti nei suoli più contaminati, sono in accordo con i risultati derivanti dalle estrazioni chimiche che mostrano una bassa disponibilità al cadmio ed un basso uptake da parte dei microrganismi.

Nella seconda parte della tesi l'uso della graminacea *Holcus lanatus* L. come potenziale specie da utilizzare nella aided-phytoremediation è stata testata preliminarmente in un esperimento in cultura idroponica per quattro settimane.

Le piante sono state coltivate precedentemente nei suoli AGIR, rispettivamente nei suoli più contaminati (38.7 mg Cd totale kg^{-1} p.s.) e nei suoli di controllo (0.7 mg Cd totale kg^{-1} p.s.). La soluzione nutriente utilizzata nella coltura idroponica è stata contaminata in modo da avere la stessa concentrazione di cadmio scambiabile dei suoli AGIR e stesso pH (pH 7 e pH 5.5, rispettivamente). L'uptake di cadmio da parte della piante ed il suo immagazzinamento è stato misurato nei fusti, foglie e radici. Inoltre l'attività guaiacol-perossidasi (GuPx) è stata misurata nelle foglie insieme al contenuto in clorofilla e carotenoidi totale. Queste analisi hanno permesso di quantificare l'effetto dell'esposizione al cadmio sulle piante. Le piante cresciute nella soluzione non contaminata hanno riportato una biomassa superiore ed una concentrazione di cadmio inferiore. L'attività GuPx era superiore nelle piante cresciute nella soluzione nutritiva contaminata in risposta agli stress ossidativi determinati dall'esposizione al cadmio e dal suo accumulo nei tessuti della pianta. Il contenuto in clorofilla e carotenoidi era chiaramente superiore nelle piante cresciute nella soluzione non contaminata. In questo esperimento il pH non ha determinato nessun effetto significativo.

Il cadmio si è accumulato nelle piante soprattutto nelle radici con una bassa traslocazione nei fusti. Questo risultato è importante perché conferma la possibilità di utilizzare *H. lanatus* nella tecnica di aided-phytoremediation.

Gli stessi suoli contaminati AGIR sono stati poi utilizzati per un esperimento di phytoremediation, utilizzando tre minerali inorganici con la funzione di bloccare e stabilizzare il cadmio nel suolo e renderlo quindi non disponibile per le piante ed i microorganismi. I minerali utilizzati sono stati: beringite+iron grit (5%+1% v/v, pH 8.0), sepiolite (6% v/v, pH 8.5) e bentonite (6%v/v, pH 3.5).

L'efficacia di questi ammendanti nell'abbattere la frazione di cadmio disponibile, è stata valutata utilizzando un campionatore di soluzione circolante (Rhizon MOM). In particolare la disponibilità del cadmio è stata valutata nei primi giorni dopo l'aggiunta degli ammendanti al terreno (0, 2, 4, 7, 14 giorni) e dopo un lungo periodo di residenza (6 mesi). La concentrazione di cadmio nella soluzione circolante è stata ridotta nei terreni trattati con sepiolite fra il 50%-e 98.7%, con la beringite+iron grit fra il 30.4% e 84.6%. I terreni trattati con la bentonite invece hanno mostrato un incremento della concentrazione di cadmio disponibile, molto probabilmente legato all'abbassamento del pH del suolo.

L'efficacia dei minerali, inoltre, è stata valutata utilizzando tre diverse specie di piante con una diversa tolleranza al cadmio (non tollerante: *Phaseolus vulgaris* L., medio tollerante: *Holcus lanatus* e tollerante: *Lactuca sativa* L. var "smeraldo"). Stress ossidativi e parametri biometrici sono stati misurati. Nessun sintomo evidente di stress, riconducibile alla contaminazione da cadmio è stato rilevato nelle piante. Nonostante i minerali abbiano quindi confermato la loro efficienza nella riduzione del cadmio disponibile nel suolo, le condizioni di crescita che gli ammendanti creano, possono risultare più sfavorevoli degli effetti del cadmio stesso. In particolare la carenza di rifornimento idrico e la costipazione del terreno nei suoli trattati con la bentonite hanno avuto un effetto molto negativo sulla crescita della *Lactuca sativa*, indipendentemente dalla concentrazione di cadmio presente nel terreno. Viceversa *H.lanatus* non ha mostrato nessuna diminuzione di biomassa su nessun terreno. Questo risultato rappresenta una ulteriore conferma del suo possibile impiego nella aided phytostabilization.

Parole chiave: bentonite, beringite, biomassa microbica, biosensore, cadmio, essudati radicali, *Holcus lanatus* L. , *Lactuca sativa* L., *Phaseolus vulgaris* L., phytoremediation, sepiolite, *in situ* stabilizzazione, soil remediation, rizosfera, suoli contaminati, soluzione circolante.

CHAPTER ONE

GENERAL INTRODUCTION

1. THE RHIZOSPHERE

The rhizosphere was defined by Hiltner (1904) as the soil volume directly or indirectly influenced by plant roots. Generally, it is comprised within 1-2 mm from the root surface, but it can extend more; examples were evidenced in the case of P or Fe deficiency, and dissolution of solid phases can occur on a larger distance. Plants may respond to the nutrient deficiency (e.g. Fe or P) which acts as signals and these signals trigger molecular mechanisms that modify cell division and cell differentiation processes within the root and have a profound impact on root system architecture (Lopez-Bucio et al., 2003).

The rhizosphere is usually divided into three different parts (Bolton et al., 1993):

Endorhizosphere: it is constituted by the interior of the root colonized by microorganisms;

Ectorhizosphere: is properly the area surrounding the root;

Rhizoplane: it is the root surface.

(see fig.1.1)

The key properties of the rhizosphere are different from those of the non-rhizosphere soil (bulk soil). Factors such as nutrient supply, element deficiency (P, Fe, Mn, S, etc) or toxicity, pH value, buffering capacity due to the presence of alkaline solid phases (such as carbonates) and soil characteristics, change the interaction between roots, soil solid phases, and microorganisms (Nannipieri et al., 2003). Similarly, the microbial community of the rhizosphere is also qualitatively and quantitatively different with respect to that of the bulk soil (Falchini et al., 2003). The characteristics of the microbial community in the rhizosphere are influenced by the rhizodeposition (considered as the release of carbon compounds from living plant roots into the surrounding soil: desquamated root cells, microbial mucilage and roots exudates, etc), and pH value, which may differ, in some cases, up to 2 units between rhizosphere and bulk soils.

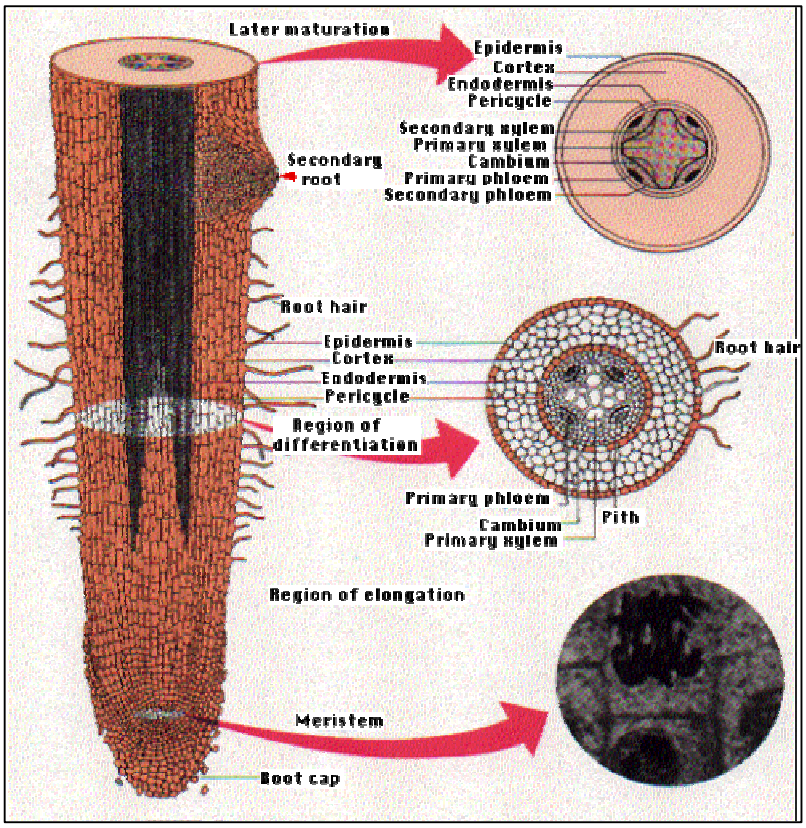


Fig.1.1Description or the different root region from http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/.

The difference in pH value between the rhizosphere soil and bulk soil is due to the influence of H^+ and OH^- (HCO_3^-) released from roots (Youssef and Chino, 1990, Marschner, 1995), or (and) to the CO_2 evolution or (and) to the uptake of NH_4^+/NO_3^- by plant roots. The importance of the pH variation within soil is in the difference of conditions (micro and macro nutrient supply) for the microbial community that are correlated to the pH value (Marschner, 1995). Continuous release of organic compounds from roots causes a colonization by microorganisms on the surface and around the root (Uren, 2000). However, roots are not only a passive target for soil organisms because some of the compounds secreted by plant roots can also act as chemio-attractants inducing the movement of specific microbes towards the roots (Uren, 2000, Scarponi, 2005). Rhizodeposits are generally defined as the "organic compounds released from alive and healthy root" (Uren and Reinsenauer, 1988). Employing this definition, the difficulty of recognizing the difference between the "real" exudates and the compounds released from others sources (e.g. secretions, excretions) is avoided.

In Table 1.1 are listed the main compounds secreted by roots.

Table 1.1. Root products (Uren et al., 2000).

Class of compounds	Single components
Sugars	Arabinose, glucose, fructose, galactose, maltose, raffinose, rhamnose, ribose, sucrose, xylose
Amino acids and amides	all 20 proteinogenic amino acids, aminobutyric acid, homoserine, cystathionine, mugineic acid phytosiderophores (mugineic acid, deoxymugineic acid, hydroxymugineic acid, epi-hydroxymugineic acid, avenic acid, distichonic acid)
Aliphatic acids	Formic, acetic, butyric, propionic, malic, citric, isocitric, oxalic, fumaric, malonic, succinic, maleic, tartaric, oxaloacetic, pyruvic, oxoglutaric, maleic, glycolic, shikimic, cis-aconitic, trans-aconitic, valeric, gluconic
Aromatic acids	p-hydroxybenzoic, caffeic, p-coumaric, ferulic, gallic, gentisic, protocatechuic, salicylic, sinapic, syringic
Miscellaneous phenolics	Flavonols, flavones, flavanones, anthocyanins, isoflavonoids
Fatty acids	Linoleic, linolenic, oleic, palmitic, stigmasterol
Enzymes	Amylase, invertase, cellobiase, desoxyribonuclease, ribonuclease, acid phospatase, phytase, pyrophosphatase, apyrase, peroxidase, protease
Miscellaneous	Vitaminis, plant growth regulators (auxins, cytokinins, gibberellins), alkyl sulphides, ethanol, H^+, K^+, nitrate, phosphate, HCO_3^-

Within root exudates, organic acids are really important because they play an important role in many plant-soil interaction processes. Organic acids are low-molecular weight C, H and O containing compounds which are found in all organisms and with one or more carboxyl groups. The properties of the organic acids are related to the dissociation properties and number of carboxylic groups of the acid (Marschner, 1995). Depending on soil pH and pKa, organic acids can carry varying negative charges, thereby allowing the complexation of metal cations in soil solution and the displacement of anions from the soil matrix. For this reason, they are also involved in many soil processes (Jones, 1998). Below examples are reported. The role of organic acids and cadmium in soil will be discussed deeply in the next paragraph. :

- *Organic acid release under Fe deficiency:*

Plant under Fe deficiency normally occurs on calcareous soils (pH > 7.0) due to the insolubility of Fe at high pH (Marschner, 1995). Organic acids such as citrate and malate are potent ligands of Fe in soil and induce the dissolution of previously unavailable insoluble ferric oxyhydroxides (Gerke, 1992; Jones et al., 1996).

Iron deficiency has been generally shown to cause increases in the organic acid concentrations in roots, stem exudates and leaves of different plant species. Increases in

organic acid concentrations in roots of Fe deficient plants are fairly ubiquitous, and occur both in Strategy I and Strategy II plant species (Abadia et al., 2002).

Strategy I plants increase soil Fe solubility by releasing protons and reductants/chelators, such as organic acids and phenolics, into the rhizosphere, while Strategy II plants are characterized by the secretion of ferric chelating substances (phytosiderophores) coupled with a specific Fe^{3+} chelate uptake system (Jian, 2005).

-Organic acid release under phosphorus deficiency:

Under phosphorus deficiency plants have evolved two broad strategies for P acquisition and use in nutrient-limiting environments: (1) those aimed at conservation of use; and (2) those directed toward enhanced acquisition or uptake (Vance et al., 2001). The release of organic acids belongs to the latter, in which plants strategies lead to enhanced uptake of P by increased production and secretion of phosphatases, greater root growth along with modified root architecture, expansion of root surface area by prolific development of root hairs (Vance et al., 2003).

When white lupin is P deficient, cluster roots synthesize and exude striking amounts of malate and citrate. Grown under P-deficiency, cluster roots exude 20- to 40-fold more citrate and malate than P-sufficient roots (Vance et al., 2003). Plants as pigeon pea (*Cajanus cajan* L.), radish (*Raghanus sativus* L.), rape (*Brassica nigra* L), barley (*Hordeum vulgare* L.), rice (*Oryza sativa*), and red clover (*trifolium pratense* L. cv. Hamidori) increased the release of organics acids such as citric, malic, malonic, oxalic, succinic, and tartaric under P-deficiency (Johnson et al., 2006).

- Role of organic acids in aluminium detoxification:

Excretion of organic acids by Al-resistant crop plants in response to Al exposure is now well established (Delhaize et al., 1993; Miyasaka et al., 1991; Pellet et al., 1995, Ma et al., 2001).

When soils become acidic, Al is solubilized into the trivalent cation, Al^{3+}. Some plant species have evolved mechanisms to tolerate Al stress. These mechanisms involved the release of organic acids such as citrate, oxalate and malate acid. The two mechanisms differ for the rapidity of response to the Al exposure. Plants with Pattern I (wheat and buck wheat) within 15-30 minutes after the exposure release malate and oxalate (response suggests that Al activates a pre-existing mechanism and that the induction of novel proteins is not required). In plants with Pattern II, the release of organic acid is in delay of 4-10 h (this suggest that in plants from pattern II the Al-tolerance mechanisms needs to be activated and required protein induction) (Ma et al., 2001).

The amount and the composition of the root exudates are influenced by many environmental factors (Vancura, 1963, Guckert et al., 1991, Mench et al., 1988a, Mench et al., 1988b).

Moreover, the root is always growing and behaves differently at different growth phases: especially young root apical parts release root exudates in high quantities. The factors involved in the variation of the composition of root exudates can be shortly summarized as follows (Brimecombe et al., 2001):

- Plant species
- Plant growth stage
- Presence of microorganisms
- Temperature
- Water availability
- Light intensity
- CO_2 concentration
- Nutrients supply/contaminant presence
- Soil texture

The strong interactions between plants, microorganisms and soil constituents in the rhizosphere make difficult to study the different components separately (fig.1.2).

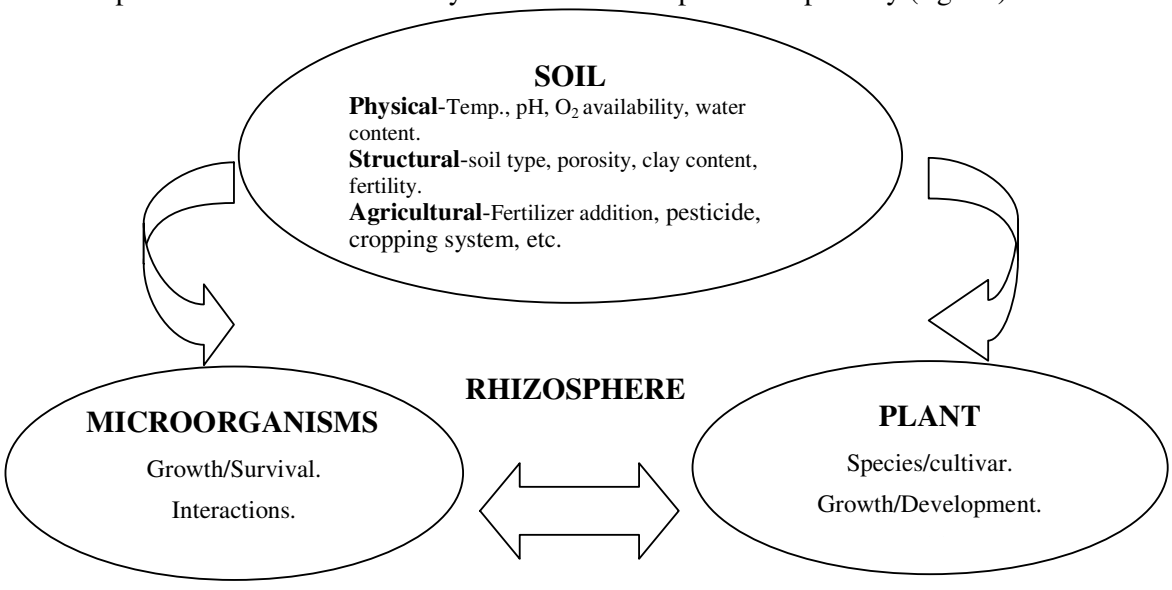


Fig.1.2 Soil-plant-microorganisms interactions (Brimecombe et al., 2001).

Several methods were developed to study the rhizosphere (Table 1.2) (Uren et al., 2000).

Table 1.2. Some experimental soil-plant systems adopted for studying rhizosphere process

System	Soil sampling	Limitations	Advantages
Open field or pot experiments	Handled shaking or gentle digging up root apparatus to detach the adhering soil or direct scraping of soil relatively near the root surfaces (rhizosphere soil), followed by careful brushing or washing off by deionized water for rhizoplane soil	Total soil volume regarded as rhizosphere soil; results not comparable due the subjectivity of the operator	Equipment cheap and easily reproducible
Polyvinyl chloride cylinders +nylon gauze + device for continuous water supply	Soil slices at measurable distance from soil-root interface.	High bulk density if soil sampled. Nutrient uptake thought an induced root hairs surface	Study of rhizosphere effect over a time and distance gradient from the soil-root interface
Stainless steel box divided by a nylon gauze into various vertical compartments at different distance by the roots	Rhizosphere and bulk initially compartmentalized	Soil-root interface scarcely resolved. Apparatus time expensive and difficult to build up	No particular constraints for growth

These methods represent options developed to simulate or reproduce the rhizosphere and to answer some scientific questions. All these methods have both advantages and disadvantages. To answer to our scientific question we chose the method developed by Badalucco and Kuikman (2001) and Falchini et al. (2003): the rhizosphere is simulated using a plastic ring standing on a Petri plate with an aluminium foil in between. Inside the ring, fresh soil is added and pressed to precise density (1.4 g cm^{-3}). On the top of the soil a cellulose paper filter (Whatman 41) is added. On the paper filter different solutions can be added in order to mimic root exudates. The synthetic root exudates used in this thesis will be named MRE (Model Root Exudates). This device has been proven to produce a gradient of MRE at increasing distance from the MRS (Model Root System) (Falchini et al., 2003). The effects of the produced gradient are underline by the analysis made on the sample slices obtained by cutting the sample in thin layers, with a special slicer. The whole system described above has been named MRS (fig. 1.3).

Previous studies (Renella et al., 2005) and our results confirm that the incubation units used (MRS) created a low molecular weight organic compounds gradient from the root surface these results were in according with a previous study based on the release of ^{14}C-labelled compounds (Falchini et al., 2003). In fact, the stimulation of soil

microorganisms was mostly observed within the 0–2 mm soil portion (rhizosphere soil) whereas the bulk soil was only negligibly affected by the organic compounds.

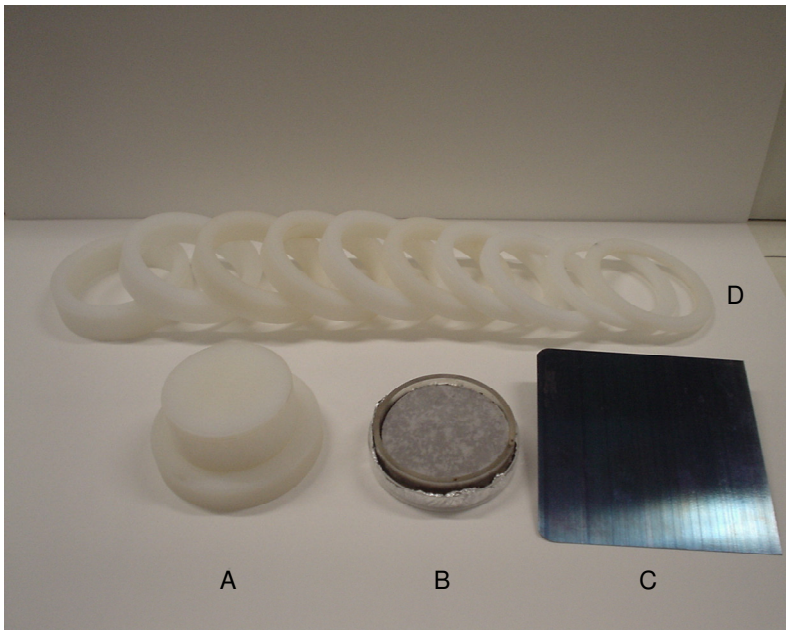


Fig. 1.3. The components of the used rhizosphere simulating system; A: Plexiglas piston, B:an incubation unit, C:the cutter, D: the different height rings.

Because of the high complexity of the rhizosphere environment, the mineralization of the different root exudates and their stimulatory effects on microbial activity have been approached, studying the effects of single low molecular weight organic molecules in simple systems mimicking the rhizosphere environment (Kozdroj and van Elsas, 2000; Badalucco and Kuikman, 2001; Falchini et al., 2003; Baudoin et al., 2003; Landi et al., 2006; Renella et al., 2005a), These studies have confirmed that the active or passive release of root exudates is the one of the main factor controlling the microbial activity. Furthermore Renella (et al.2005) used the MRS system in order to study the microbial and hydrolase activities in the rhizosphere using a mixture of different MRE the results for the mixture was not clear.

Negative effects of metals on the microbial catabolic capacity of soil have been widely documented (Tyler et al., 1989; Reber, 1992; Wenderoth and Reber, 1999). In the rhizosphere of metal-contaminated soils, these effects would be due to different mechanisms, such as a delay in the mineralization of root exudates (Haanstra and Doelman, 1984; Nordgren et al., 1988), and/or a reduced microbial metabolic efficiency

due to a greater diversion of metabolic energy from biosynthesis to maintenance in metal-stressed soil microorganisms (Chander and Joergensen, 2001; Renella et al., 2005b, Vig et al., 2003). Formation of metal root exudates complexes, and/or increased Cd availability may also reduce the mineralization capacity in the rhizosphere. Low molecular weight organic acids and amino acids can form Cd-complexes (Krishnamurti et al., 1997) which are not readily mineralizable by soil microorganisms (Brynhildsen and Rosswall, 1989; Renella et al., 2004). However, reduced microbial mineralization in the rhizosphere of Cd-contaminated soils has been also observed in the presence of glucose which has no chelating ability (Renella et al., 2005a).

1.1. *A definition of bioavailability*

The term bioavailability needs to be introduced and focus on cadmium in soil. A definition of bioavailability might be: ” the potential amount of Cd in soil able to be in contact with and uptake by living organisms” (http://www.contaminated-land.org/glossary.htm). The USEPA (http://iaspub.epa.gov/trs/trs_proc_qry.navigate_term?p_term_id=16299&p_term_cd=TERMDIS) mentionned that bioavailability is operationally defined as “A measure of the physicochemical access that a contaminant has to the biological processes of an organism”. This fraction depends from the organism itself and to the different exposure pathways, for example for microorganisms, it is both a fraction in the soil solution and a fraction bound to the solid phases, but this fraction is only a quantification of the speciation of cadmium in soil and an evaluation of exposure. In fact this amount does not explain the Cd toxicity. The toxicity is related to biological mechanisms involved directly or indirectly in the exposed organisms. These include the various reactions between macromolecules (proteins, membrane lipids, DNA, RNA, etc), small molecules, and the xenobiotic (e.g. Cd): replacement of another metal (Cd/Mn, Cd/Zn, etc), binding of Cd by SH groups and oxidation of such sulphydril groups, lipid peroxidation, etc. Thus the toxicity depends on the biological processes and various genes impacted or able to react to the concentration and speciation of the metal present in the exposed microorganisms.

Evidence from field studies suggests that under long-term metal stress there is a change in the genetic structure of the soil microbial community, without necessarily any increase in the metal tolerance (Giller et al., 1998). Fritze et al. (2000) reported an increase in the actinomycete PLFA signatures and a decrease in the fungal PLFA signatures in laboratory-incubated forest soils amended with 400 and 1000 mg Cd kg^{-1}

(as $CdCl_2$ or CdO). Griffiths et al. (1997) also reported a shift in microbial communities in an agricultural sandy loam soil, spiked with $CdSO_4$ at 1800 mg kg^{-1} soil, DNA hybridization and PLFA patterns. Generally, actinomycetes are more resistant to Cd than Gram negative and Gram positive bacteria in that order (Vig et al., 2003). The concept of bioavailability and toxicity of metal, can not be explain by a single parameter but only a battery of relevant tests, rather than just one single assay, involving important microbial activities should therefore be included in ecotoxicity studies. Summarising the Cd bioavailable fraction represents the key factor in risk assessment analysis, and varies with time, soil type, speciation, ageing, nature of applied metal salt, organisms and other environmental variables (Vig et al., 2003).

Studies carried out on the toxic effects of Cd on soil microorganisms and their activities reported contrasting results. Many of these studies were short-term studies often limited to a single soil type and conducted under controlled laboratory conditions. There is a paucity of reliable field data on Cd alone, since most field studies on Cd-microorganism interactions in soils are based on sewage sludge containing multimetals and organic substances (Vig et al., 2003). Renella et al. (2004) reported a study on Cd as alone contaminant in a long-term field experiment (AGIR). In conclusion, this study showed that low amounts of bioavailable Cd are present even in high Cd-contaminated soils and that such low concentrations of bioavailable Cd do not affect the structure of the most representative eubacterial species of the total soil microbial community. Effects were observed on microbial functions such as respiration and enzyme activities. This may have been due to physiological adaptation of soil microflora rather than permanent changes in the composition of the bacterial community.

The available fraction or soil solution Cd, and not the total concentration of Cd, seems to correlate well with the toxicity parameters (Lombi et al., 2001).

2. CHEMISTRY AND TOXICITY OF CADMIUM IN SOIL

2.1 Physical and chemical characteristics

General properties of cadmium:

Cadmium (Cd): Latin cadmia, Greek kadmeia - ancient name for calamine, zinc carbonate. Cadmium was first discovered by Stromeyer (1817) as an impurity in zinc carbonate.

Periodic Classification: IIB between Zinc and Mercury

Atomic Number :48

Atomic Weight: 112.41

Electron Configuration: 4d10

Properties: Cadmium has a melting point of 320.9°C, boiling point of 765°C, specific gravity of 8.65 (20°C), and a valence of 2. Cadmium is a soft blue-white metal

Oxidation states: (1), 2

Coordination number, Cd(II): 2, 3, 4, 5, 6, 7, 8

Uses: Cadmium is used in alloys with low melting points. It is a component of bearing alloys to given them a low coefficient of friction and resistance to fatigue. Most cadmium is used for electroplating. It is also used for many types of solder, for Ni_Cd batteries, and to control atomic fission reactions. Cadmium compounds are used for black and white television phosphors and in the green and blue phosphors for colour television tubes. Cadmium salts have wide application. Cadmium sulphide is used as a yellow pigment (Table 1.3).

Table 1.3 Cadmium Consumption in the Western World by Application (1990)

Application	% total consumption
Nickel-cadmium batteries	55
Cadmium pigments	20
Cadmium-bearing stabilizers	10
Cadmium coatings	8
Cadmium-containing alloys	3
Miscellaneous uses	4

Toxicity of trace metals and uptake by living organisms were correlated with the free ion or labile metal forms (soluble complexes, exchangeable) in waters and soil solutions (Sauve et al., 1996). The understanding of the equilibrium of Cd complexes in soil is essential in order to predict its solubility, the exposure of soil organisms and thus its potential toxicity.

Behaviour of cadmium in soil is similar to zinc. Interactions of trace metals with the soil solid phase include chemisorption on minerals, precipitation with different anions, co-precipitation in minerals, and complexation with organic ligands (McBride, 1989). The relative strengths of Zn^{2+} and Cd^{2+} complexes are often similar, but Cd forms are usually stronger (Aylett, 1979). However Zn has more affinity for O despite Cd that has more affinity for S. Cadmium has the same valence and similar ionic radius as calcium (Cd^{2+} 0.097 nm, Ca^{2+} 0.099 nm)(Aylett, 1979). Cadmium competes with Ca at level of Ca channels and intracellular Ca binding proteins (Rivetta et al., 1997). It was also proposed that Cd uptake and toxicity to animals might be caused by an interaction with Ca (Rivetta et al., 1997).

2.2. Sources of Cadmium

Cadmium is scarce over the Earth's crust, and has an average concentration of about 0.2 mg kg^{-1}. However the cadmium natural polluted sites are extremely localized and these data might be considered just an indication; in fact the relative abundance may vary in wide ranges. Cadmium is allocated mainly in minerals like olivine, sulphur and Zn oxides, Zn sulphide (e.g. sphalerite and wurtzite contain Cd up to 5%). Also Biotite and Riebeckite contain significant Cd amounts. The average of Cd content calculated on 10634 different French soil layers was 0.39 mg Kg^{-1} (D. Baize, INRA Orleans, 2004, http://etm.orleans.inra.fr/). In Italy the Cd content in agricultural soils ranged between 0.3 mg Kg^{-1} (Piemonte) and 1.3 mg Kg^{-1} (Emilia Romagna) (APAT Piemonte, 2004).

In comparison with other trace elements (e.g. zinc, chromium, copper, lead, and cobalt), the concentration of cadmium in soil is relatively low. Despite its low concentration, the hazard of cadmium is extremely toxic at very low concentrations. Although the natural cadmium contaminated soils are mainly associated with marine black shales (Aylett, 1979) or Jurassic calcareous rocks (D.Baize, INRA Orleans, 2004, http://etm.orleans.inra.fr/) and thus located only in particular areas. In the last two decades the Cd content of the pedosphere has increased. This increase is mostly due to the dry deposition from the atmosphere, estimated in about 3 g ha^{-1} yr^{-1} in the century (Jones and Johnston, 1989), from the use phosphate fertilizers (the average of content is

75 mg Cd kg^{-1} of P), and from sewage sludge (Jensen and Bro-Rasmussen, 1992). As an example in Table 1.4 are reported air emissions of cadmium. Cadmium is associated with zinc in sphalerite (ZnS) and the smelting of this ores is one of the highest sources of cadmium in the environment.

Table 1.4 Current air quality. Source: ENTEC, 2001

Country	Conc (ng/m^3)	Date	Location	Descr of loc	Emission rate (kgpa)	Ref
Austria	below detection limit		Bohler- Edelstahl			Bohler-Edelstahl, 2000
Finland	below 1	1998	Outokumpu, Tornio	Measurements at nearest housing, 1.5km from site. (TSP sample)		Outokumpu, 2000
Germany	16.8	1998	Duisburg – Meiderich	Vicinity of steel industry. It is understood that the high concentrations could be due to a nearby zinc plant.		Position Paper
Germany	1.6	1999 (1/99 to 10/99)	Duisburg-Bruckhausen	700m windward of steel mill		Position Paper
Poland	0.50 (2)	Assumed 1999	Huta Lucchini Warszawa, Warszawa	Peak concentration		Biprohut, 2000
	2.2	Assumed 1999	Huta Ostrowiec	Ostrowiec town		Biprohut, 2000
UK	0.77	1999/2000	Corus, Rotherham	Nearest sensitive receptor where max concs occur - 600m north east of site boundary	54	DETR, 2000 (1) and UK Steel
UK	No data	1999	Corus, Stockbridge	-	12	UK Steel
UK	No data	1999	ASW Tremorfa, Cardiff	-	22	UK Steel
UK	No data	1999	Avesta Sheffield	-	60	UK Steel

Notes

1. Preliminary results for the first 8 months of a 12 month project - running mean
2. Company estimate of annual average concentration based on 30 minute average figure of 2ng/m^3.

Cadmium is also an impurity found in Pb and Cu minerals from where it is released as dust from glass industries; it is present in batteries (cadmium oxide), pigments (cadmium sulphide), and it is used as a plastic stabilizer and cadmium stearate (Tables 1.5, 1.6).

Table 1.5. Estimates of atmospheric cadmium emissions (tonnes/year) on a national, regional and worldwide basis form (International programme on chemical safety, 1992)

Source	United Kingdom	EEC	Worldwide
Natural sources	ND	20	150-2600
Non-ferrous metal			
Production			
mining	ND	ND	0.6-3
zinc and cadmium		20	920-4600
copper	3.7	6	1700-3400
lead		7	39-195
Secondary production		ND	2.3-3.6
Production of cadmium-containing			
Substances	ND	3	ND
Iron and steel production	2.3	34	28-284
Fossil fuel combustion			
coal	1.9	6	176-882
oil		0.5	41-246
Refuse incineration	5	31	56-1400
Sewage sludge incineration	0.2	2	3-36
Phosphate fertilizer manufacture	ND	ND	68-274
Cement manufacture	1	ND	8.9-534
Wood combustion	ND	ND	60-180
TOTAL EMISSIONS	14	130	3350-14 640

Table 1.6. Cadmium consumption by end-uses (Nordic Council of Ministers Cadmium Review, 2003)

Application	Western World 1990 [1)]		EU about 2000 [2)]	
	tonnes Cd/year	%	tonnes Cd/year	%
Ni-Cd batteries	9,100	55	1,900	73
Pigments	3,300	20	300-350	12
Stabilisers	1,650	10	150	6
Plating	1,320	8	200	8
Alloys	500	3	30-40	1
Other	660	4	-	-
Total	16,500	100	1,930-1,990	100

2.3 Mobility of Cd in the soil

The main pollution problems involving cadmium are correlated with the fate of cadmium in soil. In sandy soil with low pH, cadmium has low affinity for soil metal-sorbing phases (e.g. oxides, humified organic matter) (Elzinga et al., 1999), and therefore a great potential for transfer through the food chains (Basta et al., 2005; Chaney and Ryan, 1994; Chaney et al., 1999). However, Cd has a high affinity for phosphates, carbonates, Mn oxides such as birnessites and other phyllomanganates. Presence of different mineral nutrients, cations or anions in the soil type, minerals not well cristallized and pH can also alter the bioavailability of Cd by complexation, sorption or desorption processes (Naidu et al., 1994, 1997; Bolan et al., 1999). Because soil is such a complex system, it is difficult to make broad generalizations on the effect of ligands in solution on the sorption of Cd and hence on the availability of Cd (Vig et al., 2003). Metals in soil exist as soluble, insoluble and exchangeable forms, and as organic and inorganic complexes; and the different forms of metals have different mobility and bioavailability (Adriano et al., 2004).

The process of identifying and quantifying the different species of metals in a sample is referred to as speciation (Ure and Davidson, 2002). The toxicity of metals toward living organisms has been shown to be best correlated with the free ion or labile metal form in waters (Neal and Sposito, 1986) and soil solutions (Sauve et al., 1996). This is in agreement with free metal ion hypothesis, which states that the bioavailability of trace metals is related to the activity of the free aquo ion (Alma and Singh, 2001).

But this hypothesis may not be valid in all situations. McLaughlin et al. (1997) found, for instance, that the uptake of Cd and Zn by lettuce grown in a ligand-buffered nutrient

solution was increased in the presence of organic ligands. This was ascribed to either the metal–ligand complexes being taken up intact by the roots or the complex formation affecting the diffusional limitations to free metal uptake in the unstirred root zone and in the apoplast. Mench and Martin (1991) reported that root exudates of *Nicotina spp*. enhanced the solubility of Cd, and this resulted also in increased bioavailability of Cd to the three Nicotiana species used as test crops (Alma and Singh, 2001). The cadmium distribution between a solid phase and pore water of a soil is commonly described by equilibrium partitioning but the total dissolved metal concentration does not necessarily correspond to the amount available to organisms, as describe in the bioavailability paragraph.

In fig. 1.4 the sources of cadmium and its fate in the different pools in soil-plant system is reported.

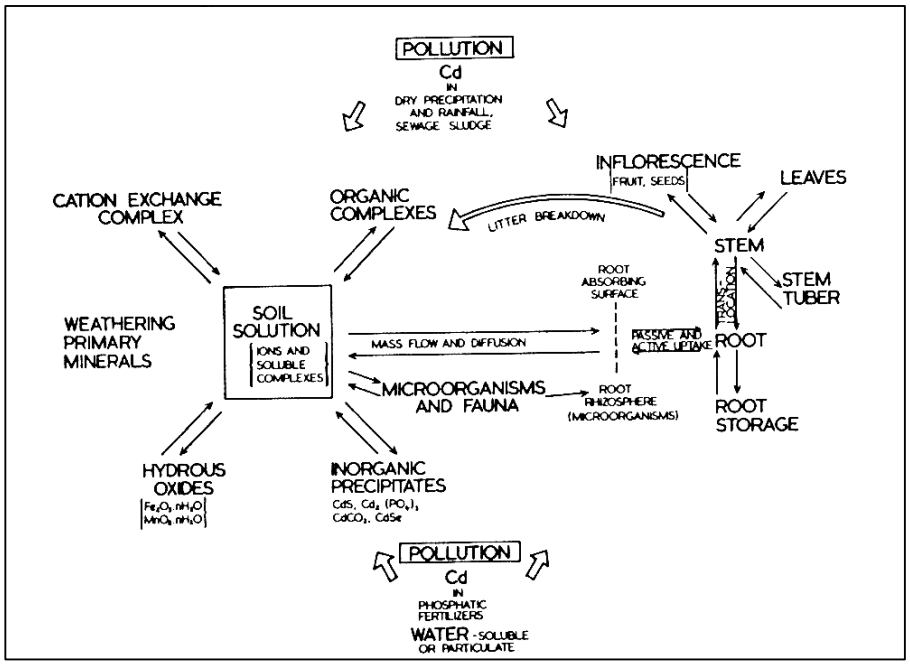


Fig. 1.4. Cadmium in the soil-plant system (Aylett, 1979).

Because cadmium is bound to the soil, its transport to water systems is delayed and hence cadmium is also less available to plant uptake. The major binding mechanisms for Cd, Pb, and Cu (which are all cationic) are complexation to humic substances and adsorption to clay minerals and oxides. Below two possible chemical reactions are sketched, illustrating the complexation of metals to humic substances. In the first reaction, cadmium is complexed to one deprotonated carboxylate group, whereas the second reaction shows copper being complexed to one carboxylate and one neighbouring phenolate group (Gustafsson, 2005).

$$R\text{-}COO^- + Cd^{2+} \leftrightarrow R\text{-}COOCd^+$$

$$\begin{matrix} R\text{-}COO^- & R\text{-}COO \\ \vdots + & Cu^{2+} \leftrightarrow \vdots \\ R\text{-}O^- & R\text{-}O \end{matrix} \quad Cu$$

Plants and microorganisms also play an important role in the mobility of cadmium in soils. In fact, roots can alter the pH value, the redox potential, and accumulate organic matter. These changes in the soil characteristics may, directly and indirectly, alter the mobility of cadmium and nutrients.

For instance, carboxylic acids and protons released from plants may react directly with mineral surfaces and release nutrients into the soil solution, or they enhance organic matter and hence the microbial Mench et al., 1988a, b, Dakora et al., 2002, Uren, 2000). At the same time, root exudates, e.g. phytosiderophores (PS), may increase plant uptake of Zn, Ni, Cd and Cu from contaminated soils, and thus alter the mobility of cadmium. Since Zn and Cd are both members of the IIb group of the periodic table, PS also have been speculated to mediate Cd uptake in grain crops that accumulate this metal (Shenker et al., 2001). PS are efficient Cd-mobilizing agents in soil and they serve as efficient mediators for Cd uptake by graminaceous plants but plants that are highly efficient in PS release under iron stress, such as barley and wheat, may not necessarily accumulate more Cd from contaminated soils than iron-inefficient species and cultivars (Shenker et al., 2001).

The type of soil significantly affects the total amount of LMWOAs (low molecular weight organic acids) just like plant cultivar, physiological condition and age of the plants, and many others properties.

Cielinski et al. (1998) demonstrated that the high Cd accumulation in durum wheat cultivar Kyle corresponded to more total LMWOAs in the rhizosphere soil than in that of the low Cd accumulating cultivar Arcola. The higher concentrations of LMWOAs in the rhizosphere soil of the Kyle cultivar suggest that more Cd was maintained into the soil solution as Cd-LMWOAs complexes, which in turn resulted in increased Cd uptake by the Kyle cultivar. These results suggest that the differing levels of LMWOAs present in the rhizosphere soil play an important role in the solubilization of particulate-bound Cd into soil solution, and its subsequent phytoaccumulation by the high and low Cd accumulating cultivars.

The inorganic pollutants are taken up by roots via membrane transporter proteins. In fig. 1.5 and 1.6 the membrane transporters localized on the plasmic membranes and the

tonoplast (IRT, ZIP, Nramp, etc.) transporters are reported. These “gates” controlled the influx and efflux of Cd in roots, and thereafter in other plant tissues, and all these mechanisms (including the detoxification and storage mechanisms) explained the differences between cultivars. Several membrane transporter exist in plants, *Arabidopsis thaliana*, for instance, has 150 different cation transporters, and 14 transporters for sulfate alone (Hawkesford, 2003; Axelsen et al., 2001). The main role of transporters is to uptake the nutrients from soil solution and the chemically similitude (e.g. Cd and Zn) may cause an inadvertently uptake of inorganic pollutants or excessive uptake of nutrients (and became toxic themselves, e.g., nitrate, phosphate, copper, manganese, zinc). Cadmium, and in general inorganic pollutants, usually exist as ions and cannot pass membranes in great amount without the aid of membrane transporter proteins when membranes are not damaged. Because uptake of cadmium depends on a discrete number of membrane proteins, their uptake is saturable, following Michaelis Menten kinetics. Individual transporter proteins have unique properties with respect to transport rate, substrate affinity, and substrate specificity (low affinity transporters tend to be more promiscuous) (Marschner, 1995). These properties may be subject to regulation by metabolite levels or regulatory proteins (e.g., kinases). Furthermore, the abundance of each transporter varies with tissue-type and environmental conditions, which may be regulated at the transcription level or via endocytosis. As a consequence, uptake and movement of cadmium in plants is complex species-and conditions-dependent processes, and difficult to capture in a model.

The presence of rhizosphere microbes can affect plant uptake of cadmium. For instance, mycorrhizal fungi can both enhance uptake of essential metals when metal levels are low and decrease plant metal uptake when metals are present at phytotoxic levels (Rufyikiri et al., 2000). Also soil bacteria, play an important role in the mobility of metals in soils; generally all the soil bacterial increase metal uptake into plants. This may simply be related to metabolic function and excretion of wastes into the rhizosphere (Angle et al., 2002). However, was found that specific bacteria within the rhizosphere of hyperaccumulators preferentially increase metal uptake into plants at a much higher rate than microbes that do not colonize the rhizosphere of hyperaccumulators (Angle et al., 2002).

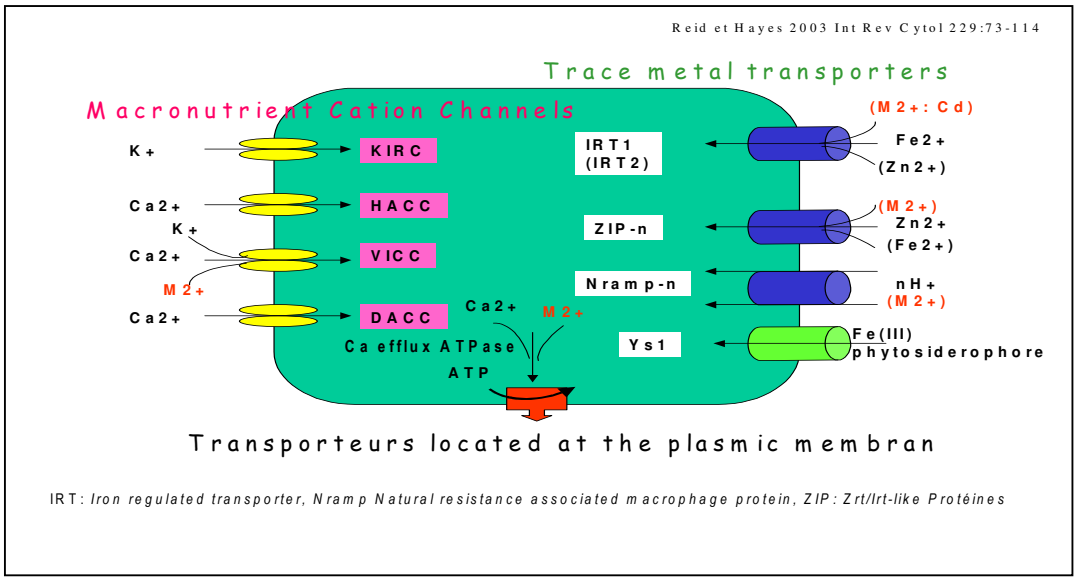


Fig.1.5 Trace metal transporters located at the plasmatic membrane

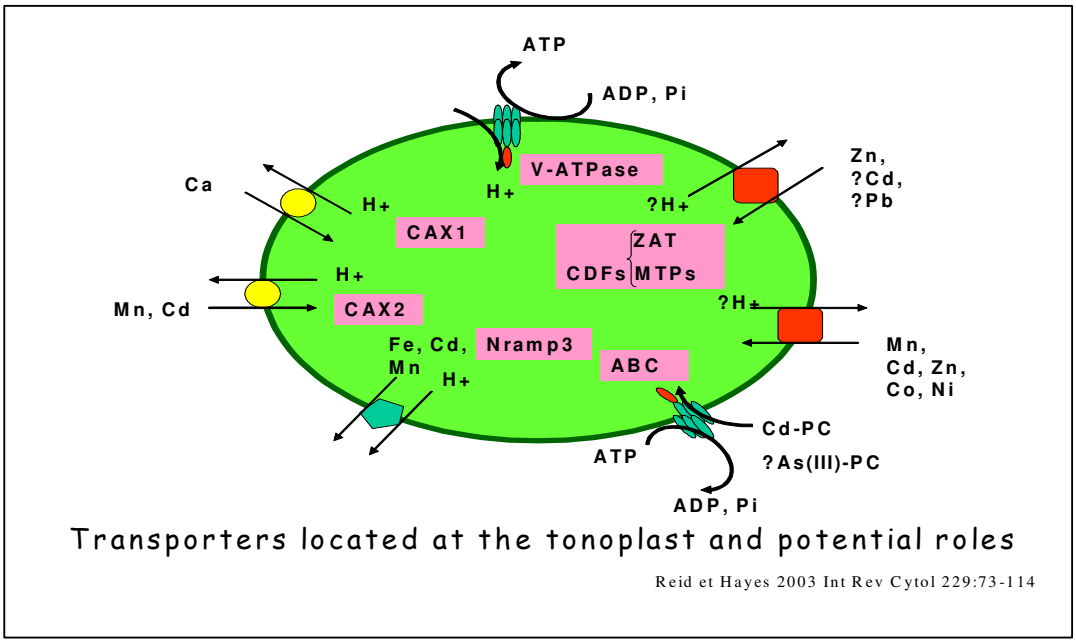


Fig.1.6 Trace metal transporters located at the tonoplast membrane

Once inside the root, cadmium may move in the different parts of the plant, and be translocated from the root system to the edible parts. The first studies on the distribution and chemical behaviour of Cd^{2+} in tissues of plants, lead with soybean plants (cv. Williams) (Cataldo et al., 1981), showed that Cd is strongly retained by roots, with only 2% of the accumulated Cd being transported to leaves; as much as 8% (of the 2%) was transported to seeds during seed filling. More recent studies verified that the

accumulation of Cd in plants tissue is strictly dependent on the plant species and the form of Cd uptake and the competition with other cations in soil: Ramos et al. (2002) showed that in *Lactuca* sp. Cd was accumulated in leaves mainly in cell wall fraction (64%) and this accumulation was fairly independent of Cd level in nutrient solution. The lowest Cd concentration (12-14%) was found in chloroplasts for both Cd levels tested. The increase in Cd concentration in the external medium caused an increase in Mn uptake and translocation to the shoots of lettuce plants, in contrast to the behaviour of the other essential micronutrients, and an increase in Mn content in the chloroplasts, suggesting an interaction between Cd and Mn at the chloroplast level (Ramos et al., 2002).

When the Cd is traslocated in the leaves, generally, cadmium content increases more in older leaves than younger ones, and in leaves more than in roots (but there are different behaviour: for example *Nicotiana tabacum* or *Thlaspi caerulescens* accumulated more in aerial plant parts). The movement of cadmium through different parts of the plant is one of the important factors that must be taken in account in its risk assessment. In fact the larger amount of Cd absorbed by humans and stored in the different parts of the organism (liver, kidneys, and bones) is derived from food (see table 1.7), hence the estimate of the solubility and bioavailability of cadmium is of paramount importance for the human health.

Cadmium uptake may change with agronomic practices, tillage, set-aside and crop rotation. Other factors affecting the uptake of cadmium by plants are reported in table 1.8.

Table 1.7 Cadmium content of certain foodstuffs, source: UK total Diet study 2004

Food Group	Mean Cd concentrations (mg/kg fresh weight)
Bread	0.015
Miscellaneous Cereals	0.014
Carcase Meat	0.001
Offal	0.041
Meat Product	0.007
Poultry	0.001
Fish	0.013
Oils & Fats	0.001-0.002
Eggs	0.001-0.003
Sugar & Preserves	0.011
Green Vegetables	0.014
Potatoes	0.025
Other Vegetables	0.009
Canned Vegetables	0.005
Fresh Fruits	0.001
Fruit Products	0.001
Beverages	0.00015-0.00019
Milk	0.00007-0.0001
Dairy Products	0.001-0.002
Nuts	0.06

Table 1.8. Factors affecting Cd uptake from soil by plants. Adapted from M.J. McLaughin et al., "Managing Cd concentration of Agricultural land", paper presented at the OECD Cd Workshop held in Saltsjöbaden (Sweden), 16-20 October 1995

pH ↓	uptake ↑
soil salinity ↑	uptake ↑
concentration of Cd ↑	uptake ↑
metal sorption by soil ↑	uptake ↓
organic matter ↑	generally uptake ↓
cation exchange capacity (CEC)↑	uptake ↓
clay, Fe and Mn oxides ↑	uptake ↓
macronutrients ↑	uptake may ↑ or ↓
temperature ↑	uptake ↑
aeration (e.g. flooding)	uptake ↓
micronutrients (e. g. Zn ↓)	uptake ↑

It is well known how Cd availability is strictly correlated with the main soil characteristics such as: CEC, pH value, organic matter content, soil texture (Gray, 1999; Christensen, 1984; Naidu et al., 1994; Mc Bride et al., 1997; Jauerta et al., 2002; Baize, 1997; Baize and Tercé 2002).

The persistence and mobility of cadmium in soils is determined largely by the extent of its sorption by soil particles. The soil pH is often the most important chemical property

governing cadmium sorption, precipitation, solubility and availability. The cadmium sorption rises with soil pH increase, and decreases with an increase in ionic strength of the electrolyte solution (Naidu et al., 1994). Therefore, both pH effect and chemical adsorption/fixation might be involved in the reduction of cadmium mobility. Low pH favours solubility, and hence mobility, and in addition, organic ligands may enhance the solubility of cadmium.

Microbial communities might be involved in changing the availability of cadmium in soils, and hence be involved in the reduction of its toxicity, through their reduction, accumulation, and *in situ* immobilization by extra cellular precipitation (Collard et al., 1994; Vig et al., 2003). The different behavior between fungi and bacterial is explained in the 3.3.4 paragraph.

2.4 Technique of speciation of cadmium in soil

The speciation of trace metal fraction in soil has been variously approached to predict their availability to plant and animals. Most of the techniques have involved single or multiple extraction steps to dissolve metal pool of interest.

Briefly the first method used to measure metals in soil was a generic partial extraction of soils by various dilute reagents that remove amounts of elements which could be empirically correlated to the plant response (McBride et al., 1997; Krishnamurti et al., 1997). The main pitfall of this method is the assumption that the toxicity of metals is correlated with their total amount. Extractants as nitric acid, hydrochloridric acid (Warman and Thomas, 1976) were approximated to the total extraction of metals. New and more efficient methods were developed to obtain a value of the labile fraction of the metals that is better correlated with the portion that is taken by the plants (single-step extraction), or to extract specific chemical pools (sequential extraction) to assess potential phytoavailability:

Single-step extraction: single solvent extractions

This approach has been standardized and accepted by the Commission of the European Communities (Ure et al., 1993), and this is surely an advantage The lack of such approach is possibility of the re-adsorption of metal during extraction with some reagents, but Krishnamurti et al. (2000) found a good correlation between a single extraction based on a 1M NH_4NO_3 solution and the bioavailable cadmium in soil. In this thesis, a single step extraction, described above, was used.

Sequential extraction: was introduced by Tessier et al. (1979). The chemical speciation of metals in soil is achieved by sequential extraction procedures using soil constituent-selective solvents (Tessier et al., 1979; Sposito et al., 1982; Quevauviller et al., 1998). These protocols have allowed the quantification of recalcitrant (bound to clay and ordered crystalline oxides) and labile (free, exchangeable and organically bound) metal pools.

However the metal pools should be considered in a dynamic equilibrium between the three fractions (labile, non labile, and free ions). The size of the pools is affected by several factors: pH, the ionic strength of the extractant (Naidu et al., 1994), the contact time between Cd and soils (Gray et al., 1998), and the competition with other metal ions (Christensen, 1987a, b), as well as on the presence of inorganic or organic ligands (Sposito, 1994).

Soil Moisture Samplers: Phytotoxicity depends also on the concentration of the metals in pore water. The plants are exposed to metal through the uptake of pore water thus inducing decrease in local metal concentrations and supply by diffusion. Among the commercially available soil moisture samplers, we used the Rhizon MOM (fig.1.7). The porous plastic material wets spontaneously and has standard 0.1 micron pores. The samplers are supplied with coextruded tubing (PE inside/PVC outside) 1 mm internal diameter, luer connector and protective cap. The sampler is reenforced by a wire. Dissolved organic and inorganic matter will not absorb on the soil moisture sampler. Rhizon MOM samplers extract the soil pore water and the determination of trace metals can be made with ICP-MS (Inductively Coupled Plasma – Mass Spectrometry). An useful correlation with available cadmium for plant uptake was verified by Chaudri (2000). This technique is easy to use, low cost, and rapid; furthermore the Rhizon soil moisture sampler inhibits the entrance of microorganisms in the samples of soil pore water. The lack of this technique is the impossibility to determine the moment of the equilibrium between the Rhizon and the soil solution. No evidences may prove this equilibrium.

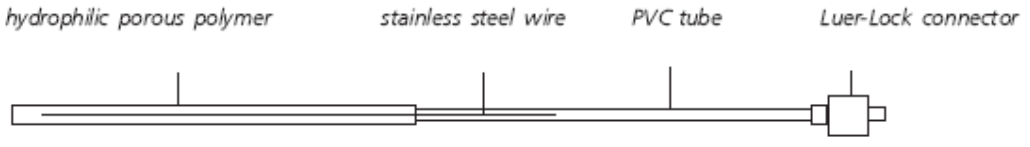


Fig. 1.7 Rhizon soil moisture sampler.

Diffusive gradient in thin-films (DGT) system (Zhang and Davison, 1995; Harper et al., 1998; Hooda et al., 1999) has been developed to measure the metal availability in soils. DGT device consists of a layer of an ion exchange resin (Chelex 100) embedded in a hydro gel. The basic assumption behind DGT-method is that diffusion of metal thought a porous matrix is the same process that organisms use to absorb elements from the soil. When the DGT-unit is placed into a soil, metal will accumulate in the resin gel layer and the diffusive flux of metal ions can be quantified (Koster et al., 2004).

In all the different techniques explained above the samples obtained need to be analyzed in the laboratory and need to be digested under high temperature, pressure and acidic conditions to free the metal ions in solution as a prerequisite for the application of all those methods (Corbisier, 1999). After the digestion the samples are analyzed with the classical analytical methods ICP/AES and ICP/MS or AAS. The limit of this technique is in the soil moisture required. The efficiency of DGT fallen with a soil moisture under the 60% of WHC (water holding capacity). It has been demonstrated that DGT is a powerful tool to predict cadmium uptake in water sediment and well mimic the cadmium uptake in soil (Nolan et al., 2005, Koster et al., 2004).

Miniaturised stripping sensor: is a ion selective electrode, based on the polarography theory (Palchetti et al., 2004). This method is fast, low cost and in rapid development and improvement, but at the moment this technique is not yet well correlated to the bioavailability of cadmium in real soil samples.

Biosensor

A biosensor is a living organisms in which a measurable signal is product in the presence or absence of the investigated compound or condition. The signal is encoded by a reporter gene. The whole-cell biosensor can be divided into three groups dependent on the mechanisms of the reporter genes: non-specific, semi-specific and specific.

In the non-specific biosensor usually the reporter genes *lux*CDABE operon from *V. fischeri* is used to detect the toxicity in soils. The contaminant presence is related to the decrease in light emitted from the biosensor. The decrease is correlated to the toxicity and to the inhibition effects of the contaminant present in the samples on the biosensor. These kinds of biosensor are non-specific because the signal decrease, in addition of the negative effects of the contaminant, but also depend on others factors (pH, salinity, and nutrient supply).

The semi-specific biosensors contain a reporter gene fused to a stress regulated promoter and are therefore induced by various conditions stressful for the biosensor organisms. These kinds of biosensor are not used in the environmental studies.

In the specific biosensor the reporter gene is genetically insert, without the promoter, in the bacterial biosensor. The specific contaminant inducing the encoding of a repressor or an activator protein correlated to the reporter gene. The reporter gene is therefore transcribed from the promoters that are regulated by repressor/activator protein sensitive only to specific compounds. In the case of metals when the intracellular metal passes through the bacteria cellular wall, the metal binds to a specific site and became the *lux*-promoter. A measurable signal, proportional to the free ion concentration of metal is then emitted (Mergeay et al., 2003, Belkin et al., 2003).

Biosensor may evaluate the effects of chemical and physical parameters on changes in metal availability. Moreover the biosensor potentiality is in the possibility to insert the reporter gene in different parts of the microbial DNA code and obtain a genetically modified bacterial able to correlate the presence of a general compounds (C, N, P, organics and xenobiotic) to a measurable signal. Since the first experiments (Mergeay et al., 1978, Corbisier et al., 1999) with biosensors, many studies have been performed in soil. Such studies proven that biosensors are excellent tools for estimating the toxicity of metals in soil (Tibazarwa et al., 2001, Dawson et al., 2005).

Among whole-cell microbial, the biosensor BIOMET® was chosen in the work presented here. This biosensor creates at VITO (Mol-Belgium) is based on the *Ralstonia metallidurans* CH34 strain AE1433 which is a facultative chemolithotrophic bacterium Gram-negative carrying two large plasmids, pMOL28 and pMOL30, hosting operons conferring resistance to different heavy metals such as *czc*, *cnr* and *ncc* genes, that code resistance mechanisms for cadmium, zinc and cobalt, thallium, copper and lead, nickel, mercury and chromate, respectively.

The reporter gene is a promoteless *lux* (*lux* CDABE) from *Vibrio fischerii* inserted into the mega-plasmid pMOL28. When the intracellular Cd concentration exceeds a threshold, expression of the *czc* operon is induced and the *lux* gene is co-expressed with production of light, which is detectable in a luminometer.

Despite the advantages of the biosensor measurements, the use of whole-cell bacterial biosensors for assessing the metal bioavailability in the rhizosphere presents some problems:

1) *Low details on the conditions of the natural environment of the biosensor*: pH, temperature, incubation time, medium and reagents can influence the performance of the biosensor. The best performance is obtained in condition similar to the natural environment of bacterial.

2) *Short lifetime*. The possibility to prolong the experiment over the C-sources added with the medium, and also the use of reconstituted media in the bioassay, represent a disadvantage.

3) *The possible 'false negative' responses due to cytotoxic metal concentrations or adverse soil properties* (i.e. pH). In our case this problem did not occur because soils were contaminated with known Cd concentrations.

4) *The possible 'false positive'* responses due to the presence of other metals (i.e. Zn) and/or to the presence of stimulating organic molecules.

3. CADMIUM INTERACTIONS WITH PLANTS

3.1. Cadmium and nutrient uptake by plants

To grow and complete the life cycle, plants must acquire not only macronutrients (N, P, K, S, Ca, and Mg), but also essential micronutrients such as Fe, Zn, Mn, B, Ni, Cu, and Mo. Plants have evolved highly specific mechanisms to take up, translocate, and store these nutrients. Although the active root uptake mechanism is selective, plants can take some contaminants such as non-essential metals (e.g. cadmium or mercury). The mechanisms of elemental uptake by plants are:

Diffusion: Simple diffusion means that the molecules can used the channels for macronutrients and pass through the membrane, driven by a concentration gradient; this limits the maximum possible concentration of the molecule inside the cell (or outside the cell if it is a waste product). The effectiveness of diffusion is also limited by the diffusion rate of the molecule, but a too high concentration of contaminants in the medium may caused the broken of the membrane due to oxidative stress.

Facilitate passive transportation: Facilitated diffusion utilizes membrane protein channels to allow charged molecules (which otherwise could not diffuse across the cell membrane) to freely diffuse in and out of the cell. These channels come into greatest use with small ions like K^+, Na^+, and Cl^-. The speed of facilitated transport is limited by the number of protein channels available.

Active transportation: Active transport requires expenses of energy to transport molecules from one side of the membrane to the other. Active transport is the only type of mechanisms that can transport molecules against a concentration gradient.

Similarly to facilitated transport, active transport is limited by the number of transporter proteins. It is generally believed that Cd uptake by non-accumulator plants represents opportunistic transport by a carrier for another divalent cation such as Zn^{2+}, Cu^{2+} or Fe^{2+}, or via cation channels for Ca^{2+} and Mg^{2+} (Welch and Norvell, 1999). Though it should be noted that most studies to date have examined Cd transport at uncharacteristically high concentrations of Cd in the experimental medium. The wheat cDNA *LCT1* has been shown to mediate uptake of both Ca and Cd, when expressed in *Saccharomyces cerevisiae* (Clemens et al., 1998, 2001). Members of the ZIP gene family are capable of transporting transition metals including Fe (II), Zn, Mn, and Cd (Guerinot, 2000). The Fe(II) transporters such as IRT1 (a member of ZIP) and Nramp have been shown to be capable of transporting several metals including Cd in *Arabidopsis thaliana* (Korshunova et al., 1999; Thomine et al., 2000). Furthermore, the Zn transporter, ZNT1, recently cloned from the Prayon ecotype of *T. caerulescens*, has been shown to mediate low-affinity uptake of Cd (Pence et al., 2000). There are also numerous studies showing inhibitory effects of Ca, Zn, Cu or Mn on Cd uptake by higher plants or algae (Smeyers-Verbeke et al., 1978, Tripathi et al., 1995). It is not known if these mechanisms are also responsible for Cd uptake in the hyperaccumulator *T. caerulescens*, particularly the Ganges ecotype.

Cd may enter into the plants roots with different mechanisms but it's not possible to identify a privileged mechanisms. Uptake of Cd^{2+} across the plasma membrane of root cells has been shown to occur via a concentration-dependent process exhibiting saturable kinetics (Blaudez D et al., 2000). The saturable nature of Cd uptake in these studies suggests that Cd is taken up via a carrier-mediated system. Conversely, for istance, Cd uptake in barley is not under metabolic control but is primarily controlled by diffusion (Cutler & Rains, 1974).

3.2. Cadmium tolerance, detoxification and accumulation by plants

Where does the Cd taken up go into the plant?

The fate of the absorbed cadmium depends on the plant characteristics, because each plant species shows a different level of tolerance to metals and different response to the onset of stress.

The concept of tolerance, detoxification and accumulation are linked. Metal tolerance can be defined as the ability of a plant to survive and reproduce on sites that are toxic or

inimical to most other plants because the soil contains elevated concentrations of one or more metals (Macnair and Baker, 1994). This definition of tolerance requires demonstration of the phytotoxicity of the soil and the ability of a plant to grow and reproduce without any sign of toxicity on this phytotoxic soil, where most other plants would stop growing normally and show signs of toxicity (Macnair and Baker, 1994).

The toxicity is manifested by an interaction between genotype and its environment (Mcnair et al., 2000). Plants possess a range of cellular mechanisms that are involved in the detoxification of metals and thus tolerance to the metal stress. Cadmium for instance is generally stored in the cell wall or in vacuole. The strategies to survive in Cd contaminated condition adopted by plants are:

Avoidance in which theoretically the contaminant is immobilized into the soils with e.g. root exudates, phytosiderophores and changing in the membrane permeability to avoid the entrance of metals into the cell and also simply changing its root location.

Tolerance: the metal ions cross the plasma membrane and enter in the cell but here the plants avoid the harmful effect of metals with e.g. binding metals ions with organic acids, peptides or storage in the cell walls or into the vacuole (Chardonnens, 2000). The role of thiols, phytochelatines, metallothionines must be taken in account. In figure 1.8 is reported the range of the potential cellular mechanisms available for the metal detoxification and tolerance in plants.

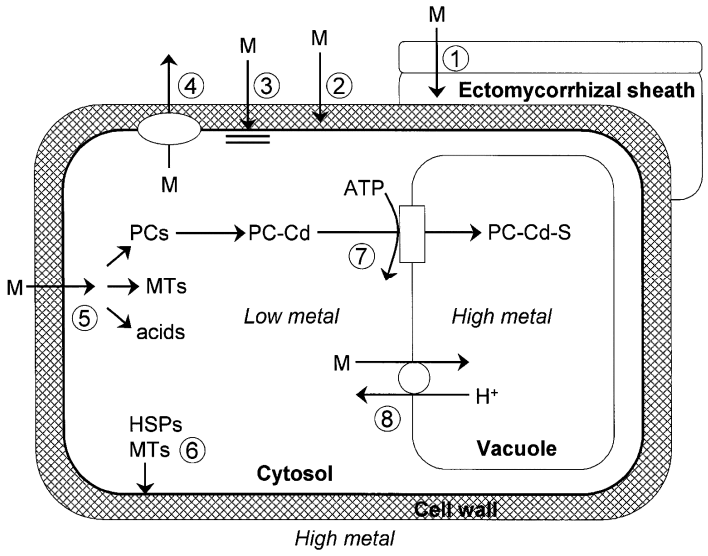


Fig.1.8. Summary of potential cellular mechanisms available for metal detoxification and tolerance in higher plants. 1. Restriction of metal movement to roots by mycorrhizas. 2. Binding to cell wall and root exudates. 3. Reduced influx across plasma membrane. 4. Active efflux into apoplast. 5. Chelation in cytosol by various ligands. 6. Repair and protection of plasma membrane under stress conditions. 7. Transport of PC-Cd complex into the vacuole. 8. Transport and accumulation of metals in vacuole (Hall, 2002, modified from Marscher, 1995).

1. Mycorrhizas: although not always considered in general reviews of plant metal tolerance mechanisms, mycorrhizas, and particularly ectomycorrhizas that are

characteristic of trees and shrubs, can be effective in ameliorating the effects of metal toxicity on the host plant (Hall, 2002; Marschner, 1995). The mechanisms employed by the fungi at the cellular level to tolerate metals are probably similar to some of the strategies employed by higher plants, namely binding to extracellular materials or sequestration in the vacuolar compartment (Hall, 2002).

2. Binding to cell wall: the cell wall may show a binding properties towards metals but this mechanism of metal tolerance has been a controversial one.

Küpper et al. (2001) reported results on a quantitative study of cellular compartmentation of cadmium in *A. halleri.* A large concentration of Cd and Zn was found in the root. Both Cd and Zn seem to form precipitates with P in the apoplast of the rhizodermis but their concentrations in the rest of the root are generally low. This result is in contrast with Hall (2002) which stated that although the root cell wall is directly in contact with metals in the soil solution, adsorption onto the cell wall must be of limited capacity and thus have a limited effect on metal activity at the surface of the plasma membrane.

One related process concerns the role of root exudates in metal tolerance. Root exudates have a variety of roles (Marschner, 1995) including that of metal chelators that may enhance the uptake of certain metals. The clearest example of a role for root secretions in tolerance is in relation to organic acids (released via active transportation or via diffusion) and the detoxification of the light metal Al (Ma et al., 2001). The role of low molecular weight organic acids and amino acids, such as citric, malic and histidine are potential ligands for metals and so could play a role in tolerance and detoxification (see paragraph 2.3 and 2.4) (Clemens, 2001)

3. Plasma membrane: the loss of functionality of plant plasma membrane is one of the first symptoms derived from the onset metal stress. The tolerance may involve the protection of plasma membrane integrity against metal damage or the repair of plasma membrane after the damage (Salt et al., 1998).

This process might involve heat shock proteins and/or metallothioneins (Hall, 2002).

4. Heat shock protein (HSPs)*:* heat shock proteins (HSPs) characteristically show increased expression in response to the growth of a variety of organisms at temperatures above their optimal growth temperature. They are found in all groups of living organisms, can be classified according to molecular size and are now known to be expressed in response to a variety of stress conditions including metals (Hall, 2002, Vierling, 1991, Lewis et al., 1999).

5. and 6. Phytochelatins and Metallothioneins: chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of metal detoxification

and tolerance. Higher plants contain two major types of cysteine-rich, metal-binding peptides, the phytochelatins (PCs) and the metallothioneins (MTs).

The phytochelatins are a family of metal-complexing peptides that have a general structure (γ-Glu Cys)$_n$-Gly where n=2–11, and are rapidly induced in plants by metal treatments (Rauser, 1995; Zenk, 1996; Cobbett, 2000; Goldsbrough, 2000). The role of PCs in the Cd detoxification has been supported by a range of biochemical and genetic evidence. Howden et al. (1995 a, b) isolated a series of Cd-sensitive mutants of *Arabidopsis* that varied in their ability to accumulate PCs; the amount of PCs accumulated by the mutants correlated with the degree of sensitivity to Cd. However there are more controversial data published by Schat et al. (2000) in which was shown that the role of PCs depends on plant species and ecotypes like for *Silene vulgaris*. Furthermore studies on *Thlaspi caerulescens* by Ebbs et al. (2001) reported that the increased PC production is not the primary mechanisms by which Cd tolerance is achieved in the Prayon ecotype of *T. caerulescens* . While synthesized in response to Cd exposure, PCs were generally present at lower levels than in the non-accumulator *T. arvense.* Phytochelatins may have a secondary role in Cd tolerance, possibly related to sequestration of Cd in leaf cell vacuoles.

Metallothioneins (MTs) are gene-encoded polypeptides that are usually classified into two groups. Class 1 MTs possess cysteine residues that align with a mammalian (equine) renal MT; Class 2 MTs also possess similar cysteine clusters but these cannot be easily aligned with Class 1 MTs (de Miranda et al., 1990; Robinson et al., 1993; Prasad, 1999). Metallothioneins genes have now been identified in a range of higher plants (Prasad, 1999). In plants, there is a lack of information concerning the metals likely to be bound by MTs, although Cu, Zn and Cd have been the most widely studied (Tomsett and Thurman, 1988; Robinson et al., 1993; Goldsborough, 2000). However, it has been reported that MT2 mRNA was strongly induced in *Arabidopsis* seedlings by Cu, but only slightly by Cd and Zn (Zhou and Goldsbrough, 1994, Cobbett and Goldsbrough, 2002). Thus the role of MTs remains to be established. They could clearly play a role in metal metabolism, but their precise function is not clear; they may have distinct functions for different metals and different exposure level (Hamer, 1986). Alternatively, they could function as antioxidants, although evidence is lacking (Dietz et al., 1999), while a role in plasma membrane repair is another possibility (Salt et al., 1998).

7. and 8. Vacuolar compartmentalization: earlier studies showed that the vacuole is the site for the accumulation of a number of metals including Zn and Cd (Ernst et al., 1992; De, 2000). One well-documented example of the accumulation of Cd and PCs in the

vacuole involved an ABC transporter. This accumulation appears to be mediated by both a Cd/H^+ antiporter and an ATP-dependent ABC transporter, located at the tonoplast (Salt and Wagner, 1993; Salt and Rauser, 1995; Rea et al., 1998); the stabilization of the Cd-PC complex in the vacuole involves the incorporation of acid-labile sulphide. In the fission yeast, a Cd-sensitive mutant has been isolated that is able to synthesize PCs, but is unable to accumulate the Cd-PC-sulphide complex (Ortiz et al., 1992); the mutant has a defect in a gene (hmt 1) that encodes an ABC-type transporter. Similar transporters may well be involved in Cd compartmentalization in higher plants (Salt and Rauser, 1995; Rea et al., 1998; Hall, 2002; Metwally, 2003).
Although PCs may play some role in Cd detoxification in *S. vulgaris*, PC production in greater amounts is not the mechanism that results in increased Cd tolerance. Thus, although evidence for the role for PCs in detoxification is strong, especially for Cd, these peptides may play other important roles in the cell, including essential metal homeostasis, sulphur metabolism or, perhaps, as anti-oxidants (Rauser, 1995; Dietz et al., 1999; Cobbett, 2000). Their participation in the detoxification of excess concentrations of some metals may be a consequence of these other functions (Steffens, 1990). The final step in Cd detoxification, certainly in the fission yeast and probably in higher plants, involves the accumulation of Cd and PCs in the vacuole (Salt et al., 1998; Schat et al., 2000).

3.3. Toxicity of cadmium

3.3.1 Toxicity of cadmium on plants

Cadmium toxicity can occur in many acidic Cd-contaminated soils. Cadmium mobilization in soil can be mediated by rhizosphere acidification, but to some extent also by complexation with carboxyl acids, or phytosiderophores. The high mobility of Cd in soil is due to its scarce affinity to be bound by organic matter, and it is associated with high rates of uptake and accumulation in some plant species. This is an important aspect from the ecotoxicological point of view. The accumulation of Cd induces a series of stress factors in plants. The visual symptoms of cadmium toxicity depend on the plant species e.g.: chlorosis, necrosis, leaf epinasty, yield decrease, red-brownish discoloration or orangeous dots on maize leaf and brown dots on stalks. Non-visual symptoms are biomass reduction and changes in mineral composition (Van Assche and Clijsters, 1990) and symptoms at the sub-cellular level (Lagriffoul et al., 1998; Sanità di Toppi and Gabrielli, 1999). However, these symptoms mostly characterize high levels

of phytotoxicity. A better understanding of the Cd effects on plants requires more sensitive parameters, such as cellular metabolic compounds that may reflect the physiological and biochemical state of the plant. Cadmium directly or indirectly inhibits physiological processes such as respiration, photosynthesis, water relations and gas exchange (Van Assche and Clijsters, 1990). Changes in cellular metabolism can be observed even at low levels of Cd, before visual symptoms become evident. Enzyme activities have been used as early diagnostic criteria to evaluate the phytotoxicity of metal-contaminated soils (Mench et al., 1994; Vangronsveld and Clijsters, 1994). One of the main toxic effects of trace metals, such as Cd, is the oxidative stress, linked with a lipid peroxidation of cellular membranes (De Vos et al., 1991; Ernst et al., 1992).

Study made on *Phaseolus vulgaris L.* (Smeets et al., 2005) exposed to an environmentally realistic concentration of cadmium (2 μM CdSO4) induced cellular redox disequilibrium and caused oxidative stress. Plant cells respond to elevated levels of oxidative stress by activating their antioxidative defence systems. The first group of enzymes involved in this defence are the ROS quenching enzymes (reactive oxygen species) such as peroxidases, catalases and superoxide dismutases (Smeets et al., 2005). However Foyer and Noctor (2005) recently revised the concept of oxidative stress: in contrast to this pejorative or negative term, implying a state to be avoided, ROS are used by plants as second messengers in signal transduction cascades in processes as diverse as mitosis, tropisms and cell death, their accumulation is crucial to plant development as well as defence. The syndrome would be more usefully described as 'oxidative signalling', that is an important and critical function associated with the mechanisms by which plant cells sense the environment and make appropriate adjustments to gene expression, metabolism and physiology.

3.3.2 Oxidative stress in plants

Oxidative stress results from several effects, such as cellular damage, caused by active forms of oxygen (e.g. superoxide ($O^{-}{}^{2}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), and singlet oxygen ($^1O_2$) (Kappus, 1985).

As already noticed above, the visual stress parameters are undoubtedly useful to understand toxicity of trace elements in soil, but often the visible symptoms are shown only at very high concentrations (Lagriffoul et al., 1998). More sensitive tools to detect the phytotoxicity of Cd are available. Chlorophyll contents and enzyme assay could be used to better understand the Cd effects on plants. Lagriffoul et al. (1998) demonstrated

that the chlorophyll and carotenoid contents in young maize plants, which were cultivated in a nutrient solution containing realistic (1-10 μM) increasing Cd concentrations decreased. Therefore, chlorophyll and carotenoid content can be considered sensitive parameters of oxidative stress induced by Cd contaminated soils Furthermore Lagriffoul (1998) has found that Apx activity is an early biochemical biomarker of Cd accumulation and toxicity in maize leaf. This reduction suggests two possible mechanisms: first, Cd may alter the chlorophyll biosynthesis by inhibiting protochlorophyllide reductase; second, Cd may alter the photosynthetic electron transport by inhibiting the water-splitting enzyme located at the oxidising site of photosystems (Lagriffoul et al., 1998). Studies on dwarf bean (Van Assche et al., 1988) showed a cadmium threshold exposure: when leaf metal content exceeded this toxic threshold value, shoot growth became inhibited and at the same time the activities of glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, isocitrate dehydrogenase, malic enzyme, glutamate-oxaloacetate transminase, and peroxidase, increased (Smeets et al., 2005).

Cadmium might preferentially accumulate in chloroplasts and inhibit photosynthesis at several levels: CO_2-fixation, stomatal conductance, chlorophyll synthesis, electron transport and enzymes of the Calvin cycle (Ernst, 1980). In this thesis, both chlorophyll and carotenoid analysis were performed.

Superoxide and hydrogen peroxide can inactivate various macromolecules directly. However, the conversion to hydroxyl radicals, catalyzed by transition metals, is a much more toxic risk (Inzè and Montagu, 1995).

Against the AOS (active oxygen species), plants posses several detoxification mechanisms: the antioxidants divide in enzymes (e.g. superoxide dismutases, glutathione peroxidases, glutathione reductases, ascorbate perroxidases and catalases) and non-enzymatic (e.g. carotenoids, ascorbic acid, E vitamins, and thiols) (Hall, 2002). Non-tolerant plants show different oxidative stresses due to the toxicity of metals. For instance, a toxicity effect is the inhibition of cytoplasmic enzyme activity, or leaf chlorosis due to competition between metal ions and nutrients (e.g. Cd/Mn, Cd/Ca, Cd/Zn, Cd/P), (Welch and Norvell, 1999; Smeyers-Verbeke et al., 1978; Tripathi et al., 1995).

In this thesis, tolerant, medium, and non-tolerant Cd plants such lettuce, *Holcus lanatus* and dwarf bean were used: some lettuce cultivars are Cd-tolerant plants and able to accumulate high levels of Cd in its edible aboveground biomass compared with other crops (Brown et al., 1996; Florijn et al., 1992), and it is widely used in comparative soil toxicity assessment experiments. *H. lanatus* represents a medium-tolerant plant, it is

widely used in laboratory (Symeonidis et al., 1992; Gaf et al., 1992; Hartley-Whitaker et al., 2002) and in this thesis it was used both in hydroponics and pot experiments. Dwarf bean, is a non-tolerant plant, and it is often used as an indicator of the capacity of soil amendments in lowering the phytoavailability of cadmium in soil (Van Assche et al., 1998; Mench et al., 2000; Mench et al., 2003).
In Chapter 5, the characteristics of the plants will be explained in more details.

3.3.3 Cadmium toxicity for human health

An element is not toxic, but the dose received and speciation, and sensitivity of the receptor result in toxicity. However cadmium is a potential hazard included in the Group I of the International Agency for Research on Cancer (IARC) classification, causing both chronic and acute diseases. Cadmium accumulation in living organisms is toxic in many inorganic and organic forms, such as cadmium acetate, cadmium carbonate, cadmium chloride, and cadmium stearate, and it enters the body mainly by inhalation or ingestion. The carcinogenic potential of cadmium is not yet well demonstrated, and different studies on such risks have reached contrasting results. The large use of cadmium in the industry has increased the exposure in even non-occupationally exposed people, for whom the most important sources are cigarette and foods (e.g. leafy vegetables and offals, cereals, potato tubers) (IARC).
The major route of exposure to cadmium for the non-smoking people is via food. Differently tobacco is an important source of cadmium for smokers, as tobacco plants can accumulate Cd if grown in polluted soils and even in non-contaminated soils. Average daily intakes from food in most areas not polluted with cadmium are 10-40 μg/d (WHO 1992; Llobet et al., 2003). In polluted areas, the value has been found to be several hundred μg per day. In non-polluted areas, uptake from heavy smoking may equal cadmium intake from food. The World Health Organisation (WHO) has established a provisional tolerable weekly intake of 7 μg/kg body weight (table1.9 and fig.1.9).

Table1.9. Cadmium concentrations in main vegetables and staple crops from various regions of the United States. A Report on the Plant Uptake of Metals from Fertilizers, Washington State Department of Agriculture Pesticide Management Division, 2001.

Crop	Sample size	Cadmium concentration (µg/kg wet weight)		
		Median	Minimum	Maximum
Rice	166	4.5	<1	230
Peanuts	320	60	10	590
Soybeans	322	41	2	1,110
Wheat	288	30	<1.7	207
Potatoes	297	28	2	180
Carrots	207	17	2	130
Onions	230	9	1	54
Lettuce	150	17	1	160
Spinach	104	61	12	200
Tomatoes	231	14	2	48

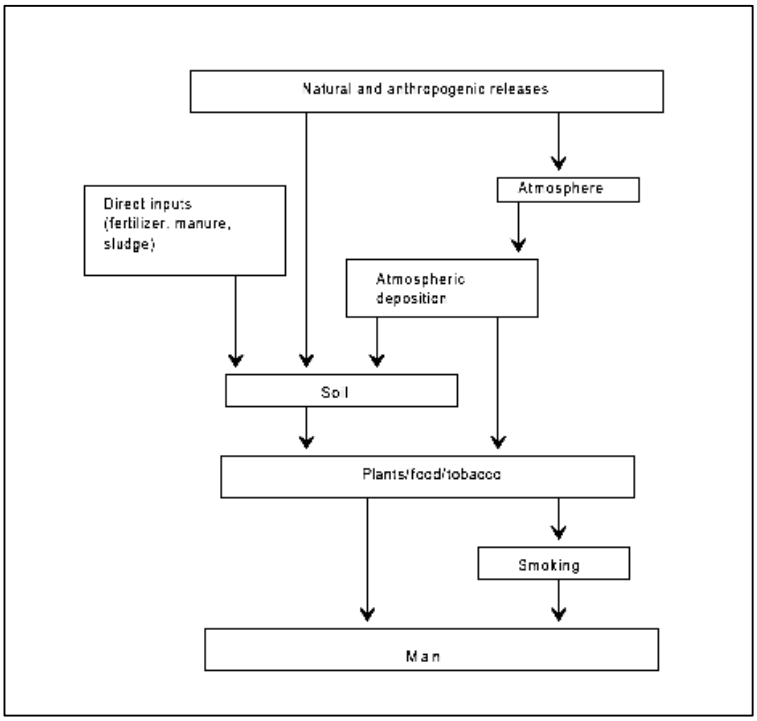


Fig. 1.9. Human exposure pathways (WHO 1992).

3.3.4 Cadmium toxicity to soil microorganisms

Soil microorganisms are the first biota to be influenced by the presence of contaminants. It is generally accepted that metal induced stress can cause an increase in biomass respiration, because under stressful conditions microorganisms use a relatively larger part of the energy to the maintenance of the biochemical functions, or due to the less efficient metabolism of microbial communities (Mergeay, 1995). Cadmium is considered to be the metal having one of the most adverse effects on enzyme activity and microbial biomass in metal contaminated soils (Smith, 1996), but also Hg, Ag, Cu, Ni and Tl are strong inhibitors.

Changes in the microbial community may concern a reduction of microbial biomass carbon and nitrogen, biochemical activity, enzymes activities (Kandeler et al., 2000; Khan and Scullion, 1999). Enzymes activities such as α- e β-glucosidase, phosphatase, urease, protease, arylsulphatase, and aspartase are inhibited by trace elements (Tabatabai, 1977; Juma and Tabatabai, 1977; Al-Khafaji and Tabatabai, 1979; Eivazi and Tabatabai, 1990; Senwo and Tabatabai, 1999).

Renella et al. (2005b) reported that alkaline phosphomonoesterase, arylsulphatase and protease activities were significantly reduced in Cd-contaminated soils whereas acid phosphomonoesterase, β-glucosidase and urease activities were unaffected by Cd.

However, microorganisms are often found to recover after an initial inhibition by high metal inputs (Holtan-Hartwig et al., 2002). Two factors may contribute to such recoveries. One is a gradual decrease in metal availability due to immobilisation reactions in soil. Second and more important, metal tolerant organisms may replace the metal sensitive ones within each functional group, thus changing the microbial community's composition and increasing its metal tolerance (Holtan-Hartwig et al., 2002). A decline in microbial diversity may reduce the resilience of soil communities towards other natural or anthropic perturbations (Degens et al., 2001).

In Gram-positive bacteria, the Cd-resistance is typically encoded by P-type ATPase efflux pumps, whereas in Gram-negative bacteria a three-component cell membrane efflux protein is responsible for ejection of Cd^{2+} ions out of the cells: czcCBAxRS Cd, Zn and Co, chromosome and pMOL28 and pMOL30 megaplasmid encoding cation/proton transmembrane antiporter pumps (Mergeay, 2000). The ability of such bacterial to survive at high heavy metals concentration is encoded into defined genes. These genes are organized in operons in the DNA.

4. *IN SITU* REMEDIATION OF METAL CONTAMINATED SOILS

Contamination of soils may require very long time to run out. Therefore rapid reduction of soil toxicity requires remediation actions. Current remediation strategies such as excavation, thermal treatments, chemical soil washing or soil remove/replace are expensive, site-destructive and may cause a temporary but significant increase in the mobilization of metals from contaminated soils. In the last decade, among metal remediation strategies, green plants and natural remediation process has been widely used (Adriano et al., 2004). Phytoremediation is the use of plants to extract, sequester, or detoxify environmental contaminants. Metal-tolerant hyperaccumulator plants may be useful to phytoremediate contaminated soils. *Thlaspi caerulescens* has been widely used (Chaney et al., 1999; Zhao et al., 2001). Phytovolatilization (plants take up volatile metals ans organic compounds and transpire or diffuse contaminat or modified form of contaminant out of roots, leaves or stems, from US EPA 2000) is another potential to remediate contaminated soils (e.g. Hg, Se) using transgenic phytovolatilizing species (Adriano et al., 2004). In some cases, the toxicity of metals in soils was reduced without the human interference. This kind of remediation is called natural remediation (Adriano et al., 2004). The natural remediation is a very long process and alone may not be sufficient in mitigating the risk from metals. To accelerate and help the process a non-invasive technique has been developed (aided-phytostabilisation), by adding to the soil inexpensive amendments that are cost-effective and minimally invasive (Vangronsveld et al., 1995; Mench et al., 1994). The effectiveness of this technique in the reducing of cadmium (and other metals) has been reported in several studies (Vangronsveld et al., 1995; Lombi et al., 2001; Brigatti et al., 2000; Garcìa-Sanchez et al., 1999; Banat et al., 2002; Mench et al., 1994, 2000, 2003; Saha et al., 2002). In table 1.10, several tentatives to reduce the toxicity of metals in soils are reported. The main intent of these technique is not to change the total concentration but to reduce the bioavailable fractions (Adriano et al., 2004)

Table 1.10.List of compounds used to reduce the metal phytotoxicity (Adriano et al., 2004).

Hydroxyapatite	Increased immobilization hrough cation exchange, adsorption, surface complexation, precipitation and co-precipitation
Rock phosphate	Increased immobilization through adsorption and precipitation
K2HPO4	Increased immobilization through phosphate-induced adsorption and precipitation
KH2PO4	Increased immobilization through phosphate-induced adsorption
Ca(H2PO4)2	Increased immobilization through phosphate-induced adsorption
(NH4)2HPO4	Increased adsorption due to an increase in pH, precipitation of Cd3(PO4)2
CaCO3	Increased immobilization through adsorption and precipitation; decreased plant uptake
Ca(OH)2	Decreased bioavailability
CaO	Decreased phytoavailability
MgCO3	Decreased phytoavailability
CaMgCO3	Decreased phytoavailability
Milorganite	Decreased phytoavailability
LSB, N-Viro; ADB, AADB	Adsorption by inorganic components, metal –organic matter complex formation
SS	Increased adsorption and complexation
BS	Increased the affinity of inorganic fraction of BS treated soil for Cd adsorption
CM	Cd in soil solution was bound in fast dissociating metal complexes
PMS and BS	PMS and BS decreased Cd adsorption, but increased Cd in ryegrass
Composted leaves	Increased bioaccumulation of Cd by earthworm
CM, PM, N-Viro	Increased Cd in the organic fraction
SDSS	Increased adsorption

LSB, lime stabilized biosolid; AADB, anaerobic digested biosolid; ADB, aerobically digested biosolid; BS, biosolid; SS, sewage sludge; CM, cattle manure; PM, poultry manure; PMS, paper mill sludge; SDSS, secondary digested sewage sludge.

Significant reduction of the toxicity induced by metals in contaminated soils has been achieved using natural or synthetic minerals like sepiolite (Brigatti et al., 2000), beringite, iron oxides, and steel shots (Mench et al., 2000). Studies with sepiolite suggest that the amount of metal sorbed by the mineral increases for smaller cations. The sepiolite sorption efficiency sequence is $Pb^{2+}<Cd^{2+}<Co^{2+}<Zn^{2+}<Cu^{2+}$ for a single-metal solutions, and $Pb^{2+}=Co^{2+}<Cd^{2+}<Zn^{2+}<Cu^{2+}$ for multiple-metal solution (Brigatti et al., 2000). The advantage of this technique is the low cost and the high effect obtained in reducing the availability of cadmium in soil pore water. The drawback of this technique is the high impact on soil characteristics (e.g. pH, water availability, and soil compression) that may reduce the number of tolerant plant species that can growth in these soil characteristics.

In this thesis, the efficiency of different minerals in lowering the toxicity Cd in contaminated soils is discussed. Across natural minerals it seems that studies on bentonite used in Cd contaminated soils was not deeply studied. Several studied on bentonite were made in laboratory condition and based on the chemically adsorption (Brigatti et al., 2000; Banat et al., 2002). Therefore this thesis aimed to determine the ability of bentonite, which is widely available and low cost, in reducing the Cd mobility in contaminated soils. Efficiency of bentonite was compared with those of beringite and sepiolite.

Beringite is a mixture of modified aluminosilicates that originates from the fluidized bed burning of coal refuse (mine pile material) from the former coal mine in Beringen, Belgium (Vangronsveld et al., 2000) and it's not considered a mineral.

Table1.11. Bentonite main characteristics from Minerology database (http://webmineral.com/)

General Bentonite Information	
Chemical Formula:	Na0.5Al2(Si3.5Al0.5)O10(OH)2·n(H2O)
Composition:	Molecular Weight = 389.27 gm
	Sodium 2.95 % Na 3.98 % Na2O
	Aluminum 17.33 % Al 32.74 % Al2O3
	Silicon 25.25 % Si 54.02 % SiO2
	Hydrogen 1.04 % H 9.26 % H2O
	Oxygen 53.43 % O
Bentonite Crystallography	
Axial Ratios:	a:b:c =0.5755:1:1.6797
Cell Dimensions:	a = 5.14, b = 8.93, c = 15, Z = 2; beta = 99.54° V = 678.98 Den(Calc)= 1.90
Physical Properties of Bentonite	
Cleavage:	[001] Perfect
Color:	White, Gray, Reddish white, Brownish white, Pale yellow.
Density:	2 - 2.3, Average = 2.15
Diaphaniety:	Translucent to subtranslucent
Habit:	Earthy - Dull, clay-like texture with no visibile crystalline affinities, (e.g. howlite).
Hardness:	1-2 - Between Talc and Gypsum
Optical Properties of Bentonite	
Gladstone-Dale:	CI meas= -0.101 (Poor) - where the CI = (1-KPDmeas/KC) CI calc= -0.246 (Poor) - where the CI = (1-KPDcalc/KC) KPDcalc= 0.2731,KPDmeas= 0.2413,KC= 0.2192
Optical Data:	Biaxial (-), a=1.494-1.503, b=1.525-1.532, g=1.526-1.533, . bire=0.0300-0.0320, 2V(Calc)=20, 2V(Meas)=9-16. Dispersion none.

Table 1.12. Sepiolite main characteristics from Minerology database (http://webmineral.com/)

General Sepiolite Information	
Chemical Formula:	$Mg_4Si_6O_{15}(OH)_2 \cdot 6(H_2O)$
Composition:	Molecular Weight = 613.82 gm
	Magnesium 15.84 % Mg 26.26 % MgO
	Silicon 27.45 % Si 58.73 % SiO2
	Hydrogen 1.97 % H 17.61 % H2O
	Oxygen 54.74 % O
	100.00 % 102.61 % = TOTAL OXIDE
Sepiolite Crystallography	
Axial Ratios:	a:b:c =0.4996:1:0.1964
Cell Dimensions:	a = 13.43, b = 26.88, c = 5.281, Z = 4; V = 1,906.43 Den(Calc)= 2.14
Physical Properties of Sepiolite	
Cleavage:	[001] Perfect
Color:	Grayish white, Yellowish white, Bluish green white, Reddish white, Gray.
Density:	2
Diaphaniety:	Opaque
Habit:	Earthy - Dull, clay-like texture with no visible crystalline affinities, (e.g. howlite).
Habit:	Fibrous - Crystals made up of fibers.
Hardness:	2 - Gypsum

5. LEGISLATION

All the recent published “guidelines” or recommendations for the application of sewage sludge placed great emphasis on metals, especially on Cd concentration in agricultural soils.

In order to prevent the build up of Cd concentrations, the EU established mandatory limits (tables 1.13, 1.14 and 1.15). According to the legislation, the maximum Cd concentration ranges between 1 and 3 mg kg^{-1} soil. In the table 1.15 the EU CEC1986 shows the UE limit in the use of sewage sludge in agricultural fields. The directive regulates the use of sewage sludge in Italy and France are respectively reported in DM 471/99 and 8/1/1998 directive, respectively. The different recommendations all aim to minimise the accumulation of cadmium in soils and its transfer through the soil-plant-animal system.

Table 1.13. Legislation addressing cadmium or cadmium compounds in products /HELCOM 2002; OECD 1994/

Country/countries	Legislation
European Union*	Ban of certain uses of cadmium and cadmium compounds as stabilisers in plastics, colorants in plastics and paint etc. Also ban on certain uses of cadmium-plating and on the use of cadmium in cosmetics. Restrictions on the content of cadmium in packaging materials. Batteries and accumulators containing more than 0.025% cadmium by weight must be labelled aimed at separation followed by recycling or special disposal. Limits on the release of cadmium from toys and ceramic articles intended to be in contact with foodstuffs. A general ban on the use of cadmium in new electrical and electronic equipment is planned to take effect as of July 1, 2006 /EU 2000a/. A ban on cadmium in vehicles except for certain applications is going to take effect as of July 1,, 2003 . /EU 2000b/.
Estonia*	Ban on import/use of batteries and accumulators containing more than 0.025% cadmium by weight.
Denmark, Sweden*	Tax on batteries and accumulators containing more than 0.025% cadmium by weight.
Austria, Belgium, Denmark, Finland, Germany, Portugal, Norway and Sweden*	Limit on the content of cadmium in fertilizers.
Japan, Switzerland	Limit on the content of cadmium in fertilizers.
Canada	Limits on the release of cadmium from toys, ceramics and equipment
Australia	Limits on the release of cadmium from toys
USA	Legal arrangements on labelling and recycling of NiCd-batteries exist in several states. Cadmium is not allowed in pesticides //
Norway	Restrictions on use of cadmium in pigments, stabilizer and surface treatment. Limits on the content of cadmium in packaging and batteries.
* Regarding individual Member States and applicant countries of the European Union, these countries are only mentioned specifically if more restricted legislation than EU-legislation is in force.	

Table 1.14. Maximum concentration of metals allowed in agricultural field under sewage sludge treatments, EU (CEC 1986).

Metal	Limit CEC 1986 (mg/Kg)	
	pH 5-7	pH > 7
Cd	1-3	
Cr	100-150	
Hg	1-3	
Cu	50-140	200
Pb	50-300	
Ni	30-75	110
Zn	150-300	450

Table 1.15 EU mandatory limit for agricultural soils (CEC 1986).

Elements	Soils of urban zones (mg Kg^{-1})	soils of industrial (mg Kg^{-1})
As	20	50
Cd	2	15
Cr VI	2	15
Co	20	250
Cu	120	600
Pb	100	1000
Hg	1	5
Ni	120	500
Se	3	15
Sn	1	350
Zn	150	1500

6. LABORATORY AND FIELD EXPERIMENTS

Prior studies

Present knowledge on the effects of Cd accumulation in soil arises mainly from two types of experiments: laboratory incubations and long-term field trials.

Advantages and pitfalls of both options have been widely discussed by several authors (Tyler et al., 1989; Brookes, 1995; Giller et al., 1998).

Laboratory incubation experiments allow mainly the study of the chemical behaviour and of the acute effects of single metals on the biological and biochemical properties of the soil.

Field trials aiming at evaluating the residual effects on soil microorganisms and plants have been set up in different countries. Long-term field experiments which have provided the most consistent information on the effects of chronic soil contamination by metals on soil micro-organisms and plants in Europe are located at Rothamsted (United Kingdom) (Brookes, 1995; Chander and Brookes, 1991), Ultuna and Gusum (Sweden) (Tyler 1989), Braunschweig (Germany) (Fliessbach et al., 1994; Fliessbach and Reber, 1992) and Bordeaux (France) (Juste and Mench, 1992; Mench et al., 1994; Weissenhorn et al., 1995; Jarausch-Wehrheim et al., 1999).

Obviously, the most reliable results are those of long-term field trials because generally acute responses to fresh addition of metals do not model the long-term effects on soil microorganisms (Renella et al., 2002).

Generally long-term field trials have been established around industrial site or on soils long-term amended with sewage sludge. In this situation, soils are generally contaminated by several metals making difficult to assess the impact of specific metals. For example high concentrations of Cd are generally associated with high Cu and Zn

contamination (Juste and Mench, 1992). So far, the knowledge on the specific toxicity of Cd in soils has been seldom assessed. Soils from the AGIR long-term experiment located at Bordeaux, France, offer the unique opportunity to assess the Cd toxicity to soil microorganisms and plants because the only contaminant over the UE limits is cadmium (Broos et al., 2005).

REFERENCES

Abadia, J., Lopez-Millan, A. F., Rombola, A., Abadia, A. 2002. Organic acids and Fe deficiency: a review. Plant and Soil 241, 75–86.

Adriano, D.C., Wenzel, W.W., Vangronsveld, J., Boland, N.S., 2004. Role of assisted natural remediation in environmental cleanup. Geoderma 122, 121–142.

Al-Khafaji, A.A., Tabatabai, M.A., 1979. Effects of trace elements on arylsulphatase activity. Soil Science 127, 129-133.

Alma, R., Singh, B. R., 2001. Heavy metals in the environment plant uptake of Cadmium-109 and Zinc-65 at different temperature and organic matter levels. Journal of Environmental Quality 30, 869–877.

Angle, J, Delorme, T., Abu-Shanab, R., Chaney, R., Van Berkum, P., Soil Microbial Effects on Heavy Metal Uptake into Hyperaccumulators. Proceedings of New Phytologist Symposium, September 30, 2002, pp 154-163.

Axelsen, K.B., Palmgren, M.G., 2001. Inventory of the superfamily of P-type ion pumps in *Arabidopsis*. Plant Physiology. 126, 696–706.

Aylett, B. J., 1979. The Chemistry, Biochemistry and Biology of Cadmium. Elsevier, North-Holland, New York.

Badalucco, L., Kuikman, P.J., 2001. Mineralization and immobilization in the rhizosphere. In: Pinton, R., Varanini, Z., Nannipieri, P. (Eds.), The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface. Marcel Dekker, New York, pp 141–196.

Banat, F., Al-Asheh, S., Abu-Aitah, L., 2002. Examination of the effectiveness of physical and chemical activation of natural bentonite for the removal of heavy metal ions from aqueous solutions. Adsorption Science and Technology 20 (2), 151-167.

Basta, N.T., Ryan, J.A., Chaney, R.L., 2005. Trace element chemistry in residual-treated soil: key concepts and metal bioavailability. Journal of Environmental Quality 34 (1), 49-63.

Baize, D., 1997. Teneurs totales en éléments traces métalliques dans les sols (France). Références et stratégies d'interprétation. INRA Éditions, Paris. 410 p.

Baize, D., M. Tercé, 2002. Les éléments traces métalliques dans les sols. Approches fonctionnelles et spatiales. INRA Éditions, Paris. 570 p.

Baudoin, E., Benizri, E., Guckert, A., 2003. Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere. Soil Biology & Biochemistry 35, 1183–1192.

Belkin, S., 2003. Microbial whole-cell sensing systems of environmental pollutants Current Opinion in Microbiology 6, 206–212.

Blaudez D., Botton B., Chalot M., Cadmium uptake and subcellular compartmentation in the ectomycorrhizal fungus *Paxillus involutus*. Environmental Microbiology (2000), 146**,** 1109-1117.

Bolan, N.S., Naidu, R., Khan, M.A.R., Tillman, R.W., Syers, J.K., 1999. The effects of anion sorption on sorption and leaching of cadmium. Australian Journal Soil Research 37, 445–460.

Bolton, H., Fredrickson, J. K., Elliott, L. F., 1993. Microbial ecology of the rhizosphere. In: Soil microbial ecology. Metting F.B. (ed.) M. Dekker New York (publ.), pp. 27-63.

Brigatti, M.F.,. Lugli, C., Poppi L., 2000. Kinetics of heavy-metal removal and recovery in Sepiolite, Applied Clay Science 16, 45-57.

Brimecombe, M.J., De Leij, F.A., Lynch, J.M., 2000. Mineralization and immobilization in the rhizosphere. In: The Rhizosphere, Piton, R., Varanini, Z., Nannipieri, P., Marcel, Dekker, Inc., New York, pp. 95-141.

Brynhildsen, L., Rosswall, T., 1989. Effects of cadmium, copper, magnesium, and zinc on the decomposition of citrate by a *Klebsiella* sp. Applied and Environmental Microbiology 55, 1375–1379.

Broos, K., Uyttebroek, M., Mertens, J., Smolders, E. 2004. A survey of symbiotic nitrogen fixation by white clover grown on metal contaminated soils. Soil Biology & Biochemistry 36, 633–640.

Brookes, P.C., 1995. Use of microbial parameters in monitoring soil pollution by heavy metals. Biology and Fertility of Soils 19, 269-279.

Brown, S.L., Chaney, R.L., Lloyd, C.A., Angle, J.S., Ryan, J.A., 1996. Relative uptake of cadmium by garden vegetables and fruits grown on long-term biosolid-amended soils. Environmental Science Technology 30, 3508–3511.

Cataldo, D.A., Garland, T.R., Wildung, R.E., 1981. Cadmium distribution and fate in soybean plants. Plant Physiology 68, 835–839.

Chander, K., Joergenesen, R.G., 2001. Decomposition of ^{14}C-glucose in two soils with different amounts of heavy metal contamination. Soil Biology & Biochemistry 33, 1811–1816.

Chaney, R.L., Ryan, J.A., 1994. Risk based standards for arsenic, lead and cadmium in urban soils. DECHEMA, Frankfurt, Germany.

Chaney, R.L., Li, Y-M., Angle, J.S., Baker, A.J.M., Reeves, RD., Brown, S.L., Homer, F.A., Malik, M., Chin, M., 1999. Improving metal-hyperaccumulators wild plants to develop commercial phytoextraction systems: Approaches and progress. In: Phytoremediation of Contaminated Soil and Water, eds. N. Terry and G.S. Banuelos, CRC Press, Boca Raton, FL.

Chardonnens, A., 2000. The role of vacuolar compartmentalization in the mechanisms of naturally selected zinc and cadmium tolerance. PhD thesis Department of Ecology and Ecotoxicology of Plants, Faculty of Biology, Vrije Universiteit, De Boelelaan 1087, 1081 HV, The Netherlands.

Chaudri, A.M., Allain, C.M.G., Barbosa-Jefferson, V.L., Nicholson, F.A., Chambers, B.J., McGrath, S.P., 2000. A study of the impacts of Zn and Cu on two rhizobial species in soils of a long-term field experiment. Plant and Soil 221, 167–179.

Chlopecka, A., Adriano, D.C., 1996. Mimicked in-situ stabilization of metals in a cropped soil: bioavailability and chemical form of zinc. Environmental Science Technology 30, 3294–3303.

Christensen, T.H., 1984a. Cadmium sorption at low concentration. II. Reversibility, effects of changes in solute composition, and effects of soil aging. Water Air and Soil Pollution 21, 105-114.

Christensen, T.H., 1987b. Cadmium sorption at low concentration. VI. A model for zinc competition. Water Air and Soil Pollution 34, 304-314.

Clemens, S., 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212, 475-486.

Clemens, S., Antosiewicz, D.M., Ward, J.M., Schachtman, D.P., Schroeder, J.I., 1998. The plant cDNA *LCT1* mediates the uptake of calcium and cadmium in yeast. Proceedings of the National Academy of Sciences, USA 95, 12043-12048.

Cieslinski, G., Van Rees, K.C.J., Szmigielska, A.M., Krishnamurti, G.S.R., Huang, P.M., 1998. Low-molecular-weight organic acids in rhizosphere soils of durum wheat and their effect on cadmium bioaccumulation, Plant and Soil. 203, 109–117.

Cobbett, C.S., 2000. Phytochelatin biosynthesis and function in heavy-metal detoxification. Current Opinion in Plant Biology 3, 211–216.

Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: Roles in Heavy Metal Detoxification and Homeostasis. Annual Review of Plant Biology 53, 159-182.

Collard, J.M., Corbisier, P., Diels, L., Dong, Q., Jeanthon, C., Mergeay, M., 1994. Plasmids for heavy metal resistance in Alcaligenes eutrophus CH34: mechanisms and applications. FEMS Microbiology Reviews. 14. 405-414.

Corbisier, P., van der Lelie, D., Borremans, B., Provoost, A., de Lorenzo, V., Brown, N.L., Lloyd, J.L., Csoregi, E., Johansson, G., Mattiasson, B., 1999. Whole-cell and protein-based biosensor for detection of bioavailable heavy metals in environmental samples. Analytica, Chemica Acta 387, 235-244.

Commission of the European Communities, 1986. Council directive on the protection of the environment, and in particular the soil, when sewage sludge is used in agriculture. Official Journal European Community L81 (Annex 1A), 6-12.

Cutler, J. M. & Rains, D. W. (1974). Characterization of cadmium uptake by plant tissue. Plant Physiology 54, 67-71

Dakora, F.D., Phillips, D.A., 2002. Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant and Soil 245, 35–47.

Dawson, J., Campbell, C. D., Towers, W., Cameron, C.M., Paton, G.I., 2005. Linking biosensor responses to Cd, Cu and Zn partitioning in soils Environmental Pollution *in press.*

De, D.N., 2000. Plant cell vacuoles. Collingwood, Australia. CSIRO Publishing.

Delhaize, E., Craig, S., Beaton, C.D., Bennet, R.J., Jagandish, V.C. and Randall, P.J. 1993. Aluminium tolerance in wheat (*Triticum aestivum* L). I. Uptake and distribution of aluminium in root apices. Plant Physiology 103, 685-693.

de Miranda, JR., Thomas, MA., Thruman, DA., Tomsett, AB., 1989. Metalliothionein genes from the flowering plant *Mimulus guttatus.* FEBS Letters. 260, 277-280

De Vos, P., Claessens, F., Winderickx, J., Van Dijck, P., Celis, L., Peeters, B., Rombauts, W., Heyns, W., Verhoeven, G., 1991. Interaction of androgen response elements with the DNA-binding domain of the rat androgen receptor expressed in *Escherichia coli*. Journal of Biological Chemistry 266, 3439–3443.

Dietz, K-J., Baier, M, Krämer. U., 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad MNV, Hagemeyer J, eds. Heavy metal stress in plants: from molecules to ecosystems. Berlin: Springer-Verlag, pp. 73–97.

Eivazi, F., Tabatabai, M.A. 1990. Factors affecting glucosidase and galactosidase activities in soils. Soil Biology & Biochemistry 22, 891-897.

Elzinga, E.J., J.J.M. van Grinsven, and F.A. Swartjes. 1999. General purpose Freundlich isotherms for cadmium, copper and zinc in soils. European Journal of Soil Science 50, 139–149.

Ernst, W.H.O., Verkleij, JAC., Schat, H., 1992. Metal tolerance in plants. Acta Botanica Neerlandica 41, 229–248.

Falchini, L., Naumova, N., Kuikman, P.J., Bloem, J., Nannipieri, P., 2003. CO_2 evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation. Soil Biology & Biochemistry 36, 775–782.

Fliessbach, A., Reber, H.H., 1992. Effects of long-term sewage sludge applications on soil microbial parameters. In: Hall, J.E., Sauerbeck, D.R., L'Hermite, P. (Eds), Effects of Organic Contaminants in Sewage Sludge on Soil Fertility, Plants and Animals. Document no. EUR14236. Office for Official Publications of the European Community, Luxembourg, pp. 184–292.

Fliessbach, A., Martens, R., Reber, HH., 1994. Soil microbial biomass and microbial activity in soils treated with heavy metal contaminated sewage sludge. Soil Biology & Biochemistry 26 (9), 1201-1205.

Florijn, P.J., Nelemans, J.A., Vanbeusichem, M.L., 1992. The influence of the form of nitrogen nutrition on uptake and distribution of cadmium in lettuce varieties, Journal of Plant Nutrition 15 (11), 2405-2416.

Foyer, C.H., Noctor, G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant, Cell and Environment 28, 1056–1071.

Fritze, H., Perkiomaki, J., Saarela, U., et al., 2000. Effect of Cd-containing wood ash on the microflora of coniferous forest humus. FEMS Microbiology Ecology 32, 43–51.

Gaf, H., Ajm, B., Wart, CF., 1992. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of *Holcus lanatus* ital. L. Acta Botanica Neerlandica 41 (3), 271-281.

Garcìa-Sanchez, A., Alastuey, A., Querol, X., 1999. Heavy metal adsorpition by different minerals: application to the remediation of polluted soils. The Science of the Total Environment 242, 179-188.

Gerke, V., (1992). Evolutionary conservation and three-dimensional folding of the tyrosine kinase substrate annexin II. In The Annexins (ed. S. E. Moss). Portland Press, London, pp. 47-59.

Giller, K.E., Witter, E., McGrath, S.P., 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biology & Biochemistry 30, 1389–1414.

Goldsbrough, P., 2000. Metal tolerance in plants: the role of phytochelatins and metallothioneins. In: Terry N, Banuelos G, eds. Phytoremediation of contaminated soil and water. CRC Press LLC, pp. 221–233.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M., 1998. Sorption and desorption of cadmium from some New Zealand soils: effect of pH and contact time. Australian Journal of Soil Research 32, 199-216.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M., 1999. Solubility, sorption and desorption of native and added cadmium in relation to properties of soils in New Zealand. European Journal of Soil Science 50, 127-137.

Griffiths, B.S., Diaz-Ravina, M., Ritz, K., McNicol, J.W., Ebblewhite, N., Baath, E., 1997. Community DNA hybridisation and %GqC profiles of microbial communities from heavy metal polluted soils. FEMS Microbiology Ecology 24, 103–112.

Guckert, A., Chavanon, M., Mench, M., Morel, J.L., Villemin, G., 1991. Root exudation in *Beta vulgaris* : A comparison with *Zea mays*. In Plant roots and their environment, McMichael, B.L., H. Persson Eds., Developments in agricultural and managed-forest ecology 24, Elsevier Sciences Publishers, Amsterdam (NL), pp. 449-455.

Guerinot, ML., 2000. The ZIP family of metal transporters, Biochimica Biophysica Acta. 1465, 190-198.

Gustafsson, J.P., 2005. Modelling metal binding in soils, project. KTH.

Haanstra, L., Doelman, P., 1984. Glutamic acid decomposition as a sensitive measure of heavy metal pollution in soil. Soil Biology & Biochemistry 16, 595–600.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance, Journal of Experimental Botany 53 (366), 1-11.

Hamer, D.H., 1986. Metallothionein. Annual Review of Biochemistry 55, 913–951.

Hawkesford, M.J., 2003. Transporter gene families in plants: the sulphate transporter gene family-redundancy or specialization? Physiology Plant 117, 155–163.

Harper, M.P., Davison, W., Zhang, H., Tych, W., 1998. Kinetics of metal exchange between solids and solutions in sediments and soils interpreted from DGT measured fluxes. Geochimica Cosmochimica Acta 62 (16), 2757–2770.

Hartley-Whitaker, J., Woods, C., Meharg, A.A., 2002. Is differential phytochelatin production related to decreased arsenate influx in arsenate tolerant *Holcus lanatus*? New Phytologist 155 (2), 219-225.

Hiltner, L., 1904. Uber neue Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie. Arbeiten der Deutschen Landwirtschaftgesellschaft. 98, 59–78.

Holtan-Hartwig, L., Bechmann, M., Hoyas, TR., Linjordet, R., Bakken, LR., 2002. Heavy metal tolerance denitrifying communities: N_2O dynamics. Soil Biology & Biochemistry 34**,** 1181-1190.

Hooda, P.S., Zhang, H., Davison, W., Edwards, C., 1999. Measuring bioavailable trace metals by diffusive gradients in thin films (DGT): soil moisture effects on its performance in soils. European Journal of Soil Science 50, 285–294.

Inzé, D., Montagu, M.V., 1995. Oxidative stress in plants Current Opinion in Biotechnology . 6, 153-158.

Jarausch-Wehrheim, B., Mocquot, B., Mench, M., 1999. Absorption and translocation of sludge-borne zinc in field-grown maize (*Zea mays* L.). European Journal Agronomy 11, 23-33.

Jauerta, P.,. Schumacherb, T. E., Boeb, A., Reese, R. N., 2002. Rhizosphere acidification and cadmium uptake by strawberry clover. Journal of Environmental Quality 31, 627-633.

Jensen, A., Bro-Rasmussen, F. 1992. Environmental cadmium in Europe. Review of Environmental Toxicology 125, 101-181

Jian, M., 2005. Plant root responses to three abundant soil minerals: silicon, aluminum and iron. Critical Reviews in Plant Sciences 24 (4), 267-281(15).

Jones, D.L., 1998. Organic acids in the rhizosphere-a critical review. Plant and Soil 205, 25-44.

Jones, D.L., Darrah, P.R., 1996. Re-sorption of organic-compounds by roots of *Zea mays* L. and its consequences in the rhizosphere. 3. Characteristics of sugar influx and efflux. Plant and Soil 178, 153–160.

Jones, K.C., Johnston, E.A., 1989. Cadmium in ceral grain and herbage from long-term

experimental plot at Rothamsted, UK. Environmental Pollution 57, 199-216.

Johnson, S.E., Loeppert, R.H., 2006. Role of organic acids in phosphate mobilization from iron oxide. Soil Science Society of America Journal
70, 222-234.

Juma, N.G., Tabatabai, M.A., 1977. Effects of trace elements on phosphatase activity in soils. Soil Science Society of America Journal 41, 343-346.

Juste, C., Mench, M., 1992. Long-term application of sewage sludge and its effects on metal uptake by crops. In D.C. Adriano (Ed) Biogeochemistry of trace metals, CRC Press Inc., Boca Raton, Florida, pp. 159-193.

Kappus, H., 1985. Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. In: Sies H. (Hrsg): Oxidative Stress. Academic Press, New York, pp. 273-310.

Khan, M., Scullion, J., 1999. Microbial activity in grassland soil amended with sewage sludge containing varying rates and combinations of Cu Ni and Zn. Biology and Fertility of Soils 30, 202–209.

Kandeler, E., Tscherko, D., Bruce, K.D., Stemmer, M., Hobbs, P.J., Bardgett, R.D., Amelung, W., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil. Biology and Fertility of Soils 32, 390-400.

Koster, M, Reijnders, L., van Oost, N.R., Peijnenburg, W.J.G.M., 2005. Comparison of the method of diffusive gels in thin films with conventional extraction techniques for evaluating zinc accumulation in plants and isopods, Environmental Pollution 133, 103–116.

Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., Pakrasi, H.B., 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Molecular Biology 40, 37–44.

Kozdroj, J., van Elsas, J.D., 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches. Soil Biology & Biochemistry 32, 1405–1417.

Krishnamurti, G.S.R., Huang, P.M., van Rees, K.C.J. Kozak, L.M., Rostad H.P.V. 1995. Speciation of particulate-bound cadmium of soils and its bioavailability. The analyst 120, 659-665.

Krishnamurti, G.S.R., Huang, P.M., van Rees, K.C.J. 1997a. Kinetics of cadmium release from soils as influenced by organic acids: Implication in cadmium availability. Journal of Environmental Quality 26: 271-277.

Krishnamurti, G.S.R., Smith, L.H., Naidu, R., 2000. Method for assessing plant-available cadmium. Australian Journal of Soil Science 38(4) 823 – 836.

Krishnamurti, G.S.R., Huang, P.M., Kozak, L.M., Rostad, H.P.W., van Rees, K.C.J., 1997b. Kinetics of cadmium release from soils as influenced by organic acids: implications for cadmium availability. Journal of Environmental Quality 26, 271–277.

Kupper, H., Lombi E., Zhao, F.J., McGrath, P., 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri*. Planta 212, 75-84.

Lagriffoul, A., Mocquot, B., Mench, M., Vangronsveld, J., 1998. Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress related enzymes in young maize plants (*Zea mays* L.), Plant and Soil 200, 241–250.

Lagriffoul, A., 1998. Biomarqueurs métaboliques de la toxicité du cadmium chez le maïs (*Zea mays* L.). Mécanismes de tolérance, relation dose-effet et précocité de la réponse. Thèse de Doctorat d'Ecotoxicologie, Université Bordeaux I.

Landi, L., Valori, F., Ascher, J., Renella, G., Falchini, L., Nannipieri, P. 2006. Root exudate effects on the bacterial communities, CO_2 evolution, nitrogen transformations and ATP content of rhizosphere and bulk soils. Soil Biology & Biochemistry 38 (3), 509-516.

Lewis, S., Handy, R.D., Cordi, B., Billinghurst, Z., Depledge, M.H., 1999. Stress proteins (HSPs): methods of detection and their use as an environmental biomarker. Ecotoxicology 8, 351–368.

Llobet, J.M., Falco, G., Casas, C., Teixido, A., Domingo, J.L., 2003. Concentrations of arsenic, cadmium, mercury, and lead in common foods and estimated daily intake by children, adolescents, adults, and seniors of Catalonia, Spain. Spain Journal Agricultural Food Chemistry 51, 838–842.

Lombi, E., Zhao, F.-J., Zhang, G., Sun, B., Fitz, W., Zhang, H., McGrath, S.P. 2002. In situ fixation of metals in soils using bauxite residue: chemical assessment. Environmental Pollution 118, 435–443.

Lòpez-Bucio, J., Cruz-Ramìrez, V., Estrella, L.H., 2003. The role of nutrient availability in regulating root architecture. Current Opinion in Plant Biology 6, 280–287.

Ma, J.F., Ryan, P.R., Delhaize, E., 2001. Aluminium tolerance in plants and the complexing role of organic acids. Trends in Plant Science 6, 273–278.

Macnair, M.R., Tilstone, G.H., Smith, SE., 2000. The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G, eds. Phytoremediation of contaminated soil and water. CRC Press LLC, pp. 235–250.

Macnair, M.R., Baker, A.J.M., 1994. Metal tolerance in plants: evolutionary aspects. In: Farago ME, ed. Plants and the chemical elements. Weinheim, Germany: VCH, pp. 68-86.

McLaughlin, M.J., Smolders E., Merckx R., Maes, A., 1997. Plant uptake of Cd and Zn in chelator-bufferred nutrient solution depends on ligand type. In T. Ando (ed.) Plant nutrition-For sustainable food production and environment. Kluwer Academic Publ., Tokyo, Japan, pp. 113–118.

Marschner, H., 1995. Mineral nutrition of higher plants, Academic Press London (publ.), 889 p.

McBride, M.B., Sauvé, S., Hendershot, W., 1997. Solubility control of Cu, Zn, Cd and Pb in contaminated soils. European Journal of Soil Science 48, 337-346.

Mench, M., Morel, J.L., Guckert, A., 1988a. Action des métaux [Cd(II), Cu(II), Pb(II), Zn(II)] sur la production d'exsudats racinaires solubles chez le maïs (Zea mays L.). Agronomie 8 (3), 237-241.

Mench, M., Morel, J.L., Guckert, A., Guillet, B., 1988b. Metal binding with root exudates of low molecular weight. Journal Soil Science 39 (3), 521-527.

Mench, M., Martin, E., 1991. Mobilization of cadmium and other metals from two soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L., and *Nicotiana rustica* L. Plant and Soil 132, 187–196.

Mench, M, Fargues, S., 1994. Metal uptake by iron efficient and inefficient oats. Plant and Soil 165, 227-233.

Mench, M., Martin, E., Solda, P., 1994. After effects of metals derived from a highly metal-polluted sludge on maize (*Zea mays* L.). Water, Air, Soil Pollution 74, 1-15.

Mench, M., Bussiere, S., Boisson, J.,Castaing, E., Vangronsveld, J.,Ruttens, A., De Koe, T., Bleeker, P., Assunção, A., Manceau, A., 2003. Progress in remediation and revegetation of the barren Jales gold minespoil after in situ treatments. Plant and Soil 249, 187–202.

Mench, M., Manceau, A., Vangronsveld, J., Clijsters, H., Mocquot, B., 2000. Capacity of soil amendments in lowering the phytoavailability of sludge-born zinc. Agronomie 20, 383-397.

Mench, M., Vangronsveld, J., Didier, V., Clijsters, H., 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environmental Pollution 86, 279–286.

Mergeay, M., 2000. Bacteria adapted to industrial biotopes: Metal-resistant Ralstonia. In: Storz G., Hengge-Aronis R. (Eds), “Bacterial Stress Responses”, pp. 403-414.

Mergeay, M. 1995. Heavy metal resistances in microbial ecosystems. Molecular Microbial Ecology Manual 6. 1. 7, 1-17.

Mergeay, M., Houba, C., Gerits, J., 1978. Extrachromosomal inheritance controlling resistance to cadmium, cobalt, copper and zinc ions: evidence from curing in a Pseudomonas [proceedings]. Arch. Int. Physiol. Biochem. 86, 440-442.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., van der Lelie, D., Wattiez, R., 2003. Ralstonia metallidurans, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes, FEMS Microbiology Reviews 27 (2-3), 385-410.

Metwally, A., Finkemeier, I., Georgi, M., Dietz, K., 2003. Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiology 132, 272-281.

Miyasaka, S.C., Buta, J.G., Howell, R.K., Foy, C.D. 1991. Mechanism of aluminum tolerance in snapbeans. Plant Physiology 96, 737-743.

Naidu, R., Kookana, R.S., Sumner, M.E., Harter, R.D., Tiller, K.G., 1997. Cadmium sorption and transport in variable charge soils: a review. Journal of Environmental Quality 26, 602–617.

Naidu, R., Bolan, N.S., Kookana, R.S., Tiller, K.G., 1994. Ionic strength and pH effects on the sorption of cadmium and the surface charge of soils. European Journal of Soil Science 45, 419–429.

Nannipieri, P., Ascher, J.,Ceccherini, M.T., Landi, L., Pietramellara, G., Renella, G., 2003. Microbial diversity and soil functions European Journal of Soil Science. 54, 655.

Neal, R.H; Sposito, G., 1986. Effects of soluble organic matter and sewage sludge amendments on cadmium sorption by soils at low cadmium concentrations. Soil Science Soscak. 142, (3), 164-172.

Nolan, A.L., Zhang, H., Mike, McLaughlin, J., 2005. Prediction of zinc, cadmium, lead, and copper availability to wheat in contaminated soils using chemical speciation, diffusive gradients in thin films, extraction, and isotopic dilution techniques Journal of Environmental Quality; 34, 496-507.

Nordgren, A., Bååth, E., Sodertstrom, B., 1988. Evaluation of soil respiration characteristics to assess heavy metal effects on soil microorganisms using glutamic acid as a substrate. Soil Biology & Biochemistry 20, 949–954.

Ortiz, D.F., Kreppel, L., Speiser, D.M., Scheel, G., McDonald, G., Ow, D.W., 1992. Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. EMBO Journal 11, 3491–3499.

Palchetti, I., Laschi, S., Mascini, M., 2005. Miniaturised stripping-based carbon modified sensor for in field analysis of heavy metals, Analytical Chimica Acta 530 (1), 61-67.

Pellet, M.D., Grunes, D.L., Kochian, L. V., 1995. Organic acid exudation as an aluminum tolerance mechanism in maize (*Zea mays* L.). Planta 196, 788-795.

Pence, N.S., Larsen, P.B., Ebbs, SD., Letham, D.L.D., Lasat, M.M., Garvin, D.F., Eide, D., Kochian, L.V., 2000. The molecular physiology of heavy metal transporter in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proceedings of the National Academy of Sciences, USA 97**,** 4956–4960.

Prasad, M.N.V., 2004. Heavy metal stress in plants: from molecules to ecosystems. Berlin: Springer-Verlag, 462 p.

Quevauviller, Ph., 1998. Operationally defined extraction procedures for soil and sediment analysis I. Standardization. Trends in Analytical Chemistry
17, 289–298.

Ramos, I., Esteban, E., Lucena, J.J., Garate, A., 2002. Cadmium uptake and subcellular distribution in plants of *Lactica* sp. Cd-Mn interaction. Plant Science 162, 761–767.

Rauser, W.E., 1995. Phytochelatins and related peptides. Structure, biosynthesis and function. Plant Physiology 109, 1141–1149.

Reber, H.H., 1992. Simultaneous estimates of the diversity and the degradative capability of heavy-metal-affected soil bacterial communities. Biology and Fertility of Soils 13, 181–186.

Rea, P.A., Li, Z.-S., Lu, Y.-P., Drozdowicz, Y.M., 1998. From vacuolar GS-X pumps to multispecific ABC transporters. Annual Review of Plant Physiology and Plant Molecular Biology 49, 727–760.

Renella, G., Landi, L., Nannipieri, P., 2002. Hydrolase activities during and after the chloroform fumigation of soils as affected by protease activity. Soil Biology & Biochemistry 34, 51–60.

Renella, G., Landi, L., Nannipieri, P., 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311–315.

Renella, G., Egamberdiyeva, D., Landi, L., Mench, M., Nannipieri, P. 2006. Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biology & Biochemistry. (*in press*).

Renella, G., Landi, M., Nannipieri, P., 2005. Hydrolase synthesis in Cd-contaminated soils. Soil Biology & Biochemistry. 37, 133–139.

Renella, G., 2001. The effect of low molecular weight organic compounds on heavy metal bioavailability and toxicity in the rhizosphere. PhD thesis, University of Florence.

Rivetta, A., Negrini, N., Cocucci, M., 1997. Involvement of Ca^{2+}-calmodulin in Cd^{2+} toxicity during the early phases of radish (*Raphanus sativus* L.) seed germination. Plant, Cell and Environment 20, 600–608.

Robinson, N.J., Tommey, A.M., Kuske, C., Jackson, PJ., 1993. Plant metallothioneins. Biochemical Journal 295, 1–10.

Rufyikiri, G., Declerck, S., Dufey, J.E., Delvaux, B., 2000. Arbuscular mycorrhizal fungi might alleviate aluminum toxicity in banana plants. New Phytologist . 148, 343–52.

Saha, U.K., Taniguchi, S., Sakurai, K., 2002. Simultaneous adsorption of cadmium, zinc and lead on hydroxyaluminum- and hydroxyaluminosilicate-montmorillonite complexes, Soil Science Society of America Journal 66, 117-128.

Salt, D.E., Rauser, W.E., 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiology 107, 1293–1301.

Salt, D.E., Smith, R.D., Raskin, I., 1998. Phytoremediation. Annual Review of Plant Physiology and Plant Molecular Biology 49, 643–668.

Salt, D.E., Wagner, G.J., 1993. Cadmium transport across tonoplast of vesicles from oat roots. Evidence for a Cd^{2+}/H^{+} antiport activity. Journal of Biological Chemistry 268, 12297–12302.

Sanita` di Toppi, L., Gabbrielli, R., 1999. Review: Response to cadmium in higher plants, Environmental and Experimental Botany 41, 105–130.

Sauve, S., Cook, N., Hendershot, W.H., McBride, M.B., 1996. Linking plant tissue concentrations and soil copper pools in urban contaminated soils. Environmental Pollution 94, 153–157.

Scarponi, L., 2005. Biochimica Agraria, interazioni suolo-pianta, Patron Editore, 695-749.

Schat, H., Llugany, M., Bernhard, R., 2000. Metal-specific patterns of tolerance, uptake and transport of heavy metals in hyperaccumulating and nonhyperaccumulating metallophytes. In: Terry N, Banuelos G, eds. Phytoremediation of contaminated soil and water. CRC Press LLC, 171–188.

Senwo, Z.N., Tabatabai, M.A. 1999. Aspartame activity in soils: Effects of trace elements and relationships to otehr amidohydrolases. Soil Biology & Biochemistry 31, 213-219.

Symeonidis, L., Karataglis, S., 1992. The effect of lead and zinc on plant-growth and chlorophyll content of *Holcus lanatus* L. Journal of agronomy and crop science-zeitschrift fur acker und pflanzenbau. 168 (2), 108-112.

Smeyers-Verbeke, J., De Graeve, M., Francois, M., De Jaegere, R., Massart, D.L., 1978. Cd uptake by intact wheat plants. Plant, Cell and Environment. 1, 291–296.

Shenker, M., M. Fan, T.W., Crowley, D.E., 2001. Plant and environment interactions phytosiderophores influence on cadmium mobilization and uptake by wheat and barley plants. Journal of Environmental Quality 30, 2091-2098.

Smeets, K., Cuypers, A., Lambrechts, A., Semane, B., Hoet, P.,Van Laere, A., Vangronsveld, J., 2005. Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application, Plant Physiology and Biochemistry 43, 437–444.

Smith, S.R., 1996. Agricultural Recycling of Sewage Sludge and the Environment. CAB International, Wallingford.

Sposito, G., Lund, L.J., Chang, A.C., 1982. Trace metal chemistry in arid zone field soils amended with sewage sludge. I. Fractionation of Ni, Cu, Zn, Cd and Pb in solid phases. Soil Science Society of America Journal 46, 260–264.

Sposito, G., 1994, Chemical equilibria and kinetics in soils: New York, Oxford University Press, 268 p.

Steffens, J.C., 1990. The heavy metal-binding peptides of plants. Annual Review of Plant Physiology and Plant Molecular Biology 41, 553–575.

Tabatabai, M.A., 1977. Effects of trace elements on urease activity. Soil Biology & Biochemistry 9, 9-13.

Tessier, A., Campbell, P.G.X., Bisson, M. 1979. Sequential extraction procedure for the speciation of particular trace metals. Analytical Chemistry 51, 844-850.

Tibazarwa, C., Corbisier, P., Mench, M., Bossus, A., Solda, P. Mergeay, M., Wyns, L., van der lelie, D., 2001. A microbial biosensor to predict bioavailable nickel in soil and its transfer to plants. Environmental Pollution 113, 19-26.

Tomsett, A.B., Thurman, D.A., 1988. Molecular biology of metal tolerances of plants. Plant, Cell and Environment 11, 383–394.

Thomine, S., Wang, R., Ward, J.M., Crawford, N.M., Schroeder, J.I., 2000. Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proceedings of the National Academy of Sciences, USA 97, 4991–4996.

Tyler, G., Balsberg-Pahlson, A.M., Bengtsson, G., Bååth, E., Tranvik, L., 1989. Heavy-metal ecology of terrestrial plants, micro-organisms and invertebrates. Water Air and Soil Pollution 47, 189–215.

Tripathi, R.D., Rai, U.N., Gupta, M., Yunus, M., Chandra, P., 1995. Cadmium transport in submerged macrophyte *Ceratophyllum demersum* L. in presence of various metabolic inhibitors and calcium channel blockers. Chemosphere 31, 3783–3791.

Ure, A.M., Quevauviller, P.H., Muntau, H., Griepink, B., 1993. Speciation of heavy metals in soils and sediments. An account of the improvement and harmonization of extraction techniques undertaken under the auspices of the BCR of the Commission of the European Communities, International Journal Environment Analitycal Chemistry. 51, 135-151.

Ure, A., Davidson, C.M., 2002. Introduction. In: Chemical Speciation in the Environment, 2nd edn (eds A.M. Ure, C.M. Davidson), Blackwell Science, Oxford, pp. 1–5.

Uren, N.C., 2000. ”Types, Amounts, and possible function of coumpounds relased into the rhizosphere by soil-grown plants”. The Rizosphere, Piton R., Varanini Z., Nannipieri P., Marcel Dekker, Inc., New York, pp. 19-41.

Uren, N.C., Reisenacher, H.M., 1988. The role of root exudates in nutrient acquisition. In Advances in Plant Nutrition, Volume 3 (ed. by B. Tinker & A. Läuchli), Praeger, New York, pp. 79-114.

Van Assche, F., Clijsters, H., 1990. Effects of metals on enzyme activity in plants. Plant, Cell and Environment 13, 195–206.

Van Assche, F., Cardinaels, D., Clijsters, H., 1988. Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose-response relations in Phaseolus vulgaris L. treated with zinc and cadmium. Environmental Pollution 52, 103-115.

Vance, C.P., Uhde-Stone, C., Allan, D.L., 2003. Phosphorus acquisition and use: critical adaptations by plants for securing a nonrenewable resource. New Phytologist 157, 423-447.

Vance, CP., 2001. Symbiotic nitrogen fixation and phosphorus acquisition: plant nutrition in a world of declining renewable resources. Plant Physiology 127, 390–397.

Vancura, V., 1964. Root exudation of plants I. Analysis of root exudates of barley and wheat in their initial phases of growth. Plant and Soil 21, 231–248.

Vangronsveld, J., Mench, M., Lepp, N.W., Boisson, J., Rutten, SA., Edwards, R., Penny, C., Van der lelie, D., 2000. In situ inactivation and phytoremediation of metal- and metalloid contaminated soils: field experiments. In: Bioremediation of contaminated soils, Wise, J., Trantolo, D., Cichon, E., Inyang, H., Stotmeister,U., Eds. Marcel Dekker Inc, New York, pp. 859-884.

Vangronsveld, J., Clijsters, H., 1994. Toxic effects of metals. In: Plants and the Chemical elements. Ed. M. Farago. VCH Verlagsgesellshaft,Weinheim, Germany, pp. 149-177.

Vangronsveld, J., Sterckx, J., Van Assche, F., Clijsters, H., 1995. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. Journal of Geochemical Exploration 52, 221-229.

Vierling, E., 1991. The roles of heat shock proteins in plants. Annual Review of Plant Physiology and Plant Molecular Biology 42, 579–620.

Vig, K., Megharaj, M., Sethunathan, N., Naidu, R., 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review. Advances in Environmental Research. 8, 121-135.

Warman, P.R., Thomas, R.L. 1976. Effect of poultry manure additions on the extractability of an added metal. Communications in Soil Science and Plant Analysis 7, 405-409.

Weissenhorn, I., Mench, M., Leyval, C., 1995. Bioavailability of heavy metals and arbuscular mycorrhiza in a sewage-sludge amended sandy soil. Soil Biology & Biochemistry 27 (3), 287-296.

Welch, R.M, Norvell, W.A., 1999. Mechanisms of cadmium uptake, translocation and deposition in plants. In: McLaughlin MJ, Singh BR, eds. Cadmium in soils and plants. Dordrecht: Kluwer Academic Publishers, pp. 125–150.

Wenderoth, D.F., Reber, H.H., 1999. Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. Soil Biology & Biochemistry 31, 345–352.

Youssef, R.A., Chino M., 1989. Root-induced change in the rhizosphere of plants. I. pH changes in relation to the bulk soil. Soil Science and Plant Nutrition 35, 461–468.

Zenk, M.H., 1996. Heavy metal detoxification in higher plants - a review. Gene. 179, 21–30.

Zhang, H., Davison, W., 1995. Performance characteristics of diffusion gradients in thin films for the in situ measurement of trace metals in aqueous solution. Analytical Chemistry. 67 (19), 3391-3400.

Zhou, J., Goldsbrough, PB., 1994. Functional homologs of fungal metallothionein genes from Arabidopsis. The Plant Cell 6, 875–884.

CHAPTER TWO

THE AIMS OF THE THESIS

The aims of the thesis were:

i) to assess the effects of increasing exposure to Cd on the biomass and the activity of soil microorganisms in the rhizosphere

ii) to assess the chemical mobility and the bioavailability of Cd in the rhizosphere of soils long-term contaminated only by Cd

iii) to assess the effects of increasing exposure to Cd on plant species having different tolerance level and use of different inorganic amendments for reducing the chemical mobility and phytotoxicity of Cd in soils long-term contaminated only by Cd.

To achieve the aims, the following experimental set up and the following methods have been used:

The rhizosphere environment was simulated using the incubation units set up by Kuikman and Badalucco, according to Falchini et al. (2003). The low molecular weight organic compounds used to mimic the supply of organic compounds by living root were: glucose, citric acid and glutamic acid.

The impact of Cd on soil microorganisms was determined by measuring soil microbial biomass C and N contents, microbial respiration and inorganic N pools. The chemical mobility and bioavailability of Cd in the rhizosphere were determined by single chemical extractions using 1M NH_4NO_3 and the BIOMET® whole-cell bacterial biosensor.

The capacity of inorganic amendments to reduce Cd availability was studied using beringite combined with iron grit, sepiolite and bentonite. The reduction of Cd concentration in soil pore water was monitored using the Rhizon MOM soil moisture sampler at 0, 2, 7, and 14 days after the addition of inorganic amendments in to soil samples.

The effect of inorganic amendments in lowering the availability of cadmium in soils was monitored using three different plants species, i.e. dwarf bean, lettuce, and velvet grass, grown in remediated soils.

The next chapters are presented here as scientific articles but no manuscripts has been yet revised from the scientific journals.

CHAPTER THREE

BIOSENSOR USE TO QUANTIFY CADMIUM BIOAVAILABILITY IN THE RHIZOSPHERE

Valori Federico [a], Landi Loretta [a], Mench Michel [b], Renella Giancarlo [a] and Nannipieri Paolo [a]

[a] Department of Soil Science and Plant Nutrition University of Florence, P.le delle Cascine, 28, 50144, Florence, Italy

[b] UMR BIOGECO INRA 1202, Ecologie des Communautés, Université Bordeaux 1, Bât B8, RdC Est, 002, Avenue des Facultés F-33405 Talence, France

1. INTRODUCTION

A biosensor is a biological device for determining the availability of one (or several) chemical compound(s) to one or several organisms and /or the concentration of chemical compound(s) or it/their chemical species in a medium. The whole bacterial biosensors were developed to determine, in a medium, the bioavailability of chemical compounds and/or their concentration or chemical species. Whole bacterial biosensors are genetically modified by paring a gene that is capable to generate a measurable signal when stimulated from the physical and chemical change that occurs when the contaminant enters into the biosensor. The genes which expression is a measurable response indicating the degree of chemical or physical change is called reporter gene (Strosnider, 2003; Biran et al., 2003; Tauriainen et al., 2000; Turpeinen et al., 2003; Daunert et al., 2000).

The bioavailable fraction is the fraction of the total concentration of the chemical present in soil that is in contact and available for uptake by an organism (Klaassen, 2001).

In assessing the Cd toxicity to the microbiota, it is important to distinguish between the total Cd concentration in the environment and the Cd amount available for microbial uptake and accumulation. Cadmium accumulation is highly toxic for the cells as it is not an essential element required to mediate cellular metabolic reactions. Cadmium enters the microbial cells mainly through the same systems involved in the transport of

essential cations such as Zn, Mg, Ca and Mn (Nies, 1999), or use some transport systems for Fe and Mn (IRT, Nramp) as described in the introduction of this thesis.

Only the fraction that enters into the cell should be considered potentially toxic (Strosnider, 2003). The availability of Cd, or any other contaminant, is controlled by the physico-chemical and biological characteristics of the environment into which the contaminant is deposited, and by the chemical speciation of the contaminant. In the rhizosphere, the metal bioavailability is controlled by several soil factors including e.g. soil pH, texture, types of microorganisms and the particular abundance of LMWOAs (low molecular organic weight acids) which may originate from root exudates, soil microorganisms (including mycorhiza), and soil organic matter (oxidation, degradation, ect). Gray et al. (1998) reported the link between bioavailability (mobility and solubility) and cadmium toxicity. There is a good relationship between the chemically labile fractions (e.g. water soluble, soil pore water, and exchangeable) and the root absorption of trace elements (Kostera et al., 2005).

Conversely, the link between metal mobility and bioavailability to soil microorganisms is still not clear. Furthermore although the chemically labile pools of metals such as Cd, Ni and Zn were correlated to their content in plants, the relationship between these pools and the responses of soil microflora is still unclear.

Developments in molecular biology techniques have delivered bacterial biosensors responding to metal entry into the microbial cell (Hansen et al., 2001; Kohler et al., 2000). Biosensors have the advantages to measure potential amount of Cd in soil able to be in contact with and/or taken by living organisms and, as described in the paragraph 1.1, this fraction is termed bioavailable. Several chemical and physical methods were used to estimate the Cd bioavailability in soil or water sediments. These methods were already explained in the introduction of this thesis (see chapter 1, par.2.4). Briefly the methods are: single or sequential extraction (Tessier et al., 1979; Ure and Davidson, 2002; Krishnamurti et al., 2000), soil moisture samples (Rhizon), DGT (Hooda et al., 1999), screen electrode (Palchetti et al., 2004) and biosensors (Strosnider, 2003; Biran et al., 2003).

In the last decades rising interest was focused on bioindicators which are on-site measurements of metal ions levels (Ji and Silver, 1992; Corbisier et al., 1999; Paton et al., 1995; Tauriainen et al., 1997; Roberto et al., 2002).

The most common reporter gene used in biosensors was the *luc* from firefly *Photinus pyralis*. The *luc* operons produce the enzyme luciferase, capable of generating the luminescence. The activation of the gene results in the transcription or reading of the *luc* operon. Luciferase enzymes catalyze the oxidation of D-luciferin, its substrate, which

binds on the enzyme's active site. The light produced can be measured with different methods, including luminometer (Daunert et al., 2000).

There are few studies using long-term field trials which reported the effects of Cd as the sole metal on the composition and the activity of the soil microflora (Renella et al., 2004). Less information is available on the effect of roots exudates on Cd bioavailability in the same Cd-contaminated soils. This study aimed to assess the specific effects of long-term field exposure to elevated concentrations of Cd as the sole contaminant on its bioavailability in sandy soil, this soil type being potentially a worse case with usually highest Cd mobility compared to loamy and clay soils. Biosensor BIOMET® and model root system (MRS) were used to determine Cd bioavailability in sandy soils from long-term field plots containing increasing Cd concentrations and treated with various MRE. The expected results from this experiment were the direct correlation between the presence and concentration of some compounds (i.e. glucose, citric acid and glutamic acid), normally founded in the root exudates, and the bioavailability of cadmium in the rhizosphere. The ligand properties of citric and glutamic acids are well known, but no studies were reported using biosensor technique to find the effect of these compounds in the rhizosphere of Cd-contaminated soils.

1.1 The BIOMET® Biosensor

In work presented here the BIOMET® biosensor has been used.

The BIOMET® biosensors were developed in VITO (Vlaamse Instelling voor Technologisch Onderzoek, Mol, Belgium (Mergeay et al., 1978).

Microorganisms from very highly contaminated soils develop different metal resistance mechanisms (Mergeay et al., 1978). In Gram-positive bacteria, the Cd-resistance is typically encoded by P-type ATPase efflux pumps, whereas in Gram-negative bacteria a three-component cell membrane efflux protein is responsible for ejection of Cd^{2+} ions out of the cells: czcCBAxRS Cd, Zn and Co, chromosome and pMOL28 and pMOL30 megaplasmid encoding cation/proton transmembrane antiporter pumps (fig.3.1) (Mergeay, 2000). The ability of such bacteria to survive at high metal concentrations is encoded into defined genes. These genes are organized in operons in the DNA. Starting from these particular bacteria the suggestive idea to develop microorganisms genetically modified by coupling bacteria to transducers that convert a cellular response into detectable signals became reality (Mergeay et al., 1978; Mergeay, 2000; Strosnider, 2003; Biran et al., 2003).

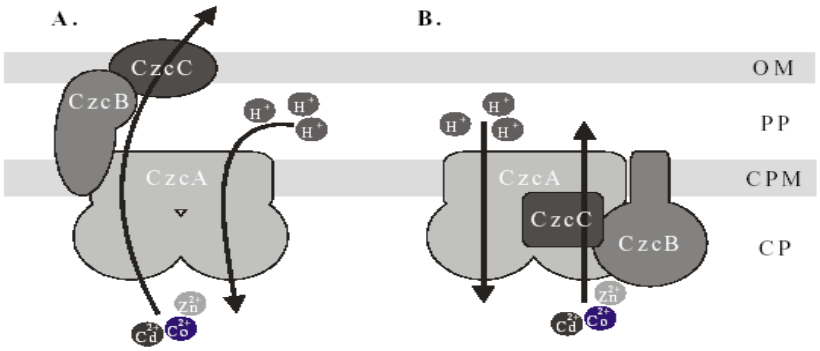


Fig. 3.1 czcCBAxRS Cd, Zn and Co, chromosome and pMOL28 and pMOL30 megaplasmid encoding cation/proton transmembrane antiporter pumps in *Ralstonia metallidurans*.

The BIOMET® is based on *lux* reporter gene. The *lux* reporter gene is similar to the *luc*-gene based biosensor, explained above: the BIOMET® uses a relevant soil bacterium (*Ralstonia metallidurans*) which is able to defend itself well against contaminant (metal) potentially harmful when one of its chemical species is present at concentration above a threshold value. The bacterium is modified, so that it gives a light reaction when specific metals go into the cell. Measuring the quantity of light is simple and the test is highly reproducible. Moreover, apart from grinding a coarse solid sample, no other sample preparation is needed. The bacterium is brought into direct contact in a suspension with the sample to be tested (Mergeay et al., 2003).

In *Ralstonia metallidurans*, the *czc* genes are inserted with the promoterless *lux CDABE* gene of *Vibrio fisherii* (fig. 3.2) so that the recombinant strain AE 1433 emits bioluminescence specifically upon Cd induction. One of the advantages of BIOMET® that must be underlined is that the result of bioavailable cadmium shows up only in the measurement of Cd that enters into the biosensor cell, so it correctly represents the measurement of Cd uptake and its toxicity.

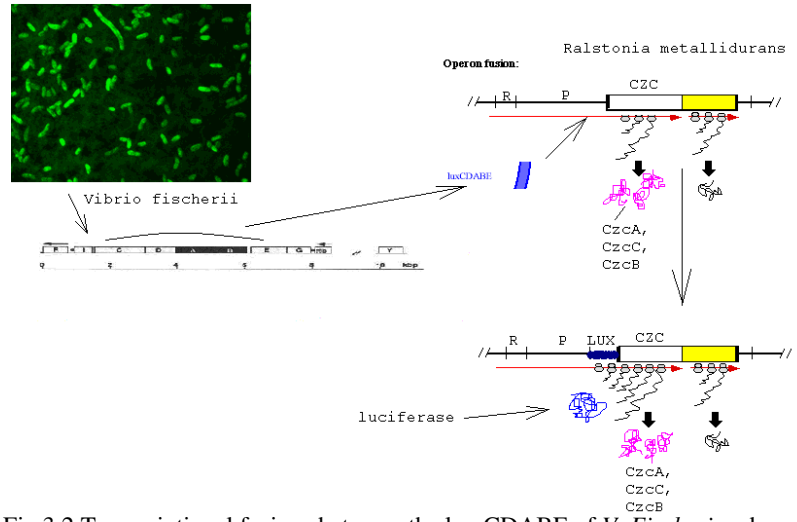


Fig.3.2 Transcriptional fusions between the lux CDABE of *V. Fischeri* and promoters specifically induced by metals in *R. metallidurans* CH34 carrying plasmid-borne and chromosomal resistance genes to different metals

2. MATERIALS AND METHODS

2.1. *Soils*

Soils are classified as sandy-clay, Arenic Udifluvent. The AGIR field experiment was managed by Unite´ d'Agronomie, INRA, Bordeaux, France (Renella et al., 2004). The cadmium contamination of the soil was achieved as described below.

The density of the 0–30 cm soil layer was determined and it was calculated as dry soil (fine fraction <2 mm) per ha and per m^2. The different plots (6 m X 3 m) have been designed at the experimental site in 1987. Then, the amount of $Cd(NO_3)_2$ (Rectapur Quality, Prolabo) to be added to each plot was calculated for contaminating the 0–30 cm soil layer at Cd concentrations of 0, 0.09, 0.18, and 0.36 mmol kg^{-1} soil; it was spread on the soil surface and mixed by rototilling at 0–30 cm depth in the 1988–1990 period. The soil was ploughed at 0–30 cm depth through the years to ensure an even distribution of Cd within the soil layer. Chemical analyses demonstrated that the target Cd contaminations were reached. The background Cd concentration in the control soil was ~0.006 mmol kg^{-1}. The soils have been kept under grass and maize cultivation. In table 3.1 the main characteristics of AGIR soils are reported (Renella et al., 2001, 2005).

Table 3.1. Main characteristics of AGIR soils

Plots	P16	P11	P6	P1
Clay (%)	17.5	17.5	17.5	17.5
Silt (%)	15.0	15.0	15.0	15.0
Sand (%)	67.5	67.5	67.5	67.5
PH (H_2O)	7.4	7.2	7.1	6.8.
Total Cd (mg Cd/Kg soil)	0.7	9.8	18.9	38.7
TOC (%)	0.95	0.25	0.47	0.49
N tot (%)	0.10	0.03	0.04	0.04

The four AGIR soils (plots P1, P6, P11 and P16) were sampled in the 0-0.30 cm soil layer in October 2004, air-dried, sieved (<2mm, stainless steel and wood), and stored in the dark at the constant temperature of 4 °C, prior to the experiments.

2.2. *Soil treatments and incubation conditions*

Seventy g of moist soil samples equivalent to 63 g dry weight (dw) were placed in plastic cylinders (68 mm diameter, 20 mm height), carefully pressed by a stainless steel piston to a fixed density of 1.4 g dm^{-3} and incubated at 25° C and watered with distilled water to the 50% water holding capacity (WHC) for 7 days prior to treatments with different root exudates. Thereafter the incubation units were covered by a cellulose round filter paper (Whatman 41, 68 mm diameter) for mimicking the root surface (fig.3.3), and moistened with 1 mL of deionised water (control) or root exudates solutions containing glucose, citric acid, or glutamic acid, to give a rate of 200 mg of root exudates-C kg^{-1} soil. This rate is similar according to the amounts of C that a root is able to release per g of soil (Uren et al., 2000). Soils were incubated in the dark at 25 °C for 0, 4, 7 and 14 days. During the incubation, four equal doses of root exudates equivalent to 200 mg C kg^{-1} soil were added after 0, 2, 4 and 7 days of incubation. After root exudates additions, the soil moisture content did not exceed 55% of WHC for all soils. At the end of each selected incubation periods, soils were sampled from 0-2 mm (rhizosphere soil) and > 4 mm (bulk soil) distance from the root surface, as described by Falchini et al. (2003), (figs.3.4 and 3.5), and analysed for bioavailable Cd. All the treatments and analytical measurements were triplicated.

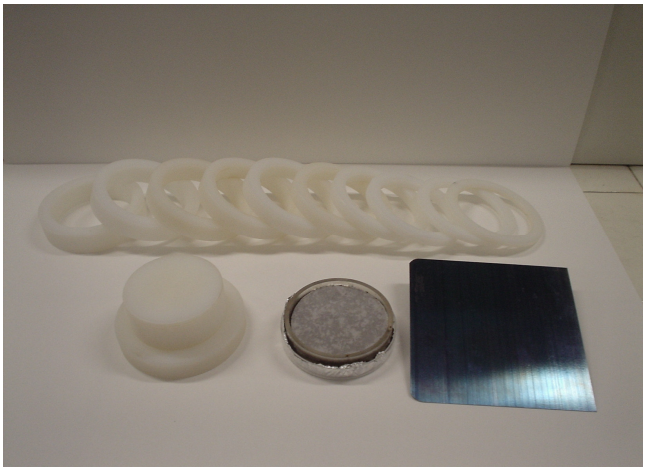


Fig 3.3 The sample unit, in the centre with all the tools necessary for the preparation of samples.

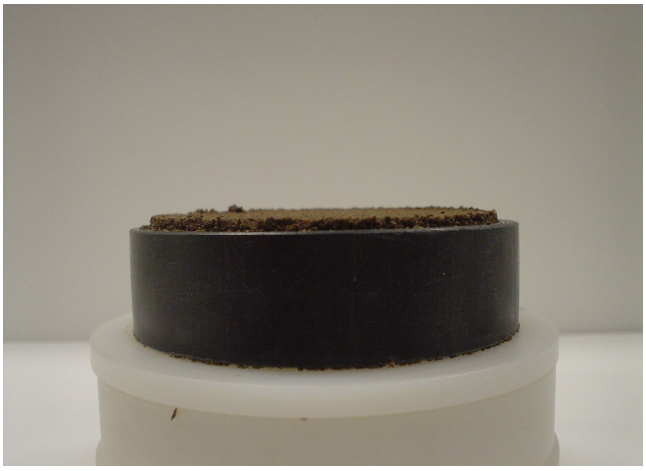


Fig 3.4 Piston pushing the soil sample to obtain soil layers either a rhizosphere or bulk soil sample

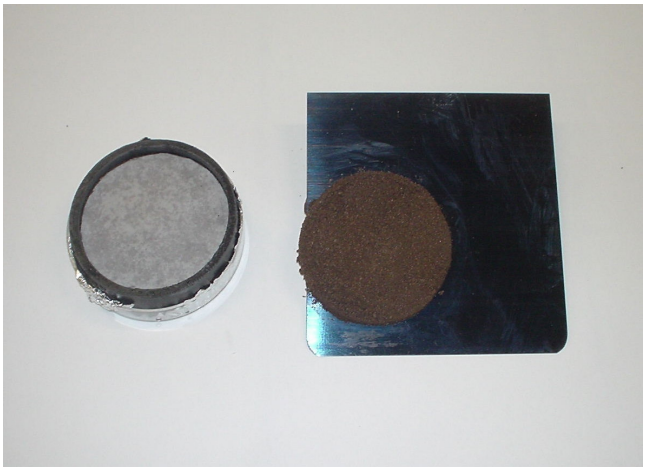


Fig 3.5. Soil obtained from the cut

2.3. *Cadmium bioavailability*

Cadmium bioavailability was estimated by using the BIOMET® biosensor based on *R. metallidurans* AE1433 carrying the *czc* operon coding for Cd, Zn and Co resistance, in transcriptional fusion with the *luxCDABE* operon of *V. fischerii* (Corbisier et al., 1999). When the intracellular Cd concentration exceeds a threshold value, expression of the *czc* operon is induced and the *lux* gene is co-expressed with production of bioluminescence. The light signal was detected using an Anthos LUCY1 luminometer (Anthos Labtech b.v., Heerhugowaard, The Netherlands) at 23°C (Corbisier et al., 1999). Soil samples (5 g fresh soil) were suspended with 30 mL of reconstitutional medium (RM) containing 0.1 % of gluconate as C-source medium and stirred for 10 minutes. Four mL of the suspension were transferred to a glass vial of 20 mL, and two dilutions, 1:2 and 1:4 were made with the RM medium. Freeze dried bacterial cells were incubated overnight at 30 °C in rich medium 869 containing 20 µL of tetracycline. The cultures were centrifuged at 4000 rpm and the pellets were re-suspended in rich medium 869 (Vanbroekhoven et al., 2003). Twenty µL of soil suspension were put into wells of a black 96-well microtitre plate. All wells were then added with 180 µL of a diluted culture (final OD660 of 0.1% in RM-GL) of the AE1433 biosensor strain was added to each well. Other twenty µL of soil suspension were added with 180 µL of a diluted culture (final OD660 of 0.1% in RM-GL) of the *R. metallidurans* AE864 biosensor strain used as a positive control. The AE864 biosensor is a constitutively light emitting strain and taken in account the measurement of a decrease in light emission due to the possible toxicity of the suspensions of soil and/or to the quenching of light due to the soils particles and eventual stimulatory effects of the MRE. Eight reaction samples containing deionised water were included in the test as negative control. The calibration curve was prepared with cadmium solution standards (3 µM, 6 µM, 12 µM, 24 µM, 48 µM, 96 µM, and 192 µM) from a 1M $CdCl_2$ stock solution. The measurement of samples, standards and control strain samples were repeated twice. The bioluminescence emitted (ALU) and the optical density (OD620 nm) of the culture was measured over 16 h at 30 min intervals, and processed using the MIKROWIN software (Mikrotec Laborsysteme, Overath, Germany), (Corbisier et al., 1999). The induction of bioluminescence (presented as the signal to noise, ratio S/N) was calculated as the light production value (S) found for the soils tested, divided by the light production value found for the bacterial exposed to water (R) and of the control biosensor strain AE864. Induction was considered significant when the S/N ratio was

greater than 2 while a S/N value below 2 indicated that the sample do not contain a bioavailable concentration.

Net S/N ratio was also calculated by subtracting the S/N values of control soils from those of soils treated with root exudates.

The strain used, AE1433 and AE864, gave us information about the nature of the toxicity in the samples. AE1433 strain is metal-sensitive while AE864 is metal-resistant. In all samples, the toxicity is due to inorganic forms of cadmium, in fact there is no decrease in the AE864 metal resistant strain, it means that toxicity is not caused by organic compounds (Vanbroekhoven et al., 2003).

Treatments with citric acid and glutamic acid did not change the soil pH value (table 4.2). Furthermore during the tests with the BIOMET, the pH of RM medium used in the assays was pH ≈7.

2.4. *Statistical analysis*

Statistical analysis was undertaken to investigate possible relationship between the biosensor results, cadmium content in bulk and rhizosphere soil and different soil treatments. The LSD values (Tukey-Kramer test, P level < 0.05) were calculated to assess the significance of differences of the means (n = 3). The significant differences within the experiment are discussed in the results paragraph.

3. RESULTS

3.1. *Bioavailable Cd*

The amount of bioavailable Cd signalled by the BIOMET biosensor varied among the soils, treatments and incubation times. Bioavailable Cd concentrations were low compared to the total Cd concentrations, indicating a low availability of Cd for bacteria. For soil from plot 16 (0.7 mg total Cd kg^{-1}), in all treatments the signal-to-noise ratio values were not significantly different from 1, in both the rhizosphere and bulk soils (table 3.2). For soil from plot 11, higher signal-to-noise ratio values were detected for the citrate and glutamate treatments after 4d in both rhizosphere and bulk soil. In the rhizosphere soil layer, glutamic acid after 4d of incubation increased significantly Cd bioavailability compared to other treatments (table 3.3). Citric acid and glucose treatments after 7d did not increase Cd bioavailability. In soil from plot 6 (18.9 mg total Cd kg^{-1}) and plot 11 (9.8 mg total Cd kg^{-1}), the signal-to-noise ratio was <2 for all treatments and incubation times in both rhizosphere and bulk soil (table 3.4), and thus no bioavailable Cd was detected. In the case of plot 11 in which S/N were >2, there were no statistically significant. Only in soils from plot 1 (38.7 mg Cd kg^{-1}), values significantly higher than 2 were found in the glucose, citrate and glutamate treatments after 4d and 7d (only for citrate and glutamate treatments) in both the rhizosphere and bulk soils (table 3.5). At times 0 and 14d the S/N values of all treatments were higher than 2, but the difference among the treatments however were not significant ($p>0.05$).
In plot 1 the influence of the treatments at T=7d was significant for all treatments both in the rhizosphere and bulk soil. The effect at T=14d did not show a marked difference within the different treatments. In soil from plot 1, the signal-to-noise ratio values ranged between 2.06 and 2.76 for the control treatment for all the incubation times in

both the rhizosphere and bulk soil (table 3.6). Glucose treatments ranged between 1.86, at T=7d and 2.84 at T=4d. Figure 6 reported the net value of S/N ratio obtained after the subtraction of the control soil value to the other treatments for the plot 1. Figure 7 reported the cadmium bioavailability value (mg Cd Kg^{-1} soil) corresponding to the net S/N values reported in fig.3.6.

The value below zero means that there was no influence of the treatments on the Cd bioavailability. In the rhizosphere soil, the glucose treatments had an influence only after 4d of incubation. Thereafter the effect disappeared (fig.3.7). In bulk soil, no effects of glucose were observed. The effect of citric acid and glutamic acid treatments on the Cd bioavailability was observed in the rhizosphere and bulk soil, with a greater effect of citric acid in the rhizosphere (the value of bioavailable Cd increased from 4 mg Cd Kg^{-1} at 4d to about 10 mg Cd Kg^{-1} at T=7d).

Bioavailable Cd released due to glutamic acid dose from 2 mg Cd Kg^{-1} at T=4d to about 7 mg Cd Kg^{-1} at T=7d and then decreased from 2 mg Cd Kg^{-1}. In bulk soil, the trend was similar for citric acid and glutamic acid but the values were lower. In Fig.3.6 and 3.7 the graph of bioavailable Cd of Plot 16 and 1 were reported.

Table 3.2 Plot 16 (0.7 mg Cd kg^{-1}), signal to noise value, S/N >2 represented a sample detected by biosensor as toxic. No significant differences were found in all the treatments.

	RHIZOSPHERE				BULK SOIL			
UNTREATED	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,375	1,296	1,377	1,251	1,375	1,222	1,252	1,219
av. mg Cd eq. / kg dr.soil	1,035	1,202	1,233	2,750	1,035	0,884	0,804	1,584
St.Dev	0,229	0,035	0,127	0,141	0,229	0,110	0,027	0,093
St.Dev mg Cd eq. / kg dr.soil	0,229	0,138	0,406	0,169	0,229	0,437	0,089	0,152
GLUCOSE	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,375	1,131	1,439	1,213	1,375	1,304	1,248	1,134
av. mg Cd eq. / kg dr.soil	1,035	0,840	3,108	1,101	1,035	2,102	1,547	0,628
St.Dev	0,229	0,096	0,040	0,116	0,229	0,092	0,078	0,043
St.Dev mg Cd eq. / kg dr.soil	0,229	0,245	0,619	0,230	0,229	0,425	0,540	0,173
CITRIC A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,375	1,349	1,270	1,059	1,375	1,508	1,388	1,107
av. mg Cd eq. / kg dr.soil	1,035	2,442	2,371	0,317	1,035	4,016	1,785	0,500
St.Dev	0,229	0,063	0,063	0,071	0,229	0,059	0,271	0,053
St.Dev mg Cd eq. / kg dr.soil	0,229	0,317	0,286	0,039	0,229	0,711	1,011	0,273
GLUTAM.A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,375	1,586	1,375	1,142	1,375	1,558	1,285	1,104
av. mg Cd eq. / kg dr.soil	1,035	4,603	1,732	0,906	1,035	6,555	1,415	0,568
St.Dev	0,229	0,143	0,016	0,257	0,229	0,076	0,137	0,093
St.Dev mg Cd eq. / kg dr.soil	0,229	0,686	0,292	0,161	0,229	0,515	0,606	0,163

Table 3.3. Parcel 11(9.8 mg Cd kg^{-1}), signal to noise value, S/N >2 represented a sample detected from biosensor as toxic. No significant differences were found in all the treatments.

	RHIZOSPHERE				BULK SOIL			
UNTREATED	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,633	1,572	1,709	1,348	1,633	1,630	1,695	1,502
av. mg Cd eq. / kg dr.soil	3,244	5,569	2,753	1,449	3,244	3,701	2,722	2,491
St.Dev	0,325	0,163	0,115	0,146	0,325	0,076	0,067	0,092
St.Dev mg Cd eq. / kg dr.soil	0,325	3,299	0,937	0,838	0,325	2,785	0,762	1,100
GLUCOSE	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,633	1,698	1,938	1,241	1,633	1,568	1,832	1,484
av. mg Cd eq. / kg dr.soil	3,244	1,987	2,747	1,227	3,244	3,868	5,446	1,767
St.Dev	0,325	0,079	0,130	0,076	0,325	0,057	0,108	0,154
St.Dev mg Cd eq. / kg dr.soil	0,325	2,097	1,068	1,032	0,325	0,607	0,709	0,117
CITRIC A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,633	1,941	1,635	0,995	1,633	1,945	1,750	1,099
av. mg Cd eq. / kg dr.soil	3,244	2,534	2,719	0,604	3,244	2,484	3,151	0,738
St.Dev	0,325	0,135	0,090	0,079	0,325	0,208	0,097	0,014
St.Dev mg Cd eq. / kg dr.soil	0,325	2,246	2,430	1,590	0,325	0,095	0,283	0,110
GLUTAM.A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,633	2,288	1,955	1,156	1,633	2,057	1,804	1,121
av. mg Cd eq. / kg dr.soil	3,244	3,385	4,012	1,013	3,244	3,684	4,281	1,225
St.Dev	0,325	0,165	0,067	0,079	0,325	0,078	0,110	0,023
St.Dev mg Cd eq. / kg dr.soil	0,325	0,353	1,559	1,075	0,325	0,908	4,338	0,754

Table 3.4. Plot 6 , (18.9 mg Cd kg^{-1}), signal to noise value, S/N >2 represented a sample detected by biosensor as toxic. No significant differences were found in all the treatments.

	RHIZOSPHERE				BULK SOIL			
UNTREATED	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,410	1,618	1,252	1,635	1,410	1,502	1,214	1,737
av. mg Cd eq. / kg dr.soil	3,130	2,002	1,701	4,030	3,130	1,597	0,501	2,228
St.Dev	0,233	0,050	0,081	0,055	0,233	0,111	0,098	0,219
St.Dev mg Cd eq. / kg dr.soil	0,233	0,161	0,218	0,150	0,233	0,270	0,157	0,286
GLUCOSE	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,410	1,627	1,345	1,920	1,410	1,494	2,070	1,673
av. mg Cd eq. / kg dr.soil	3,130	3,339	2,254	6,045	3,130	2,363	9,506	2,443
St.Dev	0,233	0,176	0,298	0,391	0,233	0,121	0,880	0,211
St.Dev mg Cd eq. / kg dr.soil	0,233	0,559	0,848	0,172	0,233	1,432	0,686	0,654
CITRIC A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,410	1,475	1,887	1,647	1,410	1,295	1,456	1,470
av. mg Cd eq. / kg dr.soil	3,130	2,123	4,588	2,159	3,130	2,372	3,410	1,165
St.Dev	0,233	0,240	0,223	0,024	0,233	0,079	0,056	0,038
St.Dev mg Cd eq. / kg dr.soil	0,233	0,913	0,892	0,181	0,233	0,164	0,275	0,364
GLUTAM.A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	1,410	1,517	1,823	1,384	1,410	1,385	1,733	1,547
av. mg Cd eq. / kg dr.soil	3,130	2,490	4,328	1,727	3,130	1,676	4,929	1,360
St.Dev	0,233	0,036	0,076	0,160	0,233	0,137	0,130	0,268
St.Dev mg Cd eq. / kg dr.soil	0,233	0,498	0,305	0,547	0,233	0,781	1,898	0,266

Table 3.5. Plot 1 (38.7 mg Cd kg^{-1}) signal to noise value, S/N >2 represented a sample detected by biosensor as toxic. (*, significant statistical values p<0.05)

	RHIZOSPHERE				BULK SOIL			
UNTREATED	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	2,361	2,226	2,060	2,426	2,361	2,290	2,081	2,760
av. mg Cd eq. / kg dr.soil	5,812	5,348	3,820	7,843	5,812	6,462	3,914	6,562
St.Dev	0,662	0,279	0,181	0,284	0,662	0,299	0,146	0,314
St.Dev mg Cd eq. / kg dr.soil	0,840	0,966	0,275	2,832	0,840	3,832	0,745	1,765
GLUCOSE	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	2,361	2,836	1,853	2,794	2,361	2,485	1,738	2,639
av. mg Cd eq. / kg dr.soil	5,812	8,040	3,089	6,451	5,812	5,162	3,074	6,116
St.Dev	0,662	0,409	0,229	0,540	0,662	0,206	0,090	0,465
St.Dev mg Cd eq. / kg dr.soil	0,840	1,330	0,837	0,744	0,840	0,952	0,482	2,302
CITRIC A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	2,361	3,991	6,008*	3,324	2,361	2,852	4,2408*	3,418
av. mg Cd eq. / kg dr.soil	5,812	9,338	13,920*	10,568	5,812	7,090	11,259*	10,121
St.Dev	0,662	0,946	1,372	0,339	0,662	0,419	0,664	0,261
St.Dev mg Cd eq. / kg dr.soil	0,840	2,144	3,437	0,808	0,840	1,960	2,009	1,349
GLUTAM.A.	T0	T4	T7	T14	T0	T4	T7	T14
avarage (av.) S/N	2,361	3,478	4,903*	3,468	2,361	3,597	4,385*	3,648
av. mg Cd eq. / kg dr.soil	5,812	7,722	10,67*	10,306	5,812	8,099	9,254*	11,081
St.Dev	0,662	0,172	0,360	0,275	0,662	0,625	0,621	0,273
St.Dev mg Cd eq. / kg dr.soil	0,840	0,974	2,849	0,982	0,840	1,902	1,978	1,487

Fig.3.6. The S/N ratio in plot 1the symbols *, indicates significant statistical values p<0.05.

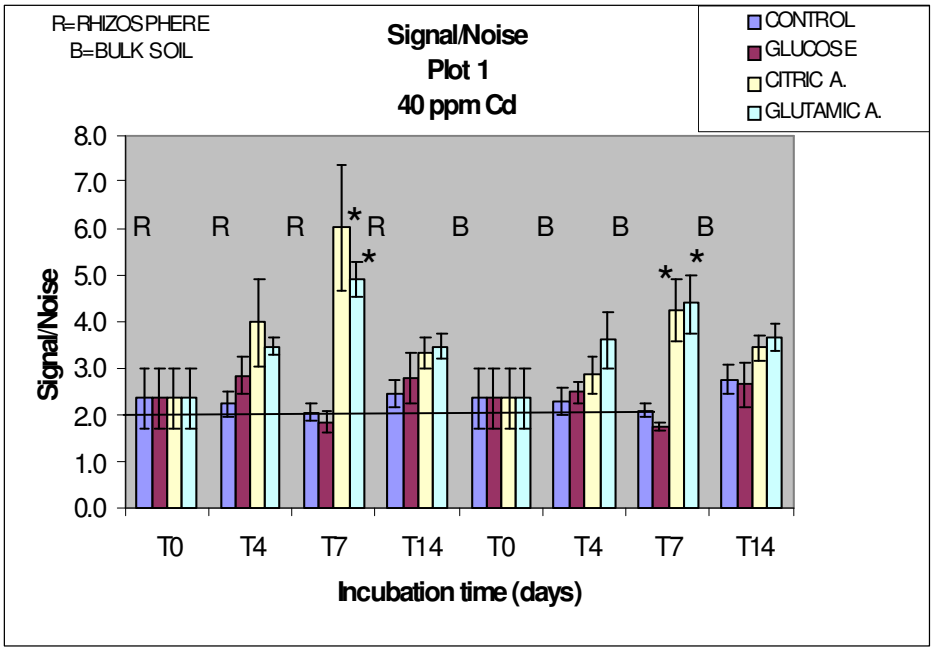


Fig.3.7. The S/N ratio in plot 16 the symbols *, indicates significant statistical values p<0.05.

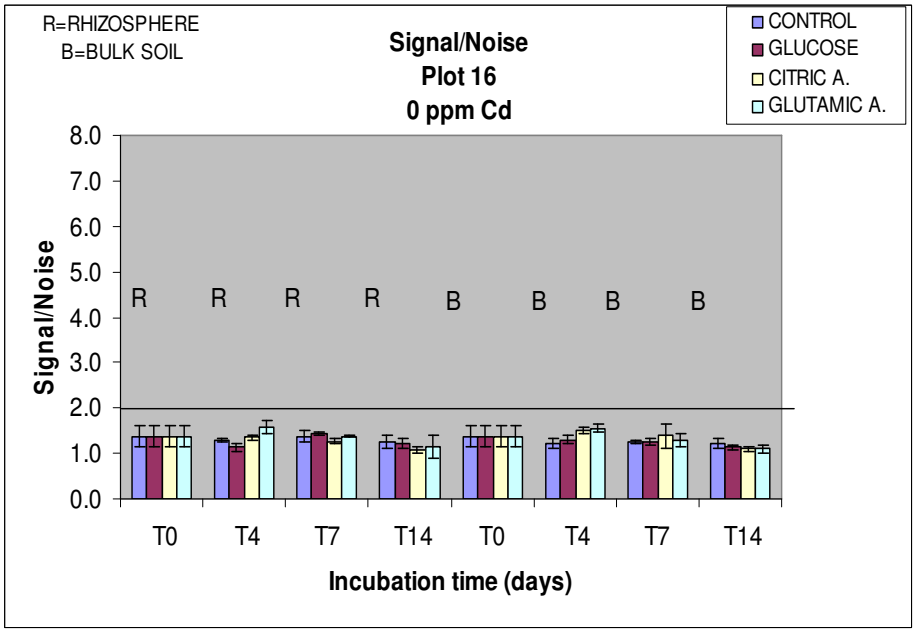


Fig.3.8. The net value of S/N ratio obtained from the subtraction of the control value to the other treatments in plot 1.

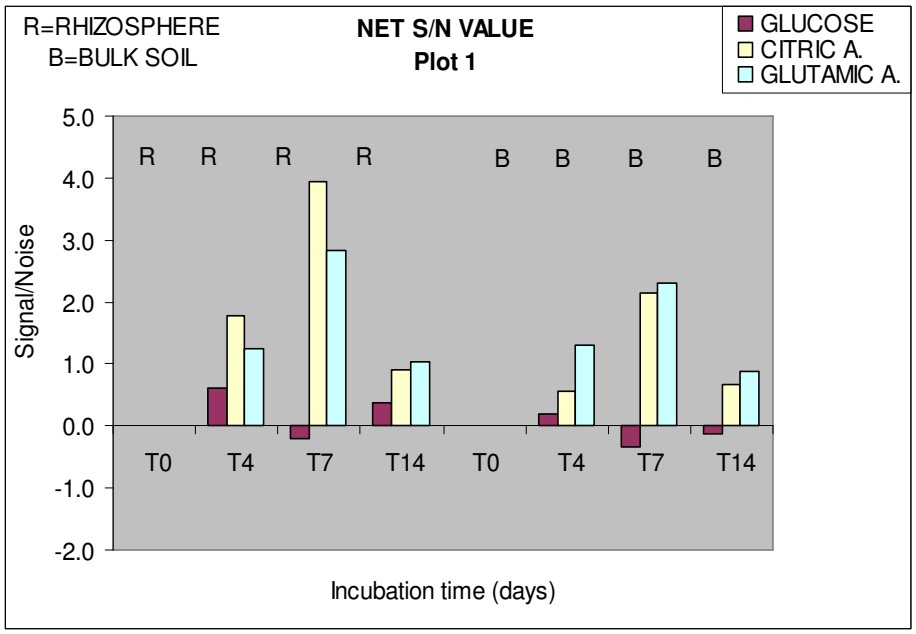
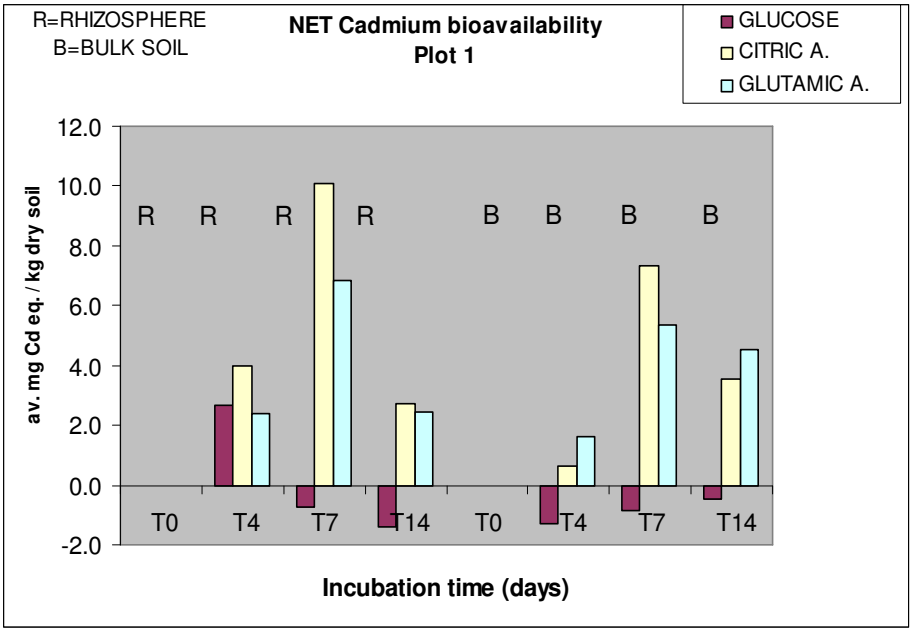


Fig.3.9. The net value of cadmium bioavailability ratio obtained from the subtraction of the control value to the other treatments in plot 1.



4. DISCUSSION AND CONCLUSION

The concentration or chemical speciation of cadmium present in soils of plots 16, 11, and 6 was insufficient to detect an influence of model root exudates on the Cd bioavailability. According to Renella et al., (2004), only for the case of soils with a total Cd content of 40 mg kg^{-1} in plot 1 there was a detection of Cd bioavailable by the BIOMET® biosensor (table 3.5). The higher Cd bioavailability value was obtained by citric acid treatments, after 7 d of incubation (table 3.5). The converted S/N value used to estimate the Cd concentration was 6.0 mg kg^{-1}, three times more compared to the control value of the same plot (2.36 mg kg^{-1}).

This value was in agreement with Chen et al. (2003), which stated that citric acids decrease the adsorption of Cd mainly due to a decrease of soil pH. The exudation of citric acids was demonstrated to be involved in the cadmium detoxification by *Aspergillus niger* (Tsekova et al., 2000). Although it is widely accepted that LMWOAs may play a role in controlling metal bioavailability due to their high concentration and metal chelating properties, the negative effects of cadmium on microorganism biomass and activities, reported by Renella et al. (2005), would be imputing to the direct capture of the citric acid complexes, including the biosensor. The higher bioavailability of cadmium in plot 1 did not found a confirmation of its negative effects in the results of microbial biomass and MRE mineralization reported in chapter four. Data on mineralization and microbial biomass did not show significant differences between soils of plot 1 treated with citric and glutamic acids and the values of the other soils with lower Cd total content.

In our experiments, even glucose which has no chelating capacity increased Cd bioavailability, possibly due to the formation of secondary metabolites with ligands properties. Another explanation would be attributed to the stimulation effects on bacterial growth and the consequentially release of CO_2, or to the stimulation of microorganisms followed by the increase in dissolved organic matter which may decrease the soil pH. Our results showed that the release of root exudates may increase Cd bioavailability to microorganisms in the rhizosphere above a total Cd content of 40 mg kg^{-1}. No similar data were previously reported in literature. However a clear effect of MRE was observed only in the most contaminated soils (P1 38.7 mg Cd kg^{-1}). The presented data are in agreement with the NH_4NO_3-exchangeable Cd results presented in the chapter four of this thesis. The higher Cd bioavailability values were obtained with

the BIOMET in soils of plot 1 treated with citric acid and glutamic acid (13.92 and 10.66 mg Cd kg^{-1} respectively). Broos et al. (2005) set up a study to assess the toxicity effects of metals in soil on symbiotic nitrogen fixation. The symbiotic nitrogen fixation was measured with ^{15}N isotope dilution in white clover (*Trifolium repens* L.) grown in potted soils. The AGIR soils (plots 1, 6, 11, and 16) were included in the Broos' contaminated soil series. The study reported that the presence of Rhizobium in AGIR soils was not affected by cadmium concentration, even in the most contaminated plots (Broos et al., 2005).

The BIOMET results were in agreement with a study carried out using three genetically modified lux-based biosensors (*Escherichia coli*, *Pseudomonas fluorescens*, and Rhizotox). The bioassays were performed in pore waters extracted from 19 contrasting soils individually spiked with Cd, Cu and Zn salts. The biosensor results did not shown Cd bioavailability in concentration below 12 mg kg^{-1} (Dawson et al., 2005).

In the modelling of Cd bioavailability in the soil-plant system the effect of root exudates should be taken in account because, as shown from the work presented here, compounds normally present in root exudates (citric acid and glutamic acid) affect the availability of cadmium, although the effect of LMWOA was detected only at high values of cadmium (40 mg kg^{-1}). These results of the biosensor suggest that for this particulary condition and soil, the limits of the maximum level of total Cd set at 2 mg/kg soil from the European legislation seems to be safe for *Ralstonia metallidurans* bacteria. However, the complexity the mobility of metals in soils and the many factors affecting the availability and uptake of contaminants from plant root suggest to be really careful with the setting of mandatory limits.

REFERENCES

Biran, I., Rissin, D., Ron, E., Walt, D., 2003. Optical imaging fiber-based live bacterial cell array biosensor. Analytical Biochemistry 315 (1), 106-113.

Broos, K., Uyttebroek, M., Mertens, J., Smolders, E., 2004. A survey of symbiotic nitrogen fixation by white clover grown on metal contaminated soils. Soil Biology & Biochemistry. 36, 633–640.

Chen, Y.X., Lin, Q., Luo, Y.M., He, Y.F., Zhen, S.J., Yu, Y.L., Tian, G.M., Wong, M.H., 2003. The role of citric acid on the phytoremediation of heavy metal contaminated soil. Chemosphere. 807-811

Corbisier, P., van der Lelie, D., Borremans, B., Provoost, A., de Lorenzo, V., Brown, N.L., Lloyd, J.L., Csoregi, E., Johansson, G., Mattiasson, B., 1999. Whole-cell and protein-based biosensors for detection of bioavailable heavy metals in environmental samples. Analytica Chemica Acta 387, 235–244.

Daunert, S., Barrett, G., Feliciano, J., Shetty, R., Shrestha, S., Smith-Spencer, W., 2000. Genetically engineered whole-cell sensing systems: Coupling biological recognition with reporter genes. Chemical Reviews 100, 2705-2738.

Dawson, J., Campbell, C. D., Towers, W., Cameron, C.M., Paton, G.I., 2005. Linking biosensor responses to Cd, Cu and Zn partitioning in soils Environmental Pollution *in Press*

Falchini, L., Naumova, N., Kuikman, P.J., Bloem, J., Nannipieri, P., 2003. CO_2 evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation. Soil Biology & Biochemistry 36, 775–782.

Gray, C.W., McLaren, R.G., Roberts, A.H.C., Condron, L.M. 1998. Sorption and desorption of cadmium from some New Zealand soils: effect of pH and contact time. Australian Journal of Soil Research 32, 199-216.

Hansen, L.H, Sørensen, S.J., 2001. The use of whole-cell biosensors to detect and quantify compounds or conditions affecting biological systems. Microbial Ecology 42, 483-494.

Jones, D.L., 1998 Organic acids in the rhizosphere - a critical review. Plant and Soil 205, 25-44.

Ji, G., Silver, S., 1992. Regulation and expression of the arsenic resistance operon from Staphylococcus aureus Plasmid pI258. Journal of Bacteriology 174, 3684-3694.

Klaassen, Curtis D., 2001. Casarett and Doull.s Toxicology: The Basic Science of Poisons, 6th Edition. New York: McGraw-Hill.

Kohler, S., Belkin, S., Schmid, RD., 2000. Reporter gene bioassays in environmental analysis. Fresenius Journal of Analytical Chemistry366, 769-779.

Kostera, M., Reijndersb, L., van Oosta, N., Peijnenburga, W., 2005. Comparison of the method of diffusive gels in thin films with conventional extraction techniques for evaluating zinc accumulation in plants and isopods. Environmental Pollution 133, 103–116.

Krishnamurti, G.S.R., Smith, L.H., Naidu, R., 2000. Method for assessing plant-available cadmium. Australian Journal of Soil Science 38 (4), 823–836.

Mench, M., Martin, E., 1991. Mobilization of Cd and other metals from soils by root exudates of *Zea mays* L., *Nicotiana tabacum* L. and *Nicotiana rustica* L. Plant and Soil 132, 187-196.

Mergeay, M., Houba, C., Gerits, J., 1978. Extrachromosomal inheritance controlling resistance to cadmium, cobalt, copper and zinc ions: evidence from curing in a Pseudomonas [proceedings]. Arch. Int. Physiol. Biochem. 86, 440-442.

Mergeay, M., 2000. Bacteria adapted to industrial biotopes: Metal-resistant *Ralstonia*. In: Storz, G., Hengge-Aronis, R. (Eds.), Bacterial Stress Responses, pp. 403–414.

Mergeay, M., Monchy, S., Vallaeys, T., Auquier, V., Benotmane, A., Bertin, P., Taghavi, S., Dunn, J., van der Lelie, D., Wattiez, R., 2003. *Ralstonia metallidurans*, a bacterium specifically adapted to toxic metals: towards a catalogue of metal-responsive genes, FEMS Microbiology Reviews. 27(2-3), 385-410.

Nies, D.H., 1999. Microbial heavy metal resistance. Applied Microbiology and Biotechnology . 51, 730-750.

Palchetti, I., Laschi, S., Mascini, M., 2005. Miniaturised stripping-based carbon modified sensor for in field analysis of heavy metals., Analytica Chimica Acta, 530, 61-67.

Paton, G.I., Campbell, C.D., Glover, L.A., Killham, K., 1995 Assessment of bioavailability of heavy metals using lux modified constructs of *Pseudomonas fluorescens*. Letters in Applied Microbiology. 20, 52–56.

Renella, G., Landi, L., Nannipieri, P., 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311–315.

Renella, G., Egamberdiyeva, D., Landi, L., Mench, M., Nannipieri, P., 2006. Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biology & Biochemistry (*in press*).

Renella, G., PhD thesis, University of Florence, 2001.

Roberto, F., Barnes, J., Bruhn, D., 2002. Evaluation of a GFP reporter gene construct for environmental arsenic detection. Talanta 58(1), 181-188.

Strosnider, H., 2003. Whole Bacterial biosensor, National Network of Environmental Management Studies Fellow, for U.S. Environmental Protection Agency Office of Solid Waste and Emergency Response Technology Innovation Office Washington, DC.

Tauriainen, S., Virta, M. Karp, M. 2000. Detecting bioavailable toxic metals and metalloids from natural water samples using luminescent sensor bacteria. Water Research 34(10), 2661-2666.

Tibazarwa, C., Corbisier, P., Mench, M., Bossus, A., Solda, P. Mergeay, M., Wyns, L., van der Lelie, D., 2001. A microbial biosensor to predict bioavailable nickel in soil and its transfer to plants. Environmental Pollution 113, 19-26.

Tsekova, K., Dentchev, D., Todorova, D., 2000. Effect of cadmium and copper on theproduction of citric acid by *Aspergillus niger*, Folia Microbiology (Praha) 45(4), 331-4.

Tessier, A., Campbell, P.G.X., Bisson, M. 1979. Sequential extraction procedure for the speciation of particular trace metals. Analytical Chemistry 51, 844-850.

Turpeinen, R., Virta, M., Haggblom, M., 2003. Analysis of arsenic bioavailability in contaminated soils. Environmental Toxicology and Chemistry 22(1), 1-6.

Ure, A., Davidson, C.M., 2002. Introduction. In: Chemical Speciation in the Environment, 2nd edn (eds A.M. Ure, C.M. Davidson), Blackwell Science, Oxford, pp. 1-5.

Zhang, H., Davison, W., 1995. Performance characteristics of diffusion gradients in thin films for the in situ measurement of trace metals in aqueous solution. Analytical Chemistry. 67 (19), 3391-3400.

CHAPTER FOUR

INFLUENCE OF ROOT EXUDATES ON THE MOBILITY OF CADMIUM AND ON BIOCHEMICAL PARAMETERS IN THE RHIZOSPHERE AND BULK SOIL

Valori Federico [a], Landi Loretta [a], Mench Michel [b], Renella Giancarlo [a] and Nannipieri Paolo [a]

[a] Department of Soil Science and Plant Nutrition University of Florence, P.le delle Cascine, 28, 50144, Florence, Italy

[b] UMR BIOGECO INRA 1202, Ecologie des Communautés, Université Bordeaux 1, Bât B8, RdC Est, 002, Avenue des Facultés F-33405 Talence, France

1. INTRODUCTION

The main rhizosphere properties and the risk for human health that is associated with the use of Cd-contaminated agricultural soils have been reviewed in chapter 1, paragraph 1.

The rhizosphere is the soil volume directly or indirectly influenced by the root presence (Uren et al., 2000). This rhizosphere soil differs in many aspects from the bulk soil due to the release of root exudates. A tentative of typical compositions of root exudates are reported in chapter 1, table 1.1. Low molecular weight organic acids (LMWOAs) represent a major fraction of root exudates (Jones, 1998). They are released from intact roots or during decomposition of dead plant material by microbial activity, and play an important role in nutrient acquisition by plants through mobilization of poorly soluble nutrients such as P and Fe (Ström et al., 2005). Root exudates may also cause a release of non-essential metals in the rhizosphere (Feng et al., 2005), sustain microbial biomass and activity in the rhizosphere (Whipps, 1990; Falchini et al., 2003), and affect the N turnover in soil (Landi et al., 2006). They are also involved in the detoxification of Al (Sandnes et al., 2005; Jones, 1998; Marschner, 1995).

Cadmium has a high mobility in soil due to its low affinity for organic matter (Strobel et al., 2005). Furthermore, the increase of dissolved organic carbon does not directly affect

the extractable amount of cadmium (Strobel et al., 2005). The Cd mobility is mostly affected by soil pH. Sauvé et al. (2000) concluded that approximately 50% of the overall variation in the distribution coefficient between soil and soil solution, for Cd could be explained by pH variations. However, no detailed information on the acidification induced by low molecular weight organic compounds of root exudates were provided. It is well established that the LMWO compounds may enhance the Cd mobility (Krishnamurti et al., 1995, 1997). A greater mobility of cadmium in the rhizosphere may in turn affect microbial biomass activity and N turnover in contaminated soil. Therefore, this work aimed to assess: i) cadmium mobility over release of LMWO root exudates ii) effects of root exudates on microbial biomass, carbon and nitrogen contents, and microbial activity in the rhizosphere of Cd-contaminated soil, and iii) effects of LMWO root exudates on N mineral pools.

Due to the rhizosphere complexity, the stimulatory effects of root exudates on microbial activity and cadmium availability were here approached by studying the effects of single synthetic low molecular weight organic molecules. Furthermore, the problem of understanding the different influences of root exudates on soils can be overcome by setting up a suitable system, which allows the formation of a concentration gradient and permits soil sampling at various distances from the rhizoplane (MRS).

The rhizosphere environment was simulated by using an incubation unit (MRS) described by Badalucco and Kuikman (2001) and Falchini et al. (2003), which was developed from a similar system used by Darrah (1991 a, b). It was shown that a concentration gradient was formed in soil inside a rigid PVC cylinder when a cellulose paper with ^{14}C-labelled compounds was located on the topsoil; the concentration of labelled compounds decreased for increasing distances from the cellulose paper. Such a system allows for creating a concentration gradient with higher concentrations close to the root surface, and soil sampling at known distances from the MRS (Badalucco and Kuikman, 2001; Falchini et al., 2003; Renella et al., 2004a; Landi et al., 2006).

2. MATERIALS AND METHODS

2.1. Soils

Soils are classified as sandy-clay, Arenic Udifluvent. The AGIR field experiment was managed by Unité d'Agronomie, INRA, Bordeaux, France (Renella et al., 2004). The cadmium contamination of the soil was achieved as described below.

The density of the 0–30 cm soil layer was determined and it was calculated as dry soil (fine fraction <2 mm) per ha and per m^2. The different plots (6 m X 3 m) have been designed at the experimental site in 1987. Then, the amount of $Cd(NO_3)_2$ (Rectapur Quality, Prolabo) to be added to each plot was calculated for contaminating the 0–30 cm soil layer at Cd concentrations of 0, 0.09, 0.18, and 0.36 mmol kg^{-1} soil; it was spread on the soil surface and mixed by rototilling at 0–30 cm depth in the 1988–1990 period. The soil was ploughed at 0–30 cm depth through the years to ensure an even distribution of Cd within the soil layer. Chemical analyses demonstrated that the target Cd contaminations were reached. The background Cd concentration in the control soil was ~0.006 mmol kg^{-1}. The soils have been kept under grass and maize cultivation. In table 4.1 the main characteristics of AGIR soils are reported (Renella et al., 2001, 2005).

Table 4.1. Main characteristics of AGIR soils

Plots	**P18**	**P16**	**P11**	**P13**	**P8**	**P6**	**P1**	**P3**
Clay (%)	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
Silt (%)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Sand (%)	67.5	67.5	67.5	67.5	67.5	67.5	67.5	67.5
$pH_{(H2O)}$	6. 7	7.4	7.2	6.1	6.3	7.1	6.8.	6.3
Total Cd (mg Cd/Kg soil)	0.7	0.7	9.8	8.7	16.0	18.9	38.7	41.0
TOC (%)	1.33	0.95	0.25	0.27	0.35	0.47	0.49	1.0
N tot (%)	0.11	0.10	0.03	0.03	0.04	0.04	0.04	0.91

The eight AGIR soils (plots P1, P3, P6, P8, P11, P13. P16 and P18), with different cadmium content and pH have been used. Soils were sampled in the 0-30 cm soil layer in March 2003, air-dried, sieved (<2mm, stainless steel and wood), and stored in the dark at 25 C° 7 days, prior to the experiments.

2.2. Experimental setup

Seventy g of moist soil samples equivalent to 63 g dry weight (dw) were placed in plastic cylinders (68 mm diameter, 20 mm height), carefully pressed by a stainless steel piston to a fixed density of 1.4? g dm^3 and incubated at 25° C and watered with distilled water to the 50% water holding capacity (WHC) for 7 days prior to treatments with different root exudates. Thereafter the incubation units were covered by a cellulose round filter paper (Whatman 41, 68 mm diameter) for mimicking the root surface (fig.3.3, 3.4, 3.5), and moistened with 1 mL of deionised water (control) or root exudates solutions containing glucose, citric acid, or glutamic acid, to give a rate of 200 mg of root exudates-C kg^{-1} soil d.w. Soils were incubated in the dark at 25 °C for 0, 4, 7 and 14 days, in 1L air tight jars containing 10 mL of 1M NaOH to trap the CO_2. During the incubation, four equal doses of root exudates equivalent to 200 mg C kg^{-1} soil were added after 0, 2, 4 and 7 days of incubation. After root exudates additions, the soil moisture content did not exceed 55% of WHC for all soils. At the end of each selected incubation periods, soils were sampled from 0-2 mm (rhizosphere soil) and > 4 mm (bulk soil) distance from the root surface, as described by Falchini et al. (2003), and analysed for available Cd, nitrogen and carbon biomass, pH, and N-inorganic pools. All treatments and analytical measurements were triplicated.

The quantities of soils obtained from the layers required a modification of biomass fumigation and extraction protocol (Vance et al., 1987). Several test experiments were done prior to the main experiments: the results showed that for these soils and this kind of experiments the variation of protocol (reduced samples weight) did not change the final results of the analysis

2.3. Soil respiration and pH

The soil pH was measured by a combined electrode in a 1:2.5 soil water suspension after equilibration according to the Italian official soil analysis (Gazzetta Ufficiale n. 248 del 21.10.99).

The CO_2 evolution from soil was measured at 4, 7 and 14 days of incubation after trapping CO_2 in a 1 M NaOH, precipitation of carbonates with 0.75 M $BaCl_2$ and automated tritration of residual NaOH with 0.1 M HCl. (Stotzky, 1965).

Net cumulative respiration was calculated by subtracting the value of CO_2-C of the untreated soils from those of the MRE-treated soils. The percentage of mineralized root exudates at 14 d was calculated between the CO_2-C cumulative respiration at 14d and the total amount of MRE added (800 mg C kg^{-1}).

2.4. Determination of the inorganic nitrogen

Inorganic nitrogen was extracted by 1M KCl (Keeney and Nelson, 1982). Briefly, 5g of soil extracted were over-run-over shacked for 1 h, with 20 mL 1 M KCl (soil:extractant ratio of 1:4). Soil suspensions were filtered through a Whatman 41 filter paper and the extracts stored at 4°C prior to the analyses. Concentrations of NH_4^+-N, NO_3^--N, were measured by a flow injection analyser (FIASTAR, FOSS). A gas diffusion mixed indicator method was used for the NH_4^+-N. The NH_4^+-N content was measured at 590 nm; NO_3^--N was determined after reduction to NO_2^- with a cadmium reduction column and reaction with sulphanilamide and N-(1-naphthyl)-ethylenediaminedihydrochloride (Keeney and Nelson, 1982).

2.5. Microbial Carbon and Nitrogen Biomass

Microbial biomass C and ninhydrin-N contents were measured by the fumigation-extraction technique according to Vance et al. (1987) and Joergensen and Brookes (1990). In our experiment, 2 g of soil were used rather than 25 g, as in the original method, due to the low amount of soil collected in the soil layers. From previous experiments, it was checked that our procedure provided similar results as the original one.

Soil was fumigated with ethanol-free chloroform for 24 h at 25°C and immediately extracted with 10 mL 0.5 M K_2SO_4 1h. Soil slurries were then filtered through Whatman 41 filter paper. The unfumigated soils were extracted in the same way, and the extracts were frozen prior to analysis. The C content of the extracts was determined

by the dichromate oxidation method and the microbial biomass C was calculated according to Vance et al. (1987): Bc = 2.22 Ec where Ec = [(organic C extracted from fumigated soil)-(organic C extracted from non-fumigated soil)] (Vance et al., 1987, Wu et al., 1990).

Biomass ninhydrin-N was measured according to Joergensen and Brookes (1990). Biomass ninhydrin-N was calculated from: [(ninhydrin-N extracted from fumigated soil) - (ninhydrin-N extracted from non fumigated soil)].

2.6. Cadmium availability

The amount of cadmium availability was determined by extractions with 1 M NH_4NO_3, using an extraction ratio of 1:20 (w/v) according to Preuss (1998). Soil slurries were filtered through Watman 41 filter paper. The concentration of Cd was determined by graphite furnace atomic absorption spectroscopy (Varian AAS SpectrAA 250 plus).

Efficiency of extraction and instrument was tested using certified reference soil REF N° CRM142R (total Cd 0.34 ± 0.04 µg/g DM) were included in each batch series. The Cd recovery was 99% compared to certified value.

2.7. Statistical analysis

The data reported are mean values of three replicates with relative standard deviations (SD). The LSD values (Tukey-Kramer test, P level < 0.05 and P<0.01) were calculated to assess the significance of the differences of each treatment during the incubation time and between the treatments of each plot.

3. RESULTS

3.1. pH

According to their initial bulk soil pH, soil samples can be divided in two soil series, i.e. neutral (P1, P6, P11, P16) or slightly acid (P3, P8, P13, and P18). The addition of root exudates did not have a marked effect on soil pH values during the incubation time (table 4.2) whatever total Cd in soil and initial bulk soil pH. No statistical differences we found within the treatments.

Table 4.2. Values of rhizospheric and bulk soil pH.

	P1				**P6**				**P11**				**P16**			
Rhizosphere	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	6.40	6.92	6.64	6.90	7.42	7.42	7.07	7.05	7.05	7.05	7.20	7.32	7.07	7.42	7.44	7.63
Glucose	6.55	6.83	6.75	6.90	7.08	6.91	6.67	6.74	7.06	7.09	7.26	7.48	7.26	7.44	7.41	7.54
Citric a.	6.43	6.69	6.90	6.60	7.28	7.28	6.82	6.69	7.05	7.17	6.94	7.20	7.12	7.33	7.57	7.64
Glutamic a.	6.79	6.74	6.76	7.01	7.21	7.21	6.88	6.87	7.12	6.95	7.01	7.10	6.98	7.44	7.55	7.40
Bulk soil	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	6.28	6.69	6.65	7.03	7.04	7.10	6.87	6.87	7.12	7.00	6.97	7.45	7.17	7.43	7.48	7.69
Glucose	6.62	6.67	7.38	6.96	7.13	6.91	7.00	6.86	7.14	6.98	7.20	7.50	7.22	7.44	7.53	7.74
Citric a.	6.49	6.75	6.71	7.00	6.93	6.91	6.99	7.08	7.10	7.09	7.04	7.58	7.16	7.39	7.48	7.70
Glutamic a.	6.77	6.73	6.89	7.42	6.86	7.06	6.98	7.01	7.09	7.01	6.93	7.28	7.43	7.38	7.35	7.36
	P3				**P8**				**P13**				**P18**			
Rhizosphere	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	6.45	6.42	6.12	6.20	6.36	n.d.	6.44	6.98	6.47	6.53	6.28	6.55	6.90	6.80	6.85	6.76
Glucose	6.50	6.26	6.32	6.36	6.18	n.d.	6.47	7.70	6.38	6.75	6.44	6.60	6.87	6.88	6.87	6.95
Citric a.	6.46	6.34	6.08	6.01	6.12	n.d.	6.41	7.02	6.51	6.55	6.42	6.46	6.80	6.88	6.88	6.87
Glutamic a.	6.49	6.16	6.86	6.85	6.21	n.d.	6.33	6.71	6.35	6.71	6.63	6.76	6.79	6.75	6.95	6.45
Bulk soil	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	6.38	6.34	6.42	6.23	6.47	n.d.	6.43	6.73	6.30	6.48	6.63	6.52	6.87	6.92	6.87	6.80
Glucose	6.39	6.29	6.41	6.36	6.40	n.d.	6.41	7.04	6.31	6.39	6.38	6.50	6.88	7.03	6.88	6.91
Citric a.	6.40	6.25	6.36	6.35	6.36	n.d.	6.66	6.75	6.37	6.44	6.43	6.28	6.89	7.02	6.88	6.60
Glutamic a.	6.38	6.16	6.16	6.60	6.53	n.d.	6.40	6.48	6.29	6.29	6.25	6.13	6.86	6.79	6.74	6.29

3.2. Soil respiration

3.2.1. Cumulative respiration

In this section we analyse the results of cumulative respiration, with reference to table 4.3 where the values for each plot P are specified.

Across the set of untreated plot soils, P1 showed lower values than P3, and the same pattern was observed for P6, in which the cumulative respiration was lower than for P8. In highly contaminated soils, acidic soils generally presented a higher mineralization than in neutral ones. Similar respiration values were also found for lower cadmium concentrations in contaminated soils. Out of 4 soils with neutral soil pH (i.e. P1, P6, P11 and P16), P16 had the highest respiration compared with the other neutral soils.

Across slightly acid soils (P3, P8, P13, P18), except for P13, respiration values were all similar, but a higher respiration in more contaminated soils was noticed.

In soils treated with glucose, all the slightly acid soils showed mainly a decrease of the cumulative respiration following the increased cadmium content. In higher contaminated soils, P3, had higher value than P1, but in P6/P8 and P11/P13 the values were similar (note also that P16 had higher values than P18).

On the other hand, no particular effect could be noticed in soils treated with citric acid. Values of the control plots (P16 and P18) were similar to those in the highest contaminated soils, i.e. P1 and P3 respectively. In soils treated with glutamic acid, P3 always had higher values compared to the other soils. Summing up, generally, the respiration was generally higher in highly contaminated soils than in medium or not contaminated soils. Furthermore, generally, in this case acid soils had values of cumulative respiration higher than neutral soils, but this difference decreased with the decreased cadmium content.

Concerning the different treatments, all the MRE were mineralized, and some differences were detected in the comparison with the untreated soils. However, no significant differences could be noticed between citric acid and glucose in one hand, and glutamic acid treatments in the other hand, even though glutamic acid treatment was normally a reason for higher mineralization.

In table 4.4, the net cumulative respiration calculated at T=14 is reported. For neutral soils, glucose was mineralized more in P3 than in P1, and more in the contaminated soils than in the uncontaminated or less contaminated ones. This pattern was more evident with glutamic acid: all the acid soils mineralized a higher percentage of glutamic acid than neutral soils, whereas in control plots this difference was only marginal. Treatments with citric acid showed opposite results: neutral and contaminated soils mineralized more than acid soils.

Table 4.3 Change in cumulative CO_2 evolved (μg CO_2-C g^{-1} d.w. soil) during incubation in untreated and treated soils. SD, standard deviation.

	Incubation Time (days)				Incubation Time (days)		
	Untreated				Glucose		
Plots	4	7	14	Plots	4	7	14
P1	27.69	51.13	57.65	P1	111.26	235.86	374.07
P3	100.15	126.99	158.75	P3	143.09	354.07	493.19
P6	29.56	74.12	96.56	P6	94.56	194.84	316.03
P8	99.34	129.20	163.99	P8	131.61	166.88	315.38
P11	19.64	41.29	70.17	P11	89.71	172.99	335.53
P13	15.15	31.05	52.40	P13	109.78	196.78	308.38
P16	50.70	83.53	153.17	P16	130.12	240.79	360.48
P18	19.68	94.94	137.45	P18	96.42	163.76	285.67
	Citric acid				Glutamic acid		
Plots	4	7	14	Plots	4	7	14
P1	126.82	248.91	348.37	P1	131.17	250.93	393.98
P3	153.11	236.31	408.62	P3	180.84	413.09	574.98
P6	123.55	208.01	372.86	P6	116.10	219.60	381.50
P8	67.77	131.36	218.24	P8	124.93	204.88	326.37
P11	181.08	302.37	459.06	P11	179.70	330.33	516.03
P13	110.83	194.02	322.59	P13	102.33	203.57	323.33
P16	57.52	234.54	380.66	P16	91.78	249.01	389.60
P18	142.28	238.90	403.98	P18	137.65	220.87	368.48

SD	Untreated			Plots	Glucose		
Plots	4	7	14		4	7	14
P1	0.17	4.52	19.92	P1	3.11	9.19	23.19
P3	4.75	8.32	26.06	P3	20.58	55.56	47.13
P6	3.01	48.82	20.94	P6	13.34	15.29	5.55
P8	4.93	23.39	19.92	P8	18.45	35.79	23.19
P11	4.91	4.91	27.24	P11	15.51	15.53	16.21
P13	3.85	5.31	9.05	P13	11.40	13.14	0.00
P16	0.01	0.73	28.02	P16	24.54	8.49	9.26
P18	1.03	1.66	27.00	P18	5.89	35.58	11.26
	Citric Acid			Plots	Glutamic Acid		
Plots	4	7	14		4	7	14
P1	5.77	5.10	7.90	P1	3.31	13.84	14.35
P3	7.99	0.33	74.98	P3	14.95	76.34	6.17
P6	6.03	9.15	6.58	P6	14.82	9.13	7.73
P8	11.06	10.60	7.90	P8	12.89	10.80	14.35
P11	3.71	3.71	41.21	P11	25.26	25.26	14.48
P13	11.21	22.91	13.28	P13	9.74	5.23	27.69
P16	7.55	66.54	29.16	P16	5.35	1.88	16.66
P18	19.20	10.79	28.35	P18	9.40	12.16	19.16

Table 4.3 a Table of statistical significance in cumulative CO_2 between the treatments and the control in the different plots (S indicates significant statistical values $p<0.05$, NS means indicates not significant statistical values $p>0.05$).

Plot1	T4	T7	T14	Plot 3	T4	T7	T14
Glucose	S	S	NS	Glucose	NS	NS	NS
Citric Acid	S	S	NS	Citric Acid	NS	NS	NS
Glutamic Acid	S	S	NS	Glutamic Acid	NS	NS	S
Plot 6	**T4**	**T7**	**T14**	**Plot 8**	**T4**	**T7**	**T14**
Glucose	NS	NS	NS	Glucose	S	NS	S
Citric Acid	NS	NS	S	Citric Acid	S	S	NS
Glutamic Acid	NS	NS	S	Glutamic Acid	S	S	NS
Plot 11	**T4**	**T7**	**T14**	**Plot 13**	**T4**	**T7**	**T14**
Glucose	S	S	NS	Glucose	S	S	S
Citric Acid	S	S	NS	Citric Acid	S	NS	S
Glutamic Acid	S	S	S	Glutamic Acid	S	S	S
Plot 16	**T4**	**T7**	**T14**	**Plot 18**	**T4**	**T7**	**T14**
Glucose	NS	NS	NS	Glucose	NS	NS	NS
Citric Acid	NS	NS	NS	Citric Acid	NS	NS	NS
Glutamic Acid	S	S	NS	Glutamic Acid	S	NS	S

Table 4.4 Net cumulative respiration (μg CO_2-C g^{-1} d.w. soil) and the percentage of mineralized substrate measured at T=14d

	Treatments		%		Treatments		%
Plot 1		**T14**	**T14**	**Plot 3**		**T14**	**T14**
	Glucose	322.53	40%		**Glucose**	391.07	49%
	Citric Acid	373.55	47%		**Citric Acid**	306.50	38%
	Glutamic Acid	404.64	51%		**Glutamic Acid**	472.86	59%
Plot 6				**Plot 8**			
	Glucose	219.48	27%		**Glucose**	218.82	27%
	Citric Acid	276.30	35%		**Citric Acid**	121.68	15%
	Glutamic Acid	284.95	36%		**Glutamic Acid**	229.81	29%
Plot 11				**Plot 13**			
	Glucose	265.36	33%		**Glucose**	238.21	30%
	Citric Acid	472.20	59%		**Citric Acid**	252.42	32%
	Glutamic Acid	445.86	56%		**Glutamic Acid**	253.16	32%
Plot 16				**Plot 18**			
	Glucose	207.31	26%		**Glucose**	132.50	17%
	Citric Acid	227.49	28%		**Citric Acid**	250.81	31%
	Glutamic Acid	236.43	30%		**Glutamic Acid**	215.31	27%

3.3. Inorganic Nitrogen pools

Ammonium exchangeable and nitrate concentrations in different soils are reported in tables 4.5 and 4.6. In the rhizosphere soil, NH_4^+-N concentrations varied among the different soils, treatments, and incubation times, and a general trend is not identifiable. Fluctuations of the values were found between different incubation times for all the treatments. Plot 1 showed significant decrease in NH_4^+-N content in soils treated with glucose and citric acid in the rhizosphere ($p<0.05$). Glutamic acid showed the expected significant increase of NH_4^+-N since the first addition. The differences between bulk soils in plot 1 were negligible for all the treatments. However, glutamic acid showed a significant increase when compared to the other treatments, although not comparable with the increase shown in the rhizosphere. The differences concerning the glutamic acid between bulk and rhizosphere soils were significant ($p<0.05$).

In the rhizosphere soils reported in plot 3, the initially NH_4^+-N content decreased after 4d in soils treated with glucose and citric acid. In plot 6, the fluctuant values in the rhizosphere of untreated, glucose, and citric acid soils were not significant, and thus no relevant effects were detected. The glutamic acid showed a significant increase in NH_4^+-N content, since T=4 in both rhizosphere and bulk soils, during the incubation time ($p<0.05$). The others treatments in bulk soils showed a significant decrease of the NH_4^+-N content at T=14 compared to values of T=7d. This pattern might be imputed to a

reduction of the MRE effect that in this case reached the bulk soils. The same effect has been reportedin previous experiments (Falchini et al., 2003). In plot 8, both in rhizosphere and bulk soils, glucose had no influence on the NH_4^+-N content. Citric acid showed a significant decrease compared with untreated soil at T=14d, in both the rhizosphere and bulk soils (table 4.5).

In plot 11, there was a significant increase at T=4d and T=7d of NH_4^+-N content in soils treated with citric acid in both the rhizosphere and bulk soils compared to T=0d ($p<0.05$). The decrease in NH_4^+-N content in citric acid-treated soils that occurred at T=14d in rhizosphere and bulk soils was not significant. The glutamic acid caused like in other plots a significant increase in NH_4^+-N content compared with the untreated soils, in both the rhizosphere and bulk soils.

In plot 13, only glutamic acid affected the NH_4^+-N content in both rhizosphere and bulk soils, while the other MRE did not show any significant influences on NH_4^+-N content.

In the control soil of plot 16, the effect of glucose and citric acid was significant after 7d of incubation, and immediately after the last MRE addition the NH_4^+-N content decreased to values lower than T=0d. Glucose and citric acid probably did not reach the bulk soil, and this explains the low differences ($p>0.05$) that were detected. Glutamic acids showed in the rhizosphere and bulk soil higher values compared to the other MRE. In plot 18, only glutamic acid increased significantly the NH_4^+-N content in both rhizosphere and bulk soils, compared to the untreated soils.

Generally, the values of NH_4^+-N in the rhizosphere and bulk soils have a similar pattern and the MRE effect was unclear.

The general pattern showed an increase of NH_4^+-N content in the first 4d, after which the decrease started. After the last MRE addition, that occurred at T=7d, a decrease of NH_4^+-N content was measured more or less in all the plots and all the treatments, except in some cases for glutamic acid (e.g. in the rhizosphere soils of plot 11 at T=14d and plot 6 at T=14d).

Cadmium seemed not to affect the NH_4^+-N content, and the slightly differences between rhizosphere and bulk soils in the different plots were an indication for it.

The pH influence on NH_4^+-N content was not clear, however, generally, NH_4^+-N concentrations were higher in neutral than acid soils, and generally increased upon increasing total Cd concentrations.

In plot 16 and plot 18 treated with citric acid, the increased started at T=7. In the glutamic acid treatment, the NH_4^+-N content obviously increased during the incubation, and it reached a maximum at T=7 d. The NH_4^+-N concentrations in the rhizosphere of

soils treated with glutamic acid were significantly higher than in the bulk, whereas no significant difference was found for the other treatments.

Table 4.5. Values of NH_4^+-N (μg g^{-1} d.w.) in the rhizosphere and bulk soils during incubation (symbols **, indicates significant statistical values p<0.05,* indicates significant statistical values p<0.01). The statistical significance reported in the table was calculated between the treatments of each plot.

	Rhizosphere					Bulk soil			
PLOT 1	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 1**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	4,10	14,98	n.d.	8,94	**Untreated**	4,43	6,64	n.d.	10,14
Glucose	10,39	6,55**	n.d.	9,05	**Glucose**	7,30	9,40	n.d.	9,49
Citric Acid	16,05	8,17**	n.d.	14,02	**Citric Acid**	10,50	8,28	n.d.	9,09
Glutamic Acid	8,13	80,46**	n.d.	106,84**	**Glutamic Acid**	8,64	10,81	n.d.	30,42**
PLOT 3	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 3**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	12,87	3,03	9,72	6,29	**Untreated**	10,85	8,93	10,73	5,01
Glucose	12,67	9,44**	9,13	7,07	**Glucose**	14,17	11,00	8,60	6,21
Citric Acid	14,66	8,67**	7,32	5,41	**Citric Acid**	15,99	9,52	8,61	15,45
Glutammic Acid	14,93	53,95**	74,79	48,23	**Glutammic Acid**	13,52	7,45	13,21	22,91
PLOT 6	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 6**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	2,52	12,11	11,03	4,05	**Untreated**	1,28	11,08	11,32	3,77
Glucose	1,84	10,10	4,44	1,91	**Glucose**	6,77	10,08	4,39	3,25**
Citric Acid	3,06	11,42	8,85	2,92	**Citric Acid**	4,64	16,64	9,10	2,73**
Glutamic Acid	5,40	77,35**	78,11**	75,30**	**Glutamic Acid**	1,05	25,04**	20,44**	14,95**
PLOT 8	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 8**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	23,87	n.d.	17,88	25,27	**Untreated**	15,86	n.d.	12,89	3,36
Glucose	16,51	n.d.	12,92	4,40	**Glucose**	14,87	n.d.	13,67	3,36
Citric Acid	19,51	n.d.	13,90	3,69**	**Citric Acid**	18,44	n.d.	15,42	3,81**
Glutamic Acid	15,36	n.d.	57,85**	32,61**	**Glutamic Acid**	15,59	n.d.	17,02	6,51**
PLOT 11	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 11**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	8,03	14,13	8,55	1,09	**Untreated**	9,79	16,72	4,28	0,60
Glucose	8,70	11,07	8,33	3,78	**Glucose**	9,24	8,90	7,88	4,25
Citric Acid	8,54	13,46**	12,03**	5,71	**Citric Acid**	6,55	11,05**	11,54**	3,73
Glutamic Acid	9,32	81,04**	42,13**	66,21**	**Glutamic Acid**	13,46	12,73	11,55	21,55**
PLOT 13	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 13**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	7,36	n.d.	6,32	4,06	**Untreated**	7,65	n.d.	6,28	3,84
Glucose	9,57	n.d.	3,74	4,34	**Glucose**	8,82	n.d.	2,84	4,43
Citric Acid	7,09	n.d.	1,77	5,04	**Citric Acid**	5,94	n.d.	2,50	4,64
Glutamic Acid	5,11	n.d.	91,32**	59,12**	**Glutamic Acid**	6,11	n.d.	12,04**	33,44**
PLOT 16					**PLOT 16**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	14,03	9,15	21,44	6,90	**Untreated**	8,04	8,62	18,99	5,23
Glucose	9,83	7,90	23,21*	4,40	**Glucose**	11,66	9,63	27,48	3,41
Citric Acid	11,13	9,03	19,90*	3,82	**Citric Acid**	8,27	9,52	27,73	12,89
Glutamic Acid	11,80	46,43**	24,71**	7,76	**Glutamic Acid**	7,91*	5,24*	21,85**	39,26**
PLOT 18	**T=0**	**T=4**	**T=7**	**T=14**	**PLOT 18**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	4,02	4,06	9,18	2,91	**Untreated**	4,52	3,97	10,56	3,60
Glucose	0,82	3,70	7,24	4,48	**Glucose**	0,58	3,81	8,14	3,99
Citric Acid	2,36	3,88	7,89	4,88	**Citric Acid**	3,37	3,57	7,72	3,89
Glutamic Acid	3,75	58,29**	74,82**	47,57**	**Glutamic Acid**	4,03	6,53*	12,62*	13,54*

n.d stands for not determined

In the rhizosphere and bulk soils, the NO_3^--N content (table 4.6) increased in the first 4d and then decreased during the incubation, (except in plot 13,). At T= 14d in general (except for soils treated with gluatmic acid), the lower values of NO_3^--N content were found. In general the values of NO_3^--N content were similar or slightly higher in the rhizosphere than in the bulk soils, and generally neutral soils presented higher values than acid soils. The glucose and citric treatments have a similar pattern. Indeed, the NO_3^--N content in the rhizosphere after 4d were generally lower (or equal in some cases of citric acid) in all the soils and treatments than in untreated soils (except plot 3 T=7d).

In the bulk soils these differences were less marked. Soils treated with glutamic acid, on the contrary, always had higher values than untreated soils (in the rhizosphere), and with higher nitrification in contaminated soils. The higher values of NO_3^--N content in soils treated with glutamic acids probably derived from the nitrification of the NH_4^+ of glutamic acids.

Table 4.6. Values of NO_3^--N (μg g^{-1} d.w) in the rhizosphere and bulk soils during the incubation (symbols **, indicates significant statistical values p<0.05,* indicates significant statistical values p<0.01). The statistical significance reported in the table was calculated between the treatments in each plot.

Rhizosphere					Bulk soil				
Plot 1	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 1**	**T=0**	**T=4**	**T=7**	**T=14**
Control	74,46	87,93	77,97	23,97	**Control**	58,37	77,95	76,38	22,89
Glucose	54,14	57,67	41,97	4,03	**Glucose**	58,10	54,95	40,76	3,72
Citric Acid	60,05	69,07*	48,91*	7,04*	**Citric Acid**	51,07	72,41	41,19	9,52
Glutamic Acid	76,18	112,10*	106,13**	78,99**	**Glutamic Acid**	60,12	110,79**	91,12	57,31
Plot 3	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 3**	**T=0**	**T=4**	**T=7**	**T=14**
Control	16,62	61,62	50,08	7,51	**Control**	17,52	62,49	56,53	5,98
Glucose	13,42	45,70	72,43	0,01	**Glucose**	17,37	38,76	75,70	0,35
Citric Acid	13,07	62,79**	63,37*	15,13	**Citric Acid**	14,89	78,27	107,65	13,87
Glutammic Acid	8,79	151,82**	168,00**	79,87	**Glutammic Acid**	11,27	73,21	69,97	71,54
Plot 6	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 6**	**T=0**	**T=4**	**T=7**	**T=14**
Control	43,49	56,78	36,38	17,59	**Control**	41,42	45,47	40,30	12,53
Glucose	43,40	32,40	11,29	0,01	**Glucose**	40,95	32,94	16,95	0,00
Citric Acid	39,79	36,80	21,66	1,58	**Citric Acid**	43,20	33,28	20,29	1,41
Glutamic Acid	41,19	59,54**	47,28**	63,52**	**Glutamic Acid**	43,83	46,78	35,80	48,14
Plot 8	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 8**	**T=0**	**T=4**	**T=7**	**T=14**
Control	41,91	n.d.	88,85	30,56	**Control**	56,65	n.d.	70,39	21,09
Glucose	46,55	n.d.	69,49	2,92	**Glucose**	39,97	n.d.	59,63	0,01
Citric Acid	33,99	n.d.	65,81*	14,28**	**Citric Acid**	49,76	n.d.	62,38	12,40
Glutamic Acid	32,19	n.d.	88,39*	68,85**	**Glutamic Acid**	42,70	n.d.	73,25	52,57
Plot 11	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 11**	**T=0**	**T=4**	**T=7**	**T=14**
Control	41,73	84,26	41,39	16,97	**Control**	43,08	80,02	39,41	17,34
Glucose	43,52	65,22	16,54**	1,62	**Glucose**	41,49	49,53	20,30	1,39
Citric Acid	39,19	42,82	22,39**	2,77	**Citric Acid**	33,13	51,49	21,87	3,25
Glutamic Acid	41,77	140,04	85,41**	65,90	**Glutamic Acid**	35,23	110,52	74,30	43,78
Plot 13	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 13**	**T=0**	**T=4**	**T=7**	**T=14**
Control	46,06	31,23	8,85	19,80	**Control**	42,42	27,74	17,75	17,47
Glucose	47,31	12,71	5,20	1,27	**Glucose**	45,55	13,16	7,20	1,07
Citric Acid	44,70	16,56	12,38	7,30	**Citric Acid**	38,00	19,33	11,28	6,36
Glutamic Acid	44,61**	35,53	35,91	46,14	**Glutamic Acid**	33,23	31,21**	30,53	37,00
Plot 16					**Plot 16**	**T=0**	**T=4**	**T=7**	**T=14**
Control	13,88	79,66	40,08	11,45	**Control**	17,05	76,26	41,31	11,48
Glucose	13,19	68,87	35,26	2,24	**Glucose**	25,25	92,81	31,30	2,55
Citric Acid	14,66	75,33**	34,25	6,10	**Citric Acid**	25,04	92,50	44,38	6,01
Glutamic Acid	16,28	109,52**	38,19	71,72	**Glutamic Acid**	16,00	93,14	39,82	59,73
Plot 18	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 18**	**T=0**	**T=4**	**T=7**	**T=14**
Control	13,29	17,89	34,17	16,34	**Control**	16,41	16,75	35,55	14,95
Glucose	11,28	1,88	1,65	0,28	**Glucose**	14,32	2,36	10,76	0,06
Citric Acid	10,37	3,24	14,54	1,48	**Citric Acid**	13,91	4,22	11,74	2,10
Glutamic Acid	10,66	52,29	51,43	72,78**	**Glutamic Acid**	14,45	54,64	52,67	58,17

3.4. Cadmium availability

The amounts of Cd extracted by 1 M NH_4NO_3 in the rhizosphere and in the bulk soil are reported in table 4.7. The solubility and availability of cadmium were compared with its total concentration, and, as expected, the highest Cd values were detected in the most contaminated soils. Broadly speaking, within the same plots, the values corresponding to the rhizosphere were similar to those of the bulk soils. No significant ($p>0.05$) differences were noticed within the same plot.

In plot 1, the efficiency of extraction ranged between 0.020% and 0.041%. Tendentially values of extracted Cd in the rhizosphere, untreated soils, were not higher than in soils treated with glucose and glutamic acid, but lower than those treated with citric acid. Values of extracted Cd in bulk soils treated with glutamic acid were higher than that of other treatments, and the differences between bulk and rhizosphere were not significant ($p>0.05$).

Soils from plot 3 showed a lower extraction ratio (ranging from 0.013% to 0.025%). In this case no differences were observed within bulk and rhizosphere soils. Despite the higher total cadmium content of plot 3 compared to plot 1 (41.0 and 38.7 mg Cd kg^{-1} soil d.w., respectively), values of extracted Cd in plot 1 are always higher than values of plot 3. The opposite results were inferred from plot 6 and 8, in which the acid soils (plot 8) have higher values than plot 6, in both rhizosphere and bulk soils (except for T=14d). Also the extraction efficiency was higher in plot 8 compared to plot 6 (maximum values were 0.046% and 0.023% respectively). In plot 6, the values within the different treatments were similar until T=7d, and then increased at T=14d; in plot 8 no differences were detected ($p>0.05$).

Plot 11 showed both in the rhizosphere and bulk soils a slightly decrease in time until T=14d, and then an increase to values twice than the initial values, but with no significance ($p>0.05$). Within the different treatments, a visible trend was not detected. In this case the efficiency of the extraction ranged between 0.020 and 0.140%, calculated in T=14d. Plot 13 showed in the rhizosphere a similar pattern between the different treatments and constant values of extracted Cd. The percentage of extraction was ranging between 0.030 and 0.051%. Plot 16 and plot 18 have the lower values of extracted Cd, and similar values in rhizosphere and bulk soils. However, the extracted fractions were higher than in the others plots, and ranged between 0.019 and 0.091% in plot 16 and 0.019 and 0.091% in plot 18, respectively. Plots with low cadmium content

(plots 11/13 and 16/18) did not show differences in the concentration of Cd extracted by NH_4NO_3.

In general the extracted Cd, considered as available and thus potentially toxic, was in all the plots very low, and in general the MRE did not affected the cadmium mobility that was more related to the soil pH values.

Table 4.7. Means and standard deviation (SD) values of Cd extracted with NH_4NO_3 1 M (μg Cd Kg^{-1} soil d.w.) in the rhizosphere and in the bulk soil during the incubation.

	Treatments	Incubation time (days)				SD			
Plot 1 (38.7 mg Cd kg^{-1} d.w.soil)									
Rhizosphere		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
	Untreated	11.333	9.750	13.000	8.333	0.3	5.3	1.0	2.3
	Glucose	10.000	9.333	10.500	8.667	1.0	0.7	1.4	0.3
	Citric acid	12.500	14.500	12.750	7.500	0.9	1.8	1.1	0.9
	Glutamic acid	11.333	14.667	11.500	7.833	3.3	0.6	1.0	0.8
Bulk soil		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
	Untreated	12.500	14.500	11.667	8.167	0.9	1.0	0.8	1.0
	Glucose	11.000	14.333	12.500	7.833	0.9	1.0	1.0	0.3
	Citric acid	8.500	13.000	11.500	7.833	5.7	0.7	0.7	0.8
	Glutamic acid	15.000	16.000	13.000	9.167	5.3	0.5	0.0	0.8
Plot 3 (41.0 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	6.967	8.800	8.067	8.067	0.6	0.0	0.6	0.6
	Glucose	6.233	9.167	8.800	5.500	0.6	0.6	0.0	4.0
	Citric acid	8.800	10.267	8.800	5.867	1.1	1.7	1.1	2.3
	Glutamic acid	9.167	8.800	7.333	8.067	1.3	1.1	1.7	0.6
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	5.133	8.433	8.067	7.700	0.6	0.6	0.6	0.0
	Glucose	5.867	8.433	8.067	6.967	0.6	0.6	0.6	2.3
	Citric acid	8.067	9.533	7.700	6.600	0.6	0.6	0.0	0.0
	Glutamic acid	8.800	8.800	8.433	8.433	1.1	0.0	0.6	0.6
Plot 6 (18.9 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	1.933	1.800	1.967	3.267	0.2	0.3	0.1	0.1
	Glucose	1.933	1.433	2.033	3.067	0.3	0.2	0.4	0.1
	Citric acid	2.267	1.567	1.400	4.200	1.2	0.3	1.2	0.3
	Glutamic acid	1.733	1.767	1.900	4.333	0.1	0.2	0.1	0.5
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	1.800	1.467	1.900	3.067	0.2	0.2	0.3	0.6
	Glucose	2.533	1.600	1.933	3.067	1.1	0.3	0.3	0.7
	Citric acid	1.933	1.733	1.867	3.400	0.2	0.2	0.4	0.5
	Glutamic acid	1.800	1.167	2.400	4.667	0.2	0.9	1.8	1.6
Plot 8 (16.0 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	5.900	n.d.	4.733	4.767	1.8	n.d.	0.8	0.9
	Glucose	7.750	n.d.	4.833	5.133	1.3	n.d.	0.4	0.4
	Citric acid	7.467	n.d.	6.333	5.867	3.0	n.d.	0.3	1.1
	Glutamic acid	5.550	n.d.	7.950	6.800	3.5	n.d.	0.1	0.3

		T=0	T=4	T=7	T=14	T=0	T=4	T=7	T=14
Bulk soil	**Untreated**	4.933	n.d.	4.400	>d.l.	0.1	n.d.	0.5	
	Glucose	4.833	n.d.	5.533	>d.l.	0.9	n.d.	0.6	
	Citric acid	6.767	n.d.	6.367	>d.l.	1.8	n.d.	1.4	
	Glutamic acid	6.800	n.d.	7.300	>d.l.	0.7	n.d.	0.6	
Plot 11 (9.8 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	4.800	2.900	2.567	9.500	0.5	0.3	0.1	3.3
	Glucose	4.867	2.500	2.367	4.800	0.5	0.5	0.3	0.3
	Citric acid	4.600	3.700	3.000	13.700	1.1	0.1	0.4	0.1
	Glutamic acid	5.600	2.833	3.067	9.900	0.2	0.2	0.5	4.7
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	4.200	2.633	2.533	5.267	0.4	0.2	0.3	0.6
	Glucose	5.067	2.567	2.567	4.733	0.2	0.2	0.2	0.6
	Citric acid	5.000	2.733	3.300	6.467	0.4	0.3	0.4	0.6
	Glutamic acid	4.867	1.967	3.300	6.533	0.4	1.7	0.5	0.5
Plot 13 (8.7 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	4.233	3.433	3.200	4.267	0.9	0.4	0.1	0.3
	Glucose	3.700	2.867	3.400	3.833	0.3	1.2	0.3	0.8
	Citric acid	4.400	3.267	4.233	3.000	1.0	0.5	0.4	1.1
	Glutamic acid	3.733	3.033	3.400	2.867	2.0	0.4	0.3	0.4
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	4.167	4.300	3.433	3.967	0.9	0.8	0.1	1.1
	Glucose	3.700	4.500	3.433	3.900	0.1	1.6	0.4	0.4
	Citric acid	4.100	3.733	3.567	3.433	0.8	0.6	0.3	0.5
	Glutamic acid	3.400	3.600	3.267	4.033	0.8	0.4	0.8	1.1
Plot 16 (0.7 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	0.133	0.333	0.500	0.433	0.1	0.1	0.1	0.2
	Glucose	0.100	0.367	0.300	0.333	0.0	0.1	0.0	0.2
	Citric acid	0.100	0.333	0.433	0.400	0.0	0.1	0.1	0.1
	Glutamic acid	0.100	0.400	0.333	0.567	0.0	0.0	0.1	0.1
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	0.100	0.333	0.400	0.367	0.0	0.1	0.1	0.1
	Glucose	0.100	0.400	0.400	0.433	0.0	0.0	0.1	0.2
	Citric acid	0.100	0.367	0.333	0.367	0.0	0.1	0.2	0.1
	Glutamic acid	0.100	0.300	0.467	0.400	0.0	0.1	0.2	0.0
Plot 18 (0.7 mg Cd kg^{-1} d.w.soil)		**T=0**	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Rhizosphere	**Untreated**	0.133	0.450	0.600	0.367	0.1	0.1	0.8	0.3
	Glucose	0.100	0.267	0.633	0.233	0.0	0.1	0.3	0.1
	Citric acid	0.100	0.267	0.333	0.267	0.0	0.1	0.3	0.2
	Glutamic acid	0.100	0.100	0.033	0.167	0.0	0.0	0.1	0.1
		T=0	**T=4**	**T=7**	**T=14**	**T=0**	**T=4**	**T=7**	**T=14**
Bulk soil	**Untreated**	0.100	0.250	0.300	0.200	0.0	0.2	0.3	0.2
	Glucose	0.100	0.100	0.367	0.067	0.0	0.0	0.1	0.1
	Citric acid	0.100	0.133	0.133	0.167	0.0	0.1	0.1	0.1
	Glutamic acid	0.100	0.100	0.033	0.200	0.0	0.0	0.1	0.0

n.d, not determined, >d.l., under detection limits

3.5. Microbial biomass, C and N contents

Microbial biomasses C and N in rhizosphere and bulk soil layer are reported in tables 4.8and 4.9. In soils from plot 1 treated with glutamic acid, in the rhizosphere, there was a visible increase of microbial biomass after 4d until 7d. Glucose treatment showed a lower increase compared to glutamic acid at T=4d. In all the treatments after the last addition of MRE (at 7d), the carbon biomass content decreased in all treatments. In the bulk soils, the effect of MRE was homogeneously lower than in rhizosphere soils, in all treatments.

Soils from plot 3 showed a different pattern: in the rhizosphere the MRE effect sustained the biomass growth until T=4d, then the same decrease of plot 1 occurred at T=7d and more evident at T=14d. The same pattern was visible in bulk soils.

The increase of carbon biomass due to glucose and glutamic acid, during the incubation at T=4d and 7d, in plot 1, was sensibly higher and significant ($p>0.05$) compared to plot 3. This difference was not significant ($p<0.05$) in bulk soils.

Soils belong to plot 6 in the rhizosphere showed the effect of MRE in glucose, citric acid and glutamic acid treatments immediately after the first addition with an increase in C-biomass. After it decreased significantly at T=14d ($p<0.05$), as reported in table 4.14. In the bulk soils, glucose did not increase the C biomass.

In rhizosphere soils from plot 8, the initially microbial biomass decreased in time. However, the values were similar and not significant ($p>0.05$) in the first 7d of incubation experiments, and thereafter a decrease in all treatments was detected. In the bulk soils, a marked decrease in C biomass at T=7d was noticed in soils treated with citric and glutamic acids. Plot 8 always had higher values of C biomass compared to plot 6 ($p<0.05$).

In plot 11, glucose increased microbial biomass C compared to untreated soils, but significant effect was only observed at 7 and 14 days. Generally in bulk soil, there was no effect of MRE, and no significant differences were observed within treatments.

Soils from plot 13 showed a similar pattern in all treatments, both in rhizosphere and bulk soils, with an increased C biomass at T=4 in rhizosphere and bulk soils ($p>0.05$). Thereafter a decrease of biomass started at T=14d and reached lower values than at T=0.

In plot 11 compared to plot 13, the microbial biomass was generally higher (except in T=4d in untreated and glucose soils) or at least equal. In the bulk soils a definable trend was not observed.

In plot 16 at T=4d citric acid and glutamic acid both in the rhizosphere and bulk soils, significantly increased the microbial biomass, also glucose increased significantly the biomass C, but only at T=7d (table.4.14). In all treatments a decrease after the last addition of MRE (T=7d) was observed.

Soils from plot 18 showed the same pattern in the rhizosphere and bulk soils. The higher significant increase in C biomass was observed in soils treated with glutamic acid at T=4d.

Glucose shown a significant increase at T=7d. The decrease which occurred in the rhizosphere in all treatments at T=14d was always significant ($p<0.05$). In bulk soils the increase of microbial biomass in soils treated with glutamic acid was significant as well as the increase at T=7d of glucose. Citric acid showed in the rhizosphere an opposite pattern, significant values of microbial biomass were detected at T=0, immediately the biomass decreased and maintained constant value until T=7d, where an additional significant decrease occurred ($p<0.05$).

The differences between plot 16 and 18 were not significant ($p>0.05$).

Generally microbial biomass increased its value during the incubation until 4-7 days, when the last MRE additions were made. Fluctuant trends were observed, but it can be affirmed that MRE increased the immobilization more in the rhizosphere than bulk soils. Cadmium concentration did not affect microbial biomass growth. The soil pH did not show a definable influence on carbon biomass. In general, for contaminated soils at similar total Cd level, the neutral soil had a higher impact than acid soils. The opposite was found in plot 8 and plot 6. In control soils, the pH did not affect the results at all.

Table 4.8. Effects of MRE on biomass C (μg C g^{-1} d.w.) in the rhizosphere and bulk soil during the incubation (symbols **, indicates significant statistical values p<0.05,* indicates significant statistical values p<0.01). The statistical significance reported in the table was calculated between the treatments in each plot.

	Rhizosphere					*Bulk soil*			
Plot 1	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 1**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	411,57	615,21	814,91	238,51	**Untreated**	416,60	641,82	752,24	247,22
Glucose	388,34	1018,64**	1561,27**	193,09	**Glucose**	357,31	400,22	664,48	104,00
Citric acid	694,12	839,53	920,61	246,41	**Citric acid**	365,09	883,51	854,23	164,04
Glutamic acid	630,62	1701,69**	1255,23**	255,74	**Glutamic acid**	266,08	525,25	1180,68	163,97
Plot 3	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 3**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	441,07	323,77	166,23	148,65	**Untreated**	416,22	430,51	176,97	114,65
Glucose	544,22	696,20	184,71	82,07	**Glucose**	483,93	695,91	126,02	80,81
Citric acid	645,76	700,52	162,96	136,55	**Citric acid**	226,99	527,28	171,52	99,85
Glutamic acid	696,95	267,24	192,77	112,09	**Glutamic acid**	305,31	348,28	220,82	116,00
Plot 6	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 6**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	333,84	420,12	127,12	122,13	**Untreated**	390,45	542,24	71,03	131,48
Glucose	355,93	741,45	159,50	180,78*	**Glucose**	418,62	317,48	77,98	125,22
Citric acid	340,46	614,45	129,18	127,29*	**Citric acid**	276,47	556,21	140,81	128,23
Glutamic acid	430,00	694,17	154,35	140,44*	**Glutamic acid**	280,77	486,27	88,89	112,08
Plot 8	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 8**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	537,67	n.d.	533,74	436,14	**Untreated**	546,34	n.d.	649,409	389,28
Glucose	847,12	n.d.	788,7	402,76	**Glucose**	885,34	n.d.	766,536	291,57
Citric acid	877,47	n.d.	667,99	150,26	**Citric acid**	876,89	n.d.	382,016	125,64
Glutamic acid	886,21	n.d.	897,14	332,9	**Glutamic acid**	1292,27	n.d.	757,339	349,27
Plot 11	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 11**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	623,95	399,03	549,97	88,75	**Untreated**	534,34	693,36	649,86	95,65
Glucose	1041,67	985,16	1886,36*	108,78*	**Glucose**	840,53	946,11	460,26	82,73
Citric acid	1024,53	1139,47	783,09*	94,73*	**Citric acid**	439,48	600,85	252,86	82,29
Glutamic acid	781,58	2035,48	1218,67*	158,29*	**Glutamic acid**	762,50	834,85	566,45	124,79
Plot 13	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 13**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	284,84	679,74	454,01	39,49	**Untreated**	320,77	761,50	635,54	56,56
Glucose	240,04	1136,13*	984,44	85,81	**Glucose**	336,56	613,39*	984,32	54,38
Citric acid	647,36	958,69*	614,92	58,48	**Citric acid**	269,24	1156,76*	734,20	34,15
Glutamic acid	195,18	1245,68*	949,07	66,47	**Glutamic acid**	374,99	1082,22*	685,27	44,64
Plot 16	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 16**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	349,36	411,31	631,29	183,19	**Untreated**	145,07	471,37	550,50	179,18
Glucose	258,06	459,71	1377,34*	279,20	**Glucose**	515,83	369,54	1028,99*	252,05
Citric acid	642,28	1321,15*	838,43	245,31	**Citric acid**	414,78	1246,52*	464,75	129,31
Glutamic acid	820,37	1425,76*	877,29	272,39	**Glutamic acid**	378,54	1443,32*	599,81	27,70
Plot 18	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 18**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	455,07	390,85	545,55	236,68	**Untreated**	563,34	470,85	631,64	219,96
Glucose	594,70	797,97	1506,31*	488,51*	**Glucose**	197,60	656,97	1237,48*	201,62
Citric acid	1071,94*	681,58*	674,62	277,20*	**Citric acid**	860,29	839,64	682,23	262,06
Glutamic acid	461,82	1187,62*	495,19	477,63*	**Glutamic acid**	845,37	1328,41*	609,52	245,46

Table 4.9 reported the microbial biomass N. In the rhizosphere, soils from plot 1, treated with citric acid showed a significant increase (p<0.05) in nitrogen biomass after 4d respect to the T=0d. The successive reduction at 7d and 14d was significant (p<0.01). The effect of glutamic acid increased significative the N biomass at T=7 (p<0.05) compare to the untreated soil. No effect of glucose was noticed. In the bulk soils, there was an increased in nitrogen biomass in soils treated with glucose which had a linear increased up to 7d, and after decreased. Glutamic acid and citric acid, generally did not affect the N-biomass content in the bulk soil.

In plot 3, glucose and glutamic acid treatments increased the biomass N, but a higher significant effect of citric acid was detected (p<0.01) at T=7d in the rhizosphere and bulk soils. In soils from plot 6, both in rhizosphere and bulk soils, citric acid at T=7d of incubation only showed significantly increase in biomass N, compared to the other

treatments. By contrast, in the rhizosphere, a significant decrease in N biomass was observed in soils treated with glutamic acid. During the incubation in soils from plot 8 no significant effect of MRE was found at 7d, but the increase of all the N-biomass content in the bulk soil at T=14 was significant ($p<0.05$). But no significance differences were found within the samples of plot 11 treated with MRE. Generally N biomass content in plot 11 showed a decrease, at T=4d, followed by an increase at T=7d. A significant increase in biomass N in the rhizosphere at T=7d relative to citric and glutamic acid was observed.

In plot 13, no statistical differences were observed neither in rhizosphere nor in bulk soil but an increase in both rhizosphere and bulk soil of microbial biomass N at T=14 in all treatments was visible.

In soils from plots 16, higher values of biomass N were observed compared to the contaminated soil, and significant differences ($p<0.05$) were reported between plot 16 and plot 1.

The MRE increased the biomass N, and the growth was sustained in all treatments for all incubation times. Whereas in soils treated with glutamic acid in the rhizosphere, a reduction at T=14d occurred.

Plot 18 did not show the same increase in N biomass after T=4d, and the reduction was significant for glucose and citric acid treatments ($p<0.05$) in the rhizosphere. At T=7d the growth was recovered (excepted in untreated soils) in both rhizosphere and bulk soils ($p<0.01$).

The trend of microbial biomass N content was fluctuant in the different treatment and plots, but generally decreased in the first days of incubation (T=4d) and increased after T=7d after the last MRE addition, and then decreased again at T=14d. This trend is independent from the MRE, rhizosphere and bulk soil.

In control soils, plot 16 and 18, despite the fluctuant pattern, the effects of MRE compared to the untreated soils were clear, and the higher effect was due to the glutamic acid treatment. Fluctuations of results, however, were within a small range.

The highest values of biomass were reported in control soils (where the Cd extracted was 0.01 μg Cd Kg^{-1} soil d.w.) compared to contaminated soils. In this case, the values were two or three times higher than in high contaminated soils, both in the rhizosphere and bulk soils. The lower values of biomass were detected in plot 1 and plot3.

In plots 18, the effect of MRE on N biomass was generally lower than in plot 16 (except T=7d). In general, the effect of MRE on nitrogen biomass content was evident after 7d, both in the rhizosphere and bulk soils, whereas glutamic acid in the rhizosphere have an high N-biomass content in all the incubation times of the experiment.

Table 4.9. Effect of MRE on microbial biomass N (μg C g^{-1} d.w.) in the rhizosphere and bulk soil during incubation (symbols **, indicates significant statistical values p<0.05,* indicates significant statistical values p<0.01). The statistical significance reported in the table was calculated between the treatments in each plot.

	Rhizosphere					***Bulk soil***			
	Incubation time (days)					**Incubation time (days)**			
Plot 1	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 11**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	33,14	12,20	49,00	19,37	Untreated	17,94	0,68	40,86	24,74
Glucose	24,53	32,10	37,11	27,43	Glucose	19,43	22,70**	55,25	19,35**
Citric acid	19,91	198,12**	31,88**	33,62**	Citric acid	0,94	22,62	37,57	21,35
Glutamic acid	55,77	35,38	217,28**	22,73	Glutamic acid	34,45	48,15	36,71	36,71
Plot 3	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 3**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	10,81	17,53	30,24	35,70	Untreated	8,46	36,56	33,11	28,64
Glucose	13,99	35,93	69,52	29,05	Glucose	2,47	27,57	36,91	21,38
Citric acid	34,45	21,91	286,13*	72,50	Citric acid	15,00	40,05	201,91**	25,87
Glutamic acid	14,87	64,02**	26,94	36,14	Glutamic acid	5,12	63,50	45,52	11,69
Plot 6	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 6**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	46,53	12,12	50,77	20,97	Untreated	32,79	25,13	33,68	27,84
Glucose	35,79	33,15	32,67	40,33	Glucose	37,72	66,43	34,93	19,81
Citric acid	27,72	62,16	171,48*	40,78	Citric acid	34,02	28,14	178,82*	23,48
Glutamic acid	37,79	74,93	18,84*	53,13	Glutamic acid	30,51	27,88	92,60	33,55
Plot 8	**T=0**		**T=7**	**T=14**	**Plot 8**	**T=0**		**T=7**	**T=14**
Untreated	32,93	n.d.	9,66	77,83	Untreated	20,48	n.d.	7,14	17,16
Glucose	37,64	n.d.	14,53	55,04	Glucose	32,18	n.d.	11,72	28,86*
Citric acid	38,12	n.d.	11,05	38,28	Citric acid	37,44	n.d.	19,33	27,68*
Glutamic acid	38,20	n.d.	11,59	106,72	Glutamic acid	28,88	n.d.	17,70	22,44*
Plot 11	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 11**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	19,38	2,58	90,64	59,11	Untreated	17,88	14,77	35,69	63,93
Glucose	31,11	2,23	42,64	71,34	Glucose	25,60	10,91	47,83	33,36
Citric acid	30,26	5,20	182,03*	66,89	Citric acid	19,51	4,03	62,51*	37,41
Glutamic acid	38,20	9,57	17,93*	173,49	Glutamic acid	50,97	5,86	41,11	31,92
Plot 13	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 13**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	31,32	21,43	60,16	27,62	Untreated	44,84	0,35	27,98	29,00
Glucose	28,12	18,56	23,68	135,70	Glucose	39,68	5,22	65,12	132,53
Citric acid	43,09	43,30	19,61	138,48	Citric acid	22,77	45,13	72,47	135,50
Glutamic acid	26,73	79,71	68,28	137,55	Glutamic acid	34,45	17,66	90,64	127,08
Plot 16	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 16**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	103,97	144,82	138,17	159,53	Untreated	158,13	180,62	130,21	165,12
Glucose	155,47	150,33	136,42	184,43	Glucose	173,39	151,11	148,43	149,68
Citric acid	156,27	178,05	138,24	162,55	Citric acid	155,32	178,77	144,97	156,97
Glutamic acid	178,04	206,77	162,33	76,05	Glutamic acid	153,33	141,87	159,43	161,12
Plot 18	**T=0**	**T=4**	**T=7**	**T=14**	**Plot 18**	**T=0**	**T=4**	**T=7**	**T=14**
Untreated	116,07	27,93	13,32	54,04	Untreated	95,14	36,46	46,38	58,30
Glucose	136,87	46,19*	178,19	74,40	Glucose	114,55	31,67	204,23*	59,66
Citric acid	112,04	43,44*	185,14	61,77	Citric acid	118,53	39,56	206,60*	36,66
Glutamic acid	183,02	208,06*	178,28	194,87	Glutamic acid	177,85	47,79	211,43*	85,92

4. DISCUSSION

The effect of glucose, citric acid and glutamic acid (compounds normally present in the root exudates) on the mobility of cadmium, microbial biomass content, soil respiration and N inorganic content, was studied by using a model root system used by Falchini et al. (2003) to simulate the diffusion of root exudates in the rhizosphere soil.

With this system, Falchini et al. (2003) observed that the addition of the substrates on a cellular filter located on the soil surface formed a gradient concentration due to their diffusion in soil. The concentration gradient of the added citric acid, glucose and glutamic acid in soil depended on both water solubility of the LMOW compound and its reactivity with soil constituents. Glutamic acids as reported to Falchini et al. (2003) diffused at a lower rate than glucose, probably because it interacted with soil matrix being a charged molecule. Similar to glutamic acid also citric acid may interact with soil matrix or organic compounds. Low molecular weight organic acids represent a stable pool for the soil organic matter resulting mainly from rhizodeposition and from microbial metabolites during soil organic matter decomposition (Renella et al., 2004). The effective sphere of influence in the rhizosphere of di- and tricarboxylic is about 0.2-1.0 mm from rhizoplane, depending on soil type, organic acid type, and time (Jones, 1998), for glucose and the monocarboxylic acids can, however, be much greater >5 mm (Jones, 1998). In this experiment, generally the effect of MRE was more marked in the rhizosphere soils than in bulk soils, and this suggested that the MRS well mimicked the release of root exudates. The microbial biomass content was generally higher in the rhizosphere of treated soils probably due to the higher concentration of root exudates in rhizosphere than in bulk soil. The results of soil respiration showed a cumulative respiration higher in treated soils, and generally higher in highly contaminated soils than in medium or not contaminated soils. Generally, within the contaminated soils, acid soils had values of cumulative respiration higher than neutral soils, but this difference decreased with the decreased cadmium content. From these results it seems that cadmium concentration influenced the soil respiration. In fact probably microbial community responded to the stress induced by metal exposure with microbial physiological adaptations to metals. As reported by Renella et al. (2004b), these adaptations rely on several mechanisms, such as precipitation of metals as phosphates, carbonates, and sulphides, physical exclusion by exopolymers, and intracellular sequestration with low molecular weight cysteine-rich proteins. Such cellular

mechanisms are energy-demanding, increase the maintenance energy and reduce the conversion of substrate into new microbial biomass and other metabolic processes (Pirt, 1975).

As expected the addition of MRE represented a C-sources and generally microbial biomass increased its value during the incubation until 4-7 days, when the last MRE additions were made. However the C biomass was generally not affected by cadmium content. These results might be explained by the cadmium availability results. In fact, LMOWA did not affect the mobility of cadmium in soil, probably due to the low C sources added. Furthermore, the low increase in microbial biomass response to the MRE addition reported in this experiment generally agreed with the results of Landi et al. (2006) who, using the same model root system and the addition of model root exudates (glucose, oxalic acid, and citric acid with a rate of 300 μg C cm^{-2}), did not showed a significant increase in ATP, an index of microbial biomass.

On the other hand, it remains to be explained why N microbial biomass was lower in soils with higher Cd concentration it should be related to the higher nitrification of glutamic acid in contaminated soils.

Glutamic acid affected nitrogen biomass content, ammonium and nitrate dynamics more than the others treatments and this is surely due to the N content of the acid itself.

The N inorganic concentration seemed not to be significantly affected by the cadmium content and generally, it was higher in neutral than acid soils.

The results of the Cd effect on the biochemical parameters were difficult to understand: despite it is well known that the dissociated carboxylic acids (holding one or more negative charges) may interact with the soil solid phase and cations through formation of metal–organic acid complexes (Sandnes et al., 2005; Uren and Reisenauer, 1988), and that the high complexing capacity of citrate would lead to a high metal sequestration resulting in a lower inhibition on microbial biomass metabolism (Renella et al., 2004b), the MRE added, as expected, were not totally mineralized. The mineralization was fluctuant. An explanation for the fluctuant values of the mineralization of MRE, despite no evidence of changing in the microflora community here, is that, in some cases, microbial functions in soil affected by soil metal contamination are often found to recover after an initial inhibition by high metal inputs (Holtan-Hartwig et al., 2002). Two factors may contribute to such recoveries. One is a gradual decrease in metal availability due to immobilisation reactions in soil. But more importantly, metal tolerant organisms may replace the metal sensitive ones within each functional group, in this way changing the microbial community's composition and increasing its metal tolerance (Almås et al., 2004; Falchini et al., 2003). In this thesis,

the negative effects of cadmium on the microbial growth were studied but no information about the changes in the structures of microbial community was considered. Values of cadmium labile pools measured by the single step extraction were not affected by the MRE. This result is in contrast with the data of Krishnamurti et al., (1995, 1997). The explanation would be that the low quantities of MRE given in soils were insufficient to change the soil condition (especially soil pH). The values obtained from bulk soils were not different from rhizosphere values, and this confirmed that in the rhizosphere soils, where the higher effect of MRE was expected, root exudates in these particularly conditions did not affect the cadmium mobility.

Sukreeyapongse et al. (2002) concluded through a flow cell release experiments that the release of cadmium is strictly dependent on soil pH values, being greater between pH<5 to pH 3. The buffer effect of AGIR soils inhibits the pH variation upon release of organic acids, and may contribute to explain the lack of consequences on Cd mobility.

In conclusion, the MRS has been demonstrated to well mimicking the release of root exudates in the rhizosphere. As already confirmed from previous studies the graduality of release of MRE has been confiremed in the reduction of the different microbial community parameters (C and N biomass, N content)(Falchini et al., 2003, Landi et al., 2006). Data obtained in this experiment indicate that, generally, low addition of root exudates to soil did not greatly influence N inorganic content, microbial biomass and respiration and did not affect solubility of Cd.

REFERENCES

Almås Å.R., Bakken L.R., Mulder J. Changes in tolerance of soil microbial communities in Zn and Cd contaminated soils. Soil Biology & Biochemistry 36, 805–813.

Badalucco, L., Kuikman, P.J., 2001. Mineralization and immobilization in the rhizosphere. In: Pinton, R., Varanini, Z., Nannipieri, P. (Eds.), The Rhizosphere. Biochemistry and Organic Substances at the Soil-Plant Interface. Marcel Dekker, New York, pp. 141–196.

Curl, E.A., Truelove, B., 1986. The Rhizosphere. Springer, Berlin. 288 p.

Darrah, P.R., 1991a. Measuring the diffusion coefficient of rhizosphere exudates in soil. I. The diffusion of non-sorbing compounds. Journal of Soil Science 42, 413–420.

Darrah, P.R., 1991b. Measuring the diffusion coefficient of rhizosphere exudates in soil. II. The diffusion of sorbing compounds. Journal of Soil Science 42, 421–434.

Falchini, L., Naumova, N., Kuikman, P.J., Bloem, J., Nannipieri, P., 2003. CO_2 evolution and denaturing gradient gel electrophoresis profiles of bacterial communities in soil following addition of low molecular weight substrates to simulate root exudation. Soil Biology & Biochemistry 36, 775–782.

Feng, M., Shan, X, Zhang, S., Wen, B., 2005. Comparison of a rhizosphere-based method with other one-step extraction methods for assessing the bioavailability of soil metals to wheat. Chemosphere 59, 939–949.

Holtan-Hartvik, L., Bechman, H., Høyas, T.R., Linjordet, R., Bakken, L.R., 2002. Heavy metals tolerance of soil denitrifying communities: N^2O dynamics. Soil Biochemistry 34, 1181–1190.

Jenkinson, D.S., 1988. Determination of microbial biomass carbon and nitrogen in soil. In J.R. Wilson (ed.), Advances in nitrogen cycling in agricultural ecosystems. Wallingford. CAB International, pp. 368-386

Joergensen, R.G., Brookes, P.C., 1990. Ninhydrin- reactive nitrogen measurements of microbial biomass in 0.5 M K_2SO_4 soil extracts. Soil Biology & Biochemistry 19, 1023-1027.

Jones, D.L., 1998. Organic acids in the rhizosphere - a critical review. Plant and Soil 205, 25-44.

Keeney, DR., Nelson, DW., 1982. 'Nitrogen-Inorganic Forms', In: Page A.L., Miller R.H. and Keeney D.R. (eds.), Methods of Soil Analysis, Part 2: Chemical and Microbiological Properties, Agronomy 9, ASA, SSSA. Madison, WI, USA. pp. 643-698.

Krishnamurti, G.S.R., Huang, P.M., van Rees, K.C.J., Kozak, L.M., Rostad H.P.V., 1995. Speciation of particulate-bound cadmium of soils and its bioavailability. The analyst 120, 659-665.

Krishnamurti, G.S.R., Huang, P.M., van Rees, K.C.J., 1997a. Kinetics of cadmium release from soils as influenced by organic acids: Implication in cadmium availability. Journal of Environmental Quality 26, 271-277.

Landi, L., Valori, F., Ascher, J., Renella, G., Falchini, L., Nannipieri, P., 2006. Root exudate effects on the bacterial communities, CO_2 evolution, nitrogen transformations and ATP content of rhizosphere and bulk soils. Soil Biology & Biochemistry 38(3), 509-516.

Marschner, H., 1995 Mineral nutrition of higher plants. Academic Press London (publ.), 889 p.

Prues, A., 1998. Action values for mobile (NH_4NO_3-extractable) trace elements in soils base on the German national standard DIN 19730.

Pirt, S.J., 1975. Principles of Microbe and Cell Cultivation. Blackwell Scientific Publication, Oxford.

Renella, G., Landi, L., Nannipieri, P., 2004a. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311–315.

Renella, G., Mench, M., van der Lelie, D., Pietramellara, G., Ascher, J., Ceccherini, M.T., Landi, L., Nannipieri, P., 2004b. Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils. Soil Biology & Biochemistry 36, 443-451.

Sandnes, A., Eldhusetb, T.D., Wollebækb, G., 2005. Organic acids in root exudates and soil solution of Norway spruce and silver birch. Soil Biology & Biochemistry 37, 259–269.

Sauvé, S., Hendershot, H., Allen, H.E., 2000. Solid–solution partitioning of metals in contaminated soils: dependence on pH total metal burden and organic matter. Environment Science Technology 34,. 1125–1131.

Stotzky, G., 1965. Microbial respiration. In: A. Black (ed.). Agronomy 9. American Society of Agronomy. Madison, WI, pp. 1550-1569.

Strobel, B. W., Borggaard, O. K., Hansen, H. C. B., Andersen, M. K., Raulund-Rasmussen, K., 2005. Dissolved organic carbon and decreasing pH mobilize cadmium and copper in soil, European Journal of Soil Science 56(2), 189-196.

Ström, L., Owenb, AG., Douglas, Godboldb, L., Jones, D., 2005. Organic acid behaviour in a calcareous soil implications for rhizosphere nutrient cycling. Soil Biology & Biochemistry 37, 2046–2054.

Sukreeyapongse, O., Holm, P.E., Strobel, B., Panichsakpatana, S., Magid, J., Hansen H.C., 2002. pH-Dependent Release of Cadmium, Copper, and Lead from Natural and Sludge-Amended Soils Journal of Environmental Quality 31, 1901-1909.

Uren, N.C., 2000. ”Types, Amounts, and possible function of coumpounds relased into the rhizosphere by soil-grown plants”. The Rizosphere, Piton, R., Varanini, Z., Nannipieri, P., Marcel Dekker, Inc., New York, pp 19-41.

Uren, N.C., Reisenauer, H.M., 1988. The role of root exudates in nutrient acquisition. In Advances in Plant Nutrition, Volume 3 (ed. B. Tinker, A. Läuchli), Praeger, New York, pp 79-114.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biology & Biochemistry 19, 703–707.

Whipps, J.M., 1990. Carbon economy. In: Lynch, J.M., (Ed.), The Rhizosphere, Wiley, New York, pp. 59–97.

Wu, J., Joergensen, R.G., Pommerening, B., Brookes, P.C., 1990. Measurement of soil microbial biomass C by fumigation-extraction - an automated procedure. Soil Biology & Biochemistry 25, 1435-1441.

CHAPTER FIVE

ASSESSMENT OF CADMIUM TOXICITY TO PLANTS AND SOIL REMEDIATION THROUGH THE AIDED-PHYTOSTABILIZATION

Valori Federico [a], Landi Loretta [a], Mench Michel [b], Renella Giancarlo [a] and Nannipieri Paolo [a]

[a] Department of Soil Science and Plant Nutrition University of Florence, P.le delle Cascine, 28, 50144, Florence, Italy

[b] UMR BIOGECO INRA 1202, Ecologie des Communautés, Université Bordeaux 1, Bât B8, RdC Est, 002, Avenue des Facultés F-33405 Talence, France

1. INTRODUCTION

High concentrations of trace elements in soil may constitute long-term health risk to ecosystem and humans. Chronic or acute cadmium exposure above a threshold level is highly toxic to humans and is considered to be a priority regarding inorganic contaminants in food (Singh, 2003). Cadmium in soil can be taken up by plants and transferred to a higher trophic level by means of plant-derived products and herbivory (Notten et al., 2005). Among the different existing strategies for remediation of contaminated soils, new options are required and the *in situ* immobilization combined with tolerant and excluders plant species (aided phytostabilization) seems to be a realistic and cost effective one (Mench et al 1994; Mench et al 2003; Singh, 2003). Natural remediation should be taken in account in the remediation strategies as it is the lowest cost effective, and it has the lowest impact on the environment, but is time consuming, and alone may not be sufficient in mitigating the risk from metals (Adriano et al., 2004). A better efficiency of the natural remediation may be obtained by its combination with the *in situ* immobilization, using inexpensive soil amendments. This combination may increase the definitive remediation of contaminated soils, and decrease time required to inactivate inorganic contaminants (Mench et al., 2003).

The stabilization (or inactivation) of the contaminant aims to reduce its mobility and decrease the labile metal fraction in the soil pore water. Thus, the stabilization may

reduce the potential risk associated with metal availability (Vangronsveld et al., 1995; Mench et al., 1994; Lombi et al., 2002). The efficiency of some adsorptive minerals on the reduction of Cd availability in contaminated soils is confirmed (Singh and Alloway, 2000; Saha et al., 2002; Brigatti et al., 2000). The adsorptive capacity of minerals for Cd sorption follows the following sequence, according to the Freundlich equation: zeolite 4A > zeolite P > zeolite Y > Australian smectite > Wyoming bentonite > beringite > natural zeolite (Singh and Alloway, 2000).

Many data on the long-term effect of mineral added to metal-contaminated soil are available (Mench et al., 2003) but the effect of added mineral has not yet been studied in the first days after the addition. The amendment effect generally is correlated with the measured of metal sorption on the minerals or with the spectrometry determination (or other chemical methods). The reliable effect should be studied directly on plants and on the cadmium labile fraction. In fact, metal contamination affected plant growth in several ways: reducing of biomass, leaf necrosis or chlorosis, oxidative stress at the sub-cellular level, and other symptoms depending from the plant species (Sanita di Toppi and Gabrielli, 1999).

This work aimed i) to compare the efficiency of three minerals to reduce Cd labile pool in eight Cd-contaminated soils (AGIR) with increasing Cd content by measuring the Cd concentration in the soil pore water, ii) to monitor the effectiveness of the 3 minerals on plant performances, by measuring chlorophyll and biometrical parameters on three different plants species transplanted sequentially in the remediated soils, and iii) to compare the Cd effect on *H. lanatus* L. growth under controlled condition in a hydroponic culture, this plant species being used in the pot experiment and having potential to be used in the field for phytostabilisation. The nutrient solution was Cd-contaminated with a Cd concentration equal to the available fraction measured in the soils from plots 1 and 3 of AGIR field experiment by $CaCl_2$ extraction (Renella, 2001).

2. MATERIALS AND METHODS

2.1. *Soil type and characteristics*

Soils are classified as sandy-clay, Arenic Udifluvent. The AGIR field experiment was managed by Unité d'Agronomie, INRA, Bordeaux, France (Renella et al., 2004). The cadmium contamination of the soil was achieved as described below.

The density of the 0–30 cm soil layer was determined and it was calculated as dry soil (fine fraction <2 mm) per ha and per m^2. The different plots (6 m X 3 m) have been

designed at the experimental site in 1987. Then, the amount of $Cd(NO_3)_2$ (Rectapur Quality, Prolabo) to be added to each plot was calculated for contaminating the 0–30 cm soil layer at Cd concentrations of 0, 0.09, 0.18, and 0.36 mmol kg^{-1} soil; it was spread on the soil surface and mixed by rototilling at 0–30 cm depth in the 1988–1990 period. The soil was ploughed at 0–30 cm depth through the years to ensure an even distribution of Cd within the soil layer. Chemical analyses demonstrated that the target Cd contaminations were reached. The background Cd concentration in the control soil was ~0.006 mmol kg^{-1}. The soils have been kept under grass and maize cultivation. In table 5.1 the main characteristics of AGIR soils are reported (Renella et al., 2001, 2005).

Table 5.1. Main characteristics of AGIR soils

Plots	**P1**	**P3**	**P6**	**P8**	**P11**	**P13**	**P16**	**P18**
Clay (%)	17.5	17.5	17.5	17.5	17.5	17.5	17.5	17.5
Silt (%)	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Sand (%)	67.5	67.5	67.5	67.5	67.5	67.5	67.5	67.5
$pH_{(H2O)}$	6.8.	6.3	7.1	6.3	7.2	6.1	7.4	6. 7
Total Cd (mgCd/Kg soil)	38.7	41.0	18.9	16.0	9.8	8.7	0.7	0.7
TOC (%)	0.49	1.0	0.47	0.35	0.25	0.27	0.95	1.33
N tot (%)	0.04	0.91	0.04	0.04	0.03	0.03	0.10	0.11

2.2. *Additives properties*

The following additives were used:

- Beringite+Iron grit, beringite is a mixture of modified aluminosilicates that originates from the fluidized bed burning of coal refuse (mine pile material) from the former coal mine in Beringen, Belgium (Vangronsveld et al., 2000). Iron grit is an industrial material used for shaping metal surfaces that contains mainly iron (97% α-Fe) and native impurities such as Mn (7710 mg kg^{-1}) (Mench et al., 2000). The iron grit used in this experiment was obtained from Wheelabrator Allevard Enterprise, France.
- Sepiolite (Sigma-Aldrich, Analytical grade) is a hydrated magnesium silicate that occurs as a fibrous chain-structure mineral in clays in several areas of the world. The major commercial deposits of sepiolite are in Spain. Sepiolite fibre characteristics vary with the source, but fibre lengths in commercial samples are generally less than 5 mm (Minerology database).

- Bentonite (Sigma-Aldrich, Analytical grade): Wyoming bentonite is composed essentially of montmorillonite clay, also known as hydrous silicate of alumina. Bentonite is capable of absorbing 7 to 10 times its own weight in water, and swelling up to 18 times its dry volume. It is a highly colloidal and plastic clay with the unique characteristic of swelling to several times its original volume when placed in water. Bentonite was formed from volcanic ash deposited in an ancient sea, and modified by geological process into the present (Mineralogy database).

Analytical grade chemicals were used throughout the investigation.
The minerals chosen in the present work were those most readily available on today's market for industrial minerals in France and Italy.
For each mineral additive and soil, triplicates were made.

2.3. *Laboratory experiments*

The eight AGIR soils (plots P1, P3, P6, P8, P11, P13, P16, and P18) were sampled in the 0-0.30 cm soil layer in October 2004, air-dried, sieved (<2mm, stainless steel and wood), and stored in the dark at 25 °C prior to experiments. For all the treatments, the additives (6% w/w of the total soil weight for sepiolite and bentonite, for beringite 5% w/w and iron grit 1% w/w) were separately well mixed with air-dried soils to obtain 1000 g soil d.w. Soil was potted with the soil moisture sampler, Rhizon MOM as described by Knight et al. (1998) and gradually watered with demineralised water to the 50% of the water holding capacity (WHC). Rhizon soil moisture samplers are an alternative for sampling soil moisture with ceramic cups. The porous plastic material wets spontaneously and has standard 0.1 µm diameter pores. The samplers are supplied with co-extruded tubing (PE inside/PVC outside) of 1 mm of internal diameter, lure connector and protective cap, and a length of 10 cm (complete with PVC/PE tubing connectors and protective caps). The sampler is reinforced by a wire. Dissolved organic and inorganic matter will not be absorbed into the soil moisture sampler. The suctions of soil pore water occurred at 1, 2, 7, 14 days and 6months after the addition of soil amendments. Initially, all the samplers have been washed with 60 mL of 2% HNO_3, followed by 60 mL of deionised water before use. Acid-washed disposable 60 mL syringes, attached to the luer-lock of the Rhizon samplers, were used to collect the

extracted soil pore water under vacuum overnight. Once extracted, the pore water was stored in acid washed plastic containers at -20 °C prior to chemical analysis.

The initial dynamics of Cd sorption by the minerals has been monitored. The cadmium concentration in the soil pore water was determined by graphite furnace atomic absorption spectroscopy (Varian AAS SpectrAA 250 plus GTA97) with a detection limit of 0.1 ng g^{-1}. The cadmium measured in soil pore water was considered as the labile pool of cadmium in soil and the Cd available for plant uptake (Krishnamurti et al., 1997). The experiment was carried out in triplicate for soils and treatments with a total of 96 samples. After the last soil pore water extraction, the pots were placed in a greenhouse.

2.4. *Plants properties and greenhouse experiment*

Plants used were: Dwarf bean (*Phaseolus vulgaris* L., cv. Aveve), Lettuce (*Lactuca sativa* L.var. "smeraldo", Blumen) and *Holcus lanatus* L. (velvet grass). Seeds of *H. lanatus* were collected the same year in uncontaminated plots of the AGIR experiments.

The plant selection was motivated by two reasons: the first was the gradient of tolerance to cadmium of the plants, i.e. Lettuce > *H. lanatus* > Dwarf bean (Mench et al., 2000; Mench et al., 2003; and Mench personal communication). The second reason was to use plants directly involved in the animals-human food chain.

The experiments in the greenhouse were carried out during the spring/autumn season and the temperature, humidity and light were in the optimal range for each plant species (dwarf bean has optimal growth in spring time, lettuce during the summer and *H. lanatus* in autumn). The greenhouse role was to avoid direct precipitation on the pots in order to avoid Cd dry deposits and to control the water content. The time elapsed between soil amendment addition and the first plant growth experiment was six months.

The life cycle of *H. lanatus* and Lettuce is around 90 d. To allow the growth of all plants during the summer both plants of *H. lanatus* and Lettuce were grown for 4 weeks before their transplantation in pots, instead of dwarf bean that has a short life cycle in this study (2-4 weeks) and thus its preliminary growth was not necessary. At the end of each phase of the experiment, before harvesting the plants, two disks of leaves with a 0.5 cm^2 area were immediately immerged in 3 mL DMF (dimethylformamide, Fluka, Analytical grade chemicals), before measuring the chlorophyll content. Densities of chlorophyll and carotenoids in the primary leaves of beans and 6^{th} leaves of lettuce and *H. lanatus* were quantified (Lagriffoul et al., 1998). Plant parts (leaves, roots, and shoots) were washed in distilled water and oven-dried at 50°C. Part of the rhizosphere

soil was immediately frozen for future analysis. Dwarf beans were sown (3 seeds per pot) in triplicates for each AGIR soils. The seedlings were grown in the greenhouse with the season status of temperature, relative humidity and light radiation respectively: Dwarf bean:T_{min}:= 5 °C-T_{max}= 30 °C; 66-69 %, 14 h light period, Lettuce T_{min}= 14 °C-T_{max}= 27 °C; 69-76%, 12 h light period, *H. lanatus* T_{min}= 6 °C-T_{max}= 21 °C; 75-80%, 10 h light period.

After sowing, the soil water status was maintained at 50% WHC with daily addition of demineralised water. After 2 weeks of growing period, bean plants were harvested. Then, after one week without fertilization to avoid any interference of fertilizers on the cadmium mobility, three plants of lettuce were transplanted in each pot. The growing period was 80 d. Soil status was maintained at 50% WHC. One week thereafter lettuces were harvested, 3 plants per pots of the last species *H. lanatus* were transplanted for 80 days of growing period.

2.5. *Hydroponic experiment*

Spontaneous plants of *Holcus lanatus* were grown on AGIR soils in both contaminated and uncontaminated. Plants from plots 1 and 3 (total Cd concentration 38.7 and 41 mg Cd Kg^{-1} and soil pH of 6.8 and 6.3 respectively) were gently collected and transplanted in pots with soils of the same plot and left in field to let the root system recovered. Plants from uncontaminated soils were collected and transplanted in plots 16 and 18 (Cd concentration 0.7 and 0.7 mg Cd/Kg, and soil pH of 7.4 and 6.7 respectively). Twelve plants for each plot were cultivated. After 4 weeks, plants were moved in laboratory, gently washed with distilled water, and placed in aerated nutrient solutions.

The device used plastic pots (PVC, 20-cm diameter, 20-cm height) containing 1500 mL of nutrient solution with vigorous air-bubbling aeration, three plants per pot, and four pots for each treatment. Hoagland-Arnon nutrient solution (Hoagland and Arnon, 1938 cited in Ewitt 1966) was used as a growth medium, and changed every 3d. The pH of the nutrient solutions of each pots was adjusted to the pH of corresponding AGIR soils (5.5 and 7 for more details see table 5.1). The nutrient solution corresponding to plots 1 and 3 was added with Cd nitrate to obtain the same $CaCl_2$ extractable–Cd concentration of the respectively AGIR soil plots (0.34 and 0.40 mg Cd Kg^{-1}). The plants were cultivated in a growth room with a 14-h light period (150 µmol m^{-2} s^{-1}). The temperature was regulated to 25°C day and 20°C night and the relative humidity was maintained at roughly 60%.

After 23d, the root elongation was measured by staining roots in a black carbon solution (1%). The root elongation was measured after one week by measuring the root growth (white coloured). After 30d, roots and shoots were harvested and washed in UHQ water. Discs for the chlorophyll content analysis were taken and placed in DMF. Leaf aliquots (1g FW, in duplicate) were immediately frozen in liquid nitrogen for gaiacol peroxidase (GuPx) analysis. Biomass and leaf area index (LAI) measurements were performed on six of the developed leaves for each plants sampled. LAI was calculated with WINDIAS software.

2.6. *Chlorophyll and carotenoid content*

The chlorophyll and carotenoid contents have been measured according to the Lichtenhaler and Wellburn (1983) and Blanke (1990) methods. Two leaf discs of 0.5 cm^2 were taken and stored in a super cooler freezer at -70°C. Discs were thereafter immerged in 3 mL of N, N-dimethylformamide (DMF) previously cooled at 4°C. The flasks were kept at 4°C in the fridge for 24 h. The absorbances were measured at 470, 647 and 664.5 nm using a UV-Vis spectrophotometer (Perkin Elmer Lambda 500).

2.7. *Guaiacol peroxidase (GuPx) activity*

Samples were immediately frozen in liquid nitrogen and stored at -80°C for enzyme analysis. The capacity (the potential activity measured *in vitro* under non-limiting reaction conditions) of the guaiacol peroxidase (GuPx; E.C.: 1.11.1.7) was measured spectrophotometrically as previously described (Van Assche et al., 1988). Enzyme capacity was expressed in mU per gram fresh weight (FW). Results of the biological evaluation are presented with reference to phytotoxicity classes (Vangronsveld et al., 1996): class 1 not toxic, class 2 slightly toxic, class 3 moderately toxic, and class 4 strongly toxic.

2.8. *Cadmium determination*

Cadmium concentration in samples of soil pore water extracted with Rhizon was determined by graphite furnace atomic absorption spectrophotometry (Varian AAS SpectrAA 250 plus GTA97) with a detection limit at 0.1 ng g^{-1}. The leaves of *H. lanatus*

were digested with a Milestones 1200 microwave following the Milestone instrument protocol:

Each sample of 0.3 g dried plant material, previously milled, were added with 5 mL of 14 M HNO_3 and 1mL of 30% vol/vol H_2O_2. The microwave program was step 1: 4 minutes at 250W step 2: 0W for 2minutes, step 3, 250W for 4 minutes, step 4, 400W for 4 minutes, last step, 2 minutes at 500W. Two blank reagents were made as background correction. All chemical reagents were ultrapure grade. Cadmium determination in plants was measured by graphite furnace atomic absorption spectrophotometry (Varian AAS SpectrAA 250 plus GTA97). Certified reference plant materials (V463, maize shoots, Cd 1.66 ± 0.32 mg/kg DM) were included in each batch series. The Cd recovery was 99% compared to certified value.

2.9. *Statistical analysis*

The LSD values (Tukey-Kramer test, p level < 0.05 marked with "*" and $p<0.01$ marked with "**") were calculated to assess the significance of differences of the means (n = 3).

3. RESULTS AND DISCUSSION

3.1. *Plant biomass: shoots and roots yielded*

Plants of dwarf bean grown in untreated soils did not show the effect of the cadmium concentration in the biomass values of shoots and roots. The biomasses of aerial plant parts from untreated soils were similar to that of plants grown in sepiolite and Ber+IG (Beringite +iron grit) treatments. The plants grown in soils treated with bentonite had lower shoots dry biomass (except P3).

Table 5.2.reported the dried weight biomass (g/plant) of the shoots and roots of the plants grown in the amendments treated soils. Ber+IG: Beringite+Iron Grit treatment. No statistical differences were detected within the different plots of each tested plants species.

LETTUCE				
SHOOTS				
	Untreated	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.009	0.046	0.083	0.087
P3	0.017	0.048	0.085	0.094
P6	0.024	0.073	0.110	0.099
P8	0.039	0.088	0.184	0.010
P11	0.020	0.085	0.137	0.220
P13	0.037	0.088	0.095	0.124
P16	0.118	0.051	0.135	0.143
P18	0.165	0.075	0.090	0.107
ROOT	**Untreated**	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.020	0.017	0.03	0.01
P3	0.006	0.015	0.02	0.01
P6	0.014	0.040	0.03	0.00
P8	0.015	0.050	0.01	0.03
P11	0.008	0.018	0.02	0.03
P13	0.010	0.009	0.02	0.01
P16	0.038	0.017	0.02	0.02
P18	0.033	0.016	0.02	0.03

DWARF BEAN				
SHOOTS				
	Untreated	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.754	0.844	0.752	0.701
P3	0.832	0.581	0.757	1.147
P6	0.592	0.773	0.721	0.688
P8	0.163	0.696	0.777	0.501
P11	0.724	0.196	0.966	0.479
P13	0.679	0.816	0.505	0.435
P16	0.703	0.642	0.887	0.400
P18	0.768	0.639	0.801	0.273
ROOT	**Untreated**	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.101	0.134	0.08	0.05
P3	0.145	0.130	0.07	0.04
P6	0.122	0.082	0.05	0.09
P8	0.140	0.053	0.08	0.05
P11	0.131	0.200	0.10	0.04
P13	0.102	0.121	0.05	0.04
P16	0.137	0.005	0.08	0.07
P18	0.080	0.002	0.07	0.07

H.lanatus				
SHOOTS				
	Untreated	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.020	0.085	0.033	0.020
P3	0.039	0.055	0.044	0.021
P6	0.005	0.084	0.025	0.047
P8	0.025	0.045	0.093	0.034
P11	0.032	0.113	0.059	0.019
P13	0.063	0.126	0.046	0.021
P16	0.088	0.136	0.103	0.053
P18	0.089	0.132	0.108	0.091
ROOT	**Untreated**	**Ber.+IG.**	**Sepiolite**	**Bentonite**
PLOT				
P1	0.005	0.020	0.01	0.01
P3	0.009	0.010	0.01	0.01
P6	0.007	0.016	0.01	0.01
P8	0.007	0.016	0.01	0.01
P11	0.007	0.020	0.01	0.01
P13	0.018	0.038	0.01	0.01
P16	0.021	0.029	0.02	0.01
P18	0.018	0.021	0.01	0.01

Table 5.3.reported the statistical differences detected within the different plots of each tested plants species.

PLOT	Dwarf bean			Lettuce			H.Lanatus		
	Ber.+IG.	Sepiolite	Bentonite	Ber.+IG.	Sepiolite	Bentonite	Ber.+IG.	Sepiolite	Bentonite
P1	NS	NS	NS	NS	NS	NS	NS	NS	NS
P3	NS	NS	NS	NS	NS	NS	NS	NS	NS
P6	NS	NS	NS	NS	NS	NS	NS	NS	NS
P8	NS	NS	NS	NS	NS	NS	NS	NS	NS
P11	NS	NS	NS	NS	NS	NS	NS	NS	NS
P13	NS	NS	NS	NS	NS	NS	NS	NS	NS
P16	NS	NS	NS	NS	NS	NS	NS	NS	NS
P18	NS	NS	NS	NS	NS	NS	NS	NS	NS

Table 5.4.reported the ratio between dried root and shoots weight biomass of the plants grown in the amendments treated soils. Ber+IG: Beringite+Iron Grit treatment.

PLOT	Dwarf bean				Lettuce				H.Lanatus			
	Untreated	Ber.+IG.	Sepiolite	Bentonite	Untreated	Ber.+IG.	Sepiolite	Bentonite	Untreated	Ber.+IG.	Sepiolite	Bentonite
P1	0,13	0,16	0,11	0,08	2,22	0,37	0,31	0,15	0,27	0,23	0,25	0,30
P3	0,17	0,22	0,10	0,03	0,35	0,31	0,21	0,10	0,23	0,18	0,17	0,43
P6	0,21	0,11	0,06	0,13	0,57	0,55	0,23	0,02	1,35	0,19	0,39	0,24
P8	0,86	0,08	0,10	0,11	0,40	0,57	0,07	2,76	0,30	0,36	0,08	0,37
P11	0,18	1,02	0,11	0,08	0,41	0,21	0,16	0,13	0,23	0,18	0,16	0,42
P13	0,15	0,15	0,09	0,10	0,28	0,11	0,21	0,12	0,29	0,30	0,20	0,52
P16	0,19	0,01	0,09	0,17	0,33	0,33	0,17	0,14	0,24	0,22	0,16	0,28
P18	0,10	0,00	0,09	0,25	0,20	0,22	0,23	0,24	0,21	0,16	0,14	0,16

In soils treated with sepiolite and bentonite, dwarf bean showed a decrease in root biomass in all the samples independent from the cadmium concentration. Plants from soils treated with Ber+IG have root biomass similar to that of plants from the untreated soils (expect of P16 and P18).

The plants of lettuce grown in untreated soils have a lower biomass in both shoots and roots as soil Cd concentration increased. The effect of cadmium gradient on the shoot biomass decreased in plants grown in soils treated with sepiolite and Ber+IG. In uncontaminated soils, *i.e*. P16 and P18, the Ber+IG addition significantly (but no differences detected if I believe the table legend) decreased lettuce shoot yield. A similar effect occurred with sepiolite addition in P18. In soils treated with bentonite, lettuce plants showed the higher values of shoot biomass (except for P8 and P6 compared to sepiolite samples).

The roots in untreated soils showed a decreased yield correlated to soil Cd concentration. The lettuces grown on Ber+IG -treated soil showed root yield only slightly higher than those grown on untreated soils. Sepiolite and bentonite treatments caused a drastically reduction of the root biomass in all plots compare to untreated and Ber+IG treated soils. *Holcus lanatus* L. plants grown in untreated soils were affected by increased Cd concentrations in both root and shoot biomasses. In the sepiolite and Ber+IG treatments, shoot yield was ameliorated compared to the untreated soils. But the root biomass in the sepiolite-treated soils did not show a positive effect. On the

contrary, the roots biomass decreased compared to the untreated samples. The Ber+IG treatments gave higher root yields compared to the sepiolite treatments. Bentonite addition did not improve the growth response of *Holcus lanatus* nor in roots neither in shoots. Thus most efficient results for *H. lanatus* were obtained with Ber+IG.

In table 5.4 the ratio between roots and shoot dried biomass is reported. In the table generally no differences were notice in the distribution of biomass and shoot biomass is between five and ten times the root biomass. Some exceptions occurred in P1 of untreated soil of lettuce in wich the root biomass is 20 times the shoot biomass.

3.2. *Cadmium availability and mineral effects*

In table 5.5 the cadmium content in soil pore water is reported.

Table 5.5 cadmium content in soil pore water, expressed as μg Cd L^{-1}, Ber+ IG: beringite + iron grit; <d.l: under detection limits.

	Incubation Time(days)						SD				
Untreated	**0d**	**1d**	**2d**	**7d**	**14d**	**180d**	**1d**	**2d**	**7d**	**14d**	**180d**
Plot 1	1.533	1.533	1.5	2.033	3.133	0.27	0.21	0.26	0.35	0.5	0.06
Plot 3	1.300	1.3	1.667	2.233	3.233	0.63	0.2	0.12	0.21	0.1	0.32
Plot 6	0.133	0.133	0.6	0.3	0.433	0.13	0.06	0.78	0	0.1	0.06
Plot 8	0.600	0.6	0.433	1.033	1.333	0.27	0.1	0.45	0.15	0.2	0.12
Plot 11	0.033	0.033	0.567	0.233	0.033	<d.l.	0.06	0.51	0.4	0.1	0
Plot 13	1.400	1.4	1.567	1.5	1.733	0.27	0.1	0.38	0.17	0.2	0.12
Plot 16	0.067	0.067	<d.l.	<d.l.	0.067	0.03	0.06	0	0	0.1	0.06
Plot 18	0.067	0.067	<d.l.	0.033	0.033	<d.l.	0.12	0	0.06	0.1	0
Ber.+IG	**0d**	**1d**	**2d**	**7d**	**14d**	**180d**	**1d**	**2d**	**7d**	**14d**	**180d**
Plot 1	1.533	1.067	1.1	0.633	0.567	0.3	0.35	0.3	0.12	0.2	0.1
Plot 3	1.300	1.3	1.2	0.9	1.5	0.2	0	0	0	0	0
Plot 6	0.133	0.367	0.367	0.433	0.333	0.27	0.06	0.12	0.06	0.1	0.12
Plot 8	0.600	0.833	0.767	0.7	0.533	0.3	0.06	0.15	0.1	0.1	0
Plot 11	0.033	<d.l.	0.067	0.1	0.067	<d.l.	0	0.06	0	0.1	0
Plot 13	1.400	0.533	0.633	0.6	0.433	0.13	0.06	0.06	0.1	0.1	0.15
Plot 16	0.067	<d.l.	0.067	0.1	<d.l.	<d.l.	0	0.06	0	0	0
Plot 18	0.067	<d.l.	0.333	0.067	0.067	<d.l.	0	0.58	0.06	0.1	0
Sepiolite	**0d**	**1d**	**2d**	**7d**	**14d**	**180d**	**1d**	**2d**	**7d**	**14d**	**180d**
Plot 1	1.533	0.667	0.6	0.667	0.6	0.17	0.06	0	0.06	0	0.12
Plot 3	1.300	0.7	1.2	1.4	1	0.6	0	0	0	0	0
Plot 6	0.133	0.033	0.2	0.2	0.233	0.07	0.06	0	0	0.1	0.06
Plot 8	0.600	0.167	0.467	0.433	0.233	0.13	0.06	0.06	0.06	0.2	0.15
Plot 11	0.033	0.033	<d.l.	<d.l.	<d.l.	0.03	0.06	0	0	0	0.06
Plot 13	1.400	0.367	0.733	0.533	0.3	0.3	0.21	0.23	0.15	0.3	0.17
Plot 16	0.067	0.067	0.067	<d.l.	<d.l.	<d.l.	0.06	0.06	0	0	0
Plot 18	0.067	0.067	0.033	<d.l.	<d.l.	<d.l.	0.06	0.06	0	0	0
Bentonite	**0d**	**1d**	**2d**	**7d**	**14d**	**180d**	**1d**	**2d**	**7d**	**14d**	**180d**
Plot 1	1.533	82	66	10	36.9	nd	17	5.2	8.19	7.1	nd
Plot 3	1.300	20	63	26.5	72.6	nd	0	0	24.8	1.9	nd
Plot 6	0.133	14	15	9.333	4.5	nd	5.29	5.2	7.77	0	nd
Plot 8	0.600	57	54	7.633	33.9	nd	60.8	9	4.67	8.4	nd
Plot 11	0.033	6	27	3.6	2.25	nd	2.83	25.5	1.27	0.6	nd
Plot 13	1.400	53	45	4.2	30.6	nd	4.24	0	2.6	0	nd
Plot 16	0.067	2	<d.l.	0.9	0.9	nd	1.15	0	0	0	nd
Plot 18	0.067	<d.l.	<d.l.	0.9	0.9	nd	0	0	0	0	nd

In all untreated soils, generally, the value of cadmium concentration in soil pore waters increased after 14d of incubation, except in P16 and P11 in which no relevant changes were detected. After 180d, the values of cadmium concentration in P11 and P16 soil were comparable with the 14d values. In the other plots, a decrease in cadmium concentration was detected. The beringite and iron grit treatment (Ber+IG) has an immediate effect on Cd concentration in soil pore water: in plot 1, the Cd concentration

since 1d after amendment addition was reduced by 30.4%, and after 180d the reduction was 80.4%. In plot 3, the reduction was 84.6% after 180d, but the reduction was not immediate. At low total Cd content in soil, i.e. plots P16 and P18, the stabilization effect of Ber+IG was higher and roughly all Cd in soil pore water was immediately immobilized. With soils treated with sepiolite, the final reduction effect in all plots (except plot 3) ranged between 76 % (plot 13) and 97.8 % (plot 11). Plot 3 showed a modest reduction at 180d around 50%.

The soils treated with bentonite in the first days after amendment addition displayed an opposite effect. The Cd concentration in soil pore water increased to very high values. The effect was more evident in acid soils. In plot 3, the value increased up to 36.9 μg Cd L^{-1}, in plot 8 up to 33.9 and in plot 13 up to 30.6 μg Cd L^{-1}. In table 5.4 the pH values of soils treated with amendments is reported.

3.3. *pH*

Table 5.6 pH values of soils treated with amendments and the pH of amendments

Untreated	Incubation Time					**Sepiolite 8.5±0.5**	Incubation Time				
PLOT	**1d**	**2d**	**1w**	**2w**	**6m**	**PLOT**	**1d**	**2d**	**1w**	**2w**	**6m**
P1	6.4	6.5	6.5	6.7	6.4	**P1**	9.2	8.7	8.6	8.5	9.1
P3	6.9	7.0	7.2	7.0	6.8	**P3**	8.4	7.5	9.0	9.0	8.7
P6	7.2	7.5	7.3	7.4	7.4	**P6**	9.4	9.1	9.2	9.1	8.9
P8	6.6	6.4	6.3	6.8	6.7	**P8**	8.9	7.8	8.5	8.7	9.1
P11	6.5	6.6	6.8	6.4	6.2	**P11**	9.1	9.0	8.0	7.8	9.0
P13	7.0	7.1	6.9	6.9	6.9	**P13**	8.6	7.5	8.1	8.3	8.4
P16	7.8	7.9	8.2	7.9	8.0	**P16**	8.9	8.6	8.4	8.4	8.5
P18	6.8	7.0	7.2	6.5	7.0	**P18**	8.4	8.9	9.0	9.1	8.6
Ber+IG 8.0±0.5	***Incubation Time***					***Bentonite 3.45±0.5***	***Incubation Time***				
PLOT	**1d**	**2d**	**1w**	**2w**	**6m**	**PLOT**	**1d**	**2d**	**1w**	**2w**	**6m**
P1	8.2	8.1	8.4	8.0	7.9	**P1**	4.2	4.4	4.5	4.7	n.d.
P3	8.7	8.5	8.6	8.3	8.5	**P3**	5.3	6.6	6.0	6.3	n.d.
P6	8.6	8.3	8.5	8.5	8.8	**P6**	4.5	4.3	4.7	5.1	n.d.
P8	8.5	8.4	8.5	8.1	7.4	**P8**	4.0	4.3	4.7	4.4	n.d.
P11	8.4	8.2	8.1	8.0	7.7	**P11**	3.1	5.0	4.5	4.6	n.d.
P13	8.6	8.5	8.7	8.5	7.9	**P13**	4.1	3.9	4.7	4.1	n.d.
P16	8.5	8.3	8.3	8.2	7.2	**P16**	4.4	4.4	4.5	5.2	n.d.
P18	8.7	8.7	8.9	8.5	8.0	**P18**	4.1	4.3	4.7	4.4	n.d.

The additives have annihilated the pH differences within the plots and the values of soil pH in plots have been driven by the additive pH values.
The pH of bentonite was 3.45. Consequently, low soil pH values surely contributed to the lower immobilization effect in soils treated with bentonite.

3.4. *Effect of amendment on the chlorophyll content*

In figure 5.1 the total chlorophyll content of Dwarf bean plants are reported.

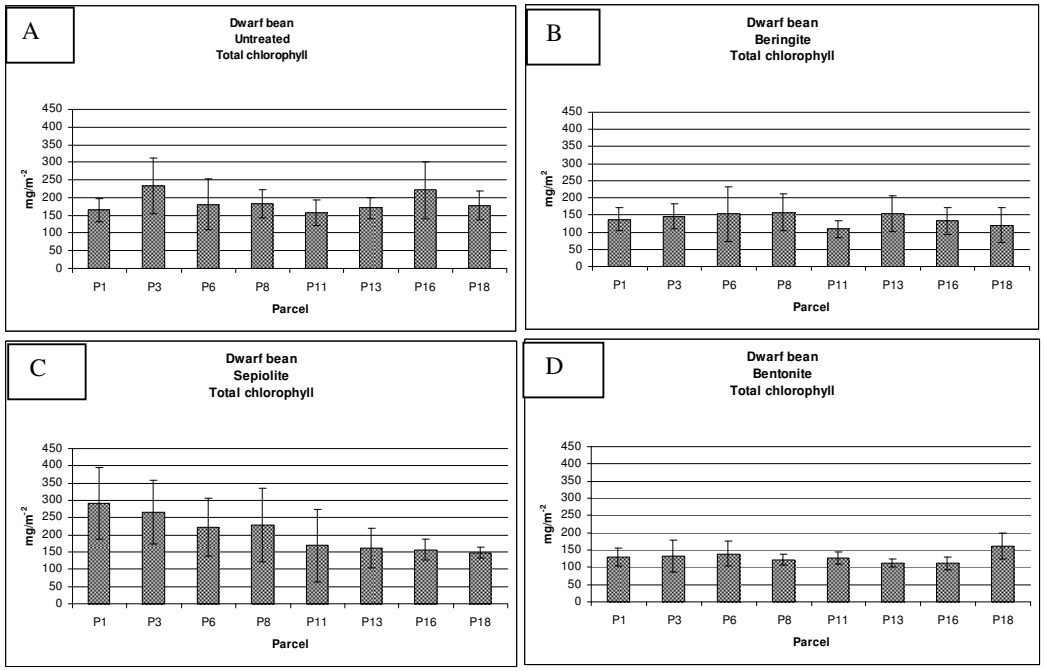


Figure 5.1. The total chlorophyll content of dwarf beans. Plants grown in A-Untreated soils, B-soils treated with Beringite+iron grit, C-soils treated with Sepiolite and D-soils treated with Bentonite

Dwarf bean leaves from plot 1 and 3 showed similar total chlorophyll content in soils treated with bentonite and beringite. Total chlorophyll content in bean leaves from soils treated with sepiolite showed a decreased gradient opposed to the cadmium concentration in soil; apparently this gradient in total chlorophyll content was not directly connected with the bean shoot yield (Table 5.2). Beans from soils treated with bentonite had the lowest values for chlorophyll content in primary leaves and this reduction would be imputing to bentonite effect on soils conditions. In particular, bentonite is capable of absorbing 7 to 10 times its own weight in water, and of swelling

up to 18 times its dry volume. Unfavourable growth condition and/or water deficiency may occur, independent from cadmium negative effects and effect of low pH.

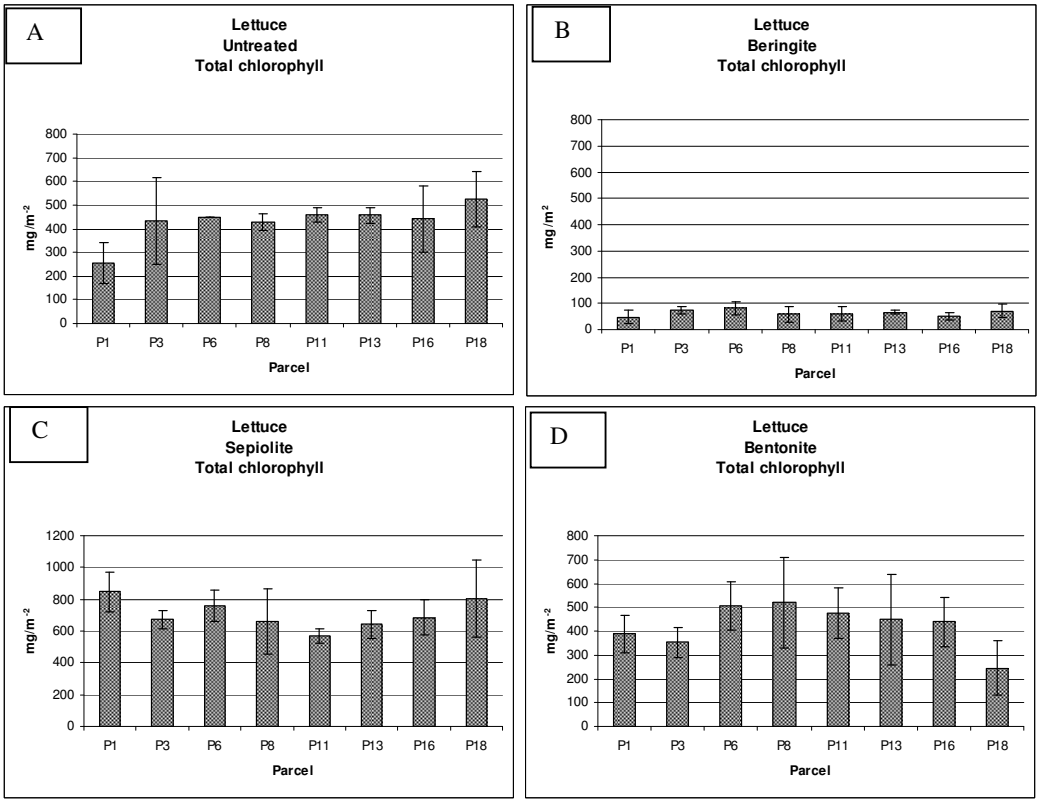


Figure 5.2.Total chlorophyll content of lettuce. Plants grown in : A-Untreated soils, B-soils treated with Beringite+iron grit, C-soils treated with Sepiolite and D-soils treated with Bentonite

In the figure 5.2, the total chlorophyll content of lettuce leaves is reported. Lettuce was affected by the cadmium concentration. In fact, for untreated soils (fig. 5.2. A), the chlorophyll content as well as the lettuce shoot yield were lower in plants cultivated on the Cd contaminated soils (especially in P1) compared to the uncontaminated soils (i.e. P16, P18).

Lettuce was less sensible to the bentonite growth condition compared to dwarf bean, and the chlorophyll content and the shoot biomass verified this behavior. In fact generally, lettuces grown in bentonite-treated soils showed chlorophyll values similar to the untreated soils and a higher shoot biomass.

Soils treated with sepiolite induced better conditions for lettuce growth as demonstrated in figure 5.2. C and in table 5.2. All lettuce leaves had total chlorophyll content higher than 600mg m^{-2}; P1 and P3 showed an increase compared to the respective untreated soils, about four times and three times respectively. Sepiolite-treated soils from plots 16 and 18 did not show different values from respective untreated soils. This indirectly

confirmed that sepiolite did not affect the growth condition of lettuce and dwarf bean. In contrast, Ber+IG effect on the chlorophyll content was high and the values were lower than all the other samples. The reason is not due to a higher biomass as reported in table 5.2. (as cell expanded in primary leaves, chlorophyll density may decrease). It would be imputing to the sorption of P in soil due to Ca from beringite and newly Fe oxides formed. In figure 5.3 the results for *H. lanatus* are reported. The positive effects of soil amendments on chlorophyll content were visible on all leaf samples. Total chlorophyll content in leaves from plants grown on Ber+IG-treated P1 soil increased by 71% compared to that from plants grown on untreated P1 soil. The effect was by 39% for Ber+IG-treated P3 and P6 soils. The other values were reported in table 5.7.Table 5.7 Percentage of increased in chlorophyll content in Holcus lanatus leaves

	beringite	**sepiolite**	**bentonite**
P1	71%	70%	26%
P3	61%	66%	50%
P6	62%	38%	40%
P8	80%	69%	62%
P11	82%	14%	64%
P13	88%	81%	78%
P16	78%	52%	34%
P18	75%	25%	24%

H. lanatus showed a higher capacity of tolerance to the conditions generated by this amendment addition. No negative effects on plant growth were noticed in all the treatments.

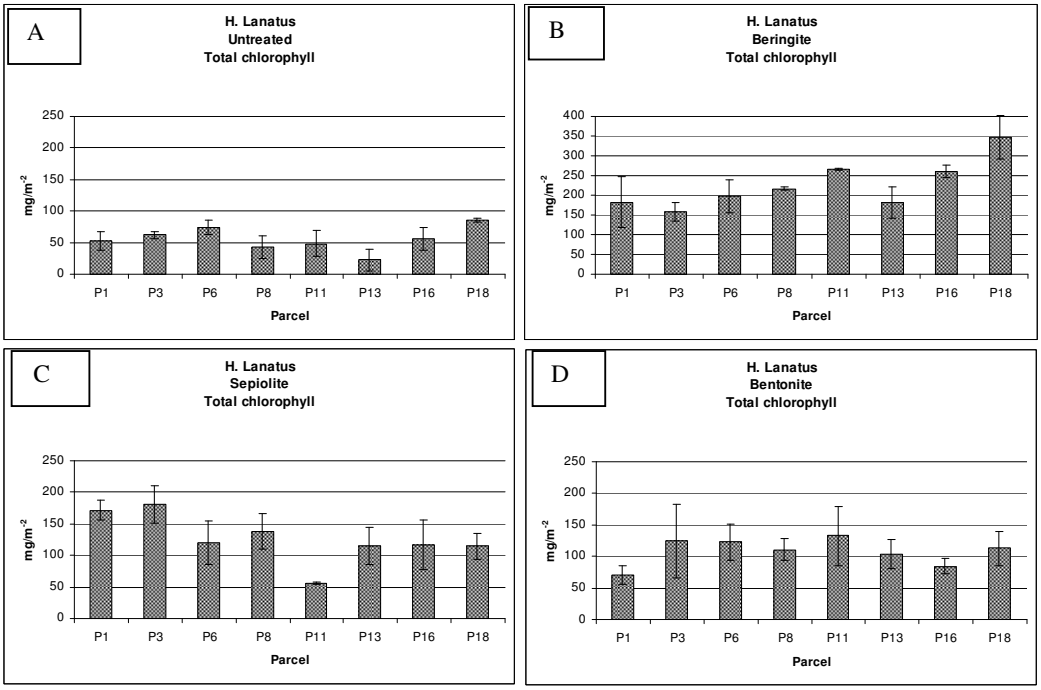


Figure 5.3. Total chlorophyll content of lettuce. Plants grown in : A-Untreated soils, B-soils treated with Beringite+iron grit, C-soils treated with Sepiolite and D-soils treated with Bentonite

3.5. *Results from plants grown in hydroponic system*

In table 5.7 the average of the oven-dried biomass of the 16 *H. lanatus* plants for each treatment are reported.

Table 5.7 Average of the dried plants (g/plant)±SD values. .The statistical analysis was performed between P1-P16 and P3and P18 (* indicates significant statistical values p<0.01).

Plot	Shoot	Root
P1	6.416± 0.152	6.732 ± 0.235
P3	7.919 ± 0.099	8.309 ± 0.125
P16	11.843* ± 0.789	12.660* ± 0.569
P18	10.211* ± 1.001	14. 683*± 1.012

The results show the negative impact of cadmium on *H. lanatus* plants. Plant growth in contaminated nutrient solution were significantly lower than plants grown in the untreated solution and there is no sensible (significant?) effect of the pH of the nutrient solution on the shoots and roots yields. These results suggest that plant transporters at the root membrane may have more influence on Cd uptake and adverse effects in plants than modulation of Cd exposure through a small change in the pH of nutrient solution.

A study has been carried out on two clones of the grass *Holcus lanatus,* one from a metal-contaminated site (Hallen Wood, Avonmouth, UK) and one from an uncontaminated site (Totley, Sheffield, UK) and reported the cadmium concentration in plants from Cd-amended hydroponic cultures (Gaf et al 1992). The Totley material displayed two-fold higher Cd concentrations in shoots than the Hallen Wood. The Totley clone showed impaired growth at relatively low Cd concentrations; the reduction in parallel of tolerance indices (TIs) to 50% occurred at an external Cd concentration of 53- µg L^{-1} compared with 94 µg L^{-1} in the Cd-tolerant Hallen Wood material (Gaf et al., 1992). In this study, transplants have been collected in the field plots. Thus it cannot be excluded that *H. lanatus* transplants collected in uncontaminated AGIR plots (P16, P18) might be more impacted by increasing Cd exposure than transplants from contaminated AGIR plots (P1, P3).

In figure 5.4 the results of chlorophyll and carotenoid content are reported.

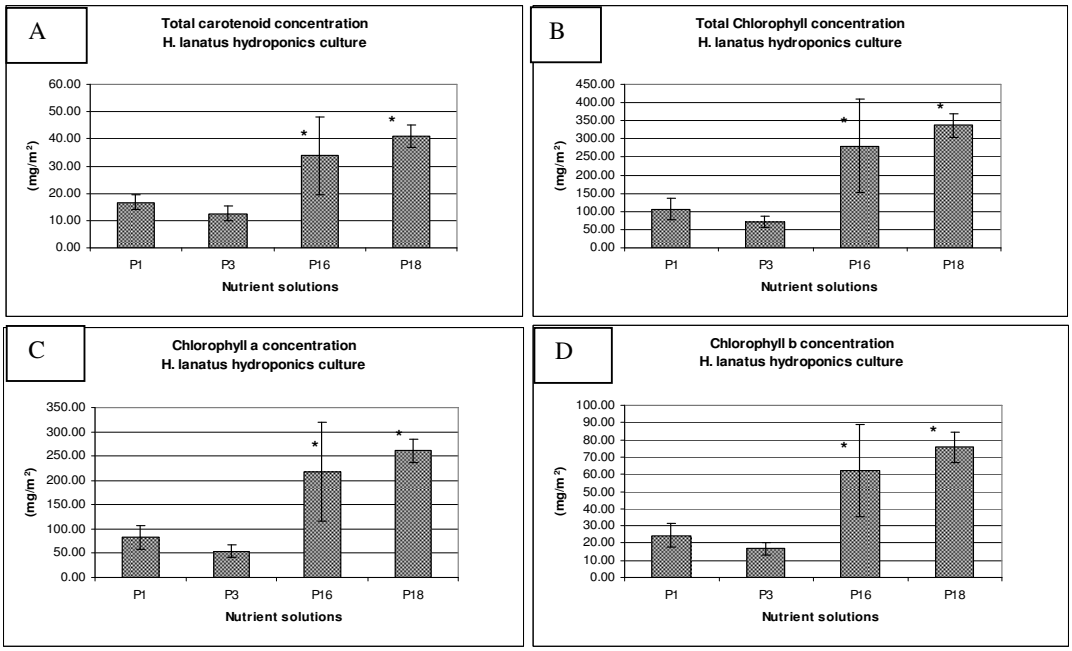


Figure 5.4. A- total carotenoid concentration, B- total chlorophyll content
C and D respectively Chlorophyll a and b content. (*, significant statistical values $p<0.05$, **, $p<0.01$)

The content of carotenoids and chlorophyll was clearly higher in *H lanatus* grown in soils not contaminated by cadmium. In figure 5.5 the total chlorophyll concentration was correlated with the GuPx capacity in leaves and Cd concentration in nutrient solution. The behavior of plants can be divided into two populations, i.e. plants grown in P1/P16 at pH ~7.1 and P3/P18 at pH ~6.5. For both populations, GuPx decreased and total chlorophyll was reduced as Cd exposure increased. In maize exposed to Cd in nutrient solution (0-25 µM), GuPx activity in leaves first increased and then level off especially in mature leaves having a higher Cd content (Lagriffoul et al., 1998). As we have only tested two different Cd concentrations, it cannot be ruled out that *H. lanatus* has a different behavior. However, in our study, it is also possible that the plant exposure to Cd increased firstly GuPx activity to a maximum point and after this threshold value as the plant metabolism is not able to maintain the antioxidative defence then the GuPx activity fall down. In fact, exposure to Cd may be too high, the GuPx activity was limited, may be due to low protein synthesis, and thus plant metabolism and growth was likely impaired due to phytotoxicity. The GuPx activity measured in leaves of plants from nutrient solution at neutral pH (7) was higher than in leaves of plants from nutrient solutions with acid pH (5.5). The GuPx activity followed the

pattern P18 > P16, P3 > P1 (2315, 2237, 1570, and 1093 mU g FW^{-1} respectively). Analysis of Cd content in leaves will be carried out and would help to explain such data.

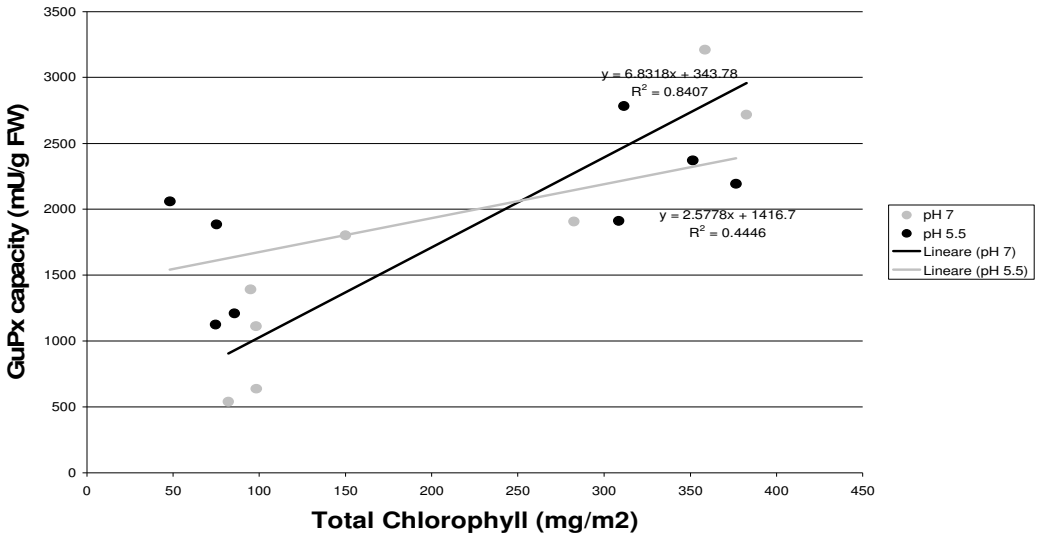


Figure 5..5 Total chlorophyll concentration vs GuPx capacity and pH

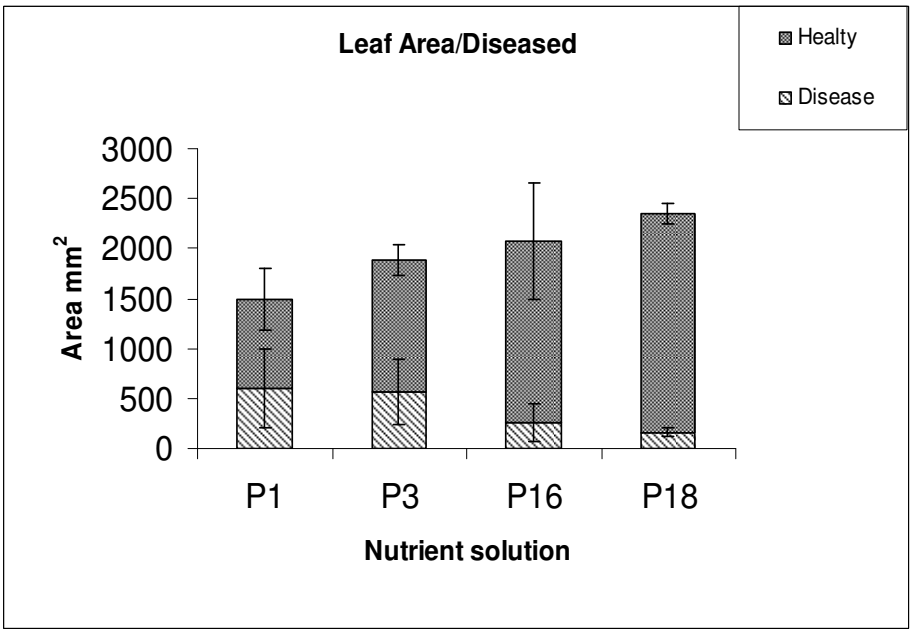


Figure 5.6 Leaf Area divided into healthy and disease surface

In fig. 5.6 the difference between healty and disease surface portion of the leaves were reported. With the term “disease” here we consider the portion of the leaves where the effects of cadmium was visible. Leaf area was calculated on the area of six developed leaves for each pot. Plants from nutrient solution P18 had the higher leaf area with those

from P16 compared to the contaminated nutrient solutions of P1 and P3. At a similar Cd concentration, plants from pots with neutral pH (P18, P3) have numerically a higher leaf area than plants cultivated in acid solutions (P16, P1) but differences were not statistically relevant. The percentage of affected area was higher in plants exposed to high cadmium concentration in P1 and P3 (Fig 5.6). In P16, the fraction of leaf area recognized as "affected" by the software WINDIAS was about 7%. In P18, it was 15%. In P1, it increased to 68% and in P3 to 42%.

In figure 5.7 the values of LA is reported. LA was calculated as the ratio between leaf area and the dried biomass of the leaf. It is an index of the growth condition of the plants, high values indicating the thinner of the leaf, high values meas thin leaves.

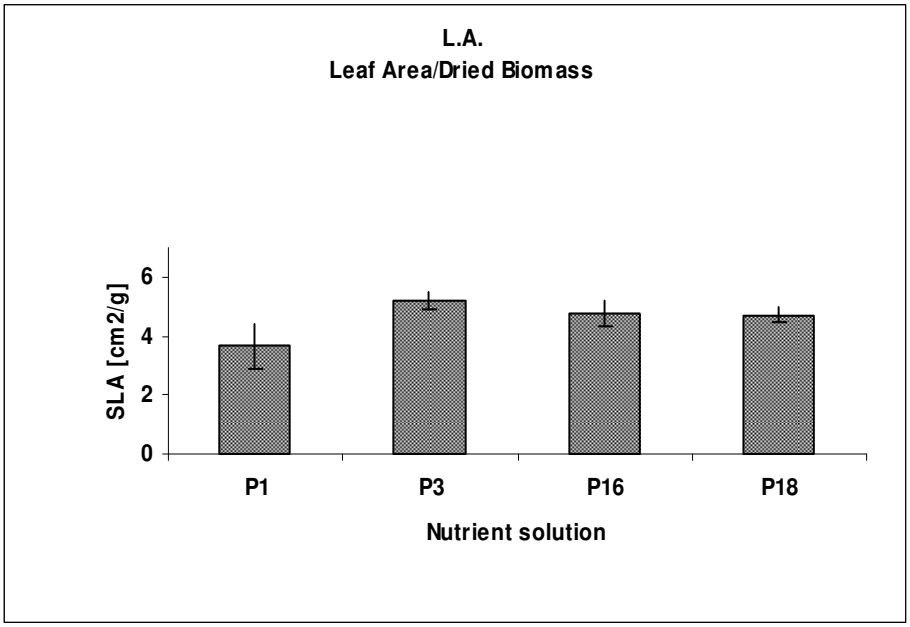


Figure 5.7 L.A., Calculated by the ratio between the Leaf Area and the dried biomass of the leaf. .

The LA values were similar. However P16 and P18 showed numerically higher values compare to P1. This result suggests that Cd affected *H. lanatus* plants as result of a reduction of biomass associated with a reduction of leaf area.

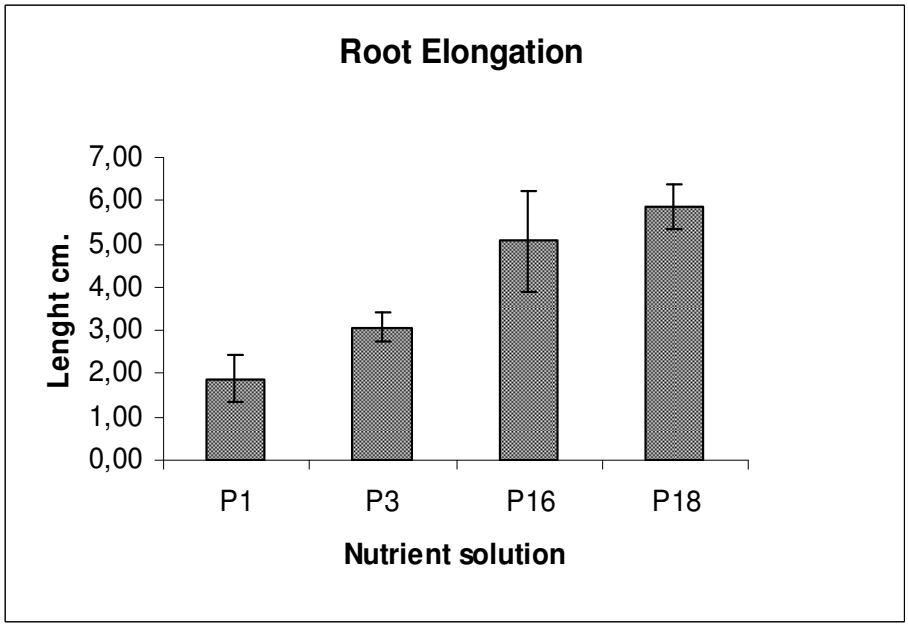


Figure 5.8 Root elongation

The root elongation reported in figure 5.8 indicated a higher root length in P18 and P16 solutions compare to P1 and P3. The difference was about 5 times for P1 and 2 times for P3. These data were in agreement with Symeonidis et al., (1992) results on *H. Lanatus*. Root length of *Holcus lanatus* L. declined rapidly with increasing lead and zinc concentrations in nutrient solution. Negative correlation was also observed between chlorophyll content and increased metal concentrations.

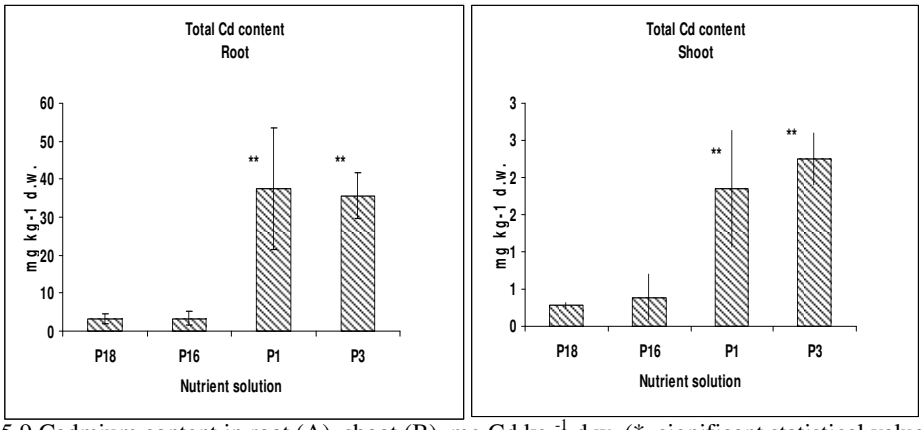


Figure 5.9 Cadmium content in root (A), shoot (B), mg Cd kg $^{-1}$ d.w. (*, significant statistical values $p<0.05$, **,$p<0.01$)

In figure 5.9 the cadmium uptake by *H. lanatus* grown in hydroponics solution is reported. Two evidences run out from the graphs above. The first was the higher storage of assimilated cadmium in the root system. This agreed with data published by Bleeker et al. (2002) on As uptake and location in *H. lanatus*. These results were in contrast

opposite to the results of Gaf et al. (1992). We demonstrated the low level of Cd translocation in the aerial parts of *H. lanatus*. Furthermore Cd accumulation in plant parts was related to its concentrations in the nutrient solutions.

4. CONCLUSION

The potential positive effect of soil amendments on Cd availability and root exposure to Cd was demonstrated by the cadmium reduction in soil pore water. The higher efficiency was demonstrated by beringite + iron grit and this confirmed other results (Mench et al., 2003.). The effect of bentonite required more attention because the lack of the 6 months results on the Cd concentration in soil pore water needs other studies to evaluate the effects of this amendment after the first days of addition. In fact several studies underlined the beneficial effect of bentonite in the inactivation of cadmium, Cd being adsorbed by bentonite (Brigatti et al., 2000; Banat et al., 2002; Singh and Alloway, 2000; Saha et al., 2002; Barbifieri et al., 2002), and probably the beneficial effects resulted after a long-term period.

Another question arisen from these preliminary results is the difficulty to avoid growth problems for sensible plants species created by the special characteristics of minerals generally used in phytostabilization studies. In this experiment, the reduction of cadmium in soil was demonstrated but however the growing condition for lettuce and dwarf bean were unfavourable on soils treated with bentonite and beringite + iron grit respectively. The combination of the two techniques, natural remediation and phytostabilization, needs more studies focused on the research of optimal plants.

The results obtained from the hydroponics experiments on *H. lanatus* confirm the results obtained in this thesis with the biosensor. This states that the bioavailability of cadmium in plot 1 and plot 3 expressed its negative effects at different biological levels, i.e. microorganisms and plants. However the reduction of some biochemical parameters in lettuce grown in the untreated soils in plot 11 and 13, with a non-detectable toxicity by the biosensor, confirmed the attention needs to transfer the results obtained with chemically or biologically assays of the bioavailability of cadmium to the higher organisms, and represent an indirect proof of the complexity of the bioavailability and toxicity of cadmium in soil.

References

Adriano, D.C., Wenzel, W.W., Vangronsveld, J., Boland, N.S., 2004. Role of assisted natural remediation in environmental cleanup. Geoderma 122, 121-142.

Banat, F., Al-Ashen, S., Abu-Aitah, L., 2002. Examination of the effectiveness of physical and chemical activation of natural bentonite for the removal of heavy metal ions from aqueous solutions. Adsorption Science Technologies 20, 151-167.

Barbafieri, F., Plassard, F., Duc, G., Petit-Ramel, M., 2002. Percolation of lead(II) and cadmium through two bentonites used as landfill liner. Chemistry and Ecology 18(18) 177-186.

Blanke, M.M., 1990. Determination of chlorophyll with DMF. Vitic. Enol. Sci. 45, 76-78.

Bleeker, P.M., Assunção, A.G.L., Teiga, P.M, De Koe, T.adn Verkleij, J.A.C., 2002. Revetation of the acidic, As contaminated Jales mine spoil tips using a combination of spoil amendments and tolerant grasses. Science Total Environment 300, 1-13.

Brigatti M.F., Lugli C., Poppi L., 2000. Kinetics of heavy-metal removal and recovery in Sepiolite. Applied Clay Science, 16, 45-57.

Ewitt, E J., 1966. Sand and water culture methods used in the study of plant nutrition. Commenwealth Bureau of Horticulture and Plantation Crops, Maidstone, UK, 547 p.

Krishnamurti, G.S.R., Huang, P.M., Kozak, L.M., Rostad, H.P.W., van Rees, K.C.J., 1997b. Kinetics of cadmium release from soils as influenced by organic acids: implications for cadmium availability. Journal of Environmental Quality 26, 271–277.

Knight, B.P., Chaudri, A., McGrath, S.P., Giller, K., 1998. Determination of chemical availability of cadmium and zinc in soils using inert soil moisture samplers. Environmental Pollution 99, 293-298.

Gaf, H., Ajm, B., Wart, CF., 1992. Cadmium tolerance and toxicity, oxygen radical processes and molecular damage in cadmium-tolerant and cadmium-sensitive clones of Holcus-lanatus L. Acta Botanica Neerlandica 41 (3), 271-281.

Lagriffoul, A., Mocquot, B., Mench, M., Vangronsveld, J., 1998. Cadmium toxicity effects on growth, mineral and chlorophyll contents, and activities of stress enzymes in young maize plants (*Zea mays* L.). Plant and Soil 200, 241-250.

Lichtenhaler, H.K., Wellburn, A.R., 1983. Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochemical Society Transactions 603 rd Meeting Liverpool 11, 591-592.

Lombi, E., Zhao, F.-J., Zhang, G., Sun, B., Fitz, W., Zhang, H., McGrath, S.P., 2002. In situ fixation of metals in soils using bauxite residue: chemical assessment. Environmental Pollution 118, 435–443.

Mench, M., Manceau, A., Vangronsveld, J., Clijsters, H., Mocquot, B., 2000. Capacity of soil amendments in lowering the phytoavailability of sludge-born zinc. Agronomie 20, 383-397.

Mench, M., Bussiere, S., Boisson, J., Castaing, E., Vangronsveld, J., Ruttens, A., De Koe T., Bleeker, P., Assunção, A., Manceau, A., 2003. Progress in remediation and revegetation of the barren Jales gold mine spoil after in situ treatments. Plant and Soil 249, 187–202.

Mench, M., Vangronsveld, J., Didier, V., Clijsters, H., 1994. Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed-silty soil. Environmental Pollution 86, 279–286.

Notten, M.J.M., Oosthoek, A.J.P., Rozema, J., Aerts, R., 2005. Heavy metal concentrations in a soil-plant-snail food chain along a terrestrial soil pollution gradient, Environmental Pollution 138, 178-190.

Renella, G., PhD thesis, University of Florence, 2001.

Renella, G., Mench, M., van der Lelie, D., Pietramellara, G., Ascher, J., Ceccherini, M.T., Landi, L., Nannipieri, P., 2004. Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils. Soil Biology & Biochemistry 36, 443-451.

Renella, G., Landi, L., Nannipieri, P., 2004. Degradation of low molecular weight organic acids complexed with heavy metals in soil. Geoderma 122, 311–315.

Renella, G., Egamberdiyeva, D., Landi, L., Mench, M., Nannipieri, P. 2005a. Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biology Biochemistry (*in press*).

Saha, U. K., Taniguchi, S., Sakurai, K., 2002. Simultaneous adsorption of cadmium, zinc, and lead on hydroxyaluminum- and hydroxyaluminosilicate-montmorillonite complexes, Soil Sci. Soc. Am. J. 66, 117-128.

Sanita` di Toppi, L., Gabbrielli, R., 1999. Review: Response to cadmium in higher plants. Environmental and Experimental Botany 41, 105–130.

Singh PK., Tewari RK., 2003. Cadmium toxicity induced changes in plant water relations and oxidative metabolism of Brassica juncea L. plants. J Environ Biol. 24(1),107-12.

Singh, B., Alloway, B.J., Bochereau, F.J.M., 2000. Cadmium sorption behavior of natural and synthetic zeolites. Commun. Soil Science and Plant Analalysis 31, 2775–2786.

Symeonidis, L., Karataglis, S., 1992. The effect of lead and zinc on plant-growth and chlorophyll content of *Holcus lanatus* L. Journal of Agronomy and Crop Science168 (2) 108-112.

Van Assche, F., Cardinaels, D., Clijsters, H., 1988. Induction of enzyme capacity in plants as a result of heavy metal toxicity: dose-response relations in *Phaseolus vulgaris* L., treated with zinc and cadmium. Environmental Pollution 52, 103-115.

Vangronsveld, J., Sterckx, J., Van Assche, F., Clijsters, H., 1995. Rehabilitation studies on an old non-ferrous waste dumping ground: effects of revegetation and metal immobilization by beringite. Journal of Geochemical Exploration 52, 221-229.

Vangronsveld, J., Mench, M., Lepp, N.W., Boisson, J., Rutten, S A., Edwards, R., Penny, C., Van der lelie, D., 2000. In situ inactivation and phytoremediation of metal- and metalloid contaminated soils: field experiments. In: Bioremediation of contaminated soils, Wise J, Trantolo D, Cichon E, Inyang H, StotmeisterU, Eds. Marcel Dekker Inc, New York, pp. 859-884.

Vangronsveld, J, Colpaert, J, Van Tichelen, K., 1996. Reclamation of a bare industrial area contaminated by non-ferrous metals: physico-chemical and biological evaluation of the durability of soil treatment and revegetation. Environmental Pollution 94, 131-140.

CHAPTER SIX

GENERAL DISCUSSION

This thesis contributes to evidence and quantify the behaviour and effects of root exudates on cadmium bioavailability and microorganisms in the rhizosphere, and to propose remediation options based on soil amendment to quench the potential increasing Cd exposure in the rhizospheric soil. After a general introduction and the aims of the thesis (chapters 1 and 2), the thesis is divided in three parts:

In the first part (chapter 3), an attempt was made to assess the specific effects of long-term field exposure to elevated concentrations of Cd, as the sole contaminant, on its bioavailability in sandy soil. In the second part (chapter 4), cadmium mobility and biochemical parameters were assessing over the release of model root exudates (MRE) in the rhizosphere using a model root system (MRS).

In the third part (chapter 5), the efficiency of three inorganic amendments, (2 out of 3 being single mineral: sepiolite, bentonite, and beringite+iron grit) in reducing Cd labile pool in eight Cd-contaminated soils and the potential use of *H. lanatus* in the aided phytostabilization were monitored.

In literature, there are few studies using long-term field trials which reported the effects of Cd as the sole metal on the composition and the activity of the soil microflora. Less information is available on the effect of roots exudates on Cd bioavailability in the same Cd-contaminated soils. Biosensor BIOMET® and MRS were used to determine Cd bioavailability in sandy soils from long-term field plots containing increasing Cd concentrations (ranging from 0.7 to 38.7 mg Cd kg^{-1} soil d.w.), and treated with various MRE (i.e. glucose, citric acid, and glutamic acid).

The expected results from this experiment were the direct correlation between the presence and concentration of MRE, normally founded in the root exudates, and the bioavailability of cadmium in the rhizosphere. The concentration or chemical speciation of cadmium present in soils of plots 16 (0.7 mg total Cd kg^{-1} soil d.w.), 11 (9.8 mg total Cd kg^{-1} soil d.w.), and 6 (18.9 mg total Cd kg^{-1} soil d.w.) were insufficient to detect an influence of model root exudates on the Cd bioavailability by the biosensor. The link between the root exudates and cadmium bioavailability to microorganisms was detected only in the case of soils with a total Cd content of 40 mg Cd kg^{-1} and especially in citric acid treatment. No similar data were previously reported in literature.

Although it is widely accepted that LMWOAs (low molecular weight organic acids) may play a key role in controlling metal bioavailability due to their high concentration and metal chelating properties, the negative effects of cadmium on microorganisms biomass and activities would be imputed to the direct capture of the citric acid Cd-complexes, including by the biosensor. However, in our experiments, even glucose, which has no chelating capacity, increased Cd bioavailability, possibly due to the formation of secondary metabolites with ligand properties. Another explanation would be attributed to the stimulation effects on bacterial growth and the subsequent CO_2 release, or to the stimulation of microorganisms followed by the increase in dissolved organic matter which may decrease the soil pH.

The results from the biosensor experiment (chapter 3) were compared to those of the MRS experiment (chapter 4), in which was demonstrated that the cadmium concentration did not affect the mineralization of root exudates (MRE) in the rhizosphere soils, the carbon biomass, and the nitrogen inorganic pools, whereas an influence on biomass nitrogen was observed in high Cd contaminated soils.

The root exudates did not affect the Cd mobility in the soils with low /medium cadmium concentration (0, 10, and 20 mg total Cd Kg^{-1}d.w.soil). In the high contaminated soils (40 mg total Cd Kg^{-1} d.w. soil), LMWOAs, particularly citric acid, has increased the cadmium labile fraction. In some cases, also glutamic acid caused an increase in Cd availability. The same effects were quantified in the biosensor results.

In the last part of the thesis (chapter 4), the Cd availability in soil pore water was measured with a soil sampler moisture Rhizon. In the untreated soils, the results showed a ratio about ten times lower than cadmium extracted with 1 M NH_4NO_3 in the MRS experiments. Two inorganic soil amendments out of 3, i.e. sepiolite and beringite+iron grit have been recognized as highly effective in the reduction of Cd concentrations in the soil pore water. Furthermore, we demonstrated that the decrease in cadmium labile fraction pools happened in the first days after soil treatments and that the stabilisation effect was still effective after six months.

Plants grown in soils treated with soil amendments showed different behaviours: Dwarf bean had a limited growth in bentonite-treated soils and it would not be imputed to the cadmium contamination but to the particular soil conditions (especially water supply and soil cloaking) that bentonite created. In contrast, *Holcus lanatus* L grew very well on bentonite-treated soils, but was less luxuriant in soils treated with beringite+ iron grit. Thus the aided phytostabilization is a valid remediation option to combine with natural remediation in the recovery of Cd contaminated soils.

These results confirm that the maximum level of Cd set at 2 mg kg^{-1} d.w. in the European legislation is safe for soil bacteria in this kind of soil. However, the complexity of metal mobility in soils and the many factors affecting the availability in soil and contaminant uptake by plant roots suggest to be really careful with the setting of mandatory limits.

In the experiment with inorganic soil amendment, the reduction of available cadmium in soil was demonstrated but however the growing conditions for lettuce and dwarf bean were unfavourable on soils treated with either bentonite or beringite+iron grit. The combination of the two techniques, natural remediation and phytostabilization, need more studies focused on the optimal plant species.

Results from the hydroponic experiments on *H. lanatus* (chapter 5) confirmed the results obtained in this thesis with the biosensor (chapter 3). This states that the Cd bioavailability in plot 1 (40 mg total Cd Kg^{-1} d.w. soil) and plot 3 (40 mg total Cd Kg^{-1} d.w. soil) expressed its negative effects at different biological levels, i.e. microorganisms and plants. However the reduction of some biochemical parameters in lettuce grown in the untreated soils in plot 11 (10 mg total Cd Kg^{-1} d.w. soil) and 13 (10 mg total Cd Kg^{-1} d.w. soil), with a non-detectable toxicity by the biosensor, confirmed the attention needs before to transfer the results obtained with chemical or biological assays of the Cd bioavailability to the higher organisms and represented an indirect proof of the complexity of Cd bioavailability and toxicity in soil.

Further studies are required to clarify the dynamics of bentonite in long-term period. Furthermore, studies carried out with MRS using real root exudates may explain the lack of Cd mobility in the rhizosphere probably due to the low C-source added with MRE. At the same time, the effectiveness of soil amendments on Cd concentration in soil solution might be assessed with Rhizon soil moisture sampler during the plant growth in order to underline the effect of root exudates on the Cd mobility.

In this thesis many newest and more accurate techniques could be use to better focus such mechanisms, like labeled ^{14}C to measured the microbial respiration and mineralization of carbon, or DNA profile to monitor the changes in the microbial community; or techniques to measure the effects of Cd on the cholophyll degradation in plants under stress condition. But it would require a larger number of samples and more complex and expensive analysis. This suggest us to first have a general screening of the problem of Cd in the rhizosphere from different point of view. In the future the works, started from the results of this thesis, will be more focused, and more specific and expensive analysis could be used.